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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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Chitinase assay;
Differential accumulation;
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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)

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