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Expression of pathogenesis related proteins in black pepper (*Piper nigrum* L.) in relation to *Phytophthora* foot rot disease

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Abstract

Foot rot caused by *Phytophthora capsici* is the most serious disease of black pepper. Though all varieties of black pepper are susceptible to this pathogen, variations do exist concerning the degree of tolerance and mechanisms of defense. The protein profiles of a relatively tolerant and a susceptible black pepper (*Piper nigrum*) variety along with that of a resistant wild species (*Piper colubrinum*) were evaluated to detect variations in the defense related proteins/enzyme expression in response to *P. capsici* infection. The SDS-PAGE analysis revealed two additional polypeptides of 16.5 and 8 kD in the leaves of the tolerant variety ('Kalluvally') on the second day after infection and these proteins expressed only on the fifth day in the susceptible 'Panniyur-1'. Variety specific proteins of molecular weight 90 and 5.5 kD were also found expressed in 'Panniyur-1' while 'Kalluvally' had unique protein bands of 14.3, 8.8, and 7.0 kD. However, *P. colubrinum* with 16 distinct bands had an altogether different banding pattern. The native protein profile obtained also indicated the expression of two additional proteins in *P. nigrum*. The over-expressed protein was characterized as β-1,3 glucanase. The intensity of expression was directly related to the level of tolerance. The role of enzymes like phenylalanine ammonia lyase (PAL), chitinase, and peroxidase in defense mechanism was also analyzed. The resistant genotype *P. colubrinum*, possessed higher enzyme activities than the *P. nigrum* varieties tested. This study thus confirmed the role of β-1, 3 glucanase and related enzymes in the defense mechanism of black pepper against foot rot disease.

Keywords: β -1, 3 glucanase, *Piper colubrinum*, *Phytophthora capsici*

Introduction

Foot rot disease of black pepper (*Piper nigrum* L.), the 'king of spices', caused by *Phytophthora capsici* results in severe crop losses. Vine mortality and crop losses up to 20 to 30 % have been reported (Balakrishnan et al., 1986). *P. capsici*, a soil-borne fungus, infects all parts of the pepper vine. All the locally released varieties and cultivars of black pepper in Kerala are susceptible to this pathogen. Although only a narrow range of variability in tolerance is reported among the cultivated types, the exotic wild species, *Piper colubrinum*, is thought to be resistant. Moreover, a partly fertile interspecific hybrid with partial resistance also has been recently developed by hybridizing *P. nigrum* and *P. colubrinum* (Vanaja et al., 2007) – a remarkable breakthrough in the introgression of disease resistance from wild species (*P. colubrinum*) to the cultivated *P. nigrum*.

Generally, the plants resist the invading phytopathogens (bacteria, fungi, or viruses) by accumulating a number of specific proteins in the intercellular spaces, which are collectively known as Pathogenesis Related (PR) proteins. These are known to be associated with the defense mechanism and PR classes 1 to 5 have already been identified (Selitrennikoff, 2001). The most extensively studied PR proteins include chitinase and β-1,3 glucanase, as they are known to inhibit the fungal

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growth by lysing the fungal cell walls (Zemanek et al., 2002). Moreover, an increased level of various oxygen derived free radicals and the resultant activation of various antioxidant enzyme systems have been reported in plants that hyper sensitively react to wound or infection (Alvarez et al., 1998). In the present study, an attempt was made to characterize the PR proteins/ polypeptides expressed in black pepper during infection and to assess the changes in various defense-related enzyme activities during *Phytophthora* infection.

Materials and Methods

Two varieties of *P. nigrum* viz. 'Panniyur-1' (susceptible) and 'Kalluvally' (tolerant) and a wild species *Piper colubrinum* (resistant) were selected for the study. Rooted cuttings grown in polybags under greenhouse conditions (4 month old) were used. Pure culture of *P. capsici* isolated from infected black pepper leaves was inoculated on carrot agar medium in petriplates and incubated at 27°C for optimum growth. Seven day old cultures having profuse growth were used for inoculation. Culture discs of uniform size (5 mm dia.) were applied on the ventral side of leaf lamina after making pinpricks (10 nos.) at the points of contact.

