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# Conditions for infection of strawberry fruit by *M. piriformis* and *Rhizopus* spp.

Solomon Agyare[1,2], Naresh Magan[2], Xiangming Xu[1*]

[1]NIAB EMR, New Road, East Malling, West Malling, Kent, ME19 6BJ, UK

[2]Applied Mycology Group, Environment and AgriFood Theme, Cranfield University, Cranfield, Bedford MK43 0AL, U.K.

*Corresponding author, email: xiangming.xu@emr.ac.uk

**Abstract**

*Botrytis cinerea*, *Mucor* and *Rhizopus* spp. are mainly responsible for post-harvest fruit rot in strawberry in the UK. However, research to date has been focused on the epidemiology and management of *B. cinerea* in strawberry (i.e., grey mould). A number of experiments were carried out to study the epidemiology of *M. piriformis* and *Rhizopus* on strawberry, including flower/fruit susceptibility, inoculum dose, *in vitro* spore germination and infection of both detached and attached fruit. The results showed that *M. piriformis* and *Rhizopus* had very similar epidemiological characteristics. Both pathogens were unable to infect flowers, but fruit became increasingly susceptible from the green development stage onwards. *In vitro* germination required near-saturation humidity conditions and was reduced in both low and high temperatures. However, infection of attached ripe fruit and subsequent rot development were relatively unaffected by temperature and relative humidity conditions that commonly occur under field conditions in the UK. On detached ripe fruit, a high spore infection potency was observed for both pathogens. Infection of fruit was completed within 9 to 24 hours. Thus, the most important factor determining the level of infection of fruit by *M. piriformis* and *Rhizopus* in UK commercial crops is probably the number of spores deposited on the fruit. Management strategies should be targeted at reducing inoculum concentrations within crops in order to minimise the risk of fruit infection by *M. piriformis* and *Rhizopus*.



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## Introduction

Postharvest strawberry losses are largely caused by *Botrytis cinerea*, *Mucor,* and *Rhizopus* spp. (Bautista-Baños et al. 2003; Feliziani and Romanazzi 2016; Harris and Dennis 1980). Infection by *Botrytis* causes grey mould in strawberry fruit while infections by *Mucor* and *Rhizopus* lead to soft rot disease (Dennis and Davis 1977; Lattanzio et al. 1996). In recent years, postharvest spoilage of strawberry (grown under protection to extend the length of the production season) caused by *Mucor* and *Rhizopus* spp. has become more common, especially in the UK. This may be partially due to a reduction of fungicide applications and the loss of some broad-spectrum crop protection chemicals. Another possible explanation is that environmental conditions under protection may be relatively more conducive to *Mucor* and *Rhizopus* infection than for *Botrytis*.

*B. cinerea* can infect almost every part of the strawberry plant including leaves, petiole, flowers, and green, white and ripe fruit (Petrasch et al. 2019; Xu et al. 2012). *Botrytis* can also remain latent in fruit and cause fruit decay at a later stage of fruit development, resulting in postharvest rots. Dennis and Davis (1977) reported that *Mucor* and *Rhizopus* caused spoilage of stored fruit especially when harvested late. Zygomycetes (i.e., *Mucor* and *Rhizopus*) were occasionally isolated from the flowers and green fruit but increased significantly as the fruit matured. In contrast, *Botrytis* was consistently found to contaminate flowers and all stages of fruit development (Harris and Dennis 1980). This was suggested by the authors to result from the fact that *Mucor* and *Rhizopus* mainly infected injured white fruit and ripe fruit, while *Botrytis* infected flowers and all stages of fruit development. There are, however, no published experimental studies directly supporting these differences.

Growth of fungi, including *Mucor* and *Rhizopus,* is affected by abiotic factors such as temperature, relative humidity (RH) and water availability (Dantigny et al. 2005, Magan 2007). Thus, the extent of fruit rot due to infection by these pathogens will be influenced by microclimatic conditions as well as by the pathogen inoculum potential and flower/fruit susceptibility. It was previously reported that *Mucor* can grow at temperatures as low as 0°C and up to 27°C (Michailides and Ogawa 1989). *Rhizopus* species are mainly mesophilic and reported to be able to grow at between 20 and 40°C (Hoffmann et al. 2013). However, this species can grow at lower temperatures of between 4 and 6°C (Siefkes-Boer et al. 2009). In general, lower temperatures tend to slow down the growth of fungal pathogens on fresh produce while high temperatures are conductive to rot formation (Michailides and Ogawa 1989). For strawberry grown under protection, the two most important environmental

variables affecting grey mould development are RH and temperature (Xu et al. 2012). Tunnel ventilation management can greatly affect RH and temperature at the canopy level. However, currently there is not sufficient knowledge and understanding of the epidemiology of *Mucor* and *Rhizopus* on strawberry to develop an integrated disease management strategy.

The present study aimed to obtain quantitative knowledge of the epidemiology of *Mucor* and *Rhizopus* on strawberry. Thus the objectives were: (a) determine flower and fruit susceptibility to *Mucor* and *Rhizopus* pathogens; (b) investigate the effect of RH and temperature on *in vitro* germination of *Mucor* and *Rhizopus* spores; (c) examine the effects of inoculum dose on infecting fruit; and (d) evaluate the effects of temperature and RH on infection of both attached and detached strawberry fruit by *Mucor* and *Rhizopus* spores.

