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ORIGINAL ARTICLE

The *in vitro* effect of gossypol and its interaction with salts on conidial germination and viability of *Fusarium oxysporum* sp. *vasinfectum* isolates

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Keywords
cotton, *Fusarium oxysporum* f. sp. *vasinfectum,* gossypol, multinomial logistic model, probability of germination, salinity, toxicity.

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Abstract

Aims: To assess the effect of different concentrations of gossypol (0, 2, 4, 10 and 20 mg l^{-1}) in combination with NaCl and Na_2SO_4 (20 mS cm^{-1}) on the conidial germination and viability of *Fusarium oxysporum* f.sp. *vasinfectum* (*Fov*).

Methods and Results: A multinomial logistic model was developed to estimate the germination probability of *Fov*. The inhibitory effect was markedly evident at the two highest concentrations of gossypol; it varied among the isolates tested and with time, and it was attenuated by the presence of sodium salts. The inhibition was temporary as the germination probability increased after 8 h. Fluorescent staining revealed that gossypol either killed the conidia or retarded the elongation of the germ tubes.

Conclusion: *Fov* showed the ability to overcome gossypol inhibition over time, and the inhibitory effect is reduced under saline conditions. Differential responses among *Fov* isolates to the presence of gossypol suggest that gossypol tolerance is genetically determined in the pathogen.

Significance and Impact of the Study: This study suggests that selecting for high plant gossypol cultivars would have minimal effect on the overall *Fov* resistance of cotton. A new statistical model was developed to explore the statistical significance of plant–pathogen interactions.

Introduction

Fusarium oxysporum f.sp. v*asinfectum* (*Fov*) causes severe crop losses in all the cotton-producing areas of the world, including Australia. As a soil inhabitant, it survives for long periods as chlamydospores. Once they germinate, the pathogen infects whole or wounded roots (Rodriguez-Gálvez and Mendgen 1995). The mycelium invading the root tissues eventually reaches the xylem vessels causing vascular occlusion and disruption of water transport by the production and release of conidia carried out in the sap stream and concurrent deposition of secondary compounds by the plant. The characteristic wilt symptoms are chlorosis and necrosis of the leaves accompanied by vascular browning of xylem. The end result is plant death or a physiologically compromised plant that will produce less lint than a healthy plant and that lint will be of inferior quality. The occurrence and severity of cotton wilt outbreaks is a function of the density of the pathogen in the soil, the prevailing environmental conditions and the genetic resistance of the plants (DeVay *et al.* 1997; McFadden *et al.* 2004). With the exception of Australia, the severity of *Fusarium* wilt outbreaks is exacerbated by nematodes (De Vay *et al.* 1997).

In contrast to the biochemical mechanisms employed by *Fov* during infection (Lockwood 1960; Suresh *et al.* 1984; Turco *et al.* 1999), the most visible means of defence deployed by the cotton plant is the production of tannins and phytoalexins into the vascular tissues resulting in the vascular occlusion and visible 'browning'

characteristic of *Fusarium* wilt infections (Bugbee 1970; Kaufman *et al.* 1981; Harrison and Beckman 1982; Mace *et al.* 1985). In fact, the level of chemical compound deposition and the vessel wall thickening correlates strongly with susceptibility (Shi *et al.* 1992). Under identical pathogen pressures, more tolerant varieties will present with less vascular occlusion and browning than susceptible varieties, so while it is clear that these mechanisms are an important component of the defence system in cotton, excessive vascular browning is evidence that the defence system, as a whole, has been fundamentally compromised. Nonetheless, the established toxicity of the phytoalexin compounds deposited in cotton vascular systems in response to *Fov* invasion suggests that selection for new phytoalexin chemotypes in cotton could lead to an improvement in *Fov* resistance (Shi *et al.* 1992).

In cotton plants, the phytoalexin compounds are terpenoid aldehydes produced by the xylem parenchyma near the infected vessels (Bell *et al.* 1975; Kaufman *et al.* 1981; Harrison and Beckman 1982). The primary terpenoid aldehyde, gossypol, is a dimeric or *bis*-naphthalene secondary metabolite constitutively sequestered in 'pigment glands' (lysigenous cavities) found in almost all plant tissues except the vascular tissues.

The apparent involvement of terpenoid aldehydes in the cotton resistance to *Fusarium* wilt pathogens was observed in bioassay studies that demonstrated that synthesis of gossypol and related compounds increase in cotton plants soon after infection, with the highest rate of accumulation in either the resistant cultivars or in the older tissues (Bell 1967; Kaufman *et al.* 1981). The role of gossypol and two key pathway intermediates, desoxyhemigossypol (dHG) and hemigossypol (HG), in the plant defence was explored in greater detail in *Fov*-infected cotton plants (Zhang *et al.* 1993; Dowd *et al.* 2004) and in other cotton–pathogen interactions (Howell *et al.* 2000; Townsend *et al.* 2006). These studies suggest that higher constitutive levels of terpenoid aldehydes or more rapid induction of terpenoid aldehydes could reduce the deposition of other occluding compounds and thus increase the tolerance of cotton to *Fov* invasion.

Recent experimental work with synthetic cotton hexaploid with low-gossypol seeds and high-gossypol plants (Vroh *et al.* 1999) highlight the fact that the exact role of terpenoid aldehydes as phytoalexins in the cotton constitutive defence system is still not fully understood. Extracts of xylem tissue containing terpenoid aldehydes are demonstrably toxic to *Fov* and to other cotton pathogens (Bell 1967; Kaufman *et al.* 1981; Zhang *et al.* 1993; Puckhaber *et al.* 2002), but the *in vitro* toxicity of terpenoid aldehydes on *Fov* has not been studied.