Extraction of foliar protein and PAGE analysis

Development of specific symptoms was confirmed on the second day after infection and the leaf tissue (0.5 g) collected from healthy and infected plants (1 cm around the necrotic lesion) was macerated in a mortar at 4°C with phosphate-citrate buffer (pH 2.8, 1 ml g^{-1} tissue). The buffer contained 0.005% β-mercaptoethanol, 0.05 M-polymethanesulphonylfluoride (PMSF), and 0.5 M cystein-HCl. Extracts were centrifuged at 15 000 rpm for 10 min. at 4°C. Supernatants were saved for electrophoretic analysis. The protein concentrations in the samples were determined by the method of Lowery et al. (1951).

For SDS-PAGE analysis, the sample protein and SDS-gel loading buffer were mixed (1:1 v/v) and heated at 100°C for 3-5 min. Samples having about 25 μg equivalent protein were loaded in each well on 10% SDS-polyacrylamide gel. Electrophoresis was carried out at room temperature on constant current of 20 mA for 1 h using the Hoefer Mighty Small Electrophoresis System (Laemmli, 1970). The protein separated on the gel was visualized by silver staining (Sigma-Aldrich, USA). For native PAGE, the extracts (50 μg per well) were electrophoresed on 8% acrylamide gel at 4°C using anionic (pH 8.9) and cationic (pH 4.3) buffer systems as per Laemmli (1970) and Hames (1981) respectively. The electrophoresis was carried out at 10 mA for 2 h at 4°C and later stained with Coomassie Brilliant Blue G 250 for observing the protein profiles.

Enzyme assays

Leaf tissue (0.5 g) was collected over ice at various time intervals from 4 h after inoculation on the fifth day. Samples were homogenized by grinding with liquid nitrogen in a mortar and then extracted at 4°C with 0.1 M sodium acetate buffer (pH 5.0; 1:1 w/v) containing various protease inhibitors. Homogenates were centrifuged at 10 000 rpm for 30 min. at 4°C and the supernatant was used as the enzyme source.

Total β-1,3 glucanase activity was measured spectrophotometrically by the laminarin dinitrosalicylic acid method (Pan et al., 1991). The assay system contained 62.5 μL crude enzyme extract and 62.5 μL 4% laminarin. Incubation was carried out at 40°C for 10 min. The reaction was then stopped by the addition of 375 μL dinitrosalicylic acid solution and heating over boiling water bath for 10 min. The resultant colored solution was diluted with 4.5 ml of distilled water, vortexed, and absorbance measured at 500 nm. The enzyme activity was expressed as micromole glucose formed per mg protein per 10 min.

Phenylalanine ammonia lyase (PAL) activity was assayed by the method suggested by Sadasivam and Manikam (1992) with slight modifications. The leaf tissue (1:10 w/v) was macerated with borate buffer containing 25 μL β-mercaptoethanol, 50 μL PMSF, 50 μL cystein-HCl, and 50 μL ascorbic acid using a chilled mortar and pestle. The homogenate was clarified by centrifugation at 12 000 rpm for 20 min. at 4°C.

Supernatant was used as enzyme source. The assay was initiated by the addition of 1 ml L-phenyl alanine solution to the mixture containing 0.5 ml borate buffer, 0.2 ml of enzyme solution, and 1.3 ml of distilled water. The mixture was incubated for 1 h at 32°C and the reaction stopped by adding 0.5 ml of trichloroacetic acid in the reaction system. A control was run by adding phenylalanine solution following the addition of trichloroacetic acid. Absorbance was measured at 290 nm and the enzyme activity expressed as micromole trans-cinnamic acid formed per mg protein per min.

Exochitinase activity was assayed as per the method described by Jeuniaux (1966) with some modifications. The enzyme activity was the measure of acetyl glucosamine released from chitin in the assay system. The physiologically active leaf tissue was macerated with citrate phosphate buffer (pH 5.1; 1:2 w/v) containing 50 μL each 5 mM ascorbic acid, 0.1 M PMSF, 0.005% β-mercaptoethanol, and 100 μL 0.5 M cystein-HCl using a chilled mortar and pestle. The homogenate was centrifuged at 15 000 rpm for 15 min. at 4°C. The supernatant collected was used as the enzyme source. In the reaction system, 1 ml of chitin suspension was mixed with 1 ml buffer and 0.5 ml of crude enzyme extract and made up to 4 ml with distilled water. Incubation of the mixture was carried out at 37°C for 90 min. An aliquot (1 ml) of the incubated mixture was diluted with 1 ml distilled water, boiled for 5 min, and centrifuged at 1 000 rpm for 5 min. The acetyl glucosamine formed in the system was assayed. The reaction system contained 0.5 ml supernatant and 0.1 ml of 0.8 M potassium tetraborate, boiled for 3 min. After cooling, 3 ml of dimethylamino benzaldehyde (DMAB) solution was added, mixed, and allowed to stand for 20 min. at 37°C. The sample was cooled and the optical density read within 10 min. at 585 nm.

