Eur J Plant Pathol
https://doi.org/10.1007/s10658-018-01661-3

Role of cell wall and cell membrane integrity in imparting defense response against *Phytophthora capsici* in black pepper (*Piper nigrum* L.)

V. V. Vandana · R. Suseela Bhai · R. Ramakrishnan Nair · Shamina Azeez

Accepted: 11 December 2018

Abstract The structural defense response of a black pepper line (04-P24) showing root resistance to *Phytophthora capsici* was studied in comparison with a highly susceptible line (Sreekara). Role of cell wall reinforcement and cell membrane integrity was analyzed. Cell membrane integrity was studied under hydroponic system in terms of leakage of electrolytes caused by the cell membrane damage due to pathogen entry. Root cell membrane rupture and resultant phenolic leakage were clearly visible in the form of color change of the liquid phase during the course of infection. Root leachates of Sreekara turned highly dark due to the increased level of phenol leakage which was proportional to the cell membrane damage. The root leachate was analyzed for change in conductivity, total phenols and ortho-dihydroxy (OD) phenols. Cell wall reinforcement of the vascular tissues of black pepper root upon pathogen inoculation was analyzed histochemically. Toluidine blue O and Maule staining differentiated the intensity of lignin deposition in the root cells of both lines and it was comparatively stronger in the resistant line. Scanning electron microscopy revealed that hyphae of *P. capsici* are not penetrating the root of 04-P24 supporting the finding that roots of this line don't support *Phytophthora* infection.

Keywords Black pepper · Cell membrane · Cell wall · Conductivity · Lignin · Phenols · *Phytophthora capsici*

Highlights 1. The study was carried out in *Phytophthora capsici* susceptible (Sreekara) and resistant (04-P24, shows root resistance to the pathogen) black pepper lines.
2. The role of cell membrane integrity and cell wall reinforcement in imparting resistance to *P. capsici* was studied and found that increased cell membrane integrity and higher lignification of root cells (mainly vascular tissues) play crucial role in root resistance of 04-P24 to *P. capsici*.
3. In our previous paper published in the journal PMPP (Vandana et al., 2014), the role of lignin in root resistance of 04-P24 was clearly described. The results obtained in this study confirm the above finding.

V. V. Vandana · R. Suseela Bhai · R. Ramakrishnan Nair
ICAR-Indian Institute of Spices Research, Marikkunnu P O, Kozhikode, Kerala 673012, India

R. Suseela Bhai (✉)
Division of Crop Protection, ICAR-Indian Institute of Spices Research, Marikkunnu P O, Kozhikode, Kerala 673012, India
e-mail: rsbhai@rediffmail.com

e-mail: suseela@spices.res.in

S. Azeez
ICAR- Indian Institute of Horticultural Research, Hessaraghatta Lake Post, Bangaluru, Kozhikode, Karnataka 560089, India

Published online: 26 January 2019

Introduction

Cell wall-associated plant defense is important in basal resistance and the cell wall acts as a protective barrier against pathogen penetration (Wang et al. 2015). Basal resistance seems to be suppressed by virulent pathogens but boosted in induced and in race-specific resistance (Pellizzari et al. 1970). Immediately after the attempted penetration of a pathogen, the host plant prepares for the cell wall reinforcement (induced structural defense mechanism) which can improve host resistance (Guest

and Brown 1997). If the host cell can repair and/or reinforce its cell walls quickly, it can reduce the penetration efficiency of the pathogen.

Modification of the cell wall serves as barriers for the progression of pathogens (van Kan 2006) and is an important defense mechanism of flowering plants against necrotrophs (Asselbergh et al. 2007; Curvers et al. 2010). Reinforcement of the cell wall involves accumulation of phenolic compounds (lignifications), suberization etc. at the penetration sites. This makes the cell wall less vulnerable to degradation by cell wall-degrading enzymes released by the intruders. Lignin also acts as an impermeable barrier for free movement of nutrients causing starvation of the pathogen.

The cell membrane acts as "signal sensors" for external stimuli and on receiving these signals, change in membrane permeability is induced causing the acidification or alkalinization of extra-cellular medium and subsequent activation of defense genes (Guest and Brown 1997). Therefore, the maintenance of cell-membrane integrity is essential for the survival of plants under biotic stress conditions. Cell membrane damage in infected plants induces HR response as reported by Heath (2000). Change in membrane permeability takes place in the early disease process in the seedlings of wheat in response to brown rust (Singh 2006).

Plant roots exude an array of small-molecular-weight compounds into the rhizosphere. Root exudate contains secondary metabolites (Uren 2000; Bertin et al. 2003) having direct defensive qualities (Jackson and Ilamurugu 2014). Pathogen recognition by the host plant root results in root secretion of antimicrobial compounds.

Phytophthora foot rot ('quick wilt) is one of the major diseases identified in Black pepper (*Piper nigrum* L.). The causal organism for foot rot disease, *Phytophthora capsici,* is one of the most serious threats to black pepper cultivation in India. It is a soil-borne pathogen and attacks the roots, stems, leaves, branches and fruit spikes of black pepper vine. The pathogen is spread by water and soil cultivation operations in pepper gardens. The pathogen can infect both mature and immature plants. Crop loss due to this disease has been identified as a major constraint in black pepper production (De Waard 1986).

Although various chemical and biocontrol measures are available to control foot rot disease in black pepper, development of resistant cultivars is the best solution to tackle this problem. Identifying resistance sources is an effective and long-lasting disease-management strategy. Recently, at ICAR-IISR, an open- pollinated progeny of black pepper (04-P24) obtained from a moderately resistant line (IISR-Shakthi) showed root resistance to *Phytophthora* species by all means of screening (Bhai et al. 2010). Even after repeated inoculations, the plant showed resistance to *Phytophthora* which may be due to structural defense barriers. This article focuses on testing this structural defense barriers (first-level defense barriers) associated with this OP progeny in resisting the *P. capsici* invasion. Since the plant is showing resistance to root infection, the main focus is given to understanding the first-level defense barriers, viz., cell wall and cell membrane integrity.

Materials and methods

Study on the role of cell membrane integrity

Plant material and pathogen inoculation

P. capsici susceptible (Sreekara) and resistant (04-P24, OP progeny of IISR Shakthi) lines of black pepper (*Piper nigrum* L.) were used in this study. The plants were multiplied using the serpentine propagation method (Thankamani 2008). Single-node black pepper cuttings were grown in sterile potting mixture (soil: sand: farm yard manure, 2:2:1) in polythene bags of size 20 × 10 cm under greenhouse conditions, maintaining a temperature of 20–25 °C with 12 h photoperiod. Plants of 4–5 leaf stage were used for studying the cell membrane integrity.

The *P. capsici* isolate 06–04 maintained in the National Repository of *Phytophthora*, ICAR-IISR, Kozhikode, Kerala was used for inoculation. The isolate was sub-cultured and maintained in carrot agar (CA) medium (Griffin 1977).

Determination of cell membrane integrity

Cell membrane integrity was studied by inoculating plants with *Phytophthora* inoculum under a hydroponic system. This was done by two methods. In the first method sterile de-ionized water was used as the inoculation medium whereas in the second method Hoagland solution (HS) was used. In both cases cell membrane integrity was studied by analyzing the root leachates in the medium by cell wall disruption due to pathogen attack. The root leakage study was done using the method described by Li et al. (2013).

5:5:90) for 24 to 48 h (Johansen 1940). The fixed segments were washed with distilled water (5 h), dehydrated in an ethyl alcohol and tertiary-butyl alcohol (TBA) series (Johansen 1940) and embedded in paraffin wax (60 °C). Thin cross sections of 12 µm sizes were cut using a rotary microtome (Leitz Microtome, No. 1512). The cross sections were stained using Toluidine blue O (O'brien et al. 1964), Maule staining (Faulkner and Kimmins 1975) and Sudan black B staining (Wang et al. 2007). The stained sections were mounted in a drop of Canada balsam, covered with a cover-slip and dried at 60 °C in an oven for at least 3 days for preparation of permanent slides (Eltahir and AbuReish 2010). Observations were recorded under a Leica DM 5000B microscope and photographs were taken.

Scanning electron microscopy (SEM)

The portions of roots where the *P. capsici* hyphae was found to be attached were removed from the root system and processed for SEM analysis. Specimens were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) in screw-top vials, washed 3 times in 0.1 M phosphate buffer (pH 7.2), post-fixed in 2% osmium tetroxide in 0.2 M phosphate buffer, rinsed in 0.1 M phosphate buffer (pH 7.2), dehydrated in 30, 50, 70, 80, 90, 95 and 100% ethanol and critical-point-dried (CPD). SEM of the sample was carried out at the Department of Nanotechnology, National Institute of Technology, Kozhikode, Kerala, India..

Statistical analysis

The data were subjected to two-way analysis of variance using ANOVA. Means were compared by Duncan's test using statistical package SAS software (Version 9.3). Differences among various treatment means were separated by least significant difference (LSD) at the 0.05 level of probability. Analysis of correlation ($p < 0.05$) of conductivity with other parameters tested was also performed.

Results

Collection and analysis of root leachate

When plants were kept inoculated under the hydroponic system, color change was observed in the liquid phase after challenging with the pathogen. Leakage of phenolic compounds by the root upon stress conditions led to the change of color of the liquid phase. Four to 5 days after inoculation, the color change was prominent in the susceptible line when compared to the resistant line. No color change was observed in root leachates of uninoculated plants. The color intensity was more in the liquid phase of pathogen-inoculated Sreekara plants compared to its resistant counterpart (Figs. 1, 2 and 3). Fig. 3 shows color change of HS upon inoculation with *P. capsici*. Under stress conditions cell membrane integrity is disturbed and this results in increased membrane leakage. Hence the conductivity of root leachate was analyzed to express the change in membrane integrity since they are positively correlated. Upon pathogen inoculation on root, the root exudes anti-pathogenic compounds to its surroundings. Hence the root leachates were analyzed for anti-pathogenic phenolics.

