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Journal of Stress Physiology & Biochemistry, Vol. 10 No. 1 2014, pp. 37-44 ISSN 1997-0838
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ORIGINAL ARTICLE

Use of Phenols, Peroxidase and Polyphenoloxidase of Seed to Quantify Resistance of Cotton Genotypes to Damping-off Incited by *Fusarium oxysporum*

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Received August 12, 2013

A greenhouse test was conducted in 2011 and 2012 growing seasons at Giza Agricultural Research Station to evaluate the reaction of six cotton genotypes to damping-off incited by *Fusarium oxysporum*. Damping-off incidence on the genotypes ranged from 70-88%. In general, the genotypes could be divided into highly susceptible, susceptible, and moderately susceptible. Data for damping-off incidence and level or activity of some biochemical components (phenols, peroxidase, and polyphenoloxidase) were entered into a computerized linear regression analysis. The analysis contrasted seven predictive models by using the biochemical components, singly or in combination, as biochemical predictors. It was evident that models nos. 2 and 6 were the best models for predicting incidence of damping-off. The superiority of these models was attributed to their high R^2 values (0.748 and 0.902, respectively) and the significance of their F. values (P = 0.026 and P = 0.031, respectively). The results of the present study suggest that peroxidase alone or both peroxidase and polyphenoloxidase, which may or may not parts of damping-off resistance mechanisms, can be used as biochemical markers to predict resistance to damping-off incited by *F. oxysporum*.

Key words: Fusarium oxysporum, Gossypium barbadense, peroxidase, phenol, polyphenol oxidase

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Key words: Fusarium oxysporum, Gossypium barbadense, peroxidase, phenol, polyphenol oxidase

Fusarium wilt is a destructive disease of cotton in many cotton – growing countries of the world including Australia, USA, Israel, Tanzania, and China, (Feng *et al*., 2000). Several reports indicate that *Fusarium oxysporum* (Schlecht.) emend Snyd and Hans makes up a major portion of the fungal flora in soil which causes vascular wilt in susceptible cultivars (Watkins, 1981; Chen *et al*., 1985; Hillocks, 1992; Davis *et al*., 1996). For instance, Gordon (1956) found that *F. oxysporum* was by for most prevalent species of *Fusarium*, as it represented approximately 67% of *Fusarium* spp. in Canadian soil. Meyer (1967) showed that relative abundance of *F. oxysporum* may be as high as 8-10% of the soil

total fungal population. In the rhizosphere, the relative abundance of *F. oxysporum* may reach 43% of the total microfungal population. On the root surface or in the superficial layers, *F. oxysporum* is even more abundant, and its frequency among isolates may reach 97%.

F. oxysporum plays an important role in the etiology of cotton seedling disease in Egypt causing pre- and post emergence damping-off and seedling root rot (Abd-Elsalam *et al*., 2006). *F. oxysporum* is of widespread distribution in the Egyptian soil (Aly *et al*., 1996) and it is easily and frequently isolated from cotton seedlings infected with damping-off. For example, Jakob (1969) isolated 97 isolates of *Fusarium* spp. from seedlings of Egyptian cottons infected with damping-off. These isolates were classified as *F. oxysporum* (56.7%), *F. moniliforme* (23.7%), *F. solani* (9.3%), *F. orthoceras* (5.2%), *F. scirpi* (4.1%), and *Fusarium* sp. (1%). *F. oxysporum* and *F. moniliforme* were the only species capable of infecting and killing cotton seedlings, other species were categorized as wound parasites of saprophytes. *F. oxysporum* was the predominant species (71.7%) in a random sample of 46 *Fusarium* isolates tested for pathogenicity. The pathogenic isolates of *F. oxysporum* represented 10.9% of the total isolates and 38.5% of the total pathogenic isolates (Aly *et al*., 1996). In another study (Aly *et al*., 2000), *F. oxysporum* was the predominant species (52.7%) in a random sample of 55 *Fusarium* isolates from Upper Egypt tested for pathogenicity. The pathogenic isolates of *F. oxysporum* represented 40.0% of the total isolates and 56.4% of the total pathogenic isolates. *F. oxysporum* was the predominant species (60.9%) in a random sample of *Fusarium* spp. recovered from cotton seedling infected with damping-off in Lower Egypt (Abd-Elsalam *et al*., 2006).

