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Induction of PR-proteins and defense related enzymes in black pepper due to inoculation with *Phytophthora capsici**

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ABSTRACT: The activities of phenylalanine ammonia lyase (PAL) and ß1,3 glucanase in both leaf and root tissues of three black pepper varieties (tolerant P24; and susceptible Panniyur[1] and Subhakara) were determined in healthy and *Phytophthora capsici* infected tissues. Infection generally enhanced both of these enzyme activities. SDS-PAGE study revealed the induction of PR-proteins in the infected tissues. Western blotting with anti-tobacco ß1,3 glucanase antibody confirmed the presence of these isoforms in the leaf extract. Among the three varieties studied, the *Phytophthora* tolerant P24 expressed higher rate of these defense-related enzymes/proteins.

Key words: PAL, ß1,3 glucanase, PR-proteins, *P. capsici*, black pepper

Induction of peroxidase, polyphenol oxidase, phenylalanine ammonia lyase (PAL) and pathogenesis-related proteins (PR-proteins) activities is one of the important aspects of the defense mechanisms of plants against the invading pathogens. PR-proteins include proteinase inhibitors or extracellular forms of glucanase and chitinase (Shivaraj and Pattabiraman, 1981; Carr and Klessig, 1989). Greater activity of PAL has been demonstrated in incompatible responses. In soybean infected with *P. cinnamomi* (Bhattacharyya and Ward, 1988), *Eucalyptus* spp. with *P. megasperma* (Cahill and McComb, 1992) and in *Capsicum annum* with *P. capsici* (Mozzetti *et al.*, 1995) increase of PAL activity has been recorded. Although soybean inoculated with *Phytophthora megasperma* f.sp. *glycinea* showed greater activity of PAL (Bönner and Griesebach, 1982), Partridge and Keen (1977) did not detect significant increase in PAL after inoculation of wound with this pathogen. Kombrink *et al.*, (1988) identified several PR- proteins in potato leaves which were inoculated with *P. infestans.* Infection by *P. megasperma* f. sp. *glycinea* greatly induced the synthesis and accumulation of ß-1,3 glucanase in hypocotyls and in leaves of soybean seedlings (Yi and Hwang, 1996). *P. capsici* infection induced the synthesis and accumulation of ß1,3 glucanases and chitinases in the stem tissues of pepper plant (Kim and Hwang, 1994). Pepper plants treated with DL-ß-amino-n-butyric acid and/or inoculated with *P. capsici* showed accumulation of ß-1,3 glucanases and chitinases in stem tissues. The 34 kDa ß-1,3 glucanase isolated from a pepper stem inhibited the hyphal growth of *P. capsici in vitro* (Kim and Hwang, 1997). Xue *et al.* (1998) reported the systemic induction of peroxidase, 1,3 ß glucanase and chitinase and related it with the resistance of bean plants to binucleate *Rhizoctonia* species. The synergistic activity of glucanase with chitinase was reported in pea and in tobacco (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993). Transgenic potato plants expressing soybean ß1,3 endoglucanase gene exhibited an increased level of resistance to *P. infestans* (Borkowska *et al.* 1998). The present study examines the role of PAL, 1,3 ß glucanases and PR-proteins in the reaction of three black pepper (*Piper nigrum* L.) varieties, viz., P24, Subhakara and Panniyur I to *P. capsici* infection.

MATERIALS AND METHODS

Plant material

Four months old black pepper cutting (P24, Panniyur 1 and Subhakara) raised in polybags of 18 × 15 cm size containing 3 kg of potting mixture in the green house were used in this study. Among the three varieties, P 24 is tolerant to *P. capsici* whereas the other two are susceptible (Sarma *et al.*, 1994).

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Fungal pathogen and inoculation procedure

An isolate of *P. capsici* (97-55), the foot rot pathogen of black pepper obtained from National Repository of *Phytophthora,* Indian Institute of Spices Research, Calicut was used. Healthy leaves were detached from the plants and kept in the humid chamber. The leaves were inoculated with zoospore suspension (10^6 zoospores/ml) of *P. capsici* and observed for the development of symptom. Likewise, black pepper cuttings along with roots were dipped in the zoospore suspension for 24 hrs and then incubated in a humid chamber for 24 hrs. After 48 h of inoculation, the tissues were taken for enzyme studies.