Isozyme analysis

Extraction of peroxidase enzyme from infected and uninfected leaves was carried out at 4°C using 0.2 M phosphate buffer at pH 7.4 containing various antioxidants and protease inhibitors. The extract was then centrifuged at 15 000 rpm for 15 min. and supernatant used as the enzyme source. Fifty microgram protein equivalent of the supernatant was loaded on to 8% polyacrylamide gel and electrophoresed at 4°C at 15–20 mA for a period of 2 h in a Hoefer Electrophoresis System. The gel after electrophoresis was stained with sodium acetate buffer (pH 5.1) containing 3% H_2O_2 and 0.1% benzidine. The isoforms of peroxidase were visualized and documented.

Western blot analysis

The protein separated by SDS-PAGE was electroblotted on to a nitrocellulose membrane (0.45 μm) using the Biorad Mini Gel Blotting System, following the procedure of Sambrook et al. (1989). The membrane was then allowed to probe with tobacco β-1, 3 glucanase polyclonal antiserum (1:5000) raised in rabbits, followed by goat anti rabbit antibody tagged with alkaline phosphatase enzyme (1:2500, Bangalore Genei). The antigen-antibody reaction in the membrane was observed by the addition of BCIP/NBT solution (ready to use solution; Bangalore Genei).

Results

Foliar symptoms were observed on the second day in 'Panniyur-1' and on the 3rd day in 'Kalluvally' as dark brown necrotic spots at the infected regions. The lesion size increased gradually on subsequent days and the whole leaf lamina became discolored within 6 days in 'Panniyur-1' and within 7 days in 'Kalluvally'. *Piper colubrinum* never expressed such foliar symptoms upon infection by the fungus (Fig. 1).

SDS-PAGE analysis of foliar proteins at specific intervals indicated distinct banding pattern for *P. nigrum* ('Kalluvally') and *P. colubrinum* (Fig. 2). Up to 24 h after incubation, both healthy and infected plants of the two species gave the same banding pattern. Altogether 15 bands were observed for the two varieties of *P. nigrum*, of which 10 were common. Variety specific proteins of molecular weight 90 and 5.5 kD were found expressed in 'Panniyur-1' while 'Kalluvally' had unique protein bands of 14.3, 8.8, and 7.0 kD. Altogether different banding pattern was

Figure 1: *Piper nigrum* variety 'Panniyur-1' (A) and *P. colubrinum* (B) infected with *Phytophthora capsici.*

observed for *P. colubrinum* with 16 distinct bands, of which only six (43.0 kD, 22 kD, 13 kD, 11.5 kD, 10.kD, and 4 kD) were shared by the *P. nigrum* varieties.

After 48 h of inoculation, one additional band of 16.5 kD was found expressed in the infected leaf of 'Kalluvally'. On the 3rd day one more additional band of 8 kD protein expressed in 'Kalluvally'. Up to the 4th day, healthy and infected plants of 'Panniyur-1' and *P. colubrinum* recorded similar banding pattern, as on the first day. On the 5th day, however, infected 'Kalluvally' continued to express the 16.5 and 8 kD protein bands and the same bands were found newly expressed in the infected leaves of 'Panniyur-1' also. Banding profile of *P. colubrinum* was similar on all these days.

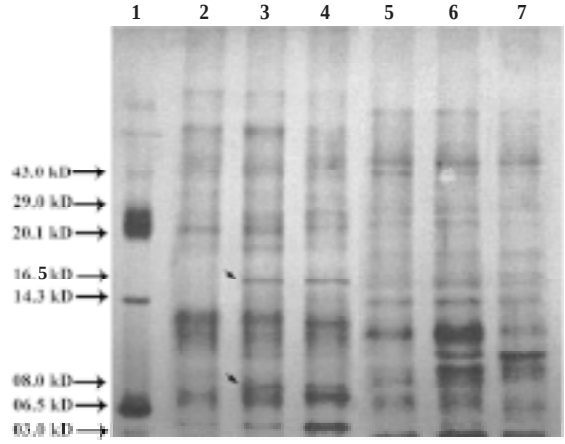

Figure 2: SDS–PAGE analysis of pathogenesis related proteins isolated from *P. nigrum* ('Kalluvally') after infection with *Phytophthora capsici*. Lane 1-Marker; Lane 2-Healthy 'Kalluvally'; Lane 3 and 4 - Infected 'Kalluvally'; Lane 5-Healthy *Colubrinum*; Lane 6 and 7- Inoculated *Colubrinum.*

