4. MATERIALS AND METHODS

4.1. Plant Materials

Sesamum orientale L.

*Sesamum orientale* (sesame) of Pedaliaceae is an erect, branched annual herb (Fig. 5a). Flowers are axillary, tubular, two lipped with a pink or white corolla. The fruit is a cylindrical capsule containing 50-100 seeds and grooved deeply with a short, trigonal beak.

Seeds of *Sesamum orientale* L. var. *thilarani* collected from Regional Agricultural Research Station (RARS), Kayamkulam was the experimental material. Healthy plantlets were raised from seeds at the green house conditions, Department of Botany, University College, Thiruvananthapuram. The identity confirmed by comparing with the voucher specimen at Jawaharlal Nehru Tropical Botanical Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram and a voucher specimen deposited in the herbarium at JNTBGRI.

Alternaria sesami (Kawamura) Mohanty and Behera

*Alternaria sesami* belongs to the class Deuteromycetes and family Dematiaceae. Light brown, slender, septate and profusely branched hyphae
Fig. 5 a. Plant material *Sesamum orientale* L. var. *thilarani*
5 b. *Alternaria sesami* (Kawamura) Mohanty and Behera
form the mycelium which ramify along the intercellular and intracellular spaces of host tissue. Asexual spores are conidia, produced singly or in chains. The spores are long, tapered with transverse and longitudinal septae (Fig. 5b).

The leaf spot disease in sesame caused by *Alternaria sesami* appear as small, round to irregular brown water soaked lesions of 1-8 mm in diameter with concentric rings mainly on the leaf lamina, stem and pods.

*Mikania scandens* (L.) Willd.

*Mikania scandens* (Asteraceae), known as climbing hemp weed having long-petioled opposite leaves with small homogamous flower heads (Fig. 5c).

*Salvinia molesta* D.Mitch.

*Salvinia molesta* (kariba weed), free floating aquatic fern, with slender stems, floating leaves, and root-like structures. The fronds paired and round to oval in shape, with dense, waxy hairs on the upper surface (Fig. 5d).

*Senna alata*, the candle bush of Caesalpiniaceae (Fig. 5e), *Lantana camara* (big sage) a small perennial shrub with tubular flowers of Verbenaceae (Fig. 5f), *Vitex negundo* (Lamiaceae) (five-leaved chaste tree) (Fig. 5g), *Lawsonia inermis* (henna) of Lythraceae (Fig. 5h), *Chromolaena odorata*, is a tropical flowering shrub of Asteraceae (Fig. 5i) and *Adhatoda vasica* (Acanthaceae), a shrub with lance-shaped leaves (Fig. 5j) were used as plant based fungicides. Microbial agents namely *Pseudomonas fluorescens*, soil borne Gram-negative rod shaped bacteria (Fig. 5k) and *Trichoderma*
Fig. 5c. *Mikania scandens* (L.) Willd.
5d. *Salvinia molesta* D.Mitch.
Fig. 5 e-j. (e) Senna alata (L.) Roxb. (f) Lantana camara L.
(g) Vitex negundo L. (h) Lawsonia inermis L.
(i) Chromolaena odorata (L.) R. M. King & H. Rob.
(j) Adhatoda vasica (L.) Nees.
Fig. 5 (k) *Pseudomonas fluorescens* Migula.
(l) *Trichoderma harzianum* Rifai.
*Harzianum*, a fungal species (Fig. 51) were used as biocontrol agents against *Alternaria* leaf spot disease in sesame. The talc based formulations of *Trichoderma harzianum* and *Pseudomonas fluorescens* were collected from Kerala Agricultural University, Vellayani, Kerala.

### 4.2. Isolation and identification of the pathogen

Leaves of sesame (*Sesamum orientale* L.) showing typical symptoms of *Alternaria* leaf spot were collected from RARS fields. The infected leaves were cut into small pieces and surface sterilized for 30 sec using 1:1000 mercuric chloride solutions. The leaf bits were washed thoroughly in sterile distilled water for thrice to remove traces of mercuric chloride. The sterilized leaf bits were placed on potato dextrose agar (PDA) medium and incubated at room temperature. In PDA slants pure colonies were developed and sporulation occurred within 7 days. PDA slants containing pure culture were used for further studies. Subculturing of the pathogen was also done in PDA medium, maintained at 4°C and recultured once in 30 days.

Pathogenicity of the fungus was tested by inoculating healthy sesame plants at different growth stages. For *in vitro* fungal inoculation studies, sesame plants were inoculated with 20 μl of *Alternaria sesami* conidial suspension (1×10³ conidia ml⁻¹) prepared from pure culture by drop method. Sterilised water (20 μl) used for mock inoculation. The inoculated plants, along with their respective healthy controls were maintained at room temperature and covered
with transparent polyethylene bags for providing high humidity to facilitate successful penetration of the pathogen into sesame. Leaves from control and pathogen inoculated plants were harvested from 1<sup>st</sup> to 13<sup>th</sup> day after inoculation for all analytical, biochemical and molecular analysis.

The pathogen was identified up to species level, based on their cultural and morphological characters. Identification of the fungus was further made after examining conidia under microscope from mature pure culture of the fungus obtained from infected leaves of sesame. Stage and ocular micrometer were used to measure the size of conidia, length of beak and number of septa. The recorded data compared with those of the standard measurements given by Ellis (1971) to identify the pathogen.

4.3. Histochemical Localization

4.3.1. Localization of superoxide anions (O<sub>2</sub><sup>−</sup>)

Superoxide anions localized following the protocol of Ogawa <i>et al.</i> (1997). Thin sections of infected and control leaf tissue were incubated in 10 mM sodium phosphate buffer (pH 7.8) containing 0.25 mM Nitro Blue Tetrazolium (NBT) for 30 min. Localization of superoxide anions was detected as blue formazan deposits.

4.3.2. Localization of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)

Histochemical localization of H<sub>2</sub>O<sub>2</sub> was done by staining the tissues with Tetramethyl benzidine (TMB) reagent, as per the method of Ros Barcelo
(1998), with slight modifications. Fresh, thin sections of infected and healthy leaf tissues were incubated in 50 mM Tris-acetate buffer (pH 7), containing 0.1 mg/ml TMB-HCl, for 15 to 20 min. The sections observed under light microscope and H₂O₂ localization detected as blue deposits. Controls were performed in the presence of catalase (200 U/ml), 1.0 mM ascorbate and by blanching the sections in 80°C hot water before staining with TMB.

4.3.3. Localization of lignin

Lignin was localized using the Wiersner test as described by Ros Barcelo (1998). Thin sections of control and infected leaf tissues were soaked in 1.0 (w/v) phloroglucinol in 25:75 (v/v) HCl: ethanol for 10 to 15 min. Deep red depositions in the tissue indicated the presence of lignin.

4.3.4. Localization of peroxidase

Peroxidase enzyme localized following the procedure of Bestwick et al. (1998) with slight modifications. Thin sections of infected and control leaves were first washed in Tris buffer (pH 9) for 10 min. The sections were then transferred to freshly prepared incubation medium containing 10 mg/ml diaminobenzidine (DAB) and 5 mM H₂O₂ dissolved in 5 ml Tris buffer (pH 9) for 10 to 15 min and directly examined under light microscope. Polymerized DAB detected as deep brown deposits, which indicated the localized enzyme.
4.4. Electron microscopy

4.4.1. Scanning electron microscopy (SEM) of leaf samples

The scanning electron microscopic (SEM) preparations of samples were done following the method of Anilkumar and Murugan (2013). The infected and control leaf samples of *S. orientale* subjected to SEM processing:

**Fixation** - Tissue pieces of infected leaves fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (*pH* 7.4) for 3 h. After fixation, the tissue pieces washed thrice thoroughly in 0.1 M phosphate buffer for 5 min.

**Dehydration** - The fixed tissue pieces dehydrated through acetone series for 25%, 50%, 75% and 90% for 10 min each. Finally, the tissues kept in 100% acetone for 30 min at room temperature.

**Critical Point Drying (CPD)** - Critical point drying done to overcome the damage caused by the passage of a phase boundary through specimen. During CPD, the specimen water was replaced by liquid CO$_2$ at 31°C and 71.8 atmospheric pressure for 20 min. CPD of dehydrated specimen was done in a critical point drier (H.C.P-2 Hitachi). After CPD, the tissue pieces subjected to mounting followed by gold coating for observation.

**Mounting** - The dehydrated tissue samples mounted on metallic stubs using adhesive tapes. The mounted stubs coated with gold in a sputter coat (E-101-Hitachi). The coated specimens observed under SEM (S-2400, Hitachi).
4.4.2. Transmission electron microscopic (TEM) analysis

Transmission electron microscopy (TEM) adopted in the infected and control leaf tissue of *S. orientale* (Fang *et al.*, 1987). The steps involved in tissue processing are as follows:

**Fixation**

Small pieces of leaf tissue (1 mm$^3$) were fixed in 3% cold glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 6 h at 0-4°C. The fixed tissue pieces cut again into thin pieces and fixation continued for 30 min. The fixed tissue pieces washed four times thoroughly in cold 0.1 M cacodylate buffer (pH 7.4) for 30 min.

**Incubation**

Fixed tissues equilibrated in 0.1 M 2-amino-2-methyl-1, 3 propanediol (AMP) buffer at pH 9.0, prior to incubation in the medium.

**Osmication**

The incubated tissue pieces again fixed in 2% osmium tetroxide in 0.05 M cacodylate buffer at pH 7.4 over night in a refrigerator. Osmicated tissue pieces washed five times thoroughly in 0.05 M cacodylate buffer for 10 min each. The tissue subjected to two washings in cold distilled water for 30 min each.

**Dehydration**

The osmicated tissues dehydrated in a graded ethanolic series of 25%, 50%, 70% and 90% for 15 min. Finally, the tissues dehydrated in 100% acetone for 30 min at room temperature.
Infiltration

Dehydrated tissue pieces infiltrated in embedding medium. Spurr's low viscosity resin was used as the embedding medium. It was prepared by the method of Spurr (1969). The composition of the resin was as follows: (a) vinyl cyclohexane dioxide (VCD) - 10.0 g; (b) diglycidyl ether of polypropylene glycol (flexibilizer - DER 736) - 6.0 g; (c) non-etyl succinic anhydride (hardner - NSA)-26.0 g; (d) dimethyl amino ethanol (accelerator-DMAE) - 0.4 g. The accelerator added lastly after gentle mixing the other components. The complete medium mixed thoroughly by stirring. The infiltration done in a series of 25% resin + 75% acetone; 50% resin + 50% acetone; for 3 h each at room temperature. The tissue kept in 75% resin + 25% acetone over night. As the last step in infiltration, the tissue pieces were kept in 100% resin for 4 changes each with 2 h at room temperature. Continuous mild agitation was given to the tissues during all steps of infiltration using a rotator.

Embedding

The infiltrated tissue pieces embedded in 100% Spurr's epoxy resin filled in embedding moulds. Specimens sunk to the bottom of the media. The embedded moulds polymerized in an oven at 70°C for 24 h. The polymerized blocks were ready for ultra thin sectioning.