Analysis of root leachates in the liquid phase

In sterile de-ionized water

Analysis of root leachates in sterile de-ionized water was carried out until the 8th DAI since the inoculated plant of the susceptible line wilted completely on the 8th DAI.

Membrane conductivity

Upon inoculation with *P. capsici*, the conductivity of the water phase showed a significant increase from the 3rd day onwards in the susceptible line and from the 4th day onwards in the resistant line. Considering the uninoculated negative controls, conductivity of root leachates of the susceptible line remained at par with that of the resistant line throughout the experiment. In both lines a gradual and steady increase of conductivity could be observed during the course of infection. The highest increase was noticed on the 8th DAI in both lines viz. 10 and 4-fold increase in Sreekara and 04-P24 respectively and it was around 2.2 fold higher in Sreekara as compared to its resistant counterpart. Only during the later days of infection (5–8 DAI), did the conductivity of negative and positive controls show significant difference (Fig. 4a).

Collection of root leachates and analysis

The plants grown as described earlier (Sreekara and 04-P24) were carefully removed with intact root system from the polythene bags and washed thoroughly in running tap water to remove the adhering soil particles. These plants were again washed with sterile distilled water. Individual plants were placed in 250 ml sterile bottles with the roots completely submerged in sterile de-ionized water (150 ml / bottle). Each bottle was inoculated with *P. capsici* inoculum plugs (5 mm discs, five numbers) and incubated at 25 °C for 8 days. The water in the bottle was observed for color change, if any, in response to root infection. To analyze the root leachates, the samples were drawn from the 1st day of inoculation until the 8th DAI (day after inoculation). The samples were filtered through a double layer of Whatman No. 1 filter paper and analyzed for changes in conductivity, total phenols and OD phenols. Uninoculated plants served as negative control for *Phytophthora* growth and sterile de-ionized water with the same number of *Phytophthora* discs as positive control. Carrot agar discs alone in sterile distilled water served as absolute control. The color change of the liquid phase indicates the membrane leakage. The experiment was conducted in triplicate. The same procedure was used for collecting the root leachates in HS medium. Here, instead of de-ionized water, sterile HS (Hoagland and Arnon 1950) was used as the liquid phase.

Determination of change in membrane conductivity of black pepper root cells during the course of P. capsici infection

The change in conductivity is directly proportional to the membrane damage induced in the plant cells. The change in conductivity of the liquid phase was measured every 24 h using a conductivity meter (EUTECH Instruments cyberscan con 11) and the values were recorded in microSiemens (μS).

Determination of total phenols and OD phenols in black pepper root leachates

The total phenol was estimated using the modified Folin-Ciocalteau method described by Eynck et al. (2009). To 1 ml of sample, 1 ml of Folin-Ciocalteau reagent was added and incubated for 3 min at room temperature. 1 ml of 1 M sodium carbonate was added to this and incubated in a rotary shaker at room temperature. After 45 min, absorbance was read at 725 nm in a spectrophotometer and quantified against a standard of gallic acid. The total phenolic content was reported as gallic acid equivalents based on a calibration curve.

OD phenols were estimated using the method described by Gutfinger (1981). For estimation, to a 1-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 read 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.

Histochemical changes

Pathogen inoculation and sampling

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 using a cork borer and were kept for sporulation in sterile distilled water under continuous light for 48 h at 24 ± 1 °C. The sporulating discs were used for plant inoculation. These sporulating discs were placed on the root surface of each intact plant grown in the polythene bags after removing some soil near the root system without injuring the root and then the inoculated root system was covered with soil. Uninoculated plants served as control. Three biological replications were maintained.

From the *P. capsici* inoculated and uninoculated plants, root samples were drawn and subjected to histochemical analysis. For each sampling, plants were uprooted, the root system was washed thoroughly in running tap water and cut into slices of around 1 cm size. Three biological replications were maintained for each treatment. Uninoculated plants served as control.

Light microscopy

Freehand sections (transverse sections) of root tissue of both *P. capsici* inoculated and uninoculated plants were stained using lactophenol cotton blue and were observed under bright-field microscope (Nikon-Eclipse Ci-L) and photographs were taken. Microtome sections (cross sections) of the root were used for Toluidine blue O (TBO), Maule and Sudan black B staining. For making microtome sections, the segmented root tissues were fixed in FAA fixative (formalin: acetic acid: ethyl alcohol;

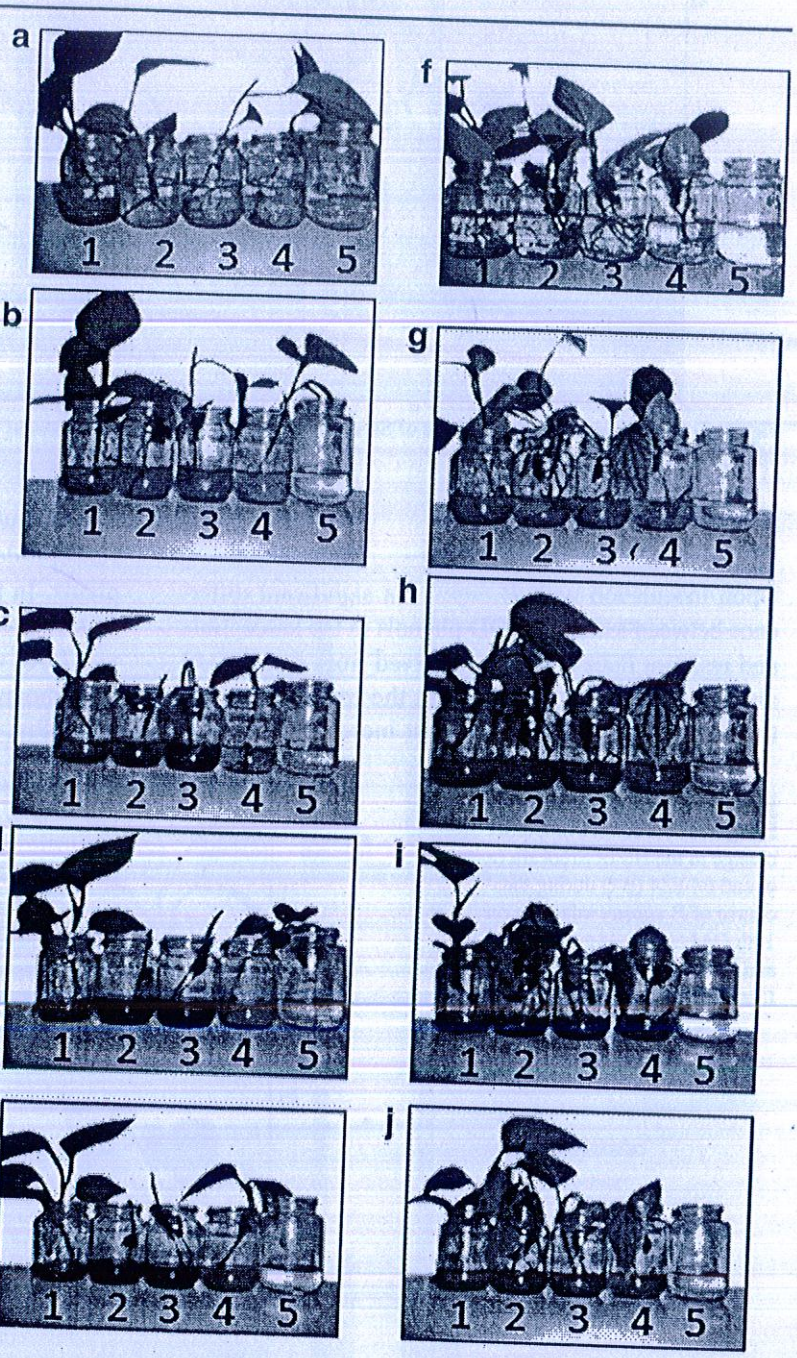

Fig. 1 Color change in the water phase of Sreekara (**a** – **e**) and 04-P24 (**f** – **j**) during the course of *P. capsici* infection, on 0th DAI (A, F), 5th DAI (B, G), 6th DAI (C, H), 7th DAI (D, I) and 8th DAI (E, J). Flasks 1: negative control, 2–4: inoculated plants, 5: positive control

Total phenols

The total phenol content in water phase of inoculated plants increased significantly from the 1st day onwards in the susceptible line. In the resistant line, during the first 2 days total phenols of both inoculated and uninoculated plants were at par, and thereafter a significant increase was noted until the 8th DAI. The highest total phenol content was detected on the 8th DAI in root leachates of pathogen-inoculated plants of both lines, ~ 3.1 and 1.4 fold high in susceptible and resistant lines, respectively. In both lines, until the 4th DAI, total phenol content of both negative and positive control remained at par and after it increased significantly (Fig. 4b). However, the highest phenolic content was observed in the susceptible line compared to the resistant line.

