International Journal of Food Microbiology, Volume 130, Issue 3, 15 April 2009, Pages 213-218

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

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Keywords: Aspergillus flavus, *Fusarium verticillioides*, water activity, 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, 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 fumonsins (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 M_1 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 at 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 AFB_1/g of culture media) and low (0.3 ng AFB_1/g of culture media) AFB_1 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 $NaNO_3$ (0.23%), $MgSO_4 \cdot 7H_2O$ (0.06%), K_2HPO_4 (0.17%) and KH_2PO_4 (0.13%). Carbon sources were incorporated into the media at a final concentration of 9.1×10^{-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; $NaNO_3$ 2 g; KCl 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01; K_2HPO_4 1 g; $ZnSO_4 \cdot 7H_2O$ 0.001 g; $CuSO_4 \cdot 7H_2O$ 0.005 g; agar 15 g; H_2O to 1 L) was used for *A. flavus* and Potato Dextrose Agar (PDA; infusion from potatoes 200g; dextrose 15g; agar 20g; H_2O 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 10^6 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

189 of the marginal conditions for growth; at 30°C the number of CSs used by *F.*
190 *verticillioides* was significantly higher at 0.90 with respect to all the other a_w
191 levels tested.
192
193 *Aspergillus flavus* strains A 2057 (low AFs produced) and A 2082 (non-
194 producer) used fewer CSs at 0.90 a_w and 25 and 30°C than the other two *A.*
195 *flavus* strains, which grew with almost all the CSs tested. The number of CS
196 used at 0.90 was significantly lower with respect to 0.98 a_w at all temperatures,
197 with the exception of 30°C and 0.87 and 0.95 a_w. A 2092 and A 2097 showed
198 significant differences only at 20°C and 0.98 a_w, with respect to all the other a_w
199 treatments at each temperature.
200
201 All the strains preferentially utilised carbohydrates and amino acids and
202 subsequently, only at the higher temperatures and a_w levels, fatty acids (Figure
203 2). All the *A. flavus* strains preferentially used carbohydrates more than amino
204 acids, in all the treatment conditions, while *F. verticillioides* grew similarly with
205 both types of CSs. Regarding the two starch components, amylopectin was the
206 most used one by all the strains studied, especially at 25-30°C by *A. flavus* and
207 at 20-25°C by *F. verticillioides*. Differences were observed between *A. flavus*
208 strains regarding amylose and *F. verticillioides* grew only occasionally using
209 amylose as a CS (data not shown).
210
211 The NOIs for each strain of *A. flavus* with *F. verticillioides* are summarised in
212 Figure 3. *F. verticillioides* nutritionally dominated over all the strains of *A. flavus*

213 at 20°C and 0.95 a_w, over the low AFB_1 producer strain (A2057) and over the
214 high AFB_1 producer strain (A 2097) at 0.98 a_w and against the other two (A
215 2092, A 2097) at 25°C and 0.95 a_w. *Aspergillus flavus* always nutritionally
216 dominated *F. verticillioides* at 30°C and high and reduced a_w (0.98, 0.87 a_w).
217 The two AFB_1 producers dominated at 0.95 a_w, A 2092 also at 0.90 a_w and A
218 2082 also at 25°C and 0.98 a_w. In most conditions the fungi studied occupied
219 different niches. Co-existence between *A. flavus* with *F. verticillioides* was only
220 found for strain A 2082 at 20°C and 0.98 a_w (Figure 3).
221
222 The comparison between toxigenic and non-toxigenic strains of *A. flavus,*
223 regarding the usage of CS, showed that the non-producer strain A 2097 was
224 more competitive than A 2082 since it was able to use more CSs. In fact, A
225 2097 was dominant in many ecological conditions when compared with both the
226 toxigenic strains; it was efficient at 20°C and dominated at almost all the a_w
227 levels tested (Figure 4). Based on the nutritional utilisation patterns and the
228 NOIs there appeared that to be an interaction between the strains with the non-
229 toxigenic strains being dominant at 25-30 °C at high or low a_w.
230
231 **4. Discussion**
232 Temperatures between 20-30°C are typical for the maize growing season from
233 flowering to harvesting. The a_w between 0.98 and 0.87 is usually found in
234 kernels from early dough to full ripe stage (Battilani et al., 2007; Zorzete et al.,
235 2008). In almost all these conditions, both mycotoxigenic strains of *A. flavus*, A
236 2057 and A2092, were able to grow with at least a few CS.

