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Time course expression studies during *Musa* - *Pratylenchus coffeae* interaction

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ABSTRACT

Understanding of molecular mechanisms underlying host-nematode interactions is of primary importance in devising strategies to control nematode. Cultivars with differential reactions to nematode and the optimum time of sampling are the important criteria to study the host-nematode interactions. Based on the investigation on rate of *P. coffeae* population buildup in the roots, root lesion index, root weight and percentage of healthy root, Karthobiumtham and Nendran were identified as the most resistant and susceptible cultivars respectively among the ten cultivars used in this study. The phenol-metabolizing enzymes, *viz.*, peroxidase and polyphenol oxidase as well as total phenols content and pathogenesis related protein, *viz.*, β-1,3-glucanse were assessed in the roots of *P. coffeae* inoculated resistant and susceptible banana cultivars. The activities of peroxidase, β-1, 3-glucanase and content of total phenol were higher in the roots of uninoculated resistant cultivar than susceptible one. In post-inoculation stage, a steady increase in enzymes activities was noticed from 2 Days After Inoculation (DAI) onwards with maximum activity on 6 DAI for peroxidase and polyphenol oxidase, 4 DAI for total phenols content and 8 DAI for β-1,3 glucanase. Except total phenols content, the level of enzyme activities was higher even up to 12 DAI than the uninoculated control plants of both cultivars. The PPO activity was again reconfirmed by estimating the time course expression of PPO transcript level. Based on the time course studies on enzyme assays and mRNA level the present study suggested that the samples should be collected within 8 DAI of nematode for understanding the molecular mechanism of *Musa*-nematode interaction.

Key words: *Pratylenchus coffeae*, banana, root lesion index, enzymes.

INTRODUCTION

Nematodes are considered as the most important pest of banana and plantain and crop losses caused due to the root-lesion nematode (*Pratylenchus coffeae*) in banana is reported to be 44.4%. As the cost of nematode management in banana and plantain using nematicides such as aldicarb, phorate and carbofuran is quite prohibitive, development of nematode resistant cultivars is a promising strategy for managing nematodes and could contribute to improved productivity at a lesser cost with no pollution to environment. Pinochet *et al.* (1) stated that some resistance genes available in wild species or land races could be used as parental lines, which will facilitate the incorporation of nematode resistance into superior breeding material. It is apparent that developing nematode resistant banana varieties could be possible either through classical or molecular breeding taking into account the difficulties inherent in triploid breeding. Similarly, Van den Perg *et al.* (2) also revealed that a lack of knowledge pertaining to resistance mechanisms in banana complicates the development of resistant banana varieties. Understanding of molecular mechanisms underlying host-nematode interactions is of primary importance in devising strategies to control nematodes. Wise *et al.* (3) stated that the success of the host-pathogen interactions studies is mainly based on the suitable genotypes and optimum time of sampling. An analysis of changes in biochemical constituents of healthy and infected roots of resistant and susceptible cultivars would help in understanding the resistant mechanism. Hence, this present study was undertaken to identify the suitable nematode resistant and susceptible banana cultivars based on the pot culture screening and to find out the optimum time of sampling based on the enzyme activity and /or transcript level of defense related genes.

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MATERIALS AND METHODS

Suckers of uniform size of ten banana cultivars, *viz.*, FHIA 1 (AA), Karthombiumtham (ABB), Nendran (AAB), Anaikomban (AA), Kunnan (AB), Pisang Jari Buaya (AA), Pisang Lilin (AA), Yangambi KM 5 (AAA), Rastahli (AAB) and Calcutta 4 (AA) were planted in individual earthen pots containing sterilized mixture of soil, sand and farm yard manure (2:1:1). Five replications of each accession were arranged in a

completely randomised block design and watered daily. Same set of five uninoculated suckers of each accessions were maintained as control. *P. coffeae* which were extracted from axenic culture maintained on carrot discs in 1% bactoagar medium were used for inoculation. After one month of planting the experimental pots were inoculated with 3000 infective juveniles of *P. coffeae* through four holes made at different depths around the base of the plant leaving the control pots. Three months after inoculation, the plants were carefully removed and washed to remove the adhering soil particles. Plant height, pseudostem girth, total number of leaves, root weight, number of healthy and infested roots, root length and visual observations on root-lesion indices were recorded based on 1 to 5 scale. Then the nematode population in soil and root was taken as per the method described by Sundararaju and Sudha (4).

