Influence of root-knot nematode infestation on antioxidant enzymes, chlorophyll content and growth in *Pogostemon cablin* (Blanco) Benth.

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Plants adapt themselves to overcome adverse environmental conditions, and this involves a plethora of concurrent cellular activities. Physiological experiments or metabolic profiling can quantify this response. Among several diseases of *Pogostemon cablin* (Blanco) Benth. (Patchouli), root-knot nematode infection caused by *Meloidogyne incognita* (Kofoid and White) Chitwood causes severe damage to the plant and hence, the oil production. In the present study, we identified *M. incognita* morphologically and at molecular level using sequenced characterized amplified region marker (SCAR). *M. incognita* was artificially inoculated at different levels of second stage juveniles (J₂) to examine the effect on Patchouli plant growth parameters. Peroxidase and polyphenol oxidase enzyme activity and changes in the total phenol and chlorophyll contents in *M. incognita* was also evaluated in response to infection. The results have demonstrated that nematode infestation leads to increased peroxidase activities in the leaves of the patchouli plants and thereby, increase in phenolic content as a means of defence against nematode infestation. Chlorophyll content was also found decreased but no changes in polyphenol oxidase enzyme activity.

**Keywords:** Chitwood, GAE, Nematode infestation, Patchouli plant, Peroxidases (EC. 1.11.1.7), Polyphenol oxidase (EC 1.14.18.1), Phenols, SCAR.

**Pogostemon cablin** (Blanco) Benth. (Syn. *P. patchouli*) (Lamiaceae) commonly known, as Patchouli is a patchouli-oil producing plant, and it is mainly distributed and cultivated in Indonesia, Malaysia, India and China¹. The essential oils from Patchouli are used as a fixative for heavy perfumes and soaps and it also possess several therapeutic properties, such as anti-depressant, anti-inflammatory, antiseptic, aphrodisiac, astringent, carminatives, diuretic, febrifuge, insecticides, fungicides sedative and tonic²,³.

Patchouli plants suffers greatly from various diseases like leaf blight, wilt, viral infection and nematode infestation but among these, root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood], is the most important and resulting in a considerable loss in oil yield⁴. Nematode infestation is considered one of the severe challenges facing the expansion of patchouli plantations. Root-knot nematodes are considered to be one of the most economically important and complex groups of obligate, sedentary endoparasitic nematodes with wide host range distributed worldwide and parasitize thousands of higher plant species including monocotyledons, dicotyledons, herbaceous and woody plants⁵,⁶. The second stage juveniles (J₂) penetrate the roots and migrate to the vascular cylinder, stimulate metabolic pathways in the root, and enhance nutrient transport towards the induced root gall⁷,⁸. The galled roots are unable to take up water and nutrients resulting in stunting of the plant and the severely affected plants often wilt readily⁹.

Plants produce a high diversity of natural products with a prominent function in the protection against predators and pathogens. These mechanisms include pre-existing physical and chemical barriers, as well as inducible defence response in the form of induction of defence-related enzymes that become activated upon pathogen infection¹⁰. In parasitic nematodes, they are particularly crucial for digestion of host tissues and evasion of host immune responses¹¹. Pathogen infection and various abiotic stresses lead to overproduction of reactive oxygen species (ROS) such as superoxide anion (O₂⁻), singlet oxygen (¹O₂), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH)
are produced continuously as by products of various metabolic pathways are highly reactive and toxic. They cause damage to proteins, lipids, carbohydrates and DNA resulting in oxidative stress. In order to limit oxidative damage under stress, plants have developed a series of detoxification systems that break down the highly toxic ROS. The important enzymes involved in removing ROS in plant cells are SOD, catalases and peroxidases. Peroxidase comprises one important class of pathogenesis related (PR) proteins (PR-9) implicated in “defence responses,” in which an important role is to catalyze the formation of phenolic radicals at the expense of H$_2$O$_2$. Phenolic compounds in plants play a vital role in their defence system, particularly redox response and free radical scavenging. Further, accumulation of phenols at the site of infection is characteristic in plant defence response, and causes rapid cell death and prevents penetration of pathogens. Increase in phenolic compounds due to M. incognita infection has been reported in several studies. Chlorophyll is an important biomolecule which led to photosynthesis, and allows plants to absorb energy from light. Nematode infection reduces chlorophyll content and ultimately the carbohydrate supply to the nodule resulting lower nitrogen fixation.