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

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

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 patters 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

285 the other CSs. This is why they are utilized first. In contrast, other CSs may be
286 also utilized although at a lower rate and also resulting in a greater lag time
287 prior to growth. These CSs may perhaps be not utilised within the experimental
288 time frame.
289
290 The ability of the two species to assimilate different CS reflects their
291 competitiveness under specific ranges of environmental conditions. However,
292 only extreme conditions were linked to the nutritional dominance of one of the
293 two tested species while in almost all cases *A. flavus* and *F. verticillioides*
294 occupied different niches independently from the *A. flavus* strain used in this
295 study. This suggests that colonisation of maize by these fungi results in different
296 populations occupying different niches and could result in the presence of both
297 aflatoxins and fumonisins.
298
299 The intra-species variability, between toxigenic and non-toxigenic *A. flavus*
300 strains and CS utilization patterns, did not appear to be related to the actual
301 ability to produce AFs (data on AFs come from other studies); in fact, pairs of
302 the four strains studied showed a very similar behaviour. This type of
303 information can be useful in understanding the ecophysiology of *A. flavus* and
304 its relationship with *Fusarium* section *Liseola* species. It may also be useful in
305 screening competitive non-producer strains of either species for the
306 development of competitive exclusion approaches for developing natural control
307 systems. This can also include specific ranges of relevant environmental factors
308 and maize specific CS. It may also be possible to integrate this approach by

309 also incorporating potential for niche exclusion of mycotoxigenic species in the
310 presence of the crop protection chemicals often used, e.g. fungicides. Recent
311 work by Arroyo et al. (2008) has demonstrated the usefulness of understanding
312 these interactions between mycotoxigenic species and strains of *P. verrucosum*
313 (ochratoxin producer) and other species in the presence and absence of
314 different concentrations of preservatives such as propionate and sorbate. This
315 approach can be included to obtain a better understanding of the fluxes in niche
316 overlap and exclusion between these important mycotoxigenic species in
317 maize.
318
319 **Acknowledgements**
320 This work was supported by the Italian Ministry of Agricultural Policy (AFLARID
321 project).
322
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420

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)
Aminoacids	
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
Carbohydrates	
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
Fatty acids	
Oleic acid	2
Linoleic acid	2
Palmitic acid	2

444

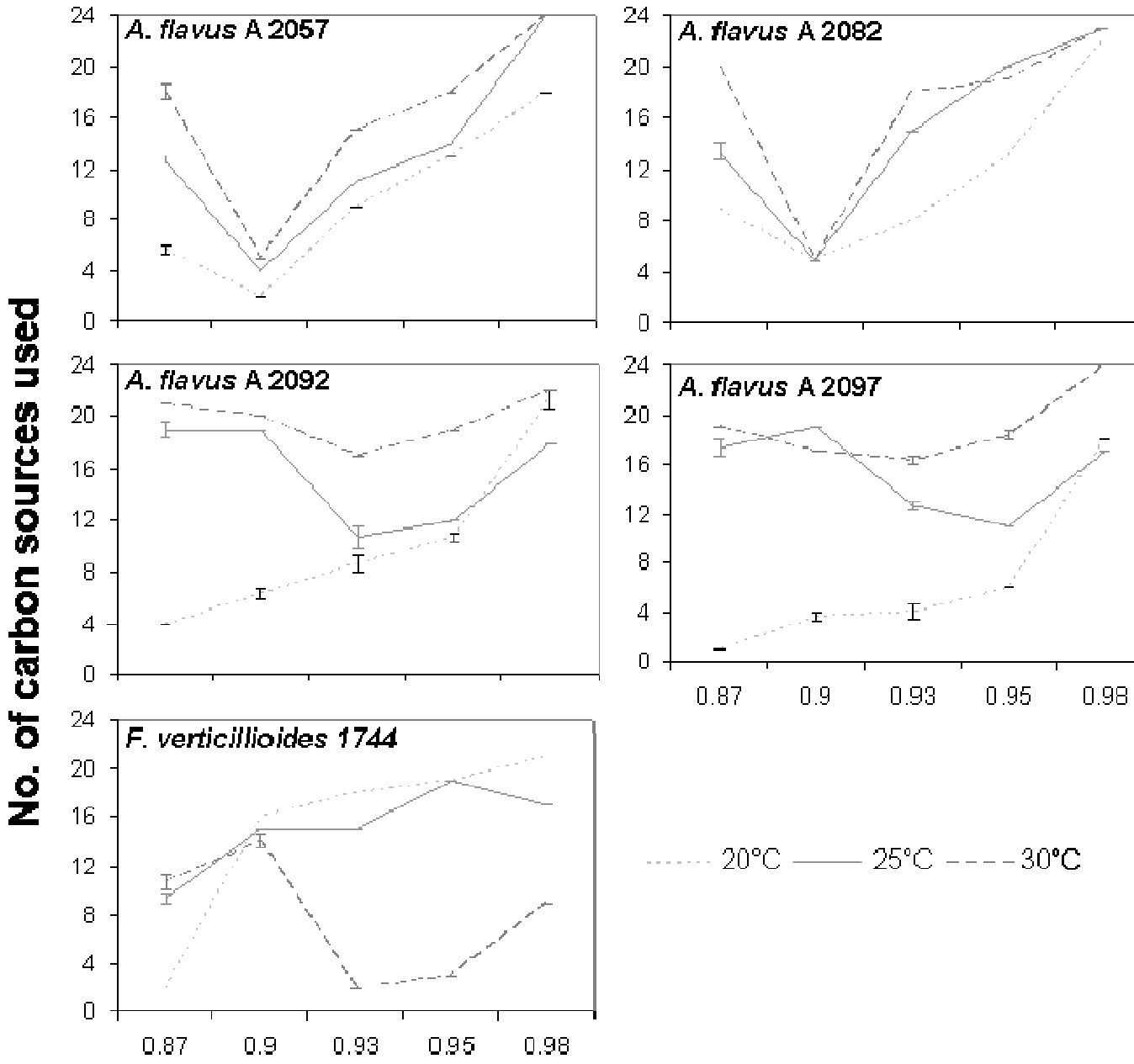
445 Table 2 - Number of wells positive for fungal growth out of 360 prepared for
446 each fungus (24 carbon sources, 5 available waters and 3 temperatures) after
447 different hours of incubation (36, 60 and 120).
448