Suckers of identified resistant and susceptible cultivars were pot cultured under green house conditions. After one month of planting individual plastic cups with provision of hole on the side were placed by removing the soil near the plants in pots then single root was selected in each plant and inserted through the hole into the plastic cup and those cups were filled with potting mixture. Each cup was inoculated with 5000 active root lesion nematodes. The root samples were collected at 0, 1, 2, 4, 6, 8, 10 and 12 DAI from the nematode inoculated cups, and frozen by using liquid nitrogen and kept in -80°C.

The enzymes activity assay on peroxidase, polyphenol oxidase and β-1,3-glucanase and estimation of total phenols content was carried out as per the methodology given by Sadasivam and Manickam (5). The frozen root samples of nematode inoculated (2, 4, 6, and 8 DAI) as well as uninoculated of both resistant and susceptible cultivars were ground to a fine powder in liquid nitrogen, homogenized and total RNA was extracted using the modified protocol of Clendennen and May (6). The RNA yield and quality were determined spectrophotmetrically at 260 and 280 nm. The RNA integrity was checked on 1.5% (w/v) agarose formaldehyde gel.

Poly (A) + RNA was purified from the total RNA using Qiagen kit. First strand cDNA was synthesized from mRNA by reverse transcription with oligo-(dT) primers according to manufacturer's protocol (Promega). The first strand cDNA was diluted 1/10 with water and was used as template for PCR. Primer sequences used for amplification were PPO (5'GACCGCATGTGGTACTTGTG 3'; 5' GGGATCTCGACGTCTTGGTA3') and Musa 28S rRNA (AY651067) (5'-ACATTGTCAGGTGGGGAGTT-3'; 5'-CCTTTTGTTCCACACACGAGATT-3'). The PCR cycling profile was denaturation at 94°C for 1 min., annealing at 64°C for 1 min. and an extension at 72°C for 1 min. for 30 cycles. The PCR reaction products were resolved on agrose gel (1%) visualized by ethidium bromide staining under UV and the image was captured using Alpha imager. The intensity of bands was determined by densitometric analysis of gels using AlphaEase FC software.

RESULTS AND DISCUSSION

Use of suitable resistant and susceptible accessions is the fourth most important criteria to study the host pathogen interaction. Hence eight resistant/tolerant accessions and two susceptible accessions were chosen based on previous study on field evaluation trial and used for pot culture screening against *P. coffeae* to reconfirm their degree of resistance. Hence in this study, the nematode population build up was assessed taking into account the root and soil population after 90 days of inoculation. The rate of population build up was very high (>100) in all the accessions except Karthombiumtham and Calcutta 4. The susceptibility was expressed as the extent of damage to the root system and the density of nematodes sustained. In root samples, least nematode population was recorded in cultivar Karthobiumtham followed by Calcutta 4 (Table 1), which emphasizes that the incompatibility of these cultivars to *P. coffeae*.

Significant differences were recorded for root-lesion index among the ten accessions. Among the cultivars minimum lesion index of 2 was recorded in Karthobiumtham and Calcutta 4, whereas, maximum lesion index of 5 was recorded in Nendran. Even though Pisang Jaribuaya and Yangambi KM 5 are considered as resistant accessions in the previous study on field evaluation, the intensive pot culture screening studies revealed that these cultivars are coming under the moderately resistant category due to root-lesion index of 3. Maximum number of infected roots, nematode population in soil and root samples was recorded in cv. Nendran, which suggested that the compatible interaction of this cultivar with *P. coffeae*. In general the growth response of the plants such as height, pseudostem girth and total number of leaves were reduced in nematode inoculated cultivars than the uninoculated one, irrespective of the cultivars. But the degree of reduction varies depending on the cultivars (Data not shown). These alterations in growth might be due to the energy demands of the host-parasite interaction. Minimum and maximum value of percentage decreased over uninoculated plants for some of the root traits namely number of roots, root weight and length of root was recorded

Table 1. Reaction of banana accessions on root parameters and nematode population on inoculation of root lesion nematode.

