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Effects of amino acids in cotton seeds against the resistance to Fusarium wilt disease

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ABSTRACT

In a greenhouse study, the sensitivity of 12 cotton genotypes was evaluated against the Fusarium wilt disease. The genotypes showed considerable variation in Fusarium-wilt resistance (0.83 to 80.25%). The genotypes could be divided into four groups, i.e. highly resistant, moderately resistant, moderately susceptible and highly susceptible. The HPLC analysis of amino acid cotton genotype seeds revealed the presence of 17 amino acids, but their occurrence in seeds varied with genotype. None of the individual amino acids was significantly correlated with resistance to Fusarium wilt. Data for resistance rating and concentrations of amino acids were submitted to stepwise-multiple regression statistical analysis. Using the predictors supplied by stepwise regression, an eight-factor model was constructed to predict the Fusarium-wilt resistance. The model showed that differences in Fusarium-wilt resistance were associated with the 8 amino acids (isoleucine, valine, proline, phenylalanine, lysine, histidine, tyrosine, and cysteine), which accounted for 100% variation in resistance. Thus amino acids composition of cottonseed may provide a supplementary assay to greenhouse tests to quantitatively distinguish between the Fusarium-wilt resistant and susceptible genotypes.

Key words: Biomarkers, fusarium-wilt, genotypes*, gossypium barbadense*, resistance, seeds, susceptible

INTRODUCTION

Fusarium wilt (*Fusarium oxysporum* f. sp. *vasinfectum*) of cotton (*Gossypium* spp.) causes serious yield losses in Egyptian cottons (*Gossypium barbadense*) since 1950s (4). Thereafter, an extensive cotton-breeding programme was initiated to develop cultivars resistant to this disease. The cotton genotypes were screened in greenhouses in soil infested with the wilt fungus. There is a strong relationship between amino acids and *Fusarium* spp. diseases particularly with *F. oxysporum* (the disease organism of vascular wilt). Amino acids directly effects the physiological activities (germination of conidia, elongation of germ tubes, and production and efficiency of enzymes) related to the pathogenicity of *Fusarium* spp. For instance, Mehta *et al.* (10) tested amino acids for the secretion of pectolytic and cellulolytic enzymes. They reported complete inhibition of polygalacturonase (PG) synthesis with leucine and phenylalanine in *F. oxysporum* and *F. moniliforme*, respectively. Leucine showed complete inhibition of pectinmethyl-

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galacturonase (PMG) synthesis in *F. oxysporum*. In *F. moniliforme*, cysteine and phenylalanine also inhibited the PMG synthesis. Leucine, phenylalanine and tryptophan completely inhibited the cellulase production in *F. moniliforme* and also controlled the synthesis of total cellulase in *F. oxysporum*. Leaves of 40-days-old healthy and wilted lentils were collected and analyzed for amino acids composition. The concentration of total free amino acids increased considerably in leaves infected by *F. oxysporum* f. sp. *lentils* but there were no qualitative changes in free amino acids in infected leaves (13). Frequency and pathogenicity of *F. oxysporum* f. sp. *vasinfectum* (the Fusarium wilt pathogen of cotton), decreased with increase in years of cropping of resistant cultivars. Root exudates from a resistant cultivar inhibited the colony growth and formation and germination of chlamydospores, but it was not true with exudates from a susceptible cultivar. Spectrophotometric analysis showed that exudates from the resistant cultivar contained certain amino acids which are not present in exudates from the susceptible one. These results suggested that build up of root exudates from resistant cultivars in soil over the years inhibited the chlamydospores formation and germination. Thus, reducing the pathogen populations *F. oxysporum* f. sp. *vasinfectum* (16). Mahakul *et al.* (9) studied the formation and germination of hyphal chlamydospores in *F. oxysporum* f. sp. *ciceri*, the vascular wilt pathogen on chickpea. Slide germination tests revealed that exudates from a susceptible host cultivar highly stimulated both chlamydospore germination and germ tube growth compared with resistant cultivar. The same was observed when germination in soil was studied.

Biochemical analysis indicated the presence of higher concentrations of amino acids in exudates of susceptible cultivar than in exudates of resistant cultivar. Jha and Chattoo (7) suggested that chimeric polyprotein expression has the potential to provide broad-spectrum disease resistance in rice. No appreciable difference in the level of phenolics was detected in the exudates of two cultivars. Thus, the differential effect of exudates on spore germination was probably not due to the exudation of antifungal compounds (such as phenolics, from the resistant host cultivar), but may be caused by difference in the levels of nutrients (such as amino acids), excreted through the roots of cultivars. Liu (8) analyzed seedlings of 14 upland cotton varieties at 3-leaf stage for their amino acid contents. When inoculated with *F. oxysporum* f. sp. *vasinfectum*, the resistant genotype seedlings had 15-19% lower total free amino acids and the susceptible genotype seedlings has 13-20% higher total free amino acids than their respective non-inoculated controls. Total amino acids decreased in both resistant and susceptible genotypes.

