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3 **Environmental factors modify carbon nutritional patterns and niche**
4 **overlap between *Aspergillus flavus* and *Fusarium verticillioides* strains**
5 **from maize**

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16 *temperature, carbon sources.*

Abstract

This study examined the utilization patterns of key Carbon sources (CS, 24: including key sugars, aminoacids and fatty acids) in maize by strains of *Aspergillus flavus* and *Fusarium verticillioides* under different water activity (a_w , 29 0.87-0.98 a_w) and temperature (20-35°C) values and compared the niche overlap indices (NOI) that estimate the *in vitro* carbon source utilization profiles (Wilson and Lindow, 1994). The ability to grow in these key CS in minimal media was studied for 120 hrs in 12 hr steps. The NOI was calculated for inter-species (*F. verticillioides* – *A. flavus*) and for intra-species (*A. flavus* - *A. flavus*) using CS utilisation patterns over the range of interacting environmental conditions. 30°C, over the whole a_w range examined, was found to be optimal for utilization of the maximum number of CS by *A. flavus*. In contrast, for *F. verticillioides* this was more so at 20°C; 25°C allowed a suboptimal usage of CS for both species. NOIs confirmed the nutritional dominance of *A. flavus* at 30°C, especially at lower a_w levels and that of *F. verticillioides* at 20°C, mainly at 0.95 a_w . In other conditions of a_w , based on CS utilization patterns, the data indicated that *A. flavus* and *F. verticillioides* occupied different ecological niches. The variability in nutritional sources utilization between *A. flavus* strains was not related to their ability to produce aflatoxins (AFs). This type of data helps to explain the nutritional dominance of fungal species and strains under different environmental conditions. This could be useful in trying to find appropriate natural biocontrol microorganisms to compete with these mycotoxigenic species.

1. Introduction

Maize is a very important staple crop world wide and in Europe. It is harvested at between milky and fully ripe stage for starch production, food and feed purposes and more recently biofuel. Thus contaminant mycotoxins, such as aflatoxins and fumonisins, need to be minimised to meet the legislative requirements (EC Regulation Nr. 1881/2006 and Nr. 1126/2007).

The major mycotoxigenic fungi colonising ripening maize are *Aspergillus flavus* and *Fusarium verticillioides* which produce aflatoxins (AFS) and fumonisins (FBs) respectively. The most common in north Italy is *F. verticillioides*, but in 2003 and 2004, because of very hot summer temperatures and moisture stress, there was a severe outbreak of aflatoxin contamination of maize for animal feed resulting in significant aflatoxin M₁ contamination in milk, (Battilani et al., 2005; Battilani et al., 2008; Piva et al., 2006; Zorzete et al., 2008).

There has been interest in trying to understand the ecological conditions which determine the dominance of individual species. Thus there is concern in understanding pathogen-pathogen interactions where they are both mycotoxin producing species (Magan and Aldred, 2007a; Magan and Aldred, 2007b).

It has been suggested that *Fusarium* species are very competitive and that kernels initially infected by *F. verticillioides* may be resistant to later infection by *A. flavus* (Wicklow et al., 1988). Nevertheless, it is not unusual to find crops with both AF and FB contamination in field surveys (Battilani et al., 2005).

Environmental factors, such as water availability (a_w) and temperature, affect the interactions and competitiveness of spoilage and mycotoxigenic fungi (Lee and Magan, 1999; Marìn et al., 1995; Marìn et al., 1998a; Marìn et al., 1998b; Magan et al., 2003; Sanchis and Magan, 2004). The co-existence of microorganisms on plant surfaces is also mediated by nutritional resource partitioning (Wilson and Lindow, 1994) and the utilisation pattern of CSs could be used to study the niche overlap. Wilson and Lindow (1994) showed that Niche Overlap Indices (NOI) > 0.90 were indicative of coexistence between species or strains in an ecological niche, while scores of <0.90 represented occupation of separate niches. Recently, Arroyo et al. (2008) showed that NOIs and relative CS utilisation patterns were significantly influenced by interactions between a_w , pH and level of preservatives used for controlling food spoilage moulds in intermediate bakery products.