It has been suggested that a variety of substances contained in plant cells are involved in resistance or susceptibility to infection by pathogens. Among these are phenols, peroxidase, and polyphenoloxidase (Agrios, 2005).

However, from practical standpoint, apart from peroxidase (Reuveni *et al*., 1992), no attempts have been made to utilize these substances, in healthy genotypes, as biochemical markers to predict resistance to diseases in breeding programs. Therefore, the main objective of the present study was to develop statistical models to predict incidence of damping-off, incited by *F. oxysporum*, on cotton genotypes by using these substances in healthy seeds as biochemical predictors.

MATERIALS AND METHODS

Cotton genotypes

Cotton genotypes (*Gossypium barbadense* L.) used in the present study were obtained from Cotton Research Institute, Agric. Res. Cent., Giza.

Isolation of *Fusarium oxysporum*

Isolation, purification, and identification of the isolates used in the present study were carried out at Cotton Pathology Section, Plant Path. Res. Inst., Agric. Res. Cent., Giza, Egypt.

Reaction of cotton genotypes to damping-off

Substrate for growth of each isolate was prepared in 500-ml glass bottle contained 50 g of sorghum grains and 40 ml of tap water. Contents of bottles were autoclaved for 30 minutes. Isolate inoculum, taken from one-week old culture on PDA, was aseptically introduced into the bottle and allowed to colonize sorghum for three weeks. The inoculum used in the present test for soil infestation was a mixture of equal parts (w/w) of 20 isolates of *F. oxysporum*. Autoclaved clay loam soil was infested with the mixture of isolates at the rate

of 50 g/kg of soil. Infested soil was dispensed in 10-cm-diameter clay pots and these were planted with seeds of the tested genotypes (10 seeds/pot). Pots were randomly distributed on a greenhouse bench under a temperature regime ranged from 23±3°C to 33±2.5°C. Percentages of infected seedlings were recorded 45 days after planting. The reactions of the genotypes to damping-off were evaluated twice with almost the same results.

Chemical analysis

Random samples of seeds, taken from the same seeds used in planting the greenhouse experiment, were used for the chemical analysis, which was carried out as follows:

Assay of total phenols

Soluble phenols in fresh samples were extracted according to Dihazi *et al*. (2003). A known weight of fresh samples was selected and extracted with 80% cold methanol (v/v) for three times at 90°C. The combined extracts were collected and filtered through Whatman No. 1 filter paper. After filtration, the filtrate was made up to a known volume with cold methanol. A known volume of the extract (0.5 ml) was added to 0.5 ml folin-Cicalteu reagent and shaked well. The mixture was allowed to stand for 3 min. One ml of saturated sodium carbonate solution (25 g Na_2CO_3 were dissolved in 1000 ml distilled water at 70-80°C, cooled down, and filtered was added to the mixture and shaked well. The mixture was allowed to stand for 60 min. The optical density was measured at 725 nm UV-Vis spectrophotometer. The quantity of total phenolic compounds was calculated according to the standard curve of gallic acid (99.5%) and expressed as mg/100 g fresh weight.

Extract of enzymes

Two grams of fresh samples were homogenized in cold phosphate buffer (0.5 M at pH 6.5). The homogenate was centrifuged at 1000 rpm for 10 min. The pigments were removed from the supernatant by adsorbing on activated charcoal and filtered. The filtrate was completed to a known volume and used to determine enzyme activity.

