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EVALUATION OF RESISTANCE, ENZYMATIC RESPONSE, AND PHENOLIC COMPOUNDS IN ROOTS OF F_1 CUCUMBER HYBRIDS TO *FUSARIUM OXYSPORUM* F. SP. *RADICIS-CUCUMERINUM*

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ABSTRACT

Cucumber (*Cucumis sativus* L.) is widely cultivated in many parts of the world. Its production is significantly affected by Fusarium root and stem rot, which is caused by the fungus *Fusarium oxysporum* f. sp. *radicis-cucumerinum*, a major disease of cucumber in some regions.Most of the management programs for the control of Fusarium root and stem rot are not successful; therefore, growing resistant cultivars may be the best solution. Use of disease-resistant cultivars is a key to environmentally friendly and economically sustainable disease control in modern crop production. To find resistance sources, 10 F_1 cucumber hybrids were screened against *F. oxysporum* f. sp. *radicis-cucumerinum.* Total phenolic content (PC), soluble peroxidase (POX), and polyphenol oxidase (PPO) activities were evaluated at 0, 4, and 8 days after inoculation. Significant differences were observed between resistant and susceptible genotypes in increase of total PCs and enzymatic activities at fourth day after inoculation. These findings can be used in breeding programs of cucumber aiming at creating F_1 cultivars resistant to Fusarium root and stem rot.

Key words: Fusarium root and stem rot, cucumber, polyphenol oxidase, peroxidase, total phenolic

INTRODUCTION

Two formae speciales of *Fusarium oxysporum* have been described, namely, *Fusarium oxysporum* f. sp. *cucumerinum* J.H. Owen (FOC) (Owen, 1995), causing Fusarium wilt, and, recently, *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (FORC), causing Fusarium root and stem rot (Vakalounakis 1996). Both pathogens are morphologically identical, with the same host range, but expressing slightly different disease symptoms (Lievens et al. 2007; Vakalounakis 1996). Fusarium root and stem rot can be seen in most cucumber growing regions around the world (Neshev 2008; Lauenstein 1955; Martyn 1996; Jenkins & Wehner 1983). In Iran, the presence of the disease has been reported in Jiroft, Yazd, and Varamin in years 2003 and 2004 (Shahriari & Zare 2006), and recently, it is the most important disease of greenhouse cucumber in Iran (Shahriari & Zare 2006).

F. oxysporum is a soil-borne fungal pathogen that infects the plant by penetrating at the root zone, colonizing the vascular tissue, and reducing the water conductance and transpiration of the host plant (Hall et al. 2013). After the host plant dies, resting structures are formed as thick-walled chlamydospores (Beckman 1987). The formation of chlamydospores provides the pathogen with the possibility to reside and persist in soil until the time they are chemotropically stimulated by the root exudates to germinate and infect the host roots (Hsu & Lockwood 1973; Griffin 1981). It has been reported that there can be three races of the pathogen based on their pathogenicity to a differential set of cucumber genotypes (Armstrong et al. 1978). It seems that the

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spread of the races around the world is caused by transfer of infected seeds (Jenkins & Wehner 1983).

The most effective way for controlling Fusarium wilt in cucumber is the development of resistant cultivars (Netzer et al. 1977; Peterson et al. 1982). According to Agrios (1988), organisms apply resistance as an ability to lessen, completely or partially, the effect of a pathogen or other damaging factors. Crop loss by Fusarium wilt disease Race 1 and 2 has been significantly reduced by using resistant cultivars in Europe and the United States (Martyn, 1996); however, the lack of a robust resistant cultivars against Race 3 still limits cucumber production throughout Asian countries: Japan (Namiki et al. 1994), Korea (Ahn et al. 1998), China (Vakalounakis et al. 2004), and within Australia.

Constitutive enzymatic reactions to pathogen infection concern changes in glutathione S-transferases (GST), peroxidases (POX), and phenylalanine ammonia lyase (PAL) activity upon pathogen attack (Klessig et al. 1998). Furthermore, changes in the types and levels of cell wall proteins, proteinase inhibitors, hydrolytic enzymes and pathogenesis-related proteins, and phytoalexin biosynthetic enzymes seem to play big role in Fusarium wilt defense (Yang et al. 1997; Klessig et al. 1998).

