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EFFECT OF SEED MYCOFLORA ON INCIDENCE OF FUSARIUM WILT DISEASE IN COTTON GENOTYPES

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ABSTRACT

Thirteen cotton (*Gossypium barbadense* L.) genotypes were evaluated for Fusarium-wilt incidence, under greenhouse conditions, in 2008 growing season. The genotypes were divided into 5 distinct groups, *i.e.* resistant, moderately resistant, moderately susceptible, susceptible, and highly susceptible. The genotypes showed considerable variation in healthy seedlings, which ranged from 0.00% on genotype 491/2002 to 90.08% on genotype 507/2002. A total of 13 fungi were isolated from the nonsterilized seeds of the 13 genotypes. The isolated fungi were *Alternaria alternata, Aspergillus flavus, A. niger, Aspergillus* sp.*, Chaetomium* sp.*, Cladosporium* sp., *Fusarium moniliforme, F. oxysporum, Nigrospora* sp.*, Penicillium* sp., *Rhizopus stolonifer, Stemphylium botryeosum,* and *Trichoderma* sp. Genotypes 27/99 and 72/99 yielded the highest number of fungi (8 fungi), while 31/99 yielded the lowest number (3 fungi). The other genotypes yielded a number of fungi ranged from 4 to 6. *Rhizopus stolonifer* was the only fungus, which was isolated from all the tested genotypes. The mean percentage of fungal recovery from seeds of the 13 genotypes showed that *A. flavus* (24.77%), *A. niger* (60.46%), *Penicillium* (18.15%), and *R. stolonifer* (65.38%) were the most dominant fungi isolated from the seeds. Other fungi occurred at frequencies ranged from 0.15 to 9.69%. Data for healthy seedlings (dependent variable) and frequencies of the fungi isolated from the seeds (independent variables or predicators) were entered into a computerized stepwise multiple regression analysis. Using the predicators supplied by stepwise regression, a six-variable model was constructed to predict healthy seedlings. This model showed that the differences in healthy seedlings were due largely to the effects of *R. stolonifer, Cladosporium, F. oxysporum, Nigrospora, F. moniliforme,* and *A. alternata*, which collectively accounted for 96.10% of the total variation in healthy seedlings- that is, the total variation in wilt incidence. The model also showed that *R. stolonifer* followed by *Cladosporium* were the most important seedborne fungi contributing to the variation in healthy seedlings. As far as we know, the results of the present study demonstrated, for the first time, that seed mycoflora play an important role in modifying the reaction of cotton genotypes to FOV. Therefore, it is suggested that the role of seed mycoflora should be considered more than it has been in the past for understanding the variations, among cotton genotypes, in resistance or susceptibility to Fusarium wilt disease.

INTRODUCTION

Fusarium wilt (*Fusarium oxysporum*. Schelecht f.sp. *vasinfectum*, (Atk.) Snyd. and Hans.) of cotton (*Gossypium* spp.) was first described by Atkinson (1892) in the USA. The earliest report of the disease outside the USA came from Egypt (Fahmy, 1927), where it spread rapidly with the release of the Sakal cultivar during the 1920s. Fusarium wilt now occurs in all the main cotton-growing areas of the world (Watkins, 1981). *Fusarium oxysporum* f.sp. *vasinfectum* (FOV) caused serious losses in the commercial Egyptian cottons (*G. barbadense* L.) in the late fifties. Since then, an extensive cotton-breeding program was initiated to develop cultivars resistant to the disease.

The economic value of cottonseed is greatly influenced by the presence of fungi in the seed. Fungi or associated metabolites may reduce the vigor of planting seed (Halloin and Bourland, 1981; Davis, 1982), increase the amount of free fatty acid in the seed thereby reducing the quality of the oil (Roncadori *et al.*, 1971), or produce mycotoxins that render the seed unsuitable for consumption (Diener *et al.*, 1976).