## Materials and methods

### Fungal isolates

Two isolates each of *M. piriformis* (MS and EMR36/5/8) and *Rhizopus* (EMR30/8 and EMR7/5/8) were cultured separately on potato dextrose agar (PDA) in 9 cm Petri plates. They were all isolated from decaying strawberry fruit in the UK, and their identity was previously confirmed with ITS sequencing and morphological characteristics (Agyare 2016). ITS sequences failed to classify the two *Rhizopus* isolates into species. A BLAST search showed that EMR7/5/8 shared > 98% of sequence identifies with *R. stolonifera*, and six other *Rhizopus* sequences in the database. Although colony and spore characteristics of both EMR30/8 and EMR7/5/8 were very similar, ITS sequences suggested that they likely originated from two different species.

Prior to the experiments, slope cultures of the four isolates were kept at 4°C for long-term storage. To produce spores for experimental studies, each isolate was cultured separately on PDA Petri plates for 3-5 days at 21°C in dark. Spore suspension was then made for each isolate separately and concentrations were adjusted following haemocytometer counts by diluting where necessary with sterile distilled water. Equal volumes (same concentration) of the two *Mucor* spore suspensions were then mixed together to obtain the *Mucor* spore suspension and the two *Rhizopus* isolates were similarly mixed to obtain the *Rhizopus* inoculum. Following inoculation, flowers/fruit were assessed for disease development with the causal agent determined based on presence and characteristics of mycelia (Agyare 2016).

**Susceptibility of strawberry flower and fruit**

Potted plants of cvs. Amesti and Sonata were arranged in 12 blocks in a glasshouse compartment with misting facilities. Each block consisted of two rows, one row for each cultivar (four plants), giving eight plants per block. Four blocks were randomly assigned for inoculation with *M. piriformis*, four with *Rhizopus* and the remaining four as the non-inoculated control. On each plant, there were at least one flower, one green fruit, one white fruit and one ripe fruit; individual flowers or fruit were tagged with labels of different colours for inoculation treatments. Each tagged flower or fruit was individually sprayed with the appropriate spore suspension mix for five seconds (delivering approximately 1 ml inoculum of $10^6$ spores $ml^{-1}$) using a hand-held fine-sprayer. Similarly, each flower or fruit of the non-inoculated control plants was sprayed with sterilised distilled water (SDW).

The plants were misted for four hours immediately after inoculation; each plant was then covered with a plastic bag for another 20 hours to maintain high RH. The plants were kept in the compartment (20-25°C) for another 3 days after the plastic bags were removed. All inoculated flowers and fruit were then harvested (i.e., four days after inoculation); those showing visual symptoms of decay were recorded and discarded. All the remaining non-symptomatic flowers and fruit were surface sterilised in 5% sodium hypochlorite for 15 minutes and then rinsed several times with SDW. Fruit were then placed on a piece of moist tissue paper (well separated from each other) within a tray covered with a plastic bag. Flowers were placed on pieces of sterile moist filter paper in 90 mm Petri dishes (covered with lids). The flower/fruit samples were incubated under ambient conditions (20-25°C) for 1-3 weeks; they were inspected daily for disease development with the causal agent identified based on visual assessment of rotting/fungal characteristics. Any diseased flower/fruit was removed immediately after initial detection. The experiment was conducted three times.

**Effect of inoculum dose on infection of detached fruit**

Four inoculum concentrations ($10^2$,$10^3$,$10^4$ and $10^5$ spores $ml^{-1}$) were used to study the effect of inoculum dose on infection of detached fruit by *M. piriformis* and *Rhizopus*. Detached ripe fruit of cv. Lusa were inoculated with a hand-held fine sprayer as described previously in a flow cabinet and left to dry for one hour; each fruit received ca. 100, 1 000, 10 000 or 100 000 spores (i.e., approximately 1 ml spore suspension of the spore concentration). The inoculated fruit were incubated under ambient conditions (20-25°C) as described previously for 5 days before disease assessment. There were 50 fruit for each inoculum dose and

pathogen combination. In addition, there was a control treatment with fruit inoculated with SDW. The experiment was conducted twice.

**Effect of relative humidity and temperature on *in vitro* spore germination**

Germination of *M. piriformis* and *Rhizopus* spores was studied at three temperatures (10, 21 and 31°C) under 100, 99, 97, 95, 91 and 85% RH. These RH levels were achieved using water agar amended with 0, 0.3, 0.9, 1.5, 2.6 and 4 M NaCl (Xu et al. 2001). Three glass slides (Menzel-Glaser cover glasses 22 × 22 mm) were placed on the lid of each Petri dish (9 cm). Two 10 µl droplets of spore suspension were placed separately on each glass slide and dried in a flow cabinet (ca. 25 minutes). The lid was then covered with the appropriate NaCl amended plate inverted and sealed with Parafilm®; the plates were then placed in an incubator set to one of the three temperatures. In addition, there was an additional (free-water) treatment; slides containing inoculum droplets were immediately placed in a water agar plate without NaCl amendment and sealed immediately without air-drying.