As Avdiushko et al. stated (1993), POX catalyzes the formation of lignin and other oxidative phenols that are involved in the formation of defense barriers and reinforcement of the cell structure. It is determined that activities of polyphenol oxidase (PPO) and POX increase in response to infection by pathogen (Khatun et al. 2009; Madadkhah et al. 2012). Morkunas and Gmerek (2007) reported that POX is involved in defense systems of yellow lupinus against the attack of pathogen such as *F. oxysporum*. In similar manner, polyphenol antioxidants play a role in preventing reactive oxygen species (ROS) damage by scavenging free radicals (Torres et al. 2006), altering cell wall composition, and accumulating antimicrobial secondary metabolites which are important in systemic acquired resistance (Ryals et al. 1996). Anand et al. (2007) showed the enhancement of defense enzymes activity and defense-inducing phenolic compounds in the cucumber (*Cucumis sativus* L.) treated with *Pseudoperonospora cubensis* and *Erysiphe cichoracearum*.

On the basis of the study conducted on cucumber so far, it has been found that pathogen infection results in the increase in the PPO levels (Chen et al. 2000). Enzyme activity of resistant melon genotypes increases in response to infection by the pathogen, but no significant differences were observed in susceptible genotypes (Madadkhah et al. 2015).

The aim of the present investigation was to find relationship between resistances against Fusarium root and stem rot and enzymes activity, total phenolic content (PC), and fresh and dry weights of shoot and root in 10 F_1 cucumber hybrids.

MATERIALS AND METHODS

Plants and cultivation

Ten F_1 cucumber hybrids were used in this study. Before sowing, seeds were surface disinfected with sodium hypochlorite (1%) for 1 min and then rinsed in sterile distilled water. Seeds were planted in cell-type plastic growing trays; one seed per cell (10 cm in diameter), filled with a sterile potting mix of peat and perlite (1:1) and were grown under greenhouse conditions (24–28 °C, 16 h day/8 h night, via natural lighting plus high-pressure Sodium Lamps to supply an average lighting level of 10 000 Lux).

Plant inoculation

F. oxysporum f. sp. *radicis-cucumerinum* used in this study was first isolated from Agricultural and Natural Resources Research Center, Varamin, Iran, and identified by using the key described by Nelson et al. (1983) and after Shahriari et al. (2011). Single spores were cultured on potato dextrose agar (PDA) at 22 ± 1 °C with a photoperiod of 14/10 h day/night for 7 days, and before seedlings inoculation, the spores were subcultured on PDA at 25 °C for 7 days with a 12 h photoperiod to induce faster germination. Next, microconidia were rinsed from the surface of the medium with 10 ml sterile water, and the resulting suspension was filtered through two layers of sterile cheesecloth to remove mycelial fragments. Finally, the filtrate was diluted with distilled water to obtain a concentration of 10^6 spores/ml controlled with a hemacytometer.

Table 1. Response of F_1 hybrids to inoculation with Fusarium root and stem rot using the root-dipping method (Fresh Weights – FW, Dry Weights – DW)