Sectioning

The tissue blocks were sectioned by an ultratome (NOVA, LKB). Ultra thin sections were mounted on clean copper grids carefully viewed and
photographed using a transmission electron microscope (TEM), (JEOL, 100SS).

4.5. Analytical methods

Leaf tissues of sesame from 1, 3, 5, 7, 9, 11 and 13 days after infection were subjected to all the analytical and biochemical studies and also compared with respective controls.

4.5.1. Quantification of superoxide anions (O$_2^{-}$)

Superoxide anions were quantified following the method of Doke (1983). 1 g infected and control leaf tissues homogenized in a prechilled mortar using 50 mM sodium acetate buffer containing 10 mM NaCl (pH 6.5). The homogenate filtered by double-layered cheesecloth and centrifuged at 10000 rpm for 10 min. The supernatant collected and made up to a known volume. An aliquot (0.1 ml) of the extract taken and mixed with the assay reagent containing 0.01 M potassium phosphate buffer (pH 7.8) with 0.05% nitro blue tetrazolium sodium salt (NBT) and 10 mM sodium azide (NaN$_3$). The assay mixture incubated for 30 min and the initial absorbance at 580 nm taken in a spectrophotometer. Final absorbance was taken after heating the mixture at 85°C for 15 min.

4.5.2. Quantification of hydrogen peroxide (H$_2$O$_2$)

H$_2$O$_2$ concentrations of the experimental tissues estimated as per the procedure of Bellincampi et al. (2000) with some modifications. It was based on
the peroxidase induced oxidation of Fe$^{2+}$, followed by the reaction of Fe$^{3+}$ with 
xylenol orange. 1 g tissue was homogenized in 10 ml cold 10 mM phosphate 
buffer (pH 7) using a prechilled mortar and pestle. The homogenate filtered and 
centrifuged at 10000 rpm for 10 min. The supernatant collected and made up to 
a known volume using the buffer. 1.5 ml of the extract was added to an equal 
volume of assay reagent containing 500 μM ammonium ferrous sulphate, 50 
mM H$_2$SO$_4$, 200 μM xylenol orange and 200 mM sorbitol. The assay mixture 
incubated for 45 min and the absorbance of the Fe$^{3+}$ xylenol orange complex at 
560 nm was observed.

4.5.3. Quantification of lipid peroxidation (LPX)

The level of lipid peroxidation in the cells measured in terms of 
malondialdehyde (MDA) content determined by the thiobarbituric acid (TBA) 
reaction as described by Zhang and Kirkham (1996). 0.4 g tissue of the 
samples homogenized in 4 ml 0.1% (w/v) trichloroacetic acid (TCA). The 
homogenate centrifuged at 10,000 rpm for 10 min. 1 ml of the supernatant was 
diluted 1:5 (v/v) with 20% (w/v) trichloroacetic acid containing 0.5% (w/v) TBA. 
The mixture heated at 95°C for 30 min and cooled in an ice bath, followed by 
centrifugation at 10,000 rpm for 10 min, and the absorbance of the supernatant 
at 532 nm was measured. The nonspecific absorption value at 600 nm 
subtracted from the 532 nm reading. The malondialdehyde concentration 
calculated using the extinction coefficient 155 mM/cm.
4.5.4. Quantification of primary metabolites

4.5.4.1. Quantification and fractionation of carbohydrates by Gas Liquid Chromatography (GLC)

The oven dried (105°C overnight) infected and control leaves were used for estimation of sugars. Soluble sugars were extracted in 80% (v/v) ethanol, the extract was transferred to 5 ml pear shaped flasks then completely dried in vacuum and stored in desiccator until used for silylation as described by Holligan and Drew (1971). In this method, each sample dissolved in 0.8 ml anhydrous pyridine, and 0.2 ml of N-trimethylsilylimidazole added as the silylating reagent. The flasks kept in water bath at 60-70°C for 30 min. After cooling, the samples transferred to injection vials for gas liquid chromatography analysis (GLC). The peak areas of both samples and standards were compared corresponding to retention time of standard sugars for qualitative as well as quantitative estimation of sugars. Standard sugars (fructose, glucose, sorbitol, inositol and sucrose) used as references.

4.5.4.2. Isolation and estimation of protein

Total protein was estimated by modified Lowry's method (1951). 1 g finely chopped leaf tissue was homogenized in 0.1 M phosphate buffer (pH 7). After centrifugation, 5 ml Tricarboxylic acid (TCA) was added to the supernatant. The precipitated protein was dissolved in 0.1 N NaOH. 5 ml alkaline copper sulphate solution was added to an aliquot of the sample,
followed by 0.5 ml Folin's reagent and the mixture was kept undisturbed for 30 min. Finally the absorbance was measured at 650 nm. Total protein was calculated from the standard curve of BSA.

4.5.4.3. Estimation of total free amino acids

Total free amino acids were determined using the method of Moore and Stein (1948). 1 g tissue refluxed in 80% methanol for 10 min and subsequently homogenized. The homogenate centrifuged at 7500 rpm for 10 min. The supernatant collected and made to known volume using 80% methanol. 0.1 ml of the supernatant mixed with 5 ml ninhydrin reagent, shaken well and boiled for 10 min. The absorbance read at 570 nm.

4.5.4.4. Quantification of proline

Free proline accumulation was determined using the method of Bates et al. (1973). 0.5 g tissue homogenized with 3% sulfosalicylic acid (SA) and kept for 15 min at 4°C. The homogenate was centrifuged at 3000 rpm for 20 min. 500 μl supernatant was treated with 1:1 acetic acid ninhydrin solution and boiled for 1 h. The mixture extracted with toluene and proline quantified spectrophotometrically at $A_{520}$ nm from the organic phase.

4.5.5. Total Pigments

Total chlorophyll estimated by the methods of Vicas et al. (2010). For pigment extraction, 1 g of infected and control leaves were homogenized with
80% acetone. The homogenate centrifuged at 12000 rpm, for 10 min at 4°C. The supernatant separated and the pigment content was determined by measuring the absorbance at 663.6 nm, 663.8 nm and 665.2 nm for chlorophyll a and 652.0 nm, 646.6 nm and 646.8 nm for chlorophyll b using spectrophotometer. Carotenoids estimated by the method of Vicas et al. (2010). An aliquot of 0.5 ml extract taken and mixed with 4.5 ml of 80% acetone and the optical density measured at 480, 645, 652 and 663 nm and used for calculating the total carotenoids.

**4.5.6. Quantification of total phenol**

Total phenol content in the samples estimated by the method of Mayr et al. (1995). 1 g tissue finely chopped into small pieces and refluxed in 80% methanol for 10 min. Subsequently it was homogenized with mortar and pestle. After cooling, the homogenate filtered and centrifuged at 10000 rpm for 10 min. For the estimation of total phenols, supernatant was used.

An aliquot of the sample pipetted out and made up to 3 ml with 80% methanol. 0.5 ml Folin-Ciocalteau reagent added and kept for 3 min. 2 ml 20% Na₂CO₃ added to the mixture and kept in boiling water bath for 1 min. The white precipitate was removed by centrifuging it for few min and the absorbance of the clear light blue solution was recorded at 650 nm against the reagent blank containing 3 ml 80% methanol, 0.5 ml Folin’s reagent and 2 ml 20% Na₂CO₃.
The reaction between phenols and an oxidizing agent phosphomolybdate in Folin-Ciocalteau reagent resulted in the formation of a blue complex. A standard graph of phenols constructed with pyrocatechol by taking absorbance against different concentration. The total phenols $g^{-1}$ tissue calculated from the standard graph.

4.5.6.1. Estimation of fractionated free and cell wall-bound phenolics

Free and cell wall-bound phenolics were determined according to Haddadchi and Gerivani (2009). Infected sesame leaves and control (0.5 g) were extracted in 50% methanol (12 v/v) for 90 min at $80^\circ$C. The extract centrifuged at 14000 g for 15 min and the supernatant taken for free phenolic determination using the Folin-Ciocalteu's phenol reagent. The pellet was sponified with 2 ml of 0.5 N NaOH for 24 h at room temperature to release the bound phenolics, neutralized with 0.5 ml 2 N HCl and centrifuged at 14000 g for 15 min. The supernatant taken for bound phenolic determination using the Folin-Ciocalteu's assay. 100 $\mu$l of the methanol and NaOH extracts were diluted to 1 cm$^3$ with water and mixed with 0.5 ml 2.0 N Folin-Ciocalteu's reagent and 2.5 ml of 20% Na$_2$CO$_3$. After 20 min at room temperature, absorbance of samples was measured at 725 nm with a spectrophotometer. Phenolic concentration in the extracts was determined from standard curve prepared with gallic acid.
4.5.6.2. Reverse phase high performance liquid chromatography (RP-HPLC) of phenols

Quantitative fractionation of various phenolic acids in the samples were studied by RP-HPLC analysis. Phenolic acids extracted from fresh tissues in aqueous methanol used for the study.

**Preparation of the sample**

1 g fresh, finely chopped tissue reflexed in boiling 80% methanol for 10 min. The tissue was homogenized, filtered through cheese cloth and centrifuged at 15000 rpm for 10 min. The resultant supernatant made up to 5 ml with 80% methanol and used for RP-HPLC analysis.

**Procedure of RP-HPLC**

A modified method of Beta et al. (1999) followed for HPLC analysis. An HPLC system (Waters Associates) equipped with a 7725 Rheodyne injector and Waters 510 HPLC pump, 486 tunable absorbance detector and Millennium 2010 software data module used for the study. An HPLC column of 4.6 × 250 mm id reverse phase (RP) C8 used for the fractionation of phenolic acids. Potassium hydrogen phosphate and acetonitrile in a ratio of 75:25 was used as the mobile phase for the isocratic elution. An elution period of 20 min with a flow rate of 0.8 ml/min was given. 10 μl of the sample was injected and the absorbance at 254 nm was recorded. Standard phenolic acids such as gallic, vanillic, p-hydroxybenzoic, ferulic, chlorogenic, sinapic, coumarate, phloroglucinol, catechol and cinnamic acids were injected separately in to the column.
Phenolic acids in the sample were identified by comparing with the retention time of the standard. Height of the peaks was taken for quantification. Concentration of the standard and height of the standard peaks were taken as the standard parameters.

4.5.7. Potassium leakage

The leaf membrane damage was estimated on the basis of K⁺ leakage (Valentovic et al., 2006). Twenty leaf discs (1.0 cm in diameter) incubated in deionized water at 25°C for 24 h (L1) and the K⁺ concentration in the leach determined by flame photometry. Then, the discs were boiled for 1 h and the K⁺ concentration was measured again (L2). The percentage of membrane damage (MD) was estimated according to the equation MD (%) = (L1/L2) x 100.