Methods to enable timely detection and accurate identification of economically important plant parasitic nematodes species, particularly root-knot nematodes are crucial before proper research on nematode resistance and management studies can be conducted. The extensive morphological variation among and within root-knot nematode (RKN) species complicates their proper identification. The present investigation is based on incidence of root-knot nematode infection caused by M. incognita in Patchouli. Study involves morphological identification of nematodes using PCR based M. incognita specific SCAR markers. We also determined changes in the phenol, chlorophyll, peroxidase and polyphenol oxidase enzyme in the leaves of Patchouli after infection with M. incognita, as these parameters could be the contributing factors for the possible defence mechanism in Patchouli.

Materials and Methods

Nematode isolation and identification

Isolation was also done from 10-15 gm infected Patchouli roots. Roots of infected patchouli plants from CSIR-NEIST campus were collected and washed with running tap water. The roots were then chopped into small pieces placed into a closed container containing sodium hypochloride (NaOCl) and shaken vigorously for 2 min. The mixture was again passed through mesh sieves of different sizes (400, 300, 250, 150 and 60 µm) and eggs were collected in the final sieve and incubated in distilled water at 25°C for 2 to 3 days. The freshly hatched J$_2$ larvae were used for further experiments.

Identification of M. incognita was done by morphological observations based on J$_2$, male stylet knobs; features of the female perineal pattern and excretory pore position/stylet length ratio matched typical traits of M. incognita. Female perineal pattern was observed by dipping infected patchouli roots in 0.9% NaCl to prevent female bursting then excised the females in the neck region and removed the body content. Posterior body with perineal pattern was removed, stained with acid fuchin-lactophenol covered with cover glass slide and observed under phase contrast microscope.

Identification using scar (sequence characterized amplified region) marker

Nematode (M. incognita) DNA was extracted according to the method described by Cein. Single egg mass of M. incognita was collected from galls of infected Patchouli roots and inoculated in the roots of tomato plant. After 5 months of infection several hundreds of J$_2$ stage M. incognita were collected from roots of tomato plants and DNA isolated. Isolated DNA was stored in 4°C for further use. The isolated DNA was subjected to SCAR primer amplification as reported by Zilstra et al. PCR amplification was done using M. incognita specific Finc and Rinc primer which generates 1200 bp fragment. PCR conditions were 95°C for 2 min followed by 45 cycle of 94°C for 30 s, 54°C for 1 min, 72°C for 2 min and final extension of 72°C for 7 min. Another SCAR set of primer Fjav and Rjav were used as false positive control taking same PCR conditions as Finc and Rinc except for annealing temperature at 61°C.

Pathogenicity test

Cuttings of Patchouli were planted in polybags. After 3 months, plantlets were transplanted in earthen pots containing 2 kg of steam sterilized soil. After one wk of adaptation, seedlings were inoculated with 500, 1000 and 1500 J$_2$ stage J$_2$ larvae of M. incognita per pot respectively, by drenching with water containing inoculums. Each treatment has got 5 replicates and was repeated twice. After 3, 5 and
7 months, plants were uprooted gently from pots and observations were taken. The measurement was performed on root length, root weight, shoot length, shoot weight, number of galls, number of egg masses, number of larvae and females per root system.

**Total chlorophyll content determination**

Fresh leaves (1 g) was cut and homogenized in 10 mL of double distilled water. An aliquot (0.5 mL) was taken and extracted with 4.5 mL of chilled 80% acetone. Supernatant was collected by centrifugation and absorbance was measured at wavelength 645 nm and 663 nm. Acetone (80%) was used as blank. Total chlorophyll content was calculated using the following formula:

\[
\text{Total chlorophyll (mg/liter)} = 0.0202 \times \text{OD}_{645 \text{ nm}} + 0.00802 \times \text{OD}_{663 \text{ nm}}
\]