Within each plate, one slide was taken out 4, 8 or 24 h after incubation and assessed for spore germination under a binocular microscope (25×); 100 spores were randomly selected for assessment of germination for each inoculum droplet. Before the assessment, a drop of thymol was placed on each dried inoculum spot to stop the spores from further germination. Spores were considered to have germinated when the germ tube was longer than the diameter of the spore (Magan, 1988). The experiment was conducted twice.

**Infection of detached fruit under different humidity levels**

Saturated salt solutions in desiccators were used to maintain different levels of RH conditions (45% - potassium carbonate, 75% - sodium chloride, 85% - potassium chloride, and 98% -potassium sulphate) (Winston and Bates 1960). Each salt solution was poured into a desiccator. Excess solid undissolved salt was maintained in each desiccator to ensure that the target RH level was maintained. The lid was sealed with paraffin to ensure airtightness. For comparison, there were also positive (inoculated) and negative (non-inoculated) control treatments (incubated in a desiccator with water to achieve 100% RH). For both pathogens, the spore concentration was $10^3$ spores $ml^{-1}$; individual fruit were inoculated with a hand-held fine-sprayer as described before. The fruit were then dried in a flow cabinet for 1 h, transferred to the desiccators (on a metal mesh – approximately 2 cm above the salt solution or water) and incubated under ambient conditions (20-25°C).

Each treatment consisted of 100 fruit, except the two control treatments (each with 40 fruit). Twenty fruit from each treatment (excluding the controls) were taken randomly after 3, 6, 9, 24 and 48 h incubation in the desiccators; they were immediately surface sterilised with 5% sodium hypochlorite solution for 15 minutes and rinsed several times with SDW. The fruit were then incubated as described previously and assessed daily for disease development for 7 days. For *Mucor*, ripe fruit of cv. Elsanta were used in one inoculation and yellow-ripe fruit of cv. Elsanta in the other. For *Rhizopus*, ripe fruit of cv. Albion were used in one inoculation and yellow-ripe fruit of cv. Elsanta in the other. The experiment was carried out only once.

**Inoculation of attached ripe fruit under different relative humidity and temperature conditions**

Ripe fruit on potted plants of cv. Sonata were inoculated with *M. piriformis* or *Rhizopus* and then incubated under one of five conditions: RH 45% at 25°C (vapour pressure deficit [vpd] 1.743 kPa), 60% at 25°C (vpd 1.268 kPa), 75% at 25°C (vpd 0.792 kPa), 75% at 15°C (vpd 0.426 kPa) and 90% at 15°C (vpd 0.171 kPa). The spore concentration used was $10^3$ spores $ml^{-1}$ for both pathogens.

For each condition, ripe fruit from 40 plants were inoculated with *M. piriformis*, 40 plants with *Rhizopus*; 10 plants were used as controls (inoculated with SDW). Each potted plant had at least two ripe fruit at the time of inoculation. Individual fruit was inoculated as described previously and then the plants were moved to a growth cabinet (Sanyo Gallenkamp PLC SGC170.PFX.J, with temperature of fluctuation ± 1.0°C and RH ± of 3%) set at one of the five conditions. Plants inoculated with the same pathogen (or SDW) were grouped together inside the cabinet in order to reduce accidental cross-contamination. Twenty fruit inoculated with either *Mucor* or *Rhizopus* were harvested randomly 4, 8, 24 and 48 h after inoculation. Fruit on the control plants were only sampled after 48 h. The sampled fruit were surface sterilised with 5% sodium hypochlorite, rinsed several times with SDW and then incubated on moist tissue paper in a tray as described previously and incubated under ambient conditions (20-25°C) for 5 days before assessment. The experiment was conducted twice.

**Data analysis**

Data were analysed within the GLM (generalised linear model) framework to determine the treatment effects on fungal spore germination and disease development. In the GLM

analysis, the error distribution was assumed to follow a binomial distribution and the logit was used as the link function. For *in vitro* germination and fruit-infection condition studies, a quasi-binomial distribution was used because of over-dispersion in the data. When testing treatment effects or comparing treatment levels, the deviance test (chi-square test) based on nested models was used. In all experiments (except for the inoculation of attached fruit in cabinets), repeated experiments were treated as a blocking factor. For the cabinet inoculation study, data were pooled from two replicate inoculations before analysis since individual conditions were repeated at different times (hence cannot be treated as a block factor). Statistical significance was assessed at the level of 0.05, 0.01 and 0.001. All analyses were carried out in R version 3.6.0 (R Project Team 2019).

## Results

### Susceptibility of strawberry flower and fruit

In non-inoculated plants, none of the flowers or fruit developed *Mucor* or *Rhizopus* rot symptoms at harvest four days after being treated with SDW; however, some ripe fruit showed *Botrytis* rot symptoms. Similarly, none of the inoculated flowers and green fruit of the cultivars examined (cvs. Amesti and Sonata) developed *Rhizopus* or *Mucor* rot four days after inoculation (Table 1). In contrast, most of the inoculated ripe fruit showed visual symptoms of *Mucor* or *Rhizopus* rot four days after inoculation (Table 1); more than 80% of the inoculated ripe fruit developed rot symptoms at harvest or post-harvest.

