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Full Length Research Paper

Changes in total phenol, total protein and peroxidase activities in melon (*Cucumis melo* L.) cultivars inoculated with *Rhizoctonia solani*

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The intensive exploration of melon crops in Sistan region favors the occurrence of root diseases such as the *Rhizoctonia* canker, caused by *Rhizoctonia solani*. In this study, eighteen melon cultivars were challenged against the pathogen aiming at selecting those potentially useful for breeding programs and/or integrated disease management. Seeds were planted in infested soil (50 mg of colonized rice grains kg^{-1} of soil). None of the tested melon cultivars was immune to *R. solani*. However, cultivars Sfidak khatdar and Ghandak demonstrated high resistance to *R. solani* and cultivars Sfidak bekhat, Mollamosai, Hajmashallahi and Ahmady were moderately resistant to *R. solani*, while all other melon cultivars were moderately to highly susceptible to *R. solani*. In order to determine resistance mechanism against *R. solani* in melons, the activities of total phenol, total protein and peroxidase in two melon cultivars Ghandak (as resistant) and Sadri (as susceptible) were determined at 0, 24, 48 and 72 h after inoculation. Inoculated resistant cultivar roots had always higher content of total phenol, total protein and peroxidase than the corresponding inoculated susceptible cultivar roots. The results indicated that there was a relationship between resistance in Ghandak and accumulation of total phenol, total protein and peroxidase.

Key words: *Rhizoctonia solani*, resistance, melon, total phenol, total protein, peroxidase.

INTRODUCTION

Diseases rank among the main challenges to the melon agribusiness development. Diseases hamper commercial initiatives for melon production and export, and, therefore, limit investments that would generate capital and jobs (Viana et al., 2001). The intensive melon growing in the Sistan region of Iran favors the occurrence of root diseases including the *Rhizoctonia* canker, caused by *Rhizoctonia solani* Kühn (Safarnezhad, 2004). The canker symptomatology varies widely. *R. solani* can damage different parts of the plant, causing seed, root, and fruit rot; dumping off, and stem canker. All of these diseases lead either to a premature plant death and/or decrease in yield (Bruton, 1998; Geetha et al., 2005). R. *solani* control is extremely difficult, taking into account that it is a soil-borne pathogen that combines high saprophytic competitive ability to a wide host range (Blancard et al., 1991; Bruton, 1996). Michereff et al. (2008) challenged twenty melon genotypes with *R. solani* and reported that genotypes Sancho, AF-1805, Athenas, AF-682, Torreon and Galileo were highly resistant to two *R. solani* isolates. To avoid the disease, farmers often abandon infested areas and migrate to non-infested fields. This practice causes large economic losses, due both to the devaluation of abandoned areas and to the need of re-installing the production infrastructure in the new fields. In this framework, the use of resistant cultivars is a strategic measure in the integrated

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management of the Rhizoctonia canker. Despite the relevance of the disease, apparently there are no reports of melon genetic resistance to the Rhizoctonia canker. To start filling the gap, this study assessed the reaction of 20 melon genotypes to *R. solani*, aiming at subsidizing future melon breeding programs. Concurrently, we intended to select cultivars potentially useful for breeding and for integrated disease management.

Plants possess a range of active defense responses that contribute to resistance against a variety of pathogens. They respond to fungal pathogen attack by activating various defense responses that are associated with the accumulation of several factors like defense related enzymes and inhibitors that serve to prevent pathogen infection. The interaction between the pathogen and host plant induces some changes in cell metabolism, primarily in the enzyme activities, including that of phenylalanine ammonia lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO), lipoxygenase, superoxide dismutase, and β-1,3-glucanase (Babitha et al., 2004; Girish and Umesha, 2005; Kini et al, 2000; Shivakumar et al., 2002). Plant-pathogen interactions are mediated by a complex network of molecular and cytological events that determine a range between susceptibility and resistance. The level of defense-related enzymes is known to play a crucial role in the degree of host resistance. Increase in activity and accumulation of these enzymes depends mainly not only on the inducing agent, but also on the plant genotype, physiological conditions and the pathogen.

