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Effect of solute, matric potential and temperature on *in vitro* growth and sporulation of strains from a new population of *Aspergillus flavus* isolated in Italy

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ABSTRACT

The effect of temperature and different solute (Ψs) and matric potentials (Ψm) on growth and sporulation of three aflatoxigenic strains of *Aspergillus flavus* isolated from contaminated maize in northern Italy was determined. The Ψs of maize-based media were modified ionically (NaCl) and non-ionically (glycerol) and the Ψm with PEG 8000 in the range -1.4 to -21.0 MPa at 25 and 30°C. Both temperature and Ψs/Ψm stress had statistically significant effects on growth rates of the three strains. The higher fungal growth was registered at 30°C and -1.4 and -2.8 MPa. *A. flavus* strains were more sensitive to Ψm than Ψs stress with limits of -9.8 MPa and -14 to -18 MPa respectively. Sporulation was significantly influenced by Ψs potential, solute type and temperature. This suggests that these aflatoxigenic strains of *A. flavus* isolated from aflatoxin-contaminated maize are probably able to colonise rapidly crop debris at prevailing temperatures and water stress conditions. This type of information on the ecology of aflatoxin producing *A. flavus* strains isolated in Italy will contribute to the development of a systems model to predict their activity in crop residue and colonisation of maize grain.

Keywords: *Aspergillus flavus*; water potential; fungal growth; sporulation; temperature

1. INTRODUCTION

Aspergillus section *Flavi* is the major group of fungi associated with aflatoxin contamination in several agricultural commodities. Three species of this section are known to produce aflatoxins (AFs): *A. flavus*, *A. parasiticus* and *A. nomius*, with the first two important in the colonisation of crops such as maize, peanuts and nuts

(Payne, 1998). They are spread in hot and dry geographic areas where they are often able to colonise and contaminate crops rapidly.

In 2003, for the first time, high aflatoxin contamination, above the European legal limits both in maize kernels (20 μg kg^{-1} and 5 μg kg^{-1} for feed and food, respectively) and in milk (0.05 μg kg^{-1}), were found Italy and it was signalled as an emerging problem in Europe (Piva et al.,). This was due to the extremely hot and dry weather conditions registered in Italy during summer, very conducive for AFs contamination (Payne, 1998; Klich *et al.*, 1994).

A. flavus overwinters in soil on crop debris and this represents the main source of primary inoculum for maize plant. Conidia are dispersed aerially and deposited on corn ears enabling germination and infection to be initiated.. The maize growth stage most susceptible to infection is early browing (Payne, 1992), that happens in late June-early July in Italy, with high temperatures, frequently higher than 30°C. The two major factors that influence soil populations of this fungus are soil temperature and moisture (Payne, 1998). Tolerance of both Ψs and Ψm stress are important for survival and for growth to occur in crop debris and in soil (Magan, 1988). Solute stress is imposed by ionic changes due to salt, and non-ionically due to water binding by components on crop residue or plant parts. Matric stress is due to water adsorption and surface tension phenomena in soil; it causes restricted solute transport and it limits growth responses. Growth variations in solute or matric stress conditions can also be due to nutritional imbalances, specific ion effects or to the decreased water content that restrict solute transport (Adebayo & Harris, 1971).

A. flavus can grow between 12 and 48°C and at water potentials (Ψt) as low as – 35 MPa (Klich *et al.*, 1994). Available water in maize debris was reported as variable between 0.40 and 0.83 during summer in Italy (Rossi et al., 2008).

Interactions between Ψt stress and temperature are fundamental because they represent the two-dimensional niche in which fungi may be able to effectively germinate, grow and actively compete for the allocation of the available resources (Marin *et al.*, 1998).