The aim of this study was (a) to compare Italian strains of *A. flavus* and *F. verticillioides* based on their ability to use CSs in maize and (b) to calculate their relative NOIs under different water activities and temperatures to understand the potential conditions under which nutritional dominance occurs. This could be beneficial for a better understanding of the reasons why aflatoxins and sometimes fumonisins contamination in maize is predominant. This information may also be useful as a basis for designing control systems, biological or chemical, as part of a prevention strategy in the maize ecosystem.

2. Materials and Methods

2.1 Fungal strains

Experiments were conducted using 5 fungal strains isolated from maize kernels in Northern Italy. One strain was *F. verticilloides* (ITEM 1744), a confirmed fumonisin producer (Moretti et al., 1995), and four strains of *A. flavus*: A 2092 and A 2057, high (1156 ng AFB₁/g of culture media) and low (0.3 ng AFB₁/g of culture media) AFB₁ producers respectively; and A 2097 and A 2082 non-AF producers (Giorni et al., 2007). *Aspergillus* strains were held in the culture collection of the Institute of Entomology and Plant Pathology, Università Cattolica del Sacro Cuore of Piacenza (Italy; code MPVP) and the taxonomic identities were confirmed by Food Science Australia (CSIRO, Sydney, Australia). Conidial heads and conidia of A2057 were described as atypical, but had other attributes which confirmed it as *A. flavus*.

2.2 Microtitre plate preparation

Sterile microtitre plates (24 wells, IWAKI, Japan) with a well capacity of 1 mL and a lid were used. A minimal medium was prepared with NaNO₃ (0.23%), MgSO₄·7H₂O (0.06%), K₂HPO₄ (0.17%) and KH₂PO₄ (0.13%). Carbon sources were incorporated into the media at a final concentration of 9.1x10⁻³ g C mL⁻¹ well⁻¹ (carbon equivalent to 2% (w/v) glucose); each well of the plate was filled with 700 µL of one CS solution. Carbon sources tested represent the principal chemical components of maize kernels and they are listed in Table 1.

The water activity (a_w) of the CS treatments was modified to five values: 0.87, 0.90, 0.93, 0.95 and 0.98 a_w by adding different amounts of NaCl (Dallyn and Fox, 1980). The pH was regulated to 6 using a phosphate buffer (10nM, Sigma) (Dawson et al., 1987). The experiments were conducted twice with three replicates per treatment per strain.

2.3 Spore suspension preparation and inoculation

Spores from 7 day old cultures grown on Czapek Agar (CZ; sucrose 30 g; NaNO₃ 2 g; KCl 0.5 g; MgSO₄·7H₂O 0.5 g; FeSO₄·7H₂O 0.01; K₂HPO₄ 1 g; ZnSO₄·7H₂O 0.001 g; CuSO₄·7H₂O 0.005 g; agar 15 g; H₂O to 1 L) was used for *A. flavus* and Potato Dextrose Agar (PDA; infusion from potatoes 200g; dextrose 15g; agar 20g; H₂O to 1L) for *F. verticillioides*, were harvested (with sterile water) and individually placed into sterile Universal bottles containing 20 mL of distilled water. Bottles were shaken vigorously for 3 minutes and centrifuged in a bench top microfuge for 15 minutes at 3000 rpm. After discarding the supernatant, they were washed three times with 20 mL of sterile water. After the third washing, spores were resuspended with the treatment buffer-NaCl sterile solution and the concentration adjusted to 10⁶ spores mL⁻¹. Wells were inoculated with 100 µL of the spore solution. Microtitre plates without inoculum were prepared and incubated as additional controls. All plates were closed with Parafilm[®] and incubated at 20, 25 and 30°C. The presence or absence of fungal growth was checked at 12 hr intervals for up to 120 hrs. The wells were all checked with a microscope to determine whether growth had

occurred (yes/no); wells were scored positive when fungal mycelium was detected.