4.5.8. Cell wall composition analysis

4.5.8.1. Estimation of Pectic substances

Pectic substance quantified following the method of Southgate (1976a). 5 g of sesame leaf powder extracted with 0.5% ammonium oxalate for 2 h at 85°C. The extraction repeated four times. The filtrate combined and made slightly acidic by 1N HCl. Four volumes of ethanol added to the filtrate by constant stirring. After the precipitate had settled decanted the supernatant through a weighed glass filter crucible. The precipitate transferred to the crucible and washed with 70% methanol. The difference in weight showed the quantity of pectic substance.
4.5.8.2. Estimation of Hemicellulose

The leaf residue after the extraction of pectic substance was treated with KOH at different concentrations (5% and 24%) for 24 h. The flask was flushed with nitrogen during alkaline treatment. The KOH extraction repeated thrice and the residue filtered off in a glass crucible. The combined filtrate acidified with acetic acid and four volumes of methanol. The precipitate filtered with a weighed glass crucible. The precipitate washed with acetone. The crucible was dried and weighed. The difference in weight was taken as hemicellulose content (Southgate, 1976a).

4.5.8.3. Estimation of Cellulose

After the extraction of hemicellulose, the same leaf residue dissolved in 24% KOH washed thoroughly with water followed by acetone, dried and weighed. The weighed residue was hydrolysed in 1N H₂SO₄ for 2 h and filtered in a weighed ash less filter paper. The paper was washed with water, acetone and then dried and re-weighed. The paper ignited and the ash weighed. The weight of ash deducted from the weight of residue after dilute acid hydrolysis and the value recorded obtained as cellulose content (Southgate, 1976a).

4.5.8.4. Quantification of lignin

Lignin content of the samples was estimated by acetyl bromide method by recording the absorbance at 280 nm (Iiyama and Wallis, 1988). 0.5 g tissue
homogenized in 10 ml methanol. The homogenate centrifuged at 5000 rpm for 5 min at room temperature. After decanting the supernatant, the pellet subjected to sequential washing with methanol, ethyl alcohol and water with three washes in each followed by centrifugation. The pellet was taken and dried at 70°C in a hot air oven over night. 10 mg of dried tissue solubilized with 3 ml 25% acetyl bromide in acetic acid. The mixture kept for 30 min at 70°C in a water bath, and then cooled at room temperature. 0.9 ml 2 N NaOH added to it followed by 0.1 ml hydroxylamine hydrochloride. The resulting reddish brown solution diluted with 1% acetic acid, centrifuged at 5000 rpm for 5 min and the absorbance at 280 nm measured immediately. A standard graph of lignin was prepared with dehydro coniferyl alcohol polymerizate (DHAP).

4.5.9. FT-IR spectroscopy

The leaves of infected and control tissue (approximately 3-4 cm) were pooled as two separate samples. The samples immediately dried in oven for 2 days at 60°C. Tablets for FTIR spectroscopy were prepared in agate mortars, by mixing leaf powder (2 mg) with KBr (1:100 p/p). The absorbance spectra measured between 300 and 4500 cm⁻¹. At least three spectra obtained for each sample (Anilkumar et al., 2012).

A FTIR spectrometer (FTIR Shimadzu Prestige 21) used to collect spectra. Spectra obtained in 32 scans co-added, 4000 resolution, and 2.0
gains. The parameters for the Fourier self-deconvolution were a smoothing factor of 15.0 and a width factor of 30.0 cm⁻¹. De-convolved and second-derivative spectra calculated for Fourier self-deconvolution and the bands selected and normalized to unity with Omnic 7 software. Curve-fitting of the original spectra was performed with Origin 7 software. The band position of functional groups monitored with Knowitall 7.8 software. The spectral region between 3000 and 2800 cm⁻¹ selected to analyze lipids. The spectral region between 1800 and 1500 cm⁻¹ selected to analyze proteins. The spectral region between 1200 and 1000 cm⁻¹ selected to analyze carbohydrates. Triplicate experiments (N = 3) were conducted and spectra from the first two times of experiments were used for establishment of chemometric models and the spectra from the third time of experiment were used for model validation.

4.6. Isolation and assay of antioxidant enzymes

4.6.1. NADPH oxidase (NOX)

NADPH oxidase catalyzes the production of superoxide anions, a free radical. Isolation and assay carried out following the procedure of Bestwick et al. (1998). 1 g infected and control leaf tissues were taken and finely chopped into small pieces and homogenized in 10 ml 50 mM phosphate buffer pH 6.8, containing 1% (w/v) sodium metabisulphate, 0.5% (w/v) PVPP. The homogenate filtered through double-layered cheesecloth and centrifuged at 16000 rpm for 30 min at 4°C. The filtrate collected in a fresh test tube and the
volume made to 10 ml using the extraction buffer. A 0.5 ml of aliquot of the sample assayed in a freshly prepared 2.5 ml of assay reaction buffer containing 50 mM sodium acetate, 0.15 mM NADPH, 20 mM MnCl₂ and 2.5 mM dichloro phenol (DCP). Absorbance recorded at 340 nm on a time scan of 10 sec intervals for 5 min.

4.6.2. Superoxide dismutase (SOD)

The Superoxide dismutase enzyme (SOD) removes O₂⁻⁻ by catalyzing its dismutation, one O₂⁻⁻ being reduced to H₂O₂ and another to O₂.

\[ 2 \text{O}_2^{\cdot-} + 2\text{H} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

The enzyme extracted and assayed following the method of Fridovich (1997) with some modifications. 1 g finely chopped tissue was washed thoroughly and homogenized in 10 ml extraction buffer containing 2.6 mM potassium phosphate, 0.1 mM EDTA and 1% PVPP (w/v) at pH 7.8. The homogenate filtered through double-layered cheesecloth and the filtrate taken as the source of enzyme for assay.

The activity of SOD measured as the inhibition of the rate of reduction of cytochrome C by the superoxide radical observed at 550 nm.

Cytochrome C (oxidized) + O₂ → Cytochrome C (reduced) + O₂

The superoxide radical enzymatically produced by the reaction

\[ \text{Xanthin} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{XOD}} \text{Uric acid} + \text{O}_2^{\cdot-} + \text{H}^+ \]
An aliquot of the sample assayed in a reaction cocktail containing 2.6 mM extraction buffer, 10.7 mM EDTA, 1.1 mM cytochrome C solution, 0.108 mM xanthin solution (total 3 ml volume) at pH 7.8 at 25°C. Two tests were carried out during the assay. The uninhibited test was done without adding the enzyme source i.e., without SOD, the assay mixture contained 2.8 ml reaction cocktail, 0.1 ml xanthin oxidase (XOD) and 0.1 ml deionized water taken in a 3 ml cuvette, mixed by gentle inversion and recorded the increase in A₅₅₀ nm for approximately 5 min. It was followed by the inhibition test, where the assay mixture contained 2.8 ml reaction cocktail, 0.1 ml XOD and 0.1 ml sample, taken in a 3 ml cuvette, which was given a gentle inversion for mixing and recorded the increase in A₅₅₀ nm for 5 min. The absorbance, A₅₅₀ nm min⁻¹ was obtained by using the maximum linear rate for both the uninhibited and inhibited tests. A blank maintained without adding XOD and sample. SOD activity measured as per the following calculations.

\[
\text{% inhibition} = \frac{\Delta A_{550}\text{nm/min of uninhibited} - \Delta A_{550}\text{nm/min of inhibited}}{\Delta A_{550}\text{nm/min of uninhibited} - \Delta A_{550}\text{nm/min of blank}} \times 100
\]

\[
\text{Unit ml}^{-1} \text{enzyme} = \frac{\% \text{ inhibition (df)}}{(50 \%) \times 0.1}
\]

Where,  

- df - dilution factor  

- 0.1 - volume in ml of enzyme  

- 50% - Inhibition rate of the Cytochrome C reduced as per the unit definition
One unit of SOD defined as the rate of reduction of cytochrome C by 50% in a coupled system, using xanthin and xanthin oxidase at pH 7.8 at 25°C in a 3 ml reaction mixture.

4.6.3. Catalase (CAT)

For CAT isolation, 1 g leaf tissue was ground and homogenized in 20 ml ice-cold extraction buffer (100 mm KH₂PO₄ or K₂HPO₄ (pH 7.8), 300 mg polyvinyl pyrrolidone (PVP), 1% (v/v) Triton X-100 and 5 mM ascorbate (Schwanz et al., 1996). The homogenate centrifuged at 25,000 rpm (20 min, 4°C). The supernatant used for the determination of catalase.

In catalase assay, the reaction mixture consisted of 50 mM K-phosphate buffer (pH 7.0) containing 10 mM H₂O₂ (0.95 ml) and enzyme extract (0.05 ml). Immediately after adding the enzyme to the buffer, the initial rate of absorbance at 240 nm was determined. The CAT activity measured at A₄₂₀ for H₂O₂ decomposition rate using the extinction coefficient of 40 mM⁻¹ cm⁻¹ according to Lijun et al. (2005).

4.6.4. Peroxidase (POX)

Peroxidase was isolated and assayed following the method of Goliber (1989). 1 g tissue homogenized in 10 ml cold 0.1 M sodium phosphate buffer pH 6, in a pre chilled mortar and pestle. The homogenate filtered through two-layered cheesecloth and centrifuged at 18000 rpm for 20 min at 4°C. The supernatant used for determining enzyme activity.
Peroxidase activity was assayed using guaiacol as substrate (Ingham et al., 1998). The reaction mixture consisted of 2 ml 0.1 M phosphate buffer pH 7.0; 1 ml 20 mM guaiacol; 50 µl 10 mM H₂O₂ and 40 µl enzyme. Guaiacol oxidized by hydrogen peroxide and the increase in absorbance measured spectrophotometrically at 470 nm for 10 min at 30°C. A set of samples containing reaction mixture without guaiacol was taken as the control. One unit of POX is the amount of enzyme required to oxidise 1 µM guaiacol by H₂O₂ at test condition.

4.6.4.1. Fractionation and assay of peroxidase – cytosolic and cell wall bound

Soluble (cytosolic) and ionic bound (cell wall bound) peroxidase were isolated from fresh tissues by modifying the method of Ingham et al. (1998). Fresh tissue (1 g) homogenized in 10 ml cold 0.1 M phosphate buffer (pH.6) containing 100 mg polyvinyl poly pyrrolidone (PVPP) to act as a phenolic scavenger. The homogenate was filtered through two layered cheesecloth, centrifuged at 20000 rpm for 20 min at 4°C. The supernatant recovered contained the soluble fraction of POX. The pellet of the above experiment used to extract ionic bound POX. The pellet washed thoroughly in 0.1 M phosphate buffer. The insoluble residue further extracted with 10 ml 0.1 M phosphate buffer containing 1 M NaCl and 10 mg PVPP. The homogenate containing bound POX filtered through cheesecloth and was recovered by centrifugation at 20000 rpm for 20 min at 4°C.
4.7. Isolation and assay of H⁺-ATPase

Plasma membrane H⁺-ATPase was isolated from leaf samples of control and infected S. orientale using the protocol of Gallagher and Leonard (1982). The isolation buffer used was 0.1M phosphate buffer (pH 7). The reaction mixture consists of 3 mM ATP, 3 mM MgSO₄, 50 mM KCl and 30 mM Tris HCl. The pH of the buffer is 7. The reaction initiated by adding 100 µl of extract to the reaction mixture at 25°C and terminated after 30 min by the addition of 0.9 ml of 5% sodium dodecylsulphate (SDS) to the reaction mixture. In control, the extract added after termination of reaction. The release of inorganic phosphate (Pi) was determined according to the method of Fiske and Subbarow (1925). After termination of the reaction, the solution was mixed with 0.1 ml of 1-amino-1-naphthol-sulphonic acid (0.125% in 15% NaHSO₃ 1% Na₂ SO₃). This mixture was shaken at 25°C for 30 min. The absorbance recorded at 750 nm. The protein content of the enzyme was estimated by Bradford method.