F_1 hybrids	FORC +/-	Shoot FW (g)	Plant FW loss (%)	Shoot DW (g)	Plant DW loss (%)	Root FW (g)	Root FW loss (%)	Root DW (g)	Root DW loss (%)
B12 × A10	-	11.39 ± 0.57bc	69.10 ± 0.02c	2.78 ± 0.15 b	68.70 ± 0.27c	2.70 ± 0.13 cd	69.26 ± 1.38c	0.56 ± 0.02g	69.64 ± 1.25c
	+	3.52 ± 0.17h		0.87 ± 0.05g		0.83 ± 0.05k		0.17 ± 0.01ef	
B12 × A4	-	14.27 ± 0.29a	57.11 ± 0.03d	3.47 ± 0.03a	57.06 ± 1.07bd	3.35 ± 0.12a	56.71 ± 0.16d	0.68 ± 0.06a	51.48 ± 1.23e
	+	6.12 ± 0.16fg		1.49 ± 0.05ef		1.45 ± 0.06i		0.33 ± 0.01i	
B12 × A11	-	11.06 ± 0.67c	53.07 ± 1.98e	2.75 ± 0.12de	54.18 ± 1.11e	2.65 ± 0.12d	54.00 ± 1.18e	0.56 ± 0.02g	44.64 ± 3.03f
	+	5.19 ± 0.28g		1.26 ± 0.03f		1.22 ± 0.04j		0.31 ± 0.02de	
B12 × B11	-	11.52 ± 0.61bc	31.25 ± 5.70g	3.00 ± 0.12b	32.00 ± 0.45g	2.89 ± 0.14bc	35.64 ± 2.28g	0.62 ± 0.03d	41.93 ± 0.85g
	+	7.92 ± 0.19de		2.04 ± 0.20cd		1.86 ± 0.05fg		0.36 ± 0.01k	
B12 × A9	-	12.58 ± 0.53b	31.64 ± 0.49g	3.05 ± 0.11b	22.62 ± 1.58h	2.94 ± 0.14bcd	30.61 ± 2.20h	0.63 ± 0.05c	53.96 ± 3.05d
	+	8.60 ± 0.30d		2.36 ± 0.44c		2.04 ± 0.04ef		0.29 ± 0.13cd	
A9 × A11	-	11.12 ± 0.95c	37.59 ± 6.07f	2.85 ± 0.16b	41.75 ± 0.88f	2.73 ± 0.10bcd	41.39 ± 2.05f	0.44 ± 0.02h	40.90 ± 1.34g
	+	6.94 ± 0.02ef		1.66 ± 0.07e		1.60 ± 0.03hi		0.26 ± 0.01cd	
A9 × B11	-	12.27 ± 0.63bc	27.14 ± 0.08 h	3.11 ± 0.14b	30.86 ± 4.30g	2.98 ± 0.12b	28.52 ± 0.77h	0.59 ± 0.01f	25.42 ± 1.25 i
	+	8.94 ± 0.38d		2.15 ± 0.08c		2.13 ± 0.08e		0.44 ± 0.02h	
B11 × A11	-	11.97 ± 0.57bc	38.52 ± 6.80f	2.90 ± 0.16b	39.65 ± 1.72f	2.79 ± 0.13bcd	39.43 ± 1.18f	0.60 ± 0.03e	36.67 ± 3.02h
	+	7.36 ± 0.35e		1.75 ± 0.05gh		1.69 ± 0.11gh		0.38 ± 0.03i	
A11 × A4	-	12.54 ± 0.50b	74.64 ± 1.29b	3.07 ± 0.13b	74.59 ± 1.09b	2.98 ± 0.13b	74.16 ± 1.14b	0.64 ± 0.03b	71.87 ± 1.08b
	+	3.18 ± 0.16h		0.78 ± 0.02g		0.77 ± 0.03k		0.18 ± 0.01ef	
A4 × A10	-	12.59 ± 0.53b	81.10 ± 0.02a	3.07 ± 0.15b	81.11 ± 0.55a	2.96 ± 0.17b	81.08 ± 0.21a	0.63 ± 0.04c	80.95 ± 1.81a
	+	2.38 ± 0.14h		0.58 ± 0.01g		0.56 ± 0.03kl		0.12 ± 0.01f	

Data are average of three replicates, each containing three seedlings. Values within a column followed by the same letter are not significantly different (Duncan's test, p = 0.01).
% weight loss: amount (%) of weight loss in + FORC to no FORC

Table 2. Influence of inoculation with Fusarium root and stem rot on peroxidase (POX), polyphenol oxidase (PPO) enzymes, and phenolic compounds (PCs) in the F_1 hybrids. Comparisons were made for each hybrid separately