Accession	Root weight (g)			Root length (cm)			Total No. of roots			Healthy roots (I)	Infected roots (I)	Root lesion index (I)	Nematode population (I)	
	UI	I	% decrease over UI	UI	I	% decrease over UI	UI	I	% decrease over UI				Soil (25°cc)	Roots (5 g)
FHIA-1	96	87.6	8.75	29	27.3	5.86	52.3	49.6	5.16	29	20.6	3.3	74 (8.61)	132 (11.83)
Karthobiumtham	141	130	7.80	26	25	3.84	56.5	54.6	3.36	38.6	16	2	71 (8.40)	80 (8.90)
Nendran	136	88	35.29	13.5	10.6	21.48	54	47.5	12.03	3.5	44	5	101 (10.06)	216 (14.61)
Anaikkomban	96	74.5	22.39	14	12	14.28	52.3	49.5	5.35	17	32.5	3	190 (13.84)	138 (11.69)
Kunnan	100	89.9	10.1	25	23.6	5.6	42.6	40.6	4.69	27.6	13	2.3	19 (4.27)	116 (10.75)
Pisang Jaribuaya	178	161	9.55	28	26.3	6.07	66.2	62.6	5.43	26.6	36	3.6	64 (7.99)	169 (12.85)
Pisang lilin	48	45	6.25	26	25	3.84	48.1	45.6	5.19	12.3	33.3	3	43 (6.54)	114 (10.64)
Calcutta-4	20	17.6	12	18.1	17.3	4.41	13	12	7.69	6.7	5.3	2	49 (6.90)	85 (9.14)
Yankambi KM-5	31	27	12.90	23	21.3	7.39	31.2	29.3	6.08	10.6	18.7	3.3	63 (7.89)	119 (10.77)
Rasthali	25	19.6	21.6	9.8	8.3	15.30	34	29	14.70	15	14	3.3	52 (7.24)	196 (14.02)
Mean	87.8	73.85		21.23	18.26		45.2	41.7		18.63	23.33	2.93	72.6 (8.17)	135.33 (11.49)
CD	7.95	5.84		5.33	4.02		4.46	2.5		3.36	3.36	2.32	0.68	0.47

Figures in parentheses are square root transformed values; I = P. coffeae inoculated, UI = Uninoculated

in cv. Karthobiumtham and cv. Nendran respectively (Table 1). Altogether, the data on root lesion index, nematode population in roots, percentage of infected roots, and root weight of the cultivars stated that Karthombiumtham and Nendran can be considered as the most resistant and susceptible cultivars respectively against *P. coffeae*.

Identified resistant (Karthobiumtham) and susceptible (Nendran) cultivars were used for studying the biochemical basis of nematode resistance. The constitutive activity peroxidose (PO) was significantly higher in the resistant cultivar than in the susceptible cultivar (Fig. 1a). High constitutive levels of peroxidase have previously been reported in the *Foc*-resistant banana hybrid. Following inoculation of the nematode, a sudden increase in activity was observed immediately after nematode inoculation (1 DAI) only in resistant cultivar not in susceptible one. In both cultivars, the activity of PO is high even up to 12 DAI in nematode inoculated plants than the uninoculted plants with the maximum activity observed on 4 in resistant cultivar and on 6 DAI in susceptible cultivar. Even though the activity of PO was on par at 6 DAI in both cultivars a sudden declining trend was observed in susceptible cultivar than the resistant cultivar. Peroxidase is important in the formation of phenolic compounds that lignify host cell walls and vascular cells and have been implicated in protecting banana against infection by root pathogen (Ploetz, 7).

The PPO activity was less in the roots of unioculated resistant cultivar than those of susceptible cultivar (Fig. 1b). An increase in trend on enzyme activity was observed at 2 and 3 DAI in resistant and susceptible cultivar respectively. But there was a significant and sudden increase in enzyme activity in resistant cultivar from 4 up to 12 DAI with the maximum activity on 6 DAI. Similar trend was also noticed in susceptible cultivar but which was significantly lesser than the resistant one. The increase in activity levels of PPO was greatest in banana roots of resistant variety inoculated with the root lesion nematode, postulated that the accumulation PPO may help to protect plants against infection by root pathogens.