POXs are oxido-reductive enzymes that participate in the wall-building processes such as oxidation of phenols, suberization, and lignification of host plant cells during the defense reaction against pathogenic agents (Chittoor et al., 1999; Kolattukudy et al., 1992). Accumulation of lignin and phenolic compounds has been correlated with disease resistance in a number of plant-pathogen interactions. These include wheat/*Puccinia graminis* f. sp. *tritici* (Beardmore et al., 1983), tomato/*Verticillium albo-atrum* (Robb et al., 1987) and rice/*Xanthomonas oryzae* pv. *oryzae* (Reimers and Leach, 1991). Enhanced POX activity has been correlated with resistance in rice (Reimers et al., 1992; Young et al., 1995), wheat (Flott et al., 1989), barley (Kerby and Somerville, 1989) and sugarcane (McGhie et al., 1997) following the inoculation with phytopathogens. POX activity was rather delayed or remained unchanged during the compatible interaction in susceptible plants.

Compounds such as phenols, salicylic acid and benzothiadiazole-7-carbothioic acid S-methyl ester (BTH) are able to induce systemic acquired resistance against a wide range of microbial pathogens in a variety of plants (Sticher et al., 1997). Polyphenols are a part of the complex immune system, which can be acquired in tissues under stress (Feucht, 1994). Contrary to animals, plants cannot synthesize antibodies for defense, but can produce numerous phenolic substances – phytoalexins. These are secondary metabolites that inhibit and kill pathogenic organisms (Bennett and Wallsgrove, 1994). The involvement of phenols in plant disease resistance is based to a large extent on their cytotoxicity, which is associated with their oxidation products (Aver'yonav and Lapikova, 1994). In this study, we identified sources of resistance to *R. solani* isolated from the Sistan region of Iran among a collection of Iranian melon cultivars. In addition, the roles of total phenol, total protein and peroxidase in resistance of melon cultivars to Rhizoctonia collapse disease were discussed.

MATERIALS AND METHODS

Screening of cultivars

Eighteen melon cultivars (Table 1), including Ghandak, Sfidak khatdar, Sfidak bekhat, Mollamosai, Nabijani, Shadegan, Zard evanaki, Moshi, Soosky, Jajrood, Hajmashalahi and Khaghani were collected from several regions of Iran to determine their resistance to *R. solani*. The most virulent isolate of *R. solani* deposited in the Culture Collection of the University of Zabol, was used for this study. *R. solani* was grown on sterilized rice grains (50 g) in Erlenmeyer flasks that were kept for ten days in an incubator at 25°C with constant luminosity (Michereff et al., 2008). The colonized substrate was placed in paper bags and dried for 48 h at 30°C with constant luminosity. Later, the substrate was ground in a blender for 5 min and weighed to prepare aliquots for incorporation into the soil. Sterilized sandy-clay soil was transferred to pots (20 × 20 cm) 21 days after sterilization and infested with *R. solani* (50 mg of colonized substrate per 1 kg of soil). Melon seeds were sown immediately after soil infestation at a density of 10 seeds per pot. The control treatment consisted of seeds sown in non-infested soil. The plants were kept in a greenhouse at an air temperature ranging from 27–35°C.

The experiment was performed using a completely randomized design with three replications. Cultivars were evaluated daily for emergence and, 15 days after sowing, disease severity was assessed using the following scale (Noronha et al., 1995) adapted for melon roots: 0 = symptomless; 1 = small lesions on the hypocotyls; 2 = large lesions on the hypocotyls, but no constriction; 3 = full hypocotyl constriction, showing damping-off; and 4 = non-emerged seeds and/or plantlets. The average disease severity was calculated for each cultivar and was used to cluster the cultivars into five reaction classes: 0 = similar to immune (SI); 0.1 - 1.0 = highly resistant (HR); 1.1 - 2.0 = moderately resistant (MR); 2.1 - 3.0 = susceptible (SU); and 3.1 - 4.0 = highly susceptible (HS). The disease severity index (DSI) in each pot was calculated according to McKinney (1923) using the function DSI = S (score on the scale × frequency) / (total number of units × maximum score on the scale) × 100 and data from the disease scale.

Plant material, pathogen, inoculation and experimental design

The most susceptible and resistant melon cultivars infected with *R. solani*, were used for understanding biochemical mechanism of disease resistance. Seeds of two melon cultivars were planted in pots containing pasteurized soil in the greenhouse after surface sterilization with sodium hypochlorite. Two weeks after germination, all plants were carefully extracted from the pots. Inoculum preparation was carried out based on the method by Michereff et al. (2008). Overall, 72 seedlings of most resistant melon cultivar were transplanted to pots containing infected soil with *R. solani* and 72

Table 1. Reaction of melon cultivars to *Rhizoctonia solani*.