Native-PAGE analysis with anionic buffer system (pH 8.9) did not give any positive results. When cationic buffer system (pH 4.3) was used, the proteins got separated with distinct banding pattern for the healthy and infected *P. nigrum* variety 'Panniyur 1' (Fig. 3). Two proteins with Rm values 0.46 and 0.615 were observed in the healthy *P. nigrum* (lanes 1, 2, and 3) and were shared with infected *P. nigrum* (lanes 4 and 5). However, these proteins were over-expressed in the infected plants. Two additional proteins of Rm values 0.53 and 0.74 were found newly expressed in the infected *P. nigrum.*

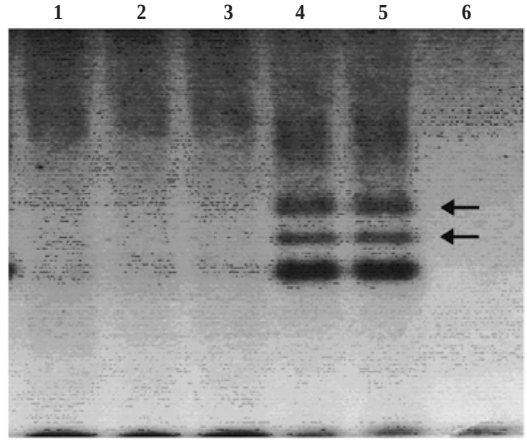

Figure 3: Native PAGE analysis of pathogenesis related proteins from *P. nigrum* after infection with *Phytophthora capsici*. Lane 1, 2 and 3: Healthy *P. nigrum*; Lane 4 and 5: Infected *P. nigrum*; Lane 6: *Phytophthora* protein.

Upon infection by the fungus, β-1, 3 glucanase activity in *P. nigrum* varieties showed an increasing trend over a period of 3 to 5 days with relatively higher levels in tolerant 'Kalluvally' (1.70 µmol/mg protein/10 min.) than the susceptible 'Panniyur-1' (0.262 µmol/mg protein/10 min.). The resistant genotype *P. colubrinum*, possessed still higher enzyme activity than the *P. nigrum* varieties, but did not show any temporal change in the activity pattern following infection (Fig. 4A). Results of the western blot analysis also revealed that the 16.5 and 8 kD proteins expressed in the infected 'Panniyur-1' and 'Kalluvally' varieties immunologically reacted to tobacco β-1, 3 glucanase polyclonal antiserum (Fig. 5, lanes 3 and 5) and was observed as clear bands on the membrane, confirming the role of glucanase in the disease reaction.

Two fold increase in phenylalanine ammonia lyase

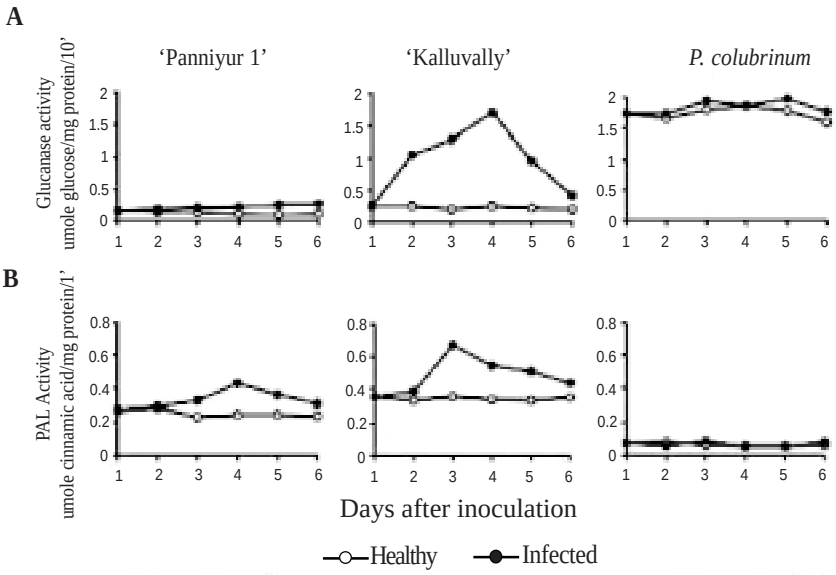

Figure 4: Comparison of β, 1-3 glucanase (A) and phenyl alanine ammonia lyase (B) activity during *Phytophthora* infection in *Piper nigrum.*