The level of β-1,3-glucanase activity was same in both resistant and susceptible cultivar (Fig. 1c). Even though the activity of β-1,3-glucanase activity significantly increased in the nematode inoculated roots of resistant (1 DAI) and susceptible (2 DAI) cultivars. The high level of glucanase activity was noticed in resistant cultivar on 8 DAI and significantly on par up to 12 DAI. The induction of early and strong activity of PR proteins in resistant plants is part of the resistance mechanism to the plants to the pathogens. Similar trend was noticed in case of susceptible cultivar but significantly lesser than the resistant cultivar. A high level of glucanase transcript accumulation in nematode infected resistant cultivar was also observed in tomato (Joosten and Wit, 8).

In general, the level of phenol content was higher in uninoculated resistant cultivar (0.92 mg/g of fresh root tissue) than susceptible cultivar, which recorded 0.7 mg/g of fresh root tissue. The total phenols content in resistant and susceptible cultivars after inoculation of *P. coffeae* revealed that quantity of phenols progressively increased on all the days, highest being on 8 DAI (Fig. 1d), but a sudden increase in total phenols content was observed from 2 DAI in resistant cultivar against slow response in susceptible cultivar. This indicates that, rapid expression of defense responses would occur in an incompatible interaction. Mateille (9) compared host reactions to nematodes in *Musa* AAA cultivars and found that there was a higher concentration of cells containing phenols in Gros Michel, which is generally recognized as being less susceptible. Nicholson and Hammerschmidt (10) evidenced that among molecules that actively contribute to plant defense against pathogen invasion, phenolics play a key role in limiting extension of pathogens in host tissues.

The enzyme activities of PPO during host-nematode interaction were again reconfirmed by quantifying the mRNA level of PPO in both resistant and susceptible cultivars. The transcript of PPO was found in both resistant and susceptible cultivars of uninoculated root samples, indicating the constitutive expression of PPO gene, whereas the mRNA levels of PPO were higher in roots of resistant as compared to susceptible plants (Fig. 1). The differences in PPO transcript level among resistant and susceptible banana roots prompted the consideration of the potential effects of PPO on *P. coffeae*.

The steady increase of PPO transcript levels upto 6 DAI was noticed in nematode inoculated resistant than susceptible roots. The level of transcript of PPO was low in resistant compared to susceptible cultivar on 8 DAI. The increasing trend in transcript level of PPO is similar to that of PPO enzyme activity. The similar trend of expression of mRNA and their enzyme activity at different time interval suggested that there is no post-translational modification occurred in this protein during the nematode infestation. Time course studies of total phenols content, enzyme assay for peroxidise, PPO, 1,3 β-glucanase and transcript level of PPO demonstrated that the biochemical mechanism got activated soon after nematode inoculation in resistant cultivar than susceptible one. The initial responses were very important in deciphering the fate of the nematode plant relationship. The resistant