Protein extraction

The leaf tissues were ground with phosphate buffer pH 7.0 containing 15-mM 2-mercaptoethanol in a cold room. The extract was mixed with five volumes of cold acetone and left overnight at -20°C. The resulting mixture was centrifuged at 10,000 rpm for 20 min at 4°C The pellet was suspended in phosphate buffer pH 7.0 and was used as the enzyme source. The total protein content of the enzyme extract was determined using Lowry's method (Lowry *et al.,* 1951).

Enzyme activity studies

Phenylalanine Ammonia lyase (PAL)

PAL activity was determined spectrophotometrically as transcinnamic acid formed during the enzyme reaction (Brueske, 1980). One gram of leaf tissue was extracted in two ml of cold 0.1 M potassium phosphate buffer pH 8.0 and filtered through muslin cloth. The filtrate was centrifuged at 10,000 rpm at 4°C for 20 min, and the supernatant was used as a crude enzyme extract. The reaction mixture contained 0.1 ml of enzyme extract, 0.4 ml of 0.1 M borate buffer pH 8.8 and 0.5 ml of 12 mM L-phenylalanine. The reaction mixture was incubated for 1 h at 32°C. The reaction was stopped by addition of 1 M trichloroacetic acid. The absorbance was read at 290 nm in a Shimadzu UV-visible spectrophotometer. The enzyme activity was expressed on fresh weight basis as nmol of transcinnamic acid released per min per gram of leaf tissue.

β-1,3 glucanase

Total ß-1,3 glucanase activity was colorimetrically assayed by the laminar-indi nitrosalicylic method (Pan *et al.,* 1991). The enzyme extract (62.5 μl) was added to 62.5 μl of 4% laminarin (Sigma, USA) and incubated at 40°C for 10 min. The reaction was stopped by the addition of 375 μl of dinitrosalicylic acid reagent and by heating for 5 min in a boiling water bath. The resulting coloured solution was diluted with 4.5 ml of distilled water, vortexed and its absorbance was measured at 500 nm. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as μmol equivalent glucose released per hour per gram fresh weight of tissue.

SDS-PAGE

SDS-PAGE was carried out according to Laemmli (1970) with 12% acrylamide and 1%(w/v) SDS. Samples containing 25-30 μg of protein were loaded in the wells. Protein molecular weight markers (Merck) comprising of cytocrome C (12.3 kDa), myoglobin (17.2 kDa), carbonic anhydrase (30.0 kDa), ovalbumin (42.7 kDa), albumin (66.2 kDa) and ovotransferin (78.0 kDa) were also co-electrophorased with the sample. Electrophoresis was done for 2-3 hr at a constant current of 15 mA (Pharmacia Biotech, EPS 600). Protein bands were visualised by staining with coomassie brilliant blue R 250 (Fig.1) and by nonammonical silver staining method (Ausubel *et al.,* 1995).

Western blot analysis

In order to identify ß 1,3 glucanase isoforms, the samples were subjected to western blot analysis (Winston et.al. 1987). An anti-tobacco ß 1,3 glucanase antiserum, a polyclonal antibody raised in rabbit was used as primary antibody.

RESULTS

PAL activity

In the infected leaves, there was significant increase in PAL activity. Among the three varieties studied, P24 showed higher PAL activity (38.4%) in the leaves on infection with *P. capsici.* There was a significant difference in PAL activities in roots among the varieties studied and in P24, it was significantly higher. In the infected root tissues, there was no increase in enzyme activity after 48 hrs (Table 1).

