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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

Fig. 1. Progress of *P. capsici* infection in Sreekara (susceptible) from 1st to 10th days after inoculation.

citrate 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 0.2 ml enzyme extract and 0.5 ml substrate, 1% carboxy methyl cellulose (CMC). The reaction was set up by incubating at 55 °C for 15 min and then stopped by the addition of alkaline copper reagent and boiling for 10 min on a water bath. Enzyme activity was determined by measuring absorbance at 620 nm 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.

Light microscopy

Histological study to visualize the presence of pathogen inside the tissue was conducted on roots of *P. capsici* inoculated plants. Fresh hand cut sections (transverse sections) of root portion – where pathogen found attached – were made, of which thin, uniform sections were selected and stained in lactophenol cotton blue (fungal specific stain) to see the presence of *P. capsici* hyphae and sporangia. Experiment was conducted in triplicates. The stained preparations were observed under bright field microscope (Nikon – Eclipse Ci-L) and photographs were taken.

Statistical analysis

Data were analyzed for significant differences by analysis of variance (ANOVA) with the statistical package SAS software (Version 9.3) and subjected to mean separation by the Least Significant Difference (LSD) test, $p < 0.05$.

Results

Plants were inoculated with *P. capsici* and samples were drawn from 1 to 10 days after inoculation (DAI). On each time sampling plants were uprooted and observed for symptom development. It was observed that up to seven days there were no signs of foot rot symptom development (root infection) on any of the inoculated plants, suggesting a period of 7 days for the establishment of *P. capsici*. The symptom appeared on the 8th day on Sreekara, where roots started showing rot symptoms and the severity of the rot (lesion) increased gradually (Fig. 1). But 04-P24 didn't develop any signs of infection even after 10th day of inoculation (Fig. 2).

Membrane conductivity

Root

Under uninoculated condition, membrane conductance of both susceptible and resistant lines was at par. After *P. capsici* inoculation, in resistant line increased membrane conductance was observed from 4th day onwards but in susceptible line, a significant increase was observed from 7th day onwards and remained high till 10th day in both lines (Fig. 3a). Highest increase was seen on 7th day in susceptible line (~1.6 fold) and on 10th day in resistant line (~1.6 fold).

Stem

Membrane conductance of stem remained at par in both lines under uninoculated condition. After pathogen inoculation, in both lines, stem conductivity showed a sharp shoot up at the 2 DAI, the increase being around 1.9 and 2 folds in susceptible and resistant

Fig. 2. Progress of *P. capsici* infection in 04-P24 (resistant) from 1st to 10th days after inoculation.

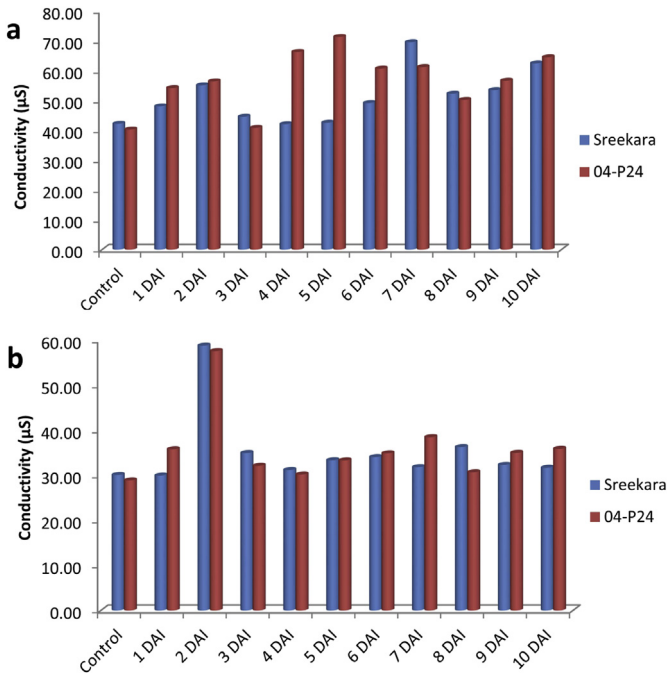

Fig. 3. Variation in conductivity in root (a) and stem (b) of uninoculated and *P. capsici* inoculated black pepper plants (*P. capsici* susceptible Sreekara and resistant 04-P24) at regular time intervals. (Control: uninoculated plants, and 1 to 10 DAI: inoculated plants from 1st to 10th day after inoculation). LSD ($p < 0.05$) for interaction – treatment × days after inoculation – are 7.93 in both root and stem.

lines respectively and there after a sudden decrease was noticed in both the cases (Fig. 3b).