activity (Fig. 4B) was observed in var. 'Panniyur 1' (0.45 μmol cinnamic acid/mg protein/min.) within three days after infection and thereafter the activity decreased. In 'Kalluvally', the PAL activity was relatively greater than that of 'Panniyur-1', having a peak two days after infection (0.75 μmol cinnamic acid/mg protein/min.). In *P. colubrinum* the PAL activity was very low compared to both *P. nigrum* varieties, and was not found affected by inoculation with the fungus.

Results of the experiment carried out with different concentrations of crude enzyme and substrates and with different incubation periods revealed that the exochitinase activity is negligible in black pepper. Likewise, at no stage of infection, chitinase activity could be detected in the plants studied. Isoforms of the peroxidase enzyme analysed (Fig. 6) clearly indicated that a high molecular isoform of peroxidase enzyme was expressed in *P. nigrum* varieties within 24 h after inoculation. Many other isoforms were also found over-expressed in the infected plants. Peroxidase activity was low in *P. colubrinum* and it did not show any induction/over-expression during infection.

Discussion

It is well established that the plants when challenged by phytopathogens like bacteria, viruses, or fungi often synthesize a specific group of proteins called PR proteins as a defense mechanism. Such proteins accumulate intercellularily and their concentration is said to be high around the infected tissues. Results of the study showed that in the relatively tolerant *P. nigrum* variety 'Kalluvally', which showed delayed symptom development, one PR protein expressed on the second day after inoculation.

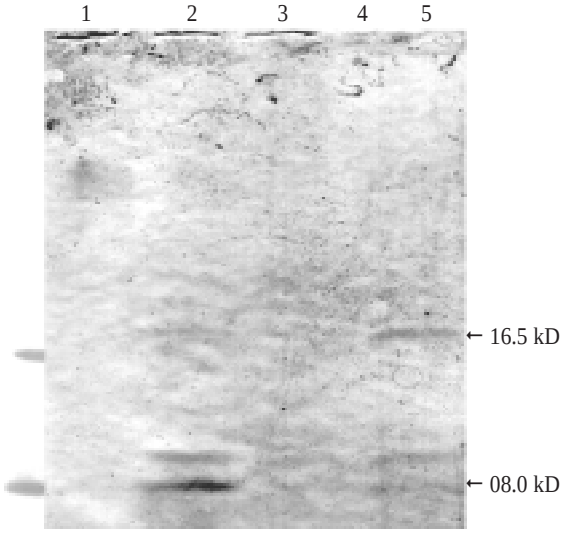

Figure 5: Western blot analysis for the detection of β -1, 3 glucanase. Lane 1: Marker; Lane 2: Healthy 'Panniyur-1'; Lane 3: Infected 'Panniyur-1'; Lane 4: Healthy 'Kalluvally'; Lane 5: Infected 'Kalluvally'.

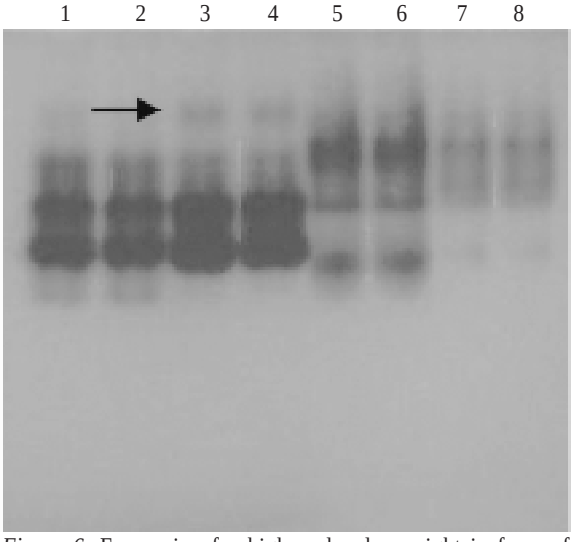

Figure 6: Expression for high molecular weight isoform of peroxidase in black pepper leaves infected with *Phytophthora capsici*. Lane 1 and 2: Uninfected *P. nigrum*; Lane 3 and 4: Infected *P. nigrum*; Lane 5 and 6: Healthy and infected 'Kalluvally'; Lane 7 and 8: Healthy and inoculated *P. colubrinum.*