Cultivar	Disease severity	Reaction	Severity (%)
Jajrood	2.330[c]*	SU	60.27[c]
Termeh	3.010[b]	SU	76.27[b]
Soosky	2.997[b]	SU	76.03[b]
Janati	3.047[b]	SU	76.23[b]
Shadegan	2.320[c]	SU	55.47[cd]
Sadri	3.500[a]	HS	87.63[a]
Hajmashalahi	1.777[d]	MR	45.27[e]
Ahmady	1.77[d]	MR	45.21[e]
Zard evanaki	2.497[c]	SU	61.63[c]
Chappat	2.384[c]	SU	60.63[c]
Khaghani	3.053[b]	SU	76.37[b]
Zaboly	2.403[c]	SU	61.57[c]
Moshi	3.519[a]	HS	87.50[a]
Mollamosai	1.217[e]	MR	28.03[f]
Ghandak	1.000[e]	HR	25.00[f]
Nabijani	2.320[c]	SU	50.37[de]
Sfidak khatdar	1.000[e]	HR	25.00[f]
Sfidak bekhat	1.212[e]	MR	28.01[f]

*Mean in the same column followed by the same letter do not differ significantly ($P \leq 0.01$) according to the DSMRT.

seedlings of most resistant melon cultivar were transplanted to pots containing pasteurized soil (control). Eight seedlings were transplanted to each pot. Seedlings of most susceptible melon cultivars were also transplanted as described above. All pots were kept in the greenhouse at 30°C and irrigated twice a week. Pot culture of the two cultivars with four treatments was laid out factorial experiment in completely randomized design (CRD) with three replications (one pot per replication). The sampling was done at 0, 24, 48 and 72 h after inoculation from end roots. Finally, samples were stored at −30°C.

Total phenol content

Total soluble phenol content in melon roots was extracted according to Seevers and Daly (1970). Root samples of 1 g were ground with a pestle and mortar in 16 ml of 80 % methanol. The extract was filtered through cloth and centrifuged at 15000 rpm for 5 min. The supernatant was used for assay of total phenol. The reaction mixture contained 0.5 ml of extract mixed with 7 ml of distilled water and 0.5 ml of Folin-Ciocalteu's reagent. After 3 min, 1 ml of saturated sodium carbonate solution was added and then the volume solution was made up to 10 ml with distilled water. After 1 h, the intensity of the blue colour was measured at 725 nm in a Spectronic 501 spectrophotometer against a blank. Total soluble phenol content was standardized against caffeic acid and absorbance values were converted to mg of phenols per g of fresh weight tissue root. The experiment was conducted in triplicates and repeated three times.

Total protein content

Melon roots for each sample were mixed and a total of 1 g was weighed out and placed in an ice cold mortar. The tissue was homogenized in liquid nitrogen with a pestle. About 1 ml of 50 mM sodium phosphate (pH 7.0) as an extraction buffer was added to the tissue and extraction was done on ice. Homogenate was transferred into an Eppendorf tube and centrifuged at 2400 rpm for 10 min and at 6800 rpm for 15 min at 4°C. The supernatant was transferred into a fresh tube and kept at −30°C until use. The total soluble protein was determined based on the method of Bradford (1976). Bovine serum albumin (BSA) fraction VI was used as a standard. The absorbance was measured at 595 nm using a Spectronic 501 spectrophotometer. Each protein sample was assayed in triplicates and standard curve was made each time.

Peroxidase activity

POX activity was measured in melon root extracts according to Reuveni (1995). Briefly, 1 ml of the reaction mixture contained 20 µg protein, 25 mM citrate–phosphate buffer (pH 5.4) and 1 mM guaiacol as an electron donor. The reaction was initiated by adding 10 µL of 30% H_2O_2 (Merck Co., Germany). Increase in absorbance was measured at 475 nm, 25°C for 30 s using a Spectronic 501 spectrophotometer. The enzyme kinetic was linear for the first 30 s. The results were expressed as an increase in A/min per µg protein. Potassium cyanide was used as an inhibitor for POX.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) and mean separations by Duncan's multiple range test (DSMRT), using MSTAT-C software v.11.0; a *P* value of 0.01 was considered to be significant.

RESULTS AND DISCUSSION

Immunity to *R. solani* was not recorded for any of the

Table 2. Total phenol specific activity in root of melon cultivars.

