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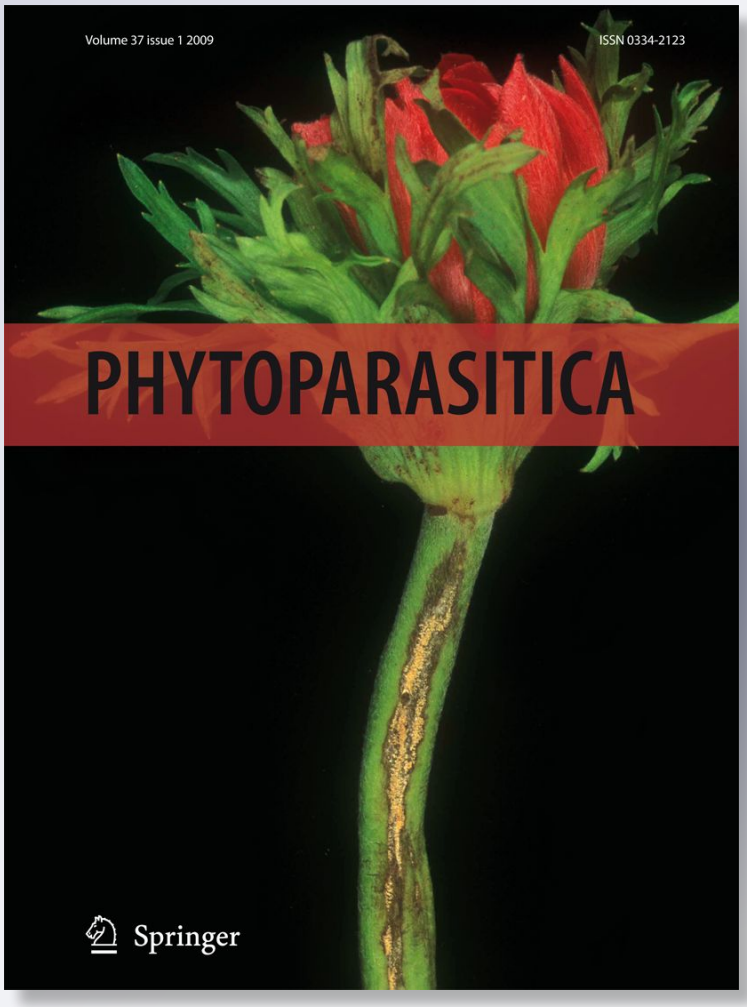

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Activity of ß-1,3-glucanase and ß-1,4-glucanase in two potato cultivars following challenge by the fungal pathogen *Alternaria solani*

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Abstract Early blight of potato, caused by *Alternaria solani*, is a ubiquitous disease in many countries around the world. Our previous screening of several Iranian potato cultivars found that variation in resistance exists between two cultivars: 'Diamond' and 'Granula'. Cultivar Diamond is more resistant to multiple isolates of *A. solani* when compared to cv. Granula. Furthermore, we have found that different pathogen isolates have varying degrees of infection. We monitored the activities of two pathogen-related (PR) glucanase proteins in Diamond and Granula in response to two isolates of *A. solani* with different degrees of virulence. ß-1,3-glucanase and ß-1,4-glucanase activities were recorded in healthy and diseased leaves of potatoes up to 10 days after inoculation. Their activities were found to be higher in diseased leaves when compared to those of uninfected leaves. Our data suggest that significantly reduced activities of theses enzymes in potato could be related to a lower degree of resistance or an increased ability of a more aggressive isolate to suppress PR protein expression.

Keywords Early blight · Pathogen-related protein · *Solanum tuberosum*

H. Shahbazi (✉)
Young Researchers Club, Arak Branch,
Islamic Azad University,
Arak, Iran
e-mail: sah.shahbazi@yahoo.com

H. Aminian · N. Sahebani
Department of Plant Protection, Aboryhan Campus,
Tehran University,
Tehran, Iran

D. A. Halterman
U.S. Department of Agriculture,
Agricultural Research Service,
Vegetable Crops Research Unit,
Madison, WI 53706, USA

Introduction

Potato (*Solanum tuberosum* L.) is considered one of the most important vegetable crops grown worldwide. Early blight (EB), caused by *Alternaria solani*, affects primarily leaves of potato, but under the right conditions it can also cause dry rot in storage of tubers infected through harvest wounds (Valmeekam et al. 2001). *A. solani* is a widespread and important pathogen of potatoes in many countries, including Iran. EB is common under optimum conditions, *viz.* high temperature, humidity, and advanced physiological age of the plant, resulting in serious damage in potato fields. In order to reduce fungicide applications to control EB, integration of host resistance and modification of cultural practices is necessary.