ß-1,3 glucanase activity

ß-1,3 glucanase activity was significantly higher in all infected leaf tissues. The variety, P24 showed markedly higher enzyme activity in both healthy and infected tissues than other varieties. No significant dif-

Table 1. PAL and ß 1,3 glucanase activities in healthy and *P. capsici* inoculated leaves and roots of black pepper

Variety	Enzymatic activity in			
	Leaves		Roots	
	PAL (in nmol trans-cinnamic acid/min/g of leaf tissue)	ß 1,3 glucanase (in µmol equivalent glucose released/h/g of fresh weight)	PAL (in nmol trans-cinnamic acid/min/g of leaf tissue)	ß 1,3 glucanase (in µmol equivalent glucose released/h/g fresh weight)
P 24 (H)	2.6	209.3	4.2	90.2
P 24 (48 HAI)	3.6 (38.4%)	357.9 (70.9%)	1.9	115.0 (27.5%)
Panniyur 1 (H)	2.7	173.3	2.4	75.4
Panniyur 1 (48 HAI)	3.5 (27.2%)	205.2 (18.4%)	1.9	90.1 (19.5%)
Subhakara (H)	3.1	188.4	3.8	87.5
Subhakara (48 HAI)	3.6 (13.4%)	297.0 (57.6%)	2.4	92.3 (5.6%)
CD at 0.05%	0.48	16.50	0.13	

* Figures in parentheses denotes the percentage increase in enzyme activity
H: Healthy tissue 48 HAI: 48 hours after inoculation

ference was observed in enzyme activities in the healthy tissues of Subhakara and Panniyur 1. In roots, ß 1,3-glucanase activity increased due to infection. In healthy roots, P24 showed significantly higher activity than the other two varieties. There was a marked increase in ß 1,3-glucanase activity in Panniyur 1 also due to infection, but the level was lesser than P24 (Table 1).

Protein Profile

Total leaf protein profiles from the leaf extracts of the three varieties obtained by SDS-PAGE revealed differences among the varieties. There was marked difference in total number and intensity of proteins between these varieties. Analysis using AlphaImager Documentation and Analysis System revealed that three proteins with molecular weights of 27, 34 and 38 kDa were induced in P24 variety up on infection. The 34-kDa protein was seen only in P24. In susceptible varieties like Subhakara and Panniyur 1 there was no such marked increase in protein after infection. In susceptible variety, Subhakara only the disintegration of many proteins was noticed in the infected tissues (Fig.1). Similarly in roots, induction of proteins due to infection was noticed. In the infected roots of P24, three proteins with molecular weights of 20, 26 and 31 kDa were induced, whereas in Panniyur 1, four proteins with molecular weights of 22, 24, 31, 33 kDa were induced. In Subhakara, only the disintegration of proteins was noticed (Fig.2).

Western blot using anti-tobacco ß 1,3 glucanase antiserum confirmed the occurrence of glucanase isoforms in the leaf extracts.

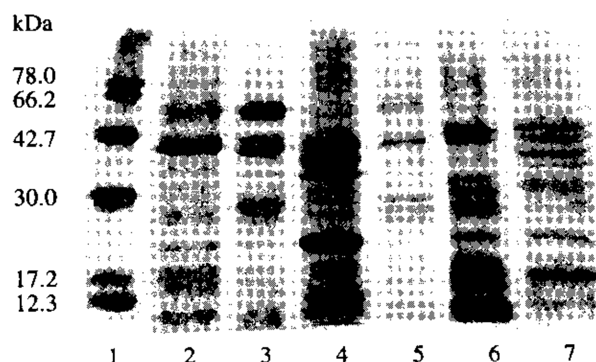

Fig. 1. SDS-PAGE showing the protein profile in black pepper leaf extracts Lane 1: Marker; Lane 2: P24(H); Lane 3: P24(I). Lane 4: Panniyur 1 (H); Lane 5: Panniyur 1 (I); Lane 6: Subhakara (H); Lane 7: Subhakara (I)