In light of the recent elucidation of the phytoalexin detoxification pathways in fungi, including *formae speciales* of *F. oxysporum* (Curir *et al.* 2000; Pedras and Ahiahonu 2005), an assessment of the mode of action of terpenoid aldehydes is needed in order to evaluate the role of terpenoid aldehydes in the cotton–*Fov* interaction. Here we assess the *in vitro* effect of the primary terpenoid aldehyde, gossypol, on the conidial germination of several *Fov* isolates. Previous assays established that the presence of gossypol in the medium did not alter mycelial growth rates *in vitro* (Turco, unpublished data) suggesting that toxic effect of terpenoid aldehydes may be limited to the conidial germination. It is also clear that salinity can exacerbate wilt disease in cotton (Ragazzi *et al.* 1994; Turco *et al.* 2002), so we also sought to ascertain the interaction between NaCl and Na_2SO_4 levels and the effect of gossypol on conidial germination. A multinomial logistic model (McCullagh and Nelder 1989) was developed to describe the categorical response 'germination time' as a function of gossypol and salt concentrations in the medium. The full factorial design used permitted the simultaneous study of the experimental factors (NaCl, Na_2SO_4 and gossypol levels) and their interactions. Using generalized linear model we were able to model the probability of the response instead of transformed counts.

Materials and methods

Fungal strains and culture conditions

Five strains of *Fov* present in the collection of Dipartimento di Biotecnologie Agrarie – Sezione di Patologia vegetale, Università di Firenze, were chosen for the experiments: strains 16421, 36198, 116616 later labelled as 141146, strain Cuanza and strain Chinese (Table 1). The monoconidial cultures were maintained on one-fourth strength of potato dextrose agar (PDA; Sigma-Aldrich Co., St. Louis, MO, USA) and then transferred on full-strength PDA medium to obtain the mother colony and the conidial suspensions for further tests.

Table 1 *Fusarium oxysporum* f.sp. *vasinfectum* isolates used in this study

Isolates	Virulence*	Source	Origin
16421	+++	ATCC®, USA	South Carolina (USA)
36198	++	ATCC®, USA	Brasil
141146	+++	CBS, The Netherlands (original number 116616)	Ivory Coast
Cuanza	+	A. Ragazzi	Angola
Chinese	+	A. Ragazzi	China

*The level of virulence from high (+++) to low (+) is indicated (Turco, personal communication, unpublished data).

Preparation of media enriched in gossypol

To obtain a stock solution, gossypol (95% pure; Sigma-Aldrich Co.) was dissolved in 95% ethanol. The suspension was filter-sterilized through a 0·2-μm Sarstedt® filter and stored at +4°C in the dark. After cooling to 45°C in a water bath, the ethanol solution was added to sterilized PDA medium to obtain a final gossypol concentration of 0, 2, 4, 10 and 20 mg l^{-1}. To evaluate conidial germination, one 2-ml aliquot of the treated medium was poured into 60-mm petri dishes. All dishes were allowed to dry under laminar flow hood and were stored in the dark at +4°C until used. To evaluate the indirect effect of the solvent on the target parameters, PDA medium treated with 95% ethanol solution (2 μl ml^{-1}) was included in the test. PDA dishes without gossypol were also prepared as control.

Effect of gossypol on conidial germination

To test the toxicity of gossypol on conidial germination, one 100-μl aliquot of a *Fov* conidial suspension (1×10^4 conidia ml^{-1} of sterile distilled water) was pipetted onto each dish and spread uniformly on media surface using a sterilized glass flat-needle. The suspension was allowed to dry in the laminar flow hood for 30 min in the dark, and the dishes were sealed with parafilm and were incubated at 25°C ± 0·2 in the dark.

Conidia viability

The viability of conidia was further evaluated by induced fluorochromasia. A 100-μl aliquot of conidial suspension (1×10^4 conidia ml^{-1}) was added to 900 μl of potato dextrose broth (PDB) enriched with 20 mg l^{-1} of gossypol and without gossypol. After 8 h, the conidial suspensions were treated with fluorescein diacetate (FDA) solution for 10–60 min (Kasten 1981) and observed under a fluorescent light using a Leitz Laborlux S microscope. Only viable conidia fluoresce, while the nonviable ones fail to fluoresce because of loss of integrity of their cell membranes.

Interaction between salinity and gossypol on conidial germination

Following the protocols described in Ragazzi *et al.* (1994) and Turco *et al.* (1999), 12 g l^{-1} of NaCl (205 mmol l^{-1}) or 17 g l^{-1} of Na_2SO_4 (120 mmol l^{-1}) were added to the PDA medium to a final EC (electrical conductivity) of 20 mS cm^{-1}. The media was then sterilized at 120°C for 15 min. After cooling, the gossypol solution was added to the experimental plates. Two sets of control plates (with only ethanol and with no additive) were prepared at the same time.

Experimental design

Conidial germination was assessed four times at 2-h intervals using a Zeiss Axioskop microscope (40× magnification). Four random fields of view were observed for each replicate (100 conidia in total) of each experimental treatment (*Fov* isolate × gossypol level × salinity level). Each experimental treatment combination was replicated twice. The conidia were considered germinated if the germ tube was greater than half the conidial diameter. The entire experiment was conducted twice.

Statistical analysis

The design of the experiment followed a full factorial design with three factors and time (summarized in Table 2). For the purpose of the analysis, the time axis was partitioned into five time intervals (0, 2, 4, 6, 8 and after 8 h) so that each statistical unit belongs to a germination time window.