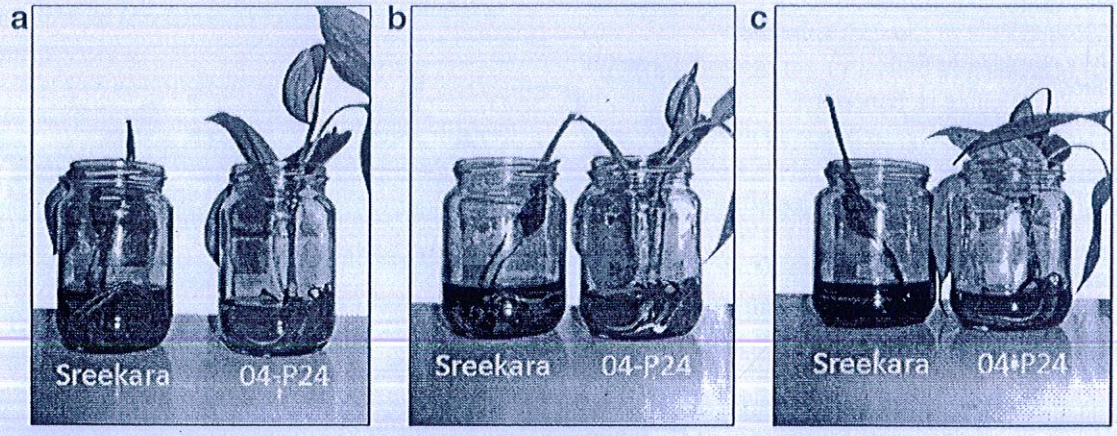

Fig. 2 Color change in the water phase of Sreekara and 04 - P24 during the course of *P. capsici* infection, on 6th DAI (**a**), 7th DAI (**b**) and 8th DAI (**c**)

OD phenols

Upon inoculation with *P. capsici*, a significant difference between leakage of OD phenols in the susceptible and resistant lines could be observed from 6 to 8 DAI and at all 3 days, it was high in the root leachate of the susceptible line. The highest increase of around 4.7 fold was noticed on the 8th DAI in inoculated susceptible plants as compared to the uninoculated plants. In the resistant line also the highest increase was noticed at 8 DAI, but it was 2.9 fold only. From 3 to 8 DAI, OD phenol content increased significantly in uninoculated plants compared to the positive controls (Fig. 4c).

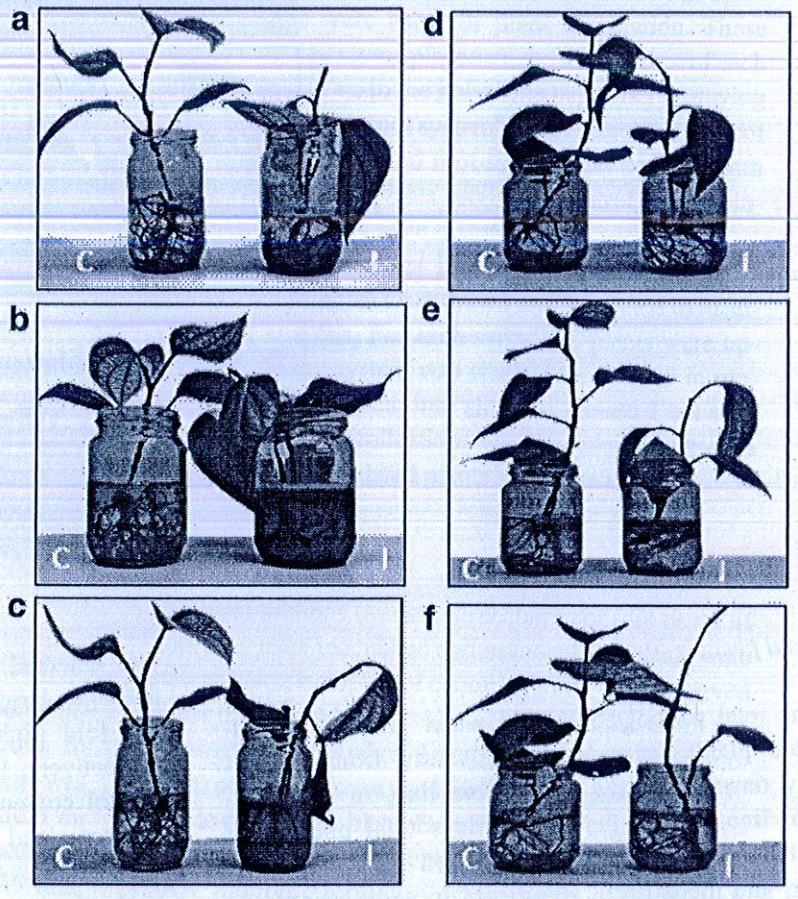

Fig. 3 Status of plants in Hoagland solution (HS). Color change in the HS of Sreekara (**a-c**) and 04-P24 (**d-f**) during the course of *P. capsici* infection on 10th DAI (A, D), 11th DAI (B, E) and 12th DAI (C, F). C: Control, I: Inoculated plants

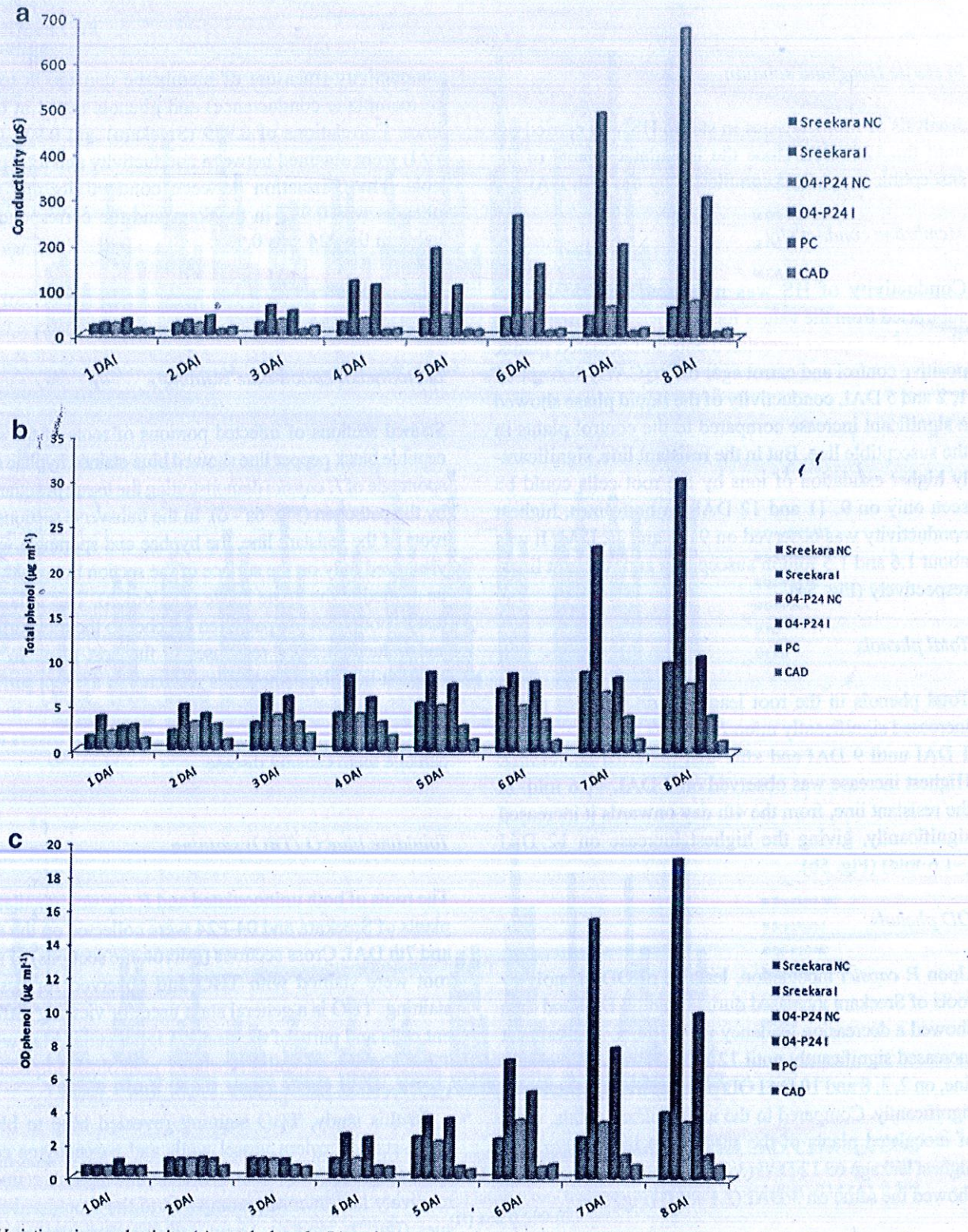

Fig. 4 Variations in conductivity (**a**), total phenols (**b**) and OD phenols (**c**) in root leachates of uninoculated and *P. capsici*-inoculated black pepper plants (*P. capsici* susceptible Sreekara and resistant 04-P24) at regular time intervals. NC: Negative Control, I: inoculated plants, PC: Positive Control, CAD: Carrot Agar Discs and 1 to 8 DAI: 1st to 8th day after inoculation. LSD ($p < 0.05$) for interaction - treatment x days after inoculation - 21.78 (A), 1.200 (B) and 0.377 (C)

In sterile Hoagland solution

Analysis of root leachates in sterile HS was carried out until the 12th DAI since the inoculated plants of the susceptible line wilted completely by the 12th DAI.

Membrane conductivity

Conductivity of HS was measured separately and subtracted from the values for conductivity of the liquid phase of *P. capsici* inoculated and uninoculated plants, positive control and carrot agar discs (CAD). Except on 1, 2 and 5 DAI, conductivity of the liquid phase showed a significant increase compared to the control plants in the susceptible line. But in the resistant line, significantly higher exudation of ions by the root cells could be seen only on 9, 11 and 12 DAI. In both lines, highest conductivity was observed on 9, 11 and 12 DAI. It was about 1.6 and 1.5 fold in susceptible and resistant lines, respectively (Fig. 5a).

Total phenols

Total phenols in the root leachates of Sreekara plants increased significantly upon *P. capsici* inoculation from 1 DAI until 9 DAI and after that it started decreasing. Highest increase was observed on 8 DAI, ~1.6 fold. In the resistant line, from the 4th day onwards it increased significantly, giving the highest increase on 12 DAI (~1.6 fold) (Fig. 5b).

OD phenols

Upon *P. capsici* inoculation, leakage of OD phenols by roots of Sreekara increased during 1 and 2 DAI and then showed a decreasing tendency until 7 DAI. Thereafter it increased significantly until 12 DAI. But in the resistant line, on 2, 3, 8 and 10 DAI OD phenols leakage increased significantly. Compared to the uninoculated plants, roots of inoculated plants of the susceptible line showed the highest leakage on 12 DAI (3.3 fold) and the resistant line showed the same on 3 DAI (2.1 fold) (Fig. 5c).

