

CRANFIELD UNIVERSITY

MICHAEL BINDER

DEVELOPMENT OF A *BOTRYTIS* SPECIFIC IMMUNOSENSOR;
TOWARDS USING PCR SPECIES IDENTIFICATION

SCHOOL OF ENGINEERING

PhD Thesis

Academic Year: 2010 - 2013

Supervisor:

Prof. Leon A. Terry

Prof. Ibtisam E. Tothill

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ABSTRACT

Botrytis species affect over 300 host plants in all climate areas of the world, at both pre and post-harvest stages, leading to significant losses in agricultural produce. Therefore, the development of a rapid, sensitive and reliable method to assess the pathogen load of infected crops can help to prescribe an effective curing regime. Growers would then have the ability to predict and manage the full storage potential of their crops and thus provide an effective disease control and reduce post-harvest losses.

A highly sensitive electrochemical immunosensor based on a screen-printed gold electrode (SPGE) with onboard carbon counter and silver / silver chloride (Ag/AgCl) pseudo-reference electrode was developed in this work for the detection and quantification of *Botrytis* species. The sensor utilised a direct sandwich enzyme-linked immunosorbent assay (ELISA) format with a monoclonal antibody against *Botrytis* immobilised on the gold working electrode. Two immobilisation strategies were investigated for the capture antibody, and these included adsorption and covalent immobilisation after self-assembled monolayer formation with 3-dithiodipropionic acid (DTDPA). A polyclonal antibody conjugated to the electroactive enzyme horseradish peroxidase (HRP) was then applied for signal generation. Electrochemical measurements were conducted using 3,3', 5,5'-tetramethylbenzidine dihydrochloride / hydrogen peroxide (TMB/H₂O₂) as the enzyme substrate system at a potential of -200 mV. The developed biosensor was capable of detecting latent *Botrytis* infections 24 h post inoculation with a linear range from 150 to 0.05 µg fungal mycelium ml⁻¹ and a limit of detection (LOD) as low as 16 ng ml⁻¹ for covalent immobilisation and 58 ng ml⁻¹ for adsorption, respectively. Benchmarked against the commercially available *Botrytis* ELISA kits, the optimised immuno-electrochemical biosensor showed strong correlation of the quantified samples ($R^2=0.998$).

The second stage of the development combined the biosensor with a DNA testing procedure in order to detect and discriminate between the *Botrytis* species. Real-time polymerase chain reaction (PCR) was used to identify the pathogen-derived DNA with primers specific to *Botrytis* species causing neck rot in onion. Two DNA sensor designs

were developed and optimised as a function of various parameters. The DNA sensor format employing two biotinylated primers employs neutravidin immobilised on the gold working electrode as a binding molecule for the amplified pathogen DNA, followed by streptavidin-peroxidase reagent for signal generation. A second DNA sensor platform based on thiol-C6 and biotin-labelled amplified DNA premixed with streptavidin-HRP conjugate was also developed in this study, reducing the total assay time to 10 min. The biosensor was capable of detecting pathogen DNA of *Botrytis* species associated with neck rot (*B. allii*, *B. aclada* and *B. byssoides*) down to a concentration of 100 fg template DNA, which is close to the LOD of the PCR aided DNA amplification process. Furthermore, the DNA assay procedure is not affected by plant tissue and the universal design of the DNA sensor offers a wide range of potential applications if used with DNA primers specific to other pathogens.

As part of this work, the developed immuno and DNA sensor platforms were tested in a large field trial. Onion bulbs (n=215) were monitored over a period of 4 months to establish the incidence of bulb rot in store. Onion neck tissue samples (n=71), collected at harvest, were tested to quantify the pathogen load using the immunosensor. Employing a threshold of 0.1 µg ml⁻¹ for positive detection, the immunosensor was capable of predicting neck rot with a success rate of 90 % with only 1 % false negative samples. Furthermore, the DNA sensor tested with onion tissue demonstrated its capability to distinguish between infected and non-infected samples with a success rate of 95 % (n=40).

A microfluidic system with two housing designs was developed for the immunosensor to incorporate all test components needed for a complete *Botrytis* test device. Gold nanoparticles labelled with polyclonal antibody-HRP conjugate were employed as a strategy for signal enhancement. This technique further reduced the assay sensitivity to a LOD of 71 ng ml⁻¹ that is suitable for detecting *Botrytis* infections in onion samples prior to showing visible symptoms. This new prototype system enables a simple and sensitive detection of *Botrytis* infections in the field.

Keywords: Biosensor, Neck Rot, Fungus, Gold Nanoparticles, Real-Time PCR

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NOTATION

Symbols (unit):

%	Percent
r.h.	Relative humidity
cm	Centimetre [10^{-2}]
mm	Millimetre [10^{-3}]
μm	Micrometre [10^{-6}]
nm	Nanometre [10^{-9}]
pm	Picometre [10^{-12}]
fm	Femtometre [10^{-15}]
M	Mole/Molar

Abbreviations:

Ab	<u>A</u> ntib <u>o</u> dy
Ag	<u>A</u> ntigen
bp	<u>B</u> ase <u>P</u> air
BSA	<u>B</u> ovine <u>S</u> erum <u>A</u> lbumin
CAD	<u>C</u> omputer <u>A</u> ided <u>D</u> esign
CV	<u>C</u> yclic <u>V</u> oltammetry
% CV	<u>C</u> oefficient of <u>V</u> ariation [%]
dNTP	<u>D</u> esoxy <u>N</u> ucleotides <u>T</u> riphosphate
dsDNA / ssDNA	<u>d</u> ouble <u>s</u> tranded / <u>s</u> ingle <u>s</u> tranded <u>D</u> eoxyribo <u>N</u> ucleic <u>A</u> cid
E-Bot LFS	<u>E</u> lectrochemical <u>B</u> otrytis <u>L</u> ateral- <u>F</u> low <u>S</u> ensor
EDC	N-(3-Dimethylaminopropyl)-N'-Ethylcarbodiimide Hydrochloride
EDTA	<u>E</u> thylenediaminetetraacetic <u>A</u> cid
ELISA	<u>E</u> nzyme <u>L</u> inked <u>I</u> mmunosorbent <u>A</u> ssay
GEP	<u>G</u> el <u>E</u> lectrophoresis
HRP	<u>H</u> orseradish <u>P</u> eroxidase

LFD	<u>L</u> ateral- <u>F</u> low <u>D</u> evice
LFiA	<u>L</u> ateral- <u>F</u> low i <u>m</u> muno <u>c</u> hromatographic <u>A</u> ssay
LOC	<u>L</u> ab <u>O</u> n <u>C</u> hip
LOD	<u>L</u> imit <u>o</u> f <u>D</u> etection
LOQ	<u>L</u> imit <u>o</u> f <u>Q</u> uantification
IgG	<u>I</u> mmunoglobulin <u>G</u>
MCH	<u>M</u> agnetic <u>C</u> apture <u>H</u> ybridisation
NC	<u>N</u> itro <u>C</u> ellulose
NHS	<u>N</u> - <u>H</u> ydroxy <u>s</u> uccinimide
PBS / PBST	<u>P</u> hosphate <u>B</u> uffered <u>S</u> aline / with <u>T</u> ween
(RT-) PCR	(<u>R</u> eal <u>T</u> ime) <u>P</u> olymerase <u>C</u> hain <u>R</u> eaction
PDA	<u>P</u> otato <u>D</u> extrose <u>A</u> gar
PDB	<u>P</u> otato <u>D</u> extrose <u>B</u> roth
PDMS	<u>P</u> oly- <u>D</u> imethyl <u>s</u> iloxane
PLA	<u>P</u> rupe <u>L</u> actose <u>A</u> gar
RFLP	<u>R</u> estriction <u>F</u> ragments <u>L</u> ength <u>P</u> olymorphism
SAM	<u>S</u> elf- <u>A</u> ssembled <u>M</u> onolayer
SEM	<u>S</u> canning <u>E</u> lectron <u>M</u> icroscopy
SP(G)E	<u>S</u> creen- <u>P</u> rinted (<u>G</u> old) <u>E</u> lectrodes
spp.	Species
SPR	<u>S</u> urface <u>P</u> lasmon <u>R</u> esonance
TAE	Tris <u>A</u> cetate- <u>E</u> DTA
TBS	<u>T</u> ris <u>B</u> uffered <u>S</u> aline
TE	<u>T</u> ris- <u>E</u> DTA
TEG	<u>T</u> riethylene <u>G</u> lycol
TMB	3,3',5,5'-Tetramethylbenzidine Dihydrochloride Hydrate
UK	<u>U</u> nited <u>K</u> ingdom
USA	<u>U</u> nited <u>S</u> tates of <u>A</u> merica
UV	<u>U</u> ltra <u>V</u> iolet

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1 INTRODUCTION AND LITERATURE REVIEW

1.1 PROJECT BACKGROUND

Botrytis species, also known as grey mould (Figure 1.1), infect many host plants in all climate areas of the world, at both pre and post-harvest stages, leading to significant losses within the agricultural industry (Elad et al., 2004). In some years, when weather conditions are most favourable, losses can be as high as 15 to 40 % of the annual crop with disastrous impact on local farmers (Maude et al., 1986; Elad et al., 2004; Vrind, 2005). Neck-rot of onions (*Allium cepa*) is one appearance of *Botrytis* infections, representing the primary storage disease of onions (Coolong et al., 2008). The total losses in the UK caused by internal defects of onions account for more than £4.6 million per year due to poor quality and substandard produce (O'Connor et al., 2010). Considerable efforts have therefore been invested in protecting crops pre and post-harvest, resulting in an annual worldwide market value of anti-*Botrytis* products of £450 million with an economically-speaking increasing trend (Elad et al., 2004; Vrind, 2005).



Figure 1.1: Scanning electron microscopy of a grey mould spore (*Botrytis cinerea*) germinating on a strawberry leaf (Garcia, 2013).

Botrytis infections of onions grown from seed with an infection rate of <10 % can spread in the field causing an epidemic of neck rot with overall infection rate of 20 to 50 % within the crop (Maude and Presly, 1977a; Maude, 1983; Stewart and Franicevic, 1994). Post-harvest topping of the foliage may also be a source of inoculum (Maude et al., 1984; O'Connor et al., 2010). Moreover, the requirements in quality for the UK are much higher than the standards in the rest of the European Union (EC1509/2001) including a retail tolerance level of 2 to 4 % for internal defects. Batches with a higher percentage of internal defects are downgraded or rejected. The standard treatment to control neck-rot disease is the fungicide Hy-TL (Thiabendazole and Thiram) that has an off-label approval since 2002 (O'Connor et al., 2010). In particular, the fungicide is not supported by any label recommendation and the risk of losses or damage caused by the use of the product lies with the user. Alarming for growers in Europe, Hy-TL was prohibited due to the recent pesticide withdrawal of the European Union (91/414/EEC) in January 2010, with no alternative seed treatment in sight.

Great efforts have been conducted to control neck-rot disease, such as the treatment of seeds with experimental fungicides, hot water or vapour in order to reduce external infections (Green, 2009). Post-harvest, *Botrytis* infections can be cured by drying the onion bulbs before storage to close the neck tissue, reduce water loss and increase storage life (Maude et al., 1984). However, drying onions before storage for several weeks is energy intensive. Hence, reducing curing temperatures can help to cut energy usage with no loss of quality in the product (Downes et al., 2009).

The fungal infection rate is highly dependent on various factors, including humidity, initial pathogen load of the seed and infection rate in field (Maude, 1983; Maude et al., 1986). A high curing temperature may not be appropriate for all geographic regions and growing seasons with alternating weather conditions. A sensitive and reliable method for the quantification of the pathogen load, pre or post-harvest can give the growers the potential to employ a customised curing regime of the onion bulbs, based on the local pathogenic risk. Growers would have the ability to assess the full storage potential of their crops, reduce post-harvest losses and energy costs and provide effective disease control.

1.2 *BOTRYTIS* SPECIES AND THEIR ENVIRONMENT

Botrytis diseases are the most common and widely distributed pathogens of vegetables, fruits, and field crops throughout the world. The optimum growth condition for the pathogen is a temperature of 18 to 23 °C and high humidity (>50 % r.h.). However, the fungus is also active at low temperatures from 0 to 10 °C, causing considerable losses in stored crops (Agrios, 2005b). There are over 20 genera of *Botrytis* (Beever and Weeds, 2004) including 22 classified species and one hybrid (Staats et al., 2005) (Table 1.1). Seven *Botrytis* species have been reported to infect onions (Beever and Weeds, 2004; Staats et al., 2005). *B. porri* typically infects the bulbs of garlic and leeks. Recently, Du Toit et al. (2002) also isolated this pathogen from onion crops and seeds. Nevertheless, *B. porri* only occasionally infects onions and is consequently not a common pathogen.

B. cinerea can cause superficial leaf fleck after the leaves have been infested by other leaf-blighting pathogens. Furthermore, *B. cinerea* has been described by Clark and Lorbeer (1973) as the cause of brown stain (Figure 1.2). However, the symptoms induced by *B. cinerea* have little effect on the plant health and growth. *B. squamosa*, *B. tulipae*, *B. aclada*, *B. allii* and *B. byssoidea* have been determined to cause neck rot; the last three of them are considered to be the primary source of the disease (Yohalem et al., 2003; Lorbeer et al., 2004; Chilvers and Du Toit, 2006).



Figure 1.2: Brown stain on the outer dry scales of an onion bulb caused by *B. cinerea* (Chilvers and Du Toit, 2006).

Table 1.1: *Botrytis* species recognised by Hennebert (1973), their common disease name, typical hosts and host plant species. The second part of the table provides an overview of species that infect onions (Beever and Weeds, 2004; Staats et al., 2005; Chilvers and Du Toit, 2006).

Species	Common Disease Name	Typical Host/Tissue Specificity	Host-Plant Species
<i>B. fabae</i> Sardiña	Chocolate spot	Leaves of bean	<i>Vicia spp. L.</i> , <i>Pisum spp.</i>
<i>B. calthae</i> Hennebert		Stem of marsh-marigold	<i>Caltha palustris</i>
<i>B. ranunculi</i> Hennebert		Buttercup	<i>Ranunculus spp. L.</i>
<i>B. ficariarum</i> Hennebert		Buttercup	<i>Ficaria verna</i>
<i>B. pelargonii</i> Roed		Leaves of geranium	<i>Pelargonium spp. L.</i>
<i>B. paeoniae</i> Oud.	Peony blight	Stems of cultivated peonies	<i>Paeonia spp. L.</i>
<i>B. hyacinthi</i> Westerd. and Beyma	Hyacinth fire	Leaves of hyacinth	<i>Hyacinthus spp. L.</i>
<i>B. elliptica</i> (Berk.) Cooke	Lily fire	Leaves, stems, and flowers of cultivated lilies	<i>Lilium spp. L.</i>
<i>B. globosa</i> Raabe	Neck rot	Wild garlic	<i>Allium ursinum.</i>
<i>B. narcissicola</i> Kle B. Ex Westerd. and Beyma	Smoulder mould	Bulbs of narcissus	<i>Narcissus spp. L.</i>
<i>B. polyblastis</i> Dowson	Narcissus fire	Leaves of narcissus	<i>Narcissus spp. L.</i>
<i>B. galanthina</i> (Berk. and Br.) Sacc.	Blight	Snowdrop	<i>Galanthus spp. L.</i>
<i>B. convoluta</i> Whetzel and Drayton	<i>Botrytis</i> rhizome rot	Rhizomes of cultivated iris	<i>Iris spp. L.</i>
<i>B. croci</i> Cooke and Massee	Crocus blight	Leaves of cultivated crocus	<i>Crocus spp. L.</i>
<i>B. gladiolorum</i> Timm. / <i>B. draytonii</i> (Budd. and Wakef.) Seaver	Gladiolus blight	Stems of cultivated gladiolus	<i>Gladiolus spp. L.</i>
<i>B. sphaerosperma</i> Buchw.	Leaf blight	Three-cornered leek (White-flowered Onion)	<i>Allium triquetrum</i>
<i>SPECIES INFECTING ONIONS:</i>			
<i>B. cinerea</i> Pers. / <i>B. fuckeliana</i> (de Bary) Whetzel	Gray mould, brown stain, leaf blight, umbel blight	Fallen leaves, fruits and flowers	>235 plant species
<i>B. tulipae</i> Lind	Tulip fire	Leaves, stems, and flowers of cultivated tulips	<i>Tulipa spp. L.</i>
<i>B. porri</i> Buchw.	-	Bulbs of garlic, leek, onion seed crops and onion seed (Du Toit et al., 2002)	<i>Allium spp. L.</i>
<i>B. squamosa</i> J. C. Walker	Leaf blight, sclerotial neck rot, umbel blight	Leaves and bulbs of onion	<i>Allium cepa</i>
<i>B. byssoidea</i> Walker/ <i>B. allii</i> (Sawada) Yamamoto (Hybrid)	Mycelial neck rot	Bulbs of onion, garlic and leek	<i>Allium spp. L.</i>
<i>B. allii</i> ^a (Munn) Yohalem	Gray mould / sclerotial neck rot	Bulbs of onion, garlic and leek	<i>Allium spp. L.</i>
<i>B. aclada</i> ^a (Fresen.) Yohalem	Gray mould / sclerotial neck rot, scape blight, and umbel blight	Bulbs of onion, garlic and leek	<i>Allium spp. L.</i>

^a 2003 recognised as two different species (Yohalem et al., 2003).

Recent molecular and taxonomic studies have indicated that *B. aclada*, *B. allii*, and *B. byssoidea* are valid neck-rot species infecting onions (Nielsen et al., 2001; Yohalem et al., 2003; Khan et al., 2013). The main difference between the species, including the different number of their mitotic chromosomes, is the size of their asexual spores called conidia. *Botrytis aclada* has slightly smaller conidia than *B. allii* Munn. *Botrytis byssoidea* instead produces the biggest conidia (Chilvers and Du Toit, 2006). Yohalem et al. (2003) identified two different clone fragments of *B. allii* that are identical with those of *B. aclada* and *B. byssoidea*. A theory emerged suggesting that during a simultaneous infection of an onion, a hybridisation between *B. aclada* and *B. byssoidea* occurred. An indication for this theory is also the fact that *B. allii* has 32 mitotic chromosomes, whereas *B. aclada* and *B. byssoidea* have 16.

The genetic variation of the *Botrytis* species infecting onions was investigated in 2001 and 2002 by Nielsen et al. using an UP-PCR (universal primer polymerase chain reaction). The sexual reproduction of *B. squamosa* recombines the chromosomes, resulting in a high diversity of the fungal genome. By contrast, *B. aclada* and *B. allii* demonstrate clonal spreading, showing effects in less genetical variations. Moreover, the three different samples of *Botrytis byssoidea* examined from the USA, the Netherlands and the UK were identical (Nielsen et al., 2001; Beever and Weeds, 2004).

1.3 NECK-ROT DISEASE

B. squamosa and *B. tulipae* have also been associated with neck rot, but these species are not typically the primary cause of the disease (Chilvers and Du Toit, 2006). Three species, however, are exclusively associated with neck rot in onions and are therefore considered as the primary causal agent of the disease; *B. byssoidea* (J. C. Walker), *B. aclada* (Fresenius) and *B. allii* (Munn) (Yohalem et al., 2003; Lorbeer et al., 2004; Chilvers and Du Toit, 2006). Though *B. allii* and *B. aclada* are the most common source of neck rot, in some years *B. byssoidea* has been more frequently isolated from diseased onion bulbs within the UK (Presly, 1985b). However, the primary source of neck-rot disease is debatable and still remains unknown.

1.3.1 INFECTION PATHWAYS

There are three different pathways a fungus can spread: sclerotia, mycelia and conidia. The disease cycle of *Botrytis* (Figure 1.3) is complex and its investigation is still a challenge. All species of *Botrytis* can form sclerotia that are long-term survival structures. The fungus overwinters mainly as sclerotia, but also as mycelium or conidia on previously infected onion debris in the soil, in cull piles, refuse dumps and in the trash in storage sheds (Walker, 1926; Tichelaar, 1967). These mechanisms allow the fungus to survive for considerable periods in the bulbs or on the seeds. In practice, both strategies are used simultaneously by the fungus, resulting in lack of differentiation between mycelial and micro-sclerotial surviving (Holz et al., 2004).

For short-term propagules in the field the mycelial system of *B. aclada*, *B. Byssoides*, and *B. allii* develop asexual conidia. Their survival is highly dependent on temperature, moisture, bacterial activity and sunlight exposure. Mainly UV light is influencing the mortality of conidia (Rotem and Aust, 1991).

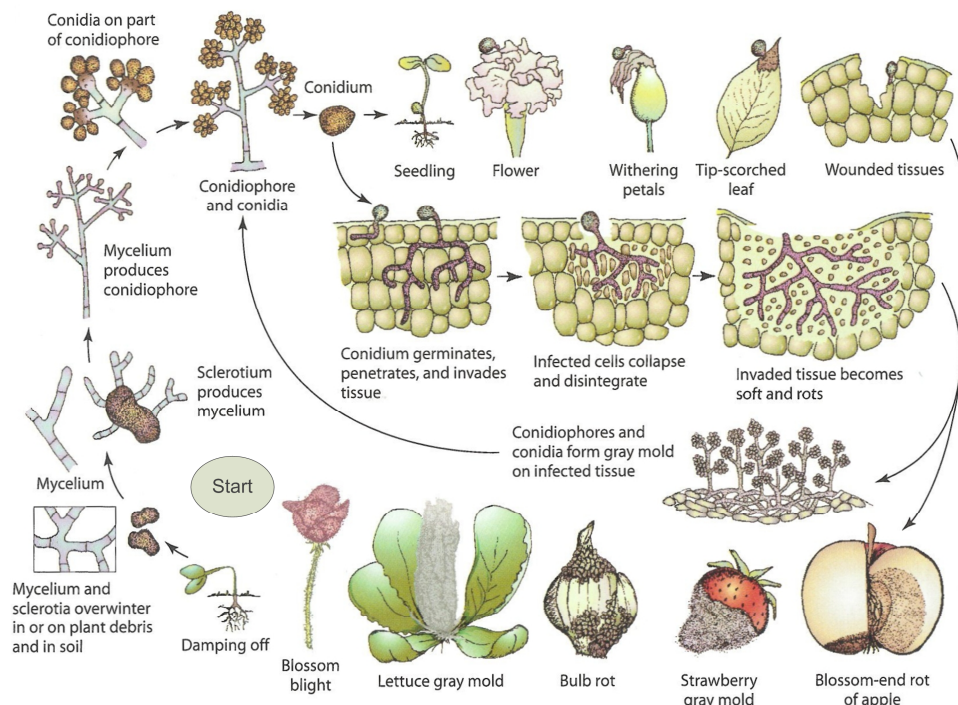


Figure 1.3: Disease cycle of *Botrytis* grey mould (Agrios, 2005b).

Research during the 70s and 80s showed that neck rot caused by *B. allii* and *B. aclada* is a seed-borne disease (Maude and Presly, 1977a; Maude, 1983; Brewster, 1994; Stewart and Franicevic, 1994; Lorbeer et al., 2004). Although sclerotia have been reported to survive in soil and on debris for two years, trials suggested that sclerotia are not the primary inoculum pathway of the disease (Maude et al., 1982). Due to the limited genetic analysis methods at that time, a separation between *B. allii* and *B. aclada* was not possible. This, however, has occurred in the last decade through molecular analysis (Nielsen et al., 2002; Yohalem et al., 2003; Staats et al., 2005; Khan et al., 2013). Though *Botrytis* species are not infecting the seeds directly, the primary inoculum pathway of neck rot is onion seeds contaminated with sclerotia, mycelia or conidia. Under normal storage conditions (10 °C, 50 % r.h.) the mycelium of *B. allii* and *B. aclada* can survive over three years on the surface as well as under the seed coat, still being viable to infect when their germination is declining (Maude and Presly, 1977a; Khan et al., 2013). However, the exact circumstances of the infection pathway are still a controversial issue (Maude and Presly, 1977a; Kritzman, 1983; Maude, 1983; Lorbeer et al., 2004; Chilvers and Du Toit, 2006).

The spread of the disease was first investigated by Tichelaar (1967) through microscopic monitoring of stained leaf samples (staining described by Preece, 1959) and autoradiography of leaves infected by radioactively marked mycelium. Radioactive conidia, or after inoculation radioactive mycelium, were obtained by growing the fungus on potato dextrose agar (PDA) added with phosphor isotope ³²P that could be tracked by an autoradiographic picture of the leaves using x-rays (Figure 1.4). Later the spread of the disease was also investigated by Maude (1977), who described the correlation between infected seeds and neck rot in a large field trial.

The pathogen is located on the outer surface of the seed as well as internally. An indication for the high proportion of the internal infected seed is the fact that an external treatment of the seed with fungicide can reduce the infection rates, but the disease cannot be eradicated (Maude and Presly, 1977a). The fungus was observed by Maude and Presly (1977a) in the majority of stained seedlings as mycelium growing from the seed coat into the living tissue of the cotyledon leaf tips.

During the green leaf stage, the fungus may either remain quiescent in the epidermis or, if moisture conditions are favourable, may grow further over the leaf surface, occasionally entering a stoma pore (Figure 1.4 A) (Tichelaar, 1967). Thereafter, when the leaves become senescent and yellowish, the fungus spreads rapidly intracellular. Autoradiographs (Figure 1.4 B) and plating out the infected leaves show that the fungus grows downwards in the tissue, still symptomless, eventually invading the neck of the onion bulb via the leaves that emerge directly from the top of the neck (Tichelaar, 1967; Maude and Presly, 1977a). Depending on the site of inoculation on the leaf, the fungus can be traced in the neck tissue and in bulb scales four to seven weeks after inoculation by treating the bulbs using a stain that contains 0.02 % methyl-red in 95 % ethanol (Tichelaar, 1967).



Figure 1.4: (A) Microscopic monitoring of *B. allii* on the leaf tissue under 300x magnification. (B) Autoradiograph of an inoculated leaf; mycelium growing from the site of inoculation (black circle) towards the neck (Tichelaar, 1967).

Further evidence for the theory that the infection pathway occurs through the leaves is the observation that phenolic compounds in onion tissue inhibit the growth of the fungus (Strange, 2003; Agrios, 2005a). These molecules occur in lower quantities in leaves than in the bulb scales, making a direct infection of the bulb less likely.

1.3.2 DEVELOPMENT OF THE DISEASE

Botrytis neck rot is, as its name indicates, a decay of the neck area of onion bulbs. It can grow asymptotically and is generally not observed until the plant is close to harvest. The lack of symptoms may occur because the mycelium remains restricted to the colourless epidermal cells in the initial stages of infection (Tichelaar, 1967). First symptoms of neck rot occur one to two months post-harvest (Hennebert, 1973; Maude and Presly, 1977a). Problematic for quality control by retailers and industry is neck rot, since it is an internal infection. Bulbs may appear flawless, thus the disease is only visible after cutting the sample (Figure 1.5 A). First indication is a softening of the tissue induced through water soaking, followed by brown discoloration. As the decay advances, the tissue of the onion loses firmness so that some regions of the bulb appear sunken (Figure 1.5 C). In the end stadium, white mycelium and black sclerotia, an irregularly shaped kernel-like body (Figure 1.5 B arrow), may appear between the scales and the outer surface. Greyish mould formed by conidiophores and conidia may also indicate the disease (Figure 1.5 D). Rarely, under very humid conditions, the symptoms are visible before harvest (Tichelaar, 1967). In this case, sporulation of *B. allii* or *B. aclada* is visible on the surface (Figure 1.5 E) or by pulling back the outermost fleshy scale (Figure 1.5 G). Neck rot may also be observable by an abnormal development of the plant directly compared with healthy onions (Figure 1.5 F).

B. byssoidea and *B. squamosa* are also considered to cause neck rot (Walker, 1925b). Symptoms of *B. byssoidea* infection are quite similar to those caused by *B. allii* or *B. aclada*. The main difference is the structural growth of the fungus, including more mycelial growth and few or no sclerotia and conidia being present. Although *B. byssoidea* is only occasionally reported to cause neck rot (Walker, 1925b; Lorbeer et al., 2004), the distribution and significance of *B. byssoidea* as a neck-rot pathogen may be underestimated compared with *B. allii* and *B. aclada* due to the difficulty of species identification (Presly, 1985b; Chilvers and Du Toit, 2006).

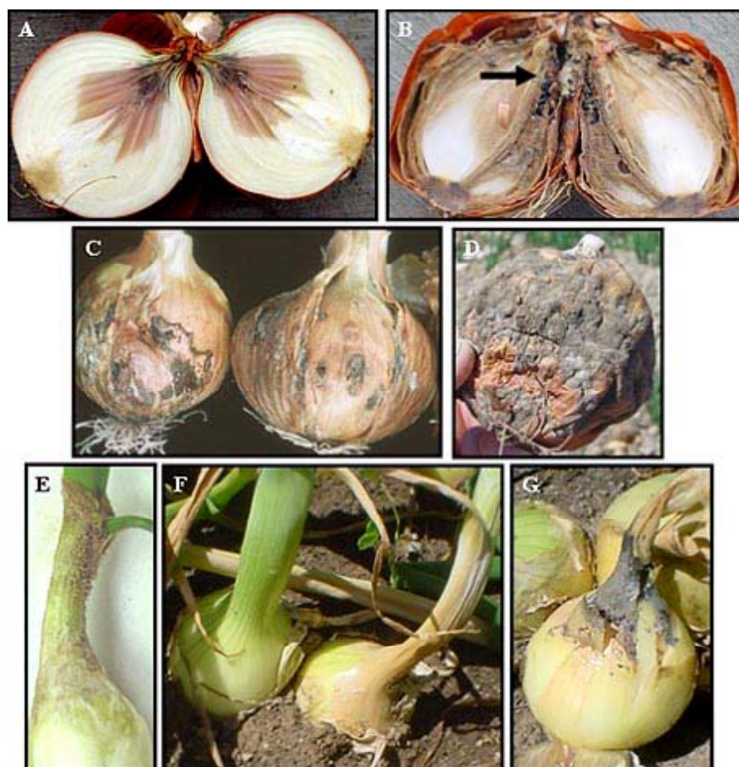


Figure 1.5: Common post-harvest symptoms (A to D) and less common pre-harvest (E to G) symptoms, caused by *Botrytis aclada* and *B. allii* on onion bulbs (Chilvers and Du Toit, 2006).

The onion bulbs infected with *Botrytis* develop small sclerotial neck rot, but just as continuum of onion leaf blight pathogenicity. Generally, symptomless *B. allii* infections may be present in living tissue as mycelium growing on the leaves and only become manifest in storage. However, Steward and Mansfield (1984) observed that under certain conditions the infection site of the pathogen can be traced by a lesion on the leaf.

1.3.3 SPREAD OF THE DISEASE

During the 70s, a large epidemic of neck rot was observed in the UK. The major source of the disease was infected seed comprising an infection rate of up to 70 % (Maude and Presly, 1977a). However, normally the infection rate of commercial onion seed rarely exceeds more than 20 %. The main mechanism of the fungus spreading is conidia, but it can also occur directly through the mycelium. The conidia are produced by conidiophores (Figure 1.6) located in the necrotic leaf tissue.



Figure 1.6: *Botrytis* conidiophores with conidia (Blog, 2010).

The disease can spread throughout the field through conidia carried by the wind to other hosts with a range of up to 270 m (Maude and Presly, 1977a). Mechanical topping of the foliage after harvest may also be a source of infection (Maude et al., 1984). Weather conditions have a strong influence on the incidence of neck rot. Long periods with high humidity (>80 % r.h.) and high frequency of rain, especially towards the end of June, increase the production and release of *Botrytis* spores (Maude et al., 1986). Due to the fact that bulbs with dry necks are more resistant to the pathogen (Walker, 1925a), high humidity especially at harvest decelerates the drying and consequently increases the risk of disease. Furthermore, experiments conducted by Tichelaar (1967) demonstrated that the fungus can infect plants regardless of their age and stage of development.

Once in storage, conidiophores present on the dry outer scale of the onion bulb do not seem to penetrate into the bulbs (Maude and Presly, 1977a). These scales act like a natural barrier to the spread of the fungus by contact. An indication for this trend is also the observation that *B. allii* does not invade the onion bulb when its neck tissue is dry (Walker, 1925a).

1.3.4 CURING NECK ROT

Great efforts have been conducted to contain the disease. Until recently, the standard treatment to prevent neck-rot disease was the fungicide Hy-TL (Thiabendazole 225 g l⁻¹ and Thiram 300 g l⁻¹ (Myles, 2003)) that has an off-label approval since 2002. Infected seed lots were sprayed with the fungicide to decrease the pathogen load. However, Hy-TL is no longer allowed for the treatment of seed due to the recent pesticide withdrawal by the European Union (91/414/EEC) in January 2010. Alternatively, fungicide disinfectants can be used (Green, 2009). The main disadvantages of these solutions are the reduction of the germination efficiency. A non-chemical solution to decrease the pathogen load is hot water treatment of the seed. In this case, the seed is first pre-soaked followed by a hot-water clearance for a defined period (Green, 2009).

Antagonistic fungal infections were also evaluated for suppression of the sporulation of *Botrytis allii*. *Alternaria alternata*, *Chaetomium globosum*, *Ulocladium atrum* and *U. chartarum* were reported to suppress the sporulation of *B. allii* almost completely under continuously wet conditions (Köhl et al., 1995). Two fungi (*C. rosea* and *U. atrum*) were further investigated in a field trial by Yohalem et al. (2004), but these antagonists were not reported to stop the progress of *B. aclada* from necrotic into the fresh leaf tissue.

Post-harvest, *Botrytis* infections can be cured by drying the onion bulbs before storage to close the neck tissue, reduce water loss, and increase storage life (Maude et al., 1984). This procedure can be carried out at different temperatures ranging from 20 to 28 °C (Downes et al., 2009; Chope et al., 2012). Currently the standard curing practice for onions in the UK is drying the crop at 28 °C for six weeks (65 to 75 % r.h.) before storage (Downes et al., 2009). However, drying onions before storage for several weeks is energy intensive and may favour bacterial rot. Hence, reducing curing temperatures may help to cut energy usage with no quality loss of the product (Downes et al., 2009).

1.3.5 OTHER ONION ROTS

Besides neck rot, a variety of other diseases and disorders affect onion crops. The most common diseases are bacterial rots and fungal basal rots (Chaput, 1995; George, 2005). Where neck rot only shows pre-harvest symptoms after long periods of humid weather or when a high pathogen load is present during growth, bacterial rot develops a far more aggressive progression of the disease. Several bacterial species such as *Pseudomonas* and *Erwinia spp.* cause onion rot, which is favoured by humid, warm weather and mechanically damaged tissue (Chaput, 1995). Bacterial rot is also known as “soft rot”, or “sour skin”. Initial foliar symptoms are tan or brown, partially rotten tissue that develops a characteristic strong sour smell. Onions with internal soft rot often appear normal, but if the bulb is dragged or squeezed, the decayed inner tissue slides out through the neck or base region (Figure 1.7 A). Other symptoms are a slippery skin and decayed or water-soaked scales, mostly surrounding a mechanical injury.

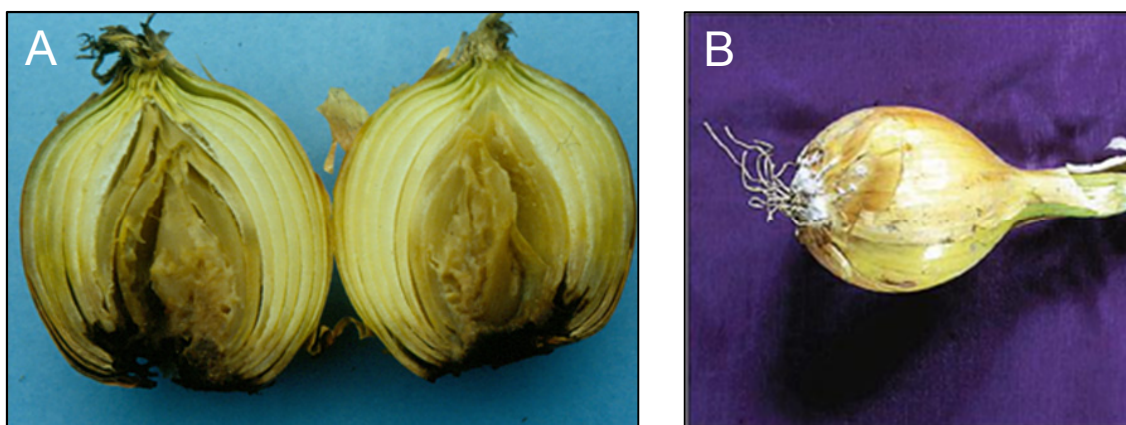


Figure 1.7: (A) Bacterial soft rot caused by *Pseudomonas* and *Erwinia spp.* (Chaput, 1995). (B) Outer symptoms of *Fusarium* base rot (Delahaut and Stevenson, 2004).

Basal rot in onions is caused by the soil-borne pathogen *Fusarium oxysporum* (Delahaut and Stevenson, 2004). In general, this pathogen attacks the basal plate of the onion plant, resulting in a decay of the roots and a subsequent collapse of the plant in the end stadium of the disease. The mycelial growth of the pathogen is white with cork like sclerotia developing upon the base of the plant and a pink to brown coloration of the infected tissue (Figure 1.7 B). Secondary *Botrytis* or bacterial infections may develop in the affected area once the tissue is decayed and susceptible. Generally *Fusarium*

oxysporum favours high growing temperatures between 25 and 28 °C and is therefore more active during the summer season, but was also reported to cause severe infections at 22 °C (Larkin and Fravel, 2002; Pio and Macedo, 2007).

1.4 DETECTION METHODS FOR *BOTRYTIS*

Detection and quantification of *Botrytis* species causing neck rot was until recently only practicable through plating out the infected material, for example onion leaves, bulb tissue or pure conidia on agar media. Although these methods are still practiced, they are limited by effort, duration and the skills of the operator. Other methods are now available that are faster and more sensitive, such as molecular tests and immunological methods.

1.4.1 CONVENTIONAL AGAR ASSAYS

After incubating the fungus on a chosen agar media, the identification occurs through macroscopic and microscopic examination of sclerotia, conidiophores and conidia, based on their size, shape and colour. Generally, potato dextrose agar (PDA) or prune lactose agar (PLA) is used to grow the fungus. These media are quick in production and easy to handle. If the repeatability of the nutrient consistency is the main objective, pectin agar is favourable. For the isolation of the fungus from onion plants PLA is the best choice due to its low pH that facilitates the growth of *B. allii* (Maude and Landahl, 2011).

The most selective and differential agar, although difficult in production, is the Kritzman agar (Kritzman and Netzer, 1978). This media is based on the capability of the fungus to produce dark brown pigments with their extracellular enzyme named polyphenol oxidase using tannic acid as a substrate. A comparison of the most common agar media is listed in Table 1.2. To avoid microbial overgrowth, bactericides and other inhibiting substances are added to the agar solution.

Table 1.2: Efficiency of four agar media for determining *Botrytis* infections in seed (Metcalf, 1998; Chilvers and Du Toit, 2006).

Characteristic assessed	Agar Medium ^a			
	PDA	PLA	Kritzman	Pectin
Repeatability of nutrient consistency	+ ^b	+	+++	+++
Freedom from contaminant fungi	+	+	+++	++
Internationally accepted for the detection of <i>Botrytis</i> spp.	+	+++	+++	+
Ease of preparation	+++	+	+	++
Ease of identifying <i>Botrytis</i>	++	++	+++	++

^a PDA = Potato dextrose agar, PLA = Prune lactose agar, Kritzman = Agar medium developed by Kritzman and Netzer, Pectin = Pectin agar

^b + = Low, ++ = Medium, +++ = High.

1.4.2 ALTERNATIVE METHODS

One method to induce sporulation of latent *Botrytis* infection was described by Du Toit et al (2004). The first step involve placing the whole onion plant in a plastic bag and keep the sample in a dark environment for four to seven weeks at a temperature of 5 to 10 °C. Next, samples were cut longitudinally and stored in transparent plastic containers filled with moist paper towels and incubated for five to seven days with a 12 h day-night rhythm at 24 to 28 °C. The result of this process is the development of conidiophores and conidia in a macroscopic scale in case of an infection (Figure 1.8).

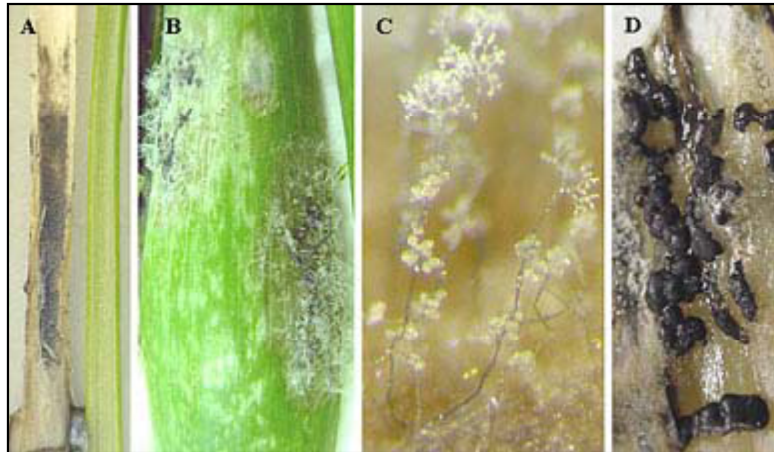


Figure 1.8: Detection of *Botrytis* as described by Chilvers and du Toit (2006).

- (A): Dense sporulation on an onion scape (non-infected scape on the right).
 (B): White mycelium and grey sporulation.
 (C): Conidiophores and conidia (48× magnification) of *B. aclada* or *B. allii*.
 (D): Sclerotia and sporulation of *B. aclada* / *B. allii* on an infected scape.

Another detection method of neck rot is described by Tichelaar (1967) and Kritzman (1983). This method is based on the artificial trigger of latent infections by changing the environmental conditions of the onion bulbs infected by *B. allii* and *B. aclada*. The switch after two days storage at 4 °C to 16 days at 15 °C induces a pH alteration of the infected tissue affected by enzymatic activity of the fungus. This can be visualised with an alcoholic solution of bromocresol-green and methyl-red. When an infected onion is cut into halves and methyl-red is applied, the affected tissue turns crimson because of the acid reaction (pH 4.2); in contrast the colour of the healthy part of the scale is yellow-orange (pH 6.6) (Tichelaar, 1967).

A different approach for the detection of *Botrytis cinerea* in strawberries was described by Vandendriessche et al. (2012) and Zhu et al. (2013). In order to detect the fungal growth, the change in volatile composition was monitored as a function of the degree of *B. cinerea* infection using multi-capillary column-ion mobility spectrometry (HS MCCeIMS) (Vandendriessche et al., 2012) and an electronic nose, containing an array of 10 different metal oxide sensors (Zhu et al., 2013). Both studies aimed at monitoring the change in volatile composition of strawberries with multivariate data analysis in order to identify potential biomarkers specific to *B. cinerea* and other fungi. Although the evolution in volatile composition during *B. cinerea* infections could be followed,

these methods are time consuming and not very sensitive and specific as volatiles produced by the fungi may be overshadowed by the fruit volatiles (Vandendriessche et al., 2012; Zhu et al., 2013). Therefore, a good differentiation between the sources of volatiles needs to be established in future work.

1.4.3 LATERAL-FLOW IMMUNOCHROMATOGRAPHIC ASSAY

The first one-step rapid lateral-flow immunochromatographic assay (LFiA), better known as lateral-flow devices (LFD), was a pregnancy test launched in 1988. The test was affected by vibration, gave a high incidence of false non-pregnant results and had low sensitivity results (Gossel and Mahalik, 1993). To date the LF-diagnostic is an important part of the quick and easy-in-use home testing. The basic components of a LFD are porous membranes, antibodies, and a signal-generating system that can be evaluated optically. The typical setup of a LFD is depicted in Figure 1.9.

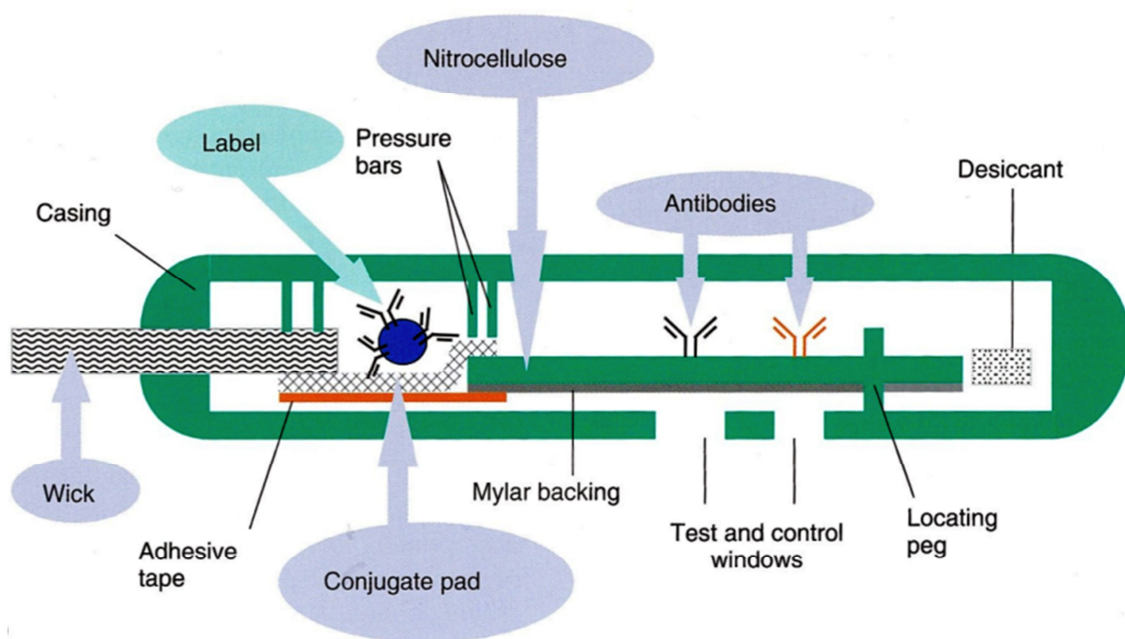


Figure 1.9: Typical architecture of a lateral-flow device (Davies et al., 2007b).

The wick captures and directs the sample to the conjugate pad by capillary forces. When a pipette is part of the equipment, the wick is generally integrated inside the device. In this case, the plastic casework also has the function of a well, directing the sample to the

first capillary element and preventing a spillage of the analyte to the interior of the housing. The flow is sustained by small overlaps of the test strip components and resisted by viscose drag and capillary forces generated by these materials. When using a low volume LFiA, some components may be additionally covered by a clear adhesive film in order to reduce evaporative loss.

After the antibodies were liberated from the matrix through the sample, they bind a specific region on the analyte molecule. The assay moves onto the membrane to the immobilised second target specific antibody. Downstream a second control antibody line is plotted on the nitrocellulose membrane that contains often anti-species conjugates. This test line aims at capturing all antibodies or signal generating elements that fail to bind to the test line. If the test result is negative, the second antibody line should still be observable as an assay control measure. For a positive result, both lines are visible.

There are two main LFD systems defined by the size of the analyte molecule. The sandwich assay is used when the molecule is large enough to be bound by two antibodies simultaneously. In contrast, the competitive assay is used for small molecules such as drugs or metabolites that can only be captured by one antibody. For the detection of *Botrytis*, the sandwich format is more suitable by reason of the large size of the fungal antigen.

The principal task of the label is to generate a signal detectable by eye or an electronic reader. Their size is limited by the pore diameter of the membrane and they should show low tendency to non-specific binding. One signal-generating system is a spherical, blue-dyed polystyrene latex (Figure 1.10 A) with a typical diameter of approximately 0.4 μm (Davies et al., 2007b). In this case, the latex is mainly embedded in an amorphous sugar protein matrix designed to avoid adhering to the conjugate pad. This prevents the latex aggregation upon drying and maintains the functionality of the antibodies during the one to three years shelf life. Additionally, the nitrocellulose membrane is coated with surface-blocking substances to prevent non-specific binding of the proteins. In order to maintain a consistently strong colour of the control line for a

positive test result, the conjugate pad often contains an excess amount of latex beads that can bind easily to the control line. At present, surfactant-free lattices are available increasing the electrostatic stability of the antibodies in suspensions through a greater surface charge density. To customise lattices for their specific application, nearly all colours of dyes are presented. For their production, the beads are washed in the presence of dissolvent dye. The readout of the test is visual, but can also be improved by using an UV/visible reader.

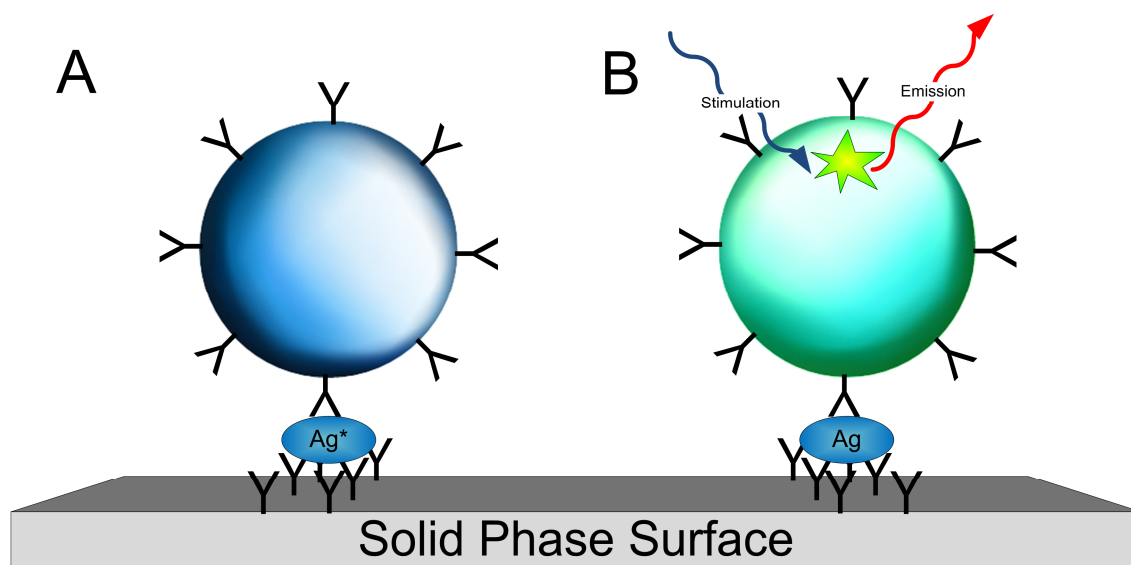


Figure 1.10: Polystyrene (A) and fluorescence (B) latex labels used for LFiAs.

*Ag = Antigen

For an optical readout, fluorescence lattices are available (Figure 1.10 B). Technical requirements for the readout are a wavelength-specific light source and adapted filter. Depending on the Stokes-shift, the difference between the absorbed and emitted wavelength can be as low as 10 to 20 nm. To produce fluorescent lattices, the beads are loaded with organic fluorophores, but this technique has never been used for a commercial LFiA. Para- and ferromagnetic particles have also been attempted for LFDs. However, their magnetic attraction resulted often in an aggregation of the beads. Therefore, superparamagnetic lattices have been developed (Figure 1.11 A) that only become magnetic when exposed to a magnetic field. The readout of the test line may be visual, as magnetic elements have a black to brown colour, depending on their oxide loading and composition, but more likely combined with a commercial magnetic reader.

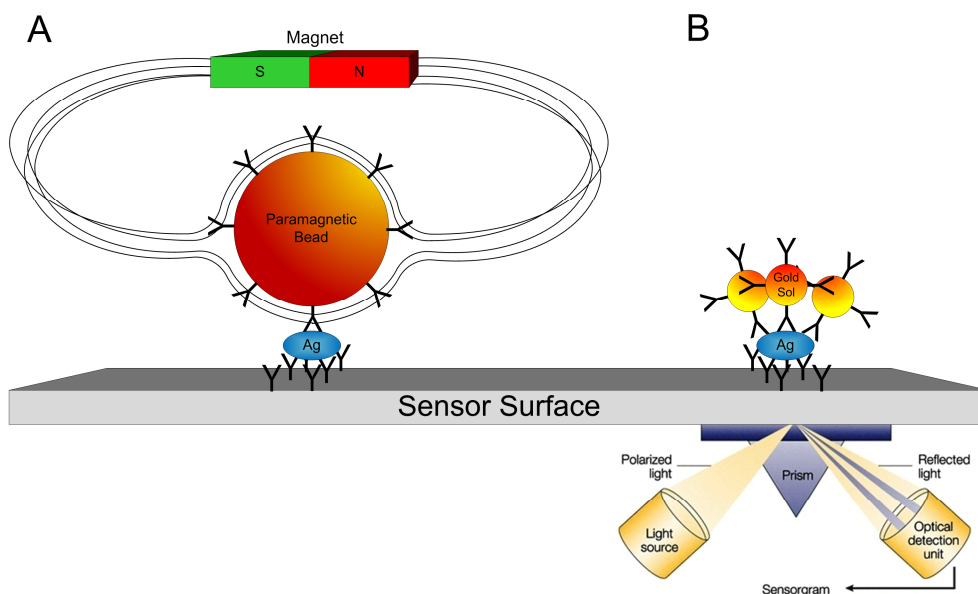


Figure 1.11: Schematic illustration of two label techniques: Superparamagnetic beads (A) and gold sol (B). Possible read out method for gold sol is surface plasmon resonance (SPR).

Beside latex, gold sol is a preferred label (Figure 1.11 B). One advantage is its natural colour with a strong optical absorption band making dyeing process become redundant. The metal character of gold sol can be also used for an optical readout by surface plasmon resonance (SPR). The adsorbed photons set up collective oscillation of the conduction electrons (plasmon) which extend to the surface as a surface plasmon wave (Davies et al., 2007b). The frequency of this resonance is diameter dependant and is measured by a detector. Gold particle diameters can be tuned reliably and routinely from 1 nm to 200 nm (Grabar et al., 1997). In general, sols employed in LFiAs have a diameter ranging from 20 to 60 nm and absorb green light (520 to 540 nm) making them appear red in white light (Glomm, 2005). Typically, Ø40 nm sol is used for the production of the red test line. Another advantage of gold sol coated with antibodies is the easy observation when aggregation occurs. This is apparent when the surface-to-surface distance in the colloidal suspension is less than the particle diameter of the particles. A colour change from red-to-pinkish/purple to blue/purple is the result that can be easily spotted as a quality control measure (Elghanian et al., 1997). Compared to latex beads, the gold sol has a ten times smaller diameter leading to a higher curvature. As a result, the binding site of the approximately 15 nm long antibody to the antigen is more exposed resulting in an increased binding accessibility of the antibodies.

Forsite Diagnostics (Sand Hutton, UK) offers an on-site LFD diagnostic kit for *Botrytis* (Figure 1.12). With Forsite being an original equipment manufacturer (OEM), the LFD diagnostic kit can also be purchased from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK). Recently, EnviroLogix (Portland, USA) released the QuickStix kit designed for the detection of *Botrytis* in wine grape juice. Both LFDs are utilising the monoclonal antibody BC-12 raised by Meyer and Dewey (2000). The BC-12 antibody employed for the fungal detection is specific to surface proteins generated by growing hyphens of *Botrytis* species. A comparative study conducted by Dewey et al. (2013) showed that both LFDs have a similar limit of detection and are capable of detecting *Botrytis* infections in wine. However, the EnviroLogix LFD is designed as a dipstick, whereas the Forsite LFD has a plastic housing.



Figure 1.12: Forsite Diagnostics (Sand Hutton, UK) on-site LFD kit for *Botrytis*.

1.4.4 ELISA

The detection and quantification of proteins or other antigen can occur through the enzyme-linked immunosorbent assay (ELISA) that is an established technique. In this method, the visualisation of the target antigen is achieved through a colour-generating enzyme covalently linked to a specific antibody. The colour formation is proportional to the concentration of antibodies bound to the analyte and can be used for quantification by reading the absorbance. There are many variations of ELISA techniques. The most common ones are the indirect and the sandwich format.

Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK) offers qualitative or semi-quantitative commercial ELISA kits for *B. allii* and *B. cinerea*. Standards are not available for the detection of *Botrytis*, but a positive control represented by freeze-dried fungus is used. The difference between the *Botrytis* kits is based on the antibodies used for the detection of the pathogen. The *B. allii* ELISA kit includes polyclonal antibodies produced by immunising rabbits with the antigen. In contrast, the *B. cinerea* ELISA kit is applying the monoclonal antibody raised from the hybridoma line BC-12 developed by Meyer and Dewey (2000). Furthermore, ADGEN Phytodiagnostics offers a reagent set comprising the probing antibody and anti-species conjugate (goat anti-rabbit antibodies linked to alkaline phosphatase). Positive and negative control, as well as the polyclonal *B. allii* antibody and the monoclonal antibody BC-12 can be ordered separately.

For the detection of *Botrytis* the indirect ELISA method with antigen directly immobilised to the well is used by the commercially available ELISA kits (Figure 1.13 A). Next, the specific antibodies (mono or polyclonal) bind to the surface of the antigen. For the detection of the antigen-antibody complex, a second anti-species antibody is added to the assay. This antibody linked with the enzyme recognises the target specific antibody and binds to its Fc-region.

Alternatively, if enzyme-conjugated antibodies are available, a direct ELISA can be accomplished (Figure 1.13 B). An advantage of this format is the lack of an additional incubation step of the anti-species conjugate. This format was used to test the quality of the antibody-horseradish peroxidase (HRP) conjugate needed for the immunoassay (please see Chapter 2).

A more specific detection and quantification can be achieved by the sandwich ELISA format (Figure 1.13 C). This practice requires two antibodies specific to different epitopes of the antigen. In contrast to the format employed by the commercially available ELISA kits, the first antibody instead of the antigen is immobilised to the well surface. The antigen is added in the second step and binds to the captured antibodies. Finally, the second antibody is added to the well that binds to another region of the analyte and generates the signal required for detection. This format was adopted for the immunosensor development.

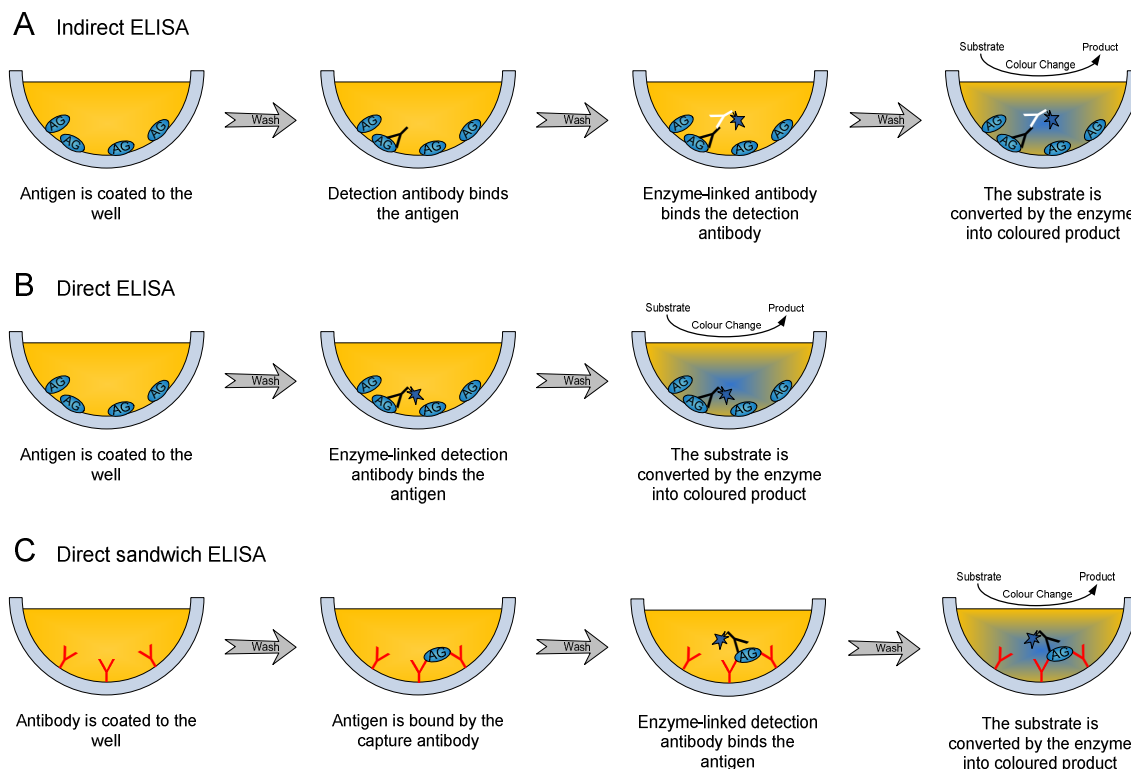


Figure 1.13: Comparison of the indirect ELISA (A) that is employed by the commercial *Botrytis* ELISA kits, the direct ELISA format (B) used for the immunoassay development and the direct sandwich format (C) that was also adopted for the biosensor development.

Meyer and Spotts (2000) developed a quantitative ELISA for the detection of *Botrytis cinerea* in pear stems using the monoclonal antibody BC-12. Infections were found to be visible at a fungal concentration of over $200 \mu\text{g g}^{-1}$ stem tissue. Latent infections usually showed a *Botrytis* concentration of less than $35 \mu\text{g g}^{-1}$ stem tissue. An indirect competitive ELISA method using the monoclonal antibody BC-12 was developed by Fernandez-Baldo et al. (2011) by immobilising *B. cinerea* antigen to microtiter plates utilising glutaraldehyde as a cross-linking agent. The limit of detection was calculated at $0.97 \mu\text{g ml}^{-1}$. This assay was used to quantify *B. cinerea* infections in grapes, pears and apples after several days of incubation.

1.4.5 PCR

There are several limitations of immunological detection methods available for *Botrytis* detection. Polyclonal antibodies often cross-react with other fungi and the monoclonal antibody BC-12 is specific to all *Botrytis* species. This is on one hand beneficial since a wide range of applications is feasible, however, if the application requires to target only particular *Botrytis* species, genetic detection methods are required using polymerase chain reaction (PCR).

For many analytical procedures, a high quantity of DNA is necessary. In retrospect, PCR (Figure 1.14) revolutionised genetic detection since it allows amplifying a target DNA section exponentially in a simple and rapid way. Essential for the specificity of the PCR are the flanking regions of the DNA called primers. Furthermore, all four deoxynucleotide triphosphates (dNTPs) and a heat stable DNA polymerase is needed that can synthesise the complementary DNA-nucleotides to the target region. After heating the DNA in order to separate the two DNA strands, hybridisation occurs allowing the primers to bind to the ends of the target regions after cooling. The DNA polymerase extends the primer oligonucleotides by fitting the complementary dNTPs just beyond the end of the sequence. An automated thermocycler can repeat the heating and cooling steps until the desired number of copies is achieved.

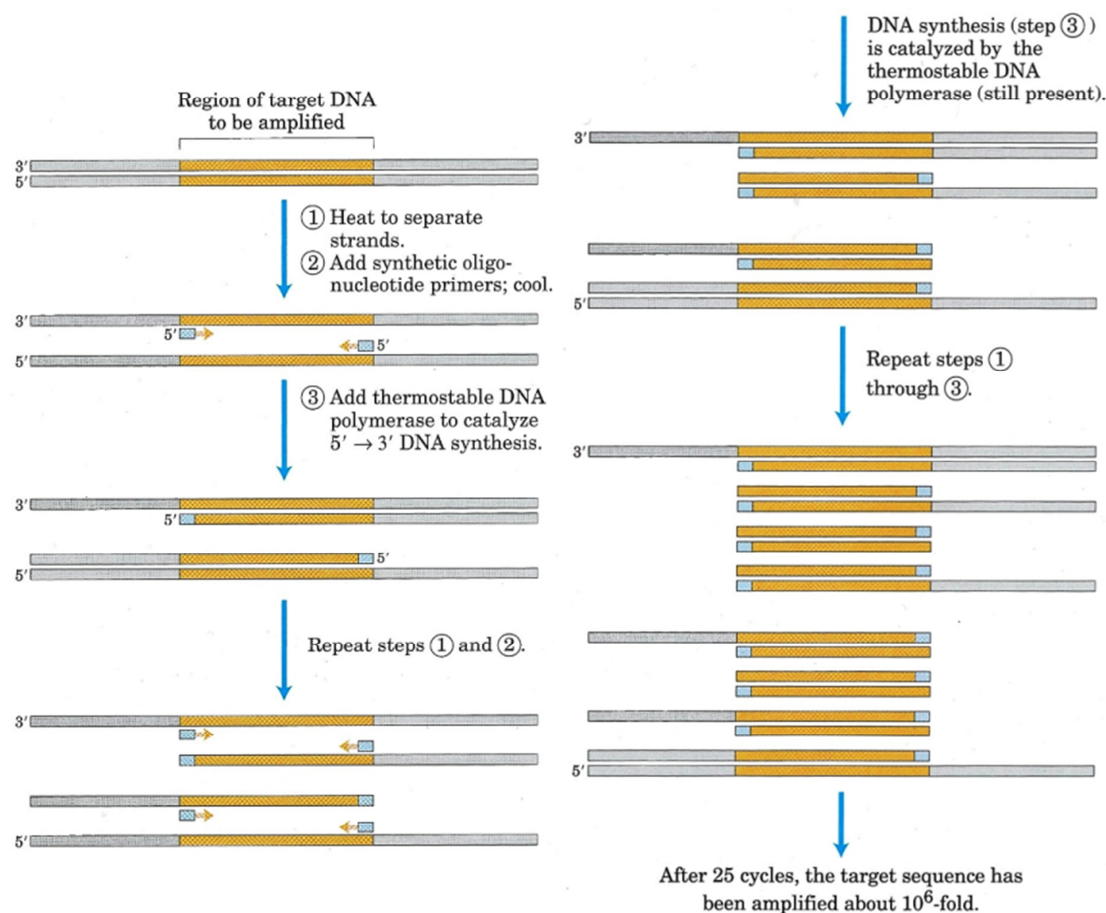


Figure 1.14: Principle of a polymerase chain reaction (PCR) to amplify a target DNA segment (Lehninger et al., 2005).

Nielsen et al. (1999) developed the first PCR assay specifically designed for the detection of *B. aclada* and *B. allii* with primers amplifying the ribosomal internal transcribed spacer region (ITS). In 2002, they described a PCR-RFLP system based on a sequence characterised amplified region (SCAR) from a random amplified polymorphic DNA (RAPD) fragment. These universal primers are capable of genetically differentiating between the five species of *Botrytis* (*B. aclada*, *B. allii*, *B. byssoidea*, *B. cinerea* and *B. squamosa*).

Using the *ApoI* restriction enzyme the fungal DNA is cut into fragments with a species-specific length:

B. aclada = 413 bp
B. byssoidea = 298 bp
B. squamosa = 269 bp

B. allii = 413 + 298 bp
B. cinerea = 250 bp

The following restriction fragments length polymorphism (RFLP) detection is based on the different lengths of the digested fragments that occurs due to species polymorphism in the ITS region of the pathogen. The fungal species can be determined through the specific bands visualised after gel electrophoresis (Figure 1.15).

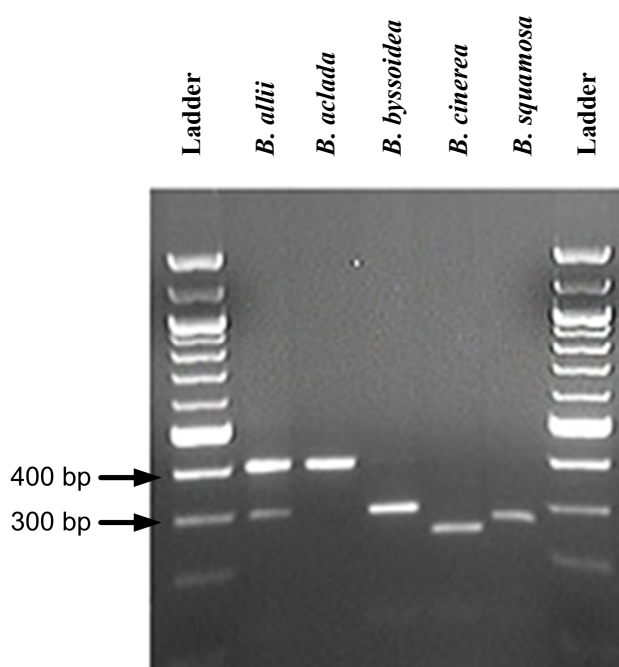


Figure 1.15: PCR-RFLP analysis using the protocol described by Nielsen et al. (2002) designed to distinguish between the five *Botrytis* spp. (*B. aclada*, *B. allii*, *B. byssoidea*, *B. cinerea* and *B. squamosa*) which were isolated from onion plants and seed, respectively (Chilvers and Du Toit, 2006).

The detection limit of the procedure developed by Nielsen et al. (2002) is between 1 and 10 pg of pure fungal DNA. Another advantage of this PCR-based method is the fast detection time. Field trials performed by Nielsen et al. (2002) showed that the detection of *B. aclada* is possible 48 h post inoculation with no visible signs of *Botrytis* infection present on the leaf pieces. However, the extraction efficiency of the PCR is not perfect. The probability to obtain a false negative result increases with the sample size (Dewey and Yohalem, 2004; Walcott et al., 2004). The non-linear coherence of sample size and DNA extraction caused by PCR-inhibitory compounds (phenolics and tannins) in seed and onion tissue is the main disadvantage of the PCR.

To overcome this drawback, Walcott et al. (2004) developed a magnetic capture hybridisation (MCH) PCR protocol for seed-borne *Botrytis* spp. The SCAR-DNA regions described by Nielsen et al. (2002) were coated to magnetic beads in order to capture the fungal DNA. Afterwards the PCR inhibiting substances contained in the crude seed extract can be removed by washing, resulting in a better amplification rate of the PCR using the primers described by Nielsen et al. (2002). A ten-fold increase of the MCH-PCR in sensitivity compared with the PCR-RFLP assay described by Nielsen et al. (2002) was reported. However, the agar assay yielded higher detection frequencies of *B. aclada* for seed lots with low infection levels (2.1 %) compared to the MCH-PCR assay (Chilvers and Du Toit, 2006), and the MCH-PCR assay lacked adequate reproducibility caused by insufficient detection frequency (40 %) of the PCR product (Chilvers et al., 2007).

Hence, the MCH-PCR developed by Walcott et al. (2004) has no ability to quantify the incidence of infection in individual seed lots. Therefore, Chilvers et al. (2007) developed a real-time fluorescence PCR using SYBR Green chemistry to detect and quantify the most common species associated with neck rot (*B. aclada*, *B. allii*, and *B. byssoidea*) in onion seed. To design the primer pair, the nuclear ribosomal intergenic spacer (IGS) regions of target and non-target *Botrytis* spp. were sequenced and aligned. After optimisation, the primer pair was specific to *B. aclada*, *B. allii*, and *B. byssoidea* and does not amplify the related species *B. cinerea*, *B. porri*, and *B. squamosa*, as well as onion DNA and isolates of 15 other fungal species commonly found associated with onion seed (Chilvers et al., 2007). The extraction of the DNA from the seed was performed by a DNA silica-based adsorption and extraction kit from Qiagen after entailed maceration of onion seed in liquid nitrogen using a mortar and pestle. The high copy number of the ribosomal DNA leads to an increase of the detection level (10 fg of DNA) compared to the conventional PCR, amplifying DNA (1-10 pg) with a low copy number as designed by Nielsen et al. (2002).

The seed assay performed by Chilvers et al. (2007) is presented in Table 1.3. A quantification of the fungus in seed is still a challenge due to the fluctuant overall efficiency (12 to 260 %) of both the DNA extraction and the real-time PCR amplification (Chilvers et al., 2007). In concrete terms, with higher number of conidia added per seed, the observed amount of fungal DNA decreases and does therefore not reach the expected amount of DNA. Consequently, a linear plotting of the PCR results to quantify the disease is possible for pure fungal DNA, but not capable of quantifying the fungus in a seed assay.

Table 1.3: Efficiency of the PCR assay described by Chilvers et al. (2007) determined by adding different numbers of conidia of *Botrytis allii* to samples of 25 seed from a *Botrytis*-“free” onion seed lot, followed by seed maceration, DNA extraction and real-time PCR assay.

Conidia added ^a /seed ^b	Mean observed neck rot, <i>Botrytis</i> DNA detected/seed ^c [fg]	Expected amount of neck rot <i>Botrytis</i> DNA/seed ^d [fg]	Efficiency ^e [%]
0	0	0	-
1	99	38	260
10	185	380	49
100	446	3,800	12
1,000	6,288	38,000	17
10,000	45,520	380,000	12

^a Number of *B. allii* conidia added.

^b Seed prior to grinding in liquid nitrogen and DNA extraction.

^c Observed quantity of *Botrytis* DNA/seed detected using a real-time PCR assay.

^d Estimated *B. allii* genome weight based on the assumption of one nucleus per conidium and an average ascomycete genome size of 36×10^6 bases, which is approximately 38 fg.

^e Estimated DNA extraction and real-time PCR efficiency, calculated per extract as:
[(observed quantity of DNA)/(expected quantity of DNA)]x100.

For direct detection and quantification in onion tissue, Coolong et al. (2008) developed a quantitative real-time PCR assay for *Botrytis aclada*, which is not affected in performance by the presence of onion tissue. The assay uses TaqMan probe-based chemistry to detect an amplicon from the L45-550 region of *B. aclada* while using a DNA sequence from the onion serine acetyl transferase gene (SAT1) as a control. The detection limits for *B. aclada* and onion were $10 \text{ pg } \mu\text{l}^{-1}$ of genomic DNA and $1 \text{ } \mu\text{g}$ of lyophilised *B. aclada* mycelium (Coolong et al., 2008).

1.5 IMMUNOLOGICAL SENSORS

Immunochemical assays or immunoassays are analytical tests based on the selective and sensitive antibody-antigen interaction (Tothill, 2003a). The term biosensor is general and applies to a variety of techniques. Immunosensors represent a major part of the area, since antibodies are the most commonly used bio receptors (Caygill et al., 2010). Clark and Lyons (1962) pioneered the amperometric biosensor development by immobilising glucose oxidase to a platinum electrode using a dialysis membrane. This analytical scheme was then transferred to the commercial glucose oxidase analyser that builds a multi-billion dollar market (Sungur et al., 2004; Palchetti et al., 2009). In general, sensors are devices that incorporate a recognition element with a signal transducer. The signal varies dynamically with the concentration of target analytes. If the binding takes place on the transducer surface, the measurement of the signal produced by the antibodies can occur through a selective transduction of the receptor-analyte reaction. The converted information results in a quantifiable electrical or optical signal that is further processed by the detector (Figure 1.16). In most cases, the receptor is in close vicinity or immobilised on the transducer surface.

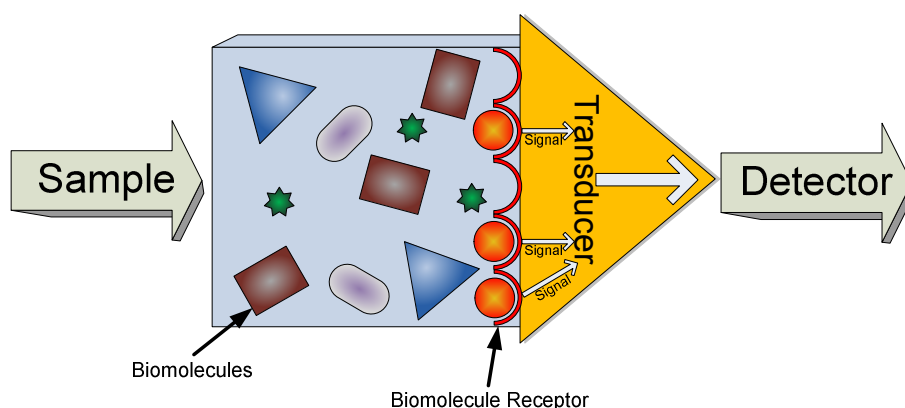


Figure 1.16: Principle of operation of biosensors.

Immunological biosensors are generally classified according to their transducer technology: electrochemical, optical and microgravimetric detection systems (Wang et al., 2008b). If the physical or chemical signal is directly detected by the transducer with no additional labels, the sensor is declared to be a direct immunosensor. In contrast, an indirect immunosensor detects the signal produced from bio-reactive labels.

1.5.1 OPTICAL IMMUNOSENSORS

As an alternative to the electrochemical approaches, optical methods can be conducted or utilised for a wide range of applications. Reasons for their success are a low sensitivity to electronic interference and the possibility of non-destructive operation. Like electrochemical immunosensors, the optical applications can be divided into two types of approaches, direct and indirect immunosensors, depending on the labelled signalling molecule.

Surface plasmon resonance (SPR) is the most common direct optical detection method. The working principle is described in Figure 1.17. A laser beam is focussed through a prism to a thin gold film. At a critical angle of incidence to the gold surface, the laser beam induces a resonance evanescent wave in the plasmons (electrons) of the gold film. The wave is not restricted by the gold film sending an evanescent field to the area beyond the surface with an exponential decay. Therefore, the environment beneath the gold surface affects the refractive index of the region. Binding the target analyte to a previously immobilised recognition element, or adsorption to the metal surface, results in a change of the resonance angle which can be detected in real time.

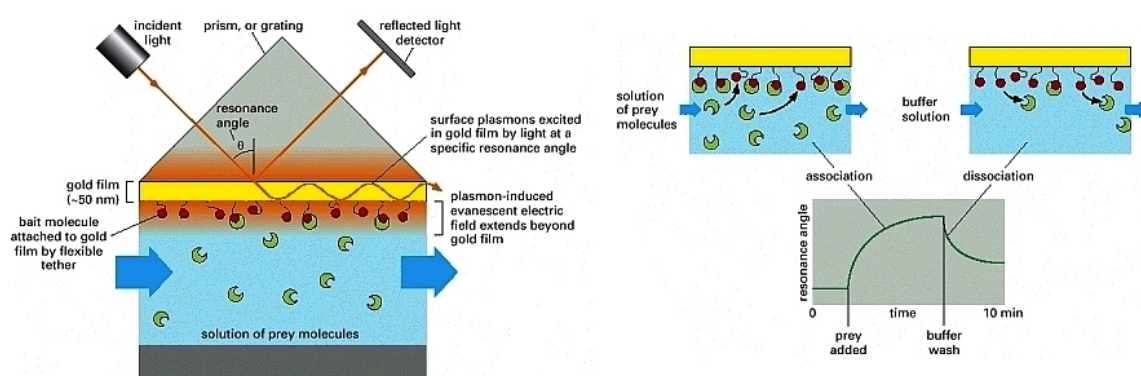


Figure 1.17: Working principle of surface plasmon resonance (Alberts et al., 2002).

There are several emerging application areas for SPR systems including, food analyses, drug detection, development of immunosensors and the study of protein interactions (Karlsson, 2004). The major advantage of the SPR is the lack of required labels. Furthermore, the immunological reaction can be traced in real time, which is very

important for the study of dynamic processes. Challenges are the reduction of non-specific adsorption and improvement of the sensitivity to analytes of low molecular weight. A recent example for the use of SPR technology is the detection of *Phakopsora pachyrhizi* in leaf extract developed by Mendes et al. (2009). This fungus causes Asian rust in soybeans. The assay procedure included cysteamine-coupling chemistry of the antibodies to self-assembled monolayer (SAM). The detection limit was $0.8 \mu\text{g}$ fungal mycelium ml^{-1} with a linear detection range ranging from 3.5 to $28 \mu\text{g ml}^{-1}$.

Fluorescence immunosensors are also capable of detecting molecular binding interactions (Davis et al., 2007). Fluorescence labelled antibodies or antigens (indicators) are immobilised on the transducer surface being in most cases an optical fibre or chip (Figure 1.18). The indicator has strong adsorption or fluorescence, if an interaction with the analyte occurs. Through lighting the optical sensor under total internal reflection with a wavelength specifically matched to the maximum adsorption of the indicator molecules, the evanescent wave at the surface will produce a detectable fluorescence signal. Photodiodes are often used to convert the optical information into an electrical signal which can be further processed.

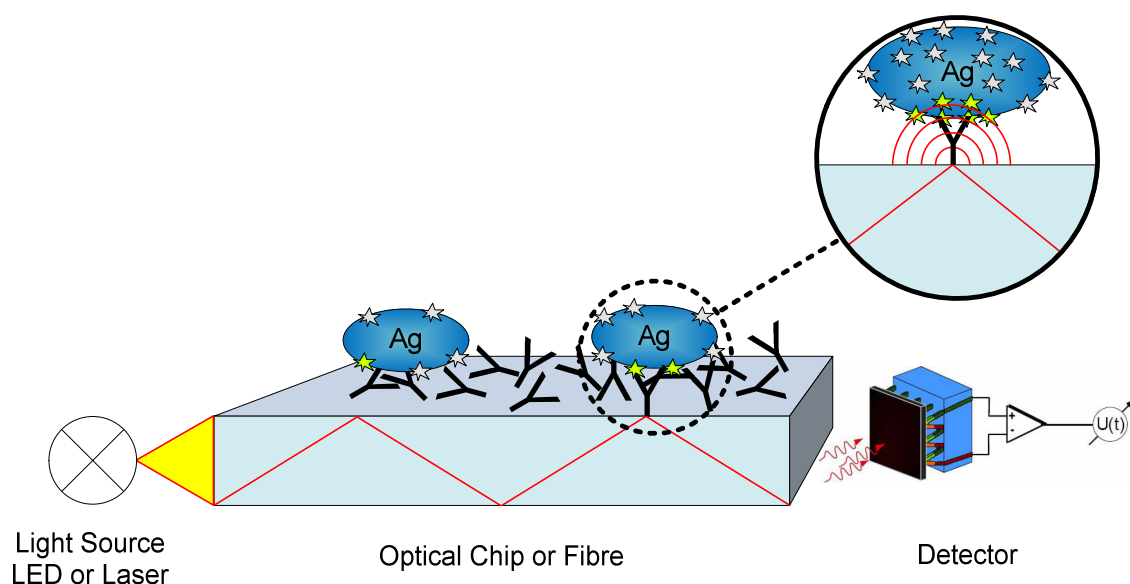


Figure 1.18: Working principle of an optical immunosensor using fluorescently labelled antigens. A light source emits beams that are transmitted by an optical fibre until the detection unit is reached and the evanescent wave at the surface is generating a fluorescent signal of the indicator molecule.

1.5.2 MICROGRAVIMETRIC IMMUNOSENSORS

This sensor type can combine the high sensitivity of piezoelectric detection and the specificity of immunological reactions. Furthermore, the device is low in cost, simple to handle and detects the immunological interaction in real time. The general working principle of these sensors is the detectable change in the oscillation frequency of the microgravimetric transducer, mainly known as quartz crystal microbalance (QCM) (Figure 1.19). Frequency variations can be caused by mass loading or changing interfacial properties (i.e. surface roughness, viscoelasticity).

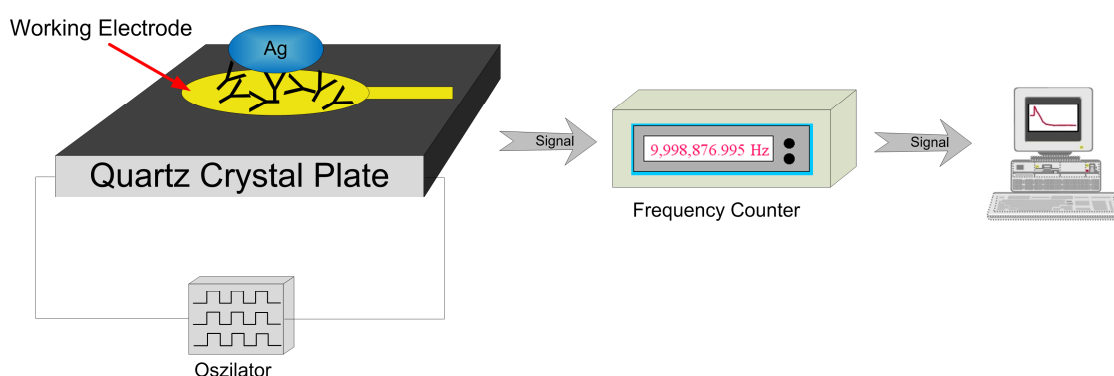


Figure 1.19: Working principle of a quartz crystal microbalance (QCM).

This technique was successfully used for the detection of bacteria (Gram positive/negative, shapes and cell arrangements) (Das et al., 2003; Salam et al., 2013), exploring the differential leucocytes antigens for the immunophenotyping of leukemia (Wang et al., 2004) and prostate specific antigen based diagnosis of cancer (Uludag and Tothill, 2010b). To improve the intrinsic sensitivity, QCM was combined with immunomagnetic separation by magnetic particles which capture the target cells in this case *Salmonella typhimurium* (Kim et al., 2003), or gold nanoparticles for signal amplification (Uludag and Tothill, 2010a; Salam et al., 2013).

QCM technique was used by Miecinikas et al. (2007) for a study of microbiological activity of *Aspergillus Niger* during long-term exposure to atmospheric pressure and aluminium colonisation. The fungus was immobilised on a 15 μm thick aluminium foil that was glued with epoxy resin to the quartz surface and the mass increase generated by

various phenomena, such as development of biomass, secretion of metabolites, water uptake by the colony and microbially induced corrosion, was monitored by recording the frequency shift of the sample (Miecinskas et al., 2007).

1.5.3 SCREEN-PRINTED ELECTROCHEMICAL SENSORS

The current market trend in diagnostic is tending to low cost, portable and rapid off-site detection methods, alleviating the need to send a sample to a specialist laboratory (Tothill, 2003b). To achieve these goals, screen-printed electrodes (SPE) are the most suitable biosensors, as they are portable, easy to use and low in production costs (Davies et al., 2007a). Furthermore, using a disposable sensor overcomes the “fouling” of the electrode, which leads to a loss in sensitivity and reproducibility (Alvarez-Icaza and Bilitewski, 1993). If required, reuse of the device is possible if the system is designed for such applications, and biosensors were reported capable of performing up to 1500 measurements over a period of seven days (Tothill et al., 1997). Among the various technologies available to produce miniaturised low-cost devices, the thick-film technology, in particular screen-printing is the dominant method to mass-produce electrochemical transducers (Pan et al., 1999; Palchetti et al., 2009). Alternatively, thin-film printing techniques are applied for the production of biosensors such as ink-jet printing or photolithography. These techniques can produce thinner biosensors (125 μm photolithography; 500 μm thick-film), or yield better resolution (1 to 5 μm , ink jet; 0.1 to 0.5 mm thick-film), but are on the other hand more complex and expensive in production (Davies et al., 2007a).

Screen-printing is an established and widely used method that utilises stencil forms or marks to print ink layers on a plastic or ceramic substrate. Generally, SPE are made from templates which allow the simultaneous production of three tracks per sensor, becoming the working, reference and counter electrodes. However, also two-electrode sensors can be used, such as the glucose sensor. Recently screen-printed electrochemical sensors were successfully employed to detect lung cancer biomarkers (Altintas and Tothill, 2013), lactate (Hirst et al., 2013) and cardiac markers (Fakanya and Tothill, 2012).

1.5.4 IMMOBILISATION STRATEGIES OF ANTIBODIES

Since immunosensors usually measure the signals resulting from the specific immunoreactions between the analytes and the immobilised antibodies or antigens, it is clear that the immobilisation procedure of the biomolecule on the surface of base transducers should play an important role in the construction of immunosensors (Wang et al., 2008b). The strength of the interaction must be sufficiently stable to perform at least a single assay. Especially the washing steps within the assay procedure increase the risk of mobilising the antibodies and consequently dismantle the assay. Another important issue is the functionality of the antibodies. The orientation is crucial for its binding ability and directed immobilisation that prevents the attachment of the binding region to the surface is advantageous. In general immobilisation strategies can be separated into two basic areas: covalent and non-covalent interaction, with multipoint electrostatic attachment of the sensing biomolecule to either a polyanionic or polycationic layer representing the most common approach (Millner et al., 2009).

1.5.4.1 Adsorption

In commercial immunoassays, the antibodies are usually immobilised simply by adsorption, this being a non-covalent immobilisation method (Wortberg et al., 1997). Many interactions between the protein and the surface can occur (Figure 1.20) leading to an irreversible attachment. Once the summed attractive forces exceed more than approximately $10kT$, the protein is essentially irreversibly absorbed (Davies et al., 2007b). For immunoassay applications, especially the strong hydrophobic interaction to the surface is relevant. The adsorption of the antibody or protein to materials like gold, plastic and latex is so powerful that a low protein concentration, typically 10 to $100 \mu\text{g ml}^{-1}$, is needed to coat the surface (Ekins et al., 1990). When coating nitrocelluloses, the antigen binding capacity increases with the surface coverage, however, this effect inverts when the pores are blocked by an overload of antibodies or proteins.

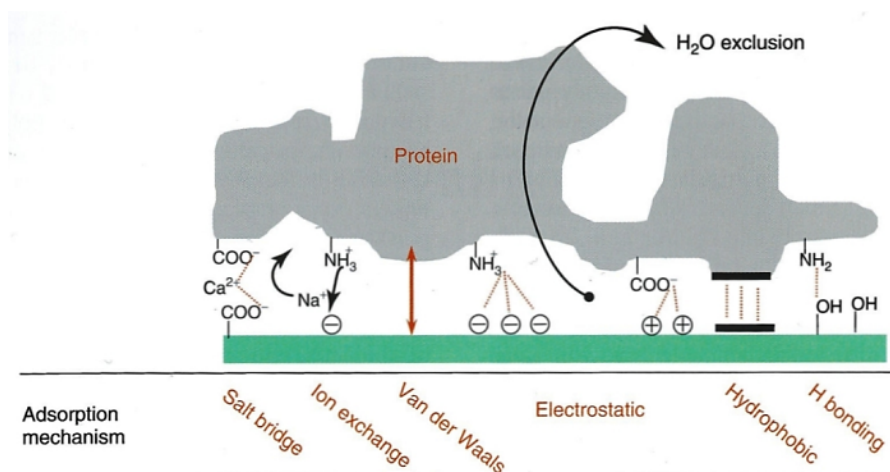


Figure 1.20: Overview of the protein adsorption mechanisms (Davies et al., 2007b).

The main advantage of adsorption used for immobilisation is the simple process and the wide field of potential applications. On the other hand, functionality of the antibodies is not warranted and the antibodies can be removed more easily during the washing steps needed in immunoassays leading to a higher variation in signal. Through the random orientation on the surface, denaturation of the protein or attachment of their binding site to the surface can occur.

1.5.4.2 Covalent Immobilisation and Polymer Layer

In general, covalent coupling (Figure 1.21 B) is preferred in experimental approaches over adsorption (Figure 1.21 A) due to enhanced stability of the antibodies (Wortberg et al., 1997). While it is easy to give reasons for employing this more complex immobilisation strategy, many of these are unreasonable given the paucity of publications that give experimental support to them, specifically to increase the loading of antibodies to the surface; to prevent desorption of the antibodies by other materials (proteins) in the sample; to orientate the antibodies and thereby increase the binding capacity of the surface (Davies et al., 2007b). Many scientists developed and published complex procedures of covalent binding, but they lack to demonstrate what fraction of the antibody exactly was linked to the desired surface and what benefit they offer compared with the control (adsorbed) group (Marks et al., 2007). However, this coupling procedure may be an advantage to reduce non-specific binding and increase the stability of the antibody (Furuya et al., 2006).

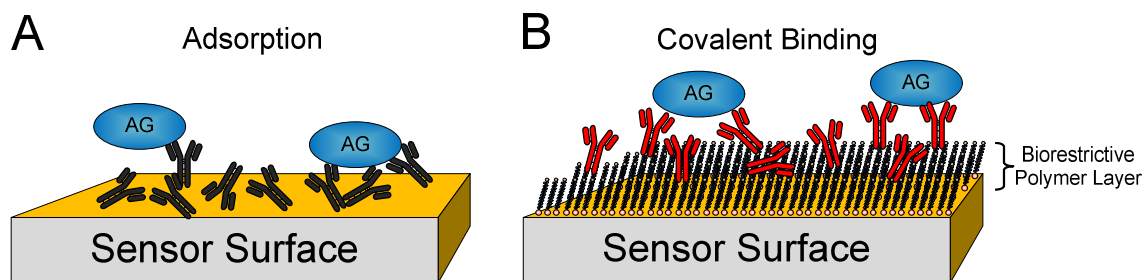


Figure 1.21: Comparison of the immobilisation strategies adsorption (A) and covalent binding (B).

Covalent immobilisation includes the coupling of a reactive group of the protein, for example the carbohydrate moiety located on the bottom Fc-region of the antibody, to a specific substrate. To construct such a surface, the label is first coated with a layer of hydrogel, typically of a hydrophilic polymer such as dextran, polyethylene glycol, or ethoxylated celluloses either by adsorption or via chemical linkers (thiols and salines), which will bind to gold or silica based surfaces (Davies et al., 2007b). This layer of bio-resistive polymer covers the surface with the aim at preventing the denaturation of the antibodies. Furthermore, the 3D polymer structure increases the surface area resulting in a better access of the antigen to the antibody binding site. Another outcome of this layer is the flexible, high hydrophilic surface that reduces non-specific binding of other proteins (Ekins et al., 1990). However, the thickness of the bio-resistive polymer layer can also decrease detection sensitivity if the layer exceeds a specific thickness.

More recently, self-assembled monolayers (SAM) are gaining increased attention due to their ability to chemisorb spontaneously building regularly orientated surface structures (Park et al., 2004; Millner et al., 2009). For the application of SAMs on gold surfaces, hydrocarbon compound with thiol groups are used resulting in an immediate sulphur-gold bond, and hydrophobic alkane chains construct a stable hydrophobic layer (Prime and Whitesides, 1993). All SAMs have in common the presence of reactive groups, such as carboxyl (R-HCO_2), thiol (R-SH), or amin (R-NH_2) in various forms, which can be coupled to other molecules. Common SAMs that are employed for gold coupling in QCM, SPR and biosensor applications are 3-mercaptopropionic acid

(MPA) (Park et al., 2004), 3-dithiodipropionic acid (DTDPA) (Salam and Tothill, 2009a), 11-mercaptoundecanoic acid (MUDA) (Uludag and Tothill, 2010b; Altintas et al., 2012) and 16-mercaptohexadecanoic acid (16- MUDA) (Salam et al., 2013).

Challenging for the covalent immobilisation is to prevent a subsequent adsorption of the antibodies and to maintain biological activity. Though a reactive group of the antibody is on one side covalently coupled to the surface, the antibody does not lose the ability to adsorb (Davies et al., 2007b). Studies performed by Williams and Blanch (1994) with radioactive tagged antibodies demonstrated that 75 % of the covalently immobilised antibodies on a silicon surface were simply adsorbed. However, Williams and Blanch (1994) also monitored a better bond between the antibody and the surface resulting in a decreased antibody loss after the washing steps. An elegant way to overcome these obstacles is the use of half-antibody fragments as described by Billah et al. (2010): By subjecting the disulphide bridge that represents a hinge between the two heavy chains of the antibody to a controlled reduction process, the free SH can be used to provide an oriented immobilisation on the sensor surface with the antibody recognition site freely available.

1.6 ELECTROCHEMICAL TECHNIQUES FOR IMMUNOSENSORS

Most of the developed immunosensors can be classified to the group of electrochemical devices. They present the advantages of high sensitivity of an electrochemical transducer and the selectivity inherent to the use of immunochemical interactions (Tothill, 2003b). Low manufacturing costs and their portable design are further key roles for their success. Their basic operating principle is the electrochemical detection of electroactive compounds by means of the transducer. These compounds can be labelled with immunoagents or markers, such as enzymes or metal ions. Electrochemical devices usually monitor the current at a fixed voltage (amperometry), the voltage at zero current (potentiometry), or measure conductivity or impedance changes (Tothill, 2001).

1.6.1 TRANSDUCER TECHNOLOGIES

Potentiometric transducers are an established technique with many commercial products. The operation point for the transducer is at near zero current flow between the interfaces of the two electrodes (Poghossian and Schöning, 2007). At this point, the ion activity is logarithmically proportional to the change in electrode or membrane potential. More recently, the techniques known from the semiconductor industry are used for the development of ion-selective field-effect transistor (ISFET). To convert a semiconductor in a biosensor, the metal gate is substituted through an ion-sensing membrane that can detect the potential change as a result of the immunochemical reaction of the antibodies immobilised on the gate and the antigens.

Capacitance transducers are common solutions for immunosensing assays. The basic working principle is the detection of the changing dielectric behaviour of the conducting polymer modified layer coated on the electrode surface, depending on its thickness and dielectric characteristics. Sensitivity and detection limit is increasing with a decreasing thickness of the layer. Watson-Craik et al. (1989) developed a conductance and capacitance measuring technique for the detection of food-borne fungi in media which comprised soya peptone and yeast extract.

The working principle of conductometric and impedance transducers is the detectable disturbance of the molecules orientation in equilibrium. Impedance is the total electrical resistance to the flow of an alternating current being passed through a given medium (Tothill, 2001). Cations and anions of an electrolyte within a homogeneous phase interact until the total force affecting any particle is equal to zero. Depending on the distance of the molecules to the monocharged layers near the electrode, which can be seen as two charged plates of a capacitor, a polarisation occurs. If an antigen binds to an antibody, the impedance change of the system can be measured.

1.6.2 AMPEROMETRY

Due to low detection levels and an inexpensive design, amperometric biosensors are the most popular immunosensing format. The working principle is based on removal

(oxidation) and addition (reduction) of electrons from a molecule or atom. Though theoretically every molecule or atom can be oxidised or reduced if sufficient energy is applied to the system, in practice only electroactive substances which can be oxidised or reduced in easily available energy ranges are used in amperometry (Arya et al., 2007). The flow of charges can be measured at the conducting electrode which has a constant applied potential. A variety of electrode materials have been exploited, including noble metals, graphite, semiconductors, electronically conductive polymers, paper, and conducting salt (Lowe, 2007). The sensor generally includes a complex measuring system with three different electrodes (Figure 1.22), although most of the commercially used devices, like the glucose sensor, are two-electrode systems.

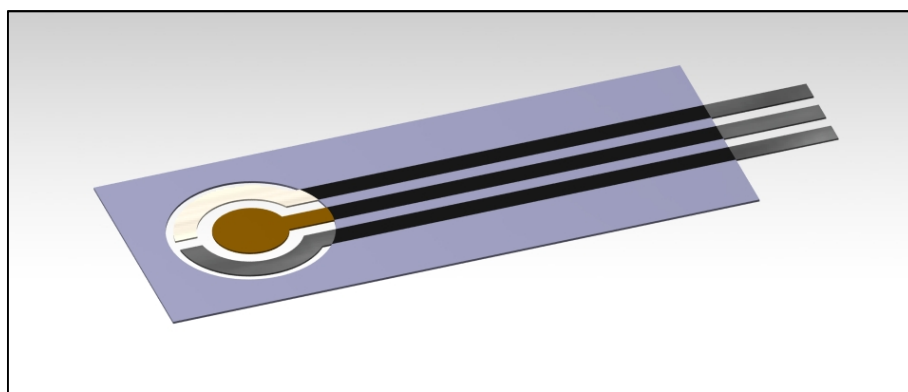


Figure 1.22: Example of a typical amperometric immunosensor (Cranfield design).

Generally, the biological sensing element is immobilised on the “working electrode”. As soon as a potential is applied, the electroactive substance will be oxidised or reduced and relinquishes electrons to the surface. Without a closed circuit provided by the second (counter or conductive) electrode, this would eventually lead to an increasing potential difference. The flow of electrons generated through the oxidation at the working electrode and the reduction process at the counter electrode is the resulting current of the biosensor. This signal can be measured and correlated to the presence of the target analyte. The reference electrode is the third electrode in the system. Silver/silver chloride (Ag/AgCl) is the most common manufacturing material, but some applications also use a saturated calomel (Hg/HgCl_2) electrode (White and Turner, 1997). Its known potential against the standard hydrogen electrode provides a stable reference against the working electrode.

As most of the proteins are not electrochemically active, there are hardly any direct applications for enzyme amperometric sensing. The electrical bridge between the redox centre of enzymes that resists direct electrical communication and the electrode can be established by using synthetic or biologically active charge carriers, the mediators (Arya et al., 2007). However, certain enzymes or redox proteins such as HRP and alkaline phosphatase can exhibit electrical communication by overcoming the steric insulation of the active site by the protein matrix. These systems can catalyse the substrate in such manner that electrons are either produced or utilised in a redox reaction. Typically, the reaction products are H_2O_2 or O_2 that are reduced or oxidised at the electrode generating electrical current (Arya et al., 2007). To increase the sensitivity of the sensor, signal amplification can be performed. By immobilising the antibody and the redox active biomolecule to gold sol (Salam and Tothill, 2009a) or liposomes with a biotin bridge (Alfonta et al., 2001) the signal can be improved by capturing more biomolecules to the antigen. Recent renewable amperometric immunosensors were developed by using a paraffin graphite-antigen composite paste electrode which can be regenerated by polishing the surface (Liu et al., 2001).

1.6.3 CYCLIC VOLTAMMETRY

In 1948, Randles and Sevcik independently described the technique of cyclic voltammetry (CV) (Oldham et al., 2002). This technique is one of the basic methods used in electrochemistry, since it is easy to perform and provides quick and useful information about the system, like redox potential, electrochemical rate constant, active surface area of the sensor and stability. The working principle of cyclic voltammetry is based on sweeping the potential at a set scan rate v between two potentials (E_1 and E_2) as illustrated in Figure 1.23. If required, an initial potential E_0 with a corresponding initial potential time can be applied to the system, which is different from the actual start potential E_1 . The scan rate defines the gradient of the linear function and consequently the time needed to switch between the two potentials. Depending on the number of measurements required during the scan period, the step potential [V] can be adjusted accordingly.

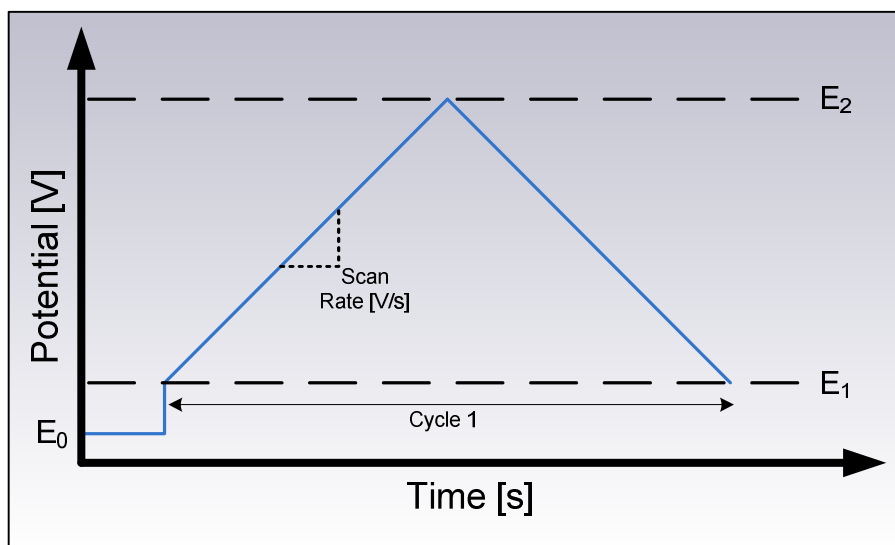


Figure 1.23: Variation of the applied potential between E_1 and E_2 as a function of time. E_0 represents an initial potential that is applied before the cycle starts.

As the potential alters, the current output voltammogram determines the redox potentials at which different electrochemical processes take place. The forward scan produces a current peak that increases as the potential reaches the oxidation potential of the analyte, but then falls as the bulk concentration is depleted close to the sensor surface (Arya et al., 2007). The resulting cyclic voltammogram illustrated in Figure 1.24 is characterised by several parameters. The anodic and cathodic peak current and their corresponding peak potentials provide the basis for the diagnostics developed for analysing the cyclic voltammetric response. As a potential is approached to the redox active solution, it results in an exponential increase of the anodic current until the anodic peak is reached. At that point potential is sufficiently positive to convert the reduced form of the bulk solution to the oxidised form resulting in a decay of the current. During the backward scan, the reaction is reversed and will produce a reduction peak in similar shape but with inverted polarity.

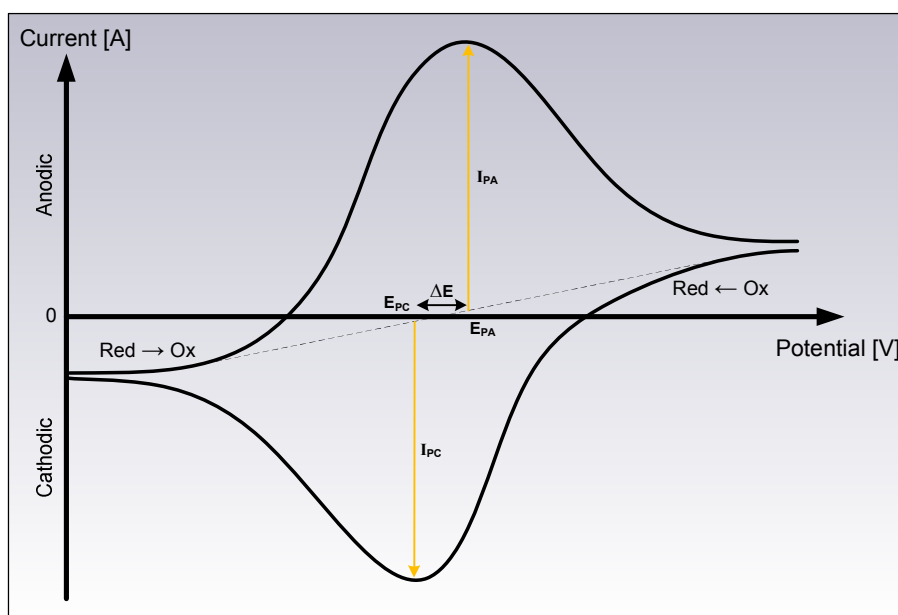


Figure 1.24: Schematic cyclic voltammogram for a reversible redox couple. Ox = Oxidised Form, Red = Reduced Form, $I_{P\ C/A}$ = Cathodic / Anodic Peak Current, $E_{P\ C/A}$ = Corresponding Cathodic / Anodic Peak Potential, $\Delta E = E_{PA} - E_{PC}$

For slow scan rates, the diffusion of the electrons is in equilibrium. In this case, the potential difference between reduction and oxidation peak is ideally $56/n$ to $59/n$ mV, with n the number of electrons involved in the redox reaction and the ratio between the cathodic and anodic peak being 1 (Mueller and Adams, 1961; Arya et al., 2007). In practice the difference is typically higher than 100 mV (Arya et al., 2007), where an increase of ΔE indicates a non-reversible reaction. The peak current of a reversible system can be used for characterisation of the sensor incorporating the Randles-Sevcik equation (White and Turner, 1997; Arya et al., 2007):

$$I_p = 2.69 \cdot 10^5 A C \sqrt{n^3 D \nu} \quad (\text{Equation 1.1})$$

I_p = Peak current

A = Area of the electrode

C = Concentration of the species

n = Number of electrons transferred

D = Diffusion coefficient

ν = Scan rate

According to the Randles-Sevcik equation, the peak current I_p is direct proportional to the active area A of the electrode, the bulk concentration C and the square root of the scan rate v .

1.6.4 CHRONOAMPEROMETRY

Chronoamperometry is a measurement method where the current is monitored as a function of time. Generally, this technique is divided into two steps. The initial potential E_1 is set to a value at which no faradic reaction occurs (Figure 1.25 A). After a defined time the potential is set to a potential E_2 at which the surface concentration of the electroactive substance is effectively zero. If the solution is unstirred, the mass transport will be provided only by diffusion of the reacting species. For a stationary working electrode the chronoamperogram (Figure 1.25 B) reflects the change in the concentration gradient of the diffusion layer above the surface. If it is assumed that linear diffusion to a planar electrode is occurring, the resulting current profile can be described by adapting Fick's second law of one-dimensional diffusion, which is known as the Cottrell equation (Cottrell, 1903; Compton and Banks, 2007):

$$I(t) = n F A C_0 \sqrt{\frac{D}{\pi t}} \quad (\text{Equation 1.2})$$

$I(t)$ = Current over time

n = Number of electrons transferred

F = Faraday constant

A = Area of the electrode

C_0 = Initial concentration of the species

D = Diffusion coefficient

t = Time

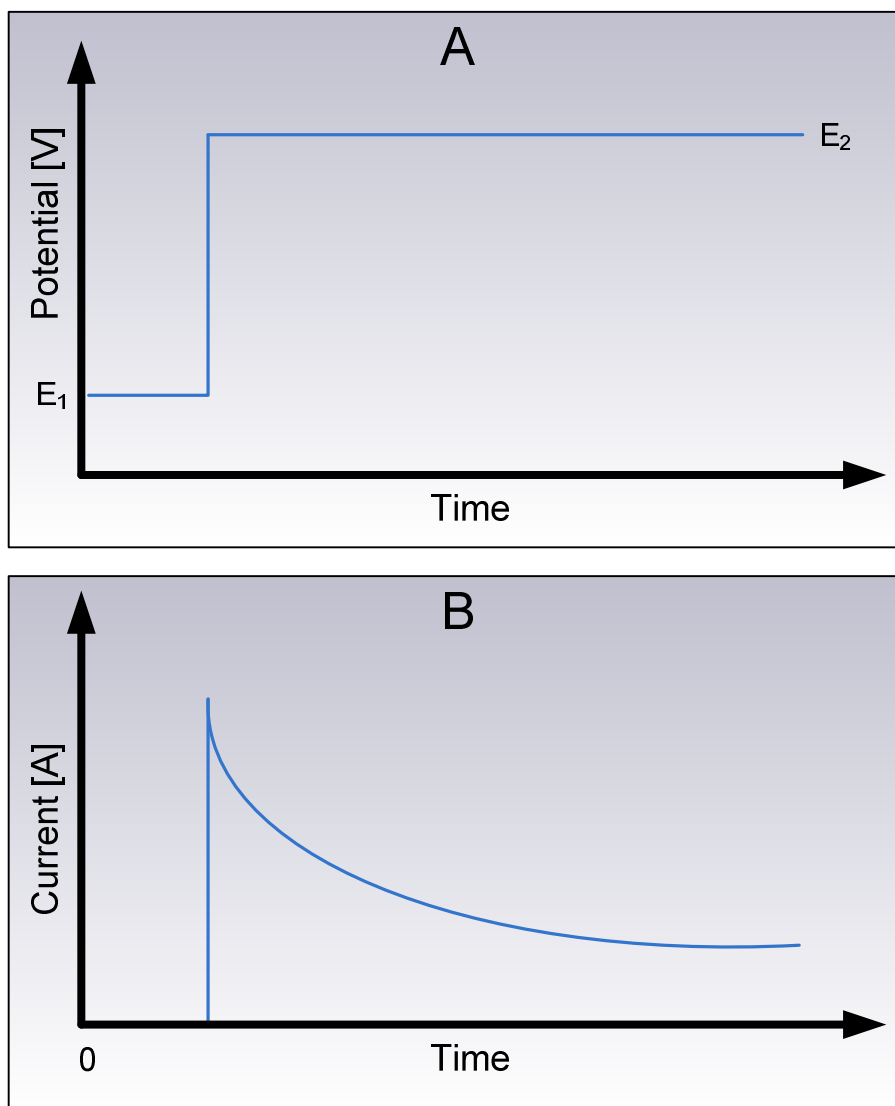


Figure 1.25: (A) Chronoamperometric principle of stepping from the initial potential E_1 to the second potential E_2 . (B) Illustrates the corresponding response of the current as a function of time.

A typical chronoamperogram is shown in Figure 1.25 (B). According to the Cottrell equation, the current decays with time proportional to $t^{-1/2}$. Depending on the enzyme activity, the reaction will reach eventually a steady state. This current is proportional to the amount of analyte performing for example immunoassays, or direct proportional to the converted substrate. Under constant conditions, the measured steady state current can be used for quantification of the analyte.

1.7 OVERVIEW OF THE DETECTION METHODS

Over the last years, a large number of detection methods for *Botrytis* have been developed. All have advantages and disadvantages (Table 1.4). Consequently, there is no standard method employed for the detection of *Botrytis*, as all the methods are relevant.