The incidence of *Mucor* and *Rhizopus* remained similar to each other irrespective of the cultivars and fruit development stage. Overall, the incidence of *Mucor*/*Rhizopus* four days after inoculation differed between the two cultivars ($P < 0.05$) and between the four fruit development stages ($P < 0.001$). However, deviance testing based on the nested models showed that there were no interactions between cultivar and fruit age on the incidence of *Mucor*/*Rhizopus*. The overall incidence of *Mucor*/*Rhizopus* was lower on cv. Sonata (6%/7%) than on cv. Amesti (24%/21%). The *Mucor*/*Rhizopus* incidence on inoculated ripe fruit (68%/62%) was greater ($P < 0.001$) than on white fruit (4%/8%), which was in turn greater ($P < 0.05$) than on both green fruit and flowers (0%/0%).

After post-harvest incubation, more fruit developed rot symptoms. Many flowers/fruit from the control plants developed various post-harvest rot symptoms, including *Botrytis*, *Penicillium, Mucor* and *Rhizopus*, with *Botrytis* being predominant. With the exception of two green fruit of cv. Sonata inoculated with *Rhizopus*, inoculated flowers or green fruit did

not develop post-harvest *Mucor* or *Rhizopus* rot symptoms. Inoculated white or ripe fruit showed a higher ($P < 0.001$) incidence of post-harvest *Mucor*/*Rhizopus* rots than the non-inoculated fruit (Table 1). As for the pre-harvest incidence, the total *Mucor*/*Rhizopus* incidence was much higher ($P < 0.001$) on inoculated ripe (90%/86%) than on white (34%/36%) fruit.

**Effect of inoculum dose on infection of detached fruit**

Fig. 1 shows the percentage *Mucor* or *Rhizopus* rot of detached ripe fruit post-incubation after inoculation with various concentrations of *M. piriformis* or *Rhizopus* spores. The overall incidence of *Mucor* rots differed ($P < 0.001$) among the inoculum concentrations. None of the non-inoculated fruit developed symptoms of *Mucor* rot. In contrast, 51% of fruit inoculated with 100 spores developed *Mucor* rot, which did not differ significantly from those fruit that received 1000 spores (54%). The incidence of *Mucor* rot on the fruit that received 10,000 spores (66%) was higher ($P < 0.05$) than those receiving 100 or 1000 spores but lower ($P < 0.001$) than those receiving 100,000 spores (89%).

Similar trends were observed for the fruit inoculated with various concentrations of *Rhizopus* spores (Fig. 1). Only one out of the 80 non-inoculated fruit developed symptoms of *Rhizopus* rot, compared to > 67% of those inoculated with *Rhizopus* spores. Inoculation of fruit with 100,000 spores led to an incidence of 91% *Rhizopus;* greater ($P < 0.001$) than the other three inoculum concentrations studied (in the range of 67% and 79%), which did not differ significantly.

**Effect of relative humidity and temperature on *in vitro* germination** In general, more spores of *M. piriformis* germinated in free water (control) than at any other RH conditions at the three temperatures examined (Fig. 2). The overall level of germination was very low (< 7%) for most conditions even after 24 h incubation. Relative humidity levels close to saturation led to higher germination rates (16% to 39%), especially at 21°C after 24 h (Fig. 2). The differences in germination between different RH levels were small at both 10 and 31°C.

GLM analysis was applied only to the germination data assessed at 24 h. The percentage germination of *M. piriformis* spores after 24 h did not differ between 10°C and 31°C but was much lower ($P < 0.001$) than in the 21°C temperature treatment. The percentage germination after 24 h did not differ between 85%, 91% and 95% RH, or between 97%,

99%, 100% RH and "freely available water". Overall, higher RH values (> 95%) led to greater ($P < 0.01$) germination than those ≤ 95% RH (Fig. 2).

Similar results were also obtained for *Rhizopus* (Fig. 2) but there were also several noticeable differences: (1) significant ($P < 0.01$) differences between 10°C and 31°C, albeit small; (2) greater differences among RH levels (85% = 91% [$P < 0.01$] < 95% = 97% < [$P < 0.05$] < 99% = 100% < [$P < 0.001$] free water); and (3) higher germination rates at near-saturation RH at all three temperatures. The highest spore germination was recorded in free water at 21°C (47%) after 24 h (Fig. 2).

**Infection of detached fruit under different humidity levels**

Fig. 3 shows the effect of different RH conditions on the incidence of infection of detached fruit with *M. piriformis* or *Rhizopus* 5 days after inoculation at 20-25°C. In addition to *Mucor* and *Rhizopus* rot, other rots were also observed in both inoculated and non-inoculated fruit with grey mould caused by *Botrytis* being predominant.

