Biochemical Evaluation of Resistance Responses of Potato to Different Isolates of *Alternaria solani*

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ABSTRACT

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The resistance phenotypes of nine potato cultivars to five isolates of *Alternaria solani*, causal agent of early blight, were studied after inoculation and growth under greenhouse conditions. We identified potato cultivars with both susceptible and resistant phenotypes as well as *A. solani* isolates with varying degrees of aggressiveness. Two potato cultivars and two pathogen isolates were selected for biochemical analysis of phenol production and peroxidase activity after inoculation. Phenol compounds were evaluated 2, 4, 6, and 8 days after inoculation, while

peroxidase activities were monitored daily for 10 days. Native polyacrylamide electrophoresis was used to identify one protein with peroxidase activity in extracts taken 6 days after inoculation. Significantly higher peroxidase activity as well as total phenol content in potato was correlated with resistance in the Iranian potato cultivar Diamond. Variability of responses within the same cultivar to different isolates of *A. solani* suggests genotypic diversity between isolates that results in phenotypic diversity for pathogen aggressiveness.

Additional keywords: disease resistance, host defense responses, plant peroxidase, plant phenolics.

Early blight, caused by the necrotrophic ascomycetous fungus *Alternaria solani* (Ellis & G. Martin) Sorauer, is one of the most common diseases of potatoes, especially where potatoes are grown under irrigation. The disease causes yield losses through defoliation of the plants and severe epidemics of early blight may reduce potato yields by as much as 20 to 30% (7,32). Early blight can also impact the quality of the harvested tubers. Fungicides used to control the disease are expensive and frequently inefficient (5). The development of potato varieties resistant to early blight remains one of the best strategies for long-term control of this disease.

To date, no monogenic traits conferring resistance to *A. solani* have been found in potato. Resistance is thought to be correlated with plant age since susceptibility increases as plants grow older (9,28). In tomato, secondary plant metabolites correlated with early blight resistance include phenolic compounds (tannins, flavonols, and phenols) in leaves and stems (1). In addition, the fruit of resistant tomato varieties contain a higher amount of phenolic compounds than those from susceptible varieties (1). The constitutive expression of phenols, which are thought to function as preformed inhibitors, is associated with nonspecific basal resistance to multiple pathogens in all plant species (26). In addition to phenolics, the production of reactive oxygen species, such as hydrogen peroxide and superoxide, also has an integral role in pathogen defense (17). Peroxidase enzymes are important in the production of hydrogen peroxide and have been linked to increased disease resistance in plants (2). Peroxidase also shows affinity to substrates involved in cellular lignification and the

products of its activity have direct antimicrobial activity in the presence of hydrogen peroxide (29).

In Iran, early blight is a ubiquitous disease that can cause serious crop losses under optimum conditions including high temperature, humidity, and susceptibility and physiological ages of the cultivars. The primary objective of our study was to identify sources of potato early blight resistance and characterize biochemical responses to different isolates of *A. solani*.

MATERIALS AND METHODS

Cultural conditions. Potato seeds (*Solanum tuberosum* L.) were planted in plastic pots (30 cm diameter) containing soil and perlite under greenhouse conditions (18 to 25°C) at Tehran University. Tubers were treated with thiabendazole (tekto WP60%, Golsam Gorgan Company, Tehran) before planting.

***A. solani* isolation and spore production.** Potato leaves infected with *A. solani* were collected in 2008 from different locations and potato cultivars from Arak Town (Markazi Province) in Iran. Five isolates of *A. solani* were recovered from sections of potato leaves with typical early blight lesions. The leaf sections were surface-sterilized in a 1% sodium hypochlorite solution for 1 min, rinsed with sterile distilled water, placed on potato dextrose agar (PDA), and incubated under continuous light at 20 ± 1°C. Hyphal tips of mycelium that grew from diseased tissue were transferred to petri dishes containing 15 ml PDA. Hyphal-tip sections were incubated for 7 days at 24 ± 1°C on PDA under 16 h/day near-ultraviolet light (310 to 400 nm). Single spores from hyphal-tipped fungal colonies were transferred to PDA dishes to obtain pure cultures of *A. solani* (N isolate, AM isolate, M isolate, A isolate, and H isolate). The isolates were maintained on PDA at 20 ± 1°C for 7 days.