Table 1.4: List of advantages and disadvantages of relevant detection methods for *Botrytis*.

Detection Method	Advantages	Disadvantages
Agar assay	• Easy to use • No specific devices required • Technically low demanding • Separation between dead and viable fungus	• No quantification • Separation of <i>B. spp.</i> difficult • Time-consuming process (10 to 14 days)
ELISA	• Faster than agar assays (16 h) • Semi-quantification method • Commercially available	• Cross-reactions to other <i>B. spp.</i> • High detection level
PCR	• Early detection (48 h post inoculation) • Specific for <i>B. spp.</i> / separation possible • High sample screening capacity (up to 100 reactions per assay) • Assay time 1 to 3 h • Low detection level (10 fg DNA)	• Quantification not technically matured • No commercial standard available • Technically highly demanding • No field use
LFD	• Easy to use • Cheap products • Field use possible • Assay time ca. 15 min	• Cross-reactions to other <i>B. spp.</i> • High detection level • Quantification difficult
Amperometric immunosensor	• Easy to use • Low detection limit • Sensitive • Field use possible • Assay time 15 min to 2 h	• No commercial standard for <i>B. spp.</i> available • Technically highly demanding

Agar assay (Figure 1.26 A), being the first technique, is easy to perform, but offers no quantification of the disease. Furthermore, the process is very time consuming (two weeks) and the morphological classification of the *Botrytis* species is very difficult to perform and requires a trained operator.

ELISA (Figure 1.26 B) offers a fast and commercially established detection. Semi-quantification is also achieved by comparing the test results to standards. Compared to PCR and amperometric sensors, ELISA assays lack the sensitivity. Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK) offers a qualitative or semi-quantitative commercial ELISA IDENTIKIT for *B. allii* and *B. cinerea*.

LFDs (Figure 1.26 C) are disposable products that are easy to use, not restricted to the laboratory, and cheap in production. Forsite Diagnostics (Sand Hutton, UK) and EnviroLogix (Portland, USA) offer an on-site diagnostic kit for *Botrytis* using the monoclonal antibody BC-12. These LFDs can currently be used for discriminating between *Botrytis* infections and bacterial rot in onions.

PCR (Figure 1.26 D) is an established technique in genetic applications. This method is the only one which is capable of separating all five *Botrytis* spp. that cause neck rot. Its low detection level is also the best achieved in this comparison, but also too sensitive by producing false positive results with dead or non-viable fungus. Furthermore, this technique is technically highly demanding and restricted to laboratory use.

Amperometric immunosensors (Figure 1.26 E) are sensitive, cheap in production and offer a low detection level. The disposable design combined with a portable reader is highly suitable for field use but more sensitive than LFDs. The development of a commercial standard is technically high demanding and time consuming.

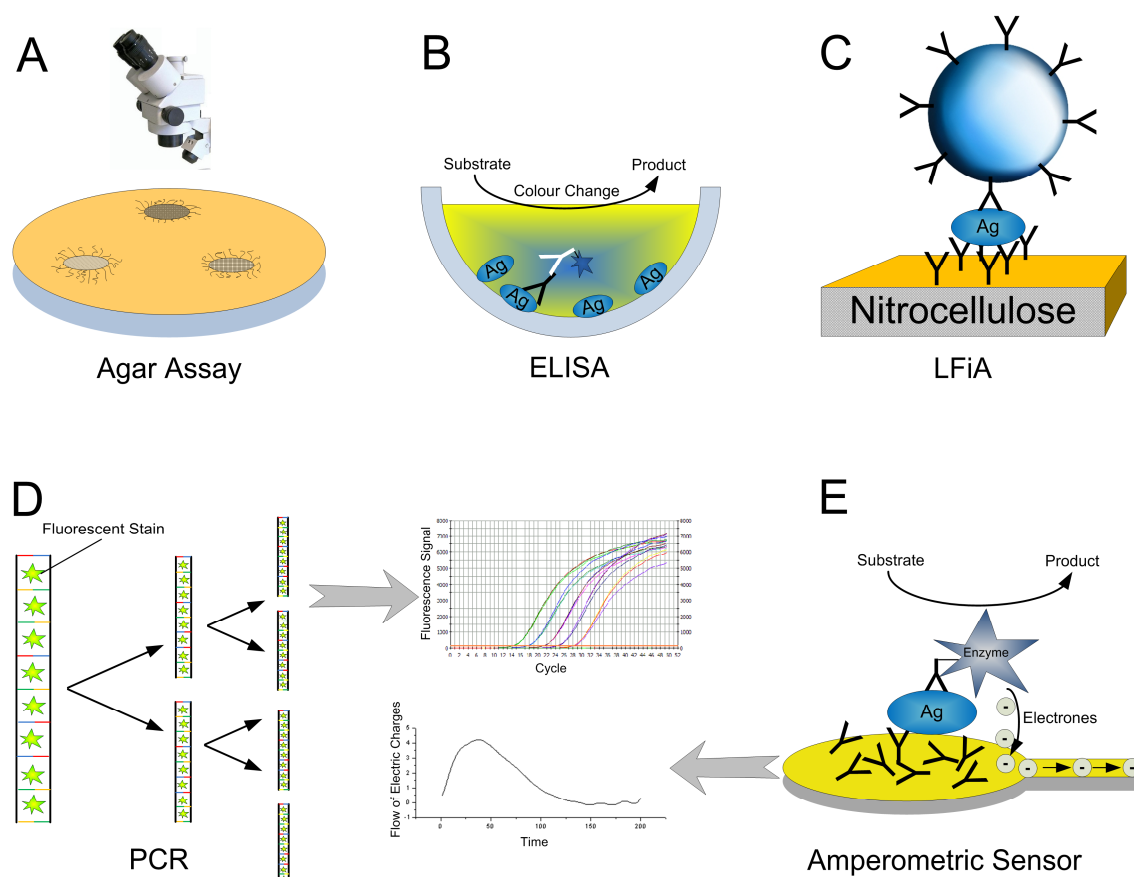


Figure 1.26: Overview of the detection methods for neck rot: Agar assay (A), ELISA (B), LFIA (C), PCR (D) and amperometric sensor (E).

An overview of the current detection methods of the relevant *Botrytis* species used in this study can be found in Table 1.5. The list includes research methods found in the literature as well as commercially available detection devices and kits. Beside conventional agar assays, only the *Botrytis* LFD from Forsite Diagnostics and EnviroLogix, as well as the ADGEN *B. cinerea* and *B. allii* ELISA kits are purchasable.

Table 1.5: Overview of the current detection methods for relevant *Botrytis* species. The list comprises commercially available methods as well as research methods found in the literature.

Target Species	Detection Technique	Description	LOD	Reference
<i>B. aclada</i> , <i>B. allii</i> , <i>B. byssoidea</i> , <i>B. cinerea</i> and <i>B. squamosa</i>	PCR	PCR detection and RFLP differentiation of <i>Botrytis</i> spp.	1-10 pg (DNA)	(Nielsen et al., 2002)
<i>B. aclada</i> , <i>B. allii</i> and <i>B. byssoidea</i>	Real-time PCR	Real-time quantitative PCR assay for estimation of the pathogen load in onion seeds.	10 fg (DNA)	(Chilvers et al., 2007)
<i>B. aclada</i>	Real-time PCR	Real-time quantitative PCR assay for estimation of the pathogen load in onion bulb tissue.	1 µg ml ⁻¹ 10 fg µl ⁻¹ (DNA)	(Coolong et al., 2008)
<i>B. cinerea</i>	Immunosensor	Quantitative competitive immunosensor (BC-12 Ab) for the detection of <i>B. cinerea</i> in fruits (tested with pears, apples, grapes).	8 ng ml ⁻¹	(Fernandez-Baldo et al., 2010)
<i>B. aclada</i>	ELISA	Indirect competitive ELISA (polyclonal Ab) for the detection of <i>B. aclada</i> in onion bulbs.	Not specified	(Linfield et al., 1995)
<i>B. cinerea</i>	ELISA	Quantitative indirect ELISA (BC-12 Ab) for the detection of <i>B. cinerea</i> in fruits (tested with pears).	1-10 µg ml ⁻¹	(Meyer et al., 2000)
<i>B. cinerea</i>	ELISA	Quantitative indirect competitive ELISA (BC-12 Ab) for the detection of <i>B. cinerea</i> in fruits (tested with pears, apples, grapes).	0.97 1 µg ml ⁻¹	(Fernandez-Baldo et al., 2011)
<i>B. cinerea</i> , <i>B. allii</i>	ELISA	Commercially available <i>B. cinerea</i> (BC-12 Ab) and <i>B. allii</i> (polyclonal Ab) ELISA kits from ADGEN Phytodiagnostics (Auchincruive, UK).	1-50 µg ml ^{-1 a}	/
<i>B. cinerea</i>	LFD	Commercially available <i>B. cinerea</i> (BC-12 Ab) LFD Forsite Diagnostics (Sand Hutton, UK) and EnviroLogix (Portland, USA).	7.5 µg ml ^{-1 a}	/

^a = Not specified by the manufacturer. LOD based on tests performed in this study.

1.8 AIMS AND OBJECTIVES

Botrytis infections lead to significant losses in the field and during storage. Commercial detection methods for *Botrytis* are available, however, due to their low specificity, sensitivity and laboratory-based design, the search for better tests continues. The demand for detection devices that are inexpensive, sensitive and can be accessed by farmers and growers, alleviating the need for laboratory-based instruments, is constantly increasing. The advances in biosensor technology started to provide simple, rapid and sensitive analyte testing systems that can revolutionise the *Botrytis* detection eliminating many flaws of current detection systems.

Furthermore, a rapid, sensitive and reliable method to assess pathogen load, pre or post-harvest can give the growers the potential to employ a customised curing regime of the onion bulbs, based on the local risk of neck-rot disease. Growers would have the ability to access the full storage potential of their crops, reduce post-harvest losses, fungicide use, energy costs and provide an effective disease control. Hence, this research aimed at developing an immunosensor for early detection and quantification of *Botrytis* infections in the field. The second stage of the development focused on combining the biosensor with a DNA testing procedure in order to detect and discriminate between the *Botrytis* species employing the polymerase chain reaction (PCR) method. In addition, both detection methods were tested in a large field trial to investigate the correlation of the pathogen load of *Botrytis* and the incidence of neck-rot infections in the field. To bring the developed system closer to a commercially successful device, a complete detection system was developed including a microfluidic, an electrochemical signal generating and detection system utilising screen-printed electrodes and a customised housing comprising all elements mentioned above.

This project was subdivided into four objectives:

I. Development of a Botrytis-specific immunosensor.

- Testing of different types of screen-printed electrodes.
- Sensor design and formats.
- Evaluation of covalent and non-covalent immobilisation strategies.
- Testing the feasibility of nanomaterials for signal enhancement.
- Optimisation of the procedure and assay conditions.
- Performance studies and comparison of the developed immunosensor to commercial detection methods.

II. Development of a DNA biosensor for Botrytis.

- DNA extraction procedure.
- Evaluation of primers suitable for the detection of neck-rot infections.
- Identifying the pathogen DNA using real-time PCR and detect the PCR product by using RFLP analysis and SPGEs.
- Evaluation of various DNA sensor designs.
- Optimisation of the DNA sensor response.
- Testing the performance of the developed DNA sensor.

III. Field trial to investigate the correlation of the pathogen load and the incidence of neck rot in store.

- Field trial design and storage experiment to assess the incidence of neck-rot disease.
- Testing the capability of the developed immuno and DNA sensor to detect and quantify *Botrytis* infections in field conditions using onion samples.
- Comparative studies with commercial detection methods.

IV. Development of a microfluidic system.

- Designing a lateral-flow sample delivery systems that can be combined with the Cranfield design screen-printed electrodes.
- Testing the feasibility of nanomaterials for signal enhancement.
- Optimisation of the assay procedure.
- Performance studies of the immunosensor with the lateral-flow sample delivery system.
- Design of a customised housing based on the current sensor system.
- Improving the design of the sensor, lateral-flow sample delivery system and housing.

A systematic summary of the objectives of this project is illustrated in Figure 1.27.

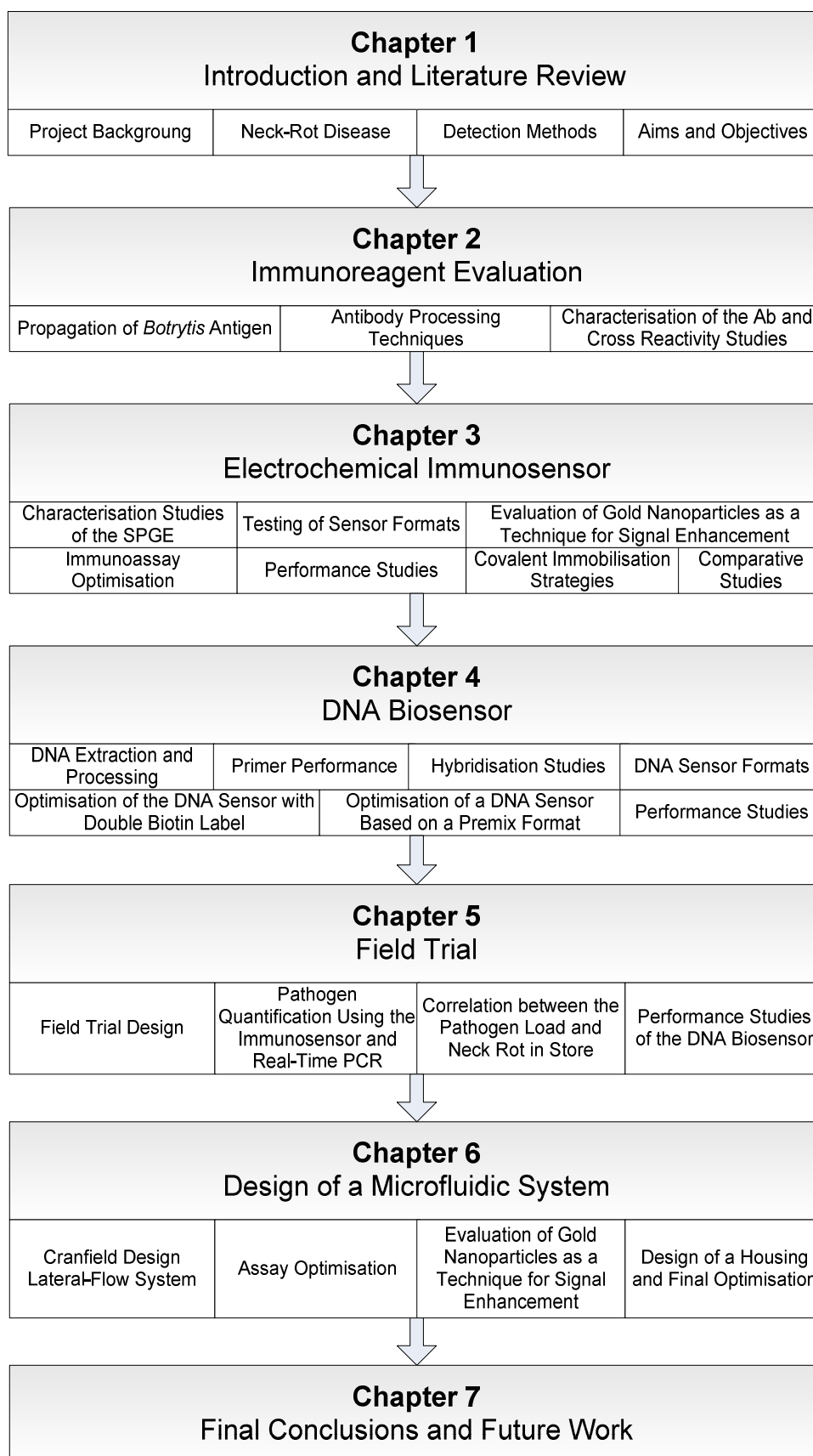


Figure 1.27: Flowchart outlining the phases of this study.

CHAPTER TWO

IMMUNOREAGENT EVALUATION

2 IMMUNOREAGENT EVALUATION

2.1 INTRODUCTION

Immunological detection methods are highly dependent on the quality and the availability of suitable antibodies. Generally, there are two different techniques for the production of antibodies. Polyclonal antibodies are a mixture of antibodies specific to several surface structures (epitopes) of an antigen. They are produced by immunising animals, usually rabbit, mouse, goat or chicken, with a pure form of the target antigen. In order to increase the immune reactions adjuvants, for instance pathogenic DNA or proteins are added to the antigen. These antibodies, especially the more specific IgG (immunoglobulin G), are extracted from the blood and purified for further use.

In contrast, the monoclonal antibodies are specific to one epitope of an antigen. They are gained by the fusion of an antibody-producing B-cell from an immunised animal with a myeloma cell. After screening and selecting the target cell, this hybrid cell line (hybridoma cell) is capable of growing in cultures for nearly unlimited time, producing antibodies specific to one epitope of the antigen.

This Chapter describes the acquisition of the suitable antibody for the sensor development and the different techniques used for the production of the fungal antigen and growing and maintaining of the *Botrytis* cultures. Furthermore, the antibodies for the immunoassay development are evaluated regarding concentration, conjugation efficiency to HRP, specificity and cross reactivity.

2.2 MATERIALS

2.2.1 GENERAL CHEMICALS AND INSTRUMENTATION

Albumin from bovine serum (BSA), HPLC-water, phosphate buffered saline tablets (PBS, 0.01 M phosphate buffer, 0.137 M sodium chloride and 0.0027 M potassium chloride, pH 7.4), Tween 20, sulphuric acid (H₂SO₄, 95-98 %), isopropanol (99.6 %) and potassium chloride were purchased from Sigma (Dorset, UK). Coating buffer (02-002) was acquired from ADGEN Phytodiagnostics (Auchincruive, UK). Sure Blue 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB) solution and milk blocking solution concentrate were purchased from KPL (Gaithersburg, USA). The goat anti-rabbit and goat anti-mouse IgG-antibodies conjugated to HRP were purchased from Invitrogen (Paisley, UK). The monoclonal antibody BC-12 was acquired from Forsite Diagnostics (Sand Hutton, UK) and the polyclonal *B. allii* antibody was obtained from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK). Antibodies were conjugated to HRP using the Lightning-Link conjugation kit purchased from Innova Biosciences Ltd. (Cambridge, UK), and purified with a Pierce conjugate purification kit purchased from Thermo Scientific (Cramlington, UK).

Absorbance measurements for ELISA were performed using the Fluostar Galaxy reader (BMG, Offenburg, Germany). Solid phase 96 immuno wells were acquired from NUNC Maxi Sorp (NUNC Thermo Fisher Scientific, Roskilde, Denmark). Incubation at 37 °C was performed under controlled environment provided by an incubator/shaker model iEMS 1415 (Labsystem, Helsinki, Finland). The vortex mixer model Genie 2 G-560E was purchased from Scientific Industries (Bohemia, USA).

2.2.2 FUNGAL CULTURES AND MEDIA

Potato dextrose agar (CM0139, 4 g l⁻¹ potato extract, 20 g l⁻¹ dextrose and 15 g l⁻¹ agar) and technical agar number 3 (P0013) with high protein concentration, were purchased from OXOID (Basingstoke UK). Potato dextrose broth (PDB) was purchased from Sigma (Dorset, UK). Cellophane membranes (P400) were obtained from Cannings, Ltd. (Bristol, United Kingdom).

Porcelain mortar and pestle (Ø70 mm) were purchased from Scientific Laboratory Supplies (Nottingham, UK), 30 ml filter tube porosity 0 (160 to 250 µm) (Robu Glassware) and 20 mm pre-filter disc porosity 1 (100 to 160 µm) and porosity 3 (16 to 40 µm) were purchased from Jencons (Lutterworth, UK).

Three species of *Botrytis* are considered as the primary causal agents of neck rot: *B. aclada*, *B. allii*, and *B. byssoidea* (Yohalem et al., 2003; Lorbeer et al., 2004; Chilvers and Du Toit, 2006). Therefore, the work was focused on all three species in this research. Furthermore, *B. cinerea*, as the most common *Botrytis* species, was also used for affinity testing. An overview of the fungal species used is listed in Table 2.1.

Table 2.1: *Botrytis* species, their source and growth media.

Species	Source	Catalogue Number:	Agar Medium
<i>B. allii</i>	CABI (Wallingford, UK)	IMI 147186	PDA ^a , onion agar ^b
<i>B. aclada</i> AII (<i>B. allii</i>)	CBS- KNAW (Utrecht, Netherlands)	CBS 103.23	PDA, onion agar
<i>B. aclada</i>	CBS- KNAW (Utrecht, Netherlands)	CBS 260.71	PDA, onion agar
<i>B. byssoidea</i> (<i>J.C. Walker</i>)	CBS- KNAW (Utrecht, Netherlands)	CBS 104.23	PDA, onion agar
<i>B. cinerea</i>	Plant Research International (Wageningen, Netherlands)	BC 143	PDA

^a PDA = Potato dextrose agar.

^b Agar containing freeze-dried onion powder.

For cross-reactivity studies, common fungi represented by *Fusarium oxysporum* (CBS192.35, Utrecht, Netherlands), *Penicillium verrucosum* (OTA11, environmental isolate) and *Aspergillus niger* (N1, environmental isolate) were acquired from the Mycology Group of Cranfield University (Vincent building, Cranfield, UK).

2.3 METHODS

2.3.1 PROCESSING OF FUNGAL CULTURES

2.3.1.1 Agar Media

For the preparation of the potato dextrose agar (PDA), 39 g l⁻¹ PDA powder (OXOID, Basingstoke, UK) was mixed as recommended by the company with distilled/deionised water (dH₂O). The final agar media contained 4 g l⁻¹ potato extract, 20 g l⁻¹ dextrose and 15 g l⁻¹ agar.

To provide a natural substrate for the fungus and induce a rapid growth and sporulation, an experimental onion agar based on the leaf extract agar described by Chilvers and Du Toit (2006) was used. Instead of dried onion leaves, freeze-dried onion powder was utilised as a nutrient source. For its production, onions of the variety super sweet or red baron were cut into small cubes and freeze-dried at -40 °C for 3 to 5 days using a vacuum freeze-dryer (Scanvac, model Cool Safe 110-4, Lyngby Denmark). The drying process was monitored by recording the weight of the onions. After reaching their constant dry weight, the freeze-dried onions were ground to a fine powder with a mortar and pestle and stored in the freezer for further use. For the production of the agar, 16 g l⁻¹ of technical agar powder (P0013, OXOID, Basingstoke UK) with high protein concentration was mixed with 15 g l⁻¹ freeze-dried onion powder in dH₂O.

After mixing the reagents, the PDA and onion agar solutions were autoclaved for 45 min at 120 °C. To avoid bacterial growth of the suspension after sterilisation, 1 g l⁻¹ streptomycin sulphate salt (Sigma, Dorset UK) was added. Eventually, 20 ml agar solution was dispensed to each petri dish (Ø90 mm, 20 mm height) under a sterile environment provided by a laminar flow cabinet (Sanyo, model MLR-350HT, Japan).

2.3.1.2 Growing and Maintaining the *Botrytis* Cultures

The fungal cultures were grown and maintained regularly in a controlled environment provided by an incubator (Sanyo, model MLR-350HT, Japan) at 23 °C with a 12 h day-night photoperiod. Depending on the demand of the fungus, each species had at least five culture replicates maintained on PDA and onion agar. An exception to this is *B. cinerea* which was exclusively grown on PDA.

For the replication of the fungus, a square piece of agar (approximately 1 cm²) containing fungal mycelium was cut from the plate with a spatula and placed in the middle of a new petri dish. After each step, the spatula was dipped in 70 % isopropanol and sterilised with a propane/butane gas stove. After six to eight weeks, new replicates of the fungal cultures were produced. However, the old cultures were stored in the incubator and used as an antigen source for assays. The separation of *Botrytis allii* and *aclada* is difficult due to overlapping spore sizes and can occur just genetically (Nielsen et al., 2002). To avoid cross contamination between these species, several additional disinfection steps of the spatula were carried out including heat treatment and dipping in isopropanol. Furthermore, these two species were not plated out in consecutive order.

Twice a year, the *Botrytis allii*, *B. aclada* and *B. byssoidea* cultures grown on onion agar and PDA were cross-transferred to the alternative agar media. Occasionally changing the agar media was reported to help the sporulation and prevent the degradation of cultures grown for longer periods (Nakasone et al., 2004).

There are several methods discussed in the literature for the long-term preservation of fungal cultures, such as sclerotisation, oil overlay, or immersion in distilled water, agar strips, soil and sand. However, the most common method applied for the preservation of fungal cultures for less than five years is freezing the culture at -20 to -80 °C (Nakasone et al., 2004). In general, vigorously growing and sporulating cultures survive the freezing process better than less vigorous strains. To increase the success rate of freezing 10 to 20 % glycerol were added to the sterile deionised water (Kitamoto et al., 2002; Nakasone et al., 2004). However, Nielsen et al. (2002) used 15 % glycerol for the preservation of *Botrytis* cultures. Therefore, this concentration was added to the dH₂O

and autoclaved for 45 min at 120 °C. Spores were harvested by adding 5 ml of the 15 % glycerol suspension to each agar plate and gently scraping off the spores with a small sterile glass mortar head. The spore solution of each of the two separate agar plates per species was collected with a pipette and four times 1 ml was aliquoted to sterile, 1.5 ml screw cap Eppendorf tubes before storing at -80 °C.

2.3.1.3 Propagation of the *Botrytis* Antigen

As there are no commercial *Botrytis* standards available, four different techniques were used to produce the antigen. The easiest and fastest way to obtain the antigen is to take the raw fungus directly from the agar plate by scraping it off with a spatula. To avoid contamination when performing this method, old agar plates were used for the generation of the antigen.

An alternative method for the production of *Botrytis* antigen standards for immunoassays is the use of freeze-dried mycelium (Meyer and Dewey, 2000). For its production, the fungus was incubated for three to six weeks in a climate chamber in order to produce a maximum amount of biomass. To sustain the following freeze-drying process performed with the vacuum freeze dryer (Scanvac, model Cool Safe 110-4, Lyngø, Denmark) at -40 °C for 7 days, the plates were pre-treated several times with liquid nitrogen to reduce the initial temperature. Afterwards, the agar from the petri dishes was ground in a clean fume cupboard with a mortar and a pestle in the presence of liquid nitrogen.

In order to compensate for the lack of purity of the freeze-dried sample, a thin, sterile cellophane membrane (P400; Cannings Ltd., Bristol, UK) was placed on top of the agar plates as reported by Marin et al. (2010). After the inoculation, the fungus was grown for six weeks until the surface of the membrane was completely covered with mycelium. With the membrane the fungus could be removed easily from the agar plate without traces of agar (Figure 2.1).

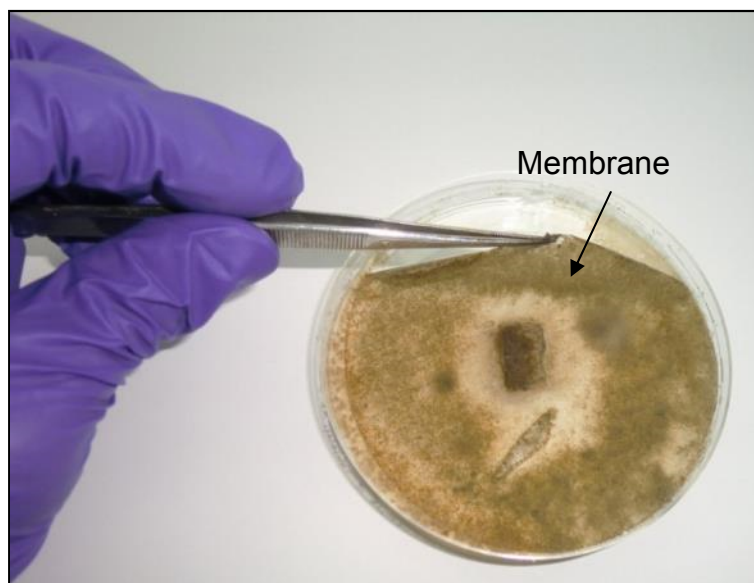


Figure 2.1: Removal of the fungal mycelia using a thin cellophane membrane layer on top of the agar plate.

A different technique to compensate the lack of purity is growing the fungus in liquid culture. This method was used by Chilvers et al. (2007) and Coolong et al. (2008) to grow *Botrytis* species associated with neck rot. Potato dextrose broth (PDB) was produced by adding 24 g l⁻¹ powder to dH₂O according to the manufacturer's recommendation and the solution was autoclaved for 45 min at 120 °C. Spores were then harvested by adding 5 ml of sterile water to each agar plate and gently scraping off the spores with the head of a small sterile glass mortar. A 200 µl of spore solution was then added to petri dishes (Ø90 mm, 20 mm height) containing 20 ml PDB and incubated for four weeks at 23 °C with 12 h day-night cycle. As sporulation for *B. byssoides* is very low, thin mycelial mats were removed using a sterile scalpel and added to the PDB. After the incubation period, the fungus was vacuum freeze dried at -40 °C for 7 days (Scanvac, model Cool Safe 110-4, Lynge, Denmark). The mycelial matt of each plate was then scraped off with a sterile spatula and transferred to a 50 ml centrifuge tube. Five ml of liquid nitrogen were added to the tube in order to facilitate the grinding process. The mycelium was ground by adding five sterile steel beads to the centrifuge tube and shaken with a vortex mixer in the presence of liquid nitrogen. The gained powder was kept in glass bottles at 4 °C for subsequent use.

To perform an assay, the fungal antigen was weighed, added to a plastic bottle comprising five steel grinding beads and the required amount of buffer was added to produce a stock solution. To grind the fungus, the bottle was shaken three times for 1 min using a vortex mixer (Scientific Industries, Bohemia, USA) at the highest setting (8), followed by 10 s of manual shaking. A concentrated stock solution with a range from 1 to 10 mg ml⁻¹ was then produced, which was further diluted to the required working strength.

2.3.1.4 Production of Spore Solutions

To harvest spores, 2 to 5 ml of dH₂O autoclaved for 45 min at 120 °C was added to the agar plate and were mobilised by gently scraping off the spores with a small sterile glass mortar head performing repeating round movements. The resulting spore solution was collected with a sterile pipette tip and transferred to a 50 ml centrifuge tube. Depending on the amount of harvested spores, the stock solution was diluted 1:10 to 1:100 to produce 1 ml of test solution. The concentration of this solution was then estimated by counting the spores in the defined squares of the haemocytometer. The accuracy of this method was increased by repeating the spore count and by quantifying numerous test solutions. Once the quantity was estimated, the spores were diluted to their final working strength using sterile dH₂O.

If a purified spore solution was required without mycelial fragments, the spores were vacuum filtered using a 30 ml glass filter tube with the porosity 0 (160-250 µm) (Robu Glassware), combined with a changeable sterile 20 mm pre-filter disc with the porosity 3 (nominal pore size 16 - 40 µm) purchased from Jencons (Lutterworth, UK). This filter is permeable for the smaller sized spores that have a length of less than 15 µm (Chilvers and Du Toit, 2006) but filters the bigger sized mycelium cells with a length of 50 to 120 µm.

2.3.2 PROTEIN DETERMINATION USING BRADFORD PROTEIN ASSAY

The polyclonal antibodies purchased from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK) are quantified in “ADGEN-Units”, where one ADGEN-Unit is equal to the required antibody concentration for two ELISA wells. The concentration of the antibodies is considered as being confidential. The concentration of the monoclonal antibody BC-12 (Forsite, Sand Hutton, UK) was also unknown. Therefore, a Bradford protein assay was performed to determine the antibody concentration which is essential for the sensor development.

The basic working principal of the Bradford protein assay is the binding of a dye (Coomassie Brilliant Blue) to proteins, which results in a shift of the absorption maximum of the dye from 465 to 595 nm (Bradford, 1976). For the quantification of the protein, bovine serum albumin (BSA) standards (0, 0.1, 0.5, 1.0 and 1.4 mg ml⁻¹) were prepared. According to the assay procedure provided by Sigma (Dorset, UK), the linear range of the assay is between 0.1 and 1.4 mg ml⁻¹. Due to the unknown dilution media of the antibodies, the standards were diluted in PBS and water to achieve two comparable standard curves. Standards were assayed in triplicates; antibody solution in duplicates. The antibody quantification occurred through adding 5 µl of antibody sample and protein standard to 200 µl of Bradford reagent (Sigma, Dorset, England). Samples and Bradford reagent were mixed gently several times and incubated at room temperature for 30 min, before the absorbance was recorded at a wavelength of 600 nm using the Fluostar Galaxy reader (BMG, Offenburg, Germany).

2.3.3 CONJUGATION OF THE ANTIBODIES TO HRP

Neither the monoclonal antibodies (Forsite, Sand Hutton, UK), nor the polyclonal antibodies (ADGEN Phytodiagnostics, Auchincruive, UK) can be purchased in a conjugated form. For a direct detection of the fungus, the antibodies were conjugated to HRP using a Lightning-Link conjugation kit purchased from Innova Biosciences Ltd. (Cambridge, UK). After adding the Lightning-Link modifier in a ratio of 1:10 to the antibody solution, the antibody sample was pipetted directly to the HRP conjugation mixture. To maximise the binding procedure of the antibodies to HRP, the sample was

incubated for 4 h at room temperature. For better distribution of the HRP, the solution was re-suspended gently by withdrawing and re-dispensing the liquid every hour using a pipette. To stop the reaction, quencher reagent was added in a ratio of 1:10. According to the Lightning-Link protocol, the efficiency rate is 99 % and the conjugate can be used 30 min after production. Further purification steps are not necessarily needed.

2.3.4 PURIFICATION OF THE ANTIBODIES USING A NICKEL-CHELATED AGAROSE COLUMN

Though the antibodies conjugated to HRP can be used directly after conjugation, non-specific binding and assay performance can be improved by purifying the antibody-HRP mix. The Lightning-Link conjugation kit from Innova Biosciences Ltd. (Cambridge, UK) covers an antibody concentration range between 0.5 and 5 mg ml⁻¹ for optimal results. However, the wide range of antibody concentrations and the efficiency rate of 99 % are only achievable by providing an environment with excess HRP. Subsequent coupling of the HRP to immobilised molecules on the sensor surface, such as antibodies and proteins, is not expected as the reaction is quenched after the conjugation process. However, non-specific binding to the sensor surface is gradually increasing with the HRP concentration.

Therefore, a Pierce conjugate purification kit purchased from Thermo Scientific (Cramlington, UK) was used to eliminate free enzyme from the HRP conjugate. According to the manufacturer's specification, the kit uses a nickel-chelated agarose column to bind IgG antibodies through interaction between the immobilised nickel-chelated resin in the agarose gel and a histidine-rich cluster in the Fc region of mammalian IgG.

To purify the antibodies, the nickel-chelated agarose column was first equilibrated by adding 10 ml of tris-buffered saline (TBS, pH 7.2, 25 mM tris, 0.15 M NaCl) and allowing the solution to drain through the column. The column was primed with 2 ml of activator buffer that contains an aqueous solution of Ni⁺² in TBS and was incubated for 10 min at room temperature. Unbound Ni⁺² was removed by washing with 10 ml TBS.

Afterwards, 0.5 to 2 ml of the antibody-HRP conjugate was applied to the column. The conjugated IgG antibodies were bound by the nickel-chelated agarose and excess HRP was removed by adding 5 ml of TBS. To mobilise the antibodies from the column, 1.7 ml elution buffer containing EDTA in TBS was applied. A blue band developed, which indicated the elution front of the antibody conjugate. Additionally, 1.5 ml of elution buffer was subdivided in 3 x 500 µl portions used to elute the blue band containing the antibodies from the column to an Eppendorf tube.

Immediately afterwards, the 1.5 ml antibody stock was applied to the polyacrylamide desalting column included in the kit, which was previously equilibrated by adding 30 ml of PBS and allowing the solution to drain through the column. Finally, 500 µl PBS were added to the column and each of a total of 16 fractions were collected in separate Eppendorf tubes. Fractions containing HRP conjugate were identified by adding 2 µl of each fraction to 1 ml of TBST and adding 5 µl of the diluted fractions to a 96 well ELISA plate containing 100 µl per well Sure Blue 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB) solution (KPL, Gaithersburg, USA). The colour reaction was incubated for 15 min in the dark and stopped using 50 µl of 0.5 M sulphuric acid.

After the purification process, the nickel-chelated agarose column was washed with 20 ml of TBS and stored in 2 ml of activation buffer. The polyacrylamide desalting column was regenerated by applying 10 ml of PBS. The kit was stored at 4 °C and can be reused for up to ten purifications.

2.3.5 ELISA METHOD FOR *BOTRYTIS*

The ELISA method developed for the detection of *Botrytis* was based on a modified version of the Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK) IDENTIKIT for *B. allii*. The *Botrytis* sample was mixed with ELISA kit coating buffer (02-002, ADGEN Phytodiagnostics, Auchincruive, UK) to produce the target concentration. Afterwards, 100 µl of the sample solution were dispensed in a microtiter plate NUNC Maxi Sorp F96 immuno well (NUNC Thermo Fisher Scientific, Roskilde, Denmark), and incubated overnight at 4 °C in order to immobilise the antigen to the plate. After washing the plate three times with PBST (phosphate buffered saline with 0.05 % Tween 20), the surface was blocked. For the blocking process, milk concentrate (KPL, Gaithersburg, USA) was diluted 1:10 in PBST and 150 µl per well were added and the plate was incubated for 1 to 2 h at 37 °C using an incubator/shaker (Labsystem model iEMS 1415, Helsinki, Finland). The surface was subsequently washed three times with PBST. For the following step, 100 µl per well of 10 µg ml⁻¹ detection antibody were added and incubated for 2 h at 37 °C. After washing the unbound antibodies three times with PBST, the goat anti rabbit and goat anti-mouse IgG-antibody conjugated to HRP (Invitrogen, Paisley, UK) were diluted as recommended 1:1000 to 1:4000 resulting in an antibody concentration of approximately 0.1 to 0.3 µg ml⁻¹, and 100 µl were dispensed to each well.

KPL milk concentrate was diluted 1:40 in PBST and used as a carrier protein for the dilution buffer of the detection antibody and anti-species conjugate. Between all steps, three washing cycles with PBST were performed to remove the unbound material. To induce colour generation of the HRP, 100 µl of Sure Blue TMB substrate was added to each well and incubated for 10 to 30 min at room temperature, followed by 50 µl of 0.5 M sulphuric acid to stop the enzyme reaction. The detection occurred by reading the absorbance of the assay at 450 nm. The limit of detection (LOD) was calculated by plotting the absorbance value of the negative plus three times its standard deviation against the resulting standard curve of the assay. Alternatively, if the data were normalised by subtracting the negative reading, three times the standard deviation of the negative control was used, respectively.

2.3.6 CHARACTERISATION OF THE ANTIBODIES

2.3.6.1 Performance of the Monoclonal Antibody BC-12 in the *Botrytis* LFD

The *Botrytis* lateral-flow devices (LFD) acquired from Forsite (Sand Hutton, UK) diagnostic was tested against *B. cinerea*, *B. allii*, *B. aclada* and *B. byssoidea*. Approximately 20 mg mycelium of each species were removed from the agar plates and added to the extraction buffer provided by the LFD kit. The sample was ground as described in Section 2.3.1.3 and 80 μ l were added to the sample pad. *B. allii* spore solution was produced according to the protocol provided in Section 2.3.1.4. The concentration was determined with a haemocytometer as 60×10^6 spores ml^{-1} . The stock was further diluted 1:10 in PBST and dropped on the LFD for testing.

To construct the standard curve, *B. allii* and *B. cinerea* concentrations ranging from 500 to 10 $\mu\text{g ml}^{-1}$ were applied to the LFD. The line reading occurred by using a LFD reader (LFDR 101, Forsite, Sand Hutton, UK) 15 min after applying 80 μ l of the sample to the wick according to the manufacturer's recommendation. The line of each LFD was read three times in a period of less than 30 s to build an average. Each concentration was measured using three LFDs.

2.3.6.2 Specificity of the Antibodies against Relevant *Botrytis* Species

The specificity of the monoclonal (Forsite, Sand Hutton, UK) and polyclonal (ADGEN, Auchincruive, UK) antibodies to *Botrytis* species considered as the primary cause of neck rot (*B. allii*, *B. aclada* and *B. byssoidea*) as well as *B. cinerea* was tested using the indirect ELISA method as described in Section 2.3.5. Therefore, two different samples of each fungus were combined to a total weight of approximately 20 mg mycelium. To minimise variations regarding structural growth and sporulation, 10 mg sample material was extracted from fungus grown on onion agar and the other part from PDA as substrate. The analyte was diluted to a concentration of 1 mg ml^{-1} in coating buffer (02-002) taken from the *B. allii* ELISA kit (Neogen Europe Ltd. – ADGEN Phytodiagnostics, Auchincruive, UK). Afterwards, the plastic bottle including the sample and five steel grinding beads was shaken three times for 1 min using a vortex mixer (Scientific Industries, Bohemia, USA) at the highest setting (8), followed by 10 s

of manual shaking. One hundred μl of each ground sample were assayed by diluting the analyte in a range from 1 mg ml^{-1} to $8\text{ }\mu\text{g ml}^{-1}$. The negative control was represented by the commercial *B. allii* ELISA kit negative control acquired from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK), which includes freeze-dried leaf extracts. The concentration of the detection antibodies was adjusted to $10\text{ }\mu\text{g ml}^{-1}$. The corresponding anti-species antibody conjugated to HRP (Invitrogen, Paisley, UK) was diluted 1:3000 according to manufacturer's recommendation.

2.3.6.3 Cross-Reactivity Studies against *Fusarium*, *Penicillium*, and *Aspergillus* Species

Cross reactivity of the monoclonal (Forsite, Sand Hutton, UK) and polyclonal (ADGEN, Auchincruive, UK) antibodies was screened against common fungi represented by *Fusarium oxysporum* (CBS192.35), *Penicillium verrucosum* (OTA11, environmental isolate) and *Aspergillus niger* (N1, environmental isolate) using indirect ELISA method. Pure mycelium was removed from the surface of the agar plate and diluted to a target concentration of 1 mg ml^{-1} . The ELISA procedure was followed as described in Section 2.3.5, with a detection antibody concentration of $10\text{ }\mu\text{g ml}^{-1}$ and a corresponding anti-species conjugate concentration of $0.3\text{ }\mu\text{g ml}^{-1}$. As negative control, BSA was prepared in PBS to match the sample concentration.

2.3.7 ESTIMATION OF THE PATHOGEN LOAD OF INFECTED ONION PLANTS USING INDIRECT ELISA

In order to estimate the pathogen load of onion leaves infected with *Botrytis allii*, two onion sets were grown for three months in a greenhouse and inoculated with *B. allii* spore solution. In this stage, the plants had three leaves; the first was inoculated with $500\text{ }\mu\text{l}$ of 20×10^6 spores ml^{-1} , the second was inoculated with $500\text{ }\mu\text{l}$ of 2×10^5 spores ml^{-1} and the third leaf was marked as control. The spore solution was produced as described in Section 2.3.1.4 and quantified using a haemocytometer. Spores were inoculated only to the first 10 cm of the leaf. The inoculation site was previously labelled with a permanent marker in order to locate the site after subsequent growth of

the leaf. The onion samples were grown for 14 days exposed to weather. The weather conditions in August 2011 during the two weeks were mostly humid with frequent showers and temperatures ranging from 14 to 19 °C at night and 19 to 25 °C during day.

After this incubation period, allowing the fungus to grow, the plants were harvested and every leaf was packed separately in a sealed bag and stored at -80 °C. A flow chart showing the experimental design and sample extraction procedure used for the solid phase ELISA tests is illustrated in Figure 2.2. Initially, the samples were cut with a pair of scissors in three pieces; the tip section including the marked inoculation (10 cm + 1 cm), the middle section (10 cm) and the neck section (10 cm). The samples weight was measured between 0.25 and 0.45 g. Afterwards, the leaves were ground for 1 min in addition of 10 ml g⁻¹ coating buffer from the *B. allii* ELISA kit (ADGEN Phytodiagnostics, Auchincruive, UK) using a 70 mm porcelain mortar and pestle (Scientific Laboratory Supplies, Nottingham, UK). The samples were held for 30 min at room temperature to allow the antigen to diffuse into the buffer. Afterwards, the sample was filtered through a 30 ml glass filter tube with the porosity 0 (160-250 µm) (Robu Glassware) using a changeable sterile 20 mm pre-filter disc with the porosity 1 (100 to 160 µm) purchased from Jencons (Lutterworth, UK). To help the filtering process and to avoid sample loss, a vacuum pump was used to collect the sample to a sterile 15 ml centrifuge tube. For each sample a separate filter and 30 ml glass filter-tube combination was used to avoid contamination.

The ELISA procedure was followed as described in Section 2.3.5, with a detection antibody concentration of 10 µg ml⁻¹ and a corresponding anti-species conjugated concentration of 0.3 µg ml⁻¹. To improve the signal strength, the TMB substrate was incubated for 1 h at room temperature. The samples were assayed in triplicates. For quantification, the absorbance value at 450 nm was plotted against a serial dilution of *B. allii* mycelium processed as described in Section 2.3.1.3, ranging from 4 to 0.03 µg ml⁻¹.

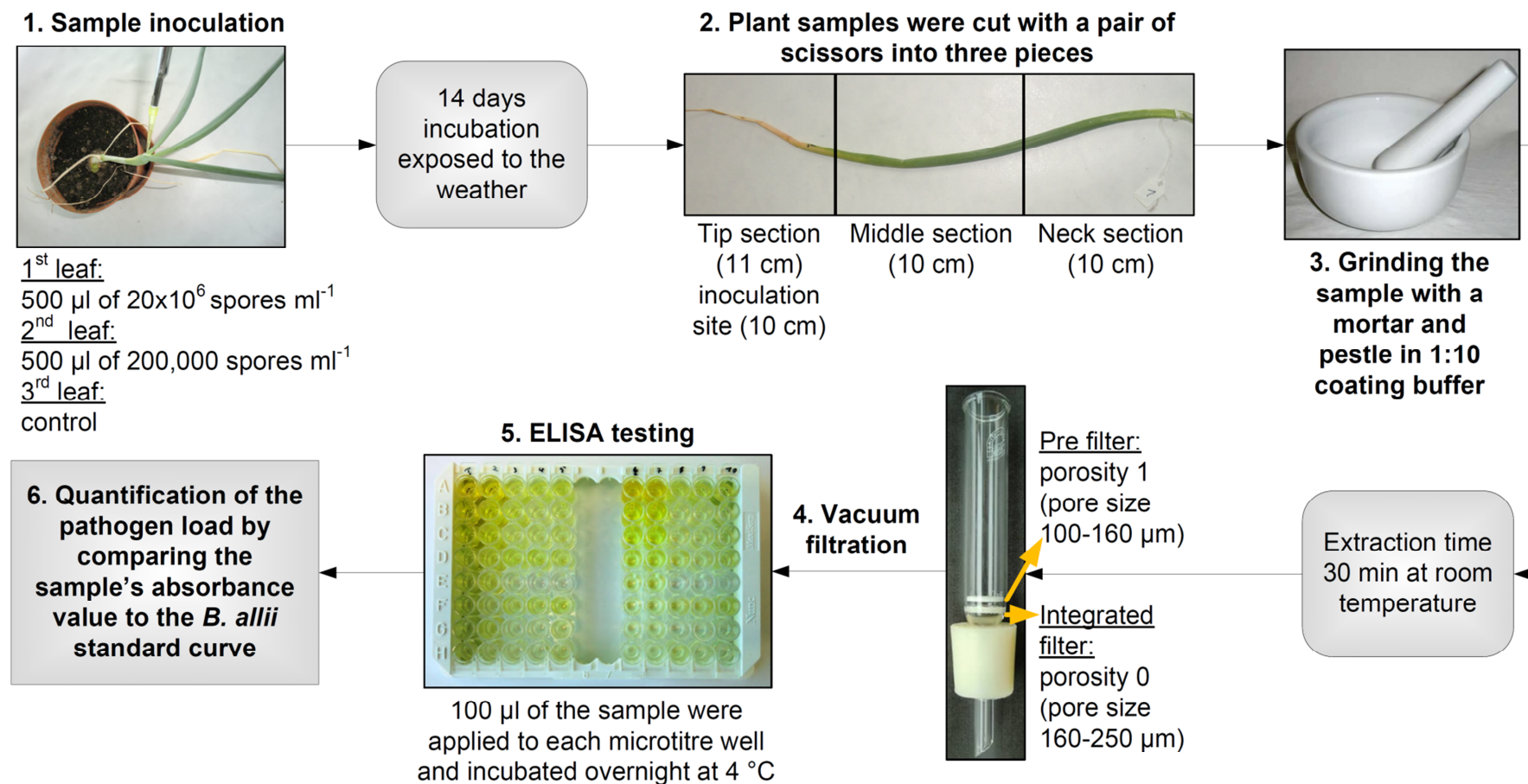


Figure 2.2: Flow diagram of the sample processing procedure used for the quantification of the pathogen load in onion samples.

2.4 RESULTS AND DISCUSSION

2.4.1 PROPAGATION OF THE *BOTRYTIS* ANTIGEN

The morphology of fungi varies when grown on agar plates and is therefore widely used by mycologists for classification. Figure 2.3 visualises the hyphal growth patterns of the *Botrytis* cultures used for this study. Differences were observed between the fungal species and even between various isolates of the same species. Especially *B. byssoides* showed more mycelial growth and few or no sclerotia and conidia being present confirming previous findings by Presly (1985a) and Chilvers et al. (2006). Since variation in appearance and growth is common, there is no standardised method for growing, maintaining and processing of *Botrytis* (Dewey and Yohalem, 2004). Several methods described in the literature are listed in Table 2.2. However, these methods vary according to their application and are not directly transferrable to the requirements of this research. Therefore, different techniques were evaluated and applied for this study.

Four different techniques were used to produce the antigen as described in Section 2.3.1.3. The easiest and fastest method to obtain the antigen is to take the raw fungus directly from the agar plate by scraping it off with a spatula. This technique is fast, simple, and results in the most natural form of the fungus. Further advantage is the high response when tested in an ELISA assay using monoclonal as well as polyclonal antibodies (Figure 2.4, Legend a). Disadvantages of this method are: possible contaminations of the agar plate, the fungus is difficult to weigh and the quantity and quality of the antigen can vary.

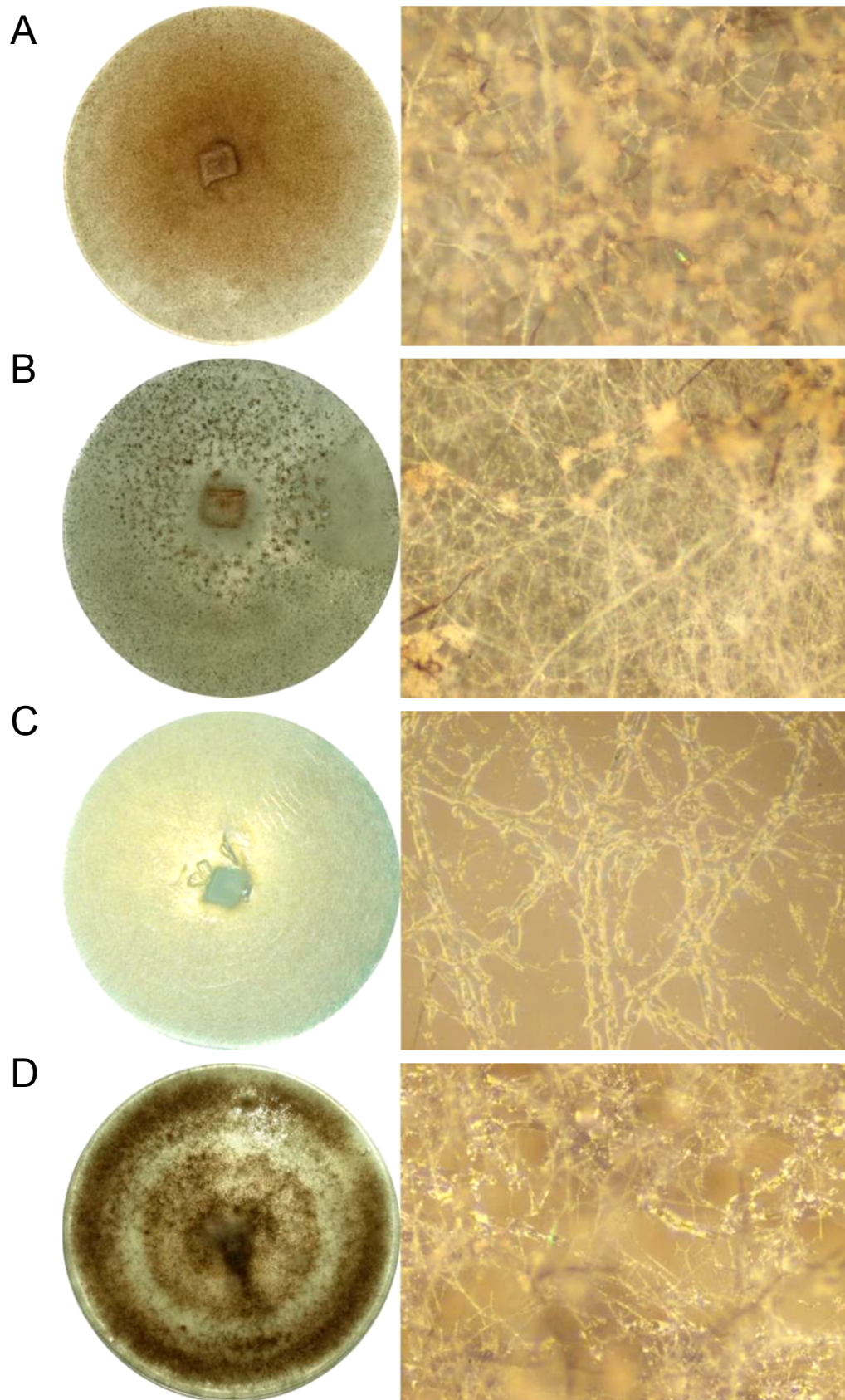


Figure 2.3: Appearance of isolates of *Botrytis allii* (A), *B. aclada* (B), *B. byssoidea* (C) and *B. cinerea* (D) on agar and the corresponding mycelia structure.

In order to compensate the lack of purity of the freeze-dried sample, a different technique was tested. A thin, sterile cellophane membrane was placed on top of the agar plates. With the membrane, the fungus could be removed easily from the surface, without traces of agar. Furthermore, the signal strength is higher compared with the unpurified freeze-dried mycelium gained by grinding the whole agar plate (Figure 2.4, Legend b). Unfortunately, it was not possible to remove the fungus from the membrane and the amount of fungal biomass was very low resulting in a difficult quantification. This method was found to be the most practical for the extraction of pathogen DNA used for the studies performed in Chapter 4.

Growing the fungus on liquid culture resulted in a high immunological response of the monoclonal as well as polyclonal antibodies, which is comparable to the raw fungus directly scraped off the agar plate (Figure 2.4, Legend c). However, this method offers the advantage that big batches can be produced and stored without the variation of quantity and quality resulting from different structural hyphal growth patterns of the fungus grown on the agar medium. Therefore, this method was used to produce a big batch of antigen, which was subsequently used for the immunosensor development.

Freeze-dried agar plates (-40 °C for 7 days) were the least suitable source of antigen. Although the signal strength of the freeze-dried mycelium is lower compared to the raw fungus, directly removed from the agar plate (Figure 2.4, Legend d), the antigen is easier to quantify, store and can be produced in large quantities. The lower signal is due to the agar media within the powder. Although the actual composition of the product is proprietary, most likely this method is used by ADGEN Phytodiagnostics (Auchincruive, UK) to produce the positive control of the commercial *B. allii* ELISA kit, since the produced antigen shows nearly identical texture and immunologic response in ELISAs. However, due to the lack of purity, this method was not further used in this study.

Table 2.2: Overview of growing and processing methods used for *Botrytis* species.

Species	Agar Media	Application	Growing the <i>Botrytis</i> spp.	Sample Extraction	Reference
<i>B. aclada</i> , <i>B. cinerea</i> ,	PDA	Real-time PCR	PDA petri dish. <i>B. aclada</i> was transferred to potato dextrose broth and cultured for 7 days at 20 °C.	Mycelium was harvested by passing the liquid culture through a 0.45 µm filter and ground in liquid nitrogen with a mortar and pestle.	(Coolong et al., 2008)
<i>B. aclada</i> , <i>B. allii</i> , <i>B. byssoidea</i> , <i>B. cinerea</i> , <i>B. porri</i>	PDA (22 °C)	Real-time PCR	PDA petri dish. Conidia or agar cubes were transferred to 50 ml tubes with potato dextrose broth. The culture was grown at 20 °C.	Freeze-dried mycelium was harvested by vacuum filtration, frozen and ground to powder for 4 s using a 2 ml screw cap tube with ceramic spheres and a Savant machine.	(Chilvers et al., 2007)
<i>B. aclada</i> , <i>B. allii</i> , <i>B. byssoidea</i> , <i>B. cinerea</i> , <i>B. porri</i> , <i>B. squamosa</i>	PDA (21 °C)	PCR	PDA petri dish or PDA petri dish covered with cellophane membrane. The fungus was grown for 5 to 7 days in the dark.	The mycelium was scraped off from the cellophane membrane using a scalpel and ground in liquid nitrogen with a mortar and pestle.	(Nielsen et al., 2002)
<i>B. cinerea</i>	PDA (21 °C)	ELISA	PDA petri dish. Grapes were squashed with a garlic press, filtered, and the juice was autoclaved. Cubes of PDA culture were added and grown for 12 days.	Mycelial mats were removed, snap-frozen in liquid nitrogen, blended and freeze-dried.	(Meyer et al., 2000) (Meyer and Dewey, 2000)
<i>B. aclada</i>	PLA (20 °C)	ELISA	PLA petri dish. <i>B. aclada</i> was grown in Czapek Dox liquid culture for 14 days at 20 °C with 6 h of orbital shaking at 100 rev. min ⁻¹ .	Spores were harvested by washing the plate with sterile dH ₂ O. Mycelium was obtained by removing the mats from the liquid culture, dried with filter paper and ground in liquid nitrogen with a mortar and pestle.	(Linfield et al., 1995)

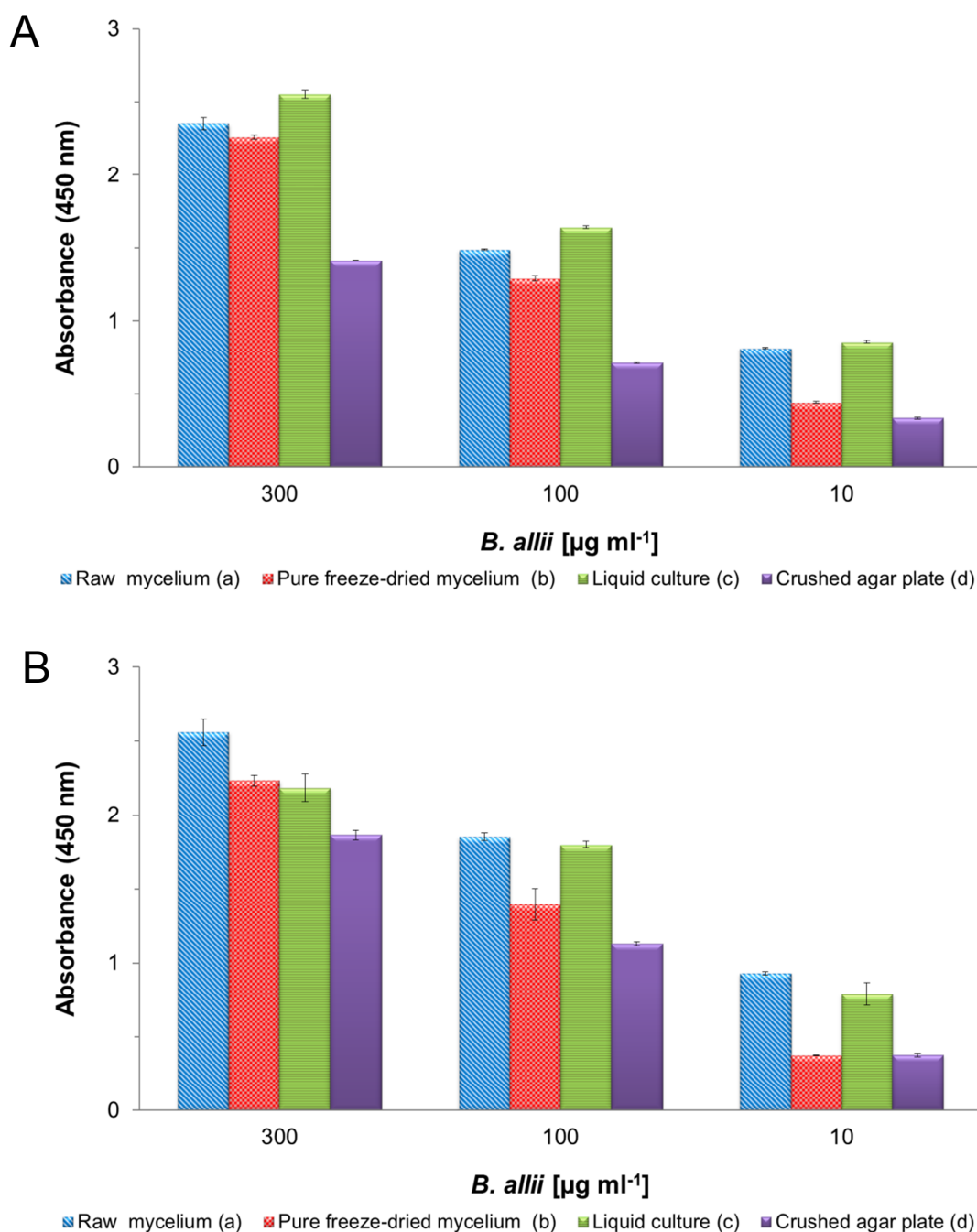


Figure 2.4: Comparison of the signal measured at 450 nm using different antigen production techniques. Indirect ELISA was performed by adsorbing the fungus on to solid phase 96 well plates overnight at 4 °C. After blocking, 100 μl of 10 $\mu\text{g ml}^{-1}$ (A) monoclonal antibodies and (B) polyclonal antibody were applied to each well, followed by 0.3 $\mu\text{g ml}^{-1}$ goat anti-species antibody conjugated to HRP. The bars represent standard error of the means (n=3).

(a) = Raw *B. allii* scraped off directly from the agar plate.

(b) = Pure *B. allii* mycelium gained by growing the fungus on an agar plate with a cellophane membrane.

(c) = Pure freeze-dried *B. allii* mycelium gained by growing the fungus on liquid culture.

(d) = Ground freeze-dried *B. allii* agar plate.

2.4.2 DETERMINATION OF THE CONCENTRATION OF THE ANTIBODIES

To quantify the protein concentration of the antibody solutions, the Bradford (1976) protein assay was used. Different dilutions of the antibody solutions acquired from Forsite (Sand Hutton, UK) and ADGEN Phytodiagnostics (Auchincruive, UK) were prepared in either water or PBS. The concentration of the antibodies was determined by comparing the absorbance at 600 nm of the unknown sample and its 1:10 dilution against both standard curves of the known protein BSA (Figure 2.5).

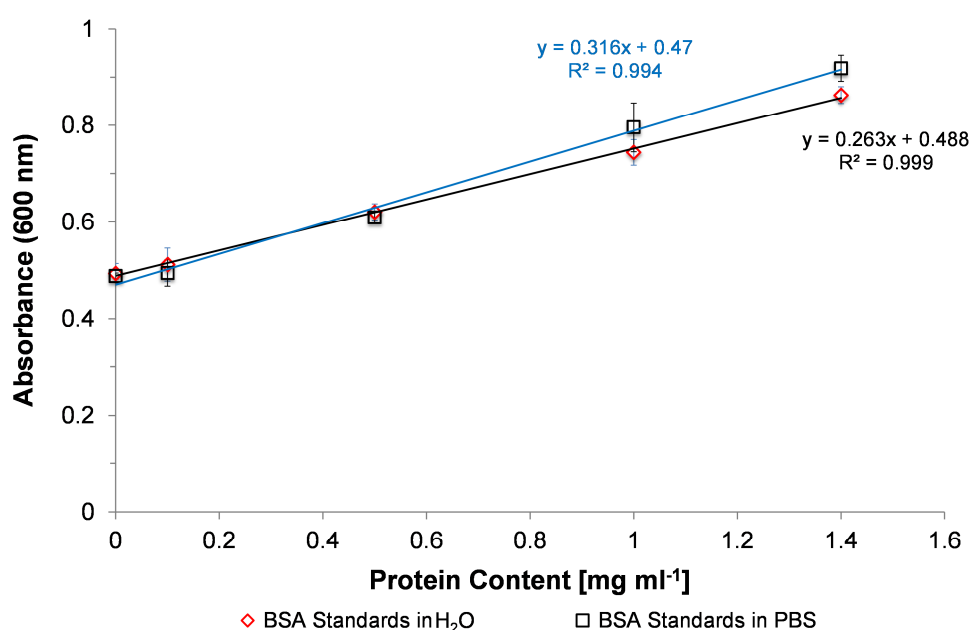


Figure 2.5: Standard curves for protein quantification conducted by diluting BSA in PBS and deionised water. The bars represent standard error of the means (n=3).

The monoclonal antibody BC-12 showed an absorbance of 0.86 undiluted, 0.50 (1:10 diluted in water) and 0.52 (1:10 diluted in PBS). This resulted in an average concentration of 1.27 mg ml⁻¹ protein content. For the polyclonal antibody from ADGEN Phytodiagnostics the calculated average concentration was 1.1 mg ml⁻¹ protein, determined from an absorbance of 0.77 undiluted, 0.51 (1:10 diluted in water) and 0.52 (1:10 diluted in PBS).

2.4.3 CONJUGATION OF THE ANTIBODIES TO HRP

In order to apply the antibodies in a direct ELISA detection, they need to be conjugated to an enzyme label. The conjugation efficiency of the antibodies was determined using a direct ELISA format with *B. allii* mycelium diluted to three different concentrations (300, 100 and 10 $\mu\text{g ml}^{-1}$). The procedure was followed according to the ELISA method described in Section 2.3.5., without the need of anti-species conjugate for signal generation. The results of the absorbance reading at 450 nm are presented in Figure 2.6.

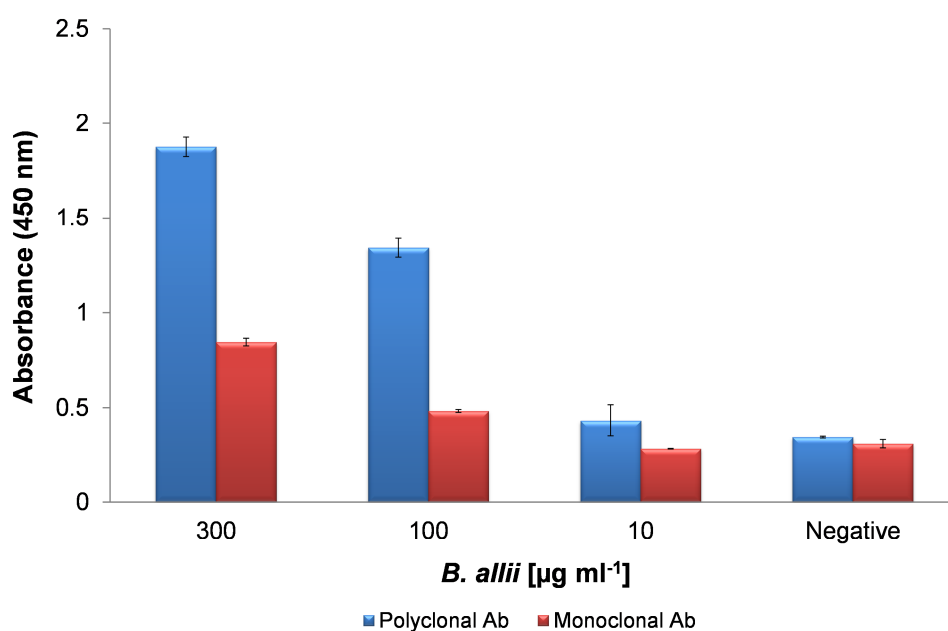


Figure 2.6: Conjugation efficiency test of the polyclonal and monoclonal antibody to HRP using a direct ELISA format (450 nm). *B. allii* mycelium with a concentration of 300, 100, 10 and 0 $\mu\text{g ml}^{-1}$ was coated to the well and used as an antigen source. The bars represent standard error of the means (n=2).

Though the protein content of the monoclonal antibody BC-12 solution has a slightly higher concentration than the polyclonal antibody (see Section 2.4.2), the absorbance value achieved was lower by more than 50 % compared with the polyclonal antibodies. As the conjugation was carried out at the same time with comparable protein antibody concentrations, the possible reason for this difference in signal generation may be the lower antibody concentration of the BC-12 antibody solution or a lower affinity to the antigen.

2.4.4 PURIFICATION OF THE ANTIBODIES USING A NICKEL-CHELATED AGAROSE COLUMN

Purifying antibodies from free enzyme can increase assay sensitivity and improve the signal-to-noise ratio (Boorsma and Kalsbeek, 1975). Therefore, a Pierce conjugate purification kit purchased from Thermo Scientific (Cramlington, UK) was used to eliminate free enzyme from HRP conjugate. In the first step, the excess HRP was removed by immobilising the antibodies to the nickel-chelated agarose column through interaction between the nickel-chelated resin in the gel and the Fc region of the IgG, followed by removing the unbound HRP by adding TBS. Afterwards, the conjugated antibodies were eluted and collected with an Eppendorf tube.

In the second step, the antibodies were desalted using a polyacrylamide desalting column and collecting a total of 16 fractions in separate Eppendorf tubes. Fractions containing HRP conjugate were identified by adding a diluted sample of each fraction to the HRP substrate TMB in a 96 well ELISA.

The absorbance measurements at 450 nm of the collected fractions (Figure 2.7) show an absorbance value of 3.3 for the desalted fractions six and seven, and 1.7 and 1.9 for the fractions five and eight. According to the manufacturer's protocol, the three fractions with the highest absorbance values should be pooled. However, the purification resulted in four fractions comprising the purified antibody HRP conjugate. Consequently, these fractions were combined to a purified antibody stock solution. The protein content of the purified conjugate was measured using Bradford protein assay and resulted in a protein content ranging from 0.22 mg ml⁻¹ (500 µl antibody solution) to 0.48 mg ml⁻¹ (1 ml conjugate). After establishing the concentration, 1:100 KPL (Gaithersburg, USA) milk concentrate was added to the antibody stock solution.

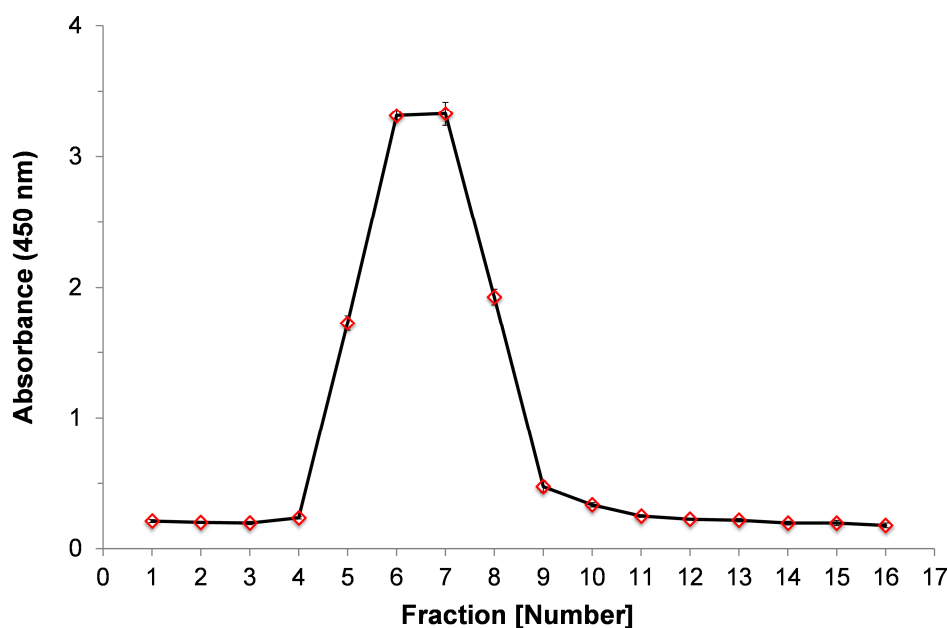


Figure 2.7: Absorbance (450 nm) of the collected antibody fractions after the purification and desalting procedure using the nickel-chelated agarose and desalting columns. Measurements were taken after 15 min incubation time of the TMB solution. The bars represent standard error of the means (n=3).

2.4.5 CHARACTERISATION OF THE ANTIBODIES

Polyclonal antibodies raised against mycelial fragments suffer from lack of specificity and strong cross-reactions to other fungi and extracts from plant tissue (Dewey, 1996). The first successful production of polyclonal antibodies against *B. allii* was accomplished by Linefield (1995). Rabbits were immunised with freeze-dried spores, cell wall and cytoplasmic fractions of the fungus, resulting in the production of polyclonal antibodies. This antiserum did not cross-react with healthy onion tissue, *B. porri*, or *B. squamosa*, but showed some minimal recognition to fungi from other genera, such as *B. byssoides* and *B. cinerea* (Linfield et al., 1995).

The first successfully raised monoclonal antibody (BC-KH4) against *Botrytis cinerea* was developed by Bossi and Dewey (1992). Although the hybridoma line was capable of detecting all species of *Botrytis*, it also showed cross reactivity with other unrelated fungi such as *Aspergillus niger* (Dewey and Cole, 1996). Therefore, a new hybridoma cell line specific to *Botrytis* species was raised. Mice were immunised with surface washings of *B. cinerea* plates mixed in a ratio of 1:1 with supernatant of a non-specific

hybridoma cell line (BC-EC7), which was used for co immunisation. This technique is effectively used to increase the percentage of hybridoma cell lines secreting taxonomically specific monoclonal antibodies that are often as low as 1 % (Meyer and Dewey, 2000). Eventually, the performances of four hybridoma cell lines were evaluated using an ELISA against the most common *Botrytis* species and other common fungi. The best cell line that showed the highest signal and lowest cross reactivity (Figure 2.8 A) was chosen for commercial production by the authors. As illustrated in Figure 2.8 (B) the monoclonal antibodies raised from the BC-12 hybridoma cells, which are used in this work, have a high affinity against surface proteins along the length of the growing hyphae.

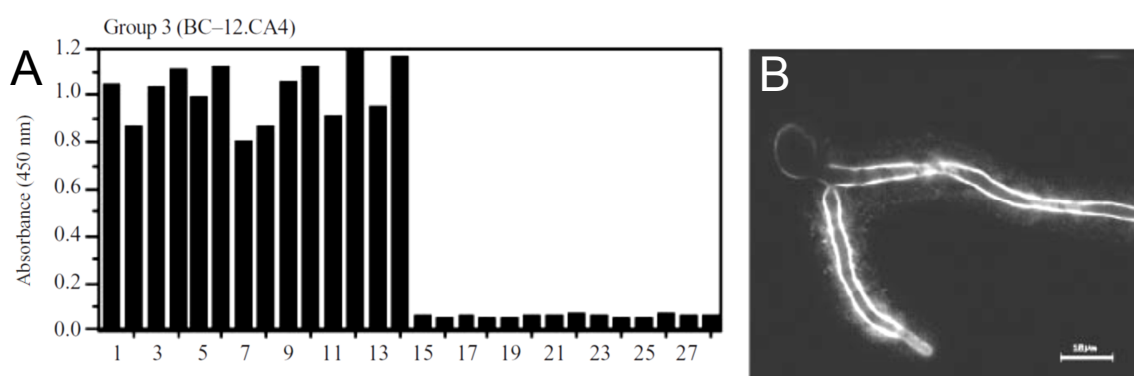


Figure 2.8: (A) Antibodies raised from BC-12 hybridoma cells tested using indirect ELISA method against surface washings of *Botrytis* spp. (col. 1-14) and unrelated fungi (col. 15 to 28). (B) Illustrates an immune fluorescence photograph of the antibody against *B. cinerea* (P-6g) spores germinated on glass slides (Meyer and Dewey, 2000).

(1) *B. byssoides*; (2) *B. allii* 42108; (3) *B. allii* HRI isolate 2; (4) *B. allii* HRI isolate 3; (5) *B. allii* HRI isolate 4; (6) *B. cinerea* P-9g; (7) *B. cinerea* IS700; (8) *B. cinerea* AG11; (9) *B. cinerea* B0510; (10) *B. cinerea* B14; (11) *B. cinerea* P-6g; (12) *B. fabae*; (13) *B. squamosa* 9; (14) *B. squamosa* 32; (15) *Alternaria. Infectoria*; (16) *A. alternata*; (17) *Aspergillus niger*; (18) *Aureobasidium pullulans*; (19) *Cladosporium macrocarpum*; (20) *C. herbarum*; (21) *Coniella fragaria*; (22) *M. hiemalis*; (23) *Stemphylium* sp.; (24) *Trichoderma harzianum*; (25) *T. viride*; (26) *Ulocladium* sp. 739; (27) *Ulocladium atrum* 738; (28) *Ulocladium atrum* 385.

2.4.5.1 Antibodies and Their Availability

There are two commercially available antibodies specific to *Botrytis*: a polyclonal antibody available from the ADGEN *B. allii* ELISA kit and a monoclonal antibody BC-12 developed by Meyer and Dewey (2000), which was acquired from Forsite Diagnostics (Sand Hutton, UK).

Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK) offers a qualitative or semi-quantitative commercial ELISA IDENTIKIT for *B. allii*. These antibodies can also be purchased separately. The antibodies are polyclonal and raised in rabbits. Further information on the antibodies regarding cross reactivity and specificity to other *Botrytis* species are not available. ADGEN Phytodiagnostics (Auchincruive, UK) also offers an ELISA IDENTIKIT for *B. cinerea* using the monoclonal antibody BC-12. Like the polyclonal antibodies against *B. allii*, the monoclonal antibody BC-12 can be ordered separately in various quantities.

2.4.5.2 Performance of the Monoclonal Antibody BC-12 in the *Botrytis* LFD

Initially, the performance of the Forsite LFD (Sand Hutton, UK) was tested using a portable line reader (Figure 2.9 B). The LFD employs the monoclonal antibody BC-12 in a sandwich format with latex as an optical label. The results of the specificity studies are illustrated in Figure 2.9, showing positive detection of all *Botrytis* species tested by using mycelium as the antigen. As previously discussed, the monoclonal antibody BC-12 used for the fungal detection is specific to surface proteins generated by growing hyphens. A detection of spores is therefore not possible, and this was also shown when the LFD was tested using *B. allii* spore solution.

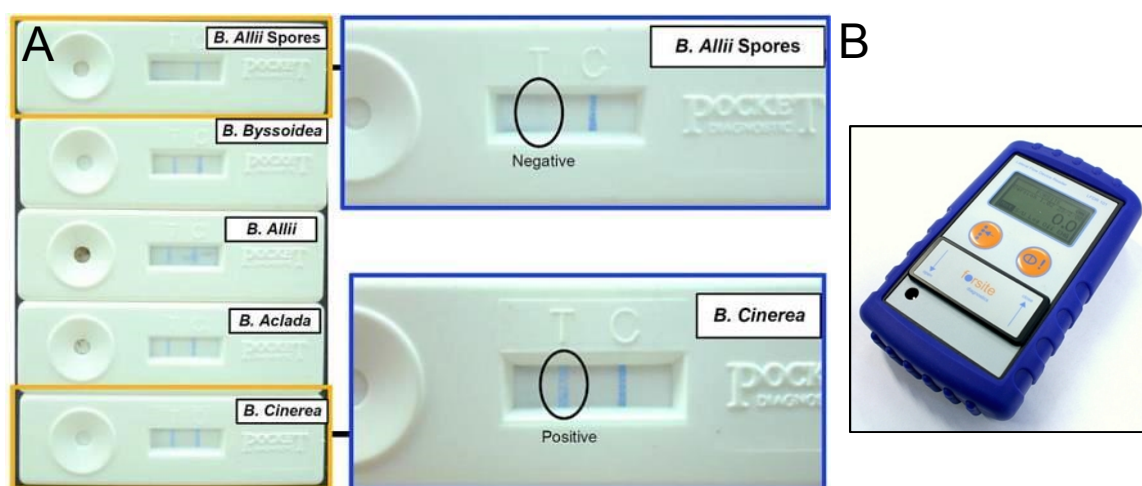


Figure 2.9: (A) Specificity test of the *Botrytis* lateral-flow device produced by Forsite Diagnostics against mycelium samples from *Botrytis* species (*B. allii*, *B. aclada*, *B. byssoides*, *B. cinerea*) and *B. allii* spores using (B) the portable reader (LFDR 101, Forsite, Sand Hutton, UK).

Lateral-flow devices can be also combined with optical line readers in order to quantify the response of the LFD. Using a reader specifically designed for this LFD, a quantification of the line signal is possible, allowing a quantification of the fungus by constructing a standard curve (Figure 2.10). The LFD performed well with a logarithmic response showing a correlation coefficient of 0.988. Calculating the limit of detection (LOD) as negative reading plus 3x its standard deviation resulted in a LOD of $7.5 \mu\text{g ml}^{-1}$. The same test was also performed using *B. cinerea* as a source of antigen, showing nearly identical results with a LOD of $7.8 \mu\text{g ml}^{-1}$. These findings are in accordance with the studies performed by Dewey et al. (2013), using the Forsite LFD combined with an optical reader as a tool to detect *Botrytis cinerea* in table and dessert wines.

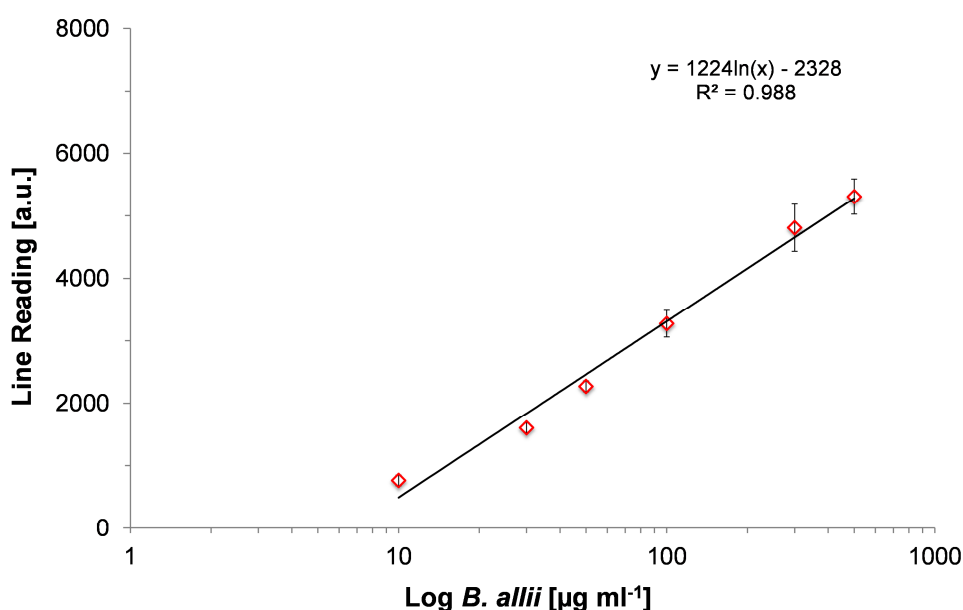


Figure 2.10: Standard curve of the Forsite *Botrytis* LFD plotted against various concentrations of *B. allii* antigen using the monoclonal antibody BC-12. The line reading was performed 15 min after pipetting 80 μl of the sample to the wick. The bars represent the standard error of the means. Assay was performed in triplicates with three consecutive readings per LFD.

2.4.5.3 Specificity of the Antibodies against Relevant *Botrytis* Species

The monoclonal antibody BC-12 acquired from Forsite and the polyclonal antibody from ADGEN Phytodiagnostics (Auchincruive, UK) were tested to confirm their capability to detect the primary species causing neck rot (*B. allii*, *B. aclada* and *B. byssoidea*) as well as *B. cinerea* which is the most common species of *Botrytis*. Mycelium samples of the four fungal species were serially diluted and assayed using a concentration ranging between 1 mg ml⁻¹ to 8 µg ml⁻¹. The colour change was generated by using goat anti-mouse and goat anti-rabbit antibody conjugated to HRP.

Both antibodies were capable of detecting all four species of *Botrytis* tested in this assay. The polyclonal antibody, being specific to several epitopes, does not show much variation in the signal response between the different *Botrytis* species (Figure 2.11 A). The resulting difference may be caused by inaccuracies of the sample extraction from the agar plate. Assay saturation was observed with both the monoclonal as well as polyclonal antibody, causing a decrease in signal when very high concentrations of antigen were used.

The variation of the immunological response of the monoclonal antibody BC-12 to the tested *Botrytis* species (Figure 2.11 B) is most likely caused by the high specificity of the monoclonal antibody to the surface proteins of the mycelia. Differences in hyphal growth patterns of the four species grown on agar are therefore affecting this specific antibody more than the polyclonal antibody. An indication for this finding is also the high immunological response to *B. byssoidea*. This species shows a dense mycelial growth with few or no spores, offering more antibody binding sites in comparison with the loose growing mycelium covered by conidia which is characteristic for *B. allii*, *B. aclada*, and *B. cinerea*. However, the sensitivity of the monoclonal antibody BC-12 is comparable to that of the polyclonal antibody.

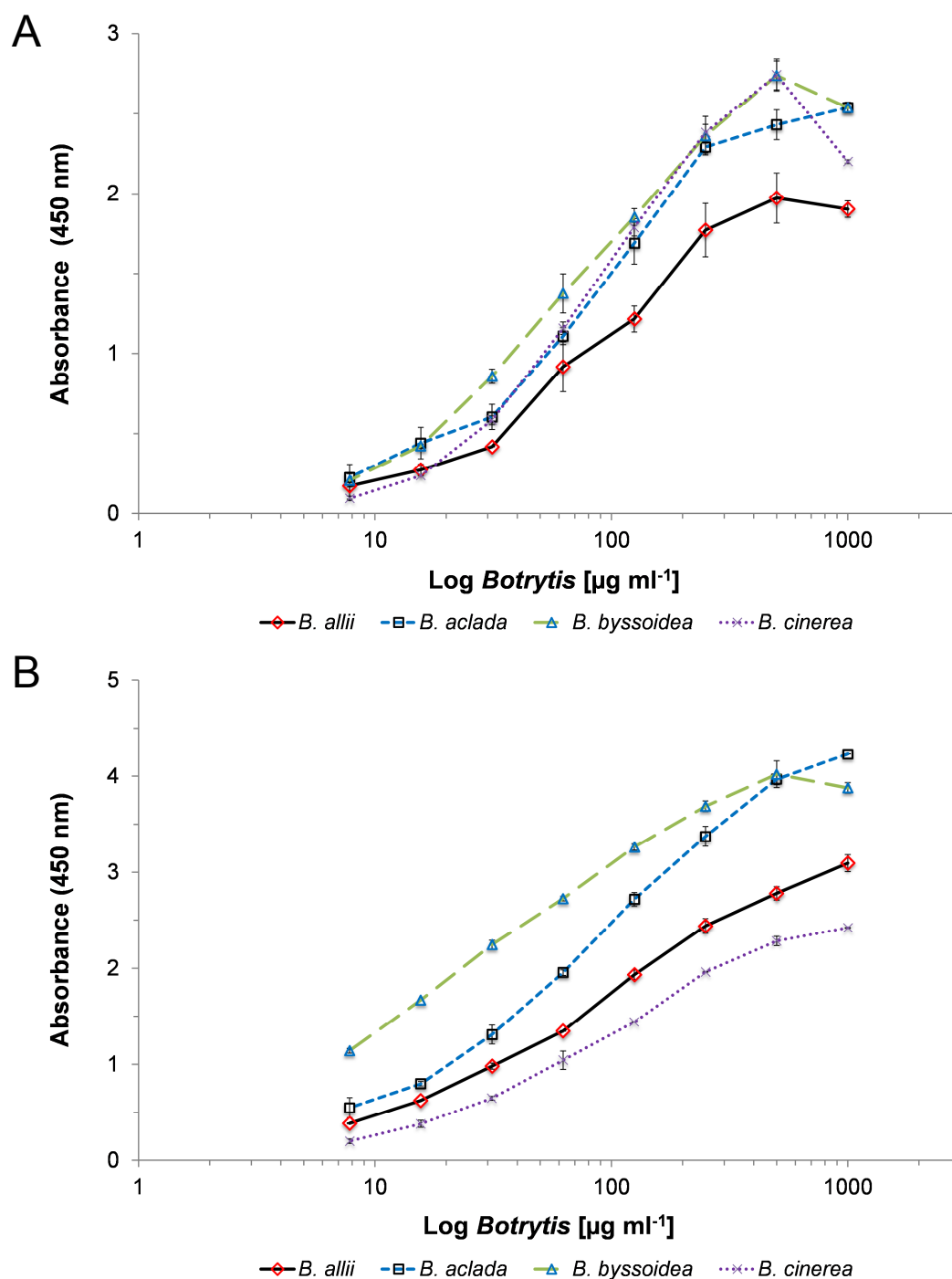


Figure 2.11: Normalised indirect ELISA response at 450 nm of two different detection antibodies ($10 \mu\text{g ml}^{-1}$) to four *Botrytis* species (*B. allii*, *B. aclada*, *B. byssoidea* and *B. cinerea*) serially diluted to a concentration ranging between 1000 and $8 \mu\text{g ml}^{-1}$. The bars represent standard error of the means ($n=3$).

(A) Polyclonal antibody used for detection followed by goat anti-rabbit conjugate.

(B) Monoclonal antibody BC-12 as detection antibody and goat anti-mouse conjugate used for signal generation.

2.4.5.4 Cross-Reactivity Studies against *Fusarium*, *Penicillium* and *Aspergillus* Species

Cross-reactivity studies were performed against *Fusarium oxysporum* (CBS192.35), which is a soil-borne pathogen causing basal rot in onions (see Section 1.3.5) and other widely spread fungi represented by *Penicillium verrucosum* (OTA 11) and *Aspergillus niger* (N1, environmental isolate). Onion basal rot caused by *Fusarium oxysporum* generally occurs when soil temperatures are very warm (optimum 28 °C) and is therefore more common as an onion pathogen in areas with warmer climates, such as Spain (Agrios, 2005a). However, fungal basal rot was found to be the third most common onion disease after neck rot and bacterial infections within the field trials performed during this study (see Section 5.4.1.1).

Both, the monoclonal as well as the polyclonal antibody, showed high specificity to all *Botrytis* species tested by using the ELSA assay developed in this work (Figure 2.12). The cross reactivity and specificity studies performed by Meyer and Dewey (2000) of the monoclonal antibody BC-12 were confirmed in this experiment. The BC-12 hybridoma cell line showed no cross reactivity with other common fungi represented by *Fusarium oxysporum*, the cause of onion basal rot, *Penicillium verrucosum* and *Aspergillus niger* (Figure 2.12 A).

Figure 2.12 (B) illustrates the indirect ELISA results of the polyclonal antibodies. Unlike the BC-12 antibody, the polyclonal antibodies showed cross reactivity with *Fusarium oxysporum*, *Penicillium verrucosum* and *Aspergillus niger*. The highest absorbance value regarding cross reactivity was achieved by *Fusarium oxysporum* and *Aspergillus niger* with approximately 50 % of the absorbance compared to the *Botrytis* species (Table 2.3).

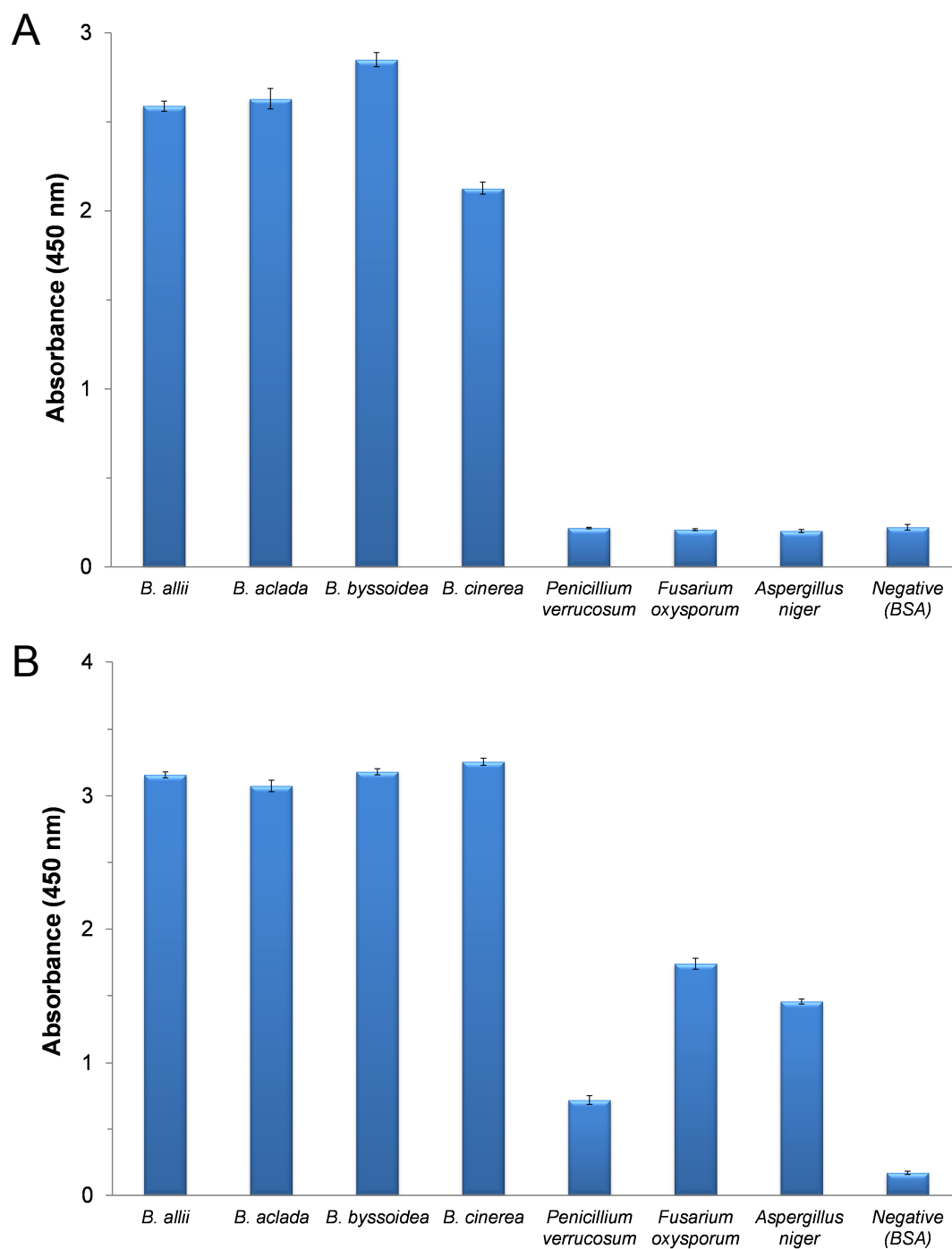


Figure 2.12: Indirect ELISA response of two different detection antibodies with a concentration of $10 \mu\text{g ml}^{-1}$ to four *Botrytis* species (*B. allii*, *B. aclada*, *B. byssoidea* and *B. cinerea*) and other common fungi represented by *Fusarium oxysporum*, *Penicillium verrucosum* and *Aspergillus niger*. Fungal concentration is 1 mg ml^{-1} . The bars represent the standard error of the means ($n=3$).

(A) Monoclonal antibody BC-12 as detection antibody and goat anti-mouse conjugate used for signal generation.

(B) Polyclonal antibody used for detection followed by goat anti-rabbit conjugate.

Table 2.3: Indirect ELISA response at 450 nm and the relative activity of the polyclonal antibody to *Botrytis* species (*B. allii*, *B. aclada*, *B. byssoidea* and *B. cinerea*) and other common fungi represented by *Fusarium oxysporum*, *Penicillium verrucosum* and *Aspergillus niger*.

Fungal Species	Absorbance	Standard Deviation	Relative Activity [%] ^a
<i>B. cinerea</i>	3.254	±0.046	100.0
<i>B. byssoidea</i>	3.179	±0.039	97.6
<i>B. allii</i>	3.156	±0.039	96.8
<i>B. aclada</i>	3.074	±0.074	94.2
<i>Penicillium verrucosum</i>	0.717	±0.057	17.9
<i>Fusarium oxysporum</i>	1.743	±0.070	51.1
<i>Aspergillus niger</i>	1.456	±0.032	41.8
Negative control (BSA)	0.166	±0.021	0.0

^a Relative activity in % compared to the highest absorbance value achieved by *B. cinerea*. The data were normalised by subtracting the negative reading (BSA) from the absorbance.

As the polyclonal antibodies are raised by injecting the fungal antigen to an animal thus evocating an immunological reaction, the resulting antibodies are specific to several different epitopes of the antigen. Because of the multiplicity of antigenic sites, a heterogeneous mixture of different antibodies of varying specificity is produced (Tothill, 2003a). Especially the raise of polyclonal antibodies from mycelial fragments is very difficult due to the lack of specificity and strong cross reactions with other fungi (Dewey, 1996). Some surface structures are shared by several fungal species resulting in similar epitopes that are detected by the antibody. As a result, the polyclonal antibodies showed cross reactivity with *Fusarium oxysporum*, *Penicillium verrucosum* and *Aspergillus niger*. However, on the other hand their capability to detect several epitopes is beneficial regarding signal strength. The high absorbance values of the polyclonal antibodies are constant independently of the applied species of *Botrytis*. Therefore, in order to ensure that the developed sensor in this work is specific to *Botrytis* species, it is recommended to use the monoclonal antibody as capture molecule, while the polyclonal can be used as the detection antibody.

2.4.6 ESTIMATION OF THE PATHOGEN LOAD OF INFECTED ONION PLANTS USING INDIRECT ELISA

The target sensitivity of the biosensor depends on the pathogen load of infected onion plants. This information is crucial for the future development of the sensor. However, at the current stage, the literature cannot provide answers to this question. Therefore, a quantification experiment with inoculated onion plants was designed to estimate the pathogen load. Two onion sets grown for approximately three months under controlled conditions provided by the greenhouse were inoculated with *B. allii* spore solution. High humidity is directly correlated to an increased risk of neck-rot infections (Maude and Presly, 1977b). Considering these findings, the onion plants were exposed to weather instead of growing them in the greenhouse after inoculation.

2.4.6.1 Permeability of the Filter to *B. allii* Antigen

The sample extraction protocol supplied with the *B. allii* ELISA kit (ADGEN Phytodiagnostics, Auchincruive, UK) suggests filtering the samples through a layer of muslin or other cotton materials after grinding with a mortar and a pestle. Though this method is inexpensive and easy to perform, there are several drawbacks. Muslin has a rough fibre structure with varying pore sizes. Antigen molecules are more likely to adsorb to a rough surface compared to a smooth material like glass that can also provide a filter system with a defined pore size.

In order to improve the sensitivity and reproducibility of the sample extraction, the sample was filtered through a 30 ml glass filter tube with the porosity 0 (160 to 250 μm) (Robu Glassware) using a changeable, sterile 20 mm pre-filter disc with the porosity 1 (100 to 160 μm) purchased from Jencons (Lutterworth, UK). The 30 ml glass filter tube has an integrated filter that cannot be changed. This filter was chosen to have the largest pore size of 160 to 250 μm . The 20 mm pre-filter disk with the pore size of 100 to 160 μm is changeable and prevents the integrated filter to be jammed by plant material. This porosity was chosen for the test, as it is permeable for mycelial cells. Though the exact dimensions of *Botrytis* mycelium cannot be found in the literature, the approximate numbers can be estimated by extrapolating the size of the mycelium based

on the defined size of their conidia which is well known and an established characteristic for each species using microscopic images. Regarding these figures, a mycelial cell was estimated to have a cylindrical shape with a diameter of 15 to 30 μm and a length of 40 to 70 μm .

To test the permeability of the sample processing system for *Botrytis* antigen, a filtration experiment was performed by comparing the absorbance value of an antigen solution before and after filtration using the polyclonal as well as the monoclonal antibody BC-12. The absorbance values after filtration (Figure 2.13) vary by 1.48 % for the polyclonal and 0.98 % for the monoclonal antibody BC-12. Both values are within the standard deviation of the assay and indicate that the filtration system is permeable for the *Botrytis* antigen.

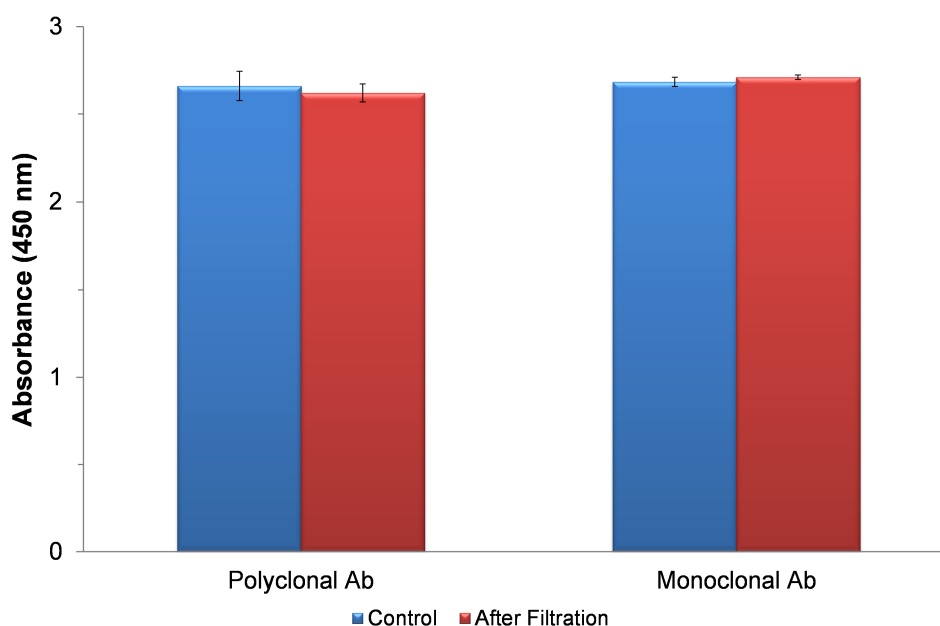


Figure 2.13: Indirect ELISA response of two different detection antibodies with a concentration of $10 \mu\text{g ml}^{-1}$ followed by the corresponding anti-species conjugate ($0.3 \mu\text{g ml}^{-1}$) to *Botrytis allii* antigen ($100 \mu\text{g ml}^{-1}$), before and after filtration. The fungal solution was vacuum filtered, using a 30 ml glass tube with the filter porosity 0 (160 to 250 μm) in combination with a sterile 20 mm pre-filter disc with the porosity 1 (100 to 160 μm). The bars represent standard error of the means ($n=3$).

2.4.6.2 Quantification of the Pathogen Load

The onion samples were grown for 14 days outside the greenhouse exposed to weather, allowing the fungus to grow and spread from the leaf tip towards the bulb. The leaves of the inoculated plants were subsequently tested using the commercially available *B. allii* and *B. cinerea* ELISA kits (ADGEN Phytodiagnostics, Auchincruive, UK). The absorbance values of the leaf sections as well as the corresponding standard curve are presented in Figure 2.14. The two onion plants tested within this study show a common pattern. The tip section comprising the site of inoculation have the highest pathogen load which was calculated at $4.7 \mu\text{g g}^{-1}$ onion tissue (polyclonal) and at $3.0 \mu\text{g g}^{-1}$ onion tissue for the monoclonal antibodies. The high value is caused by the mycelial growth and not by the *B. allii* spores, as spores cannot be detected by both antibodies (see Section 2.4.5). The pathogen load is gradually decreasing towards the bulb with an average of $3.5 / 1.2 \mu\text{g g}^{-1}$ *B. allii* on onion tissue (polyclonal / monoclonal antibodies) for the middle section, and $2.9 / 1.1 \mu\text{g g}^{-1}$ onion tissue (polyclonal / monoclonal antibodies) for the neck section of the onion leaf. All samples of the infected leaves showed a higher absorbance value compared with not inoculated onion leaves provided by three different plants grown under controlled environment in the greenhouse, as well as the negative control of the ADGED *B. allii* ELISA kit.

The results of this ELISA test are in accordance with the findings of Tichelaar (1967) and Maude and Presly (1977a), who observed *B. allii* infections as mycelium growing extra and intracellular over the onion leaves, by using radioactively marked conidia as well as plating and staining techniques. They estimated the fungus to reach the onion bulb after approximately four to eight weeks, depending on the site of inoculation. However, their findings were based on mature onion plants that have an average leaf size of 40 to 70 cm. The more sensitive ELISA results showed that the fungus could be detected in all three sections of the leaf two weeks post inoculation.

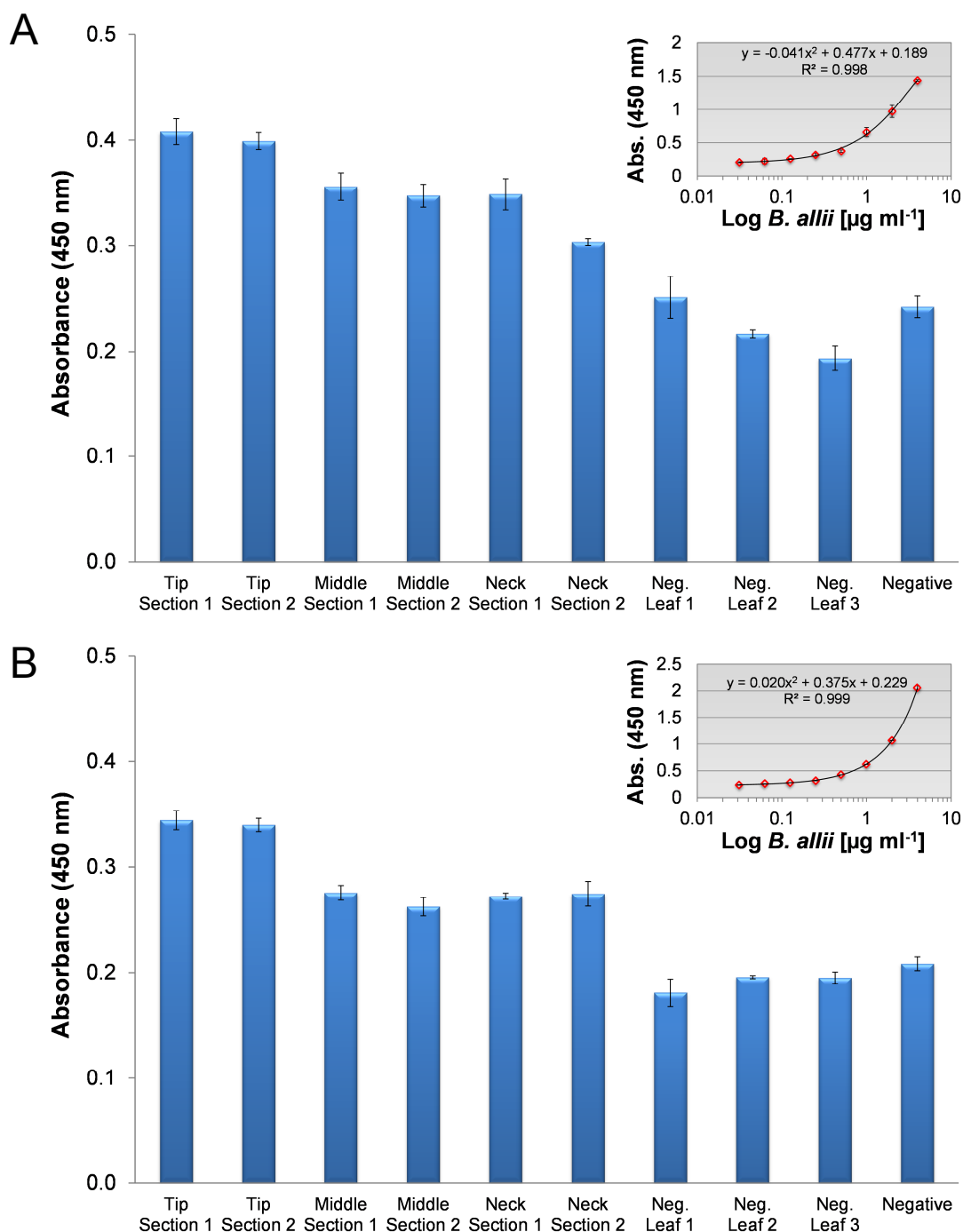


Figure 2.14: Indirect ELISA response of sampled leaf sections using (A) polyclonal antibody ($10 \mu\text{g ml}^{-1}$) for detection followed by goat anti-rabbit conjugate ($0.3 \mu\text{g ml}^{-1}$) and (B) the monoclonal antibody BC-12 ($10 \mu\text{g ml}^{-1}$) for detection followed by goat anti-mouse conjugate ($0.3 \mu\text{g ml}^{-1}$) for signal generation. The tip section was inoculated with 10^5 *B. allii* spores and incubated for two weeks exposed to weather. The pathogen load of each plant section was then quantified by plotting the absorbance value to a *B. allii* standard curve (embedded graph). The bars represent standard error of the means (n=3).

Meyer and Spotts (2000) used a quantitative ELISA for the detection of *Botrytis cinerea* in pear stems using the monoclonal antibody BC-12. Infections were found to be visible at a fungal concentration over $200 \mu\text{g g}^{-1}$ stem tissue. Latent infections usually showed a *Botrytis* concentration of less than $35 \mu\text{g g}^{-1}$ stem tissue. Fernandez-Baldo et al. (2010; 2011) quantified the pathogen load of *B. cinerea* in pears, apples and grapes with a competitive indirect ELISA method. Like Meyer and Spotts (2000), the monoclonal antibody BC-12 was used for the assay. The pathogen load of *B. cinerea* was found to be approximately $100 \mu\text{g g}^{-1}$ fruit tissue after four days of incubation at 25°C and approximately $700 \mu\text{g g}^{-1}$ fruit tissue after 10 days of incubation. However, *B. cinerea* infections are more vigorous and only symptomless in the early stage of the development (Sanzani et al., 2012; Saito et al., 2013). Comparing these numbers to the ELISA results showing a pathogen load of approximately 3.5 to $1 \mu\text{g B. allii}$ per gram leaf tissue, the biosensor has to show a much lower limit of detection of at least $1 \mu\text{g g}^{-1}$ onion tissue for the quantification of *B. allii* infections compared with *B. cinerea*.

2.5 CONCLUSIONS

The Bradford method was successfully used to quantify the protein content of an unknown solution. The accuracy of the method is adequate for the determination of the protein concentration of the antibody solution.

Studies on the characteristic of *Botrytis* species and the growth performance of the fungus using different agar substrates are of particular importance when applied to the sensor development. Different antigens gained directly from the agar plate, or indirectly, using a protocol for freeze-drying the fungus, result in various signal strength, depending on the purity of the antigen. Despite comparative studies, it is still not clear what the best source of antigen for the use in immunological detection methods is. For this work, three different methods were found to be the most suitable for the production of fungal antigen. Raw mycelium scraped off from the agar plate or grown on liquid cultures was used for immunological studies, whereas for the extraction of pathogen DNA, the fungus was grown on agar containing a thin cellophane membrane.

Testing the conjugation efficiency of the monoclonal antibodies to HRP in comparison with the polyclonal antibodies showed that lower signals were achieved compared with the polyclonal conjugate. Therefore, the polyclonal conjugate was chosen for the subsequent purification process in order to remove excess HRP and was used as detection antibody for the sensor development. A sandwich format where the monoclonal antibodies are used to capture the fungus and the polyclonal antibodies conjugated to HRP for detection is suggested as a good format for the sensor development.

The polyclonal antibodies purchased from the commercial ELISA kit (ADGEN Phytodiagnostics) as well as the monoclonal antibody BC-12 (Forsite, Sand Hutton, UK) are capable of detecting all three species considered as the primary cause of neck rot. Furthermore, other *Botrytis* species, for example *B. cinerea*, are also recognised by the antibodies. The sensor, which will be developed based on using these antibodies, will therefore incorporate various applications for the detection of *Botrytis* and will not be limited to the detection of neck rot. Furthermore, a DNA sensor will be developed in this study to complete the immunosensor result by identifying the fungal species using PCR with primers targeting neck-rot species.