Defense responses of plants against biotic stresses include a multitude of tools, including various types of proteins with putative protective functions. Expression of a group of proteins, commonly referred to as

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pathogenesis-related (PR) proteins, plays an important role in plant defense against pathogens and in general adaptation to stressful environments (Edreva 2005). An important common feature of most PR proteins is their antifungal effect, with some also exhibiting antibacterial, insecticidal, nematicidal, and antiviral action (Van Loon et al. 2006). Activity of PR proteins can be generally attributed to their hydrolytic, proteinase-inhibitory and membrane-permeabilizing abilities. Thus, hydrolytic enzymes such as β-1,3-glucanases, chitinases, and proteinases are important for weakening and decomposing fungal cell walls that contain glucans, chitin, and proteins (Selitrennikoff 2001; Van Loon and van Strien 1999).

Early blight resistance in tomato is correlated with a higher and more rapid induction of the PR proteins chitinase and *ß*-1,3-glucanase following challenge by the pathogen *A. solani* (Lawrence et al. 1996, 2000). *ß*-1,4-glucanase may also play an important role in plant–pathogen interactions since a reduction of this enzyme in transgenic tomato fruit resulted in increased resistance in the necrotroph *Botrytis cinerea* (Flors et al. 2007). The objective of the present study was to investigate the changes in ß-1,3-glucanase and ß-1,4-glucanase following an infection by *A. solani* in potato using both resistant and susceptible host cultivars and two pathogen isolates with differing degrees of aggressiveness. We found that both of these factors have a significant impact on the expression of ß-glucanase activities after inoculation.

Methods

Plant growth conditions Certified seed tubers of potato (*Solanum tuberosum* L.) cvs. 'Diamond' and 'Granula' were planted in plastic pots (30 cm diam) containing peat and perlite under greenhouse conditions (18–25°C) at Tehran University. Potato seed consisted of small whole tubers weighing ~50 g each. Tubers were treated with thiabendazole (tekto WP 60%, Golsam Gorgan Company, Tehran, Iran) prior to planting to prevent tuber rot.

Alternaria solani inoculum preparation Potato leaf samples, infected with the *A. solani* fungus, were collected in 2008 from commercial potato fields in Iran. Two isolates of *A. solani* were recovered from sections of potato leaves with EB lesions. One isolate, named "A", had been characterized previously as being highly aggressive on potato and a second isolate, named "N", was less aggressive (Shahbazi et al. 2010). The isolates were maintained on PDA at 20°C for 7 days. To induce sporulation, cultures were grown for 6 days at 23–25°C on PDA under near-ultraviolet light (310–400 nm) with 16 h of daylight. Conidia were collected by washing plates with sterile water and the resulting spore suspension was adjusted to a concentration of 10^6 spores ml^{-1}.

Plant inoculations and disease evaluations Four plants (45 days old) of each potato cultivar were inoculated with each isolate of *A. solani* and a water control. To inoculate, the aerial portion of plants was sprayed with the previously prepared conidial suspension (10^6 spores ml^{-1}) or sterile water as a control and covered with plastic bags for 24 h to accelerate infection. The plants were then allowed to grow under normal conditions in the greenhouse (Pelletier and Fry 1989).

Extraction of endo-ß-glucanase enzymes Random samples of infected leaves (0.5 g) were harvested each day for 10 days after inoculation (dai). Leaves were ground using a mortar and pestle in liquid nitrogen. One ml of 0.05 mM sodium acetate buffer (pH 5.0) was added per 0.5 g fresh weight of leaf sample. The extract was then poured into a 2 ml plastic tube and centrifuged at 14,000 × *g* for 20 min at 4°C. The supernatant (enzyme extract) was collected in a clean plastic tube and stored at −20°C for subsequent analysis (Madhaiyan et al. 2004). Protein concentration was determined according to Bradford (1976) with bovine serum albumin as a standard.