Assay of peroxidase activity

Peroxidase (EC1.11.1.7) was assayed following the method of Kar and Mishra (1976) with slight modification. Five ml of the assay mixture contained 300 µM of phosphate buffer (pH 6.8), 50 µM Catechol, 50 µM H_2O_2, and 1 ml of crude enzyme extract were prepared. After incubation at 25°C for 5 min., the reaction was stopped with the addition of 1 ml of 10% H_2SO_4. The colour intensity was red at 430 nm and the enzyme activity was expressed as enzyme activity/gram fresh weight/h.

Assay of polyphenoloxidase activity

Polyphenoloxidase (EC 1.14.18.1) was assayed according to the method described by Kar and Mishra (1976) with slight modification. Five ml of the assay mixture contained 125 µM of phosphate buffer (pH 6.8), 100 µM pyrogallol, and 1 ml of crude enzyme extract. After incubation at 25°C for 5 min., the reaction was stopped with the addition of 1 ml of 10% H_2SO_4. The colour intensity was read at 430 nm, and the enzyme activity was expressed as enzyme activity/gram fresh weight/h.

Statistical analysis

The experimental design of the greenhouse experiment and the laboratory tests was a randomized complete block with three replicates (blocks). Analysis of variance (ANOVA) of the data was performed with MSTAT-C. Duncan's multiple range test was used to compare genotypes means. Linear regression analysis was used to evaluate the relationship between the biochemical components

(independent variables) and incidence of damping-off (dependent variable). Regression analysis was performed with a computerized program (SPSS version 13).

RESULTS AND DISCUSSION

F. oxysporum caused pre germination decay of the seed, decay of the seedling on the way to soil surface (pre emergence damping-off), partial or complete girdling of the emerging seedling at or near the soil surface ("Sore shin" or post emergence damping-off), and seedling root rot.

Environmental conditions in the greenhouse were favorable for unrestricted development of *F. oxysporum*. The soil was sterile, temperature was optimal most of the time, the sterile soil was infested with highly pathogenic isolates, and the inoculums density was relatively high. Under these conditions, it is unlikely that any susceptible seedlings would have escaped detection in the test. In general, the tested genotypes could be divided into three groups, i.e. highly susceptible, susceptible, and moderately susceptible (Table 1).

The occurrence of major losses from cotton seedling damping-off incited by *F. oxysporum* is not uncommon in all cotton-producing areas in Egypt. These losses vary over years and locations but characteristically result in poor stands. Stands may be replanted if severely damaged and, even if damage is not severe enough for replanting, it may make weed control and other cultural practices difficult for the remainder of the season. Replanting, poor stands and seedling development, and weed competition ultimately affect plant maturity, fiber quality, and seed cotton yield (Kappelman, 1977). Thus, the widespread use of seed-dressing fungicides for controlling the disease has become indispensable under Egyptian condition. While effective fungicides are available (El-Samawaty, 1999), it is becoming increasing evident that their widespread use is associated with some problems, such as the potential harmful effect on non-target organisms, the development of resistant races of the pathogens, and the possible carcinogenicity. Other problems include gradual elimination and phasing out of some compounds (Zaki *et al*., 1998).

Use of cotton cultivars with damping-off resistance can resolve all these problems. However, successful screening for damping-off resistance in cotton requires the development of a reliable method for quantification of resistance. This method should meet two requirements. It should be independent of the pathogen, and should reflect the genetic differences among genotypes. The biochemical components of cottonseed (Table 2) may meet these requirements for several reasons. The involvement of these compounds in resistance or susceptibility is well documented in the literature as previously mentioned in the introduction. The occurrence of each, except phenols, varied with the genotype (Table 2). They can be determined rapidly and with small amounts of cottonseed, therefore, large number of genotypes can be tested without sacrificing the seeds.