Correlation analysis (conductivity, total phenols, and OD phenols)

Correlation analysis was carried out with the values for analyzed parameters in sterile de-ionized water. A highly positive correlation was found between the conductivity (measure of membrane damage in terms of membrane conductance) and phenols tested in both lines. Correlations of 0.979 (Sreekara) and 0.921 (04-P24) were obtained between conductivity and total phenols. The correlation between conductivity and OD phenols was 0.992 in Sreekara and the corresponding value in 04-P24 was 0.981.

Light microscopy

Lactophenol cotton blue staining

Stained sections of infected portions of roots of the susceptible black pepper line showed blue-stained hyphae and sporangia of *P. capsici* demonstrating the tissue penetration by the pathogen (Fig. 6a - d). In the transverse sections of roots of the 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. 6e - h). This 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.

Toluidine blue O (TBO) staining

The roots of both uninoculated and *P. capsici*-inoculated plants of Sreekara and 04-P24 were collected on the 4th and 7th DAI. Cross sections (microtome sections) of the root were stained with TBO and observed for tissue staining. TBO is a general stain used for viewing different cells and parts of the tissue. Lignin stains blue with TBO. Other cell wall-associated phenolic compounds stain green to blue green.

In this study, TBO staining revealed blue to blue green-stained xylem vessel walls and parenchyma cell walls (Fig. 7). These cells of uninoculated plants stained relatively less intensely compared to the inoculated tissues (Fig. 7a, and d). Upon pathogen inoculation cell walls of xylem vessel and parenchyma cells stained more strongly compared to the corresponding uninoculated plants in both lines (Fig. 7b-f). Moreover these cells stained weakly for lignin in the susceptible line compared to the corresponding cells from the resistant line (Fig. 8).

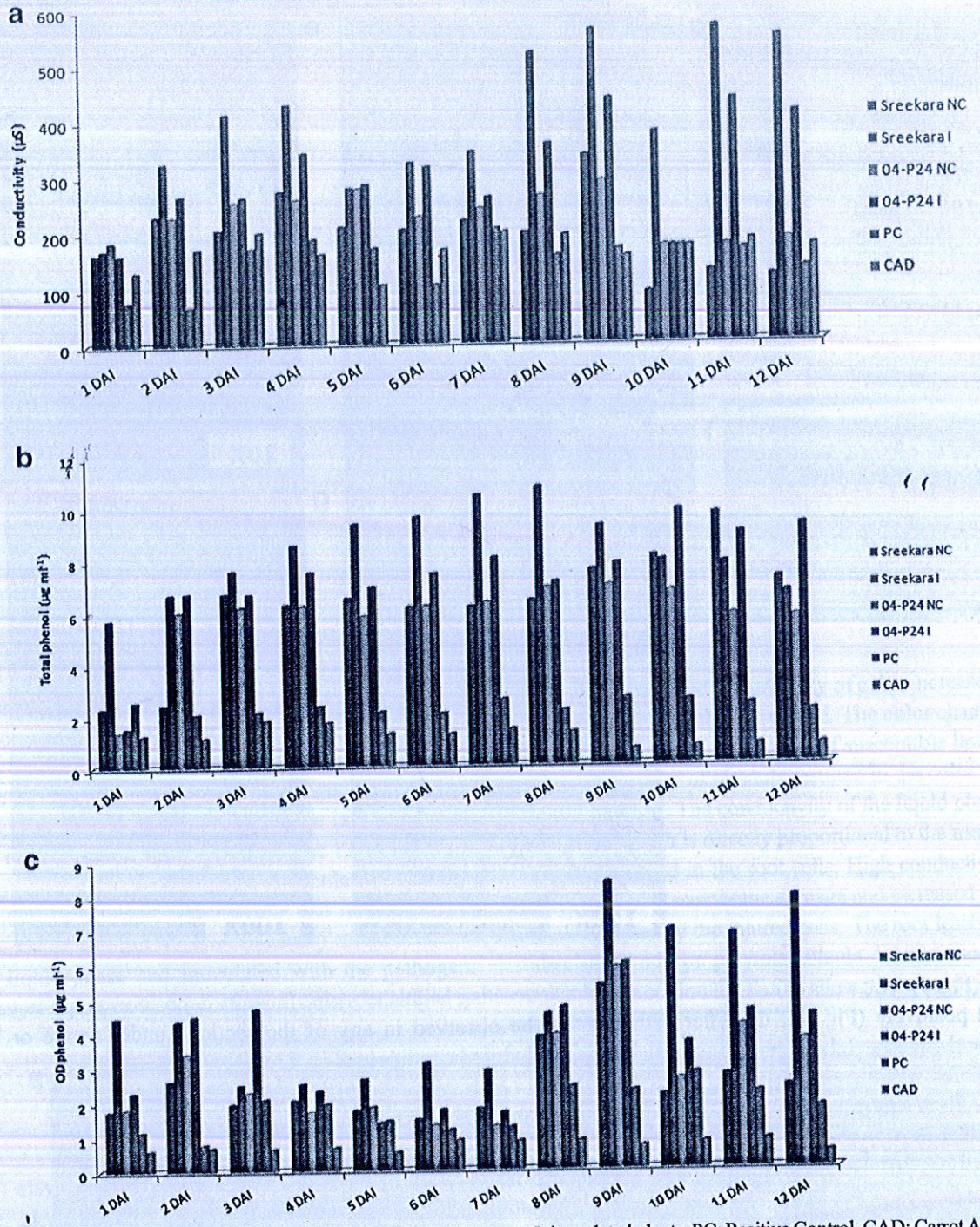

Fig. 5 Variations in conductivity (**a**), total phenols (**b**) and OD phenols (**c**) in root leachates of uninoculated and *P. capsici* inoculated black pepper plants (*P. capsici* susceptible Sreekara and resistant 04-P24) at regular time intervals. NC: Negative Control, I: inoculated plants, PC: Positive Control, CAD: Carrot Agar Discs and 1 to 12 DAI: 1st to 12th day after inoculation. LSD ($p < 0.05$) for interaction - treatment x days after inoculation −114 (A), 0.998 (B) and 0.599 (C)

Maule staining

This is a specific staining technique for visualization of lignin. KMnO4 is used for staining. This specifically reacts with syringyl lignin and results in a reddish brown color. The use of Maule reaction revealed brown-stained xylem vessel walls and adjacent parenchyma cell walls in the root cells of both *P. capsici* susceptible and

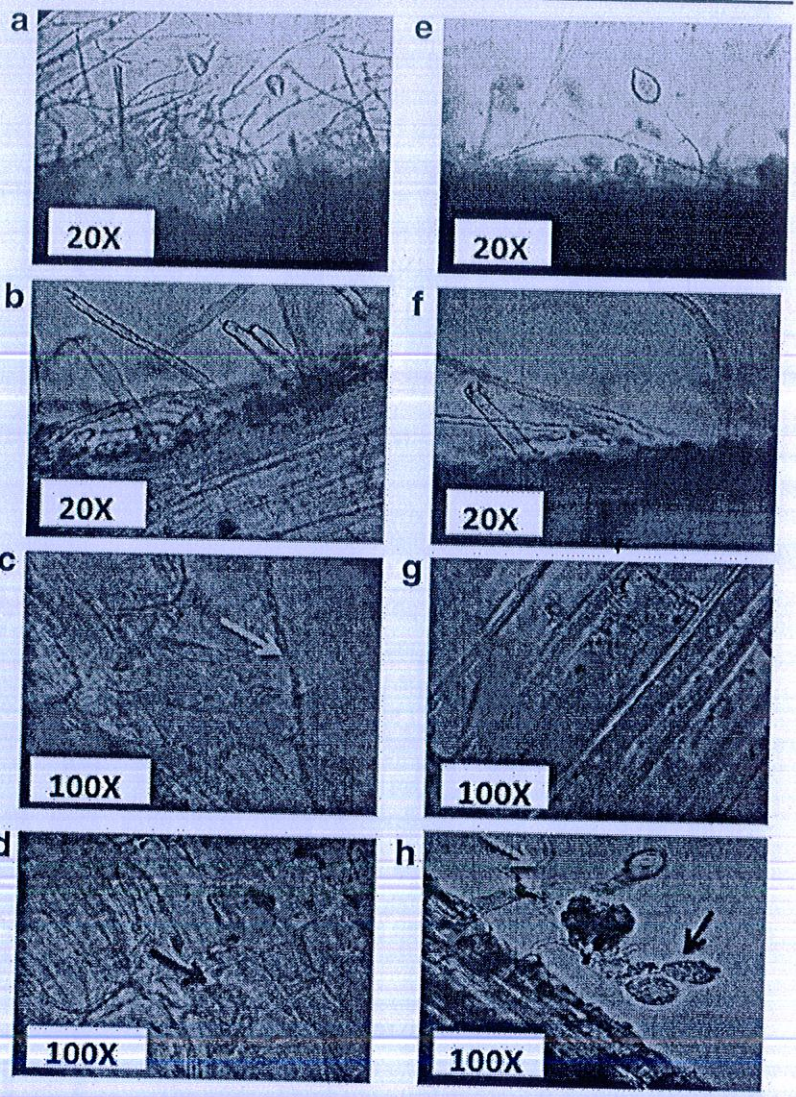

Fig. 6 Transverse sections of intact roots of Sreekara (**a** –**d**) and 04-P24 (**e** – **h**) inoculated with *P. capsici* at 4 DAI. Root surface colonized with pathogen (A, B and E, F). Hyphae (C) and Sporangia (D) are seen inside the root tissue of Sreekara. The inner tissue of root of 04-P24 found devoid of them (G). Red arrow: sporangia and yellow arrow: the hyphae

resistant lines (Fig. 9). The walls of cells of uninoculated plants stained positively (Fig. 9a, d). After pathogen inoculation, both lines exhibited a stronger staining response compared with the corresponding uninoculated plants (Fig. 9b-f) and this response was stronger in inoculated 04-P24 roots than in its inoculated susceptible counterpart (Fig. 10). Hence, lignin deposition was mainly confined to vascular tissue, especially xylem vessels.