F_1 hybrids	Day after inoculation	PPO (ΔOD 515/min/mg protein)	Average PPO (ΔOD 515/min/mg protein)	POX (ΔOD 475/min/mg protein)	Average POX (ΔOD 475/min/mg protein)	PCs (mg/g)	Average PCs (mg/g)
B12 × A10	0	0.40 ± 0.03 c	0.47 ± 0.08 de	27.67 ± 0.57 c	32.45 ± 6.36 e	6.75 ± 0.01 b	7.25 ± 1.76 ab
	4	0.55 ± 0.02 a		39.68 ± 1.52 a		9.21 ± 0.03 a	
	8	0.46 ± 0.01 b		30.00 ± 1.13 b		5.79 ± 0.02 c	
B12 × A4	0	0.44 ± 0.01 c	0.57 ± 0.12 bcd	30.66 ± 2.08 c	40.20 ± 11.98 d	5.71 ± 0.03 c	7.47 ± 2.13 ab
	4	0.69 ± 0.01 a		53.61 ± 0.57 a		9.83 ± 0.02 a	
	8	0.58 ± 0.01 b		36.32 ± 1.52 b		6.78 ± 0.04 b	
B12 × A11	0	0.44 ± 0.01 c	0.56 ± 0.11 cd	30.01 ± 1.00 c	40.32 ± 12.11 d	5.86 ± 0.07 c	7.71 ± 2.14 a
	4	0.67 ± 0.01 a		53.64 ± 1.15 a		10.1 ± 0.02 a	
	8	0.57 ± 0.02 b		37.31 ± 1.52 b		7.16 ± 0.03 b	
B12 × B11	0	0.54 ± 0.03 c	0.69 ± 0.15 ab	39.67 ± 0.58 b	49.55 ± 18.86 b	4.38 ± 0.03 c	7.20 ± 4.40 ab
	4	0.83 ± 0.01 a		71.32 ± 1.15 a		12.28 ± 0.03 a	
	8	0.71 ± 0.01 b		37.66 ± 1.52 c		4.96 ± 0.01 b	
B12 × A9	0	0.54 ± 0.01 c	0.68 ± 0.14 abc	35.33 ± 0.57 c	45.99 ± 16.22 c	4.41 ± 0.03 b	6.20 ± 4.05 bc
	4	0.82 ± 0.02 a		64.65 ± 1.52 a		10.22 ± 0.02 a	
	8	0.69 ± 0.01 b		38.00 ± 1.01 b		3.99 ± 0.01 c	
A9 × A11	0	0.51 ± 0.01 c	0.65 ± 0.14 abc	34.69 ± 1.15 c	46.66 ± 16.38 bc	4.50 ± 0.02 b	6.24 ± 3.90 bc
	4	0.79 ± 0.01 a		65.30 ± 2.08 a		10.08 ± 0.03 a	
	8	0.66 ± 0.02 b		40.00 ± 1.05 b		4.15 ± 0.03 c	
A9 × B11	0	0.55 ± 0.01 c	0. 7 ± 0.14 a	51.02 ± 1.00 b	54.45 ± 19.72 a	4.28 ± 0.02 c	7.23 ± 4.60 ab
	4	0.83 ± 0.01 a		75.66 ± 2.08 a		12.54 ± 0.02 a	
	8	0.72 ± 0.01 b		36.67 ± 1.15 c		4.89 ± 0.01 b	
B11 × A11	0	0.50 ± 0.01 c	0.64 ± 0.12 abc	32.33 ± 1.52 c	43.54 ± 14.55 c	5.53 ± 0.04 c	7.99 ± 2.58 a
	4	0.78 ± 0.01 a		60.00 ± 1.00 a		10.68 ± 0.03 a	
	8	0.64 ± 0.01 b		38.31 ± 0.58 b		7.77 ± 0.03 b	
A11 × A4	0	0.35 ± 0.01 c	0.42 ± 0.07 e	27.65 ± 1.52 c	31.98 ± 5.13 e	5.99 ± 0.03 b	5.90 ± 1.95 c
	4	0.48 ± 0.02 a		37.64 ± 2.51 a		7.81 ± 0.02 a	
	8	0.42 ± 0.01 b		30.65 ± 1.15 b		3.92 ± 0.03 c	
A4 × A10	0	0.32 ± 0.01 c	0.39 ± 0.06 e	26.33 ± 1.15 c	29.33 ± 4.09 e	6.45 ± 0.02 b	5.85 ± 2.19 c
	4	0.43 ± 0.01 a		34.00 ± 1.00 a		7.69 ± 0.04 a	
	8	0.41 ± 0.01 b		27.65 ± 1.15 b		3.41 ± 0.01 c	

Data are average of three replicates, each containing three seedlings. Values within a column for each hybrid separately followed by the same letter are not significantly different (Duncan's test, p = 0.01).