Whereas in the susceptible var. 'Panniyur-1', this protein expression was delayed up to the fifth day and the symptom development was relatively faster. Further, the resistant *P. colubrinum*, which natively possessed these proteins, developed no symptoms at all. It is thus confirmed that the expression of the specific PR proteins is positively related to the tolerance of black pepper to *P. capsici*. Moreover, the same molecular weight bands (16.5 and 8.0 kD) were always found expressed in *P. colubrinum* signifying their potential role in imparting resistance to the pathogen.

It was also found that β-1, 3 glucanase enzyme activity increased significantly in *P. nigrum* during infection and there was high native activity of this enzyme in *P. colubrinum*. Since the cell wall of *P. capsici* consisted largely of glucan, which makes up 80-90% of the dry weight, it can be inferred that β-1,3-glucanase and β-1,4-glucanase produced by the host were involved in pathogen suppression (Erwin and Ribeiro, 1996). Glucanases may thus have a significant role in the tolerance mechanism of black pepper to *Phytophthora* infection. Induction of multiple forms of β-1,3 glucanase enzyme due to pathogenic attack has been reported in several plant species. For example, potato leaves infected with *P. infestans* have shown high glucanase activity. This enzyme solubilizes elicitor active glucan molecule from the fungal cell wall and also induces other defense enzymes (McDowell and Dangal, 2000). Infection of *Capsicum annuum* with *P. capsici* led to the accumulation of β-1,3-glucanases and it was much more pronounced in the resistant species (Egea et al., 1999). It is confirmed from the chitinase assay that no signal has been raised in black pepper that lead to chitinase gene activation. The cell wall of the *Phytophthora* does not contain chitin and may explain why the plant did not express chitinase enzyme activity upon infection by *P. capsici.*

The enhanced peroxidase as well as PAL activity might be the result of hypersensitive reaction often seen in many plants due to wounding or infection. Thus, the early expression of a high molecular weight peroxidase isoform and the early spurt in PAL activity hyper-sensitively reacting to *P. capsici* may have direct role in the induction of signal transduction pathway leading to the over-expression of glucanase enzyme. *P. colubrinum*, which did not react hyper-sensitively to *Phytophthora*, had low activity for PAL and peroxidase. The glucanase activity in this species was higher than *P. nigrum* varieties and upon infection with fungus the activity did not change. This observation clearly indicates that PAL and peroxidase activity during hypersensitive reactions in black pepper due to *P. capsici* infection induce the ß-1, 3 glucanases.

Separation of the proteins by native-PAGE for basic proteins has revealed the nature of stable proteins in the pH 2.8 extract of black pepper. Similar observations have been reported in stressed xanthinic tobacco leaf extract, in which four basic PR proteins were identified upon native-PAGE (Houge and Asselin, 1987). It has been suggested that ethylene mediates the signal transduction pathway for the induction of basic PR proteins (Odjakova and Hadjivanava, 2001). The native proteins in black pepper resolved only in the basic system of electrophoresis. The polypeptides derived from these proteins on SDS-PAGE analysis showed the presence of two additional peptides of 16.5 and 8 kD, which upon western blot analysis, reacted positively with glucanase

polyclonal antiserum. This indicates the possibility that the two polypeptides may be part of the basic glucanase. A two dimensional study of these proteins will give more information regarding the native basic protein from which the two polypeptides were generated. In addition, the possibility of ethylene to act as second messenger in black pepper needs further explorations.

Overall, this study revealed the role of different PR proteins and enzymes in the disease reaction of black pepper with respect to *Phytophthora* foot rot infection. The high native activity of glucanase enzyme in the resistant genotype, the increased activity upon infection in the susceptible genotype, and the positive reaction in the western blot analysis confirmed the positive role of the enzyme in imparting disease resistance. Chitinase enzyme was not found involved in the disease reaction and the role of PAL was not comparable in the two species studied.

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