Nearly all ripe fruit (whether inoculated or uninoculated with *M. piriformis*) developed rot symptoms (Fig. 3). Although inoculation led to a greater proportion of ripe fruit with *Mucor* symptoms (except those incubated at 100% RH for 48 h) than the control treatment (Fig. 3), this difference was not statistically significant. The level of *Mucor* rot was much lower on inoculated yellow (30%) than on ripe (75%) fruit (Fig. 3, $P < 0.001$). At least 50% of the inoculated yellow fruit remained healthy after post-harvest assessment whereas the corresponding value for ripe fruit was <5%. RH did not significantly affect development of *Mucor* rot on either ripe or yellow fruit. The duration of incubation under the different RH levels conditions affected *Mucor* incidence only on yellow fruit. The incidence was greater for 24 h (40%) and 48 h (39%) than after 3 h (23%), 6 h (23%) and 9 h (19%) ($P < 0.01$).

Most ripe fruit inoculated with *Rhizopus* decayed irrespective of the initial RH level (Fig. 3). Neither the initial RH nor the duration of incubation under the RH treatments significantly affected development of *Rhizopus* rot on ripe fruit. The incidence of *Rhizopus* was much lower on inoculated yellow (39%) than ripe (84%) fruit (Fig. 3, $P < 0.001$). At least 50% of inoculated yellow fruit remained healthy after post-harvest assessment, whereas the corresponding value for ripe fruit was <13%. Both RH and the initial incubation duration affected development of *Rhizopus* rot in yellow fruit ($P < 0.01$). Thus, initial incubation under 45% and 75% RH led to lower incidences (25% and 26%) than under 85% RH (48%), 98% RH (48%) and 100% RH (65%) ($P < 0.01$). Incidence of *Rhizopus* was

greater for the initial incubation duration of 9 h (40%), 24 h (45%) and 48 h (52%) than 3 h (28%) and 6 h (23%)($P < 0.01$).

**Inoculation of attached ripe fruit under different RH and temperature combinations**

The post-harvest level of *Mucor* or *Rhizopus* rots on those attached fruit inoculated with *Mucor* or *Rhizopus* at five RH-temperature (vpd) combinations is given in Fig. 4. The number of non-inoculated fruit for a given vpd level ranged from 40 to 42.

The number of fruit inoculated with *M. piriformis* for a given vpd level ranged from 103 to 174 with the incidence of *Mucor* ranging from 26% (0.171 kPa) to 42% (0.792 kPa). Inoculation with *M. piriformis* led to a greater ($P < 0.001$) number of fruit with *Mucor* (32%) rots than the control (1%). The differences between the five vpd levels were just significant at the level of $P = 0.05$, primarily due to the greater incidence at vpd of 0.792 kPa than the other four vpd levels (Fig. 4). The duration of the initial vpd conditions following inoculation did not affect *Mucor* development.

The number of fruit inoculated with *Rhizopus* for a given vpd level ranged from 101 to 188; the incidence of fruit with *Rhizopus* rots ranged from 34% (0.426 kPa) to 51% (0.171 kPa). Inoculation with *Rhizopus* led to a greater number of fruit with *Rhizopus* (42%) rots than the non-inoculated control (0%) ($P < 0.001$). Neither the vpd nor the initial incubation duration significantly affected *Rhizopus* development.

## Discussion

A number of experiments were carried out to study the epidemiology of *M. piriformis* and *Rhizopus* on strawberry which have shown that they are unlikely to be able to infect flowers and green fruit, but that fruit become increasingly susceptible from the green stage onwards. Although *in vitro* germination of both *M. piriformis* and *Rhizopus* required near-saturation RH conditions and was lower at both low and high temperatures, infection of ripe fruit and subsequent rot development were not significantly affected by temperature and RH that are often encountered under commercial production conditions (15 to 30°C and 50 to 95% RH) in the UK.

Unlike *B. cinerea*, neither *M. piriformis* nor *Rhizopus* could infect flowers. Only white and ripe fruit were susceptible to infection by these two pathogens. In addition to post-harvest rot development, both *Mucor* and *Rhizopus* rots could develop on ripe fruit within four days of inoculation. These results on fruit susceptibility are supported by recent field

surveys across a number of sites (including both open-field and protected crops) in the UK (Agyare 2016). In the survey, *Mucor* and *Rhizopus* were isolated only from white and ripe fruit whereas spoilage agents isolated from flowers and green fruit were mainly *Botrytis* and *Penicillium*. The present study has demonstrated that both *M. piriformis* and *Rhizopus* could readily infect intact strawberry fruit from the white stage onwards. This contrasts with previous speculation that *Mucor* and *Rhizopus* mainly infect wounded white and ripe fruit (Harris and Dennis 1980).