Susceptibility of cotton to Fusarium wilt is commonly affected by environmental factors and can also be modified by associated microorganisms. To date, as far as we know, no attempts have been made to evaluate the potential role of cotton seedborne fungi in incidence of Fusarium wilt. An understanding of this potential role could lead to practical measures for control of the disease.

The main objectives of this investigation were to identify fungi associated with seeds of some Egyptian cotton genotypes, and to evaluate their relationship to incidence of Fusarium wilt.

MATERIALS AND METHODS

Evaluation of cotton genotypes for incidence of Fusarium wilt

Thirteen experimental genotypes were evaluated in the present study (Table 1). These genotypes were submitted by Cotton Breeding Section, Cotton Research Institute, Agric. Res. Center, Giza. The inoculum used in the present test was a mixture of equal parts (w/w) of 50 isolates of FOV race 3. These isolates were obtained from the fungal collection of Cotton Pathology Lab., Plant Path. Res. Inst., Agric. Res. Center, Giza. Autoclaved clay loam soil was infested with the mixture of the isolates at a rate of 10 g/kg of soil. Substrate for growth of each isolate was prepared in 500-ml glass bottles. Each bottle contained 50 g of sorghum grains and 40 ml of tap water. Contents of the bottle were autoclaved for 30 minutes. Isolates inoculum, taken from one-week old culture on PDA, was aseptically introduced into the bottle and allowed to colonize sorghum for 3 weeks. Infested soil was dispensed in 10-cm-diameter clay pots and these were planted with 10 seeds per pot. There were 5 replications (pots) for each genotype.

Pots were distributed on a glasshouse bench in a randomized complete block design of 5 replications. The greenhouse was equipped with a heating system assuring that the minimum temperature in the greenhouse was maintained at 28^{o}C; however, due to the lack of a cooling system, the maximum temperature was out of control fluctuating from 30 to 35^{o}C depending on the prevailing temperature during the day (The test was conducted on January and February, 2008). Percentage of infected seedlings was recorded 40 days from planting date. The infected seedlings included the dead seedlings and the surviving seedlings, which showed external or internal symptoms (Aly *et al*., 2000).

Isolation of seedborne fungi:

Occurrence of seedborne fungi was determined by the standard blotter method (ISTA, 1993). Ten nonsterilized seeds for each cotton (*Gossypium barbadense* L.) genotype were selected at random and placed on three

layers of damp 9-cm Whatman No. 1 filter paper in a Petri dish and each was replicated ten times. The plates were incubated in 12-hr light and 12-hr darkness at 20±2°C for 7 days. After incubation, each colony was examined macroscopically or microscopically for identification to genus or species level according to Gilman (1966), Booth (1971), or Barnett and Hunter (1979). Isolation frequency of each fungus was expressed as the percentage of seeds from which the fungus grew. If more than one fungus grew from the same seed, each was counted.

Table 1: Reaction of 13 cotton genotypes to artificial infection by *Fusarium oxysporum* f.sp. *vasinfectum* (race 3) under greenhouse conditions in 2008.

Genotype	Healthy seedlings [a] (%)	Disease reaction [b]
201/2003	61.21	Ms
43/2003	87.34	R
27/99	20.76	S
Giza 74	29.06	S
51/99	65.85	MS
72/99	21.67	S
Pima X Giza 80	70.47	MR
31/99	60.53	MS
30/2003	86.51	R
427/2002	70.69	MR
507/2002	90.08	R
491/2002	0.00	HS
514/2002	80.63	R

[a] Healthy seedlings = 100 – wilt incidence. The following formula was used for calculating wilt incidence: [infected seedlings/emerging seedlings] x 100. Infected seedlings included the dead seedlings and the surviving seedlings, which showed external and internal symptoms or only internal symptoms.

[b] Disease reactions are resistant (R), moderately resistant (MR), moderately susceptible (MS), susceptible (S), and highly susceptible (HS).