Sudan black B staining

The stain Sudan black B is used for staining lipids in the cell walls of plant cells. In this study, the cross sections (microtome sections) of Sreekara and 04-P24 roots were stained in Sudan black B for analyzing the deposition of suberin on cell walls. Cell wall stains dark blue on reacting with the stain. Suberin deposition could not be observed in any of the sections under native or induced conditions (Fig. 11).

Scanning electron microscopy (SEM)

The portions of roots where *P. capsici* hyphae were attached to the surface were drawn for SEM analysis. Scanning electron micrographs of roots of Sreekara and 04-P24 inoculated with *P. capsici* are shown in Fig. 12 and in Fig. 13, respectively. In both lines, hyphae of *P. capsici* were found attached to the surface of the roots (Fig. 12a-c, 13b, c, and f). In the root of Sreekara, germination of *P. capsici* zoospores and subsequent attachment of the germinated hyphae on root surface were clearly observed (Fig. 12). Root damage caused by the pathogen (Fig. 12a-c) and penetration of hyphae into the

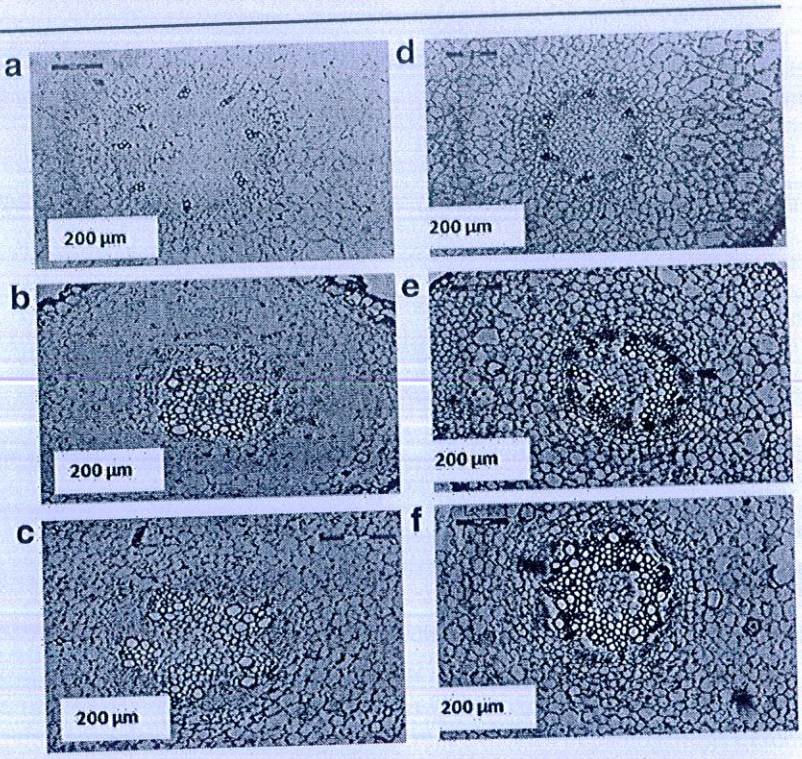

Fig. 9 Intensity of lignification in root cells of black pepper in response to *P. capsici* infection - response of Sreekara (**a-c**) and 04-P24 (**d-f**) to Maule staining. A and D: uninoculated roots of Sreekara and 04-P24 respectively; B and E: root tissue drawn on 4 DAI; C and F: drawn on 7 DAI. Characteristic brown staining of xylem vessel walls and parenchyma walls denote lignifications of cell walls of these cells

response to biotic stress they are exuded at higher rates due to membrane damage (Buonaurio 2008). There are many reports on plant cell membrane damage caused by biotic stresses (Benhamou and Bélanger 1998; Singh 2006; Trivedi and Singh 2014). Hence in this study, a new technique was adopted to assess the membrane integrity of root cells in *P. capsici* inoculated and uninoculated plants of both susceptible and resistant black pepper lines. The plant roots were completely immersed in a liquid phase and inoculated with the pathogen. During the progress of infection, a color developed in the liquid phase and the intensity of color increased with increase in the incubation period. The color change was prominent and high in the case of susceptible line (Figs. 1, 2, 3). Leakage of phenols resulted in the color change of the medium. The conductivity of the liquid phase was measured which is directly proportional to the membrane damage induced in the root cells. High conductivity denotes increased membrane damage and increased leakage of electrolytes by the injured cells. The root leachate was also analyzed for phenols. Plants secrete more antimicrobial compounds in the rhizosphere to defend the

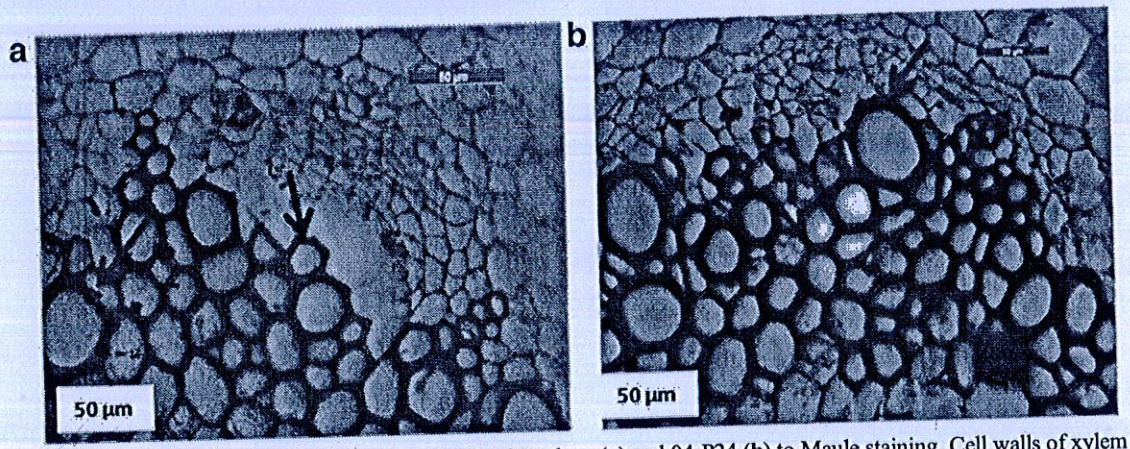

Fig. 10 Difference in the histological response of root cells of Sreekara (**a**) and 04-P24 (**b**) to Maule staining. Cell walls of xylem vessels and parenchyma of 04-P24 root stained more strongly (indicated by red arrow) than the corresponding cells of Sreekara root

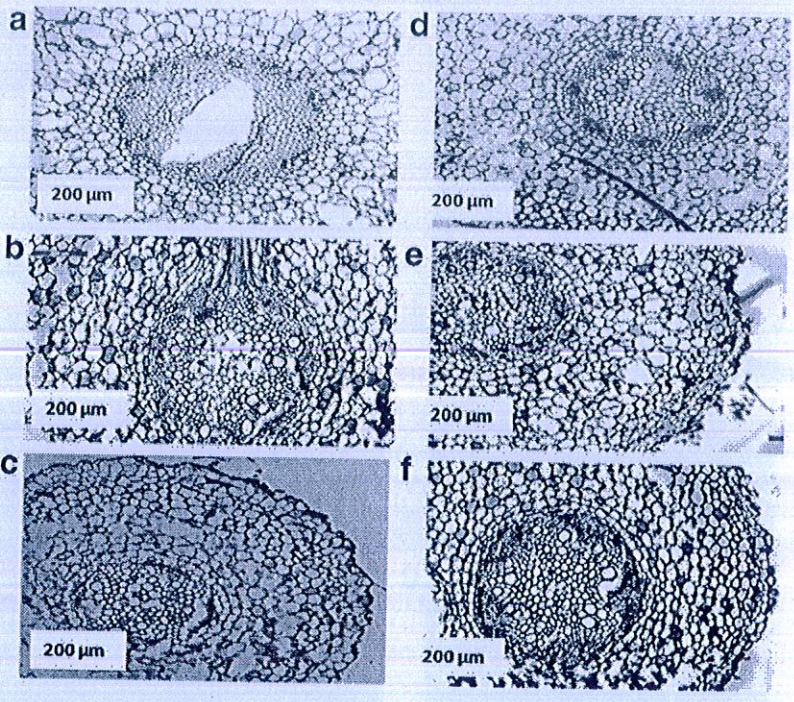

Fig. 7 Intensity of lignification in root cells of black pepper in response to *P. capsici* infection Sreekara (**a-c**) and 04-P24 (**d-f**). A and D: uninoculated roots of Sreekara and 04-P24 respectively; B and E: root tissue drawn on 4 DAI; C and F: drawn on 7 DAI. Characteristic blue or blue green staining of xylem vessel walls and parenchyma walls denote lignin and/or other cell wall associated phenols

injured portion (Fig. 12f) are clearly observed in Sreekara. In 04-P24, *P. capsici* hyphae attached to the surface of the root but penetration into the root tissue was not noticed (Fig. 13 b, c, and f).

Discussion

In this study, the structural defense response of black pepper roots to *P. capsici* infection was studied in *Phytophthora*-resistant black pepper line 04-P24 (Bhai et al. 2010) in comparison with the susceptible line Sreekara. Role of cell membrane integrity and cell wall reinforcement (important structural barriers to pathogen ingress) were studied here. The overall result showed increased cell wall strengthening and cell membrane integrity in the resistant line compared to its susceptible counterpart.

