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ORIGINAL ARTICLE

Differential induction of chitinase in *Piper colubrinum* in response to inoculation with *Phytophthora capsici*, the cause of foot rot in black pepper

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KEYWORDS

Chitinase assay;
Differential accumulation;
Piper;
Phytophthora capsici;
Reverse transcription

Abstract Plant chitinases have been of particular interest since they are known to be induced upon pathogen invasion. Inoculation of *Piper colubrinum* leaves with the foot rot fungus, *Phytophthora capsici* leads to increase in chitinase activity. A marked increase in chitinase activity in the inoculated leaves was observed, with the maximum activity after 60 h of inoculation and gradually decreased thereafter. Older leaves showed more chitinase activity than young leaves. The level of chitinase in black pepper (*Piper nigrum* L.) upon inoculation was found to be substantially high when compared to *P. colubrinum*. RT–PCR using chitinase specific primers revealed differential accumulation of mRNA in *P. colubrinum* leaves inoculated with *P. capsici*. However, hyphal extension assays revealed no obvious differences in the ability of the protein extracts to inhibit growth of *P. capsici in vitro*.

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1. Introduction

Plants respond to attack by pathogenic microorganisms by the induction of expression of a large number of genes encoding diverse proteins, many of which are believed to have a role in defense. Outstanding amongst the induced genes are those encoding chitinases, which hydrolyses chitin, a polymer of N-acetyl-glucosamine found in fungal cell walls and insect cuticles. Furthermore, various chitinase preparations inhibit the growth of many fungi *in vitro* by causing lysis of hyphal tips, especially in combination with β-1,3-glucanase (Broekaert et al., 1988; Broglie et al., 1991; Schiumbaum et al., 1986) and chitinase has been shown to accumulate around fungal hyphal material *in planta* (Benhamou et al., 1990; Wubben et al., 1992). All plants analyzed to date contain multiple forms of chitinase which have been divided into several classes on the basis of their structural and functional properties (Collinge et al., 1992). Endochitinases have been described and characterized for several plant species (Boller, 1988). These enzymes are of particular interest in studies of plant resistance against fungal pathogens, as the natural source is often present in fungal hyphae but absent in plants.

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Piper colubrinum Link, belongs to the family *Piperaceae* and is a distant relative of the cultivated black pepper, *Piper nigrum* L. This plant has gained biotechnological significance because it has been found to be resistant against a number of plant pathogens, viz., *Phytophthora capsici*, the causal organism of foot rot disease in black pepper, nematodes like *Meloidogyne incognita* and *Radopholus similes*, which causes heavy crop loss to black pepper cultivation (Nambiar and Sarma, 1977; Ramana and Mohandas, 1987; Devasahayam, 2000). Interspecific hybridization using *P. colubrinum* for the transfer of disease resistance genes to cultivated pepper was unsuccessful due to incompatibility problems. *P. colubrinum*, the plant with multiple resistance, thus can be biotechnologically utilized for transferring the resistance genes to cultivated black pepper varieties against the specific pathogens.

Identification and isolation of chitinase gene from *P. colubrinum* would be useful as this gene may be used to increase defense activity against a wide range of pathogens attacking spice crops viz., *Colletotrichum* in black pepper, *Fusarium* and *Ralstonia* in ginger and nematodes like *Radopholus* and *Meloidogyne* which attack major spices. The present study is aimed to study the induction of chitinase and its activity in *P. colubrinum* Link upon challenging it with *P. capsici* Leonian.

2. Methods

2.1. Plant and pathogen materials

P. colubrinum and *P. nigrum* plants were maintained in the green house. *P. capsici* were cultured on carrot agar plates and allowed to grow for 48 h in darkness. The agar buds of size 50 mm were cut and inoculated on the lower surface of the leaves with a layer of wet cotton over it. The inoculated plants were maintained in the same condition for 3 days with intermittent wetting. The leaves were detached from *P. colubrinum* at intervals of 12, 24, 48, 60, 72 and 96 h of inoculation. Black pepper (Var. Karimunda) leaves inoculated with *P. capsici* was kept for 48 h under the same conditions. Uninoculated plants were also maintained under the same conditions in the green house and was used as control. Young leaves (first and second) and old leaves (sixth and seventh) of *P. colubrinum* were also inoculated separately on different plants and kept for 48 h.