Total phenols

Root

Total phenols of uninoculated resistant plants were around 1.3 fold high as compared to susceptible plants. Upon challenge inoculation, a significant increase in total phenols was seen in susceptible line, while in resistant line, except on second day, higher content of total phenols observed compared to uninoculated plants. The greatest increase, around 3.5 fold, was obtained on 3rd day in susceptible line and around 2.2 fold, in resistant line on 6th day (Fig. 4a).

Stem

Uninoculated resistant line showed approximately 1.5 fold higher total phenols as compared to their susceptible counterparts. At post inoculation stage, a significant increase was observed in susceptible line till 9th day, the highest increase was observed on 3rd day (~2.6 fold). In resistant line, significantly high phenol content was obtained from 3rd day onwards and the highest increase of around 1.9 fold was obtained on 9th day (Fig. 4b).

Orthodihydroxy phenols

Root

OD phenols remained at par in both lines before pathogen inoculation. After inoculation, a significant increase of OD phenols

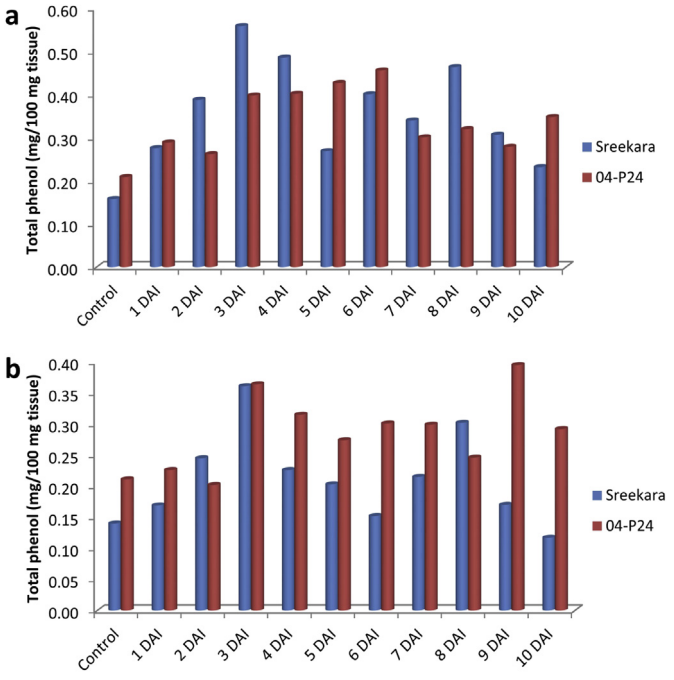

Fig. 4. Total phenol content in root (a) and stem (b) of uninoculated and *P. capsici* inoculated black pepper plants (*P. capsici* susceptible Sreekara and resistant 04-P24) at regular time intervals. (Control: uninoculated plants, and 1 to 10 DAI: inoculated plants from 1st to 10th day after inoculation). LSD ($p < 0.05$) for interaction – treatment × days after inoculation – are 0.051 (a) and 0.022 (b).

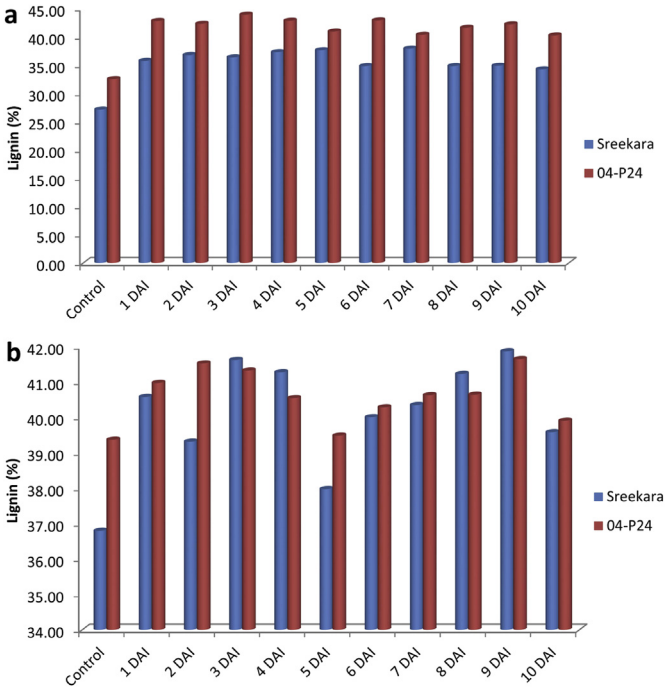

Fig. 6. Lignin content (ABSL) in root (a) and stem (b) of uninoculated and *P. capsici* inoculated black pepper plants (*P. capsici* susceptible Sreekara and resistant 04-P24) at regular time intervals. (Control: uninoculated plants, and 1 to 10 DAI: inoculated plants from 1st to 10th day after inoculation). LSD ($p < 0.05$) for interaction – treatment × days after inoculation – are 2.906 (a) and 1.665 (b).

was seen from 3rd day in resistant line compared to the uninoculated plants. In resistant line, highest increase in OD phenol quantity was on 5th day (~2.8 fold) and it was on 4th day (~2 fold) in susceptible line (Fig. 5a).