2.4 Calculation of Niche Overlap Index (NOI)

Results of CS utilisation were used to calculate a Niche Overlap Index (NOI) (Wilson and Lindow, 1994). The index was computed for both the target pathogen (A; *F. verticillioides* or toxigenic strains of *A. flavus*) and compared with the other strains (B; different *A. flavus* strains or the non-aflatoxin producing strain of *A. flavus*):

NOI_{A/B} = Nr. of CS used by both strains / total Nr. of CS used by species A (1)

NOI_{B/A} = Nr. of CS used by both strains / total Nr. of CS used by species B (2)

The NOI values were between 0 and 1 and it defines whether fungi co-exist, i.e., use common CS (NOI_{A/B} and NOI_{B/A} >0.9), occupy separate niches (NOI_{A/B} and NOI_{B/A} <0.9) or one strain dominates (NOI_{A/B} >0.9 and NOI_{B/A} <0.9 strain A nutritionally dominate and *vice versa*) (Wilson and Lindow, 1994; Arroyo et al., 2008).

2.5 Data analysis

A three factor randomised complete block design ANOVA (ANalysis Of VAriance) was applied to data on CS used by different strains using the statistical and data management package MSTAT-C (MSTAT-C, 1991). This software is beneficial for experimental design, managing, transforming and analyzing data. The randomised complete block design is a statistical analysis

in which each block contains a complete set of treatments; since each treatment occurs once within each block, treatments can be compared within blocks, and so block-to-block (gradient) variations does not effect the treatment comparison (Clewer and Scarisbrisk, 2001).

The percentage of CSs used was computed and arcsine transformation, appropriate for observations which are proportions (Fowler and Cohen, 1990), was applied before data analysis.

3. Results

The number of CSs used by the fungi increased in time. During the first 36 hrs after incubation the number of CSs utilized was very limited but significantly increased after 60 hours. Subsequently, up to the end of the incubation period (120 hrs) the number of CSs used by the fungi remained almost unvaried from those recorded after 60 hrs, with variations between 1 and 4% (Table 2). For this reason, only data on CSs used after 60 hrs incubation were considered for statistical analyses. Minor variations were observed between replicates, but the data was very similar for the two repetitions carried out. Thus, overall data across both sets of experiments were combined and the means are presented (Figure 1).

The 5 strains used a different number of CS at all the treatment environmental conditions (Figure 1). At 30°C the number of CSs used was the highest for all the strains of *A. flavus*, while for *F. verticillioides* the number of CSs used at 20°C was maximum, except at 0.87 a_w, where only 2 CSs were used because

of the marginal conditions for growth; at 30°C the number of CSs used by *F. verticillioides* was significantly higher at 0.90 with respect to all the other a_w levels tested.

Aspergillus flavus strains A 2057 (low AFs produced) and A 2082 (non-producer) used fewer CSs at 0.90 a_w and 25 and 30°C than the other two *A. flavus* strains, which grew with almost all the CSs tested. The number of CS used at 0.90 was significantly lower with respect to 0.98 a_w at all temperatures, with the exception of 30°C and 0.87 and 0.95 a_w . A 2092 and A 2097 showed significant differences only at 20°C and 0.98 a_w , with respect to all the other a_w treatments at each temperature.

All the strains preferentially utilised carbohydrates and amino acids and subsequently, only at the higher temperatures and a_w levels, fatty acids (Figure 2). All the *A. flavus* strains preferentially used carbohydrates more than amino acids, in all the treatment conditions, while *F. verticillioides* grew similarly with both types of CSs. Regarding the two starch components, amylopectin was the most used one by all the strains studied, especially at 25-30°C by *A. flavus* and at 20-25°C by *F. verticillioides*. Differences were observed between *A. flavus* strains regarding amylose and *F. verticillioides* grew only occasionally using amylose as a CS (data not shown).