The cell membrane integrity was studied as a measure of membrane leakage. During pathogen entry into the host cell, the membrane is ruptured. This results in leakage of cell components into the surrounding medium, either soil or water. The leachate mainly contains ions, phenols (anti-microbial) and nutrient components like carbohydrates and amino acids. Under natural conditions these are exuded by plant roots. However, in

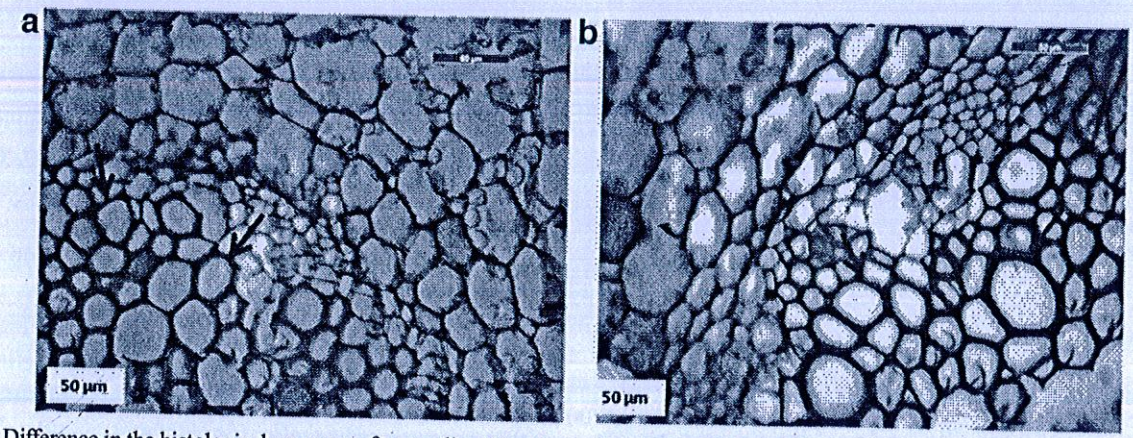

Fig. 8 Difference in the histological response of root cells of Sreekara (**a**) and 04-P24 (**b**) to TBO staining. Cell walls of xylem vessels and parenchyma of 04-P24 root stained more strongly (indicated by red arrow) than the corresponding cells of Sreekara root

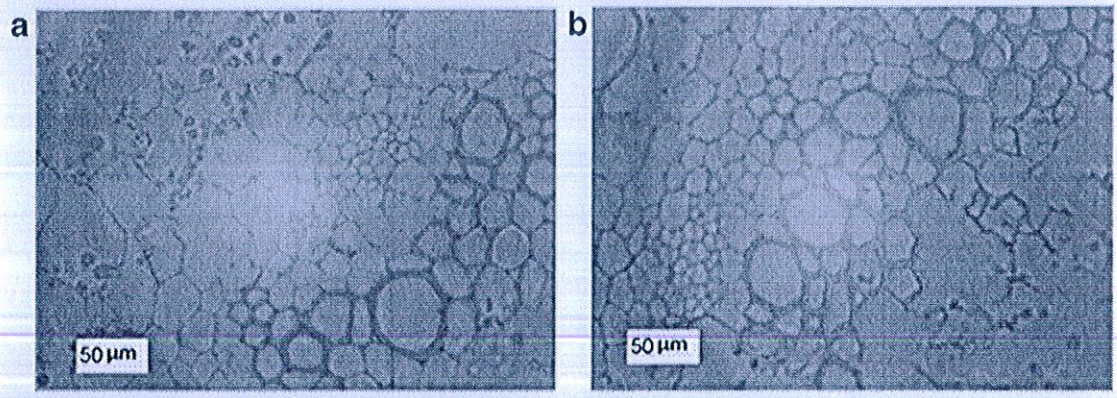

Fig. 11 Histological response of root cells of Sreekara (**a**) and 04-P24 (**b**) to Sudan black B staining

root from soil-borne pathogens. In both lines, total phenols and OD phenols increased with increase in days after inoculation but the increase was significantly high in root leachates of Sreekara during later days of infection (Fig. 4b and c, 5b and c) since infection was very prominent in Sreekara during this period.

Sterile de-ionized water was used as liquid phase for this study. But in water, nutrient deficiency was a problem which also resulted in stress-induced membrane damage in root cells of both lines. This might be the reason for the slight color change observed in root leachates of uninoculated plants during later days. Hence de-ionized water was replaced with HS in the next set of experiments. HS lengthened the period of survival of plants until 12 days as compared to 8 days in water and also the root infection was delayed in HS giving notable color change of the medium from the 9th day onwards. In HS, the results obtained for conductivity and phenols were not consistent with those obtained in water phase.

Due to the non availability of adequate methods, the role played by the root exudates in pathogenesis of root-infecting bacteria and fungi has not been fully studied (Bais et al. 2003, 2004). Considering the difficulty in collecting root exudates in their native form, the hydroponic method described by Li et al. (2013) was adopted in this study. The exudation of phenolic compounds by black pepper root in response to *P. capsici* infection was visually very clear in this experiment, i.e. the color

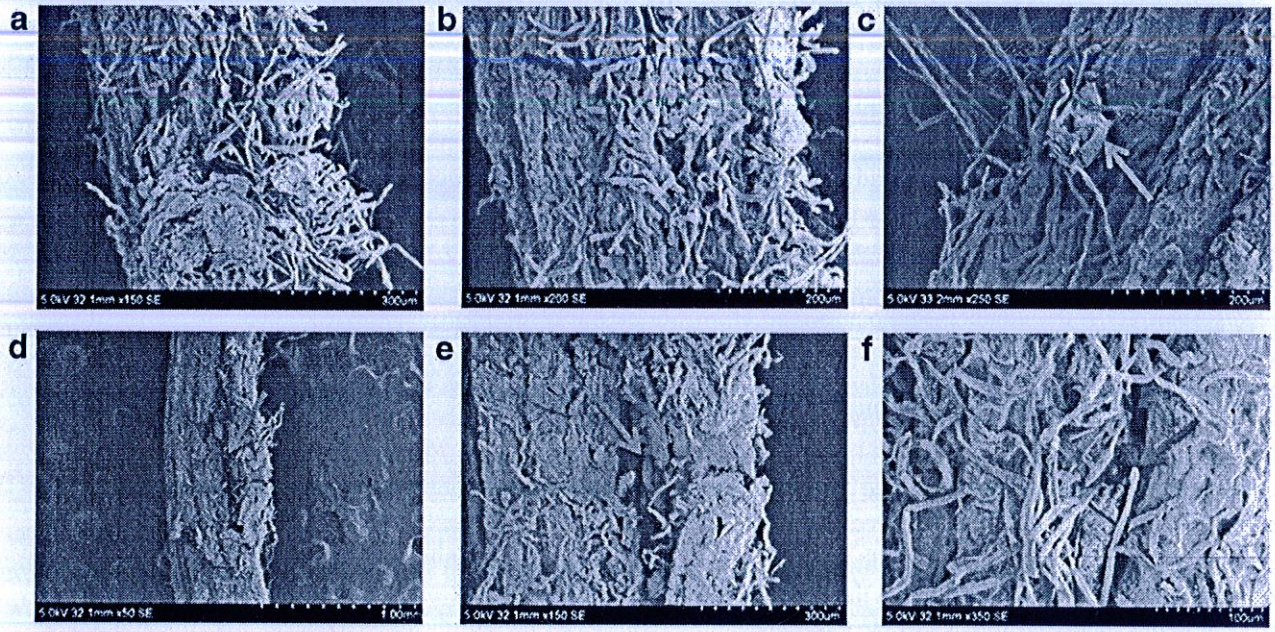

Fig. 12 SEM images showing attachment of hyphae of the pathogen on root surface of Sreekara root inoculated with *P. capsici*. **a** – **c**: attachment of hyphae of the pathogen on root surface; **d** – **f**: injury caused on root surface by pathogen penetration. Red arrow: hyphae, blue arrow: Injured site; green arrow: germinating spore and orange arrow: penetrating hyphae

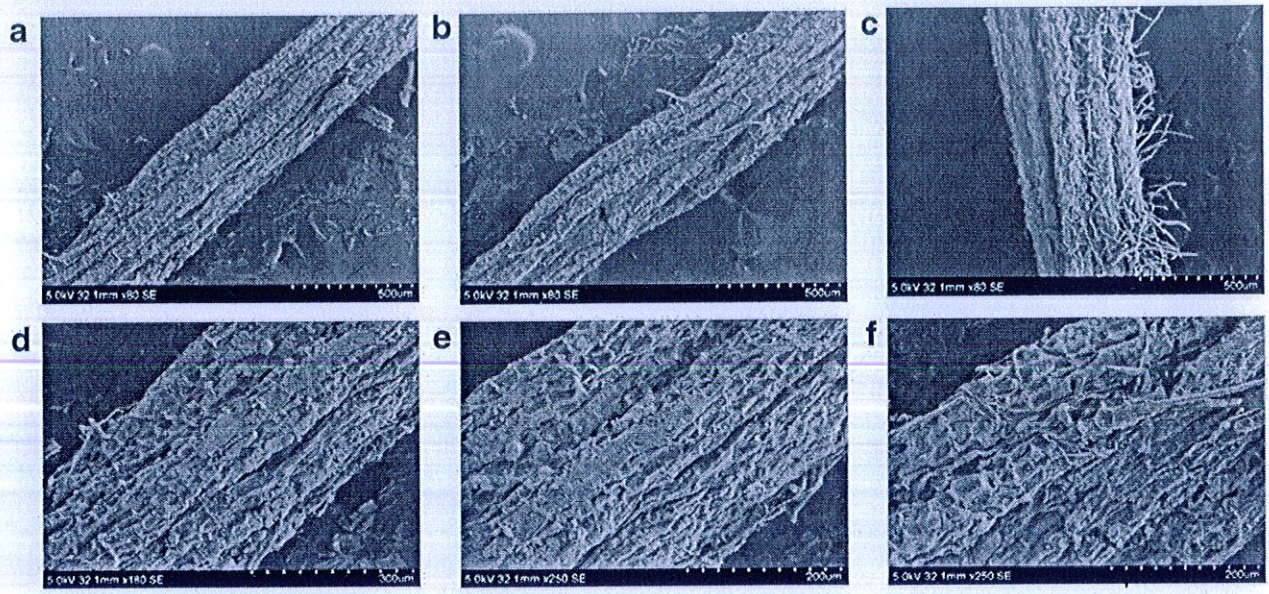

Fig. 13 SEM images of 04-P24 root inoculated with *P. capsici*. **a**, **b** and **d**, **e**: the parts of intact root surface devoid of any hyphae. Attachment of hyphae of the pathogen on root surface is shown in **c** and **f**. Red arrow: hyphae

development started on the 6th DAI in Sreekara and by the 7th DAI in 04-P24 in water phase and then increased until the 8th DAI. A plant resistant to biotic stress secretes anti-microbial compounds in higher concentration than the susceptible plants (Dixon 2001). In this study also, the uninoculated plants of 04-P24 exuded more OD phenols (6 and 7 DAI) compared to the uninoculated Sreekara plants.