Some studies have been conducted on the biology of *A. flavus* to determine favourable ecological parameters able to promote growth, especially in the USA (Kheiralla *et al.*, 1992; Truckless *et al.*, 1988). They showed that 25-30°C were optimal for growth of *A.* Section *flavi* strains (Giorni *et al.*, 2007; Kheiralla *et al.*, 1992; Nesci *et al.*, 2004; Sanchis & Magan, 2004). However, only one paper has compared the effect of Ψs and Ψm stress on growth of *A. flavus* strains (Nesci *et al.*, 2004) and none on sporulation.

Interestingly, it has been suggested by Calvo *et al.* (2002) that sporulation capacity and secondary metabolite production by *A. flavus* and *A. nidulans* are linked by the same induction pathways and influenced by environmental factors. They have provided information on this with regard to pH, temperature and carbon/nitrogen sources, but no studies have been conducted considering solute or matric stress.

The objective of this study was to obtain information on the capacity of three aflatoxigenic *A. flavus* strains collected in northern Italy, to grow and sporulate under different interacting Ψs/Ψm stress and temperature combinations.

2. MATERIALS AND METHODS

2.1 Fungal strains and media preparation

Three *A. flavus* strains (MPVP A 2052, A 2073 and A 2092) stored in the fungal collection of the Università Cattolica del Sacro Cuore, Institute of Entomology and Plant Pathology, isolated from maize grown in Italy, previously characterised as able to produce AFB_1 and AFB_2,, were used. They were selected to represent the whole population collected; they belong to the three clusters drawn with all strains characteristics (Giorni *et al.*, 2007).

The medium was a maize-based agar with 3% maize flour and 2% agar with Ψt of approx. -1.4 MPa (a_w=0.99) measured with a Hygroskop-BT (Rotronic Instrument Corp.).The Ψs was modified ionically with NaCl (Lang, 1967) and non-ionically with glycerol (Dallyn & Fox, 1980) to values reported in Table 1.

The Ψm was modified using Polyethylene glycol 8000 (PEG 8000). Known amounts of PEG 8000 were added according to the equation of Michel and Kaufmann (1973) as detailed by Magan (1988), to obtain target Ψm (Table 1). Sterile circular discs (ø 8.5 cm) of capillary matting were placed in sterile 9 cm Petri dishes containing approx. 15 mL of cooled medium. The matting was overlayed with sterile discs of polyester fibre and cellophane (P400, Cannings Ltd., Bristol, U.K.).

2.2 Fungal growth and sporulation

Spores of the 3 strains of *A. flavus*, obtained from a 7 day old Czapek dox agar culture, were suspended in 1% peptone-water, shaken vigorously and spread onto plates of the basic medium. Plates were incubated over night at 25°C to allow spore germination. The different Ψs and Ψm plates were inoculated centrally with an agar plug obtained using a 4 mm surface-sterilised cork borer. Four replicates were prepared of each treatment. Plates of the same Ψs/Ψm were sealed in polyethylene bags and incubated at 25 and 30°C (12 hours day light). These temperatures were selected because optimal for fungal growth and common during the crucial period for sporulation in field.

The diameter of all colonies was measured daily in two orthogonal directions until one strain covered the whole plate for a maximum of 14 days. The extension rate (mm day^{-1}) was computed for each treatment.

Data on sporulation were obtained in relation to Ψs stress only. Petri dishes were inoculated as previously described and incubated for 7 d; colonies were washed with 5 ml of sterile water added with 0.05% Tween 80 and the spore production determined with a haemocytometer as detailed by Parra *et al.* (2004). The experiment was carried out with three replicates per treatment.

2.3 Data analysis

Two dimensional profiles were drawn using Excel (Microsoft Office 2000) to show the effect of time and Ψs/Ψm on fungal extension. Radial extension in different treatments and for all the strains were rescaled in the range 0 - 1 considering 85 mm (diameter of Petri dishes used) as the maximum possible extension for the tested strains.