ß-1,3-glucanase assay The method of Abeles *et al.* (1971) was used to determine *ß*-1,3-glucanase activity in leaves on each of 10 dai. An aliquot of the enzyme extract (30 µl) was added to 30 µl of 4% Laminarin (Sigma Chemical Co., St. Louis, MO, USA) as the substrate and the tubes were placed in a 40°C water bath to initiate enzyme reaction. After 30 min, the reaction was terminated by adding 187 µl of dinitrosalicylic acid as a reagent to measure reducing sugars and the tubes were placed in a 100°C water bath for 5 min. Samples without incubation at 40°C were used as blanks. The absorbance was read at 500 nm in a UV–VIS spectrophotometer. *ß*-1,3-glucanase activity was expressed as mg glucose equivalent released per mg protein per minute.

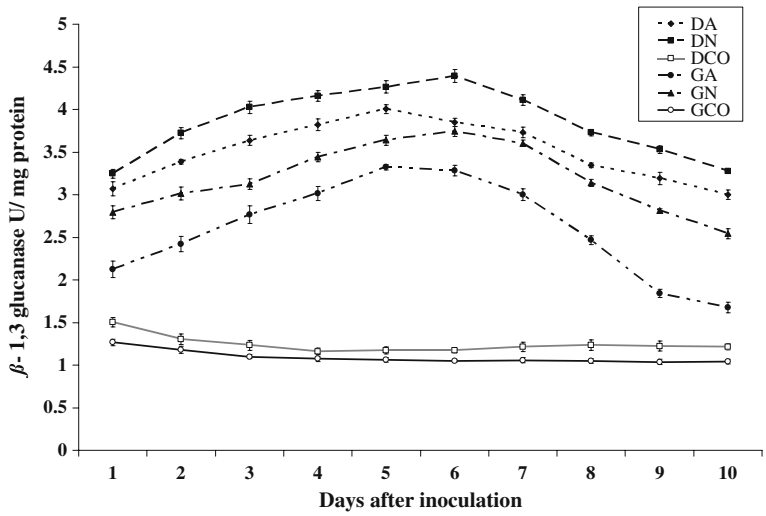

Fig. 1 *ß*-1,3-glucanase activity in leaves of potato cultivars Diamond and Granula after inoculation with *Alternaria solani*. DCO: Diamond sprayed with sterile water; GCO: Granula sprayed with sterile water; GA: Granula sprayed with *A. solani* isolate A; DA: Diamond sprayed with *A. solani* isolate A; DN: Diamond sprayed with *A. solani* isolate N; GN: Granula sprayed with *A. solani* isolate N. Results are the means of four replications. Bars denote standard error

ß-1,4-glucanase assay For the *ß*-1,4-glucanase assay, samples of leaves were analyzed on days 2, 4, 6, and 8 after inoculation. An aliquot of the enzyme extract (100 µl) was added to 100 µl of 1% carboxy methyl cellulose (Sigma Chemical Co.) as substrate and the tubes were placed in a 50°C water bath to initiate enzyme reaction. After 30 min, the reaction was stopped by adding 600 µl of dinitrosalicylic acid as reagent to measure reducing sugars and tubes were placed in a 100°C water bath for 5 min. Samples without incubation at 50°C were used as blanks. The absorbance was read at 500 nm in a UV–VIS spectrophotometer. *ß*-1,4-glucanase activity was expressed as mg glucose equivalent released per mg protein per minute (Yedidia et al. 2000).

Analysis of results All experiments were carried out using a factorial model of completely randomized experimental design in four replicates per treatment. In the *ß*-1,3-glucanase activity study, the statistically factorial model was 2×3×10 (two potato cultivars × three treatments: two *A. solani* isolates and sterile water × 10 days). In the *ß*-1,4-glucanase activity study, the statistically factorial model was 2×3×4 (two potato cultivars × three treatments × 4 days). The statistical analysis was performed using SAS v9 (Statistic Analysis System) software (Littell et al. 1996). Analysis of variance was performed on all data sets. Duncan's test was used to show significant differences (P=0.05) between treatments. All data are presented as means. Analyses of variance for both experiments were significant at the 1% level (P<0.01).