2.2. Preparation of colloidal chitin

Colloidal chitin was prepared from commercial chitin by the method of Wen et al. (2002) with a few modifications. Five grams of chitin powder was added slowly into 60 ml of concentrated HCl and kept at 4 °C overnight with vigorous stirring. The mixture was added to 2 l of ice cold 95% ethanol with rapid stirring and kept overnight at 25 °C. The precipitant was collected by centrifugation at 5000 rpm for 20 min at 4 °C and was washed several times with sterile distilled water through a filter paper placed on a funnel fitted with glass wool, till the pH of colloidal chitin became neutral (pH 7.0). The powder was later dried and stored at 4 °C for future applications.

2.3. Extraction of chitinase

Twenty grams of leaf material was homogenized to a fine powder in liquid nitrogen using a pestle and mortar. The powder was suspended in an extraction buffer consisting of 0.1 M acetate buffer (pH 5.0) containing 0.1% (W/V) each of ascorbic acid and sodium sulphite and 5% PVP. The homogenates were squeezed through a muslin cloth and was centrifuged at 12,000 rpm at 4 °C for 30 min. The supernatant was incubated at 37 °C and quickly cooled to 4 °C. Ammonium sulfate was added to the supernatant at 90% saturation and stirred at 4 °C for 2 h followed by centrifugation at 12,000*g* at 4 °C for 20 min. The precipitate collected after saturation was redissolved in acetate buffer and dialyzed against the same buffer overnight. Protein concentrations were measured according to Lowry's method using bovine serum albumin as the standard.

2.4. Chitinase activity

Colloidal chitin was used as the substrate. 0.1 ml of protein extract was added to 1.0 ml of 0.1% colloidal chitin in 50 mM acetate buffer (pH 5.0) at room temperature to start the enzyme reaction. After 30 min, the reaction was stopped by heating at 100 °C for 5 min. The reducing ends which were released by the samples were determined according to the method of Somogyi (1952). The preheated extracts (100 °C for 5 min) were used as controls. One unit of chitinase activity was defined as an amount capable of releasing reducing ends corresponding to 1 μg GlcNAc from colloidal chitin at pH 5.0 in 1 h.

2.5. RNA isolation

Leaves from inoculated and uninoculated plants of *P. colubrinum* were used for RNA isolation. RNA isolation was carried out using a modified guanidinum thiocyanate–phenol chloroform method (Johnson et al., 2005). The quality of RNA was checked by resolving the RNA on 1% denaturing agarose gel stained with ethidium bromide and the quantification was done using a spectrophotometer by taking the absorbance at 260 and 280 nm. Primers were designed based on the conserved sequence motifs of chitinase gene. The highly conserved amino acid sequence SHETTGG – found in almost all characterized chitinases – was used to synthesize primers for amplifying chitinase specific gene from *P. colubrinum*.

2.6. RT–PCR

Reverse transcription and PCR amplification of cDNA was carried out using 1 μg of RNA according to Johnson et al. (2005). Oligo $dT_{(18)}$ primer was used in the first strand synthesis and the reaction volume was set to 15 μL. RNA and primer were denatured by incubating at 75 °C for 2 min, followed by incubation at 37 °C for 1 h and 75 °C for 5 min. After incubating the tubes at 37 °C for 10 min, the thermocycler was paused and 1 μL of MMLV reverse transcriptase (100 U/μL) (Ambion, USA) was added and mixed before continuing incubation. Only 10% of the cDNA synthesized (2 μL), was used for subsequent PCR amplification. To the tube containing 2 μL of the first strand reaction mixture, 2.0 μL of 10X DNA polymerase buffer (Ambion, USA), 1.0 μL of dNTP mix (10 mM), 0.75 μL of $MgCl_2$ (50 mM), 1.25 μL Oligo $dT_{(18)}$ (250 ng/reaction), 2.0 μL of chitinase specific primer (2 μM) (Bangalore Genei, India) and 0.4 μL of Super Taq DNA Polymerase (5U/μL)

(Ambion, USA) were added and the volume was made up to 20 µL using nuclease free water. PCR amplification was carried out using the following thermocycling conditions: 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 2 min, annealing at 40 °C for 2 min and 72 °C for 3 min. The 40th cycle was followed by a final extension step at 72 °C for 10 min.