The analysis of variance (ANOVA) of all the collected data was carried out using the statistical package MSTAT-C (Michigan State University, ver. 1, 1991, East Lansing, MI, USA) and means were compared using the Tuckey test to determine their statistical significance. An experimental design with three factors (strain, temperature and water stress) in a randomised complete block was used to analyse extension data and logarithmically transformed sporulation data [ln (value+1)].

3. RESULTS AND DISCUSSION

3.1 Solute and matric stress effects on growth

The colony covered the whole plate in 7 and 13 days, respectively with Ψs and Ψm stress. ANOVA showed a significant effect ($P<0.01$) of all the main factors (strain, temperature and water stress type) on the colony extension (Table 2).

No growth was observed at -21.0 MPa and the extension rate at 25 and 30°C was similar under Ψs stress (< 7.0 MPa water stress) regardless of the solute used (Fig. 1).

In Ψm stress conditions, the extension rate was generally about 50% of that measured with Ψs stress (Fig. 1). The best temperature among those tested was 30°C, with no difference found between -1.4 and -2.8 MPa, while limits for growth were about -14.0 and 21.0 MPa, respectively for Ψm and Ψs stress.

Comparing the effect of Ψs and Ψm stress on mycelial extension, - 2.8 MPa was optimal under both imposed types of water stress and not significantly different from the unmodified media (-1.4 MPa). The Italian strains showed the ability to grow down to -14.0 MPa in ionically modified Ψs medium, while under Ψm stress this was limited to -9.8 MPa. The strains seemed to be more tolerant to both types of imposed water stress than those from Argentina previously examined. In fact, Nesci *et al.* (2004) reported no growth at Ψs and Ψm < -14.0 MPa stress.

Significant differences in tolerance of Ψs and Ψm stress indicate a higher sensitivity to this factor. This was also supported by the time required to reach the maximum extension. The lower tolerance to Ψm stress confirms the greater difficulty involved in extracting water from soil pores and the consequent limited solute transport (Adebayo & Harris, 1971); as a consequence, soil colonisation would probably occur over a narrower range of water availability when compared to that which occurs in maturing maize ears where Ψs stress is more important. This difference in sensitivity was previously observed for Argentinean strains of *A. flavus* (Payne, 1992) and also with other species such as *Alternaria alternata* and some basidiomycetes (Adebayo & Harris, 1971; Boddy, 1983; Whipps & Magan, 1987; Magan *et al.*, 1995). In contrast, limited differences were observed in tolerance to Ψs and Ψm stress for ochratoxigenic strains of *A. ochraceus* (Lee & Magan, 1999; Ramos *et al.*, 1999).

Two dimensional profiles were drawn based on Ψs or Ψm x time interactions (Fig. 2) and differences between optimum and marginal conditions were observed as well as lag phase necessary for fungal development.

At marginal incubation time (2 days) and Ψs (-21 MPa), no growth was observed in both modified media; with more water stress, growth was influenced by solute type, with an optimum at 5 days and -2.8 MPa with ionically modified media and 6 days and -1.4 to -2.8 MPa when glycerol was added (Fig. 2a).

Italian aflatoxigenic strains have an optimal extension rate profile similar to that found in the USA for isolates from groundnuts and maize. However in that study germination/growth has been reported till to -32.2 MPa, but only after more than 40 days incubation (Sanchis & Magan, 2004). Our interest was in how rapidly growth could occur and the Italian strains were unable to do so at -21 MPa after 7 days incubation.

3.2 Solute stress effects on sporulation

Strain, temperature and water stress all significally ($P<0.01$) affected *A. flavus* conidia production. Two strains were similar ($1.3 \cdot 10^5$ conidia cm^{-2} colony) while the other (A 2092) produced significantly more conidia ($3.7 \cdot 10^5$ conidia cm^{-2} colony). Furthermore, sporulation was significantly higher at 25°C than at 30°C ($3.4 \cdot 10^5$ versus $1.4 \cdot 10^5$).