Table 1 Mean comparison of glucanase activity in leaves of potato after inoculation with *Alternaria solani**

Cultivar	*ß*-1,3-glucanase	*ß*-1,4-glucanase
Diamond	3.285 A	3.108 A
Granula	3.167 B	2.830 B

*Within columns, means followed by a different letter differ significantly at P=0.05

Results

We have previously shown that the potato cv. Diamond exhibits increased resistance to *A. solani*

Table 2 Mean comparison of *ß*-1,3-glucanase activity in leaves of potato cultivars after inoculation with *Alternaria solani*

Dai	Mean*
5	3.506 A
4	3.406 AB
6	3.385 AB
7	3.316 BC
2	3.290 BC
8	3.240 BCD
3	3.166 CD
9	3.098 DE
1	2.938 EF
10	2.911 F

* Means with a common letter do not differ significantly at P=0.05

Table 3 Mean comparison of glucanase activities in leaves of potato cultivars after inoculation with different isolates of *Alternaria solani**

Isolate	*ß*-1,3-glucanase	*ß*-1,4-glucanase
N	3.610 A	3.404 A
A	3.451 B	3.165 B
CO	2.616 C	2.338 C

*Within columns, means followed by a different letter differ significantly at $P=0.05$

Table 4 Mean comparison of *ß*-1,4-glucanase activity in leaves of potato cultivars after inoculation with *Alternaria solani*

Dai	Mean*
4	3.405 A
6	3.030 B
2	2.927 C
8	2.515 D

* Means with a different letter differ significantly at $P=0.05$

when compared to the susceptible cv. Granula (Shahbazi et al. 2010). Although quantification of symptoms was not calculated, the whole plant inoculations done in these experiments confirmed these results, since cv. Diamond exhibited fewer disease symptoms than cv. Granula (data not shown). In order to determine whether glucanase activity correlated with the disease resistance phenotypes, we performed enzyme assays using extracts from inoculated leaves over a period of 10 days. In these experiments, cv. Diamond exhibited higher *ß*-1,3-glucanase activity than did cv. Granula after inoculation with *A. solani* (Fig. 1). The difference between *ß*-1,3-glucanase activities in the two cultivars in response to different isolates of *A. solani* and the water control was found to be significant (Table 1). In both cultivars the highest *ß*-1,3-glucanase activity was observed on the fifth dai, followed by the fourth and sixth, while the lowest activity was detected 10 dai (Table 2). Interestingly, there was also a significant difference between *ß*-1,3-glucanase levels elicited by the A isolate, the N isolate, and water control in each cultivar (Fig. 1; Table 3). The N isolate, which is the less aggressive, elicited higher levels of *ß*-1,3-glucanase than the more aggressive A isolate throughout the experimental time course (Table 3). The levels elicited by both isolates were significantly higher than in water-inoculated plants. In plants that were not inoculated with *A. solani*, levels of *ß*-1,3-glucanase remained higher in cv. Diamond than in Granula throughout the experimental time course, suggesting a difference in basal levels of the enzyme in each cultivar.

ß-1,4-glucanase activity was also significantly higher in *A. solani*-inoculated leaves compared to water-inoculated leaves (Fig. 2; Table 3). In addition, *ß*-1,4-glucanase in cv. Diamond was significantly higher than cv. Granula 2, 4, 6, and 8 dai (Table 1). In both cultivars, the level of *ß*-1,4-glucanase activity increased between the second and fourth days and then decreased 6 and 8 dai (Table 4).

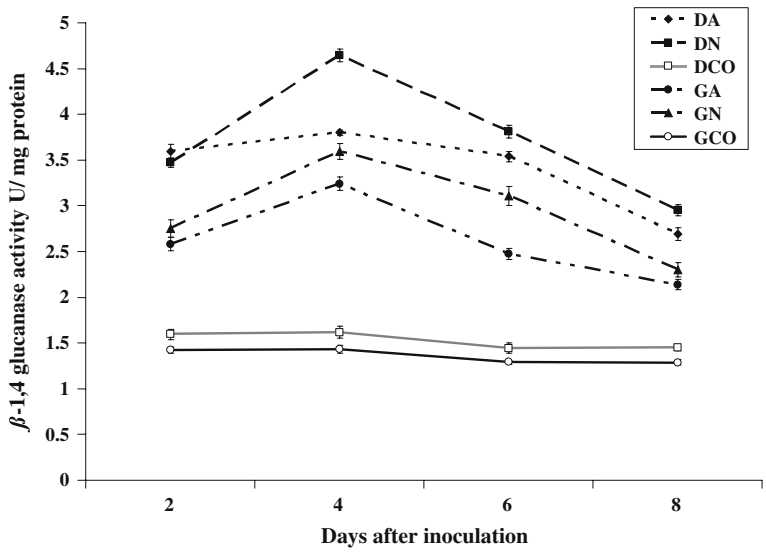

Fig. 2 *ß*-1,4-glucanase activity in leaves of potato cultivars Diamond and Granula after inoculation with *Alternaria solani*. DCO: Diamond sprayed with sterile water; GCO: Granula sprayed with sterile water; GA: Granula sprayed with *A. solani* isolate A; DA: Diamond sprayed with *A. solani* isolate A; DN: Diamond sprayed with *A. solani* isolate N; GN: Granula sprayed with *A. solani* isolate N. Results are the means of four replications. Bars denote standard error