White/yellow (maturing) fruit were much less susceptible to *M. piriformis* and *Rhizopus* than ripe fruit. This difference was expressed in several ways. Firstly, the overall level of rot incidence was lower on inoculated white than on inoculated ripe fruit. Secondly, a greater proportion of white fruit than ripe fruit, that had been inoculated when attached, developed rotting after post-harvest incubation. Thirdly, infection of detached yellow fruit increased with the initial incubation duration under the higher RH conditions whereas on detached ripe fruit an incubation period as short as 3 h resulted in a similar level of fruit rotting as after 48 h. The lower susceptibility of white/yellow fruit to both *M. piriformis* and *Rhizopus* than ripe fruit was also independent of RH levels at 20-25°C as shown by the inoculation of detached fruit. This difference in susceptibility is consistent with a previous isolation study (Harris and Dennis 1980) that showed an increase in the number of *Mucor* and *Rhizopus* strains isolated from late season ripe fruit than from green or white fruit earlier in the season. However, this difference in the number of isolated fungal strains among fruit stages could be partially due to differential inoculum strength between sampling times. The present study excluded this potential effect of inoculum dose as each flower or fruit of different maturity stages received approximately the same number of spores. This age related susceptibility to pathogens (i.e., ontogenic resistance) is commonly observed in many pathosystems (Develey-Rivière and Galiana 2007; Ficke et al. 2002; Li and Xu 2002; Xu and Robinson 2010). In addition, there were significant differences between cvs. Amesti and Sonata in their susceptibility to *M. piriformis* and *Rhizopus*; however, such differences were small relative to the difference between ripe and white fruit. Thus, this cultivar difference could be ignored in practical disease management. On ripe fruit, as few as 100 spores per fruit resulted in at least 50% of fruit with rot symptoms for both *M. piriformis* and *Rhizopus*, indicating a high potency of these pathogens to infect such fruit.

*In vitro* germination of *M. piriformis* and *Rhizopus* spores showed similar relationships with temperature and RH. Germination requires RH levels near saturation; this effect was more pronounced at 21°C than either low (10 °C) or high (31°C) temperatures. Spores could

readily germinate within four hours; further increases in germination thereafter were usually small unless under high RH conditions at 21°C. These results suggest that under conducive conditions spores of both *M. piriformis* and *Rhizopus* can germinate and may consequently cause infection within 4-8 hours of being in contact with the fruit. Compared to *M. piriformis*, more *Rhizopus* spores germinated under high RH at 31°C, consistent with the temperature profile of *Rhizopus* and *Mucor* species (Hoffmann et al. 2013; Michailides and Ogawa 1989). Most *Rhizopus* spp. are mesophilic and grow consistently over the range of 20-40°C while *Mucor* are often limited to 0-27°C.

Inoculation of both detached (yellow and ripe) and attached (ripe) fruit indicated that infection of fruit was not significantly affected by the climatic conditions examined. Although there were significant effects of RH on infection of detached yellow fruit by *Rhizopus* and of vpd on infection of attached ripe fruit by *M. piriformis*, such effects were very small relative to the overall level of fruit rot resulting from the inoculation. The time required for achieving close to maximum infection/rot was very short. On detached ripe fruit, 3 h was sufficient for both *M. piriformis* and *Rhizopus*; on detached yellow fruit, 9 h and 24 h was sufficient for *Rhizopus* and *M. piriformis*, respectively; on attached ripe fruit, 4 h was sufficient. The five levels of vpd (combinations of RH and temperature) were chosen to represent commonly occurring conditions for commercial strawberry production in the UK. In addition, RH in the boundary layer of the fruit surface could be close to saturation, particularly given the uneven nature of such surfaces, and may hence support infection. Thus, the present study suggests that climatic conditions are unlikely to be the main factor limiting infection of fruit by *M. piriformis* and *Rhizopus* for the main strawberry production season in the UK.

The two *Rhizopus* isolates used in the present study probably came from two species as their exact species identities could not be resolved with confidence based on the ITS sequences and morphological characteristics. Thus, it was not possible in the present study to attribute the observed results to one particular *Rhizopus* species. Nevertheless, the present results are still valuable in informing the epidemiological characteristics of *Rhizopus* spp. causing strawberry fruit decay and illustrating the high-degree of similarities between *M. piriformis* and *Rhizopus* spp. in causing strawberry fruit (soft) rots.

In summary, fruit become increasingly susceptible to *M. piriformis* and *Rhizopus* as they mature from the green stage onwards. However, such age-related resistance may have only a limited value for practical disease management since in most of the fruit production period there is a mixture of fruit at different maturity stages. Infection of fruit could be

completed within 9-24 hours under the prevailing climatic conditions in strawberry crops. Infection of fruit by *M. piriformis* and *Rhizopus* is unlikely to be constrained by climatic conditions in the UK commercial strawberry production. Thus, the most likely factor determining the level of infection of fruit by *M. piriformis* and *Rhizopus* is the number of spores deposited on the fruit surface. Consequently, crop hygienic management, especially of crop debris, is of paramount importance in order to reduce the inoculum load and pressure. In addition, rapid cooling and post-harvest cool-chain management may also help to slow down post-harvest development of latent infection, as demonstrated for the post-harvest development of grey mould in raspberry fruit (O'Neill et al. 2012).

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**Compliance with ethical standards**

Conflict of interest: The authors confirm no conflict of interest.

Ethical approval: This research did not involve any human participants, and/or animals.

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Figure 1. Percentage of fruit rot after inoculation of detached ripe strawberry fruit (cv. Lusa) with different concentrations of *M. piriformis* or *Rhizopus* spores under ambient conditions, pooled over two repeat experiments. Treatments (i.e., inoculum dose for the same pathogen) with the same lower case letter above the bar did not differ significantly ($P > 0.05$) from each other based on the deviance test (Chi-square) of nested models in the GLM analysis.