A multinomial logistic model was developed to study the conditional probability of germination as a function of the treatment combinations. The log-odds of the probability of germination in time window j against time window 1 given treatment variables are:

$$\log\left(\frac{p_j}{p_1}\right) = x^T\beta$$

where $x^T$is a row vector of covariates and beta is the vector of parameters for the linear predictor (McCullagh and

Table 2 Statistical tests for the selected model

Source of variation	*df*	Deviance	Residual *df*	Residual deviance	*P*(>|Chi|)
Isolate : time	16	241	1875	8267	3·173e-42
Medium : time	8	207	1867	8061	2·643e-40
Gossypol : time	16	3377	1851	4684	0
Isolate : medium : time	32	461	1878	4223	1·55e-77
Isolate : gossypol : time	64	269	1723	3953	5·228e-27
Medium : gossypol : time	32	71	1691	3882	8·7913–05
Isolate : medium : gossypol : time	128	297	1563	3585	1·995e-15

Nelder 1989). Vector elements $(p_1, p_2, p_3, p_4, 1 - p_1 - p_2 - p_3 - p_4)$ are parameters of the multinomial likelihood function for the number of germinated conidia in time interval *j*. Model selection was performed using the Akaike Information Criterion (AIC) and components of the optimized model were tested for inclusion in the final model through likelihood ratios. Model fitting was performed in R (R Development Core Team 2005). Estimated treatment probabilities were calculated from point estimates of betas and plotted according to several factors combinations.

Graphs were produced using Systat v. 10 (SPSS Inc., Richmond, CA, USA) along with Power Point (Microsoft Office XP).

Results

Effect of gossypol on conidial germination on nonsalt-amended PDA

Germination of the *Fov* conidia was clearly influenced by the presence of gossypol in the agar medium. The germination probability (GP) was negatively associated with increasing gossypol concentration.

As expected the highest GP was observed on untreated agar medium (control, 0 mg l^{-1}), followed by an appreciable decline at the first gossypol level tested (2 mg l^{-1}). The GP decreased with increasing concentration of gossypol and the minimum value was evident at 20 mg l^{-1} (the fourth level tested) for all time windows (Fig. 1). Exceptions were observed at 2 h, at which time the GP difference between the control and the 2 mg l^{-1} was highly significant but no variation on the GP was detected between the 2 and the 4 mg l^{-1} concentration.

Figure 1 also shows that the conidia of all *Fov* isolates had high probability of germinating within 2 h; the fewest conidia germinated in the 4-h time window, increasing again to appreciable values although lower than observed during the first two hours. The only exception to this pattern was a few gossypol × time interactions. In particular, the highest GP values were observed at 6 h for the control (0 mg l^{-1}) and for 2 mg l^{-1} of gossypol. Unexpectedly at 8 h, there was a slight increase in the GP at the highest concentration of gossypol. The statistical analysis showed that variation in the GP among *Fov* isolates was more strongly correlated with time than with gossypol concentration in the media.

Differences in the GP were observed among the isolates. *Fov* 141146 had the highest GP value for the experimental control – 2 h (0·14) treatment and the lowest value for the 4 h – 20 mg l^{-1} treatment (0·03) (Fig. 2a). A peak in the GP was observed at 6 h for all the gossypol

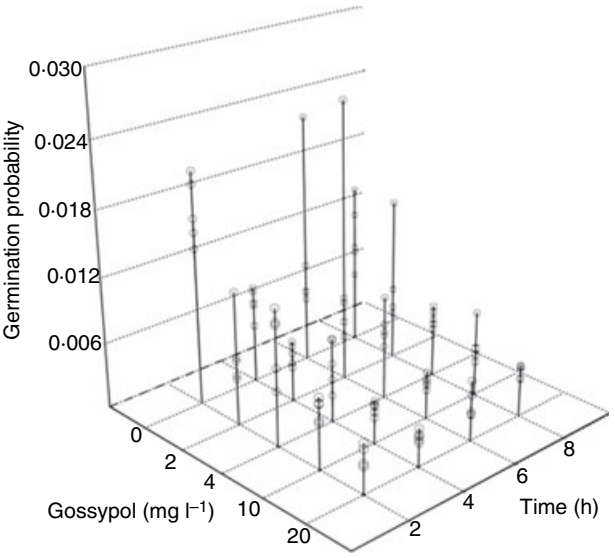

Figure 1 Probability of germination of *Fusarium oxysporum* f.sp. *vasinfectum* (*Fov*) isolates, on different times, on nonsalt-amended potato dextrose agar (PDA) enriched with increasing concentrations of gossypol. Each vertical segment shows five points, one for each isolate.

concentrations tested except the 10 mg l^{-1}, and in contrast to the common decline in the GP at 4 h, a modest increase in the GP was observed at 4 mg l^{-1} gossypol. At 8 h, no differences on GP were observed among control and the two lowest gossypol concentrations.

The Cuanza *Fov* isolate responded to the treatments differently (Fig. 2b). A negative and linear correlation between gossypol concentration and the GP was observed, with the lowest values at 20 mg l^{-1} and 6 h. Between 4 and 6 h, a decline in the GP was still observed for all treatments. At 8 h the GP increased. The highest value was observed in the control; the GP values were lower for all the gossypol treatments. At 2 mg l^{-1} gossypol the GP was 50% of the control value. The GP increased to 0·08 at 10 g l^{-1} before dropping to 0·04 at 20 mg l^{-1}.

Fov isolate 16421 responded to the treatments in yet another fashion (Fig. 2c). GP was highest after 2 h on untreated medium, declined by at least 75% at the 2 mg l^{-1} gossypol (0·06), rose to 0·11 for the next concentration and then continuously declined with increasing concentrations of gossypol. A linear decrease in the GP from 2 to 6 h was observed, except for the highest gossypol concentration (20 mg l^{-1}) where the GP changed minimally across all the time points. There was aberrant peak at 2 mg l^{-1} gossypol at 8 h (Fig. 2c).

Comparisons to the 95% ethanol controls indicated that the ethanol had no effect on *Fov* conidial germination probabilities.