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

449
450

451 Figure 1

452



453

454 Figure 2

455

		0.87			0.90			0.93			0.95			0.98		
A. flavus **(A 2057)**		**C**	**A**	**FA**	**C**	**A**	**FA**	**C**	**A**	**FA**	**C**	**A**	**FA**	**C**	**A**	**FA**
	20°C	30	30		20			50	40		80	50		100	60	30
	25°C	70	50		20	10		80	30		90	50		100	100	100
	30°C	90	70	30	50			80	50	30	90	60	70	100	100	100
A. flavus **(A 2082)**																
	20°C	50	40		40	10		60	20		80	50		100	90	70
	25°C	70	50	30	40	10		90	50		100	80	30	100	90	100
	30°C	100	80	30	40	10		90	50	100	100	60	70	100	90	100
A. flavus **(A 2092)**																
	20°C	10	30		20	30	30	50	20	30	80	30		90	100	70
	25°C	90	70	70	90	70	70	60	50		80	40		100	60	30
	30°C	100	80	70	100	70	70	80	50	100	80	70	100	80	100	100
A. flavus **(A 2097)**																
	20°C		10		10	30		30	10		50	10		90	80	
	25°C	90	70	30	90	70	70	80	50		70	40		100	60	
	30°C	100	70	30	90	60	30	90	50	70	90	80		100	100	100
F. verticillioides **(1744)**																
	20°C	20			80	70		70	80	70	90	90		100	70	100
	25°C	50	30	30	70	70		70	70		90	90		70	90	
	30°C	60	40		80	50	70	10	10		10	10	30	30	50	30

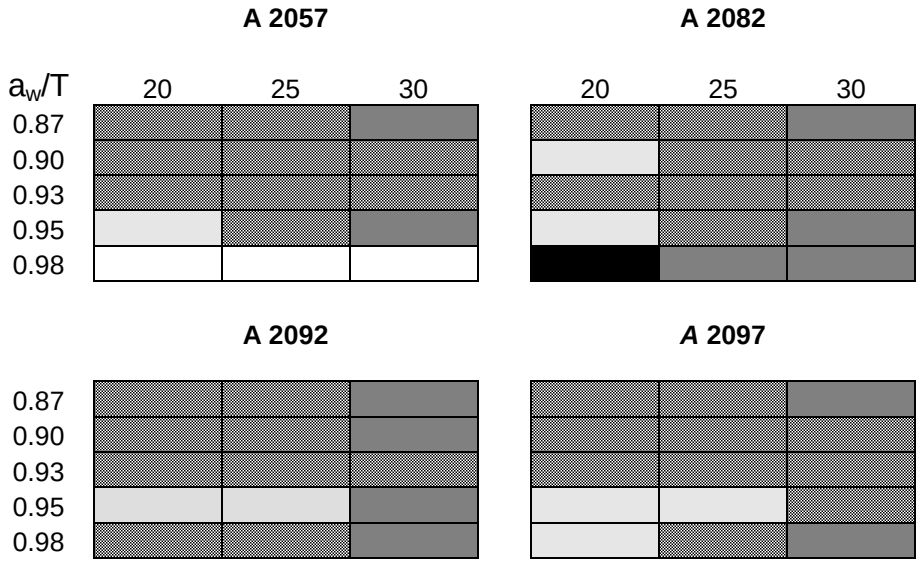
no growth observed

456

457

458 Figure 3

459



460

461

462

Figure 4

***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			

Environmental factors modify carbon nutritional patterns and niche overlap between Aspergillus flavus and Fusarium verticillioides strains from maize.

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2009-04-15

Paola Giorni, Naresh Magan, Paola Battilani, Environmental factors modify carbon nutritional patterns and niche overlap between Aspergillus flavus and Fusarium verticillioides strains from maize, International Journal of Food Microbiology, Volume 130, Issue 3, 15 April 2009, Pages 213-218
http://dx.doi.org/10.1016/j.ijfoodmicro.2009.01.032