The PCR products were resolved on 15% polyacrylamide gels stained with ethidium bromide, electrophoresed at 80 V for 3 h and photographed under exposure to UV light. The banding pattern was observed and the molecular weight of the differentially expressed band was calculated.

2.7. Antifungal assay

A square (1 cm^2) of agar with growing fungus was placed into the middle of a petri dish with carrot agar. When the fungal mycelium reached a diameter of 5 cm, sterile filter paper discs of Whatmann were positioned adjacent to the fungal colony margin. The discs were saturated with 100, 150 and 200 µg of the protein extract. Sterile extraction buffer was used as controls. The plates were then incubated in the dark for a period of 24 h. The plates were observed for any fungal inhibition after 24 h and evaluated visually by comparing with the controls. This experiment was repeated three times and the data was pooled.

3. Results and discussion

The chitinase activity of the extracts of *P. colubrinum* leaves harvested at different time intervals after inoculation is shown in Fig. 1. Maximum levels of chitinase activity in *P. colubrinum* was observed 60 h post inoculation after which the levels of chitinase gradually decreased. Very little activity was detected in the uninoculated control plants. Accumulation of chitinase against *Phytophthora* has been reported earlier by several workers (Meins and Ahl, 1989; Schroder et al., 1992; Siefert and Grossman, 1997). Differential accumulation of chitinase isoforms in pepper stems infected with compatible and incompatible isolates of *P. capsici* was reported by Kim and Hwang (1994) and Hwang et al. (1997). Infection of potato leaves by the late blight fungus *P. infestans* lead to a strong increase in chitinase activity (Buchter et al., 1997).

In black pepper (susceptible variety), the chitinase activity of the inoculated plants (48 h) was much higher compared to *P. colubrinum*. The base level of chitinase in black pepper also was substantially higher. The chitinase activity of black pepper upon 48 h of inoculation was found to be 40% higher than that of *P. colubrinum* under the same conditions (data not shown). The presence of higher chitinase activities in susceptible potato varieties was reported by Schroder et al. (1992). The overall timing and the magnitude of induction was similar in the resistant and susceptible potato varieties. Pegg and Young (1981) suggested that presence of higher amount of the enzyme in the susceptible variety may be due to a larger number of plant cells responding to the invading fungus as compared to the resistant variety. The presence of higher quantity of chitinase in susceptible tomato compared to the resistant variety was reported by Ferraris et al. (1987). The reason for this differential accumulation was postulated due to the larger fungal biomass that accumulated in susceptible interactions. Plant chitinases may be acidic or basic, vacuolar or cytoplasmic, constitutive or induced by various stresses including disease challenge. So there is often a base level of chitinase activity in different species and then this level can go up after a stress event and this may be due to different chitinase genes being activated (Taylor et al., 1990; Mc Fadden et al., 2001).

To analyze the differential activity of chitinase, the response was analyzed on young and old leaves. While enzyme activity in the young leaves (top of the plant) was found to be less, increasing amounts of chitinase were present in the old leaves (Fig. 2). This result is in accordance with similar reports in tobacco (Shinshi et al., 1987), potato (Schroder et al., 1992) and other plants (Knogge et al., 1987; Mauch et al., 1988; Metraux and Boller, 1986), where chitinase gene expression also increased with the age of the leaf.