Plant inoculations and evaluations. Single spore isolates of *A. solani* were grown on PDA for 6 days at 24 ± 1°C under 16 h/day near-ultraviolet light (310 to 400 nm) in order to induce sporulation. Conidia from these in vitro cultures were collected by

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washing with sterile water and diluted to a concentration of 10^6 spores ml^{-1} (3,25).

Certified seed tubers of *S. tuberosum* 'Diamond', 'Granula', 'Agrida', 'Milva', 'Lady rozeta', 'Satina', 'Maradona', 'Sante', and 'Santana' potatoes were planted. Four plants (45 days old) of each cultivar were inoculated separately with each isolate of *A. solani* or a sterile water control. The plants were sprayed with the conidial suspension or sterile water and covered with clear plastic bags for 24 h to increase humidity and accelerate infection. The plants were grown under normal conditions in the greenhouse at 26°C (3,28). The intensity of infection was recorded using the scale described in Table 1 (30). The two cultivars with the lowest and highest early blight infection scores and the two *A. solani* isolates with the least and greatest aggressiveness were selected for further evaluations.

Phenol assay. Leaf tissue (0.5 g) was ground in liquid N_2 with a mortar and pestle. It was extracted separately in 8 ml of 80% methanol. The extracts were centrifuged at $4,000 \times g$ for 5 min (24). To determine the amount of total phenols, 1 ml of leaf extract was placed in test tubes containing 5 ml of distilled water, 250 μl of Folin, and 1 ml of 20% sodium carbonate. The test tubes were stirred and readings were taken after 60 min using an absorbance spectrophotometer at 720 nm. Total phenol was expressed as the milligrams per gram of leaf fresh weight (24).

Peroxidase assays. Leaf tissue was ground in liquid N_2 with a mortar and pestle. Approximately 0.5 g of tissue was transferred to 1 ml of extraction buffer (0.1 M sodium phosphate, pH 6.0). Extracts were centrifuged at $14,000 \times g$ for 20 min at 4°C and the supernatant (enzyme extract) was transferred to a new tube on ice until assayed (23). Protein concentration was determined according to Bradford (4) with bovine serum albumin as a standard.

Enzyme extracts (containing 30 μg of total protein) were added to 30 μl of 200 mM guaiacol and 25 mM citrate phosphate (pH 5.4). To each sample 30 μl of 30% H_2O_2 was added and the absorbance was read at 470 nm in a UV-VIS spectrophotometer. The activity was calculated as the rate of increase of absorbance per minute, and expressed as unit per milligram of protein (14).

Native PAGE. Isozymes of peroxidase were analyzed by native polyacrylamide gel electrophoresis (PAGE) (31). Samples were prepared by homogenizing 0.5 g of leaflets from *A. solani* or water-inoculated plants. *Alternaria*-only samples were prepared from liquid-grown cultures and treated the same as plant tissue samples. Electrophoretic separation was performed under non-denaturing conditions using a 6% stacking gel and 12% separation gel containing Tris-glycine buffer, pH 8.3. Gels were incubated in 25 mM citrate-phosphate buffer (pH 5.4) containing guaiacol for 30 min and enzyme bands were visualized with a 1% solution of H_2O_2 . The gels were photographed and the R_f values of each band were calculated (distance run by isoenzyme/distance run by bromophenol blue).

Analysis of results. All experiments were carried out using a factorial model of a completely randomized experimental design in four replicates per treatment. In greenhouse evaluations, the factorial model was 9×5 , in total phenol determinations it was $2 \times 3 \times 4$ (two potato cultivars $\times$ three treatments: two *A. solani* isolates and sterile water $\times$ 4 days), and in peroxidase assays it was $2 \times 3 \times 10$ (two potato cultivars $\times$ three treatments $\times$ 10 days). Statistical analysis was performed using SAS version 9 (20). Analysis of variance was performed on all data sets. A Duncan test with a probability of 0.05 was used to show significant differences between treatments. All data are presented as means. Analyses of variance for greenhouse, peroxidase activity, and total phenol evaluations were significant at the 1% level ($P < 0.01$).