The monoclonal antibody BC-12 showed no cross reactivity with *Fusarium oxysporum*, which is a soil-borne pathogen causing basal rot in onions, and other widely spread fungi represented by *Penicillium verrucosum* and *Aspergillus niger*. The high specificity of the BC-12 hybridoma cell line was confirmed, resulting in a high qualification of the monoclonal antibody BC-12 for the use as a capture antibody. The polyclonal antibody was found to have 51 % cross reactivity with *Fusarium oxysporum* and 42 % to *Aspergillus niger*. Therefore, their application as the detection antibody in the sandwich assay will be recommended, since this will ensure the specificity of the sensor to *Botrytis* species that will be developed in this work. The application of these heterogeneous antibodies for detection will also ensure a steady signal, which will not be affected by structural differences of the *Botrytis* species.

Initial ELISA tests demonstrated that infected onion bulbs showing visible signs of neck rot, have a pathogen load of 1 to 3 mg g⁻¹ onion tissue and symptomless leaf infections of 3.5 to 1 µg g⁻¹ onion tissue. Furthermore, the quantification of the pathogen load depends on the *Botrytis* species and antigen processing of the fungus used for the standard curve, the antibody and the sample processing technique. Standardised test methods for the quantification of *Botrytis* infections are not available. Therefore, the pathogen load can only be estimated and should not be seen as an absolute numbers. However, based on the ELISA results, the target limit of detection is at least 1 µg *Botrytis* per gram onion tissue or 0.1 µg ml⁻¹ using a 1:10 sample/buffer dilution factor for the extraction procedure.

CHAPTER THREE

ELECTROCHEMICAL IMMUNOSENSOR FOR *BOTRYTIS*

3 ELECTROCHEMICAL IMMUNOSENSOR FOR *BOTRYTIS*

3.1 INTRODUCTION

The current market trend is tending to low cost, portable and rapid off-site detection methods, alleviating the need to send a sample to a specialist laboratory (Tothill, 2003b). To achieve these goals, biosensors are the ideal solution to fulfil these requirements in a modern detection system and are therefore fast becoming a major contender in the race for commercially successful, robust, immune-based detection methods. In particular, screen-printed electrodes (SPE) are the most suitable biosensor format as they are portable, easy to use, and offer the advantage of a highly sensitive electrochemical transducer (Davies et al., 2007a).

The fundamental principle of electrochemical immunosensors is the ability of some enzymes to catalyze a substrate in such manner that electrons are either produced or utilised in a redox reaction. However, the vast majority of enzymes do not have the ability of direct electron transfer. This difficulty may be caused by the location of the electroactive region of the enzyme, which is often located deep within its structure, or due to effects connected with surface immobilisation or orientation of the molecule (Newman and Turner, 2007). In the early 80s, a group of scientists based at Cranfield and Oxford Universities, realised that employing redox couples, known as mediators, can eliminate many problems associated with electron transfer of the enzymes, which turned out to be in retrospect a forerunner of the glucose sensor (Cass et al., 1984). Mediators work as an electron shuttle between the redox centre of the enzyme and the electrode. However, certain enzymes or redox proteins can exhibit electrical communication by overcoming the steric insulation of the active site by the protein matrix. With electrode supports, a biocatalytic transformation of the enzyme can be stimulated electrochemically, which can drive the substrate close enough to the enzyme's active site to make non-mediated electron transfer possible (Arya et al., 2007). These sensor systems employ electroactive enzymes, such as HRP or alkaline phosphatase, and alleviate the need of a mediator, giving them an edge in the race for robust immune-based biosensors.

This Chapter describes the development of an electrochemical immunosensor for *Botrytis* using screen-printed gold electrodes (SPGE) based on the schematic diagram shown in Figure 3.1. Immobilised antibodies on the gold working electrode capture the fungus contained in the sample prior to the addition of the HRP-labelled secondary antibodies for detection and quantification of the pathogen load. For signal generation, the TMB/H₂O₂ (3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate / hydrogen peroxide) system was used in combination with the enzyme horseradish peroxidase (HRP) (Figure 3.1 B). The induced change of the reduction current of the TMB-HRP-H₂O₂ system was measured using an electrochemical analyser and the detection was performed by chronoamperometric measurement at an applied potential of -0.2 V, versus silver / silver chloride (Ag/AgCl) pseudo-reference electrode. The resulting current can be directly related to the amount of the HRP and consequently proportional to the concentration of *Botrytis*.

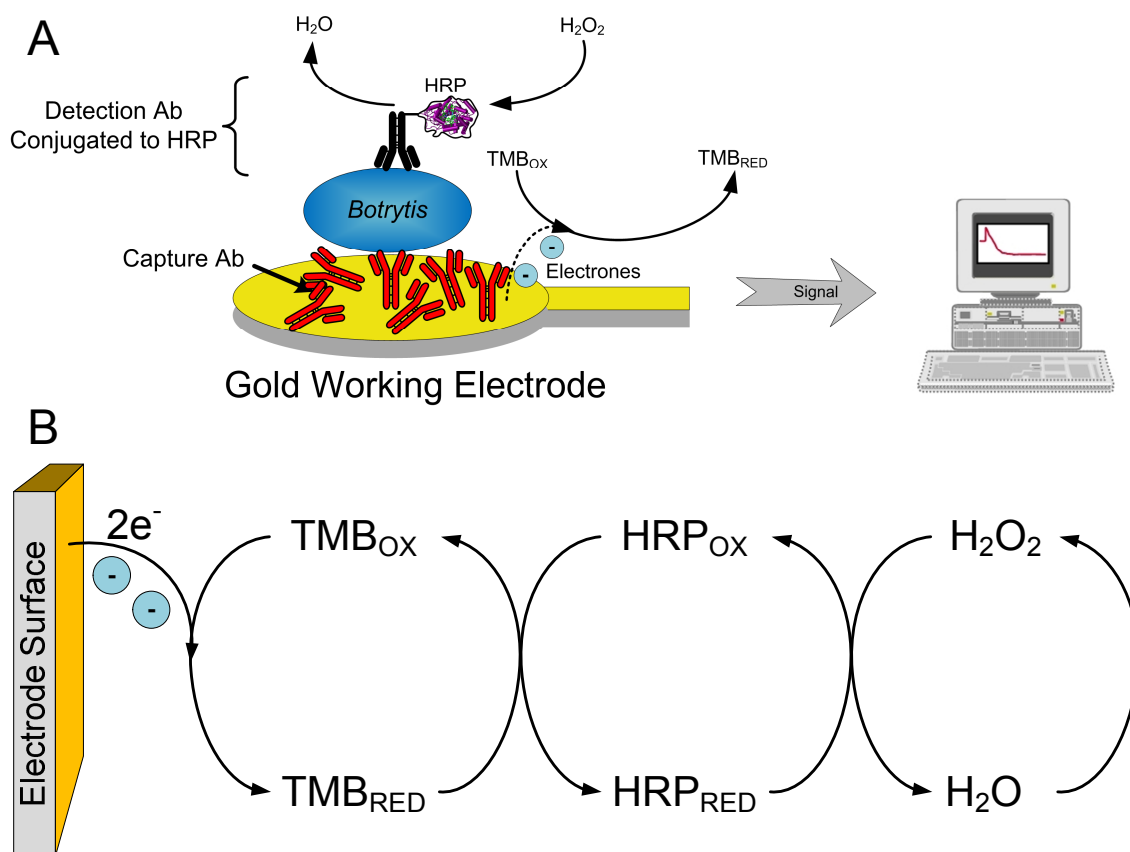


Figure 3.1: Working principle of the immunosensor with HRP as electroactive species (A), using the TMB-HRP-H₂O₂ redox couple system for signal generation (B).

Most common examples of the enzymes employed in amperometric detection are glucose oxidase, lactate oxidase, peroxidase and alkaline phosphatase (Arya et al., 2007). In this study, horseradish peroxidase (HRP) was selected as the enzyme label and TMB/H₂O₂ as the electroactive substance for signal generation. This enzyme system operates mediator free and combines very fast reaction kinetics and high substrate turnover rates, resulting in a strong signal generation in a relatively short time span, with good biological performance (Mackey et al., 2007; Kandimalla et al., 2008; Parker and Tothill, 2009). Its stable and compact design allows an effective conjugation to antibodies or gold nanoparticles. TMB is neither mutagenic nor carcinogenic and it provides a more sensitive substrate for HRP than traditional substrates such as O-phenylenediamine (OPD) and 2,2'-azino-bis-(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS) and is therefore the most used substrate for HRP in commercial assays (Porstmann and Kiessig, 1992; Fanjul-Bolado et al., 2005).

These key features of the TMB-HRP-H₂O₂ redox couple combined with a sensitive detection are beneficial to achieve the final goal of this study namely to develop a rapid, portable and affordable biosensor to assess the pathogen load of *Botrytis*. Employing this biosensor system can help to inform the curing regime to further suppress neck-rot disease caused by *Botrytis* species.

3.2 MATERIALS

3.2.1 GENERAL CHEMICALS AND INSTRUMENTATION

Albumin from bovine serum (BSA), horseradish peroxidase (HRP) type II/VI, HPLC water, phosphate buffered saline tablets (PBS, 0.01 M phosphate buffer, 0.137 M sodium chloride and 0.0027 M potassium chloride, pH 7.4), Tween 20, potassium chloride, 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB), hydrogen peroxide solution (30 % w/w) in dH₂O, carbonate-bicarbonate buffer (0.05 M, pH 9.6), phosphate-citrate buffer (0.05 M, pH 5.0), sodium citrate (pH 8.1), sodium acetate, sulphuric acid (H₂SO₄, 95-98 %), potassium ferricyanide (K₄ Fe (CN)₆ · 3H₂O), 3,3-dithiodipropionic acid (DTDPA) and 11-mercaptopundecanoic acid (MUDA) were purchased from Sigma (Dorset, UK). ELISA coating buffer (02-002) was acquired from ADGEN Phytodiagnostics (Auchincruive, UK). Sure Blue TMB solution and milk blocking solution concentrate were purchased from KPL (Gaithersburg, USA).

The goat anti-rabbit and goat anti-mouse IgG antibody conjugated to HRP were purchased from Invitrogen (Paisley, UK). The monoclonal antibody BC-12 was acquired from Forsite Diagnostics (Sand Hutton, UK) and the polyclonal *B. allii* antibody was obtained from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK). Antibodies were conjugated to HRP using the Lightning-Link conjugation kit purchased from Innova Biosciences Ltd. (Cambridge, UK) and purified with a Pierce conjugate purification kit purchased from Thermo Scientific (Cramlington, UK). N-hydroxysuccinimide (NHS) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) were purchased from Thermo Scientific (Cramlington, UK). Gold nanoparticles size Ø20, Ø40 and Ø60 nm were purchased from BBI International (Cardiff, UK).

Porcelain mortar and pestle (Ø70 mm) were purchased from Scientific Laboratory Supplies (Nottingham, UK), 30 ml filter tube porosity 0 (160 to 250 µm) (Robu Glassware) and 20 mm pre-filter disc porosity 1 (100 to 160 µm) were purchased from Jencons (Lutterworth, UK).

Absorbance measurements for ELISA were performed using the Fluostar Galaxy reader (BMG, Offenburg, Germany). Solid phase 96 immuno wells were acquired from NUNC Maxi Sorp (NUNC Thermo Fisher Scientific, Roskilde, Denmark). Incubation at 37 °C was performed under controlled environment provided by an incubator/shaker model iEMS 1415 (Labsystem, Helsinki, Finland). The vortex mixer model Genie 2 G-560E was purchased from Scientific Industries (Bohemia, USA). STUART rotatory and roller mixer (model MRP-465-020V) were purchased from Fisher Scientific (Loughborough, UK).

All electrochemical measurements were conducted using a 4-channel analyser PGSTAT10 (Figure 3.2 A) purchased from Autolab (Metrohm, Runcorn, UK) with the general-purpose electrochemical software GPES 4.9.007 (Figure 3.2 D) for single and multichannel readings. The measured signal was converted to a digital output by the USB box (Figure 3.2 C). For the reading, the screen-printed electrodes were plugged into boxed connectors (Figure 3.2 B) purchased from Drop Sens (Oviedo, Spain).

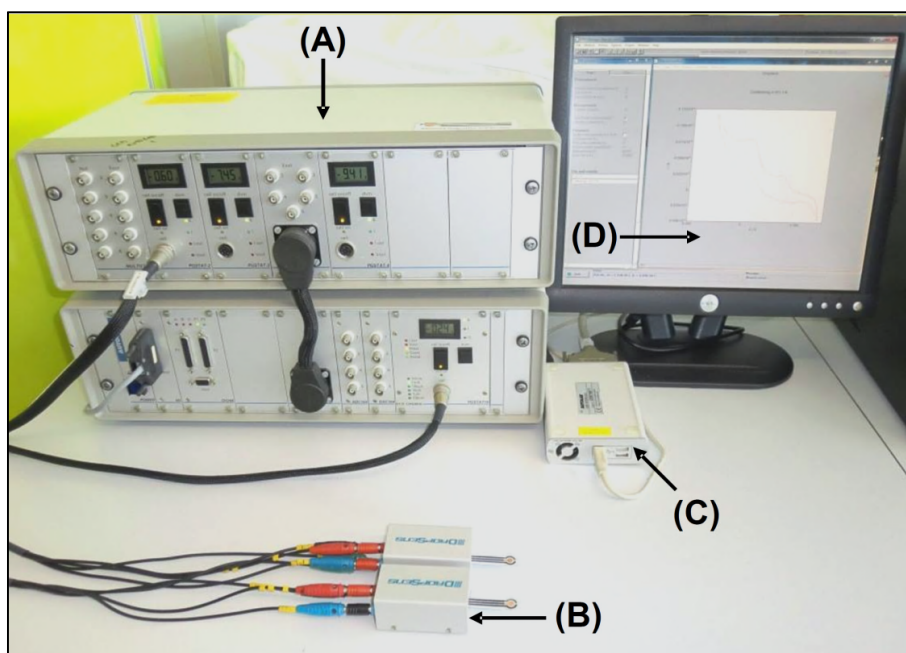


Figure 3.2: Electrochemical measurement setup: The sensor is attached to the Drop Sens box connectors (B). The signal of each channel is measured individually using the Autolab PGSTAT10 analyser (A), converted by the USB box to a digital signal (C) and displayed through the GPES 4.9.007 software (D).

3.2.2 SCREEN-PRINTED GOLD ELECTRODES

The various types of sensors for the assay development were designed by Cranfield University and printed at DuPont Microcircuit Materials (Bristol, UK). The screen-printed gold electrodes (Figure 3.3) consisted of a gold working electrode (0.226 cm^2 planar area), carbon counter and Ag/AgCl reference electrode. Table 3.1 comprises a list of the ink compositions used for the production of each sensor type. Electrodes were printed onto $125 \text{ }\mu\text{m}$ thick PET (polyethylene terephthalate) sheets. The base carbon print (7102 and BQ221) had been IR dried, the subsequent inks had been box-oven dried at $140 \text{ }^\circ\text{C}$ for 30 min. Before the assay was performed, the sensor was heat-treated using the Carbolite oven model PN 120 (200) (Hope, UK).

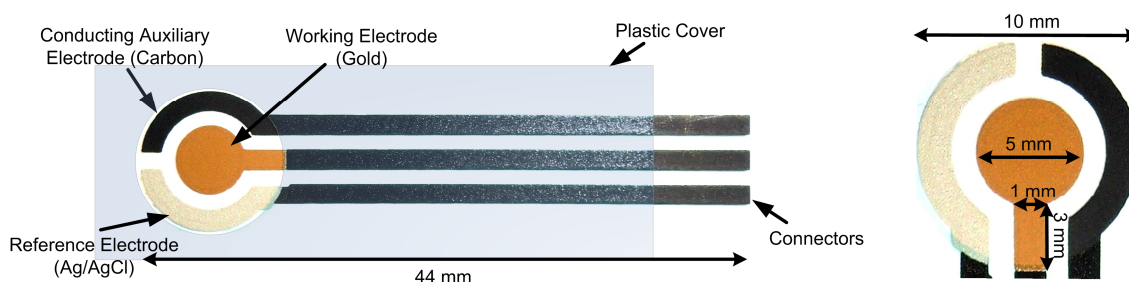


Figure 3.3: Screen-printed gold electrode with carbon counter electrode and silver/silver chloride reference electrode (printed at DuPont, Bristol, UK).

Table 3.1: Overview of the three DuPont sensor types JA, JC and JD and their composition.

Building Sequence	Sensor Type		
	JA	JC	JD
Carbon	7102	7102	BQ221
Gold	BQ331	BQ331	BQ331
Ag/AgCl	5870	5880	5880
Encapsulation	5036 blue	5036 blue	5036 blue

Figure 3.4 provides an overview of the screen-printing process used for the production of the electrodes used in this study. The Cranfield design screen-printed sensor is using a three-electrode system comprising a gold working electrode, a carbon counter and a silver-silverchloride reference electrode. The main focus of the project is to assess the pathogen load in field conditions to provide disease control, making the application of SPGEs ideally suitable for the detection of *Botrytis*.

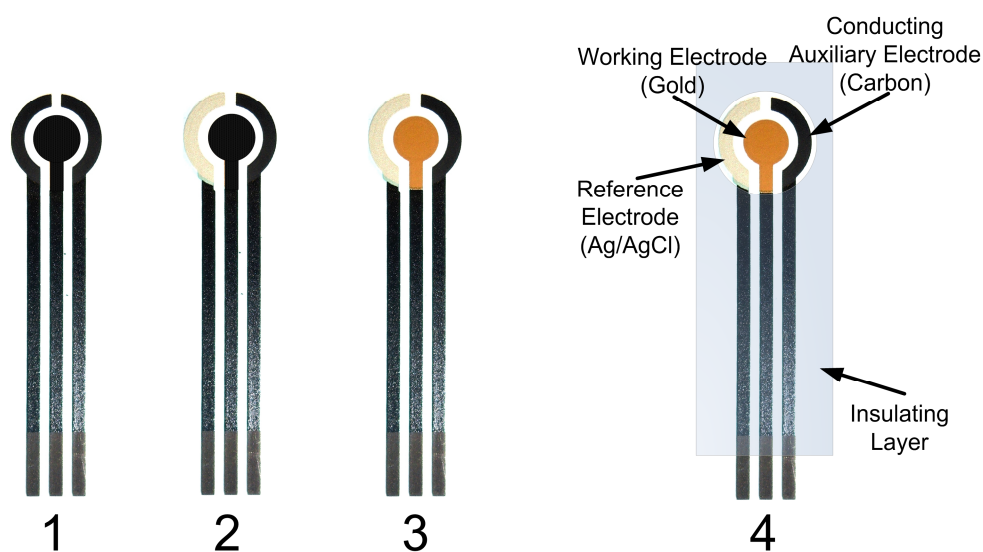


Figure 3.4: Production process of the Cranfield design screen-printed electrodes used in this study. (1) Application of the carbon ink to the plastic base. (2) Ag/AgCl reference electrode coated to the carbon. (3) Print of the gold working electrode. (4) Cover of vital parts of the electrode with a protective insulating layer.

3.3 METHODS

3.3.1 ELECTROCHEMICAL MEASUREMENTS AND DATA ANALYSES

Two different techniques were used for the analysis and evaluation of the screen-printed gold electrodes (SPGE), cyclic voltammetry and chronoamperometry. All electrochemical measurements were performed with a 4-channel analyser PGSTAT10 purchased from Autolab (Metrohm, Runcorn, UK) with the general-purpose electrochemical software GPES 4.9.007 for single and multichannel readings.

3.3.1.1 Cyclic Voltammetric Analyses

Cyclic voltammetry was used to characterise the surface of the gold working electrode of the different sensor types using potassium ferricyanide ($\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$) and 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate TMB. These substances can be electrochemically reduced and oxidised producing reduction and oxidation peaks. The peak search in the cyclic voltammogram was carried out employing the software GPES 4.9.007. Generally, the peaks of a potassium ferricyanide cyclic voltammetry are not completely symmetric to the 0-ampere x-axis. A basic minimum-maximum search would not reflect the precise values of the peaks. Therefore, a linear baseline was first plotted to both the anodic and cathodic peak before the peak location and height were determined (Figure 3.5). However, this method was not suitable for the cyclic voltammogram of TMB as the shape of the cathodic and anodic peaks is not uniform. Here, the x-axis was used as baseline for the peak search.

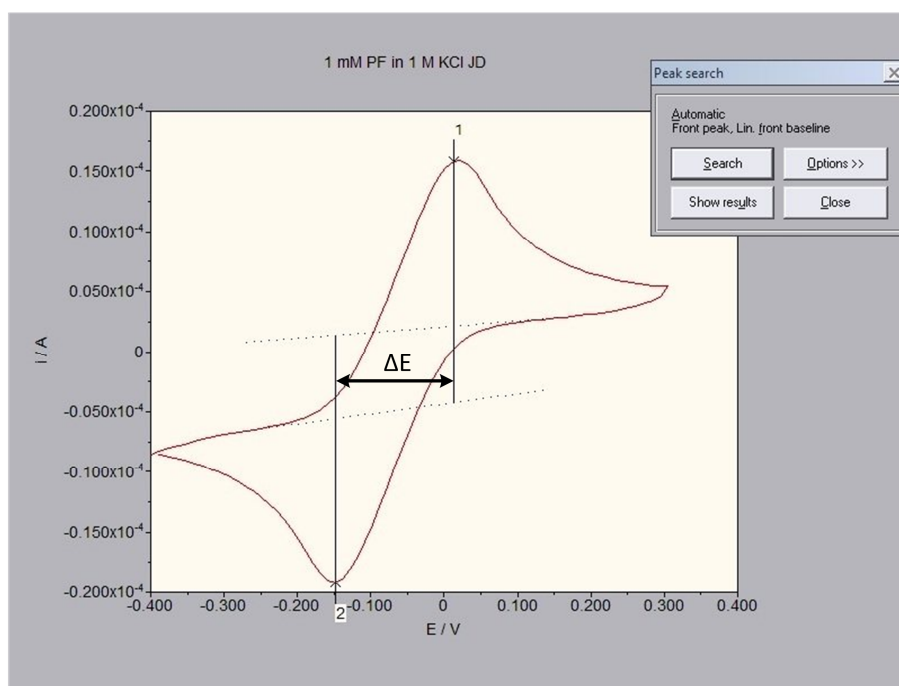


Figure 3.5: Cyclic voltammogram of a JD sensor with a plotted linear baseline for peak scanning using the GPES 4.9.007 software.

3.3.1.2 Chronoamperometric Analyses

The chronoamperometric reading of the sensor was carried out using the step amperometry function of the GPES software. Chronoamperometric studies performed in this Chapter showed that the optimal step potential for the signal generation of the TMB/H₂O₂-HRP system is -0.2 V. This potential was applied 10 s after the start of the reading and held for further 90 s. The quantification of the fungus occurred through plotting the steady state current after 100 s against the fungal concentration. Occasionally, when high numbers of replicates were required with limited time for the reading, a fast measurement was performed using the value after 70 s. After each scan, the raw data were exported to Excel for further processing. In general, for fast reading of multiple sensors, two electrodes were measured at the same time using the multichannel software with two Autolab analysers utilised alternatingly. In this case, the substrate was applied to the sensor electrodes simultaneously using two pipettes or an eight-channel multipipette with two tips. All experiments were performed in a laboratory environment with a room temperature of 18 to 22 °C.

3.3.1.3 Non-Linear Regression and Statistical Evaluation

Non-linear regression was performed using the generalised reduced gradient (GRG) method (Lasdon et al., 1978; Smith and Lasdon, 1992) of the SOLVER analysis tool. Curve fitting and statistical calculations were accomplished as described by Brown (2001) with x being the independent, y the dependent variable, n the number of data points or replicates and μ the average:

$$\text{Degree of freedom:} \quad df = n_{\text{Data}} - n_{\text{variables}} \quad (\text{Equation 3.1})$$

$$\text{Standard deviation:} \quad SD = \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n - 1}} \quad (\text{Equation 3.2})$$

$$\text{Standard error:} \quad SE = \sqrt{\frac{\sum_{i=1}^n (y_i - y_{Fit})^2}{df}} \quad (\text{Equation 3.3})$$

Standard error of means:
$$\text{SEM} = \frac{s}{\sqrt{n}} \quad (\text{Equation 3.4})$$

Coefficient of variation:
$$\text{CV} [\%] = \frac{s}{\mu} \cdot 100 \quad (\text{Equation 3.5})$$

Correlation coefficient:
$$R^2 = 1 - \frac{\sum_{i=1}^n (y_i - y_{Fit})^2}{\sum_{i=1}^n (y_i - \bar{y})^2} \quad (\text{Equation 3.6})$$

Several variations of the commonly used four parameter non-linear function that can be found in the literature for the standardisation of the immunoassay response, are all functional if not algebraic equivalents (Finney, 1974; Tijssen, 1985; van Vunakis and Tijssen, 1987). However, in order to provide a more general form than the four parameter fit function, Finney (1983) firstly described the five parameter non-linear function (Equation 3.7). By adding a compensation factor for asymmetry, the fit of the standard curve is in many cases more accurate than the more static four parameter logistic equation (Warwick, 1996). For a perfect symmetric fit the compensation factor e approximates to one and the five parameter fit function turns into a normal four parameter non-linear function. To construct a standard curve for the sensor, the data of the immunoassay response were normalised by subtracting the average of the negative control before fitting a five parameter logistic equation described by Warwick (1996):

$$Y = d + \left[\frac{(a - d)}{\left(1 + \left(\frac{p_{a50}}{C} \right)^b \right)^e} \right] \quad (\text{Equation 3.7})$$

Y = Response

a = Minimum response

d = Maximum response

p_{a50} = Concentration at the EC50 value

C = Concentration

b = Slope

e = Compensation factor for asymmetry

3.3.2 CHARACTERISATION STUDIES USING CYCLIC VOLTAMMETRY

For the electrochemical characterisation of the different screen-printed gold electrodes cyclic voltammetry was carried out using the electroactive mediator potassium ferricyanide ($\text{K}_3\text{Fe}[\text{CN}]_6$). Different scan rates (10 mV s^{-1} to 100 mV s^{-1}) as well as varying potassium ferricyanide concentrations were evaluated to achieve the best signal characterisation. Furthermore, the TMB- H_2O_2 substrate system was analysed.

The scan bandwidth was adjusted depending on the location of the cathodic and anodic peak. The cyclic voltammetric measurements were repeated four times for each sensor. Due to the slow diffusion rate of the molecules in the solution, the values of the first scan were not used for characterisation. After the first scan, equilibrium between diffusion and required electrons occurred resulting in reproducible values for the 2nd to 4th scan. These three scans were averaged and used for characterisation. The step potential was set to 10 mV.

3.3.2.1 Evaluation of Different Potassium Ferricyanide Concentrations

To investigate the influence of the potassium ferricyanide to the cyclic voltammetry characterisation of the electrodes, different test concentrations from 50 to 1 mM were evaluated in order to achieve the best signal for the sensor characterisation. The peak heights of the scans were compared at a constant scan rate of 50 mV s^{-1} . For each potassium ferricyanide concentration, a new sensor (JD) was used. To obtain the different potassium ferricyanide concentrations, a 100 mM stock solution was prepared in 1 M KCl. This solution was further diluted with 1 M KCl to the final test concentration of the potassium ferricyanide reagent. For the measurement 100 μl were dropped on the sensor surface covering all three electrodes. The scan bandwidth was adjusted depending on the location of the cathodic and anodic peak.

3.3.2.2 The Effect of Different Scan Rates to the Reversibility of the Potassium Ferricyanide Reaction

Cyclic voltammetry analyses with scan rates of 10, 20, 50, 70 and 100 mV s^{-1} were conducted against potassium ferricyanide (1 mM in 1 M KCl). The scan bandwidth was set to a constant value ranging from -0.4 V to +0.3 V. For the measurement of each scan rate 100 μl of the potassium ferricyanide concentration were dropped on a new JD sensor covering all three electrodes with liquid.

3.3.2.3 Characterisation of Different Sensor Types

Through the collaborative work with DuPont Microcircuit Materials (Bristol, UK), different combinations of ink type were examined, which were used to fabricate the screen-printed sensors. In order to identify the sensor with the best performance, cyclic voltammetric measurements with three replicates of each electrode type (JA, JC and JD) were performed against potassium ferricyanide and TMB. The potassium ferricyanide concentration was 1 mM in 1 M KCl solution. TMB was prepared in 25 mM phosphate citrate buffer / 0.05 M KCl working buffer to a final concentration of 4 mM. The scan rate was 20 mV s^{-1} and the scan bandwidth was constantly set between -0.4 V to +0.3 V and -0.5 to 0.8 V for the TMB, respectively. For the measurement, 100 μl of the solution was dropped on a new sensor covering all three electrodes with the liquid.

3.3.2.4 TMB- H_2O_2 Substrate System

The electrochemical biosensor developed in this study utilises the TMB/ H_2O_2 substrate system for signal generation. The amperometric signal is highly sensitive and the signal can be disturbed by ionic interference (Compton and Banks, 2007). Therefore, the reagents used in the signal generating system were tested separately using cyclic voltammetry analysis with a scan bandwidth ranging from -0.5 to 0.8 V and a scan rate of 20 mV.

For the preparation of the TMB-H₂O₂ substrate, 0.05 M phosphate citrate buffer (pH 5.0) was added to 0.1 M KCl in equal volumes to prepare the working buffer for the substrate. The buffer was prepared as described in Section 3.3.3.3 with a final TMB and H₂O₂ concentration of 4 mM and 0.06 %, respectively. Before the cyclic voltammetric measurements were conducted, 100 µl of the TMB, H₂O₂ and TMB+H₂O₂ substrate were dropped on the sensor surface, covering all three electrodes.

3.3.3 DEVELOPMENT OF AN ELECTROCHEMICAL IMMUNOSENSOR USING SPGE

3.3.3.1 Scanning Electron Microscopy (SEM)

Surface analysis of the SPGE was conducted using a scanning electron microscope (model 7231, Oxford instruments, UK), operated at a potential of 20 kV with a maximum magnification of x100,000. The properties of the sample material, such as conductivity and topography, have a high impact on the maximum resolution of the SEM. Non-conductive samples cannot be visualised with a SEM and therefore, a 10 to 20 nm gold or carbon layer was coated on the sample. The gold coating occurred by exposing the sample to a gold plasma for approximately 3 min. For the carbon coating, a graphite wire was heated by passing a high current, which resulted in the vaporisation of carbon particles. The sample was exposed to the particles for approximately 10 min, allowing the free carbon particles to produce a uniform layer on the surface of the sample. The electric circuit was then completed by dropping a conductive paste from the electrode on the sample holder. All coating processes of the sample were conducted in a vacuum chamber with a controlled nitrogen flow.

For images of the SPGE without biological sample material immobilised on the surface, the gold working electrode was connected to the sample holder using conductive paste. As the gold working electrode is conductive by itself, the SEM was performed directly, without the need of a conductive layer.

3.3.3.2 Chronoamperometric Analyses of the HRP / TMB-H₂O₂ Substrate System

Different step potentials were applied to the HRP / TMB-H₂O₂ system in order to evaluate the optimum value for future chronoamperometric measurements. A stock solution of 10 ng ml⁻¹ goat anti-mouse IgG antibodies conjugated HRP was prepared in phosphate citrate buffer (pH 5.0). As the HRP is reacting instantly with the TMB-H₂O₂ signal generating system, the stock solution was diluted directly on the sensor with the TMB-H₂O₂ substrate to the final concentration of 1 ng ml⁻¹ (100 µl/sensor). The TMB H₂O₂ substrate was prepared as described in Section 3.3.3.3 with a TMB and H₂O₂ concentration of 4 mM and 0.06 %, respectively. In order to establish a baseline current for each potential, each sensor was first measured only with the TMB-H₂O₂ substrate. This signal was then subtracted from the reading with added HRP for normalisation.

After the optimum step potential was established, the HRP/TMB-H₂O₂ substrate system was further investigated using different IgG-HRP conjugate concentrations of 100, 50, 10 and 1 ng ml⁻¹. Similar to the previous experiment, the HRP solution was diluted to its final concentration in TMB-H₂O₂ substrate on the sensor just before the start of the chronoamperometric measurement.

3.3.3.3 Assay Procedure for Screen-Printed Gold Electrodes

Prior to the immobilisation of the antibodies, the sensor was heat treated in an oven (Carbolite, model PN 120 (200), Hope, UK) for 30 min at 120 °C to clean the surface. To provide a saturated surface with a maximum amount of immobilised antibodies, the working electrode was coated with 15 µl capture antibodies with a concentration of 100 µg ml⁻¹ using carbonate-bicarbonate buffer (0.05 M, pH 9.6). The incubation time for this step was 2.5 to 3 h at 37 °C, allowing the antibodies to adsorb to the surface of the sensor. In general, all incubation steps were carried out under controlled humid environment. The volume required to cover the working electrode is 15 to 20 µl. Excluding the following blocking step which requires a volume of 50 µl to cover all three electrodes, this value was constant for the following assay steps. After each incubation step, the surface was washed gently with PBST (phosphate buffered saline with 0.05 % Tween 20), followed by rinsing with deionised water. Tween 20, being a

surfactant, reduces the surface tension of the liquid and leads to a spread of the sample drop on the sensor. Therefore, PBS without Tween 20 was used for the dilution of antibodies and samples.

To reduce non-specific binding of the antibodies, the sensor surface was blocked using a milk protein concentrate (KPL Inc., Gaithersburg, USA) diluted 1:10 in PBS. The incubation time was at least 1 h at 37 °C. After washing, various amounts of fungal samples were then prepared in PBS, 20 µl were dropped on the working electrode and incubated for 1 h at 37 °C. The negative control was represented by the commercial *B. allii* ELISA kit negative control from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK), which includes freeze-dried leaf extracts. In between, the surface was washed with PBST, followed by rinsing with deionised water. A period of 1 h was also used for the following incubation of the detection antibody. The purified detection antibodies were diluted to a working strength of 80 µg ml⁻¹ in buffer containing 1:40 milk protein concentrate (KPL Inc., Gaithersburg, USA), which was used to reduce non-specific binding of the antibody to the plastic tube.

If the antibodies are labelled with HRP (direct format) the substrate can be added at this stage of the assay, after washing the unbound material with PBST. For the indirect format, an anti-species conjugate has to be applied to the sensor after washing and incubated for 30 to 60 min at 37 °C in order to bind to the detection antibodies.

For the preparation of the TMB-H₂O₂ substrate, a stock of working buffer was prepared by adding 0.05 M phosphate citrate buffer (pH 5.0) to 0.1 M KCl in equal volumes. A part of this buffer was also used to dilute a 30 % (w/w) hydrogen peroxide solution in a ratio of 1:10, resulting in a 3 % (w/w) hydrogen peroxide working buffer mix. The second stock was prepared by adding 150 µl of dH₂O to every 1 mg of TMB powder. To produce 1 ml of the final substrate, 20 µl of the 3 % (w/w) hydrogen peroxide stock as well as 20 µl of the TMB-water stock is diluted in 960 µl of working buffer. This results in a final concentration of 4 mM TMB and 0.06 % H₂O₂ in phosphate citrate – KCl buffer. The substrate was produced in batches of 1 or 1.5 ml and covered in aluminium foil as TMB solution is photo reactive.

Since the washing and reading of the sensors is time consuming, in general the assay was subdivided in batches of approximately 10 to 16 sensors fitted in separate incubation trays. A 15 to 20 min time offset was maintained between the batches using a stopwatch. Employing this modification, the incubation times between the batches were more comparable and improved the inner and inter-assay performance. Before starting the amperometric measurement, 100 μl of the TMB- H_2O_2 substrate were dropped on the sensor surface covering all three electrodes. After dropping the substrate on the electrode, the pipette tip was changed to avoid contamination of the TMB- H_2O_2 stock with mobilised HRP from the sensor surface. To minimise the variations in the sensor signal due to poor substrate quality, a fresh TMB- H_2O_2 substrate was mixed for every inner assay sensor batch or after 30 min of use.

3.3.3.4 Preparation of the Gold Nanoparticle Antibody-HRP Conjugate

Gold nanoparticles with $\text{Ø}40 \text{ nm} \pm 3.2 \text{ nm}$ particle size were purchased from BBI International (Cardiff, UK). The colloid solution with a concentration of 9×10^{10} particles per millilitre (in H_2O), was stored in the dark at 4°C and not modified prior to use. The antibody-colloid gold conjugate was prepared according to a modified procedure described by Uludag and Tothill (2010a).

The adsorption process of the antibodies to the gold particles was initialised by adding 2.5 μl of 0.2 M NaOH to 500 μl of colloid solution in order to create a pH of 9.0. For the subsequent signal generation of the conjugate, 5 μl of 1 mg ml^{-1} HRP (Type II/IV, Sigma, Dorset, UK) was added to the Eppendorf tube, followed by 60 min of incubation time at room temperature protected from light. For better diffusion of the particles, all incubation steps were carried out under continuous shaking using a rotator (Stuart, UK) at 10 rpm and an angle of 20° . Next, 5 μl of 1 mg ml^{-1} detection antibody was added and incubated for another 60 min. After this period, the Eppendorf tube was centrifuged for 20 min at 4°C , allowing the gold colloid to settle down in a separate layer on the bottom of the tube. The centrifugation results in a separation of the loosely packed red sediment of the antibody and HRP-labelled gold nanoparticles and a clear supernatant comprising unbound antibody and HRP. To concentrate the colloid, approximately 500 μl of the supernatant were carefully extracted with a pipette.

To reduce the unoccupied gold surface which leads to an increase of non-specific binding while performing the sensor assay, the nanoparticles were blocked using 15 μl of 10 mg ml^{-1} BSA prepared in PBS. The protein solution also acts as a stabiliser of the immune-gold colloid, preventing the non-specific adsorption of the particles to each other or the wall of the Eppendorf tube while stored. To guarantee a reproducible concentration of the produced stock solution, the remaining volume containing the gold conjugate was re-suspended to a total volume of 70 μl , adding 30 to 45 μl of 0.05 M PBS.

Generally, the conjugated gold particles were produced one day before usage due to better performance after 24 h of storage at 4°C. The gold particles showed a shelf life of at least two weeks without significant loss in activity. The stability of the gold colloid was tested by adding 20 μl of 2.5 M NaCl to 200 μl 1:10 diluted modified gold colloid solutions in PBST. If flocculation occurs, the binding of the proteins to the gold particles was not successful and the procedure has to be repeated.

3.3.4 EVALUATION OF GOLD NANOPARTICLES AS A TECHNIQUE FOR SIGNAL AMPLIFICATION

3.3.4.1 Visualisation of the Gold Nanoparticles Using Scanning Electron Microscopy

Scanning electron microscopy images of the Ø40 and Ø60 nm gold nanoparticles were performed using the equipment and protocol described in Section 3.3.3.1. First, the monoclonal antibody BC-12 (100 $\mu\text{g ml}^{-1}$) was immobilised on the sensor surface, followed by 1 h blocking with 50 μl milk protein concentrate diluted 1:10 in PBS and then 300 $\mu\text{g ml}^{-1}$ of *B. allii* antigen was applied. The Ø40 and Ø60 nm gold particles were labelled with polyclonal antibodies and HRP as described in Section 3.3.3.4. Next, 20 μl of this solution were applied to the sensor surface and incubated for 1 h at 37 °C. Before the SEM images were taken, unbound particles were removed by washing with dH₂O.

3.3.4.2 Optimisation of the Gold Nanoparticles Production

The production of the gold nanoparticles is a complex procedure using various parameters. Due to the numerous possible combinations of variables, an optimisation by using biosensors is not practicable, as the maximum number per assay is limited to 40 sensors. On the other hand, 96 well solid phase ELISAs are inexpensive, less time consuming and therefore more suitable for screening large numbers of replicates. The solid phase ELISA was performed as described in Section 2.3.5, with antibody and HRP-labelled gold nanoparticles used for detection. Several parameters were tested in various combinations, such as gold nanoparticles size (Ø20, Ø40 and Ø60 nm), applied order, volume and incubation time of the antibody and HRP applied, type of antibody (poly or monoclonal), HRP type (II and VI), gold nanoparticles volume extracted, incubation method (roller or rotator mixer), dilution factor of the particles and pH. The variations were then screened against four *B. allii* concentrations ranging from 1 mg ml⁻¹ to 10 µg ml⁻¹ as well as the negative control represented by 10 mg ml⁻¹ BSA.

3.3.4.3 Performance Studies of the Optimised Gold Nanoparticles Assay

The performance of the optimised gold nanoparticles assay was tested against various concentrations of *B. allii*. The Ø40 nm gold colloid was produced as described in Section 3.3.3.4 with 5 µl HRP VI and 10 µl of polyclonal antibodies incubated for 1 h using a rotator mixer.

3.3.5 IMMUNOSENSOR OPTIMISATION

3.3.5.1 Incubation Time

The incubation time for the capture and detection antibodies are crucial for a good assay performance and was therefore evaluated using SPGE following the protocol described in Section 3.3.3.3. Due to the wide range screened within this study, the number of *B. allii* antigen concentrations was limited to 100 and 0 µg ml⁻¹. Initially, 50 µg ml⁻¹ of the monoclonal antibody BC-12 was incubated for 30 min to 4 h followed by 50 µg ml⁻¹ conjugated polyclonal antibody incubated for 1 h. Afterwards, a capture antibody incubation time of 3 h was combined with varying conjugate incubation times (15 min

to 2 h). In order to compensate the influence of non-specific binding to the sensor signal, the current was normalised by subtracting the reading of the negative control of each tested value.

3.3.5.2 Detection Antibody Concentration

For the signal generation, the polyclonal antibody purchased from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK) was conjugated to the enzyme HRP as described in Section 2.3.3. While keeping the capture antibody concentration constant ($50\ \mu\text{g ml}^{-1}$), the detection antibody applied was varied using eight concentrations of purified antibodies ranging from 10 to $120\ \mu\text{g ml}^{-1}$ and seven concentrations of unprocessed conjugate ranging from 10 to $100\ \mu\text{g ml}^{-1}$. According to the Lightning-Link protocol used for the conjugation process, the efficiency rate is 99 % and further purification steps are not necessarily needed. However, purifying antibodies from free enzyme can increase assay sensitivity and improve signal-to-noise ratio (Boorsma and Kalsbeek, 1975). Therefore, a Pierce conjugate purification kit with nickel-chelated agarose binding column was used to eliminate free enzyme from HRP conjugate, and the performance of the purified and unpurified conjugate was compared using *B. allii* concentrations of 100, 10 and $0\ \mu\text{g ml}^{-1}$. The assay was performed as described in Section 3.3.3.3.

3.3.5.3 Optimisation of the Coating Buffer

Different coating buffers were tested to evaluate the most suitable one for the immobilisation of the monoclonal capture antibody BC-12. All buffers had an alkaline to near neutral pH, as this pH range has been reported to give best results for the immobilisation of antibodies by adsorption (Malone et al., 1996; Chen et al., 2007b; Salam and Tothill, 2009b). ELISA coating buffer (02-002, pH 9) was purchased from ADGEN Phytodiagnostics (Auchincruive, UK). Carbonate-bicarbonate buffer (0.05 M, pH 9.6) and sodium citrate (pH 8.1) and phosphate buffered saline (pH 7.4) were acquired from Sigma, (Dorset, UK).

The buffers were tested with a coating antibody concentration of $50 \mu\text{g ml}^{-1}$, followed by purified HRP-conjugated polyclonal antibodies ($50 \mu\text{g ml}^{-1}$) and fungal concentrations of 100, 10 and $0 \mu\text{g ml}^{-1}$. The assay was performed in triplicates as described in Section 3.3.3.3.

3.3.5.4 Capture Antibody Concentration

Various concentrations of the monoclonal antibody BC-12 (10, 30, 50, 70, 100, 150 and $200 \mu\text{g ml}^{-1}$) obtained from Forsite Diagnostics (Sand Hutton, UK), were coated on the sensor using physical adsorption. The antibodies were previously diluted in carbonate-bicarbonate buffer to their final concentration before dropping $15 \mu\text{l}$ on the gold working electrode. For signal generation, $80 \mu\text{g ml}^{-1}$ of purified polyclonal antibody conjugated to HRP was used with fungal concentrations of 100, 10 and $0 \mu\text{g ml}^{-1}$. The assay was performed as described in Section 3.3.3.3.

3.3.5.5 Standard Curve of the Optimised Biosensor

After accomplishing the optimisation process, a standard curve was created by testing the biosensor response to *B. allii* concentrations ranging from $0.05 \mu\text{g ml}^{-1}$ to 1 mg ml^{-1} . The assay was performed as described in Section 3.3.3.3 with a capture antibody concentration of 100 g ml^{-1} followed by $80 \mu\text{g ml}^{-1}$ of purified polyclonal antibody conjugated to HRP for signal generation. After reading, the current was normalised by subtracting the negative reading and plotted against the *B. allii* concentration. For standardisation, a five parameter non-linear function described by Finney (1983) was fitted to the data points using the generalised reduced gradient (GRG) method (Lasdon et al., 1978; Smith and Lasdon, 1992). Non-linear regression and statistical calculations were performed as described by Brown (2001). The limit of detection (LOD) was calculated as the negative reading plus three times its standard deviation.

3.3.6 PERFORMANCE STUDIES OF THE BIOSENSOR

To test the performance of the biosensor with onion samples, a quantification experiment was designed using onion discs grown on selective agar, produced as described by Kritzman and Netzer (1978) (see Section 1.4.1). After removing the outer scale of UV sterilised onion sets (variety Forum), the onion was cut longitudinally into 3 pieces and placed on Kritzman agar. Each disc was weighted and 100 $\mu\text{l g}^{-1}$ of spore solution with a concentration of 100,000 spores ml^{-1} was dropped on the onion disc, resulting in a sample concentration of 10,000 spores g^{-1} onion tissue. The spore solution was produced as described in Section 2.3.1.4 and mycelial fragments were removed by using a 30 ml glass filter tube with the porosity 0 (160-250 μm) (Robu Glassware) combined with a changeable sterile 20 mm pre-filter disc with the porosity 3 (nominal pore size 16 to 40 μm). A controlled environment provided by an incubator (Sanyo, model MLR-350HT, Japan) at 20 °C with a 12 h day-night photoperiod was used to allow the fungus to grow on the surface of the onion disc.

Three samples were processed after 0, 1, 2, 3, 4, 5 and 7 days of incubation using the protocol described in Section 2.3.7. In addition, the samples were pre-filtered with a sample extraction bag purchased from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK), which comprises a heavy duty outer and a synthetic intermediate filter layer to remove large grained plant material that can block the glass filter tube and inhibit pipetting. To compensate the weight loss by evaporating water, the added buffer used for the sample preparation was based on their weight measured before inoculation. After extraction, the samples were stored individually at -20 °C until testing.

The samples used for the biosensor were also tested using the commercially available *B. allii* and *B. cinerea* ELISA Kit (ADGEN Phytodiagnostics, Auchincruive, UK), which utilises the same antibodies employed in this study. The ELISA procedure was followed as described in Section 2.3.5, with a detection antibody concentration of the polyclonal and monoclonal antibodies of 10 $\mu\text{g ml}^{-1}$ and a corresponding anti-species conjugated concentration of 0.3 $\mu\text{g ml}^{-1}$. To improve the signal strength, the TMB substrate was incubated for 30 to 40 min at room temperature in the dark.

3.3.7 EVALUATION OF COVALENT IMMOBILISATION TECHNIQUES

3.3.7.1 Covalent Coupling Procedure Using Thiol-Coupled SAMs

Self-assembled monolayers (SAM) were produced using two polymer compounds: 3-dithiodipropionic acid (DTDPA) and 11-mercaptoundecanoic acid (MUDA). The coupling procedure is based on a modified protocol described by Uludag and Tothill (2010b). The polymers were weighted and diluted to their target concentration of 5 to 50 mM in ethanol. Covalent coupling is based on the formation of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) esters, which form a covalent bond with reactive amine groups of the antibody under elimination of N-hydroxysuccinimide (NHS) (Van Delden et al., 1997). The technique using EDC-NHS coupling chemistry for the antibody immobilisation is illustrated in Figure 3.6. One μl of the polymer was dropped onto the sensor surface using a pipette. The thiol group builds an immediate sulphur-gold bond and hydrophobic alkane chains construct a stable, regularly orientated hydrophobic layer (Prime and Whitesides, 1993). Excess polymer was gently removed by rinsing the electrode with dH_2O .

Following the self-assembly of the monolayer, the reactive carboxyl group was chemically coupled to the amine group of the antibodies by using EDC and NHS activation. Therefore, 100 μl aliquots of 0.4 M EDC and 0.1 M NHS were produced and stored at $-20\text{ }^\circ\text{C}$ for further use. EDC-NHS solutions were combined in a ratio of 1:1, 20 μl were dropped on the working electrode and incubated for 15 min at room temperature allowing the activation of the sulphide monolayer. After washing the electrode with dH_2O , 15 μl of 100 $\mu\text{g ml}^{-1}$ capture antibodies in 0.05 M acetate buffer (pH 5.5) were dropped on the working electrode and incubated for 2.5 h at $37\text{ }^\circ\text{C}$. The following steps were performed using the general assay procedure described in Section 3.3.3.3.

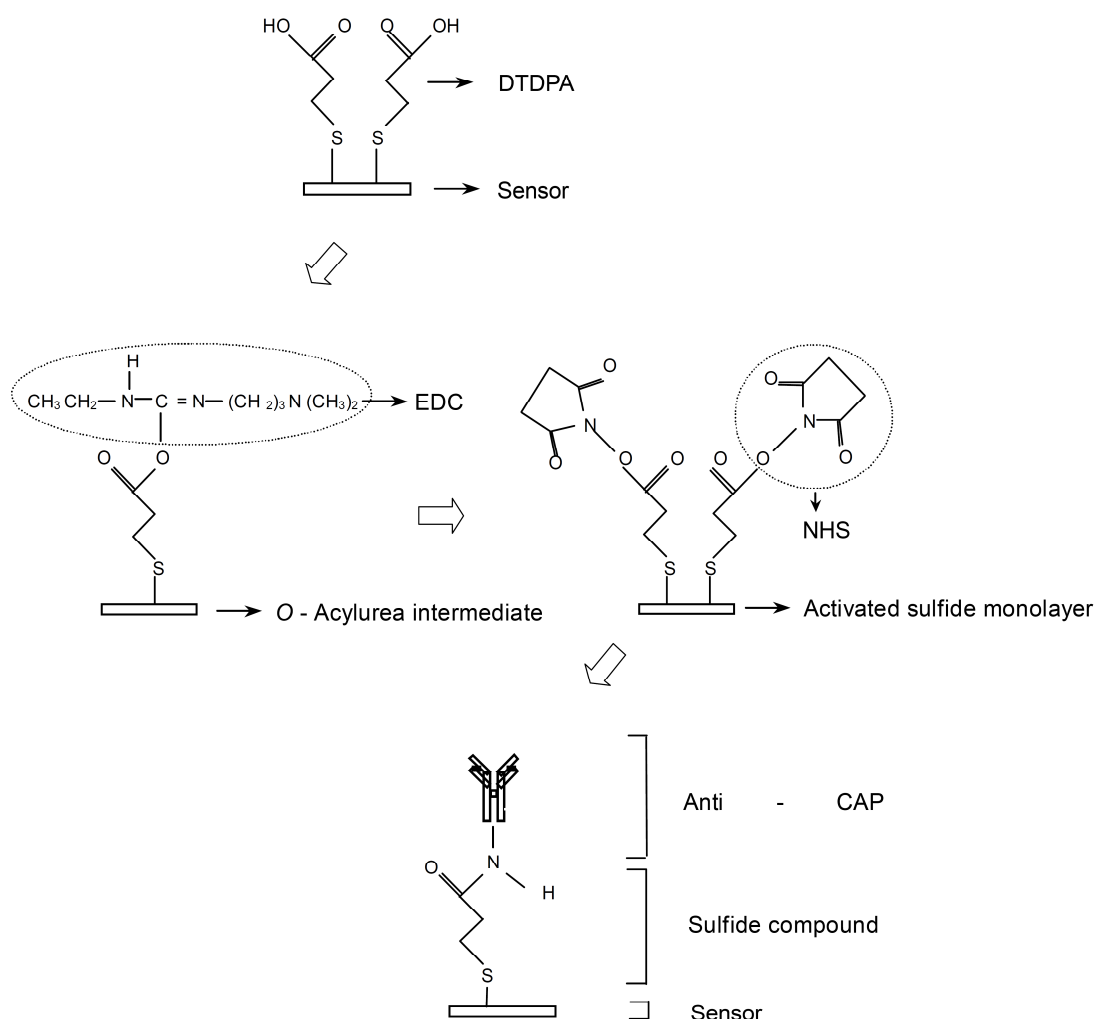


Figure 3.6: EDC-NHS coupling mechanism used for the immobilisation of the capture antibodies to the gold working electrode of the biosensor (Park et al., 2004).

3.3.7.2 Characterisation of the Self-Assembled Monolayers

The thiol-coupled SAMs were characterised using cyclic voltammetry (CV) and scanning electron microscopy (SEM). SEM was performed as described in Section 3.3.3.1 using gold coating. To construct the polymer layer, 3 x 1 µl of 50 mM polymer solution were dropped on the working electrode and rinsed with dH₂O.

Cyclic voltammetry analysis was performed as described in Section 3.3.1.1 using 1 mM potassium ferricyanide diluted in 1 M KCl at a scan rate of 20 mV s⁻¹. Calculation of the active surface area and the scan bandwidth was equivalent to Section 3.4.1.3. The sensors were coated overnight in a 9 cm petri dish containing the 5 and 50 mM 11-MUDA and 3-DTDPA solution at room temperature in the dark.

3.3.7.3 Influence of Covalent Coupling Using SAM on the Immobilised Capture Antibody Load and Optimisation of the Monolayer Concentration

An experiment was designed to evaluate the surface loading of covalently and non-covalently immobilised antibodies using HRP-conjugated anti-species secondary antibodies. First, 100 µg ml⁻¹ of the monoclonal antibody BC-12 were covalently immobilised to the sensor surface following the protocol described in Section 3.3.7.1. The SAM was constructed using various parameters: 1 µl and 3x1 µl of 50 mM DTDPA, 1 µl and 3x1 µl of 5 mM MUDA. Furthermore, some sensors were coated for 1 h in a petri dish filled with 5 ml of 50 mM DTDPA and 5 and 50 mM MUDA, respectively. After blocking, instead of the antigen, 10 µg ml⁻¹ goat anti-mouse conjugate were added and incubated for 1 h at 37 °C. The sensor protocol was followed as described in Section 3.3.3.3, modified by adding only 5 µl of the conjugate, in order to avoid a false positive signal produced by anti-species conjugate non-specifically bound to the area outside the gold working electrode. Unbound conjugate was removed by washing the sensors three times with PBST, followed by rinsing with dH₂O, before adding the substrate (8 mM TMB, 0.12 % H₂O₂ in 1:1 phosphate citrate – KCl buffer). The control was represented by the adsorbed group.

Various DTDPA and MUDA concentrations ranging from 1 to 100 mM were then assayed against *B. allii* concentrations of 100, 10 and 0 µg ml⁻¹ to evaluate the most suitable one for the construction of the polymer layer on the gold electrode. Covalent immobilisation strategies were performed as described in Section 3.3.7.1 with a monoclonal capture antibody concentration of 100 µg ml⁻¹, followed by 80 µg ml⁻¹ purified polyclonal conjugate. The sensor assay procedure was performed according to Section 3.3.3.3.

3.3.7.4 Washing Resistance of Covalent and Non-Covalent Coupling Procedures

Covalent immobilisation strategies claim to decrease the loss of proteins during the washing steps performed during the assay (Leckband and Langer, 1991; Williams and Blanch, 1994). To evaluate this statement, an assay with covalent and non-covalent coupling techniques was designed using controlled washing steps. To construct the SAM, 1 μl of 50 mM DTDPA and 5 mM MUDA solution was applied to the working electrode. The washing steps after the immobilisation of the capture antibody (100 $\mu\text{g ml}^{-1}$) and the blocking step were performed with a pipette by dropping three and seven times 500 μl on the sensor electrodes. Furthermore, a group was washed for 10 s using a standard narrow mouth LDPE bottle. The general setup was identical to the assay described in Section 3.4.7.2. The performance of the covalently coupled antibodies was compared to the adsorbed group.

3.3.7.5 Comparison of Covalent Coupling Procedures Using DTDPA and MUDA SAM to Passive Adsorption

A comparative study of covalent coupling procedures, using 50 mM DTDPA and 5 mM MUDA thiol layers and passive adsorption as described in Section 3.3.3.3 was conducted. Covalent immobilisation strategies were performed as described in Section 3.3.7.1. The monoclonal capture antibody concentration was 100 $\mu\text{g ml}^{-1}$ followed by 80 $\mu\text{g ml}^{-1}$ purified polyclonal conjugate. Five different fungal concentrations were used (300, 100, 10, 1 and 0.1 $\mu\text{g ml}^{-1}$) to determine the assay performance.

3.3.7.6 Standard Curve of the Biosensor Employing Covalent Coupling to DTDPA Self-Assembled Monolayer

After accomplishing the optimisation process, a standard curve for covalent immobilisation was constructed by testing the biosensor response to *B. allii* concentrations ranging from $0.03 \mu\text{g ml}^{-1}$ to $500 \mu\text{g ml}^{-1}$. Covalent immobilisation to 50 mM 3-DTDPA layer was performed as described in Section 3.3.7.1. The assay was performed as described in Section 3.3.3.3 with a capture antibody concentration of $100 \mu\text{g ml}^{-1}$ followed by $80 \mu\text{g ml}^{-1}$ of purified polyclonal antibody conjugated to HRP for signal generation. The current after 100 s was normalised and plotted against the *B. allii* concentration. For standardisation, a five parameter non-linear function described by Finney (1983) was fitted to the data points using the generalised reduced gradient (GRG) method (Lasdon et al., 1978; Smith and Lasdon, 1992). Non-linear regression and statistical calculations were performed as described by Brown (2001). The limit of detection was calculated as the negative reading plus three times its standard deviation.

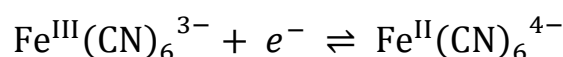
3.3.8 COMPARATIVE STUDIES OF THE SPGE WITH THE COMMERCIAL ELISA KITS

The biosensor response was compared to the commercial *B. allii* and *B. cinerea* ELISA kits purchased from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK). *B. allii* samples ranging from 2.5 to $150 \mu\text{g ml}^{-1}$ were diluted in PBS and quantified following the procedure described in Section 2.3.5. The standard curve for the ELISA kits was constructed using a serial dilution of *B. allii* antigen ranging from 200 to $1.6 \mu\text{g ml}^{-1}$. The SPGE assay was performed as described in Section 3.3.3.3 with a capture antibody concentration of $100 \mu\text{g ml}^{-1}$ followed by $80 \mu\text{g ml}^{-1}$ of purified polyclonal antibody conjugated to HRP for signal generation. Covalent immobilisation to 50 mM 3-DTDPA layer was performed as described in Section 3.3.7.1.

3.4 RESULTS AND DISCUSSION

3.4.1 CHARACTERISATION STUDIES USING CYCLIC VOLTAMMETRY

Cyclic voltammetry analysis is an established method used to characterise the working electrode surface of screen-printed gold electrodes used in this work. Potassium ferricyanide was utilised as reactive substance for the redox reaction as showed below:



For characterisation of the screen-printed electrode, the cathodic and anodic peak current and the location of their corresponding potential were used (Figure 3.7). The cathodic and anodic peak current was employed to calculate the active surface area of the electrode employing the Randle-Sevcik equation. A small cathodic and anodic peak separation ΔE indicates that the electrochemical reaction is quasi reversible. The ideal ΔE value for a perfectly reversible redox reaction of potassium ferricyanide is 56 to 59 mV and the ratio between the cathodic and anodic peak is 1 (Mueller and Adams, 1961; Arya et al., 2007).

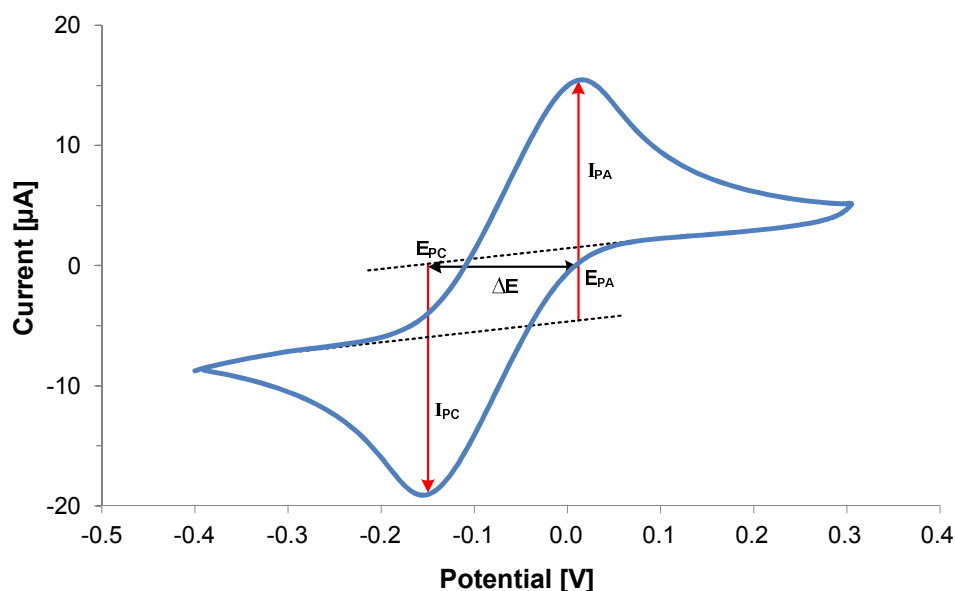


Figure 3.7: A cyclic voltammogram for the JD gold screen-printed electrode using a 1 mM potassium ferricyanide solution in 1 M KCl at a scan rate of 20 mV s⁻¹.

$I_{P\ C/A}$ = Cathodic / anodic peak current;

$E_{P\ C/A}$ = Potential applied at the cathodic / anodic peak; $\Delta E = E_{PA} - E_{PC}$

3.4.1.1 Evaluation of Different Potassium Ferricyanide Concentrations

To investigate the influence of the potassium ferricyanide on the cyclic voltammetry characterisation of the electrodes, different test concentrations ranging from 50 to 1 mM were evaluated. The resulting cyclic voltammogram of the potassium ferricyanide concentrations is presented in Figure 3.8. With higher concentrations, the shift between the anodic and cathodic peak potential rises (Table 3.2) making the redox reaction more irreversible. An ideal reversible cyclic voltammetric one-electron reaction has a ΔE value of approximately 56 mV (Mueller and Adams, 1961). However, in practice the difference is typically 100 mV and higher (Arya et al., 2007).

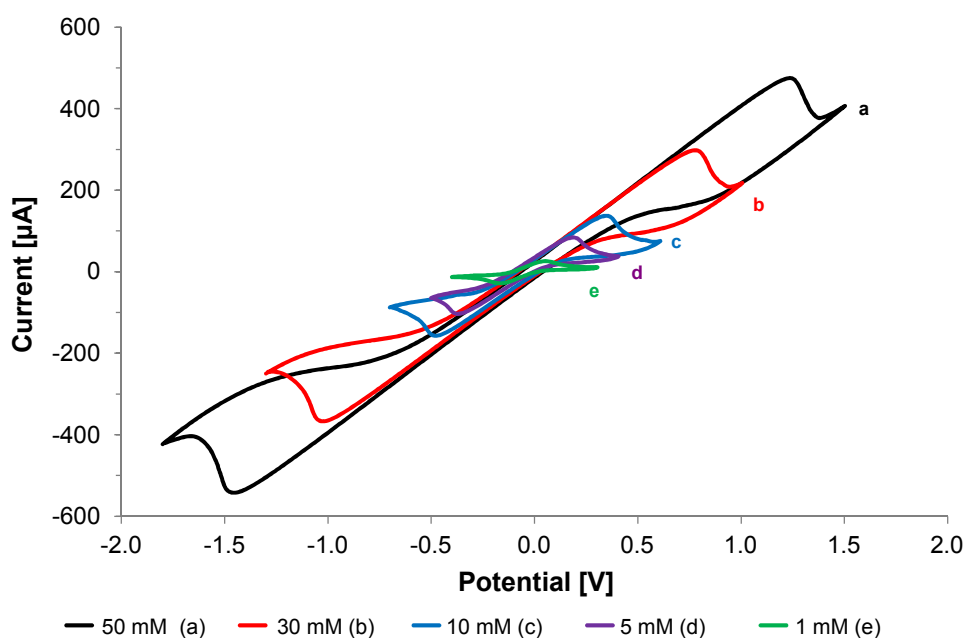


Figure 3.8: The influence of the various potassium ferricyanide concentrations on the cyclic voltammetry signal of a JD SPGE.

Table 3.2: Cyclic voltammetric analyses of different potassium ferricyanide concentrations in 1 M KCl with a scan rate of 50 mV s⁻¹. The standard deviation was calculated using three scans.

	Potassium Ferricyanide Concentration				
	50 mM	30 mM	10 mM	5 mM	1 mM
I_{PA} [μA] ^a	575 ±4.0	353 ±9.7	167 ±2.9	90 ±0.8	35 ±0.5
E_{PA} [V] ^b	1.23 ±0.02	0.77 ±0.02	0.34 ±0.01	0.18 ±0.01	0.05 ±0.00
I_{PC} [μA] ^a	-534 ±1.4	-359 ±3.2	-172 ±1.0	-105 ±0.2	-31 ±0.0
E_{PC} [V] ^b	-1.44 ±0.01	-1.01 ±0.01	-0.48 ±0.01	-0.37 ±0.00	-0.16 ±0.01
ΔE [V] ^c	2.68 ±0.02	1.79 ±0.02	0.82 ±0.01	0.55 ±0.01	0.22 ±0.01

^a I_{P C/A} = Cathodic / anodic peak current

^b E_{P C/A} = Potential applied at the cathodic / anodic peak

^c Peak distance ΔE = E_{PA} - E_{PC}

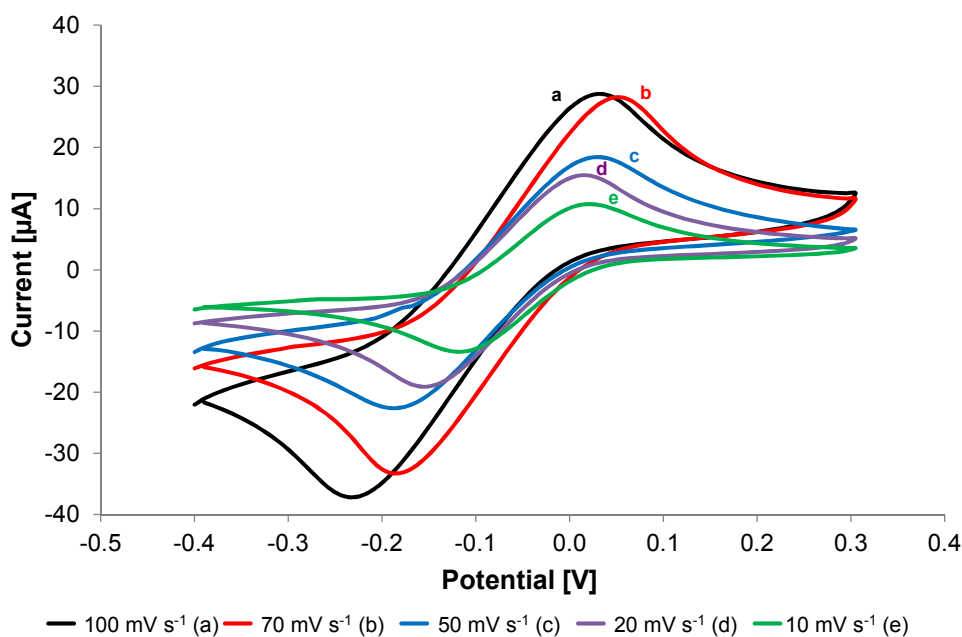
For the utilised JD sensors (DuPont, Bristol, UK) a concentration of 1 mM showed the best compromise between signal strength, reproducibility and reversibility of the reaction, with the smallest standard deviation and ΔE value. Consequently, this concentration was selected for future characterisation experiments.

3.4.1.2 The Effect of Different Scan Rates on the Reversibility of the Potassium Ferricyanide Reaction

In order to find the most appropriate scan rate for the characterisation of the electrodes, different scan rates from 10 to 100 mV s⁻¹ were evaluated. Previous experiments indicated that a potassium ferricyanide concentration of 1 mM in 1 M KCl results in the best reproducibility and reversibility of the redox reaction. Following the Randles-Sevcik equation, the peak current is directly proportional to the square root of the scan rate. As illustrated in Table 3.3 with the corresponding cyclic voltammograms presented in Figure 3.9, the SPGE performed well. The cathodic and anodic peak separation ΔE was found to be 138 mV at 10 mV s⁻¹, which indicates that the electrochemical reaction is quasi reversible.

Table 3.3: Cyclic voltammetric analyses at scan rates ranging from 100 mV s⁻¹ to 10 mV s⁻¹. Standard deviation was calculated using three scans for each scan rate.

	100 mV s ⁻¹	70 mV s ⁻¹	50 mV s ⁻¹	20 mV s ⁻¹	10 mV s ⁻¹
I_{PA} [μA] ^a	32.55 ±0.77	34.97 ±0.60	34.59 ±0.50	19.94 ±0.14	15.43 ±0.13
E_{PA} [V] ^b	0.023 ±0.00	0.05 ±0.01	0.053 ±0.00	0.013 ±0.00	0.02 ±0.02
I_{PC} [μA] ^a	-37.76 ±0.20	-34.60 ±0.27	-30.68 ±0.03	-20.30 ±0.20	-14.83 ±0.09
E_{PC} [V] ^b	-0.229 ±0.00	-0.181 ±0.01	-0.165 ±0.01	-0.155 ±0.01	-0.118 ±0.01
ΔE [V] ^c	0.252 ±0.00	0.231 ±0.01	0.218 ±0.01	0.168 ±0.01	0.138 ±0.02
I_{PA} / I_{PC} ^d	0.86	1.01	1.13	0.98	1.04
A_{active} [%] ^e	66.33	78.45	87.08	84.89	90.28

^a I_{P C/A} = Cathodic / anodic peak current^b E_{P C/A} = Potential applied at the cathodic / anodic peak^c Peak distance ΔE = E_{PA} - E_{PC}^d Ratio between the cathodic and anodic peak current.^e Ratio between the active area calculated by the Randles-Sevcik equation and the geometric surface.**Figure 3.9:** Cyclic voltammograms using the electroactive mediator potassium ferricyanide (1 mM) with different scan rates ranging from 100 to 10 mV s⁻¹.

The calculation of the active surface area of the gold working electrode was based on the Randles-Sevcik equation below. Especially the determined active surface area (A) of the screen-printed gold electrode is a good indication of the signal quality, which is important for future sensor assays.

$$I_p = 2.69 \cdot 10^5 A C \sqrt{n^3 D \nu} \quad (\text{Equation 1.1})$$

The diameter of the gold working electrode is 5 mm, including the rectangular squared gold surface below the main electrode, with the length " a " of 3 mm and the width " w " of 1 mm, the geometric area A is calculated at 0.226 cm². The bulk concentration of potassium ferricyanide C is 1 mM l⁻¹, the number of electrons n involved in the $\text{Fe(CN)}_6^{3-} \leftrightarrow \text{Fe(CN)}_6^{4-}$ system is one, the diffusion coefficient D of $\text{K}_3\text{Fe(CN)}_6$ is 7.6x10⁻⁶ cm² s⁻¹ (Morrin et al., 2007) and the scan rate ν varies from 0.1 V s⁻¹ to 0.01 V s⁻¹. The resulting peak current I_p was calculated as the average of the cathodic and anodic peak current $I_{pC/A}$.

The following calculation shows exemplarily the calculation method of the active area of a screen-printed gold electrode for a scan rate of 20 mV s⁻¹:

$$I_p = 2.69 \cdot 10^5 \cdot A_{\text{active}} \cdot C \sqrt{n^3 D \nu} \quad \rightarrow \quad A_{\text{active}} = \frac{I_p}{2.69 \cdot 10^5 C \sqrt{n^3 D \nu}}$$

$$A_{\text{active}} = \frac{\left(\frac{19.94 + 20.3}{2}\right) \cdot 10^{-6} \text{A}}{2.69 \cdot 10^5 \frac{(\text{As})^{\frac{3}{2}}}{\text{mol} \cdot \text{J}^{\frac{1}{2}}} \cdot 10^{-3} \frac{\text{mol}}{\text{L}} \cdot \sqrt{1^3 \cdot 7.6 \cdot 10^{-6} \frac{\text{cm}^2}{\text{s}} \cdot 0.02 \frac{\text{V}}{\text{s}}}}$$

$$A_{\text{active}} = \frac{\left(\frac{19.94 + 20.3}{2}\right) \cdot 10^{-6} \text{A}}{2.69 \cdot 10^5 \frac{(\text{As})^{\frac{3}{2}}}{\text{mol} \cdot \text{J}^{\frac{1}{2}}} \cdot 10^{-6} \frac{\text{mol}}{\text{cm}^3} \cdot \sqrt{1^3 \cdot 7.6 \cdot 10^{-6} \frac{\text{cm}^2}{\text{s}} \cdot 0.02 \frac{\text{J}}{\text{As}^2}}}$$

$$A_{\text{active}} = 0.19185 \text{ cm}^2$$

$$A_{\text{active}} [\%] = \frac{A_{\text{active}}}{A_{\text{geometric}}};$$

$$\text{With: } A_{\text{geometric}} = \pi r^2 + a \cdot w = 3.142 \cdot \left(\frac{0.5}{2} \text{ cm}\right)^2 + 0.3 \text{ cm} \cdot 0.1 \text{ cm} = 0.226 \text{ cm}^2$$

$$A_{\text{active}} [\%] = \frac{0.19185 \text{ cm}^2}{0.226 \text{ cm}^2} \cdot 100 = \underline{\underline{84.89 \%}}$$

Using a scan rate of 10 mV s^{-1} showed the best results regarding the reversibility of the reaction with the lowest peak-to-peak separation ΔE and the highest active surface area with a value of over 90 %. However, with a total cycle time of nearly 10 min per sensor, the measurement time was found to be too long for the characterisation of the sensors, as multiple sensors were measured to ensure a statistically significant value. Regarding this, a scan rate of 20 mV s^{-1} was chosen as the best compromise between measurement time and reproducibility. Furthermore, the oxidation and reduction peaks are better defined and show the lowest standard deviation, resulting in a ratio of the anodic and cathodic peaks of 0.98.

3.4.1.3 Characterisation of Various Sensor Types

In order to find the sensor with the best performance, three replicates of each type (JA, JC and JD) were tested against potassium ferricyanide and TMB- H_2O_2 . The precise composition of the different sensors examined in this work is listed in Section 3.2.2. Previous experiments indicated that a potassium ferricyanide concentration of 1 mM in 1 M KCl with a scan rate of 20 mV s^{-1} results in the best reproducibility of the redox reaction. The results of the analysis are presented in Table 3.4 with the corresponding cyclic voltammograms in Figure 3.10. The calculation of the active surface area occurred as described in Section 3.4.1.2.

Table 3.4: Overview of cyclic voltammetric analyses of the three DuPont sensor types JA, JC and JD at a scan rate of 20 mV s^{-1} against 1 mM potassium ferricyanide. Standard deviation represents three sensors of each type with three scans per sensor.

	JA	JC	JD
$I_{PA} [\mu A]^a$	15.71 ± 0.90	19.10 ± 0.21	20.29 ± 0.34
$E_{PA} [V]^b$	0.056 ± 0.05	0.022 ± 0.01	0.014 ± 0.00
$I_{PC} [\mu A]$	-17.63 ± 0.23	-19.47 ± 0.33	-20.48 ± 0.27
$E_{PC} [V]$	-0.123 ± 0.06	-0.162 ± 0.01	-0.154 ± 0.01
$\Delta E [V]^c$	0.179 ± 0.08	0.184 ± 0.01	0.168 ± 0.01
I_{PA} / I_{PC}^d	0.89	0.98	0.99
$A_{active} [\%]^e$	70.34	81.37	86.00

^a $I_{P C/A}$ = Cathodic / anodic peak current

^b $E_{P C/A}$ = Potential applied at the cathodic / anodic peak

^c Peak distance $\Delta E = E_{PA} - E_{PC}$

^d Ratio between the cathodic and anodic peak current

^e Ratio between the active area calculated by the Randles-Sevcik equation and the geometric surface

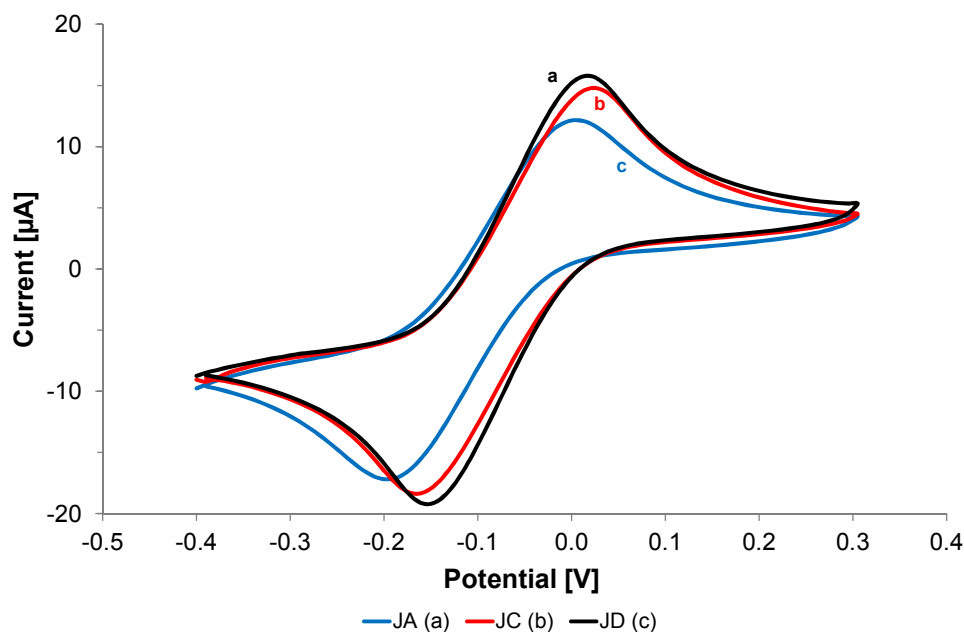


Figure 3.10: Cyclic voltammograms of DuPont SPGE (JA, JC and JD) at a scan rate of 20 mV s^{-1} using a 1 mM potassium ferricyanide solution. The graphs represent the average of three sensors with three scans per sensor.

In particular, the sensors JC and JD performed very well, with an active surface area of 81.4 % (JC) and 86.0 % (JD) (Table 3.4). In terms of reproducibility, peak ratio, active surface area and ΔE value, the JD sensor, as the newest sensor generation of DuPont Microcircuit Materials (Bristol, UK), showed the best performance. This finding was also confirmed comparing the performance of the sensor types with TMB (Figure 3.11). The signal strength of the JD sensors is 7 % (JD-JA) and 20 % (JD-JC) higher for the 1st anodic peak, 87 % (JD-JA) and 47 % (JD-JC) higher for the 2nd anodic peak and 43 % (JD-JA) and 46 % (JD-JC) higher for the cathodic peak current (Figure 3.11 embedded graph). Due to its superior performance, the JD sensor type was selected for the future assay development. However, the JA and JC sensors were still employed for basic research.

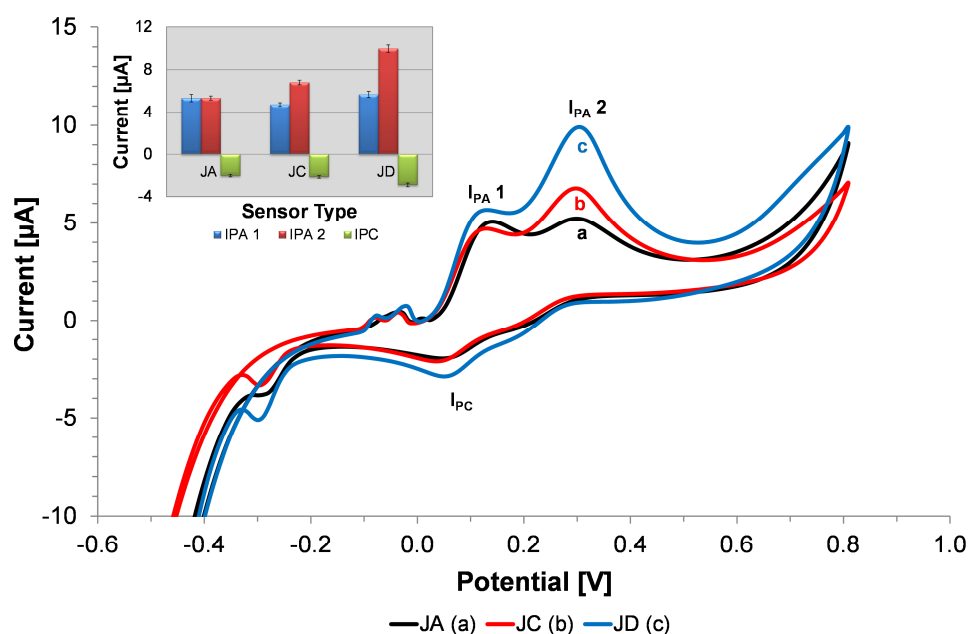


Figure 3.11: Cyclic voltammogram of DuPont SPGE (JA, JC and JD) using 4 mM TMB at a scan rate of 20 mV s⁻¹. The embedded graph visualises the maximum current of the cathodic and anodic peaks. Data are plotted as the average of three sensors, bars represent the standard error of the means (n=9).

$I_{PA\ 1/2}$ = First and second anodic peak current; I_{PC} = Cathodic peak current.

3.4.1.4 HRP / TMB-H₂O₂ substrate system

The components of the TMB/H₂O₂ substrate system for the signal generation of the biosensor were characterised using cyclic voltammetry with a bandwidth ranging from -0.5 to 0.8 V. One hundred μ l of 4 mM TMB, 0.06 % H₂O₂ and 4 mM TMB + 0.06 % H₂O₂ substrate was dropped on the sensor surface covering all three electrodes. Reagents were prepared in 25 mM phosphate citrate - 0.05 M KCl working buffer. The scan rate was constant at 20 mV s⁻¹.

The cyclic voltammogram of the TMB/H₂O₂ signal generating system is illustrated in Figure 3.12. The H₂O₂ solution alone shows only two small anodic peaks at a potential of -0.15 V and -0.02 V. The second anodic peak is also visible in the cyclic voltammogram of TMB, ranging between -0.08 and -0.01 V. On the cathodic side, there is one peak at a potential of -0.35 V. These small peaks are due to Ag and Cl ions of the Ag/AgCl reference electrode, which interact with the working buffer containing electrolytes (Horányi et al., 1983) and do not occur when measuring the complete TMB-H₂O₂ substrate system.

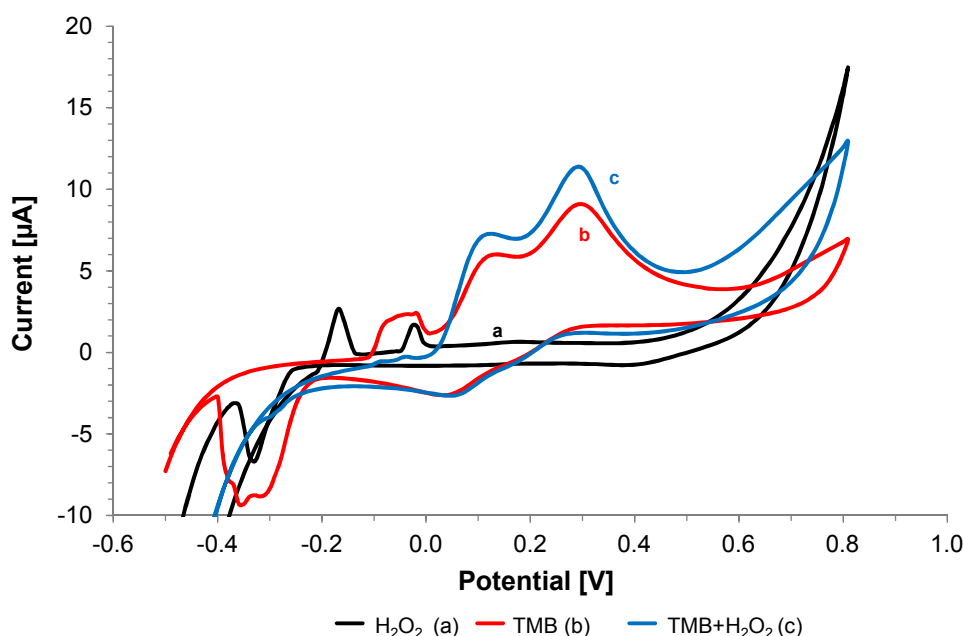


Figure 3.12: Cyclic voltammogram of (a) 0.06 % H₂O₂, (b) 4 mM TMB, and (c) biosensor substrate containing 4 mM TMB + 0.06 % H₂O₂ in 25 mM phosphate citrate - 0.05 M KCl working buffer. The signals are plotted as the average of three sensors.

3.4.2 DEVELOPMENT OF AN ELECTROCHEMICAL IMMUNOSENSOR USING SPGE

3.4.2.1 Scanning Electron Microscopy (SEM)

The morphology of the sensor surface is a key performance factor, as several surface interactions are needed to perform a successful immunoassay. Microscopic techniques like scanning electron microscopy (SEM), scanning tunnelling microscopy and transmission electron microscopy (TEM) become therefore an important complementary tool for surface structure characterisation (Davis et al., 1998).

The scanning electron microscopy of the JD SPGE shows a homogeneous distribution of the gold ink with round ink granules, producing a maximised surface area (Figure 3.13). There are two sizes of ink particles. The larger sized particles have a diameter of approximately $3 \pm 0.5 \mu\text{m}$. The smaller sized particles ($\varnothing$ ca. 0.1 to 1 μm) cluster the area between the big sized ink particles. High surface area resulting from the three dimensional ink granules has a direct impact on the maximum amount of biomaterial immobilised on the working electrode and was reported to improve assay performance (Noh and Tothill, 2006). At a magnification of x100,000 the surface roughness of the gold particles is visible, showing smooth ditches approximately 10-50 nm deep (Figure 3.13 D).

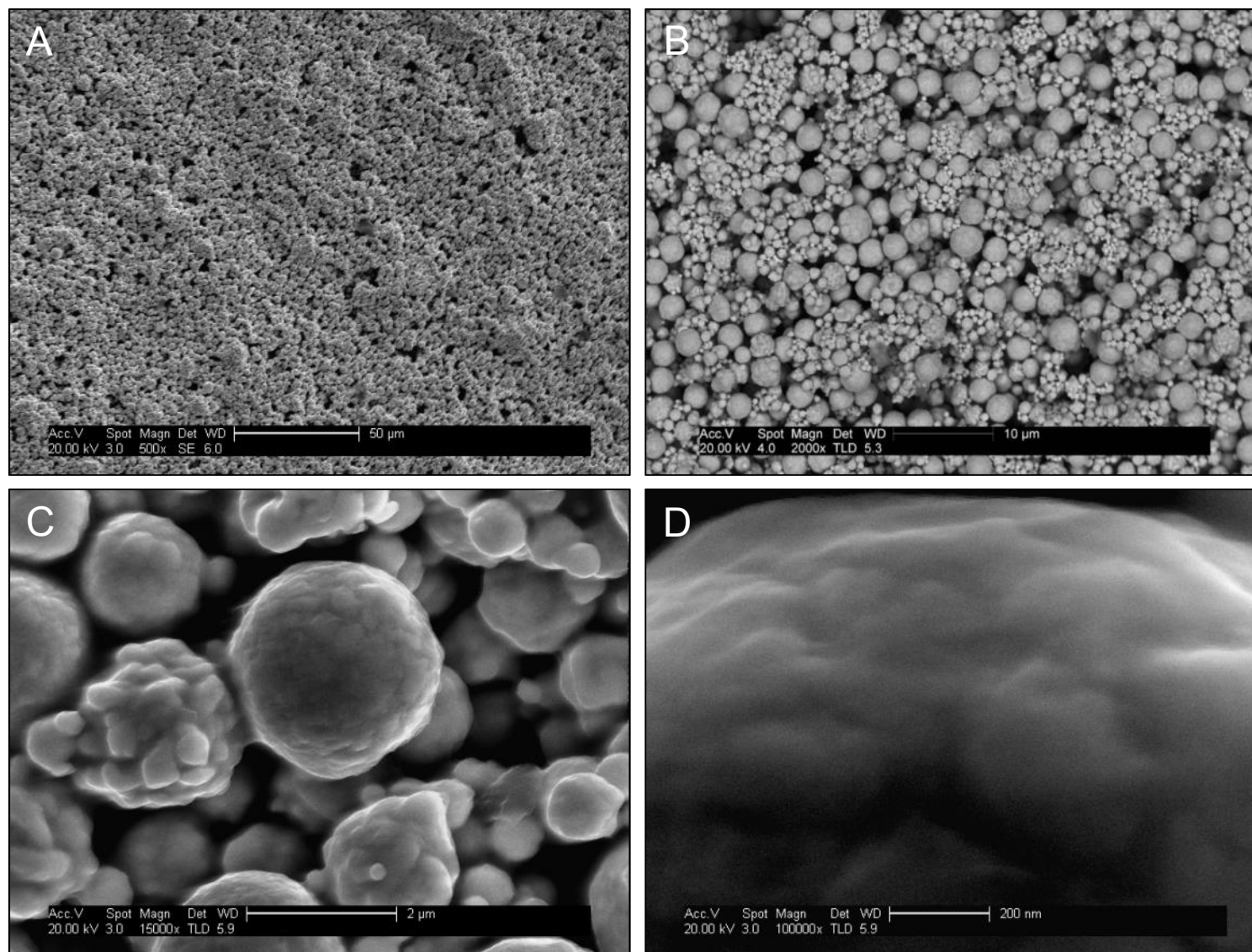


Figure 3.13: SEM of a DuPont SPGE using a magnification of (A) x500, (B) x2,000, (C) x15,000 and (D) x100,000.

Essential for good SEM images of non-conducting materials is the coating with a nanolayer. Two techniques were evaluated gold plasma and vaporised carbon coating. However, the resolution achieved with the carbon coating was not as detailed as the one achieved with gold plasma coating (Figure 3.14). The carbon layer was clearly visible as a white thin film on the sensor surface (Figure 3.14 B, arrow). Furthermore, this coating technique is with approximately 15 min per sample more time consuming than the gold nanocoating. Considering these drawbacks, the gold plasma coating was used for non-conductive samples.

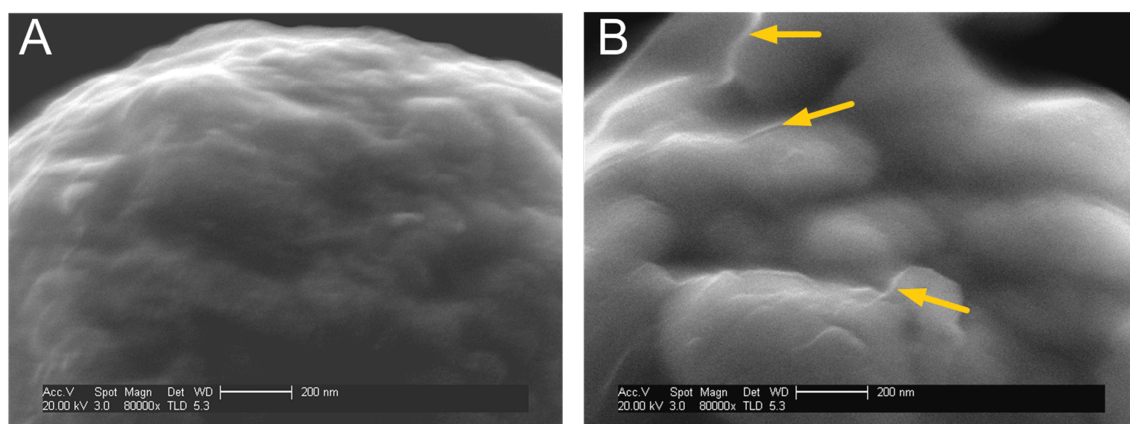


Figure 3.14: SEM of a bare SPGE with a magnification of x80,000 using gold plasma (A) and carbon coating (B). Arrows indicate the visible carbon layer.

3.4.2.2 Chronoamperometric Analysis of the HRP / TMB-H₂O₂ Substrate System

The applied step potential for chronoamperometric measurements highly depends on the type of electrode used (gold, carbon, modified graphite), the composition of the counter and reference electrode, the enzyme-substrate system and also the manufacturing company (Arya et al., 2007; Wollenberger et al., 2008). Therefore, the step potential for chronoamperometric measurements has to be analysed separately for every biosensor system to select the optimal potential to apply to the assay.

Different step potentials ranging from -0.4 to +0.3 mV were evaluated for the HRP / TMB-H₂O₂ system in order to achieve the best signal to noise ratio. TMB-H₂O₂ substrate (4 mM / 0.06 %) was prepared in phosphate citrate / KCl buffer. The measurement was normalised by first measuring the baseline current of the TMB-H₂O₂ system at each step potential, before adding 10 µl of 10 ng ml⁻¹ goat anti-mouse IgG antibodies conjugated HRP to 90 µl substrate.

Cyclic voltammetric measurements of the HRP / TMB-H₂O₂ system (Section 3.3.2.4) suggested a cathodic peak at 0.03 V. However, these findings are not in accordance with the chronoamperometric measurements of the HRP / TMB-H₂O₂ system (Figure 3.15). If a potential of 0.03 V is applied to the HRP / TMB-H₂O₂ system, the current is not significantly higher than the background current (-1 ± 0.7 µA) after applying the HRP conjugate to the substrate. Instead, a potential of -200 mV vs. Ag/AgCl reference electrode resulted in a cathodic peak of -5.5 µA. This potential was then used for further stepamperometric measurements.

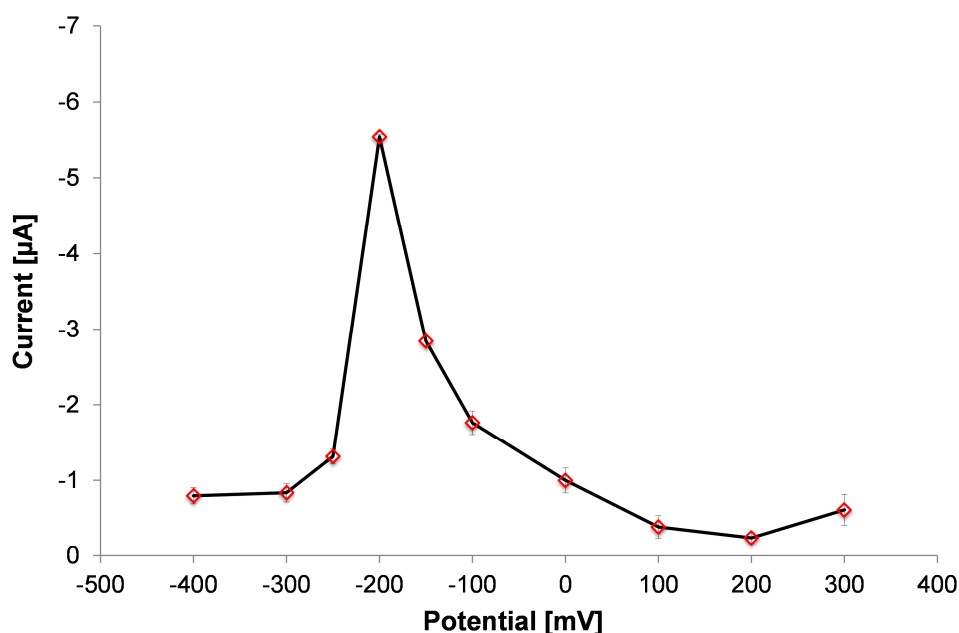


Figure 3.15: Chronoamperometric reading after applying different step potentials to the HRP / TMB-H₂O₂ system. The TMB-H₂O₂ background signal was subtracted from each potential measurement before the IgG-HRP conjugate (final concentration 1 ng ml⁻¹) was added to the electrode. The measurements were taken after 100 s, the bars represent standard error of the means (n=3).

After the optimum step potential was established, further stepamperometric measurements were conducted using varying IgG-HRP concentrations (1, 10, 50, and 100 ng ml⁻¹) with constant TMB-H₂O₂ concentration. Figure 3.16 illustrates the signal development of the HRP / TMB-H₂O₂ system with increasing enzyme concentration. The electrochemical response of the HRP-TMB system is linear with a R^2 value of 99.7 for HRP concentrations up to 50 ng ml⁻¹. With signals over 10 μ A, the linearity decreases reaching the steady state of the reaction, reducing the correlation coefficient to 97.8.

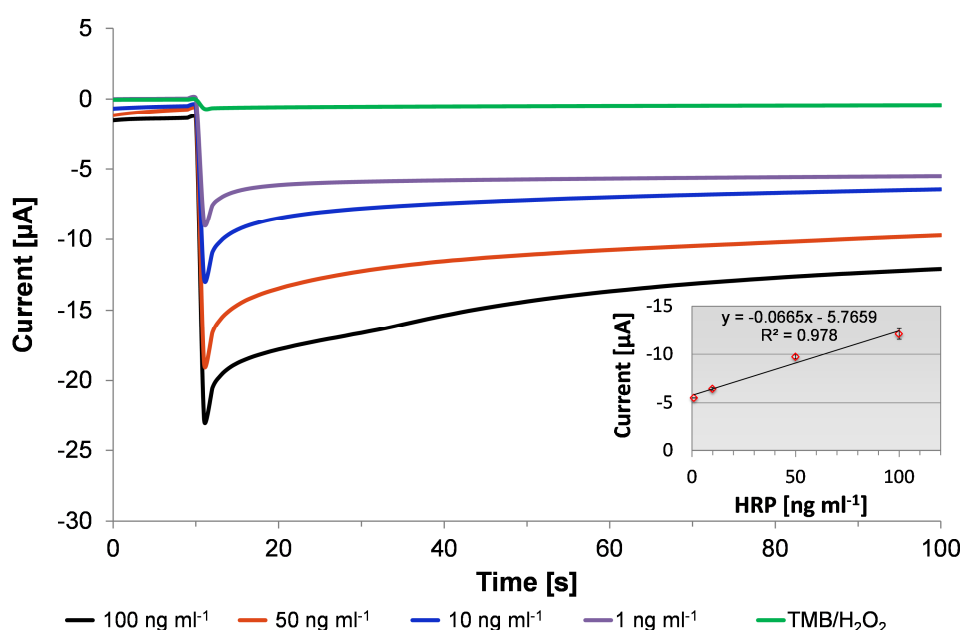


Figure 3.16: Biosensor signal at a step potential of -200 mV vs. Ag/AgCl using varying IgG-HRP conjugate concentrations (1, 10, 50, and 100 ng ml⁻¹) to TMB-H₂O₂ background. The embedded graph shows the steady state current after 100 s as a linear function of the HRP concentration. The bars represent standard error of the means (n=3).

The signal saturation can have varying reasons. In general, HRP has very fast reaction kinetics and substrate turnover rates (Mackey et al., 2007). Assuming that the diffusion coefficient of the reduced and oxidised forms is constant and equal, limiting factors for the enzyme turnover is the substrate concentration of the diffusion layer (Lyons, 2007). However, high substrate excess resulting from increased substrate concentrations was reported to lead to inhibiting effects on the HRP enzyme activity and unstable assays

(Edman et al., 1999). In order to find a balance between signal strength, assay performance and reproducibility of the HRP / TMB-H₂O₂ system, a TMB-H₂O₂ concentration of 4 mM and 0.06 % was used for sensor signals below 20 μ A. In rare cases when the signal was constantly higher than this threshold, the TMB-H₂O₂ concentration was doubled to 8 mM and 0.12 % in order to provide sufficient TMB-H₂O₂ substrate for the enzyme reaction.

3.4.3 EVALUATION OF SENSOR FORMATS

3.4.3.1 Indirect ELISA Format Using Anti-species Antibodies

The first sensor format evaluated is the indirect sandwich ELISA (Figure 3.17). The monoclonal as well as the polyclonal antibodies have been used alternately as capture and detection antibodies in a comparable assay performed at the same time. When the monoclonal was coated to the sensor surface, the polyclonal antibody was used for detection and vice versa. For signal generation, anti-mouse and anti-rabbit antibodies (Invitrogen, Paisley, UK), raised in goat as a host animal, conjugated to HRP were used.

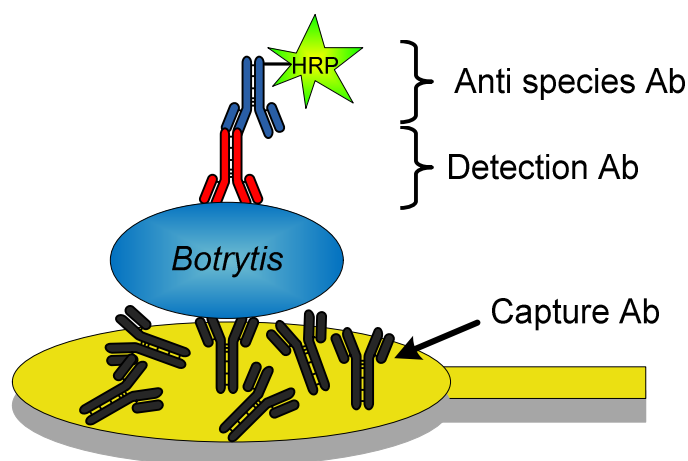


Figure 3.17: Schematic illustration of an indirect sandwich ELISA format on a SPGE.

The assay was performed using three different concentrations of pure *B. allii* fungus (10, 100, 300 $\mu\text{g ml}^{-1}$) and the negative control, which was represented by the commercial negative control from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK). The protocol was followed as described in Section 3.3.3 with an antibody concentration of 50 $\mu\text{g ml}^{-1}$ for coating and a detection antibody concentration of 10 $\mu\text{g ml}^{-1}$. The anti-species conjugate against mouse (for monoclonal antibody BC-12) and the conjugate against rabbit (for the polyclonal antibody from ADGEN), both labelled with HRP, were adjusted to 1 $\mu\text{g ml}^{-1}$ which represents the lowest dilution factor recommended by the company.

The results of the assay are illustrated in Figure 3.18. Both formats showed a separation of the different fungal concentrations. The maximum signal generated using the monoclonal antibody BC-12 used as the detection antibody followed by the goat anti-mouse antibodies conjugated to HRP was -12.5 μA compared to -5.66 μA of the polyclonal antibodies and goat anti-rabbit conjugate. However, a bigger part of the signal is produced by non-specific binding of the anti-mouse antibodies to HRP indicated by the higher current of the negative control, which was -6.22 μA for the goat anti-mouse and -3.77 μA for the goat anti-rabbit antibodies. Furthermore, the separation between the different fungal concentrations is significantly better with a 5.5 μA range between the highest and lowest concentration.

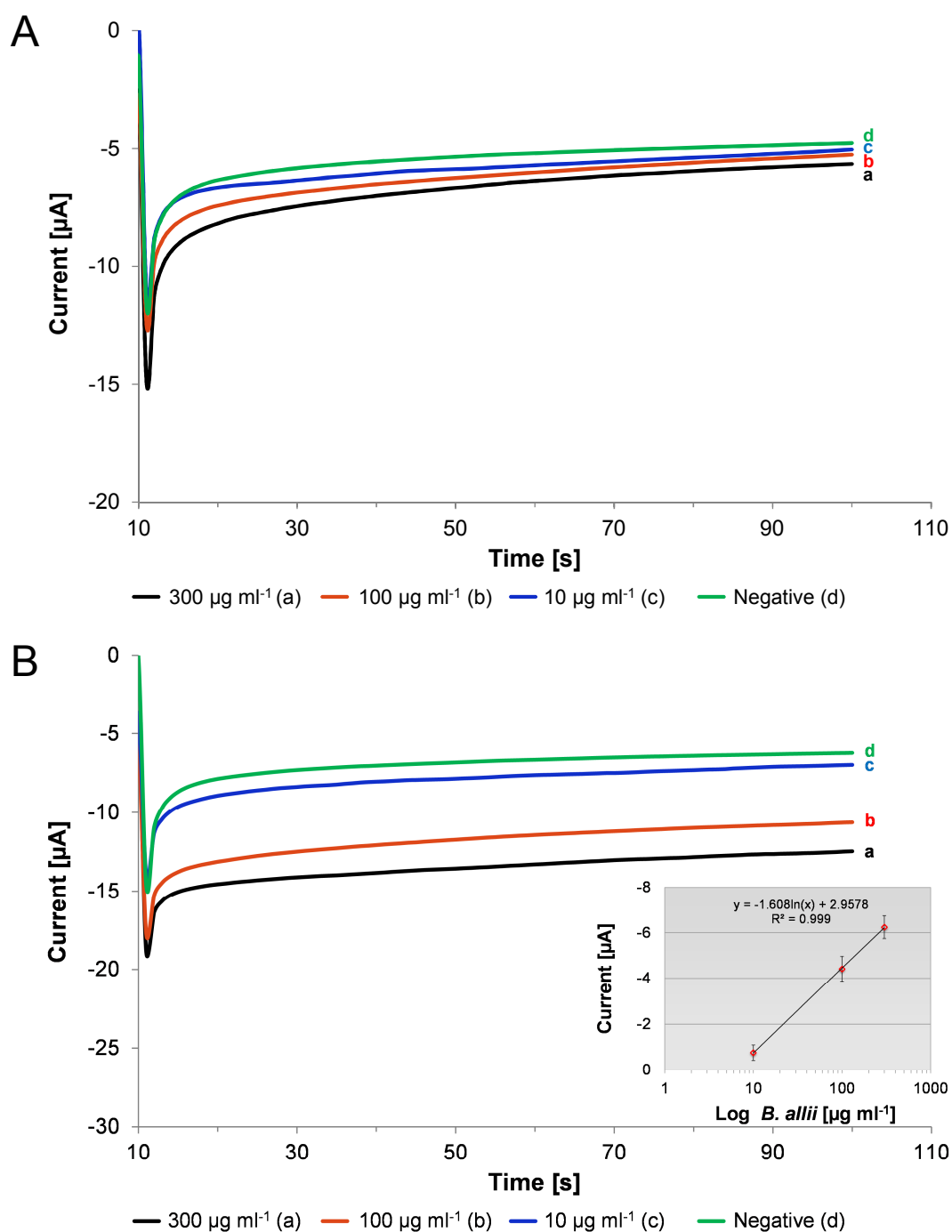


Figure 3.18: Indirect sandwich ELISA format with (A): monoclonal antibody BC-12 as capture and polyclonal antibodies as detection, and (B): polyclonal antibodies as capture, and monoclonal antibody BC-12 as detection with the embedded graph showing the linear range. The signal represents the average of two sensors for each concentration, the bars represent the standard error of the means.

The standard curve for the fungal concentration between 0 and 300 $\mu\text{g ml}^{-1}$ is presented in Figure 3.18 B (embedded graph). The detection limit for the indirect ELISA format with polyclonal capture antibodies and monoclonal antibody BC-12 used for detection is calculated at approximately 40 $\mu\text{g ml}^{-1}$. The high correlation coefficient of $R^2 = 0.999$ for the fitted curve as well as the low standard deviation of the goat anti-mouse antibodies indicate a better reproducibility of this assay format compared to the goat anti-rabbit antibodies used for the signal generation.

3.4.3.2 Direct ELISA Format with HRP Conjugated Antibodies

The indirect ELISA has a major disadvantage. As the signal is not generated directly, the anti-species antibodies conjugated to HRP have to be added to the sensor, leading to the need of an additional incubation step. An improvement is represented by the direct format (Figure 3.19), where the enzyme is covalently coupled to the detection antibody as this will reduce the steps used to conduct the assay. The procedure used for the conjugation of the antibodies to HRP was carried out using the Lightning-Link conjugation kit purchased from Innova Biosciences Ltd. (Cambridge, UK) as described in Section 2.3.3.

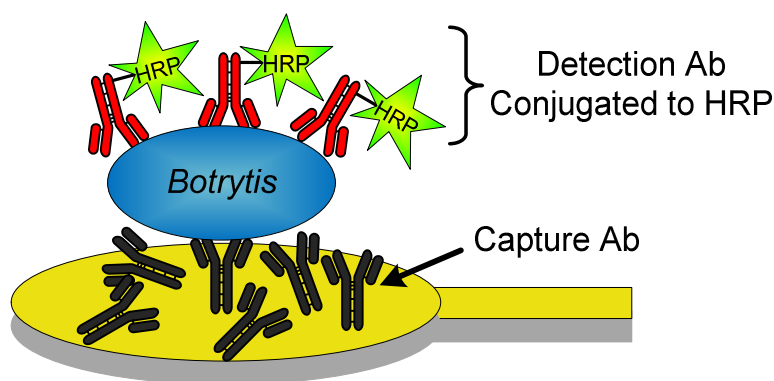


Figure 3.19: Schematic illustration of a direct sandwich ELISA format performed on a SPGE.

Similar to the indirect format, raw *B. allii* fungus was used as antigen with three different concentrations (10, 100, 300 $\mu\text{g ml}^{-1}$). The protocol was followed as described in Section 3.3.3, with an antibody concentration of 50 $\mu\text{g ml}^{-1}$ for the capture and 10 $\mu\text{g ml}^{-1}$ for detection antibody. Previous tests performed with the conjugated

antibodies (Section 2.4.3) demonstrated that the monoclonal antibody BC-12 conjugated to HRP is producing a 50 % lower signal than the polyclonal conjugate. Therefore, a sandwich format was accomplished using the monoclonal antibody BC-12 as capture and the polyclonal antibody conjugated to HRP for detection.

The chronoamperometric signal of the direct ELISA format is illustrated in the embedded graph of Figure 3.20 with the corresponding plot of the steady state current against fungal concentration shown in Figure 3.20. The assay performed well with a standard deviation as low as 0.01 μA for the negative control and a % CV value of 5 % at a fungal concentration of 10 $\mu\text{g ml}^{-1}$. This results in a limit of detection (LOD = negative control plus three times its standard deviation) calculated at approximately 2 $\mu\text{g ml}^{-1}$. To improve the signal strength for future assays a higher detection antibody concentration can be applied.

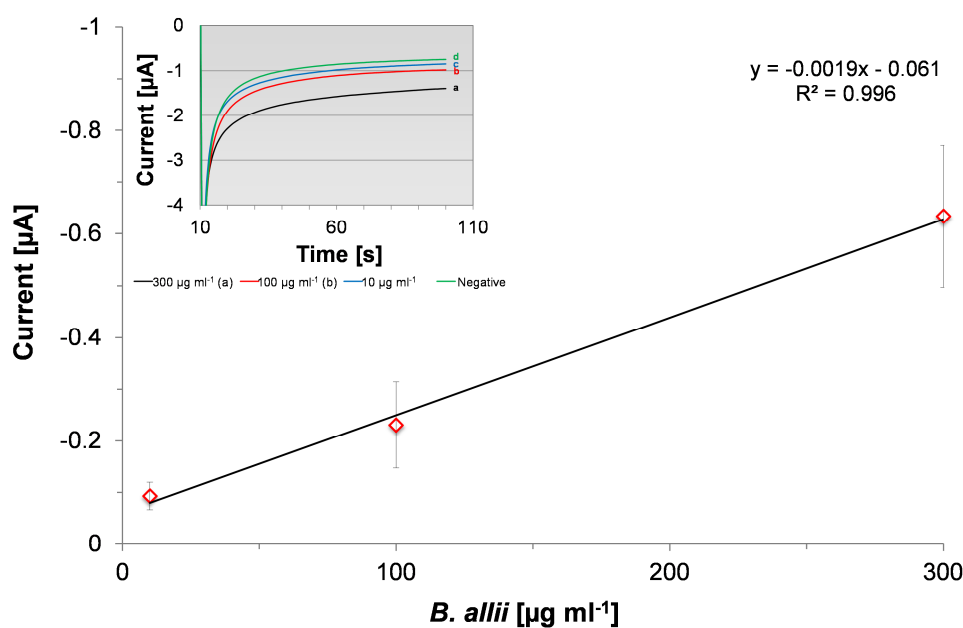


Figure 3.20: Direct ELISA format using JD SPGEs with monoclonal antibodies BC-12 as capture ($50 \mu\text{g ml}^{-1}$) and polyclonal antibodies conjugated to HRP as detection ($10 \mu\text{g ml}^{-1}$). The steady state current after 100 s measured at -200 mV was plotted against *B. allii* concentration (10, 100 and 300 $\mu\text{g ml}^{-1}$) to construct a standard curve. The embedded graph shows the averaged chronoamperometric signal for each concentration; the bars represent the standard error of the means ($n=3$).