The NOIs for each strain of *A. flavus* with *F. verticillioides* are summarised in Figure 3. *F. verticillioides* nutritionally dominated over all the strains of *A. flavus*

at 20°C and 0.95 a_w , over the low AFB₁ producer strain (A2057) and over the high AFB₁ producer strain (A 2097) at 0.98 a_w and against the other two (A 2092, A 2097) at 25°C and 0.95 a_w . *Aspergillus flavus* always nutritionally dominated *F. verticillioides* at 30°C and high and reduced a_w (0.98, 0.87 a_w). The two AFB₁ producers dominated at 0.95 a_w , A 2092 also at 0.90 a_w and A 2082 also at 25°C and 0.98 a_w . In most conditions the fungi studied occupied different niches. Co-existence between *A. flavus* with *F. verticillioides* was only found for strain A 2082 at 20°C and 0.98 a_w (Figure 3).

The comparison between toxigenic and non-toxigenic strains of *A. flavus*, regarding the usage of CS, showed that the non-producer strain A 2097 was more competitive than A 2082 since it was able to use more CSs. In fact, A 2097 was dominant in many ecological conditions when compared with both the toxigenic strains; it was efficient at 20°C and dominated at almost all the a_w levels tested (Figure 4). Based on the nutritional utilisation patterns and the NOIs there appeared that to be an interaction between the strains with the non-toxigenic strains being dominant at 25-30 °C at high or low a_w .

4. Discussion

Temperatures between 20-30°C are typical for the maize growing season from flowering to harvesting. The a_w between 0.98 and 0.87 is usually found in kernels from early dough to full ripe stage (Battilani et al., 2007; Zorzete et al., 2008). In almost all these conditions, both mycotoxigenic strains of *A. flavus*, A 2057 and A2092, were able to grow with at least a few CS.

237

238 Thirty Celsius degrees was found to be optimal for *A. flavus*, with most of CS
239 used at all the a_w values. These data are partially in agreement with Marìn et al.
240 (1998b); indeed, they found *F. moniliforme* (= *F. verticillioides*) was always
241 nutritionally dominant at 25°C and $a_w > 0.95$, while in the present study 2 strains
242 of *A. flavus* were nutritionally dominant at 0.98 a_w and occupied separated
243 nutritional niches with respect to *F. verticillioides* at 0.95, while the other 2 *A.*
244 *flavus* strains were nutritionally dominated by *F. verticillioides* at 0.95 a_w but
245 occupied separate nutritional niches with respect to *F. verticillioides* at 0.98 a_w .

246

247 The behaviour observed with CS utilization pattern was confirmed when only
248 carbohydrates were considered. Low molecular weight carbohydrates were
249 frequently used, in agreement with the report of optimal growth of *A. flavus*
250 using glucose and maltose as CS (Massoud et al., 1999). Amylopectin, a
251 relevant component representing around 60% of kernel dry matter, was used at
252 almost all a_w levels at 20 and 25°C by *F. verticillioides* and at 25 and 30°C by *A.*
253 *flavus*. This is relevant also in relation to toxin production, because Bluhm and
254 Woloshuk (2005) showed the importance of amylopectin content in the medium
255 in relation to FBs synthesis. There are no other related reports on stimulation of
256 such components on mycotoxin production. Nevertheless, Woloshuk et al.
257 (1997) underlined that the best inducers of AFs biosynthesis are CS readily
258 metabolised via glycolysis and that amylose has a role in the induction of AFs
259 biosynthesis

260

The different use of CS at different temperatures was also confirmed by the NOIs, with nutritional dominance of *A. flavus* always observed at 30°C, especially at extreme a_w , and nutritional dominance of *F. verticillioides* at 20°C, mainly at 0.95 a_w . This demonstrates that total and common CS compounds utilized by each fungus and NOI can be modified by environmental conditions such as a_w and temperature. We found *A. flavus* dominant over *F. verticillioides* at 0.87 and 0.98 a_w , while Marin et al., (1998a) found < 0.96 a_w to be conducive to nutritional dominance by Aspergilli.