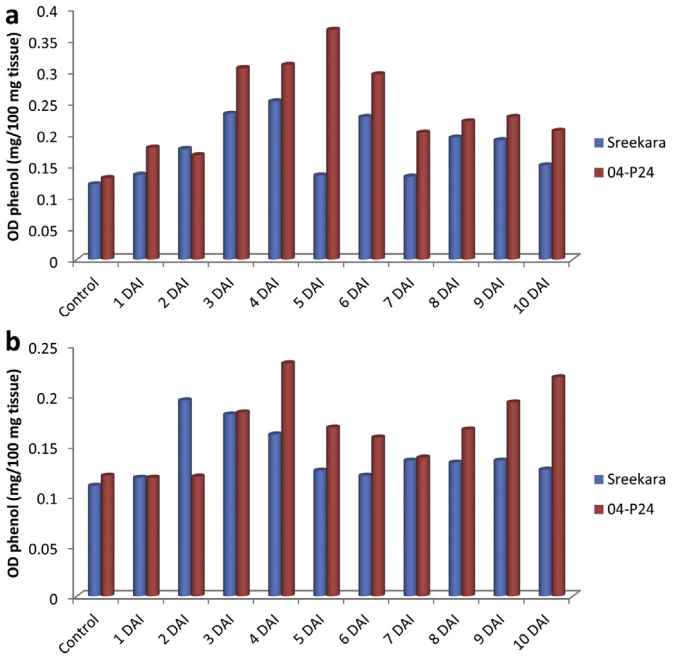

Fig. 5. OD phenol content in root (a) and stem (b) of uninoculated and *P. capsici* inoculated black pepper plants (*P. capsici* susceptible Sreekara and resistant 04-P24) at regular time intervals. (Control: uninoculated plants, and 1 to 10 DAI: inoculated plants from 1st to 10th day after inoculation). LSD ($p < 0.05$) for interaction – treatment × days after inoculation – are 0.051 (a) and 0.022 (b).

Stem

In both lines, under uninoculated condition, there was no significant difference in OD phenol content in both lines. After the pathogen inoculation, the resistant line showed similar trend as seen in inoculated roots. The highest induction of around 1.8 and 2.1 folds were obtained in susceptible line (2nd day) and resistant line (4th day) respectively (Fig 5b).

Lignin (ABSL) content

Root

There was high lignin content in uninoculated resistant line (around 1.2 fold) compared to uninoculated susceptible plants. In inoculated plants of both lines, a significant increase was noticed from 1st day onwards as compared to their uninoculated plants. The highest increase was around 1.4 fold in susceptible line and 1.3 fold in resistant line at 5 and 3 DAI respectively (Fig. 6a).

Stem

The lignin content of resistant plants was significantly high (~1.1 fold) in relation to susceptible line under uninoculated state. After *P. capsici* inoculation, a significant rise was observed in susceptible line but in resistant line only at 2, 3 and 9 DAI, a noticeable change observed. In both lines greatest increase was obtained on 9th day (~1.13 and 1.05 folds respectively) (Fig. 6b).

Defense related enzymes

Peroxidase

Root. Under uninoculated and inoculated condition, resistant line exhibited increased enzyme activity (~1.2 and 1.3 folds respectively). In both lines, inoculated plants showed significantly greater peroxidase activity as compared to their uninoculated control

plants. In susceptible line, from 2nd day onwards and in resistant line, from 1st day onwards significant rise in enzyme activity was observed and remained high throughout the study. Highest activity was recorded on 10th day in susceptible line and on 9th day in resistant line – approximately 2.8 and 2.5 folds respectively (Fig. 7a).

Stem. In uninoculated plants, peroxidase activity remained at par in susceptible and resistant lines. After inoculation, a significant increase could be observed in both lines (from 4th day onwards in susceptible line and from 1st day onwards in resistant line) compared to their uninoculated plants. Susceptible line recorded highest activity on 10th day and the resistant line on 6th day (~2.1 and 3.1 folds respectively) (Fig. 7b).