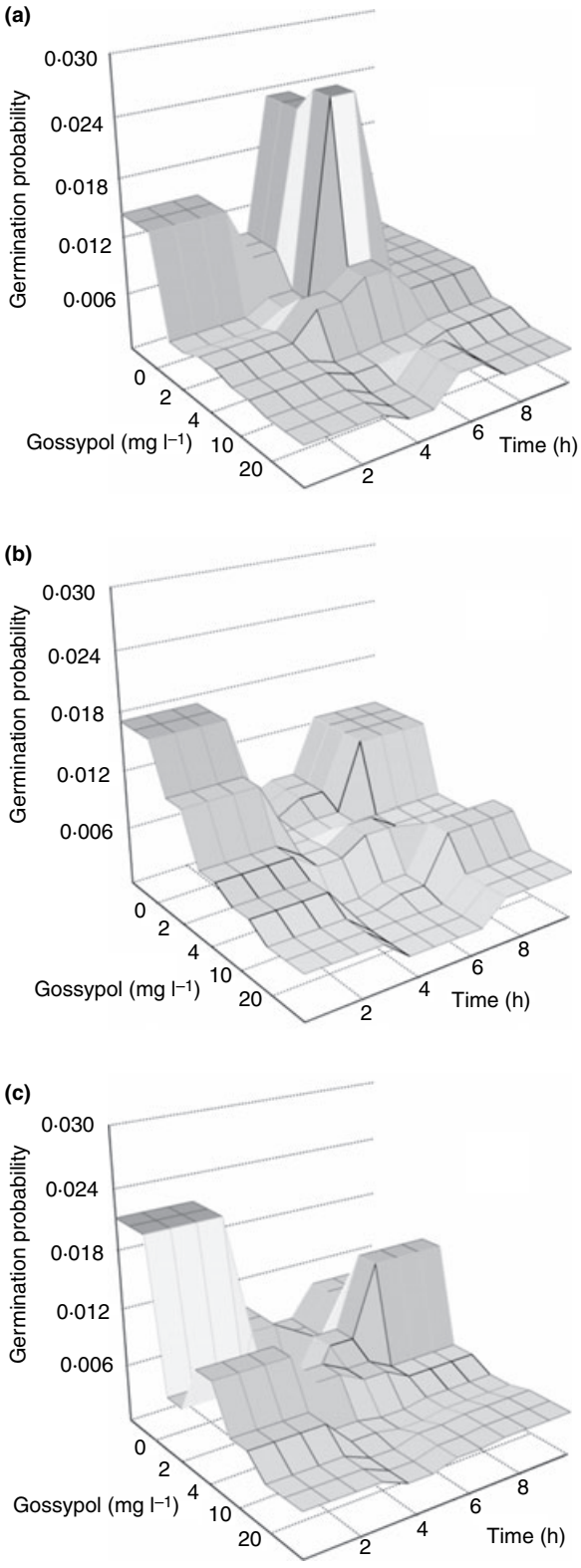

Figure 2 Probability of germination, on different times, of isolates 141146 (a), Cuanza (b) and 16421 (c) on nonsalt-amended potato dextrose agar (PDA) enriched with increasing concentrations of gossypol.

Effect of gossypol on conidial germination on salt-amended PDA

The *Fov* conidial germination percentages changed when NaCl or Na_2SO_4 were added to the agar medium and the effect was a function of the gossypol concentration–time interactions.

The GP on NaCl-enriched medium was the highest for the control at 2 h and rapidly declined with increasing concentrations of gossypol (Fig. 3a). As was observed before, the GP fell at the 4- and 6-h time points, increasing again at 8 h. At all time points, the decrease on the GP was negatively correlated with the gossypol concentration; however, an unexpected peak was observed for both the 10 and 20 mg l^{-1} gossypol treatments at 8 h. When the media composition was modified by adding Na_2SO_4, conidial germination probabilities were higher overall. The trend was stronger among the control treatments than in the gossypol-amended treatments. The Na_2SO_4 GPs were more similar to those observed on NaCl-enriched medium than to the nonsalt-amended treatments, with the exception of isolate 16421 at 2 mg l^{-1} gossypol × 8 h. No significant differences were observed between 10 and 20 mg l^{-1} gossypol at 8 h and at 2 and 6 h (Fig. 3b).

The *Fov* isolates reacted differently to the two different salts. As an example, Fig. 4 shows the GPs of isolate 16421 in response to NaCl and to Na_2SO_4. On NaCl-enriched medium (Fig. 4a), the GP rapidly declined at 2 h from the untreated to the first gossypol concentration (2 mg l^{-1}), remaining unchanged until 4 mg l^{-1}, and then declining again to 0·03. In contrast, a decrease was observed between 4 and 6 h for all the gossypol concentrations tested; the lowest value (0·03) was observed at 4 h – 20 mg l^{-1}. The probability increased again to appreciable values, except the control (0·06), at 8 h. As Fig. 4b shows, a stepwise decrease was evident on Na_2SO_4-enriched medium with increasing concentrations of gossypol with deviations at 2 mg l^{-1} gossypol × 2 h (50% of the control value) and 2 mg l^{-1} × 8 h. The lowest value (0·02) observed occurred in the 20 mg l^{-1} gossypol treatment.

The isolate Cuanza's response on NaCl-enriched medium was less linear than observed before (Fig. 5a). This was most evident at 8 h, at which time there were no differences between the control and the 20 mg l^{-1} gossypol treatments, but there were clear reductions in GP for all the intervening gossypol concentrations. The relation between GP and gossypol concentration on Na_2SO_4-amended medium was more linear (Fig. 5b). No GP decline was observed for the 2 mg l^{-1} concentration at 4 h but it increased 2 h later (6 h) reaching a value of 0·16 but then fell to 0·03 at 8 h, lower than the value