Previous studies with the ochratoxigenic species *Penicillium verrucosum* and competing fungi *in vitro* and on wheat grain showed that this mycotoxigenic species co-existed with *F. culmorum* and *Alternaria alternata* and *A. ochraceus* at 0.99 to 0.95 a_w and 15°C based on colony interactions (Cairns et al., 2003; Magan et al., 2003). This was similarly confirmed by NOI of *P. verrucosum* relative to these other species and the xerophilic species *Eurotium repens* at 0.99 a_w where NOIs were >0.90 indicative of coexistence with these species. However, at lowered a_w levels (0.90), when compared with *E. repens* then based on nutritional utilisation patterns and NOI, they occupied different niches. The inference was that *P. verrucosum* was not as competitive as other spoilage fungi in primary resource capture on wheat grain at >0.95 a_w , although it may alter resource quality and influence secondary colonisation.

Interestingly, the type of CS was also very important and relevant since carbohydrates, much easier to degrade, allowed faster fungal growth rates than

the other CSs. This is why they are utilized first. In contrast, other CSs may be also utilized although at a lower rate and also resulting in a greater lag time prior to growth. These CSs may perhaps be not utilised within the experimental time frame.

The ability of the two species to assimilate different CS reflects their competitiveness under specific ranges of environmental conditions. However, only extreme conditions were linked to the nutritional dominance of one of the two tested species while in almost all cases *A. flavus* and *F. verticillioides* occupied different niches independently from the *A. flavus* strain used in this study. This suggests that colonisation of maize by these fungi results in different populations occupying different niches and could result in the presence of both aflatoxins and fumonisins.

The intra-species variability, between toxigenic and non-toxigenic *A. flavus* strains and CS utilization patterns, did not appear to be related to the actual ability to produce AFs (data on AFs come from other studies); in fact, pairs of the four strains studied showed a very similar behaviour. This type of information can be useful in understanding the ecophysiology of *A. flavus* and its relationship with *Fusarium* section *Liseola* species. It may also be useful in screening competitive non-producer strains of either species for the development of competitive exclusion approaches for developing natural control systems. This can also include specific ranges of relevant environmental factors and maize specific CS. It may also be possible to integrate this approach by

also incorporating potential for niche exclusion of mycotoxigenic species in the presence of the crop protection chemicals often used, e.g. fungicides. Recent work by Arroyo et al. (2008) has demonstrated the usefulness of understanding these interactions between mycotoxigenic species and strains of *P. verrucosum* (ochratoxin producer) and other species in the presence and absence of different concentrations of preservatives such as propionate and sorbate. This approach can be included to obtain a better understanding of the fluxes in niche overlap and exclusion between these important mycotoxigenic species in maize.

Acknowledgements

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Figure captions

Fig. 1 - Carbon sources used by the four strains of *Aspergillus flavus* and one of *Fusarium verticillioides* grown on a minimal media adjusted at 5 water activity values and incubated at 20, 25 and 30 °C.

Fig. 2 - Percentage of usage of carbon sources typology (carbohydrates, aminoacids and fatty acids) by all the strains tested at the different conditions of temperature (20, 25 and 30°C) and a_w (0.87, 0.90, 0.93, 0.95 and 0.98).

Fig. 3 - Schematic representation of NOI for the different conditions of temperature (20, 25 and 30°C) and a_w (0.87, 0.90, 0.93, 0.95 and 0.98) of the strains of *A. flavus* with *F. verticillioides* as the target pathogen.

Fig. 4 - Schematic representation of NOI for the different conditions of temperature (20, 25 and 30°C) and a_w (0.87, 0.90, 0.93, 0.95 and 0.98) of *A. flavus* non-aflatoxins producers (A 2082 and A 2097) used in the experiment with respect to toxigenic strains of *A. flavus* (A 2057 and A 2092) only. Grey cells represent the nutritional dominance of the non-toxigenic strains.