Beta-1,3-glucanase

Root. Before pathogen inoculation, roots from both susceptible and resistant lines had showed similar enzyme activity. After inoculation, a significantly higher activity of β-1,3-glucanase was noticed in susceptible line from 2nd to 10th day. While in resistant line, except at 1 and 3 DAI, inoculated roots showed increased enzyme activity during the progress of infection. The greatest increase was on 2nd day (~1.7 fold) in susceptible line whereas in resistant line, it was on 7th day (~1.5 fold) (Fig. 8a).

Stem. Among the black pepper lines, resistant line showed approximately 1.2 fold enhanced enzyme activity under uninoculated condition. Significant increase in enzyme activity was observed in inoculated plants of both lines from 2nd day and remained high throughout the study. Susceptible line showed greatest activity on 6th day and the resistant line, on 4th day – around 1.7 and 1.4 folds respectively (Fig. 8b).

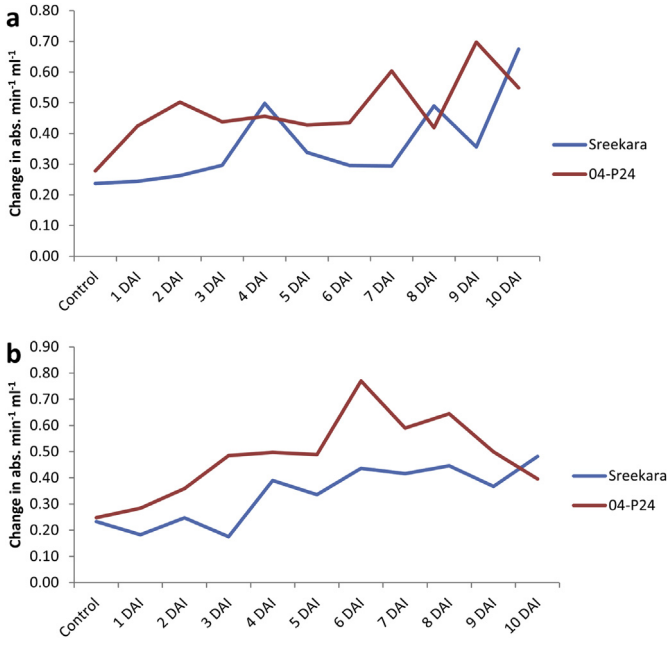

Fig. 7. Time-course activity of peroxidase in root (a) and stem (b) of uninoculated and *P. capsici* inoculated black pepper plants (*P. capsici* susceptible Sreekara and resistant 04-P24) at regular time intervals. (Control: uninoculated plants, and 1 to 10 DAI: inoculated plants from 1st to 10th day after inoculation). LSD ($p < 0.05$) for interaction – treatment × days after inoculation – are 0.022 in both root and stem.

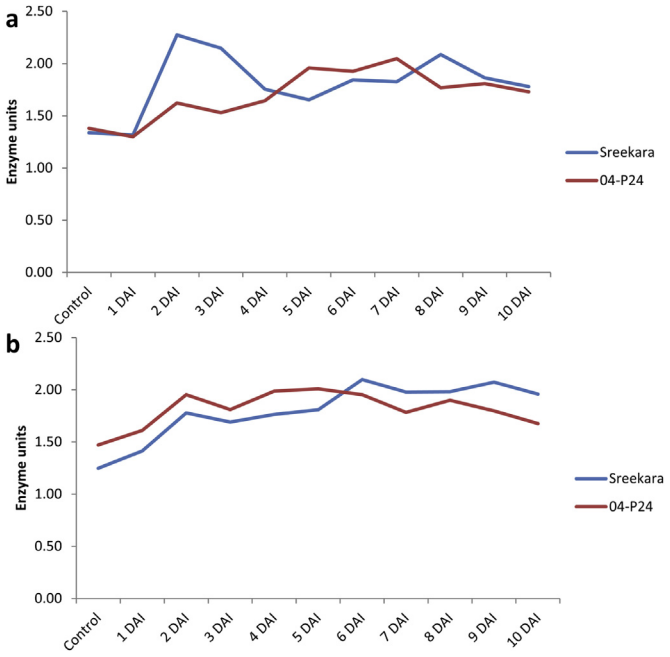

Fig. 8. Time-course activity of β-1,3-glucanase in root (a) and stem (b) of uninoculated and *P. capsici* inoculated black pepper plants (*P. capsici* susceptible Sreekara and resistant 04-P24) at regular time intervals. (Control: uninoculated plants, and 1 to 10 DAI: inoculated plants from 1st to 10th day after inoculation). LSD ($p < 0.05$) for interaction – treatment × days after inoculation – are 0.255 (a) and 0.202 (b).