3.4.3.3 Direct ELISA Format with the Application of Gold Nanoparticles

The initial tests of the gold nanoparticles assay (Figure 3.21) were performed with three different fungal concentrations (1, 0.5 and 0.1 mg ml⁻¹) of pure *B. allii* fungus. Monoclonal as well as the polyclonal antibodies have been used alternately as capture and detection antibodies in a comparable assay performed at the same time. The gold conjugate was produced according to the protocol described in Section 3.3.3.4, with monoclonal as well as polyclonal antibodies conjugated to Ø40 nm gold particles, previously labelled with HRP.

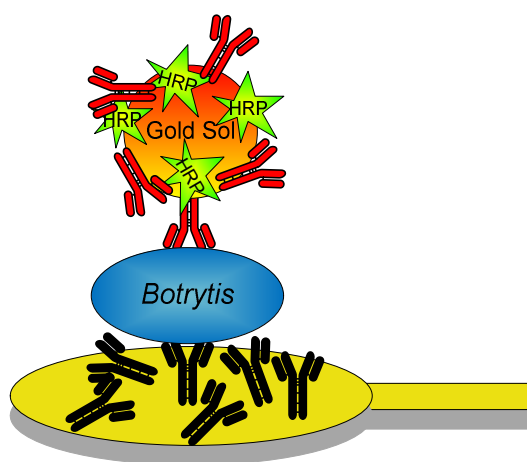


Figure 3.21: Schematic illustration of a direct sandwich ELISA format employing HRP and antibody-labelled gold nanoparticles on SPGE.

The chronoamperograms illustrated in Figure 3.22 show that both formats performed well using the gold colloid. Though the polyclonal antibodies used for the detection showed a non-linear effect at higher concentrations, this format produced better signal to noise ratio and the signal separation between the fungal concentrations was with 1.1 μ A, 0.73 μ A higher than the maximum signal separation achieved by conjugating the monoclonal antibody BC-12 to the gold sol. Following tests confirmed this observation. The non-linearity can be explained with steric hindrance of the antigen, preventing the binding of the fungus to the antibody, when very high concentrations are applied to the sensor. This effect was also observed in ELISA tests using the same fungal concentrations (Section 2.3.6.2). However, this behaviour is uncritical for the sensor design, because generally the concentrations of naturally infected onion plants and bulbs are between 100 and 300 μ g ml⁻¹.

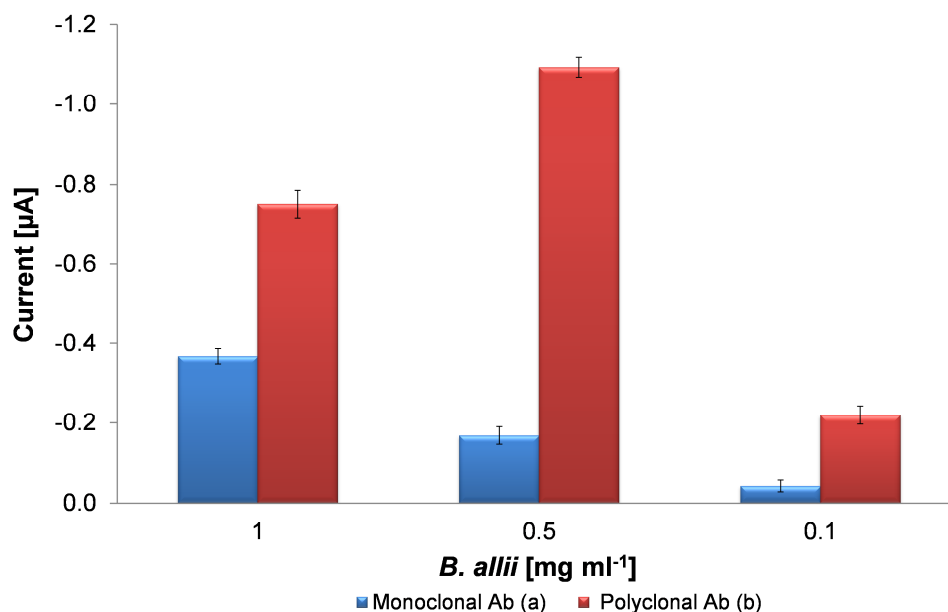


Figure 3.22: Normalised direct ELISA response performed with JD SPGE using various antibody combinations as capture molecule and for signal generation utilising Ø40 nm gold particles labelled with HRP. (a) Monoclonal antibody BC-12 was used as capture molecule and polyclonal antibodies were used for detection and (b) polyclonal antibodies used as capture molecule followed by the monoclonal antibody BC-12 for detection. The bars represent the standard error of the means (n=3).

In order to make the results more comparable to previous performed direct and indirect sensor assays, the antigen was diluted to a target concentration of 10, 100, and 300 µg ml⁻¹ and used in a sensor assay with the monoclonal antibody as capture molecule and the polyclonal antibody used for detection. Figure 3.23 illustrates the chronoamperometric signal of the direct ELISA format using Ø40 nm gold particles with the corresponding plot of the steady state current against the fungal concentration. The limit of detection (LOD = negative control plus three times its standard deviation) for this assay was calculated at 1.4 µg ml⁻¹. The assay performed well, showing a small standard deviation resulting in an average % CV value of 9.8 ± 0.05 % and a good signal separation of 0.39 µA. These promising results confirm the ability of the gold nanoparticles to generate an amperometric signal which is comparable to the conventional format using antibodies conjugated to HRP. To improve the signal strength and detection limit, further experiments have to be performed to optimise the preparation of the gold conjugate.

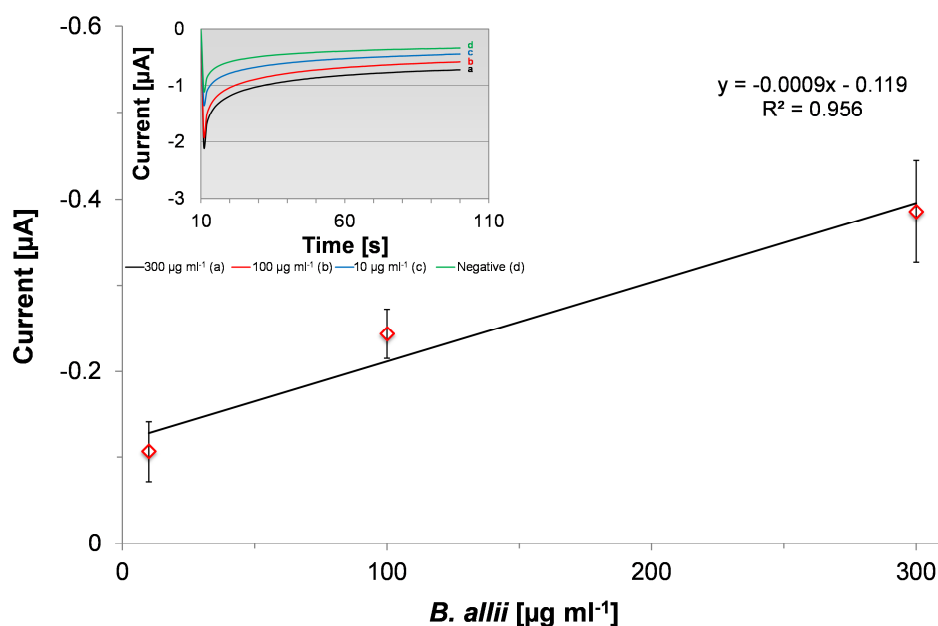


Figure 3.23: Standard curve of the direct ELISA format with monoclonal antibody BC-12 ($50 \mu\text{g ml}^{-1}$) as capture reagent and polyclonal antibody conjugated to $\text{Ø}40 \text{ nm}$ gold particles labelled with HRP for detection. Measurements were taken after 100 s at a potential of -200 mV after 100 s and plotted against various *B. allii* concentrations. The embedded graph shows the averaged chronoamperometric signal of three sensors for each concentration; the bars represent the standard error of the means ($n=3$).

3.4.3.4 Comparison of the Sensor Formats

Several assay formats were evaluated in this Chapter to enhance the sensor sensitivity. Table 3.5 provides an overview of the tested sensor formats. The calculated LOD does not represent a final value as the assays were not optimised. However, these experiments give essential information about the basic trend for further assay developments.

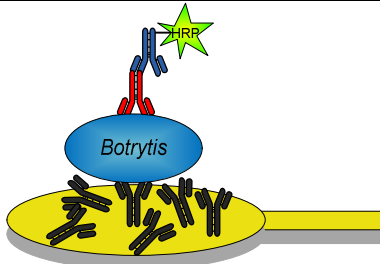
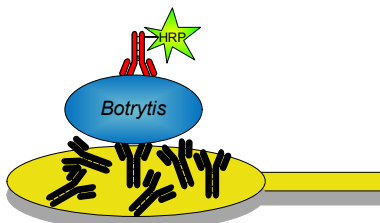
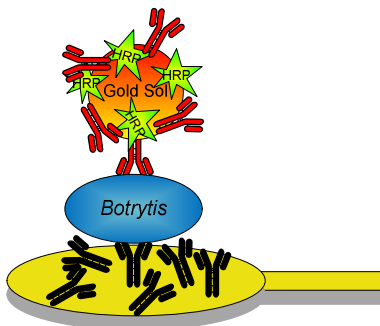
The indirect ELISA format using the goat anti-mouse antibodies produced a better signal separation compared to the goat anti-rabbit antibodies when applied to the biosensor. This can be caused by a lower affinity of the anti-species conjugate and does not give information about the quality of the detection antibody. Especially considering that the direct assay formats showed an opposite result giving better performance while using the monoclonal antibody BC-12 as capture and the polyclonal antibody for detection.

Compared to the indirect format using anti-species conjugate, the direct format, with conjugated detection antibodies or gold nanoparticles, eliminates one incubation step. This leads to an easier, less time-consuming assay procedure which is desirable for a commercial application. The best signal generation was achieved using monoclonal antibodies as capture and conjugated polyclonal antibodies for detection. Further experiments have to be conducted to improve the signal strength of the assay. This assay format accomplished the best signal reproducibility and sensitivity, resulting in a low detection limit of $2 \mu\text{g ml}^{-1}$ and was therefore chosen for further optimisation.

The direct format incorporating gold nanoparticles performed well, showing a small standard deviation for low concentrations and a good signal separation. These promising results confirm the ability of the gold nanoparticles to generate an amperometric signal, which is comparable to the conventional format, using antibodies conjugated to HRP. Though the detection limit is lower than the direct format with conjugated antibodies, there are remaining challenges regarding the reproducibility and signal strength of the assay.

Except for the indirect ELISA with the conjugated goat anti-mouse antibodies, the polyclonal antibodies from the *B. allii* ELISA kit performed better as detection antibodies, compared to the monoclonal antibody BC-12. However, the poor performance in the indirect ELISA format can be caused by a lower affinity of the goat anti rabbit anti-species conjugate. Furthermore, the polyclonal antibody showed some cross reactivity with other fungal species (Chapter 2.3.6.3), especially *Fusarium oxysporum*, and *Aspergillus niger*. Though these fungal species normally do not infect the neck tissue of an onion plant, the application of the developed biosensor can be enhanced by using a monoclonal antibody as capture molecule with no tendency for cross reactivity. The application of the polyclonal antibody for detection will also ensure a steady signal which will not be affected by structural differences of the *Botrytis* species and will have superior signal strength compared to the monoclonal antibody used for detection.

Table 3.5: Overview of the sensor formats evaluated for the biosensor development.

Assay Format	Capture Ab	Detection Ab	LOD [$\mu\text{g ml}^{-1}$]	Schematic of the Assay*
Indirect ELISA	BC-12	Polyclonal Ab followed by conjugated goat anti-rabbit	160	
	Polyclonal Ab	BC-12 Ab followed by conjugated goat anti-mouse Ab	40	
Direct ELISA	BC-12	Polyclonal Ab conjugated to HRP	2	
	Polyclonal Ab	BC-12 conjugated to HRP-labelled Ø40 nm gold particles	33	
Direct ELISA with gold nanoparticles	BC-12	Polyclonal Ab conjugated to HRP-labelled Ø40 nm gold particles	1.4	

*  = Capture Ab;  = Detection Ab;  = Anti species Ab;  = Conjugated HRP.

3.4.4 EVALUATION OF GOLD NANOPARTICLES AS A TECHNIQUE FOR SIGNAL AMPLIFICATION

Nanomaterials such as gold nanoparticles, carbon nanotubes, latex nanoparticles, and micromagnetic beads have been used to improve the immunosensor's performance (Li et al., 2002; Karman et al., 2007; Fernandez-Baldo et al., 2010). Recently, gold nanoparticles (nano-Au) have gained much attention mainly because of their capability to increase the specific surface area with simultaneous good biocompatibility and no needed chemical modification prior to the bioconjugation (Li et al., 2002). Their strong capability to absorb biomolecules, such as protein and DNA, without loss of their biological activity, leads to a wide range of applications (Hyatt, 1989). The result is an amplification of the signal due to the ability of the gold sol to absorb more than one HRP to the surface (Salam and Tothill, 2009a; Uludag and Tothill, 2010a). Consequently, the signal produced per bound antibody is higher in comparison to the conventional direct format (Figure 3.24). Therefore, the use of gold nanoparticles for the sensor assay was evaluated for the use in the biosensor assay.

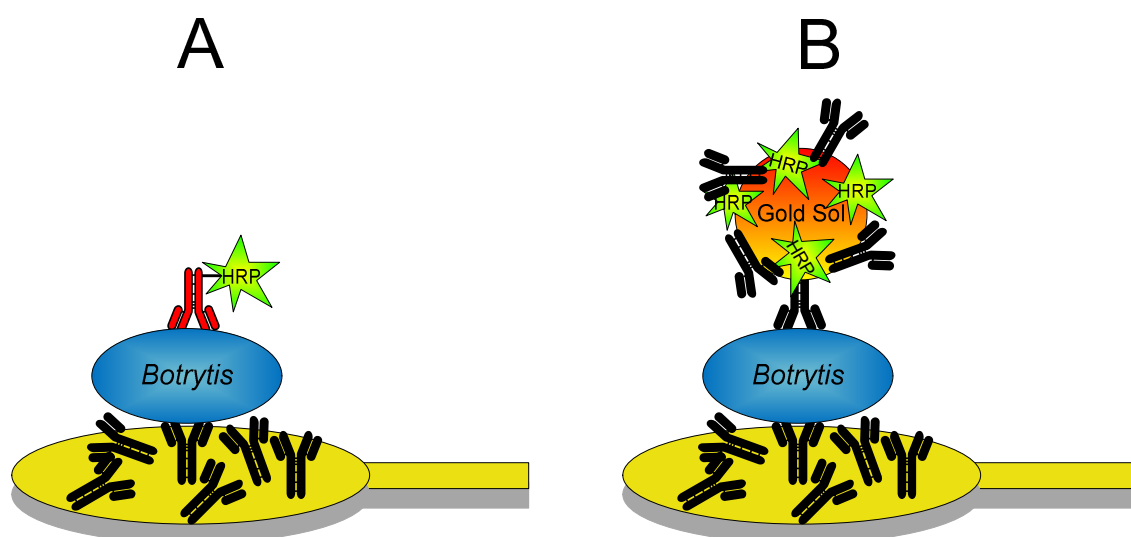


Figure 3.24: Comparison of the conventional direct format with conjugated antibodies (A), to the direct application using gold nanoparticles labelled with antibodies and HRP (B).

3.4.4.1 Visualisation of the Gold Nanoparticles Using Scanning Electron Microscopy

The SEM images after performing the sensor assay with gold nanoparticles are illustrated in Figure 3.25. Immobilising proteins on the working electrode affects the resolution of the SEM images, as they inhibit the current flow of the scanning electrons. A gold coating to increase the conductivity and the resolution was not practicable, as the 20 nm layer covered the gold particles in the same manner. Subsequent visualisation was not possible using a coating.

The gold nanoparticles are visible as bright dots attached to the uncondutive and therefore black antigen (Figure 3.25 B-D). The imaging software of the SEM comprises a measuring mode which calculates the length of the magnified objects. This function was used to identify the gold particles on the working electrode, by estimating the diameter of the particles (Figure 3.25 B and D). The SEM images confirmed the ability of the Ø40 and Ø60 nm particles to bind to the antigen.

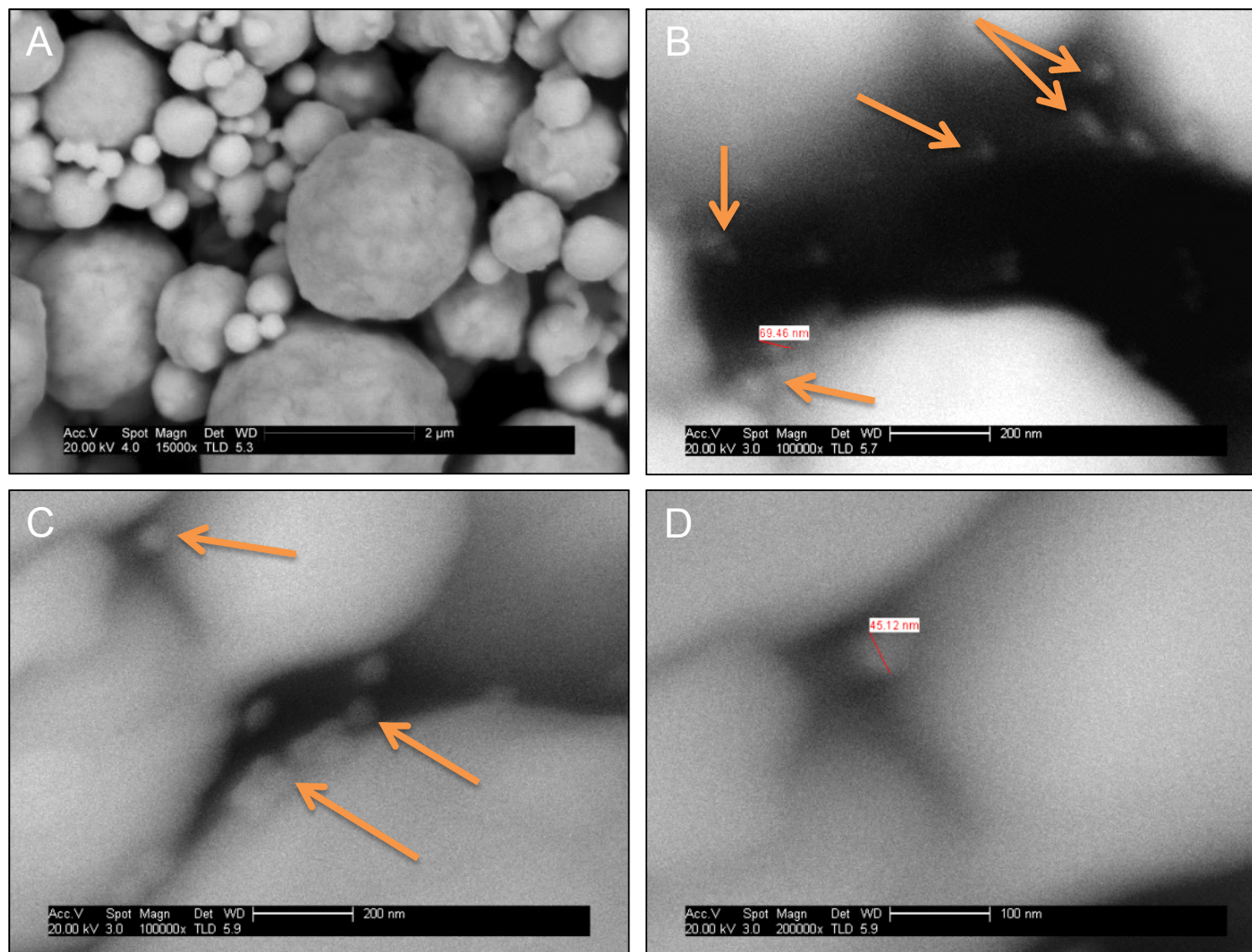


Figure 3.25: SEM of the working electrode with Ab and HRP labelled 40 and 60 nm gold nanoparticles used for detection. Monoclonal Ab ($100 \mu\text{g ml}^{-1}$) were immobilised on the sensor surface, followed by blocking and the addition of $300 \mu\text{g ml}^{-1}$ of *B. allii* antigen.

(A) $\times 15,000$ with $\varnothing$ 60 nm gold;
 (B) $\times 10^5$ with $\varnothing$ 60 nm gold;
 (C) $\times 10^5$ with $\varnothing$ 40 nm gold and
 (D) $\times 2 \cdot 10^5$ with $\varnothing$ 40 nm gold.

3.4.4.2 Optimisation of the Gold Nanoparticles Production

The gold nanoparticle-conjugate production process was screened by varying key parameters such as particle size, order, volume and incubation time of the antibody and HRP applied, type of antibody and HRP, extracted volume of the particles, mixing method, dilution factor and pH. The averaged normalised ELISA response and the corresponding manufacturing and test parameters are listed in Table 3.6. Previous tests using the conjugated poly and monoclonal antibodies (Section 2.4.3) showed a good ELISA response. To match this performance, an absorbance of approximately 0.5 or higher should be achieved. However, none of the tested parameter combination matched the signal strength of the HRP conjugate, the highest absorbance after normalisation was found to be 0.105.

One significant source of variation was spotted. When the plate was washed by using a pipette, the signal increased to an absorbance value of 0.388. However, this washing method is not very efficient and resulted in non-linear effects of the absorbance caused by non-specific binding. Longer incubation time as well as overnight coating at room temperature and 4 °C did not result in a significant increase in signal.

However, some trends were observed after monitoring the overall performance. First, using 20 nm gold particles showed a lower averaged signal, compared to the bigger sized 40 and 60 nm gold particles. Second, increasing the concentrations of the antibodies or HRP did not improve the signal strength significantly. A possible reason for this observation is the two-step incubation process. If the gold nanoparticles are first coated with a high concentration of antibodies, the secondly incubated HRP may not be able to adsorb to the already covered surface of the antibodies or vice versa. However, applying the antibodies and HRP at the same time did not enhance the signal either. Varying the pH by adding various volumes of NaOH, reducing the dilution factor of the gold nanoparticles, as well as their concentration did not lead to better performance.

Table 3.6-1: Optimisation of the gold nanoparticles production using direct ELISA method.

Au Size [nm]	<i>B. allii</i> [$\mu\text{g ml}^{-1}$]	Order and Volume HRP and AB	Incubation Method	1 st and 2 nd Incubation Time [h]	Type HRP	Volume Gold [μl] / Supernatant Removed [μl]	Au-NP Dilution	Max. ^b Absorbance [$\times 10^3$]
20	300, 100, 50, 10	5 μl HRP first, 5 μl Ab second	roller mixer	1, 1	II	500 / 470	01:20	32
40	300, 100, 50, 10	5 μl HRP first, 5 μl Ab second	roller mixer	1, 1	II	500 / 470	01:20	92
40	300, 100, 50, 10	5 μl HRP first, 5 μl Ab second	rotator mixer	1, 1	VI	500 / 470	01:20	69
40 (nb) ^a	300, 100, 50, 10	5 μl HRP first, 10 μl Ab second	rotator mixer	1, 1	VI	500 / 470	01:20	105
40 (nb)	300, 100, 50, 10	5 μl HRP first, 5 μl Ab second	roller mixer	1, 1	II	500 / 470	01:20	29
40 (nb)	300, 100, 50, 10	5 μl Ab first, 5 μl HRP second	roller mixer	1, 1	II	500 / 470	01:20	13
40 (nb)	300, 100, 50, 10	5 μl Ab first, 5 μl HRP second (in CBB)	roller mixer	1, 1	II	500 / 470	01:20	aggregation
40 (nb)	300, 100, 50, 10	Ab + HRP at the same time (5 μl each)	rotator mixer	3	II (nb)	500 / 470	01:10	16
40 (nb)	300, 100, 50, 10	Ab + HRP at the same time (20 μl each)	rotator mixer	2	II (nb)	500 / 470	01:10	54
40 (nb)	300, 100, 50, 10	5 μl HRP first, 20 μl Ab second	rotator mixer	1, 1	II (nb)	500 / 470	01:10	44
40 (nb)	300, 100, 50, 10	5 μl Ab first, 20 μl HRP second	rotator mixer	1, 1	II (nb)	500 / 470	01:10	53
20 (nb)	300, 100, 50, 10	5 μl Ab first, 20 μl HRP second	rotator mixer	1, 1	VI	500 / 470	01:10	25
40 (nb)	300, 100, 50, 10	5 μl HRP first, 5 μl Ab second	rotator mixer	1.5, 1.5	VI	500 / 470	01:10	19
40 (nb)	1000, 300, 100, 50	HRP + Ab at the same time (5 μl each)	fridge	overnight fridge	II	500 / 470	01:10	23
40 (nb)	1000, 300, 100, 50	HRP + Ab at the same time (5 μl each)	roller mixer	overnight roller mixer	II	500 / 470	01:10	40
40 (nb)	1000, 300, 100, 50	5 μl HRP first, 5 μl monoclonal Ab second	rotator mixer	1, 1	VI	500 / 470	01:10	33
60	1000, 300, 100, 50	HRP + Ab at the same time (20 μl each)	rotator mixer	1, 1	VI	500 / 470	01:10	58

Table 3.6-2: Optimisation of the gold nanoparticles production using direct ELISA method.

Au Size [nm]	<i>B. allii</i> [$\mu\text{g ml}^{-1}$]	Order and Volume HRP and AB	Incubation Method	1 st and 2 nd Incubation Time [h]	Type HRP	Volume Gold [μl] / Supernatant Removed [μl]	Au-NP Dilution	Max. ^b Absorbance [$\times 10^3$]
60	1000, 300, 100, 50	10 μl Ab first, 50 μl HRP second	rotator mixer	1, 1	II	500 / 470	01:10	43
60	10 ³ , 300, 100, 50	2.5 μl HRP first, 5 μl Ab second	rotator mixer	1, 1	II	250 / 235	01:10	86
40	1000, 300, 100, 50	5 μl HRP first, 5 μl Ab second, 30 min ELISA incubation	rotator mixer	1, 1	II	500 / 470	01:10	14
40	1000, 300, 100, 50	5 μL HRP first, 5 μl Ab second, 60 min ELISA incubation	rotator mixer	1, 1	II	500 / 470	01:10	51
40	500, 300, 100, 50	5 μl HRP first, 20 μl Ab second	roller mixer	1, 1	II	500 / 500	01:10	51
40 (nb)	500, 300, 100, 50	50 μl AB first, 25 μl HRP second, 30 μl BSA for blocking	roller mixer	1, 1	II	500 / 500	01:10	91
40 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second	roller mixer	1, 1	VI (nb)	500 / 500	01:10	79
60 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second	roller mixer	1, 1	II	500 / 500	01:10	71
20 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second	rotator mixer	1, 1	VI (nb)	500 / 500	undiluted	47
60 (nb)	500, 300, 100, 50	2.5 μl HRP first, 10 μl Ab second	rotator mixer	1, 1	II	250 / 250	01:10	68
60 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second	rotator mixer 10 rpm	1, 1	II	500 / 500	01:10	/
60 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second	rotator mixer 20 rpm	1, 1	II	500 / 500	01:10	/
60 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second	roller mixer 20 rpm	1, 1	II	500 / 500	01:10	/
60 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second, blocking with 50 μl BSA	rotator mixer 20 rpm	1, 1	II	500 / 500	01:10	33

Table 3.6-3: Optimisation of the gold nanoparticles production using direct ELISA method.

Au Size [nm]	<i>B. allii</i> [$\mu\text{g ml}^{-1}$]	Order and Volume HRP and AB	Incubation Method	1 st and 2 nd Incubation Time [h]	Type HRP	Volume Gold [μl] / Supernatant Removed [μl]	Au-NP Dilution	Max. ^b Absorbance [$\times 10^3$]
40 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second, blocking with 50 μL BSA	rotator mixer 20 rpm	1, 1	II	500 / 500	01:10	14
40 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second, blocking with 50 μl BSA	roller mixer 20 rpm	1, 1	II	500 / 500	01:10	31
60 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second, blocking with 50 μl BSA	roller mixer 20 rpm	1, 1	II	500 / 500	01:10	37
60 (nb)	500, 300, 100, 50	2x5 μl HRP, 2x5 μl Ab	rotator mixer	1, 1	II	1000 / 1000	Undiluted	/
60 (nb)	500, 300, 100, 50	5 μl HRP, 20 μl Ab	rotator mixer	1, 1	VI	500 / 500	01:10	25
60 (nb)	500, 300, 100, 50	5 μl HRP, 5 μl Ab, blocking with milk before centrifugation	rotator mixer	1, 1	II	500 / 500	01:10	24
60 (nb)	500, 300, 100, 50	5 μl AB first, 10 μl HRP second, glass tube	rotator mixer 20 rpm	1, 1	VI	500 / 500	01:10	41
60 (nb)	500, 300, 100, 50	5 μl HRP and 10 μl Ab at the same time, glass tube	rotator mixer 20 rpm	1	II	500 / 500	01:10	45
60 (nb)	500, 300, 100, 50	5 μl HRP, 5 μl Ab, washing with pipette	rotator mixer	1, 1	II	500 / 500	01:10	344
40 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second, 1.25 μl NaOH	rotator mixer	1, 1	II	500 / 500	01:10	/
40 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second, 2.5 μl NaOH (standard)	rotator mixer	1, 1	II	500 / 500	01:10	88
40 (nb)	500, 300, 100, 50	5 μl HRP first, 5 μl Ab second, 5 μl NaOH	rotator mixer	1, 1	II	500 / 500	01:10	33

^a nb = New batch^b Average absorbance [$\times 10^3$] of the highest response achieved in duplicates. If absorbance < 10 after normalisation, the value was considered as not significant (/).

3.4.4.3 Performance Studies of the Optimised Gold Nanoparticles Assay

After optimisation, the parameter combination using Ø40 nm gold colloid with 5 µl HRP VI and 10 µl of polyclonal antibodies incubated for 1 h using a rotator mixer showed the best absorbance value and was tested in a sensor assay presented in Figure 3.26. The gold nanoparticles performed well, showing a correlation coefficient of 0.995 and an average coefficient of variation of 7.9 %. The LOD could be further reduced to 0.72 µg ml⁻¹. However, the gold nanoparticles do not show an equivalent signal strength compared to the direct format using conjugated polyclonal antibodies. After normalisation, the resulting signal is with 1.2 µA lower than the values achieved using the conjugate by over 50 %.

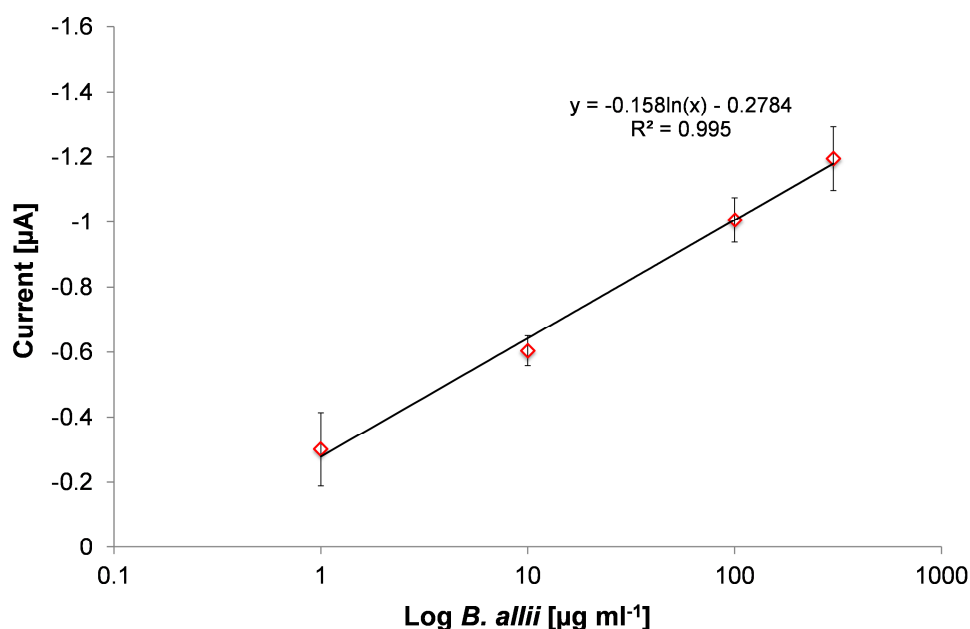


Figure 3.26: Chronoamperometric response of Ø40 nm gold particles labelled with polyclonal antibodies and HRP. The gold colloid (500 µl) was produced by adding 5 µl HRP VI to the nanoparticles and incubated for 1 h at room temperature protected from light using a rotator mixer. Afterwards, 10 µl of polyclonal antibodies were added and incubated for another 60 min, before centrifugation and blocking the particles with BSA. The gold colloid was assayed with *B. allii* concentrations ranging from 300 to 1 µg ml⁻¹. The bars represent standard error of the means (n=3), LOD (neg+3xSD) was calculated at 0.72 µg ml⁻¹, average CV=7.9 %.

Possible reasons for the lack of signal strength are the washing steps performed during the assay. Gold particles have a high weight due to their solid metal filling. If a gold particle is assumed to have a spherical shape with the density of gold being 19.3 g cm^{-3} (Hammer et al., 1994), the mass of an uncoated $\text{Ø}40 \text{ nm}$ gold particle is 0.65 fg and 2.2 fg for a $\text{Ø}60 \text{ nm}$ gold particle, respectively. A HRP conjugated IgG with a molar mass of approximately 200 kDa (Mimori et al., 1981; Kabanda et al., 1995), with 1 Da being equal to $1.66 \times 10^{-24} \text{ g}$ (Hammer et al., 1994), the mass is calculated at $0.33 \times 10^{-3} \text{ fg}$. Comparing these numbers, the gold particles have a 10^3 to 10^4 -fold higher mass and a bigger surface area than conjugated antibodies, resulting in an increased risk of removing the molecule during the washing steps.

Antibody-labelled gold particles are an established technique for visual readout processes in LFDs (Glomm, 2005; Davies et al., 2007b; Zhang et al., 2009). Here, the risk of mobilising the particle is significantly lower, as there are no washing steps during the assay and the flow through the membrane is with 1 to 1.3 cm s^{-1} relatively slow. Considering these facts, the incorporation of a lateral-flow membrane for the sample delivery to the working electrode is more suitable for the incorporation of gold nanoparticles as a signal enhancement technique.

Gold nanoparticles have also been used successfully for signal enhancement in QCM and SPR assays (Wang et al., 2008a; Salam and Tothill, 2009a; Uludag and Tothill, 2010b; Bedford et al., 2012). However, these techniques are different from electrochemical detection methods. The mass of the gold particles is directly correlated to the signal strength. This results in a 10^3 to 10^4 -fold higher mass if an IgG labelled gold particle binds to the antigen on the sensor, compared to unconjugated IgG only.

For the electrochemical detection, an additional enzyme label has to be coated to the particle for signal generation. Therefore, the signal enhancement depends on the number of HRP molecules bound to the particles. The number of immobilised proteins is limited by the surface area of the particles and can be estimated by calculating the density limit for single molecule surface patterning using the equation described by Schwartz and Quake (2007). Assuming a maximum circle packing density limit of 91% , the

maximum number of immobilised molecules (n_{Max}) can be estimated by comparing the surface area of the particles to the planar area of the molecule:

$$n_{Max} = 0.91 \cdot \frac{A_{Nanoparticles}}{A_{Molecule}} \quad (\text{Equation 3.8})$$

Antibodies have different sizes due to variations in morphologic structure resulting in variation of size between 10 to 18 nm long and 8 to 11 nm wide (San Paulo and García, 2000). The maximum surface area of a spherical gold particle ($A=4\pi r^2$) is calculated at 1,257 nm² (Ø20 nm), 5,027 nm² (Ø40 nm) and 11,310 nm² (Ø60 nm), the planar area of an antibody is approximately 200 nm². Using the equation described by Schwartz and Quake (2007), the maximum number of immobilised antibodies is 5 for a Ø20 nm particle, 22 for a Ø40 nm particle and 51 for a Ø60 nm particle. For HRP immobilisation, these numbers are approximately three-fold higher, as the molecule is smaller. Although the equation described by Schwartz and Quake (2007) provides an estimate of the best achievable signal-enhancing factor, this is a theoretical approach, assuming that ideal conditions occur resulting in a complete coverage of the surface area. In practice, the orientation of the molecules is random on the surface of the particle reducing the maximum coverage significantly.

Ding et al. (2010) reported a 100-fold increase in sensitivity compared with the normal ELISA method, by using 10 nm gold particles for the development of an electrochemical biosensor for the detection of hepatitis B. However, Ding et al. (2010) lacked to demonstrate the improvement in signal strength produced by the 10 nm gold particles, compared to the normal ELISA control group. Furthermore, Ding et al. (2010) immobilised antibodies already conjugated to HRP to the 10 nm gold particles in their studies. Although, the use of 10 nm gold particles for signal enhancement is questionable, the use of antibodies conjugated to HRP could be another approach to improve the signal strength of the immunoassay using nanoparticles. By providing an environment with an excess of HRP during the labelling process, the mobilisation of a part of the gold particles during the washing is acceptable, as there are remaining conjugated antibodies and IgG-HRP labelled gold particles for signal generation.

3.4.5 IMMUNOSENSOR OPTIMISATION

In this study, various parameters were tested and optimised in order to boost the performance of the immunoassay by using an adapted protocol of the checkerboard or matrix method (Malone et al., 1996). The most straightforward approach is to establish basic conditions to provide a stable assay before introducing the matrix to further optimise the assay conditions (Malone et al., 1996). However, the matrix method is only practicable with three or less variables, as the statistical number or replicates needed grow exponentially. To restrict the number of variables, one parameter was kept constant while varying the second parameter and vice versa. In order to achieve the best biosensor response, capture and detection antibody concentration were investigated, as well as several other parameters including experiments for discovering the most suitable coating buffers, detection antibody processing techniques and incubation times were conducted and are discussed in this section.

3.4.5.1 Incubation Time

A successful immobilisation of the capture antibody to the sensor surface is crucial, as all following steps depend on an optimised amount of capture antibody bound to the sensor surface. However, the sensor assay used in this study is a direct sandwich ELISA format. Therefore, a performance study was performed to identify the optimum incubation time for both the capture and detection antibody. Initially, 50 $\mu\text{g ml}^{-1}$ of the monoclonal antibody BC-12 were incubated for 30 min to 4 h followed by 50 $\mu\text{g ml}^{-1}$ conjugated polyclonal antibody incubated for 1 h. Afterwards, a capture antibody incubation time of 3 h was combined with varying conjugate incubation times (15 min to 2 h).

Figure 3.27 shows the impact of varying incubation times to the sensor signal. For the capture antibody (Figure 3.27 A), the assay lacks performance at an incubation period of less than 2 h. Obviously this period is not sufficient to ensure a strong bound of the antibodies to the sensor surface, resulting in a current of less than 0.5 μA after normalisation using a fungal concentration of 100 $\mu\text{g ml}^{-1}$ (Figure 3.27 A, embedded graph). The increase in sensor signal reaches a plateau between 2 to 4 h. An incubation time of 2.5 to 3 h was adopted for the passive immobilisation of the capture antibody for all future assays.

Overnight incubation at 4 °C by pipetting 20 μl of the capture antibody was also tested with negative results. Although, the sensors were stored in a closed ELISA plate incubation tray on a layer of wet paper towels in order to provide saturated humid environment, the coating liquid was found to evaporate during storage, leading to the denaturation of the antibodies. Increasing the volume of coating liquid to 30 or 50 μl per sensor compromises the accuracy of the antibody deployment on the working electrode, since a spillage over the other electrodes is likely. Therefore, overnight incubation was not used for the coating antibody immobilisation.

In contrast to the immobilisation process of the capture antibody, the binding of the detection antibody to the antigen takes place within minutes and is not critical regarding assay performance (Figure 3.27 B). Based on the normalised data (Figure 3.27 B, embedded graph), an incubation time of 1 h shows the best electrochemical response and was therefore used for the future assay development. Further evidence for the quick bond between antibody and antigen is the observation that the difference between 1.5 h and 15 min incubation corresponds to 30 % loss in signal strength. However, after exceeding an incubation time of 1.5 h, the signal is reduced after normalisation due to increased non-specific binding of the HRP conjugate to the sensor surface. These findings were also adopted for the antigen incubation time, since the antibody antigen interactions are constant if the affinity of the antibody is comparable (Friguet et al., 1985; Beatty et al., 1987; Malone et al., 1996). For our studies, ELISA and sensor tests performed in Chapter 2 and 3 showed that the difference between the affinity of the polyclonal detection and the monoclonal capture antibody to *Botrytis* is less than 10 %.

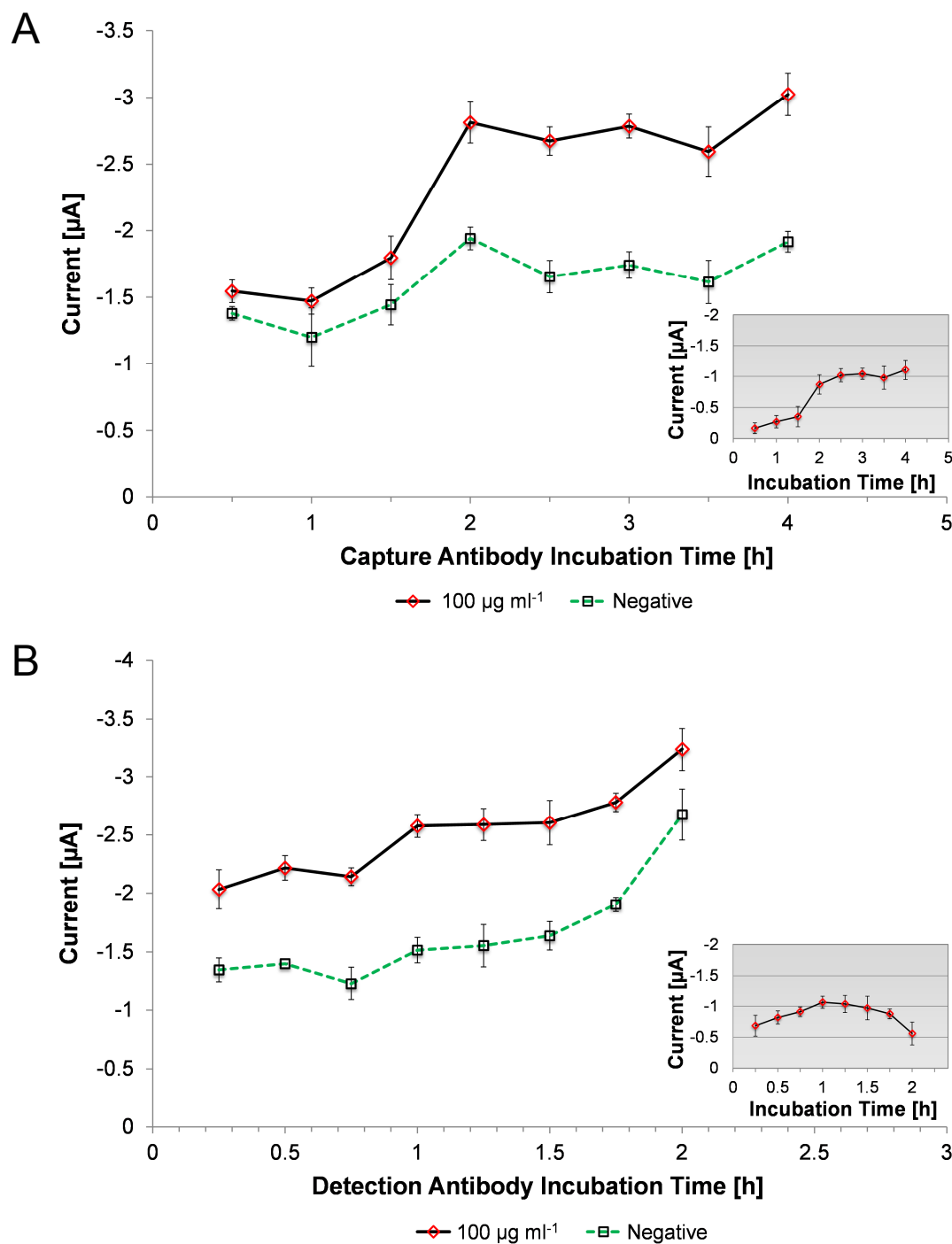


Figure 3.27: Effect of different incubation times of (A) capture antibody BC-12 and (B) polyclonal HRP conjugate, both applied in a concentration of 50 µg ml⁻¹ on the sensor signal using SPGE. First, the capture antibody was incubated for 0.5 to 4 h at 37 °C with a constant conjugate incubation of 1 h. Afterwards, the capture antibody was incubated for 3 h, while varying the detection antibody incubation from 0.25 to 2 h using a *B. allii* concentration of 100 and 0 µg ml⁻¹. The embedded graphs show the normalised current of each concentration, bars represent the standard error of the means (n=3).

3.4.5.2 Detection Antibody Concentration

For the examination of different detection antibody concentrations, the capture antibody concentration of the monoclonal antibody BC-12 was kept at a constant value of $50 \mu\text{g ml}^{-1}$ while varying the concentration of the polyclonal HRP conjugate applied in unprocessed and purified form.

As expected, the signal strength increases with the antibody concentration (Figure 3.28). However, non-specific binding is proportional to the HRP concentration and increases as well with the detection antibody concentration indicated by the rising current of the negative control. After normalisation (Figure 3.28 embedded graphs), the purified conjugate shows an up to $0.7 \mu\text{A}$ higher current compared to the unprocessed antibodies, as higher antibody concentrations can be applied to the sensor without increasing the negative control by the excess of HRP. The enzyme labelled detection antibody should be optimised to obtain a maximum response for the specific binding of the antigen with a signal close to equilibrium in a steady state, together with low non-specific binding (Malone et al., 1996). For the unpurified conjugate (Figure 3.28 A), the current peaks at a concentration of 25 to $50 \mu\text{g ml}^{-1}$ before the signal drops with higher concentration after subtracting the negative control. This tendency is also in accordance with the purified conjugate (Figure 3.28 B), but here the antibody concentration is ranging from 40 to $100 \mu\text{g ml}^{-1}$.

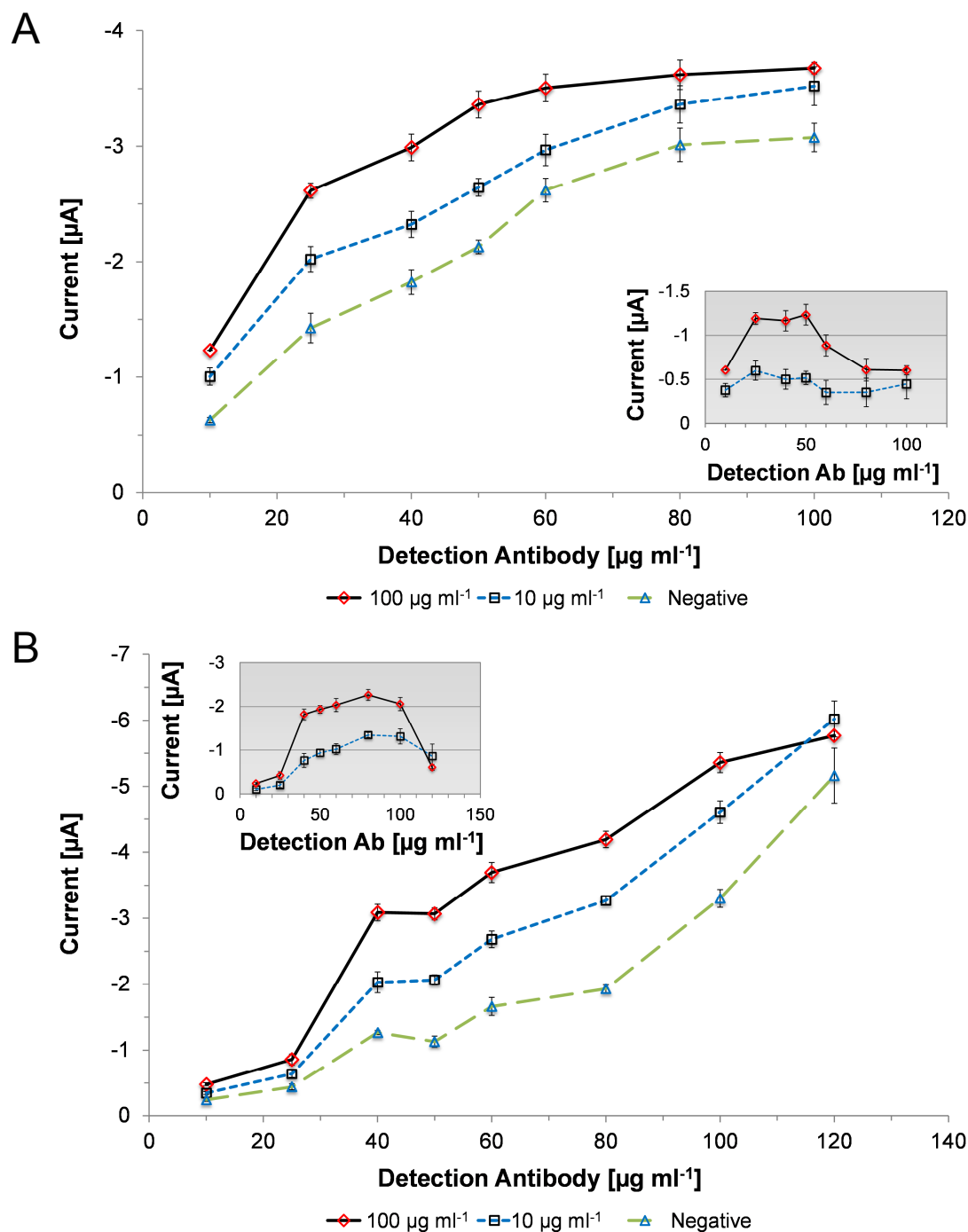


Figure 3.28: Sensor signal current against various concentrations of the (A) unprocessed and (B) purified polyclonal conjugate. As capture molecule, 50 $\mu\text{g ml}^{-1}$ of the monoclonal antibody BC-12 were immobilised on the sensor surface. The embedded graphs show the normalised current of each concentration; the bars represent the standard error of the means ($n=4$).

For further testing, two concentrations of the purified conjugate were chosen to perform a sensor assay with five fungal concentrations (300, 100, 10, 1 and 0 $\mu\text{g ml}^{-1}$). Previous experiments (Figure 3.28 B) indicated that an antibody concentration of 50 and 80 $\mu\text{g ml}^{-1}$ resulted in the best compromise between signal strength and standard deviation, with %CV values of 4 to 8 % and a signal strength of approximately 2 μA after normalisation.

The electrochemical response of the two purified antibody concentrations 50 and 80 $\mu\text{g ml}^{-1}$ to the fungal concentrations is plotted in Figure 3.29. After fitting a logarithmical curve to both graphs, the limit of detection (LOD = negative + 3xstandard deviation) was calculated at 0.99 $\mu\text{g ml}^{-1}$ *B. allii* for 50 $\mu\text{g ml}^{-1}$ and 0.42 for 80 $\mu\text{g ml}^{-1}$ purified conjugate, respectively. Due to the lower LOD and up to 0.5 μA higher signal, a purified detection antibody concentration of 80 $\mu\text{g ml}^{-1}$ was chosen for further development and optimisation.

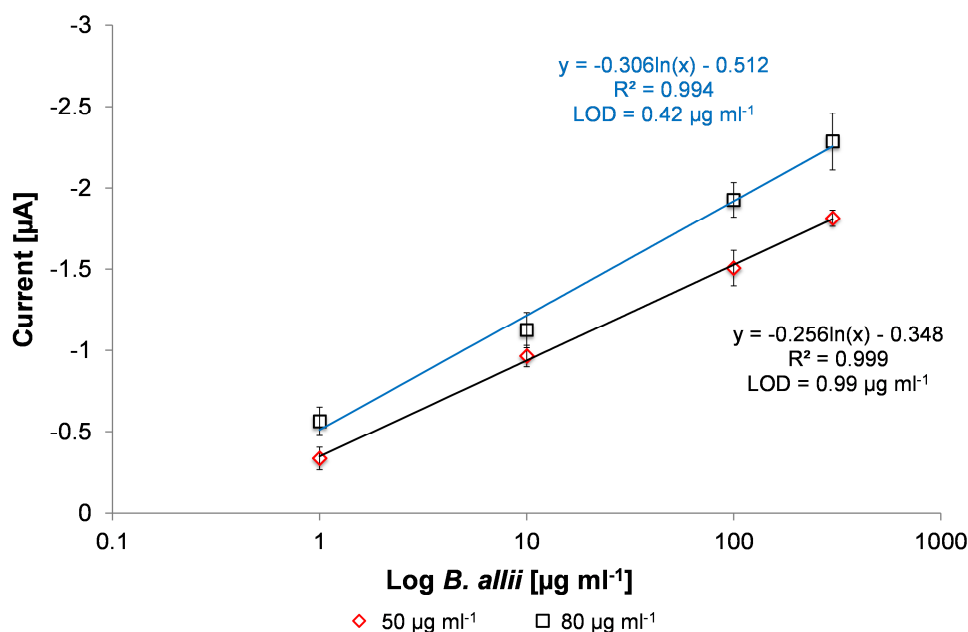


Figure 3.29: Normalised direct ELISA response of the purified polyclonal conjugate concentrations 50 and 80 $\mu\text{g ml}^{-1}$ to the *B. allii* concentrations 300, 100, 10, 1 and 0 $\mu\text{g ml}^{-1}$. As capture molecule, 50 $\mu\text{g ml}^{-1}$ of the monoclonal antibody BC-12 were immobilised on the sensor surface. LOD = limit of detection, bars represent the standard error of the means (n=4).

3.4.5.3 Optimisation of the Coating Buffer

The immobilisation of the antibodies can occur through many interactions of the proteins to the gold surface, such as ionic exchanges, electrostatic or hydrophobic interactions and hydrogen or thiol bounds (Davies et al., 2007b). Some scientists claim adsorption to be relatively independent of ionic strength, buffer type or pH (Geerligs et al., 1988; Porstmann and Kiessig, 1992), whilst others hold an opposite view (Tsang et al., 1980; Liu et al., 1999; Tan et al., 2005).

In this study *B. allii* ELISA Kit coating buffer (02-002, pH 9), carbonate-bicarbonate buffer (CBB, 0.05 M, pH 9.6), sodium citrate buffer (SCB, pH 8.1), phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.137 M sodium chloride and 0.0027 M potassium chloride, pH 7.4) were tested with a capture antibody concentration of 50 $\mu\text{g ml}^{-1}$. All buffers had an alkaline to near neutral pH, as this pH range has is well established to give best results for the adsorption of antibodies (Malone et al., 1996; Chen et al., 2007b; Salam and Tothill, 2009b).

The sensor signal obtained by the different coating buffers are illustrated in Figure 3.30. Carbonate bicarbonate buffer showed the best performance with a good signal to noise ratio for both *B. allii* concentrations, followed by PBS with an approximately 10 % lower signal. The coating buffer adopted from the *B. allii* ELISA Kit (pH 9) also performed well regarding the peak signal, but also showed a higher negative control. Lowest signal to noise ratio, signal strength as well as with 14 % highest CV value made sodium citrate buffer (pH 8.1) the least suitable for coating the antibodies to the working electrode.

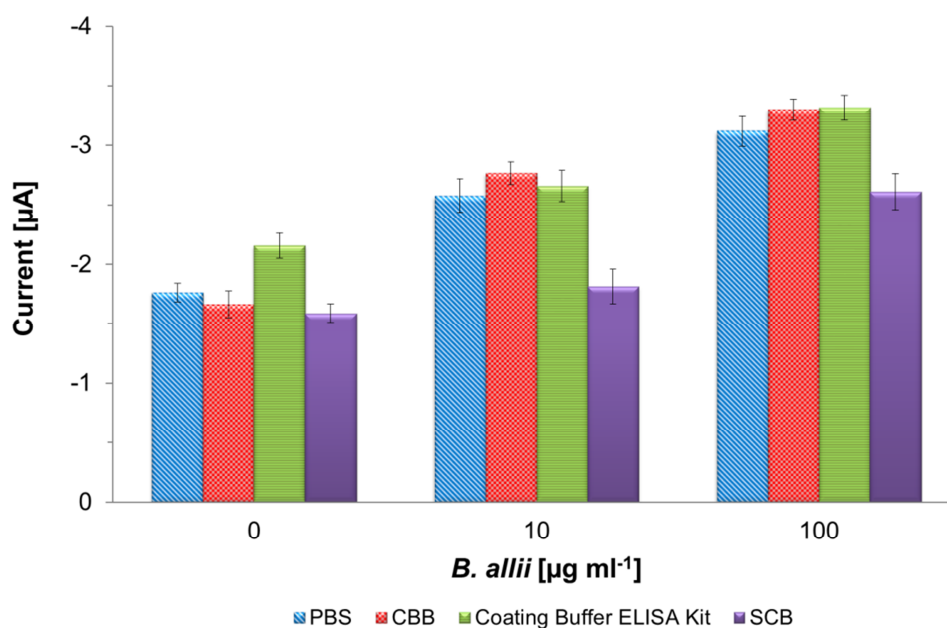


Figure 3.30: Influence of different capture antibody buffers to the sensor signal. To perform a direct ELISA, 50 µg ml⁻¹ of the monoclonal antibody BC-12 were diluted in phosphate buffered saline (PBS, pH 7.4), carbonate-bicarbonate buffer (CBB, pH 9.6), *B. allii* ELISA Kit coating buffer (pH 9) and sodium citrate buffer (SCB, pH 8.1). As secondary antibody, purified polyclonal antibodies conjugated to HRP (80 µg ml⁻¹) were applied to the sensor to complete the direct ELISA format. The bars represent the standard error of the means (n=3).

3.4.5.4 Capture Antibody Concentration

The concentration of capture antibodies is crucial for the assay performance and was optimised for an antibody concentration ranging from 10 to 200 µg ml⁻¹ using three different concentrations of *B. allii* (100, 10 and 0 µg ml⁻¹). For signal generation, 80 µg ml⁻¹ purified polyclonal antibodies conjugated to HRP were used. With higher antibody concentration, more antigen binding sites are available, resulting in an increased sensitivity (Figure 3.31). However, saturation is reached at a capture antibody concentration of 50 µg ml⁻¹ for a fungal concentration of 10 µg ml⁻¹ and 100 µg ml⁻¹ for 100 µg ml⁻¹ *B. allii*. From an economic point of view, 50 µg ml⁻¹ offers the best compromise between sensitivity and invested antibodies, as further doubling the amount of antibodies only results in approximately 20 % increase in signal at a fungal concentration of 100 µg ml⁻¹. For this study, the main focus is to achieve best immunoassay sensitivity. Therefore, a coating antibody concentration of 100 µg ml⁻¹ was chosen for future assay development.

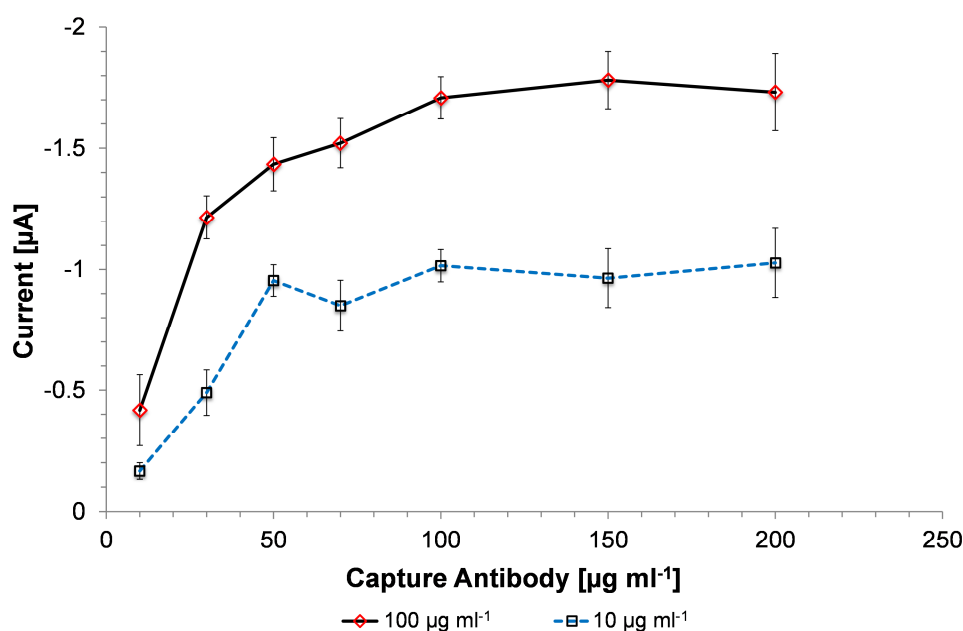


Figure 3.31: The normalised sensor current against various concentrations of the monoclonal antibody BC-12 immobilised on the sensor surface as capture molecule. The assay was performed using purified polyclonal antibodies conjugated to HRP ($80 \mu\text{g ml}^{-1}$) and tested with 100 and $10 \mu\text{g ml}^{-1}$ fungal mycelium. The bars represent the standard error of the means ($n=4$).

3.4.5.5 Standard Curve of the Optimised Biosensor

A standard curve of the biosensor response ranging from a *B. allii* concentration of $0.05 \mu\text{g ml}^{-1}$ to 1 mg ml^{-1} was constructed using the optimised immunoassay described in Section 3.3.5. For standardisation, the data of the immunoassay response were normalised, before fitting a five parameter logistic equation described by Warwick (1996):

$$Y = d + \left[\frac{(a - d)}{\left(1 + \left(\frac{p_{a50}}{C}\right)^b\right)^e} \right] \quad (\text{Equation 3.7})$$

Y = Response

d = Maximum response

C = Concentration

e = Compensation factor for asymmetry

a = Minimum response

p_{a50} = Concentration at the EC50 value

b = Slope

Figure 3.32 shows the standard curve of the optimised immunoassay. The linear range of the immunoassay was found to be from 0.1 to 300 $\mu\text{g ml}^{-1}$, resulting in a calculated LOD (negative+3xSD) as low as 0.058 $\mu\text{g ml}^{-1}$ and an average coefficient of variation of 7.8 %. The achieved LOD is well suitable for detecting *Botrytis* infections prior to showing visible symptoms and is lower than all commercially available detection methods which were tested with 7.5 $\mu\text{g ml}^{-1}$ (Forsite LFD) and 1-10 $\mu\text{g ml}^{-1}$ (ADGEN ELISA).

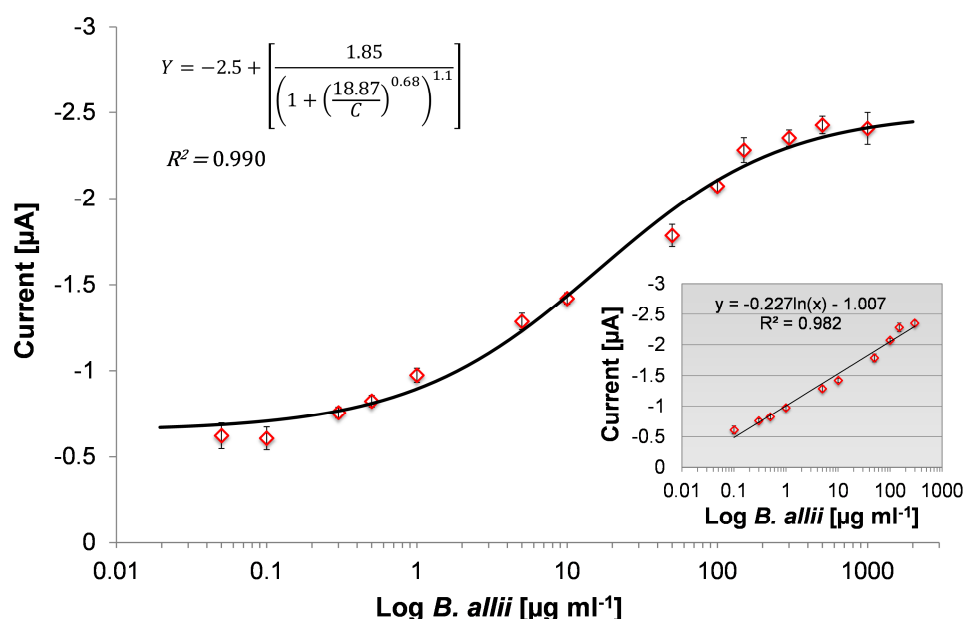


Figure 3.32: Standardised biosensor response of the optimised immunoassay against various concentrations of *B. allii* antigen. The monoclonal antibody BC-12 ($100 \mu\text{g ml}^{-1}$) was immobilised on the sensor surface as capture molecule. For detection, $80 \mu\text{g ml}^{-1}$ of purified polyclonal antibodies conjugated to HRP were applied to the sensor. Measurements were taken after 100 s at a potential of -200 mV. The embedded graph shows the linear range of the assay, bars represent the standard error of the means (n=5), average CV=7.8 %.

3.4.6 PERFORMANCE STUDIES OF THE BIOSENSOR

To study the performance of the optimised biosensor described in Section 3.3.5, a quantification experiment was designed using onion samples inoculated with spore solution. Onion discs were grown on selective agar, produced as described by Kritzman

and Netzer (1978) for 1 to 7 days, allowing the fungus to grow on the surface and subsequently quantified using the biosensor. The monoclonal antibody raised from the BC-12 hybridoma cells, which is used in this study, has a high affinity against surface proteins along the length of the growing mycelium (Meyer and Dewey, 2000). As demonstrated in Section 2.4.5, spores are not detected by the antibody. This ability provides excellent conditions for quantification experiments, as the inoculation source on the plant does not contribute to the quantified pathogen load.

3.4.6.1 Effect of Onion Tissue on the Commercial ELISA Kit and Biosensor Response

The same samples used for the biosensor were also tested using the commercially available *B. allii* and *B. cinerea* ELISA Kit (ADGEN Phytodiagnostics, Auchincruive, UK), which utilises the antibodies employed in this study. Unlike the leaf quantification experiment performed in Section 2.3.7, the *B. allii* standard curve was diluted in onion standard obtained by following the sample extraction procedure used for this experiment. To avoid possible contamination, the outer scales of the onion sets were UV treated, disinfected and removed, so that only the inner scales were used for further processing. Figure 3.33 compares the response of the normal standard curve diluted in buffer compared to the onion extract standard solution. The high disturbance of the onion bulb tissue prevented a successful quantification of the onion samples. The inhibited absorbance response was consistent throughout all ELISA assays and independent from the antibody uses.

In contrast to the commercially available *B. allii* and *B. cinerea* ELISA kits (ADGEN Phytodiagnostics, Auchincruive, UK), the difference between the sensor signal of the *B. allii* antigen diluted in onion extract and buffer after normalisation (Figure 3.34) is maximally 10 % at a fungal concentration of $1 \mu\text{g ml}^{-1}$ and is within the standard deviation of the assay.

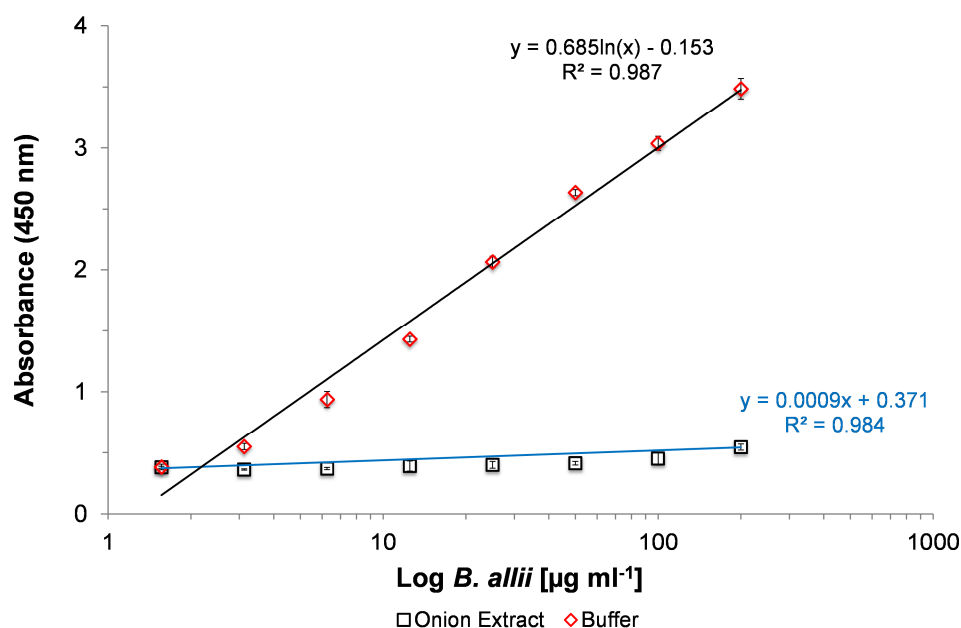


Figure 3.33: Comparison of the absorbance at 450 nm achieved by the commercial *B. allii* ELISA kit of standards produced in buffer and buffer comprising onion extract gained from onion bulbs. The indirect ELISA was performed by coating the well overnight at 4 °C with a serial dilution of *B. allii*. Afterwards, the polyclonal detection antibody ($10 \mu\text{g ml}^{-1}$) was used followed by goat anti-rabbit antibodies ($0.3 \mu\text{g ml}^{-1}$) for signal generation. The bars represent the standard error of the means ($n=3$).

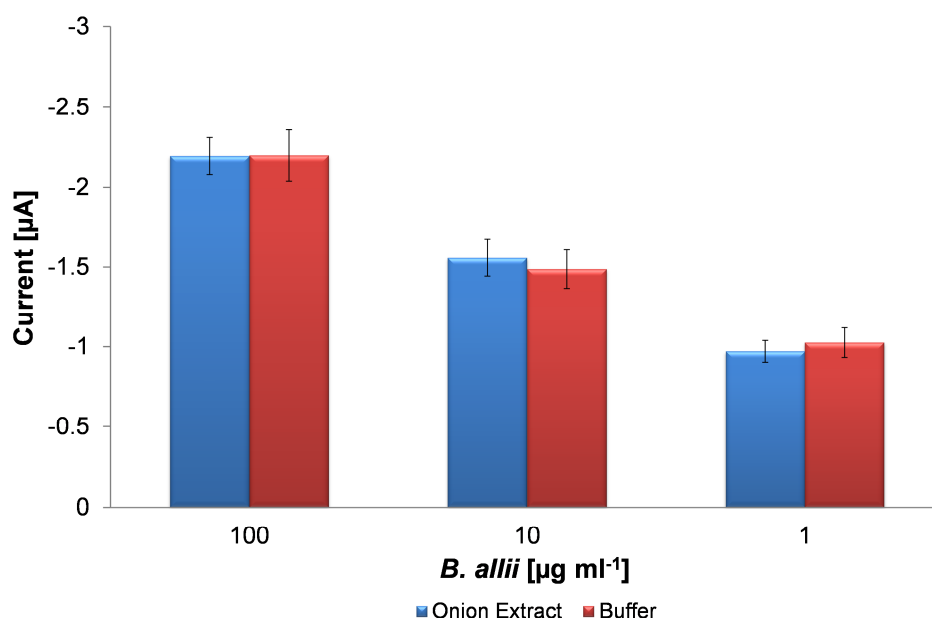


Figure 3.34: Normalised biosensor response against *B. allii* antigen diluted in onion extract and buffer. The monoclonal antibody BC-12 ($100 \mu\text{g ml}^{-1}$) was immobilised on the sensor surface as capture molecule. For detection, $80 \mu\text{g ml}^{-1}$ of purified polyclonal antibodies conjugated to HRP were applied to the sensor. The bars represent the standard error of the means ($n=3$).

The poor performance of the commercially available *B. allii* and *B. cinerea* ELISA kit is not caused by the applied antibodies as the same antibodies are used for the biosensor development. Furthermore, the solid phase ELISA has been used successfully several times within this study. More likely, the lack of performance is due to the ELISA format which varies from the one used for the biosensor development. The ADGEN ELISA requires a direct coating of the antigen to the well overnight. Following this protocol, the antigen competes inevitably with other non-specific intracellular proteins to cover the available space of the well, before adding the detection and secondary antibody for signal generation (Figure 3.35 A). In contrast, the biosensor uses a sandwich format (Figure 3.35 B), where the non-specific proteins that potentially inhibit the immunoassay are removed during the washing steps.

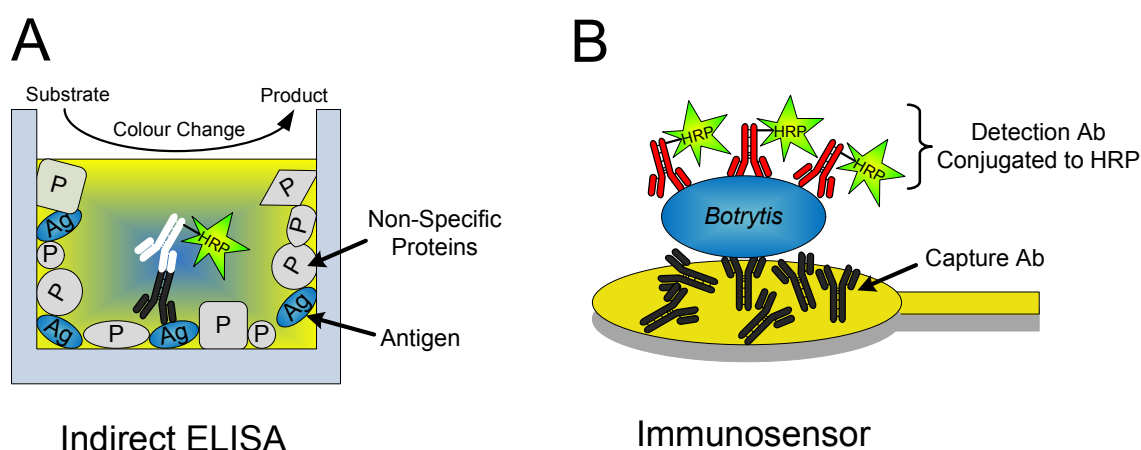


Figure 3.35: Comparison of (A) the indirect ELISA format with antigen and proteins directly immobilised to the polyacrylamide well plate and (B) the direct sandwich format used for the biosensor development.

3.4.6.2 Quantification of the Pathogen Load Using the Electrochemical Biosensor

The results obtained by quantifying the pathogen load of the onion disc samples are summarised in Figure 3.36. The selective Kritzman agar showed a visible colour change three days post inoculation indicating the growth of *B. allii* (Figure 3.36 A). In contrast to the commercial ELISA kits, the biosensor showed good performance with no inhibiting effects on the onion tissue. The fungus could be detected in two out of three samples 24 h post inoculation demonstrating the fast growth of the fungal spores. Three samples were considered as not detected based on the LOD and a confidence level of 95 % of the negative control (day 0), one sampled after 24 h, one sampled after 48 h and one sampled after 72 h of incubation. However, none of these samples showed visible growth of the fungus or staining of the selective Kritzman agar indicating an inhibited growth or a not successful inoculation. As expected, with increasing incubation time the fungal mycelium is growing exponentially, peaking at a fungal concentration of $502 \mu\text{g ml}^{-1}$ after seven days. The maximum CV value was recorded with 17 % and the average CV was calculated at 7.5 %.

This is the first report of an immunological method demonstrating the ability of detecting a *Botrytis* infection 1 day post inoculation. Fernandez-Baldo et al. (2010; 2011) quantified *B. cinerea* in inoculated pears, grapes and apples with $10\text{-}14 \mu\text{g ml}^{-1}$ four days post inoculation. Nielsen et al. (2002) detected *B. aclada* infections on onion leaves 48 h post inoculation, while Coolong et al. (2008) detected the pathogen 3 days after inoculating onion discs, both using PCR. However, quantification is delicate using genetic methods due to the fluctuating efficiency of DNA extraction and PCR amplification (Chilvers et al., 2007). Immunoassays provide a more reliable and consistent method to quantify the pathogen load.

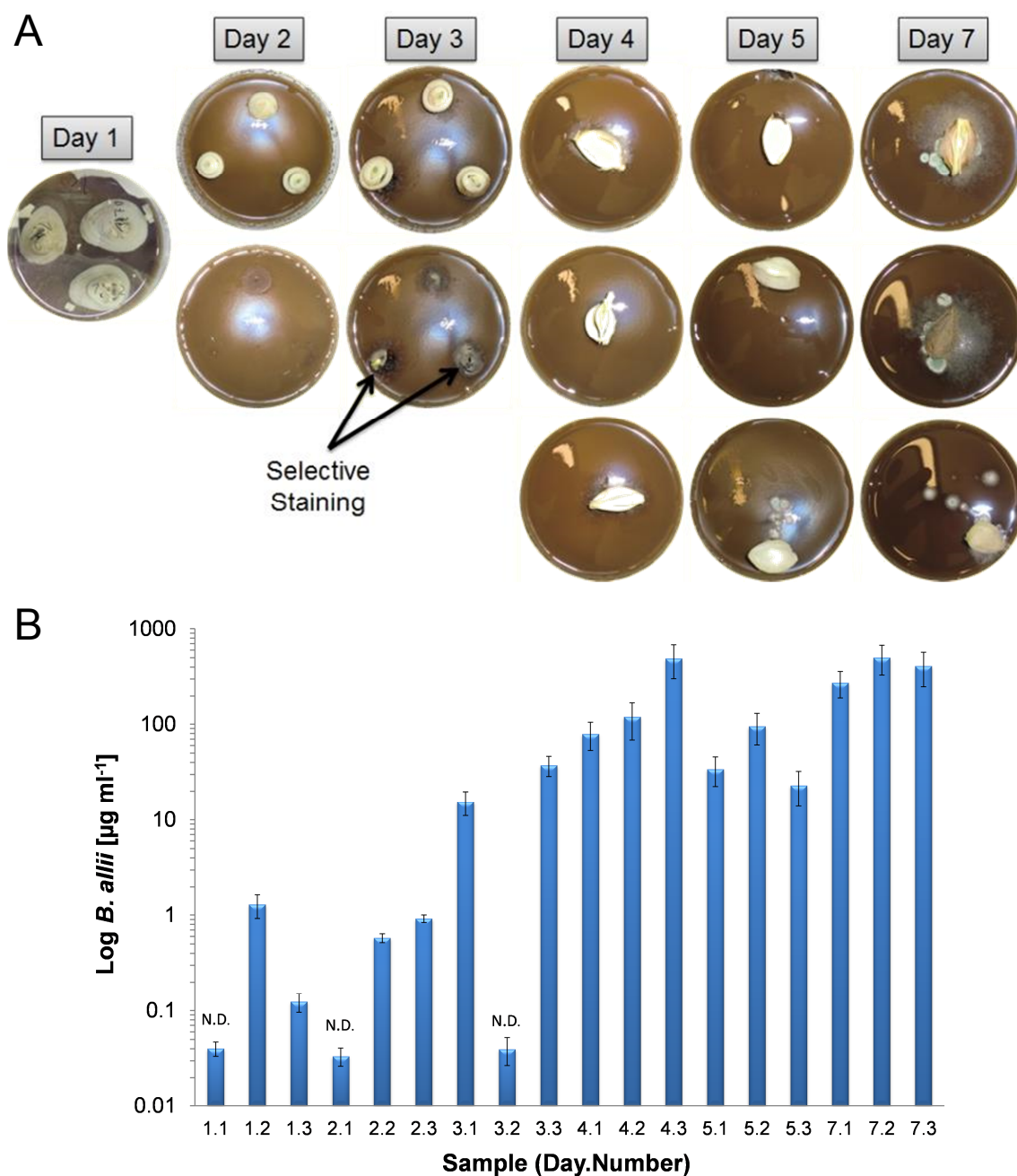


Figure 3.36: Performance studies of the biosensor. (A) Onion sets were inoculated with 10,000 spores g^{-1} onion tissue and incubated for 1-7 days on selective Kritzman agar. (B) Three onion samples were subsequently quantified after 1, 2, 3, 4, 5 and 7 days using the biosensor. The monoclonal antibody BC-12 ($100 \mu\text{g ml}^{-1}$) was immobilised on the sensor surface as capture molecule. For detection, $80 \mu\text{g ml}^{-1}$ of purified polyclonal antibodies conjugated to HRP were applied to the sensor. The bars represent the standard error of the mean ($n=4$), N.D.= not detected.

3.4.7 EVALUATION OF COVALENT IMMOBILISATION TECHNIQUES

The optimised immunoassay using passive adsorption for the immobilisation of the capture antibodies performed well, with benchmark detection limit, high reproducibility and no interference with onion tissue. The biosensor meets all targets of a sensitive detection method for *Botrytis* infections. However, especially the washing steps performed throughout the assay yield a high source of variation. To compensate this flaw, covalent immobilisation techniques have been evaluated, as covalently immobilised antibodies were reported to show a 3 % decrease in antibody loss per wash (Leckband and Langer, 1991; Williams and Blanch, 1994).

Covalent coupling has been controversially discussed in the literature. Some scientists claim that covalent coupling improves the stability of the antibodies (Wortberg et al., 1997), reduces non-specific binding of other proteins (Ekins et al., 1990) and increases the number of immobilised antibodies (Furuya et al., 2006), whilst others do not see reasons to employ this more complex immobilisation strategy as it lacks to demonstrate the benefit they offer compared to the control (adsorbed) group (Williams and Blanch, 1994; Davies et al., 2007b; Marks et al., 2007).

In order to chemically couple the antibodies, a polymer layer is first immobilised on the sensor surface, comprising reactive groups such as carboxyl (R-HCO_2), thiol (R-SH), or amin (R-NH_2). These self-assembled monolayers (SAM) have the ability to chemisorb spontaneously building regularly orientated two-dimensional surface structures (Millner et al., 2009). For the application of SAMs on gold surfaces, hydrocarbon compound with thiol groups are used resulting in an immediate sulphur-gold bond and hydrophobic alkane chains construct a stable hydrophobic layer (Prime and Whitesides, 1993). These techniques are well established in QCM and SPR applications.

There are seemingly unlimited numbers of polymer molecules that can be employed for covalent coupling. They vary in the length of the hydrocarbon chain, the reactive groups and the chemical structure. Especially the length of the carbon chain is an important factor for the success of the assay. If the bio-resistive polymer layer exceeds a critical

thickness, the detection sensitivity decreases, as electron transfer to the sensor surface is inhibited. For this study, a polymer with a short hydrocarbon chain 3,3-dithiodipropionic acid (DTDPA, Figure 3.37 A) and one with a long hydrocarbon chain, named 11-mercaptoundecanoic acid (MUDA, Figure 3.37 B) were selected for further evaluation.

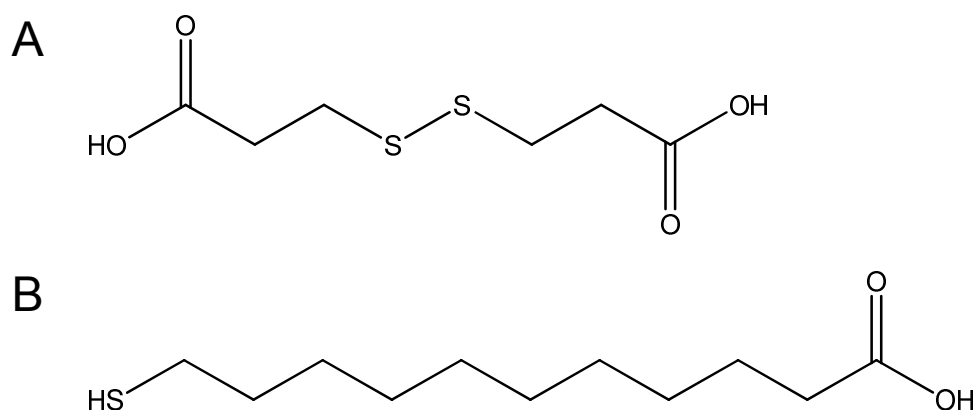


Figure 3.37: Chemical formula of (A) 3,3-dithiodipropionic acid (DTDPA) and (B) 11-mercaptoundecanoic acid (MUDA).

3.4.7.1 Characterisation of the Self-Assembled Monolayers

The 11-MUDA and 3-DTDPA layer was visualised using SEM (Figure 3.38). The polymer clustered especially in the areas between the gold ink particles of the working electrode. Comparing the morphology of the 3-DTDPA (Figure 3.38 B) with 11-MUDA (Figure 3.38 D) under a 50,000-fold magnification, the shorter 3-DTDPA constructs a scale like overlapping layer structure, whereas the longer chained 11-MUDA forms a smooth, glue like layer in between the ink particles. The larger hydrocarbon chain of the 11-MUDA has been reported to favour to build of a functionalised layer for sensor application (Chaki and Vijayamohanan, 2002). Unlike polished SPR or QCM chips, the formation of a monolayer is visibly inhibited by the rough surface of the ink, especially in between the particles, where removing the excess layer by washing is difficult. However, the resulting three-dimensional cluster also increases the surface area which can be beneficial for the subsequent immobilisation of the antibodies.

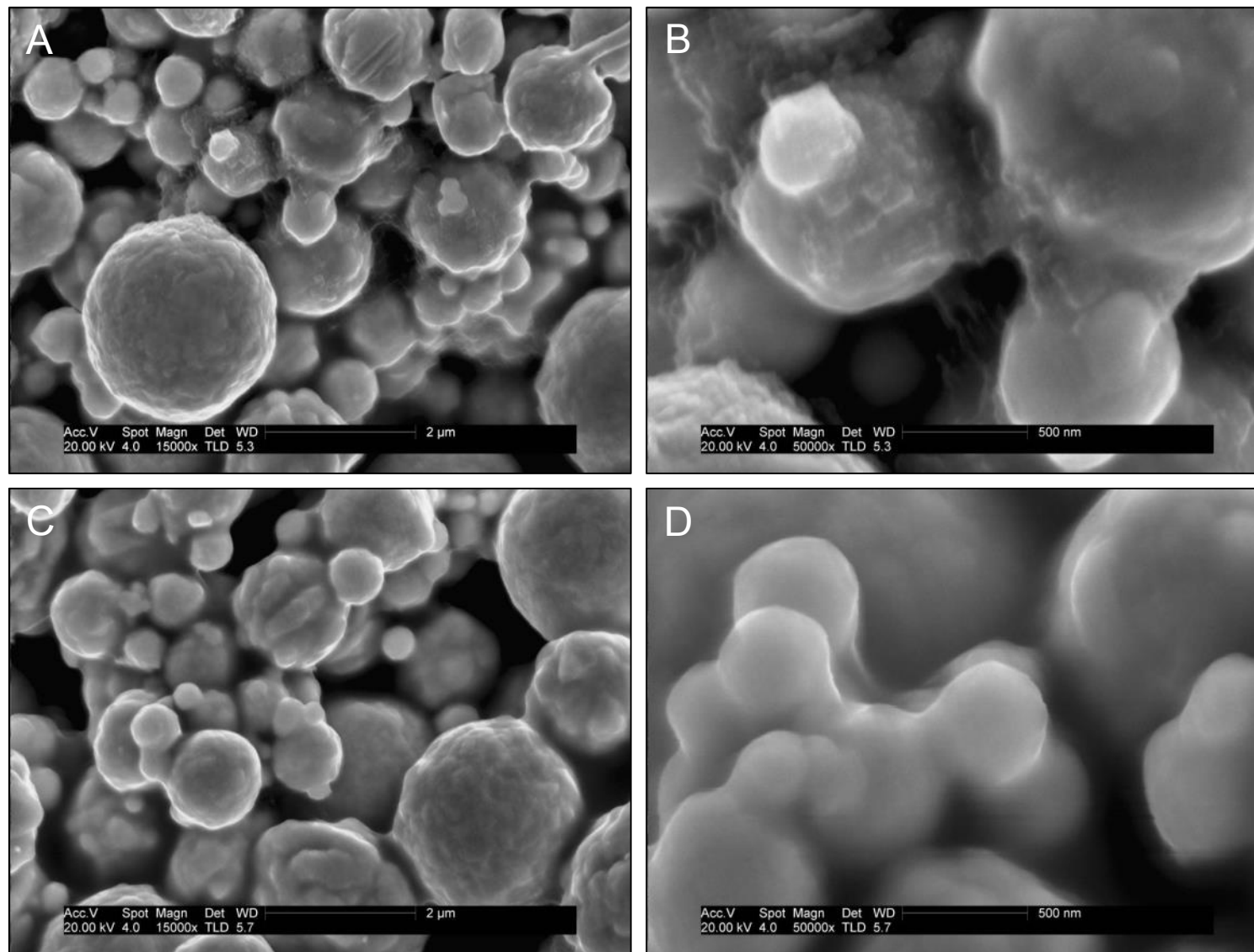


Figure 3.38: SEM of the working electrode of a DuPont SPGE covered with 1 µl of 5 mM MUDA and DTDPA solution using a magnification of (A) x15 k, DTDPA (B) x50 k, DTDPA (C) x15 k MUDA (D) x50 k MUDA

As previously discussed, the length of the carbon chain is an important factor for the success of the assay. If the bio-resistive polymer layer exceeds a critical thickness, the detection sensitivity decreases as the polymer impedes the flow of electrons to the sensor surface. Therefore, a study was performed using the electroactive mediator potassium ferricyanide, in order to evaluate the electrochemical behaviour of the polymer layer in various concentrations. The results are presented in Figure 3.39.

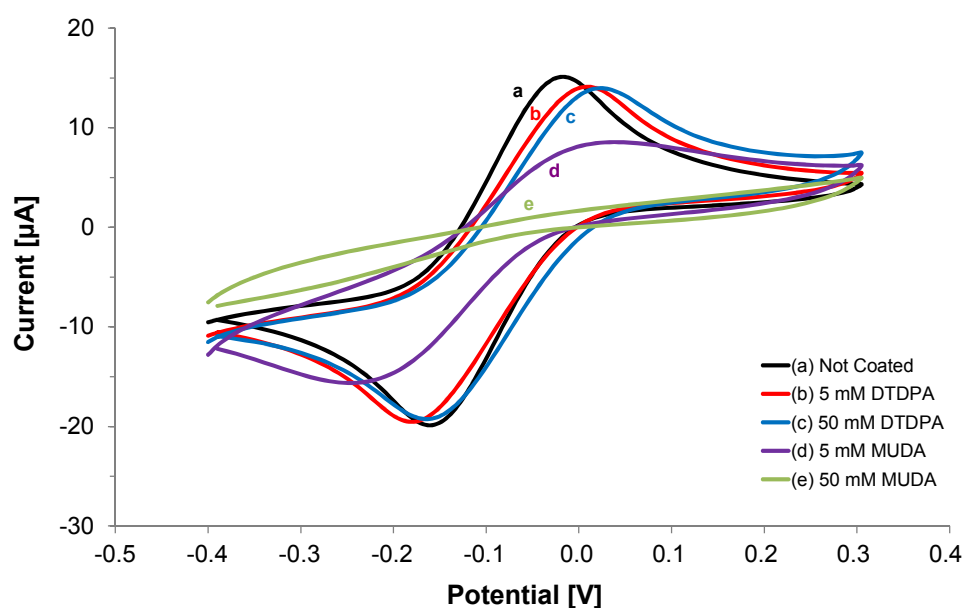


Figure 3.39: Cyclic voltammograms using the electroactive mediator potassium ferricyanide (1 mM in 1 M KCl) against (a) bare electrode and electrode coated with (b) 5 mM DTDPA, (c) 50 mM DTDPA, (d) 5 mM MUDA and (e) 50 mM MUDA. Graph shows the average of three scans per sensor in triplicates at a scan rate of 20 mV s^{-1} .

The resulting cathodic / anodic peak current, their position and ratio as well as the calculated active surface area is listed in Table 3.7. The cyclic voltammogram of the 11-MUDA, using a concentration of 50 mM inhibited the electron transfer to the sensor layer in such a way that the cathodic and anodic peaks could not be identified by the GPES software. Instead, the active surface area of 27 % was calculated based on the maximum and minimum value referred to the x-axis of the scans. However, at a 5 mM 11-MUDA concentration, cathodic and anodic peaks could be detected and the active surface area of 51 % showed that thus the longer chained polymer layer has an impeding effect, the electron transfer to the sensor surface is still functional.

Considering these results a 11-MUDA concentration of 5 mM was used for further testing. The final concentration has to be evaluated based on the performance in immunoassays.

3-DTDPA, comprising a 3C hydrocarbon chain, only decreased the active surface area by less than 2 %, even when applied in high concentrations. These promising results demonstrate the ability of the shorter 3-DTDPA polymer to provide a functional electron transfer to the surface of the electrode. The concentration of the 3-DTDPA applied to produce the nano layer does not affect significantly the active surface area and can be therefore used in high concentrations if required.

Table 3.7: Cyclic voltammetric analyses of the polymer layers constructed with 5 mM and 50 mM 3-DTDPA and 11-MUDA, as well as uncoated SPGE at a scan rate of 20 mV s⁻¹. The data represent the average and standard deviation of three sensors with three scans per sensor.

	Bare electrode		5 mM DTDPA		50 mM DTDPA		5 mM MUDA^f		50 mM MUDA	
I_{PA} [μA]^a	20.35	±0.33	20.20	±0.77	20.30	±1.43	8.94	±0.71	4.97 ^g	±0.39
E_{PA} [V]^b	-0.017	±0.01	0.006	±0.01	0.021	±0.02	0.033	±0.03	/	/
I_{PC} [μA]^a	-21.16	±0.22	-20.91	±0.47	-20.42	±0.41	-15.47	±0.41	-7.91 ^g	±0.58
E_{PC} [V]^b	-0.160	±0.01	-0.177	±0.02	-0.160	±0.02	-0.259	±0.02	/	/
ΔE [V]^c	0.143	±0.01	0.184	±0.02	0.181	±0.03	0.292	±0.03	/	/
I_{PA} / I_{PC}^d	0.96		0.97		0.99		/		/	
A_{active} [%]^e	87.57		86.72		85.90		51.49		27.17	

^a I_{P C/A} = Cathodic / anodic peak current

^b E_{P C/A} = Potential applied at the cathodic / anodic peak

^c Peak distance ΔE = E_{PA} - E_{PC}

^d Ratio between the cathodic and anodic peak current

^e Ratio between the active area calculated by the Randles-Sevcik equation and the geometric surface

^f I_{P C/A} and E_{P C/A} referred to x-axis

^g Max / min cathodic / anodic current since no peak was detected by the software

Initial studies were performed by immobilising the polymer layer overnight using a 9 cm petri dish filled with 5 ml of polymer diluted in ethanol. Though the chemisorption of the thiol group to the gold surface occurs spontaneously within seconds, a longer incubation time can result in a more homogenous monolayer (Chaki and Vijayamohanan, 2002). However, overnight incubation has several drawbacks. The ethanol softens the cover layer of the screen-printed electrodes resulting in an increased risk of mechanical damage. Secondly, the layer covers the whole sensor surface, including vital parts of the biosensor, such as the connectors and the counter and reference electrodes. Therefore, a different coating strategy had to be found.

To facilitate the production of the nanolayer, the immobilisation of the polymer was performed by pipetting 1 μ l of 5 and 50 mM DTDPA solution one and three times to the sensor surface. One μ l was chosen as this volume expands evenly over the gold surface of the working electrode without reaching the reference and counter electrode. Figure 3.40 illustrates the cathodic peak current obtained by using the instant coating technique compared to the overnight coating. The variation between overnight coating and applying the monolayer directly is with 0.6 % (5 mM DTDPA) and 2.1 % (50 mM DTDPA) negligible. These results confirm the ability of the polymer to form an immediate sulphur-gold bond with hydrophobic alkane chains constructing a stable hydrophobic layer (Prime and Whitesides, 1993). Furthermore, there was no significant improvement between applying the polymer solution once and multiple times to the working electrode. This more time-consuming technique was proved to be redundant and as a result not used for future construction of the SAM.

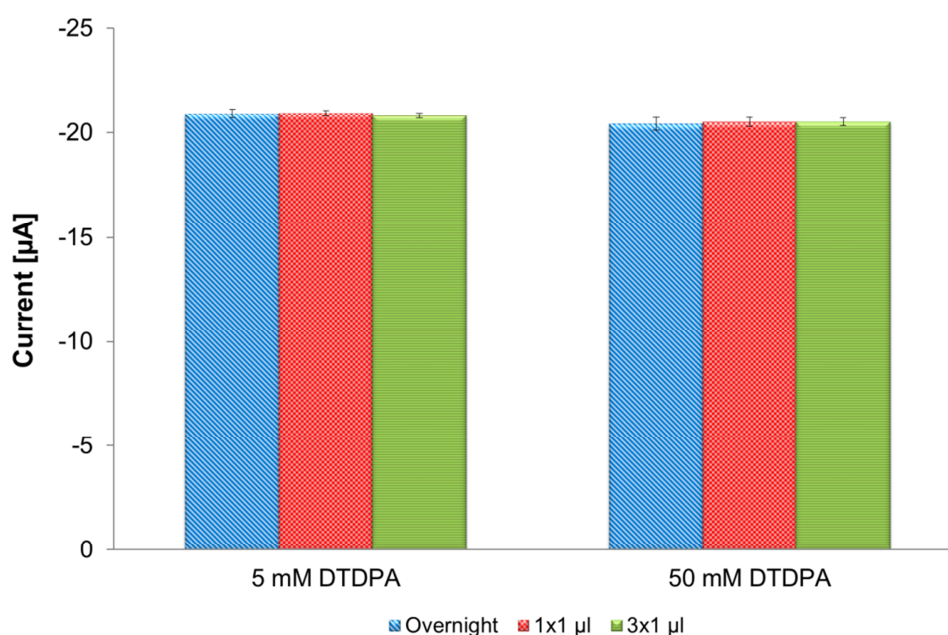


Figure 3.40: Validation of the different techniques for the deposition of the SAM using overnight coating, and pipetting 1 µl of the solution 1x and 3x to the gold working electrode. The columns represent the cathodic peak current using the electroactive mediator potassium ferricyanide (1 mM in 1 M aqueous KCl solution) against 5 mM DTDPA and 50 mM DTDPA. The average signal and standard error of the means were obtained at a scan rate of 20 mV s^{-1} ($n=3$).

3.4.7.2 Influence of Covalent Coupling Using SAM on the Immobilised Capture Antibody Load and Optimisation of the Monolayer Concentration

The successful construction of an immunosensor depends on having an effective procedure for immobilising the bio-recognition element to the transducer surface (Billah et al., 2010). Covalent strategies using SAM were reported to facilitate the immobilisation of the antibodies resulting in an increased surface loading (Furuya et al., 2006). There are few direct methods to monitor the antibody concentration on the surface, such as antibodies tagged with a marker. One possibility is to radioactively mark the antibodies using isotopes such as ^{125}I (Williams and Blanch, 1994). However, this method is not in accordance with the University's health and safety regulations.

A non-hazardous alternative is to use fluorescent markers. Goat anti-rabbit (DyLight 488 nm) and Goat anti-rabbit (DyLight 633 nm, Piercent, Northumberland, UK) were directly immobilised on the sensor surface and visualised with a confocal microscope. Though fluorescent markers are widely used in bioscience, this method was not transferrable to biosensor applications. The gold sensor surface reflected the incoming light in such manner that, although various filter combinations were applied, a distinction between coated and uncoated sensors was not applicable.

Therefore, an experiment was designed using the indirect evaluation of the capture antibody load using HRP-conjugated anti-species secondary antibodies. The HRP labelled secondary antibody simulates a direct format without the use of the antigen. Furthermore, the electrochemical detection also examines the permeability of the polymer layer for electrons. The results of this study are presented in Figure 3.41. According to the electrochemical response of the indirect detection method used in this study, the surface load of covalent coupled antibodies is higher by 77 % (3-DTDPA) and by 40 % (11-MUDA) in comparison to the adsorbed control. The higher response may have been caused by the three-dimensional structure of the polymer observed in Section 3.4.7.1. Another possibility is the decreased loss during washing (Leckband and Langer, 1991; Williams and Blanch, 1994).

Regarding the concentrations used for this assay, as cyclic voltammetric findings suggested (see Section 3.4.7.1), 50 mM MUDA showed an impeding effect, inhibiting the electron transfer to the working electrode. This resulted in a signal lower by 35 % for the 50 mM MUDA solution, whereas the difference in signal strength for the 5 and 50 mM DTDPA group coated overnight was with 4.5 % relatively small. This result is in accordance with the voltammetric analysis performed in the previous Section. However, a polymer concentration of 50 mM DTDPA performed better by approximately 5 % considering the signal strength and was therefore chosen for further testing.

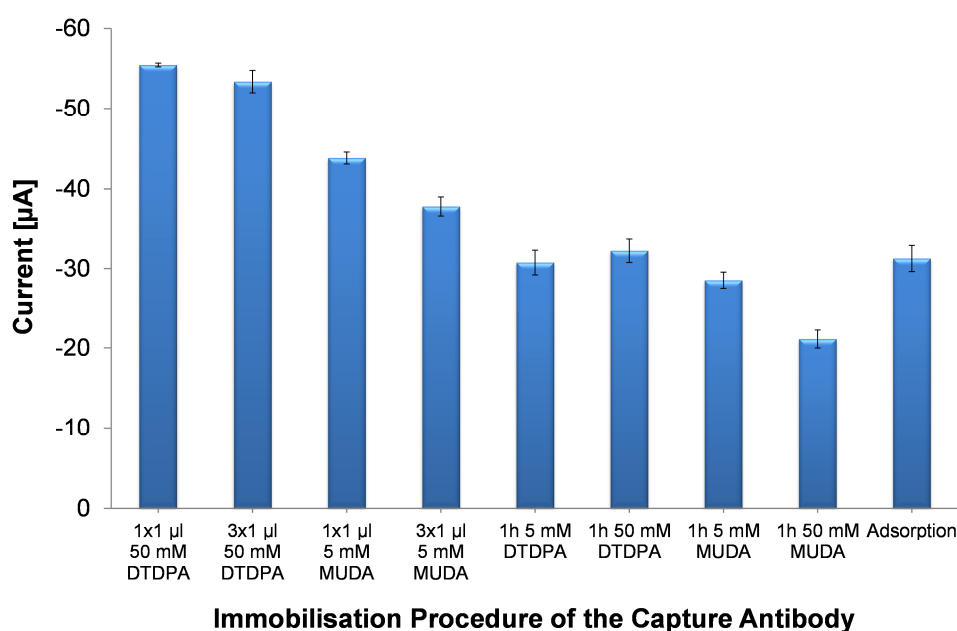


Figure 3.41: Evaluation of the capture antibody load immobilised on the working electrode using covalent coupling with 5 and 50 mM MUDA and DTDPA as well as passive adsorption. First, $100 \mu\text{g ml}^{-1}$ of the capture antibody BC-12 were applied to the working electrode, followed by blocking and $5 \mu\text{l } 10 \mu\text{g ml}^{-1}$ anti-species HRP conjugate. The polymers were applied in three groups by dropping $1 \mu\text{l}$ on the working electrode, $3 \times 1 \mu\text{l}$ and incubating the SPGEs in polymer solution with various concentrations. The bars represent standard error of the means ($n=4$).

As previously discussed, the construction of the SAM by bathing the electrode in polymer may produce a more homogenous layer, but also has a major disadvantage. Vital parts of the electrode, such as counter and reference electrode, are also covered with polymers through non-specific binding to the carbon and Ag/AgCl electrode. This drawback was confirmed in this experiment leading to a significant drop in signal caused by inhibited current flow. Independent of the immobilised polymer, the group with SAMs applied only to the working electrode showed up to 70 % higher current. Furthermore, applying only $1 \mu\text{l}$ to the electrode without repeating this procedure three times resulted in a better surface load and higher assay reproducibility indicated by lower standard deviation. This finding was consistent for 3-DTDPa as well as 11-MUDA layer and was standardly used for further assays.

For best performance of the covalent coupling procedure was subsequently optimised as a function of various DTDPA and MUDA concentrations ranging from 1 to 100 mM in a full sensor assay. Previous findings using cyclic voltammetry (see Section 3.4.7.1) suggested that high concentrations of 11-MUDA (50 mM) have an impeding effect on the current flow, whereas the short chained 3-DTDPA is applicable in concentrations over 50 mM without a loss in active surface area. Figure 3.42 summarises the results obtained for these experiments. MUDA (Figure 3.42 A) applied in a concentration of 50 mM impeded the current flow in such a manner that the signal strength was lower by 48 % compared to the MUDA concentrations of 1 to 10 mM. Though 1 mM had the highest signal value, 5 and 10 mM showed a 72 % better signal separation between the antigen concentrations of 100 and 10 $\mu\text{g ml}^{-1}$ *B. allii* which is crucial for a quantitative method. There was no significant difference between the MUDA concentrations of 5 and 10 mM suggesting that a uniform polymer layer was produced. However, 5 mM MUDA had a lower standard deviation by 40 % and was therefore chosen for future assays.

Unlike MUDA, DTDPA did not show an impeding effect on the electrode if applied in concentrations >50 mM (Figure 3.42 B). However, 10 mM did not yield sufficient monomers to form a uniform layer and provide an enhanced signal. As excess polymer is removed during the washing step after the formation of the SEM, no significant difference was recorded between 50, 70 and 100 mM. However, 50 mM yielded the best values for the signal separation (+13 %). This concentration was subsequently used for the covalent coupling procedure.

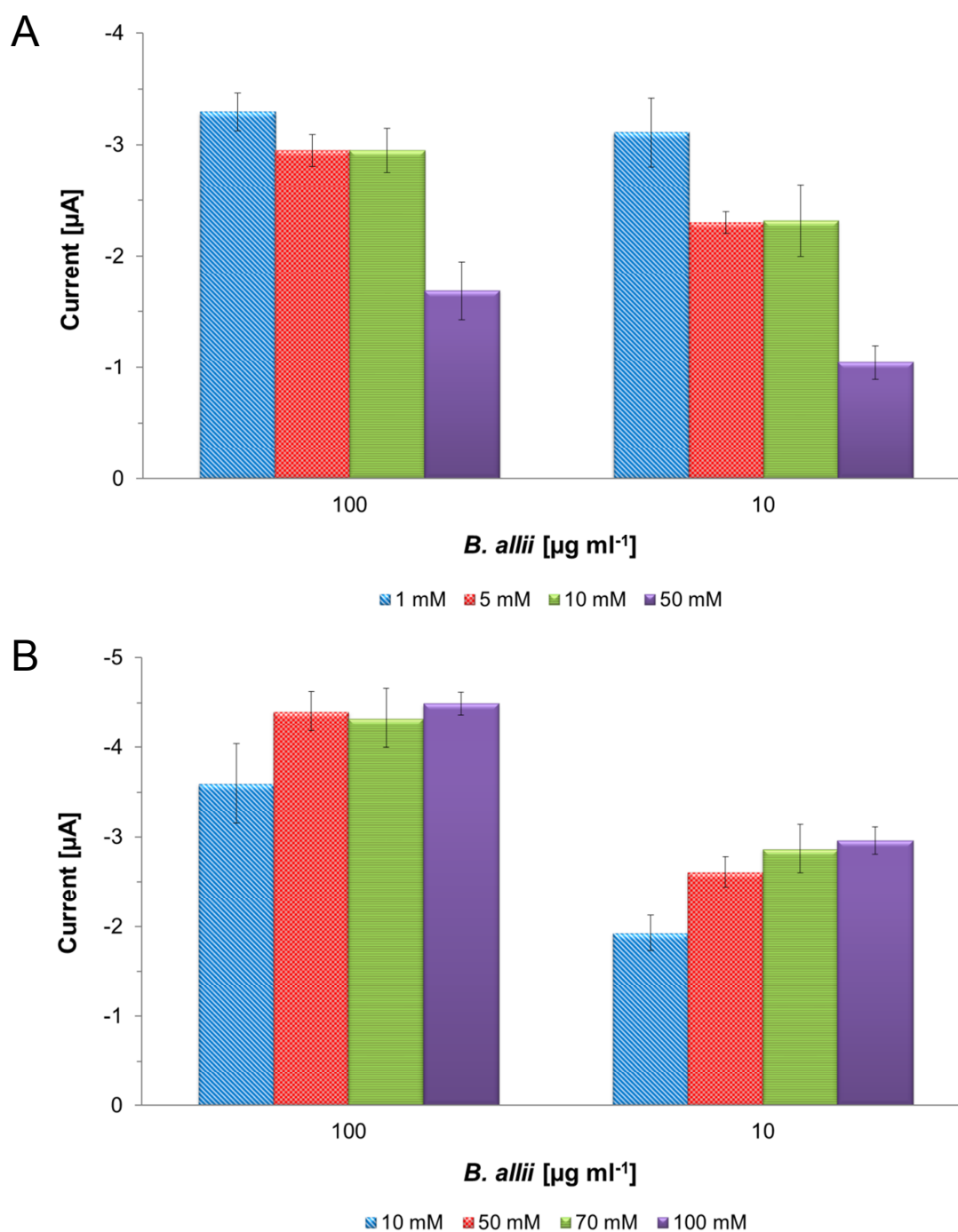


Figure 3.42: Comparison of the normalised sensor signal after 70 s using covalent coupling with (A) MUDA concentrations of 1, 5, 10 and 50 mM and (B) DTDPA concentrations of 10, 50, 70 and 100 mM against 100 and 10 $\mu\text{g ml}^{-1}$ *B. allii*. The monoclonal capture antibody BC-12 (100 $\mu\text{g ml}^{-1}$) was chemically attached to the SEMs employing EDC-NHS coupling chemistry followed by 80 $\mu\text{g ml}^{-1}$ of purified polyclonal conjugate for signal generation. The bars represent the standard error of the means (n=4).

3.4.7.3 Washing Resistance of Covalent and Non-Covalent Coupling Procedures

The biggest benefit of the application of covalent immobilisation strategies is the chemical bond between antibodies and gold surface connected through the SAM. Covalently coupled antibodies were reported to show decreased antibody loss during washing (Leckband and Langer, 1991; Williams and Blanch, 1994). This claim was tested in this study by detecting the surface load of the antibodies after performing controlled washing steps by applying anti-species conjugate that binds the immobilised capture antibody.

Williams and Blanch (1994) reported that covalent coupling procedures reduced the loss per wash by 3 % compared to the adsorbed control. However, this numbers can vary depending on the surface and immobilisation strategy used. These findings were confirmed within this study. Figure 3.43 shows the residual signal after performed multiple washings steps. Both covalent coupling procedures did not suffer a decrease in signal after 3 and 7 washing steps with 500 μ l of PBST, with no significant variation in signal (<1.5 %). In contrast, the signal strength of the adsorbed control decreased by 8 %. The signal drop after 10 s continuous washing was determined with 16 % (3-DTDPA) and 34 % (11-MUDA). However, the washing steps within an assay are normally less than 3 s per electrode and more comparable with the 7x 500 μ l washed group. Surprisingly, the adsorbed group showed an increase in signal of the 10 s washing group. The excessive washing for 10 s may have removed large parts of the blocking proteins allowing the anti-species conjugate to non-specifically bind to the surface and increase current flow.