At the stage of the first true leaves, seedlings were removed from growing trays and roots were washed under running tap water. Thereafter, the roots were inoculated by dipping in the conidial suspension, swirling for 10 s, and then replanting back into the same trays. Pots were transferred to a greenhouse with temperature of 20–28 °C (16 h day/8 h night) (Martyn & McLaughlin 1983; Beckman 1987). Nine seedlings of each hybrid were inoculated, and roots of three plants were dipped in sterile water as control. At zero, fourth, and eighth day after inoculation, total PC, soluble POX, and PPO activities were evaluated. The resistance evaluation was expressed in fresh and dry weights of aerial parts and roots at the end of the fourth week. The percentages of weight losses in comparison with control were calculated.

Extraction of POX and PPO and activity assay

The extraction and purification were done according to Janda et al. (2003) method. Root samples (1 g) were ground with mortar and pestle in liquid nitrogen and homogenized in 1 ml sodium phosphate buffer (pH 7.0). The extracts were transferred into 2 ml Eppendorf tubes and centrifuged at 14,000 rpm for 20 min at 4 °C in a refrigerated centrifuge and then the supernatant was stored at −80 °C for analyzing POX and PPO enzyme activities and determining protein concentration. The bovine serum albumin, guaiacol, and proline were obtained from Sigma-Aldrich, Co. Protein concentration was determined according to Bradford (1976) with bovine serum albumin as a standard. POX activity was measured using method proposed by Janda et al. (2003), observing the absorbance change at 475 nm for 1 min at 25 °C in a ultraviolet–visible (UV–vis) spectrophotometer (Perkin Elmer-lambda 25). The activity of PPO was measured according to Chen et al. (2000) method. The change in absorbance was monitored at 515 nm for 1 min at 25 °C.

Extraction and assay of phenolic compounds

Frozen roots (0.5 g) from each replicate were homogenized with 10 ml of 80% acidified methanol, and the extracts were left for 24 h at room temperature before centrifuging at 15 000 for 10 min. One milliliter of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin–ciocalteu reagent, and the solution was kept at 25 °C for 3 min. Then 1 ml of a saturated solution of Na_2CO_3 and 1 ml of distilled water were added, and the mixture was incubated for 1 h at 25 °C. The absorption of the developed blue color was measured using UV–vis spectrophotometer (Perkin Elmer-lambda 25) at 725 nm using as a blank water and reagent only. Caffeic acid (Fluka, Buchs, Switzerland) was used as reference phenolic compound. The total phenolic compounds of samples were expressed in milligrams of caffeic acid per gram of root weight (Swain & Hillis 1959).

Experimental design and data analysis

Both experiments were performed as a factorial based on completely randomized design with two factors and three replications, each containing three seedlings. In the first experiment (Table 1), ten F_1 hybrids (factor 1) were infected or not with pathogen (factor 2). In the second experiment (Table 2), F_1 hybrids was factor 1 and number of days after inoculation with FORC was factor 2. Analysis of variance, and means comparison for statistical significances were assayed using the Duncan's test at $p = 0.01$. All calculations were done by means of the SAS software (version 9.1).

RESULTS

Resistance of F_1 hybrids seedlings to *F. oxysporum* f. sp. *radicis-cucumerinum*

The symptoms of disease on susceptible plants were observed at 8–10 days after inoculation. Symptomatic plants were usually killed within 15–20 days. Reduction of growth and yellowing was observed on resistant plants.