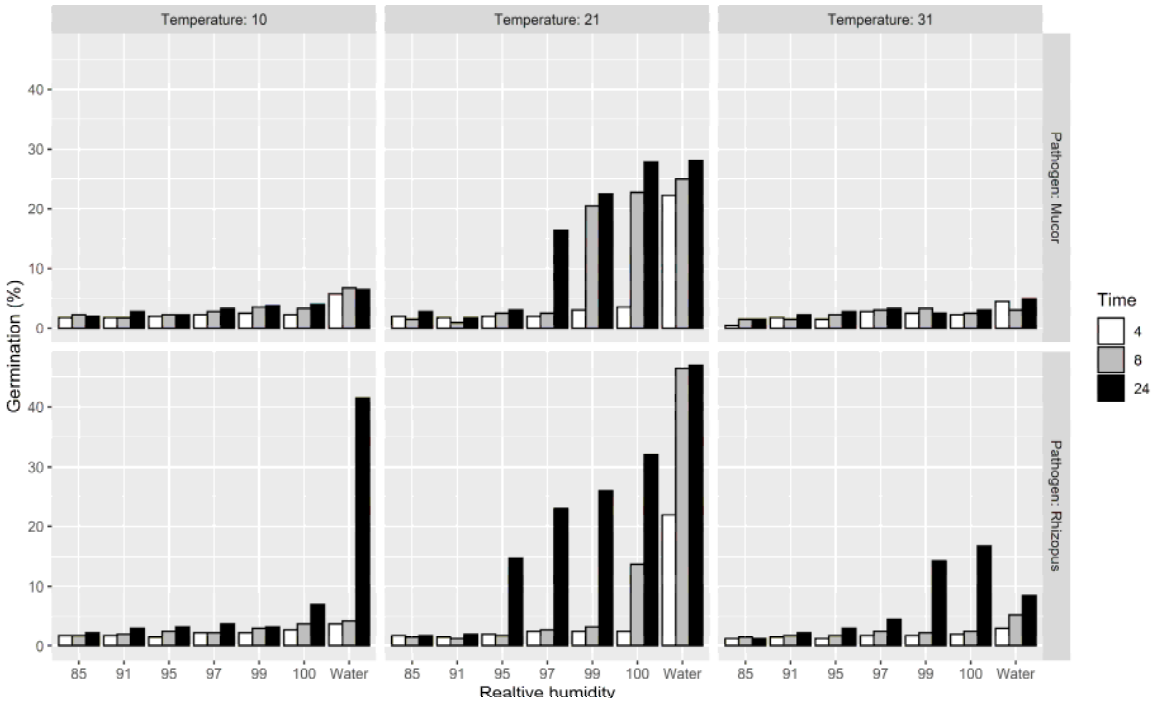


Figure 2. Percentage of *in vitro* germination of *M. piriformis* and *Rhizopus* spores under different levels of relative humidity at three temperatures (10, 21 and 31°C) assessed 4, 8 and 24 h, and pooled over two repeat experiments. Specific relative humidity levels were achieved with water agar amended with an appropriate amount of NaCl.

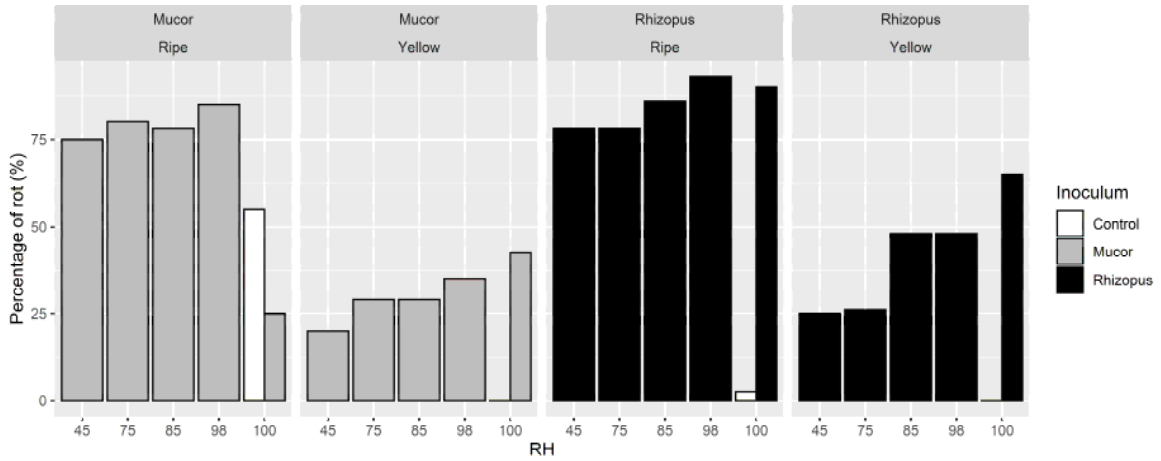


Figure 3. Percentage of fruit rot after inoculation of detached yellow or ripe fruit with *M. piriformis* or *Rhizopus* spores incubated under different relative humidity conditions (RH 45-100%) at 20-25°C in desiccators containing saturated salt solutions to achieve the desired relative humidity. Inoculated fruit were destructively sampled 3, 6, 9, 24 and 48 h after inoculation. The non-inoculated fruit (control) were only incubated under 100% RH and sampled at 48 h. Those fruit that did not develop visual rot symptoms at the time of sampling were surface-sterilised and incubated for post-harvest rot development at 20-25°C.