Statistical analysis of the data:

Pearson's correlation coefficient was calculated to evaluate the degree of association between each of the isolated fungi and healthy seedlings. Stepwise regression technique with greatest increase in R^2 as the decision criterion was used to describe the effect of seedborne fungi on healthy seedlings. Statistical analysis was performed with a computerized program.

RESULTS AND DISCUSSION

External symptoms of Fusarium wilt were evident on the susceptible seedlings of the tested genotypes 20 days after planting. These seedlings were usually killed within 25 to 30 days after planting or they might survive showing external wilt symptoms on cotyledons. The symptoms were discrete areas of vein discoloration on the cotyledonary leaves, usually began at the margin, turn yellow or brown, eventually, the entire leaf wilted.

A distinctive characteristic of Fusarium wilt is dark brown discoloration of the root and stem xylem. However, there is no consensus of

opinions regarding the diagnostic importance of this vascular discoloration for judging susceptibility to Fusarium wilt in a seedling test. For example, Armstrong and Armstron (1978) stated that vascular discoloration was a questionable standard for judging susceptibility to wilt in a seedling test. Zink *et al.* (1983) found no clear relationship between the severity of external symptoms in surviving muskmelon seedlings and the extent and degree of internal vascular discoloration. On the other hand, Salgado *et al.* (1994) used vascular discoloration as a criterion for judging susceptibility of tepary bean seedlings to Fusarium wilt. Osman (1996) found highly significant positive correlation between vascular discoloration of cotton seedlings (cultivar Giza 74) and each of wilt incidence ($r = 0.93$, $p<0.01$) and wilt severity ($r = 0.98$, $p<0.01$). In the present study, we used rigorous criteria for disease rating. According to these criteria, the seedlings were considered healthy only if they were completely free of any internal and external symptoms. Thus, the seedlings were considered susceptible if they showed internal discoloration even though they were free of any external symptoms.

Environmental conditions in the greenhouse were favorable for unrestricted development of the wilt fungus. The soil was autoclaved, the temperature was optimal most of the time, and the inoculum density was relatively high. Thus, these conditions resulted in 0.00 % healthy seedlings on genotype 491/2002, which is known as highly susceptible (Table 1) (A.A. Aly, *personal observations*). In general, the tested genotypes could be divided into five distinct groups, *i.e.* resistant, moderately resistant, moderately susceptible, susceptible, and highly susceptible (Table 1). The genotypes showed considerable variation in healthy seedlings, which ranged from 0.00% on genotype 491/2002 to 90.08% on genotype 507/2002.

A total of 13 fungi were identified among the 13 genotypes that were tested (Table 2). No single genotype yielded all the 13 fungi. Genotypes 27/99 and 72/99 yielded the highest number of fungi (8 fungi), while 31/99 yielded the lowest number (3 fungi). The other genotypes yielded a number of fungi ranged from 4 to 6. *R. stolonifer* was the only fungus, which was isolated from all the tested genotypes.

The mean percentage of fungal recovery from cottonseeds (Table 1) showed that *A. flavus* (24.77%), *A. niger* (60.46%), *Penicillium* sp. (18.15%), and *R. stolonifer* (65.38%) were the most dominant fungi isolated from the nonsterilized cottonseeds. Other fungi occurred at frequencies ranged from 0.15 to 9.69%. The dominance of *A. niger* relative to the other fungi isolated from cottonseeds is consistent with the findings of Simpson *et al.* (1973) who found that *A. niger* was a dominant fungus at several locations, infecting up to 23% of the seeds. *Penicillium* and *Rhizopus* are among the fungi involved in cotton boll rot and may cause deterioration in fiber quality and favourable environmental conditions (Abd El-Rehim *et al.*, 1993). *Alternaria* has been reported as a dominant member of the mycoflora of cottonseed by Davis (1977). However, it was listed as an infrequent fungus by Roncardori *et al.* (1971), and was present in more than 10% of the seeds from only one location in the study by Simpson *et al.* (1973). Klich (1986) found *A. alternata* in more than 10% of the seed. In the present study, *A. alternata* was found in 1.08% of the seed. Generally, fusaria were major components of the fungal flora in the

earlier studies (Roncardori *et al.*, 1971 and Simpson *et al.*, 1973). In the present study, *F. moniliforme* and *F. oxysporum* were found in 0.38 and 0.85% of the seed, respectively. However, one should keep in mind that taxonomic changes in the genus *Fusarium* makes comparisons to earlier studies difficult.*Cladosporium* and *R. stolonifer* were the only fungi, which significantly correlated with healthy seedlings (Table 3).

