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Biochemical defense responses of black pepper (*Piper nigrum* L.) lines to *Phytophthora capsici*

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ABSTRACT

A study on biochemical factors involved in black pepper defense response against *Phytophthora capsici*, was carried out in *P. capsici* susceptible (Sreekara) and resistant (04-P24, shows root resistance to the pathogen) black pepper lines. Seven important factors – change in membrane conductance, total phenols, orthodihydroxy (OD) phenols, lignin and defense related enzymes (peroxidase, β-1,3 glucanase and β-1,4 glucanase) – were studied under uninoculated and pathogen (*P. capsici*, isolate 06-04) inoculated condition to know the preformed and induced responses. The pathogen was inoculated (soil inoculation) and plants were observed for changes, at 24 h intervals for 10 days. On 8th day after inoculation symptoms started appearing on Sreekara and increased the severity till 10th day. Both root and stem samples were subjected for biochemical analysis. Of the factors analyzed, it was found that membrane conductance, OD phenol, lignin and peroxidase activity play significant role in root resistance to *P. capsici* in 04-P24. Light microscopy of the portion of root – where pathogen found attached – was also done.

Introduction

Black pepper (*Piper nigrum* L.) is known as the 'King of spices', dominating 34% of the world spice trade in volume. The major diseases identified in this crop are *Phytophthora* foot rot ('quick wilt') and slow decline disease ('slow wilt'). *Phytophthora capsici*, the causal organism for foot rot disease, is one of the most serious threats to black pepper cultivation in India. Crop loss due to this disease has been identified as a major constraint in its production. Plants possess both preformed and inducible mechanisms to resist pathogen invasion. Pathogen must overcome the morphological barriers, secondary metabolites (phytoanticipins), and antimicrobial proteins to invade a plant. Increased cell permeability is associated with host response to the pathogen, and the magnitude of increase is greatest in hypersensitive host–pathogen combination.

Earlier workers have screened around one million seedlings and identified one progeny from a black pepper cultivar Perambramundi viz. IISR Shakti, as moderately resistant to *Phytophthora* infection [7]. The mechanism of resistance in this line was reported to be due to the early activation of defense enzyme viz. phenylalanine ammonia lyase (PAL) and induction of pathogenesis related (PR) proteins such as β-1,3, glucanase [42]. Recently another open pollinated (OP) progeny (04-P24) raised from IISR Shakti showed root resistance to *P. capsici* by all means of screening [7]. Even after repeated inoculations, the plant showed resistance to *P. capsici* root infection. An integrated disease management strategy incorporating resistant cultivars will be the ultimate solution to tackle the problem of crop loss due to *Phyophthora* infection. Hence identifying resistance sources to multiple infections becomes imperative as an effective and long term disease management strategy. So far, a detailed study on *Phytophthora* resistance (biochemical factors) of black pepper has not been conducted. So the present study is aimed to elucidate the biochemical mechanisms of resistance in OP progeny 04-P24 having root resistance in comparison with a highly susceptible variety Sreekara. In black pepper, some preliminary studies were already carried out by different researchers on biochemical defense parameters against *Phytophthora*. Since 04-P24 is an OP progeny of IISR Shakti and in this variety, the role of membrane conductance, total phenols and defense related enzymes (beta-1,3-glucanase and peroxidase) had already been studied, these parameters were included in our study also. The role of OD phenols and lignin were not done so far in 04-P24. In many plants, these two factors play definite role in host plant defense against different pathogens. OD phenols are resistant factors

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because they become highly reactive upon oxidation by polyphenol oxidase and peroxidase to the corresponding quinines, which are toxic to the pathogen or which inactivate enzymes including hydrolytic enzymes produced by plant pathogenic fungi [10,33]. Similarly the cell wall of *Phytophthora* is formed of cellulose, an attempt was also made to study the possible role of beta-1,4-glucanase in defense against this pathogen. So the defense related biochemical parameters viz. change in membrane conductivity, total phenols, orthodihydroxy (OD) phenols, lignin and enzymes like peroxidase, β-1,3 and β-1,4 glucanases during *P. capsici*-black pepper interactions are examined to find out possible correlation with disease resistance.

Materials and methods

Plant material and pathogen inoculation

Plant material

P. capsici susceptible (Sreekara) and resistant (04-P24, OP progeny of IISR Shakti) lines of black pepper (*P. nigrum* L.) were used in this study. The plants were multiplied using serpentine propagation method. Single node cuttings were grown and maintained in sterile porting mixture (soil: sand: cow dung, 2:2:1) in polythene bags of size 20 × 10 cm under green house conditions and maintained. Plants of 4–5 leaf stage were selected for conducting the experiment.

Pathogen

The *P. capsici* isolate 06-04 maintained in National Repository of *Phytophthora*, IISR, Kozhikode was used for inoculation. The isolate was sub-cultured and maintained in carrot agar (CA) medium.