4.8. Isolation and assay of phenylalanine ammonia lyase (PAL)

Isolation of PAL was made following the method of Morrison et al. (1994). 0.5 g of finely chopped fresh tissue weighed and freeze-dried for 2 h. The frozen tissues homogenized in 10 ml 0.1 M Tris-HCl buffer containing 20 mM 2-mercaptoethanol and 0.5% polyethylene glycol (PEG) at pH 7.6 in a pre-chilled
mortar and pestle. The homogenate filtered through double-layered cheesecloth and the filtrate centrifuged at 20000 rpm for 20 min, in a cold centrifuge at 4°C.

The activity of PAL was estimated by the method of Whetten and Sederoff (1992). The activity related to the amount of cinnamic acid formed by the action of the enzyme on the substrate. The reaction mixture contained 2.5 ml 12 mM phenylalanine in 0.1 M Tris-HCl buffer (pH 8.5) and 0.5 ml enzyme extract. The reaction allowed to proceed for 30 min at room temperature and stopped by the addition of 2 ml 2 N HCl. The reaction mixture extracted with 3 ml toluene by vortexing for 10 sec and centrifuged the mixture at 1000 rpm for 5 min to separate the phases. The OD of the cinnamic acid recovered recorded at 290 nm against a blank of toluene alone. A standard graph of absorbance as a function of cinnamic acid concentration was drawn using reaction mixtures that contain known volumes of cinnamic acid, but no enzyme. These mixtures extracted and examined spectrophotometrically for enzyme assays. Cinnamic acid released in the sample estimated from the standard graph of cinnamic acid. One unit of PAL activity is equivalent to the μg of cinnamate released by the deamination of L-phenylalanine under ambient condition.

4.9. Extraction and assay of polyphenol oxidase (PPO)

PPO activity was determined as per the method of Mayer et al. (1965). Leaf samples (1 g) were homogenized in 2 ml 0.1 M sodium phosphate buffer
(pH 6.5) and centrifuged at 16000 g for 15 min at 4°C. The supernatant was taken as the enzyme source. The reaction mixture consisted of 200 μl of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 200 μl of 0.01 M catechol were added and the change in O.D. was recorded at 30 s interval up to 3 min at 495 nm. The enzyme activity was expressed as changes in O.D. at 495 nm min⁻¹ mg⁻¹ protein.

4.10. Isozyme analysis

For isozyme analysis using native polyacrylamide gel electrophoresis (PAGE), crude enzyme extracts electrophoresed in a resolving gel of 10% acrylamide concentration and stacking gel of 5% acrylamide concentrate-ion. Protein staining and destaining were done according to the method described by Laemmli (1970).

After the electrophoresis, the gel was subjected to peroxidase staining. Peroxidase isoenzyme staining was done according to the method described by Ros Barcelo (1987). After electrophoresis, the gel was washed twice (for 5 min each) in 0.05 mM sodium acetate buffer, pH 5.5. The gel transferred into the freshly prepared solution containing 1 mg ml⁻¹ diaminobenzidene in the above buffer. The colour reaction started by the addition of 0.05 ml 6% H₂O₂. The gel was allowed to remain in the solution till the brown bands were visible. The reaction stopped by the addition of 5% acetic acid and washed several times with water to remove traces of substrate. The gel was stored in 5% ethanol.
Isozymes of CAT visualized on gels by the method of Woodbury et al. (1971). H$_2$O$_2$ reduces potassium ferricyanide to potassium ferrocyanide, the peroxide is oxidized to molecular oxygen. Ferric chloride reacts with ferrocyanide to form stable, insoluble prussian blue pigment. After acrylamide gel electrophoresis, the gels were washed thrice in distilled water to remove the buffer from the gel’s outside surface, where staining occurs. The gels were then transferred to a closed tray containing 0.003% H$_2$O$_2$ for 10 min. Then the gels briefly rinsed with distilled water and placed in a 10% solution of ferric chloride and potassium ferricyanide (freshly prepared) for 10 min. The staining solution was then poured off and stored in distilled water. They should remain in the dark except when being studied.

SOD activity detected on gels using the photochemical procedure of Beauchamp and Fridovich (1971). SOD was localized by soaking the gels in 2.45 x 10$^{-3}$ M nitro blue tetrazolium for 20 min, followed by an immersion, for 15 min, in a solution containing 0.028 M tetra methyl ethylenediamine, 2.8 x 10$^{-5}$ M riboflavin, and 0.036 M potassium phosphate at pH 7.8. The gels were then placed in a tray and illuminated for 5 to 15 min. During illumination the gels became uniformly blue except at positions containing superoxide dismutase. Illumination discontinued when maximum contrast between the achromatic zones and the general blue colour had been achieved. The gels were then photographed. The different types of SOD differentiated by performing the
activity stains in gels previously incubated in 3 mM I⁻¹ KCN or 5 mM I⁻¹ H₂O₂ as described by Asada et al. (1975).

For polyphenol oxidase, gel was equilibrated for 30 min in 0.1% phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0), followed by 10 mM catechol in the same buffer. The addition of catechol, followed by a gentle shaking resulted in appearance of dark brown discrete protein bands (Jayaraman et al., 1987). After staining, the gel washed with distilled water and photographed.

For esterase (EST) and acid phosphatase, leaves were ground in 0.01 M Tris, 0.08 M glycine, 0.005 M cysteine, and 20% sucrose at pH 8.3. Ion exchange resin Dowex 1 x 8 (0.4 g / 1 g fresh tissue) added to the extraction buffer to eliminate polyphenols. Homogenates centrifuged at 10,000 rpm for 10 min. The supernatant used as a source of enzymes. Anodally migrating isoforms of esterase and acid phosphatase were resolved on 7.5% polyacrylamide slabs as separating gel with 3% stacking gel by the electrophoretic system of Angelov (2003). Cathodal isoforms of EST run on 7.5% separating gel and 3% stacking gel according to Reisfeld et al. (1961). The length of the separating gel was 6 cm and stacking gels were 1.5 cm long. Electrophoresis conducted at 200 V/25 mA for the basic gels and at 150 V/45 mA for the acidic gel system. Electrophoresis of cathodal esterase carried out until the indicator dye, pyronin G, reached the gel end (1 front). The duration of anodal
electrophoresis was 1.25 fronts of indicator bromphenol blue for EST and 1.5 fronts for acid phosphatase. Staining protocols performed as mentioned in Angelov (2003).

4.11. Extraction and assay of antioxidants and antioxidant enzymes of ascorbate – glutathione cycle

4.11.1. Quantification of ascorbate (Asc) and dehydroascorbate (DHA)

Asc and DHA measured according to Logan et al. (1998) with minor modifications. Briefly, 1 g of experimental materials were ground in 1 ml of ice cold 6% (v/v) HClO₄. The extract centrifuged for 10 min in 10,000 rpm at 4°C and supernatant immediately assayed for Asc and DHA. 100 μl of the extract was neutralized with 30 μl 1.5 mM Na₂CO₃. Asc was assayed spectrophotometrically at 265 nm (ε = 14 mM cm⁻¹) in 100 mM potassium phosphate buffer (pH 5.6), before and after 15 min incubation with 5 units of ascorbate oxidase. For total ascorbate, 100 μl of extract was neutralized with 30 μl 2 mM Na₂CO₃ and incubated for 30 min at room temperature with equal volume of 20 mM GSH in 100 mM Tricine–KOH (pH 8.5). DHA content calculated as the difference between total ascorbate and Asc.

4.11.2. Estimation of reduced glutathione (GSH) and oxidized glutathione (GSSG)

The levels of reduced and oxidized glutathione were estimated fluorimetrically (Hissin and Hilf, 1976). 1 g leaf tissue ground in 1 ml 25% H₃PO₃
and 3 ml 0.1 M sodium phosphate–EDTA buffer (pH 8.0). The homogenate centrifuged for 20 min at 10,000 rpm and the supernatant used for the estimation of GSH and GSSG. The supernatant further diluted five times with sodium phosphate–EDTA buffer (pH 8.0). The final assay mixture (2.0 ml) contained 100 µl of the diluted supernatant, 1.8 ml phosphate–EDTA buffer and 100 µl O-phthalaldehyde (1 mg ml⁻¹). After thorough mixing and incubation at room temperature for 15 min, the solution read at 420 nm after excitation at 350 nm.

An aliquot of 0.5 ml of the supernatant incubated at room temperature with 200 µl 0.04 M N-ethylmaleimide for 30 min to interact with the GSH present in the supernatant. To this mixture, 4.3 ml 0.1 N NaOH was added. A 100 µl of this mixture taken for the measurement of GSSG, using the procedure outlined for GSH assay, except that 0.1 N NaOH used as the diluent rather than phosphate–EDTA buffer (Hissin and Hilf, 1976).

4.11.3. Extraction and assay of antioxidant enzymes

Extraction of enzymes carried out at 4°C. All the experimental samples (0.5 g) were ground and homogenized in 20 ml ice-cold extraction buffer (100 mm KH₂PO₄ or K₂HPO₄ (pH 7.8), 300 mg polyvinyl pyrrolidone (PVP), 1% (v/v) Triton X-100 and 5 mM ascorbate (Schwanz et al., 1996). The homogenate centrifuged at 25,000 rpm (20 min, 4°C). The supernatant used for the
determination of glutathione reductase (GR), monodehydro ascorbate reductase (MDHAR) and ascorbate peroxidase (APX). The protein content of all the enzymes estimated according to the method of Bradford (1976).

**Ascorbate peroxidase (APX)**

In ascorbate peroxidase assay, the reaction mixture consisted of 100 mM K-phosphate buffer (pH 7.0) (0.25 ml), 1 mM ascorbate (0.25 ml), 0.4 mM EDTA (0.25 ml), 0.19 ml distilled water, 10 mM H₂O₂ (0.01 ml) and enzyme extract (0.05 ml). The reaction started by adding H₂O₂ and the oxidation rate of ascorbate measured by the initial rate of decrease of absorbance at 290 nm. The molar absorption coefficient of ascorbate (2.8 mM cm⁻¹) used to calculate the activity of enzyme (Yanagida et al., 1999).

**Monodehydro ascorbate reductase (MDHAR)**

In MDHAR assay, the reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.0), 0.1 mM NADH, 4 mM ascorbate and 0.05 ml enzyme extract. The reaction started by adding NADH and the oxidation rate of ascorbate measured by the initial rate of decrease of absorbance at 340 nm. The molar absorption coefficient of NADH (6.2 Mm cm⁻¹) was used to calculate the enzyme activity (Song et al., 2005).