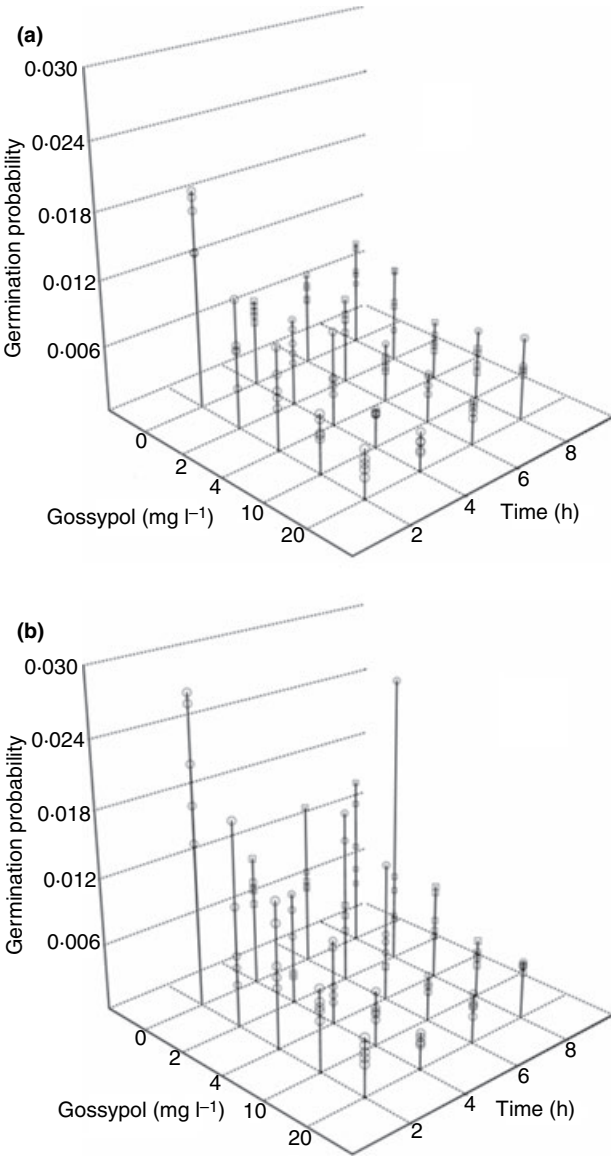

Figure 3 Probability of germination, on different times, of *Fusarium oxysporum* f.sp. *vasinfectum* (*Fov*) isolates on agar media enriched with 12 g l^{-1} (205 mmol l^{-1}) of NaCl (a) or 17 g l^{-1} (120 mmol l^{-1}) of Na_2SO_4 (b) at increasing concentrations of gossypol. Each vertical segment shows five points, one for each isolate.

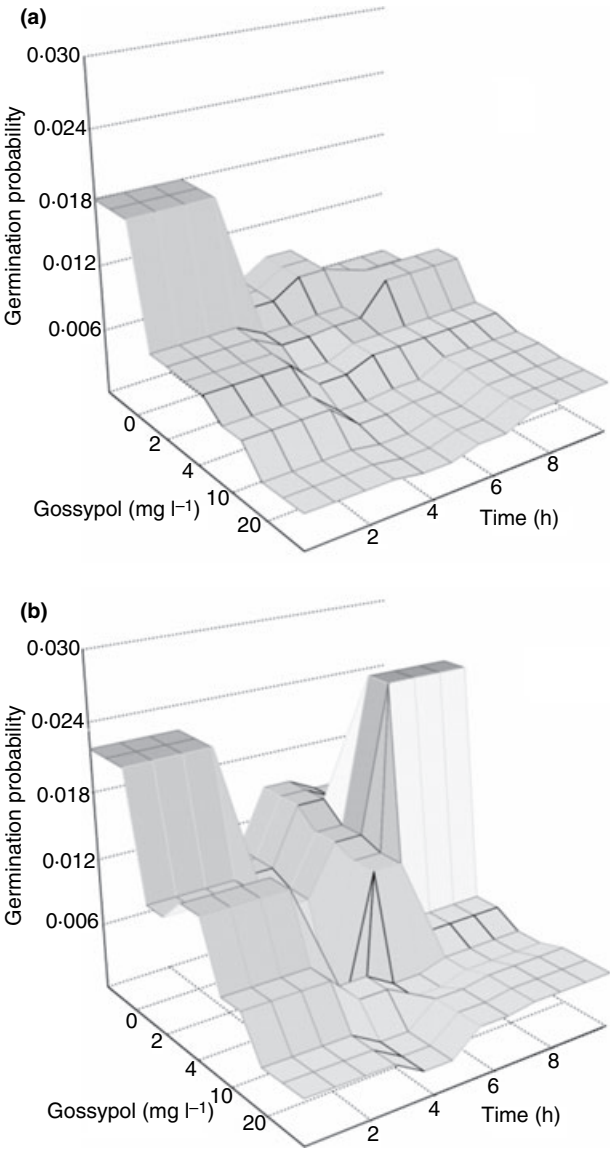

Figure 4 Probability of germination, on different times, of *Fusarium oxysporum* f.sp. *vasinfectum* (*Fov*) isolate 16421 on NaCl- (a) or Na_2SO_4- (b) enriched media supplemented with increasing concentrations of gossypol.

observed for the control but almost equal among the four concentrations tested.

Germination time variation

Conidia that did not germinate by the 8-h time point were expected to germinate soon thereafter. Nevertheless, we cannot exclude that after the end of the observation time (8 h) some conidia did not germinate, thus in the last time windows, the score is because of both late and inviable conidia. Under the assumption that the number of conidia unable to germinate is very low after 8 h, it is not necessary to correct the GP values for nonviable conidia. In Fig. 6, the probability of germination after the 8-h time point is estimated for each media type and gossypol concentration. Five points are shown for each treatment combination and they refer to *Fov* isolates.