Table 2: Frequencies (%) of fungi isolated from cottonseeds of 13 cotton genotypes.

Genotype	Isolation frequency (%) of [a]												
	Alternaria alternata	*Aspergillus flavus*	*A. niger*	*Aspergillus* sp.	*Chaetomium* sp.	*Cladosporium* sp.	*Fusarium moniliforme*	*F. oxysporum*	*Nigrospora* sp.	*Penicillium* sp.	*Rhizopus stolonifer*	*Stemphylium botryeosum*	*Trichoderma* sp.
201/2003	0.0	50.0	76.0	0.0	0.0	0.0	0.0	2.0	0.0	6.0	46.0	0.0	0.0
43/2003	2.0	26.0	94.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	48.0	0.0	0.0
27/99	2.0	98.0	84.0	0.0	0.0	0.0	0.0	1.0	10.0	34.0	80.0	10.0	0.0
Giza 74	0.0	86.0	100.0	0.0	0.0	0.0	4.0	2.0	0.0	0.0	78.0	0.0	0.0
51/99	0.0	32.0	78.0	0.0	0.0	0.0	0.0	0.0	0.0	14.0	100.0	2.0	0.0
72/99	0.0	12.0	80.0	0.0	0.0	0.0	1.0	4.0	0.0	18.0	100.0	22.0	2.0
Pima X Giza 80	0.0	0.0	86.0	48.0	0.0	0.0	0.0	0.0	2.0	8.0	98.0	10.0	0.0
31/99	0.0	22.0	48.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
30/2003	0.0	38.0	90.0	0.0	0.0	0.0	0.0	0.0	0.0	36.0	10.0	24.0	0.0
427/2002	0.0	8.0	50.0	0.0	0.0	0.0	0.0	2.0	0.0	20.0	66.0	24.0	0.0
507/2002	2.0	0.0	0.0	0.0	20.0	0.0	0.0	0.0	0.0	50.0	20.0	26.0	0.0
491/2002	8.0	0.0	0.0	0.0	0.0	4.0	0.0	0.0	0.0	46.0	80.0	6.0	0.0
514/2002	0.0	0.0	0.0	0.0	0.0	2.0	0.0	0.0	0.0	2.0	24.0	2.0	0.0
Mean	1.08	24.77	60.94	3.69	1.54	0.46	0.38	0.85	1.09	18.15	65.38	9.69	0.15

[a] Frequency (%) of fungi isolated from 100 nonsterilized seeds from each genotype by the standard blotter method and examined 7 days from incubation at 20ºC ± 2 and alternative cycle of cool white light/darkness.

Data for healthy seedlings and frequencies of the fungi isolated from the nonsterilized seeds were entered into a computerized stepwise multiple regression analysis. The analysis constructed a predictive model by adding predicators, in this case, frequencies of the isolated fungi, to the model in order of their contribution to R^2. The analysis was effective in eliminating those variables with little or no predictive value by incorporating into the model only those variables that made a satisfactory significant contribution to the R^2 value of the model (Podleckis *et al*., 1984). Using the predictors supplied by stepwise regression, a six-variable model was constructed to predict healthy seedlings (Table 4). This model showed that the differences in healthy seedlings were due largely to the effects of *R. stolonifer, Cladosporium, F. oxysporum, Nigrospora, F. moniliforme,* and *A. alternata*

(Table 5), which collectively accounted for 96.10% of the total variation in healthy seedlings. The model also showed that *R. stolonifer* followed by *Cladosporium* were the most important seedborne fungi contributing to the variation in healthy seedlings – that is, the variation in wilt incidence (Table 5).