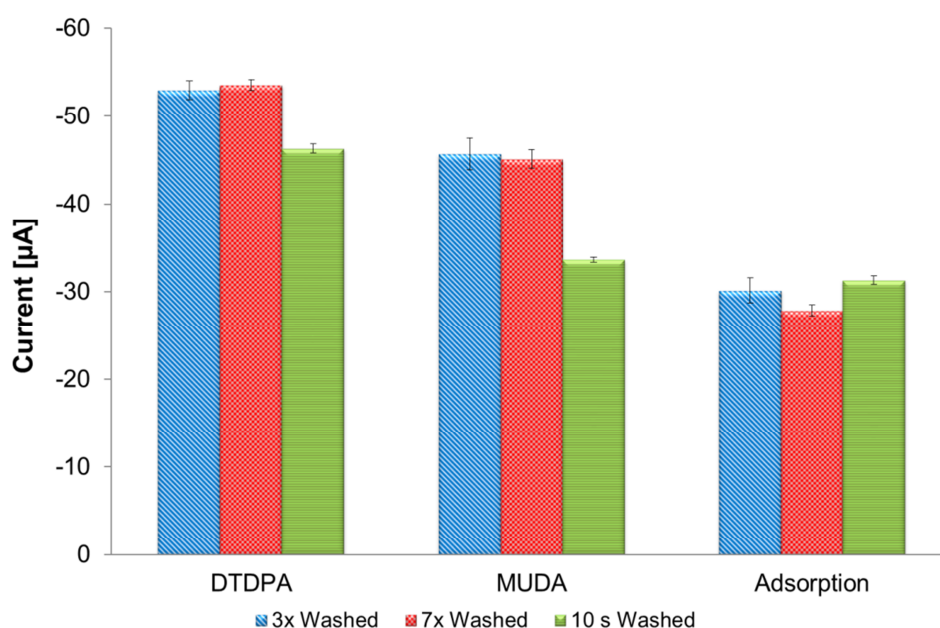


Figure 3.43: Remaining capture antibody load after performing controlled washing procedures (3x 500 μ l, 7x 500 μ l and 10 s continuous wash) using covalent coupling with 5 mM MUDA and 50 mM DTDPA SAMs, as well as passive adsorption. First, 100 μ g ml⁻¹ of the capture antibody BC-12 was immobilised to the working electrode, followed by blocking and 5 μ l of 10 μ g ml⁻¹ anti-species HRP conjugate. The bars represent the standard error of the means (n=4).

3.4.7.4 Comparison of Covalent Coupling Procedures Using DTDPA and MUDA SAM to Passive Adsorption

Immunoassays were conducted to determine the effectiveness of the formation of the covalent bond between the two thiolated polymer layers 3-DTDPA and 11-MUDA and the capture antibodies compared to the adsorbed control group. Covalent coupling is based on the formation of EDC esters which form a covalent bond with reactive amine groups of the antibody under elimination of NHS (see Section 3.3.7.1). The normalised assay response of the covalent and non-covalent immobilisation strategies are compared in Figure 3.44.

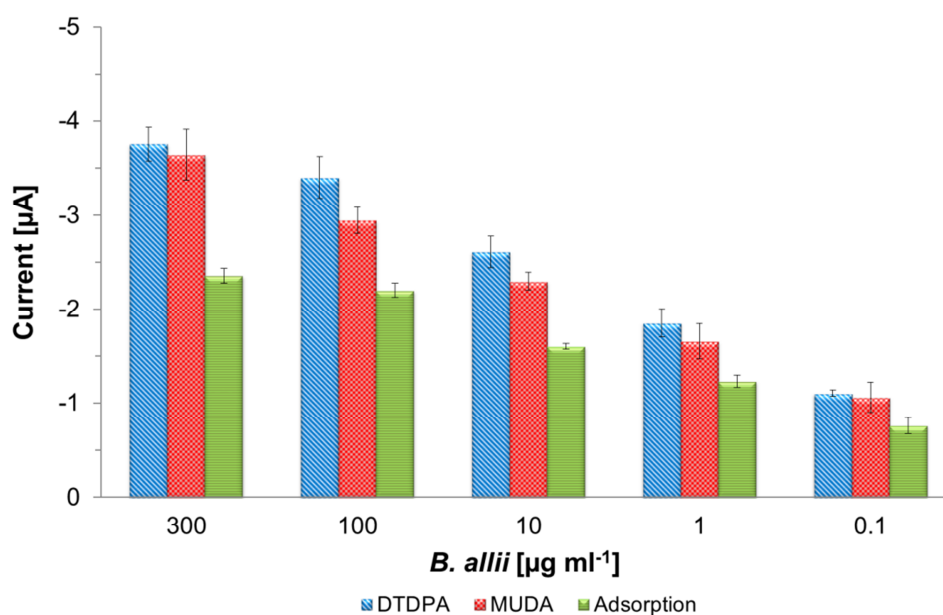


Figure 3.44: Comparison of the normalised sensor signal after 70 s using covalent coupling with 5 mM MUDA and 50 mM DTDPA EDC-NHS activated polymer layers, as well as passive adsorption used for the immobilisation of the monoclonal capture antibody BC-12 ($100 \mu\text{g ml}^{-1}$). For detection, $80 \mu\text{g ml}^{-1}$ of purified polyclonal antibodies conjugated to HRP were applied to the sensor. The bars represent the standard error of the means ($n=4$).

The signal strength of 3-DTDPA as well as 11-MUDA modified SPGEs was found to be higher by 44 % compared with the adsorbed control. Both covalent immobilisation procedures performed well, with a high linear correlation and an average coefficient of variation of 5.8 % (DTDPA) and 6.9 % (MUDA). Focussing on the assay performance of the two polymer layers used for covalent coupling, DTDPA showed a 3 to 13 % higher signal compared to MUDA. Furthermore, the correlation coefficient is higher, demonstrating a better quality logarithmical range for detection, as well as with 21 ng ml^{-1} a lower LOD (MUDA 43 ng ml^{-1}). However, both covalent immobilisation strategies led to a more sensitive assay response compared with the adsorbed group, which was found to have a LOD of 58 ng ml^{-1} (see Section 3.4.5.5).

3.4.7.5 Standard Curve of the Biosensor Employing Covalent Coupling to DTDPA Self-Assembled Monolayer

Self-assembled monolayers were reported to provide an effective method to protect the antibodies on the sensor surface, thereby facilitating a strong ligand immobilisation with increased surface load and wash resistance, as well as minimised non-specific adsorption and maintain biological activity (Williams and Blanch, 1994; Furuya et al., 2006; Millner et al., 2009; Altintas et al., 2012). These claims were confirmed in this Chapter and after optimisation of polymer concentration a standard curve for the assay was constructed presented in Figure 3.45.

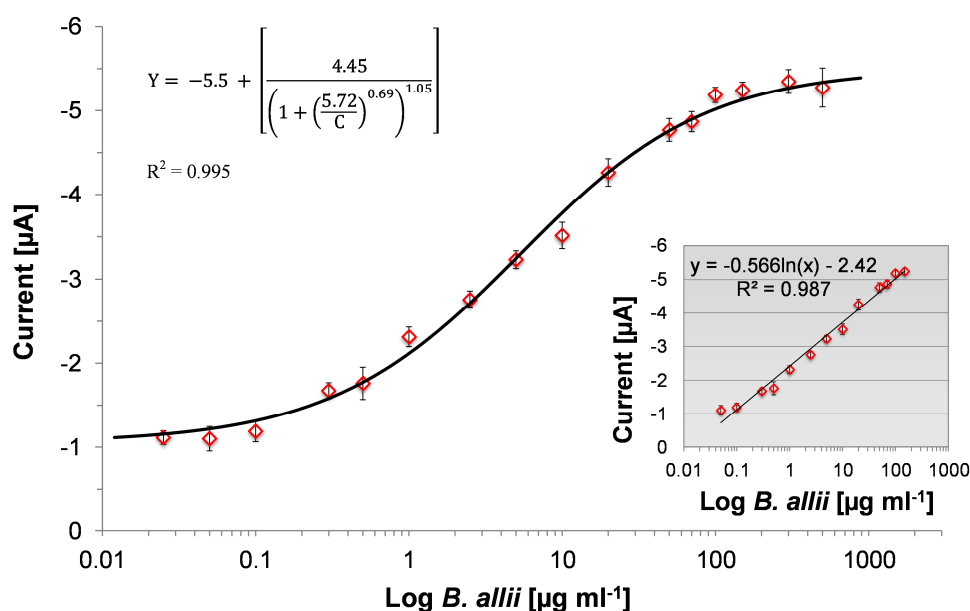


Figure 3.45: Normalised amperometric immunosensor response against various concentrations of *B. allii* antigen using covalent immobilisation of the monoclonal capture antibody BC-12 (100 µg ml⁻¹) to a DTDPA (50 mM) polymer layer. Polyclonal antibody-HRP conjugate (80 µg ml⁻¹) was then applied to the sensor, followed by adding the TMB-H₂O₂ substrate for signal generation. Measurements were taken after 100 s at a potential of -200 mV. Embedded graph shows the linear range of the assay, the bars represent the standard error of the means (n=5), average CV=5.6 %.

With the aid of covalent immobilisation, the sensitivity of the immunosensor was found to be enhanced when compared to that achieved using passive adsorption ($\text{LOD}=58 \text{ ng ml}^{-1}$, see Section 3.4.5.5). The increased signal strength, wash resistance and surface load combined with better reproducibility of the assay resulted in an improved LOD (negative+3xSD) of 16 ng ml^{-1} and a limit of quantification (negative+10xSD) of 34 ng ml^{-1} fungal mycelium. The obtained sensitivity is suitable for detecting *Botrytis* infections prior to showing visible symptoms, producing a powerful tool for pre and post-harvest disease control. However, from a manufacturer's point of view, the detection limit achieved using passive adsorption might be sufficient for a commercial use without the need to employ the more complex covalent coupling procedure.

3.4.8 COMPARATIVE STUDIES OF THE SPGE WITH THE COMMERCIAL ELISA KITS

The developed screen-printed immunosensor based on covalent immobilisation was tested against the commercial *B. allii* and *B. cinerea* ELISA kits purchased from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK). Previous experiments conducted in Section 3.4.6.1 showed that the commercial ELISA kits are highly affected by plant tissue and lack the ability to quantify *Botrytis* samples in the presence of non-specific proteins. Therefore, standards and samples were assayed in PBS for this comparison.

Both ELISA kits as well as the amperometric method were capable of quantifying the various concentrations of the *B. allii* samples. The results obtained are summarised in Figure 3.46, which show consistency in the determination of the *Botrytis* samples with high correlation between the amperometric and spectrophotometric method (slope = 0.985; $R^2 = 0.998$).

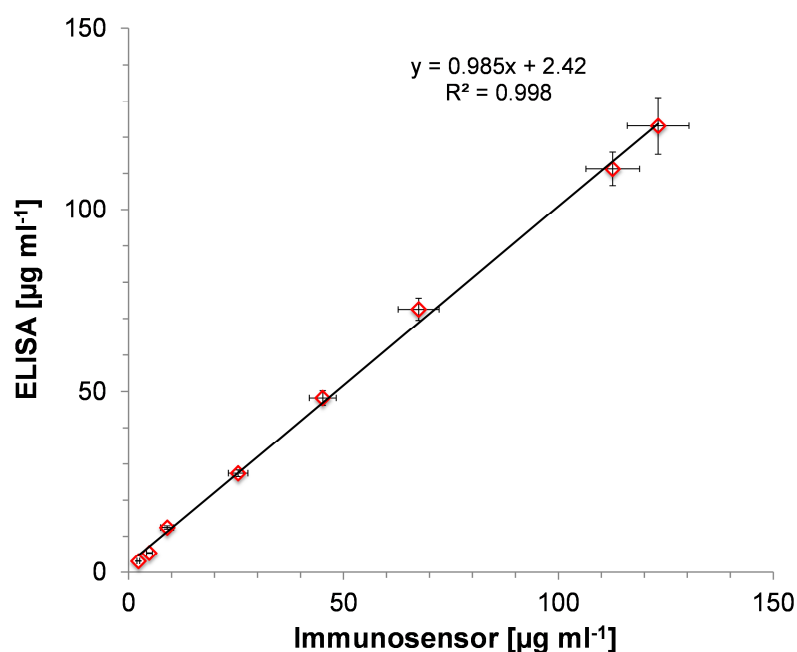


Figure 3.46: Correlation of the developed amperometric immunosensor and the commercial *B. allii* and *B. cinerea* ELISA assays using various concentrations of *B. allii* standards. ELISA was performed in quadruplicate, data are plotted as average concentration determined by the two ELISA kits. The bars represent the standard error of the means (n=5 immunosensor; n=8 ELISA).

The average LOD (negative+3xSD), was calculated at $0.96 \mu\text{g ml}^{-1}$ for the ELISA method and is in accordance with other ELISA methods for *Botrytis* detection described in the literature, which offer a calculated LOD of approximately $1 \mu\text{g ml}^{-1}$ (Meyer et al., 2000; Fernandez-Baldo et al., 2011). The intra-assay variation on quantifying the samples had an average error of 18 % for the commercial ELISA kits and 12.1 % for the biosensor. The average inter-assay variation on the samples was found to be 11.8 %, which meant that there was close agreement between the different methods that were used.

3.5 CONCLUSIONS

Three different sensor types have been compared using cyclic voltammetry against potassium ferricyanide and TMB. JD showed in both cases the best performance with the smallest standard deviation and the highest active surface area. This sensor was used for the future immunoassay development.

Several sensor formats were evaluated for the immunosensor development: the indirect ELISA using anti-species conjugate, the direct format with detection antibodies conjugated to HRP and the detection antibodies conjugated to HRP-labelled gold sol. The indirect ELISA format has several drawbacks. Though the signal strength was superior, the assay lacked reproducibility, resulting in a poor detection limit of $40 \mu\text{g ml}^{-1}$. Furthermore, the indirect format needs an additional incubation step for the secondary antibodies, which will increase the time required for the assay. Considering these facts, this assay was not chosen for the sensor development, but was still in use for the 96 well plate ELISA.

The gold nanoparticles production was optimised using solid phase 96-well ELISA and the LOD was further reduced to $0.72 \mu\text{g ml}^{-1}$. SEM images demonstrated the capability of the gold particles to bind to the fungal antigen. However, this technique could not match the signal strength and LOD of the direct format using HRP-conjugated detection antibodies. Reasons for the lack of performance can be the washing steps performed during the assay. Incorporating a lateral-flow sample delivery may reduce the drag force applied by the washing fluid and improve the signal strength. Another approach to overcome the lack of amplification is to immobilise HRP-conjugated antibodies on the gold colloid. By providing an environment with an excess of HRP during the labelling process, the loss of gold particles during the assay and washing steps can be compensated by the numerous HRP molecules on the gold sol and the conjugated antibodies.

Showing a superior performance, the direct sensor format with HRP-conjugated polyclonal antibodies was further optimised by evaluating the influence of key parameters, such as incubation time, capture and detection antibody concentration and buffers used for immobilisation. Applying purified conjugate further enhanced the sensitivity of the assay. A standard curve was constructed, showing an LOD as low as 58 ng ml^{-1} . A subsequent test of the optimised biosensor using onion samples, tested after varying days of *B. allii* incubation, demonstrated the capability to detect the fungus in two out of three samples 24 h post inoculation. The biosensor showed excellent performance throughout the whole range of pathogen loads detected in this study. Furthermore, unlike the commercially available *B. allii* and *B. cinerea* ELISA kits, which utilise an indirect format, the superior sandwich format of the biosensor was not affected by onion tissue.

To improve assay performance and reproducibility, covalent immobilisation strategies were tested in this study using two polymer layers; 3-dithiodipropionic acid (DTDPA) and 11-mercaptoundecanoic acid (MUDA). Tests showed increased capture antibody load as well as washing resistance compared to the adsorbed control. Furthermore, both EDC-NHS activated SAMs led to an improved LOD compared to the adsorbed control. However, DTDPA performed better regarding signal strength (+13 %) coefficient of variation (-1 %) as well as calculated LOD (16 ng ml^{-1} DTDPA, 43 ng ml^{-1} MUDA). After finalising the assay conditions a standard curve for the covalent immobilisation was constructed achieving a benchmark limit of detection of 16 ng ml^{-1} and a limit of quantification (negative+10xSD) as low as 34 ng ml^{-1} fungal mycelium. The obtained sensitivity is suitable for detecting *Botrytis* infections prior to showing visible symptoms, producing a powerful tool for pre and post-harvest disease control.

CHAPTER FOUR

DEVELOPMENT OF A DNA BIOSENSOR FOR *BOTRYTIS*

4 DEVELOPMENT OF A DNA BIOSENSOR FOR *BOTRYTIS*

4.1 INTRODUCTION

Based on recent progress in genomics, the interest in DNA detection devices has increased drastically opening various fields of application for high-efficient analysis of DNA sequences (Pänke et al., 2008). Without any doubt, the introduction of the polymerase chain reaction (PCR) induced a new area in DNA analysis, as this technique made the specific amplification of small quantities of DNA feasible. Combining this technique with biosensors turned out to be in retrospect a forerunner of a new generation of DNA-based biosensors which can detect, analyse and quantify target DNA. Commercial systems which are easy to use and portable are expected to revolutionise healthcare and molecular diagnostics (Laureyn et al., 2007).

An electrochemical DNA biosensor is defined as a device that utilises oligonucleotides, coupled to a transducer to generate a detectable signal. Hybridisation-based approaches (Figure 4.1 A) are the most common DNA biosensors (Kerman et al., 2009). Here, one DNA strand (probe DNA) is immobilised on the sensor surface. By contact of the capture probe with the complementary target probe, hybridisation occurs forming double stranded DNA which leads to a detectable signal.

Detection of the hybridisation event can be achieved by different transducer principles, such as mass accumulation (Uludag et al., 2010; Altintas et al., 2012; Kaewphinit et al., 2012), changes in optical density (Lawrie et al., 2010; Melnik et al., 2011) or detection of labels (Cao et al., 2006; Valis et al., 2006). Among other techniques, electrochemical methods have recently emerged as the most attractive due to their simplicity, low instrumentation costs, possibility for real-time and label-free detection and generally high sensitivity (Lucarelli et al., 2008). There are basically four different electrochemical detection techniques applied for DNA biosensors: oxidation / reduction of nanoparticle tagged oligonucleotides, oxidation peak current of electroactive DNA bases, such as guanine or adenine, electrochemical signal produced by labels, for

instance metal cations that bind single and double stranded DNA and enzymatically induced electrochemical signal using a substrate mediator system with enzymatically tagged DNA hybrids (Kerman et al., 2009). This research is focusing on the enzyme based electrochemical approach for DNA detection using the HRP / TMB- H_2O_2 substrate system (Figure 4.1 B).

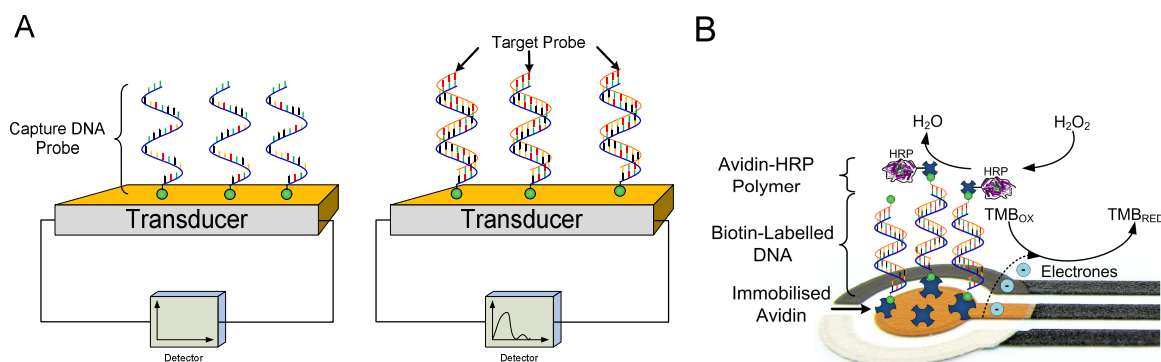


Figure 4.1: Schematic illustration of a DNA biosensor. (A) The capture probe is immobilised on the transducer surface. After adding the target probe, hybridisation occurs. This information is converted by the transducer to a detectable signal. (B) DNA biosensor format developed in these studies utilising an enzyme-based electrochemical detection system using the HRP / TMB- H_2O_2 substrate system.

PCR and real-time (RT-) PCR applications are well-established techniques and widely used in biotechnology. Real-time PCR has the major advantage that the fluorescent dye in the master mix visualises the amplification process and enables further analysis. On the other hand, these devices need an optical readout system making the instruments more expensive compared to a normal thermocycler. However, PCR is in general a laboratory-based technique, as it requires specialised machinery and equipment. However, there is increased interest in portable microsystems for DNA analysis (Tothill, 2001). New development in battery-operated portable thermocyclers for PCR and real-time PCR applications have emerged that are capable of DNA amplification in field conditions (Pal and Venkataraman, 2002; Lagally et al., 2004; Hsieh et al., 2006). Figure 4.2 illustrates the combined PCR-electrochemical DNA biosensor detection cycle used for sample testing in field.

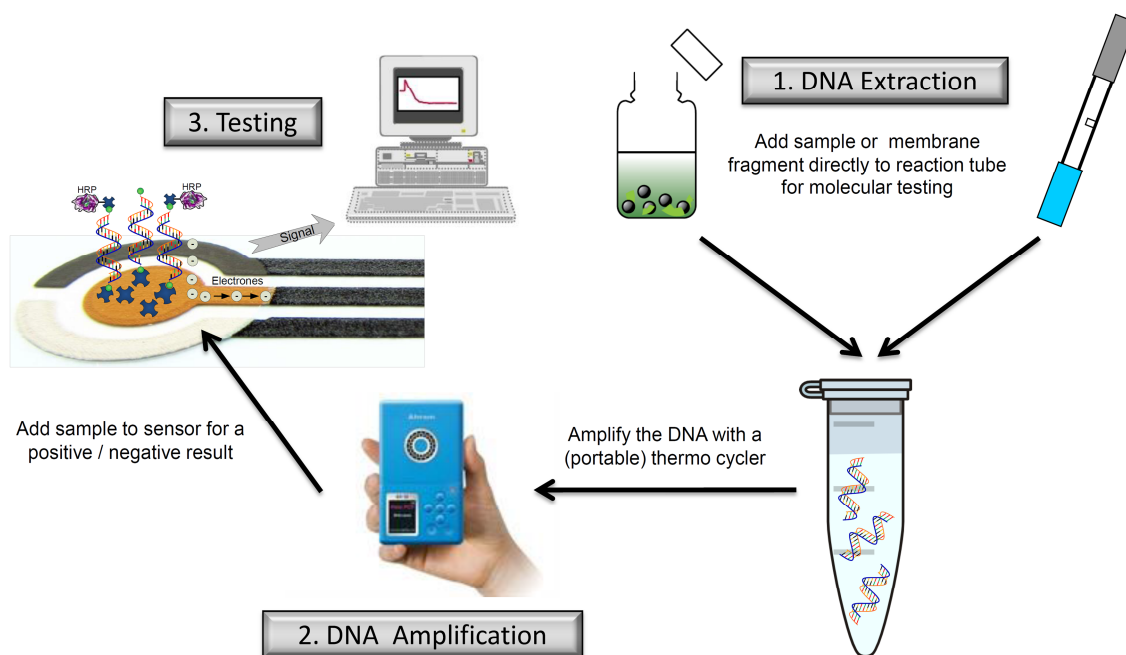


Figure 4.2: Flow diagram for the sample-testing procedure using a PCR-biosensor in field conditions.

This Chapter is focussing on the development of a portable detection method for *Botrytis* DNA in field. Genetically based methods offer superior specificity that immunological detection cannot provide. On the other hand, DNA testing methods for *Botrytis* suffer from fluctuating DNA extraction efficiency and quality making an absolute quantification of the pathogen load an unsolved challenge (Chilvers et al., 2007; Coolong et al., 2008). This study aims at combining the specificity and sensitivity of genetic methods with the sensitive and reliable technique of electrochemical biosensing by producing a portable, inexpensive and disposable analysing system for PCR-amplified DNA. After quantifying the pathogen load using a biosensor the sample can be further examined genetically, by extracting the DNA from the buffer or a LFD membrane fragment. Using primers that target a defined sequence of the pathogen, PCR amplification enables to produce rapidly up to 10^6 to 10^9 copies of the pathogen DNA (Blair and Zajdel, 1992). The PCR product is then subsequently tested using disposable SPGE, giving a positive or negative response within minutes.

4.2 MATERIALS AND INSTRUMENTATION

The primer sequences (Table 4.1) and biotin-HRP conjugate were purchased from Invitrogen (Paisley, UK). Modified CRT-primers sequences comprising 5'-biotin, 5'-biotin-TEG and 5'-thiol-C6, streptavidin–peroxidase conjugate, tris-EDTA (ethylenediaminetetraacetic acid) buffer (1.0 M tris-HCl, 0.1 M EDTA, pH 8.0), DNase/RNase free dH₂O, tris acetate-EDTA buffer (40 mM tris-acetate, 1 mM EDTA, pH 8.3) phosphate buffered saline tablets (PBS, 0.01 M phosphate buffer, 0.137 M sodium chloride and 0.0027 M potassium chloride, pH 7.4), Tween 20, potassium chloride, 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB), hydrogen peroxide solution (30 % w/w) in H₂O, carbonate-bicarbonate buffer (0.05 M, pH 9.6), phosphate-citrate buffer (0.05 M, pH 5.0) and Eppendorf PCR tubes (0.2 ml) were acquired from Sigma (Dorset, UK). ApoI restriction digest (1,000 units) and the corresponding NE buffer 3 (50 mM tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, pH 7.9) were delivered by New England Biolabs (Hitchin, UK). Neutravidin binding protein was acquired from Pierce Thermo Scientific (Cramlington, UK). Milk blocking solution concentrate was purchased from KPL (Gaithersburg, USA).

Table 4.1: Primer sequences used in this study.

Name	Sequence (5'-3')	Purification Grade	Reference
BA2f	GTGGGGGTAGGATGAGATGATG	RP ^a	(Nielsen et al., 2002)
BA1r	TGAGTGCTGGCGGAAACAAA	RP	(Nielsen et al., 2002)
CRT1f	TCACCGGGAGCTATCATAGGC	RP / RP-HPLC ^b	(Chilvers et al., 2007)
CRT2r	GAGCTAGCGCATTTGAAAGC	RP / RP-HPLC	(Chilvers et al., 2007)

^a RP = Reverse-phase cartridge purification. The full-length oligos are retained in the column, while the truncated sequences are washed off.

^b RP-HPLC= Reverse-Phase High Performance Liquid Chromatography. This purification system operates on the same principle as a reverse-phase cartridge, but provides a higher purity due to increased resolution of the HPLC.

Filter tips size 1000, 200, 20 and 10 μ l were purchased from Greiner Bio-One Ltd. (Gloucestershire, UK). Pico Drop P10 (10 μ l) UV pipette tips used for DNA quantification were purchased from Cambridge Bioscience (Cambridge, UK). TrackIt cyan orange GEP loading was acquired from Invitrogen (Paisley, UK). SafeView nucleic acid stain was purchased from NBS Biologicals Ltd. (Huntingdon, UK). Agarose (genetic analysis grade) and ultrapure HPLC grade dH₂O were purchased from Fisher Scientific (Loughborough, UK).

dNTP Mix, HotStarTaq Plus DNA polymerase, SYBR Green PCR kit strip tubes (0.1 ml) for the Rotor Gene real-time PCR machine, GelPilot 100 bp ladder, DNeasy Plant Mini kit and RNase-Free water were acquired from Qiagen (Crawley, UK). Precellys tough microorganism lysing tubes (VK05, 2 ml) comprising 0.5 mm glass beads, and 5.0 mm zirconium oxide beads (80 g) were purchased from Stretton Scientific (Stretton, UK).

Spectrophotometric DNA quantification was accomplished using the Picodrop (model: Piopet-01, Hinxton, UK). Real-time PCR amplification of the DNA was performed using the Qiagen (Crawley, UK) Rotor Gene real-time PCR machine (model: 5-Plex) with the Q series software (version: 2.0.2). Data were subsequently analysed and processed using the Q series software, Excel and TeeChart office (version: 2.0).

PCR amplification and reagent incubation were carried out using thermocyclers acquired from Techne (model: TC-512, Stone, UK) and Biometra (model: 070-601, Göttingen, Germany). Gel electrophoresis was performed using a basin (model Sub-Cell-GT) and a Power-Pac-Basic power unit, both purchased from Biorad (Hemel Hempstead, UK). UV-pictures of the gel were taken using a Syngene GBox i Chemi XR camera system (Synoptics Ltd., Cambridge, UK) with the processing software Gene Snap (version: 7.09).

Electrochemical measurements and biosensor related machinery and equipment are equivalent to the materials and methods described in Chapter 3.

4.3 METHODS

4.3.1 PRODUCTION OF DNA TEMPLATE

4.3.1.1 DNA Extraction

Fungal species were grown on PDA covered with a thin, sterile cellophane membrane (P400; Cannings, Ltd., Bristol, United Kingdom) as described in Section 2.3.1.3. After 6 weeks of incubation, the membrane comprising the fungus was removed, vacuum freeze-dried (Scanvac, model Cool Safe 110-4, Lyngø Denmark) at -40 °C for 7 days and stored at -20 °C for further use.

The sample extraction procedure was based on a modified protocol provided as part of the DNeasy Plant Mini DNA extraction kit acquired from Qiagen (Crawley, UK). Several DNA extraction methods can be found in the literature, such as the cetyltrimethylammoniumbromide (CTAB) method (Lee and Taylor, 1990; Møller et al., 1992), or phenol extraction procedures (Ellington and Pollard, 1999). However, though these methods are cheaper to perform and show a high efficiency for the extraction of DNA from lyophilised mycelium, the project is focussing on onion plants which have been reported to contain inhibiting compounds affecting the DNA extraction and PCR amplification (Chilvers et al., 2007). The DNeasy Plant Mini DNA was recommended by the Cranfield Mycology group to be the benchmark extraction procedure for fungal and plant DNA, as the kit includes several filtration steps using spin columns in order to enhance the quantity and purity of the gained DNA.

The protocol was initiated by adding 0.1 mg sample into a 2 ml lysing Precellys tough microorganism lysis tubes (VK05, 2 ml) comprising 0.5 mm glass beads. To facilitate the tissue breakdown, the tube was snap frozen for 30 s in liquid nitrogen and a 5 mm zirconium oxide bead (80 g) was added to the tube. The sample was then ground using a tissue lyser (Bertin technologies, model: Precellys 24) for 2x30 s with 5 s break in between the cycles at a speed of 6,000 rpm according to the manufacturer's recommendation for fungi.

For the following DNA extraction, 400 µl AP1 buffer and 4 µl RNase were added to the lysed cells and incubated for 10 min at 65 °C using a heated sample block or water bath. Afterwards, 130 µl AP2 buffer were added and incubated for 5 min on ice. To separate the buffer comprising the DNA from the tissue, the sample was centrifuged for 5 min at 14,000 rpm and the supernatant was transferred into the QIAshredder spin column. After centrifugation (2 min at 14,000 rpm), the flow-through fraction was transferred to an 1.5 ml Eppendorf tube and 1.5 times the sample volume of AP3 buffer was added to the solution and mixed using a pipette. Batchwise, 650 µl of the mixed solution was transferred to the DNeasy mini spin column and centrifuged for 1 min at 8,000 rpm until the whole solution was filtered. Two times 500 µl of AW buffer solution were added to the column and centrifuged for 1 min at 8,000 rpm (1st step) and 2 min at 14,000 rpm (2nd step). The spin column comprising the DNA was carefully removed from the collection tube and transferred to a new 1.5 ml Eppendorf tube. For the elution of the DNA, 75 µl of the AE buffer (10 mM tris-Cl, 0.5 mM EDTA, pH 9.0) were added drop by drop to the membrane, incubated for 5 min at room temperature and centrifuged for 1 min at 8,000 rpm. The final elution was repeated to obtain a total of 150 µl purified DNA. The DNA was quantified as described in the following section before storage at -20 °C.

4.3.1.2 DNA Quantification

A Pico Drop P10 spectrophotometer was used to quantify the DNA yield by monitoring the absorbance between 230 and 340 nm. To estimate the concentration of the DNA, the absorbance at 260 nm was used by the software to extrapolate the DNA load ($A_{260}=1=50 \mu\text{g ml}^{-1}$). Furthermore, the absorbance ratio between 260 and 280 nm was monitored, giving crucial information about the purity and quality of the extracted DNA. The Pico Drop spectrophotometer employs customised UV-permeable pipette tips (P10 UVpette). Throughout all measurements, the sample volume was constantly set to 2.5 µl as recommended by the manufacturer. Before performing the quantification and after 20 consecutive measurements, the spectrophotometer was calibrated using AE elution buffer (10 mM tris-Cl, 0.5 mM EDTA, pH 9.0) from the DNeasy plant mini kit.

4.3.2 PCR AMPLIFICATION PROCEDURE

4.3.2.1 Primer Reconstitution and PCR Preparation

After their delivery, the primers were reconstituted in TE buffer to a stock primer concentration of 100 μM . Next, the primers were further diluted in UV sterilised, ultrapure HPLC-grade dH_2O to aliquots of 5 μM for PCR and 10 μM for real-time PCR as recommended by the manufacturer's protocol and stored at $-20\text{ }^\circ\text{C}$ for further use. In general, the components used to set up the PCR reaction were kept on ice in order to maintain their functionality and insure a gentle defrosting process. To prevent contamination, all DNA-based pipetting steps were performed using hydrophobic filter tips. Furthermore, the preparation of the negative control and master mix was set up in an area, different from the one used for DNA extraction, sample preparation and product analysis according to the manufacturer's recommendation.

4.3.2.2 PCR Assay

The PCR template was set up according to the recommendations of the HotStar Taq Plus protocol (Qiagen, Crawley, UK). This PCR kit comprises a recombinant 94 kDa Taq DNA polymerase that requires an initial activation step (5 min at $95\text{ }^\circ\text{C}$). Afterwards, the PCR protocol was set up according to a modified procedure based on the RFLP protocol described by Nielsen et al. (2002). The 3-step PCR procedure was initiated by a 30 s denaturation at $94\text{ }^\circ\text{C}$ followed by 30 s annealing at $60\text{ }^\circ\text{C}$. The length of the *Botrytis* template sequence is approximately 500 bp. Therefore, the extension time was set to 30 s, as the elongation rate of the HotStar Taq DNA polymerase is 2 to 4 kilobase per minutes. The cycle was repeated 30 times followed by a 3 min final elongation at $72\text{ }^\circ\text{C}$. All reagents, concentrations and volumes required to set up a 25 μl reaction are listed in Table 4.2.

Table 4.2: Composition of a 25 μ l PCR reaction performed using a thermocycler.

Component	Volume [μ l]	Concentration
Q solution ^a	5	5x concentrated
PCR buffer/ Coral Load PCR buffer ^b	2.5	10x concentrated, contains: tris-Cl, KCl, (NH ₄) ₂ SO ₄ , 15 mM MgCl ₂ , pH 8.7.
Forward primer	2.5	500 nM (Nielsen et al., 2002)
Reverse primer	2.5	500 nM (Nielsen et al., 2002)
dNTPs	0.5	400 μ M of each dNTP
Taq polymerase	0.1	1 unit
Sample DNA	11.9	< 1 μ g

^a Q solution modifies the melting behaviour and reduces PCR inhibiting secondary structures of the DNA.

^b Contains gel-loading reagent and two dyes for subsequent GEP.

4.3.2.3 Real-Time PCR Assay

Using the fluorescent dye SYBR Green, real-time PCR enables to monitor the amplification of the DNA template without the need of further analysis steps like gel electrophoresis. The Qiagen (Crawley, UK) SYBR Green kit contains a master mix, which comprises all vital reaction components such as dNTPs, HotStar Taq Plus DNA polymerase and SYBR Green in an optimised concentration. Similar to the normal PCR protocol, an activation step (5 min at 95 °C) for the polymerase was needed.

Primers and real-time PCR protocol was based on the studies performed by Chilvers et al. (2007), with a 10 s denaturation at 95 °C, 15 s annealing at 65 °C and 15 s elongation at 72 °C. For the real-time PCR amplification of the primers designed by Nielsen et al. (2002), the cycle parameters were adopted from Section 4.3.2.2. Template DNA was amplified for 25 to 50 cycles. Before the melt curve was measured between 55 °C and 95 °C using a 0.5 °C s⁻¹ ramp, a second denaturation step was included (95 °C for 30 s). Table 4.3 provides an overview of the reagent volumes and final concentrations

required to set up a 25 µl reaction for the real-time PCR using the Rotor Gene system (Qiagen, Crawley, UK). The volumes applied are based on the recommendations of the Rotor Gene real-time PCR handbook (Qiagen, Crawley, UK).

Table 4.3: Composition of a 25 µl real-time PCR reaction using the Qiagen Rotor Gene system.

Component	Volume [µl]	Concentration
SYBR Green master mix	12.5	Contains dNTPs, Taq polymerase, SYBR Green fluorescent dye.
Forward primer	2	800 nM (Chilvers et al., 2007)
Reverse primer	2	800 nM (Chilvers et al., 2007)
DNase, RNase free dH ₂ O	4	/
Sample DNA	4.5	< 1 µg

4.3.2.4 Performance Studies of the Neck Rot and RFLP Primers

The LOD and specificity of the primers developed by Nielsen et al. (2002) and Chilvers et al. (2007) were tested following the real-time PCR protocol described in Section 4.3.2.3. Primers were tested against *B. allii* (Cabi, IMI 147186), *B. aclada* (CBS 103.23), *B. byssoidea* (CBS, 104.23) and *B. cinerea* (Plant Research International, BC 143). To establish the LOD, *B. allii* DNA was extracted as described in Section 4.3.1 and diluted to target concentrations ranging from 10 fg to 100 ng for the neck-rot primers and 0.1 fg to 100 ng for the RFLP primers. Ct graphs and analysis were performed using the Rotor Gene Q series software and the normalised cycle threshold value of 0.1 and 0.01.

4.3.3 RFLP ANALYSIS AND GEL ELECTROPHORESIS PROTOCOL

The RFLP analysis described by Nielsen et al. (2002) is based on the selective digestion of the ApoI restriction enzyme New England Biolabs (Hitchin, UK). The concentration

of ApoI is defined in units, with one unit (0.1 μ l) being the equivalent of the amount needed to digest 1 μ g DNA in 1 h at 50 °C using a reaction volume of 50 μ l. Table 4.4 provides an overview of the components and concentrations required for the RFLP analysis. Amplified DNA was transferred to a new PCR tube and incubated in a thermocycler for 3 h or overnight at 50 °C. To prevent over digestion during storage, the restriction enzyme was heat-deactivated for 20 min at 80 °C after the incubation.

Table 4.4: Volumes and concentrations used for the RFLP analyses of *Botrytis* spp. The total volume per sample was 25 μ l.

Component	Volume [μ l]	Concentration
Amplified sample DNA	20 μ l	80-140 ng μ l ⁻¹
NE Buffer 3	2.5 μ l	10x concentrated, contains: 50 mM tris-HCl, 100 mM NaCl, 10 mM MgCl ₂ , 1 mM dithiothreitol, pH 7.9
BSA	0.25 μ l	100x concentrated (200 μ g ml ⁻¹)
ApoI	0.5 μ l	5 units
DNase, RNase free dH ₂ O	1.75 μ l	

In order to visualise the amplified product of the PCR and to separate the fragments according to their length, gel electrophoresis (GEP) was performed. 1.5 % agarose was added to TAE buffer and slowly heated for approximately 10 min on a hot plate (50 % power). In order to ensure the dissolving of the agarose powder, the solution was mixed using a magnetic stirrer. Before casting the solution to the mould, 5 μ l SafeView (NBS Biologicals Ltd.), a non-toxic DNA stain, were added per 100 ml gel. Depending on the size of the gel tray, 70 to 150 μ l agarose solution were produced. After cooling, 10 μ l PCR sample as well as 6 μ l GelPilot 100 bp ladder were loaded to the gel slots. The DNA separation was performed using a Biorad power unit. To improve the resolution, first 50 V were applied to the gel for 20 min, allowing the DNA to enter the gel homogeneously, before the potential was increased to 100 V for 40 to 100 min. Pictures of the gel were taken using a Syngene GBox with a short-wave band pas filter.

4.3.4 DEVELOPMENT OF A DNA SENSOR USING SPGE

4.3.4.1 Assay Procedure for the DNA Biosensor

The assay procedure of the DNA biosensor was based on the experience and results gained with the development of the immunological assay described in Chapter 3. Prior to the immobilisation of the streptavidin capture molecule, the sensor was heat-treated in an oven (Carbolite, model PN 120 (200), Hope, UK) for 30 min at 120 °C to clean the surface. Previous studies performed in Section 3.4.5.4 indicated that the maximum achievable protein surface loading of the working electrode (0.226 cm² planar area) is peaking at a protein concentration of approximately 100 µg ml⁻¹. In order to provide a saturated environment, this streptavidin concentration was diluted in carbonate-bicarbonate buffer (CBB, 0.05 M, pH 9.6) and incubated for 2.5 to 3 h at 37 °C, allowing the molecule to adsorb to the surface of the sensor. The volume required to cover the working electrode is 15 µl. Excluding the following blocking step, which requires a volume of 50 µl to cover all three electrodes, this value was kept constant for the following assay steps. However, the yielded volume of PCR DNA product is low (25 µl per reaction). Therefore, the volume of amplified PCR product applied to the sensor was limited to 10 µl instead. After each incubation cycle, the surface was washed gently with deionised water and dried with nitrogen gas.

To reduce non-specific binding, the sensor surface was blocked using 50 µl milk protein blocking concentrate (KPL Inc., Gaithersburg, USA) diluted 1:10 in PBS. The incubation time was at least 1 h at 37 °C. After washing the DNA amplified as described in Section 4.3.2 with a biotin label on both primers, was dropped on the working electrode and incubated for 1 h at 37 °C forming a strong streptavidin-biotin bond. A period of 1 h was also used for the following incubation of the streptavidin-labelled HRP used for signal generation. The purified detection molecule was diluted to a working strength of 50 µg ml⁻¹ in PBS buffer containing 1:40 milk protein concentrate to decrease non-specific binding to the tube. The TMB-H₂O₂ substrate and reading of the sensor signal was performed as described in Section 3.3.3.3 and 3.3.1, respectively.

The premix format employed the CRT1f primer tagged with a thiol- C_6 and the CRT2r primer with a biotin label. The assay was initiated by adding streptavidin-HRP conjugate directly to the amplified DNA product to obtain a target concentration of $20 \mu\text{g ml}^{-1}$ in 1:100 milk protein concentrate. The mixture was incubated for 2 h at 37°C to allow the streptavidin-HRP conjugate to bind to the biotin tagged reverse primer CRT2r. Next, the amplified DNA product was added directly to the sensor with no prior surface modification and incubated for 1 h at 37°C . After washing, the TMB- H_2O_2 substrate was added and the sensor signal was read as described in Chapter 3.

4.3.4.2 Hybridisation Studies Using Biotin-Labelled Complementary Sequences

Initial tests were performed using a complementary sequence with a defined concentration. The following DNA sequence and biotin-labelled primers were computed in order to match the elongated sequence using the primers described by Nielsen et al. (2002):

5'- AAA CAA AGG CGG TCG TGA GT TCCACTACT CATC ATC TCA TCC TAC CCC CGC -3'
 Biotin3'- TTT GTT TCC GCC AGC ACT CA GTAG TAG AGT AGG ATG GGG GTG -5'Biotin

The sensor assay was performed as described in Section 4.3.4.1. After immobilising the streptavidin ($100 \mu\text{g ml}^{-1}$), the surface was blocked and $20 \mu\text{M}$ of the 3'-biotin-labelled capture probe was added to the sensor and incubated for 1 h at 37°C . Though DNA hybridisation itself takes place in seconds, the following target sequence was incubated for 30 min at 37°C , allowing the capture probe to hybridise with the target DNA strands. Various concentrations of the target sequences were applied, ranging from 0.1 to $10 \mu\text{M}$, with DNase-free dH_2O used as a negative control. Afterwards, $20 \mu\text{M}$ of 5'-biotin-labelled detection probe was applied to the sensor and incubated for another 30 min at 37°C . To complete the sandwich format, streptavidin-labelled HRP ($100 \mu\text{g ml}^{-1}$) was incubated for 1 h at 37°C . DNA sequences were diluted in TE buffer, streptavidin and streptavidin-HRP in PBS.

4.3.4.3 Estimation of the DNA Concentration after PCR Amplification

The RFLP primers were used to amplify *B. allii* DNA ranging from 1 fg to 100 ng. *B. allii* DNA was extracted as described in Section 4.3.1, real-time PCR protocol was followed as described in Section 4.3.2.3. After amplification, the DNA product was relatively quantified as described in Section 4.3.1.2 using the amplified negative control to normalise the spectrometer.

4.3.4.4 Evaluation of Different Hybridisation Strategies for the Two-Step DNA Detection Using the DNA Biosensor

The high-temperature denaturation method to produce ssDNA is based on the immediate transfer of heated DNA to ice water in order to freeze the re-annealing process. First, 100 $\mu\text{g ml}^{-1}$ streptavidin was coated on the sensor surface, followed by 10 μM biotin-labelled capture probe. The double-stranded DNA product was boiled for 5 min in order to induce denaturation. Afterwards, the separated DNA was chilled rapidly in $-3\text{ }^{\circ}\text{C}$ ice water comprising saturated NaCl for 1 min. Finally, 10 μl of the DNA was transferred to each sensor and used to perform a sensor assay as described in Section 4.3.4.1. The sandwich format was constructed as previously performed in the hybridisation studies (Section 4.3.4.2).

For the labelling process of the amplified DNA, each biotin-labelled target probe was added to the PCR product to a final concentration of 10 μM , which represents an over ten-fold excess of biotin-labelled probe to primers, enhancing the probability of the ssDNA to re-anneal with the biotin-labelled probe. The PCR product was then transferred to a thermocycler in order to perform a subsequent denaturation step ($95\text{ }^{\circ}\text{C}$, 10 min) followed by 5 min annealing at $65\text{ }^{\circ}\text{C}$. The cooling slope was $1\text{ }^{\circ}\text{C s}^{-1}$, to control the annealing process. Finally, 10 μl of the DNA were transferred to each sensor and used to perform a sensor assay as described in Section 4.3.4.1. PCR was performed as described in Section 4.3.2.3 using the RFLP primers and *B. allii* as target species. Negative control was represented by DNase-free dH_2O .

4.3.4.5 DNA Sensor Formats and Primer Designs

Two primer designs were evaluated in this study and tested with the neck-rot primers CRT1f/2r. One system employed two biotin labels, one on each primer, the second approach utilised a 15-atom mixed polarity triethylene glycol (TEG) spacer at the 5'-end of each primer sequence. *B. allii* DNA was produced according to Section 4.3.1 and 10 ng DNA was used to produce amplified pathogen DNA as described in Section 4.3.2. Positive and negative samples were amplified for 25 cycles and used for the sensor assay as described Section 4.3.4.1. Neutravidin ($500\ \mu\text{g ml}^{-1}$) was immobilised on the sensor surface and used as a capture molecule for the biotin-labelled DNA, followed by $100\ \mu\text{g ml}^{-1}$ streptavidin-HRP conjugate for signal generation.

4.3.5 OPTIMISATION OF THE DNA SENSOR RESPONSE

4.3.5.1 Streptavidin-HRP Conjugate Concentration

Streptavidin-HRP concentrations ranging from 10 to $100\ \mu\text{g ml}^{-1}$ were assayed to achieve best signal performance. Positive and negative samples were amplified for 25 cycles according to Section 4.3.1 with biotin-labelled primers CRT1f/2r and used for the sensor assay as described Section 4.3.4.1. The sensor signal was monitored as a function of the streptavidin-HRP concentration to assess the highest signal separation between positive and negative samples.

4.3.5.2 Direct Detection of Immobilised Neutravidin Using Biotin-HRP Conjugate

To evaluate the load of immobilised neutravidin on the sensor surface, a system was developed and optimised utilising biotin-HRP conjugate. 500 and $50\ \mu\text{g ml}^{-1}$ neutravidin were immobilised for 2 h on the sensor surface as described in Section 4.3.4.1. The negative control was represented by $0.5\ \text{mg ml}^{-1}$ BSA. After washing the sensor surface was blocked as described in the Section 4.3.4.1 and $10\ \mu\text{l}$ of the biotin-HRP conjugate (1 , 5 , 10 and $30\ \mu\text{g ml}^{-1}$) diluted in PBS were applied to the working electrode and incubated for 1 h for signal generation. To compensate the influence of non-specific binding to the sensor signal, the current was normalised by subtracting the reading of the negative control.

4.3.5.3 Neutravidin Immobilisation Employing Adsorption and Covalent Immobilisation Techniques

Various neutravidin concentrations (10 to 1,000 $\mu\text{g ml}^{-1}$) were assayed using two immobilisation strategies: passive adsorption and covalent immobilisation to 3-DTDPA self-assembled polymer layer. The DNA sensor assay was performed as described in Section 4.3.4.1, covalent immobilisation strategies were performed according to Section 3.3.7.1. After blocking, the immobilised neutravidin was directly targeted employing the system designed in Section 4.3.5.2 with a biotin-HRP conjugate concentration of 1 $\mu\text{g ml}^{-1}$.

4.3.5.4 Evaluation of Covalent Immobilisation Strategies of Neutravidin versus Adsorption

Covalent immobilisation to 3-DTDPA layer (50 mM) compared to passive adsorption using two neutravidin concentrations (30 and 300 $\mu\text{g ml}^{-1}$). Positive and negative samples were amplified for 25 cycles according to Section 4.3.2.3. The DNA sensor assay was followed as described in Section 4.3.4.1, covalent immobilisation strategies were performed according to Section 3.3.7.1. For signal generation streptavidin-HRP conjugate (1 $\mu\text{g ml}^{-1}$) was used and the signal separation between positive and negative samples was plotted against the neutravidin concentrations of the two immobilisation strategies.

Streptavidin-HRP concentrations ranging from 10 to 100 $\mu\text{g ml}^{-1}$ were assayed to achieve best signal performance. Positive (10 ng *B. allii*) and negative samples were amplified for 25 cycles according to Section 4.3.2.3 with biotin-labelled primers CRT1f/2r and used for the sensor assay as described Section 4.3.4.1. The sensor signal was monitored as a function of the streptavidin-HRP concentration to assess the highest signal separation between positive and negative samples.

4.3.6 DEVELOPMENT OF A DNA SENSOR BASED ON A PREMIX FORMAT WITH THIOL-C6 PRIMERS

4.3.6.1 Streptavidin-HRP Conjugate Concentration

Streptavidin-HRP concentrations ranging from 0.1 to 100 $\mu\text{g ml}^{-1}$ were assayed against positive (10 ng *B. allii*) and negative samples, previously amplified for 25 cycles according to Section 4.3.2.3 with thiol-C6 and biotin-labelled primers CRT1f/2r. The sensor assay was followed as described in Section 4.3.4.1. PCR amplified DNA was incubated for 2.5 h at 37 °C allowing the streptavidin-HRP to bind to the biotin-labelled primer CRT2f before adding the DNA to the working electrode. The sensor signal was monitored as a function of the streptavidin-HRP concentration to assess the highest signal separation between positive and negative samples.

4.3.6.2 Premix Time of PCR Amplified DNA with the Streptavidin-HRP Conjugate

The incubation times of the streptavidin-HRP to the PCR amplified DNA were investigated in a range between 5 min to 2.5 h. Positive (10 ng *B. allii*) and negative samples were amplified for 25 cycles according to Section 4.3.2.3 with thiol-C6 and biotin-labelled primers CRT1f/2r. The sensor assay was followed as described in Section 4.3.4.1 with a streptavidin-HRP concentration of 20 $\mu\text{g ml}^{-1}$. In order to compensate the influence of non-specific binding to the sensor signal, the current was normalised by subtracting the background current produced by the master mix before PCR amplification.

4.3.6.3 DNA Incubation Time

The incubation time of the DNA comprising the streptavidin-HRP on the sensor was evaluated. Positive (10 ng *B. allii*) and negative samples were amplified for 25 cycles according to Section 4.3.2.3 with thiol-C6 and biotin-labelled primers CRT1f/2r. After premixing the DNA with the streptavidin-HRP conjugate (20 $\mu\text{g ml}^{-1}$) for 2 h, the master mix was dropped on the sensor and incubated for 5 min to 2 h and the sensor

assay was followed as described in Section 4.3.4.1. The current was normalised by subtracting the reading produced by the unamplified master mix.

4.3.6.4 Final Optimisation and Reduction of the Background Current

Methods to reduce the background signal represented by the current produced by the unamplified DNA master mix before PCR amplification were evaluated. PCR master mix comprising thiol-C6 and biotin-labelled primers CRT1f/2r was produced according to Section 4.3.2.3 and premixed with the streptavidin-HRP conjugate ($20\text{ }\mu\text{g ml}^{-1}$) for 2 h without PCR amplification. The unamplified master mix was used to perform a DNA sensor assay following the procedure described in Section 4.3.4.1.

The first stage involved testing whether adding the detergent Tween (0.05 %) to the wash buffers tris-EDTA and PBS result in an improved performance compared to dH_2O . Controlled washing steps were accomplished by rinsing the sensor five times with 1 ml of the washing solution using a pipette. The second experiment evaluated whether adding 1:100 milk protein solution and 0.1 mg ml^{-1} BSA to the master mix can reduce non-specific binding and improve signal to noise ratio.

4.3.6.5 LOD Test of the DNA Sensor

The limit of detection of the developed DNA sensor system was tested with *B. allii* DNA extracted according to the protocol described in Section 4.3.1 and diluted to target concentrations ranging from 100 ng to 100 fg. DNA was amplified for 25 cycles according to Section 4.3.2.3 with thiol-C6 and biotin-labelled primers CRT1f/2r. The DNA sensor assay was followed as described in Section 4.3.4.1 and the normalised sensor signal was plotted as a function of the template DNA before PCR amplification.

4.3.7 PERFORMANCE STUDIES

4.3.7.1 DNA Sensor Response Using PCR and Real-Time PCR Thermocyclers

The potential use of a low-cost thermocycler acquired from Techne (model: TC-512, Stone, UK) was evaluated and compared with a modern Rotor Gene real-time PCR machine, acquired from Qiagen (Crawley, UK). PCR master mix comprising thiol-C6 and biotin-labelled primers CRT1f/2r was produced according to Section 4.3.2.3 and amplified using the real-time PCR and low-cost thermocycler. To confirm a successful amplification, the DNA product of the low-cost thermocycler was transferred to the Rotor Gene real-time PCR thermocycler and a melt analysis was performed prior the DNA sensor assay (see Section 4.3.4.1).

4.3.7.2 Specificity Studies of the DNA Sensor Utilising the Neck-Rot Primers CRT1f/2r

The specificity of the DNA sensor was tested against *B. allii* (Cabi, IMI 147186), *B. aclada* (CBS 260.71), *B. byssoidea* (CBS, 104.23) and *B. cinerea* (Plant Research International, BC 143). The DNA was extracted according to the protocol described in Section 4.3.1 and amplified for 25 cycles according to Section 4.3.2.3 with thiol-C6 and biotin-labelled primers CRT1f/2r. The DNA sensor assay was followed as described in Section 4.3.4.1.

4.3.7.3 Specificity Studies of the DNA Sensor in Combination with the RFLP Primers BA1r/2f

The specificity of the DNA sensor was tested against *B. allii* (Cabi, IMI 147186), *B. aclada* (CBS 260.71), *B. byssoidea* (CBS, 104.23) and *B. cinerea* (Plant Research International, BC 143) in combination with the RFLP primer pair BA1r/2f. The DNA was extracted according to the protocol described in Section 4.3.1 and amplified for 25 cycles as described in Section 4.3.2.3 with biotin-labelled primers BA1r/2f. The sensor assay protocol was followed as presented in Section 4.3.4.1.

4.3.7.4 Evaluation of the Minimum Assay Time

The minimum assay time was evaluated as a function of the premix and DNA incubation time on the sensor surface. A premixing and sensor incubation time of 5, 15 and 30 min were tested to evaluate which is the shortest possible DNA sensor assay protocol. Additionally, an incubation time of 5 min was performed at room temperature. PCR master mix comprising thiol-C6 and biotin-labelled primers CRT1f/2r was produced according to Section 4.3.2.3 and premixed with the streptavidin-HRP conjugate ($20\text{ }\mu\text{g ml}^{-1}$) before performing the DNA sensor assay according to Section 4.3.4.1 employing the modified incubation times.

4.4 RESULTS AND DISCUSSION

4.4.1 DNA EXTRACTION AND QUANTIFICATION

According to the DNeasy extraction kit handbook, an A_{260}/A_{280} value of 1.71.9 is characteristic for the highest grade of pure DNA. The DNA extracted from *B. allii* and *B. aclada* was quantified between 8 and 30 ng μl^{-1} . A_{260}/A_{280} averaged with 1.81 indicating a high grade of purity. On the other hand, *B. cinerea* and *B. byssoidea* extracted DNA material was quantified between 4 and 8 ng μl^{-1} . The poor performance of the extraction was most likely due to the less successful breakdown of the cells using the lysis tubes comprising 0.5 mm glass beads.

4.4.2 PERFORMANCE STUDIES OF THE NECK ROT AND RFLP PRIMERS

Two primer pairs can be found in the literature specific to *Botrytis* species. The universal primers developed by Nielsen et al. (2002) can be used in combination with the RFLP analysis to distinguish between *B. aclada*, *B. allii*, *B. byssoidea*, *B. cinerea*, and *B. squamosa*. On the other hand, the primers described by Chilvers et al. (2007) are optimised for real-time PCR analysis of common species associated with neck rot (*B. aclada*, *B. allii* and *B. byssoidea*). Both primers can be used for the detection of *Botrytis* neck rot in onion and were used within this study.

4.4.2.1 Neck-Rot Primers

The neck-rot primers designed by Chilvers et al. (2007) were tested with the fungal species *B. allii* (Cabi, IMI 147186), *B. aclada* (CBS 103.23), *B. byssoidea* (CBS, 104.23) and *B. cinerea* (Plant Research International, BC 143) in a real time and normal PCR assay performed as described in Section 4.3.2. This primer pair, designed for real-time PCR applications, amplifies a 113 bp section of the common species associated with neck rot i.e. *B. aclada*, *B. allii* and *B. byssoidea* (Chilvers et al., 2007).

The fluorescent signal of the real-time PCR is illustrated in Figure 4.3 B. Further GEP analysis confirmed the specificity of the primers to common species associated with neck rot, as *B. cinerea* was not amplified (Figure 4.3 B). The fragments were located near the 100 bp ladder and showed an equal and defined peak at 78 °C in the melt curve (Figure 4.3 A embedded graph). Relative quantification with a normalised threshold of 0.1 resulted in a Ct-value between 7.6 and 13.9 cycles.

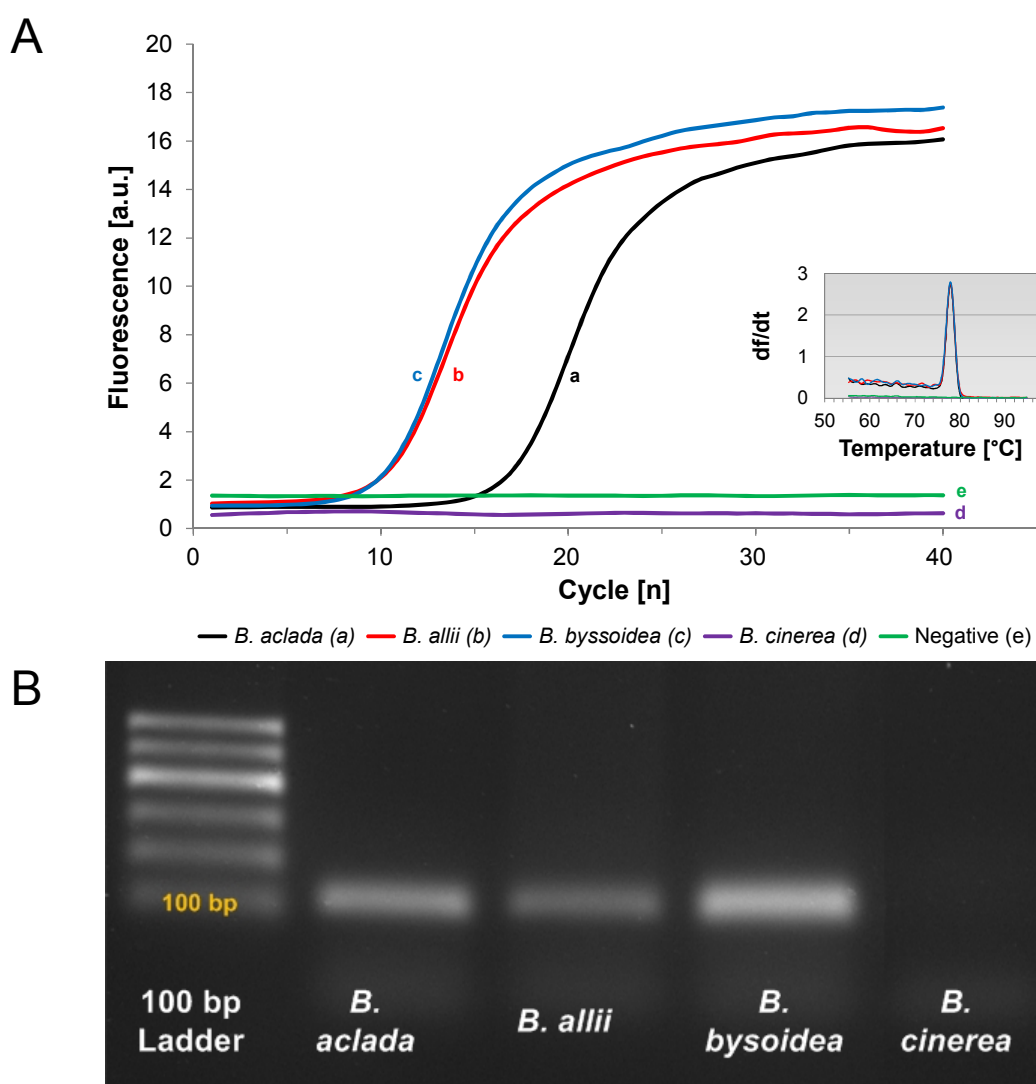


Figure 4.3: PCR amplification of common species associated with neck rot, i.e. *B. aclada*, *B. allii* and *B. bysoidea* as well as *B. cinerea*, using the primer pair described by Chilvers et al. (2007). (A) Fluorescent reading of the real-time PCR with the corresponding melt curve. (B) Gel electrophoresis analysis of the amplified product.

Furthermore, the real-time PCR assay was successfully tested with *B. allii* directly extracted from 1 mg ml⁻¹ antigen in PBS, prepared as described in Section 2.3.1.3 for the biosensor use. This method compromises the DNA extraction efficiency showing a decreased sensitivity with an approximately 10 cycles higher Ct, whereas the *Botrytis* DNA extracted from neck-rot species extracted using the Plant Mini kit averaged at a Ct-value of 17.3 cycles (norm. threshold 0.1). However, this result indicates that DNA extraction from PBS is practicable if samples used in biosensor assays require further confirmation using PCR. Additionally, the PCR amplification was successfully performed with onion samples diluted in PBS from the sensor performance experiment (see Section 3.4.6.2), incubated for two days. *B. allii* was detected in the sample, with an average Ct-value of 25.33 cycles (normalised threshold 0.1). The pathogen load of the samples was quantified using the biosensor with approximately 0.5 µg ml⁻¹. This confirms the ability of the primers to detect latent *B. allii* infections without visible signs of neck rot.

Tested against various template concentrations of *B. allii* DNA, the neck-rot primers designed by Chilvers et al. (2007) showed good performance (Figure 4.4) with a linear range between 1 pg and 100 ng template DNA (Figure 4.4 embedded graph). Chilvers et al. (2007) claim a reliable real-time PCR detection of the pathogen down to 10 fg. Though signal saturation was observed with fungal concentrations less than 1 pg, a detection limit of 10 fg pathogen DNA was confirmed within this study, since the Ct-value of the negative control was with approximately 25 to 27 cycles significantly higher.

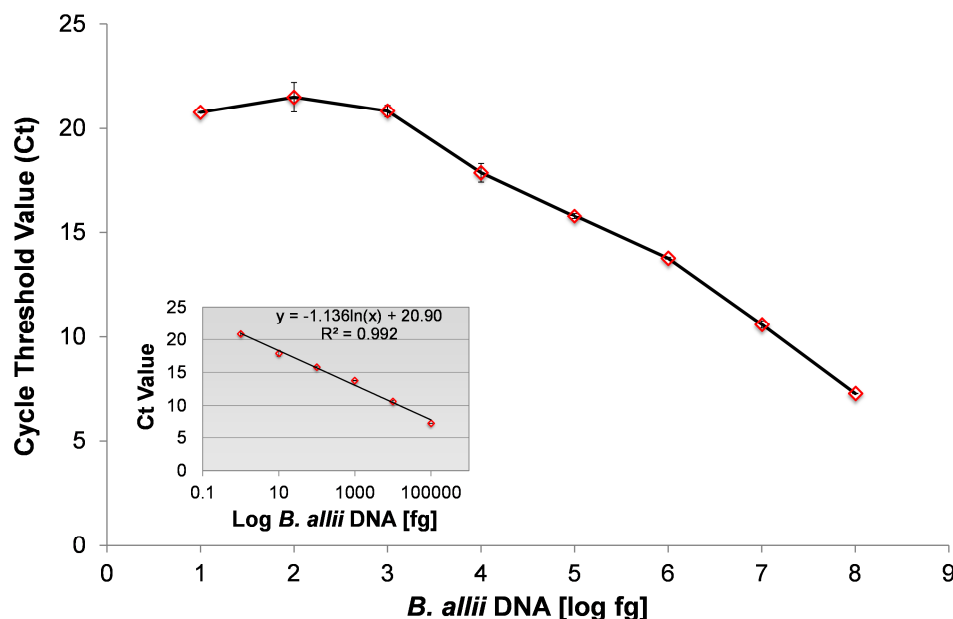


Figure 4.4: Real-time PCR performed with the neck-rot primers using various amounts of *B. allii* template DNA added per PCR, ranging from 100 ng to 10 fg plotted versus the corresponding cycle threshold (Ct 0.01 normalised signal). The embedded graph illustrates the standard curve of the linear range. The bars represent standard error of the means (n=3).

4.4.2.2 RFLP Primers

The RFLP primers designed by Nielsen et al. (2002) provide a wider range of applications as they amplify five species of *Botrytis*: *B. aclada*, *B. allii*, *B. byssoidea*, *B. cinerea*, and *B. squamosa*. This primer pair was used in a real time and normal PCR assay as described in Section 4.3.2. In general, real-time PCR assays are optimised for a fragment length of 100 to 300 bp. However, real-time PCR assays were also performed with fragment lengths of up to 1100 bp (Celius et al., 2000; Towner et al., 2008). The amplification of long product over 400 bp is strongly dependent on a good DNA quality, whereas shorter real-time PCR products, mostly used with a length of 70 to 250 bp, are more or less “independent” of their quality (Towner et al., 2008). Even if quantification is not the primary target, real-time PCR can provide a critical advantage compared to normal PCR. The amplification can be monitored directly by the fluorescent signal giving vital information about contaminations and non-specific elongation identified by performing the melt curve analysis.

The size of the expected PCR product determine the elongation time of the reaction (Blair and Zajdel, 1992). Therefore, the elongation time was increased to 30 s, instead of the 15 s of the regular real-time assay, which proved to compensate the longer fragment length allowing the HotStar Taq DNA polymerase (elongation rate 2 to 4 kbp min⁻¹) to elongate the approximately 500 bp fragment. The fluorescent reading of the real-time PCR reaction is illustrated in Figure 4.5. The RFLP primers demonstrated their ability to amplify *B. allii*, *B. aclada*, *B. byssoidea* and *B. cinerea*. However, the reaction efficiency varied showing a decreased sensitivity for *B. cinerea*, resulting in a Ct-value between 22 and 23 cycles (norm. threshold 0.01).

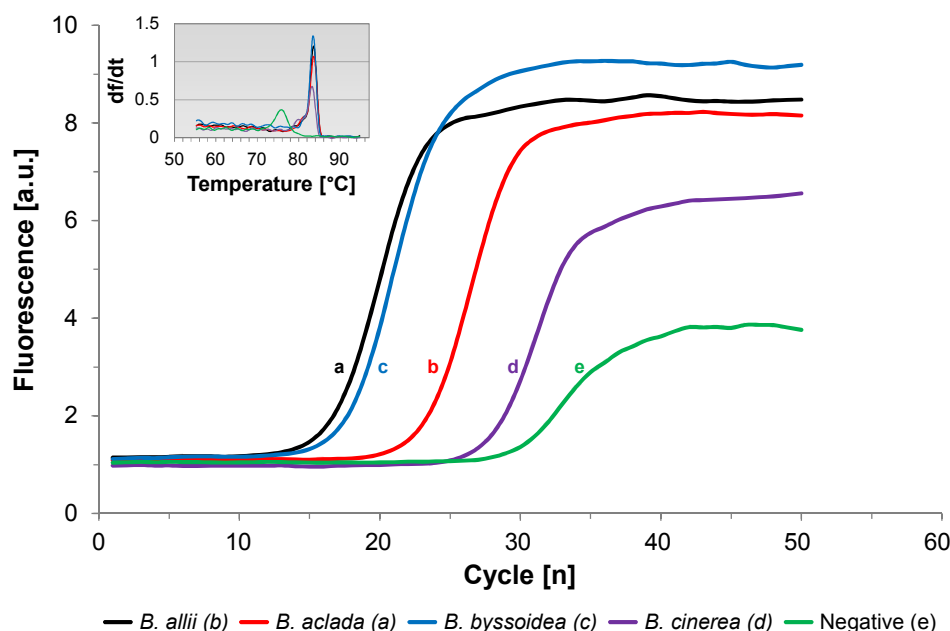


Figure 4.5: Fluorescent signal of the real-time PCR amplification of the *Botrytis* species *B. allii*, *B. aclada*, *B. byssoidea* and *B. cinerea* using the RFLP primers described by Nielsen et al. (2002). The embedded graph illustrates the corresponding melt curve.

The poor DNA amplification of *B. cinerea* can have several reasons. First to mention is the fluctuating efficiency of the DNA extraction procedure. However, the DNA quantification performed in Section 4.4.1 also indicated problems with the cell breakdown of *B. byssoidea*, resulting in approximately the same amount of extracted DNA material (4 and 8 ng µl⁻¹). In contrast, *B. byssoidea* did not show a decreased performance with an average Ct of 12 cycles (norm. threshold 0.01). A second approach

can be the lower affinity of the primers to *B. cinerea*, caused by inhibiting compounds in the DNA extract or a high tertiary structure. GEP analysis of the RFLP fragments performed by Nielsen et al. (2002) and Chilvers and Du Toi (2006) showed an approximately 40 % weaker band of *B. cinerea* and *B. squamosa* after amplification compared with the fungal species *B. allii*, *B. aclada* and *B. byssoidea* (see Section 1.4.5). These findings are in accordance with the real-time PCR response and GEP analysis (Section 4.4.3) performed within this study.

Non-specific elongation of the negative control was observed with increasing cycle number (>30). This effect was also observed using the neck-rot primers tested in the previous section. With high cycle numbers, dNTPs and primers form random double stranded structures, which are then elongated by the DNA polymerase, leading to a fluorescent signal caused by the Sybr Green dye (Heid et al., 1996b; Bell and Ranford-Cartwright, 2002). However, non-specific elongation can be distinguished from contaminations by the subsequent melt curve analysis (Figure 4.5 embedded graph). Contaminations show a low intensity peak, located at a similar temperature as the target sequence (here 84 °C), whereas non-specifically amplified DNA generally shows a peak at lower temperatures (72 to 78 °C) indicating a much shorter fragment. For the DNA sensor development the cycles were reduced to 25 to eliminate the problems associated with non-specific elongation. Hence, the reduced cycle numbers shortens the overall assay time significantly with no loss in performance since saturation of the PCR reaction is reached for positive samples.

The fluorescent reading of the real-time PCR assay using various template concentrations of *B. allii* DNA is illustrated in Figure 4.6. Nielsen et al. (2002) claimed a limit of detection between 1 and 10 pg pure fungal DNA. These numbers were confirmed within this study. The logarithmical correlation between template DNA and Ct threshold value ends with DNA concentrations lower than 10 pg eventually reaching saturation (Figure 4.6 B). The correlation coefficient was 0.991, between 0.1 and 100 ng (Figure 4.6 B, embedded graph) and $R^2 = 0.965$ if the detection range is extended to 10 pg. For this primer pair, a cycle number between 30 and 35 provides the best compromise between amount of amplified product, assay duration and non-specific elongation of the negative control.

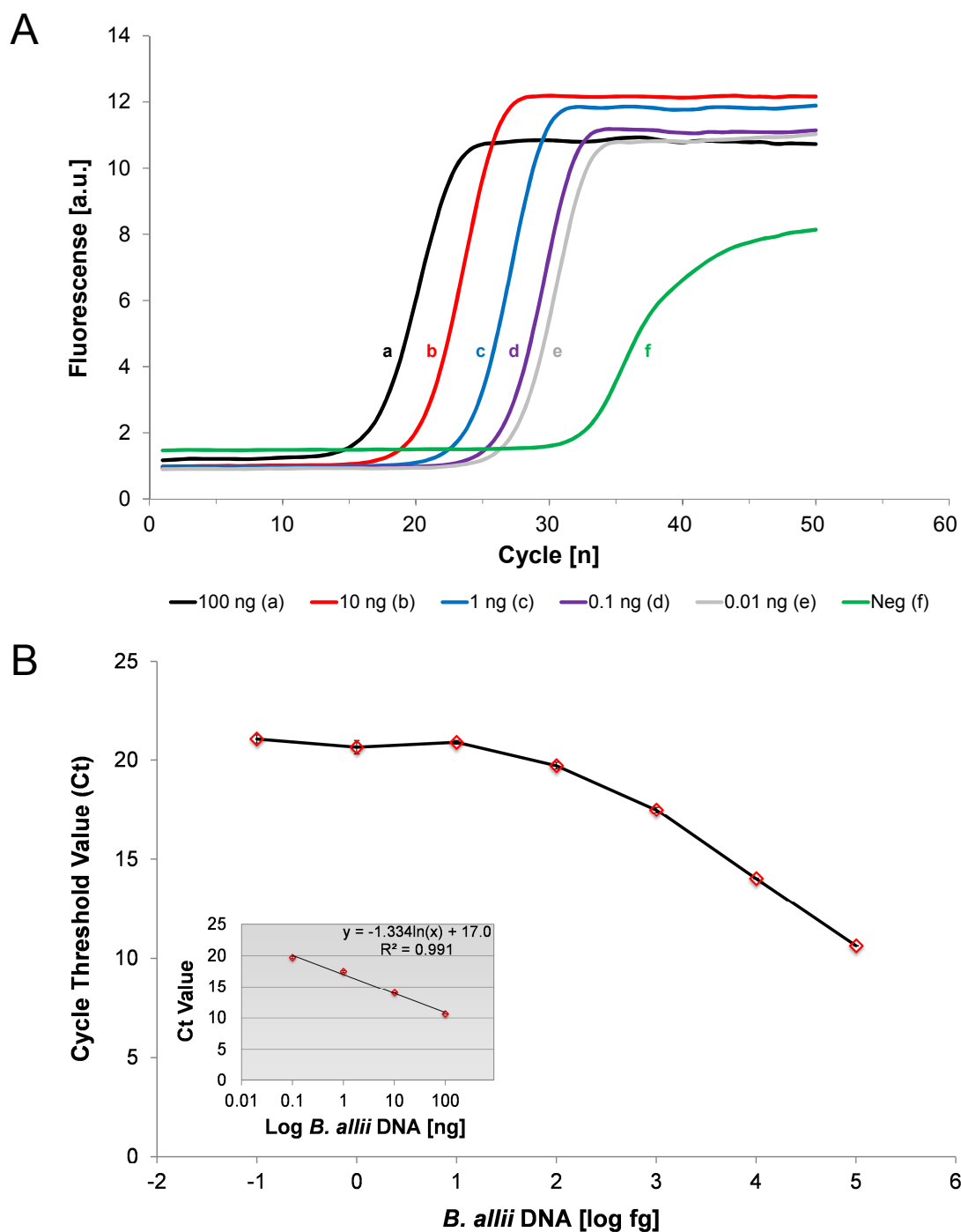


Figure 4.6: Real-time PCR performed with the RFLP primers using various amounts of *B. allii* template DNA. (A) Fluorescent reading obtained by amplifying template DNA concentration between 100 and 0.1 ng *B. allii*. (B) Log *B. allii* DNA added per PCR, ranging from 100 ng to 0.1 fg versus corresponding cycle threshold (Ct 0.01 normalised signal). The embedded graph illustrates the linear range with the corresponding standard curve of the assay. The graphs are plotted as average of three reactions, the bars represent standard error of the means (n=3).

4.4.3 RFLP ANALYSIS OF *BOTRYTIS* SPECIES

Classic differentiation between the *Botrytis* species occurred morphologically by comparing the size and hyphan growth pattern of the fungi (Chilvers and Du Toit, 2006). However, the overlapping spore size and identical structural growth makes this approach not feasible for the separation of *B. allii* and *B. aclada*. To overcome this uncertainty, Nielsen et al. (2002) designed a RFLP analysing protocol identifying the fungal species. After digesting the DNA with the ApoI restriction enzyme and GEP, the *Botrytis* spp. can be identified due to their fragment length (Figure 4.7).

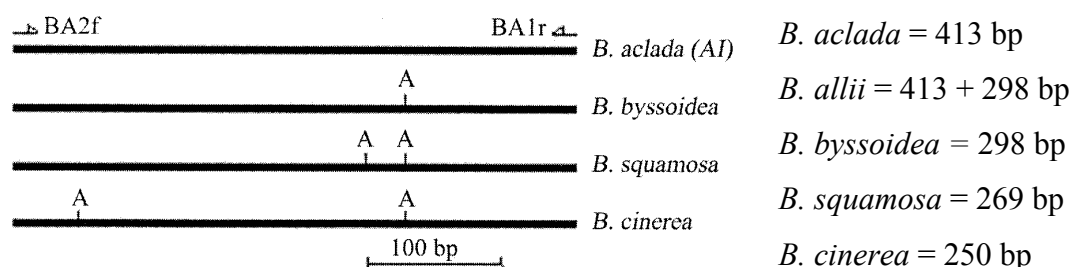


Figure 4.7: ApoI restriction map and resulting fragment length of PCR amplified *Botrytis* spp. causing disease in onions using the primers BA1r/2f described by Nielsen et al. (2002).

The *Botrytis* species *B. allii* (Cabi, IMI 147186), *B. aclada* (CBS 103.23), *B. byssoidea* (CBS, 104.23) and *B. cinerea* (Plant Research International, BC 143) were amplified as described in Section 4.3.2.3 and digested overnight using the protocol described in Section 4.3.3. Figure 4.8 shows the subsequent GEP analysis of the PCR-amplified DNA. The RFLP analysis of *B. cinerea* and *B. byssoidea* showed two bands: one identical approximately 100 bp long, and one band with approximately 250 bp for *B. cinerea* and 300 bp for *B. byssoidea*. The 300 bp fragment of *B. byssoidea* is also shared by *B. allii*. However, this species shows a second approximately 400 bp long fragment, which is also visible for *B. aclada* (Figure 4.8 A). This finding emphasises the assumption of Yohalem et al. (2003) that *B. allii* is a hybrid species emerging from a simultaneous infection of *B. aclada* and *B. byssoidea*.

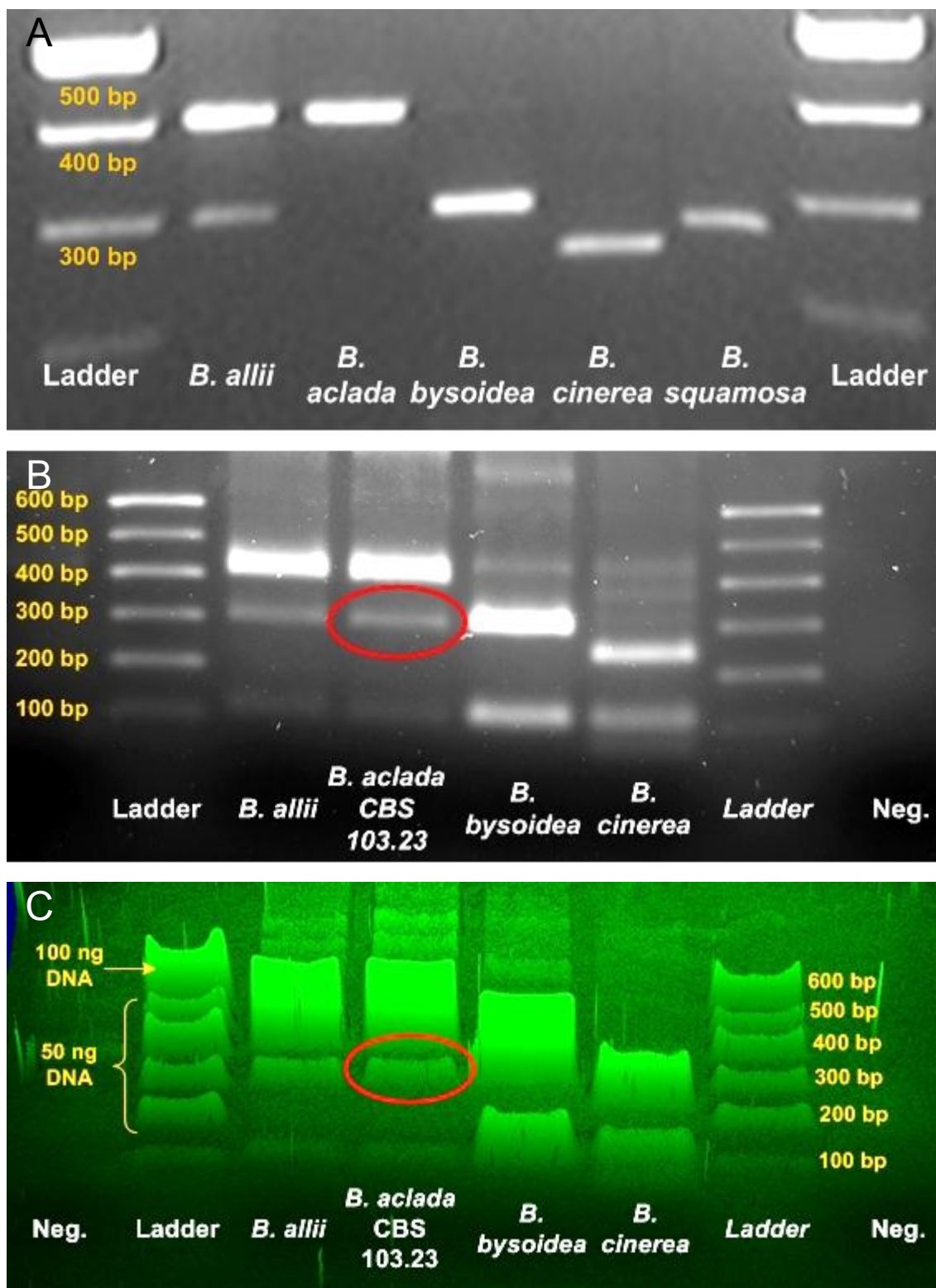


Figure 4.8: PCR-RFLP analysis using the protocol described by Nielsen et al. (2002). (A) Studies performed by Chilvers and Du Toi (2006). (B) RFLP analysis with the corresponding power spectrum (C) of the *Botrytis* species *B. allii* (Cabi, IMI 147186), *B. aclada* (CBS 103.23), *B. bysoidea* (CBS, 104.23) and *B. cinerea* (Plant Research International, BC 143).

However, considering the result of the RFLP analysis of the *B. aclada* (CBS 103.23) species used for this study, two fragments are visible (Figure 4.8 B and C, marking). The power spectrum (Figure 4.8 C) indicates a line strength that results from approximately 50 ng DNA. Furthermore, the strength of the 300 bp fragment is identical to the one shown by *B. allii* (Cabi, IMI 147186) and is too powerful to be related to contaminations. According to Verkley (2012), the CBS KnaW database curator for filamentous fungi, the CBS is not distinguishing between *B. aclada* and *B. allii* using genetic species analysis. Based on the RFLP differentiation performed in this study, the *B. aclada* species CBS 103.23 can be classified as *B. allii*. For further research, a new *B. aclada* species (CBS 260.71) was purchased from CBS- KNAW (Utrecht, Netherlands). This genus was genetically classified by Nielsen et al. (2002) as *B. aclada* within his studies. Subsequent RFLP analysis performed using the culture CBS 260.71 is presented in Figure 4.9 and identified the new culture as *B. aclada*.

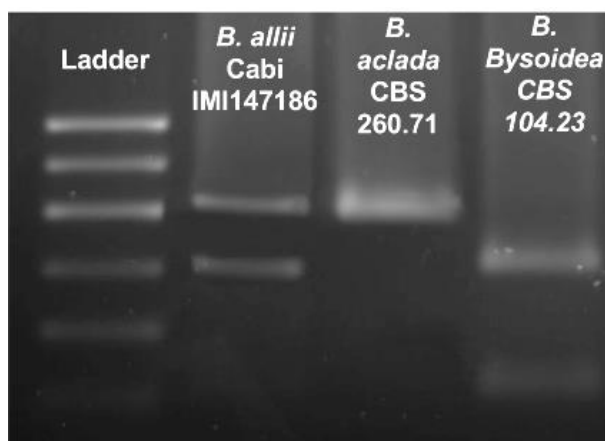


Figure 4.9: PCR-RFLP analysis using the protocol described by Nielsen et al. (2002) of the *Botrytis* species *B. allii* (Cabi, IMI 147186), *B. aclada* (CBS 260.71) and *B. byssoidea* (CBS, 104.23).

4.4.4 DEVELOPMENT OF A DNA SENSOR USING SPGE

4.4.4.1 Hybridisation Studies Using Biotin-Labelled Complementary Sequences

The hybridisation efficiency of biotin-labelled primers was tested using a complementary probe which was designed to form a sandwich assay with the single stranded target DNA. This format was chosen for initial prove of concept studies, as only synthesised, purified DNA can be applied in defined concentrations, whereas the quantification of PCR amplified *Botrytis* DNA is relative and high in error.

The assay procedure is illustrated in Figure 4.10. Streptavidin was immobilised on the working electrode to bind the biotin attached to the capture probe. Afterwards, the hybridisation occurred with various concentrations of the target probe. To complete the sandwich assay, a biotin-labelled detection probe was added to the working electrode, which hybridises specifically with the other end of the target probe. For signal generation, streptavidin-HRP was added to bind the free biotin of the detection probe and generate the signal using the TMB-H₂O₂ substrate-system.

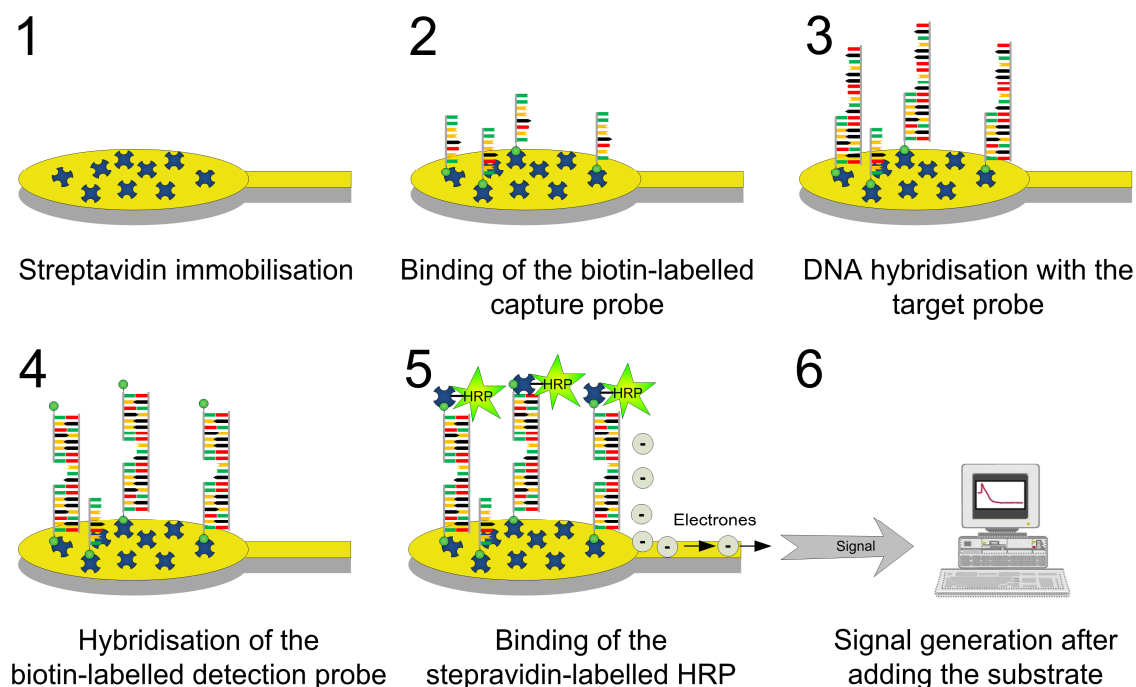


Figure 4.10: Schematics of the assay procedure used for the hybridisation studies.

Figure 4.11 shows the measured stepamperometric signal generated by the streptavidin-labelled HRP. A strong correlation between the various concentrations of target probe and sensor signal was found ($R^2 = 0.998$) in this study. These findings indicate a successful bond between biotin-labelled capture probes and target sequence, as well as between streptavidin and biotin used to immobilise the capture probe and signal generation. The LOD for this format was calculated at $0.49 \mu\text{M}$ target probe.

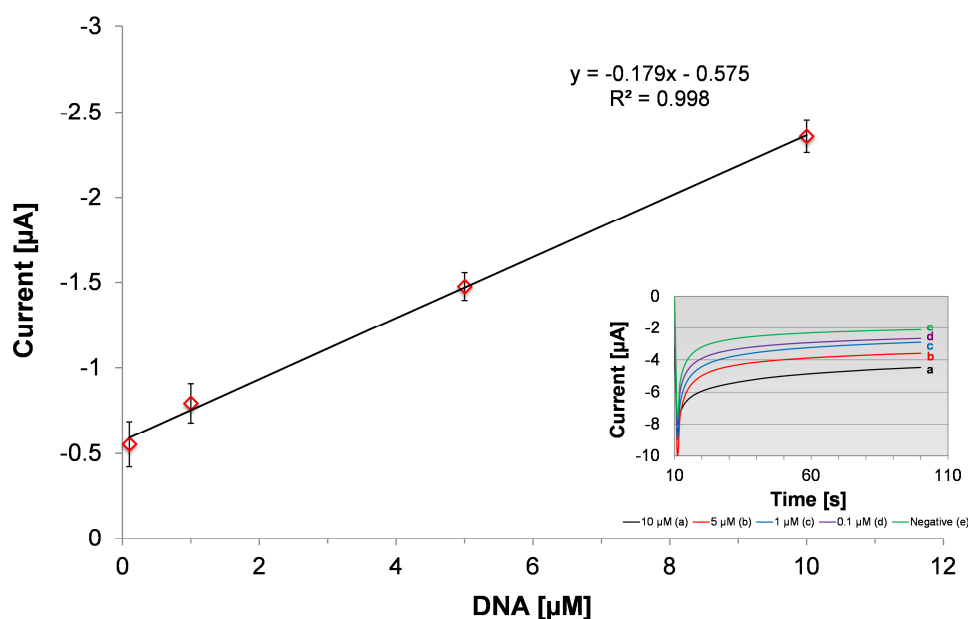


Figure 4.11: Electrochemical response after 100 s of the DNA sensor against hybridised target probe. Streptavidin ($100 \mu\text{g ml}^{-1}$) was immobilised on the working electrode, followed by $20 \mu\text{M}$ 3'-biotin-labelled capture probe. The target probe was applied in various concentrations (10, 5, 1, 0.1 and $0 \mu\text{M}$) and bound to the capture DNA, followed by 5'-biotin-labelled detection probe ($20 \mu\text{M}$). For signal generation, streptavidin-labelled HRP ($100 \mu\text{g ml}^{-1}$) was added in order to bind to the 5'-biotin. The embedded graph shows the averaged electrochemical sensor signal of each probe concentration, the bars represent the standard error of the means ($n=4$).

4.4.4.2 Estimation of the DNA Concentration after PCR Amplification

The protein kinetics of a PCR reaction can be approximated with the all-or-nothing behaviour of enzymes, if there are no inhibiting compounds present (Aoyagi, 2002). After passing the cycle threshold which is proportional to the amount of template DNA, the exponential amplification is completed after a few cycles eventually reaching

saturation after consuming the resources of the reaction (Blair and Zajdel, 1992; Heid et al., 1996a; Liu and Saint, 2002). In contrast to the immunosensor designed to distinguish between the pathogen loads, the DNA sensor only shows a positive / negative response of the PCR reaction.

The primers described by Nielsen et al. (2002) were chosen to estimate the DNA load after PCR amplification for two reasons. First, being designed for RFLP analysis, the primers amplify several *Botrytis* species offering a wider approach. Second, comparative studies of the two primers performed in Section 4.4.2 showed a lower performance, resulting in a fluorescent signal reduction by approximately 40 %, indicating a smaller amount of amplified DNA. Furthermore, the amplified sequence is approximately 500 bp, which is longer than the neck-rot primers by 400 bp. The increased distance between enzyme and sensor surface can inhibit the electron transfer and reduce the signal strength (Kerman et al., 2009).

After the real-time PCR, the amplified product was quantified using the Pico Drop spectrophotometer (Table 4.5). First, the absolute amount of the negative control after PCR amplification was measured. The resulting value of $426.3 \pm 8.94 \text{ ng } \mu\text{l}^{-1}$ was then used as a baseline for the following measurements of the amplified product. The normalised relative concentration of the amplified DNA was found to be between 138.4 and $122.5 \text{ ng } \mu\text{l}^{-1}$, indicating that the DNA concentration is relatively independent from the amount of template DNA used. In absolute terms, the difference between the highest (100 ng) and lowest (1 pg) concentration of DNA after PCR is less than 3 % after PCR amplification. Focussing on different DNA concentrations before amplification is not the major objective of the DNA biosensor development, as the PCR product is independent from the amount of template DNA added. However, a certain security level regarding the sensitivity has to be considered for the sensor development. If, for example, the PCR is interrupted before the amplification is completed, the quantity of amplified product will be significantly lower, as the steady state is not reached and the amplification is an exponential process. On the other hand, if the threshold for the biosensor is too high, it might give false positive results caused by contaminations or non-specific amplification of the negative control.

Table 4.5: Relative and absolute dsDNA quantified after 50 cycles of real-time PCR amplification using various amounts of extracted *B. allii* template DNA using the Pico Drop spectrophotometer (n=3).

<i>B. allii</i> DNA before PCR [ng]	dsDNA after PCR [ng μl^{-1}]	Baseline	Average A_{260}/A_{280}
Negative ^b	426.3 $\pm$ 8.94	AE buffer ^a	1.89
10 ²	138.4 $\pm$ 3.1	Negative	1.68
10	138.2 $\pm$ 1.15	Negative	1.47
1	138.0 $\pm$ 3.28	Negative	1.47
0.1	132.6 $\pm$ 5.02	Negative	1.50
10 ⁻²	122.5 $\pm$ 3.43	Negative	1.63
10 ⁻³	123.8 $\pm$ 4.81	Negative	1.58

^a = Elution and storage buffer used for the DNA extraction (10 mM tris-Cl, 0.5 mM EDTA, pH 9.0).

^b = Negative control after PCR amplification.

4.4.4.3 Evaluation of Different Hybridisation Strategies for the Two-Step DNA Detection Using the DNA Biosensor

Hybridisation between a capture or detection probe and the amplified target DNA fragment can only occur between single stranded DNA. However, the amplified PCR product is double-stranded DNA. Several strategies can be found in the literature to influence hybridisation. DNA hybridisation performed by varying the salt concentration in the solution is one approach (Metfies et al., 2005). Here, the salt concentration is adjusted in time-consuming trial and error experiments to reach hybridisation at a specific temperature. However, in some conditions, this can lead to an increased non-specific adsorption on the surface and mismatched pairing, as high salt concentration enhances hybridisation (Lucarelli et al., 2004; Tawa and Knoll, 2004). Furthermore, this method is highly affected by several factors, such as quantity, purity and sequence length of the DNA (Kerman et al., 2009) and is therefore not suitable for the detection of PCR-amplified products in field conditions. Alternatively, the second

DNA strand can be removed by labelling one primer with 5'-phosphate, allowing a λ exonuclease to digest one strand (Laureyn et al., 2007). However, this method is time consuming, since it requires enzyme incubation.

Two methods to label the PCR product were tested in this section. One is based on the quick transfer of denaturated DNA from boiling water to ice (Xu et al., 2001). Through the immediate reduction of thermal energy, the product remains separated as ssDNA. The other strategy is to perform a subsequent denaturation and annealing step after adding biotin-labelled probes in high concentrations. Here, the strategy is that the probes anneal first to the complementary strand, as they are shorter and have a higher affinity. The over ten-fold higher concentration of the labelled probes further increases the probability of a successful hybridisation.

The electrochemical response generated by the streptavidin-labelled HRP is presented in Figure 4.12. Both methods resulted in a higher signal of the *B. allii* DNA positive control compared to the negative. The difference in signal between a positive and negative amplification of *B. allii* was calculated at 0.46 μ A for the hot water denaturation and 3.32 μ A for the final annealing step of biotin-labelled probe primers.

However, heating and freezing dsDNA was found to be an ineffective method to produce ssDNA in order to perform a sandwich assay. A possible reason for the lack of performance and signal separation between positive and negative hybridisation is the additional incubation step needed for the annealing of the second probe. Though this method might freeze the DNA strands in a separate form by removing thermal energy, a subsequent hybridisation of the separated DNA strands is feasible after the temperature increases. Consequently, the detection probe cannot hybridise to the target sequence to complete the sandwich format. An improvement of this method might be the cooling of the DNA in liquid nitrogen (Nelson et al., 1982). However, even with this strategy, the re-hybridisation of the DNA with increasing temperature cannot be prevented.

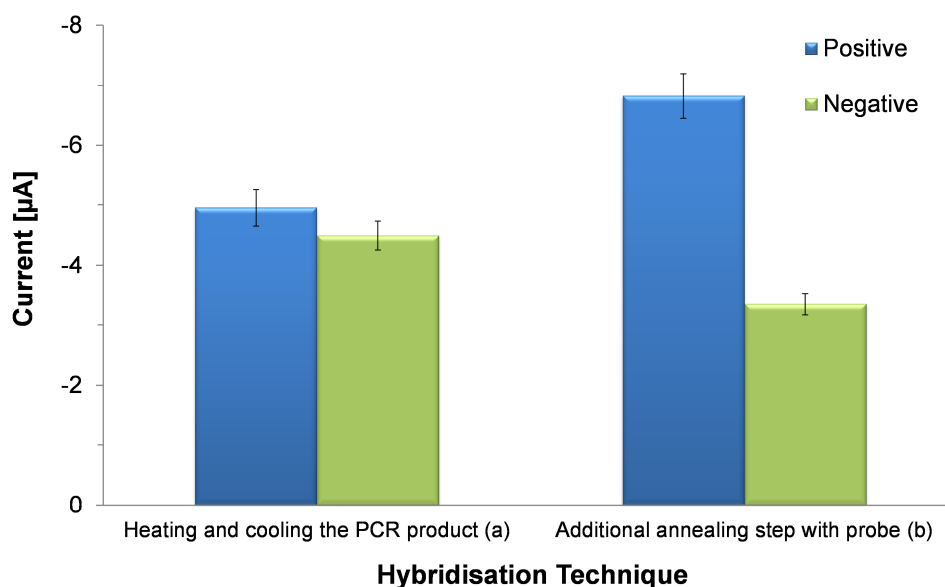


Figure 4.12: Electrochemical response of two hybridisation techniques used for the DNA sensor. (a) To separate the dsDNA, the PCR product was boiled for 5 min and transferred to ice water for 1 min. The ssDNA was immediately applied to the sensor allowing the immobilised capture probe (10 μM) to hybridise with the amplified product. After washing, the sandwich was accomplished by adding a biotin-labelled detection probe. (b) Average response achieved by adding the biotin-labelled probes to the PCR product and performing an additional denaturation (10 min, 95 $^{\circ}\text{C}$) and annealing (5 min, 65 $^{\circ}\text{C}$) step. The signal was generated by 100 $\mu\text{g ml}^{-1}$ streptavidin-HRP conjugate. The bars represent standard error of the means ($n=4$).

Better performance was achieved by performing an additional denaturation and annealing step. Having biotin labels on both sides of the DNA, the immobilisation of the DNA to the streptavidin coated to the sensor surface is improved. On the other hand, this method also increases the risk of DNA binding on both sides to the streptavidin, thus inhibiting the biotin-streptavidin coupling of the HRP molecule. However, though some DNA strands might show double binding, a decreased signal was not observed. The distinctive three-dimensional structure of the working electrode with round various sized ink particles (see Section 3.4.2.1) might help to reduce this effect, compared to a flat, polished surface area of other biosensor applications.

4.4.4.4 DNA Sensor Formats and Primer Designs

Immobilising the DNA on the sensor surface is a crucial step for the DNA sensor development, as the primers and their design provide a vital linkage between the sensor surface and the detection molecule. Biotin-avidin systems were reported to provide an effective method to create a strong bond between molecules, being the strongest reported non-covalent bond, with an unbinding force of up to 250 pN (Moy et al., 1994). They thereby facilitate a strong ligand immobilisation of DNA to biosensors (Kambhampati and Knoll, 2002; Dupont-Filliard et al., 2004; Chung et al., 2011). Previous studies performed in Section 4.4.4.3, demonstrated that a biotin-avidin system showed an encouraging performance in detecting pathogenic DNA. However, at this stage an additional annealing step would be required after adding the biotin-labelled primers to the DNA probe. Though this step is relatively short, from a manufacturer point of view, a different approach is preferable. Advances in recombinant protein design enabled the inexpensive production of heat-stable biotin, which can be tagged to DNA sequences and used directly in PCR, without the risk of protein denaturation (Kricka, 1995; Millar et al., 1995; Laureyn et al., 2007). Biotin can be ordered cost effectively as a label attached to oligonucleotides, alleviating the need of time consuming and expensive conjugation procedures.

Two primer designs were evaluated in this study and tested with the neck-rot primers CRT1f/2r. A schematic picture of the two primer designs is presented in Figure 4.13. One system employed two biotin labels, one on each primer, the second approach utilised a 15-atom mixed polarity triethylene glycol (TEG) spacer at the 5'-end of each primer sequence (Figure 4.13 C). Though TEG spacers are approximately ten times more expensive than a normal biotin label, employing this spacer molecule has been reported to enhance assay performance by facilitating the orientation and accessibility of the biomolecule (Canaria et al., 2005; Yam et al., 2006; Hermanson, 2013).

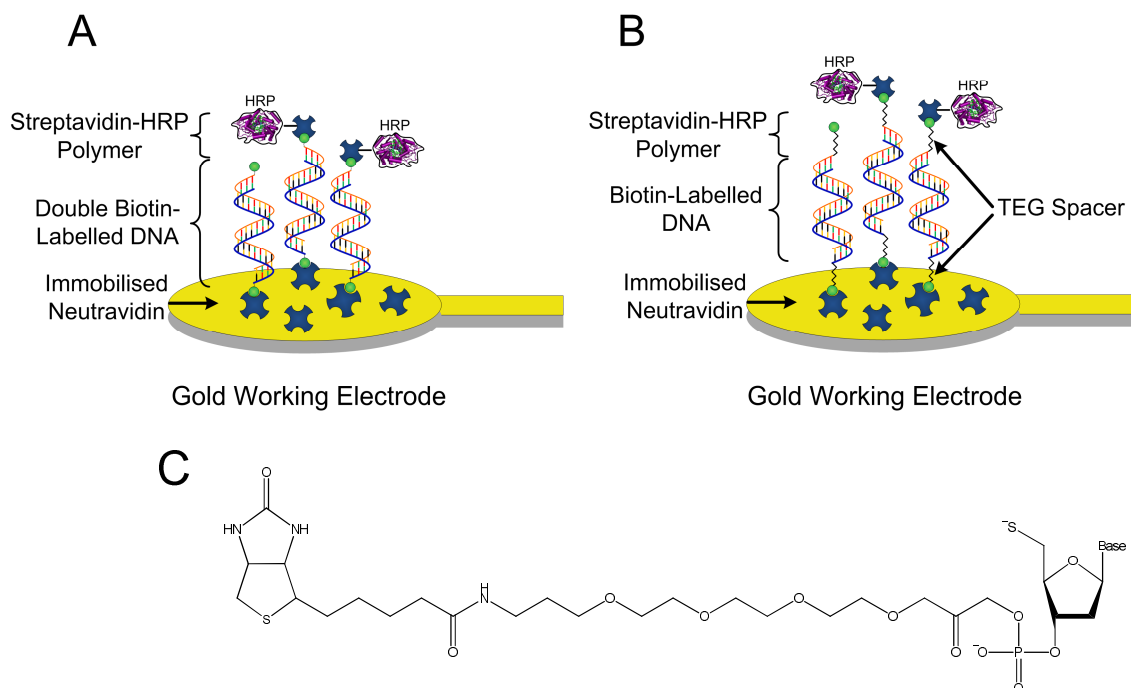


Figure 4.13: Schematic illustration of two different primer designs tested for the DNA sensor. Neutravidin was used as a capture molecule on the sensor surface to bind the pathogen-derived DNA labelled with (A) biotin and (B) biotin with a triethylene glycol (TEG) spacer. (C) Chemical formulation of a biotin TEG labelled DNA base.

A comparison of the electrochemical response of the two primers is illustrated in Figure 4.14. Surprisingly, the TEG spacer did not improve the assay performance. Hence, the separation between positive and negative samples, representing the major performance indicator for the DNA sensor, is with $1.2\ \mu\text{A}$ approximately $3\ \mu\text{A}$ lower compared to the DNA sensor employing biotin-labelled primers without the TEG spacer. On the other hand, the biotin-labelled primers without the TEG-spacer worked well and showed good signal separation between positive and negative samples, comparable with the one achieved in Section 4.4.4.3. However, employing biotin labelled primers eliminates the additional annealing step and was therefore chosen for further optimisation.

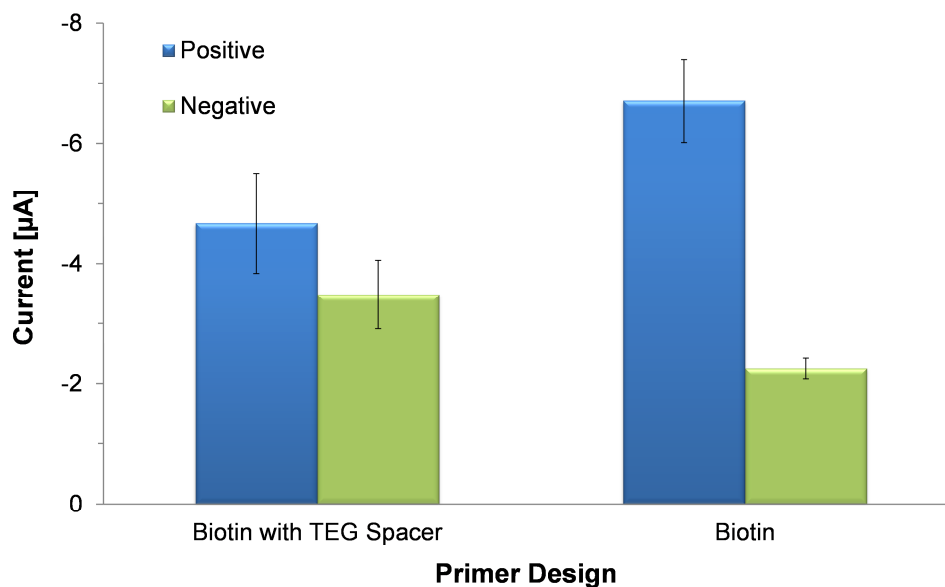


Figure 4.14: Normalised electrochemical response of two primer designs for the DNA sensor. *B. allii* DNA was amplified employing biotin-labelled primers, with and without a 15 atom triethylene glycol (TEG) spacer between the biotin and the DNA strand. For signal generation $50 \mu\text{g ml}^{-1}$ streptavidin-HRP conjugate was applied to the sensor. The bars represent the standard error of the means ($n=4$), average CV=6.9 %.

Although employing a spacer to facilitate DNA immobilisation is a well-accepted strategy, the effect of introducing a spacer molecule to increase the distance between the specific DNA sequence and the surface has rarely been assessed (Peeters et al., 2008). The underlying rationale of employing a spacer is that the further an immobilised molecule is away from the surface, the closer it is to the solution state, and the more likely it is to react freely with dissolved molecules (Wong et al., 2005; Halperin et al., 2006; Ricci et al., 2007). However, employing a TEG spacer may not be beneficial for the DNA sensor system developed in this study. Figure 4.15 shows potential sources that can inhibit the successful formation of a sandwich format needed for signal generation. TEG spacer may increase non-specific adsorption of proteins contained in the master mix that may hinder the alignment of the DNA, or adsorption may be facilitated as a result of decreased stiffness or double binding of the biotin label. Furthermore, these systems are predominantly used in applications that utilise a polished flat surface which is not given by the SPGE.

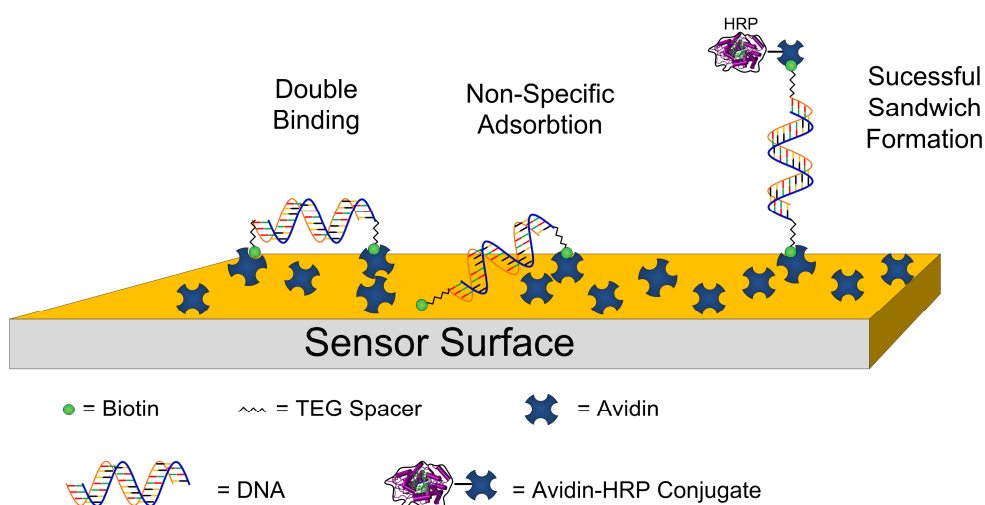


Figure 4.15: Schematics showing possible sources of decreased sensor performance caused by double binding of the biotin-TEG spacer and non-specific adsorption of the DNA compared to the completed sandwich format.

4.4.5 OPTIMISATION OF THE DNA SENSOR RESPONSE

The overall objective of the DNA sensor is to detect pathogen derived DNA in field conditions, alleviating the need to perform a real-time PCR or gel electrophoresis. Considering the fundamental principle of PCR, amplifying the DNA until saturation is reached, the concentration of amplified DNA after PCR is independent from the amount of starting material as long as the amplification is within the saturated state (Section 4.3.4.3). A quantification of the template DNA is therefore not feasible, but a sensitive and specific positive or negative result is given. In this light, the DNA sensor aims at reliably distinguishing between positive samples where the target pathogen is present, and negative samples with no pathogen or for example other pathogens which are not targeted by the utilised primers such as bacterial rot or other fungi. Consequently, the best DNA sensor performance is warranted for highest possible signal separation between positive and negative samples as well as low intra-assay variation, minimising the risk of a false positive or negative signal response.

Additionally to the normal statistical values like average, coefficient of variation and standard deviation, the data were tested for equal ($F > 0.05$) and unequal variance ($F < 0.05$) using an F -test, and subsequently verified if the groups are significantly different at a confidence level of 95 % employing a T -test.