This study aimed to evaluate the relationship between the amino acid composition of cotton genotypes seeds and resistance to Fusarium wilt and to develop a statistical model to predict the resistance to Fusarium wilt by using seeds in amino acids as biochemical predictors.

MATERIALS AND METHODS

Cotton genotypes (*Gossypium barbadense* L.) used in this study were obtained from Cotton Research Institute, Agricultural Research Center, Giza, Egypt. The isolation, purification and identification of amino acids in isolates used were done in Cotton Pathology Laboratory, Plant Pathology Agricultural Research Center, Giza, Egypt.

Sensitivity of cotton genotypes to Fusarium wilt

Substrate for growth of each isolate was prepared in 500-ml glass bottles (containing 50 g of sorghum grains and 40 ml tap water). Contents of bottles were autoclaved for 30 min. Pure culture prepared from the one-week old culture on PDA, was aseptically inoculated into the bottle and allowed to colonize sorghum for 3-weeks. The inoculum used in this test for soil infestation was a mixture of equal parts (w/w) of 50 isolates of *F. oxysporum* f. sp. *vasinfectum* (FOV) race 3. Autoclaved clay loam soil was infested with the mixture of isolates at 10 g/kg of soil. Infested soil was dispensed in clay pots (10-cm-dia) and each pot was sown with 10 seeds of test genotypes. Pots were randomly distributed on a greenhouse bench under a temperature regime [23±3°C to 33±2.5°C]. The pots were regularly watered just enough for the seedlings to survive. Healthy seedlings (%), which did not show any external or internal symptoms (3) were recorded 45 days after sowing. The sensitivity of genotypes to Fusarium wilt gave the same results. At the end of pathogenicity tests, all soil, pots and benches were sterilized as per quarantine regulations (1).

Analysis of cottonseeds amino acid composition

The amino acid composition of seed samples was determined as per High-Performance Liquid Chromatography-Pico-Tag method (6), which was performed as under:

Extraction of total amino acids: A sample corresponding to 40 mg protein was weighted into 25 x 150 mm hydrolyzed tube, aliquot (7.5 ml) of 6 N HCl was added, purged with nitrogen for 60 sec and the tube was capped immediately. The tube was placed in oven at 110°C for 24 h, removed and allowed to cool. The tube contents were quantitatively transferred to 25 ml volumetric flask and completed to volume with High Performance Liquid Chromatography (HPLC) grade water. About 1 ml solution was filtered through 0.45 µm Millipore membrane filter.

Derivatization of amino acids: Ten microliters of filtered sample in 6 x 50 mm tube was placed into drying vial and dried in a freeze-dryer workstation for 10-15 min. Aliquot (30 µl) of redried solution (mixture of 200 µl methanol, 200 µl 0.2 N sodium acetate and 100 µl triethylamine) was added to the sample tubes and redried again in the workstation. An aliquot (30 µl) of freshly prepared derivatization agent (mixture of 350 µl methanol and 50 µl phenylisothiocyanate) was added to the tube contents and allowed to react for 20 min, dried in the workstation for 15 min. Thirty µl methanol was added and dried again. 250 µl of sample diluents (Waters, USA) were added to the dried tube, vortexed and transferred to injection vials. The standard amino acid (Sigma, USA) solution was treated the same as the sample.

Separation of total amino acids derivatives using HPLC: Separation of total amino acid derivatives was done in a stainless steel Pico-Tag amino acid column (150 x 3.9 mm). Spectra Physics P2000 variable wavelength detector was adjusted to 254 nm. The amino acids were quantified by comparing the peak area with those corresponding amino acid standard solutions using the Spectra Physics Data System program.

Statistical analysis: The experimental design of the greenhouse test was randomized complete block with six replicates (blocks). Analysis of variance (ANOVA) of data was done with MSTAT-C. Duncan's multiple range test was used to compare genotype means. Linear correlation coefficient (r) was calculated to evaluate the degree of association between concentration of each amino acid and resistance of the genotypes to Fusarium wilt. Stepwise regression technique with the greatest increase in R^2 as the decision criterion was used to describe the effects of amino acids on resistance. Correlation and regression analysis was preformed with a computerized program (SPSS Version 13).

RESULTS AND DISCUSSION

Pathogenicity assays

Twenty days after planting, the external symptoms of Fusarium wilt were evident in the susceptible seedlings of test genotypes. These seedlings were killed within 25 to 30 days after planting or if survived showed external wilt symptoms on cotyledons. The symptoms showed areas of vein discolouration in cotyledonary leaves, usually began at the margin, turned yellow or brown and eventually the entire leaf wilted.