**Glutathione reductase (GR)**

In glutathione reductase assay, the reaction mixture consisted of 100
mM K-phosphate buffer (pH 7.8) (0.25 ml), 10 mM oxidized glutathione (GSSG) (0.05 ml), 0.48 ml distilled water, 1 mM NADPH (0.12 ml) and enzyme extract (0.1 ml). The assay started by addition of GSSG. GR activity was determined from the rate of NADPH oxidation measuring the absorbance decreased at 340 nm. GR activity was monitored by $A_{340}$ for NADPH oxidization as GSSG reduction according to Chen and Wang (2002).

4.12. Isolation and assay of Endonuclease

Endonuclease enzyme isolated and assayed by stop rate determin-ation method of Wang et al. (2008) with some modifications. 1 g finely chopped leaf tissue was homogenized in 10 ml extraction buffer containing 50 mM Tris HCl (pH 8) containing 1 mM MgCl$_2$ and 0.1% (w/v) BSA. The homogenate filtered and centrifuged at 10000 rpm for 10 min at 4°C. The filtrate collected in a fresh test tube and the volume was made to 10 ml using the extraction buffer. 1 ml of aliquot of the sample was assayed in 2 ml of λ phage DNA. A blank maintained by adding 2 ml distilled water without adding λ phage DNA. The initial absorbance was recorded at 260 nm and the mixture was incubated at 37°C for 30 min and the decreased absorbance at 260 nm after incubation was recorded. The difference in absorbance used to calculate the endonuclease activity.
4.13. Molecular

4.13.1. Random Amplification of Polymorphic DNA (RAPD)

4.13.1.1. Isolation and purification of DNA

Genomic DNA of fifteen sesame varieties collected from RARS, Kayamkulam was extracted and purified by CTAB method of Saghai-Maroof et al. (1984) with some modifications. DNA was isolated from the leaf samples of plantlets raised from seeds. The leaves of all the varieties were collected under chilled condition. Uniformity was maintained in age, size and position of the leaves during collection. Leaf samples were thoroughly washed with double distilled water and stored at -20°C. 1 g of tissue was taken, finely chopped and ground into a fine powder in a pre-chilled mortar using liquid nitrogen. The powdered tissue was made into a slurry using 9 ml of the extraction buffer containing 1M Tris-HCl (pH 7.5), 5M NaCl, 0.5M EDTA (pH 8), 1% CTAB and 1% β-mercapto ethanol pre heated at 65°C. The slurry was then incubated at 65°C for 90 min in a shaking water bath and precipitated with half volume of chloroform: isoamyl alcohol (24:1) by thorough vortexing for 5 min. The solution was centrifuged at 1500 x g for 10 min at room temperature (RT). The top aqueous layer was collected in a fresh centrifuge tube and again treated with chloroform: isoamyl alcohol (24:1), vortexed thoroughly for 5 min, centrifuged for 10 min at 1500 x g and the top aqueous layer was collected into a fresh tube. The DNA was precipitated out by adding equal volume of chilled isopropyl
alcohol with gentle shaking and centrifuged at 11000 x g for 5 min at 4°C. The supernatant was discarded and the DNA pellet was washed with 70% ethanol and dissolved in minimum volume of Tris-EDTA (TE) buffer (pH 8) in a sterilized eppendorff tube and stored at -20°C. The extracted DNA was purified for further use. 1μl of RNase A was added and incubated for 1 h at 37°C, followed by adding 5μl of Proteinase K and incubated for 30 min. Equal volume of equilibrated phenol was added and mixed thoroughly by vortexing. The mixture was centrifuged at 1100 x g for 5 min in a microfuge at RT. Top aqueous layer was collected and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and vortexed for 5 min. The solution was centrifuged at 11000 x g for 5 min and the top aqueous layer was collected in a fresh centrifuge tube. Equal volume of chloroform: iso amyl alcohol (24:1) was again added, vortexed for 5 min and subjected to centrifugation at 11000 x g for 5 min. The top aqueous layer was collected and 2.5 volume of chilled ethanol and 1/10th volume of 3 M sodium acetate were added to precipitate out the DNA. The mixture was kept for one hour at -20°C for maximum precipitation. Finally the mixture was centrifuged at 11000 x g for 5 min, decanted the supernatant and the DNA pellet was air dried for 15 min. The DNA pellet was washed with 70% alcohol, air-dried, dissolved in minimum volume of TE buffer and stored at -20°C for further use. An aliquot of the purified DNA was dissolved in 3 ml of TE
buffer and the absorbance was measured at 260 nm and 280 nm to check the purity of the DNA. The DNA content was determined using the standard value of DNA.

Fifteen Oligo nucleotide 10mer primers of Operon (OPD series, Operon Technologies, USA) were used for the random amplification of the genomic DNA.

Fifteen sesame varieties and the primer sequences used in RAPD analysis

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name of sesame varieties</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thilarani</td>
<td>P1</td>
<td>AACCGACGGG</td>
</tr>
<tr>
<td>2</td>
<td>KYM- 1</td>
<td>P2</td>
<td>GGGGGTGCGTT</td>
</tr>
<tr>
<td>3</td>
<td>Thilathara</td>
<td>P3</td>
<td>TGCCCTGCCT</td>
</tr>
<tr>
<td>4</td>
<td>Thilak</td>
<td>P4</td>
<td>CCAGACCTCTG</td>
</tr>
<tr>
<td>5</td>
<td>Soma</td>
<td>P5</td>
<td>AAGCTCCCCCG</td>
</tr>
<tr>
<td>6</td>
<td>Surya</td>
<td>P6</td>
<td>TACCACCCCCG</td>
</tr>
<tr>
<td>7</td>
<td>Rama</td>
<td>P7</td>
<td>GCCGGACTGT</td>
</tr>
<tr>
<td>8</td>
<td>CO-1</td>
<td>P8</td>
<td>GTCACTCCCC</td>
</tr>
<tr>
<td>9</td>
<td>TMV-6</td>
<td>P9</td>
<td>ACCGGGAAGG</td>
</tr>
<tr>
<td>10</td>
<td>TMV-7</td>
<td>P10</td>
<td>GAACCCAACC</td>
</tr>
<tr>
<td>11</td>
<td>PYR-1</td>
<td>P11</td>
<td>GTCGCCGTCA</td>
</tr>
<tr>
<td>12</td>
<td>PKDS-91</td>
<td>P12</td>
<td>TCTGGTGAGG</td>
</tr>
<tr>
<td>13</td>
<td>RT-54</td>
<td>P13</td>
<td>TGAGCGGACA</td>
</tr>
<tr>
<td>14</td>
<td>RM-94</td>
<td>P14</td>
<td>ACCTGAACGG</td>
</tr>
<tr>
<td>15</td>
<td>VRI-1</td>
<td>P15</td>
<td>TTGGCACGGG</td>
</tr>
</tbody>
</table>

4.13.1.2. Optimization assay for RAPD

Critical factors, which influence the optimization of the DNA amplification during PCR reaction, include the quality and quantity of the genomic DNA,
annealing temperature, concentrations of MgCl₂, dNTPs and Taq polymerase and the number of cycles during PCR amplification. Standardization of these factors is crucial for the elimination of defects associated with PCR amplification such as smear on running gel, nonspecific bands and false amplification. The reactions were carried out in a DNA thermocycler (MJ Research Inc. USA) using 20 µl reaction mixture. Reactions without DNA were used as negative control.

**Concentration of the components in 20 µl polymerase chain reaction mixture**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td>100ng</td>
</tr>
<tr>
<td>dNTP</td>
<td>100µM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2mM</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>2.5µl</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>2U</td>
</tr>
<tr>
<td>DNA</td>
<td>50ng</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>for 25µl mix</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>25µl</strong></td>
</tr>
</tbody>
</table>

**4.13.1.3. Agarose gel electrophoresis**

Agarose gel was casted in a Genei mini model horizontal gel apparatus by melting agarose in 1 X TAE buffer, pH 8.0 (100 mM Tris-acetate and 10 mM EDTA). Ethidium bromide (0.5 mg/ml) was added to the buffer after sufficient cooling, which fluoresces DNA. 1.5% agarose solution was poured to the
horizontal electrophoresis tank after positioning the comb, without entering any air bubble. The mould was allowed to solidify at room temperature. After 20 to 30 min, the comb was carefully removed. 1 X TAE buffer was poured into the tank and the level of the buffer was maintained up to 1 mm above the gel. 15 μl of each of reaction products was mixed with 3 μl of 6 X gel loading marker dye (0.25% bromophenol blue, 0.25% xylene cyanol and sucrose - 40% in distilled water), before loading. GeneRuler™ DNA ladder mix (MBI, Fermentas, USA) was used as the molecular weight marker. After loading the samples, the apparatus was connected to an electric field (40 V) of constant strength and direction. During the experiment, the negatively charged DNA was migrated towards anode at the neutral pH. The gel was allowed to electrophoresis, until the dye migrated up to more than half of the gel. Electric current was turned off and electrodes were removed. Gel was viewed in a UV-trans illuminator to detect DNA bands and photographed by electrophoresis documentation and analysis system (Kodak, Digital Science). Molecular weight was determined from the GeneRuler™ DNA ladder mix (Sambrook and Russel, 2001).

4.13.1.4. Data analysis

The polymorphic DNA bands that showed consistency in repeated experiments were screened according to their presence (‘1’) or absence (‘0’) in each of the genotypes. Percentage of genetic distance between the genotypes was estimated by the pair wise comparison method of Nie and Li (1979).
4.13.1.5. Cluster analysis

After calculation of all pair wise similarities between varieties, the relationships among them were expressed by performing cluster analysis using the software GENSTAT. It is then graphically represented as a dendrogram (Ford et al., 1997).

4.14. Oil content (%)

The oil content of each genotype was determined with the help of NMR (Nuclear Magnetic resonance spectrometer) and the varieties were grouped as low and high oil types (Daun, 2000).