Data for damping-off incidence and level or activity of each component were entered into a computerized linear regression analysis. The analysis constructed seven predictive models by using the biochemical components, singly or in combination, as biochemical predictors (Table 3). It is evident that models no_s. 2 and 6 are the best models for predicting incidence of damping-off. The superiority of these models is attributed to their high R^2 values and the significance of their F. values. Models 4 and 7 also show high R^2 values; however, they were excluded due to the non significance of

their F. values. One should keep in mind that the significant r values of models 2 and 6 should be interpreted with caution (Gomez and Gomez, 1984) because significant correlation do not necessarily imply causation. In other words, results of the present study suggest that peroxidase alone or both peroxidase and polyphenoloxidase in uninfected cottonseed, which may or may not parts of damping-off resistance mechanisms, can be used as biochemical markers to predict resistance to damping-off incited by *F. oxysporum*.

In practical terms, our results mean that a primary selection to eliminate susceptible genotypes can be made before planting. In this primary selection, only genotypes with low levels of peroxidase activity (in case of using model 2) or with low levels of peroxidase activity and high levels of polyphenoloxidase activity (in case of using model 6) would be retained for further evaluation under greenhouse conditions and thereby decrease the time and effort necessary for the development of resistant genotypes in breeding programs.

Table 1 : Incidence of damping-off (%) incited by F. oxysporum on six cotton genotypes and their disease categories under greenhouse conditions in Giza in 2011 and 2012 growing seasons.

Genotype	Damping-off [a] (%)	Disease category [b]
403/2002	88.33 A	HS
423/2002	83.33 AB	HS
401/2002	81.66 ABC	S
427/2002	76.66 ABC	S
494/2002	71.67 BC	MS
405/2002	70.00 C	MS

Combined pre emergence and post emergence. Each value is the mean of two growing seasons and each season included three replicates (pots). Means followed by the same letter(s) are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

Disease categories are highly susceptible (HS), susceptible (S), and moderately susceptible (MS).

Table 2 : Determination of levels and activities of some biochemical components in healthy seeds of six cotton genotypes.

Genotype	Component		
	Phenols (mg/g fresh weight)	Peroxidase (activity/h/g fresh weight)	Polyphenoloxidase (activity/h/g fresh weight)
401/2002	2.01 A	6.49 AB	11.81 CD
427/2002	1.68 A	7.42 AB	20.68 AB
403/2002	1.79 A	9.92 A	23.38 AB
405/2002	1.76 A	2.58 B	7.17 D
494/2002	1.79 A	3.86 AB	17.43 BC
423/2002	2.22 A	9.23 A	26.35 A

Each value is the mean of three replicates. Within a column, means followed by the same letter(s) are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

Table 3 : Linear regression models that describe the relationship between some biochemical components (Xs) in healthy seeds of six cotton genotypes and incidence of damping-off incited by F. oxysporum (Y) on these genotypes.

Independent variable(s) or predictor(s)	Model no.	Linear regression model	r[a]	R^{2b}	F. value	P > F
Phenols (X1)	1	Y = 49.74 + 15.38 X1	0.434	0.188	0.929	0.390
Peroxidase (X2)	*2	Y = 62.22 + 2.35 X2	0.865	0.748	11.856	0.026
Polyphenoloxidase (X3)	3	Y = 67.39 + 0.63 X3	0.642	0.412	2.800	0.170
X1 and X2	4	Y = 52.99 + 5.45 X1 + 2.21 X2	0.877	0.769	4.981	0.111
X1 and X3	5	Y = 51.53 + 9.20 X1 + 0.55 X3	0.688	0.473	1.345	0.383
X2 and X3	*6	Y 62.27 + 4.85 X2 – 0.98 X3	0.950	0.902	13.740	0.031
X1, X2, and X3	7	Y = 53.50 + 5.18 X1 + 4.70 X2 – 0.98 X3	0.959	0.920	7.710	0.117

* The best regression models for quantifying incidence of damping-off incidence by F. oxysporum on cotton genotypes.

[a] Linear correlation coefficient.

[b] Coefficient of determination.

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