A distinctive characteristic of Fusarium wilt is dark brown discolouration of root and stem xylem. Armstrong and Armstrong (3) stated that vascular discolouration is a questionable standard to judge the susceptibility to wilt in a seedling test. Zink *et al.* (17) found no relationship between the severity of external symptoms in surviving muskmelon seedlings and the extent and degree of interval vascular discoloration. However, Salgado *et al*. (14) used vascular discoloration as a criterion to judge the susceptibility of tepary bean seedlings to Fusarium wilt. Osman-Eman (11) reported a 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 this study, we used rigorous criteria (healthy, dead, morphological and anatomical features) to rate symptoms wilt caused by *Fusarium oxysporum* f. sp. *vasinfectum*. 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 greenhouse were favorable for unrestricted development of wilt fungus. The soil was sterile, temperature was optimal, the sterile soil was infested with highly pathogenic isolates, and the inoculums density was high. Under these conditions, it is unlikely that any susceptible seedlings would have escaped the detection test. Thus, the genotype 501/2002 (highly susceptible), showed almost 100% wilt symptoms (Table 1). In general, the test genotypes could be divided into four groups, i.e. highly resistant, moderately resistant, moderately susceptible and highly susceptible.

Currently, due to lack of wilt nurseries, screening of breeding materials under greenhouse conditions is the only reliable method in Egypt to distinguish the Fusarium-wilt highly resistant genotypes. The test is time-consuming and influenced by variability inherent in the experimental system. Under very favorable environmental conditions the first symptoms of disease appear on susceptible genotypes 20 days after planting and may require longer time under less favorable conditions.

Table 1. Sensitivity of 12 cotton genotypes to Fusarium wilt under greenhouse conditions in 2008 and 2009

Genotypes	Healthy seedlings (%)	Disease category [b]
514/2002	80.25 A	HR
423/2002	78.43 A	HR
488/2002	75.26 A	HR
507/2002	70.81 A	MR
427/2002	67.41[a] A	MR
405/2002	47.61 B	MS
812/2002	34.42 BC	MS
Giza 74	28.36 C	MS
449/2002	10.23 D	HS
490/2002	8.19 DE	HS
491/2002	2.78 EF	HS
501/2002	0.83 F	HS

[a] Each value is the mean of two experiments and each one included six replicates. Means followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

[b] Disease category are moderately resistant (MR), moderately susceptible (MS), highly resistant (HR), and highly susceptible (HS).

Therefore, another reliable method (either alternative or complementary to the greenhouse test) is required to identify the Fusarium-wilt highly resistant genotypes. This method should meet two requirements: (i). It should be independent of pathogen and (ii). should reflect the genetic differences among the genotypes. Amino acid composition of cottonseed may meet these requirements. For several reasons, amino acids may have a direct effect on phytopathogenic fungi, they act as fungicides or fungistatics. Amino acids may disturb the normal nitrogen metabolism of the plant resulting in fungicidal or fungistatic substances; they may also change the nutritional conditions for fungus (15).

Amino acid profiles of cotton genotypes

Amino acid profiles can be quickly determined from small quantity of seeds by using HPLC. Therefore, large number of genotypes can be analyzed without sacrificing the seeds. The HPLC analysis of amino acid composition of cottonseeds revealed the presence of 17 amino acids but the occurrence of each amino acid in the seeds varied with the genotype (Table 2).

It is well known that the type and degree of association between the characters may facilitate or complicate the selection process in breeding programmes. Selection for a character may result in improvement or deterioration in other characters, according to the type and degree of correlation. Hence, it was desirable to assess the type and degree of association between the resistance to Fusarium wilt (percentage of healthy seedlings) and the amino acid composition of cottonseed.

Pearson's correlation coefficient (r) was calculated to measure the degree of association between Fusarium wilt resistance and the composition of each amino acid (Table 3). None of the individual amino acids were significantly correlated with resistance