First of all, the GP at this time point does not seem to rely on the composition of nutritive media (PDA, NaCl-enriched PDA, Na_2SO_4-enriched PDA) as no significant

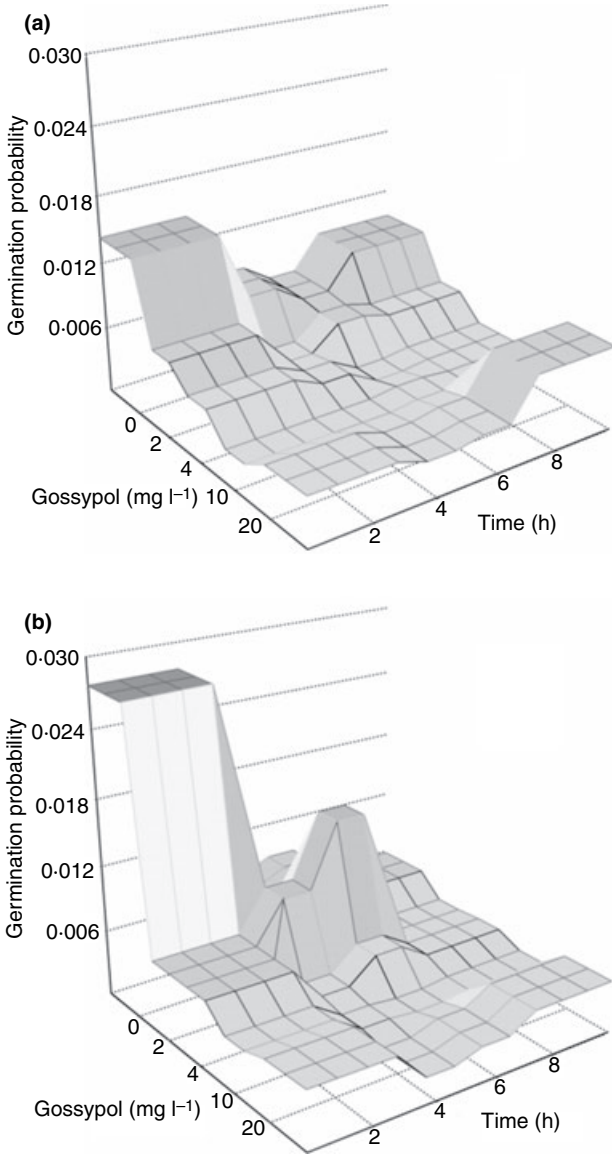

Figure 5 Probability of conidial germination, on different times, of *Fusarium oxysporum* f.sp. *vasinfectum* (*Fov*) isolate Cuanza on NaCl (a) or Na_2SO_4 (b) enriched media supplemented with increasing concentrations of gossypol.

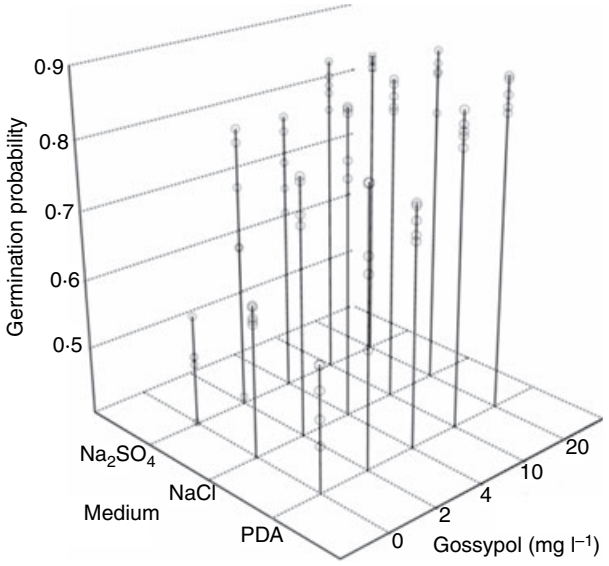

Figure 6 Probability of germination after the end of the experiment (8 h) to infinity of *Fusarium oxysporum* f.sp. *vasinfectum* (*Fov*) isolates on different agar media supplemented with increasing concentrations of gossypol. Each vertical segment shows five points, one for each isolate.

difference was observed among them for any of the concentration of gossypol tested, but what is evident is that the presence of gossypol delays the germination of conidia. This is evident in the increasing numbers of conidia germination after 8 h as the gossypol concentration increases. The GP increased in a relevant way from untreated PDA (0 mg l^{-1} of gossypol, control) to 2 mg l^{-1}, remaining unchanged at the next one concentration. The values increased again to around 0·8 for the two highest concentrations of gossypol, 10 and 20 mg l^{-1}.

Germ tube elongation, development and conidia viability

Germ tube elongation, ramification and the number of produced conidia were visually assessed under light microscope at 8 and 24 h after the beginning of the experiments. All the treatment combinations (isolate × gossypol × medium) were considered.

At 8 h, on media without gossypol (regardless of salt treatment) the elongated germ tubes and hyphae were more elongated than at 20 mg l^{-1} of gossypol (Figs 7a,b). Mycelia at 24 h were more developed, with abundant production of conidia (Fig. 7c), compared with growth on the highest concentration of gossypol (Fig. 7d).

The viability of conidia was visualized under fluorescent microscope. All the nongossypol-treated conidia (both micro- and macroconidia) fluoresced (indicative of intact cell walls) and had well-developed germ tubes (Fig. 7e). Only 60% of the conidia exposed to gossypol were viable based on the fluorescent assay (Fig. 7f). This suggests that gossypol killed almost 40% of the conidia.