Mean comparison of data showed that FORC affected fresh and dry weights of shoots and roots. The control seedlings differed with fresh and dry weight depending on the genotype. The highest fresh weight of shoots and roots was obtained in B12 × A4 plants, and the lowest fresh weight of shoots in B12 × A11 plants (Table 1). The lowest dry weight of roots had plants A9 × A11 and A9 × B11. All inoculated plants had a lower fresh and dry weight in comparison with control. Decrease expressed in percentages of control plants was from 31.6 to 81.1 (fresh weight of shoots), from 30.9 to 81.1 (dry weight of shoots), from 28.5 to 81.1 (fresh

weight of roots), and from 25.4 to 81.1 (dry weight of roots). The smallest weight losses had seedlings A9 × B11, B12 × A9, and B12 × A11 and the biggest seedlings A4 × A10, A11 × A4, and B12 × A10.

Determination of enzymatic activities and PC contents

The initial (at zero day) values of PPO and POX activities differed between genotypes (Table 2). The most susceptible genotypes had the lowest PPO activity and the highest weight losses. The enzymes activities were usually highest at day 4 and decreased at day 8 even to the level of initial values. The most resistant seedlings were characterized with the highest (about 50%) increase in enzymes activity, whereas the activity of the most susceptible genotypes increased at the day 4 by 34–38%. Very similar pattern of enzyme activity values was found for POX activity. The most resistant seedlings had initially the highest activity and its increase at day 4 rounded to 80–86%, whereas in the most susceptible ones, 29–43%. Quite different results were obtained for the initial contents of PC. Namely, there were no differences between resistant and susceptible genotypes. Increase in PC at day 4 showed the same pattern as for PPO and POX. The increase in most resistant was from 131% to 192%, whereas for most susceptible, only from 19% to 36%.

DISCUSSION

Plants have endogenous defense mechanisms that can be induced in response to attack by insects and pathogens. Here, we report significant differences of disease severity between resistant and susceptible F_1 hybrids as well as in their PCs levels and PPO and POX activities. It has been assumed that resistance to several phytopathogens could be associated with the presence of higher levels of phenols (Kushwaha & Narain 2005). According to Hammond-Kosack and Jones (1996), POX participates in the production of ROS, which are directly toxic to the pathogen. As an indirect effect, they can also decrease the spread of the pathogen by enhancing the cross-linking and lignification of the plant cell walls (Hammond-Kosack & Jones 1996). The induction of PPO activity through pathogens has been reported in a variety of plant taxa, including monocots and dicots (Chen et al. 2000).

Our results confirmed the opinion expressed by Kosuge (1969) that there are direct correlations between increase in PCs and PPO and POX activities and plant resistance. Although in our experiments, there was a significant reduction in the enzymes activity and PC at the eight day in both resistant and susceptible genotypes, the earlier induced activity might play a role in decrease of infections severity. A higher level of enzymatic activity of cell-wall-bound POX has been reported in different plant species, including cucumber (Chen et al. 2000).

Our results suggest that increase in PCs after infection apparently might play a crucial role in the resistance of F_1 hybrids to Fusarium stem and root.

The results for POX and PPO activities strongly support their role in direct defense mechanisms of cucumber against FORC. Increased POX and PPO activities were related to induce resistance to anthracnose in cucumber (Tian et al. 2008). Spore germination and mycelial growth of certain fungi is reported to be inhibited by POX (Joseph et al. 1998).

POX and PPO play a vital role in the defense mechanism against pathogens through participating in the oxidation of PCs to quinines, leading to the increase in antimicrobial activity and inhibiting pathogen progression. According to Schenk et al. (2000), the increase in PPO and POX in reaction to pathogen attack is not the definitive evidence that oxidative enzymes directly participate in plant defenses to restrict pathogen attack, because there is a complex relationship between these enzymes and several chemical compounds in the cell. However, increase in the level of PPO and POX activities and PCs occurred in both susceptible and resistant F_1 hybrids plants as a reaction to infection. The activity increase in the resistant ones was more considerable; though it could be assumed a biochemical indicator for resistance of F_1 hybrids. Results indicate significant differences between reactions of cucumber F_1 hybrids to FORC, so pathogen-derived damage can be reduced by resistance breeding.

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