Li et al. (2013) adopted the hydroponic system (water) for studying composition of root exudates of peanut. They immersed the root system of a peanut plant in sterile water for 24 h and used this water as root exudate. Here also the root exudates of black pepper were drawn in the similar way under pathogen inoculated and uninoculated conditions. In this study, secretion of phenolics was increased in both varieties under uninoculated conditions because of the abiotic stresses like anaerobic condition and nutrient deficiency in the hydroponic system. Even the uninoculated plants died of wilting in water by the 9th DAI. So the experiment was stopped by the 8th DAI. Moreover, since black pepper is a terrestrial plant, it is not well suited for growing under hydroponic conditions. To solve this problem, the experiment was replicated in HS (Hoagland and Arnon 1950) instead of water. In water, the Sreekara plants started showing rot symptoms from the 5th DAI but in HS, it was from the 9th DAI, which means the longevity of uprooted plants increased in HS. So the experiment could be prolonged until 12 DAI.

The total phenols and OD phenols were quantified in root leachates of uinoculated and pathogen inoculated plants of both varieties to confirm the exudation of phenolic compounds that resulted from the cell membrane damage in root cells. The result showed higher concentration of all the phenolics in root leachates of pathogen-inoculated Sreekara plants than in those of 04-P24. This implies the greater membrane integrity of 04-P24 root cells. The reason for increased membrane integrity in 04-P24 might be the cell wall reinforcement of root cells. This was confirmed by histological analysis, in which a highly positive correlation was observedbetween root leachates' conductivity (inversely related to membrane integrity) and all the phenol contents tested.

This study is supported by the works in barley. In barley infected with *Drechslera graminea*, increase in cell membrane injury was observed with increased days of infection (Trivedi and Singh 2014). The cell membrane injury results in electrolyte leakage. An increase in cell membrane injury was associated with disease development in barley. Electrolyte leakage is one of the initial responses of barley to infection by *D. graminea* (Trivedi and Singh 2014). According to Belanger and Bushnell (2002), fungal pathogens invade the host cell by penetrating the cell wall and, hence, if the intruding pathogen can be inhibited at this entry stage itself, cellular integrity is maintained and damage to plant tissues can be significantly reduced. The interruption of cell membrane integrity by biotic stresses increases

root exudates in the rizosphere. To counteract infection from soil, plants exude a variety of biologically active compounds into the rhizosphere. Such root exudates are known to have inhibitory action on soil-borne pathogens. Upon pathogen inoculation, synthesis of anti-microbial compounds is induced and they are released by the roots along with the naturally occurring ones. These anti-microbial compounds mainly include phenylpropanoids. Phenolics and terpenoids secreted by the roots have strong anti-bacterial and anti-fungal qualities (Buonaurio 2008; Lanoue et al. 2010; Wurst et al. 2010; Vukovic et al. 2013). Phenylpropanoids are ubiquitous phenolics (Lanoue et al. 2010), and flavonoids are one of the largest classes of phenylpropanoid-derived secondary metabolites of root exudates (Cesco et al. 2010).

The method of root exudates collection (Li et al. 2013) used in this experiment is easy to perform and reflects the changes of secretion of the whole root system. However, this method also has limitations like the problem of nutrient deficiency in water. So, HS was tested as liquid phase instead of water. Here, even though the color change in the medium due to phenolic leakage was apparently clear, the corresponding values obtained for conductivity and phenolics were not in accordance with the increase in color intensity of the medium. The reason for this may be the interference of inorganic and metal ions in the solution. Thus sterilized de-ionized water is found as a suitable liquid phase medium for root leachates study in black pepper (Bhai, unpublished). This is the first report of study on cell membrane integrity in black pepper under hydroponic conditions. Similar studies on other crops are not reported so far.

Reinforcement of cell walls of vascular tissue was observed in both susceptible and resistant line upon *P. capsici* inoculation (Figs. 8, 10). It was stronger in the resistant line. Lignification of the cell wall is one of the important first-line defense barriers to pathogen ingress in plants. The defensive lignification and suberization of the cell wall takes place faster, restricts the cleavage of its compounds, reduces the nutrient flow between plant and pathogen, and also the transport of mycotoxins (Nicholson and Hammerschmidt 1992). In this study, increased lignin deposition in the cell walls of xylem vessels and adjacent parenchyma cells of the inoculated resistant line compared to the inoculated susceptible line was observed (Fig. 10). This is in agreement with the finding of increased lignin content in roots of 04-P24 compared to the roots of Sreekara upon *P. capsici* inoculation (Vandana et al. 2014). However suberin (a polyester of hydroxy acids and dicarboxyl acids) deposition on cell walls was not observed in the roots after pathogen inoculation. Even though suberin plays a significant role in some host – pathogen interactions (Enstone et al. 2002; Huitema et al. 2004), it is reported that suberin is mainly seen in relation to wound-associated changes and at post-infection stages in plants (Pearce and Rutherford 1981). This might be the reason for the absence of suberin deposition in this study. In the stain Lactophenol cotton blue, phenol has the function of killing the fungus, lactic acid preserves the fungal structures and cotton blue stains the chitin of fungal cell walls. Lactophenol cotton blue staining (Fig. 6) and SEM analysis (Figs. 12, 13) clearly indicated that *P. capsici* is not entering into the root tissue of 04-P24 even though hyphae are found attached on the root surface.

Upon pathogen attack, plants accumulate structural phenolic compounds in the cell wall and synthesize lignin to reinforce it. Thus, the plant cell wall acts as an important defensive barrier first encountered by many pathogens before induction of intracellular defense responses (Lipka et al. 2005). Oliver et al. (2009) observed the deposition of phenolic compounds in cell walls of *Physcomitrella patens* infected with *Pythium irregulare* and *P. debaryanum*. Incorporation of phenolic compounds in the plant cell wall is an important defense mechanism of flowering plants against *Botrytis cinerea* (Ramírez et al. 2011). Jones and Dangl (2006) reported the significance of lignin deposition on the cell wall in various plant –pathogen interactions including radish - *Peronospora parasitica,* potato - *P. infestans,* wheat - *Septoria nodorum,* cucumber - *Cladosporium cucumerinum,* cucumber- *Colletotrichum lagenarium* and carrot – *B. cineria.* The role of lignin in plant resistance to biotic stress is elucidated in different pathosystems like wheat- *Puccinia graminis* (Menden et al. 2007), in wheat- *Fusarium proliferatum* (Bishop et al. 2002), in cotton - *Verticillium dahliae* (Shi et al. 2012), in melon - *Podosphaera fusca* (Romero et al. 2008) etc. Egea et al. (2001) observed the accumulation of lignin-like polymers in cell suspension cultures of *Capsicum annuum* in response to elicitation by both lyophilized mycelium and culture filtrate of *P. capsici.* All these results support the finding of this study that increased lignification of root cells of 04-P24 upon *P. capsici* inoculation is positively correlated with the root resistance of this line. The results of this study highlight the importance of increased cell wall reinforcement (vascular tissue) and

cell membrane integrity of root cells in root resistance of 04-P24 to *P. capsici*.

Acknowledgements This research was funded by the Kerala State Council for Science, Technology and Environment (KSCSTE), Council Order No. (T)364/FSHP/2010/CSTE, Thiruvananthapuram, Kerala, India. Authors acknowledge Director, ICAR-IISR, Kozhikode, Kerala, India for facilities provided. The authors gratefully acknowledge the Director, National Institute of Technology, Kozhikode for providing the SEM facility.

Compliance with ethical standards

Ethical statement This research article is not submitted elsewhere for publication and this manuscript complies to the Ethical Rules applicable for this journal.

References

Asselbergh, B., Curvers, K., França, S. C., Audenaert, K., Vuylsteke, M., Van Breusegem, F., & Höfte, M. (2007). Resistance to *Botrytis cinerea* in sitiens, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. *Plant Physiology, 144*(4), 1863–1877.

Bais, H. P., Vepachedu, R., Gilroy, S., Callaway, R. M., & Vivanco, J. M. (2003). Allelopathy and exotic plant invasion: From molecules and genes to species interactions. *Science, 301*(5638), 1377–1380.

Bais, H. P., Park, S. W., Weir, T. L., Callaway, R. M., & Vivanco, J. M. (2004). How plants communicate using the underground information superhighway. *Trends in Plant Science, 9*(1), 26–32.

Belanger, R. R., & Bushnell, W. R. (2002). *The powdery mildews: A Comprehensive treatise.* St. Paul: APS Press.

Benhamou, N., & Bélanger, R. R. (1998). Induction of systemic resistance to *Pythium* damping-off in cucumber plants by benzothiadiazole: Ultrastructure and cytochemistry of the host response. *The Plant Journal, 14*(1), 13–21.

Bertin, C., Yang, X., & Weston, L. A. (2003). The role of root exudates and allelochemicals in the rhizosphere. *Plant and Soil, 256*(1), 67–83.