In this section, various parameters were tested and optimised in order to boost the performance of the DNA immunoassay. To achieve the best biosensor response, the streptavidin-HRP conjugate concentration, the neutravidin load on the sensor surface and various immobilisation strategies employing covalent immobilisation as well as adsorption were investigated and optimised. Only if the parameter combinations tested in this section provide a considerable signal separation, as well as a P -value which is less than 5 % (T -test), a successful assay is warranted.

4.4.5.1 Streptavidin-HRP Conjugate Concentration

To complete a sandwich format for the DNA sensor, streptavidin-HRP needs to bind to the free biotin label on the 5'-end of the DNA strand to induce an electrochemical signal after adding the substrate. Streptavidin-HRP concentrations ranging from 10 to 100 $\mu\text{g ml}^{-1}$ were assayed with biotin-labelled DNA to evaluate best signal performance. Figure 4.16 visualises DNA sensor signal of the positive and negative samples against streptavidin-HRP conjugate concentrations ranging from 10 to 100 $\mu\text{g ml}^{-1}$. As expected, the more conjugate applied, the higher the current. However, the signal separation between positive and negative samples peaked at a streptavidin-HRP concentration of 50 $\mu\text{g ml}^{-1}$. The signal separation between positive and negative samples achieved using a streptavidin-HRP concentration between 10 to 70 $\mu\text{g ml}^{-1}$ were considered as significantly different at a confidence level of 95 %. Only the concentration of 100 $\mu\text{g ml}^{-1}$ failed the T -test with $P = 6.8$ % suggesting that positive and negative samples cannot be separated reliably.

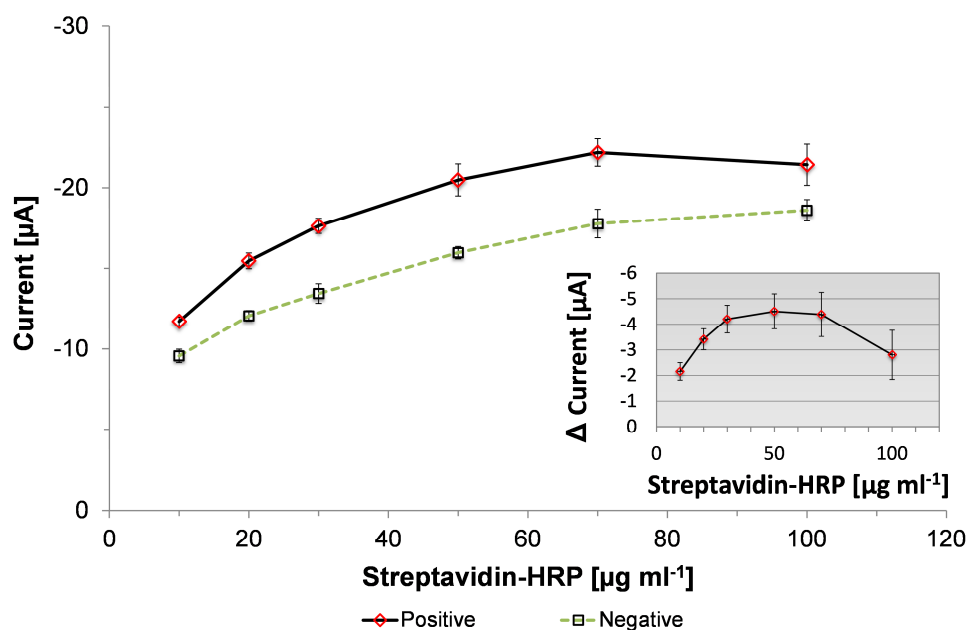


Figure 4.16: Sensor current against various concentrations (10 to $100 \mu\text{g ml}^{-1}$) of the streptavidin-HRP conjugate. Positive and negative samples were amplified for 25 cycles and captured by immobilised neutravidin. Streptavidin-HRP conjugate was then applied for signal generation. Embedded graph shows the signal separation between the positive and negative sample, bars represent the standard error of the means ($n=4$).

4.4.5.2 Direct Detection of Immobilised Neutravidin Using Biotin-HRP Conjugate

A successful immobilisation of the DNA capture molecule to the sensor surface is crucial, as all following steps depend on an optimum amount of neutravidin bound to the sensor surface. To evaluate the load of immobilised neutravidin on the sensor surface a detection system was developed and optimised utilising biotin-HRP conjugate. The biotin-labelled HRP is capable of binding directly to the neutravidin on the sensor surface, which enables this system to generate a signal that is proportional to the neutravidin load.

The study was initiated by evaluating a suitable biotin-HRP conjugate concentration which maximises the signal resolution of the immobilised neutravidin in the required range. The results obtained of this experiment are presented in Figure 4.17. Using $1 \mu\text{g ml}^{-1}$ of the biotin-HRP conjugate resulted in the highest signal separation between the two neutravidin concentrations 50 and $500 \mu\text{g ml}^{-1}$ after normalisation.

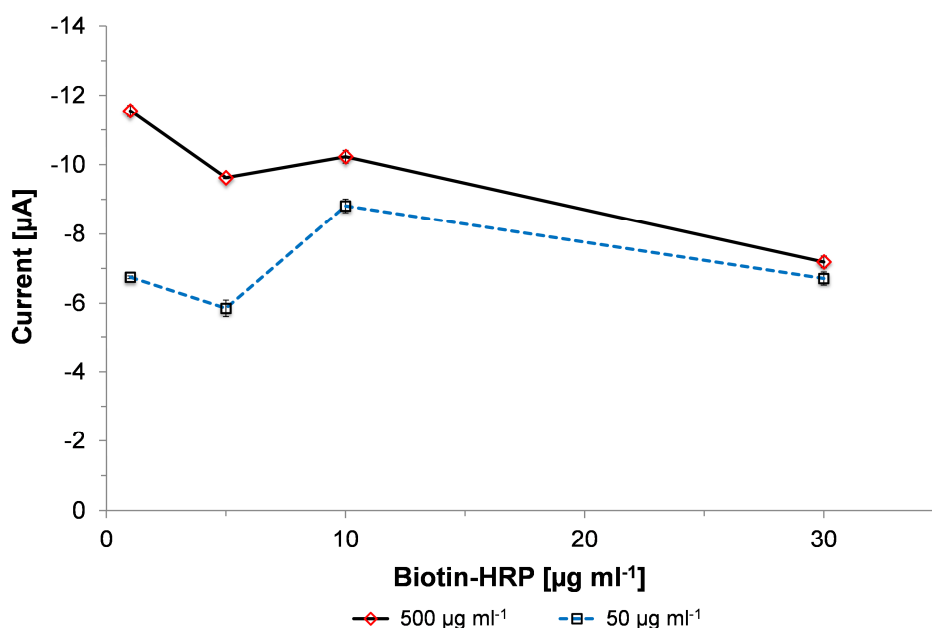


Figure 4.17: Normalised electrochemical signal after 70 s of biotin-HRP conjugate (1 to 30 $\mu\text{g ml}^{-1}$) tested with SPGE coated with 500 and 50 $\mu\text{g ml}^{-1}$ neutravidin. Protein immobilisation was performed by incubating neutravidin for 2 h at 37 °C. After blocking, the biotin-HRP conjugate was incubated on the sensor surface for 1 h. The bars represent the standard error of the means (n=3).

4.4.5.3 Neutravidin Immobilisation Employing Adsorption and Covalent Immobilisation Techniques

The direct detection system of immobilised neutravidin which was designed in the previous Section was used to assess the neutravidin load achieved employing passive adsorption and covalent immobilisation to 3-DTDPA self-assembled monolayer. The results of this experiment are presented in Figure 4.18. Previous studies performed in Section 3.4.7.2 indicate an increased surface load of antibodies using covalent immobilisation. This finding was replicated for neutravidin being the primary capture molecule immobilised on the working electrode, indicated by the higher signal compared to the adsorbed group. Another trend observed for covalent immobilisation was that the maximum surface load is reached with lower concentrations of neutravidin, peaking at a concentration of 30 $\mu\text{g ml}^{-1}$. In contrast, the adsorbed group reached a maximum surface load at neutravidin concentrations over 300 $\mu\text{g ml}^{-1}$.

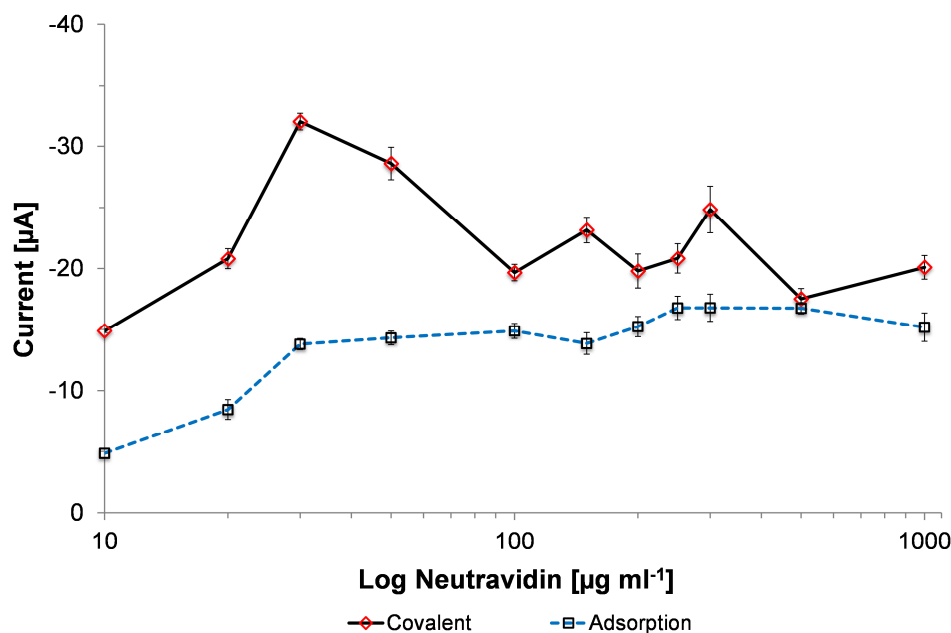


Figure 4.18: Normalised signal after 70 s of neutravidin concentration ranging from 10 to 1000 $\mu\text{g ml}^{-1}$ using adsorption and covalent coupling to 3-DTDP monolayer (50 mM). Neutravidin was incubated for 2.5 h at 37 °C followed by 1 $\mu\text{g ml}^{-1}$ biotin-HRP conjugate for signal generation. The bars represent the standard error of the means (n=4), average CV=9.1 % (covalent) and 10.6 % (adsorption).

Though the surface load employing covalent immobilisation appears to be more fluctuating compared to the adsorbed group, this technique shows an increased signal by 60 %. The peak at 30 $\mu\text{g ml}^{-1}$ may be explained by the unique structure of avidin. In contrast to antibodies where the orientation of the molecule is crucial for successful binding, avidin has four biotin binding sites evenly distributed over the molecule. The decreased surface load may facilitate the accessibility of the lateral binding sites and increase the amount of bound biotin (Hermanson, 2013).

4.4.5.4 Evaluation of Covalent Immobilisation Strategies of Neutravidin versus Adsorption

Covalent immobilisation was reported to decrease the protein loss per wash (Leckband and Langer, 1991; Williams and Blanch, 1994), improves the stability (Wortberg et al., 1997), reduces non-specific binding of other proteins (Ekins et al., 1990) and increases the surface load (Furuya et al., 2006). An enhanced surface load and wash resistance was confirmed in Chapter 3 resulting in a more sensitive immunosensor assay. Covalent

coupling to 3-DTDPA polymer layer was therefore tested as a potential immobilisation strategy for the DNA sensor assay and compared to the adsorbed control. Previous findings in Section 4.4.5.3 confirmed the increased averaged neutravidin load on the sensor surface even at neutravidin concentrations as low as $30 \mu\text{g ml}^{-1}$.

Covalent coupling as well as passive adsorption were tested in a DNA sensor assay with neutravidin concentrations of 30 and $300 \mu\text{g ml}^{-1}$. Figure 4.19 summarises the maximum signal separation between positive and negative samples achieved with both immobilisation strategies. The trend observed in Section 4.4.5.3 that covalent coupling requires lower concentrations of neutravidin was confirmed in this experiment. At a neutravidin concentration of $30 \mu\text{g ml}^{-1}$ the covalently coupled group showed a 81 % higher signal compared with adsorption. However, at a neutravidin concentration of $300 \mu\text{g ml}^{-1}$, adsorption showed with approximately $6.4 \mu\text{A}$ a 25 % higher signal. Focussing on the intra assay performance, covalent coupling provided more reproducible assay conditions, resulting in a lower coefficient of variation by 0.6 %.

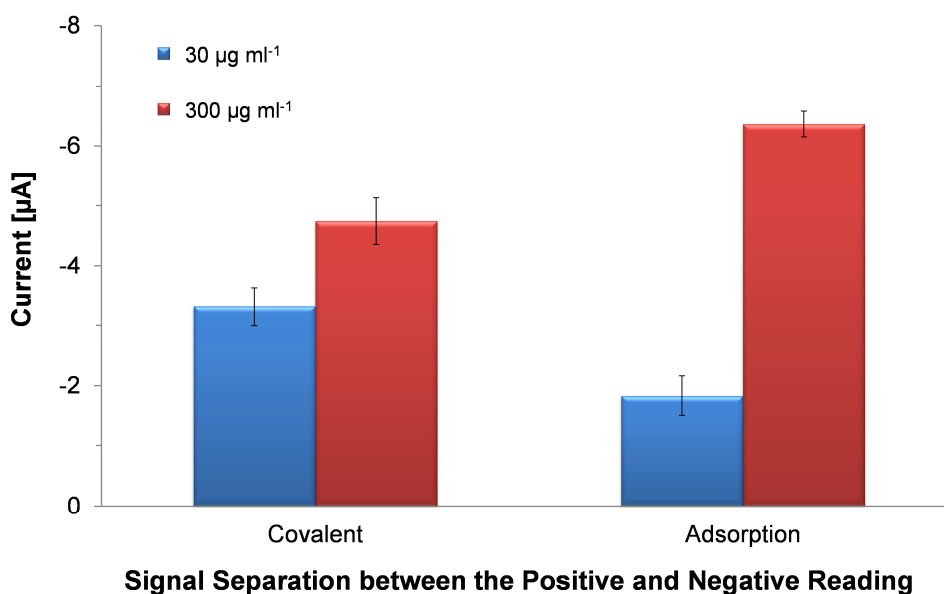


Figure 4.19: Signal separation between positive and negative samples using adsorption and covalent coupling to 3-DTDPA monolayer (50 mM). The two immobilisation strategies were tested using neutravidin concentrations of 30 and $300 \mu\text{g ml}^{-1}$ followed by $1 \mu\text{g ml}^{-1}$ streptavidin-HRP conjugate for signal generation. The bars represent the standard error of the means (n=4), average CV=7.6% for covalent immobilisation and 8.2 % for the adsorbed group.

Though covalent coupling is an established technique for surface immobilisation of avidin (Wink et al., 1997; Cosnier, 2000; Goddard and Hotchkiss, 2007), its predominant field is antibody immobilisation of immunosensors. Padeste et al. (2000) characterised covalent immobilisation of avidin conjugate on gold electrodes. Though the covalent immobilisation to the 3,3-dithiodipropionic acid bis-(*N*-hydroxysuccinimide) layer was successful, the adsorbed control provided a higher electrochemical signal. The signal enhancing effect of the increased surface load was followed by a signal enhancing of the negative control, reducing the net signal to a value which is lower compared to the adsorbed control. Another potential factor may be that the orientation of avidin with its four binding sites is not as critical in comparison to antibodies.

However, both immobilisation strategies showed good performance and reliably distinguished between positive and negative samples. The decision if covalent immobilisation is beneficial for the DNA sensor has to be assessed using a multilevel approach. If the development prioritised costs and signal performance, adsorption offers more benefits. On the other hand, if increased surface load at low concentrations of neutravidin and reproducibility is the major target, covalent immobilisation strategies are more suitable.

4.4.6 DEVELOPMENT OF A DNA SENSOR BASED ON A PREMIX FORMAT WITH THIOL-C6 PRIMERS

The optimised DNA sensor utilising neutravidin as capture molecule proved to be a reliable method to detect pathogen-derived DNA after PCR amplification. However, the two 5'-biotin labels require an avidin immobilisation step on the working electrode. To compensate this flaw, a novel premix format was developed and optimised in this Section utilising thiolated primers. In contrast to the previous format, which employs a biotin label on both sides of the DNA (Figure 4.20 A), this system is based on a thiol-C6 labelled forward primer CRT1f, whereas the reverse primer utilises a biotin molecule that can be bound by the streptavidin-HRP conjugate (Figure 4.20 B and C).

Thiol groups have been successfully used for surface immobilisation since they have the ability to form an immediate sulphur-gold bond with the biosensor surface (Prime and Whitesides, 1993; Herne and Tarlov, 1997). As a result, this primer design was chosen and tested as a potential application in the DNA sensor.

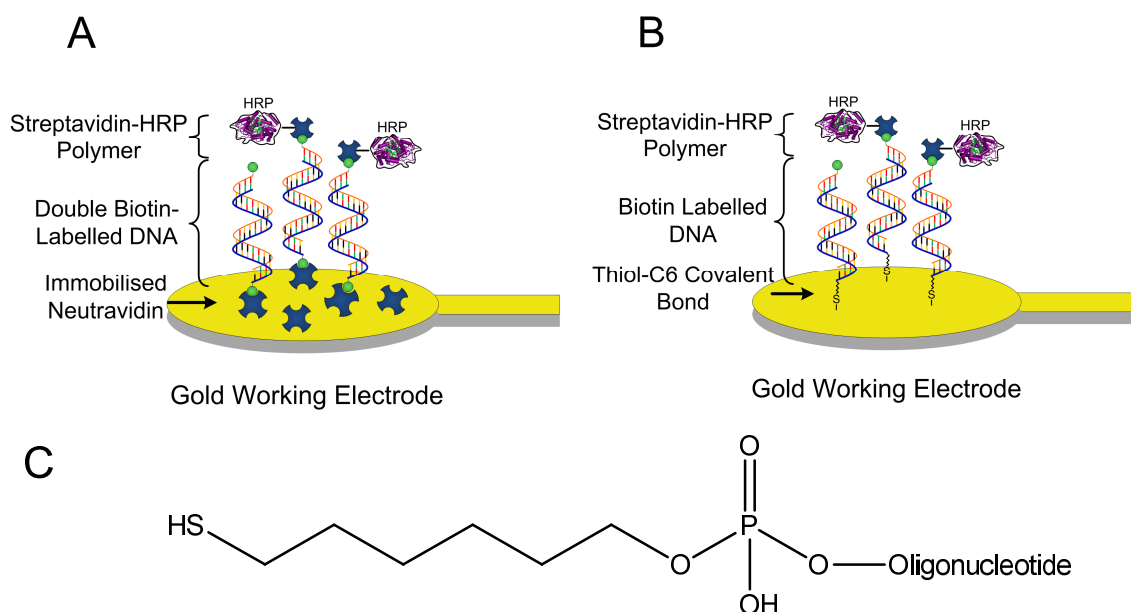


Figure 4.20: Comparison of the classic DNA sensor format employing two 5'-biotin labels (A), to the premix format with thiol-C6 primers. The sulphur group of the thiol C6 primer (C) enables a direct covalent bond to the gold working electrode without the need of prior protein immobilisation on the surface.

The interest in portable, simple to use and highly efficient DNA detection devices has increased drastically, opening various fields of application for DNA sensor developments (Tothill, 2001; Pänke et al., 2008). From a manufacturer's point of view this system has various advantages. While the classic assay operates on a step-by-step application, it is more desirable to have a premixed complex as this may save assay time and washing steps, hence, provide an improved handling. In particular, after the PCR amplification process with biotin and thiol-C6 labelled primers, streptavidin-HRP conjugate is added directly to the PCR product to bind the biotin label. Next, the DNA can be added directly to the gold working electrode of the biosensor without the need of prior surface modification. Figure 4.21 illustrates the steps involved in a DNA testing procedure based on a premix format with thiol-C6 primers.

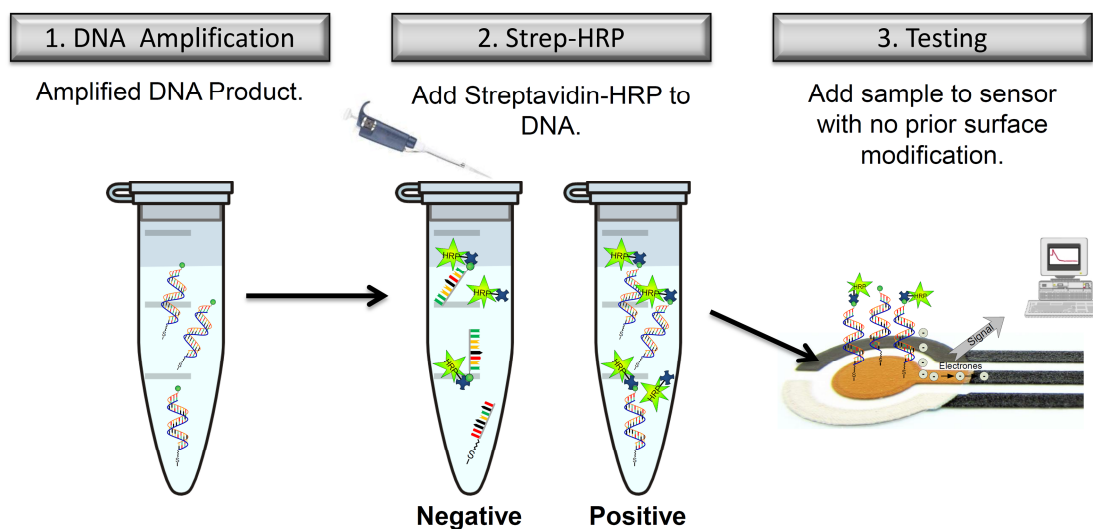


Figure 4.21: Flow diagram for the sample testing procedure using DNA biosensor based on the premix format. (1) The sample DNA is amplified using PCR in combination with thiol-C6 and biotin-labelled primers. (2) The second step involves adding the streptavidin-HRP conjugate to the DNA to bind to the biotin label. A positive sample is amplified with double stranded DNA comprising both primers: biotin on one side and thiol-C6 on the other, whereas a negative sample is not amplified and includes loose primers and DNA. (3) For testing the DNA-conjugate mix is added to the sensor and the thiol-C6 group forms a covalent bond to the sensor immobilising the DNA. After adding the substrate a signal is generated by the HRP conjugate.

4.4.6.1 Streptavidin-HRP Conjugate Concentration

Unlike the previous format which employs two biotin labels, the format with one thiol-C6 primer allows a premixing of the DNA and conjugate. During the incubation, the streptavidin-HRP binds to the free biotin label on the 5'-end of the primer CRT2f. Challenging for this assay format is to find a balance between signal strength and increased non-specific binding, which represents a major issue since the working electrode is not modified or blocked.

Streptavidin-HRP concentrations ranging from 0.1 to 100 $\mu\text{g ml}^{-1}$ were assayed with biotin-labelled DNA to evaluate best signal performance. Figure 4.22 visualises DNA sensor signal of the positive and negative samples against streptavidin-HRP conjugate concentrations. As previously discussed, in contrast to the streptavidin-HRP optimisation of the double biotin format performed in Section 4.4.5.1, the slope of the

chart is significantly steeper, as a result of non-specific binding to the unmodified sensor surface. The signal separation between positive and negative samples peaked at a streptavidin-HRP concentration of $20 \mu\text{g ml}^{-1}$ (Figure 4.22 embedded graph). From a statistical perspective, the signal separation between positive and negative samples using a streptavidin-HRP concentration between 1 to $70 \mu\text{g ml}^{-1}$ was considered as significantly different at a confidence level of 95 %, whereas 0.1 and $100 \mu\text{g ml}^{-1}$ failed the *T*-test with a *P* value of 38 % and 17 %, respectively.

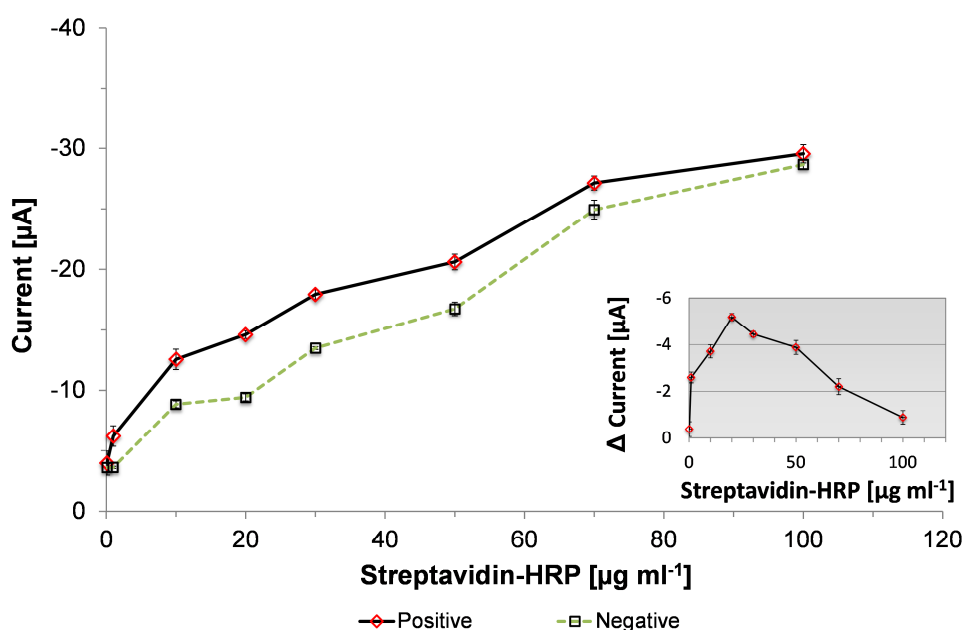


Figure 4.22: Sensor signal current against various concentrations (0.1 to $100 \mu\text{g ml}^{-1}$) of the streptavidin-HRP conjugate. Positive and negative samples were amplified for 25 cycles with thiol-C6 and biotinylated primers before adding the streptavidin-HRP conjugate directly to the DNA to bind the biotin located on the primer and incubated for 2.5 h. The master mix was then dropped on the sensor and incubated for 1 h. The embedded graph shows the signal separation between the positive and negative sample, the bars represent the standard error of the means ($n=4$), average CV=10.4 %.

4.4.6.2 Premix Time of PCR Amplified DNA with the Streptavidin-HRP Conjugate

For the premix format the incubation time is a vital parameter since a successful bond between the streptavidin-HRP conjugate and the biotin-labelled primer is the first step required for a sound assay. Unbound HRP is removed during the washing step and cannot contribute to the biosensor signal. The binding curve of the conjugate is presented in Figure 4.23. A gradual increase of the positive signal is followed by a decrease of the negative control. Over time, the free streptavidin-HRP which would contribute to non-specific binding is coupled to the biotin and can add to the electrochemical signal. After 2 h saturation is reached and no further signal improvement can be achieved. Surprisingly, even 5 min yielded with 3.4 μA enough signal separation to reliably detect positive samples. However, best signal separation was achieved with a premix time of 2 h. All incubation times were considered as significantly different with a maximum P value of 0.2 %.

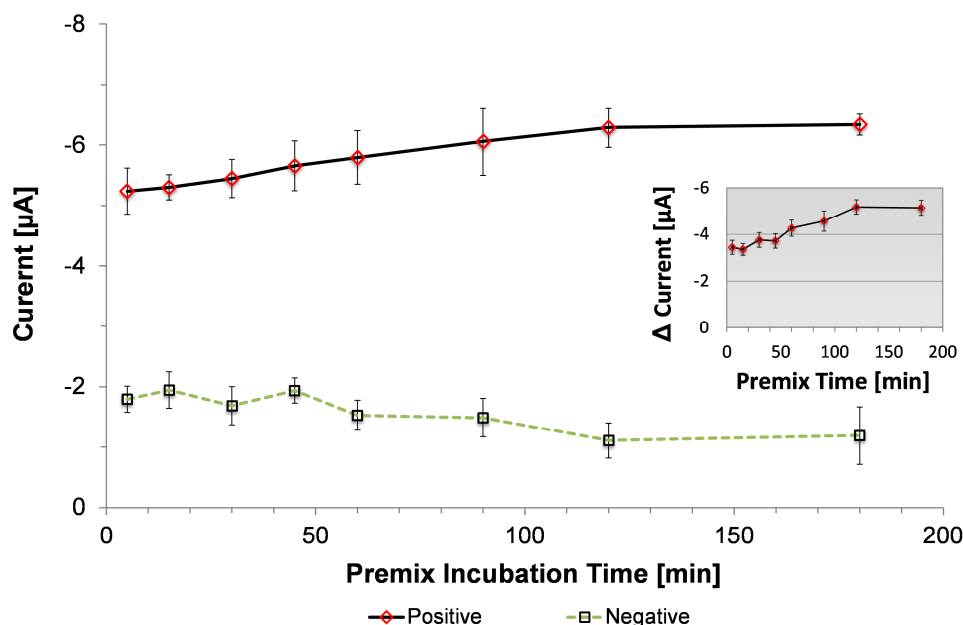


Figure 4.23: Comparison of the normalised sensor signal of various premix times (5 min to 2.5 h) of the streptavidin-HRP conjugate with the thiol-C6 and biotinylated DNA. The master mix of the positive (*B. allii*) and negative samples was then dropped on the sensor and incubated for 1 h. The embedded graph shows the signal separation between the positive and negative sample plotted against the premix incubation time, the bars represent the standard error of the means ($n=4$), average CV=7.1 %.

4.4.6.3 DNA Incubation Time

The DNA incubation time on the sensor surface was investigated in a range between 5 min and 2 h. Thiol groups have been successfully used for surface immobilisation since they have the ability to form an immediate sulphur-gold bond with the biosensor surface (Prime and Whitesides, 1993; Herne and Tarlov, 1997). However, though the chemisorption of the thiol group to the gold surface occurs spontaneously within seconds, a longer incubation time was reported to facilitate the process resulting in a more homogenous monolayer (Chaki and Vijayamohan, 2002). As discussed in Section 4.3.6.1, finding a balance between signal enhancement and increased non-specific binding is the major challenge for the thiol-C6 premix format. Figure 4.24 illustrates the signal development of the positive and negative control with increased incubation time. The signal separation (Figure 4.24 embedded graph) shows an increase until the peak is reached at an incubation time of 60 min. Zhang et al. (2002) investigated the binding ability of thiolated DNA to gold and suggested an incubation period of minimum 20 min. This finding was confirmed in this experiment. An incubation time of 15 min resulted in a four-fold increase in signal compared to 5 min. The trend shows a gradual increase, eventually reaching a plateau at an incubation period between 45 and 60 min.

Though the positive signal is gradually increasing up to an incubation time of 2 h, the signal separation is decreasing for the incubation period of 90 and 120 min. This is caused by the over proportional increase of the negative control, since longer incubation periods favour the adsorption of the unbound streptavidin-HRP conjugate to the unmodified surface. However, even 5 min incubation was sufficient to pass the *T*-test with a *P* value of 3.8 %. Only an incubation period of 120 min did not result in sufficient signal separation between positive and negative samples and failed the *T*-test with a *P* value of 22.3 %.

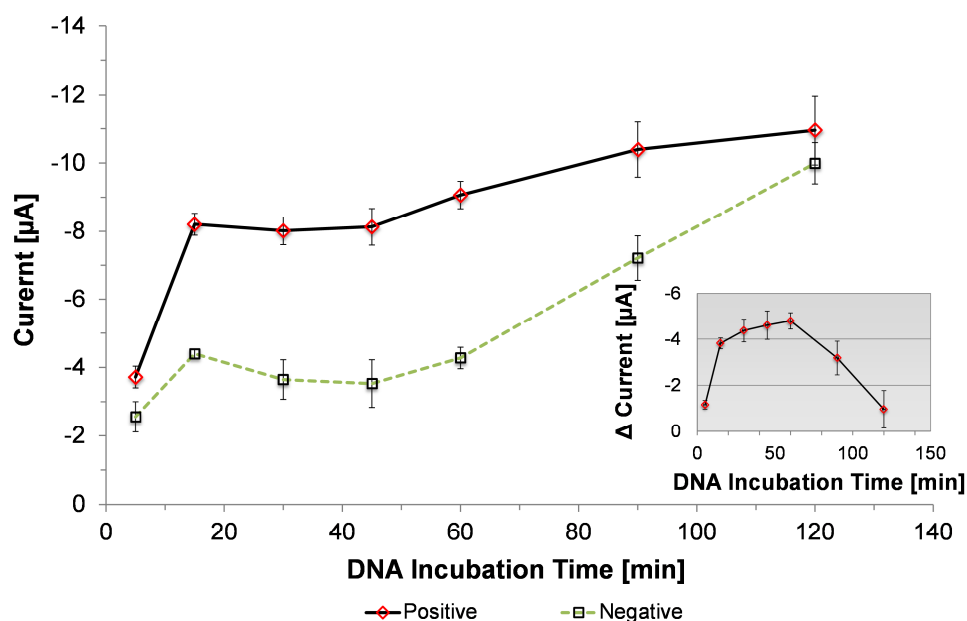


Figure 4.24: Normalised DNA sensor signal plotted against sensor incubation times ranging from 5 min to 2 h. The master mix of the positive (*B. allii*) and negative samples were PCR-amplified with thiol-C6 and biotinylated primers and premixed for 2 h with the streptavidin-HRP conjugate ($20 \mu\text{g ml}^{-1}$). Subsequently, $10 \mu\text{l}$ of the DNA-conjugate mix were dropped on the gold working electrode and the sensor signal was monitored as a function of the incubation time. The embedded graph shows the signal separation between the positive and negative samples, the bars represent the standard error of the means ($n=4$), average CV=6.9 %.

4.4.6.4 Final Optimisation and Reduction of the Background Current

Adding detergents like Tween (0.05 %) to the wash buffers or the supplement of proteins, such as milk protein solution or BSA, are established techniques to improve the assay performance by reducing non-specific binding (Kenna et al., 1985; Malone et al., 1996). These techniques were evaluated with the aim of reducing the background current generated by the unamplified master mix.

Though EDTA is the standard buffer solution for DNA and RNA in molecular biology, its washing performance in combination with Tween resulted in a higher signal compared with PBS-Tween and dH_2O (Figure 4.25 A). No significant difference was found between washing the sensor with PBS-Tween compared to the control washed with dH_2O .

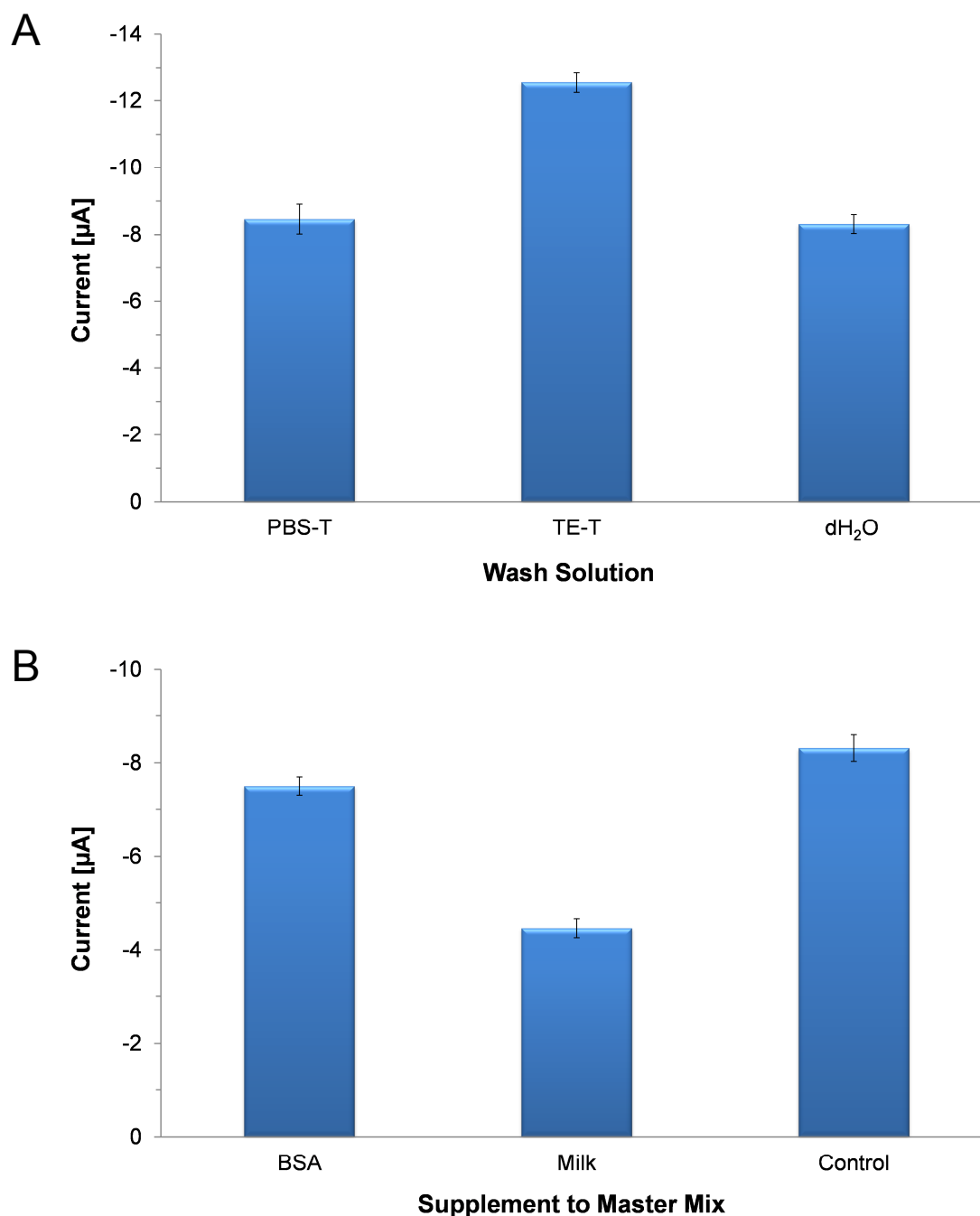


Figure 4.25: Optimisation of the background current produced by unamplified PCR master mix incubated for 2 h with streptavidin-HRP conjugate (20 mg ml^{-1}). (A) DNA sensor signal of the background current generated after performing a controlled washing step with five times 1 ml PBS-Tween, tris EDTA-Tween and dH₂O. (B) DNA sensor response of unamplified master mix comprising 0.1 mg ml^{-1} BSA, 1:100 milk protein solution and no supplement (control). The bars represent the standard error of the means ($n=4$), average CV = 7.4 % (A) / 7.0 % (B).

In contrast adding proteins to the master mix resulted in a background current lower by 10 % for BSA and a 46 % reduction when milk protein solution is added to the master mix (Figure 4.25 B). The supplement of BSA to antibodies or enzyme solutions is a well-known technique and is also recommended by the manufacturer of the ApoI restriction digest used for the RFLP analysis (see Section 4.3.3). However, the milk protein solution KPL (Gaithersburg, USA) which was standardly used for the immunoassay development showed a superior performance in reducing non-specific binding.

4.4.6.5 LOD test of the DNA Sensor

The limit of detection of the optimised PCR-DNA sensor system was tested in this Section. The electrochemical DNA sensor response as a function of various *B. allii* template DNA concentrations is illustrated in Figure 4.26. The DNA sensor showed a steady signal up to a DNA concentration of 1 pg. Pathogen detection can be performed until a DNA concentration of 100 fg is reached with a maximum *P* value of 1.2 %.

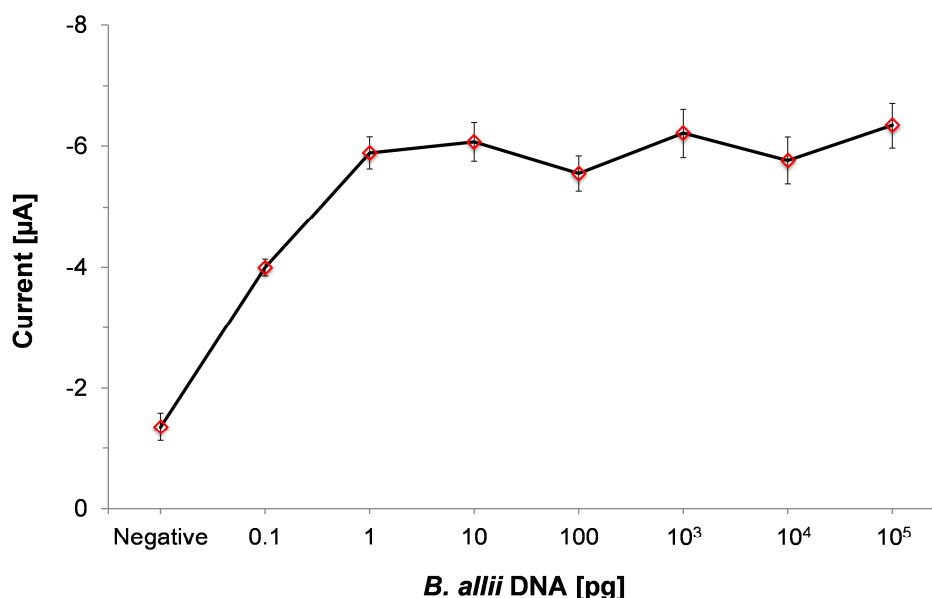


Figure 4.26: Normalised DNA sensor signal as a function of *B. allii* template DNA concentrations ranging from 100 ng to 100 fg. Template DNA was PCR amplified with thiol-C6 and biotinylated primers for 25 cycles and premixed for 2 h with the streptavidin-HRP conjugate (20 μg ml⁻¹). The bars represent the standard error of the means (n=4), average CV=3.6 %.

These encouraging results are in accordance with the LOD of the CRT1f/2r primer designed by Chilvers et al. (2007) of 10 fg, which was tested in Section 4.4.2.1. This finding suggests that the PCR amplification is the limiting factor of the DNA sensor design. As long as the PCR amplification yields enough pathogen derived DNA template, the DNA sensor is operating in a steady state, which is indifferent from the amount of template DNA, confirming the experiments performed in Section 4.3.4.3.

This is the first report on the detection of *Botrytis* DNA using an electrochemical DNA sensor. Betazzi et al. (2008) developed a disposable electrochemical DNA array for the detection of PCR-amplified DNA of hazelnut allergens in foodstuffs using alkaline phosphatase. Surface-tethered and biotinylated signalling probes were used to produce biotinylated hybrids, coupled with a streptavidin-alkaline phosphatase conjugate. The LOD of 0.1 nM l^{-1} was achieved using pulse voltammetric detection of the substrate α -naphthyl phosphate. Herrasti et al. (2013) developed a lab on chip (LOC) device for the qualitative detection of DNA using optical and electrochemical detection systems. The system showed inferior performance compared to the system developed in this study with a limit of detection of approximately 10 ng DNA.

Recently, lab on a chip (LOC) amplification of nucleic acids from various biological samples has undergone extensive development producing numerous devices that are on the market or under development (Chen et al., 2007a). Quantitative analysis is also achievable for LOC systems by integrating a real-time PCR thermocycler (Ibrahim et al., 1998; Gulliksen et al., 2004; Xiang et al., 2005; Lee et al., 2006). However, these integrated systems are an expensive investment and hence require complex chips which cannot be easily mass produced. Therefore, these systems are designed for medical diagnostics and not for price sensitive industries like agriculture. The major benefit of the DNA sensor designed in this study is the cost effective and simple combination of low-cost PCR thermocycler with a robust and sensitive amperometric detection based on screen-printed electrodes, which LOC systems currently cannot provide.

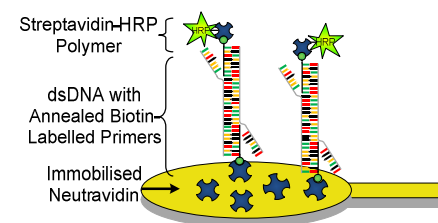
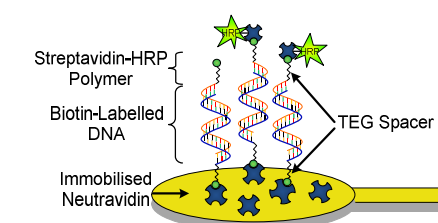
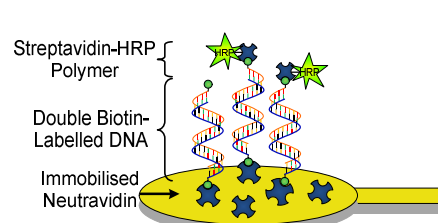
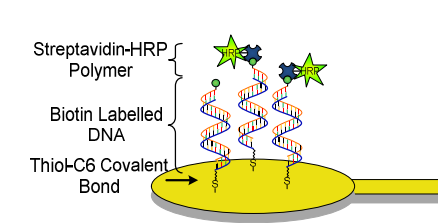
4.4.7 PERFORMANCE STUDIES

4.4.7.1 Comparative Summary of the Assay Formats Evaluated for the DNA Sensor Development

In this Chapter, four DNA sensor designs were developed and two were selected for further optimisation as a function of various parameters. The DNA sensor format employing two biotin and biotin-TEG labelled primers is based on a sandwich assay in which neutravidin is immobilised on the sensor surface as capture molecule for the DNA, followed by streptavidin-HRP conjugate for signal generation. Pathogen DNA was amplified directly using PCR or labelled by performing a final denaturation and annealing cycle after adding the primer pair. The premix format was accomplished by adding streptavidin-HRP directly to the thiol-C6 and biotin-labelled DNA before dropping the master mix on the sensor. A direct comparison of the developed preliminary and optimised assay formats is presented in Table 4.6.

The double biotin as well as the premix format proved to provide stable assay conditions for the detection of pathogenic DNA. The maximum signal separation between positive and negative samples of the double biotin format was higher by approximately 1 μ A (20 %) compared to the premix format. However, while the classic assay operates on a step-by-step application, it is more desirable to have a premixed complex as this may save assay time and washing steps, thus provide an improved handling. In this Section the performance of both optimised sensor formats were tested on a multilevel approach.

Table 4.6: Summary of the performance of various assay setups developed for the DNA sensor based on screen-printed electrodes.

Format	Best Signal Separation	Average Signal Separation	Average Coefficient of Variance	Schematic of the Assay
Two-step format with additional denaturation and annealing cycle	3.5 μ A	3.5 μ A (n=10)	12 % (n=10)	
Double biotin-TEG format	1.2 μ A	1.1 μ A (n=28)	8.3 % (n=28)	
Double biotin format	6.4 μ A	Covalent: 4.6 μ A (n=16) Adsorption: 6.1 μ A (n=24)	Covalent: 8.8 % (n=16) Adsorption: 6.9 % (n=24)	
Premix format with thiol-C6 labelled primers	5.5 μ A	4.9 μ A (n=52)	4.8 % (n=52)	

4.4.7.2 DNA Sensor Response Using PCR and Real-Time PCR Thermocyclers

The overall objective of the DNA sensor is to develop a simple to use, low-cost detection device for amplified *Botrytis* DNA, alleviating the need of real-time PCR equipment or subsequent gel electrophoresis analysis. A successful marketing of the DNA sensor requires the use of low-cost thermocyclers, where used models can be acquired in a price range between 50 and 500 pounds. In this Section, the performance of the low-cost thermocycler in combination with the optimised DNA sensor using the premix format was evaluated and compared to a state of the art real-time PCR machine. Figure 4.27 illustrates the DNA sensor response accomplished by the two DNA amplification devices. The results show strong similarity between the real-time PCR and the low-cost thermocycler with a difference in signal separation of less than 2 %. This confirms the ability of the DNA sensor to be operated as a DNA detection instrument in combination with a low-cost thermocycler.

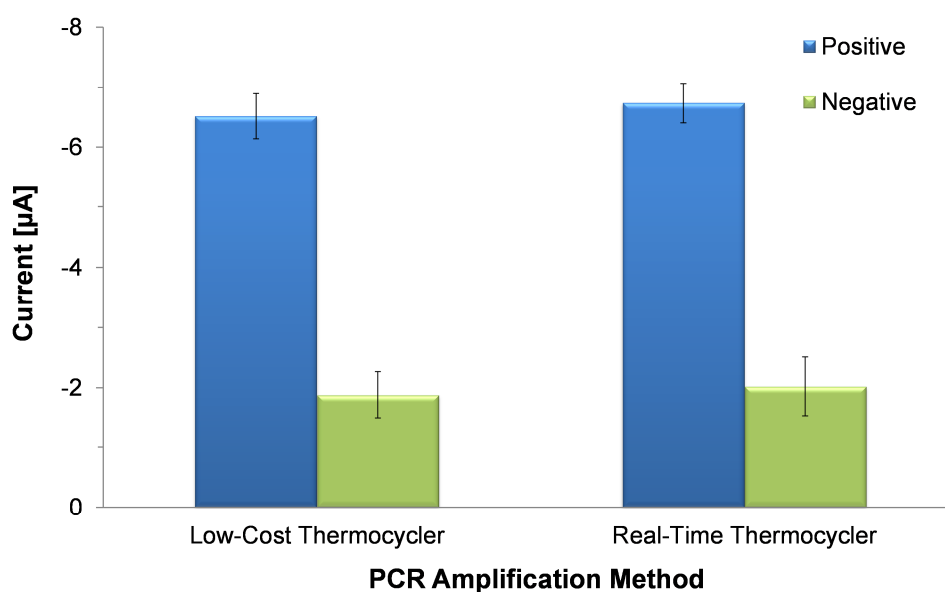


Figure 4.27: Comparison of the normalised DNA sensor achieved by using a low-cost and real-time PCR thermocycler. The master mix of the positive (*B. allii*) and negative samples were PCR amplified with thiol-C6 and biotinylated primers using a Rotor Gene real-time PCR machine (Qiagen) and low-cost thermocycler (Techne model, TC-512). After amplification, streptavidin-HRP conjugate ($20 \mu\text{g ml}^{-1}$) was added to the master mix and incubated for 2 h. The bars represent the standard error of the means ($n=4$), average CV=6.4 % (low-cost) and 7.0 % (real-time PCR) thermocycler.

4.4.7.3 Specificity Studies of the DNA Sensor Utilising the Neck-Rot Primers CRT1f/2r

The specificity of the DNA sensor was tested against common species of *Botrytis*. The CRT1f/2r primers employed for the amplification of the pathogen DNA targeted all three species associated with neck rot with no cross reactivity with the worldwide common plant pathogen *B. cinerea* (Figure 4.28). The difference between the signal response of the species is with 4.9 % within the intra-assay variation and the sensor signal produced by *B. cinerea* was not significantly different from the negative control ($P=27.2\%$). These results confirm the findings of the real-time PCR regarding the specificity of the primer pair CRT1f/2r tested in Section 4.4.2.1. In combination with this set of primers, the DNA sensor proved to be a powerful tool for pre and post-harvest disease control for early detection of neck rot in onions.

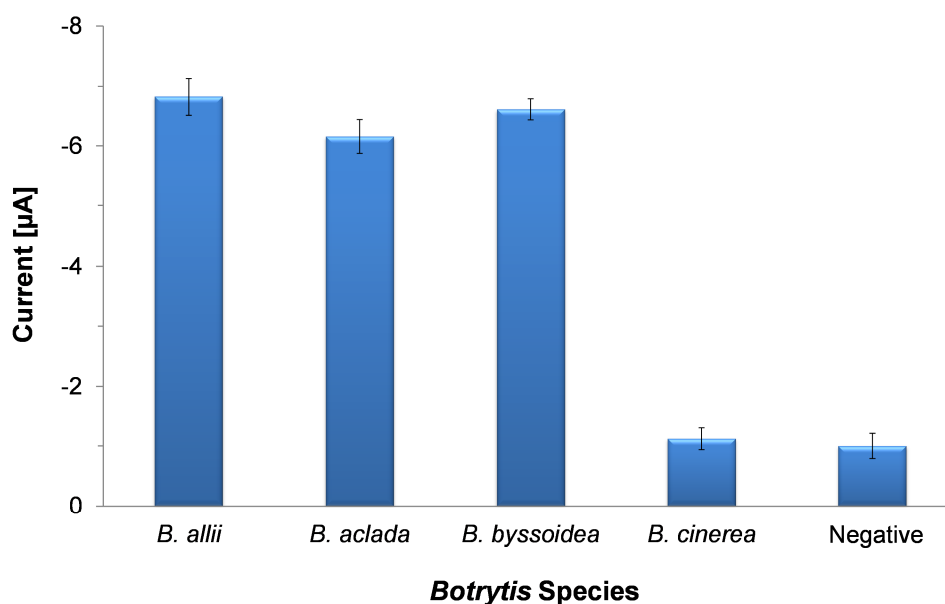


Figure 4.28: Normalised DNA sensor signal against *Botrytis* species associated with neck rot, i.e. *B. aclada*, *B. allii* and *B. byssoides* as well as *B. cinerea*, using the primer pair CRT1f/2r. *Botrytis* DNA was amplified with thiol-C6 and biotinylated primers for 25 cycles and subsequently premixed with streptavidin-HRP conjugate ($20\ \mu\text{g ml}^{-1}$). The bars represent the standard error of the means ($n=5$), average CV=4.8 %.

4.4.7.4 Specificity Studies of the DNA Sensor in Combination with the RFLP Primers BA1r/2f

A major benefit for the DNA sensor compared to the immunosensor is the enhanced specificity of the PCR that immunological methods cannot provide. Here, the specificity is determined by the primers utilised for the amplification process of the sample. As a result, the DNA sensor system can be used with various sets of primers for alterable pathogens. In this Section the DNA sensor, which was optimised for the detection of *Botrytis* neck rot employing the primers CRT1f/2r, was tested with the RFLP primers designed by Nielsen et al. (2002).

Figure 4.29 shows the electrochemical response of the DNA sensor tested against the *Botrytis* species *B. aclada*, *B. allii*, *B. byssoidea* and *B. cinerea*. The combination with the primer pair BA1r/2f enables the DNA sensor to detect the worldwide common pathogen *B. cinerea* along with the neck-rot species *B. aclada*, *B. allii*, and *B. byssoidea*, demonstrating the universal use of this system. However, the DNA-sensor response is with an average signal separation of 3.6 μA not as sensitive as the DNA-sensor assay performed with the primers CRT1f/2r. The DNA-sensor assay being optimised for the neck-rot primers may be one reason. More likely, the length of the amplified DNA product is the predominant cause for the decreased sensor signal. The primers described by Nielsen et al. (2002) are designed for RFLP analysis and amplify a 450 bp long DNA fragment, whereas the CRT1f/2r designed for real-time PCR amplify a 113 bp fragment. The increased distance to the sensor surface may impede the electron transfer. Furthermore, real-time PCR performed in Section 4.4.2 suggested an inferior DNA amplification compared to the CRT1f/2r primers. However, even with the decreased sensitivity, a reliable separation between positive and negative samples is warranted with a maximum *P* value of 0.3 % and an average coefficient of variation of 7.6 % demonstrating the robustness of the DNA assay procedure.

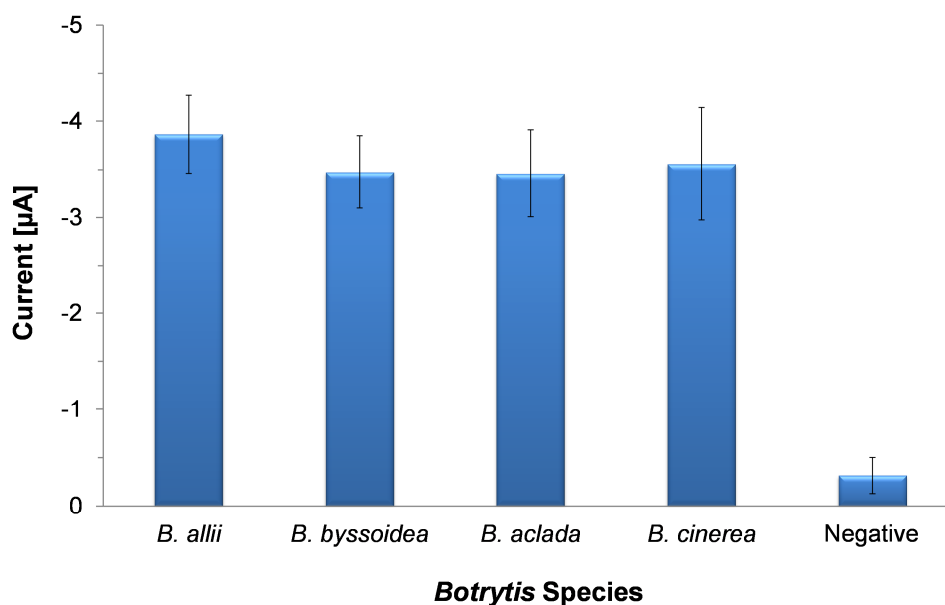


Figure 4.29: Normalised DNA sensor signal against common *Botrytis* species (*B. aclada*, *B. allii*, *B. byssoidea* and *B. cinerea*) using the primer pair BA1r/2f. *Botrytis* DNA was amplified with biotin-labelled primers for 25 cycles and dropped on the neutravidin ($300 \mu\text{g ml}^{-1}$) coated working electrode. For signal generation, streptavidin-HRP conjugate ($50 \mu\text{g ml}^{-1}$) was incubated for 1 h followed by the TMB- H_2O_2 substrate. The bars represent the standard error of the means ($n=4$), average CV=7.6 %.

These encouraging results demonstrate the wide use of potential applications of the DNA sensor system. Focussing on *Botrytis*, other primers can be combined with the DNA sensor to detect *Botrytis cinerea* (Martinez et al., 2008; Diguta et al., 2010; Saito et al., 2013) or *Botrytis squamosa* (Carisse et al., 2009). Furthermore, the DNA sensor is not limited to these applications and can be adopted for other fungal and bacterial pathogens, or as a low-cost device for medical diagnostic in developing countries.

4.4.7.5 Evaluation of the Minimum Assay Time

The assay duration is a major performance factor for commercially successful biosensors (Alvarez-Icaza and Bilitewski, 1993; Tothill, 2001; Marks et al., 2007). A fast assay response is preferable, but should not neglect challenges regarding reproducibility and sensitivity of the assay. The premix format with thiol-C6 primers provides the most favourable starting point, as previous studies performed in

Section 4.3.6.2 and 4.3.6.3 indicated that even short incubation periods result in a reliable separation between positive and negative samples.

The minimum incubation time for premixing and incubating the master mix on the sensor surface was found to be 5 min (Figure 4.30). Surprisingly, a shorter incubation period of 5 and 15 min showed higher signal separation by over 2 μA compared to 30 min (Figure 4.30 embedded graph). Performing a 5 min incubation at room temperature did also yield with 2.2 μA enough signal separation to reliably detect pathogen DNA with a maximum P value of 0.05 %. However, also the risk of false positive or negative responses doubled from to approximately 10 %, suggesting that the assay is not operated in a steady state. If shorter assay times are adopted, further studies need to be conducted to overcome this challenge.

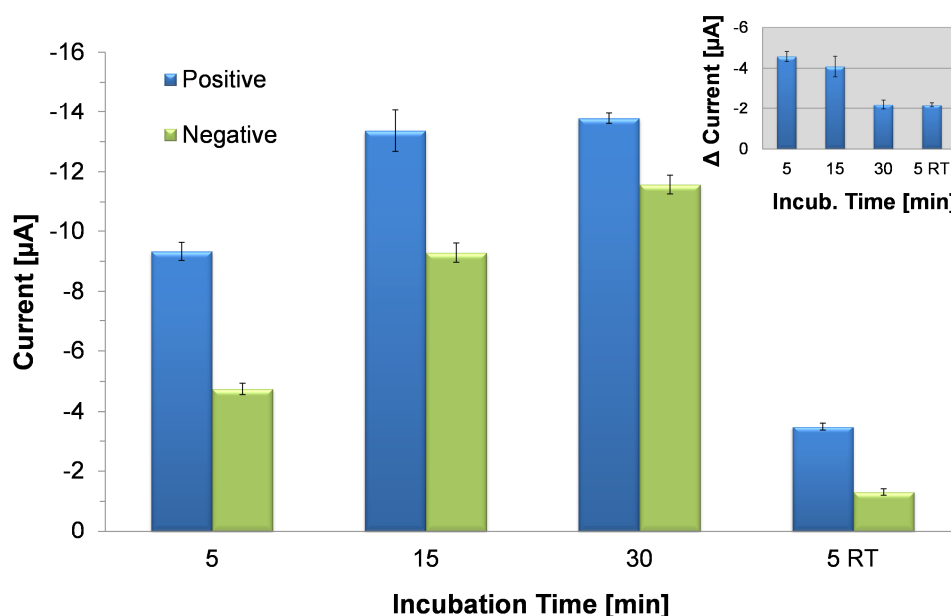


Figure 4.30: Evaluation of various premix and DNA incubation times ranging from 5 to 30 min. Positive (*B. allii*) and negative samples were amplified with the thiol-C6 and biotinylated primers and premixed for 5, 15 and 30 min with streptavidin-HRP conjugate ($20 \mu\text{g ml}^{-1}$). Subsequently, the master mix was dropped on the sensor and incubated for 5, 15 and 30 min. The embedded graph shows the signal separation between the positive and negative sample plotted against the premix and DNA incubation time, the bars represent the standard error of the means ($n=4$); average CV=7.9 %, RT = room temperature.

4.5 CONCLUSIONS

DNA was effectively extracted from all *Botrytis* species relevant for this study with A_{260}/A_{280} values indicating a high grade of purity after quantification using a spectrophotometer. Furthermore, fungal DNA was successfully extracted from onion samples and samples diluted in PBS. However, the breakdown of the fungal cells was found to be a delicate procedure leading to fluctuating extraction efficiencies between the fungal species.

The primers CRT1f/2r and BA1r/2f utilised for the amplification of *Botrytis* species were tested in real time as well as normal PCR assays. The primer pair described by Chilvers et al. (2007) was designed to target the species associated with neck rot, i.e. *B. aclada*, *B. allii* and *B. byssoidea* in a real-time PCR assays. The specificity to neck-rot species as well as the low LOD of 10 fg pathogen DNA made these primers most suitable for a DNA sensor development, as they do not amplify the related *Botrytis* species *B. cinerea* and *B. squamosa*. For a more general application, the primers designed by Nielsen et al. (2002) are appropriate, as they amplify five species of *Botrytis*: *B. aclada*, *B. allii*, *B. byssoidea*, *B. cinerea* and *B. squamosa*. Furthermore, these primers provide the only approach to distinguish between the related species *B. aclada* and *B. allii*. The RFLP analysis using the *ApoI* restriction digest and subsequent gel electrophoresis was successfully performed within these studies, demonstrating the capability of the genetic fingerprint analysis to separate the *Botrytis* species.

Numerous hybridisation techniques were tested with a complementary probe based on the amplified PCR product of the RFLP primers. Applied in varying concentration, the target probe hybridised to the DNA immobilised on the working electrode as well as to the detection probe generating an electrochemical signal, which was found to be proportional to the DNA concentration. However, this format requires single-stranded DNA, whereas the PCR product is double stranded. Therefore, two different strategies were evaluated to overcome this challenge.

The production of ssDNA by heating and immediate cooling of the PCR product was found to be inefficient. Though this method is easy to perform and practicable for field use, large parts of the ssDNA strands re-hybridised with increasing temperature before a successful annealing of the biotin-labelled capture and detection probe. A higher efficiency was accomplished by performing a subsequent denaturation and annealing cycle after adding the biotin-labelled capture and detection probe. This method showed superior signal strength and binding efficiency, as the DNA has two biotin labels facilitating the orientation of the DNA. A decreased signal due to inhibited interaction of the streptavidin-HRP dimer to the probe biotin caused by double binding of the biotin labels to the immobilised streptavidin was not observed. The distinctive three-dimensional structure of the working electrode with round, larger and smaller-sized ink particles might help to reduce this effect.

However, all these sensor formats require an additional processing step of the PCR product. Therefore, a DNA sensor assay was developed with biotin and biotin-TEG labels, where the PCR amplification is performed in the presence of the biotin-labelled primers eliminating the need of an additional hybridisation step. The DNA sensor format employing two biotinylated primers is based on a sandwich assay in which neutravidin is immobilised on the sensor surface as capture molecule for the DNA, followed by streptavidin-HRP conjugate for signal generation. Employing a TEG spacer did not improve the assay performance. Hence, the separation between positive and negative samples, representing the major performance indicator for the DNA sensor, was with 1.2 μA lower by approximately 3 μA , compared with the DNA sensor employing biotin-labelled primers without the TEG spacer. The double biotin format was then optimised as a function of various parameters. Covalent immobilisation strategies to 3-DTDPA layer were evaluated with mixed results. The decision if covalent immobilisation is beneficial for the DNA sensor has to be assessed using a multilevel approach. If the development prioritises cost effectiveness and signal performance, adsorption offers more benefits. On the other hand, if increased surface load at low concentrations of neutravidin and reproducibility is the major target, covalent immobilisation strategies are more suitable.

The second DNA sensor design developed in this study is based on a premix format, and was accomplished by adding streptavidin-HRP directly to the thiol-C6 and biotin-labelled DNA before dropping the master mix on the sensor. Though the maximum signal separation between positive and negative samples of the double biotin format was higher by approximately 20 % compared with the premixed assay, this format offers numerous advantages. While the classic assay operates on a step-by-step application, it is more desirable to have a premixed complex to reduce the total assay time to 10 min. Pathogen detection can be performed down to a concentration of 100 fg DNA which close to the limit of detection of the DNA amplification process.

Both formats provide stable assay conditions for the detection of pathogenic DNA. The DNA sensor demonstrates a promising new approach as a portable, low-cost alternative for real-time PCR detection, alleviating the need of expensive, laboratory-based equipment. In combination with the primers CRT1f/2r, the DNA sensor proved to be a powerful tool for pre and post-harvest disease control for the early detection of neck rot in onions, targeting only *Botrytis* species associated with neck rot (*B. aclada*, *B. allii*, and *B. byssoides*). The specificity of the DNA sensor is based on the primer sequence used for the amplification of the DNA and is therefore applicable to target other pathogens as well. For instance, in combination with the primer pair BA1r/2f enabled the DNA sensor to detect the worldwide common pathogen *B. cinerea* along with the neck-rot species *B. aclada*, *B. allii*, and *B. byssoides*, demonstrating the universal use of the DNA sensor.

CHAPTER FIVE

CORRELATION BETWEEN THE PATHOGEN LOAD OF *BOTRYTIS* AND THE INCIDENCE OF NECK-ROT DISEASE IN STORE

5 CORRELATION BETWEEN THE PATHOGEN LOAD OF *BOTRYTIS* AND THE INCIDENCE OF NECK-ROT DISEASE IN STORE

5.1 INTRODUCTION

The precise circumstances that lead to neck-rot infections in field are still controversially discussed in literature (Maude and Presly, 1977a; Kritzman, 1983; Maude, 1983; Lorbeer et al., 2004; Chilvers and Du Toit, 2006). Most of the research was conducted during the 70s and 80s, by investigating empirically in large field trials the influence of environmental conditions (Maude and Presly, 1977b; Maude et al., 1986), the presence of infected debris (Maude et al., 1982), drying conditions and mechanical topping of the foliage (Maude et al., 1984), infected seed (Maude and Presly, 1977a; Maude, 1983) and the spread in the field (Maude and Presly, 1977b; Presly, 1985b) on the development of neck rot in store. However, with the cheap and exhaustive use of new fungicides, the disease was thought to be controlled and as a result few new researches were conducted.

Yohalem (2004) investigated unsuccessfully the use of two fungi (*Clonostachys rosea* and *Ulocladium atrum*) in a field trial as antagonists to stop the progress of *B. aclada* from necrotic into the fresh leaf tissue. Leaves are the primary infection court for neck-rot infections, from which the fungus starts to grow towards the onion neck (Maude and Presly, 1977a). The primary source of inoculation is either soil-borne (Maude et al., 1982) or seed-borne (Maude and Presly, 1977a; Maude, 1983; Brewster, 1994; Stewart and Franicevic, 1994; Lorbeer et al., 2004). Though it is a well-known fact that neck rot caused by *B. allii* and *B. aclada*, respectively, is a seed-borne disease, this supposedly established theory is challenged. Du Toit et al. (2004), found no significant relationship between infected seed and the incidence of neck rot in store monitored over three seasons in the semiarid Columbia Basin of Washington.

Though the correlation between *Botrytis* infections and the incidence of neck rot in store is an established causal fact, very little is known about the impact of the pathogen load on the development of the disease. Hence, the correlation was not yet investigated due to the lack of sensitive and reliable detection methods for the fungus. Nowadays, advances in molecular biology and biosensor development provide new technologies, revolutionising the pathogen detection of *Botrytis*. The close collaboration to the UK onion growing industry within this project gives the unique opportunity to combine the know-how and technical resources of Cranfield University with practical work in field conditions.

The field trial aimed at investigating the precise circumstances of the development of neck-rot disease in store, based on a local pathogen load. The immunosensor and DNA-based detection systems for *Botrytis*, which were developed in this study, were used to detect and quantify the fungus pre-harvest, in order to establish a link between the pathogen load and the risk of neck-rot disease in store.

5.2 MATERIALS AND INSTRUMENTATION

Phosphate buffered saline tablets (PBS, 0.01 M phosphate buffer, 0.137 M sodium chloride and 0.0027 M potassium chloride, pH 7.4), Tween 20, potassium chloride, 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB), hydrogen peroxide solution (30 % w/w) in dH₂O, carbonate-bicarbonate buffer (0.05 M, pH 9.6), phosphate-citrate buffer (0.05 M, pH 5.0), sodium acetate, sulphuric acid (H₂SO₄, 95-98 %), primers sequences comprising 5'-thiol-C6 (CRT1f) and 5'-biotin (CRT2r) as listed in Table 4.1, streptavidin-peroxidase conjugate, tris-EDTA (ethylenediaminetetraacetic acid) buffer (1.0 M tris-HCl, 0.1 M EDTA, pH 8.0), HPLC grade dH₂O, tris acetate-EDTA buffer (40 mM tris-acetate, 1 mM EDTA, pH 8.3) and Eppendorf PCR tubes (0.2 ml) were purchased from Sigma (Dorset, UK). Sure Blue TMB solution and milk blocking solution concentrate were purchased from KPL (Gaithersburg, USA).

The goat anti-rabbit and goat anti-mouse IgG-antibody conjugated to HRP were purchased from Invitrogen (Paisley, UK). The monoclonal antibody BC-12 was acquired from Forsite Diagnostics (Sand Hutton, UK) and the polyclonal *B. allii* antibody was obtained from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK). Antibodies were conjugated to HRP using the Lightning-Link conjugation kit purchased from Innova Biosciences Ltd. (Cambridge, UK), and purified with a Pierce conjugate purification kit purchased from Thermo Scientific (Cramlington, UK). N-hydroxysuccinimide (NHS) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) were purchased from Thermo Scientific (Cramlington, UK).

Filter tips size 1000, 200, 20 and 10 µl were acquired from Greiner Bio-One Ltd. (Gloucestershire, UK). Pico Drop P10 (10 µl) UV pipette tips used for DNA quantification were purchased from Cambridge Bioscience (Cambridge, UK). SYBR Green PCR kit strip tubes (0.1 ml) for the Rotor Gene real-time PCR machine, DNeasy Plant Mini kit and RNase-Free water were acquired from Qiagen (Crawley, UK). Precellys tough microorganism lysing tubes (VK05, 2 and 7 ml) comprising 0.5 mm glass beads and 5.0 mm zirconium oxide beads (80 g) were purchased from Stretton Scientific (Stretton, UK) and were used in combination with the Precellys 24 homogenising instrument.

Absorbance readings for ELISA, electrochemical measurements and biosensor-related machinery and equipment are equivalent to the materials and methods Sections described in Chapter 3. Spectrophotometric DNA quantification, real-time PCR amplification and data analysis were performed according to Chapter 4.

5.3 METHODS

5.3.1 FIELD TRIAL DESIGN

Two field trials were performed in this study. The field site near Cardington (Bedfordshire, UK) was used to evaluate the presence of onion rots for the growing season 2012, whereas the site near Littleport (Cambridgeshire, UK) aimed at evaluating the impact of the pathogen load, tested by using the immunosensor and real-time PCR on the incidence of neck-rot disease after storage. For the Cardington field trial sets of two cultivars (Forum and Jagro) prone to neck-rot infection were planted in March 2012. Randomised plots were planted using fungicide treated (Signum and Topsin) and untreated onion sets. Averaged incidence of onion rot was established by sampling approximately 1,000 onion bulbs at harvest and after two months of storage at 15 °C. The design of the Littleport field trial is presented in the following Sections.

5.3.1.1 Sample Size and Rationale

To test the performance of the developed biosensors, a plot of approximately 350 ± 50 onions was planted near Littleport (Cambridgeshire, UK) by Cranfield University in collaboration with the Allium and Brassica Centre (Kirtton, UK). The onion seed lot used for this study was provided by Syngenta (Fulbourn, UK) and had an internal *Botrytis* infection level of approximately 6 %. The internal infection level was tested according to the standard procedure of the company; after surface sterilisation of the seed coat, the seeds were placed individually on agar media in order to visualise the fungus. Seedlings were grown in a greenhouse at the Allium and Brassica Centre until they reached a size of approximately 5 to 10 cm and planted in April 2012 in a separate plot on the far end of the field (Figure 5.1). The onion plot did not encounter any special treatment by the collaborative onion grower and was regularly sprayed with pesticides, fungicides and fertilisers.



Figure 5.1: Field trial site near Littleport (Cambridgeshire, UK). Seventeen onion plots were planted in April 2012 by Cranfield University and harvested in September. The yellow square is marking the boarder of the plot used for the biosensor studies; the picture was taken in July 2012.

5.3.1.2 Sample Inoculation with *B. allii* Spore Solution

The leaves of the onion plants were inoculated five times at monthly intervals, starting with the planting of the seedlings. To avoid cross contamination from the test plot, the inoculum (1 ml) was dropped on the plant using a pipette and distributed over the leaves with sterile gloves. The plot was divided into two parts and inoculated with a low (10^5 spores ml^{-1}) and a high (10^6 spores ml^{-1}) *Botrytis allii* spore solution produced as described in Section 2.3.1.4. To ensure successful inoculation, approximately 50 onions from the high group were additionally inoculated with 5×10^6 spores ml^{-1} one month before harvest, representing the very high-infected group.

5.3.1.3 Sample Collection and Storage Conditions

The onions were harvested in dry condition in September 2012. The sample processing of dry leaf tissue is very difficult, as the cellulose fibres develop leather-like behaviour. Therefore, the onions were harvested with most of the samples still being in the green leaf stage. The onions were harvested individually, the roots were cut and the loose outer scale was removed. The foliage was topped using a pair of scissors that was sterilised after each bulb using a paper tissue soaked in 100 % isopropanol, leaving a neck size of approximately 3 cm. At harvest, 215 onions comprising 100 from the low-infected group, 75 from the high-infected group, 25 from the very high-infected group and 15 non-infected onions were collected, numbered and stored individually in apple boxes according to their group. Non-infected onions, representing the control group, were treated with fungicides (Topsin and Maxim) and harvested from a separate plot located on the opposite side of the test field. Additionally, a sample of the corresponding neck section of each onion was collected, numbered and frozen at -80 °C for further testing. The onions were dried outside protected from rain for two days at temperatures during day between 22 and 26 °C and 17 to 19 °C at night, in order to simulate the undercutting process which is used to dry the leaves and bulbs before storage. The weather conditions during this period were sunny with no rain. The onions were subsequently stored at 4 °C and regularly monitored for signs of neck rot. If signs of rot were present, the onions were cut, photographed, weighed and the results were recorded in a database. The storage experiment was ended after four months.

5.3.1.4 Data Analyses

Data were collected in a database and processed using Excel and Graph Pad Prism (v5.03) statistical software. Grouped data were visualised in box plots and analysed by *T*- and *F*-tests at a confidence level of 95 / 99 % as well as one way ANOVA using the Prism software. Statistical calculations were performed as described in Section 3.3.1.3, data were considered valid at a confidence level of 95 %.

5.3.2 SAMPLE EXTRACTION PROTOCOL

For the sample extraction of the frozen neck tissue collected at harvest, the outer scale was removed and cut from the bottom up into thin disc slides on a clean petri dish using a sterile scalpel. The cut tissue (1.1 g) of was then transferred to 7 ml homogenising tubes containing 0.5 mm glass beads and three 5 mm zirconium oxide beads (80 g). The sample was then ground for 2x30 s at 6,000 rpm with a 10 s break between the cycles employing a Precellys 24 homogenising instrument. 0.1 g of the sample was transferred to a new 2 ml tube for further DNA extraction performed as described in Section 4.3.1.1. However, the sample forms a fine mesh and cannot be separated from the 0.5 mm glass beads used for the grinding. Therefore, the average weight of five lysis tubes was used to calculate the sample plus bead ratio required to transfer 0.1 mg sample. This was calculated at 300 mg sample glass-bead mix. Five ml PBS were added and homogenised for 2x30 s at 6,000 rpm with 10 s break in order to dissolve the fungal antigen to the buffer. The leaf mash was transferred to a 15 ml tube and the remaining sample was rinsed by adding another 5 ml PBS to the tube, resulting in a final ratio (sample to PBS buffer) of 1:10. Next, the sample was filtered using an extraction bag with a heavy duty outer and a fine intermediate filter layer. The filtered sample was transferred to a new tube and stored at 4 °C for testing. A flow diagram of the sample extraction procedure is illustrated in Figure 5.2.

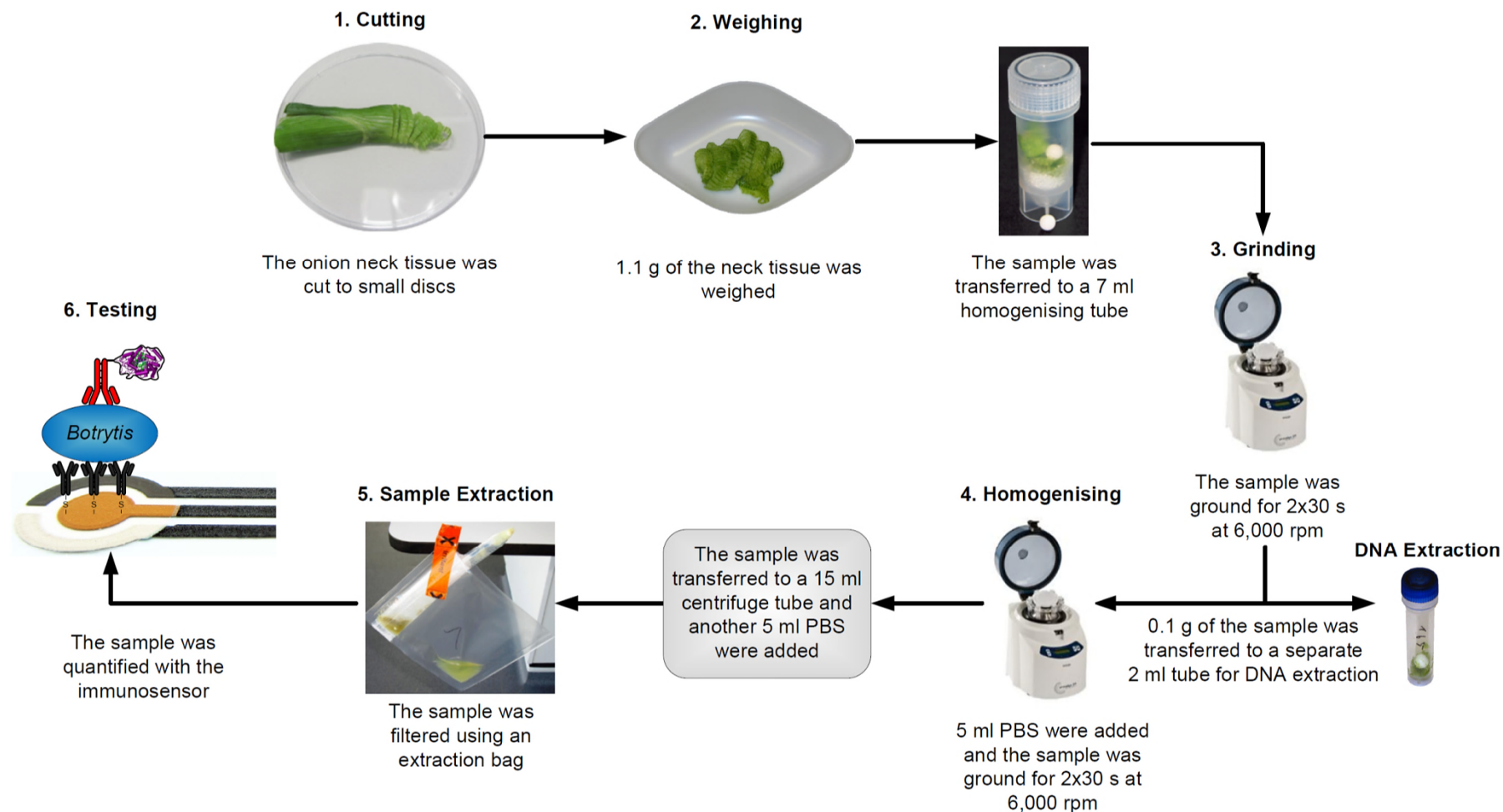


Figure 5.2: Flow diagram of the sample extraction procedure used for the quantification of the pathogen load in onion neck samples.

5.3.3 TESTING THE PRESENCE OF LATENT *BOTRYTIS* INFECTIONS USING SCANNING ELECTRON MICROSCOPY AND SELECTIVE AGAR

The fungus was visualised by scanning electron microscopy using the equipment and protocol described in Section 3.3.3.1. To provide conductivity the tissue was coated with a thin gold nanolayer by exposing the sample to plasma for approximately 3 min.

Additionally, the success of the inoculation strategy was tested in July 2012 before performing the third inoculation by visualising the presence of latent *Botrytis* infections on the leaves using selective agar produced as described by Kritzman and Netzer (1978) (see Section 1.4.1). Ten onion plants were collected from different locations of the low and from the high infected side of the plot. For each sample, an approximately 3 cm long piece of three different leaves were cut with a sterile pair of scissors and placed on the selective agar. A controlled environment provided by an incubator (Sanyo, model MLR-350HT, Japan) at 20 °C with a 12 h day-night photoperiod was used to allow the fungus to grow on the agar for 14 days.

5.3.4 QUANTIFICATION OF THE PATHOGEN LOAD USING THE IMMUNOSENSOR

Onion neck tissue samples were defrosted and the pathogen load was determined using the optimised immunosensor. The samples were processed according to the protocol described in Section 2.3.7. Covalent immobilisation to 50 mM 3-DTDPA layer was performed as described in Section 3.3.7.1. The assay was followed according to the protocol provided in Section 3.3.3.3 with a monoclonal capture antibody concentration of 100 µg ml⁻¹ followed by 80 µg ml⁻¹ of purified polyclonal antibody conjugated to HRP for signal generation. Quantification of the pathogen load was accomplished by plotting the normalised electrochemical signal after 100 s against the standard curve constructed in Section 3.4.7.5. Additionally, two standards (10 µg ml⁻¹ and negative control) were used for each batch (8 samples) to test and enhance the quality of the data fit. The assay was conducted in quadruplicates and data were considered valid at a confidence level of 95 %.

5.3.5 COMPARATIVE STUDIES WITH THE COMMERCIAL ELISA KIT AND RT-PCR

5.3.5.1 Quantification Using the Commercial ELISA Kits

The samples used for the biosensor were tested using the commercially available *B. allii* and *B. cinerea* ELISA kit (ADGEN Phytodiagnostics, Auchincruive, UK) that utilises the same antibodies employed in this study. The ELISA procedure was followed as described in Section 2.3.5, with a detection antibody concentration of the polyclonal and monoclonal antibodies of $10 \mu\text{g ml}^{-1}$ and a corresponding anti-species conjugated concentration of $0.3 \mu\text{g ml}^{-1}$. *B. allii* standards ranging from 50 to $0.4 \mu\text{g ml}^{-1}$ were serially diluted in onion leaf extract (negative control) as well as in a 1:5 dilution of the leaf extract in order to ease the influence of non-specific proteins.

5.3.5.2 Real-Time PCR Quantification of the Pathogen Load

During the sample processing of the onion neck tissue for the immunosensor (see Section 5.3.2), 0.1 g of the ground material were transferred to a separate tube and stored at -20°C for subsequent DNA analysis. The DNA was extracted and quantified following the protocol described in Section 4.3.1. For quantitative measurements, the sample DNA was normalised to a concentration of $1 \text{ ng } \mu\text{l}^{-1}$ and the Ct value at a normalised cycle threshold of 0.01 was plotted against *B. allii* DNA diluted to target concentrations ranging from 1 ng to the LOD of the primers (10 fg DNA). Real-time PCR assay was followed as described in Section 4.3.2.3 using the primers CRT1f/2r.

5.3.6 DNA TEST PROCEDURE OF THE PATHOGEN EMPLOYING THE DNA SENSOR

The DNA sensor based on the premix format was tested with DNA extracted from field trial samples. Ten samples were tested comprising five infected and five samples from the control group. DNA was extracted from onion neck tissue and *B. allii* (10 ng) as positive control according to Section 4.3.1 and amplified for 25 cycles with thiol-C6 and biotin-labelled primers CRT1f/2r following the PCR amplification procedure provided in Section 4.3.2.3. The DNA sensor assay was followed as described in Section 4.3.4.1.

5.4 RESULTS AND DISCUSSION

5.4.1 FIELD TRIAL DESIGN

Two field trials were performed in this study, one near Cardington (Bedfordshire, UK) and one near Littleport (Cambridgeshire, UK). The Cardington field trial site was used to evaluate the presence of other onion rots in comparison with neck rot, whereas the Littleport field trial aimed at evaluating the impact of the pathogen load tested by using the immunosensor and real-time PCR on the incidence of neck-rot disease after storage and therefore represents a key element of this study.

5.4.1.1 Evaluation of the Presence of Other Onion Rots

Besides neck rot, a variety of other diseases and disorders affect onion crops. However, the literature cannot provide answers to the distribution of these onion diseases. Therefore, the incidence of bulb rot was investigated during the Cardington (Bedfordshire, UK) field trial. Sampling over 1,000 onions grown from chemically treated and untreated sets, two major disease types were identified as the most common alternative diseases.

Table 5.1 provides an overview of the bulb rot present in chemically treated and untreated onions of the variety Forum and Jagro. Neck rot is with an overall average of 3.9 % the most common disease, followed by bacterial rot with 2.8 % and fungal basal rot with 2.1 %. As expected, bulb rot is more present in onions grown from untreated sets. Surprisingly, also the incidence of bulbs showing a dysmorphological shape was reduced by the fungicide treatment. Focussing on the overall number of diseased bulbs, the cultivar Jagro was with 88.1 % sound bulbs more susceptible to rot in comparison with the cultivar Forum (93.7 % sound bulbs). Figure 5.3 shows typical symptoms caused by bacterial soft rot and *Fusarium* base rot as well as the less common brown stain caused by *Botrytis cinerea*.

Table 5.1: Averaged incidence of onion rots recorded during the Cardington (Bedfordshire, UK) field trial 2012. The average was formed by sampling approximately 1,000 onions for signs of rot, separated in chemically treated and untreated groups, of the varieties Forum and Jagro.

Disease Type	Forum [%]		Jagro [%]	
	Treated	Untreated	Treated	Untreated
Sound	99.5	87.9	93.8	82.3
Neck rot	0.5	7.5	3.3	4.4
Bacterial rot	0	4.2	0.9	5.9
Fungal base rot	0	0.4	1.7	6.2
Mutant ^a	0	2.5	0.6	1.2

^a Bulbs showing a dysmorphological, not round shape.

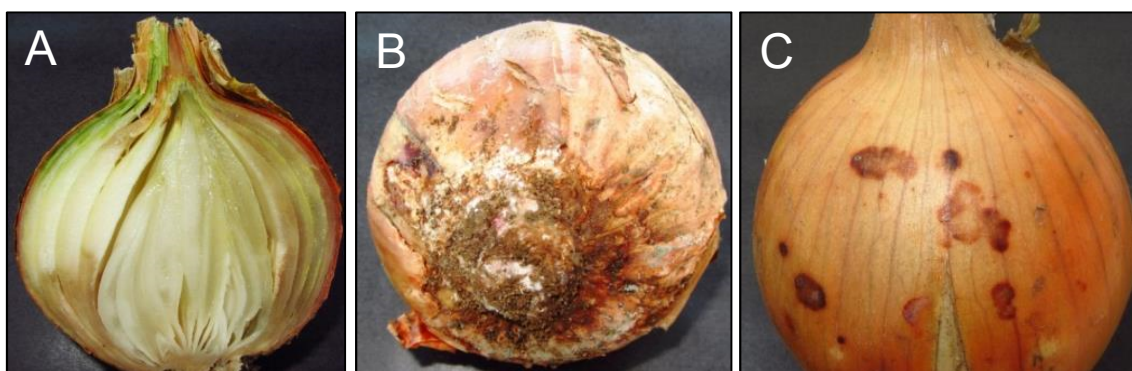


Figure 5.3: (A) Bacterial soft rot caused by *Pseudomonas* and *Erwinia* spp. (B) Outer symptoms of *Fusarium* base-rot infections. (C) Brown stain caused by *B. cinerea*. The pictures were taken during the Cardington (Bedfordshire, UK) field trial 2012.

5.4.1.2 Correlation between the Pathogen Load and the Incidence of Neck-Rot Disease in Store

The life cycle of *Botrytis* neck rot is complex and controversially discussed in literature (see Section 1.3). Its investigation is still a challenge for scientists around the world. Understanding the circumstances that lead to neck rot is therefore crucial for a

successful field trial. Neck rot is a seed-born disease (Maude and Presly, 1977a; Maude, 1983; Brewster, 1994; Stewart and Franicevic, 1994; Lorbeer et al., 2004) and therefore a seed lot with an internal infection level of approximately 6 % was used for this field trial. *Botrytis* infections of onions grown from seed with an infection rate of <10 % can spread in the field causing an epidemic of neck rot with an overall infection rate of 20 to 40 % within the crop (Maude and Presly, 1977a; Maude, 1983; Stewart and Franicevic, 1994). However, the limited storage capacity of Cranfield University required a different approach with a high level of infection in order to provide sufficient samples for the subsequent testing.

Accordingly, the plot was divided into two parts and the leaves of the onion plants were additionally inoculated with a low (10^5 spores ml^{-1}) and a high (10^6 spores ml^{-1}) *Botrytis allii* conidia solution. Since the plot did not encounter any special treatment by the collaborative onion grower, including a regular treatment with fungicides, the inoculation was repeatedly performed at monthly intervals. Furthermore, approximately 50 onions from the highly infected group were additionally inoculated with 5×10^6 spores ml^{-1} one month before harvest, representing the very high-infected group.

5.4.2 TESTING THE PRESENCE OF LATENT *BOTRYTIS* INFECTIONS USING SCANNING ELECTRON MICROSCOPY AND SELECTIVE AGAR

Since *Botrytis* infections are symptomless scanning electron microscopy was used to visualise the fungus. Figure 5.4 A provides a direct comparison between the spores of *B. allii*, and *B. aclada* and the related species *B. cinerea*. The *B. byssoidea* culture did not develop spores for the entire period of the study, which is in accordance with the findings of Presly (1985a) and Chilvers and Du Toit (2006), thus the fungal species could not be included into this comparison. Figure 5.4 B visualises a germinating *B. allii* spore using a 1,200-fold magnification, 24 h after inoculating the onion tissue. Thereafter, symptomless *B. allii* infections may be present in living tissue as mycelium (Figure 5.4 C) growing on the leaves and only become manifest in storage. From these investigations, the disease cycle can be represented as shown in Figure 5.5.

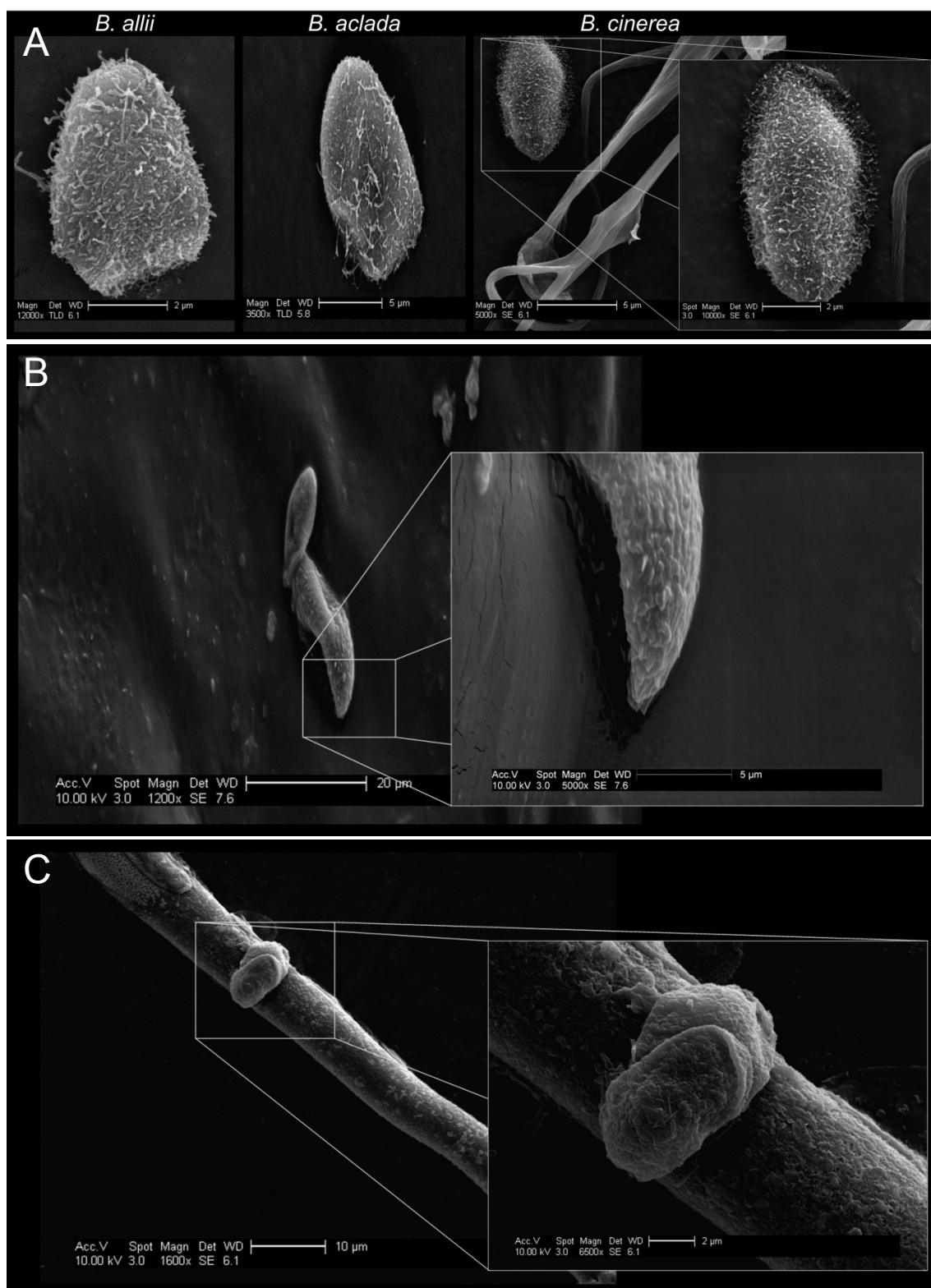


Figure 5.4: Scanning electron microscopy of (A) *Botrytis allii*, *B. aclada* and *B. cinerea* spores; (B) a germinating *Botrytis allii* spore on onion tissue 24 h post inoculation; and (C) *Botrytis allii* mycelium. The SEM was performed under vacuum after the sample surface was coated with a conductive gold nanolayer.

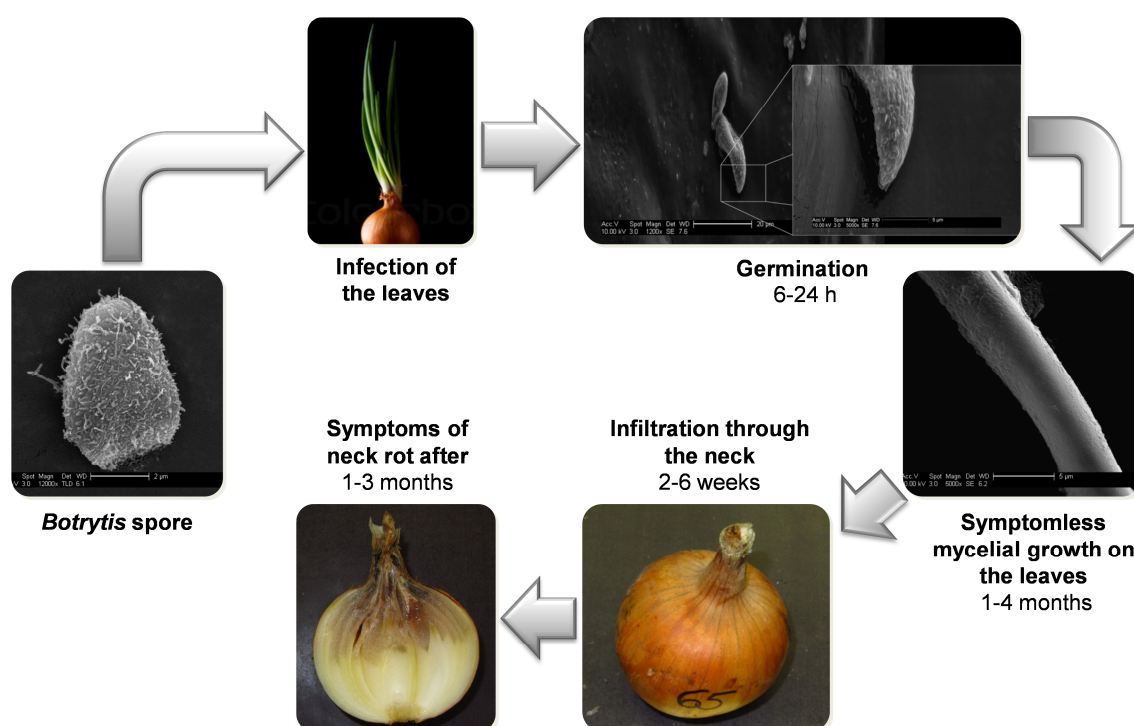


Figure 5.5: Disease cycle of neck-rot infections caused by *Botrytis*.

Additionally, the presence of viable fungus was tested after two inoculations utilising selective agar produced according to Kritzman and Netzer (1978). This media is based on the capability of the fungus to produce dark brown pigments with their extracellular enzyme using tannic acid as a substrate. Ten samples were tested comprising five samples from the low infected (sample no. 1 to 5) and five samples from the high infected side of the plot (sample no. 6 to 10). Each sample included three pieces of different leaves. Figure 5.6 shows the results of this experiment. Fungal growth was observed on all ten samples. Also other common *Fusarium* and *Penicillium* species were observed predominantly present on the samples 5, 6 and 7. Nevertheless, a selective staining was observed in nine out of ten samples indicating a successful inoculation strategy. Only sample 10 showed a strong overgrowth of non-specific fungi and selective staining could not be determined reliably.

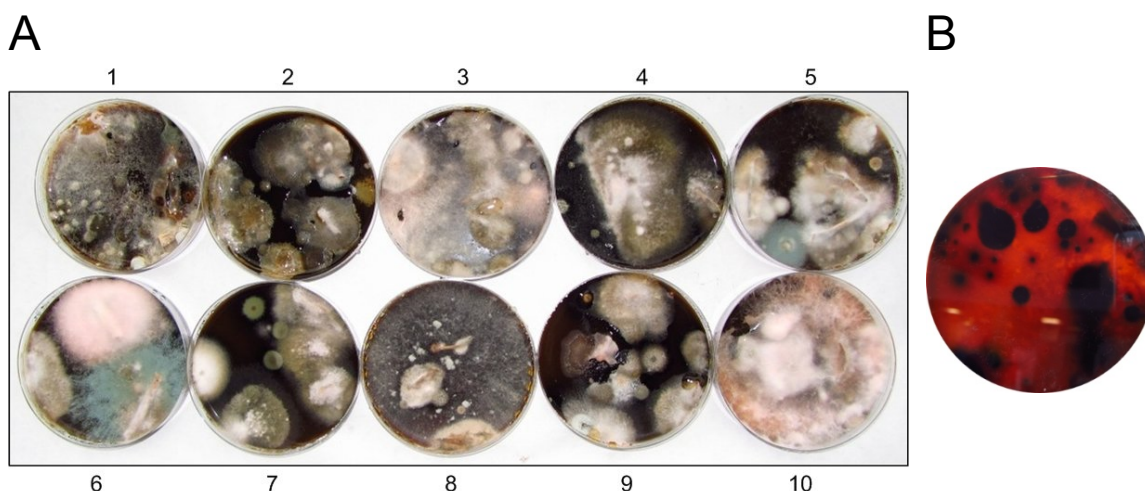


Figure 5.6: (A) Kritzman agar used to visualise the presence of symptomless *Botrytis* infections on onion leaves. Samples no. 1-5 were collected from the low infected (10^5 spores ml^{-1}) and sample no. 6 to 10 were collected from the high infected side (10^6 spores ml^{-1}) of the plot near Littleport (Cambridgeshire, UK) after two inoculations conducted one month apart. Each sample included three pieces of leaves. (B) Selective staining was produced by the extracellular enzyme polyphenol oxidase that converts tannic acid to dark brown pigments.

5.4.3 INCIDENCE OF BULB ROT AFTER FOUR MONTHS OF STORAGE

After harvesting the onion plot in September 2012, 215 onions comprising 100 from the low-infected group, 75 from the high-infected group, 25 from the very high-infected group and 15 from the control group, were numbered and stored individually according to their group. The seed from the control group was treated with fungicide and harvested from a separate plot which was not inoculated with *Botrytis*. After four months of storage at 4 °C, 96 % of the onion bulbs inoculated with a very high spore concentration, 86 % of the high, 60 % of the low and 20 % of the control group developed signs of neck-rot disease. Table 5.2 summarises the incidence of bulb rot after each month of storage with typical examples of bulb rot presented in Figure 5.7. The level of basal rot was found to be approximately 30 % for the very high and high-infected group, 20 % for the low-infected group and 13 % for the control group.

Table 5.2: Cumulated percentage of onion showing neck rot and basal rot after storage at 4 °C. Onions were grown in a separate plot in a field near Littleport (Cambridgeshire, UK) and inoculated with a very high (5×10^6 spores ml^{-1}), high (10^6 spores ml^{-1}), and low (10^5 spores ml^{-1}) concentration of *Botrytis allii* conidia solution. The seed of the control group was treated with fungicide and harvested from a separate plot.

Storage Time [Months]	Disease Type	Cumulated % Neck Rot in Groups			
		Very High (n=25)	High (n=75)	Low (n=100)	Control (n=15)
1	Neck rot	4 %	4 %	4 %	0 %
	Basal rot	4 %	4 %	3 %	0 %
2	Neck rot	46 %	30 %	14 %	0 %
	Basal rot	19 %	12 %	9 %	0 %
3	Neck rot	88 %	77 %	38 %	13 %
	Basal rot	27 %	26 %	13 %	7 %
4	Neck rot	96 %	85 %	60 %	20 %
	Basal rot	27 %	32 %	20 %	13 %

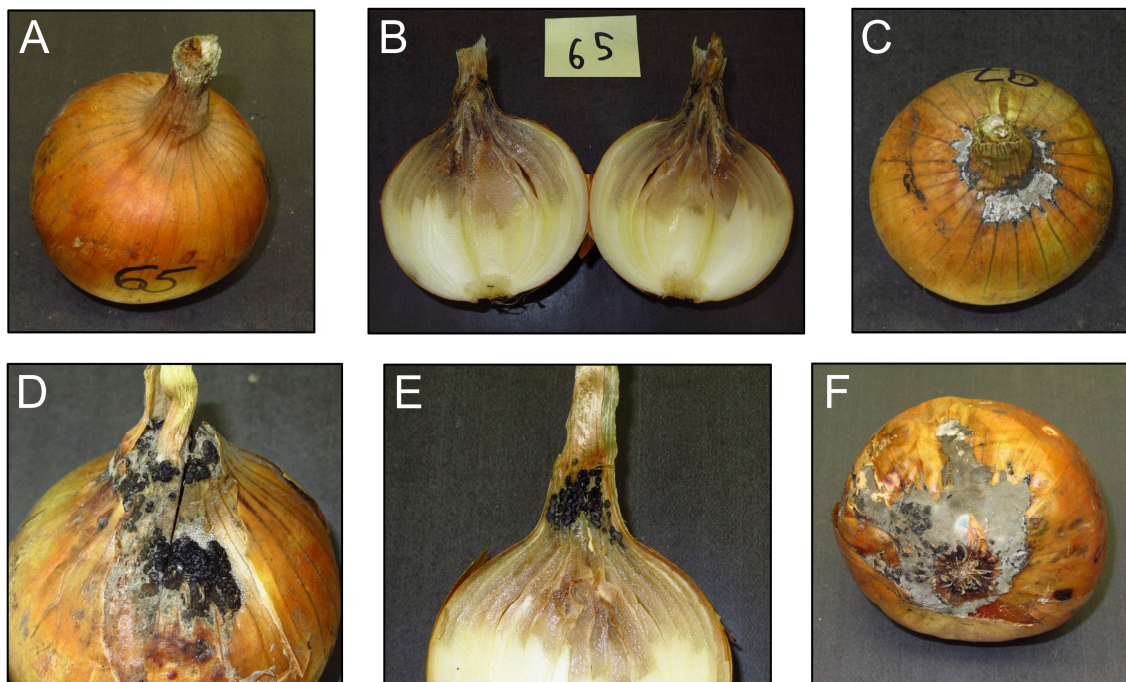


Figure 5.7: Stored onion harvested from the Littleport (Cambridgeshire, UK) filed trial 2012 with no outer symptoms of infection (A), but with neck-rot disease visible after sampling the bulb (B). Severe neck rot with sclerotia (D and E) and less common outer symptoms of neck (C) and base rot (F) caused by *Botrytis*.

A correlation between the concentration of *Botrytis allii* spore used for the inoculation of the leaves and the severity of neck-rot as well as base-rot infection of the onion bulbs after four months of storage was observed. This results in a variety of infection levels within the onion samples. However, not all the samples are suitable for further testing. Samples with severe basal rot as well as too short neck tissue were excluded, leaving approximately 120 samples suitable for testing. After the incidence of neck rot was established, a total of 71 samples of the neck tissue frozen at -80 °C comprising a balanced number of all groups were tested and the pathogen load was determined using the immunosensor and quantitative real-time PCR. The results are presented in the following Sections.

5.4.4 QUANTIFICATION OF THE PATHOGEN LOAD USING THE IMMUNOSENSOR

To test the performance of the immunosensor, samples of onion neck tissue collected and frozen at harvest were quantified using the immunosensor. The sample pool comprised a balanced number of the very high, high and low infected group as well as control samples which were harvested from a separate plot. Of further interest are also infected samples that did not develop neck rot in store. This experiment aims at investigating the precise link between pathogen load of *Botrytis* and the incidence of neck-rot disease in store.

5.4.4.1 Correlation between the Initial Inoculation of the Test Groups and Their Pathogen Load

The results of the storage experiment (see Section 5.4.3) proved a correlation between the concentration of *Botrytis allii* spore used for the inoculation of the leaves and the severity of neck-rot infection after four months of storage. These findings were confirmed in tests using the immunosensor. Comparing the median of the pathogen load of each group reflects the inoculum of *B. allii* conidia used to infect the onion leaves (Figure 5.8). However, at this stage the groups are heterogeneous and do not reflect the incidence of neck rot over time. Groups are therefore not considered as significantly different at a confidence level of 95 %, except for the control versus the high group.

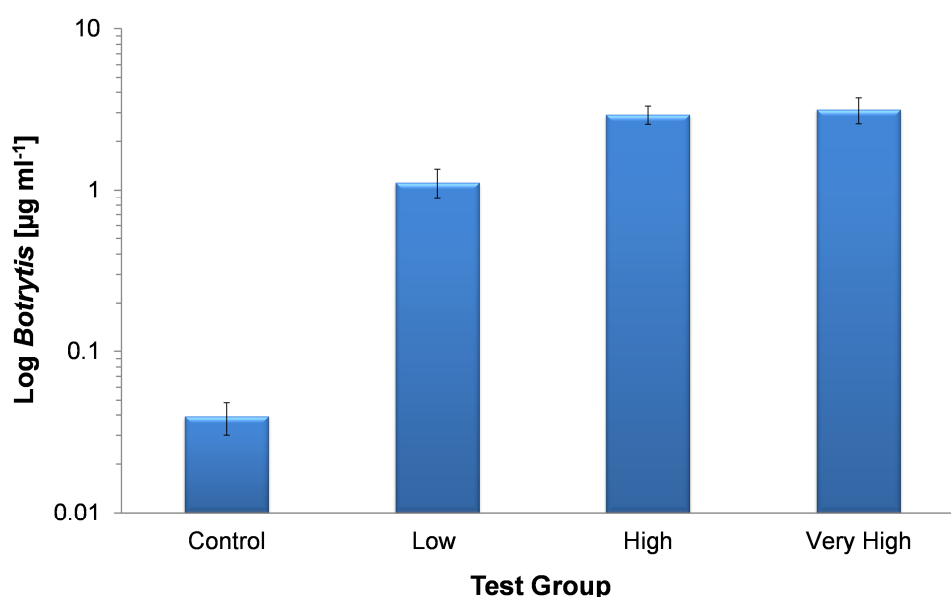


Figure 5.8: Median pathogen load based on the 25th / 75th percentile of each group quantified using the immunosensor. Test groups were inoculated five times on a monthly interval with a low (10^5 spores ml⁻¹) and a high (10^6 spores ml⁻¹) *Botrytis allii* conidia solution. The very-high infected group was additionally inoculated with 5×10^6 spores ml⁻¹ one month before harvest. The bars represent the standard error of the median (n=71).

5.4.4.2 The Influence of the Pathogen Load on the Severity of Neck-Rot Disease

Comparing the test groups to each other did not yield sufficient information to establish a direct link between the pathogen load and the incidence of neck rot in store. This is due to the heterogeneity of the groups. For instance, within the infected and control group there are samples that developed neck rot after various time spans as well as negative samples with no rot. Though on average a trend was observed between the infection grade and the initial inoculum represented by the test groups, a different approach was needed to visualise the direct impact of the pathogen load on neck-rot disease.

Figure 5.9 summarises the pathogen load of the samples relative to the time until symptoms of neck rot were visible. Here, a clear correlation between the initial inoculum and the severity of the disease was observed. The pathogen load of the groups developing neck-rot disease was found to be significantly different from the control using one way ANOVA analysis of the variance and *T*-tests at a confidence level of 99 % (except 3 vs. 4 months). However, the overall objective of the immunosensor analysis is to prescribe an effective curing regime based on the pathogenic risk. Accordingly, a threshold was established at $0.1 \mu\text{g ml}^{-1}$ in order to differentiate between infected and sound samples. The value is based on the pathogen load of the negative group (see Section 5.4.4.3) as well as the limit of quantification and variance of the assay.

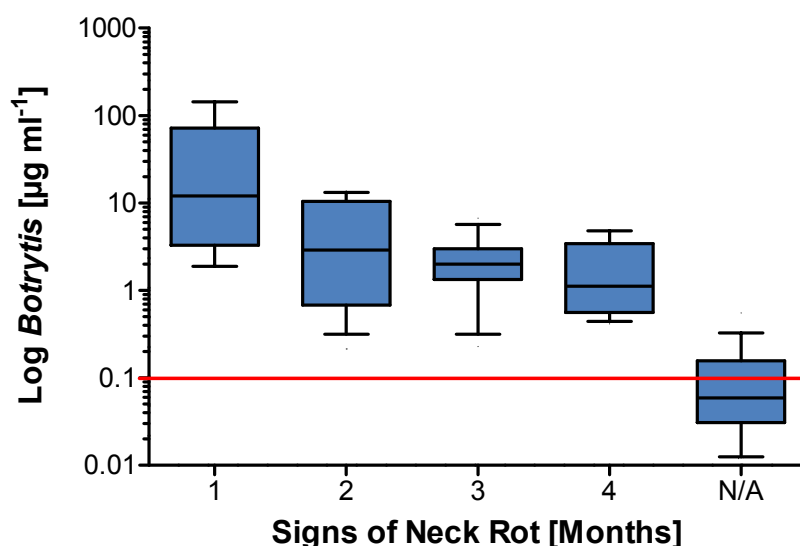


Figure 5.9: Box plot showing the distribution of the pathogen load ($n=71$) determined by the immunosensor relative to the period of months until symptoms of neck rot were visible. Boxes comprise the 25th / 75th percentile of the samples as well as the median of the group. Whiskers represent the 90th percentile of each group; the red line illustrates the threshold for positive detection.