**Total phenol contents determination**

The total phenolic content was determined using the method of Macdonald *et al.* with minor modifications. Plant samples were extracted with ethanol and dried the samples using rotary evaporator. An amount of 1 mL of ethanolic extract of concentration 1 mg/mL was mixed with 5 mL of 0.2N Folin-ciocalteau and 4 mL of 75 g/L Na$_2$CO$_3$. For blank, distilled water was used except the plant extract. The absorbance was measured at 765 nm against the blank. Standard graph was prepared using gallic acid as standard. The total content of phenolic compounds in the extract in gallic acid equivalents (GAE) was calculated by the following formula:

\[
T = C \cdot V / M
\]

where \(T\) = Total phenolic contents in microgram per milligram plant extract, in GAE; \(C\) = Concentration of gallic acid (from standard graph) in microgram per milliliter; \(V\) = Volume of the extract in milliliter; and \(M\) = Weight of ethanolic extract in milligram.

**Peroxidase activity assay (EC 1.11.1.7)**

*Plant material collection and enzyme extraction*

Leaves of patchouli both infected and normal plants were collected from CSIR-NEIST campus in cooling bags and stored in deep freeze (−80°C) until use. Homogenize the sample in ice-cold 0.1 M phosphate buffer, pH 6.0 (1:10, w/v) in a chilled mortar and pestle using white sand as abrasive. The extract were passed through cheese cloth and centrifuged at 16000 g for 20 min at 4°C and the supernatant was used as crude enzyme extract.

**Protein estimation**

Protein estimation was done according Lowry using Folin-ciocalteau reagent. Standard graph was prepared by using BSA as standard. Absorbance was taken at 660 nm and plotted the standard graph. Bovine serum albumin was used as standard.

**Peroxidase activity assay**

Peroxidase test was performed to check the activity of peroxidase in control and nematode infested plants. Peroxidase activity was carried out according to the method described by Summer and Gjessing with some modification. An amount of 1 mL of o-dianisidine (0.01 M), 0.5 mL of 20 mM H$_2$O$_2$, 1 mL of phosphate buffer (0.1 M, pH 6.0) and 2.4 mL of distilled water were mixed in a test tube. Blank was prepared accordingly but instead of H$_2$O$_2$ equal volume of distilled water was added. Incubated the reaction mixture for 30°C and start the reaction by adding 0.2 mL of crude enzyme extracts. The activity was expressed as changes in absorbance at 430 nm min$^{-1}$ mg$^{-1}$ proteins.

**Polyphenol oxidase activity assay (EC 1.14.18.1)**

*Enzyme extraction and protein estimation*

Plant samples (10 g) were cut and soaked in 10 g/L chilled sodium sulphite solution and then washed with distilled water. The washed samples were then homogenized in 20 mL of phosphate buffer for 3 min and squeezed through cheese cloth. The extract was then centrifuged at 10000 g for 10 min at 4°C. The supernatant containing the crude enzyme extract was used for enzyme assay. Protein content was estimated by Lowry method.

**Polyphenol oxidase activity assay**

The activity of polyphenol oxidase was determined based on the methods of Vamos-Vigyazo and Nadudvari-Marlcus with slight modification. 1 mL of 0.05 M catechol was prepared and 1 mL of 0.1 M phosphate buffer (pH 7.0) solution was added. To this, 3 mL distilled water was added and the enzyme reaction started by introducing 0.5 mL of crude enzyme extract of polyphenol oxidase. The change in absorbance was measured spectrophotometrically at wavelength 540 nm at a regular interval of 30 s. The activity was expressed as changes in absorbance at 540 nm min$^{-1}$ mg$^{-1}$ protein.

**Results**

**Nematode identification**

Nematode infected patchouli plants in fields showed severe yellowing, decline and heavily
damaged roots (Fig. 1a). Males of *M. incognita* appeared vermiform with wide head cap and concave labial disc with blunt stylet tip and rounded stylet knobs as well as cylindrical shaft (Fig. 1b). The perineal patterns of female *M. incognita* showed a high dorsal arch with smooth to wavy striae. Lateral line was not distinct. Striae were found bend towards the vulva (Fig. 1c). Amplification reactions with SCAR primers were visualized on 1% agarose gel. PCR with the *M. incognita* specific primer set (Finc/Rinc) resulted in 1200 bp amplified fragment in PCR but no fragments were observed when amplified with Fjav/Rjav (Fig. 2).