The maximum number of spores was produced in unmodified medium ($4.8 \cdot 10^7$ conidia cm^{-2} colony).), followed by $1.8 \cdot 10^7$ at -2.8 MPa in Ψs and it decreased significantly at each Ψs variation with a complete inhibition with ionic solute at ≥ -14.0 MPa and to $4.7 \cdot 10^5$ conidia cm^{-2} colony at -21 MPa in glycerol modified medium..

Very few studies have tried to quantify the efficacy of changing Ψs stress on conidial production.

Gervais & Molin (2003) and Parra *et al* (2004) found differences between optimal water potential conditions for growth and sporulation for *Penicillium roqueforti* and *A. niger* respectively. The strains used for testing sporulation in this study were previously tested both for growth and AFB_1 production in different temperature and water availability conditions (Giorni *et al.*, 2007). The results suggested that differences of 5°C and -0.7 MPa from the optimal conditions (25°C; -1.4 MPa) can produce a 10-15% reduction in fungal extension and a significant reduction in AFB_1 production and sporulation (65-80% and 55% respectively) (data not shown). This could be explained by results reported by Brodhagen & Keller (2006) regarding the regulation of both sporulation and mycotoxin production in *A. flavus* by G protein signalling pathways. The relationship between mycotoxin production and sporulation were also found by Mostafa *et al.* (2005) who demonstrated that most of the toxins were produced after the fungus has completed its initial growth phase and began the development stage, represented by sporulation and sclerotia formation.

Data obtained in this study is critical in building up a picture of the key factors which influence growth and sporulation of strains of this important mycotoxigenic species from northern Italy and similarly to the approach followed for *Fusarium verticillioides* (Battilani *et al.,* 2003), they will contribute in the development of a predictive model.

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Table 1 Correspondence between water potential and aw of main treatments used in this study for water stress caused by addition of solute (Ψs) or by use of matric (Ψm).

Ψs	Ψm	a_w
-1.4	-1.4	0.99
-2.8	-2.8	0.98
-7.0	-7.0	0.95
	-9.8	0.93
-14.0		0.90
-21.0		0.85

Table 2 Results of ANOVA on mean radial growth rate (mm day^{-1}) of the 3 strains grown on maize flour agar at 25 and 30°C with different solute (salt or glycerol) and matric potential (polyethylene glycol 8000) modifications.

		Radial growth (mm day^{-1})	
Factors		**Solute stress** (1)	**Matric stress** (2)
Strain	A 2092	3.30 c	1.97 a
	A 2052	3.33 b	1.45 b
	A 2073	3.37 a	1.93 a
Temperature (°C)	25	3.43 a	1.59 b
	30	3.24 b	1.97 a
		Control	
Water potential (-MPa)	-1.4	5.74 ab	2.99 a
	Non-ionic solute (glycerol)		**Matric**
	2.8	5.76 ab	
	7.0	5.24 bc	0.00 c
	14.0*	1.02 d	1.04 b
	21.0	0.00 e	3.09 a
	Ionic solute (NaCl)		
	2.8	6.07 a	
	7.0	5.05 c	
	14.0	1.14 d	
	21.0	0.00 e	

(1) measured at 7 days of incubation
(2) measured at 13 days of incubation
*: -9.8 MPa instead of –14.0 MPa for matric potential treatment
Data with different letters indicates statistically significant difference ($P<0.01$).

Legends to figures

Fig. 1 Comparison of the effect of solute potential modified with NaCl and the non-ionic solute glycerol, and metrically with PEG 8000 on growth at 25 and 30°C after 7 days of incubation. Values refer to the mean extension rate of the 3 strains used for the experiment.

Fig. 2 Comparison of two dimensional profiles of mean extension of three *A. flavus* strains on media (a) modified with ionic and non-ionic solutes (NaCl, glycerol) in relation to time and solute potential and (b) in relation to matric potential stress (modified with PEG 8000) at both 25 and 30°C. Different shading represents different growth rates.

