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RESEARCH PAPER

Morphological and biochemical responses of five tobacco cultivars to simultaneous infection with *Pythium aphanidermatum* and *Meloidogyne incognita*

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Summary. Five tobacco cultivars responded to *Meloidogyne incognita* inoculation with a 1.0–3.0 gall index. Inoculation with *Pythium aphanidermatum* resulted in a 0.33–2.66 root rot index, except cultivar RK-12 P3, which was without symptoms. The root rot symptoms became more severe in the plants concomitantly inoculated with fungus and nematode.The root rot symptoms also developed on cultivar RK-12 P3 in the presence of root-knot nematode. Soil populations of *P. aphanidermatum* were significantly greater in the presence than absence of *M. incognita*. Root-knot nematode population, however, showed a reverse trend, being less in the presence of root rot fungus. The fungus and nematode individually reduced the leaf pigments and plant growth of all cultivars except RK-12 P3 ($P \leq 0.05$), being greater in concomitantly inoculated compared with single pathogen inoculated plants. Phenol and salicylic acid contents of tobacco leaves gradually increased up to 15–20 days after planting, and thereafter, decreased. The greatest phenol and salicylic acid concentrations were recorded in tobacco plants concomitantly inoculated with the fungus and nematode.

Key words: carotenoids, chlorophylls, root rot, root-knot, salicylic acid, total phenols.

Introduction

Tobacco is a high-value non-food crop grown throughout the world, including India. The crop is regularly attacked by pathogens. Pre- and post-emergence damping off caused by *Pythium aphanidermatum* (Edson) Fitzp. is a commonly occurring disease in tobacco. The infection causes cessation of plant growth and leaves fade to pale yellow (Devaki *et al.*, 2008). Root-knot caused by *Meloidogyne* spp. is another major disease of tobacco, which may cause up to 60% yield loss depending upon the inoculum level and cultivars (Charles *et al.*, 2005). The nematode *M. incognita* is found frequently associated with tobacco in India (Khan *et al.*, 2010) and other countries (Charles *et al.*, 2005). In addition to direct damage, root-knot nematodes facilitate invasion of *Pythium* spp. and other soilborne pathogens leading to synergistic yield losses (Evans and Haydock, 1993). In the *Meloidogyne-Pythium* disease complex, root rot becomes more severe and causes extensive damage to the crop at any stage of plant growth than with *Pythium* alone (Powell, 1971; Reddy *et al.*, 2001).

In response to pathogen attack, plants produce a highly specific blend of phenolic compounds including salicylic acid (SA), resulting in the activation of various plant defense responses. These responses include the induction of local and systemic disease resistance, the potentiation of host cell death, and the containment of pathogen spread (Dempsey *et al.* 1999; Garcia *et al.*, 2001; Bari and Jones, 2009). Phenolic acids and SA are generally present in healthy plants at very low concentrations (Dixon *et al.*, 1994; Durrant and Dong, 2004), but their concentrations

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increase considerably in response to pathogen infection (Nicholson and Hammerschmidt, 1992). In incompatible interactions, hypersensitive response of plants occurs due to the programmed cell death (PCD) at the site of attempted pathogen penetration, thus preventing further proliferation of the pathogen (Staskawicz *et al*., 1992; Dempsey *et al*. 1999). However, hypersensitive response and other defense mechanisms vary with cultivar and etiology of the associated pathogen (Ryals *et al*., 1996).

The present study was carried out to measure the variation in the synthesis of phenols and SA, as influenced by host reaction (cultivars), pathogen (fungus and nematode) and disease etiology (mono- and dual pathogen application). To achieve this objective, the effects of single and concomitant inoculations of tobacco cultivars with *M. incognita* and *P. aphanidermatum* on total phenols, SA, chlorophylls, carotenoids, root rot index, soil populations of pathogens, length and dry weight of roots and shoots of tobacco cultivars were studied.

Materials and methods

Tobacco cultivars

The tobacco *(Nicotiana tabacum* L.) cultivars RK-10 P3, RK-12 P3, RK-13 P4, RK-18 P8, RK-26 P3, were procured from the Central Tobacco Research Institute (CTRI), Rajamundri, Andhra Pradesh, India.

Preparation of fungal inoculum

A pure culture of *P. aphanidermatum* was procured from the Division of Mycology and Plant Pathology, IARI, New Delhi, India. Inoculum of the fungus was prepared on sorghum grains. For inoculation, a known weight of sorghum seeds colonized with the fungus were ground with distilled water in an electric grinder and standardized to 2 g seeds/10 ml (1×10^6 cfu g^{-1} seed).

Preparation of nematode inoculum

Infected roots of eggplant were collected from a nematode culture bed having pure population of *M. incognita*. Roots were rinsed with tap water, and females and egg masses from the galled tissue were excised. Perennial pattern sections (Whitehead, 1968) of ten females excised from the root system were prepared to confirm the species of *M. incognita*. The egg masses were transferred in a course sieve lined with two layers of tissue paper. The sieve was kept on a Baermann's funnel filled with adequate amount of water, and the entire assembly was incubated at 25±2ºC for 1 week. Hatched juveniles were collected from the funnel every 24 h and stored in water in a refrigerator. Nematodes in the suspension were counted in a counting dish under a stereomicroscope (Khan, 2008) and standardized to 2000 juveniles/5 mL suspension.