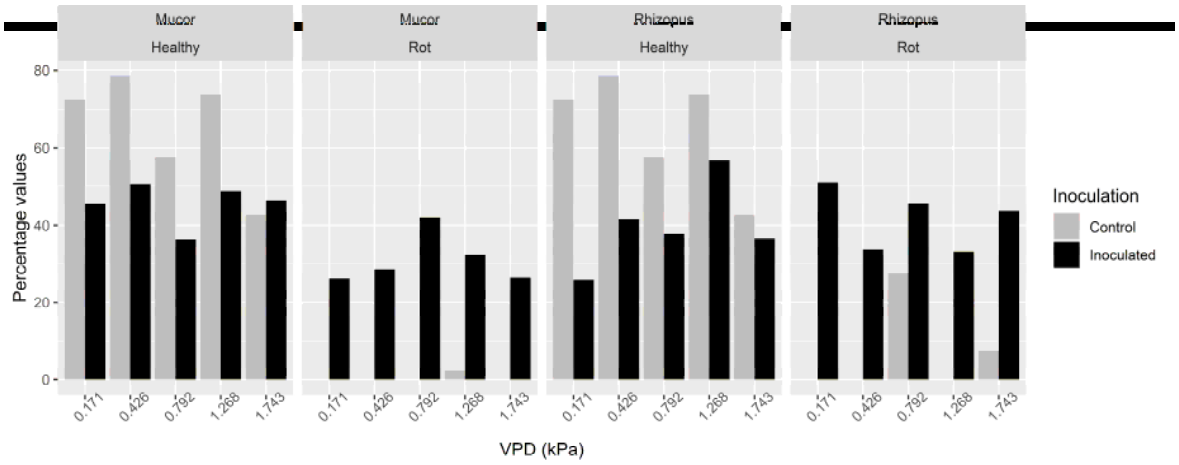


Figure 4. Percentage of healthy fruit and fruit rot resulting from inoculation of attached ripe strawberry fruit with *M. piriformis* or *Rhizopus* conidia in controlled environment cabinets under five different combinations of relative humidity (%RH) and temperature (°C) wih the following vapour pressure deficits (vpd, in kPa): RH 45% at 25°C (= 1.743 kPa), 60% at 25°C = 1.268 kPa, RH 75% at 25°C = 0.792 kPa, RH 75% at 15°C = 0.426 kPa and RH 90% at 15°C = 0.171 kPa. Inoculated fruit were sampled 4, 8, 24 and 48 h after inoculation. Non-inoculated fruit (control) were only sampled after 48 h. Those fruit that did not rot at the time of sampling were surface-sterilised and incubated for post-harvest rot development assessment at 20-25°C. Treatment conditions significantly affected development of *Mucor* ($P < 0.05$) but not *Rhizopus*.

Table 1. Average percentage (± Standard Error) of pre- and post-harvest *Mucor* and *Rhizopus* rots resulting from inoculation of attached flowers, green, white and ripe fruit of two strawberry cultivars (cvs. Amesti and Sonata) in glasshouse experiments.

| Fruit development stage | Inoculated with *Mucor piriformis* | | | Inoculated with *Rhizopus* | | | Non-inoculated control | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total fruit | Pre-harvest | Post-harvest | Total fruit | Pre-harvest | Post-harvest | Total fruit | Pre-harvest | Post-harvest |
| **cv. Amesti** | | | | | | | | | |
| Flowers | 42 | 0 | 0 | 61 | 0 | 0 | 39 | 0 | 0 |
| Green | 55 | 0 | 0 | 44 | 0 | 0 | 46 | 0 | 0 |
| White | 75 | 6.9±6.9 | 38.3±2.5 | 63 | 10.7±4.6 | 34.4±1.1 | 80 | 0 | 8.2±5.0 |
| Ripe | 75 | 67.8±8.9 | 18.0±1.9 | 63 | 67.2±8.8 | 19.6±10.2 | 87 | 0 | 8.1±3.9 |
| **cv. Sonata** | | | | | | | | | |
| Flowers | 89 | 0 | 0 | 75 | 0 | 0 | 73 | 0 | 0 |
| Green | 167 | 0 | 0 | 156 | 0 | 1.3±0.7 | 155 | 0 | 0 |
| White | 148 | 3.3±2.2 | 25.2±5.3 | 157 | 7.6±2.4 | 23.1±2.1 | 139 | 0 | 13.9±10.2 |
| Ripe | 37 | 61.3±2.8 | 26.7±5.1 | 31 | 55.4±14.4 | 27.9±14.0 | 34 | 0 | 14.9±11.9 |

Flowers/fruit were assessed for visual rot symptoms four days after inoculation; non-symptomatic flowers/fruit were then harvested, surface-sterilised, incubated for 1-3 weeks under ambient conditions and regularly assessed for fruit rot development

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# Conditions for infection of strawberry fruit by M. piriformis and Rhizopus spp.

**Agyare, Solomon; Magan, Naresh; Xu, Xiangming**

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