441 Table 1 – Carbon sources and percentage of the compound added in each well
 442 in niches overlap experiments. All compounds were prepared by Sigma
 443 (Saint Louis, MO, USA).

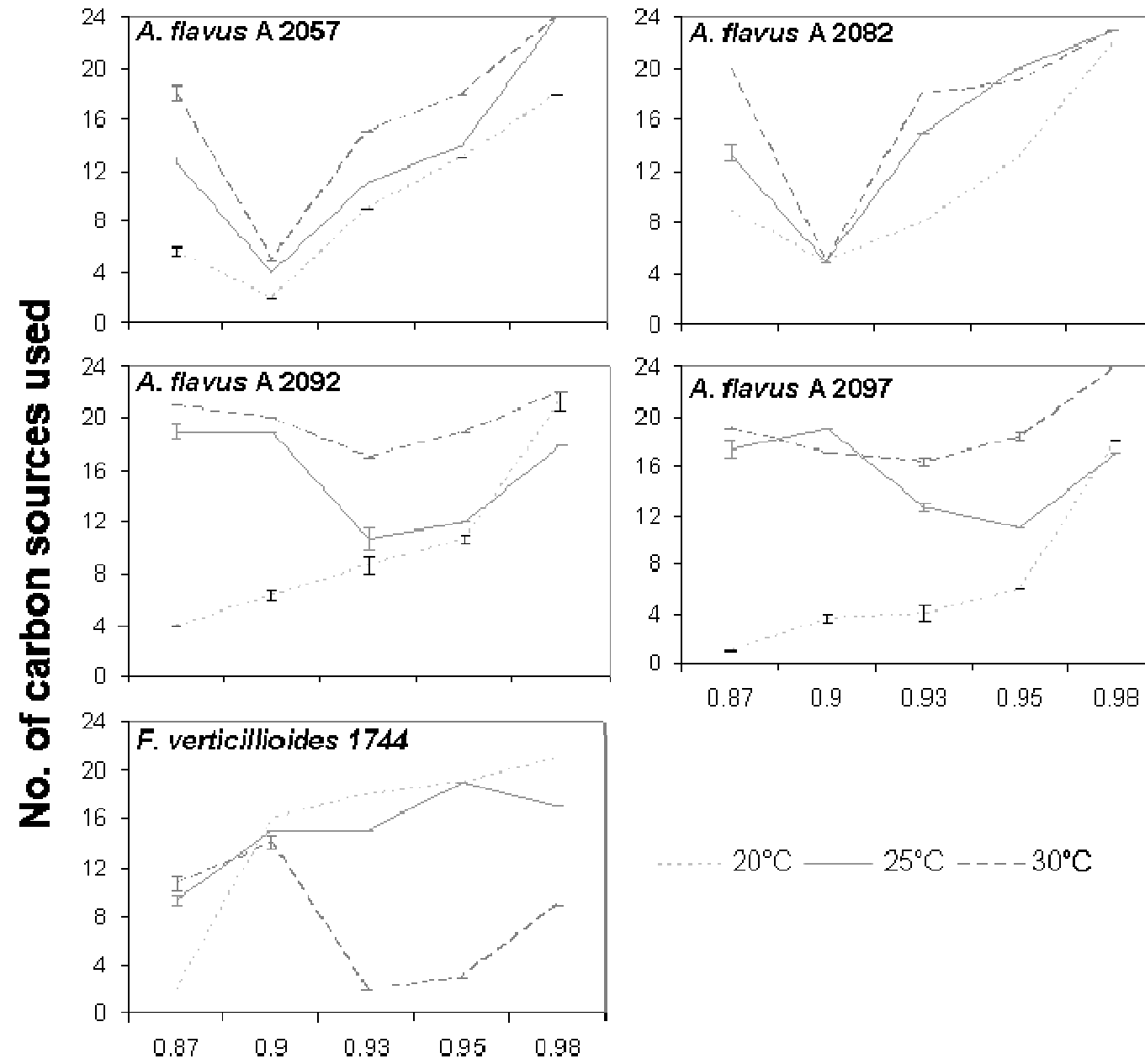
CARBON SOURCE	% compound (w/v) (equivalent to 9.1 mgC/mL)
<i>Aminoacids</i>	
L-Leucine	1.65
L-Alanine	2.25
D-Alanine	2.25
D-L-Threonine	2.25
L-Serine	2.68
D-Serine	2.68
L-Histidine	1.96
L-Proline	1.74
L-Phenylalanine	2
L-Aspartic acid	2
L-Glutamic acid	2
<i>Carbohydrates</i>	
D-Galactose	2.28
D-Raffinose	2.50
D-Glucose	2.28
D-Maltose	2.28
D-Fructose	2.28
Sucrose	2.16
D-Melibiose	2.28
Dextrin	2
Amylopectin	2
Amylose	2
<i>Fatty acids</i>	
Oleic acid	2
Linoleic acid	2
Palmitic acid	2