Pathogen inoculation

P. capsici was grown on CA for 72 h at 24 ± 1 °C. Inoculum plugs of 5 mm size were cut from the periphery of the actively growing culture, and were kept for sporulation under continuous light for 48 h at 24 ± 1 °C. The sporulated discs were used for plant inoculation. Uninoculated plants served as control. Three replications were maintained for all the treatments.

Sampling

From the inoculated plants, root and stem samples were drawn from 1 to 10 days after inoculation (DAI) at 24 h interval and subjected to biochemical analysis. For each sampling, plants were uprooted and observed for symptom development (root infection) and documented. Analysis was carried out in triplicates on triplicate samples. Uninoculated plants served as control.

Biochemical analysis

Determination of change in membrane conductivity

For determining the change in membrane conductivity, 500 mg of the sample (~1 cm long pieces) was weighed, suspended in 25 ml de-ionized water and kept at room temperature overnight. Sample was removed and the conductivity measured using conductivity meter (EUTECH Instruments cyberscan con 11). Values were recorded in micro Siemens (μS) at 20 °C.

Extraction and estimation of phenols

Total phenols were extracted according to the method described by Ref. [12]; with some modifications. 200 mg of tissue was extracted twice with 1.5 ml of 80% methanol at room temperature for 1 h with constant shaking and the extracts were filtered using Whatman no. 1 filter paper.

Total phenols, were estimated using Folin-Ciocalteau method described by Gutfinger, (1981) with some modifications. 0.5 ml Folin-Ciocalteau reagent was added to 0.2 ml of sample; after 3 min, 1 ml of saturated Na_2CO_3 solution was added and diluted to 10 ml with water. After 1 h, absorbance was read at 725 nm in a spectrophotometer and quantified against a standard of gallic acid. The phenolic content was reported as gallic acid equivalents based on a calibration curve.

OD phenols were estimated using the method described by Ref. [15]. To 0.2 ml sample, 1 ml of 0.1 M phosphate buffer (pH 6.5) and 2 ml of 5% $Na_2MoO_4.2H_2O$ solution were added. The contents were mixed and incubated for 15 min; the absorbance was measured at 350 nm in a spectrophotometer and quantified against a standard of caffeic acid. The OD phenolic content was reported as caffeic acid equivalents based on a calibration curve.

Determination of acetyl bromide soluble lignin (ABSL) content

The root and stem samples were washed dried, powdered and used for lignin estimation. Lignin was determined by acetyl bromide procedure of [17]. 10–15 mg dried powdered samples were weighed into a brown vial and 2 ml of acetyl bromide in glacial acetic acid (1:3 v/v containing perchloric acid (70%, 0.08 ml)) was added. The mixture was incubated at 70 °C for 30 min and the digested samples were transferred, with the aid of acetic acid, to 50 ml volumetric flasks containing 5 ml of 2 M sodium hydroxide and 12 ml of acetic acid. The flasks were made to the mark with acetic acid. Absorbance was measured at 280 nm and lignin content was determined using SAC (Specific Absorption Coefficient) of lignin, 20 g^{-1} cm^{-1}.

Extraction and enzyme assays

Peroxidase (EC 1.11.1.7)

Peroxidase activity was determined according to [1]. Samples (0.25 g) were extracted in 5 ml chilled 25 mM Borate: HCl buffer (pH 8.8) by grinding at 4 °C. The extract was centrifuged at 4 °C and the supernatant was used for the enzyme assay. Reaction mixture consisted of 0.5 ml enzyme extract, 3 ml of the substrate – 0.5 M pyrogallol in 0.1 M sodium phosphate buffer (pH 6) and 0.5 ml of 1% H_2O_2. Activity was determined by measuring absorbance at 20 s interval for a period of 3 min at 420 nm in a spectrophotometer, and compared against heat inactivated controls. The enzyme activity was expressed in units of change in OD min^{-1} mg^{-1} protein.

β-1,3-glucanase (E.C.3.2.1.6)

Beta-1,3-glucanase activity was assayed colorimetrically using the Nelson–Somogyi method [40] with slight modifications. Samples (0.5 g) were extracted with 5 ml of 0.1 M sodium acetate buffer (pH 5.0) at 4 °C. The extract was centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant served as the crude enzyme extract for the enzyme assay. The reaction mixture consisted of 0.075 ml enzyme extract and 0.075 ml substrate, 4% laminarin. The reaction was set up by incubating at 40 °C for 10 min and then stopped by the addition of alkaline copper reagent and boiling for 10 min on a water bath. β-1,3-glucanase activity was determined by measuring absorbance at 620 nm in a spectrophotometer, and compared against heat inactivated controls, and glucose standards. The enzyme activity was expressed in units of mg glucose released min^{-1} mg^{-1} protein.

β-1,4-glucanase (E.C.3.2.1.4)

Beta-1,4-glucanase (E.C.3.2.1.4) activity was assayed colorimetrically using the Nelson–Somogyi method [40] with slight modifications. Samples (0.5 g) were extracted with 5 ml of 0.1 M sodium