RESULTS

Greenhouse evaluations. Greenhouse infection assays of multiple potato cultivars with different isolates of *A. solani* re-

vealed significant differences between hosts. The intensity of the infection was determined by visual rating of inoculated plants using a scale from 0 to 100 based on the symptoms described in Table 1. Plants with a lower rating were considered more resistant than those with a high rating. *S. tuberosum* 'Diamond' exhibited the lowest disease severity with an average score ($\pm$ standard error) of 16 (± 2) among the five *A. solani* isolates (Fig. 1). Cultivar Granula showed the highest disease severity with an average score of 51 (± 2). Analysis of variance indicated a significant difference between responses of cultivars to *A. solani* infection. Other cultivars fell between 'Diamond' and 'Granula' with no clear separation between resistant and susceptible cultivars.

Among *A. solani* isolates used in these assays, isolate A exhibited the highest aggressiveness with an average host disease severity score of 45 (± 2). Isolate N was the least aggressive with an average host score of 24 (± 2). The other isolates exhibited an intermediate level of aggressiveness ($A > AM > M > H > N$). No immunity to *A. solani* was observed in any of the cultivars used in this study. Also no significant interactions between a specific isolate and host were observed since the isolates exhibited consistent patterns of aggressiveness. However, since 'Diamond' exhibited significantly less disease severity than 'Granula', we used these two cultivars as well as *A. solani* isolates A (most aggressive) and N (least aggressive) to assay defense response differences.

Total phenol. Total phenol content was significantly higher in pathogen-inoculated leaves when compared with healthy, water-inoculated controls (Fig. 2). Total phenol in 'Diamond' was higher than 'Granula' on all days tested. Significant differences in phenolic levels between the two cultivars were observed on all days of the assay (Table 2). In 'Granula', the level of phenolic compounds increased between the second and fourth day, and then decreased up to 8 days after inoculation. Total phenols decreased significantly in 'Diamond' between 2 and 8 days after inoculation.

Differences in total phenol were also observed over time when using isolates of *A. solani* with different aggressiveness phenotypes (Fig. 2). The phenol levels in 'Diamond' and 'Granula' were averaged 2, 4, 6, and 8 days after inoculation with *A. solani* isolates A and N. There were significant differences between phenol levels elicited by isolate A, isolate N, and the water control on all days (Table 2). The less aggressive strain N elicited more total phenol than the more aggressive isolate A. Both *A. solani* isolates elicited similar patterns of total phenol accumulation compared with the water control with an increase between 2 and 4 days after inoculation followed by a decrease over the next 4 days.

Peroxidase activity. Peroxidase activities were slightly higher in 'Diamond' when compared with 'Granula' and this difference was significant ($P = 0.0098$; Table 3). Peroxidase activity in plants inoculated with either *A. solani* isolate A or N was higher than in control plants sprayed with water (Fig. 3). When peroxidase activity was monitored over time after inoculation with *A.*

TABLE 1. Scale for evaluation of the foliar damage in potato caused by *Alternaria solani*

Rating	Description of symptoms
10	Spots on lower leaves
20	Spots on most of the lower leaves
30	Spots on all lower and some of the middle leaves
40	Clearly developed blight lesions in lower leaves
50	Blight lesions in lower leaves spread to some middle leaves
60	Blight lesions developed in all inferior and most of the middle leaves
70	Blight lesions developed in all lower and middle leaves
80	Blight lesions developed in all lower and middle leaves and spread to upper leaves
100	Total blight (death of the plant)

solani isolates in both cultivars Diamond and Granula, the highest activity was observed on the second or third day, depending on the isolate used, while the lowest activity was detected 10 days after inoculation.

Electrophoretic patterns of peroxidase activities on the sixth experimental day are presented in Figure 4. The sixth day was chosen because the peroxidase activity in 'Diamond' was similar in response to the A and N isolates, but the isoenzyme banding pattern was most dissimilar. Under native conditions, only one isoenzyme band could be detected in all plant samples and was not present in fungal samples, demonstrating that the peroxidase isoenzyme is derived from plant tissue and not *Alternaria*. The isoenzyme in 'Diamond' appeared more abundant than in 'Granula' regardless of the *A. solani* isolate used.

DISCUSSION

We have shown that resistance to *A. solani* in the resistant potato cultivar Diamond is correlated with increased host defense responses including accumulation of total phenol and elevated peroxidase activity. Resistant and susceptible cultivars Diamond and Granula, respectively, could be significantly separated not only by disease severity scores but also by quantification of total phenol and peroxidase levels.