Figure 1. Giorni *et al.*

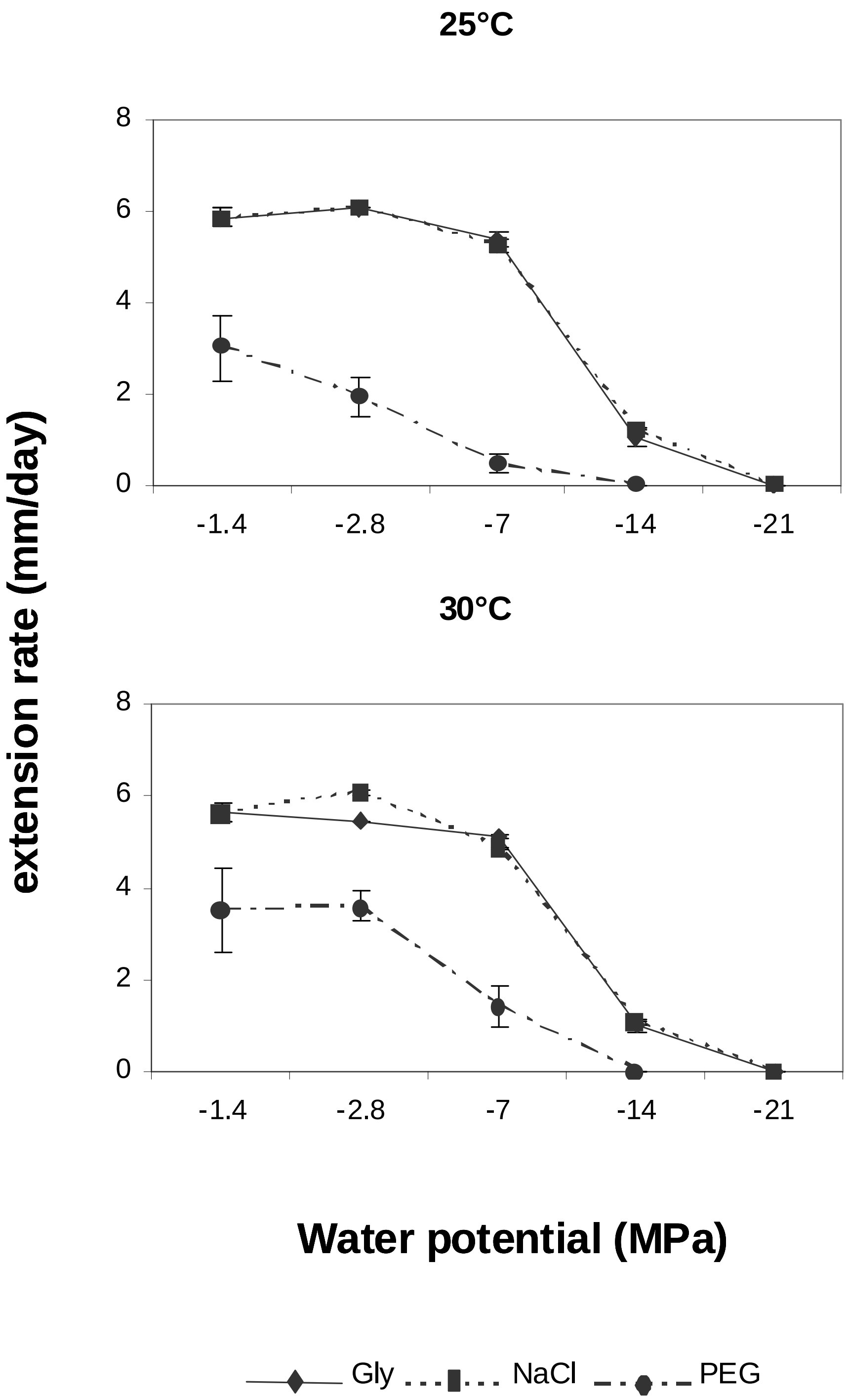

Figure 2. Giorni *et al.*