4.15. Differential Display Reverse Transcription (DDRT)

Differential display reverse transcription has been employed to detect and characterize the altered gene expressions in S. orientale varieties - Thilarani and KYM-1 by assessing their mRNA profile. The basic principle involved was the systematic amplification of mRNAs, isolated from the plant samples, by means of RT-PCR and the distribution of their 3’ terminal on a polyacrylamide gel. The procedure involved the use of a set of oligo nucleotide primers, one being anchored to the poly adenylate tail of a subset of mRNA, the other being short arbitrary sequence, anneals at several positions relative to the first primer. The subpopulations of mRNA defined by these primer pairs were amplified after reverse transcription and resolved on a DNA sequencing gel (Liang and Pardee, 1992).
Isolation of total RNA

Total RNA from the plant tissues was isolated by Guanidium Thio Cyanate (GTC) method, following the procedure of Gauthier et al. (1997) with some modifications. Tender leaf samples of the two varieties were collected from *S. orientale* with utmost care to avoid contamination, washed with sterile, deionized diethylpyrocarbonate (DEPC) treated water and immediately stored at -20°C. All autoclavable materials needed for RNA isolation were autoclaved at 121°C / 15 lb for 20 min. All non-autoclavable materials were sterilized with 3% H₂O₂ for 6 h and rinsed with sterile DPEC treated water just before use. All stock solutions and buffers were prepared with DPEC treated RNase free water. Approximately 1 g of tissue was homogenized in a pre-chilled mortar, using liquid nitrogen, into a fine powder. 5 ml of solution D (containing 4 M Guanidium thio cyanate, 25 mM sodium citrate, 0.5% sarcosyl and 0.1M β-ME) was added and the fine powder was made into slurry. The slurry was then transferred to a clean, sterile 15 ml centrifuge tube. Sequentially, 1 ml of sodium acetate (2 M pH 4), 5 ml of citrate buffer saturated phenol (pH 4.3), 2 ml of chloroform: isoamyl alcohol (24:1) were added and vortexed for 5 min between each addition. The resulting mixture was then incubated on ice for 15 min and centrifuged at 10000 x g for 10 min at room temperature. The upper aqueous phase was carefully pipetted and transferred to a fresh tube. Two volumes of 95% ethanol was added, gently vortexed for 3 min and centrifuged again for 10
min at 10000 x g. The supernatant was decanted and the RNA pellet was washed in 70% ethanol. The supernatant was again decanted after centrifugation and the RNA pellet was air dried and dissolved in minimum volume of sterile water and stored at −70°C for further use.

**Formaldehyde agarose gel electrophoresis**

The quality and purity of isolated RNA was checked by formaldehyde agarose gel electrophoresis of the RNA sample. 20 ml of RNA sample mixture was prepared with 4.5 µl RNA, 10 µl of formamide, 3.5 µl of formaldehyde and 2 µl 10 X 3-N-morpholino propane sulphonic acid (MOPS) buffer. The mixture was heated at 65°C for 10 min followed by immediate cooling on ice for 2min. 1µl ethidium bromide (EtBr) and 2 µl RNA gel loading dye were added to the mixture, vortexed and loaded into the wells of formaldehyde agarose gel, containing 3 ml of 10 X MOPS buffer, 5.25 ml of formaldehyde, 300 mg agarose and 21.75 ml of sterile water and earlier casted on to the electrophoretic apparatus (Sambrook and Russel, 1989).

**Reverse Transcription**

First strand cDNA synthesis was done by RT-PCR, using oligo dT reverse primers (HT₁₁G, HT₁₁C, HT₁₁A; Genei, Bangalore). The 20 µl reaction mixture contained 5 µl RNA, 1µl oligo dT₁₁, 1 µl 10 mM dNTPs, 4 µl of 5 X RT buffer, 100 units of M-MuLV reverse transcriptase and 8.5 µl of sterile water. The reaction was carried out for 1 h at 37°C, after an initial heating at 70°C, in
the absence of RTase and dNTPs, for 1 min, followed by cooling on ice for 2
min (Sambrook and Russel, 1989).

**PCR amplification**

**Three reverse primers and nine forward primers employed for PCR
amplification of cDNA template**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>5’</td>
<td>AAGCTTTTTTTTTTTTA</td>
</tr>
<tr>
<td>D2</td>
<td>5’</td>
<td>AAGCTTTTTTTTTTTC</td>
</tr>
<tr>
<td>D3</td>
<td>5’</td>
<td>AAGCTTTTTTTTTTG</td>
</tr>
<tr>
<td>U1</td>
<td>5’</td>
<td>AAGCTTCATTCCG</td>
</tr>
<tr>
<td>U2</td>
<td>5’</td>
<td>AAGCTTCGGCATA</td>
</tr>
<tr>
<td>U3</td>
<td>5’</td>
<td>AAGCTTGAGCTT</td>
</tr>
<tr>
<td>U4</td>
<td>5’</td>
<td>AAGCTTCGACTGT</td>
</tr>
<tr>
<td>U5</td>
<td>5’</td>
<td>AAGCTTGATTGCC</td>
</tr>
<tr>
<td>U6</td>
<td>5’</td>
<td>AAGCTTCAATCGG</td>
</tr>
<tr>
<td>U7</td>
<td>5’</td>
<td>AAGCTTCGATCGT</td>
</tr>
<tr>
<td>U8</td>
<td>5’</td>
<td>AAGCTTCTCAGCA</td>
</tr>
<tr>
<td>U9</td>
<td>5’</td>
<td>AAGCTTATCGCCT</td>
</tr>
</tbody>
</table>

Using these primers, 27 different forward and reverse primer pair combinations
were utilized for carrying out the reactions.

<p>| | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>D1U1</td>
<td>D1U2</td>
<td>D1U3</td>
<td>D1U4</td>
<td>D1U5</td>
<td>D1U6</td>
<td>D1U7</td>
<td>D1U8</td>
<td>D1U9</td>
</tr>
<tr>
<td>D2U1</td>
<td>D2U2</td>
<td>D2U3</td>
<td>D2U4</td>
<td>D2U5</td>
<td>D2U6</td>
<td>D2U7</td>
<td>D2U8</td>
<td>D2U9</td>
</tr>
<tr>
<td>D3U1</td>
<td>D3U2</td>
<td>D3U3</td>
<td>D3U4</td>
<td>D3U5</td>
<td>D3U6</td>
<td>D3U7</td>
<td>D3U8</td>
<td>D3U9</td>
</tr>
</tbody>
</table>

The reactions were performed in a 20 µl volume, containing 1 µl of 25
mM MgCl₂, 10 X PCR buffer, 1.6 µl of 25 µM dNTPs, 2 µl of 2 µM each of
primer pair, 2 units of Taq Polymerase, 7.4 µl of H₂O and 2 µl of cDNA, and the
PCR thermal profile was as follows: initial denaturation at 94°C for 2 min, 40 cycles of 94°C for 30s, 42°C for 2 min, 72°C for 30s with a final extension at 72°C for 5 min. Amplification products were separated by polyacrylamide gel electrophoresis (Sambrook and Russel, 2001).

**Polyacrylamide gel electrophoresis of DDRT products**

25 ml of resolving gel, containing 6.65 ml of 30% acrylamide mix (acrylamide: bisacrylamide, 29:1), 5 ml of 5 X TBE buffer, 0.175 ml of 10% ammonium per sulphate (APS), 8.75 µl of TEMED and 13.175 ml of deionized H₂O, was prepared. TEMED added to the mixture just before pouring into the apparatus. A teflon comb of suitable size was immediately placed on top of the gel and the gel was allowed to solidify. Just before loading the samples, the comb was removed and the wells were carefully cleaned with double distilled H₂O, followed by running buffer (TAE buffer). 20 µl each of PCR products was loaded to the wells and the gel run at 150 V. When the running front reached the bottom line, the power supply cut off and the gel carefully transferred to deionize H₂O taken in a glass tray and subjected to silver staining for visualizing the bands (Sambrook and Russel, 2001).

**Silver staining**

The gel first immersed in 100 ml of 10% acetic acid, which was used as the fixing solution, and incubated for 15 min with gentle shaking. The fixing
solution removed from the tray and the gel rinsed with deionized H₂O thrice. The gel impregnated in 100 ml of silver nitrate solution (1mg/ml silver nitrate solution with 0.375 ml of 40% formaldehyde) for 20 min with gentle shaking and two more changes of solution were done. The gel rinsed with deionized H₂O and subjected to incubation in 100 ml of developing solution containing, 0.3 M Na₂CO₃, 100 μl of Na₂S₂O₃.5H₂O (from 10 mg/ml stock) and 0.375 ml of formaldehyde. Incubation was repeated twice and the gel was immersed in stopper solution of 10% acetic acid for 2 to 3 min, followed by rinsing in deionized H₂O (Sambrook and Russel, 1989) and photographed (Kodak Digital camera).

4.16. Plant extracts/microbe based fungicides

4.16.1. Preparation of plant extracts

The aqueous plant extracts prepared by the method of Swapnalatha and Kannabiran (2006) with slight modification. For this fresh leaves of Mikania scandens (L.) Willd., Senna alata (L.) Roxb., Lantana camara L., Vitex negundo L., Lawsonia inermis L., Chromolaena odorata (L.) R. M. King & H. Rob., Adhatoda vasica (L.) Nees. and Salvinia molesta D.Mitch., were collected separately. The leaves thoroughly washed in running water followed by rinsing with distilled water and blotted with filter paper, dried under shade over a period of 10 days. The powdered dried leaves (10 g) of the above plants distilled separately in 100 ml sterile distilled water at 100°C for 1 h using soxhlet
apparatus and allowed to stand for 24 h at 4°C. The crude mixture of each plant centrifuged at 4000 rpm for 10 min and the extract filtered through Whatman No.1 filter paper into a sterile filter flask and stored in sterile condition. Subsequently, each aqueous extract of the plants screened for its fungicidal potentiality against *Alternaria sesami* by agar disc diffusion method.

### 4.16.2. *In vitro* analysis (Disc diffusion method)

The antagonistic effects of the selected plant extracts against *A. sesami* evaluated by agar disc diffusion method. Molten PDA medium (20 ml) taken in sterile petriplates were allowed to solidify. The conidial suspension of *A. sesami* uniformly spread using sterile cotton swab on the petriplates. 50 μl of each plant extract at three different concentrations (10%, 7.5% and 5%) were added to each of the Whatman No.1 filter paper disc. The systems incubated for 24 h at 28 ± 1°C. The control was prepared using sterile distilled water and pathogen only. Three replicates maintained for each treatment. The zone of inhibition of each plant extract against pathogen was recorded in millimetres (Murray *et al*., 1995).

### 4.16.3. Biocontrol agents

*Pseudomonas fluorescens* and *Trichoderma harzianum* collected from Kerala Agricultural University, Vellayani, were screened for biocontrol agents against *A. sesami* by *in vitro* dual culture method.
4.16.3.1. *In vitro* study: (Dual culture method)

The antagonistic efficacy of *Pseudomonas fluorescens* and *Trichoderma harzianum* against *A. sesami* was analysed. To study the hyperparasitism, the pathogen and antagonist inoculated in PDA plates on diametrically opposite points. For testing antagonistic properties of *P. fluorescens* and *T. harzianum*, 5 mm discs of antagonist and *A. sesami* cut from the edge of 7 days old cultures were placed 6 cm apart on potato dextrose agar (PDA) plate. Three replicates maintained for each treatment and incubated at 28 ± 2°C for 48 h. Periodical observations on the growth of the antagonist to colonize the pathogen were recorded. Monoculture plates of biocontrol agents and *A. sesami* served as control. Seven days after incubation, radial growth of test fungus and *P. fluorescens* or *T. harzianum* isolates were measured. Colony diameter of test fungus in dual culture plate was recorded and compared with control. The growth inhibition calculated by using the formula: 100 X C - T / C, where C = growth in control and T = growth in treatment (Vincent, 1947).