Discussion

Cotton plants defend themselves against pests and fungal diseases by a variety of means, one of which is the production of secondary metabolites, e.g. gossypol, that have antimicrobial and antiherbivory properties. Gossypol and other related terpenoid aldehydes are constitutively sequestered in lysigenous cavities (gossypol glands) found

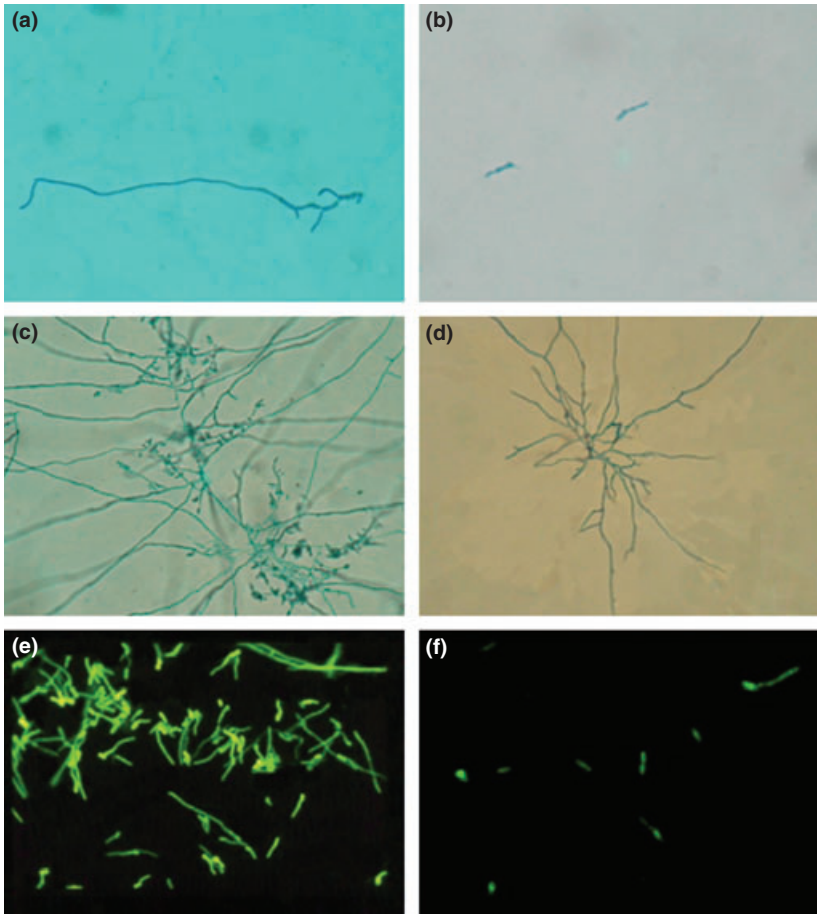

Figure 7 Germ tube elongation on non-amended (a) and gossypol-amended media (b) at time 8 h; hyphae development and ramification of both nonamended (c) and gossypol-amended media (d) 24 h after the beginning of the experiments. Viability of *Fusarium oxysporum* f.sp. *vasinfectum* (*Fov*) conidia not treated (control) (e) and treated with gossypol (f) as observed under fluorescence microscope (20× magnification).

in most plant tissues and organs. Secondary metabolite synthesis can also be induced by microbial attack.

Fov, the causative agent of cotton wilt disease infects plants mainly through roots where few gossypol-producing glands are located. The fungus reaches the apical parts of the plant by moving along vessels, where the gossypol glands are totally absent. *Fov*-immune cotton cultivars have not been found; commercial accessions currently range from susceptible to moderately tolerant. As there are reports that *Fov* attack is correlated with increases in induced terpenoid aldehyde synthesis, it was of interest to determine if the terpenoid aldehydes (represented experimentally by gossypol) really had an inhibitory effect on *Fov* conidial germination, and whether the conidia were actually killed or was conidial germination merely being delayed?

This study highlights that conidia of *Fov*, belonging to five isolates chosen represent a variety of geographic provenances and virulence level (Table 1), have a GP inversely associated to the gossypol concentration in the agar medium. At the two highest concentrations, represented by 10 and 20 mg l^{-1}, the GP was very low, averaging 3%. The inhibitory effect appeared a few hours after germination with the lowest probability of germination occurring within 4 h at the highest gossypol concentration. Even at the lowest concentration, gossypol inhibited conidial germination, suggesting that minimal amounts may constrain the fungus, delaying or blocking conidial germination and hence the infection process. These results are in agreement with those presented by Kaufman *et al.* (1981), who also found a positive correlation between the toxicity of gossypol-like compounds and level of host tolerance to *Fusarium* wilt disease. Among the four sesquiterpenoids Kaufman *et al.* (1981) tested, dHG killed the conidia and mycelium while the hemigossypol-6-methyl ether (MHG) showed the lowest toxicity to the pathogen (Zhang *et al.* 1993).

Although the inhibitory effect of gossypol was observed in all treatments, an unexpected gap was evident at the 4-h time point, suggesting a temporary reduction in the ability of conidia to germinate; 2 h later (6 h) the germination probability returned to appreciable values. The results presented here suggest a variable response over time for conidial germination to indicate some conidia

may experience a 'self-inhibition' phase (perhaps analogous to variable seed dormancy in higher plants). Several hypotheses are relevant. On agar media, where the osmotic potential is around –3 bar, some conidia might take up water more slowly than requiring a delay in the initiation of metabolism until enough water is absorbed. In general, *Fusarium* species grow best between –5 and –25 bar (Cook 1981), but osmoregulation by the fungal cell might require additional energy, a process depending on the biochemical features of each isolate. On the other hand, one of the earliest events during germination is a rapid increase in the rate of protein synthesis. This process requires energy and is essential for germ tube formation and elongation in all fungal spores (Lovett 1976). Furthermore, Macko *et al.* (1976) indicated that spores of several fungi contain germination inhibitors, which in turn, prevent protein synthesis. Consequently, these inhibitors have to be removed before the spores form a germ tube and the amount of the inhibitor present depends on the concentration of spores in the medium. An alternative hypothesis is that a self-induced toxicity occurs soon after the first conidia germinate because of the synthesis and release into the media of natural-occurring mycotoxins. This has been proposed for *Fusarium graminearum* (Eugenio *et al.* 1970). Low zearalenone-producing isolates formed high numbers of perithecia providing the evidence that the mycotoxin has a role in the regulation of sexual reproduction in *Gibberella zeae* (Hagler *et al.* 2001). If the *Fov*-produced mycotoxin degrades quickly in artificial media, it would explain the subsequent rebound in conidial germination rates. As expected, the decrease in germination probability was more marked for the nongossypol treatments (control) than it was for the gossypol-amended treatments.