Bhai, R. S., Eapen, S. J., Anandaraj, M., & Saji, K. V. (2010). Identification of *Phytophthora* and nematode-resistant source-from opens pollinated progenies of black pepper (*Piper nigrum*) using a modified protocol. *Indian Journal of Agricultural Science, 80*(10), 893–897.

Bishop, D. L., Chatterton, N. J., Harrison, P. A., & Hatfield, R. D. (2002). Changes in carbohydrate coordinated partitioning and cell wall remodeling with stress-induced pathogenesis in wheat sheaths. *Physiological and Molecular Plant Pathology, 61*(1), 53–63.

Buonaurio, R. (2008). Infection and plant defense responses during plant-bacterial interaction. *Plant-Microbe Interactions,* 169–197.

Cesco, S., Neumann, G., Tomasi, N., Pinton, R., & Weisskopf, L. (2010). Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. *Plant and Soil, 329*(1–2), 1–25.

Curvers, K., Seifi, H., Mouille, G., De Rycke, R., Asselbergh, B., Van Hecke, A., Vanderschaeghe, D., Höfte, H., Callewaert, N., van Breusegem, F., & Höfte, M. (2010). Abscisic acid deficiency causes changes in cuticle permeability and pectin composition that influence tomato resistance to *Botrytis cinerea*. *Plant Physiology, 154*(2), 847–860.

De Waard, P. W. F. (1986). Current state and prospective trends of black pepper (*Piper nigrum* L.) production. *Outlook on Agriculture, 15*(4), 186–195.

Dixon, R. A. (2001). Natural products and plant disease resistance. *Nature, 411*(6839), 843–847.

Egea, C., Ahmed, A. S., Candela, M., & Candela, M. E. (2001). Elicitation of peroxidase activity and lignin biosynthesis in pepper suspension cells by *Phytophthora capsici*. *Journal of Plant Physiology, 158*(2), 151–158.

Eltahir, A. S., & AbuReish, B. I. (2010). Comparative morphological and anatomical studies of the barks of three *Albizzia* species. *Journal of Chemical and Pharmaceutical Research, 2*(3), 260–268.

Enstone, D. E., Peterson, C. A., & Ma, F. (2002). Root endodermis and exodermis: Structure, function, and responses to the environment. *Journal of Plant Growth Regulation, 21*(4), 335–351.

Eynck, C., Koopmann, B., Karlovsky, P., & Von Tiedemann, A. (2009). Internal resistance in winter oilseed rape inhibits systemic spread of the vascular pathogen *Verticillium longisporum*. *Phytopathology, 99*(7), 802–811.

Faulkner, G., & Kimmins, W. C. (1975). Staining reactions of the tissue bordering lesions induced by wounding, tobacco mosaic virus, and tobacco necrosis virus in bean. *Phytopathology, 65*(12), 1396–1400.

Griffin, M. J. (1977). Cocoa Phytophthora workshop, Rothamsted Experimental Station, England, 24–26 may 1976. *PANS, 23*(1), 107–110.

Guest, D., & Brown, J. (1997). Plant defense against pathogens. In J. F. Brown & H. J. Ogle (Eds.), *Plant pathogens and plant diseases, Rockvale publications* (pp. 263–286). Australia: Armidale NSW 2350.

Gutfinger, T. (1981). Polyphenols in olive oils. *Journal of the American Oil Chemists Society, 58*(11), 966–968.

Heath, M. C. (2000). Hypersensitive response-related death. *Plant Molecular Biology, 44*, 321–334.

Hoagland, D.R., & Arnon, D.I., 1950. The water-culture method for growing plants without soil. Circ. 347. Univ. of Calif. Agric. Exp. Station, Berkley.

Huitema, E., Bos, J. I., Tian, M., Win, J., Waugh, M. E., & Kamoun, S. (2004). Linking sequence to phenotype in *Phytophthora*–plant interactions. *Trends in Microbiology, 12*(4), 193–200.

Jackson, K. M., & Ilamurugu, K. (2014). Metabolic profiling of rice root exudates and its impact on rhizosphere microbial dynamics under aerobic conditions. *Research Journal of Agricultural Sciences, 5*(4), 777–781.

Johansen, D. A. (1940). *Plant microtechnique* (pp. 27–154). New York: McGraw Hill.

Jones, J. D., & Dangl, J. L. (2006). The plant immune system. *Nature, 444*(7117), 323–329.

Lanoue, A., Burlat, V., Henkes, G. J., Koch, I., Schurr, U., & Röse, U. S. (2010). De novo biosynthesis of defense root exudates

in response to *Fusarium* attack in barley. *New Phytologist, 185*(2), 577–588.

Li, X. G., Zhang, T. L., Wang, X. X., Hua, K., Zhao, L., & Han, Z. M. (2013). The composition of root exudates from two different resistant peanut cultivars and their effects on the growth of soil-borne pathogen. *International Journal of Boilogical Science, 9*(2), 164–173.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., Landtag, J., Brandt, W., Rosahl, S., Scheel, D., & Llorente, F. (2005). Pre-and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. *Science, 310*(5751), 1180–1183.

Menden, B., Kohlhoff, M., & Moerschbacher, B. M. (2007). Wheat cells accumulate a syringyl-rich lignin during the hypersensitive resistance response. *Phytochemistry, 68*(4), 513–520.

Nicholson, R. L., & Hammerschmidt, R. (1992). Phenolic compounds and their role in disease resistance. *Annual Review of Phytopathology, 30*(1), 369–389.

O'brien, T. P., Feder, N., & McCully, M. E. (1964). Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma, 59*(2), 368–373.

Oliver, J. P., Castro, A., Gaggero, C., Cascón, T., Schmelz, E. A., Castresana, C., & De León, I. P. (2009). *Pythium* infection activates conserved plant defense responses in mosses. *Planta, 230*(3), 569–579.

Pearce, R. B., & Rutherford, J. (1981). A wound-associated suberized barrier to the spread of decay in the sapwood of oak (*Quercus robur* L.). *Physiological Plant Pathology, 19*(3), 359IN26–369IN31.

Pellizzari, E. D., Kuc, J., & Williams, E. B. (1970). The hypersensitive reaction in *Malus* species: Changes in the leakage of electrolytes from apple leaves after inoculation with *Venturia inaequalis*. *Phytopathology, 60*, 373–376.

Ramírez, V., Agorio, A., Coego, A., García-Andrade, J., Hernández, M. J., Balaguer, B., Ouwerkerk, P. B., Zarra, I., & Vera, P. (2011). MYB46 modulates disease susceptibility to *Botrytis cinerea* in *Arabidopsis*. *Plant Physiology, 155*(4), 1920–1935.

Romero, D., Rivera, M. E., Cazorla, F. M., Codina, J. C., Fernández-Ortuño, D., Torés, J. A., Pérez-García., A., & de Vicente, A. (2008). Comparative histochemical analyses of oxidative burst and cell wall reinforcement in compatible and incompatible melon–powdery mildew (*Podosphaera fusca*) interactions. Journal of Plant Physiology, 165(18), 1895–1905.

Shi, H., Liu, Z., Zhu, L., Zhang, C., Chen, Y., Zhou, Y., Li, F., & Li, X. (2012). Overexpression of cotton (*Gossypium hirsutum*) dirigent1 gene enhances lignification that blocks the spread of *Verticillium dahliae*. *Acta Biochimica et Biophysica Sinica, 44*(7), 555–564.

Singh, A. (2006). Cell membrane injury in flag leaf of wheat by brown rust (*Puccinia recondita* rob. Ex. Desm. f. Sp. tritici) at different nitrogen levels. *Journal of Phytological Research, 19*(1), 111–113.

Thankamani, C. K. (2008). Black pepper. In V. A. Parthasarathy, K. Kandiannan, & V. Srinivasan (Eds.), *Organic spices* (p. 250). New Delhi: New India Publishing Agency.

Trivedi, M., & Singh, A. (2014). Cell membrane injury in barley (*Hordeum vulgare* L.) associated with infection by *Drechslera graminea*, stripe disease. *International Journal of Scientific & Technology Research, 3*(2), 320–323.

Uren, N. C. (2000). Types, amounts and possible functions of compounds released into the rhizosphere by soil grown plants. In R. Pinton, Z. Varanini, & P. Nannipieri (Eds.), *The Rhizosphere: Biochemistry and organic substances at the soil interface* (pp. 19–40). New York: Marcel Dekker.

van Kan, J. A. (2006). Licensed to kill: The lifestyle of a necrotrophic plant pathogen. *Trends in Plant Science, 11*(5), 247–253.

Vandana, V. V., Bhai, R. S., & Shamina, A. (2014). Biochemical defense responses of black pepper (*Piper nigrum* L.) lines to *Phytophthora capsici*. *Physiological and Molecular Plant Pathology, 88*, 18–27.

Vuković, R., Bauer, N., & Ćurković-Perica, M. (2013). Genetic elicitation by inducible expression of β-cryptogein stimulates secretion of phenolics from *Coleus blumei* hairy roots. *Plant Science, 199*, 18–28.

Wang, G., Lin, Q., & Xu, Y. (2007). *Tetraena mongolica* maxim can accumulate large amounts of triacylglycerol in phloem cells and xylem parenchyma of stems. *Phytochemistry, 68*(15), 2112–2117.

Wang, P., Liu, X., Guo, J., Liu, C., Fu, N., & Shen, H. (2015). Identification and expression analysis of candidate genes associated with defense responses to *Phytophthora capsici* in pepper line "PI 201234". *International Journal of Molecular Sciences, 16*(5), 11417–11438.

Wurst, S., Wagenaar, R., Biere, A., & Van der Putten, W. H. (2010). Microorganisms and nematodes increase levels of secondary metabolites in roots and root exudates of *Plantago lanceolata*. *Plant and Soil, 329*(1–2), 117–126.