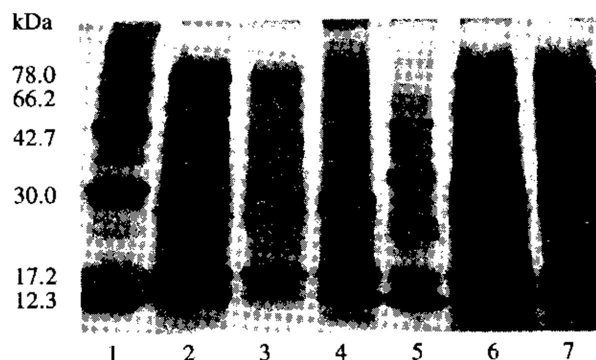

Fig. 2. SDS-PAGE showing the protein profile in black pepper root extracts Lane 1: Marker; Lane 2: P24(H); Lane 3: P24 (I); Lane 4: Panniyur 1 (H); Lane 5: Panniyur 1 (I); Lane 6: Subhakara (H); Lane 7: Subhakara (I)

DISCUSSION

Disease resistance in plants is due to both constitutive and induced mechanisms. Induction of PR-proteins is an important aspect of induced mechanism. The hydrolytic enzymes such as ß-1,3 glucanases and chitinases synthesised during pathogenesis have been implicated in the resistance of several pathosystems. PAL is activated rapidly in the initial phases whereas ß-1,3 glucanases are activated slowly and systemically (Kombrink *et al.,* 1993). Shewry and Lucas (1997) reported the role of the defense-related enzymes in disease resistance. Plants synthesize phytoalexins when microorganisms invade them. Majority of these phytoalexins are all phenolics in nature. PAL catalyzes the first reaction in the synthesis of a wide range of natural products based on the phenylpropane skeleton including lignin monomers and certain phytoalexins (Lamb *et al.,* 1989). PAL is also associated with accumulation of aromatic fungitoxic compounds (Ampomah and Friend, 1988) and lignification (Cahill and McComb, 1992).

In the present study, PAL and ß 1,3-glucanase activity increased significantly after 48 hrs of infection. Infection of tobacco mosaic virus in tobacco leaves resulted in 6-10 fold increase of PAL activity after 48 hrs (Massale *et al.,* 1980). Among three black pepper varieties studied the *Phytophthora* tolerant variety P24 showed higher activity of these defense-related enzymes. Moreover, the induction of PR-proteins was observed in tolerant line.

In a number of plant species, ß 1,3 glucanase exists in multiple forms. It is localised in both cell walls and vacuoles of ethylene-treated bean leaves (Mauch *et al.,* 1989). These enzymes solubilise elicitor-active glucan molecules from the fungal cell wall (Mauch and Staechelin, 1989) and also induce defense enzymes. When the pathogen grows initially in the intercellular space, the fungus may come in contact with ß-1,3 glucanase localized in the middle lamellae. That stimulates the activity of ß-1,3 glucanase in the infected tissues. Joosten and De Wit (1989) isolated a 35-kDa protein showing ß-1,3 glucanase activity in tomato leaves after inoculation with *Colletotrichum fulvum.* Increase in ß-1,3 glucanase activity was found in potato leaves infected with *P. infestans* (Schröder *et al.,* 1992). Higher activity of ß-1,3 glucanase was noticed in both healthy and infected tissues of tolerant variety P24 than the other two susceptible varieties in this study. Even though ß-1,3 glucanase activity increased in susceptible varieties due to infection, the level was lower than that of tolerant P24 variety. This suggests that the higher activities of this defense related enzyme in P24, might be responsible for tolerance against *P. capsici.*

Among the three varieties studied, P24 is tolerant to *P. capsici* whereas the other two are susceptible. In the order of susceptibility, the variety Subhakara is most susceptible followed by Panniyur 1. One of the reasons for tolerance of P24 could be due to the higher production of defense related products. An array of PR-proteins is reported in literature against the fungus *P. capsici.* A 34-KDa protein has been isolated and purified in *Capsicum,* which was toxic to *P. capsici* (Kim and Hwang, 1997). The production of a similar protein in black pepper *P. capsici* pathosystem suggests its possible role in the tolerance of P24. Since the defense related genes encoding ß-1,3 glucanases have already been characterised, cloned and expressed in other crops, the utility of these genes in developing transgenic black pepper gains importance.

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