Time after Ghandak Sadri inoculation	Control	Inoculated	Control	Inoculated
0	1.115	1.117^{d}*	1.017^{d}	1.019^{d}
24	1.328	1.807^{cd}	1.1^{b}	1.14^{d}
48	1.307	2.43^{cd}	1.22^{a}	1.24^{d}
72	1.547	2.501^{bc}	1.25^{a}	1.323^{cd}

*Mean in the same column followed by the same letter do not differ significantly ($P \leq 0.01$) according to the DSMRT.

cultivars studied (Table 1). Cultivars Sfidak khatdar and Ghandak were highly resistant to *R. solani*, while cultivars Sfidak bekhat, Mollamosai, Hajmashalahi and Ahmady were moderately resistant. Moreover, cultivars Nabijani, Zard evanaki, Shadgan, Soosky, Jajrood, Termeh, Janati, Cappat, Zaboly and Khaghani were susceptible and cultivars Moshi and Sadri were highly susceptible. Sfidak khatdar and Ghandak had the lowest levels of canker severity. These cultivars were not significantly different from cultivars Sfidak bekhat and Mollamosai (Table 1). Disease severity levels ranged from 25 - 87.63% (Table 1). Sfidak khatdar and Ghandak had the lowest levels of disease severity. These cultivars were not significantly different from cultivars Sfidak bekhat and Mollamosai. In conclusion, cultivars Sfidak khatdar and Ghandak (highly resistant), Sfidak bekhat, Mollamosai, Hajmashalahi and Ahmady (moderately resistant) collected from the Sistan region were resistant to *R. solani*. Therefore, these cultivars are promising sources of resistance to *R. solani* and should be a preferential choice for melon growing in infested areas.

Screening and the development of resistance to this soil-borne plant pathogenic fungus would be of major benefit to melon growers throughout the Sistan melon-producing region. Successful melon production in areas affected by *R. solani* will include breeding for resistance against this soil-borne plant pathogenic fungus, but the integration of complementary management strategies is required to maximize resistance durability. Among these strategies, field and crop rotation, as well as the destruction of crop remains, could be very effective. Sources of resistance to *R. solani* (Michereff et al., 2008) have already been identified. However, no attempt has been made to screen Iranian melon cultivars for resistance to *R. solani*. This paper is the first report of the screening of melon cultivars for resistance to *R. solani* in Iran.

The total phenol content of two melon cultivars Ghandak (as resistant) and Sadri (as susceptible) were determined at different times after inoculation with *R. solani* (Table 2). The total phenol content increased in resistant cultivar roots in response to inoculation with *R. solani* as compared to un-inoculated roots. There was no significant difference in the total phenol content between inoculated susceptible cultivar and un-inoculated control. The increase in total protein was observed in resistant cultivar roots on the very next day after inoculation with the pathogen. The increasing trend continued in resistant cultivar roots up to 72 h after inoculation. In resistant cultivars roots, the maximum total protein of 2.501 mg g^{-1}, was found in *R. solani* inoculated roots on 72 h after inoculation. Inoculated resistant cultivar roots had always higher content of total phenol content than the corresponding inoculated susceptible cultivar roots. The total phenol content was always higher in inoculated resistant cultivar roots than the un-inoculated roots (Table 2). In general, it was concluded that there was a relationship between resistance in Ghandak and accumulation of phenolic compounds. These findings are in agreement with those of other studies (Kiraly and Farkas, 1962; Lorber et al., 1972). Mandavia et al. (1997) also observed increased phenolic content in root and stem tissues of chickpeas plants that were both resistant and susceptible to wilt fungi infection.

The present study also showed that the pathogen inoculation of resistant melon cultivars resulted in increased accumulation of phenolics. It is known that the presence of phenolic compounds in plants and their synthesis in response to infection is associated with resistance (Mohammadi and Kazemi, 2002) and studies have indicated that greater accumulation of phenolics due to increased PAL activity offered protection against diseases (Geetha, et al., 2005; Girish and Umesha, 2005). Induced accumulation of phenolics into cell walls acts by strengthening the cell wall and also by releasing anti-microbial compounds, which leads to a resistance response. The amount of phenolics accumulation was greater in resistant cultivars than in susceptible cultivars in response to *R. solani* infection.