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Table 2 - Number of wells positive for fungal growth out of 360 prepared for each fungus (24 carbon sources, 5 available waters and 3 temperatures) after different hours of incubation (36, 60 and 120).

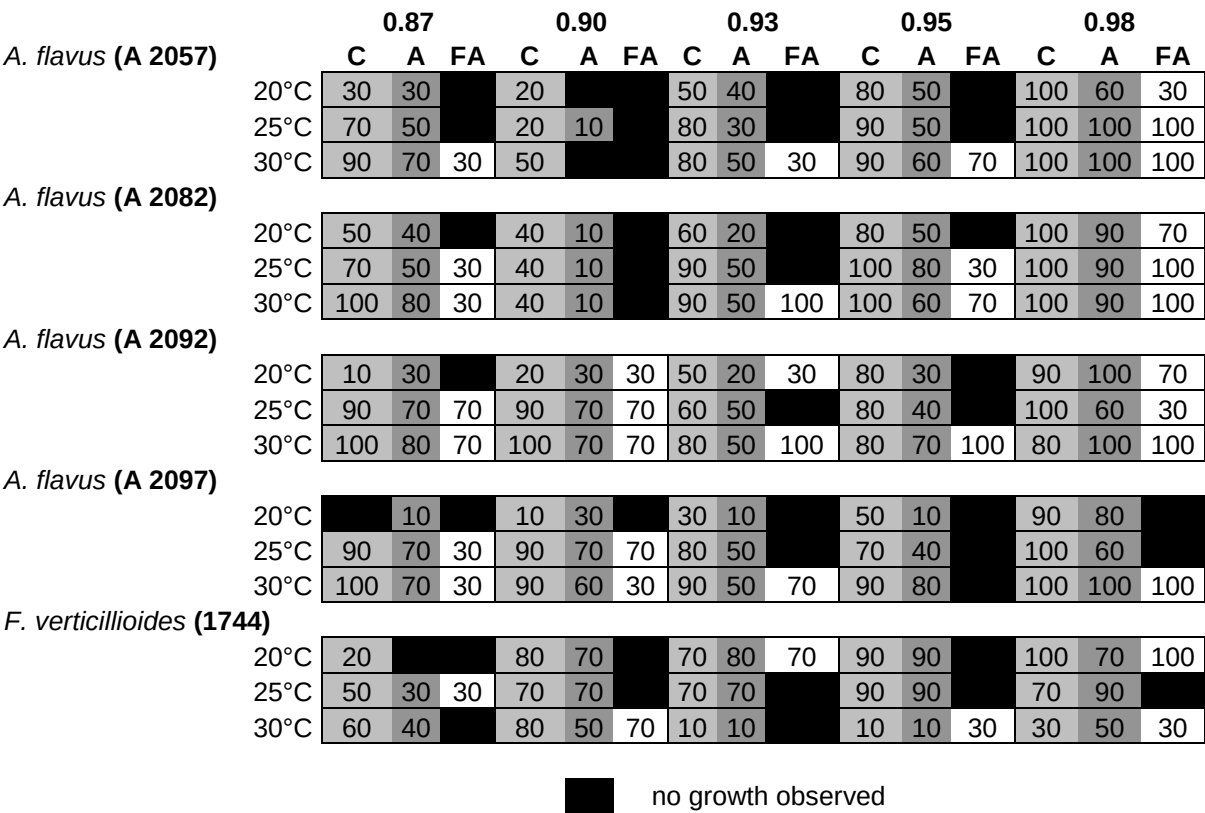
Strain	36 h	60 h	120 h
<i>A. flavus</i> A 2057	88	194	200
<i>A. flavus</i> A 2082	142	219	223
<i>A. flavus</i> A 2092	127	229	238
<i>A. flavus</i> A 2097	120	205	220
<i>F. verticillioides</i> 1744	137	190	203