To answer the question as to whether the induction of chitinase was the result of gene activation, RNA was isolated from the inoculated and non-inoculated leaves. Chitinase specific primers were designed based on the highly conserved sequence motifs obtained from the database. The highly conserved amino acid sequence SHETTGG was used to design degenerate primers for amplifying chitinase specific cDNA. The abundance of chitinase mRNA was low in control leaves whereas, 18 h post inoculation, a strong increase in chitinase was observed in the inoculated leaves. A 500 bp chitinase specific cDNA fragment was amplified in the inoculated leaves and was absent in the uninoculated control (Fig. 3). However,

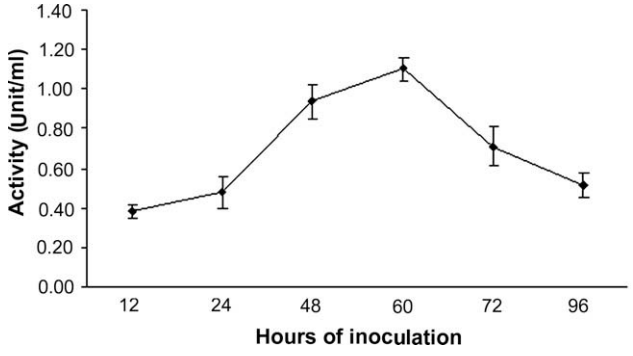

Figure 1 Chitinase activities of leaf extracts from *P. colubrinum* after 12, 24, 48, 60, 72 and 96 h of inoculation with *P. capsici*. Values presented are the means of four replicates. Error bars represent the standard errors of the mean.

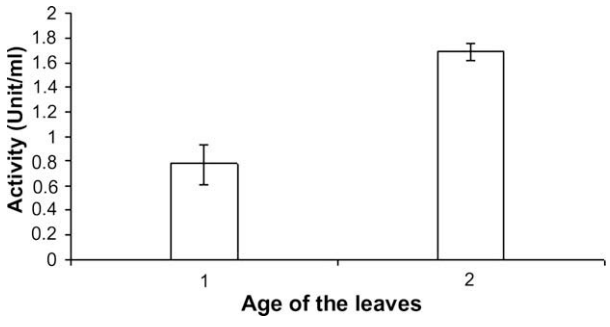

Figure 2 The chitinase activities in protein extracts of different leaves of *P. colubrinum*. 1 – young leaves, 2 – old leaves. Each mean and error bar were obtained from samples of four repeated blocks.

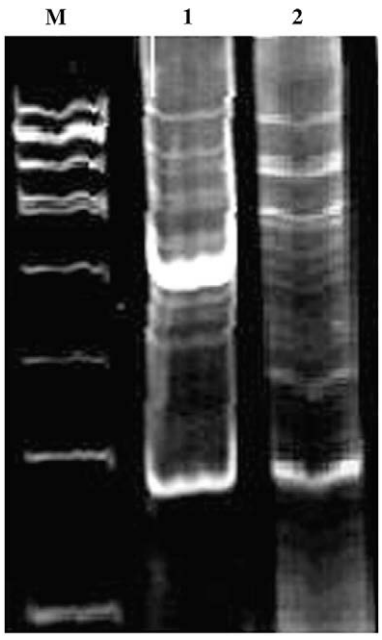

Figure 3 RT–PCR product of the RNA isolated from control and inoculated leaves of *P. colubrinum* using chitinase specific primer. M-1 Kb ladder, 1 – inoculated sample, 2 – uninoculated sample. Arrow indicates 500 bp chitinase specific band.

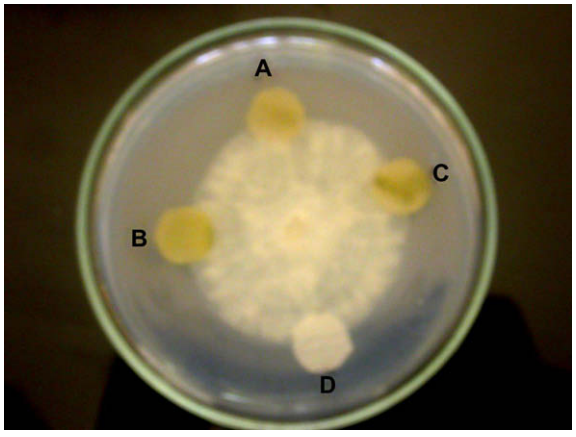

Figure 4 The effect of protein extracts from the leaves of *P. colubrinum* on the growth of *P. capsici*. Protein extracts from *P. colubrinum* was applied in amount of 100 µg (A), 150 µg (B) and 200 µg (C) on sterile filter paper discs. Extraction buffer (D) was used as negative controls.

cloning and sequencing of the cDNA is required to confirm the identity of the chitinase gene. Differential accumulation of chitinase mRNA in leaves upon challenge with pathogens were reported in a number of plants (Rasmussen et al., 1992; Vad et al., 1991; Buchter et al., 1997).