**Pathogenicity test**

*Effects of Meloidogyne incognita in plant growth*

The influences of 3 different concentrations of nematode inoculums on shoot and root length as well as on fresh weight of *P. cablin* plants are shown in (Fig. 3). The two inoculums (1000 and 1500 J₂ larvae per pot) significantly decreased the root length as compared to control plant with the lowest recorded after 7 months at 1500 J₂ inoculation level with 8.27±0.9 cm. Root weight did not change significantly in all the inoculums level after 3 months. But after 5 months (5.04±0.96 g at 1000 J₂ and 5.4±1.8 g at 1500 J₂) and 7 months (5.55±1.64 g at 1000 J₂ and 5.65±1.23 g at 1500 J₂) the root weight increased significantly compared to control plant (3.46±0.9 g and 4.72±1.17 g after 5 months and 7 months of inoculation). Shoot length increased gradually from 3rd and 7th months at their respective inoculation level but significant decrease was observed for 1500 J₂ inoculation level after 5th and 7th months as compared to control plant. In case of shoot weight, it decreased significantly in all the treatment compared to control plant though there was no significant change observed among all the 3 inoculum levels with the exception at 500 J₂ inoculums level after 5th month.

*Reproduction rate of M. incognita in patchouli roots*

The number of galls per plant and the number of females per gall increased significantly with the increase of inoculums concentration. It was observed

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Fig. 1— (a) Patchouli root infected with *Meloidogyne incognita* and arrow indicating the galls on root; (b) Adult male of *M. incognita*. Arrows indicating the stylet knobs, cylindrical shaft, concave labial disc and stylet tip; and (c) Perineal pattern of female *M. incognita* with arrows indicating the characteristic high dorsal arch, vulva and bent striae.

Fig. 2— SCAR primer amplification of *M. incognita* using Finc/Rinc.

Fig. 3— Effects on patchouli growth parameters. (a) Root length (cm); (b) Root weight (g); (c) Shoot weight (g); and (d) Shoot length (cm). [Data within each parameter sharing the same letter are not significantly different from each other at *P* ≥0.05 level according to Least Significant difference test].
that increase in number of galls/plant is directly proportional to the increase in time duration. No significant difference was found in case of number of egg mass per gall and number of larvae per gall in all the inoculation level after 3rd month but from 5th (85.40 in 500 J2 inoculation level) to 7th (685.56 in 1500 J2 inoculation level) months it increased gradually with the increase in inoculation level (Table 1).

Changes in chlorophyll content and antioxidant enzymes

Total chlorophyll content decreased gradually with the increase in inoculums level and inoculums period with lowest recorded 7.74 mg/L after 7 month at 1500 J2 inoculums level. Control plants showed normal increase in total chlorophyll content starting from 14.64 to 20.53 mg/L (Fig 4a). Peroxidase activity levels increased significantly in the leaves of Patchouli plants after infestation with *M. incognita* up to 5 months as compared to control plants. However, no significant change was observed in the 7th month. The highest change in absorbance due to peroxidase activity (0.398) was recorded in the 5 month old plants at 1500 J2 inoculums level (Fig. 4c). Similar results were noted for phenolic compounds with highest value of 31.43 GAE µg/mg protein after 5 months at 1500 J2 inoculums level but in control plant it increased gradually from 23.10 to 25.60 GAE µg per mg protein (Fig. 4b). Polyphenol oxidase enzyme did not show any significant change in any of the inoculums level and time periods. It was recorded between 0.072 to 0.092 changes in absorbance m-1 mg-1 protein (Fig. 4d).