β-1,4-glucanase

Root. In both lines, β-1,4-glucanase activity were at par before *P. capsici* inoculation. After inoculation, on 1st day itself significant, enhanced enzyme activity was shown by both lines. Except on 5th day in susceptible line and 2nd day in resistant line, significant enzyme activity was observed in inoculated root as compared to their uninoculated counterparts. The highest activity was recorded on 1st day in susceptible line (~3.8 fold) and on 10th day in resistant line (~3.4 fold) (Fig. 9a).

Stem. Among the black pepper lines, significant difference could not be observed in enzyme activity under uninoculated condition. Upon pathogen inoculation, enzyme activity increased in both lines, being the highest activity on 1st day in susceptible line (~3.6 fold) and on 8th day in resistant line (~3.2 fold) (Fig. 9b).

Light microscopy

Stained sections of infected portions of root of susceptible black pepper line showed blue color stained hyphae and sporangia of *P. capsici* demonstrating the tissue penetration by the pathogen (Fig. 10a–d). In the transverse sections of root of resistant line, the hyphae and sporangia were visualized only on the surface of the section but could not be spotted in the tissue inside (Fig. 10e–h). This result indicates that in both lines the pathogen attaches to the root surface and induces defense responses in the host plant. In the resistant line, the pathogen is restricted to the root surface and is not penetrating the healthy tissue below. But in the susceptible line, the hyphae penetrate the root tissues and damage them causing disease.

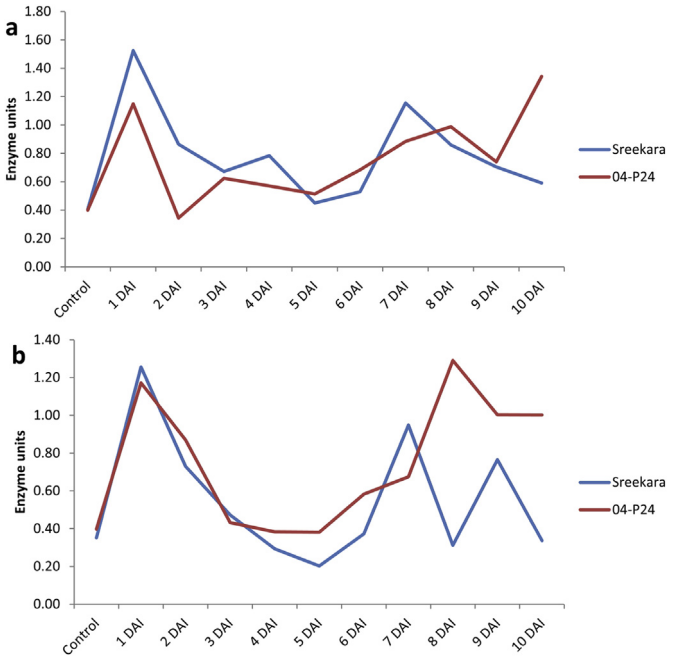

Fig. 9. Time-course activity of β-1,4-glucanase in root (a) and stem (b) of uninoculated and *P. capsici* inoculated black pepper plants (*P. capsici* susceptible Sreekara and resistant 04-P24) at regular time intervals. (Control: uninoculated plants, and 1 to 10 DAI: inoculated plants from 1st to 10th day after inoculation). LSD ($p < 0.05$) for interaction – treatment × days after inoculation – are 0.081 (a) and 0.065 (b).

Correlation analysis ($p < 0.05$)

A highly positive correlation between peroxidase and lignin (0.821), conductivity and β-1,3-glucanase (0.818) and OD phenols and lignin (0.801) were obtained in roots from resistant line. The corresponding values in roots of susceptible line were 0.524, 0.582 and 0.665 respectively (Table 1). In stem, correlations of 0.847 and 0.824 were found between β-1,3 glucanase and peroxidase, and total phenol and OD phenol respectively in resistant line, but in susceptible line, a highly positive correlation was seen between β-1,3 glucanase and lignin (0.835) and β-1,3 glucanase and peroxidase (0.823) (Table 2).

Discussion

The analysis of black pepper plants infected by *P. capsici* has revealed changes in the host cell membrane conductivity (measure of membrane permeability), in enzyme activities and in metabolite concentrations within the infected host tissues. However, due to the intricate nature of host-pathogen interactions, it is difficult to delineate the individual change caused by the plant or pathogen. In this study, an attempt was made systematically to analyze changes in some of key plant biochemical parameters in black pepper infected with *P. capsici* during the various stages of the disease progress and to correlate these changes.