To check the ability of the chitinase extract to inhibit the growth of *P. capsici in vitro*, hyphal extension assays was carried out. 100, 150 and 200 µg of protein extracts of *P. colubrinum* were applied on filter paper discs. Extraction buffer was used as negative control. No inhibition was observed in the fungal growth when compared to the control (Fig. 4). The possible reason for this may be that the effective concentration of the protein desired is much lower in the extract than that would be required. Ji and Kue (1996) showed that only purified chitinase and glucanase isolated from pathogen-induced cucumber plants inhibited fungal growth more effectively than the amount of these enzymes in the crude extract. Since crude extract or partially purified extracts are a mixture containing many compounds, it is also possible that some of such compounds can act as a nutrient or factor benefiting the fungus and negating the role of the chitinase enzyme (Moravcikova et al., 2004). Therefore the positive effect of chitinase along with other defense proteins might be masked in inhibiting fungal growth under the present test conditions. It may also be realized that the results of the *in vitro* fungal tests with plant extracts are not always relevant for the *in planta* situation (Paxton, 1991). Thus, despite an increased occurrence of chitinase enzyme in the inoculated leaves, the growth of *Phytophthora* was not effectively impaired. The inability of protein extracts from infected potato leaves with high chitinase and glucanase activities to inhibit the growth of *P. infestans in vitro* was reported by Schroder et al. (1992) and Mauch et al. (1998). Using crude extracts or purified chitinase and glucanase preparations from pea, growth of several Oomycetes, including *Phytophthora* was not inhibited. Oomycetes possess a cell wall structure of the cellulose-glucan type lacking chitin (Bartincki-Garcia, 1968). Consequently, chitinase cannot participate in the degradation and β-1,3-glucanase alone is apparently not sufficient.

However, these results do not preclude an adverse effect of chitinase either alone or in combination with β-1,3-glucanase on other fungal or bacterial pathogens, and hence a positive contribution to plant resistance in general.

Though *P. colubrinum* has been reported to be resistant to a number of plant pathogens, the plant remains biotechnologically under utilized. Research on successful isolation of resistance genes and PR proteins from this plant is still in infancy. A PCR based suppression subtractive hybridization (SSH) was used by Dicto and Manjula (2005) to identify *P. colubrinum* genes that are differentially expressed in response to the signaling molecule, salicylic acid (SA). One of the clones derived from subtracted library showed sequence homology to osmotin, a member of class-V group of pathogenesis related (PR) gene family. Girija et al. (2005a) reported successful isolation of hydroxy methyl glutaryl CoA reductase gene from *P. colubrinum*. The *hmgr* gene is also reported to confer resistance to fungal pathogens besides its role in isoprenoid biosynthesis. Successful cloning of a cDNA fragment corresponding to β-1,3-glucanase from black pepper (*P. nigrum* L.) was reported by Girija et al. (2005b). The induction of PR-proteins and defense related enzyme such as PAL and β-1,3-glucanase against *P. capsici* was observed in P24–a tolerant variety of black pepper (Stephen et al., 2001).

The present study was mainly carried out to study the activity of chitinase in *P. colubrinum* upon inoculation with *P. capsici*. *P. colubrinum*, being a multiple resistance plant, is biotechnologically important as the resistance and defense genes from this plant can be used to confer resistance to a number of plant pathogens attacking spice crops. A clear picture of the role of chitinase in disease response can often be derived using Northern analysis and using different probes specific to different chitinase genes. Most studies have shown that chitinase may not be involved directly in contributing

disease resistance but has a synergistic activity with glucanase. Once isolated, the gene may be useful in providing defense response against a number of plant pathogens affecting black pepper and other crops. This is the first report on the identification and differential accumulation of chitinase in *P. colubrinum*.

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References

Bartincki-Garcia, B., 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Ann. Rev. Microbiol. 22, 87–108.