The inhibitory effect of gossypol on conidial germination appears to attenuate after 8 h. Either the *Fov* acquires resilience, i.e. develops a means to detoxify the terpenoid aldehyde, or the gossypol breaks down naturally. Baugher and Campbell (1969) found considerably reduced amounts of free gossypol in cottonseed inoculated with *Diplodia*. This detoxification efficiency has also been observed on other fungi (Zhang *et al.* 2006). Remarkable differences in germ tube elongation, ramification and the observed number of conidia were observed under light microscope during the 8 h of the experiment and 24 hs later (Fig. 7a–d) supporting the hypothesis that gossypol delays conidial germination. In fact, no differences in mycelia growth were observed among control and the four gossypol treatments on agar medium (data not shown) at the end of the experiment. Once inside a plant, the *Fov* conidia may recognize the gossypol by way of an elicitor-receptor system as suggested by Bailey *et al.* (1998). The duration of the recognition time strongly depends on the aggressiveness (virulence) of the isolates and on the level of host tolerance. Consequently, observation under fluorescent microscope suggests that as soon as the gossypol was absorbed through the cell wall, the conidia that survive are those than can quickly activate a defence mechanism, while conidia that fail to do so die (Fig. 7e,f). This may be dependent on the conidial wall thickness. The toxic effect of the gossypol was more evident on micro- than on macro-conidia. At the highest concentration (20 mg l^{-1}), at least 40% of conidia die, suggesting that the gossypol induced a complete disorganization of both cytoplasm and nucleus accompanied by disintegration of the cell wall, as described by Zhang *et al.* (1993). It should also be noted that in these experiments (artificial system) gossypol has a direct effect on the propagules, in that it is not bound to or accompanied by any other defence compounds present in seeds and whole plants.

The presence of NaCl or Na_2SO_4 in the medium altered germination response of the conidia and also the inhibitory effect of the gossypol. Na_2SO_4 in the medium stimulates germination on untreated agar medium, where the GP ranged from 0·12 to 0·27. These are the highest values observed. Sodium is reported to stimulate reproductive as well as vegetative development of several fungal species (Mert and Dizbay 1977), including *Fusarium oxysporum* f.sp. *betae* (El-Abyad *et al.* 1988).

However, Na_2SO_4 seems to help the conidia to counteract the inhibitory effect of gossypol, enabling the initiation of germination and germ tube elongation. In fact, the incidence of wilt disease was reported to be higher in cotton plants irrigated with saline water (Turco *et al.* 2002). Sodium ions probably alter the metabolic pathway involved in the formation and elongation of germ tube in such a way as to contrast the effect of gossypol. Previous studies reported a significant change in the enzymatic activity of all the *Fov* isolates tested here on sodium-supplemented media (Turco *et al.* 1999). Over time, this 'encouraging' effect attenuates and it eventually disappears. As sodium ions are stable the observation may be attributable to: (i) the absorption and metabolism of the sodium by the conidia or (ii) sodium may be bound in nonreactive chemical complexes. Further investigations are needed to more fully understand these observations. It is clear, however, that the impact of salinity stress on conidial germination strongly depends on the composition of the salt as well as the ionic strength. This is evident in the differential response to the NaCl relative to Na_2SO_4.

The effect of gossypol on *Fov* conidial germination suggests that it is an accessory rather than key component of the cotton anti-*Fov* defence response, otherwise one would expect higher level of toxicity as well toxic effects

on the mycelium. It has been established that glandless cotton hybrids were more susceptible to *Fusarium* wilt disease compared with the low-gossypol seed ones (Turco *et al.* 2004). In these experiments, the gossypol concentrations are lower than those observed in plant tissues, ranging from 0·6% to 9% or 6 000 to 90 000 ppm of the medium. These results, therefore, do confirm that gossypol is one component of the cotton plant defence against wilt diseases, as reported elsewhere (Bell 1967, 1981; Bugbee 1970; Zhang *et al.* 1993; Daayf *et al.* 1997). Recent gene expression experiments are slowly revealing the complexity of the plant disease responses to pathogens like *Fov* and in time these studies will identify key defence response components that are amenable to modification by molecular biologist and plant breeders (Jun-Liu *et al.* 1999; Dowd *et al.* 2004; Wang and Roberts 2006).

Our results also highlight the physiological flexibility of the fungus illustrated in its ability to adapt to the inhibitory effect of gossypol after 8 h, similar to what was reported for *Asperigllus flavus* (Mellon *et al.* 2003). This is a crucial and practical point, as otherwise we would incorrectly predict that cultivars resistant or highly tolerant to *Fusarium* wilt would have higher levels of gossypol. The suggestion that high levels of gossypol may not improve *Fusarium* wilt resistance in cotton is not necessarily negative. In the cotton seed, the oils and protein meals of which are used as a cooking oil and animal feed, respectively, the gossypol is toxic to nonruminants, and therefore highly detrimental (Sunilkumar *et al.* 2006). The ideal cotton plant would be represented by a hybrid with high terpenoid aldehyde concentrations in the plant tissue but very low concentrations in the seeds. Hybrids of this type are being developed (Vroh *et al.* 1999) and while they will be of commercial interest, they could simultaneous prove useful experimentally in ascertaining the role of gossypol in the cotton plant–*Fov* interaction. One agronomic possibility could be a seed-coat treatment using a gossypol-chelate formulation to reduce *Fov* infection during the earliest stages of seed germination and growth.

Furthermore, the differential response of the *Fov* suggests that there is genetic variability among the isolates in their response to the presence of terpenoid aldehydes. In order to clarify this latest aspect further studies are needed.

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