Here, different biochemical parameters associated with plant defense response against biotic stress were analyzed in black pepper – *P. capsici* association in *Phytophthora* susceptible and resistant black pepper lines viz. Sreekara and 04-P24. A comparative study was carried out to reveal the difference in these biochemical factors in both the lines under uninoculated and inoculated conditions. Under uninoculated conditions, a significantly higher content of total phenols (1.3 fold increase), lignin (1.2 fold increase) and peroxidase activity (1.2 fold increase) was observed in the roots of resistant line as compared to those of susceptible line. In stem, total phenols, lignin, β-1,3-glucanase and β-1,4-glucanase activity were significantly high (1.5, 1.1, 1.2 and 1.1 folds increase respectively) in uninoculated 04-P24. After challenge inoculation with *P. capsici*,1.1 fold increase in membrane conductivity, 1.4 fold increase in OD phenols, 1.2 fold increase in lignin and 1.3 fold increase in peroxidase activity was recorded in 04-P24 over Sreekara root. The biochemical factors which showed significantly high activity in 04-P24 stem under inoculated condition include total phenol (1.4 fold increase), OD phenol (1.8 fold increase), and peroxidase (1.4 fold increase).

Membrane conductivity

The cell membrane damage of host tissue by pathogen intrusion and subsequent tissue softening has been suggested by Ref. [20]. Cell permeability is associated with the host response to the pathogen, and the magnitude of increase is greatest in hypersensitive host-pathogen combination. In our study, the degree of membrane integrity was assessed by the leakage of electrolytes. The mechanical wounding caused by pathogen and efflux of electrolytes in black pepper root was maximal at 5th DAI in resistant line and on 7th DAI in susceptible line under inoculated conditions (Fig. 3). In black pepper – *P. capcisi* interaction, changes in membrane conductivity were associated with infection progress [38]. In this study, the overall results showed significantly high membrane conductivity in roots of resistant line during earlier period of infection compared to the roots of susceptible line under inoculated condition (Fig. 3a). The possible explanation for this is the HR reaction at the site of pathogen entry. Sometimes HR reaction becomes unnoticeable due to involvement of single or very few cells [2]. That might be the reason for not developing visible lesion in roots of resistant line. The result of light microscopy is in agreement with this finding (Fig. 10).

Total phenols and OD phenols

In this study, the total phenols were considerably low in roots of resistant line during early days of infection when compared to inoculated susceptible plants (Fig. 4a). The explanation for this could be that the phenolics may act as substrates for the synthesis of compounds involved in disease resistance, like phytoalexins, hydroxycinnamic acids etc. [11]. But OD phenols in roots of resistant line remained high during the course of infection process, throughout the experiment (Fig. 5a). [27]; noticed high content of OD phenols in a *Phytophthora* tolerant genotype of black pepper, "Kalluvally", among all the black pepper varieties tested. The concentration of total phenols and OD phenols, showed significant changes due to infection in sorghum – *Drechslera sorghicola* interaction [21], in tea – *Sclerotium rolfsii* interaction [6], in olive tree – *Verticillium dahliae* interaction [28], in groundnut – *S. rolfsii* interaction [37] and in rice – *Cnaphalocrocis medinalis* interaction [35]. OD phenols play considerable role in increased resistance against plant pathogens and commencement of defense mechanisms like hypersensitive reaction. These reports corroborate with the observations made in this paper.

Lignin

Lignification – a structural defense mechanism – plays an important role in hypersensitive response of plants to pathogens. Peroxidase is one of the important enzymes in lignification process – as a terminal enzyme involved in the polymerization and synthesis of lignin. In our study, a highly positive correlation (0.821) was obtained between peroxidase activity and lignin content in