The total protein of two melon cultivars Ghandak (as resistant) and Sadri (as susceptible) were determined at different times after inoculation with *R. solani* (Table 3). Total protein increased in resistant and susceptible cultivars roots in response to inoculation with *R. solani* as compared to un-inoculated ones. The increase in total protein was observed on the very next day after inoculation with the pathogen. The increasing trend continued up to 72 h after inoculation. In resistant and susceptible cultivars roots, the maximum total protein of 3.626 and 2.609 mg g^{-1}, respectively, were found in *R. solani* inoculated roots on 72 h after inoculation. Inoculated resistant cultivar roots had always higher

Table 3. Total protein specific activity in root of melon cultivars.

Time after Ghandak Sadri inoculation	Control	Inoculated	Control	Inoculated
0	2.11f^{g}*	2.12^{f}	2.033^{h}	2.043^{gh}
24	2.113^{fg}	2.331^{e}	2.037^{h}	2.141^{f}
48	2.113^{fg}	3.19^{b}	2.039^{h}	2.411^{d}
72	2.118^{fg}	3.626^{a}	2.04^{gh}	2.609^{c}

*Mean in the same column followed by the same letter do not differ significantly ($P \leq 0.01$) according to the DSMRT.

Table 4. Peroxidase specific activity in root of melon cultivars.

Time after Ghandak Sadri inoculation	Control	Inoculated	Control	Inoculated
0	0.516^{e}*	0.52^{e}	0.4^{e}	0.413^{e}
24	0.518^{e}	1.343^{c}	0.405^{e}	0.902^{d}
48	0.52^{e}	1.801^{b}	0.408^{e}	0.991^{d}
72	0.523^{e}	2.219^{a}	0.41^{e}	1.381^{c}

*Mean in the same column followed by the same letter do not differ significantly ($P \leq 0.01$) according to the DSMRT.

content of total protein than the corresponding inoculated susceptible cultivar roots. The total protein was always higher in inoculated resistant and susceptible cultivars roots than the un-inoculated roots (Table 3). In general, it was concluded that there was a relationship between resistance in Ghandak and accumulation of total protein.

The peroxidase of two melon cultivars Ghandak (as resistant) and Sadri (as susceptible) were determined at different times after inoculation with *R. solani* (Table 4). Total peroxidase activity increased in resistant and susceptible cultivars roots in response to inoculation with *R. solani* as compared to un-inoculated roots. The increase in peroxidase activity was observed on the very next day after inoculation with the pathogen. The increasing trend continued up to 72 h after inoculation. In resistant and susceptible cultivars roots, the maximum peroxidase activity of 2.219 and 1.381 mg g^{-1}, respectively, were found in *R. solani* inoculated roots on 72 h after inoculation. Inoculated resistant cultivar roots had always higher peroxidase than the corresponding inoculated susceptible cultivar roots. The peroxidase activity was always higher in inoculated resistant and susceptible cultivars roots than the un-inoculated roots (Table 4). In general it was concluded that there was a relationship between resistance in Ghandak and accumulation of peroxidase. Peroxidases are implicated to have major role in plant pathogen interaction (Castillo, 1992), participate in a variety of defense mechanisms (Moerschbacher, 1992) in which H_2O_2 is often supplied by an oxidative burst, a common event in defense responses (Lamb and Dixon, 1997). The association of peroxidases with disease resistance/susceptibility to pathogen has been reported in many crops. High peroxidase activity has been associated with resistance in tomato against *Fusarium oxysporum* f. sp. *lycopersici* (Retig, 1974), in pepper to *Phytophthora capsici* (Alcazar et al., 1995), in mung bean to *R. solani* (Arora and Bajaj, 1985) and in soybean against *Phytophthora sojae* (Melger, 2006), similar to the results in this study. Several roles have been attributed to plant POX in the host-pathogen interaction, including induced resistance. In the resistant cultivar, the activity of POX was higher when compared with the un-inoculated seedlings.

It is clear from the present study that the defense-related enzymes are up-regulated in the resistant melon cultivars upon pathogen inoculation, whereas in the susceptible melon cultivars the levels of these enzymes remained unchanged or down-regulated. This was correlated with the up-regulation and down-regulation of total phenol contents in resistant and susceptible melon cultivars in the presence and absence of pathogen infection. Hence, the regulated levels of defense-related enzymes and the total phenols contents can be effectively used to screen for host resistance. This will overcome the laborious, time-consuming, labor-intensive procedure for screening melon cultivars under field conditions. Further studies on the accumulation of transcripts of these enzymes are needed to demonstrate the role of these defense-related enzymes in melon and the *R. solani* system.

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