Employing this threshold, 90 % of the samples quantified using the immunosensor with a pathogen load $>0.1 \mu\text{g ml}^{-1}$ developed neck-rot disease within four months of storage. Only one sample was tested negative but developed neck rot after three months. However, evaluating the success rate of 90 %, other factors were identified that have an impact on the incidence of neck-rot disease. Focussing on the remaining 10 % of the

group that were inoculated and tested positive for *Botrytis* infections, but did not develop neck rot, 72 % of the samples were observed to have a small, short or closed neck, suggesting that morphological differences between the onion bulbs can have an impact on the susceptibility of the bulbs to the fungal disease.

Exemplary for these findings are the samples that were inoculated with spore solution but did not develop neck rot (Figure 5.10). Sample numbers 103, 104, 112, and 123 did not yield sufficient pathogen load for a positive detection of the fungus, whereas samples 87, 105 and 128 were tested positive for *Botrytis* infections with a pathogen load ranging from 0.17 to 0.26 $\mu\text{g ml}^{-1}$. However, whether this low level of *Botrytis* is sufficient to develop neck-rot disease is questionable. In contrast, samples 64, 187 and 197 clearly showed an increased level of infection with 0.51 to 14.6 $\mu\text{g ml}^{-1}$, but did not develop neck rot in store.

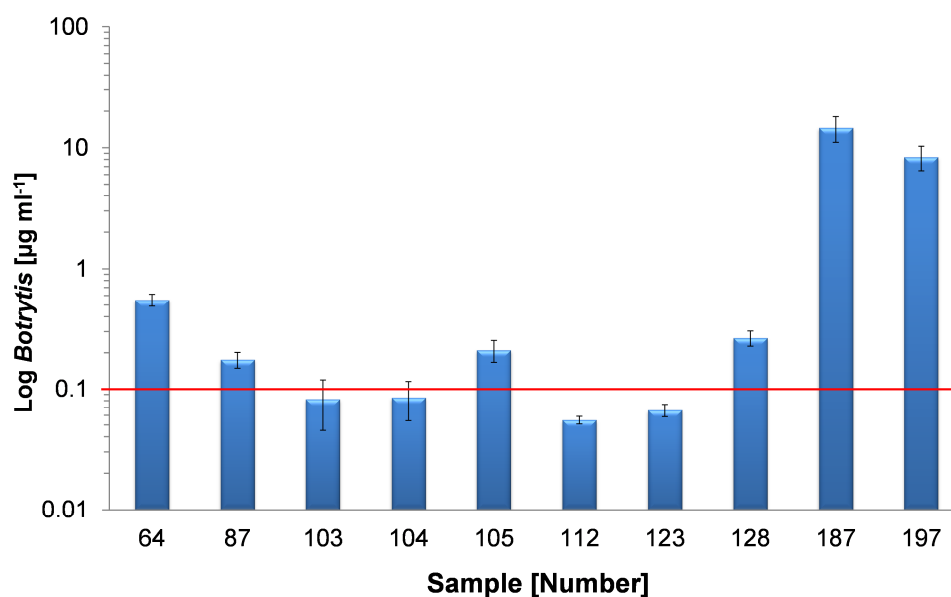


Figure 5.10: Quantitative measurements of ten onion neck samples that did not develop neck rot in store, though the onion plants were repeatedly inoculated with *B. allii* spore solution. Samples 64 and 87 were harvested from the high-infected side of the plot, whereas the remaining samples were part of the low-infected group. Pathogen load was determined using the immunosensor, the red line illustrates the threshold for positive detection, the bars represent the standard error of the means (n=4).

Evaluating the neck section, samples 187 and 197, which were tested with a high level of infections, showed a relatively closed neck for sample 187 (Figure 5.11 A) and a short neck for sample 197 (Figure 5.11 B), respectively. The leaves emerging directly from the top of the neck are the primary infection court for *Botrytis*, from which the fungus starts to grow towards the bulb (Tichelaar, 1967; Maude and Presly, 1977a). Closed or short necks were reported to inhibit the fungal growth (Maude et al., 1984; Downes et al., 2009). Breeding plants that feature a closed or short neck tissue can be a valid strategy for the control of neck rot by decreasing the susceptibility to neck-rot disease.

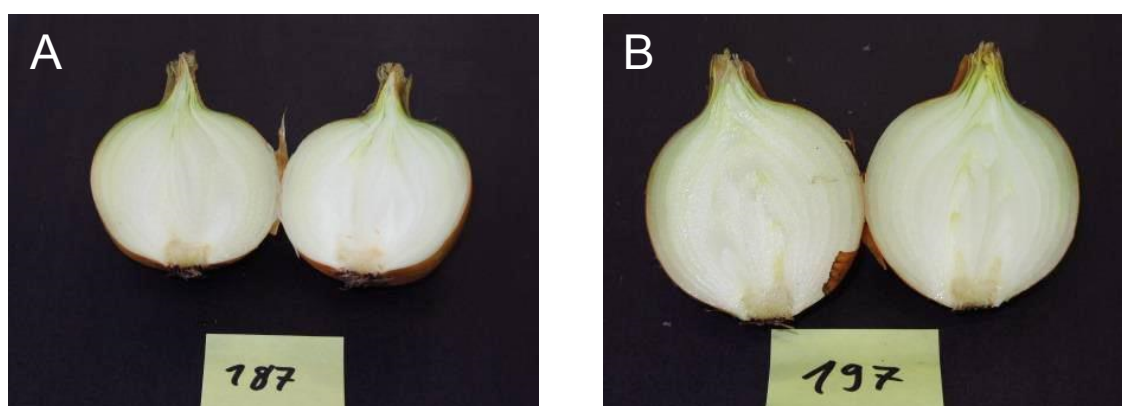


Figure 5.11: Cross section of the onion bulbs (A) 187 and (B) 197 after four months of storage at 4 °C. Leaves of the onion plants were inoculated five times with 10^5 spores before harvest.

5.4.4.3 Representative Test Procedure for Neck-Rot Disease Utilising the Immunosensor Based on the Control Group

The previous Section demonstrated the capability of the immunosensor in predicting pathogenic risk of neck rot in store with a success rate of 90 % using a threshold of $>0.1 \mu\text{g ml}^{-1}$ for pathogen detection. However, the overall objective of the immunosensor development is to prescribe a customised curing regime, based on the local pathogenic risk. Beyond doubt, the level of neck rot, as a result of numerous inoculations with *Botrytis allii* spores, was necessary regarding the need of various infection levels within the samples for testing, but does not reflect the real incidence of neck rot which is in general less than 10 % (see Section 5.4.1.1). From a developer's

point of view, a system is needed that involves the detection of the pathogen load within a rational sample size and realistic infection grade. In this Section the control group comprising 15 non-infected samples was used to simulate a testing procedure employing the immunosensor. The seeds of these samples represent a genuine pathogenic risk since they were treated with fungicide and the seedlings were planted on a separate plot, eliminating a direct contact to the infected groups. Furthermore, the onion plants were repeatedly treated with fungicides during their growth stage as part of the standard procedure to suppress neck-rot disease.

The quantitative measurements of the control group are presented in Figure 5.12. Four samples were tested positive at a threshold of $>0.1 \mu\text{g ml}^{-1}$, N4, N10, N14 and N15. The pathogen load within the positive samples is ranging from $0.33 \mu\text{g ml}^{-1}$ (N10) to $2.0 \mu\text{g ml}^{-1}$ (N15). The remaining samples, except for N7 and N3, are below the limit of quantification of approximately 50 ng ml^{-1} . The storage experiment of the corresponding onion bulb showed that without further treatment the samples N4, N14 and N15 developed neck rot in store.

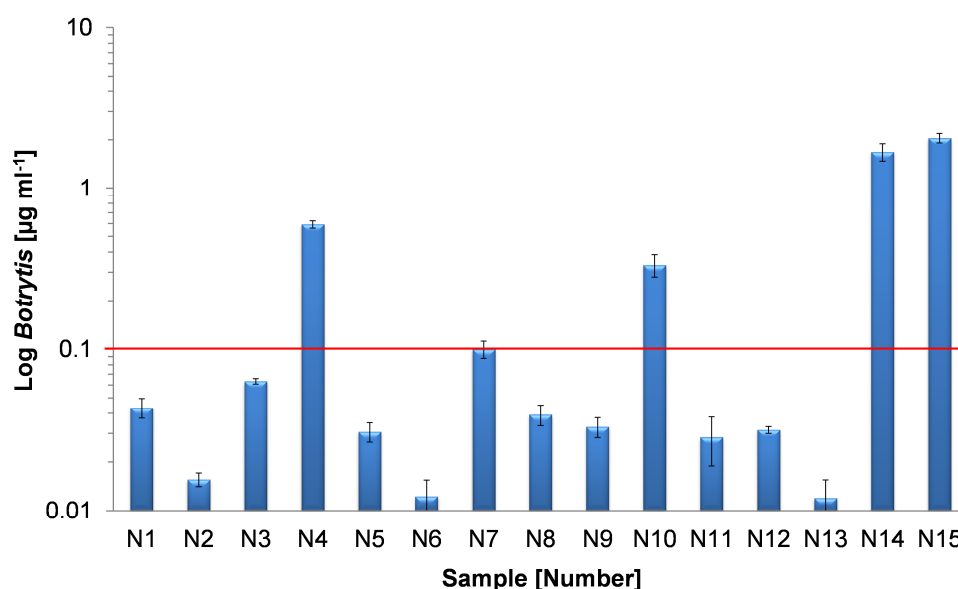


Figure 5.12: Immunosensor quantification of the pathogen load of the control group. The seed of the control group was treated with fungicide and harvested from a separate plot. The red line illustrates the defined threshold for positive detection, the bars represent the standard error of the means (n=4).

From a grower's perspective, after detecting the pathogen in 27 % of the test samples, an increased risk of neck rot can be assumed. Data from the storage experiment showed, that without further treatment, three out of four samples would develop neck-rot disease in store. However, the pathogen load was tested with samples taken before harvest. At this stage, the onion bulbs can be cured post-harvest by appropriate drying conditions. Reflecting the results of the sample testing, a high curing temperature of $>28^{\circ}\text{C}$ for 3 to 6 weeks is appropriate since the pathogen load is relatively high. Over time, expertise can base the post-harvest storage conditions on the local pathogenic risk, as high curing temperature may not be appropriate for all geographic regions and growing seasons with alternating weather conditions. High-temperature curing can promote internal defects caused bacterial rot (*Pseudomonas* spp.), thus when pathogen load is low, curing can be performed at a lower temperature, which is beneficial for quality and can reduce energy costs (Downes et al., 2009; Chope et al., 2012).

5.4.5 COMPARATIVE STUDIES WITH THE COMMERCIAL ELISA KIT AND RT-PCR

Comparative studies were performed to ascertain the quality of the developed electrochemical immunosensors in assessing the pathogen load in comparison with the commercially available ELISA kits and real-time PCR. The onion samples were analysed using the various detection methods, and the quantitative measurements were evaluated.

5.4.5.1 Quantification Using the Commercial *Botrytis* ELISA Kits

Preliminary studies using the commercial ELISA kits were performed with four samples, two were part of the very high infected group (I1 and I2) and two samples from the control group (N1 and N6). The storage experiment conducted in Section 5.4.3 showed that the positive samples developed rapidly severe neck rot in store and the negative samples were free of rot. The absorbance measurements of the samples are presented in Figure 5.13. Due to the high influence of non-specific proteins, the data were not considered as being significant at a confidence level of 95 %. All absorbance values were below the LOD of the assay (negative+3xSD) and could not be quantified.

Diluting the standards in 1:5 leaf extract did also not ease the influence of non-specific proteins. These results are in accordance with Section 3.4.6.1, where the poor format of the ELISA kits was found to provide insufficient sensitivity for the quantification of onion samples.

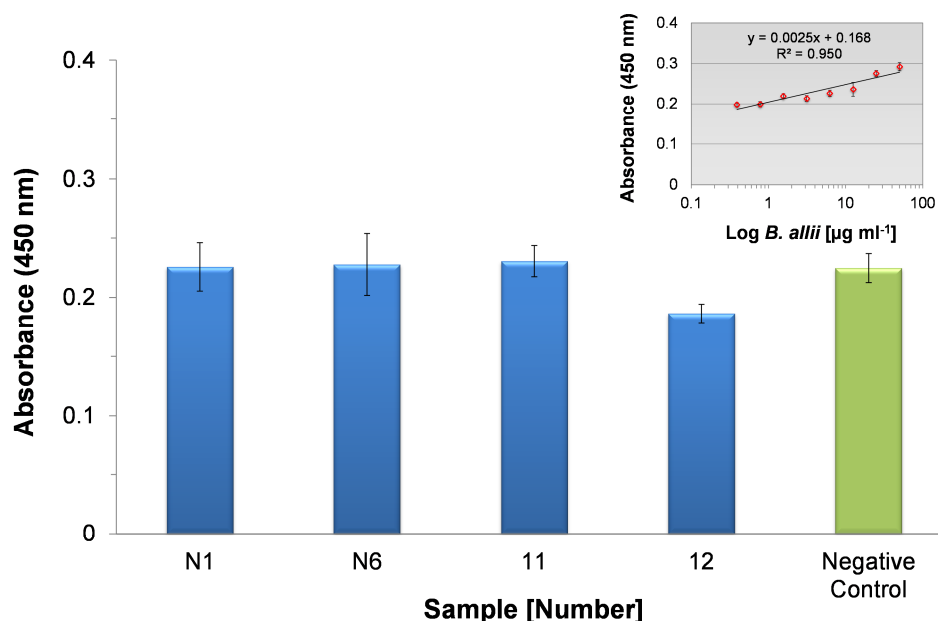


Figure 5.13: Absorbance measurements at 450 nm of the control samples N1 and N6 and high infected samples 11 and 12. The negative samples showed no rot after four months of storage, whereas the high infected samples had severe bulb rot. *B. allii* serially diluted in onion leaf extract was used to construct a standard curve for the immunoassay ranging from 50 to 0.4 $\mu\text{g ml}^{-1}$ (embedded graph). The data are plotted as average of the *B. allii* and *B. cinerea* ELISA kits, the bars represent the standard error of the means (n=4).

5.4.5.2 Real-Time PCR Quantification of the Pathogen Load

As discussed in the previous Section, the commercially available *B. allii* and *B. cinerea* ELISA kits did not yield sufficient sensitivity to quantify the pathogen load of latent *Botrytis* infections. Instead, real-time PCR proved to be the only detection system capable of ascertaining the pathogen load and providing a comparative reference for the developed immunosensor. The results of the DNA testing procedure are presented in Figure 5.14. The quantitative measurements of the various infection levels were

considered significantly different using one-way ANOVA analysis and *T*-test of the infected groups versus the control (max *P*-value 2.9 %). However, due to an overlap of the samples that developed neck rot after three months and the control, the means were not found to be statistically different with *P*=11.9 %. Similar to the immunosensor quantification, a threshold was established in order to evaluate the quality of the detection method. Employing a threshold of 1 fg fungal DNA as bottom line for positive detection, real-time PCR was capable of predicting the incidence of neck rot in store with a success rate of 89 % with only two false negative samples (3 %).

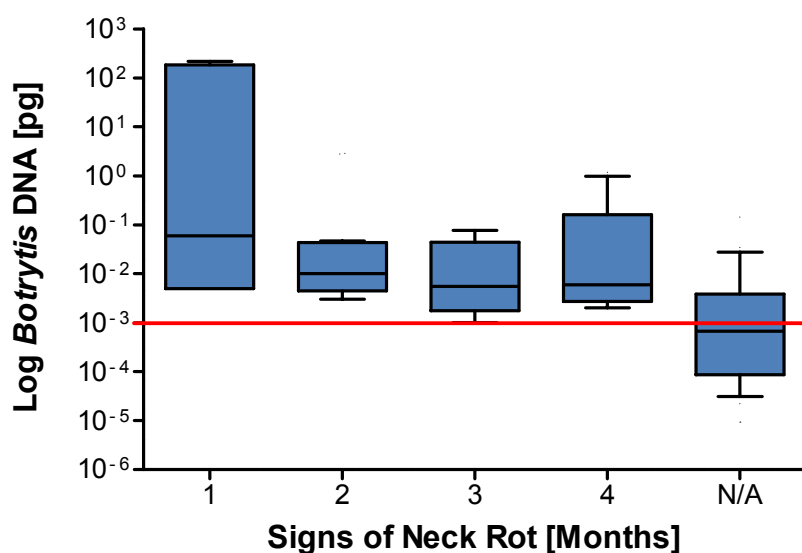


Figure 5.14: Box plot showing the distribution of the pathogen load (n=71) determined by real-time PCR relative to the period of months until symptoms of neck rot became visible. Boxes comprise the 25th / 75th percentile of the samples as well as the median of the group. The whiskers represent the 90th percentile of each group; the red line illustrates the threshold for positive detection.

Recently, the *Botrytis* research has intensified using genetic applications. PCR and real-time PCR assays were developed for the specific detection and separation of various *Botrytis* species. Nielsen et al. (2002) developed the RFLP primers with a limit of detection of approximately 1 pg of pure fungal DNA. Tests with plant material inoculated with 500 spores demonstrated the capability of the primers of detecting latent *B. aclada* infections in leaf pieces 48 h post inoculation. However, though Walcott et al. (2004) improved the DNA extraction procedure by employing magnetic capture

hybridisation (MCH), the primers BA1r/2f were not designed for quantitative measurements and therefore less sensitive compared with the primers CRT1f/2r described by Chilvers et al. (2007), which were utilised for the real-time PCR assay. The primers were designed to detect *B. aclada* spores in seed lots, alleviating the need to conduct a time-consuming agar assay. Though this method is highly sensitive, pathogenic quantification is delicate due to fluctuating efficiency of the DNA extraction and PCR amplification (Chilvers et al., 2007).

This is the first report of successful detection of neck-rot infections in field using real-time PCR. The success rate of 89 % with only few false negative samples demonstrated the potential application of real-time PCR to predict neck-rot disease in store. The fluctuating efficiency of the DNA extraction was overcome by employing a standardised sample extraction protocol and normalisation of the isolated DNA template. The quantification was achieved by constructing a standard curve based on purified pathogen DNA. However, an application of this method in field conditions is restricted due to the high demand in special equipment for the DNA testing procedure.

5.4.5.3 Correlation between Immunological and DNA Quantification of *Botrytis*

The quantitative measurements of the developed immunosensor and real-time PCR assay were compared in this Section and evaluated regarding key performance parameters. Comparing the quantitative measurements against the time until symptoms of neck rot became visible, achieved using the immunosensor (Figure 5.9) and real-time PCR (Figure 5.14), show a significant linear trend for the immunological ($R^2=0.26$) as well as the genetical detection method ($R^2=0.19$) at a confidence level of 95 % using one-way ANOVA analysis. Accordingly, both methods can be used to predict the incidence of neck rot in store based on the pathogen load estimated before harvest. Though immunological and genetic detection of *Botrytis* are fundamentally different techniques, a strong agreement ($R^2=0.30$) was found plotting the individual data against each other using double logarithmic non-linear regression (Figure 5.15).

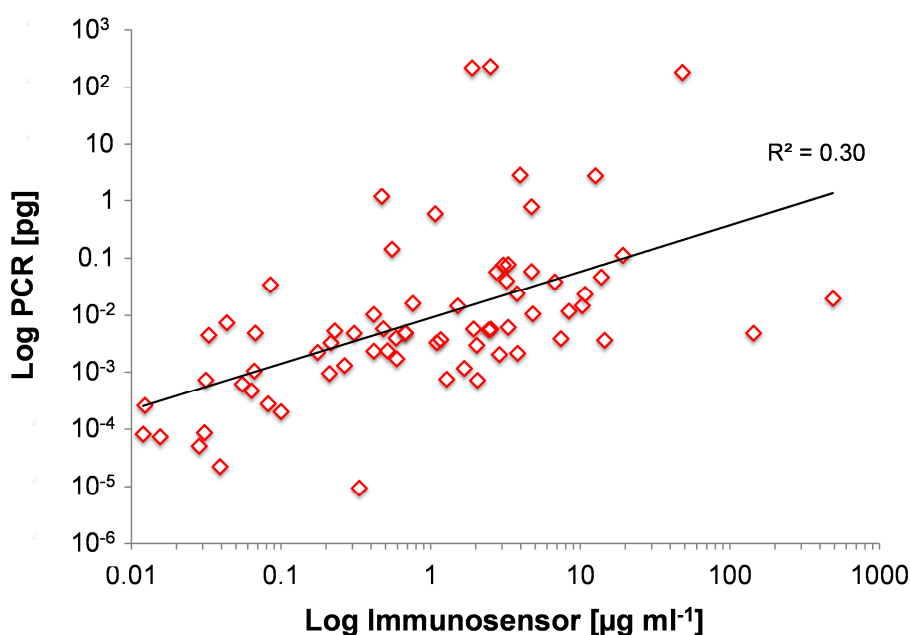


Figure 5.15: Log. / log. non-linear regression of the quantitative measurements (n=71) of the immunosensor versus real-time PCR. Immunosensor quantification is based on immunologic recognition of *Botrytis* mycelium, whereas real-time PCR detection is targeting pathogen DNA.

Evaluating the performance of both detection methods employing a threshold for positive detection, the immunosensor had a success rate of 90 % with 1 % false negative, whereas PCR predicted the incidence of neck rot in store with a success rate of 89 % and 3 % false negative samples. In retrospect, even if these numbers vary only marginally, the immunosensor proved to be a more reliable method of detecting the pathogen load in onion samples. The infected groups were tested as significantly different from the control at a confidence level of 99 % with a maximum *P*-value of 0.22 %, whereas real-time PCR showed a stronger overlap between the infected and control groups with only three out of four groups being significantly different at a lower confidence level of 95 %.

Furthermore, the threshold for positive detection in PCR assays was set to 1 fg fungal DNA, which is ten times lower than the limit of detection of the primers (see Section 4.4.2.1 performance studies). Using the LOD as a threshold instead would lower the success rate of real-time PCR to 56 %. In comparison, the threshold of the

immunosensor was defined as 100 ng ml^{-1} being six times higher than the limit of detection (16 ng ml^{-1}) and three times higher than the limit of quantification (34 ng ml^{-1}). This was achievable due to a superior distinction of the infected groups and led to a more unconstrained detection compared to PCR. Additionally, the enhanced sensitivity of the immunosensor enables a facile adaptation of the threshold value according to the application. For example, applying a threshold of $0.35 \mu\text{g ml}^{-1}$ maximises the success rate for positive detection to 96 %, however, the numbers of false negative detection increases likewise to 6 %. Real-time PCR does not offer this advantage since it was operated on the limit.

The sample processing represents another drawback of PCR. To ensure full performance of the assay, DNA must be extracted that yields high quality. This involves various purification and test steps, which are time consuming and require special equipment, such as centrifuges, 60°C incubators, spectrophotometer and filter tips (see Section 4.3.1). In contrast, the sample extraction of the immunosensor can be performed in field conditions by using a mortar and pestle or a tube comprising grinding beads, followed by rough filtration employing an inexpensive sample extraction bag. Moreover, the sample extraction protocol for immunological detection is less susceptible to contaminations.

5.4.6 DNA TEST PROCEDURE OF THE PATHOGEN EMPLOYING THE DNA SENSOR

To assume that real-time PCR is a potential alternative to the immunosensor is questionable regarding the high investment required for the machinery and special equipment needed for the analysis. However, providing information about an appropriate curing regime can also be based on the number of infected samples within a test group instead of quantitative values. If the operator requires a more specific response targeting only particular species of *Botrytis* that immunological methods cannot provide, the developed DNA sensor can be utilised as a low-cost alternative to real-time PCR.

The performance of the DNA sensor in distinguishing between positive and negative samples was tested with ten onion neck tissue samples extracted from the field trial. Infected samples contain two samples from the very high, two samples from the high, and one sample from the low-infected group as well as five samples from the non-infected control group. The DNA sensor signal is presented in Figure 5.16. All infected samples which developed neck rot in store and were tested positive for *Botrytis* using the immunosensor and real-time PCR were tested positive using the DNA sensor and showed a similar current compared with the positive control (*Botrytis allii*). Control samples which were tested negative in previous immunosensor and real-time PCR assays resulted in a low amperometric signal. The positive and negative samples passed the *T*-test with $P < 0.01$ % demonstrating the capability of the DNA sensor to reliably distinguish between infected and not infected samples.

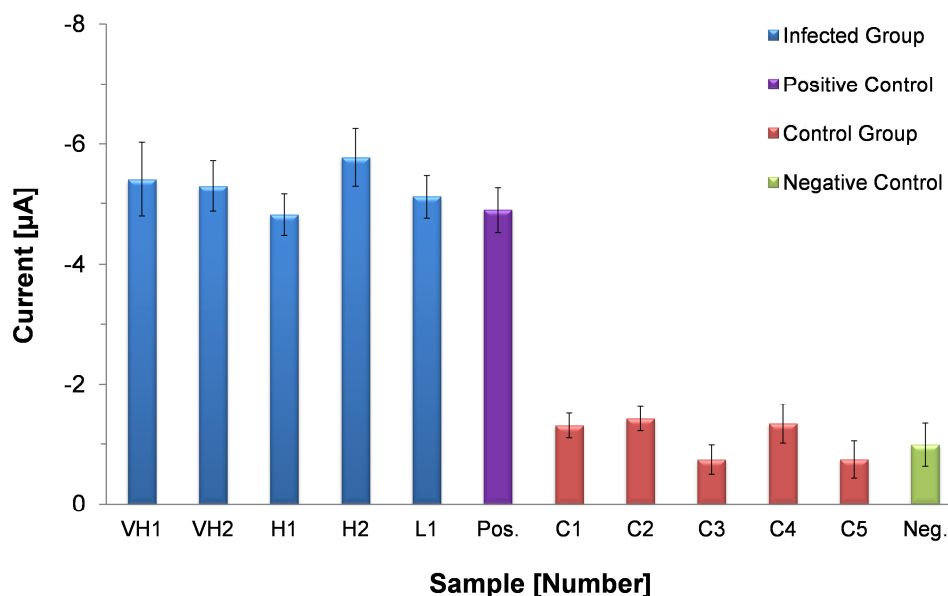


Figure 5.16: Normalised DNA sensor signal tested against samples from the very high (VH), high (H) and low (L) infected group as well as control samples (C) which did not develop neck rot in store. Template DNA, positive (*B. allii*) and negative control was amplified with thiol-C6 primers for 25 cycles and subsequently premixed for 2 h with the streptavidin-HRP conjugate (20 µg ml⁻¹). The bars represent the standard error of the means (n=4), average CV=5.3 %.

5.5 CONCLUSIONS

The factors that impact the development of neck rot in store are controversially discussed in literature and largely unknown. Though the correlation between *Botrytis* infections and the incidence of neck rot in store is an established causal fact confirmed in numerous field trials conducted during the 70s and 80s, very little is known about the precise influence of the pathogen load on the development of the disease.

A plot of approximately 350 onions were planted in Cambridgeshire and infected with *B. allii* spore solution according to their group. Latent infections were present in living tissue, which were tested positive in nine out of ten samples using selective agar, but only becomes manifest in storage. The first stage of the field trial was to establish the infection grade of the disease. After four months of storage at 4 °C, 96 % of the onion bulbs inoculated with a very high spore concentration, 86 % of the high, 60 % of the low and 20 % of the control group developed signs of neck-rot disease, proving a significant link between the initial inoculum and the severity of the disease.

The second stage involved quantifying the pathogen using the immunosensor, the commercial ELISA kits and real-time PCR with the objective to manifest the impact of the pathogen load on the incidence of neck rot in store. The ELISA kits were highly influenced by the presence of non-specific proteins and were not used for further analysis, thus reproducing the findings of previously conducted ELISA tests with plant material. In contrast, the immunosensor was capable of predicting neck rot with a success rate of 90 % with 1 % false negative sample using a threshold of 0.1 µg ml⁻¹. The threshold was based on a multilevel approach considering various factors, such as limit of detection / quantification, assay variation and quantitative measurements of the control group. However, this value can be adapted according to the application. For example, applying a threshold of 0.35 µg ml⁻¹ maximises the success rate for positive detection to 96 %, however, the numbers of false negative detection increases likewise to 6 %. From a grower's point of view, a conservative threshold of 0.1 µg ml⁻¹ is preferable since false negative samples can lead to misjudgement of the pathogenic risk, whereas positive detected samples can be cured by employing appropriate countermeasures, such as fungicides or increased drying temperature or drying period.

The fungal infection rate is highly dependent on various factors, including humidity, initial pathogen load of the seed and infection rate in field. A high curing temperature may therefore not be appropriate for all geographic regions and growing seasons with alternating weather conditions. A quantification of the pathogen load, pre or post-harvest using the immunosensor can give the growers the potential to employ a customised curing regime of the onion bulbs based on the risk of the local neck-rot infections. However, though the immunosensor successfully visualised the pathogenic risk in a genuine test group provided by the not infected control group harvested from a separate plot, additional factors were identified that have an impact on the development of the disease. Focussing on the remaining 10 % of the group that were inoculated, tested positive for *Botrytis* infections, but did not develop neck rot, 72 % of the samples were observed to have a small, short or closed neck. Breeding plants that promote these features can be a valid strategy for the control of neck rot by decreasing the susceptibility to neck-rot disease.

Alternatively, real-time PCR can be used to detect *Botrytis* infections. The quantitative measurements of the two methods showed strong agreement and the success rate of 89 % with only few (3 %) false negative samples demonstrated successfully that real-time PCR can be a powerful tool for pre and post-harvest disease control. However, real-time PCR gave inferior performance compared to the immunosensor, and assuming that real-time PCR is a potential alternative to the immunosensor is questionable considering the high investment required for the machinery and special equipment needed for the analysis. Alternatively, the DNA sensor can be used as a low-cost detection tool if the operator requires a more specific response, targeting only particular species of *Botrytis*. The DNA sensor was capable of distinguishing between DNA extracted from positive and negative samples with a success rate of 95 % (n=40), confirming the results of quantitative real-time PCR and immunosensor. Additionally, no inhibiting effect of plant tissue on the PCR amplification was observed demonstrating the robustness of the DNA sensor system.

CHAPTER SIX

DESIGN OF A MICROFLUIDIC SYSTEM FOR THE IMMUNOSENSOR

6 DESIGN OF A MICROFLUIDIC SYSTEM FOR THE IMMUNOSENSOR

6.1 INTRODUCTION

Lateral-flow immunosorbent assays (LFiAs) have dominated rapid point of care diagnostic since the first commercially available one-step test devices have been released in the late 80s (Davies et al., 2007b). These formats gained popularity in the consumer markets due to their portable and disposable design and their easy use, alleviating the need of specialist laboratories. However, the lack of quantitation was the major obstacle that has challenged the demand of existing assay formats in consumer markets (Yetisen et al., 2013). To compensate this flaw, line readers emerged for quantitative measurements. However, the provided sensitivity is often insufficient for the target applications, including the detection of latent *Botrytis* infections. Though the commercially available *Botrytis* LFDs from Forsite Diagnostics (Sand Hutton, UK) and EnviroLogix (Portland, USA) can provide a useful tool for winemakers to detect *Botrytis cinerea* (Dewey et al., 2013), the limit of detection is not sensitive enough to detect *Botrytis* infections at a low level prior to showing visible symptoms.

Lateral-flow assays are enhanced by several factors, including the labelling and signal generating system. If a simple to use and reliable lateral-flow sample delivery system can be combined with the high sensitivity of an electrochemical transducer, a powerful tool would be created that can revolutionise pathogen detection in the field.

This study aims at combining the advantages of a sensitive electrochemical detection system based on screen-printed gold electrodes with the robust design of lateral-flow devices. From a product development point of view, it is more desirable to have an assay which combines the sample introduction, filtration, antigen-antibody interaction and sample generation in one device, instead of a step-by-step approach which is predominantly used for most plate and sensor-assay formats. Crucial for the success of a portable biosensor is a customised housing which comprises all components, and hence facilitates the handling of the detection system.

6.2 MATERIALS

6.2.1 GENERAL CHEMICALS AND INSTRUMENTATION

Albumin from bovine serum (BSA), horseradish peroxidase (HRP), HPLC grade dH₂O, phosphate buffered saline tablets (PBS, 0.01 M phosphate buffer, 0.137 M sodium chloride and 0.0027 M potassium chloride, pH 7.4), Tween 20, potassium chloride, 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB), hydrogen peroxide solution (30 % w/w) in dH₂O, carbonate-bicarbonate buffer (0.05 M, pH 9.6), phosphate-citrate buffer (0.05 M, pH 5.0), 3,3-dithiodipropionic acid (DTDPA) and sodium acetate were purchased from Sigma (Dorset, UK).

The monoclonal antibody BC-12 and LFD sample extraction buffer containing surfactants was acquired from Forsite Diagnostics (Sand Hutton, UK). The polyclonal *B. allii* antibody and negative control was obtained from Neogen Europe Ltd. – ADGEN Phytodiagnostics (Auchincruive, UK). Antibodies were conjugated to HRP using the Lightning-Link conjugation kit purchased from Innova Biosciences Ltd. (Cambridge, UK) and purified with a Pierce conjugate purification kit purchased from Thermo Scientific (Cramlington, UK). N-hydroxysuccinimide (NHS) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) were purchased from Thermo Scientific (Cramlington, UK). Gold nanoparticles size Ø40 and Ø60 nm were purchased from BBI International (Cardiff, UK). Milk protein blocking solution concentrate was purchased from KPL (Gaithersburg, USA).

Electrochemical measurements and biosensor related machinery and equipment are equivalent to the materials and methods Section described in Chapter 3.

6.2.2 CRANFIELD DESIGN LATERAL-FLOW STRIP

The lateral-flow (LF) strip for the electrochemical *Botrytis* lateral-flow sensor (E-Bot LFS) was designed in this project by Cranfield University and produced by Forsite Diagnostics (Sand Hutton, UK). A Hi-Flow Plus 180 nitrocellulose membrane (25 mm long and 5 mm wide) was cast to a polyester support (235 μm thick). The Hi-Flow Plus 180 membrane has a capillary rise time for water of 180 s per 4 cm and is 130 μm thick. The monoclonal antibody BC-12 (3 mg ml^{-1}) was deposited at a reagent dispense rate of 1 $\mu\text{l cm}^{-1}$ to create the test line which is located in the middle of the membrane. In comparison to the Pocket Diagnostic *Botrytis* LFD, the Cranfield design strip has a 3 mm wide antibody line and no control line for anti-species conjugate since the reading is performed electrochemically. The line location of the Cranfield design lateral-flow strip compared with the Pocket Diagnostic *Botrytis* LFD is illustrated in Figure 6.1. Blocking was performed by immersing the membrane for 15 s in a protein solution, before passing the LF-strip through a series of vertical drying towers. The strip was completed by incorporating a glass-fibre sample pad and a cellulose-fibre absorbent pad.

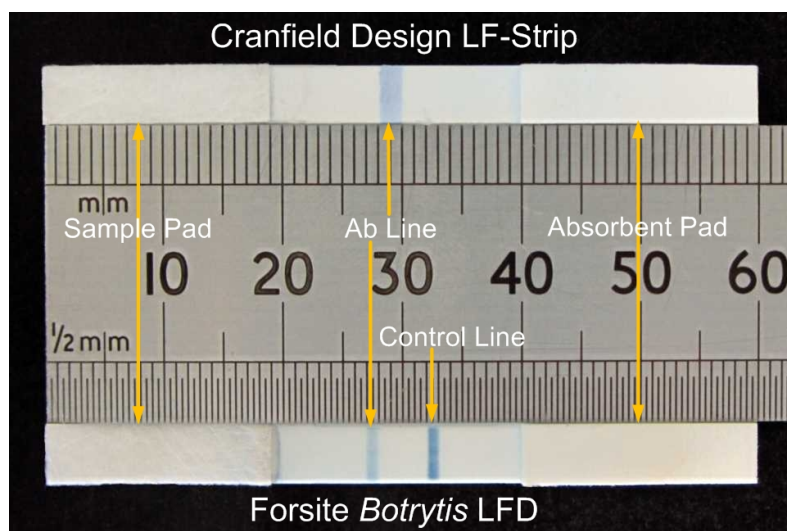


Figure 6.1: Comparison of the Cranfield design lateral-flow strip used for the sample delivery system in combination with the screen-printed gold electrodes with the Pocket Diagnostic *Botrytis* LFD commercially produced by Forsite. The antibody line of the Cranfield design LF-strip was visualised by including a conjugate pad with immobilised latex-antibody beads to the sample pad and performing an assay with 1 mg ml^{-1} *B. allii* solution.

6.3 METHODS

6.3.1 DEVELOPMENT OF A LATERAL-FLOW SAMPLE DELIVERY SYSTEM

6.3.1.1 Assay Procedure for the Electrochemical *Botrytis* Lateral-Flow Sensor

The sample extraction protocol was designed for the use in field conditions following the procedure described in Section 2.3.1.3, utilising the Forsite LFD extraction buffer in combination with five steel grinding beads. Next, the sample was premixed with purified polyclonal antibodies ($100\ \mu\text{g ml}^{-1}$) produced according to Section 2.3.3 and 2.3.4 in a ratio of 1:1 and incubated for 45 min at $37\ ^\circ\text{C}$. To enhance the stability and assay performance of the antibodies, 1:100 KPL milk protein solution was added to the PBS buffer and surfactants were not included to the antibody buffer. However, the sample extraction buffer contains a surfactant to facilitate the sample flow.

The nitrocellulose membrane is 0.5 cm wide, $130\ \mu\text{m}$ thick and 2.5 cm long with a porosity of approximately 70 %. The bed volume (minimum assay volume required) of the membrane was calculated at $12.25\ \mu\text{l}$ ($(0.5 \times 2.5 \times 0.013)\ \text{cm} \times 0.7$). However, this number does not include the bed volume of the sample pad. To minimise the amount of antibodies needed per assay, approximately 1 cm of the sample pad was cut off to reduce the bed volume of the lateral-flow strip. First, $20\ \mu\text{l}$ of the sample-conjugate mix was applied to the sample pad followed by another $10\ \mu\text{l}$ in order to allow the sample to be dragged by the capillary force into the membrane preventing an overspill. After 10 to 15 min, the electrochemical signal of the test line on the lateral-flow sensor was measured after applying $100\ \mu\text{l}$ of the TMB- H_2O_2 substrate. Electrochemical measurements and data analysis were performed as described in Section 3.3.1, the TMB- H_2O_2 substrate was produced according to Section 3.3.3.3.

6.3.1.2 HRP-Conjugate Concentration

The signal generation procedure of the E-*Bot* LFS was optimised as a function of various conjugate concentrations. The purified antibody-HRP conjugate was diluted to the target concentration ranging from 50 to $300\ \mu\text{g ml}^{-1}$ and premixed for 1 h with *B. allii* concentrations of 100, 10 and $0\ \mu\text{g ml}^{-1}$ following the protocol described in

Section 6.3.1.1, and the sensor signal was monitored as a function of the antibody-HRP concentration to assess the best performance.

6.3.1.3 Premix Incubation Time

The premix incubation time of the antibody-HRP conjugate with the sample was investigated in a range between 5 to 90 min with *B. allii* concentrations of 100 and 0 $\mu\text{g ml}^{-1}$. The sensor assay was followed as described in Section 6.3.1.1 with a conjugate concentration of 100 $\mu\text{g ml}^{-1}$. After subtracting the negative reading (normalisation), the sensor signal was plotted against the premix incubation time.

6.3.1.4 Evaluation of Additional Antibody Immobilisation on the SPGE as a Strategy for Performance Enhancement

Covalent immobilisation to 3-DTDPA layer (50 mM) and adsorption of the monoclonal antibody BC-12 to the working electrode were compared with an unmodified SPGE used for the reading of the lateral-flow strip. Immobilisation strategies were followed according to the protocol described in Section 3.3.3.3 (adsorption) and Section 3.3.7.1 (covalent immobilisation). The sensor assay was performed as described in Section 6.3.1.1 with *B. allii* concentrations of 100, 10 and 0 $\mu\text{g ml}^{-1}$ premixed with 100 $\mu\text{g ml}^{-1}$ HRP conjugate.

6.3.1.5 Effect of a Washing Step on the Sensor Performance

The impact of a washing step prior to the sensor reading was evaluated with *B. allii* concentrations of 10 and 0 $\mu\text{g ml}^{-1}$. The sensor assay was performed according to Section 6.3.1.1 with a conjugate concentration of 100 $\mu\text{g ml}^{-1}$. However, 10 min after applying the sample to the lateral-flow strip a washing step, was performed by adding 20 μl sample buffer to the pad. A period of 5 min was maintained to allow the wash buffer to travel towards the absorbent pad before the sensor signal was measured and the test group with a washing step was compared to the control.

6.3.1.6 Standard Curve of the Optimised Lateral-Flow Assay

After the optimisation process was completed, a standard curve was created by testing the electrochemical response of the E-Bot LFS against *B. allii* concentrations ranging from $0.5 \mu\text{g ml}^{-1}$ to $100 \mu\text{g ml}^{-1}$. The assay was performed as described in Section 6.3.1.1 using a conjugate concentration of $100 \mu\text{g ml}^{-1}$ premixed for 45 min with the sample. The test line of the lateral-flow strip was measured 10 min after applying the sample. The limit of detection was calculated as the current of the negative control plus three times its standard deviation.

6.3.2 EVALUATION OF GOLD NANOPARTICLES AS A TECHNIQUE FOR SIGNAL AMPLIFICATION

The performance of the lateral-flow assay in combination with gold nanoparticles was investigated. The gold colloid was produced according to a modified procedure described in Section 3.3.3.4. Gold nanoparticles ($\text{Ø}40 + \text{Ø}60 \text{ nm}$) were diluted to a concentration of $10^9 \text{ particles ml}^{-1}$ and combined in a ratio of 1:1 to produce a stock of 1 ml. The particles were concentrated by spinning the tube for 20 min at 8.000 rpm using a precooled centrifuge (4°C). The supernatant was removed ($960 \mu\text{l}$) and the concentrated particles were added to the polyclonal antibodies. At this step, two groups were created. For the immuno and lateral-flow sensor, polyclonal antibodies were conjugated to HRP using a conjugation kit, and excess HRP was subsequently purified with a conjugate purification kit (see Section 2.3.3 and 2.3.4). For the first group, the gold nanoparticles were added to unpurified conjugate prior to the purification step, which contains an excess of HRP in a ratio of 1:2 to 1:4, whereas for the second gold nanoparticles group, purified HRP conjugate was used in combination with additionally added HRP ($15 \mu\text{l}$ of 1 mg ml^{-1}).

HPLC grade dH₂O was used to produce an antibody-HRP conjugate concentration of 100 µg ml⁻¹ and pH adjustment and blocking was performed according to the standard protocol described in Section 3.3.3.4 with 2 h incubation of the particles conjugate using a roller mixer at 10 rpm. The lateral-flow procedure was followed according to Section 6.3.1.1 and the performance of the gold assay was tested against various concentrations of *B. allii* and compared to the standard assay procedure without gold nanoparticles.

6.3.3 CAD DESIGN OF THE HOUSING

CATIA v5-R21 computer-aided design (CAD) software was used to design the housing for the lateral-flow sensor system. Drawings were based on the components and dimensions of the Cranfield design lateral-flow strip produced by Forsite Diagnostics (Sand Hutton, UK), which was used to construct the *E-Bot* LFS. Additionally, an innovative optimised system was developed. The CAD drawings of the two housings in dipstick and integrated design are attached as Appendix B.

6.4 RESULTS AND DISCUSSION

6.4.1 DEVELOPMENT OF THE LATERAL-FLOW SAMPLE DELIVERY SYSTEM

6.4.1.1 HRP-Conjugate Concentration

In general, lateral-flow assays follow a premix format where the detection analyte is immobilised on the conjugate pad and released by the sample flow allowing the conjugate to bind the target antigen (Davies et al., 2007b). However, since the *E-Bot* LFS employs an electrochemical signal generation system in contrast to the optical systems most LFDs utilise, initial tests were performed to evaluate the optimum conjugate concentration to maximise the assay performance. Various antibody-HRP conjugate concentrations ranging from 50 to 500 µg ml⁻¹ were assayed with *B. allii* antigen concentrations of 100, 10 and 0 µg ml⁻¹ to evaluate which concentration leads to the best signal generation.

The electrochemical signal of the various conjugate concentrations is presented in Figure 6.2. A linear increase in the current of the negative control was observed as a consequence of non-specific binding, which is in accordance to previous findings performed in Section 3.4.5.2. However, this effect is not as strong compared to the immunosensor without lateral-flow sample delivery system, since the reading is performed after 10 min, leaving insufficient time to favour non-specific adsorption. After normalisation (Figure 6.2 embedded graph) all conjugate concentrations $>100 \mu\text{g ml}^{-1}$ showed good performance. However, applying less conjugate is favourable to reduce assay costs. Hence, conjugate concentrations $>200 \mu\text{g ml}^{-1}$ had the tendency to show a decreased performance compared to 100, 150 and 200 $\mu\text{g ml}^{-1}$, expressed by less signal separation between the fungal concentrations. Consequently, a conjugate concentration of $100 \mu\text{g ml}^{-1}$ was chosen to conduct the assay.

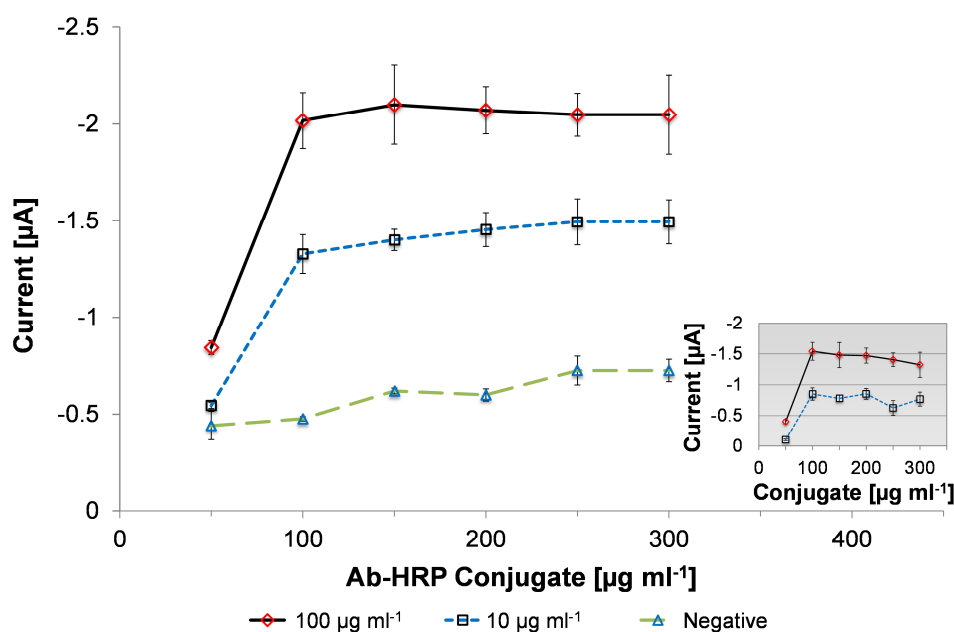


Figure 6.2: Sensor signal current against various concentrations (50 to $300 \mu\text{g ml}^{-1}$) of the antibody-HRP conjugate. Polyclonal antibody-HRP conjugate was premixed with *B. allii* concentrations 100 , 10 and $0 \mu\text{g ml}^{-1}$ and applied on the lateral-flow strip. The embedded graph shows the normalised signal of each conjugate concentration; the bars represent the standard error of the means ($n=4$).

6.4.1.2 Premix Incubation Time

A commercially successful sensor system requires a moderate assay time to facilitate the handling. Premix incubation times ranging from 5 to 90 min were assessed to evaluate the best compromise between signal performance and total assay time. The impact of varying incubation times to the sensor signal is illustrated in Figure 6.3. Though short incubation times of 5 and 15 min can be used for the detection of *Botrytis*, a premix time of >30 min resulted in an increased performance by over 40 %. The antibody antigen interaction is generally enhanced by longer incubation periods (Malone et al., 1996). This finding is in accordance with the optimisation studies performed for the immunosensor in Section 3.4.5.1, suggesting best performance after an incubation period of approximately 1 h. However, the sensor signal reaches a plateau after 45 min and therefore the shorter incubation period was chosen for the immunoassay, since adopting this value, the total assay time can be reduced to less than 1 h.

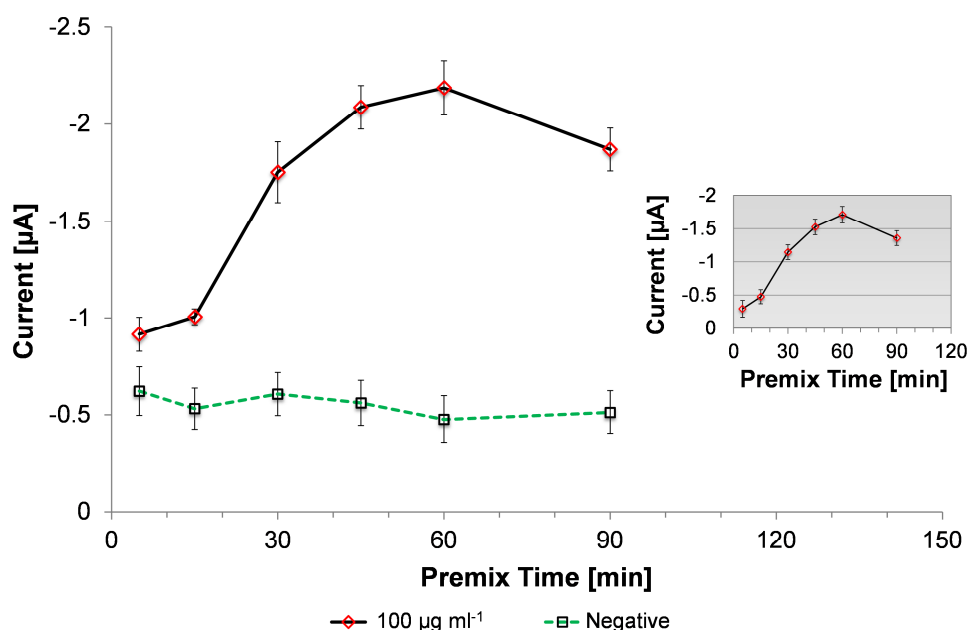


Figure 6.3: Sensor signal as a function of premix incubation times ranging from 5 to 90 min with 100 µg ml⁻¹ polyclonal-HRP conjugate. *B. allii* concentrations of 100 and 0 µg ml⁻¹ were tested with the E-Bot LFS and assessed according to their signal strength. The embedded graph illustrates the normalised signal, the bars represent the standard error of the means (n=4).

6.4.1.3 Evaluation of Adsorption and Covalent Antibody Immobilisation as a Strategy for Performance Enhancement

In contrast to the immunosensor developed in Chapter 3 where the monoclonal capture antibody BC-12 was immobilised on the gold working electrode, the E-Bot LFS employs a system adopted from LFD with capture antibodies immobilised on the test line located on the membrane. However, immobilising additional antibodies on the working electrode may be a valid strategy for performance enhancement, since the antibody capacity on the nitrocellulose membrane is limited by the internal surface area. Antibody immobilisation on the working electrode of the SPGE was performed using adsorption and covalent immobilisation to 3-DTDPA layer and compared to the unmodified sensor.

A direct comparison of the sensor signal tested with *B. allii* concentrations of 100 and 10 $\mu\text{g ml}^{-1}$ is presented in Figure 6.4. Surprisingly, both immobilisation strategies used for the additional immobilisation of the capture antibody to the working electrode could not match the signal achieved by the control represented by the unmodified sensor. It is an established fact that an increased antibody load has a direct impact on the sensitivity of immunoassays (Tijssen, 1985; Malone et al., 1996), which was also confirmed in this study (see Section 3.4.5.4). However, this generalisation was proven inadequate in the case of the electrochemical lateral-flow sensor. Additional antibodies immobilised on the working electrode may facilitate the capture of the target antigen and thus enhance sensitivity. However, the improved performance may be eliminated by the impeding effect of the proteins on the working electrode in comparison with the unmodified sensor surface where the electrons can flow freely to the gold working electrode and generate a higher signal.

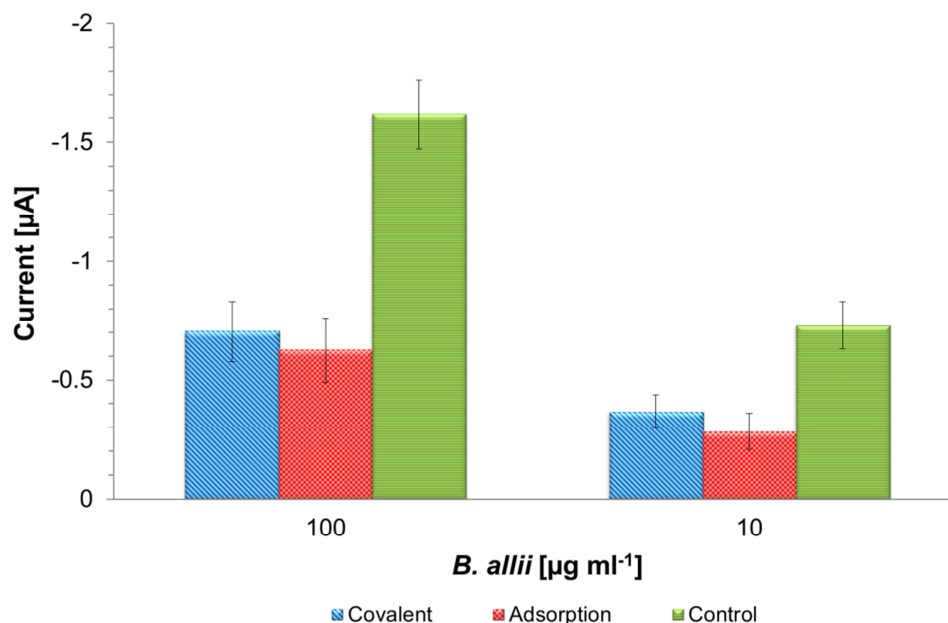


Figure 6.4: Normalised signal of the E-Bot LFS generated by using covalent immobilisation and passive adsorption of the monoclonal antibody BC-12 to the working electrode, in comparison with the control represented by a bare gold electrode with no surface modification. Covalent immobilisation to 3-DTDPA layer (50 mM) was performed using EDC-NHS coupling of the antibody BC-12 ($100 \mu\text{g ml}^{-1}$), whereas adsorption was achieved by direct immobilisation using carbonate-bicarbonate buffer (pH 9.6). The bars represent the standard error of the means ($n=4$).

6.4.1.4 Effect of a Washing Step on the Sensor Performance

Most lateral-flow assays do not employ a washing step before the reading, since the capillary force of the membrane drags the fluid with the non-specific proteins and unbound material away from the test line towards the absorbent pad in a sufficient manner. However, applying a washing step may be beneficial to remove unbound material from the test line, in order to reduce the background current and ultimately enhance the assay performance. The electrochemical signal achieved with and without employing a washing step is presented in Figure 6.5. The test group with a washing step showed a lower current of the negative control by 7 % and a reduced standard error by 35 %. However, the sensor enhancement after normalisation by less than 5 % is not significant enough, with $P=43$ % at a confidence level of 95 %, to justify the more complex assay protocol. From a manufacturer's point of view it is beneficial to design the test procedure as simple as possible with the ultimate goal of an improved handling of the test device. A washing step was therefore not adopted for the assay protocol.

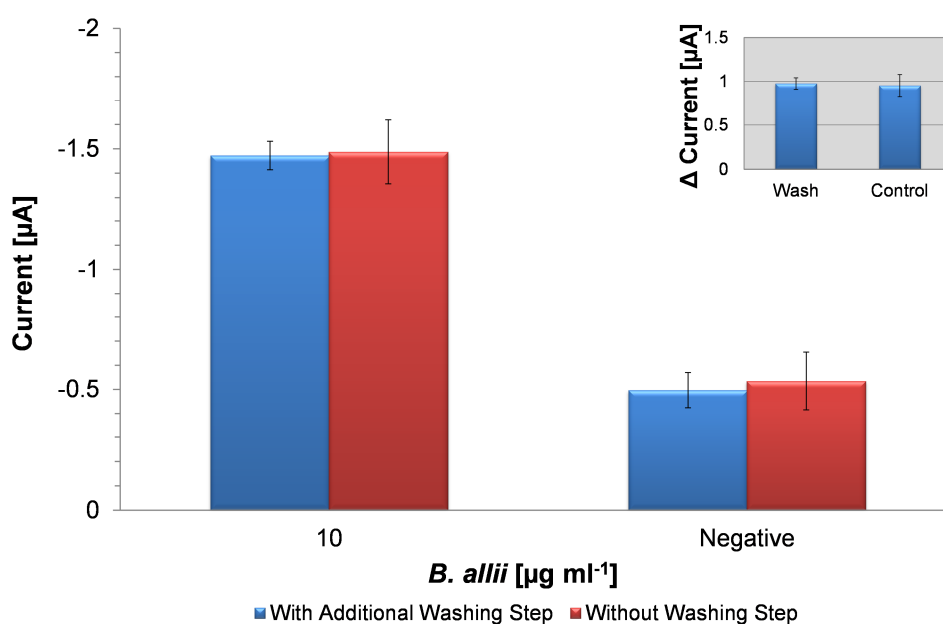


Figure 6.5: Electrochemical signal of the E-Bot LFS against *B. allii* concentrations of 10 and 0 $\mu\text{g ml}^{-1}$ with and without applying an additional washing step. Ten minutes after applying the sample to the lateral-flow strip, an additional washing step was performed for one group by adding 20 μl buffer containing 0.05 % Tween 20 to the sample pad. A period of 5 min was maintained to allow the wash buffer to be dragged towards the absorbent pad before the sensor signal was measured electrochemically. The embedded graph visualises the signal separation of each test group between the fungal concentrations 10 and 0 $\mu\text{g ml}^{-1}$, the bars represent the standard error of the means (n=4).

6.4.1.5 Standard Curve of the Optimised Lateral-Flow Assay

A standard curve of the electrochemical lateral-flow sensor was constructed using the optimised immunoassay protocol described in Section 6.4.1. *B. allii* standards ranging from 0.5 $\mu\text{g ml}^{-1}$ to 100 $\mu\text{g ml}^{-1}$ were plotted against the normalised sensor signal as presented in Figure 6.6. The E-Bot LFS showed a good performance tested against the various fungal concentrations with a calculated LOD (negative+3xSD) as low as 0.12 $\mu\text{g ml}^{-1}$ and a limit of quantification (negative+10xSD) of 0.25 $\mu\text{g ml}^{-1}$. The average coefficient of variation of 8.8 % and $R^2=0.988$ indicated good intra and inter-assay reproducibility. The obtained sensitivity is suitable for detecting *Botrytis* infections prior to showing visible symptoms.

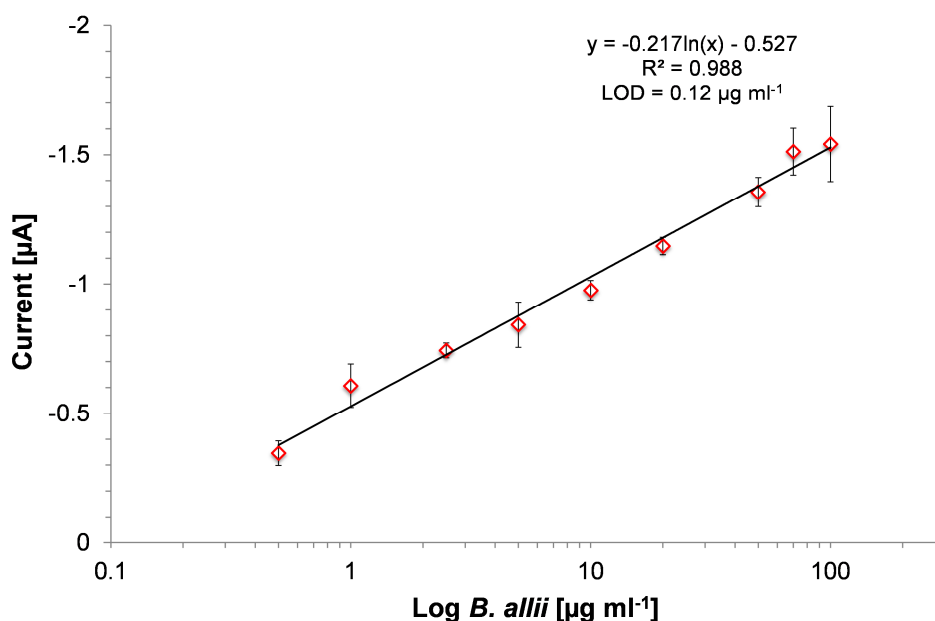


Figure 6.6: Optimised amperometric response of the E-Bot LFS against various concentrations of *B. allii* antigen. Purified polyclonal antibody-HRP conjugate ($100 \mu\text{g ml}^{-1}$) was premixed for 45 min with the sample and applied to the lateral-flow strip. After 10 min the test line comprising the immobilised monoclonal antibody BC-12 was electrochemically measured using JD screen-printed gold electrodes. TMB- H_2O_2 substrate was used for signal generation, measurements were taken after 100 s at a potential of -200 mV. The bars represent the standard error of the means ($n=4$), average CV=8.8 %.

6.4.2 EVALUATION OF GOLD NANOPARTICLES AS A TECHNIQUE FOR SIGNAL AMPLIFICATION

Antibody-labelled gold particles are an established technique for visual readout processes in LFDs (Glomm, 2005; Davies et al., 2007b; Zhang et al., 2009). One advantage is its natural colour with a strong optical absorption band making dying process become redundant. However, gold nanoparticles produced in their classic way with antibody and HRP labelled separately to the gold sol did not prove to be a valid strategy for signal enhancement having an inferior sensor signal compared with the standard assay with HRP-conjugated antibodies (see Section 3.4.4.3). Despite of these results gained from the immunosensor developed in Chapter 3, which employs a step-by-step assay format, the risk of mobilising the particle is significantly lower by

utilising lateral-flow membranes, as there are no washing steps during the assay and the flow through the membrane is relatively slow with 180 s for 4 cm. In this Section, a new approach was tested by labelling polyclonal-HRP conjugate to the gold particles using an environment with excess HRP. Two test groups were created for the gold particles enhanced assay, one with conjugated but unpurified antibodies which have an excess ratio of HRP of 1:2 to 1:4, whereas for the second group purified HRP conjugate with additionally added HRP was employed. Both test groups were diluted to match an antibody concentration of $100\text{ }\mu\text{g ml}^{-1}$ in order to provide a direct comparison with the previously optimised *E-Bot* LFS assay protocol.

The amperometric signal of the gold-aided immunoassay compared to the control is presented in Figure 6.7. The gold assay with unpurified antibody-HRP conjugate gave inferior performance (-42 %) compared to the signal of the control group without gold particles. The lower sensitivity compared with the purified conjugate tested in Section 3.4.5.2 may be one reason. Another potential reason for the lack of performance may be the conjugation protocol itself. To stop the conjugation process, a quencher reagent is added which blocks the reactive groups (see Section 2.3.3). However, as a result, the molecular interactions that are needed for the antibodies to be immobilised to the gold particles may be inhibited. Conjugating the antibodies in the presence of the gold particles could eliminate this drawback. In contrast, the gold assay utilising the purified HRP conjugate in the presence of additional HRP resulted in a signal enhancement by approximately 40 % compared with the control group that utilises the same purified HRP conjugate at a concentration of $100\text{ }\mu\text{g ml}^{-1}$ without HRP-labelled gold sol.

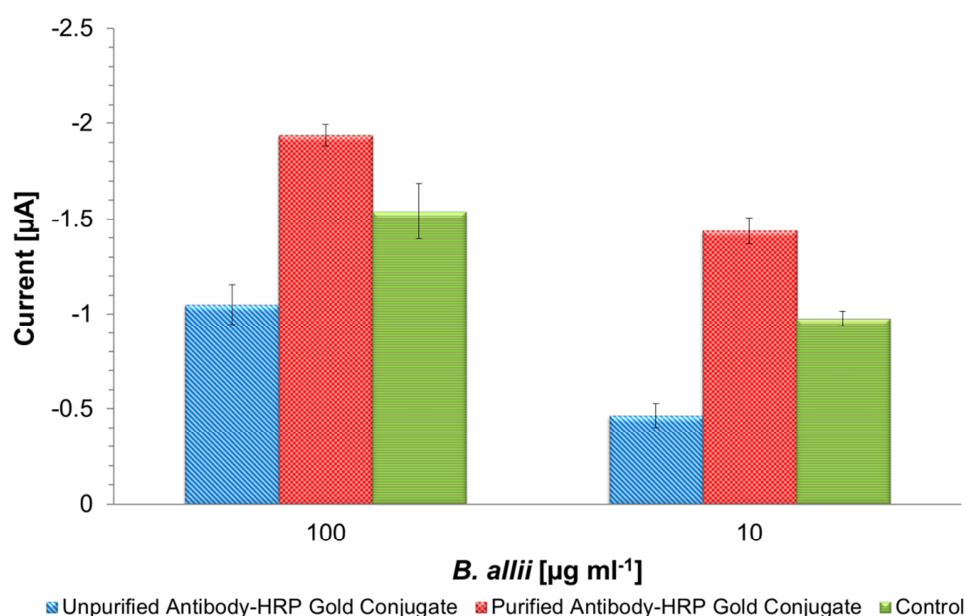


Figure 6.7: Normalised signal of the E-Bot LFS generated using gold nanoparticles labelled with conjugated unpurified polyclonal antibodies and purified HRP conjugate with additionally added HRP. The antibody-HRP conjugate of the gold particles assay was adjusted to $100 \mu\text{g ml}^{-1}$, matching the concentration of the control group without gold, but produced providing an environment with excess HRP. The bars represent the standard error of the means ($n=4$).

Ding et al. (2010) reported a 100-fold increase in sensitivity compared with the normal ELISA method by using 10 nm gold particles labelled with antibody-HRP conjugate for the detection of hepatitis B employing an electrochemical biosensor. Though the use of 10 nm gold particles is questionable since they provide a minimum surface area for the approximately 15 nm antibody-HRP conjugate, they proved that the use of HRP-labelled antibodies is a valid strategy for signal enhancement. Using HRP-labelled antibodies eliminates the drawback of the normal gold assay which immobilises antibodies and HRP separately on the gold sol. Here, unbound antibodies cannot contribute to the signal since they do not have the enzyme label needed for signal generation. As a result, the enhancement of the gold particles with numerous HRP molecules immobilised on the surface needs to compensate the loss of signal caused by non-binding of the antibodies before it can contribute to an enhanced signal.

After these encouraging results, a standard curve was constructed by assaying *B. allii* antigen ranging from 0.5 to 100 $\mu\text{g ml}^{-1}$. The resulting standard curve is presented in Figure 6.8. The use of gold particles enhanced the sensor signal on average by 23 % in comparison with the control group, resulting in a calculated LOD (negative+3xSD) as low as 0.071 $\mu\text{g ml}^{-1}$ and a limit of quantification (negative+10xSD) of 0.12 $\mu\text{g ml}^{-1}$. Though the achieved LOD is inferior compared to the immunosensor developed in Chapter 3 (16 ng ml^{-1}), it is suitable for the detection of *Botrytis* infections prior to showing visible signs of neck rot that was established in the field trial with a threshold for positive detection being 0.1 $\mu\text{g ml}^{-1}$. The lateral-flow sample delivery system further improves the handling of the test device and reduces the assay time to less than 1 h, producing a powerful tool for pre and post-harvest disease control.

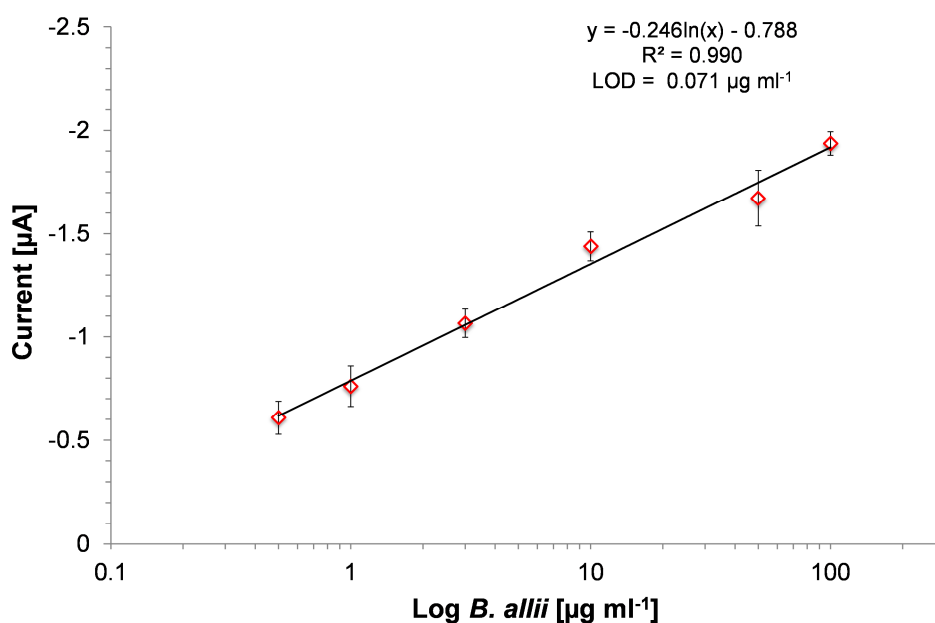


Figure 6.8: Electrochemical signal of the E-Bot LFS employing gold particles labelled with purified polyclonal antibody-HRP conjugate, produced in an environment of excess HRP, against various concentrations of *B. allii* antigen ranging from 0.5 to 100 $\mu\text{g ml}^{-1}$. The conjugate (100 $\mu\text{g ml}^{-1}$) was premixed for 45 min with the sample and applied to the lateral-flow strip. After 10 min the test line comprising the immobilised monoclonal antibody BC-12 was electrochemically measured using TMB/ H_2O_2 substrate for signal generation. Amperometric measurements were conducted after 100 s at a potential of -200 mV, the bars represent the standard error of the means (n=4), average CV=11 %.

6.4.3 DIPSTICK DESIGN BIOSENSOR HOUSING

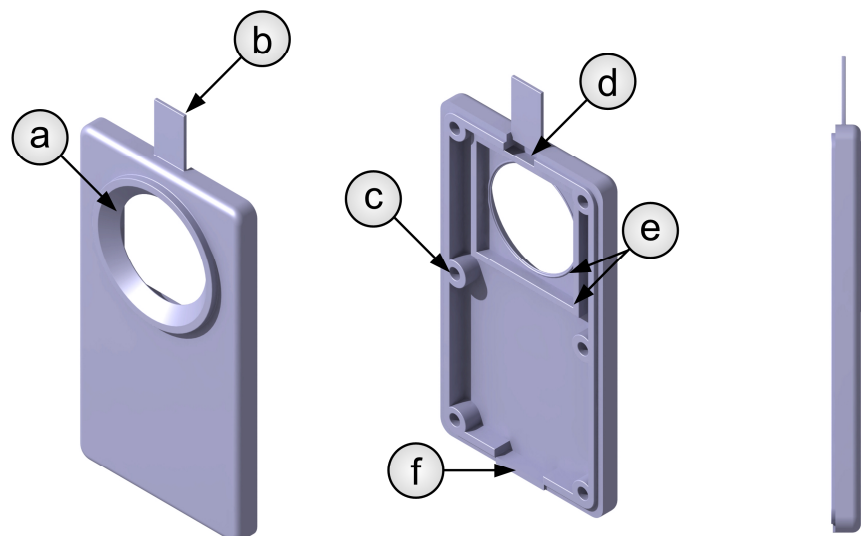
Two housings were designed for the E-Bot LFS: one based on the current screen-printed electrode and the Cranfield design lateral-flow strip produced by Forsite Diagnostics, whereas the second design was based on an optimised fully integrated system. Both housings incorporate all test components needed for a complete *Botrytis* test device and are described in this Chapter.

6.4.3.1 Upper and Lower Casing

The upper part of the casing is illustrated in Figure 6.9 A. The dipstick design is based on an external sample port for the lateral-flow strip, which is reinforced by a plastic panel (Figure 6.9 b). After the analyte is applied to the sample pad and the immunological reaction is completed by the attachment of the antigen to the test line, the TMB/H₂O₂ substrate can be applied to the large substrate port (Figure 6.9 a) that directs the substrate to the test line of the lateral-flow strip. To prevent the substrate from flooding the interior, the E-Bot LFS employs a poly-dimethylsiloxane (PDMS) sealing that is held in place by two support systems (Figure 6.9 e): one integrated in the substrate well and another in the square outer frame. To attach the two parts of the housing, the upper cassette has several pinch points (Figure 6.9 c) as well as a pressure bar for the screen-printed electrode (Figure 6.9 f).

The major function of the lower casing (Figure 6.9 B) is to accommodate the screen-printed electrode. To accomplish this task, a customised cavity (Figure 6.9 g) was designed following the dimensions of the Cranfield screen-printed electrode. Six pins (Figure 6.9 i) and a continuous groove serve as pinch points between the upper and lower casing to ensure a firm hold. Further improvement of the handling is provided by the reinforcement panel at the bottom end of the casing (Figure 6.9 h) designed for a direct attachment of the lateral-flow sensor to a standard Drop Sens (Oviedo, Spain) connector box. This feature enables an application of the system in field conditions, employing a portable reader, as well as a combination with a stationary modular Autolab (Metrohm, Runcorn, UK) electrochemical analyser capable of reading up to ten sensors at the same time.

A



B

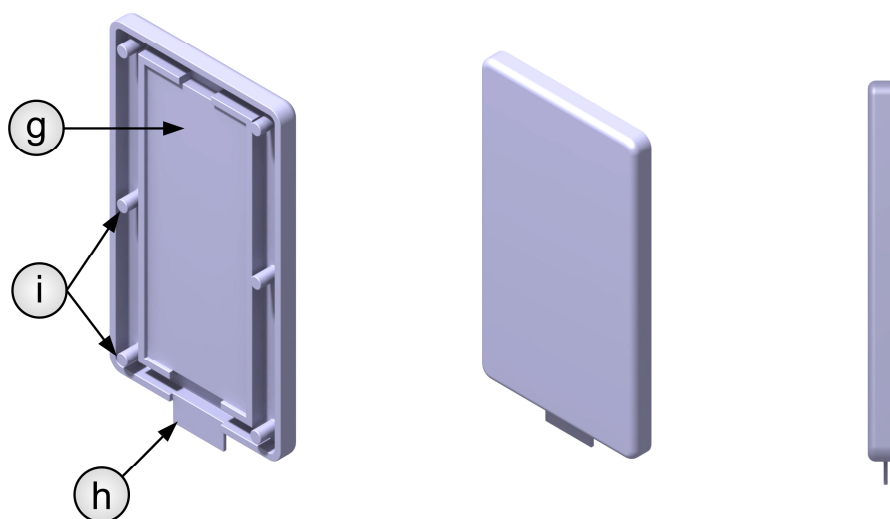


Figure 6.9: Front, rear and lateral view of the upper (A) and lower casing (B) of the E-Bot LFS in dipstick design. The upper casing features: (a) A port for the TMB/H₂O₂ substrate; (b) a reinforcement panel for the lateral-flow strip; (c) six holes for the pins of the lower casing; (d) a slot for the lateral-flow strip; (e) support for the PDMS sealing; and (f) a pressure bar for the screen-printed electrode. The lower casing features: (g) A customised cavity for the screen-printed electrode; (h) a reinforcement panel at the lower end designed for a direct attachment of the connectors to a reader; and (i) six pins which ensure a firm hold between the upper and lower part.

6.4.3.2 Electrochemical *Botrytis* Lateral-Flow Sensor in Dipstick Design

The complete dipstick design E-*Bot* LFS (Figure 6.10) comprises five parts: the upper casing, the lateral-flow strip, the PDMS sealing, the lower casing and the screen-printed electrode. Having only few parts, a fast manual or automated assembling is provided, hence reducing production costs. PDMS was chosen as a material for sealing since it is widely used for microfluidic channels and offers several advantages over other materials used for sealing: it is easily mass producible by cast moulds, inexpensive, robust and hydrophilic (Weigl et al., 2007; Dey et al., 2010). Most important, it is flexible and sticks to the encapsulation layer of the screen-printed electrodes producing a waterproof sealing. For the sample testing procedure, the lateral-flow sensor can be dipped in the analyte for a couple of seconds, allowing the sample to be dragged by the capillary force to the test line. Alternatively the analyte can be dropped to the sample pad with a pipette. After a period of 5 to 10 min, the sensor signal is measured after the TMB/H₂O₂ is added to the substrate port using an amperometric reader.

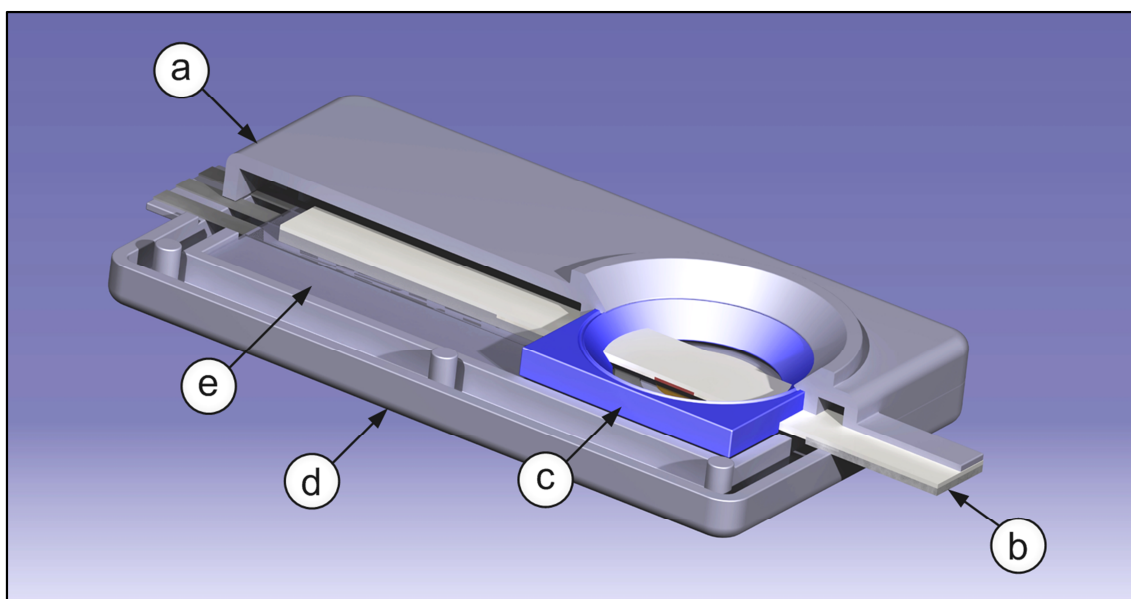


Figure 6.10: CAD drawing of the dipstick design E-*Bot* LFS system comprising the upper casing (a), the lateral-flow strip (b), a PDMS sealing (c), the lower casing (d) and the screen-printed electrode (e).

6.4.4 OPTIMISED ELECTROCHEMICAL LATERAL-FLOW SENSOR

6.4.4.1 Biosensor Housing in Integrated Design

In contrast to the dipstick design, where the sample pad is located outside of the housing, the novel design is based on a fully integrated system where the lateral-flow strip is embedded into the housing. The optimised upper casing (Figure 6.11 A) offers various additional functions apart from the substrate window (Figure 6.11 b) needed for signal generation for the dipstick design. Here, a sample well is included (Figure 6.11 a), directing the analyte to the lateral-flow strip, as well as a control window (Figure 6.11 d) for quality control. The tasks of the lower casing (Figure 6.11 B) are expanded by accommodating additionally the lateral-flow strip besides the screen-printed electrode by means of a customised cavity that immobilises, aligns and supports these vital parts of the biosensor at the same time (Figure 6.11 e).

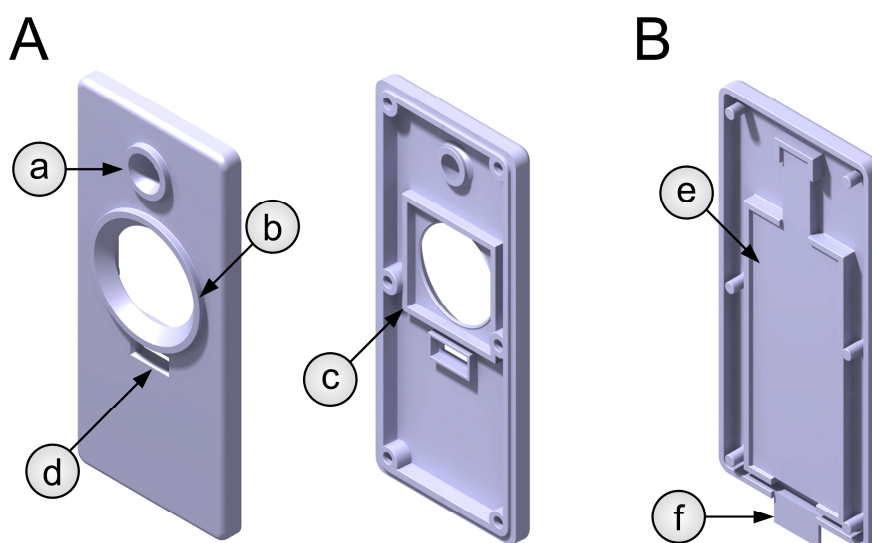


Figure 6.11: (A) Front and rear view of the upper part of the casing and (B) front view of the lower part of the casing in the fully integrated design. The housing features: (a) A sample well, (b) a port for the TMB/H₂O₂ substrate; (c) a support for the PDMS sealing; (d) a control window; (e) a cavity for the screen-printed electrode and for the lateral flow strip; and (f) a reinforcement panel for the connectors.

6.4.4.2 Improved Screen-Printed Electrodes and Lateral-Flow Strip

The main feature of the optimised screen-printed electrode (Figure 6.12 A) is the modified shape of the electrodes. Instead of a circle, the working electrode has a rectangular shape (Figure 6.12 a), increasing the surface area from currently 0.226 cm^2 to 0.353 cm^2 . Furthermore, the counter and reference electrodes (Figure 6.12 b) show a larger gap at the top end and, as a result, do not contact the lateral-flow strip and avoid an interference of the measured signal. Alternatively to the gold ink, the E-Bot LFS can be combined with cheaper carbon electrodes, as long as a comparable sensitivity is provided.

The improved lateral flow-strip (Figure 6.12 B) has no continuous backing and a larger antibody line (Figure 6.12 e) that is 5 mm instead of 3 mm wide. The hydraulic connection of the strip components is warranted by means of adhesive tapes which are only applied between the overlaps of the materials, ensuring a stable intrinsic capillary pull. Thus the test line is fully accessible facilitating the diffusion of the substrate, and hence improving the signal strength and sensitivity. A further feature of the lateral-flow strip for the fully integrated sensor is a wick (Figure 6.12 c) that pre-filters the sample. This system alleviates the need of a filtration step as performed for the immunosensor in combination with a sample extraction bag. Instead of premixing the sample with the antigen, a conjugate pad is employed (Figure 6.12 d) with immobilised antibody and HRP-labelled gold nanoparticles. The gold sol has two functions: first, generating the signal that is electrochemically measured at the test line, and second, the strong colour can be easily spotted as a quality control measure, creating a band at the control line (Figure 6.12 f) that visualises a successful assay. If required, a washing step can be performed in combination with a bigger absorbent pad (Figure 6.12 g).

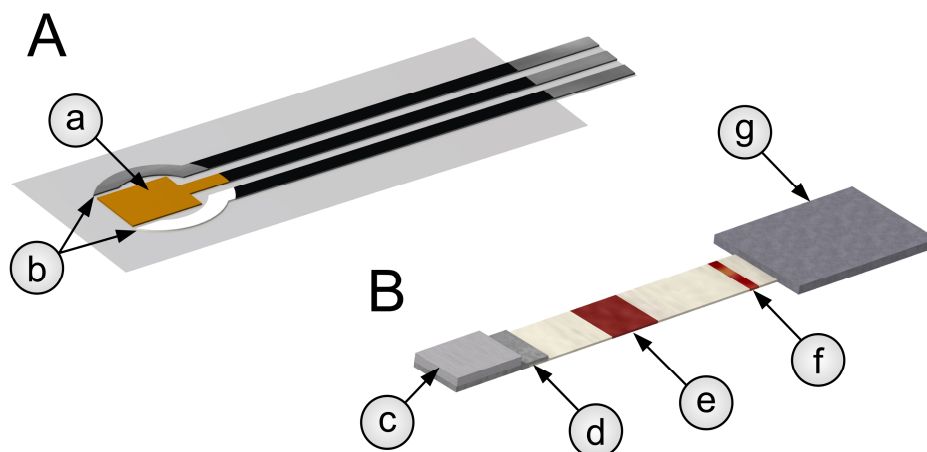


Figure 6.12: Optimised screen-printed electrode (A) and lateral-flow strip (B) used for the integrated design lateral-flow sensor. The screen-printed electrode features a modified electrode shape with a rectangular working electrode (a) and shorter counter and reference electrodes (b). The improved lateral-flow strip offers a wick (c), a conjugate pad (d), a test (e) and a control line (f), and, if required, a larger absorbent pad (g) for a potential washing step.

6.4.4.3 Optimised Integrated Design of the Electrochemical Lateral-Flow Sensor

Dipstick and lateral-flow formats have dominated rapid on-site diagnostics over the last three decades (Davies et al., 2007b). However, their oftentimes low sensitivity and the lack of quantitation have challenged the demand and success of existing assay formats in consumer markets. A commercial system which is easy to use, portable, sensitive and inexpensive can revolutionise *Botrytis* detection and other applications, such as molecular diagnostic.

The focus of the optimised E-*Bot* LFS (Figure 6.13) was to combine the experience gained from this study to produce an innovative and user-friendly biosensor system for the detection of *Botrytis*. A portable design and thus facile use in field conditions are of particular importance for a commercially successful device. Crucial for a mobile application of the E-*Bot* LFS is a portable reader making the use of laboratory-based equipment become redundant. Considering this specification, the E-*Bot* LFS can be combined with an electrochemical analysing system from Palm Sens (Utrecht, Netherlands), comprising an amperometric reader operated via laptop or PDA (Figure 6.13 B), or a Rapidlabs (Bangi, Malaysia) hand-held reader (Figure 6.13 D).

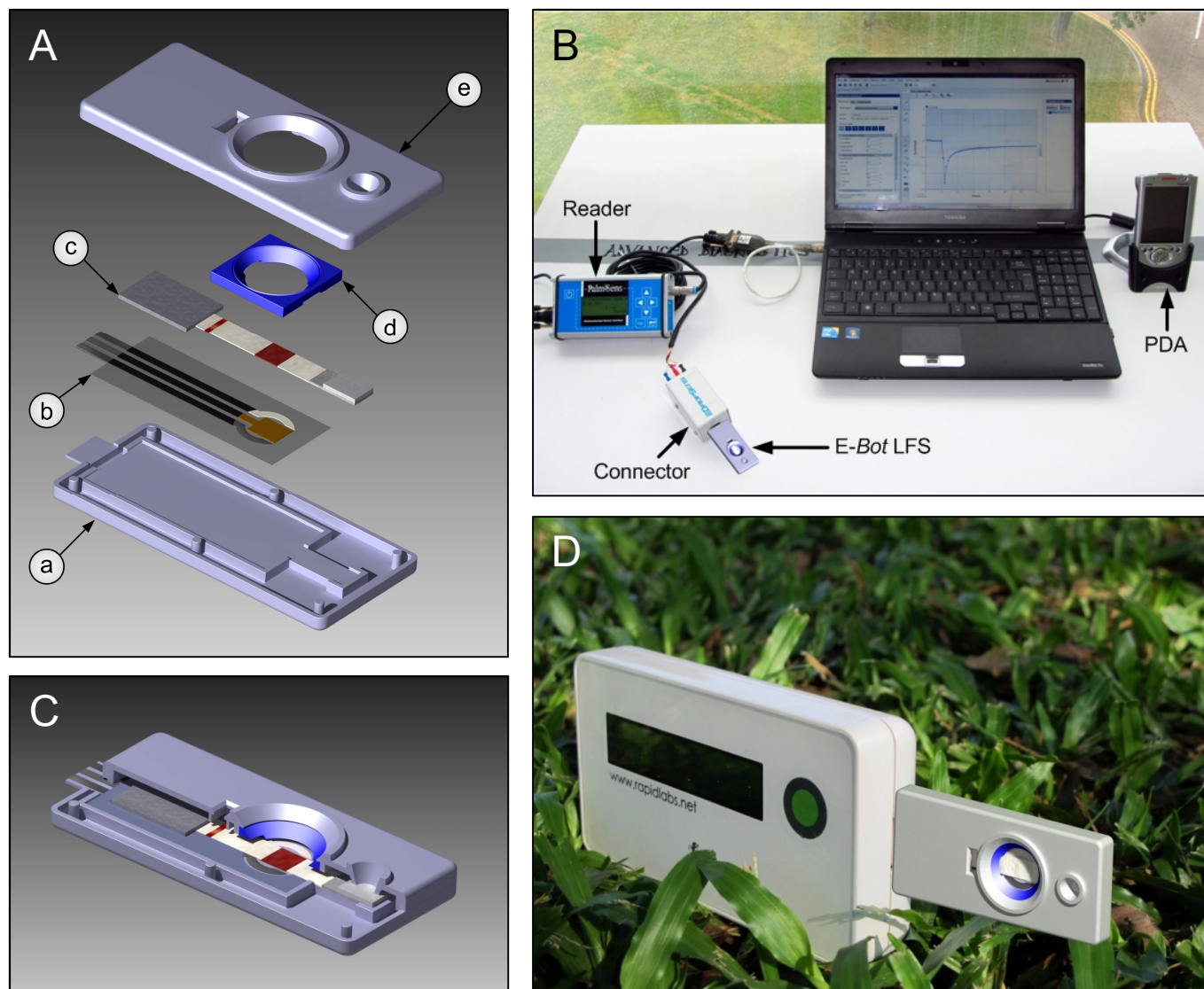


Figure 6.13: CAD drawings of the integrated design E-Bot LFS system. Figures (A) and (C) show the assembly of the sensor comprising a lower casing (a), the screen-printed electrode (b), the lateral-flow strip (c), the PDMS sealing (d) and the upper casing (e). For field use, the E-Bot LFS can be combined with (B) a Palm Sens (Utrecht, Netherlands) electrochemical analysing system, or (D) a Rapidlabs (Bangi, Malaysia) hand-held reader.

The optimised E-Bot LFS system eliminates many flaws of other detection methods for *Botrytis*. Unlike ELISA kits and the immunosensor developed in Chapter 3, the integrated microfluidic system reduces the liquid handling by the operator and incubation steps to a minimum. The improved screen-printed electrode and lateral-flow strip are designed to further enhance the assay sensitivity by increased surface area of the working electrode, an optimised electrode design and a wider test line that maximises the amount of immobilised antibodies.

Tests performed in Section 6.3.1.5 suggest that a washing step can have a positive impact on the signal strength and can lower the assay variation. If such measure is required, the new lateral-flow strip can be combined with a larger absorbent pad. However, this feature would require a manual attachment of the conjugate pad, whereas the standard manufacturing procedure involves strip components which are laminated in continuous lines and are subsequently cut into strips.

6.5 CONCLUSIONS

A lateral-flow sample delivery system was developed and tested in this Chapter that combines the advantages of the robust design and improved handling of a lateral-flow strip with the sensitive electrochemical detection system of screen-printed electrodes. The Cranfield design electrochemical lateral-flow sensor was optimised as a function of various parameters, such as conjugate concentration, premix incubation time antibody immobilisation strategies and washing steps. The optimised E-Bot LFS showed a good performance tested against the various fungal concentrations with a calculated limit of detection ($LOD = \text{negative} + 3 \times SD$) as low as $0.12 \mu\text{g ml}^{-1}$ and a limit of quantification ($LOQ = \text{negative} + 10 \times SD$) of $0.25 \mu\text{g ml}^{-1}$. Surprisingly, applying additional antibodies on the working electrode showed inferior performance compared to the bare electrode. This would open the potential use of cheaper carbon electrodes for the E-Bot LFS, as long as a comparable sensitivity is provided.

Gold particles labelled with antibody-HRP conjugate were used as a strategy for signal enhancement. Employing these nanomaterials enhanced the sensor signal on average by 23 % compared with the control group, further reducing the LOD ($0.071 \mu\text{g ml}^{-1}$) and LOQ ($0.12 \mu\text{g ml}^{-1}$). Though the achieved LOD is inferior compared to the immunosensor developed in Chapter 3 (16 ng ml^{-1}) the sensitivity of the sensor system is suitable for the detection of *Botrytis* infections prior to showing visible signs of infection. Hence, the lateral-flow sample delivery system further improves the handling of the test device and reduces the assay time to less than 1 h, producing a powerful tool for pre and post-harvest disease control. Focussing on the detection of neck-rot disease, the threshold for positive detection was established in the field trial (Chapter 5) being approximately $0.1 \mu\text{g ml}^{-1}$, demonstrating that the detection of *Botrytis* neck-rot infections is a potential application for the sensor system. However, more work is needed to test this system and further improve the sensitivity.

Two housings were developed for the microfluidic system: dipstick and fully integrated design. The dipstick design is based on the current lateral-flow strip produced at Forsite Diagnostics. The optimised E-Bot LFS design features an improved screen-printed electrode, lateral flow strip and upper and lower cassette design that is customised to incorporate all parts of the detection device. Employing a conjugate pad in combination with gold nanoparticles as an optical label also enables the incorporation of a test line that visualises a successful test and was included for the optimised housing as well as a sample well. However, both housings incorporate all test components needed for a complete *Botrytis* test device and can be combined with a portable reader for the use in field conditions, making the use of laboratory-based equipment become redundant.

CHAPTER SEVEN

FINAL CONCLUSIONS AND FUTURE WORK

7 FINAL CONCLUSIONS AND FUTURE WORK

7.1 OVERVIEW

Botrytis is an economically important pathogen in the agricultural industry. In some years, when weather conditions are most unfavourable, losses can as high as 15 to 40 % of the annual crop with a disastrous impact on producers. Considerable efforts are therefore invested in restraining *Botrytis* infections pre and post-harvest, resulting in an annual worldwide market for anti-*Botrytis* products amounting to £450 million GBP with an economically upward trend. However, the lack of innovative and sensitive detection systems which can assess the pathogen load in the field is a driving factor for continuous research.

This study covers the development of three sensor systems that aim at detecting *Botrytis* species in order to provide disease control and reduce pre and post-harvest losses. All three sensor platforms employ the highly sensitive screen-printed gold electrodes designed by Cranfield University in combination with an enzymatic electrochemical signal generating system. The immunosensor demonstrated high performance throughout a wide range of pathogen concentrations with a limit of detection that is suitable for detecting *Botrytis* infections prior the appearance of visible symptoms, and thus offering a crucial advantage over other commercial detection methods for *Botrytis*. If the detection aims at targeting only particular species of *Botrytis*, genetic methods can be employed combined with an electrochemical DNA sensor. Here, the pathogen-derived DNA is amplified using a thermocycler with primers targeting species-specific sequences. This system can be used as a portable, low-cost alternative for real time PCR detection, alleviating the need of expensive, laboratory-based equipment. Both sensor systems were tested in a large field trial enabling a sensitive detection and quantification of symptomless *Botrytis* infections with an excellent success rates >90 %. Additionally, an electrochemical microfluidic aided immunosensor was developed. The lateral-flow sample delivery system combined with a customised housing was designed to move the technology from a laboratory-based assay to a viable, commercially successful product. A patent application was formulated to protect the intellectual property of these novel biosensor systems described in this study.

7.2 ELECTROCHEMICAL IMMUNOSENSOR

The studies were initiated by conducting a comprehensive evaluation of the antibodies in combination with various sensor formats for the immunoassay development. A direct sandwich ELISA format using the monoclonal antibody BC-12 as the capture molecule followed by a polyclonal antibody conjugated to the electroactive enzyme HRP which was selected for further optimisation. One reason for the direct sandwich format was the lack of an additional incubation step for the anti-species conjugate; another was the better performance regarding reproducibility and detection limit. The antibodies employed for the immunoassay are capable of detecting *Botrytis* regardless of their species. This feature leads to a wide range of potential applications for the immunosensor such as quality control by retailers and industry, as well as detection of other pathogenic *Botrytis* species, enabling the immunosensor to be applied as a universal tool to assess *Botrytis*-derived infections in other markets. *Botrytis cinerea* for example is the most general and therefore dominant species of *Botrytis* affecting over 270 host plants. This species can cause disastrous losses of up to 40 % of the annual production of fruits and vegetables, such as strawberries, grapes and tomatoes, representing the biggest potential market for the immunosensor. Additionally, the monoclonal antibody BC-12 developed by Meyer and Dewey (2000) showed no cross reactivity with *Fusarium oxysporum* which is a soil-borne pathogen causing basal rot in onions and other widely spread fungi represented by *Penicillium verrucosum* and *Aspergillus niger*.

Two immobilisation strategies including passive adsorption and covalent coupling to thiolated self-assembled monolayer were investigated for the immunoassay development. Though the optimised immunoassay using passive adsorption for the immobilisation of the capture antibodies showed good performance with a benchmark detection limit of 58 ng fungal mycelium ml⁻¹, there was the need for further improvement. Especially the washing steps performed throughout the assay lead to a high degree of variation. To compensate this flaw, covalent immobilisation techniques have been evaluated. Employing 3-dithiodipropionic acid (DTDPA) as well as 11-mercaptoundecanoic acid (MUDA) resulted in enhanced assay sensitivity. In both cases there was continued improved signal when compared with the adsorbed group.

Covalent coupling using EDC-NHS activated DTDPA polymer layer further reduced the limit of detection to $16 \mu\text{g ml}^{-1}$. However, from a manufacturer's point of view, the detection limit achieved using passive adsorption might be sufficient for a commercial use without the need to employ the more complex covalent coupling procedure.

Gold nanoparticles with antibody and HRP labelled separately to the sol did not promote sensitivity. The lower signal strength resulted in an LOD of $0.72 \mu\text{g ml}^{-1}$ that is inferior to the normal assay employing HRP-conjugated antibodies. One of the reasons for the lack of performance may be the washing steps performed during the assay. Another approach to overcome the lack of amplification could be to immobilise HRP-conjugated antibodies to the gold colloid.

However, the obtained LOD using HRP-conjugated antibodies is suitable for detecting *Botrytis* infections prior to showing visible symptoms and therefore offers an excellent advantage over other commercial detection methods for *Botrytis*, such as ELISA kits and LFDs which offer an LOD between 1 and $10 \mu\text{g ml}^{-1}$. The field trial demonstrated the capability of the immunosensor in predicting the incidence of neck-rot disease in onion bulbs after four months of storage with a success rate of 90 %, thus producing a powerful tool for pre and post-harvest disease control. These promising results demonstrated that neck-rot disease can be predicted based on the local pathogenic risk.

7.3 DNA SENSOR FOR *BOTRYTIS*

The second sensor platform aims at detecting pathogen-derived DNA amplified by using a PCR thermocycler. Considering the fundamental principle of PCR to amplify the DNA until saturation is reached, the concentration of amplified DNA after PCR is independent from the amount of starting material as long as the amplification is within the saturated state. A quantification of the template DNA is therefore not possible, but a sensitive and specific positive or negative result is given.

Two DNA sensor formats were developed and optimised as a function of various parameters. The DNA sensor format employing two biotin-labelled primers is based on a sandwich assay in which neutravidin is immobilised on the sensor surface as capture

molecule for the DNA, followed by streptavidin-HRP conjugate for signal generation. The premix format was accomplished by adding streptavidin-HRP directly to the thiol-C6 and biotin-labelled DNA before dropping the master mix on the sensor. Though the maximum signal separation between positive and negative samples of the double biotin format was higher by approximately 20 % compared to the premixed assay, this format offers numerous advantages. While the classic assay operates on a step-by-step application, it is more desirable to have a premixed complex to reduce the total assay time to 10 min.

The double biotin format was optimised as a function of various parameters. Covalent immobilisation strategies to 3-DTDPA layer were evaluated with mixed results. The decision whether covalent immobilisation is beneficial for the DNA sensor has to be assessed using a multilevel approach. If the development prioritises cost effectiveness and signal performance, adsorption offers more benefits. On the other hand, if increased surface load at low concentrations of neutravidin and enhanced reproducibility is the major target, covalent immobilisation strategies are more suitable. However, both formats provide stable assay conditions for the detection of pathogenic DNA.

The performance of the DNA sensor detecting fungal DNA at low infection levels using PCR was tested with DNA extracted from onion neck tissue samples collected during the field trial. The DNA sensor demonstrated its capability of distinguishing between infected and uninfected samples with a success rate of 95 % confirming the results of quantitative real-time PCR and immunosensor. Furthermore, no inhibiting effects of plant tissue were observed.

The DNA sensor combined primer pair described by Chilvers et al. (2007) was capable of detecting only species causing neck rot (*Botrytis allii*, *aclada* and *byssoidea*) with DNA concentrations as low as 0.1 pg pathogen DNA, which is close to the limit of detection of the PCR amplification process, showing no unspecific elongation of related *Botrytis* species such as the worldwide common pathogen *Botrytis cinerea*. The precise circumstances of neck-rot infections are discussed controversially in the literature. Which *Botrytis* species in particular is the primary causal agent of neck rot is still

unknown and very little research has been conducted in this field since the 70s. Considering these facts, the primers described by Chilvers et al. (2007) are more suitable for detecting neck rot instead of employing primers that would only target *B. allii* or *B. aclada*. However, in general the specificity of the DNA sensor is determined by the primer sequence used for the PCR and is therefore capable of detecting various target pathogens. Tested with the primers described by Nielsen et al. (2002), the DNA sensor was specific to *Botrytis* species associated with neck rot as well as *Botrytis cinerea*, demonstrating the wide range of potential applications. These primers provide a more general approach for *Botrytis* detection, as they additionally amplify *B. cinerea* and *B. squamosa*. Focussing on *Botrytis* infections, other primers can be combined with the DNA sensor to detect particular species such as *Botrytis cinerea* (Martinez et al., 2008; Diguta et al., 2010; Saito et al., 2013) or *Botrytis squamosa* (Carisse et al., 2009). Moreover, the DNA sensor is not limited to these applications and can be adopted for other fungal and bacterial pathogens. There is future potential that this sensor system and platform could be used as a portable, low cost alternative for real time PCR detection, alleviating the need of expensive, laboratorybased equipment. Hence, another field of use would be its employment as a low-cost device for medical diagnostics in developing countries.

7.4 ELECTROCHEMICAL *BOTRYTIS* LATERAL-FLOW SENSOR

The third sensor system combines the advantages of a lateral-flow sample delivery system that is simple to use and improves handling, with the enhanced sensitivity of an enzymatic electrochemical detection system based on the Cranfield design screen-printed electrodes. The optimised E-*Bot* LFS was tested against various concentrations of *Botrytis* and showed with 0.12 $\mu\text{g ml}^{-1}$ a good limit of detection.

Surprisingly, applying additional antibodies on to the working electrode gave inferior performance in comparison with a bare electrode. This can make the use of gold electrodes redundant since the gold inks are predominantly designed for improved surface immobilisation of molecules. Alternatively, cheaper carbon electrodes may be utilised as long as a comparable sensitivity is provided.

Gold particles labelled with antibody-HRP conjugate were used as a strategy for signal enhancement. Employing these nanomaterials improved the electrochemical signal by 23 %, thus further reducing the LOD to $0.071 \mu\text{g ml}^{-1}$. Focussing on the detection of neck-rot disease, the threshold for pathogen detection was established in the field trial at approximately $0.1 \mu\text{g ml}^{-1}$, showing that the detection of *Botrytis* neck-rot infections is a potential application for the sensor system. Employing this sensor platform, growers would be able to assess the full storage potential of their crops, reduce post-harvest losses, energy costs and provide effective disease control.

However, the main focus of the system is the on-site detection of *Botrytis* infections. A major drawback of the current sensor system is the premix time needed for the antibody antigen interaction that was found to be at least 30 min. Employing a conjugate pad in combination with gold sol labelled with antibody-HRP conjugate can reduce the total assay time to less than 15 min and should therefore be given preference for a mass-produced commercial device. On the other hand, the field trial demonstrated that a sensitivity as low as $0.1 \mu\text{g ml}^{-1}$ is needed and reducing the assay time can compromise sensitivity. However, immunoassays are enhanced by several factors that can compensate for this flaw. Employing a Hi-Flow Plus 240 membrane can enhance the sensitivity compared with Hi-Flow Plus 180 membrane allowing a longer antibody antigen interaction. The gold nanoparticles assay and sensor strip design can be further optimised. The sensitivity of the screen-printed electrode is another source of improvement. Furthermore, in combination with the customised housing, membranes can be employed without a backing, which is potentially inhibiting the free diffusion of the TMB- H_2O_2 substrate. However, from a product development point of view, little research is needed to transfer the electrochemical *Botrytis* lateral-flow sensor to a viable commercial product, since this study covers many aspects of the research and development required.

7.5 LIMITATIONS

The immunosensor with immobilised antibodies on the surface of the screen-printed electrode surface is, in its current stage of development, a highly sensitive format with a benchmark limit of detection compared to other commercially available detection methods. However, it is a multistep procedure which requires the operator to add solutions on the sensor surface at different stages of the assay, thus requiring numerous incubation and washing steps, which will result in an assay taking up to 2 h to complete. To overcome this obstacle, the combination with lateral-flow systems is required to reduce assay time to less than 15 min and make the biosensor more user friendly by minimising liquid handling by the operator. From a manufacturer's point of view, the stability of the HRP conjugate on the sensor strip over a period of 6 to 12 months needs to be evaluated. Fakanya and Tothill (2012) investigated the stability of the Cranfield design screen-printed electrodes over a period of six months and found no significant loss in signal strength when using stabilisers for the antibody immobilisation. However, to ensure a minimum variation which is crucial for a commercial detection method, the long-term stability and reproducibility of the system is a major obstacle that could not be covered in this study due to the limited time.

From a commercial perspective, the electrochemical lateral-flow sensor is a viable detection device for *Botrytis*. The lateral-flow sensor in its current stage of development is a prototype comprising two parts: the Cranfield design screen-printed electrode and the lateral-flow strip for sample delivery system. Though this study covered many aspects of the research and development for a successful detection system for *Botrytis*, a commercial partner is needed for the final optimisation and production of the immunosensor as well as further research that emphasises the potential to reduce losses and thus save money. Focussing on neck-rot disease of onion bulbs, this study championed the neck-rot research in field conditions but could cover only one growing season. Further research in collaboration with growers and industrial partners is needed to create a global database and to group the results of various studies employing the developed immunosensor, in order to manifest the correlation between the pathogen load and the incidence of neck-rot disease in store. Over the years, the growers as well as industry would benefit from the experience gained, as customised countermeasures

can be applied to suppress neck-rot disease based on the local pathogenic risk. The novel threshold system developed in this study to predict neck rot in store using the pre-harvest pathogen load offers one approach for sustainable disease control. With further research covering numerous growing seasons and changing environmental conditions, a traffic light system can be established which can be used by growers to assess the local pathogenic risk. However, the threshold of $0.1 \mu\text{g ml}^{-1}$ employed in the field trial for positive detection is based on a relatively small sample size of 71 and does not represent a universally valid value for all applications. With increased sample size and alternating experimental environments, such as geographical area, various varieties and weather conditions, a continuously improved accuracy of the threshold can be achieved that may be generally acknowledged.

Assay time and variation is not a major obstacle for the DNA sensor, since this system is qualitative and the premix format with thiol-C6 labelled primers offers a total assay time of approximately 10 min. Biotin or thiol-C6 labelled primers are widely available and inexpensive. Focussing on the PCR amplification procedure the assay time (approximately 30 min) is short and with up to 100 samples at the same time, also suitable for a large sample number. For a commercial application, the optimised master mix comprising the dNTPs, polymerase and primers can be mass produced and sold as frozen ready-to-use kits. The biggest drawback of the DNA sensor is the DNA extraction procedure that is time consuming. Simplified sample extraction methods can be found in literature (Cullen and Hirsch, 1998; Kuske et al., 1998; Fukuta et al., 2003) that can be used in field conditions, alleviating the need of special laboratory-based equipment. However, employing these techniques normally results in a lower DNA quantity and quality compared with the more complex laboratory-based methods. Furthermore, the risk of false positive and negative increases with fluctuating DNA extraction efficiency. These flaws can be compensated by the PCR amplification, producing rapidly up to 10^6 to 10^9 copies of a target region of the pathogen DNA as well as the high sensitivity of the DNA sensor that offers a stable signal until the limit of detection of 100 fg pathogen DNA is reached. However, the use of a commercial DNA extraction kit is recommended in order to eliminate inhibiting effects of plant tissue and substandard DNA quality.

7.6 FUTURE WORK

The overall results achieved in this study include the development of a novel immuno and DNA-based biosensor for the detection of *Botrytis* infections in field conditions. Additionally, a prototype microfluidic system was developed and tested to move the technology to a viable, commercially successful product. However, there are several suggestions for further development to improve the biosensor surfaces for future applications:

I. *Development of a Botrytis-specific immunosensor.*

- Further research may be conducted to test various sensor platforms and to optimise sample handling and sensitivity.

II. *Development of a DNA biosensor for Botrytis.*

- New DNA extraction and PCR amplification protocols may be evaluated to obtain a robust and viable DNA-based detection system for *Botrytis*.
- The developed sensor platform may be adopted for the detection of other fungi and bacteria by employing primers that target various pathogens.

III. *Development of a microfluidic system.*

- The electrochemical lateral-flow sensor may be improved that include the microfluidic system, the screen-printed electrode, housing, assay conditions and automated data processing.
- Further research in the field using the developed prototype may be conducted in order to establish the correlation between the pathogen load of *Botrytis* and the incidence of neck rot in store creating a global database for enhanced disease control.
- The shelf life and long-term reproducibility may be investigated over a period of at least six months.

7.7 FINAL CONCLUSIONS

Neck rot and other pathogenic infections of economically important crops caused by *Botrytis* species result in considerable losses worldwide in field and during storage. Although research has intensified over the last four decades, many aspects of *Botrytis* infections are still unknown. Understanding the pathogen and environmental factors that influence the incidence of *Botrytis* infections is crucial for future disease control. Therefore, a multilevel approach is needed, combining basic research and various disease control measures, such as pre and post-harvest detection, fungicides, storage conditions and quality management at the packing line, to sustainably decrease the pathogenic risk. Nevertheless, the solutions presented in this study can be easily integrated into the quality-testing procedures of growers and distributors providing vital information about the local pathogen risk. The high sensitivity combined with the portable and inexpensive design of the developed biosensor platforms makes this approach eminently suitable for the detection of latent *Botrytis* infections, producing a powerful tool for pre and post-harvest disease control.