4.16.4. *Mikania scandens* extract as fungicide

4.16.4.1. Isolation of essential oil from *Mikania scandens* leaves

150 g *M. scandens* leaves in 1000 ml sterile distilled water was hydrodistilled in a clevenger-type apparatus for 3 h. The oil layer obtained was dried over anhydrous Na$_2$SO$_4$, the solvent was removed by rotary flash
evaporator. After three experiments, the yields were averaged on the basis of the dry weight of the material. 2 μl of the oil was injected into the GC-MS and GC-FID spectrometer (Lubaina and Murugan, 2013).

**GC-MS**

Fractionation of the components of the essential oil were carried out using an Agilent 6890 Series II gas chromatograph (Palo Alto, U.S.A.) coupled to an Agilent 5973 quadrupole mass spectrometer with electron ionization mode (EI) generated at 70 eV (ion source at 230°C and transfer line at 280°C). The GC was performed using a JandW DB-5 (5% diphenyl- 95% dimethyl silicone) capillary column (30 m x 0.25 mm i.d. x 0.25 μm film), and helium was used as the carrier gas (1 mL min⁻¹). The initial temperature was programmed from 35°C to 60°C (at 1°C min⁻¹), to 170°C (3°C min⁻¹), to 200°C (8°C min⁻¹), and to 280°C (15°C min⁻¹), and maintained at 280°C for 5 min. The injector port (splitless mode, 0.5 min) was at 250°C. Retention indices calculated with reference to n-alkanes. All compounds identified by comparison of both the mass spectra (Wiley 275 library and NIST08) and the retention index data.

**GC-FID**

The qualitative analyses of essential oil from *M. scandens* leaves was carried out using an Agilent 5890 Series II gas chromatograph coupled to an Agilent 3396A integrator equipped with a HP-1 capillary column (12 m X 0.20
mm I.D., 0.33 μm film thickness). Hydrogen used as the carrier gas (1 ml min⁻¹). Conditions used for GC-FID were identical to those used for GC-MS.

4.16.4.2. Treatment of sesame with *Alternaria sesami* and *M. scandens* extract

Treatment experiments comprises the following. Initially, the seeds of sesame pre-treated with 7.5 % *M. scandens* leaf extract by dip method for a period of 2 h. Subsequently, 30 days old seedlings treated again with 7.5 % *M.scandens* aqueous leaf extracts thrice with an interval of 7 days. 7 days after the final application of *M. scandens* extract, the treated plants inoculated with 20 μl conidial suspension of *A. sesami* (1×10³ conidia ml⁻¹) prepared from 7 days old pure culture. Control was sprayed with distilled water and 20 μl conidial suspension of *A. sesami* only (no plant extract treatment).

4.16.4.3. Enzyme analysis

After inoculation with the pathogen, sesame leaves from experimental and control plants in pot conditions were harvested at different time intervals i.e., 24, 48, 72, 96 and 120 h to assess the induction of resistance in terms of peroxidase (Goliber, 1989 ; Ingham et al., 1998), polyphenol oxidase (Mayer et al.,1965), phenylalanine ammonia lyase (Morrison et al.,1994 ; Whetten and Sederoff, 1992), free and cell wall-bound phenolics (Haddadchi and Gerivani, 2009).
4.16.5. *Salvinia molesta* extract as plant based fungicide

The aquatic fern, *Salvinia molesta* collected fresh from the Botanical garden pond, University College and kept in plastic bucket containing tap water at the green house for 7 days. Chemicult, a commercially available hydroponic supplement containing a full range of macro and micronutrients added to the tap water. This was done to ensure vigorous growth of plants for the experiment.

4.16.5.1. Isolation and bioassay of cytokinin

30 g fresh weight (FW) plant samples were surface dried using paper towel and homogenized in 100 ml 80% ethanol for 24 h at 10°C. The extracts were further sonnicated and then filtered with Whatman No. 1 filter paper, rinsed further with 100 ml 80% ethanol and dried under vacuum at 35°C. The crude extract partially purified following the protocol of Dobrev and Kaminek (2002), where each sample redissolved in 3 ml 80% ethanol and passed through an activated Sep-Pak® Plus C18 (SPE) cartridge. The eluate collected and dried under vacuum. The samples were redissolved in 5 ml 1 M formic acid and loaded onto another activated Oasis® MCX (150 mg/6 cc) cartridge. Cytokinin eluted from the cartridge using 5 ml 0.35 M NH₄OH in water followed by 5 ml 0.35 M NH₄OH in 60% methanol. These two fractions mixed and dried. The extracts were resuspended in 2.5 ml 80% ethanol and strip loaded onto Whatman No. 1 chromatography paper and developed in a descending manner
in isopropanol:ammonia:water (10:1:1, v/v/v) until the solvent front had moved approximately 30 cm. After drying at 60°C for 24 h, the chromatograms were fractionated into 10 equal Rf zones and placed in individual flasks. These assayed for cytokinin using the soybean callus bioassay (Miller, 1965).

Miller’s solidified culture media (excluding the kinetin) added to the flasks containing the cut-up chromatograms. Three pieces of approximately 1 mg 3-4 week old soybean callus placed in each flask under aseptic conditions. The callus grew for 28 days in continuous 1 μmol m⁻² s⁻¹ light at 24°C after which the callus was weighed. Triplicate set of kinetin standards was included where kinetin added to the medium at 5, 10 and 50 μg l⁻¹ concentrations, as well as a control in which no kinetin added to the medium.

4.16.5.2. High-performance liquid chromatography (HPLC)

After 3 h of extraction, the homogenate was centrifuged (15000 x g, 4°C) and the pellets were reextracted. The combined supernatants were concentrated to approximately 1.0 ml under vacuum at 35°C, diluted to 20 ml with ammonium acetate buffer (40 mM, pH 6.5) and purified using a combined DEAE-Sephadex (1.0 cm × 5.0 cm)-octadecylsilica (0.5 cm × 1.5 cm) column and immunoaffinity chromatography based on generic cytokinin (Faiss et al., 1997). The methanolic eluate from immunoaffinity chromatography columns evaporated to dryness and dissolved in 75 μl of the mobile phase for HPLC analysis. Cytokinin O-glucosides and nucleotides hydrolyzed to free bases and
ribosides by action of α-glucosidase or alkaline phosphatase respectively before subsequent HPLC-MS analysis (Novak et al., 2003).

The samples were analysed by HPLC (Waters Alliance 2690) linked to a Micromass ZMD 2000 single quadrupole mass spectrometer equipped with an electrospray interface [LC(+)ES-MS] and photodiode array detector. Isopentenyladenine (iP) and ribotide used as internal standards. Using a post column split of 1:1, the effluent was introduced into an electrospray source (source block temperature 100°C, desolvation temperature 250°C, capillary voltage +3.0 V, cone voltage 20 V) and PDA (scanning range 210-300 nm; with 1.2 nm resolution) and quantitative analysis of the different cytokinins was performed in selective ion recording mode. Quantification performed by Masslynx software using a standard isotope dilution method. The ratio of endogenous cytokinin to appropriate labelled standard was determined and further used to quantify the level of endogenous compounds in the original extract, according to the known quantity of added internal standard (Novak et al., 2003).

4.16.5.2. Experimental protocols

Sesame seeds and mature plants were pre-treated with S. molesta extract (10%) containing cytokinin similar to M. scandens treatment followed by A. sesami inoculation. The control maintained with A. sesami inoculation and
distilled water treatment. The extract treated and untreated sesame leaves after inoculation with the pathogen were harvested at different time intervals for light microscopic as well as molecular analyses in terms of MAPK-4 signalling pathway by RT-PCR using gene specific primers of MAPK-4 and the internal control actin.

**Sample preparation for light microscopic analysis**

Seven days after the final application, the *S. molesta* extract treated and untreated control plants were inoculated with 20 μl conidial suspension of *A. sesami* by drop method. The inoculated plants were covered with plastic bags to maintain high humidity for rapid penetration of the pathogen. Inoculated plants were sampled at 6, 12, and 24 h post inoculation (hpi). Thin sections of the leaf samples were taken, stained with 1% cotton blue in lactophenol and were examined using fluorescent microscope (Garg *et al.*, 2010).

**Sample preparation for molecular studies**

The 30 days old sesame plants in pots were subjected to three kind of treatments. (i) plants were treated with *S. molesta* extract containing cytokinin alone (ii) plants were inoculated with 20 μl conidial suspension of *A. sesami* alone (iii) plants were treated initially with *S. molesta* extract containing cytokinin followed by 20 μl conidial suspension of *A. sesami*. Leaves were collected from plants of each treatment for RNA isolation at 6, 12 and 24 h post
inoculation (hpi) for RT-PCR analysis. RT-PCR analysis was performed with the help of One-Step RT-PCR (Qiagen, USA) using the gene specific primer of MAPK -4 and actin as internal control under the following PCR conditions: reverse transcription at 50°C for 30 min, initial PCR activation step at 95°C for 15 min followed by 35 cycles of amplification (94°C for 1 min, 59°C for 1 min and 72°C for 1 min) with final extension at 72°C for 10 min. After the completion of RT-PCR, the amplicons were analyzed by electrophoresing them in 1.8% agarose gel electrophoresis, followed by quantification using the spot densitometry tool of Alpha Imager software.

The sequence of primers used for RT-PCR analysis

<table>
<thead>
<tr>
<th>Name of gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>Expected product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPK-4</td>
<td>GCTCTAACCAACCCCTAAGT</td>
<td>GTAACCACGCGTGAACACGTA</td>
<td>228</td>
</tr>
<tr>
<td>Actin</td>
<td>ATTCTCACCAAGTATCC</td>
<td>CATGATCTGAGTCATCTTTCT</td>
<td>200</td>
</tr>
</tbody>
</table>

Pathogenesis related proteins (PRP)

Total protein from S. molesta treated sesame plants followed by A. sesami inoculated and sesame + A. sesami inoculated were isolated and subjected to SDS-PAGE to know the band pattern of PR proteins as per the methodology described by Laemmli (1970).

4.16.5.3. SDS–PAGE

The reagents (a) Acrylamide: bisacrylamide (29:1) (1.665 ml); (b) 1.5 M Tris - HCl (1.25 ml); (c) 1% ammonium persulphate (0.5 ml); (d) 10% SDS (0.05
ml) and distilled water (1.85 ml) were taken to prepare a 5 ml separating gel mixture in SDS gel system. 5 µl of Tetra methyl ethylene diamine (TEMED) was added to the mixture, stirred well and any air trapped was removed in vacuo, just before casting the gel. 5 ml of the mixture needed to cast a slab gel of 80×70 mm size and 1 mm in thickness. The electrophoretic tank and plates rinsed thoroughly with distilled water. The teflon spacers were kept in position between the two plates and fixed closely to the electrophoretic tanks by tightening the screws of the electrophoretic apparatus. The separating gel cast within the sandwiched glass plates leaving sufficient space for the stacking gel. The separating gel layered on top with distilled water to get an even surface and kept overnight. In the next day, the surface washed carefully with distilled water to cast the stacking gel.