A. solani isolates with varying degrees of aggressiveness were identified using greenhouse infection assays. Two of these isolates, A and N, induced significantly more or less disease, respectively, regardless of the host cultivar used. Differences in disease symptomatology elicited by isolates A and N was also

Fig. 1. Disease severity scores of greenhouse-grown potato cultivars inoculated with *Alternaria solani* isolates (H, M, N, AM, or A) or water (CO). Visual disease symptoms were used to determine disease severity scores according to the scale in Table 1. Bars are presented in the following order: H, M, N, AM, A, and CO. Error bars show the standard error among four replications.

Fig. 2. Total phenol levels of potato cultivars Diamond and Granula after inoculation with *Alternaria solani*. DCO, 'Diamond' sprayed with sterile water; GCO, 'Granula' sprayed with sterile water; GA, 'Granula' sprayed with *A. solani* isolate A; DA, 'Diamond' sprayed with *A. solani* isolate A; DN, 'Diamond' sprayed with *A. solani* isolate N; and GN, 'Granula' sprayed with *A. solani* isolate N. Results are the means of four replications. Error bars denote standard error. LFW = leaf fresh weight.

consistent between inoculations for greenhouse evaluations and those for phenol and peroxidase assays. Although genotypic differences have not been established for these isolates, the presence of multiple strains of *A. solani* in potato growing regions is not uncommon (21,33). Isolates from the same host, such as potato, have been found to be more closely related to one another than those collected from another host, such as tomato (34).

TABLE 2. Variance analysis of total phenol in two cultivars after inoculation with two isolates of *Alternaria solani*^a

Source	df	Mean square	F value	P > F
Cultivar	1	0.114	149.20	<0.0001
Treatment	2	0.022	29.85	<0.0001
Day	3	0.010	6.98	0.0017
Cultivar × treatment	2	0.005	14.10	<0.0001
Cultivar × day	3	0.016	21.14	<0.0001
Treatment × day	6	0.005	7.57	<0.0001
Cultivar × treatment × day	6	0.004	5.97	<0.0001
Error	72	0.0007		

^a Plants were also treated with water as a control.

TABLE 3. Variance analysis of peroxidase activity in two cultivars after inoculation with two isolates of *Alternaria solani*^a

Source	df	Mean square	F value	P > F
Cultivar	1	0.157	5.81	0.0098
Treatment	2	0.179	6.62	0.0004
Day	9	1.020	4.14	0.0003
Cultivar × treatment	2	0.112	37.37	<0.0001
Cultivar × day	9	0.097	3.59	<0.0001
Treatment × day	18	0.308	11.28	<0.0001
Cultivar × treatment × day	18	0.242	8.88	<0.0001
Error	180	0.027		

^a Plants were also treated with water as a control.

Therefore, among isolates collected from the same host, any genetic variability that translates to differences in aggressiveness is of interest and warrants further study.

Resistance to *A. solani* in potato is associated with responses that take place after pathogen spore germination, appressoria formation, and penetration (8). Although movement and growth of the pathogen was not assayed in our experiments, the differences between total phenol and peroxidases observed in our experiments took place after the 24 to 36 h that is typically required for penetration into the leaf tissue (8) suggesting that these responses are a result of intimate contact between host and pathogen. Resistance to *A. solani* in potato is correlated with an HR-like response (defined as autofluorescence, granular cytoplasm, and thickened cell wall but no cell death) (8). However, because of the necrotrophic nature of *A. solani* it is unclear whether a complete HR (including programmed cell death) alone would be capable of inhibiting pathogen spread. It is therefore likely, and consistent with results presented here, that increased defense responses play an important role in responses to *A. solani*. Resistance to several phytopathogens has been attributed to the presence of higher levels of phenol (13,16,27). Additionally, peroxidase is involved in the production of reactive oxygen species, which are directly toxic to the pathogen or indirectly reduce the spread of the pathogen by increasing the cross linking and lignification of the plant cell walls (11). An early increase in peroxidase activity, similar to that in our study, was recorded in the tropical legume *Stylosanthes humilis* inoculated with *Colletotrichum gloeosporioides* (12), suggesting that the early induction might have a role in limiting the number of successful infections. In our study however, it is unlikely that production of peroxidase 2 or 3 days after inoculation is associated with the inhibition of penetration of the host since peroxidase activity in both cultivars Diamond and Granula was high but they showed different levels of resistance to both isolates A and N on