REFERENCES AND BIBLIOGRAPHY

- Agrios, G. N. (2005a), "How Plants Defend Themselves against Pathogens", in Agrios, G. N. (editor), *Plant Pathology* (5th ed), Dana Dreibelbis, Burlington, USA, p. 207-248.
- Agrios, G. N. (2005b), "Plant Diseases Caused by Fungi", in Agrios, G. N. (editor), *Plant pathology* (5th ed), Dana Dreibelbis, Burlington, MA, p. 510-522.
- Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002), *Molecular biology of the cell* (4th ed), Garland Science, New York.
- Alfonta, L., Singh, A. K. and Willner, I. (2001), "Liposomes Labeled with Biotin and Horseradish Peroxidase: A Probe for the Enhanced Amplification of Antigen–Antibody or Oligonucleotide–DNA Sensing Processes by the Precipitation of an Insoluble Product on Electrodes", *Analytical Chemistry*, Vol. 73, No. 1, p. 91-102.
- Altintas, Z. and Tothill, I. E. (2013), "Biomarkers and biosensors for the early diagnosis of lung cancer", *Sensors and Actuators B: Chemical*, Vol. 188, p. 988-998.
- Altintas, Z., Uludag, Y., Gurbuz, Y. and Tothill, I. E. (2012), "Development of surface chemistry for surface plasmon resonance based sensors for the detection of proteins and DNA molecules", *Analytica Chimica Acta*, Vol. 712, p. 138-144.
- Alvarez-Icaza, M. and Bilitewski, U. (1993), "Mass production of biosensors", *Analytical Chemistry*, Vol. 65, No. 11, p. 525-533.
- Angus, M. B. (2001), "A step-by-step guide to non-linear regression analysis of experimental data using a Microsoft Excel spreadsheet", *Computer methods and programs in biomedicine*, Vol. 65, No. 3, p. 191-200.
- Aoyagi, K. (2002), "PCR", in Gerstein, A. S. (editor), *Molecular Biology Problem Solver: A Laboratory Guide*, John Wiley & Sons, Inc., New York, p. 291-329.
- Arya, S. K., Singh, P. S. and Malhotra, B. D. (2007), "Electrochemical Techniques in Biosensors", in Marks, R. S., Cullen, D. C., Karube, I., Lowe, C. R. and Weetall, H. H. (editors), *Handbook of biosensors and biochips*, John Wiley, Chichester, p. 342-377.
- Beatty, J. D., Beatty, B. G. and Vlahos, W. G. (1987), "Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay", *Journal of immunological methods*, Vol. 100, No. 1, p. 173-179.
- Bedford, E. E., Spadavecchia, J., Pradier, C. M. and Gu, F. X. (2012), "Surface Plasmon Resonance Biosensors Incorporating Gold Nanoparticles", *Macromolecular Bioscience*, Vol. 12, No. 6, p. 724-739.

- Beever, R. B. and Weeds, P. L. (2004), "Taxonomy and genetic variation of *Botrytis* and *Botryotinia*", in Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. (editors), *Botrytis: Biology, Pathology and Control*, Kluwer Academic Publishers, Dordrecht, p. 29-52.
- Bell, A. S. and Ranford-Cartwright, L. C. (2002), "Real-time quantitative PCR in parasitology", *Trends in parasitology*, Vol. 18, No. 8, p. 338-342.
- Bettazzi, F., Lucarelli, F., Palchetti, I., Berti, F., Marrazza, G. and Mascini, M. (2008), "Disposable electrochemical DNA-array for PCR amplified detection of hazelnut allergens in foodstuffs", *Analytica Chimica Acta*, Vol. 614, No. 1, p. 93-102.
- Billah, M. M., Hodges, C. S., Hays, H. C., W. and Millner, P. A. (2010), "Directed immobilization of reduced antibody fragments onto a novel SAM on gold for myoglobin impedance immunosensing", *Bioelectrochemistry*, Vol. 80, No. 1, p. 49-54.
- Blair, G. and Zajdel, M. (1992), "The polymerase chain reaction: Already an established technique in biochemistry", *Biochemical education*, Vol. 20, No. 2, p. 87-91.
- Blog, F. (2010), *A Survey of Ascomycetous Holomorphs*, available at: <http://maryam2230.blogfa.com/post-147.aspx> (accessed 25th November 2011).
- Boorsma, D. M. and Kalsbeek, G. L. (1975), "A comparative study of horseradish peroxidase conjugates prepared with a one-step and a two-step method.", *Journal of Histochemistry & Cytochemistry*, Vol. 23, No. 3, p. 200-207.
- Bossi, R. and Dewey, F. M. (1992), "Development of a monoclonal antibody-based immunodetection assay for *Botrytis cinerea*", *Plant Pathology*, Vol. 41, No. 4, p. 472-482.
- Bradford, M. M. (1976), "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding", *Analytical Biochemistry*, Vol. 72, No. 1-2, p. 248-254.
- Brewster, J. L. (1994), "Interactions with other Organisms: Weeds, Pests, Diseases and Symbionts", in *Onions and other vegetable alliums*, CABI Publishing, Wellingford, UK, p. 197-202.
- Canaria, C. A., Smith, J. O., Yu, C. J., Fraser, S. E. and Lansford, R. (2005), "New syntheses for 11-(mercaptoundecyl)triethylene glycol and mercaptododecyltriethyleneoxy biotin amide", *Tetrahedron letters*, Vol. 46, No. 28, p. 4813-4816.
- Cao, Z., Li, Z., Zhao, Y., Song, Y. and Lu, J. (2006), "Magnetic bead-based chemiluminescence detection of sequence-specific DNA by using catalytic nucleic acid labels", *Analytica Chimica Acta*, Vol. 557, No. 1, p. 152-158.

- Carisse, O., Tremblay, D. M., Lévesque, C. A., Gindro, K., Ward, P. and Houde, A. (2009), "Development of a taqman real-time pcr assay for quantification of airborne conidia of *Botrytis squamosa* and management of *Botrytis* leaf blight of onion", *Phytopathology*, Vol. 99, No. 11, p. 1273-1280.
- Cass, A. E., Davis, G., Francis, G. D., Hill, H. A. O., Aston, W. J., Higgins, I. J., Plotkin, E. V., Scott, L. D. and Turner, A. P. (1984), "Ferrocene-mediated enzyme electrode for amperometric determination of glucose", *Analytical Chemistry*, Vol. 56, No. 4, p. 667-671.
- Caygill, R. L., Blair, G. E. and Millner, P. A. (2010), "A review on viral biosensors to detect human pathogens", *Analytica Chimica Acta*, Vol. 681, No. 1, p. 8-15.
- Celius, T., Matthews, J. B., Giesy, J. P. and Zacharewski, T. R. (2000), "Quantification of rainbow trout (*Oncorhynchus mykiss*) zona radiata and vitellogenin mRNA levels using real-time PCR after in vivo treatment with estradiol-17 β or α -zearalenol", *The Journal of steroid biochemistry and molecular biology*, Vol. 75, No. 2, p. 109-119.
- Chaki, N. K. and Vijayamohanan, K. (2002), "Self-assembled monolayers as a tunable platform for biosensor applications", *Biosensors and Bioelectronics*, Vol. 17, No. 1-2, p. 1-12.
- Chaput, J. (1995), *Identification of Diseases and Disorders of Onions*, report number 81003, Queen's Printer for Ontario, Ontario Ministry of Agriculture, Food and Rural Affairs.
- Chen, L., Manz, A. and Day, P. J. R. (2007a), "Total nucleic acid analysis integrated on microfluidic devices", *Lab on a Chip - Miniaturisation for Chemistry and Biology*, Vol. 7, No. 11, p. 1413-1423.
- Chen, Z., Peng, Z., Zhang, P., Jin, X., Jiang, J., Zhang, X., Shen, G. and Yu, R. (2007b), "A sensitive immunosensor using colloidal gold as electrochemical label", *Talanta*, Vol. 72, No. 5, p. 1800-1804.
- Chilvers, M. I. and Du Toit, L. J. (2006), "Detection and identification of *Botrytis* species associated with neck rot, scape blight, and umbel blight of onion", *Plant Health Progress*, [Online].
- Chilvers, M. I., Du Toit, L. J., Akamatsu, H. and Peever, T. L. (2007), "A real-time, quantitative PCR seed assay for *Botrytis* spp. that cause neck rot of onion", *Plant Disease*, Vol. 91, No. 5, p. 599-608.
- Chope, G. A., Cools, K., Hammond, J. P., Thompson, A. J. and Terry, L. A. (2012), "Physiological, biochemical and transcriptional analysis of onion bulbs during storage", *Annals of Botany*, Vol. 109, No. 4, p. 819-831.

- Chung, D., Kim, K. and Choi, S. (2011), "Electrochemical DNA biosensor based on avidin–biotin conjugation for influenza virus (type A) detection", *Applied Surface Science*, Vol. 257, No. 22, p. 9390-9396.
- Clark, L. C. and Lyons, C. (1962), "Electrode systems for continuous monitoring in cardiovascular surgery", *Annals of the New York Academy of Sciences*, Vol. 102, No. 1, p. 29-45.
- Clark, C. A. and Lorbeer, J. W. (1973), "Reaction of onion cultivars to Botrytis brown stain.", *Plant Disease Reporter*, Vol. 57, p. 210-214.
- Compton, R. G. and Banks, C. E. (2007), *Understanding voltammetry*, World Scientific Pub Co Inc, Danvers USA.
- Coolong, T. W., Walcott, R. R. and Randle, W. M. (2008), "A quantitative real-time polymerase chain reaction assay for *Botrytis aclada* in onion bulb tissue", *Hort Science*, Vol. 43, No. 2, p. 408-413.
- Cosnier, S. (2000), "Biosensors based on immobilization of biomolecules by electrogenerated polymer films", *Applied Biochemistry and Biotechnology*, Vol. 89, No. 2, p. 127-138.
- Cottrell, F. G. (1903), *Zeitschrift für physikalische Chemie, Stöchiometrie und Verwandtschaftslehre*, Vol. 42, p. 385.
- Cullen, D. and Hirsch, P. (1998), "Simple and rapid method for direct extraction of microbial DNA from soil for PCR", *Soil Biology and Biochemistry*, Vol. 30, No. 8, p. 983-993.
- Das, K., Penelle, J., Rotello, V. M. and Nüsslein, K. (2003), "Specific recognition of bacteria by surface-templated polymer films", *Langmuir*, Vol. 19, No. 15, p. 6226-6229.
- Davies, R. J., Eapen, S. S. and Carlisle, S. J. (2007a), "Glucose Measurement Within Diabetes via "Traditional" Electrochemical Biosensor.", in Marks, R. S., Cullen, D. C., Karube, I., Lowe, C. R. and Weetall, H. H. (editors), *Handbook of biosensors and biochips*, John Wiley, Chichester, p. 1111-1129.
- Davies, R. J., Eapen, S. S. and Carlisle, S. J. (2007b), "Lateral-Flow Immunochromatographic Assays", in Marks, R. S., Cullen, D. C., Karube, I., Lowe, C. R. and Weetall, H. H. (editors), *Handbook of biosensors and biochips*, John Wiley, Chichester, p. 1151-1165.
- Davis, F., Collyer, S. D. and Higson, S. P. J. (2007), "Overview of Modern Analytical Needs", in Marks, R. S., Cullen, D. C., Karube, I., Lowe, C. R. and Weetall, H. H. (editors), *Handbook of biosensors and biochips*, John Wiley, Chichester, p. 23-39.

- Davis, J. J., Halliwell, C. M., Hill, H. A. O., Canters, G. W., van Amsterdam, M. C. and Verbeet, M. P. (1998), "Protein adsorption at a gold electrode studied by insitu scanning tunnelling microscopy", *New journal of chemistry*, Vol. 22, No. 10, p. 1119-1123.
- Delahaut, K. and Stevenson, W. (2004), *Onion Disorder: Fusarium basal rot*, report number A3114, Cooperative Extension of the University of Wisconsin, Madison.
- Dewey, F. M. and Cole, L. (1996), "Monoclonal Antibody based assays for detection and quantification of *Botrytis cinerea*.", in Dehne, H. W., Adam, G., Diekmann, M., Frahm, J., Mauler-Machnik, A. and van Halteren, P. (editors), *Diagnosis and Identification of Plant Pathogens*, Kluwer Academic Publishers, Dordrecht, p. 91-94.
- Dewey, F. M., Steel, C. C. and Gurr, S. J. (2013), "Lateral-Flow Devices to Rapidly Determine Levels of Stable *Botrytis* Antigens in Table and Dessert Wines", *American Society for Enology and Viticulture*, Vol. 4, No. 2, p. 291-295.
- Dewey, F. M. (1996), "Development of immunoassays for the detection and quantification of fungi", *Developments in Plant Pathology*, Vol. 8, p. 139-146.
- Dewey, F. M. and Yohalem, D. (2004), "Detection, Quantification and Immunolocalisation of *Botrytis* Species", in Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. (editors), *Botrytis: Biology, Pathology and Control*, Kluwer Academic Publishers, Dordrecht, p. 181-194.
- Dey, P. K., Pramanick, B., RaviShankar, A., Ganguly, P. and Das, S. (2010), "Microstructuring of SU-8 resist for mems and bio-applications", *International Journal on Smart Sensing and Intelligent Systems*, Vol. 3, No. 1, p. 118-129.
- Diguta, C. F., Rousseaux, S., Weidmann, S., Bretin, N., Vincent, B., Guilloux-Benatier, M. and Alexandre, H. (2010), "Development of a qPCR assay for specific quantification of *Botrytis cinerea* on grapes", *FEMS microbiology letters*, Vol. 313, No. 1, p. 81-87.
- Ding, C., Li, H., Hu, K. and Lin, J. (2010), "Electrochemical immunoassay of hepatitis B surface antigen by the amplification of gold nanoparticles based on the nanoporous gold electrode", *Talanta*, Vol. 80, No. 3, p. 1385-1391.
- Downes, K., Chope, G. A. and Terry, L. A. (2009), "Effect of curing at different temperatures on biochemical composition of onion (*Allium cepa* L.) skin from three freshly cured and cold stored UK-grown onion cultivars", *Postharvest Biology and Technology*, Vol. 54, No. 2, p. 80-86.
- Du Toit, L. J., Derie, M. L., Hsiang, T. and Pelter, G. Q. (2002), "*Botrytis porri* in onion seed crops and onion seed", *Plant Disease*, Vol. 86, p. 1178.

- Du Toit, L. J., Derie, M. L. and Pelter, G. Q. (2004), "Prevalence of *Botrytis* spp. in onion seed crops in the Columbia Basin of Washington", *Plant Disease*, Vol. 88, No. 10, p. 1061-1068.
- Dupont-Filliard, A., Billon, M., Livache, T. and Guillerez, S. (2004), "Biotin/avidin system for the generation of fully renewable DNA sensor based on biotinylated polypyrrole film", *Analytica Chimica Acta*, Vol. 515, No. 2, p. 271-277.
- Edman, L., Földes-Papp, Z., Wennmalm, S. and Rigler, R. (1999), "The fluctuating enzyme: a single molecule approach", *Chemical Physics*, Vol. 247, No. 1, p. 11-22.
- Ekins, R., Chu, F. and Biggart, E. (1990), "Fluorescence spectroscopy and its application to a new generation of high sensitivity, multi-microspot, multianalyte, immunoassay", *Clinica Chimica Acta*, Vol. 194, No. 1, p. 91-114.
- Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. (2004), "*Botrytis* spp. and diseases they cause in agricultural systems", in *Botrytis: Biology, Pathology and Control*, Kluwer Academic Publishers, Dordrecht, p. 1-9.
- Elghanian, R., Storhoff, J. J., Mucic, R. C., Letsinger, R. L. and Mirkin, C. A. (1997), "Selective colorimetric detection of polynucleotides based on the distance-dependent optical properties of gold nanoparticles", *Science*, Vol. 277, p. 1078-1081.
- Ellington, A. and Pollard, J. D. (1999), "Preparation and analysis of DNA", in Ausubel, R. B., Brent, R., Kingston, R., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (editors), *Short protocols in molecular biology*, Wiley, New York, p. 1-52.
- Fakanya, W. M. and Tothill, I. E. (2012), *Development of a multimarker electrochemical immunosensor for cardiovascular disease detection* (unpublished PhD thesis), Cranfield University, Cranfield, UK.
- Fanjul-Bolado, P., González-García, M. B. and Costa-García, A. (2005), "Amperometric detection in TMB/HRP-based assays", *Analytical and Bioanalytical chemistry*, Vol. 382, No. 2, p. 297-302.
- Fernandez-Baldo, M. A., Messina, G. A., Sanz, M. I. and Raba, J. (2010), "Microfluidic Immunosensor with Micromagnetic Beads Coupled to Carbon-Based Screen-Printed Electrodes (SPCEs) for Determination of *Botrytis cinerea* in Tissue of Fruits", *Agricultural and Food Chemistry*, Vol. 58, No. 21, p. 11201-11206.
- Fernandez-Baldo, M. A., Fernandez, J. G., Pereira, S. V., Messina, G. A., Raba, J. and Sanz Ferramola, M. I. (2011), "Development of an indirect competitive enzyme-linked immunosorbent assay applied to the *Botrytis cinerea* quantification in tissues of postharvest fruits", *BMC Microbiology*, Vol. 11, p. 220-228.

- Finney, D. J. (1983), "Statistical Methods in Biological Assays", *Clinical Chemistry*, Vol. 29, p. 1762.
- Finney, D. J. (1974), *Statistical Methods in Biological Assays*, Griffin, London.
- Friguet, B., Chaffotte, A. F., Djavadi-Ohanian, L. and Goldberg, M. E. (1985), "Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay", *Journal of immunological methods*, Vol. 77, No. 2, p. 305-319.
- Fukuta, S., Kato, S., Yoshida, K., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M. and Ishimoto, Y. (2003), "Detection of tomato yellow leaf curl virus by loop-mediated isothermal amplification reaction", *Journal of virological methods*, Vol. 112, No. 1, p. 35-40.
- Furuya, M., Haramura, M. and Tanaka, A. (2006), "Reduction of nonspecific binding proteins to self-assembled monolayer on gold surface", *Bioorganic & medicinal chemistry*, Vol. 14, No. 2, p. 537-543.
- Garcia, I. (2013), *Botrytis cinerea: a highly infectious crop killer*, available at: http://www.canna-uk.com/botrytis_cinerea (accessed 25th November 2013).
- Geerligs, H., Weijer, W., Bloemhoff, W., Welling, G. and Welling-Wester, S. (1988), "The influence of pH and ionic strength on the coating of peptides of *herpes simplex* virus type 1 in an enzyme-linked immunosorbent assay", *Journal of immunological methods*, Vol. 106, No. 2, p. 239-244.
- George, N. A. (2005), *Plant Pathology* (5th ed) , Dana Dreibelbis.
- Glomm, W. R. (2005), "Functionalized gold nanoparticles for applications in bionanotechnology", *Journal of Dispersion Science and Technology*, Vol. 26, No. 3, p. 389-414.
- Goddard, J. M. and Hotchkiss, J. H. (2007), "Polymer surface modification for the attachment of bioactive compounds", *Progress in Polymer Science*, Vol. 32, No. 7, p. 698-725.
- Gossel, T. A. and Mahalik, M. P. (1993), "Pregnancy testing products", *U.S. Pharmacist*, Vol. 18, No. 7, p. 37-48.
- Grabar, K. C., Brown, K. R., Keating, C. D., Stranick, S. J., Tang, S. L. and Natan, M. J. (1997), "Nanoscale characterization of gold colloid monolayers: a comparison of four techniques", *Analytical Chemistry*, Vol. 69, No. 3, p. 471-477.
- Green, K. R. (2009), "Seed Treatment for the Control of Onion Neck Rot", *Seed Production and Treatment*, Vol. 24, No. 5.

- Gulliksen, A., Solli, L., Karlsen, F., Rogne, H., Hovig, E., Nordstrøm, T. and Sirevåg, R. (2004), "Real-Time Nucleic Acid Sequence-Based Amplification in Nanoliter Volumes", *Analytical Chemistry*, Vol. 76, No. 1, p. 9-14.
- Halperin, A., Buhot, A. and Zhulina, E. B. (2006), "Hybridization at a surface: The role of spacers in DNA microarrays", *Langmuir*, Vol. 22, No. 26, p. 11290-11304.
- Hammer, A., Hammer, H. and Hammer, K. (1994), *Physikalische Formeln und Tabellen* (6th ed), Lindauer, Munich.
- Heid, C. A., Stevens, J., Livak, K. J. and Williams, P. M. (1996a), "Real time quantitative PCR.", *Genome research*, Vol. 6, No. 10, p. 986-994.
- Heid, C. A., Stevens, J., Livak, K. J. and Williams, P. M. (1996b), "Real time quantitative PCR.", *Genome research*, Vol. 6, No. 10, p. 986-994.
- Hennebert, G. L. (1973), "Botrytis and Botrytis-like genera", *Persoonia*, Vol. 7, p. 183-204.
- Hermanson, G. T. (2013), "(Strept)avidin-Biotin Systems", in Hermanson, G. T. (editor), *Bioconjugate Techniques* (3rd ed), Academic Press, Boston, p. 465-505.
- Herne, M. T. and Tarlov, M. J. (1997), "Characterization of DNA Probes Immobilized on Gold Surfaces", *Journal of the American Chemical Society*, Vol. 119, No. 38, p. 8916-8920.
- Herrasti, Z., Etxabe, I., Mitxelena, J. M., Gabilondo, I., Martínez, M. P. and Martínez, F. (2013), "Development and integration of an electrochemical system in a LOC device for DNA detection", *Sensors and Actuators B: Chemical*, Vol. 189, p. 66-70.
- Hirst, N. A., Hazelwood, L. D., Jayne, D. G. and Millner, P. A. (2013), "An amperometric lactate biosensor using H₂O₂ reduction via a Prussian Blue impregnated poly(ethyleneimine) surface on screen printed carbon electrodes to detect anastomotic leak and sepsis", *Sensors and Actuators B: Chemical*, Vol. 186, p. 674-680.
- Holz, G., Coertze, S. and Williamson, B. (2004), "The Ecology of *Botrytis* on Plant Surface", in Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. (editors), *Botrytis: Biology, Pathology and Control*, Kluwer Academic Publishers, Dordrecht, p. 9-27.
- Horányi, G., Rizmayer, E. M. and Joó, P. J. (1983), "Radiotracer Study of the Adsorption of Cland Hso⁴⁻ Ions on a Porous Gold Electrode and on Underpotential Deposited Metals on Gold.", *Electroanalytical Chemistry*, Vol. 152, p. 211-222.

- Hsieh, T., Luo, C., Lee, G., Liao, C. and Huang, F. (2006), "A micromachined low-power-consumption portable PCR system", *Journal of Medical and Biological Engineering*, Vol. 26, No. 1, p. 43.
- Hyatt, M. A. (1989), *Colloidal Gold: Principles, Methods, and Applications*, Academic Press.
- Ibrahim, M. S., Lofts, R. S., Jahrling, P. B., Henschel, E. A., Weedn, V. W., Northrup, M. A. and Belgrader, P. (1998), "Real-Time Microchip PCR for Detecting Single-Base Differences in Viral and Human DNA", *Analytical Chemistry*, Vol. 70, No. 9, p. 2013-2017.
- Kabanda, A., Goffin, E., Bernard, A., Lauwerys, R. and Van Ypersele de Strihou, C. (1995), "Factors influencing serum levels and peritoneal clearances of low molecular weight proteins in continuous ambulatory peritoneal dialysis", *Kidney international*, Vol. 48, No. 6, p. 1946-1952.
- Kaewphinit, T., Santiwatanakul, S. and Chansiri, K. (2012), "Quartz crystal microbalance DNA based biosensor for diagnosis: A review", *Sensors and Transducers*, Vol. 143, No. 8, p. 44-59.
- Kambhampati, D. and Knoll, W. (2002), "Biotin-streptavidin sensor surface: A versatile platform for performing DNA hybridization interactions", in Möbius, D. and Miller, R. (editors), *Studies in Interface Science* (16th ed), Elsevier, Ulm, p. 279-316.
- Kandimalla, V.,B., Tripathi, V., S. and Ju, H. (2008), "Biosensors based on immobilization of biomolecules", in Zhang, X., Ju, H. and Wang, J. (editors), *Electrochemical sensors, biosensors, and their biomedical applications*, Academic Press, Amsterdam; Boston, p. 503-530.
- Karlsson, R. (2004), "SPR for molecular interaction analysis: a review of emerging application areas", *Journal of Molecular Recognition*, Vol. 17, No. 3, p. 151-161.
- Karman, K., Endo, T. and Tamiya, E. (2007), "Label-Free Gene and Protein Sensors Based on electrochemical and Local Plasmon Resonance Devices", in Marks, R. S., Cullen, D. C., Karube, I., Lowe, C. R. and Weetall, H. H. (editors), *Handbook of biosensors and biochips*, John Wiley, Chichester, p. 925-937.
- Kenna, J., Major, G. and Williams, R. (1985), "Methods for reducing non-specific antibody binding in enzyme-linked immunosorbent assays", *Journal of immunological methods*, Vol. 85, No. 2, p. 409-419.
- Kerman, K., Vestergaard, M. and Tamiya, E. (2009), "Electrochemical DNA Biosensors: Protocols for Intercalator-Based Hybridization in Solution and at the Surface", in Rasooly, A. and Herold, K. E. (editors), *Methods in Molecular Biology 504: Biosensors and Biodetection, Methods and Protocols*. (2nd ed), Human Press, New York, p. 99-143.

- Khan, M. I., Marroni, V., Keenan, S., Scott, I. A. W., Viljanen-Rollinson, S. L. H. and Bulman, S. (2013), "Enhanced molecular identification of *Botrytis* spp. from New Zealand onions", *European Journal of Plant Pathology*, Vol. 1, p. 1-13.
- Kim, G., Rand, A. G. and Letcher, S. V. (2003), "Impedance characterization of a piezoelectric immunosensor part II: *Salmonella typhimurium* detection using magnetic enhancement", *Biosensors and Bioelectronics*, Vol. 18, No. 1, p. 91-99.
- Kitamoto, Y., Suzuki, A., Shimada, S. and Yamanaka, K. (2002), "A new method for the preservation of fungus stock cultures by deep-freezing", *Mycoscience*, Vol. 43, No. 2, p. 143-149.
- Köhl, J., van der Plas, C. H., Molhoek, W. M. L. and Fokkema, N. J. (1995), "Effect of interrupted leaf wetness periods on suppression of sporulation of *Botrytis allii* and *B. cinerea* by antagonists on dead onion leaves", *European Journal of Plant Pathology*, Vol. 101, No. 6, p. 627-637.
- Kricka, L. J. (1995), "Labels, Labeling, Analytical Strategies, and Applications", in Kricka, L. J. (editor), *Nonisotopic Probing, Blotting, and Sequencing* (2nd ed), Academic Press, San Diego, p. 3-40.
- Kritzman, G. (1983), "Identification of latent *Botrytis allii* Munn in onion bulbs", *Crop Protection*, Vol. 2, No. 2, p. 243-246.
- Kritzman, G. and Netzer, D. (1978), "A selective medium for isolation and identification of *Botrytis* spp. from Soil and onion Seed", *Phytoparasitica*, Vol. 6, No. 1, p. 3-7.
- Kuske, C. R., Banton, K. L., Adorada, D. L., Stark, P. C., Hill, K. K. and Jackson, P. J. (1998), "Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil", *Applied and Environmental Microbiology*, Vol. 64, No. 7, p. 2463-2472.
- Lagally, E., Scherer, J., Blazej, R., Toriello, N., Diep, B., Ramchandani, M., Sensabaugh, G., Riley, L. and Mathies, R. (2004), "Integrated portable genetic analysis microsystem for pathogen/infectious disease detection", *Analytical Chemistry*, Vol. 76, No. 11, p. 3162-3170.
- Larkin, R. P. and Fravel, D. R. (2002), "Effects of varying environmental conditions on biological control of *Fusarium* wilt of tomato by nonpathogenic *Fusarium* spp.", *Phytopathology*, Vol. 92, No. 11, p. 1160-1166.
- Lasdon, L. S., Waren, A. D., Jain, A. and Ratner, M. (1978), "Design and Testing of a Generalized Reduced Gradient Code for Nonlinear Programming.", *ACM Transactions on Mathematical Software*, Vol. 4, No. 1, p. 34-50.

- Laureyn, W., Stakenborg, T. and Jacobs, P. (2007), "Genetic and other DNA-based Biosensors Applications", in Marks, R. S., Cullen, D. C., Karube, I., Lowe, C. R. and Weetall, H. H. (editors), *Handbook of biosensors and biochips*, John Wiley, Chichester, p. 1035-1053.
- Lawrie, J. L., Jiao, Y. and Weiss, S. M. (2010), "Size-dependent infiltration and optical detection of nucleic acids in nanoscale pores", *IEEE Transactions on Nanotechnology*, Vol. 9, No. 5, p. 596-602.
- Leckband, D. and Langer, R. (1991), "An approach for the stable immobilization of proteins", *Biotechnology and bioengineering*, Vol. 37, No. 3, p. 227-237.
- Lee, J. G., Cheong, K. H., Huh, N., Kim, S., Choi, J. W. and Ko, C. (2006), "Microchip-based one step DNA extraction and real-time PCR in one chamber for rapid pathogen identification", *Lab on a Chip - Miniaturisation for Chemistry and Biology*, Vol. 6, No. 7, p. 886-895.
- Lee, S. B. and Taylor, J. W. (1990), "Isolation of DNA from fungal mycelia and single spores", in Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (editors), *PCR Protocols: A Guide to Methods and Applications*, Academic Press, London, p. 282-287.
- Lehninger, A. L., Nelson, D. L. and Cox, M. M. (2005), "Lehninger principles of biochemistry", in (4th ed), W.H. Freeman, New York, p. 319-320.
- Li, Z., Jin, R., Mirkin, C. A. and Letsinger, R. L. (2002), "Multiple thiol-anchor capped DNA-gold nanoparticle conjugates", *Nucleic acids research*, Vol. 30, No. 7, p. 1558-1562.
- Linfield, C. A., Kenny, S. R. and Lyons, N. F. (1995), "A serological test for detecting *Botrytis allii*, the cause of neck rot of onion bulbs", *Annals of Applied Biology*, Vol. 126, No. 2, p. 259-268.
- Liu, G. D., Wu, Z. Y., Wang, S. P., Shen, G. L. and Yu, R. Q. (2001), "Renewable amperometric immunosensor for *Schistosoma japonicum* antibody assay", *Analytical Chemistry*, Vol. 73, No. 14, p. 3219-3226.
- Liu, W. and Saint, D. A. (2002), "Validation of a quantitative method for real time PCR kinetics", *Biochemical and biophysical research communications*, Vol. 294, No. 2, p. 347-353.
- Liu, B., Yan, F., Kong, J. and Deng, J. (1999), "A reagentless amperometric biosensor based on the coimmobilization of horseradish peroxidase and methylene green in a modified zeolite matrix", *Analytica Chimica Acta*, Vol. 386, No. 1-2, p. 31-39.

- Lorbeer, J. W., Seyb, A. M., Boer, M. and van den Ende, J. E. (2004), "*Botrytis* Species on Bulb Crops", in Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. (editors), *Botrytis: Biology, Pathology and Control*, Kluwer Academic Publishers, Dordrecht, p. 273-294.
- Lowe, R. C. (2007), "Overview of Biosensor and Bioarray Technology", in Marks, R. S., Cullen, D. C., Karube, I., Lowe, C. R. and Weetall, H. H. (editors), *Handbook of biosensors and biochips*, John Wiley, Chichester, p. 7-22.
- Lucarelli, F., Marrazza, G., Turner, A. P. F. and Mascini, M. (2004), "Carbon and gold electrodes as electrochemical transducers for DNA hybridisation sensors", *Biosensors and Bioelectronics*, Vol. 19, No. 6, p. 515-530.
- Lucarelli, F., Tombelli, S., Minunni, M., Marrazza, G. and Mascini, M. (2008), "Electrochemical and piezoelectric DNA biosensors for hybridisation detection", *Analytica Chimica Acta*, Vol. 609, No. 2, p. 139-159.
- Lyons, M. E. G. (2007), "Modeling of Biosensor Interfaces", in Marks, R. S., Cullen, D. C., Karube, I., Lowe, C. R. and Weetall, H. H. (editors), *Handbook of biosensors and biochips*, John Wiley, Chichester, p. 291-319.
- Mackey, D., Killard, A. J., Ambrosi, A. and Smyth, M. R. (2007), "Optimizing the ratio of horseradish peroxidase and glucose oxidase on a bienzyme electrode: Comparison of a theoretical and experimental approach", *Sensors and Actuators B: Chemical*, Vol. 122, No. 2, p. 395-402.
- Malone, M. D., Biddlecombe, R. A. and Law, B. (1996), "Enzyme-linked immunosorbent assay (ELISA) development and optimisation", in Law, B. (editor), *Immunoassays: A Practical Guide*, Taylor and Francis, London, p. 127-149.
- Marín, P., Jurado, M., Magan, N., Vázquez, C. and González-Jaén, M. T. (2010), "Effect of solute stress and temperature on growth rate and TRI5 gene expression using real time RT-PCR in *Fusarium graminearum* from Spanish wheat", *International journal of food microbiology*, Vol. 140, No. 2-3, p. 169-174.
- Marks, R. S., Cullen, D. C., Karube, I., Lowe, C. R. and Weetall, H. H. (2007), *Handbook of biosensors and biochips*, John Wiley, Chichester.
- Martinez, F., Corio-Costet, M. F., Levis, C., Coarer, M. and Fermaud, M. (2008), "New PCR primers applied to characterize distribution of *Botrytis cinerea* populations in french vineyards", *Vitis - Journal of Grapevine Research*, Vol. 47, No. 4, p. 217-226.
- Maude, R. B. (1983), "The correlation between seed-borne infection by *Botrytis allii* and neck rot development in store.", *Seed Science and Technology*, Vol. 11, No. 3, p. 829-834.

- Maude, R. B., Bambridge, J. M., Presly, A. H. and Phelps, K. (1986), "Effects of field environment and cultural practices on the incidence of *Botrytis allii* (neck rot) in stored onion bulbs.", *Quaderni Della Scuola Di Specializzazione In Viticoltura ed Enologia*, Vol. 2, p. 241-243.
- Maude, R.B. and Landahl, S. (2011), *Personal communication at HRI Wellesbourne Conference Centre (23rd March 2011). Participants: Maude, R. B; Landahl, S.; Smith, B.; Binder, M.*, Warwick University, Wellesbourne, UK.
- Maude, R. B. and Presly, A. H. (1977a), "Neck rot (*Botrytis allii*) of bulb onions I. Seed-borne infection in relationship to the disease in store and the effect of seed treatment", *Annals of Applied Biology (UK)*, Vol. 86, No. 2, p. 163-180.
- Maude, R. B. and Presly, A. H. (1977b), "Neck rot (*Botrytis allii*) of bulb onions II. Seed-borne infection in relationship to the disease in store and the effect of seed treatment", *Annals of Applied Biology*, Vol. 86, No. 2, p. 181-188.
- Maude, R. B., Shipway, M. R., Presly, A. H. and O'Connor, D. (1984), "The effects of direct harvesting and drying systems on the incidence and control of neck rot (*Botrytis allii*) in onions", *Plant Pathology*, Vol. 33, No. 2, p. 263-268.
- Maude, R. B., Bambridge, J. M. and Presly, A. H. (1982), "The persistence of *Botrytis allii* in field soil", *Plant Pathology*, Vol. 31, No. 3, p. 247-252.
- Melnik, E., Bruck, R., Hainberger, R. and Lämmerhofer, M. (2011), "Multi-step surface functionalization of polyimide based evanescent wave photonic biosensors and application for DNA hybridization by Mach-Zehnder interferometer", *Analytica Chimica Acta*, Vol. 699, No. 2, p. 206-215.
- Mendes, R. K., Carvalhal, R. F., Stach-Machado, D. R. and Kubota, L. T. (2009), "Surface plasmon resonance immunosensor for early diagnosis of Asian rust on soybean leaves", *Biosensors and Bioelectronics*, Vol. 24, No. 8, p. 2483-2487.
- Metcalf, D. (1998), "*Botrytis* seed testing - what the results mean?", *Onions Australia.*, Vol. 15, p. 33-34.
- Metfies, K., Huljic, S., Lange, M. and Medlin, L. K. (2005), "Electrochemical detection of the toxic dinoflagellate *Alexandrium ostenfeldii* with a DNA-biosensor", *Biosensors and Bioelectronics*, Vol. 20, No. 7, p. 1349-1357.
- Meyer, U. M. and Dewey, F. M. (2000), "Efficacy of different immunogens for raising monoclonal antibodies to *Botrytis cinerea*", *Mycological Research*, Vol. 104, No. 8, p. 979-987.
- Meyer, U. M., Spotts, R. A. and Dewey, F. M. (2000), "Detection and Quantification of *Botrytis cinerea* by ELISA in Pear Stems During Cold Storage", *Plant Disease*, Vol. 84, No. 10, p. 1099-1103.

- Miecinskas, P., Leinartas, K., Uksiene, V. and Juzeliunas, E. (2007), "QCM study of microbiological activity during long-term exposure to atmosphere-aluminium colonisation by *Aspergillus Niger*", *Journal of Solid State Electrochemistry*, Vol. 11, No. 7, p. 909-913.
- Millar, D. S., Withey, S. J., Tizard, M. L., Ford, J. G. and Hermontaylor, J. (1995), "Solid-phase hybridization capture of low-abundance target DNA sequences: application to the polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum*", *Analytical Biochemistry*, Vol. 226, No. 2, p. 325-330.
- Millner, P. A., Hays, H. C. W., Vakurov, A., Pchelintsev, N. A., Billah, M. M. and Rodgers, M. A. (2009), "Nanostructured transducer surfaces for electrochemical biosensor construction—Interfacing the sensing component with the electrode", *Seminars in cell & developmental biology*, Vol. 20, No. 1, p. 34-40.
- Mimori, T., Akizuki, M., Yamagata, H., Inada, S., Yoshida, S. and Homma, M. (1981), "Characterization of a high molecular weight acidic nuclear protein recognized by autoantibodies in sera from patients with polymyositis-scleroderma overlap.", *Journal of Clinical Investigation*, Vol. 68, No. 3, p. 611.
- Moller, E. M., Bahnweg, R., Sandermann, H. and Geiger, H. H. (1992), "A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues", *Nucleic acids research*, Vol. 20, No. 22, p. 6115-6116.
- Morrin, A., Killard, A. J. and Smyth, R. M. (2007), "Electrochemical Characterization of Commercial and Home-Made Screen-Printed Carbon Electrodes", *Analytical Letters*, Vol. 36, No. 9, p. 2021-2039.
- Moy, V. T., Florin, E. L. and Gaub, H. E. (1994), "Intermolecular forces and energies between ligands and receptors", *Science*, Vol. 266, No. 5183, p. 257-259.
- Mueller, T. R. and Adams, R. N. (1961), "Voltammetry at inert electrodes: II. Correlation of experimental results with theory for voltage and controlled potential scanning, controlled potential electrolysis, and chronopotentiometric techniques. Oxidation of ferrocyanide and o-dianisidine at boron carbide electrodes", *Analytica Chimica Acta*, Vol. 25, No. 5, p. 482-497.
- Myles, T. (2003), *Pesticides 2003*, report number 00-353-1-8204260, Pesticide Control Service, Department of Agriculture and Food, Dublin.
- Nakasone, K. K., Peterson, S. W. and Jong, S. C. (2004), "Preservation and distribution of fungal cultures", in Mueller, G.M., Bills, G. F. and Foster, M. S. (editors), *Biodiversity of Fungi*, Elsevier Academic Press, London, p. 37-47.

- Nelson, W. J., Vorgias, C. E. and Traub, P. (1982), "A rapid method for the large scale purification of the intermediate filament protein vimentin by single-stranded DNA-cellulose affinity chromatography", *Biochemical and biophysical research communications*, Vol. 106, No. 4, p. 1141-1147.
- Newman, J. D. and Turner, A. P. F. (2007), "Historical Perspective of Biosensors and Biochip Development", in Marks, R. S., Cullen, D. C., Karube, I., Lowe, C. R. and Weetall, H. H. (editors), *Handbook of biosensors and biochips*, John Wiley, Chichester, p. 41-56.
- Nielsen, K., Justesen, A. F., Jensen, D. F. and Yohalem, D. S. (2001), "Universally Primed Polymerase Chain Reaction Alleles and Internal Transcribed Spacer Restriction Fragment Length Polymorphisms Distinguish Two Subgroups in *Botrytis aclada* Distinct from *B. byssoides*", *Phytopathology*, Vol. 91, No. 6, p. 527-533.
- Nielsen, K., Justesen, A. F. and Yohalem, D. S. (1999), "PCR based detection of latent infections of *Botrytis aclada* Fres. in onion bulbs", *Petria*, Vol. 9, p. 105-108.
- Nielsen, K., Yohalem, D. S. and Jensen, D. F. (2002), "PCR detection and RFLP differentiation of *Botrytis* species associated with neck rot of onion", *Plant Disease*, Vol. 86, No. 6, p. 682-686.
- Noh, M. F. M. and Tothill, I. E. (2006), "Development and characterisation of disposable gold electrodes, and their use for lead(II) analysis", *Analytical and Bioanalytical Chemistry*, Vol. 386, No. 7-8, p. 2095-2106.
- O'Connor, D., Leon, T. and Landahl, S. (2010), *Non invasive approaches to identification and automatic rejection of internal defects in bulb onions [Feasibility Study]*, report number HL1103, Horticulture LINK Programme, Cranfield University.
- Oldham, K. B., Gavaghan, D. J. and Bond, A. M. (2002), "A full analytic treatment of reversible linear-scan voltammetry with square-wave modulation", *The Journal of Physical Chemistry B*, Vol. 106, No. 1, p. 152-157.
- Padeste, C., Grubelnik, A. and Tiefenauer, L. (2000), "Ferrocene-avidin conjugates for bioelectrochemical applications", *Biosensors and Bioelectronics*, Vol. 15, No. 9, p. 431-438.
- Pal, D. and Venkataraman, V. (2002), "A portable battery-operated chip thermocycler based on induction heating", *Sensors and Actuators A: Physical*, Vol. 102, No. 1, p. 151-156.
- Palchetti, I., Laschi, S. and Mascini, M. (2009), "Electrochemical Biosensor Technology: Application to Pesticide Detection", in Rasooly, A. and Herold, K. E. (editors), *Methods in Molecular Biology 504: Biosensors and Biodetection, Methods and Protocols*. (2nd ed), Human Press, New York, p. 169-183.

- Pan, J., Gregory, L. T. and Quintero, A. (1999), "Screen printing process design of experiments for fine line printing of thick film ceramic substrates", *Journal of Electronics Manufacturing*, Vol. 9, No. 3, p. 203-213.
- Pänke, O., Balkenhohl, T., Kafka, J., Schäfer, D. and Lisdat, F. (2008), "Impedance Spectroscopy and Biosensing", in Sheper, T., Renneberg, R. and Lisdat, F. (editors), *Biosensing for the 21st Century: Advances in Biochemical Engineering / Biotechnology*. (109th ed), Springer, Berlin, p. 195-237.
- Park, I., Kim, D., Adanyi, N., Varadi, M. and Kim, N. (2004), "Development of a direct-binding chloramphenicol sensor based on thiol or sulfide mediated self-assembled antibody monolayers", *Biosensors and Bioelectronics*, Vol. 19, No. 7, p. 667-674.
- Parker, C. O. and Tothill, I. E. (2009), "Development of an electrochemical immunosensor for aflatoxin M1 in milk with focus on matrix interference", *Biosensors and Bioelectronics*, Vol. 24, No. 8, p. 2452-2457.
- Peeters, S., Stakenborg, T., Reekmans, G., Laureyn, W., Lagae, L., Van Aerschot, A. and Van Ranst, M. (2008), "Impact of spacers on the hybridization efficiency of mixed self-assembled DNA/alkanethiol films", *Biosensors and Bioelectronics*, Vol. 24, No. 1, p. 72-77.
- Pio, T. F. and Macedo, G. A. (2007), "Optimizing the production of cutinase by *Fusarium oxysporum* using response surface methodology", *Enzyme and microbial technology*, Vol. 41, No. 5, p. 613-619.
- Poghossian, A. and Schönig, M. J. (2007), "Chemical and Biological Field-Effect Sensor for Liquids - A Status Report", in Marks, R. S., Cullen, D. C., Karube, I., Lowe, C. R. and Weetall, H. H. (editors), *Handbook of biosensors and biochips*, John Wiley, Chichester, p. 395-411.
- Porstmann, T. and Kiessig, S. (1992), "Enzyme immunoassay techniques an overview", *Journal of immunological methods*, Vol. 150, No. 1, p. 5-21.
- Preece, T.F., (1959), *A staining method for the study of apple scab infections*, Blackwell Publishing Ltd, .
- Presly, A. H. (1985a), "Methods for inducing sporulation of some *Botrytis* species occurring on onions and leeks", *Transactions of the British Mycological Society*, Vol. 85, No. 4, p. 621-624.
- Presly, A. H. (1985b), "Studies on *Botrytis* Spp. Occurring on Onions (*Allium-Cepa*) and Leeks (*Allium-Porrum*)", *Plant Pathology*, Vol. 34, No. 3, p. 422-427.

- Prime, K. L. and Whitesides, G. M. (1993), "Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide): A model system using self-assembled monolayers", *Journal of the American Chemical Society*, Vol. 115, No. 23, p. 10714-10721.
- Randles, J. E. B. (1948), "A cathode ray polarograph. Part II. - The current-voltage curves", *Transactions of the Faraday Society*, Vol. 44, p. 327-338.
- Ricci, F., Lai, R. Y., Heeger, A. J., Plaxco, K. W. and Sumner, J. J. (2007), "Effect of molecular crowding on the response of an electrochemical DNA sensor", *Langmuir*, Vol. 23, No. 12, p. 6827-6834.
- Rotem, J. and Aust, H. J. (1991), "The Effect of Ultraviolet and Solar Radiation and Temperature on Survival of Fungal Propagules", *Journal of Phytopathology*, Vol. 133, No. 1, p. 76-84.
- Saito, S., Dunne, K. J., Evans, K. J., Barry, K., Cadle-Davidson, L. and Wilcox, W. F. (2013), "Optimisation of techniques for quantification of *Botrytis cinerea* in grape berries and receptacles by quantitative polymerase chain reaction", *Australian Journal of Grape and Wine Research*, Vol. 19, No. 1, p. 68-73.
- Salam, F. and Tothill, I. E. (2009a), *Development of immunosensors for Salmonella Typhimurium* (unpublished PhD thesis), Cranfield University, Cranfield, UK.
- Salam, F. and Tothill, I. E. (2009b), "Detection of *Salmonella typhimurium* using an electrochemical immunosensor", *Biosensors and Bioelectronics*, Vol. 24, No. 8, p. 2630-2636.
- Salam, F., Uludag, Y. and Tothill, I. E. (2013), "Real-time and sensitive detection of *Salmonella Typhimurium* using an automated quartz crystal microbalance (QCM) instrument with nanoparticles amplification", *Talanta*, Vol. 115, p. 761-767.
- San Paulo, A. and García, R. (2000), "High-Resolution Imaging of Antibodies by Tapping-Mode Atomic Force Microscopy: Attractive and Repulsive Tip-Sample Interaction Regimes", *Biophysical journal*, Vol. 78, No. 3, p. 1599-1605.
- Sanzani, S. M., Schena, L., De Cicco, V. and Ippolito, A. (2012), "Early detection of *Botrytis cinerea* latent infections as a tool to improve postharvest quality of table grapes", *Postharvest Biology and Technology*, Vol. 68, p. 64-71.
- Schwartz, J. J. and Quake, S. R. (2007), "High density single molecule surface patterning with colloidal epitaxy", *Applied Physics Letters*, Vol. 91, No. 8.
- Sevcik, A. (1948), "First analytical theory of linear sweep voltammetry.", *Chem. Commun*, Vol. 13, p. 349.
- Smith, S. and Lasdon, L. (1992), "Solving large sparse nonlinear programs using GRG", *ORSA journal on computing*, Vol. 4, No. 1, p. 2-15.

- Staats, M., Van Baarlen, P. and Van Kan, J. A. L. (2005), "Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity", *Molecular biology and evolution*, Vol. 22, No. 2, p. 333-346.
- Stewart, A. and Franicevic, S. C. (1994), "Infected Seed as a Source of Inoculum for *Botrytis* Infection of Onion Bulbs in Store", *Australasian Plant Pathology*, Vol. 23, No. 2, p. 36-40.
- Stewart, A. and Mansfield, J. W. (1984), "Fungal development and plant response in detached onion, onion bulb scales and leaves inoculated with *Botrytis allii*, *B. cinerea*, *B. fabae* and *B. squamosa*", *Plant Pathology*, Vol. 33, No. 3, p. 401-409.
- Strange, R. N. (2003), "The Plant Fights Back - 1. Constitutive Defense Mechanisms", in Strange, R. N. (editor), *Introduction to plant pathology*, John Wiley & Sons Inc, Chichester, UK, p. 241-260.
- Sungur, S., Emregü, E., Günend, G. and Numanoglu, Y. (2004), "New Glucose Biosensor Based on Glucose Oxidase-immobilized Gelatin Film Coated Electrodes", *Journal of Biomaterials Applications*, Vol. 18, No. 4, p. 265-277.
- Tan, X., Li, M., Cai, P., Luo, L. and Zou, X. (2005), "An amperometric cholesterol biosensor based on multiwalled carbon nanotubes and organically modified sol-gel/chitosan hybrid composite film", *Analytical Biochemistry*, Vol. 337, No. 1, p. 111-120.
- Tawa, K. and Knoll, W. (2004), "Mismatching base-pair dependence of the kinetics of DNA-DNA hybridization studied by surface plasmon fluorescence spectroscopy", *Nucleic acids research*, Vol. 32, No. 8, p. 2372-2377.
- Tichelaar, G. M. (1967), "Studies on the biology of *Botrytis allii* on *Allium cepa*", *European Journal of Plant Pathology*, Vol. 73, p. 157-160.
- Tijssen, P. (1985), "Practice and theory of enzyme immunoassays.", in Burdon, R., H. and van Knippenberg, P., H. (editors), *Laboratory Techniques in Biochemistry and Molecular Biology*. (15th ed), Elsevier Science Publishers B.V., Amsterdam.
- Tothill, I. E. (2001), "Biosensors developments and potential applications in the agricultural diagnosis sector", *Computers and Electronics in Agriculture*, Vol. 30, No. 1-3, p. 205-218.
- Tothill, I. E., Newman, J. D., White, S. F. and Turner, A. P. F. (1997), "Monitoring of the glucose concentration during microbial fermentation using a novel mass-producible biosensor suitable for on-line use", *Enzyme and microbial technology*, Vol. 20, No. 8, p. 590-596.
- Tothill, I. E. (2003a), "On-line immunochemical assays for contaminant analysis", in Tothill, I. E. (editor), *Rapid and on-line Instrumentation for Food Quality Assurance*, Woodhead Publishing Limited, Cambridge, p. 14-39.

- Tothill, I. E. (2003b), *Rapid and on-line Instrumentation for Food Quality Assurance*, Woodhead Publishing Limited, Cambridge.
- Towner, J. S., Sealy, T. K., Khristova, M. L., Albariño, C. G., Conlan, S., Reeder, S. A., Quan, P. L., Lipkin, W. I., Downing, R. and Tappero, J. W. (2008), "Newly discovered ebola virus associated with hemorrhagic fever outbreak in Uganda", *PLoS pathogens*, Vol. 4, No. 11, p. e1000212.
- Tsang, V., Wilson, B. C. and Maddison, S. E. (1980), "Kinetic studies of a quantitative single-tube enzyme-linked immunosorbent assay.", *Clinical chemistry*, Vol. 26, No. 9, p. 1255-1260.
- Uludag, Y., Hammond, R. and Cooper, M. A. (2010), "A signal amplification assay for HSV type 1 viral DNA detection using nanoparticles and direct acoustic profiling", *Journal of Nanobiotechnology*, Vol. 8, p. art. no. 3.
- Uludag, Y. and Tothill, I. E. (2010a), *Development of an Affinity Sensor For Prostate Cancer Diagnosis* (unpublished PhD thesis), Cranfield University, Cranfield, UK.
- Uludag, Y. and Tothill, I. E. (2010b), "Development of a sensitive detection method of cancer biomarkers in human serum (75%) using a quartz crystal microbalance sensor and nanoparticles amplification system", *Talanta*, Vol. 82, No. 1, p. 277-282.
- Valis, L., Mayer-Enthart, E. and Wagenknecht, H. A. (2006), "8-(Pyren-1-yl)-2'-deoxyguanosine as an optical probe for DNA hybridization and for charge transfer with small peptides", *Bioorganic & medicinal chemistry letters*, Vol. 16, No. 12, p. 3184-3187.
- Van Delden, C., Bezemer, J., Engbers, G. and Feijen, J. (1997), "Poly (ethylene oxide)-modified carboxylated polystyrene latices-immobilization chemistry and protein adsorption", *Journal of Biomaterials Science, Polymer Edition*, Vol. 8, No. 4, p. 251-268.
- van Vunakis, H. and Tijssen, P. (1987), "Practice and theory of enzyme immunoassays", *Analytical Biochemistry*, Vol. 162, No. 1, p. 309-310.
- Vandendriessche, T., Keulemans, J., Geeraerd, A., Nicolai, B. M. and Hertog, M. L. A. T. M. (2012), "Evaluation of fast volatile analysis for detection of *Botrytis cinerea* infections in strawberry", *Food Microbiology*, Vol. 30, p. 1-9.
- Verkley, G. (2012), *Personal communication with Gerard Verkley. Database Curator filamentous fungi, CBS-Knaw Fungal Biodiversity Centre, Utrecht, Netherlands*.
- Vrind, T. A. (2005), "The *Botrytis* Problem in Figures", *Acta Horticulturae*, Vol. 669, p. 99-102.

- Walcott, R. R., Gitaitis, R. D. and Langston, D. B. (2004), "Detection of *Botrytis aclada* in onion seed using magnetic capture hybridization and the polymerase chain reaction", *Seed Science and Technology*, Vol. 32, No. 2, p. 425-438.
- Walker, J. C. (1926), "*Botrytis* neck rot of onions", *Journal of Agricultural Research*, Vol. 33, p. 893-928-928.
- Walker, J. C. (1925a), "Control of mycelial neck rot of onions by artificial curing", *Journal of Agricultural Research*, Vol. 30, p. 365-373.
- Walker, J. C. (1925b), "Two undescribed species of *Botrytis* associated with the neck rot disease of onion bulbs.", *Phytopathology*, Vol. 15, p. 708-713.
- Wang, H., Zeng, H., Liu, Z., Yang, Y., Deng, T., Shen, G. and Yu, R. (2004), "Immunophenotyping of acute leukemia using an integrated piezoelectric immunosensor array", *Analytical Chemistry*, Vol. 76, No. 8, p. 2203-2209.
- Wang, F., Wang, J., Liu, X. and Dong, S. (2008a), "Nanoparticle-amplified surface plasmon resonance study of protein conformational change at interface", *Talanta*, Vol. 77, No. 2, p. 628-634.
- Wang, H., Shen, G. and Yu, R. (2008b), "Aspects of recent development of immunosensors", in Zhang, X., Ju, H. and Wang, J. (editors), *Electrochemical sensors, biosensors, and their biomedical applications*, Academic Press, Amsterdam, p. 237-260.
- Warwick, M. J. (1996), "Standardisation of immunoassays", in Law, B. (editor), *Immunoassays: A Practical Guide*, Taylor and Francis, London, p. 150-170.
- Watson-Craik, I. A., Aidoo, K. E. and Anderson, J. G. (1989), "Induction of conductance and capacitance changes by food-borne fungi", *Food Microbiology*, Vol. 6, No. 4, p. 231-244.
- Weigl, B. H., Bardell, R. L. and Cabrera, C. (2007), "Practical Aspects of Microfluidic Devices: Moving Fluids and Building Devices", in Marks, R. S., Cullen, D. C., Karube, I., Lowe, C. R. and Weetall, H. H. (editors), *Handbook of biosensors and biochips*, John Wiley, Chichester, p. 711-729.
- White, S. F. and Turner, A. P. F. (1997), "Mediated Amperometric Biosensors", in Kress-Rogers, E. (editor), *Handbook of biosensors and electronic noses : medicine, food, and the environment*, CRC Press, Boca Raton, p. 227-244.
- Williams, R. A. and Blanch, H. W. (1994), "Covalent immobilization of protein monolayers for biosensor applications", *Biosensors and Bioelectronics*, Vol. 9, No. 2, p. 159-167.
- Wink, T. H., Van Zuilen, S. J., Bult, A. and Van Bennekom, W. P. (1997), "Self-assembled monolayers for biosensors", *Analyst*, Vol. 122, No. 4, p. 43-50.

- Wollenberger, U., Spricigo, R., Leimkühler, S. and Schröder, K. (2008), "Protein Electrodes with Direct Electrochemical Communication", in Sheper, T., Renneberg, R. and Lisdat, F. (editors), *Advances in Biochemical Engineering / Biotechnology*. (109th ed), Springer, Berlin Heidelberg, p. 20-64.
- Wong, E. L. S., Chow, E. and Gooding, J. J. (2005), "DNA recognition interfaces: The influence of interfacial design on the efficiency and kinetics of hybridization", *Langmuir*, Vol. 21, No. 15, p. 6957-6965.
- Wortberg, M., Orban, M., Renneberg, R. and Cannamm, K. (1997), "Fluorimetric Immunosensors", in Kress-Rogers, E. (editor), *Handbook of biosensors and electronic noses : medicine, food, and the environment*, CRC Press, Boca Raton, p. 369-405.
- Xiang, Q., Xu, B., Fu, R. and Li, D. (2005), "Real time PCR on disposable PDMS chip with a miniaturized thermal cycler.", *Biomedical Microdevices*, Vol. 7, No. 4, p. 273-279.
- Xu, C., Cai, H., Xu, Q., He, P. and Fang, Y. (2001), "Characterization of single-stranded DNA on chitosan-modified electrode and its application to the sequence-specific DNA detection", *Fresenius' journal of analytical chemistry*, Vol. 369, No. 5, p. 428-432.
- Yam, C. M., Deluge, M., Tang, D., Kumar, A. and Cai, C. (2006), "Preparation, characterization, resistance to protein adsorption, and specific avidin–biotin binding of poly(amidoamine) dendrimers functionalized with oligo(ethylene glycol) on gold", *Journal of colloid and interface science*, Vol. 296, No. 1, p. 118-130.
- Yetisen, A. K., Akram, M. S. and Lowe, C. R. (2013), "Paper-based microfluidic point-of-care diagnostic devices", *Lab on a Chip - Miniaturisation for Chemistry and Biology*, Vol. 13, No. 12, p. 2210-2251.
- Yohalem, D. S., Nielsen, K., Green, H. and Funck Jensen, D. (2004), "Biocontrol agents efficiently inhibit sporulation of *Botrytis aclada* on necrotic leaf tips but spread to adjacent living tissue is not prevented", *FEMS microbiology ecology*, Vol. 47, No. 3, p. 297-303.
- Yohalem, D. S., Nielsen, K. and Nicolaisen, M. (2003), "Taxonomic and nomenclatural clarification of the onion neck rotting *Botrytis* species", *Mycotaxon*, Vol. 85, p. 175-182.
- Zhang, G., Guo, J. and Wang, X. (2009), "Immunochromatographic Lateral Flow Strip Tests", in Rasooly, A. and Herold, K. E. (editors), *Methods in Molecular Biology 504: Biosensors and Biodetection, Methods and Protocols*. (2nd ed), Human Press, New York, p. 169-183.

- Zhang, Y., Austin, R., Kraeft, J., Cox, E. and Ong, N. (2002), "Insulating behavior of λ -DNA on the micron scale", *Physical Review Letters*, Vol. 89, No. 19.
- Zhu, N., Mao, S., Pan, L., Yuan, L. and Tu, K. (2013), "Early detection of fungal disease infection in strawberry fruits by e-nose during postharvest storage", *Nongye Gongcheng Xuebao/Transactions of the Chinese Society of Agricultural Engineering*, Vol. 29, No. 5, p. 266-273.

APPENDIX

A. Publications

Poster Presentations:

**Development of a *Botrytis* Specific Immunosensor;
Towards Using PCR Species Identification**

15-18th May 2012, Cancun (Mexico): 22nd World Congress on Biosensors

19th September 2012, Cranfield (UK): Post Graduate Conference

24th February 2013, Cranfield University (UK): Cranfield Nano Showcase

27-29th August 2013, Jalan Bendahara (Malaysia): AsiaSense

Oral Presentations:

**Electrochemical immunosensor for the diagnosis of *Botrytis* with PCR-based
species identification**

*12-15th May 2013, Sitges (Spain): 3rd International Conference on Bio-Sensing
Technology*

26th September 2012, Cranfield (UK): Post Graduate Conference

Newsletters:

Seed to Store: a holistic approach to controlling internal rots in onion.

*March 2011, August 2011, July 2012: Technology Strategy Board (TSB) Project
Update*

Development of a *Botrytis* Specific Immunosensor; Towards Using PCR Species Identification

Michael Binder, Leon A. Terry, Ibtisam E. Tothill*

Cranfield Health, Cranfield University, Cranfield, Bedfordshire MK43 0AL, United Kingdom

Introduction

Internal defects of onions, particularly neck rot, caused by *Botrytis* species result in considerable losses in field and during storage (Figure 1). Three species of *Botrytis* are considered the primary causal agents of neck rot: *Botrytis aclada*, *B. allii* and *B. byssoidea*. Current detection methods for *Botrytis* comprise conventional agar culture, ELISA and genetic analyses. Hence, a more rapid, sensitive and reliable method to assess the pathogen load of infected onions could help to prescribe an effective curing regime, thus reducing postharvest loss. This research describes the development of a biosensor for the early detection and identification of *Botrytis* infected crops.



Figure 1 (A-D): Common postharvest symptoms of neck rot.

Sensor Development

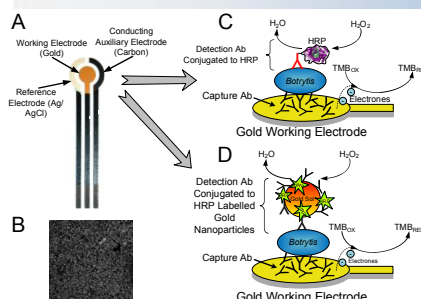


Figure 2: (A) Electrochemical immunosensor based on a screen-printed gold working electrode (SPGE), with on-board carbon counter and Ag/AgCl pseudo-reference electrode (inks are all from DuPont, Bristol, UK). A monoclonal antibody against *Botrytis* was immobilised on the surface of the gold working electrode (B) using physical adsorption. A direct sandwich enzyme-linked immunosorbent assays (ELISA) format was then developed using polyclonal antibodies conjugated to the electroactive enzyme horseradish peroxidase (HRP) (C) and to gold nanoparticles (D) for enhanced sensitivity. Electrochemical measurements were then conducted at a potential of -200 mV against on-board screen-printed Ag/AgCl pseudo-reference electrode using 3,3', 5,5' - Tetramethylbenzidine dihydrochloride/ H_2O_2 as the enzyme mediator/substrate system.

Results

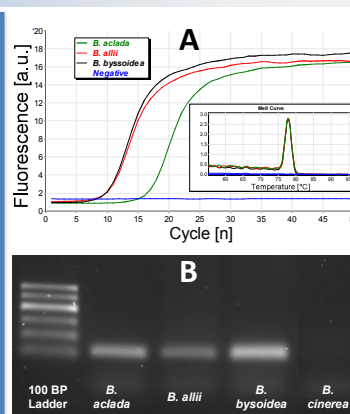
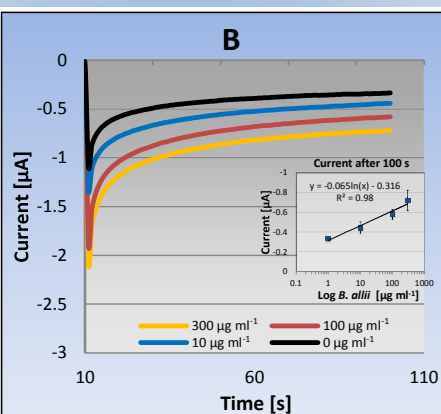
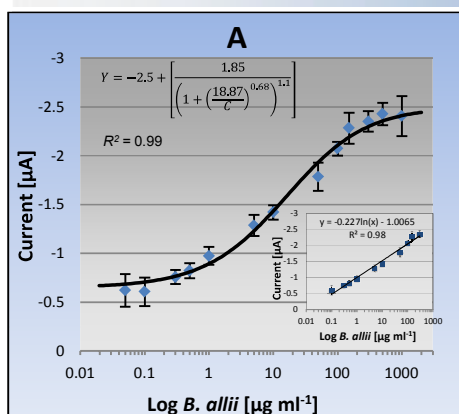


Figure 3: (A) Current vs. *B. allii* concentration achieved using direct ELISA format with polyclonal antibodies conjugated to HRP on gold working screen-printed electrodes. (B) Sensor reading of direct ELISA with polyclonal antibodies immobilised on gold nanoparticles (40 nm) labelled with HRP for detection. Measurements were taken at -200 mV vs. Ag/AgCl after 100 s, error bars represent standard deviation ($n=5$).

Figure 4: (A) Real time PCR amplification of *Botrytis* species causing neck rot with corresponding melt curve. (B) Gel electrophoresis of the PCR product.

Conclusions

We have demonstrated a promising new approach for the detection of *Botrytis* using an enhanced sensitivity electrochemical biosensor based on a sandwich ELISA format with conjugated antibodies as well as HRP labelled gold nanoparticles for signal generation. The sensor is able to detect all three species causing neck rot as well as other *Botrytis* species with a limit of detection which is less than $0.1 \mu\text{g ml}^{-1}$. This is the first report of neck rot detection using an electrochemical biosensor. Real time PCR was used to identify the pathogen DNA with primers specific to *Botrytis* species causing neck rot. This will be combined with the immunosensor in order to identify *Botrytis* species.

Acknowledgements

The authors would like to thank the Technology Strategy Board and industry partners viz. DuPont, Allium and Brassica Centre, Forsite Diagnostics, Syngenta Seeds, Polytec UK, Rustler, Moulton Bulb, South Eastern Produce, Stourgarden, Suffolk Onion Growers, G's Fresh and Thorlabs for collaboration and financial support.



BIOSENSORS2012

15-18 May 2012 • Cancun, Mexico

22nd Anniversary
World Congress on
Biosensors

Best Poster Award 2012

Poster Session 2 Winner

For an outstanding contribution at the
22nd Anniversary World Congress on Biosensors

Held in Cancun, Mexico

15-18 May 2012

Presented to

M. BINDER, L.A. TERRY, I.E. TOTHILL

For the contribution

DEVELOPMENT OF A BOTRYTIS SPECIFIC
IMMUNOSENSOR; TOWARDS USING PCR
SPECIES IDENTIFICATION

Prof. Anthony Turner
(Chairman)

David Sleeman
(Publisher)



Development of a *Botrytis* Specific Immunosensor; Towards using PCR Species Identification

Michael Binder, Leon A. Terry, Ibtisam E. Tothill*

Cranfield Health, Cranfield University, Cranfield, Bedfordshire MK43 0AL, United Kingdom

Introduction

Internal defects of onions, particularly neck rot, caused by *Botrytis* species result in considerable losses in field and during storage (Figure 1). Three species of *Botrytis* are considered the primary causal agents of neck rot: *Botrytis aclada*, *B. allii* and *B. byssoides*. Current detection methods for *Botrytis* comprise conventional agar culture, enzyme-linked immunosorbent assays (ELISA) and genetic analyses. Hence, a more rapid, sensitive and reliable method to assess the pathogen load of infected onions could help to prescribe an effective curing regime, thus reducing postharvest loss. This research describes the development of a biosensor for the early detection and identification of *Botrytis* infected crops.

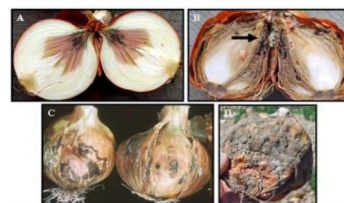


Figure 1 (A-D): Common postharvest symptoms of neck rot.

Sensor Development

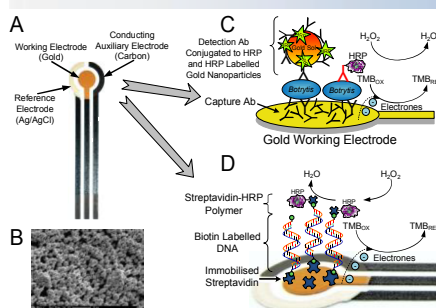


Figure 2: (A) Electrochemical biosensor based on a screen-printed gold working electrode (SPGE), with on-board carbon counter and Ag/AgCl pseudo-reference electrode. A monoclonal antibody against *Botrytis*, or streptavidin for the pathogen DNA was immobilised on the gold working electrode using the appropriate surface chemistry for the immuno and DNA sensors respectively; (B) SEM image of the gold electrode; (C) A direct sandwich ELISA was then developed for the immunosensor using polyclonal antibodies- HRP conjugate and nanoparticles for enhanced sensitivity; (D) For the DNA sensor development, *Botrytis* DNA was amplified using biotin labelled primers and applied on the sensor, followed by a streptavidin-HRP conjugate. Electrochemical measurements were then conducted using 3,3',5,5'-Tetramethylbenzidine dihydrochloride/H₂O₂ as the enzyme mediator/substrate system.

Results

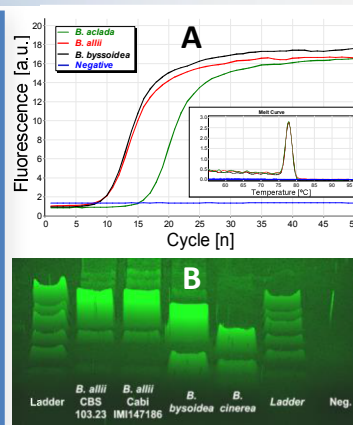
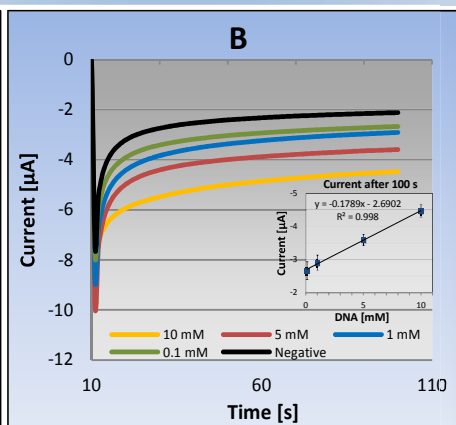
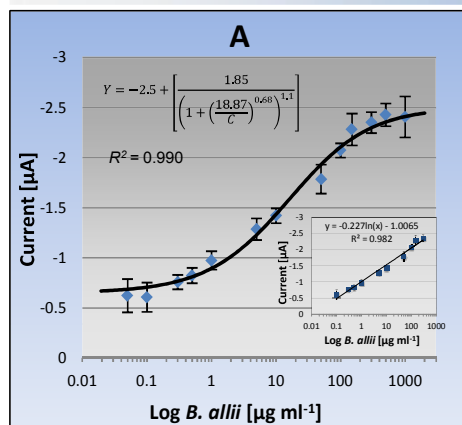


Figure 3: (A) Current vs. *B. allii* concentration achieved using direct ELISA format with polyclonal antibodies conjugated to HRP on the gold working screen-printed electrodes. (B) DNA Sensor readings using biotin labelled *B. allii* DNA, followed by a streptavidin-HRP conjugate for detection. Measurements were taken at -200 mV vs. Ag/AgCl after 100 s, error bars represent standard deviation (n=5).

Figure 4: (A) Real time PCR amplification of *Botrytis* species causing neck rot with corresponding melt curve. (B) Species differentiation using RFLP analysis.

Conclusions

We have demonstrated a promising new approach for the detection of *Botrytis* using an enhanced sensitivity electrochemical biosensor based on a sandwich ELISA format with conjugated antibodies as well as HRP labelled gold nanoparticles for signal generation. The sensor is able to detect all three species causing neck rot as well as other *Botrytis* species with a limit of detection which is 58 ng ml⁻¹. This is the first report of neck rot detection using an electrochemical biosensor. Genetic differentiation between the *Botrytis* species was performed using restriction fragments length polymorphism (RFLP) differentiation. Real time PCR was used to identify the pathogen DNA with primers specific to *Botrytis* species causing neck rot. A DNA based procedure is now being optimised to combine the biosensor with PCR techniques for early detection and identification of *Botrytis* in the field.

Acknowledgements

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The authors would like to thank the Technology Strategy Board and industry partners viz. Allium and Brassica Centre, Forsite Diagnostics, Syngenta Seeds, Polytec UK, Rustler, Moulton Bulb, South Eastern Produce, Stourgarden, Suffolk Onion Growers, G's Fresh and Thorlabs for collaboration and financial support. www.cranfield.ac.uk/health

Michael Binder

Awarded the

Prize for Best Poster

as voted for by the Judges

at the

**Cranfield Health Postgraduate
Conference**

- 2012 -

**Professor Joe Lunec
Head of School**

Michael Binder

Awarded the

**Head of School Prize
for
Best Oral Presentation**

at the

**Cranfield Health Postgraduate
Conference**

- 2013 -



**Professor Joe Lunec
Head of School**

Seed to Store: a holistic approach to controlling internal rots in onion



- **Botrytis control by Ultra Violet Radiation**

At the sub-committee meeting on 3rd February an experiment plan was discussed and agreed. Allium and Brassica Centre will provide cv. '**Alpha**' **seeds, sets and bulbs** for the **first year** trials to be conducted in the Cranfield University research glasshouse. **Alpha** was chosen for convenience since there were **spare bulbs** still at Allium and Brassica Centre and also **related seed and sets**.

Four *Botrytis allii* strains were acquired viz. IMI 292066, 42078, 147186 and Syngenta strain, and are grown in the Plant Science Laboratory led by **Dr Leon Terry**. In addition, UV equipment was purchased for trials in the laboratory and part of a new –40°C freezer was financed to keep onion samples in the future (work package 1). The idea is to **produce infected seed and sets** (both internally and externally) which can then be treated and grown on and should provide a known level of infection.

- **Immunosensor**

In order to collate and investigate **what** type of **technologies** are available for *Botrytis allii* detection, a **literature search** was conducted. It covered the scientific literature as well as products available commercially for *Botrytis* detection. This has now been concluded and a **full report** has been **written**. The **practical work** to develop the sensor (**lateral flow** strip, **ELISA** tests and **amperometric** sensor) was also initiated by ordering and purchasing the **antibodies, reagents** and also different species of *Botrytis* other than *allii* (*aclada* and *byssoidea*) needed to develop the test procedure. A visit to **Forsite Diagnostics Ltd** production facilities has been organised by **Dr Sam Tothill**, in order to discuss and initiate the development of the **lateral flow** assay and also look at the available facilities which will be used for future **test development** of an ELISA test for *Botrytis allii* using a microtitre plate. This is in order to investigate the affinity and specificity of the available antibodies for the required species (work package 2).

Seed to Store: a holistic approach to controlling internal rots in onion

• Laser Doppler Vibrometry (LDV) - Internal Defect Detection 1

A large amount of data was collected during the **feasibility study HL1103**. This was identified to provide the first database of **onion vibration signatures** to compare to this current year's tests.

It is planned that **scanning LDV data** will be re-analysed in order to extract more or different information than done so far. Firstly, **170** neck rot infected onions and control bulbs were measured repeatedly, which provided **11520** data points. Previously this data was used to calculate one value per onion: the **stiffness** value. Secondly, data of 338 "bad" onions, which created 6084 data points, are available. Here data profiles could be analysed in **more detail** - especially from the lot with high incidence of **bacterial rot**.

The **LDV instrument** for the project arrived, so the refinement of the testing protocol can now be started. (work package 3).



Figure 1 Photograph of LDV sensor head mounted on a secure tripod.

• Optical Coherence Tomography - Internal Defect Detection 2

A large amount of images were collected during the **feasibility study HL1103**. These were visually inspected and interpreted.

Good image quality is important for all image analysis techniques. Low noise, high contrast, optimal focus, maximum optical depth are essential. It is helpful if **initial** high quality **images** are **recorded**, which avoids excessive thresholding, contrast stretching, filtering and noise reduction.

The previously employed commercial **OCT** system had a wavelength of 930nm (Thorlabs). It featured a high, but fixed, resolution and high signal-to-noise (SNR). A 1330 nm research system has been constructed in the laboratory. This reaches a slightly lower SNR, but is more flexible, e.g. usable at high depth of field (lower resolution). It is also adaptable for other wavelengths.

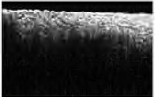
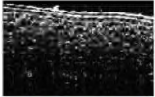
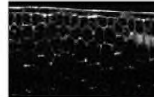
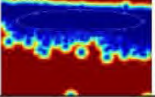
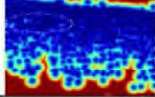
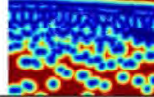
Seed to Store: a holistic approach to controlling internal rots in onion

Dr Helen Ford is currently investigating 'dynamic focus' to decouple focus (resolution) from depth of field. Initial efforts have been made for automatic data processing:

Figure 2 OCT images in gray scale and processed as distance maps

(work package 4).

Initial efforts to quantify data and obtain objective representative parameters

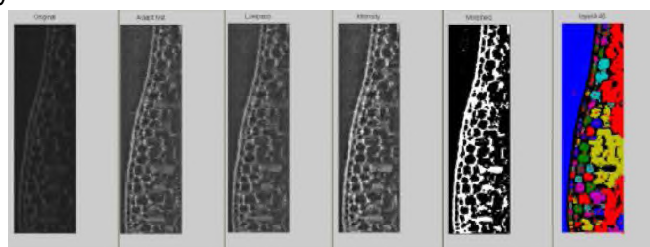
	Basal plate tissue	Inner scale	Outer scale
Original image			
Distance map (Showing user-selected region of interest)			
Mean distance (av. of 10)	0.10 (+/-0.2) pixels	1.03 (+/- 0.2) pixels	4.39 (+/-0.2) pixels

• Strategies

Dr Sandra Landahl received training in image processing. Now descriptive analysis of initial **OCT** data and optimisation of the data processing is under way:

Figure 3 OCT image processed from left to right: raw, histogram adapted, low pass filtered, intensity adjusted, black/white, area and centroid detected

(work package 5)



• Visits

Michael Binder (PhD student) and **Dr Sandra Landahl** visited **Rustler Produce** and **Moulton Bulb** sites to familiarise themselves with packing procedures and possibly identify work-flow positions where the non-destructive sensor(s) could be implemented. At the **Rustler** site a **weight grading** machine exists, which singulates the bulbs. Originally it was fitted with an optical sensor. This would be an **ideal spot** to put the LDV. Both companies reported **existing NIR sensor** that they had visited in **Norway**. Until now that sensor does not appear to perform well enough.

Cranfield 28 February 2011

Seed to Store: a holistic approach to controlling internal rots in onion



Apologies to all for the late production of this second newsletter, due to holidays and other hold ups!

Part 1 - “Non chemical” treatment of seeds and sets by Ultra Violet (UV-C), Vapour heat treatment and Ozone.

The initial studies concentrated on the effect of UV-C to target dose levels which are tolerated by seeds and sets, and in turn destructive to *B. allii* (neck rot). Destruction is measured in effect on both the mycelium (active growth) and conidia (spores). First results are available regarding in-vitro trials of efficacy of UV-C light to destroy *B. allii*. Increasing doses were tested on conidia and mycelia of four *B. allii* strains (Syngenta, IMI 292 066, 42 078, 147 186). Viability of conidia was measured by means of germination test on potato dextrose agar plates with streptomycin (PDA) for 4 days at 23° C, 12 hour fluorescent light / dark cycle. The difference in vigour between the control and irradiated sample was clearly visible (Fig. 1). In the latest trial it appeared that most conidia were inactivated at 3.2 kJ/m². Mycelium diameters were also recorded (Table 1). Experiments are ongoing and the optimum dose is yet to be established for all strains.

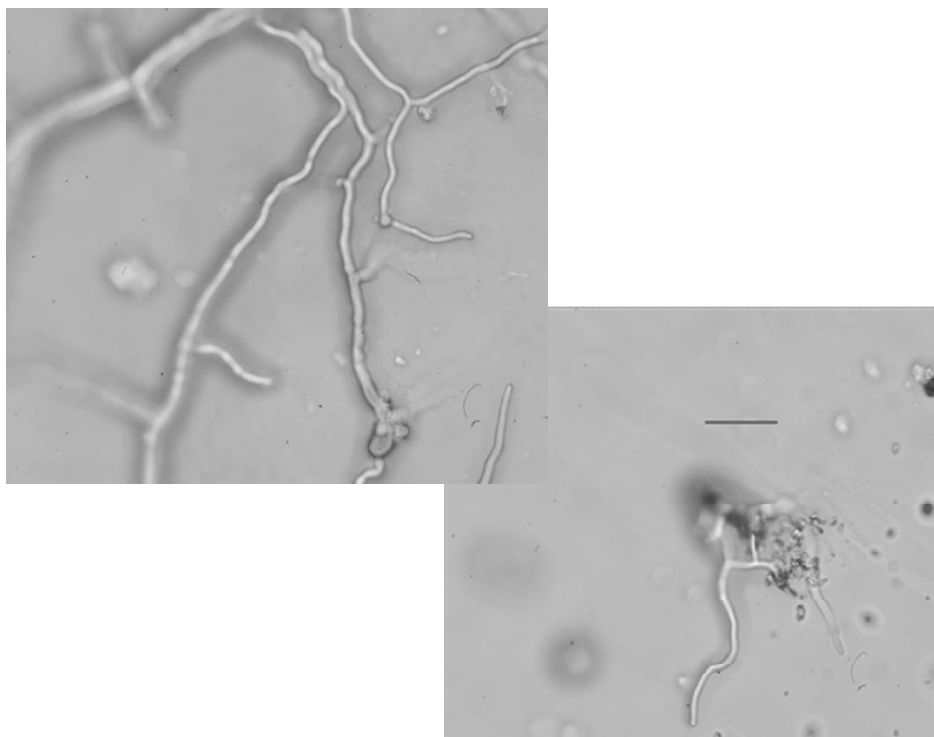


Figure 1 Photos of *B. allii* conidial germination (Syngenta strain) 4 days after irradiation with UV-C light (left control (normal growth), right 1.2kJ/m²).

Seed to Store: a holistic approach to controlling internal rots in onion

day	dose	CU	SL1	SL2	Syn.	l.s.d.
06.06.11	0.0	5.4	7.3	4.8	8.4	
	0.8	3.2	6.4	5.6	9.0	
	1.0	1.5	6.5	4.9	7.4	
	1.2	0.0	7.1	5.0	7.8	0.82
21.06.11	0.0	0.9	4.2	3.3	5.1	
	1.4	0.7	3.8	0.9	5.8	
	1.6	0.4	4.2	2.9	4.3	
	1.8	0.4	3.4	2.5	4.9	1.20
08.07.11	0.0	3.2	4.1	4.0	5.6	
	1.0	0.9	2.7	3.0	5.0	
	1.4	2.4	2.4	2.8	3.8	
	1.8	2.6	2.7	3.1	2.8	1.80
15.08.11	0.0	6.7	7.0	6.8	7.5	
	2.0	6.1	6.3	6.1	6.6	
	2.6	5.8	6.0	5.5	6.8	
	3.2	5.4	6.1	5.4	6.5	0.50

Table 1: Diameter (cm) of *B. allii* mycelium 4 days after transferring plugs onto PDA and irradiating with UV-C light. A new UV-C sensor was used from July on, so dosages (kJ/m²) were re-applied to new samples.

It can be seen from the above table that the four strains of *B. allii* (i.e. CV, SL1, etc) were adversely affected by UV-C but that higher levels will be necessary to provide effective control. Note that the first two dates were with an eventually suspect sensor, 8 July with the new sensor and 15 August with a new colony of the fungus.

Sets

Sets were inoculated (Syngenta, IMI 147 186, 100µl of 50*10³cfu/ml) and treated by UV-C irradiation (0, 6, 12kJ/m²). Onions cv. 'Alpha' (provided by A&B centre) of all treatments (unwounded control, 100µl water control and inoculated) were growing well.

Seeds

Seeds cv. 'Alpha' were externally disinfected with 5% NaDCC and Millipore water in agitator for 30 min., inoculated with two *B. allii* strains (Syngenta, IMI 147 186) and water as control and irradiated with 0, 6, 12, 18 and 24 kJ/m² UV-C light. Germination was normal and in the beginning of June seedlings were transplanted into 1l pots. They are growing well and leaf samples were collected and frozen to test if *B. allii* can be detected at this stage with our current instrumentation. These onions will be grown until leaves senesce and will replace the bulbs expected from the sets to be stored and inspected periodically for neck rot visually and by means of laser Doppler vibrometer (LDV, see WP3).

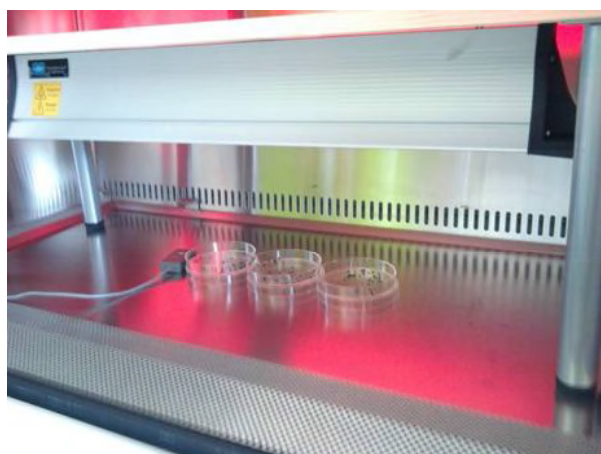


Figure 2 UV-C lamps over UV-transparent Petri dishes with onion seeds and UV-C sensor.

It can be concluded so far that much higher UV-C levels can now be used to control the fungus without affecting viability of either seed or sets.

N.B. The UV-C unit in the laboratory is at a much smaller scale than an industrial unit would be. It is scaled to fit in a laminar flow cabinet and has relatively little power (2x 20W).

Seed to Store: a holistic approach to controlling internal rots in onion

Part 2

Detection of *B. allii* in advance of visible neck rot development - Immunosensor for assessing pathogen load.

The **literature search** conducted by **Michael Binder** is now attached for all project partners and revealed that the primary inoculum pathway of neck rot is caused by onion seeds externally or internally contaminated with sclerotia (resting spores), mycelia or conidia (spores). The fungus was observed as mycelium growing from the seed-coat into the living tissue of the first leaf tips (Fig. 3). It was also observed that during the green leaf stage the fungus remains symptomless, but grows downwards in the tissue when the leaves become senescent and yellowish, eventually invading the neck of the onion bulb. Depending on the site of inoculation on the leaf, the fungus can be traced in the neck tissue and in bulb scales four to seven weeks after inoculation. Post harvest mechanical topping can also spread inoculum. This information provides an insight into when to conduct the tests and also on pathogen load in different part of the bulb.



Figure 3 Microscopic monitoring of *B. allii* on the leaf tissue (Tichelaar, 1967)

A visit to **Forsite Diagnostics Ltd** was successful in exchanging know how regarding the antibodies used in the tests and the lateral flow device (LFD) produced by the company for Botrytis detection. **Dr Sam Tothill** from Cranfield and **Dr Paul Meakin** from Forsite agreed to test the LFD device against a range of spores and mycelium samples extracted from a range of Botrytis species including *Botrytis allii*. The

results showed that the LFD test gave **negative results to spore samples** but **positive results to mycelium samples** prepared from all the Botrytis species tested (Fig. 4). These results indicated that the Forsite LFD test can detect *Botrytis allii* as well as other Botrytis species.

Cranfield also received the monoclonal antibody used in the LFD test from Forsite to test against the commercially available polyclonal antibody acquired from ADGEN Phytodiagnosics ELISA kit. An ELISA test was developed using the two different types of antibodies and the results showed that both antibodies are able to detect all three species causing neck rot (*Botrytis allii*, *B. aclada* and *B. byssoidea*). Consequently the Forsite LFD can currently only be used for discriminating between *Botrytis* infections and bacterial-rot.

From the above results both antibodies can be applied to develop the immunosensor for neck rot. The work has therefore now been initiated on the development of the immunosensors using the available antibodies.

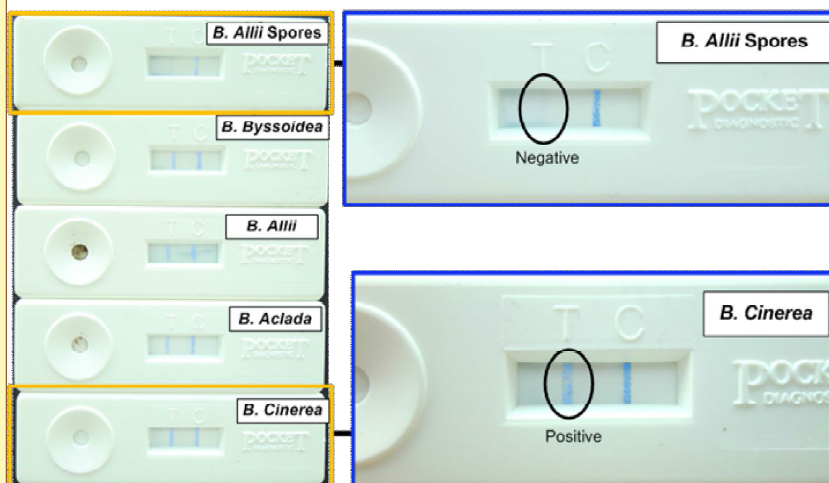


Figure 4 The lateral flow device produced by Forsite Diagnostics Ltd for Botrytis was tested for its specificity against a range of Botrytis species.

Seed to Store: a holistic approach to controlling internal rots in onion

Part 3

Elimination of defective onions on a grading line – Vibration signatures associated with internal defects in onion bulbs

Work is now starting in this area, analysing additional LDV data from the Link feasibility project and collection of data from new crop bulbs of Jagro, Shakespeare and SS1 initially.

An experimental plan was agreed to create a more controlled experimental environment: 500 fresh bulbs each of 4 batches will be provided (total = 2000 bulbs). These will be inoculated with all 4 strains *B. allii* and control (100 bulbs each). Bulbs will be stored at 3 to 4° C and not be cured. Each batch will be measured monthly with LDV (one batch per week). Data will be collected as resonant frequencies of onions in response to swept sine from shaker exiting the onion signal (Fig. 5).

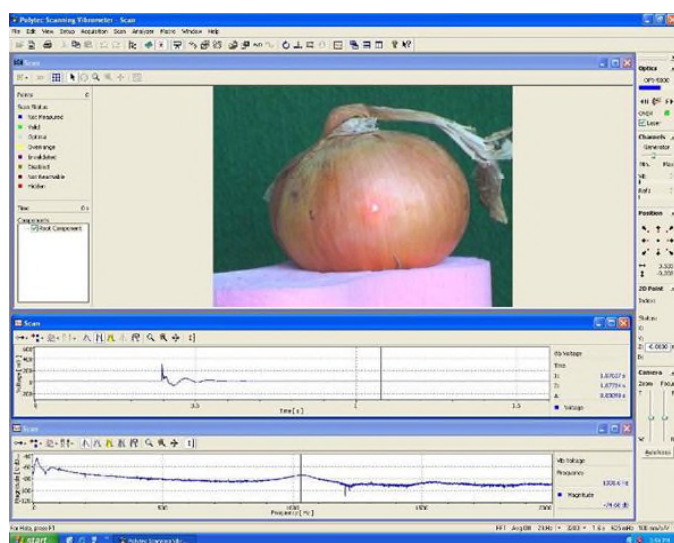


Figure 5 Screen print of a collected signal by means of LDV. Top shows image of sample with laser light reflection at measurement point. Bottom shows vibration signature and Fourier transform indicating the first resonant frequency at 1030 Hz.

Histological changes associated with internal defects using OCT

Preliminary test on onion seeds 'Alpha', both dry and then during sprouting on wet paper has been conducted and interesting images have been collected. The forward plan is to grow seeds on agar and examine the germinating seed from different directions. Plans are under way for a testing protocol of stored onions to collect images of neck rot at different development stages (Fig. 6).

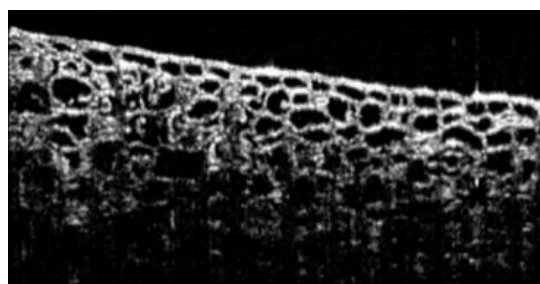
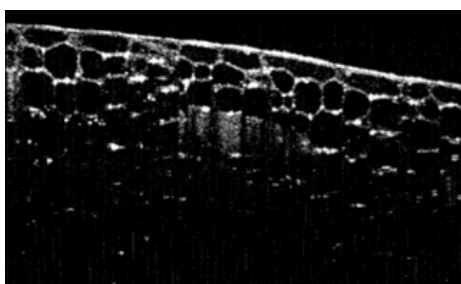


Figure 6

Left: OCT image of sound 'SS1' tissue.

Right: OCT image of infected 'SS1' tissue brown, sunken and dry in visual appearance.

N.B.

A questionnaire for packers is being prepared to collect information on disease occurrence and losses in pack houses. This database will aid screening strategies in the packing environment for the control and assessment of internal disease in onions.

Seed to Store: a holistic approach to controlling internal rots in onion



A brief progress Update in advance of the next full committee meeting on 17 July.

Dr Sam Tothill and Mr Michael Binder presented some results conducted in this work package at the 22nd Anniversary World Congress on Biosensors, 15-18 May 2012, Mexico, which was attended by more than 630 delegates worldwide. The poster was awarded the top prize for the session it was presented (with more than 1200 abstracts submitted to the congress) and gained a great deal of interest from the international biosensor community.

Poster award at the BIOSENSOR 2012 Congress.



UV-C treatment of seed and sets

- UV-C treatment of seeds to control *Botrytis allii*
Onion seed was provided by one of our partners (variety 'Solution', which had an internal infection rate of 6% (after surface disinfection). Seed was divided into five lots of 300g of which two were treated with UV-C irradiation in a custom-made UV-tunnel on 30th Jan. 2012. All seed was then film-coated either with Maxim, a new Syngenta active (1 ml/kg), or with TopsinM+Thiram (2.5 and 4 ml/kg, respectively). Modules were grown ABC and then planted on 17th and 18th April 2012 at Shottisham and Littleport, respectively, in a randomised design. All onions (Fig. 1 and 2) will be harvested and stored at 4° C for approx. 3 months, then cut and infection recorded, while a sub-sample will be measured repeatedly by means of LDV.



Fig. 1 Onion field Shottisham 07/06/12.



Fig. 2 Onion field Littleport 19/06/12.

Seed to Store: a holistic approach to controlling internal rots in onion

- UV-C treatment of sets to control *Botrytis allii*

Cv. 'Forum' and 'Jagro' sets were provided by ABC and further graded at Cranfield. For both varieties 600 sets were treated on 6th March 2012 with UV-C irradiation. Chemical treatments included Folicur+TopsinM (2 ml/l water, 4 ml/l water) and Folicur+Signum (2 ml/l water, 10 g/l water). Sets were planted on 13th March 2012 at Cople, in a randomised design. Sets were grown in one field (3 reps each cultivar, 5 treatments). All onions (Fig. 3 and 4) will be harvested and stored, then inspected, while a sub-sample will be measured repeatedly by means of LDV.



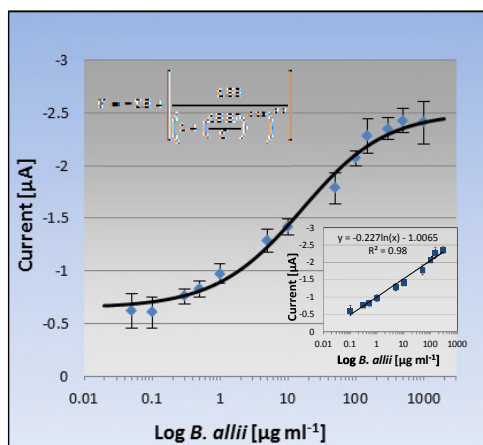
Fig. 3 One half of onion (cv. 'Forum') field Cople



Fig. 4 One half of onion (cv. 'Jagro') field Cople 07/06/12.

Detection of *B. allii* in advance of visible neck rot development - Immunosensor for assessing pathogen load

The biosensor design was finalised and a standard curve conducted for *B. allii* detection (Figure 1), showing a detection limit, which is as low as $0.06 \mu\text{g ml}^{-1}$. Initial tests comparing the biosensor against the commercial ELISA detection kit, using inoculated onion samples, demonstrated superior sensitivity and specificity of the biosensor. The work has, therefore, now been focused on the development of the PCR procedures to identify the pathogen DNA. First tests showed a specific amplification of the *Botrytis* species considered as the primary cause of neck rot (Figure 2, A), as well as the possibility of performing genetic fingerprinting to distinguish between the *Botrytis* species (Figure 2, B). The assay is now being optimised to achieve the optimal results.



A set of ~ 100 onions were planted in the field next to Littleport for use for the biosensor field trials. Inoculations with *B. allii* spores were conducted; first at planting, followed by two other inoculations one month apart. The objective of this study is to correlate the pathogen load to the incidence of neck rot in storage.

Figure 1: *B. allii* concentration against the current signal achieved using the developed biosensor. The limit of detection is $0.06 \mu\text{g ml}^{-1}$, $n=3$.

Seed to Store: a holistic approach to controlling internal rots in onion

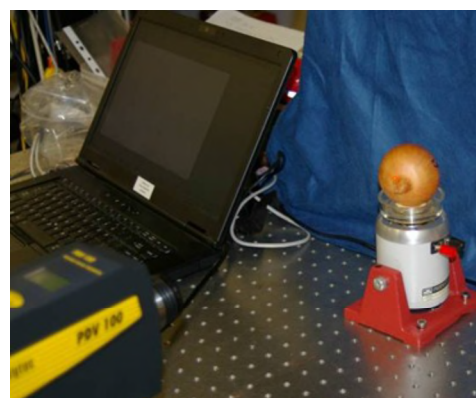


Figure 2: (A) PCR amplification of *Botrytis* species causing neck rot,
(B) Genetic fingerprinting of different *Botrytis* species

Elimination of defective onions on a grading line – Vibration signatures associated with internal defects in onion bulbs

A storage experiment was performed with three varieties of onions (viz. 'Centro', 'Arthur', 'Setton'), which were inoculated with four strains *B. allii* and control. Bulbs were stored at 3° C. Each batch was measured at 4 different dates by means of LDV. Data was collected as frequency spectra of onions in response to a swept sine from a shaker which caused the onion to vibrate (Fig. 5). When this newsletter was produced the data analysis was on-going and requests were made by project partners to model disease incidence especially at early stages. Preliminary results showed that onions with different disease severity could be separated and classified by the calculated stiffness values. Significant differences were found in the rate of frequency decrease when measured bulbs had different disease severity at the end of the experiment.

Fig. 5 Set-up for measuring vibration signatures of onions.



Histological changes associated with internal defects using OCT

In the last quarter, image-correlation techniques were implemented, for tracking cell movements and changes over short time scales. During the current reporting period, we have been refining and extending these methods, to examine the possibility of following cell changes over longer periods. To achieve this, a particular plane within the sample must be identifiable following bulk movement or distortion, which requires acquisition of 3D data.

Data sets have been acquired from healthy onions, comprising sets of about 50 parallel image planes separated by a few micrometres. The following procedures have been developed:

1. The cell shape and volume can be determined for individual cells identified on a particular image, by tracking the variation in area for this cell in different planes within the image set.

**Seed to Store:
a holistic approach to controlling internal rots in onion**

2. Using correlation of planes from 3D image sets acquired at different times, bulk movement of the whole sample, or regions within the sample, can now be periodically measured and corrected for, allowing changes in an individual image to be tracked over a much longer period than was previously possible. It would be possible, in principle, for a sample to be removed and replaced, and the original plane of cells to be re-located, though the amount of data required to achieve this would be prohibitively large in practice.

Exploit disease control in the field and diagnostic strategies at packing

It was agreed that a questionnaire for growers would be produced to collect information on disease occurrence and losses in pack houses to provide database to aid find screening strategies in the packing environment for the control and assessment of internal disease in onion. This has been sent and returned by some but not all! Situation to be reviewed in due course.

B. Technical Drawings

I. Upper Housing Dipstick design

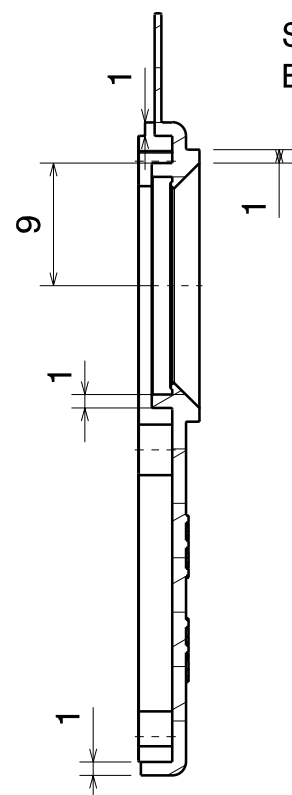
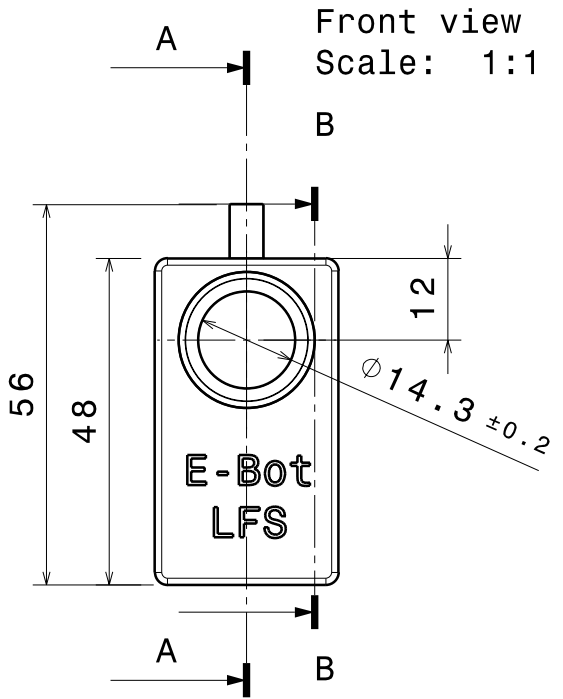
II. Lower Housing Dipstick design

III. Upper Housing Optimised design

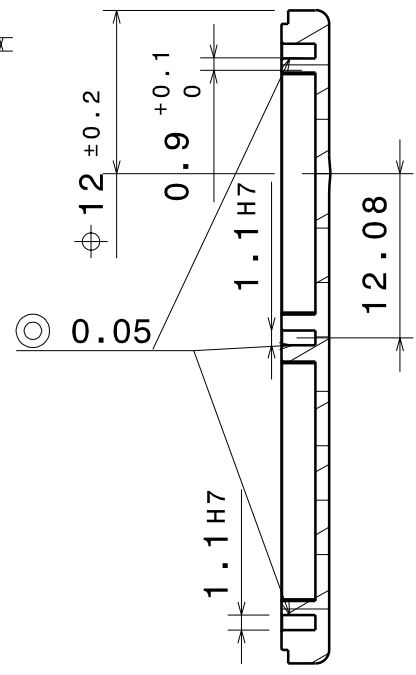
IV. Lower Housing Optimised design

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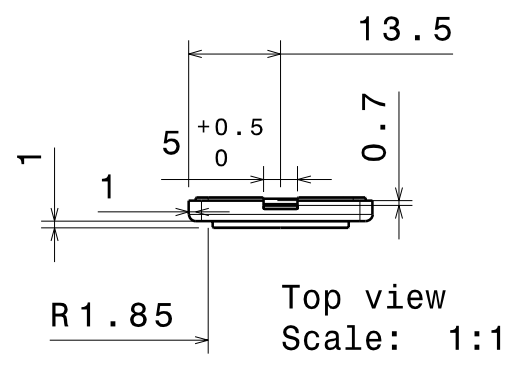
Section view A-A (Left)
B-B (Right), Scale: 2:1



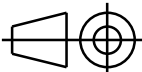
ISO Material view
Scale: 2:1



4
3
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1



Top view
Scale: 1:1

DESIGNED BY: M. Binder		Housing Upper Part	I	-
DATE: 2013			H	-
CHECKED BY: M. Seidl			G	-
DATE: 2013			F	-
SIZE A4		Dipstick Design	E	-
SCALE 1:1	Tolerance Class (IN ISO 2768-1) M	DRAWING NUMBER 2013-M.B.-v1.1	D	-
		SHEET 1 / 1	C	-
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D A



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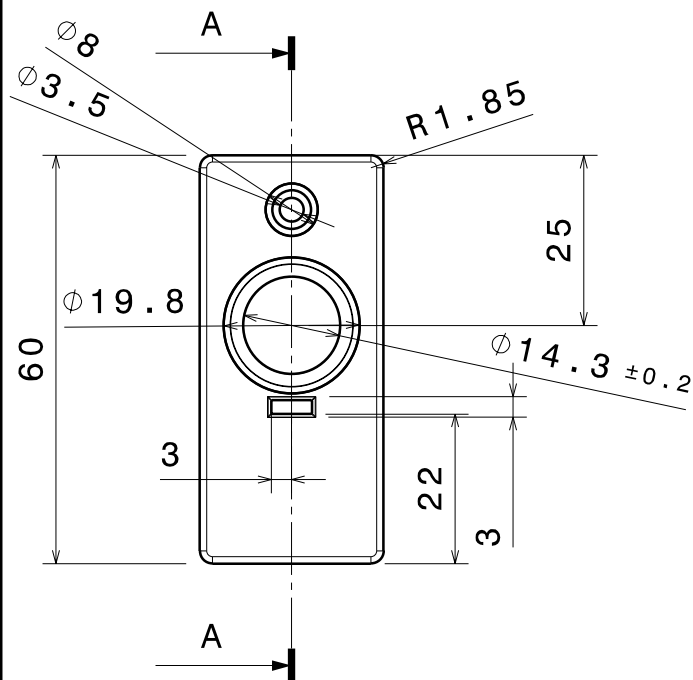
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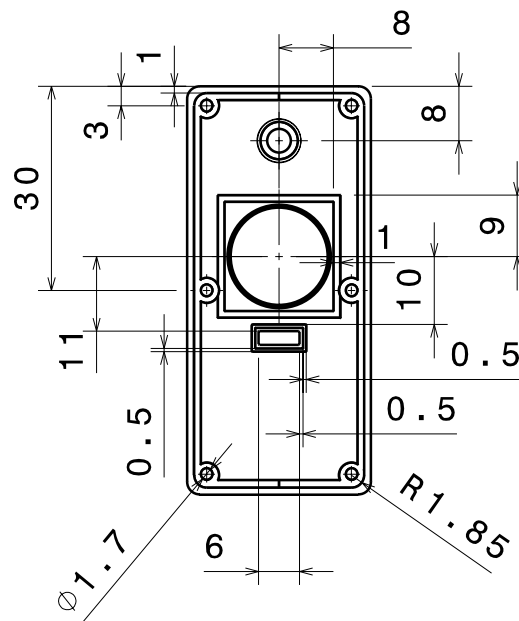
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Front view
Scale: 1:1



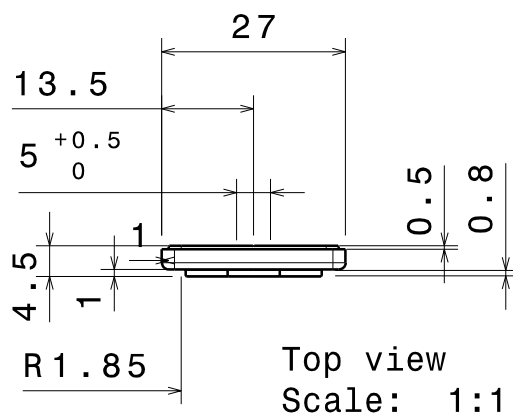
Rear view
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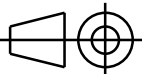
ISOMETRIC
Material view,
Scale: 2:1



4
3
2
1



Top view
Scale: 1:1

DESIGNED BY: M. Binder		Housing Upper Part	I	-
DATE: 2013			H	-
CHECKED BY: M. Seidl			G	-
DATE: 2013			F	-
SIZE A4		Optimised Design	E	-
SCALE 1:1	Tolerance Class (IN ISO 2768-1) M		D	-
DRAWING NUMBER 2013-M.B. -v2.1		SHEET 1/2	C	-
			B	-
			A	-

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D C B A

[illegible]

Technical drawing of a mechanical part (Fig. 1) showing a cross-section. The drawing includes the following dimensions and features:

- Outer diameter: $\phi 5.7$
- Inner diameter: $\phi 3.5$
- Total height: 8
- Angle: 109°
- Dimension line 'C' indicates a specific length.
- A dimension line '1' is shown at the bottom.

Technical drawing of a mechanical part (Fig. 1.10) showing a cross-section with dimensions: outer diameter $\phi 3.2$, inner diameter $\phi 1.8$, wall thickness 1, and a 45° chamfer. The part is labeled 'a'.

Optimised Design

DRAWING NUMBER

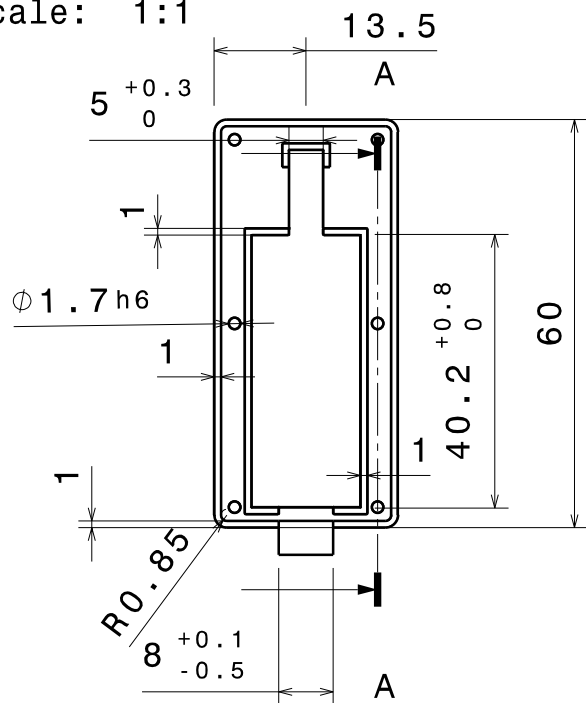
2013-M.B-v2.2

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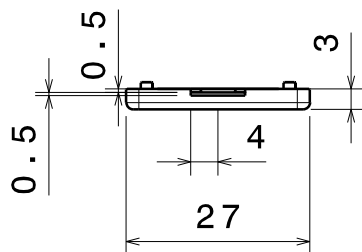
2 / 2

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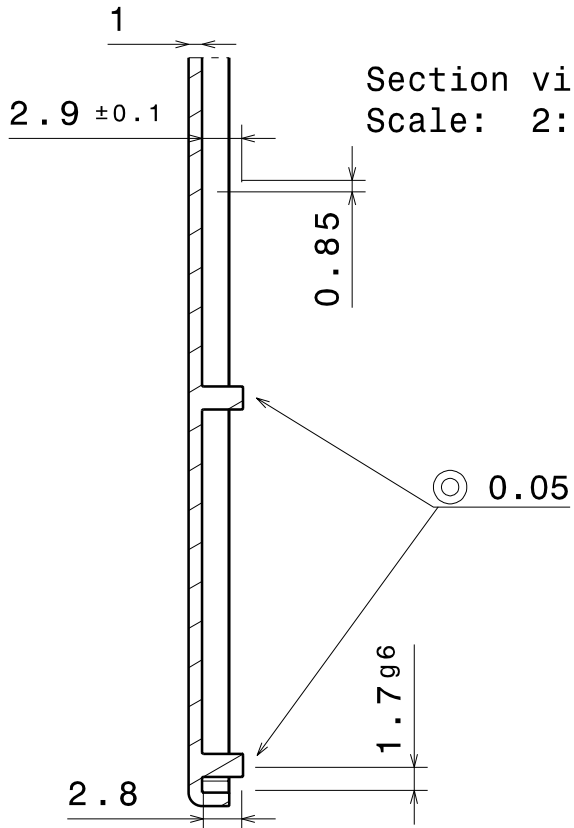
Front view
Scale: 1:1



Bottom view
Scale: 1:1



Section view A-A
Scale: 2:1



Material view
Scale 1:1



DESIGNED BY: M. Binder		Housing Lower Part		I	-
DATE: 2013				H	-
CHECKED BY: M. Seidl		Optimised Design		G	-
DATE: 2013				F	-
SIZE A4		2013-M.B. -v2.3_low		E	-
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