Benhamou, N., Joosten, M.H.A.J., de Wit, P.J.G.M., 1990. Subcellular localization of chitinase and of its potential substrate in tomato root tissues infected by *Fusarium oxysporium* f. sp. *radicis-lycopersici*. Plant Physiol. 92, 1108–1120.

Boller, T., 1988. Ethylene and the regulation of antifungal hydrolases in plants. In: Miflin, B.J. (Ed.). In: Surveys of Plant Molecular and Cell Biology, vol. 5. Oxford University Press, Oxford, UK, pp. 145–174.

Broekaert, W.F., Parijis, J.V., Allen, A.K., Peumans, W.J., 1988. Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn apple, tobacco and wheat. Physiol. Mol. Plant Pathol. 33, 319–331.

Broglie, K., Chet, I., Holiday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J., Broglie, R., 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254, 1194–1197.

Buchter, R., Stromberg, A., Schmelzer, E., Kombrink, E., 1997. Primary structure and expression of acidic (class II) chitinase in potato. Plant Mol. Biol. 35, 749–761.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., Vad, K., 1992. Plant chitinases. Plant J. 3, 31–40.

Devasahayam, S., 2000. Insect pests of black pepper. In: Ravindran, P.N. (Ed.), Black Pepper (*Piper nigrum* L.). Harwood Academic Publishers, Amsterdam, pp. 309–334.

Dicto, J., Manjula, S., 2005. Identification of elicitor-induced PR5 gene homologue in *Piper colubrinum* Link by suppression subtractive hybridization. Curr. Sci. 88, 624–627.

Ferraris, L., Abbattista, G.I., Matta, A., 1987. Activation of glycosidases as a consequence of infection stress in *Fusarium* wilt of tomato. J. Phytopathol. 118, 317–325.

Girija, D., Beena, P.S., Nazeem, P.A., Puroshothama, M.G. 2005a. Molecular cloning of cDNA fragment encoding hydroxy methyl glutaryl CoA reductase in *Piper colubrinum*. In: Peter, K.V. (Ed.), Proceedings of the National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and Strategies, 2005, Kerala Agricultural University, Thrissur, Kerala, India, pp. 303–306.

Girija, D., Beena, P.S., Nazeem, P.A. 2005b. Molecular cloning of a cDNA fragment encoding the defense related protein β-1,3-glucanase in black pepper (*P. nigrum* L.). In: Muthunayakam (Ed.), Proceedings of Kerala Science Congress, 2005, KFRI, Kerala, India, pp. 81–82.

Hwang, B.K., Sunwoo, J.Y., Kim, Y.J., Kim, B.S., 1997. Accumulation of β-1,3-glucanase and chitinase isoforms, and salicylic acid in the DL-β-amino-*n*-butyric acid-induced resistance response of pepper stems to *Phytophthora capsici*. Physiol. Mol. Plant Pathol. 51, 305–322.

Ji, C., Kue, J., 1996. Antifungal activity of cucumber β-1,3-glucanase and chitinase. Physiol. Mol. Plant Pathol. 49, 257–265.

Johnson George, K., Sandeep Varma, R., Ganga, G., Anandaraj, M., Parthasarathy, V.A., 2005. A method for isolation and reverse transcription of high quality RNA from *Piper* species. J. Spices Aromat. Crops 14, 10–14.

Kim, Y.J., Hwang, B.K., 1994. Differential accumulation of β-1,3-glucanase and chitinase isoforms in pepper stems infected by compatible and incompatible isolates of *Phytophthora capsici*. Physiol. Mol. Plant Pathol. 45, 195–209.

Knogge, W., Kombrink, E., Schmeizer, E., Hahlbrock, K., 1987. Occurrence of phytoalexins and other putative defense related substances in uninfected parsley plants. Planta 171, 279–287.

Mauch, F., Hadwiger, L.A., Boller, T., 1988. Antifungal hydrolases in pea tissue. I. Purification and characterization of two chitinases and two 1,3-β-glucanases differentially regulated during development and in response to fungal infection. Plant Physiol. 87, 325–333.