Table 3: Correlation between healthy seedlings (%) and frequencies of fungi isolated from cottonseeds of 13 cotton genotypes.

Isolation frequency (%) of	Healthy seedlings (%)
Alternaria alternata	0.0558 [a]
Aspergillus flavus	- 0.1191
Aspergillus niger	- 0.1001
Aspergillus sp.	0.1204
Chaetomium sp.	0.3165
Cladosporium sp.	- 0.5844 *
Fusarium moniliforme	- 0.2757
Fusarium oxysporum	- 0.4856
Nigrospora sp.	- 0.2215
Penicillium sp.	- 0.0997
Rhizopus stolonifer	- 0.5894 *
Stemphylium botryeosum	0.1143
Trichoderma sp.	0.0571

[a] **Linear correlation coefficient (r) is significant at $p < 0.05$ (*).**

Table 4: Stepwise regression model that describes the relationship between healthy seedlings (Y) of cotton and frequencies of fungi (X_s) isolated from 13 cotton genotypes.

Stepwise linear regression model	Coefficient of determination (R^2)	F. value [a]
$Y = 92.80 - 0.24\ \mathbf{X_{11}} - 19.44\ \mathbf{X_6} - 8.56\ \mathbf{X_8} - 4.35\ \mathbf{X_9} - 7.62\ \mathbf{X_7} + 0.49\ \mathbf{X_1}$	96.10	24.63 ***

[a] **F. value is significant at $p < 0.005$.**

Slopes of *R. stolonifer* (X_{11}), *Cladosporium* (X_6), *F. oxysporum* (X_8), *Nigrospora* (X_9), and *F. moniliforme* (X_7) were negative in the regression model, which indicate that the increase in isolation frequency of these fungi was associated with a decrease in healthy seedlings – that is, an increase in wilt incidence. This finding suggests the occurrence of a synergistic interaction between each of these fungi and FOV. This conclusion is in concert with some early reports, which indicated that some fungi could enhance incidence of Fusarium wilt in cotton. For instance, Sabet and Khan (1969) found that *Rhizoctonia solani* increased incidence of Fusarium wilt in cotton. When Risk and Mohamed (1986) inoculated cotton varieties Giza 66

and Karnak using both *R. solani* and FOV, wilt incidence was increased in Karnak but unchanged in Giza 66. In California, the phenomenon of sudden wilt was investigated by Schnathorst (1964) and found to be an interaction between FOV and *Thielaviopsis basicola*. *Trichoderma harzianum* may be an important component of cotton wilt complex along with *Meloidogyne incognita* and FOV (Yang *et al*., 1976).

Table 5: Identification of the predicators included in stepwise regression model shown in Table (4) and their relative contribution to the total variation in healthy seedlings.

Predictor	Number	Relative contribution (%)
Rhizopus stolonifer	X_{11}	34.74
Cladosporium sp.	X_6	25.32
Fusarium oxysporum	X_8	17.44
Nigrospora sp.	X_9	13.01
Fusarium moniliforme	X_7	3.46
Alternaria alternata	X_1	2.12

The *in vitro* antagonism of *A. alternata* against *F. oxysporum, F. solani,* and *F. equiseti* has been demonstrated by Rudra *et al.* (2005). Therefore, it seems reasonable to conclude that the positive slope of *A. alternata* (X_1) in the regression model could be attributed to the antagonistic activity of *A. alternata* against FOV. Another possibility is that colonization of cotton roots by *A. alternata* induced a systemic resistance in cotton seedling against FOV (Matta, 2002).

As far as we know, the results of the present study demonstrated, for the first time, that seed mycoflora play an important role in modifying the reaction of cotton genotypes to FOV. Therefore, it is suggested that the role of seed mycoflora should be considered more than it has been in the past for understanding the variations, among cotton genotypes, in resistance or susceptibility to Fusarium wilt diseases.