Consistent with ß-1,3-glucanase levels, ß-1,4-glucanase activity was higher after inoculation with *A. solani* isolate N compared to isolate A in both cv. Diamond and cv. Granula. These differences were significant at 4, 6 and 8 dai. In water-inoculated plants, levels of ß-1,4-glucanase also remained higher in cv. Diamond than in Granula throughout the experimental time course.

Discussion

We have previously shown that increased resistance in the *S. tuberosum* cv. Diamond was correlated with an increase in the amount of total phenols and peroxidase activity (Shahbazi et al. 2010). In the present study we have shown that resistance in cv. Diamond is also correlated with an increase in activities of two PR enzymes: ß-1,3-glucanase and ß-1,4-glucanase. The activity of these two proteins was significantly higher after inoculation with *A. solani* when compared with the susceptible potato cv. Granula.

In general, PR proteins are considered as inducible proteins elicited by environmental and developmental stimuli. However, an increasing body of data points to their occurrence as constitutive components in different plant organs and in seeds, irrespective of stress conditions (Buchel and Linthorst 1999; Vigers et al. 1991). For example, basic tobacco glucanase PR-2d functions developmentally in seed germination by weakening the endosperm, thus allowing the radicle to protrude (Vögeli-Lange et al. 1994). The role of PR proteins in defense against pathogens is corroborated by data about their constitutive expression in seeds and plant organs, and high fungitoxicity of seed osmotins and thaumatin-like proteins has been established (Abad et al. 1996; Vigers et al. 1992). A protective role of PR proteins can also be inferred from their accumulation in plant cell wall appositions formed against pathogen ingress, as well as from their release into fungal structures penetrating plant tissues (Jeun and Buchenauer 2001). Importantly, high constitutive expression of PR proteins results in plants with high levels of natural disease resistance. This correlation was observed in several pathosystems, such as apple infection by *Venturia inaequalis* (Gau et al. 2004), tomato infection by *A. solani* (Lawrence et al. 2000), and potato infection by *Phytophthora infestans* (Vleeshouwers et al. 2000). An increase in the accumulation of PR proteins has also been reported in inoculated resistant plants compared with susceptible plants. Differential responses of resistant/susceptible plants were reported in tomato inoculated with *Cladosporium fulvum* (Wubben et al. 1996), in potato inoculated with *P. infestans* (Tonón et al. 2002), and in apple inoculated with *V. inaequalis* (Poupard et al. 2003). Here, we have found similar results in the correlation between increased accumulation of glucanases and resistance to *A. solani* in potato. Although basal levels of glucanase were not calculated in the potato cvs. Diamond and Granula, the water-inoculated controls provided insight into differences in glucanase activity in the absence of pathogen. During our experiments, water-inoculated cv. Diamond consistently had significantly higher levels of both ß-1-3- and ß-1-4-glucanase than did cv. Granula, indicating that the former cultivar expresses higher basal levels of both enzymes. This would suggest that the resistance phenotype in cv. Diamond may be due, in part, to increased activity of ß-glucanase enzymes. However, further experiments will be necessary to determine if a correlation exists between increased basal-level glucanase expression and increased resistance to *A. solani* and whether it can be used to develop a screening assay for increased resistance to the pathogen.

Little information is available about differences between strains of *A. solani* that result in changes in virulence. Plant pathogens express many proteins, termed effectors, that are delivered into the apoplast or plant cell cytoplasm, resulting in host susceptibility (Chisholm et al. 2006). Since the role of pathogen effectors is to suppress defense responses that are elicited upon invasion, leading to host susceptibility, variation within the effector set of pathogens could lead to changes in virulence. In this scenario, aggressive or highly virulent strains of a pathogen would be able to suppress host defenses more effectively, leading to increased disease. In our experiments, we found that glucanase activity in potato was reduced in plants that were inoculated with the more aggressive isolate A, compared with glucanase activity in plants exposed to isolate N. This would suggest that the difference in virulence could, in part, be due to the ability of isolate A to suppress expression of both ß-1-3- and ß-1-4-glucanases in infected tissue, regardless of the host resistance phenotype.

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