Figure 1



454 Figure 2

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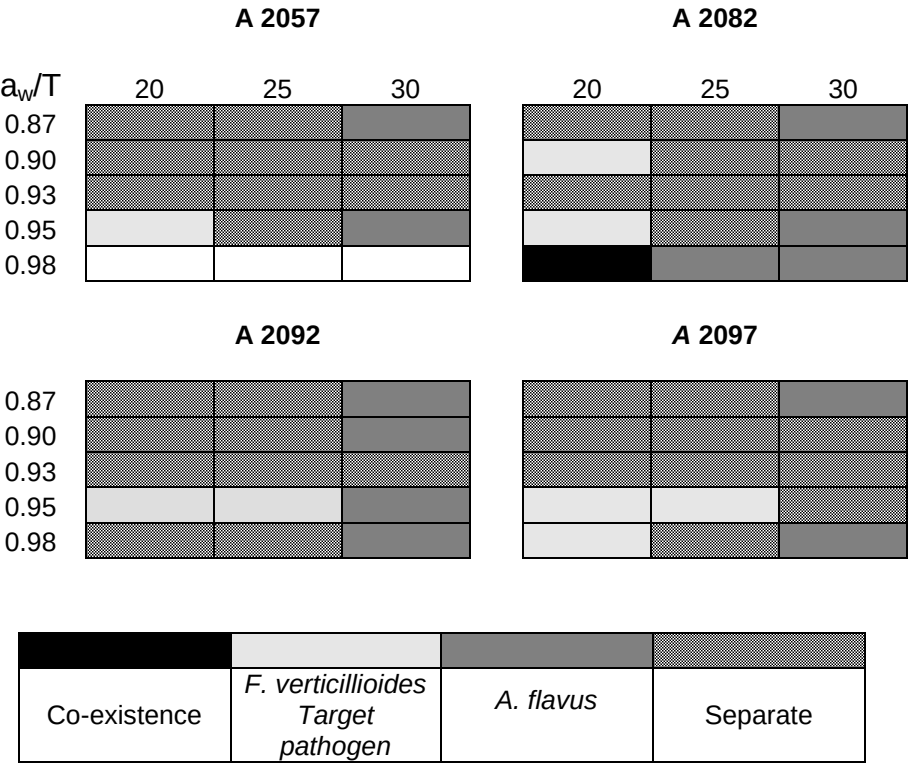


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458 Figure 3

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463 Figure 4

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A 2057 vs A 2082

	20	25	30
0.87			
0.90			
0.93			
0.95			
0.98			

A 2092 vs A 2082

	20	25	30
0.87			
0.90			
0.93			
0.95			
0.98			

A 2057 vs 2097

	20	25	30
0.87			
0.90			
0.93			
0.95			
0.98			

A 2092 vs A 2097

	20	25	30
0.87			
0.90			
0.93			
0.95			
0.98			

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