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تأثير فطريات البذرة على حدوث مرض ذبول الفيوزاريوم فى التراكيب الوراثية للقطن

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قيم ١٣ تركيب وراثى من الأقطان المصرية وذلك من حيث المقاومة أو القابلية للإصابة بمرض ذبول الفيوزاريوم ، تحت ظروف الصوبة ، خلال موسم ٢٠٠٨. قسمت التراكيب الوراثية إلى خمسة مجموعات محددة هى على النحو التالى: مقاومة ومتوسطة المقاومة ومتوسطة القابلية للإصابة وقابلة للإصابة وشديدة القابلية للإصابة. تباينت التراكيب الوراثية فيما بينها – بشكل واضح – من حيث المقاومة أو القابلية للإصابة بالمرض ، وعلى ذلك فقد تراوحت نسبة البادرات السليمة من صفر% فى التركيب الوراثي ٢٠٠٢/٤٩١ إلى ٩٠,٠٨% فى التركيب الوراثى ٢٠٠٢/٥٠٧. أظهر التقدير النوعى للفطريات المعزولة من البذور الغير معقمة للتراكيب الوراثية وجود الفطريات التالية: ألترناريا ألترناتا ، أسبرجلس فليفس ، أسبرجلس نيجر ، أسبرجلس ، كيتوميم ، كلادوسبوريوم ، فيوزاريوم مونيليفورمى ، فيوزاريوم أوكسيسبورم ، نيجروسبورا ، بنيسيليوم ، ريزوبس ستولونيفر ، ستمفيليم بوتريوزم وتريكودرما. أكبر عدد من الفطريات (٨ فطريات) أمكن عزله من كل من التركيبين الوراثيين ٩٩/٢٧ و ٩٩/٧٢ ، أما أقل عدد (٣فطريات) فقد أمكن عزله من التركيب الواثى ٩٩/٣١ ، باقى التراكيب الوراثية أعطت عند العزل منها عدداً من الفطريات تراوح ما بين ٤ إلى ٦. فطر ريزوبس ستولونيفر هو الوحيد الذى أمكن عزله من جميع التراكيب الوراثية المختبرة. أظهر التقدير الكمى أن فطريات أسبرجلس فليفس (٢٤,٧٧%) ، أسبرجلس نيجر (٦٠,٤٦%) ،بنيسيليوم (١٨,١٥%) وريزوبس ستولونيفر (٦٥,٨%) هى الفطريات الأكثر شيوعاً عند العزل من البذرة ،أم الفطريات الأخرى فقد تراوح تكرار عزلها من٠,٥ إلى ٩,٦٩%. أمكن – باستخدام أسلوب الإنحدار المتعدد المرحلى – التوصل إلى نموذج إنحدار لوصف تأثير فطريات البذرة (متغيرات مستقلة) على التباين الكلى فى النسبة المئوية لليادرات السليمة (متغير تابع). أظهر هذا النموذج أن ٩٦,١٠% من التباين الكلى فى نسبة البادرات السليمة من الممكن أن يعزى إلى تأثير فطريات ريزوبس ستولونيفر ، كلادوسبوريوم ، فيوزاريوم أكسيسبورم ، نيجروسبورا ، فيوزاريوم مونيليفورمى وألترناريا ألترناتا. كما أظهر النموذج أن فطرى ريزوبس ستولونيفر وكلادوسبوريم هما الأكثر أهمية فى التأثير على نسبة البادرات السليمة ، أى الأكثر أهمية فى التأثير على حدوث المرض. تدل نتائج الدراسة الحالية – وللمرة الأولى – على أن فطريات البذرة تلعب دوراً هاماً فى تحديد مستويات المقاومة أو القابلية للإصابة – بمرض ذبول الفيوزاريوم – فى التراكيب الوراثية للقطن ، وأن هذا الدور هو أهم مما كان يُعتقد فى السابق.