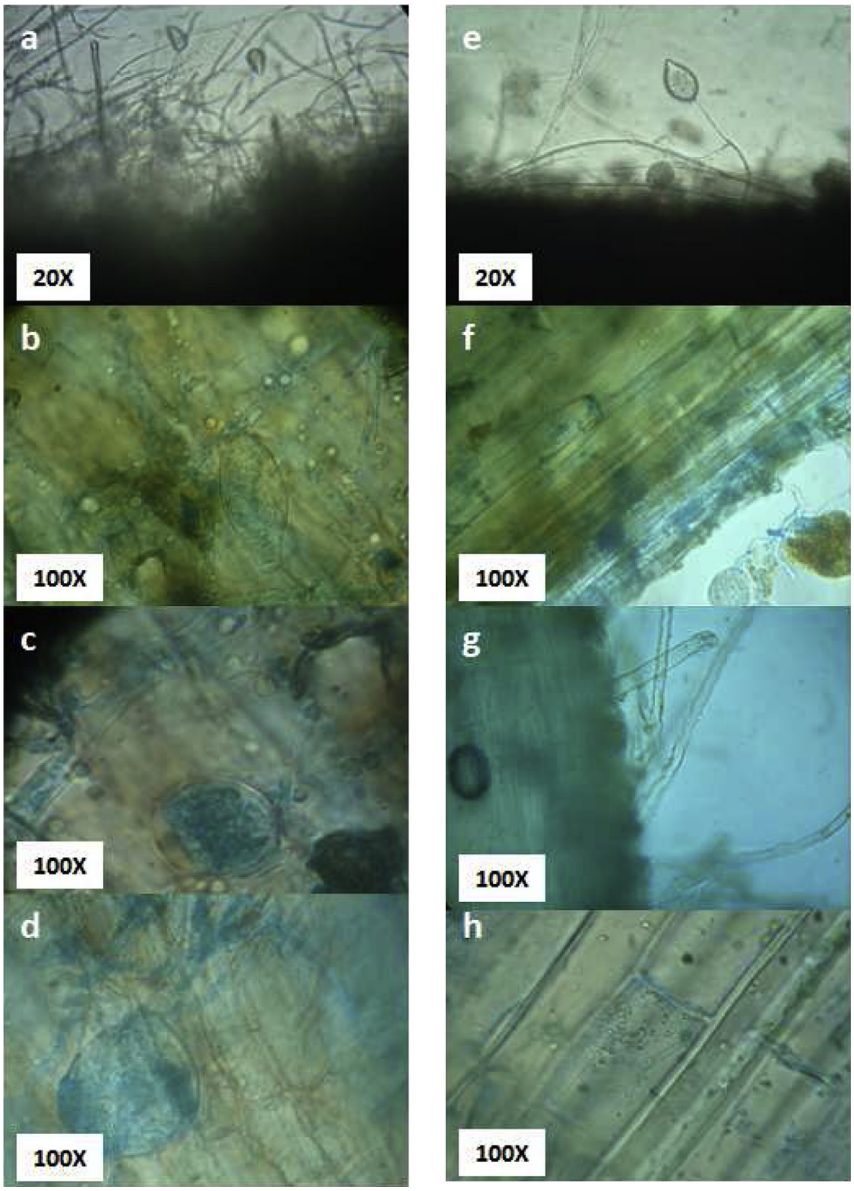

Fig. 10. Light microscopy images of transverse sections of roots of Sreekara (a–d) and 04-P24 (e–h) inoculated with *P. capsici* (sample drawn at 4 DAI). Figures a and e show the root surface colonized with pathogen (intact root). Figures b–d show the presence of *P. capsici* sporangia and hyphae inside the root tissue of Sreekara. In 04-P24, the hyphae and sporangia are found attached on the surface layer of tissue (f and g) and the inner tissue found devoid of them (h).

Table 1
Correlation matrix showing correlation ($p < 0.05$) between different biochemical defense parameters in roots of Sreekara (a) and 04-P24 (b).

	Conductivity	Total phenol	OD phenol	Lignin	β-1,3-glucanase	β-1,4-glucanase	Peroxidase
a							
Conductivity	1.000						
Total phenol	0.362	1.000					
OD phenol	0.163	0.905	1.000				
Lignin	0.608	0.833	0.665	1.000			
β-1,3-glucanase	0.582	0.870	0.748	0.799	1.000		
β-1,4-glucanase	0.581	0.525	0.285	0.698	0.400	1.000	
Peroxidase	0.486	0.446	0.477	0.524	0.495	0.205	1.000
b							
Conductivity	1.000						
Total phenol	0.546	1.000					
OD phenol	0.756	0.741	1.000				
Lignin	0.774	0.727	0.801	1.000			
β-1,3-glucanase	0.818	0.651	0.726	0.690	1.000		
β-1,4-glucanase	0.553	0.454	0.340	0.623	0.442	1.000	
Peroxidase	0.758	0.791	0.566	0.821	0.772	0.632	1.000

Table 2
Correlation matrix showing correlation ($p < 0.05$) between different biochemical defense parameters in stems of Sreekara (a) and 04-P24 (b).