**Discussion**

Morphological characteristics of root-knot nematode female, male and juvenile have been studied extensively and are still widely used to support species identification. The perineal pattern is one of the most characteristic morphological features of the genus *Meloidogyne* and can be used to distinguish species of *Meloidogyne sp*. In the present investigation, the isolated nematodes showed similar characteristics, such as structure of male head and female perineal pattern as previously described by Eisanbach et al. with significant differences with other *Meloidogyne* species. But due to wide intra-specific variability in *meloidogyne* these characters are become subjective and can lead to doubts in species identification. Hence, more species specific SCAR primers can be used successfully in straightforward, fast and reliable PCR assays to identify *M. incognita*, *M. javanica* and *M. arenaria* according to Zilstra et al. who found a similar 1200 bp fragments when amplified with Finc/Rinc SCAR primer. The SCAR based PCR method used in our study again proved the reliability of this molecular tool for authentic identification of the RKN. Our results also showed a 1200 bp product in accordance with the findings of Foure et al.

<table>
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<th>Inoculation Level (J2 larvae/plant)</th>
<th>No of galls/plant</th>
<th>No of egg masses/gall</th>
<th>No. of larvae/gall</th>
<th>No. of females/gall</th>
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<td>7</td>
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<td>40.12c</td>
<td>62.20c</td>
<td>10.50a</td>
</tr>
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Fig. 4—Effects of *M. incognita* on (a) Total chlorophyll contents; (b) Total phenolic compounds; (c) Peroxidase enzyme activity; and (d) Polyphenol oxidase enzyme activity. [Data within each inoculation level sharing the same letter are not significantly different from each other at P ≥0.05 level according to Least Significant difference test].
The present study showed decreased overall growth of patchouli evaluated under green house conditions. Previous studies viz., El-Sherif et al.\textsuperscript{37} and Chandra et al.\textsuperscript{38} have also shown that \textit{M. incognita} infection causes decrease in different growth parameters like root length, root weight, shoot length, shoot weight, in different plants. Our findings also confirms the decreased growth parameters and number of gall formation directly proportional to the increasing inoculum level as earlier stated by Khan et al.\textsuperscript{39}. However, root and shoot weight showed gradual increase up to 5 months and after that it fell lower than the control plant. These findings are in agreement with the findings of Lynd and Ansuman\textsuperscript{40} and this may due to the formation of giant cells in the roots and lower part of the shoot regions. In the present study, we confined the inoculation level below 1500 J/plant as the previous study by Kesba\textsuperscript{41} revealed that the density of \textit{M. incognita} decrease after attaining a certain level of inoculation level.

Compounds that are able to reduce the damaging effects of certain stresses may be of great importance from both theoretical and practical points of view. Pathogen-mediated defence responses involve physico-chemical processes, such as cell-wall modifications, and biochemical responses, such as the generation of reactive oxygen species. Such defence responses are of high energetic cost, and are based on modifications of the physiology and metabolism of infected plants both locally and systemically. Previously, many reports have shown that the peroxidase activity increases in a number of host-parasitic interactions. Present study also confirmed that peroxidases are elicited by the infestation of nematodes indicating their role in defence against nematode infestation. The crude peroxidase enzyme extracted from the leaves of infected \textit{M. incognita} showed more activity than the un-inoculated. These findings conformed to earlier reports by Mahfouz et al.\textsuperscript{42}. The results confirmed that the level of peroxidase activity enhancement after nematode infestation was directly correlated with the efficiency of enzyme. However, the polyphenol oxidase enzyme did not change significantly in this study, both in the 3\textsuperscript{rd} and 7\textsuperscript{th} months. Similar activity was recorded for both the months though it was found less in the 5\textsuperscript{th} month. Increases in peroxidase activity could be correlated with infection in plants as polymerization of cinnamyl alcohols to lignin is catalyzed by peroxidase lignification leading to disease resistance. Reduction in total chlorophyll contents was similar to the findings of Chahal et al.\textsuperscript{43}, which led to the disturbance in nodule function. Increased phenolic concentration infected with \textit{M. incognita} as reported earlier by Bhargava et al.\textsuperscript{44} has also been observed. The above study showed increase in phenolic compounds in early stage and later it decreased. In conclusion, the present study suggested that defence mechanism of plants nematode infestation involves peroxidase enzyme as a key player, which finally produce phenolic compounds in the disease plants. Peroxidases may be used to raise insect resistant plants against broad range of insects by transgenic approach. In general, plant defence signalling is studied using model plants. However, different plant species probably have particularities in their responses. Therefore, reinforce the relevance to study plant defence signalling in patchouli, an important tropical oil-plant and a promising economical plant.

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