Mc Fadden, H.G., Lawrence, G.J., Dennis, E.S., 2001. Differential induction of chitinase activity in flax (*Linum usitatissimum*) in response to inoculation with virulent and avirulent strains of *Melamspora lini*, the cause of flax rust. Austral. Plant Pathol. 30, 27–30.

Meins Jr., F., Ahl, P., 1989. Induction of chitinase and 1,3-β-glucanase in tobacco plants infected with *Pseudomonas tabaci* and *Phytophthora parasitica* var. *nicotianae*. Plant Sci. 98, 163–173.

Metraux, J.P., Boller, T., 1986. Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial and fungal infections. Physiol. Mol. Plant Pathol. 28, 161–169.

Moravcikova, J., Libantova, J., Matusikova, G., Nap, J.P., Mlynarova, L., 2004. Genetic transformation of Slovak cultivar of potato (*Solanum tuberosum* L.) efficiency and the behaviour of the transgene. Biologia 6, 473–479.

Nambiar, K.K.N., Sarma, Y.R., 1977. Wilt disease of black pepper. J. Plantation Crops 5, 92–103.

Paxton, D.J., 1991. Assays for antifungal activity. Meth. Plant Biochem. 6, 33–46.

Pegg, G.F., Young, D.H., 1981. Changes in glycosidase activity and their relationship to fungal colonization during infection of tomato by *Verticillium albo-atrum*. Physiol. Plant Pathol. 19, 371–382.

Ramana, K.V., Mohandas, C., 1987. Plant parasitic nematodes associated with black pepper in Kerala. Indian J. Nematol. 17, 62–66.

Rasmussen, U., Bojsen, K., Collinge, B.C., 1992. Cloning and characterization of a pathogen-induced chitinase in *Brassica napus*. Plant Mol. Biol. 20, 277–287.

Schiumbaum, A., Mauch, F., Vogeli, U., Boller, T., 1986. Plant chitinases are potent inhibitors of fungal growth. Nature 324, 365–367.

Schroder, M., Hahlbrock, K., Kombrink, E., 1992. Temporal and spatial patterns of 1,3-β-glucanase and chitinase induction in potato leaves infected by *Phytophthora infestans*. Plant J. 2, 161–172.

Shinshi, H., Mohnen, D., Meins Jr., F., 1987. Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc. Natl. Acad. Sci. USA 84, 89–93.

Siefert, F., Grossman, K., 1997. Induction of chitinase and β-1,3-glucanase activity in sunflower suspension cells in response to an elicitor from *Phytophthora megasperma* f. sp. *glycinea* (Pmg). Evidence for regulation by ethylene and 1-amino cyclopropane-1-carboxylic acid (ACC). J. Exp. Bot. 317, 2023–2029.

Somogyi, M., 1952. Determination of reducing sugars by Nelson–Somogyi method. J. Biol. Chem. 200, 245.

Stephen, R.J., Anandaraj, M., Sarma, Y.R., 2001. Induction of PR-proteins and defense related enzymes in black pepper due to inoculation with *Phytophthora capsici*. Ind. Phytopath. 54, 23–28.

Taylor, J.L., Fritzemeier, K.H., Hauser, I., Kombrink, E., Rohwer, F., Schroder, M., Strittmatter, G., Hahlbrock, K., 1990. Structural analysis and activation by fungal infection of a gene encoding a pathogenesis-related protein in potato. Mol. Plant–Microbe Interact. 3, 72–77.

Vad, K., Mikkelsen, J.D., Collinge, D.B., 1991. Induction, purification and characterization of chitinase isolated from pea leaves inoculated with *Ascochyta pisi*. Planta 184, 24–29.

Wen, C.M., Tseng, C.S., Cheng, C.Y., Li, Y.W., 2002. Purification, characterization and cloning of a chitinase from *Bacillus* sp. NCTU2. Biotechnol. Appl. Biochem. 35, 213–219.

Wubben, J.P., Joosten, M.H.A.J., Van Kan, J.A.L., de Wit, P.J.G.M., 1992. Subcellular localization of plant chitinases and β-1,3-glucanases in *Cladosporium fulvum* (syn. *Fulvia fulva*)-infected tomato leaves. Physiol. Mol. Plant Pathol. 226, 289–296.