	Conductivity	Total phenol	OD phenol	Lignin	β-1,3-glucanase	β-1,4-glucanase	Peroxidase
a							
Conductivity	1.000						
Total phenol	0.461	1.000					
OD phenol	0.762	0.786	1.000				
Lignin	0.286	0.678	0.656	1.000			
β-1,3-glucanase	0.376	0.484	0.533	0.835	1.000		
β-1,4-glucanase	0.212	0.150	0.248	0.470	0.229	1.000	
Peroxidase	0.060	0.108	0.124	0.530	0.823	0.082	1.000
b							
Conductivity	1.000						
Total phenol	0.120	1.000					
OD phenol	0.084	0.824	1.000				
Lignin	0.657	0.559	0.480	1.000			
β-1,3-glucanase	0.550	0.629	0.693	0.687	1.000		
β-1,4-glucanase	0.470	0.269	0.311	0.657	0.400	1.000	
Peroxidase	0.282	0.695	0.649	0.571	0.847	0.413	1.000

resistant line after pathogen inoculation. Correlation between tissue lignifications and disease resistance has been proved by many researchers [32,44,45]. In our study, lignin content was found to be high in roots of both susceptible and resistant lines under *P. capsici* inoculated condition and resistant line had higher induction compared to susceptible line (Fig. 6a). Similar results were reported in black pepper cell suspension culture upon elicitation with *P. capsici* elicitors [4].

Defense related enzymes

Plants respond to the presence of microbial pathogens by *de novo* synthesis of defense related enzymes. Cell wall peroxidase belongs to one of the important enzyme systems in reactive oxygen species (ROS) metabolism, generating H_2O_2 [9,13] which in turn leads to the development of an antimicrobial environment within the apoplast [34]. The induction of peroxidase activity in response to pathogen infection has already been repeatedly reported in several plant species [5,8,14,18,23,26,29–31,41]. Enhancement of peroxidase activity in root of resistant black pepper line upon *P. capsici* infection herein is in agreement with that reported for resistant cultivars of muskmelon (*Cucumis melo*) upon infection with *Pseudoperonospora cubensis* [36], in sugarcane upon *Colletotrichum falcatum* invasion [43], in *Cucumis sativus* upon inoculation with cucumber downy mildew *P. cubensis* [24,25], in green bean upon infection with *Uromyces appendiculatus* [39], in taro upon inoculation with *P. colocasia* [29], in black pepper upon inoculation with *P. capsici* [38], in black pepper cell suspension culture upon inoculation with *P. capsici* elicitors [4] and in apricot upon inoculation with *Hendersonula toruloidea and Phiaoacremonium aleophillium* [3]. In the present study, the induction of peroxidase activity in *P. capsici* – infected black pepper roots and stems of resistant and susceptible lines over their uninfected controls has been revealed (Fig. 7).

The induction of β-1,3-glucanase enzymes occurs in different plant species in response to fungal attacks [16]. Beta-1,3-glucanase has been induced in resistant cv. of celery infected with *Fusarium oxysporum* [22] and chickpea upon infection with the fungal pathogen *Ascochyta rabiei* [16]. Since the enzyme β-1,3-glucanases has potential to hydrolyze fungal and oomycete cell wall β-1,3-glucan, it has been reported to have a role in defense against invading fungal and oomycete pathogen [19] [38]. and [42] observed higher β-1,3-glucanase activity in *Phytophthora* tolerant variety P24 (IISR Shakti). In this study, the results showed that β-1,3-glucanase significantly increased in *P. capsici* inoculated roots of susceptible and resistant lines over their control plants (Fig. 8).

P. capsici has cellulose in its cell wall and so, the host plants might secrete β-1,4-glucanase to prevent this pathogen. We checked the changes in this enzyme activity in inoculated susceptible and resistant black pepper lines compared to the uninoculated plants and it was observed that β-1,4-glucanase activity varied in inoculated plants significantly. The increased enzyme may be either of plant or of pathogen origin. This has to be further confirmed in our future work. The results obtained in this study are significant in understanding the shifts in the enzyme properties with disease progression.

Hence the overall results from this study revealed that among the biochemical defense parameters, membrane conductivity, OD phenols, lignin and peroxidase play significant role in 04-P24 root resistance to *P. capsici*. All biochemical parameters analyzed here were found to be changing in stems of inoculated plants compared to uninoculated plants in both lines. This is an indication of systemic defense response in these lines against the pathogen. More research is required to establish these observations through anatomical and molecular investigations.

Contributions

- Vandana, V. V.: Research student.
- Dr. R. Suseela Bhai (Principal Scientist, Plant Pathology): Guide
- Dr. Shamina Azeez (Principal Scientist, Plant Biochemistry): Co-guide

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