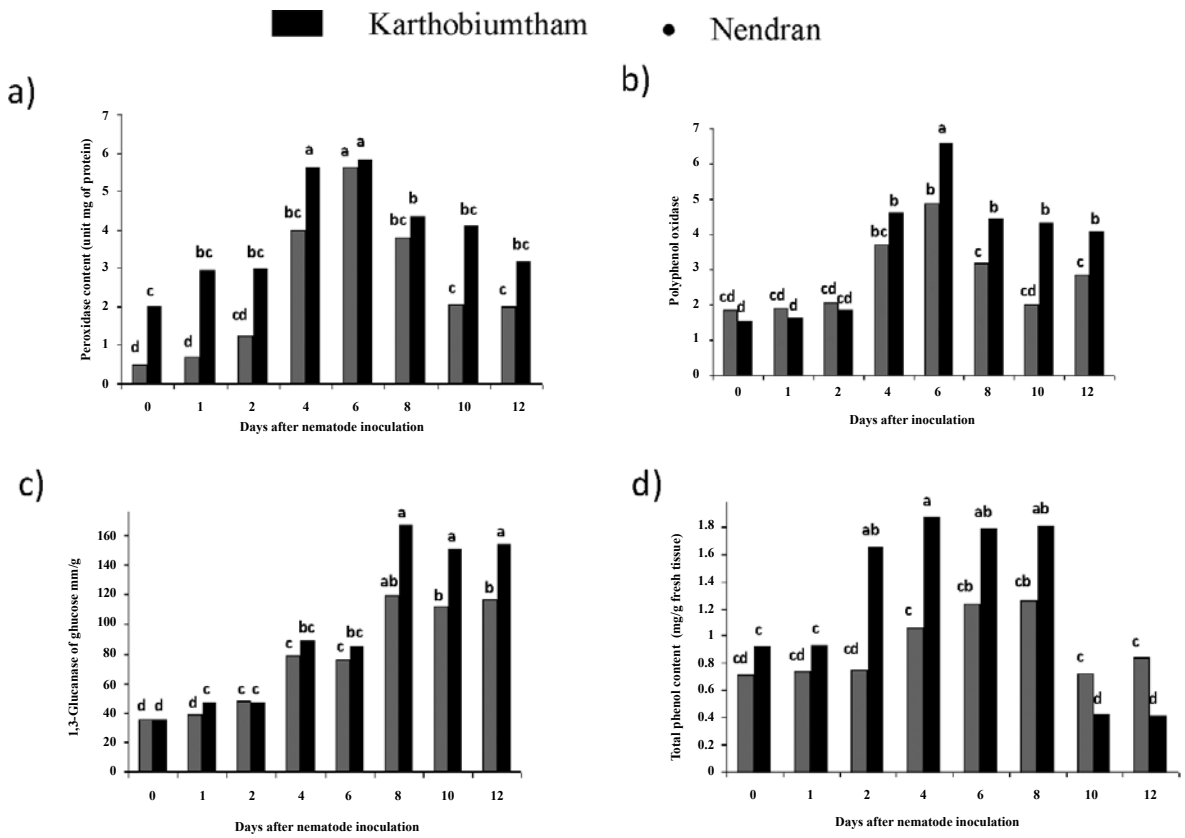

Fig. 1. Induction of defense related gene activity in cv. Karthobiumtham and Nendran plants inoculated with *P. coffeae*. **a.** Peroxidase; **b.** Polyphenol oxidase; **c.** β-1,3-glucanase and **d.** Total phenols content. Data analysed using ANOVA and the Duncan's Multiple Range Test. Bars presented with the same letter are not significantly different at P<0.05.

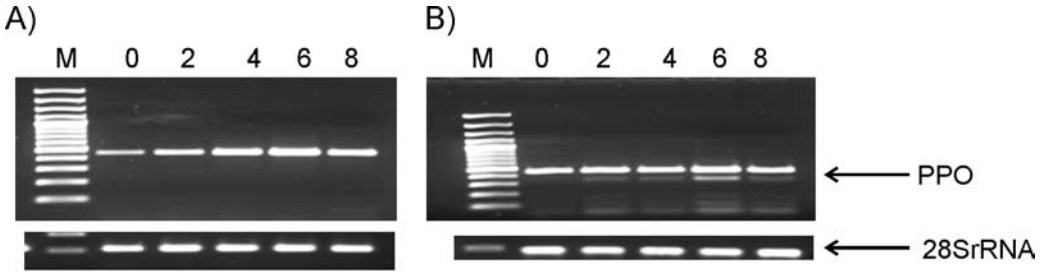

Fig. 2. Expression profiles of *Musa* poly phenol oxidase (PPO) in root samples of **(A)** cv. Karthobiumtham, and **(B)** cv. Nendran. Equal concentration of first strand cDNA synthesized from the total RNA isolated at 0, 2, 4, 6, 7 DAI of *P. coffeae* were used as templates. 28S rRNA was used to normalize the amount of templates added in PCR reactions.

cultivars showed a hypersensitive effect wherein the biochemical activity of these enzymes was very high. The maximum activity of these enzymes reached in between 6 to 8 days after nematode inoculation. Hence, this study suggested that Karthobiumtham could be an appropriate resistant *Musa* genotype and which could be used to understand the host nematode interaction by analysing the samples before 8 days after inoculation of nematode.

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