Table 2. Amino acid composition (%, w/w) of cottonseeds

Amino acid	Cotton Genotype											
	427/2002	405/2002	423/2002	514/2002	501/2002	490/2002	449/2002	488/2002	812/2002	491/2002	507/2002	Giza 74
Aspartic	0.031	0.025	0.017	0.120	0.020	0.027	0.116	0.008	0.038	0.031	0.022	0.052
Glutamic	0.208	0.099	0.215	0.183	0.137	0.027	0.072	0.024	0.229	0.125	0.157	0.153
Serine	0.027	0.018	0.024	0.043	0.020	0.027	0.037	0.029	0.030	0.027	0.021	0.069
Glycine	0.053	0.051	0.057	0.049	0.063	0.040	0.037	0.020	0.058	0.074	0.068	0.064
Histidine	0.079	0.014	0.077	0.016	0.059	0.029	0.077	0.022	0.049	0.102	0.065	0.040
Arginine	0.022	0.162	0.028	0.050	0.038	0.039	0.079	0.068	0.163	0.031	0.030	0.054
Threionine0.042	0.088	0.036	0.101	0.038	0.065	0.059	0.035	0.099	0.076	0.034	0.087	
Alanine	0.371	0.240	0.339	0.199	0.322	0.372	0.312	0.158	0.255	0.217	0.235	0.372
Proline	0.038	0.065	0.042	0.063	0.039	0.081	0.035	0.061	0.061	0.074	0.027	0.026
Tyrosine	0.043	0.071	0.038	0.009	0.054	0.075	0.068	0.068	0.042	0.073	0.090	0.050
Valine	0.040	0.037	0.039	0.064	0.040	0.052	0.044	0.043	0.032	0.036	0.052	0.045
Methionine	0.022	0.044	0.025	0.053	0.030	0.067	0.037	0.059	0.031	0.045	0.082	0.036
Cysteine	0.027	0.032	0.019	0.027	0.040	0.075	0.052	0.039	0.009	0.020	0.012	0.010
Isoleucine 0.015	0.022	0.013	0.040	0.022	0.051	0.037	0.023	0.017	0.034	0.025	0.025	
Leucine	0.060	0.043	0.057	0.077	0.055	0.070	0.061	0.054	0.068	0.041	0.045	0.063
Phenylalanine	0.073	0.084	0.077	0.116	0.092	0.086	0.052	0.063	0.056	0.077	0.070	0.087
Lysine	0.038	0.047	0.050	0.020	0.017	0.018	0.017	0.018	0.008	0.051	0.016	0.027

Table 4. Regression equation that describes the effect of amino acid content (X_s) of seeds from 12 cotton genotypes on level of Fusarium wilt resistance (Y)

	Stepwise regression model	R²[a]	F-value[b]
Y =	-59.95 – 3671.39**X14** + 5759.25**X11** + 844.21**X9** – 1265.09 **X16** + 873.54 **X17** – 174.82**X5** – 146.17**X10** – 46.59**X13**	100.00	8327.04

[a] Coefficient of determination. Relative contribution of the predictors **X14** (Isoleucine), **X11** (Valine), **X9** (Proline), **X16** (Phenylalanine), **X17** (Lysine), **X5** (Histidine), **X10** (Tyrosine) and **X13** (Cysteine) to the total variation in Fusarium wilt resistance were 17.13, 50.85, 13.82, 9.57, 7.25, 0.75, 0.58, and 0.04%, respectively.

[b] F-value was significant at $P < 0.0000$.

to Fusarium wilt. The effects of different amino acids concentrations (0, 0.6, 1.2, and 1.8 gL^{-1}) on growth and development of *F. oxysporum* f. sp. *nevium* were determined. Asparagine, methionine, leucine, glutamic acid and phenylalanine promoted the growth and development of *F. oxysporum* f. sp. *nevium* at 1.2 g L^{-1} or higher concentrations but methionine and phenylalanine inhibited the growth and development of *F. oxysporum* f. sp. *nevium* at 0.6 g L^{-1} or less concentrations (5).

Table 3. Relationship of Fusarium wilt resistance [a] of 12 cotton genotypes and amino acid composition (%) of seeds from these genotypes

Amino acid	r [b]	Amino acid	r
Aspartic	-0.048	Tyrosine	-0.360
Glutamic	0.345	Valine	0.303
Serine	-0.058	Methionine	0.151
Glycine	-0.222	Cysteine	-0.388
Histidine	-0.270	Isoleucine	-0.414
Arginine	-0.060	Leucine	0.076
Threionine	-0.188	Phenylalanine	0.109
Alanine	-0.334	Lysine	0.110
Proline	-0.174		

[a] Percentage of healthy seedlings

[b] Pearson's correlation coefficient, which measures the degree of association between Fusarium wilt resistance and composition of the designated amino acid.

Data for Fusarium-wilt resistance and concentrations of amino acids were analyzed by a computerized stepwise multiple regression analysis. The analysis constructed a predictive model by adding predictors, in this case, concentrations of amino acids, 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 significantly contributed to the R^2 value of model (12) Is this the correct reference for this?. Using the predictors supplied by stepwise regression, an eight-factor model was constructed to predict Fusarium-wilt resistance (Table 4).

This model showed that differences in resistance were associated with isoleucine, valine, proline, phenylalanine, lysine, histidine, tyrosine, and cysteine, which accounted for 100% variation in Fusarium-wilt resistance. This study demonstrates that regression models, which include amino acids as biochemical predictors, may provide a supplementary method to greenhouse tests to quantitatively distinguish between the Fusarium wilt resistant and susceptible genotypes.

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