Fig. 3. Peroxidase activity in leaves of potato cultivars Diamond and Granula after inoculation with *Alternaria solani*. DCO, 'Diamond' sprayed with sterile water; GCO, 'Granula' sprayed with sterile water; GA, 'Granula' sprayed with *A. solani* isolate A; DA, 'Diamond' sprayed with *A. solani* isolate A; DN, 'Diamond' sprayed with *A. solani* isolate N; and GN, 'Granula' sprayed with *A. solani* isolate N. Results are the means of four replications. Error bars denote standard error.

Fig. 4. Isoenzyme patterns of peroxidase identified by native polyacrylamide gel electrophoresis 6 days after inoculation. Samples are shown as follows: DCO, 'Diamond' sprayed with sterile water; GCO, 'Granula' sprayed with sterile water; GA, 'Granula' sprayed with *Alternaria solani* isolate A; DA, 'Diamond' sprayed with *A. solani* isolate A; A, *A. solani* isolate A alone; DN, 'Diamond' sprayed with *A. solani* isolate N; GN, 'Granula' sprayed with *A. solani* isolate N; and N, *A. solani* isolate N alone.

those days. Together, differences in accumulation of phenolic compounds between resistant 'Diamond' and susceptible 'Granula' as well as changes in peroxidase activity after inoculation indicate that these responses are closely associated with defense responses in cultivated potato only after penetration and intimate contact between the host and pathogen.

The fact that we observed no clear separation between resistant and susceptible potato cultivars suggests that *A. solani* resistance is not monogenic and that varying degrees of resistance are determined by the contribution of multiple defense genes. In addition to environmental variables, factors such as plant age are known to be involved in resistance to early blight (9,28). Plants tested in our greenhouse experiments were of an equally young age (45 days old) and environmental conditions were kept uniform. Therefore, disease severity score differences between cultivars are likely to be due to variation in the host genotypes. Although total phenols and peroxidase activity appear to contribute to resistance to *A. solani* in potato, other factors such as pathogenesis-related (PR) gene expression are likely involved. In tomato, polygenic resistance to *A. solani* is correlated with increased levels of the PR proteins chitinase and β -1,3-glucanase (18,19). Further analysis of the suite of host responses to early blight is necessary to determine the components critical for adequate resistance to the disease.

Resistance to *A. solani* has been a focus of potato and tomato breeders for a number of years. However, the development of resistant cultivars has been hampered by both a lack of resistant germplasm sources, the polygenic nature of inheritance, and variability among accessions of the same species (6,15). Difficulty in breeding for resistance is further compounded by the length of time it takes to make phenotypic evaluations of large numbers of individuals in the field. Detached leaf assays do not correlate well with field results (10,22). Greenhouse assays significantly speed the process, however our results suggest that an evaluation of *A. solani* isolates be made before use in greenhouse screening experiments in order to ensure that a highly aggressive isolate has been identified. Alternatively, the correlation between resistance and defense responses such as phenol production leads to the possibility of using this as a screen for potentially resistant germplasm since we would expect that cultivars with higher basal levels of phenolics are less susceptible than those with lower basal levels.

Understanding the biochemical responses that take place during resistant and susceptible interactions between cultivated potato and *A. solani* will help us to differentiate truly resistant germplasm from cultivars that might contain late-maturity traits that indirectly affect pathogen growth. Not surprisingly, we found considerable variability among resistance traits of cultivated potato varieties. Only nine Iranian potato cultivars were analyzed in our experiments and we would expect to find additional phenotypic diversity among cultivars derived from other breeding programs. Wild potato species also contain diverse sources of *A. solani* resistance traits and biochemical assays that can assist in screening for useful germplasm would be helpful. We also found considerable variation of aggressiveness among different *A. solani* isolates. This variability resulted in quantitative differences in defense responses elicited upon inoculation and suggests that a gene-for-gene interaction, where one major host gene product is

responsible for the recognition of a single pathogen gene product, does not exist between the cultivars and isolates used in these experiments.

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