The surface of the separating gel was cleaned and any droplet of water trapped, was removed. The polymerization mixture was prepared with the following constituents. (a) Acrylamide-bisacrylamide (29:1) (0.75 ml); (b) 0.5 M Tris-HCl, pH 6.8 (1.25 ml); (c) 1% Ammonium persulphate (0.5 ml) (d) 10% SDS (0.05 ml) with the addition of 2.45 ml distilled water and 5 µl TEMED to get a gel strength of 4%. In slab gel system, after the stacking gel was poured over the separating gel, a comb made up of teflon was inserted to form wells into which samples were loaded. The comb was removed after 2 h, followed by cleaning the wells with electrode buffer using a syringe and a long blunt needle.
Preparation of samples

The purified protein samples were homogenized in a solubilising buffer in the following constituents. (a) 10% Glycerol-1.0 ml; (b) 5% Mercaptoethanol-0.5 ml; (c) 2.3% SDS-2.3 ml; (d) 0.5 M Tris-HCl, pH 6.8-1.25 ml; (e) Bromophenol blue solution 0.1 ml; (f) Distilled water made up to 10 ml. The amount of buffer taken was adjusted to contain an excess of SDS, at least 40 times the amount of protein in the sample. The sample digested in a boiling water bath for 2-3 min and cooled. The wells in stacking gel were loaded with equal quantities of digested protein. The electric power supply adjusted to 60 V. When the marker dye entered the separating gel from the stacking gel, the potential difference across the plates increased to 120 V. Electrophoresis stopped when the dye front reached the anodic end of the gel, which usually needed 90-120 min. Marker protein of known molecular weight was also loaded along with sample proteins separately.

Coomassie brilliant blue staining

The gel stained with coomassie brilliant blue having the following composition, to locate the proteins. (a) Coomassie blue powder-50 mg; (b) Acetic acid-10 ml; (c) Isopropanol-25 ml and (d) Distilled water-65 ml. The gel kept in the stain overnight and destained with a solution of 10% acetic acid and 25% methanol until the background became clear. The gels washed in distilled water several times and photographed immediately.
4.16.6. *Pseudomonas fluorescens* as biocontrol agent

4.16.6.1. Treatment of sesame with *Pseudomonas fluorescens* and *Alternaria sesami*

Sesame seeds and thirty days old sesame plants in pots were pre-treated with 2% talc based formulation of *Pseudomonas fluorescens* containing an average of 2.5 to $3 \times 10^8$ cfu/g bacterial cells and the treatment was repeated up to three times with an interval of seven days. One week after the final application of biocontrol agent, the treated plants inoculated with 20 µl of *A. sesami* conidial suspension ($1 \times 10^3$ conidia ml$^{-1}$) prepared from 7 days old pure culture. Control sprayed with distilled water followed by 20 µl conidial suspension of *A. sesami*.

4.16.6.2. Enzyme analysis

Sesame leaves pre-treated with Pfl followed by pathogen inoculation and untreated control plants under pot conditions were harvested from 24, 48, 72, 96 and 120 h to assess the induction of resistance in terms of peroxidase (Goliber, 1989; Ingham *et al.*, 1998) and polyphenol oxidase (Mayer *et al.*, 1965). Activity of fungal cell wall degrading enzymes such as chitinase and β-1,3-glucanase were also evaluated.

**Assay of β-1, 3 glucanase**

1 g leaf tissue was extracted in 5 ml of 0.05 M sodium acetate buffer (pH 5.0). The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C and the
supernatant was used as enzyme source. β-1, 3-glucanase activity was assayed by the laminarin dinitrosalicylic acid method (Pan et al., 1991). The reaction mixture consisted of 62.5 μl of 4% laminarin (Sigma) and 62.5 μl of enzyme extract. The reaction was carried out at 40°C for 10 min. The reaction was stopped by adding 375 μl of dinitrosalicylic acid and heated for 5 min in boiling water, vortexed and the absorbance was measured at 500 nm.

**Assay of Chitinase**

**Preparation of Colloidal Chitin**

5 g of crab shell chitin (Sigma) was slowly added into 100 ml of cold 0.25 N HCl with vigorous stirring and kept overnight at 4°C. The mixture filtered through glass wool into 200 ml icecold ethanol at 4°C with rapid stirring. The chitin suspension was centrifuged at 10,000 rpm for 20 min and the resultant chitin pellet was washed repeatedly with distilled water until the pH became neutral (Roberts and Selitrennikoff, 1988). The concentration adjusted to 10 mg/ml.

Chitinase activity of biocontrol treated and control leaves were assayed according to the procedure developed by Boller and Mauch (1988). The reaction mixture consisted of 10 μl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin (10 mg/ml). After incubation for 2 h at 37°C, the reaction was stopped by centrifugation at 1000 x g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a reagent tube
containing 30 µl of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3% (w/v) snail gut enzyme (Helicase) for 1 h. After 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 µl 1 M sodium borate buffer (pH 9.8). The mixture incubated in a boiling water bath for 3 min and then rapidly cooled in an ice bath. After addition of 2 ml of dimethyl aminobenzaldehyde (DMAB), the mixture again incubated for 20 min at 37°C. Immediately there after the O.D measured at 585 nm. N-acetylglicosamine used as the standard. The enzyme activity expressed as µmol of GlcNAc equivalents/min/ml of bacterial culture.

**4.16.6.3. Native PAGE**

Electrophoretic separation of isozymes of Peroxidase, Polyphenol oxidase, Chitinase and β-1, 3-glucanase were carried out as per the protocol of Laemmli (1970) with some modifications. For native polyacrylamide gel electrophoresis (PAGE), a resolving gel of 8% polyacrylamide and stacking gel of 4% concentration were prepared. Samples (50 µg protein) were loaded on gel and subjected to electrophoresis. After electrophoresis, the gel separated from the plate. In the case of peroxidase, the methodology was adapted as per the protocol of Ros Barcelo described at section 4.10. Polyphenol oxidase isozyme was analysed as per the methodology of Jayaraman et al. (1987) described at 4.10.
Chitinase activity was detected by PAGE according to the method of Trudel and Asselin (1989). After native electrophoresis, gels were incubated in 150 mM sodium acetate buffer at pH 5.0 for 5 min, and then in 100 mM sodium acetate buffer at pH 5.0 containing 0.01% (w/v) glycol chitin for 30 min at 37°C. The gels were finally transferred into a solution containing 0.01% (w/v) Calcofluor white M2R (Sigma) in 500 mM Tris HCl (pH 8.9). After 5 min the brighter solution was removed and the gels were rinsed with distilled water for 1 h. Lytic zones were visualized and photographed under UV light in an alpha imager. The isozyme expression pattern of β-1, 3-glucanase activity was analyzed by the method of Grenier and Asselin (1993). The gels were incubated in 50 mM sodium acetate buffer, pH 5.0 for 3 h at 37°C. β -1, 3-glucanase activities on gels were revealed by staining the gels for 15 min with 0.025% (w/v) Aniline blue fluorochrome in 150 mM K₂HPO₄, pH 8.6 and visualized under long wave UV (365 nm) light and photographed.

4.16.7. Disease assessment by pot and field study

The disease assessment parameters namely the disease incidence (number of spots per leaf) and disease severity (% of surface infected area) were recorded after M. scandinus, S. Molesta, P. fluorescens and a synthetic fungicide Mancozeb (0.1%) pre-treatments on sesame seeds followed by mature plants and subsequently inoculated with A. sesami in pot and field conditions during the growing seasons of 2012 and 2013 and each trial replicated thrice.
4.16.7.1. Pot study

Sesame seeds and one month old sesame plants in pots were treated separately with 7.5% aqueous leaf extract of *Mikania scandens*, 10% aqueous plant extract of *Salvinia molesta*, 2% talc based formulation of *Pseudomonas fluorescens* and 0.1% Mancozeb using a hand sprayer and the treatment was repeated up to three times at one week interval. Seven days after the final treatment, the plants were inoculated with 20 μl conidial suspension of *A. sesami* by drop method. Control plants sprayed with 20 μl conidial suspension of *A. sesami* and distilled water. The experimental plants, along with their respective controls were covered with polyethylene bags for maintaining high humidity to ensure successful penetration of the pathogen into the host tissue. The disease severity was recorded one week after inoculation with the pathogen following 0-5 scale of Shrestha *et al.* (2005) with some modifications where 0= no infection, 1= up to 5% area covered by the disease, 2= 6-10% area covered, 3= 11-20% area covered, 4= 21-30% area covered, 5= 31-100% area covered.

4.16.7.2. Field trials

Similar to pot culture, field trials in plots of 2.0 m x 2.0 m (4 m²) and separated by a clean space of 0.4 m² in a Randomized Complete Block Design (RCBD) were also conducted on growing seasons of 2012 and 2013 and replicated three times in a field at Vembayam, Trivandrum, Kerala. Plant
extracts or biocontrol pre-treated sesame seeds (20 g) mixed with 100 g sandy soil were sown uniformly in the plots. The 4 weeks old sesame plants after sowing were treated with 7.5% aqueous leaf extract of *Mikania scandens*, 10% aqueous plant extract of *Salvinia molesta*, 2% talc based formulation of *Pseudomonas fluorescens* and 0.1% Mancozep separately and the treatment was repeated at one week interval until 6 WAS (weeks after sowing) using a hand held garden sprayer. Subsequently, after the final application of plant extracts, biocontrol agent, the plants sprayed with spore suspension of *A. sesami*. Plants sprayed with sterilized water followed by conidial suspension of *A. sesami* served as control. Disease assessment done one week after pathogen spray from two permanent randomly placed quadrants (50 cm x 100 cm) per plot. The total number of plants and number of infected in a quadrant were counted and the percentage of disease incidence was calculated. Disease severity recorded according to percentage of area covered following 0-5 scale of Shrestha *et al.* (2005) by assessing 10 randomly tagged plants per plot after stipulated treatments. The following formula used in determining the severity of infections.

\[
\text{Disease severity} = \frac{\text{Sum of all scores}}{\text{Number of plants scored (N)}} \times \frac{100}{\text{Highest score (5)}}
\]

Where N is the total number of assessments and 5 the maximum score on the scale.
4.17. Statistical analysis

a) Inoculation trials

ANOVA in CRD was done with two factors namely I and D. The 1st factor I has two levels, I₀ (control) and I₁ (infected), since the observations were recorded from 1st to 13th day (D) after infection for alternate days. Thus D has seven levels which were denoted as D₁, D₂, D₃, ...D₇ (Gomez and Gomez, 1984).

b) Treatment trials

ANOVA in CRD was done with two factors namely T and H. The 1st factor T has two levels, T₀ (control) and T₁ (treated), since the observations were recorded from 24th to 120th hour (h) after infection. Thus, H has five levels, which were denoted as H₁, H₂, ..H₅ (Gomez and Gomez, 1984).

In both inoculation and treatment trials, after confirming the significance of F values, the significance of the differences between the mean values of 5 replications were tested using ANOVA. Significant differences were considered at P < 0.01 probability levels.

c) Assessment trials

For disease assessment, ANOVA in CRD with 6 replications were done to compare the effectiveness of the treatments (Snedecor and Cochran, 1968).