Plant culture

Earthen pots (15 cm diameter) were filled with 1 kg soil:compost mix (3:1, v:v), and autoclaved for 15–20 min. Before planting tobacco seedlings, 10 mL fungal suspension containing 2 g colonized seeds and/or 5 mL nematode suspension containing 2000 juveniles were added to the top soil of individual pots singly or concomitantly (Tables 1 to 3). One day later, 4-week-old seedlings of tobacco were transplanted one seedling/pot. For each cultivar, eight treatments with three pots were maintained. Hence, each cultivar was grown in 24 pots. Pots were arranged in a completely randomized block design, and were regularly watered with tap water. Plants from three pots were evaluated at 0, 5, 10, 15, 20, 30, 60 and 90 d after transplanting to determine total chlorophyll contents, carotenoids, total phenols and SA in leaves. Leaf pigments were determined 15 d after transplanting. Additionally, root rot, root gall and egg mass indices, root and shoot lengths and dry weights were determined on 90-day-old plants (at harvest).

Symptoms of root rot and root-knot

Roots of 90-d-old tobacco plants inoculated with *P. aphanidermatum* and *M. incognita* singly or concomitantly were examined for brown to black root rot lesions, and severity of root rot was assessed using a 0–5 root rot index (RRI), where 0, no rotting; 1, 1–10% rotting; 2, 11–25% rotting; 3, 26–60% rotting; 4, 61–80% rotting; 5, 81–100% rotting. The roots were also examined for the presence of nematode galls and egg masses, which were assessed using 0–5 gall index and egg mass index (Taylor and Sasser, 1978), where 0, no galls or egg masses; 1, 1–2 galls or egg masses; 2, 3–10 galls or egg masses; 3, 11–30 galls

or egg masses; 4, 31–100 galls or egg masses, and 5, ≥100 galls or egg masses/root system.

Soil populations of *Pythium aphanidermatum*

Soil population, in terms of colony forming units (cfu), of *P. aphanidermatum* g^{-1} soil was determined at harvest by the dilution plate method. Suspensions from 10^{-4} dilutions were spread over on potato dextrose agar plates under a laminar flow. Three replicate plates were maintained for each treatment. The plates were incubated at 25±2ºC for 72 h, and thereafter colonies were counted using a colony counter.

Soil populations of *Meloidogyne incognita*

Population density of *M. incognita* juveniles in soil was determined at harvest using Cobb's decanting and sieving method (modified) followed by Baermann's funnel technique (Southey, 1986). Nematode suspensions collected from the Bearmann funnel were examined in counting dishes under a stereomicroscope to count nematodes, and the population was expressed as number of juveniles kg^{-1} soil.

Estimation of total phenol (TP) and salicylic acid contents

One g of leaf tissue from each of the five tobacco cultivars was collected and processed separately. The 1 g leaf sample for TP assay was homogenized in 10 mL 80% methanol and agitated for 15 min at 70°C (Zieslin and Ben Zaken, 1993). One mL of methanol extract was added to 5 mL of distilled water and 250 µL of Folin-Ciocalteau reagent (1N) and the solution was kept at 25°C for 5 min. The absorbance of the blue colour that developed was measured at 725 nm with a spectrophotometer (UV 2450, Shimadzu Japan). Catechol was used as standard. The amounts of TP were expressed as µg catechol g^{-1} fresh leaf (Sharma and Sain, 2005).

For SA, 1 g leaf sample was collected and cut into small pieces of 0.5–1.0 cm. The pieces were soaked in water overnight. The water-leaf suspension was filtered through the Watman No. 1 filter paper and extracted in ethyl acetate. The ethyl acetate fraction was taken and sodium sulphate was added to remove water, and the filtrate was then evaporated to dryness in a water bath. Ten mL methanol were added to the dried sample. Thereafter, the solution was used for recording the absorbance at 306 nm with a spectrophotometer (UV 2450, Shimadzu Japan; Shane and Kowblansky, 1968). A standard curve of SA was prepared for SA concentrations of 0, 10, 20, 30, 40, 50, 100 ppm in methanol. From the standard curve the concentration of SA in the sample was calculated according to the formula $y = mx \pm c$ (Lowery *et al.*, 1951).

Estimation of leaf pigments

Total chlorophyll and carotenoid contents of leaves were measured by grinding 1 g fresh leaf tissue from interveinal areas of tobacco plants 15 d after inoculation in 40 mL 80% acetone, using mortar and pestle. The suspension was decanted in a Buchner funnel containing two Watman no. 1 filter papers. The filtration was done with the help of a vacuum pump. The residue was ground three times after adding acetone. The suspension was again decanted in a Buchner funnel and filtered by a vacuum pump. Finally, the mortar and pestle were rinsed with acetone and the solution was also transferred in the Buchner funnel and filtered. The filtrate was transferred to a 100 mL flask added up with acetone to reach 100 mL. The optical density (O.D.) of the filtrate was measured with a spectrophotometer at 470 nm for carotenoids (Maclachlan and Zalik, 1963) and 645 and 663 nm for total chlorophyll contents (Arnon, 1949).

Statistical analyses

The experiments were conducted during two consecutive years. The data from three replicates were averaged and presented in the tables and were used to calculate percent variation over experimental controls. The data of the 2 years were analyzed separately by two factor analysis of variance (ANOVA) considering root rot fungus as factor one and root-knot nematode as factor two, using MINITAB 11.0 for WINDOWS-XP. Least significance difference (LSD) was calculated at $P \leq 0.05$. The F-values were calculated and compared with the table value at $P \leq 0.05$. Correlation analysis was performed only on data collected in the second year, to identify relationships between root rot/root-knot with biochemical parameters.

Results

Symptoms of root rot and root-knot

Inoculation with 2 g of *P. aphanidermatum* resulted in stunted growth, yellowing of foliage and root rotting of all tobacco cultivars except RK-12 P3, which did not show any symptoms. The root rot index was greatest on RK-10 P3 (2.66/2.33) and least on RK-12 P3 (1.0/1.33) in concomitantly inoculated plants in both years. Plants inoculated with 2000 juveniles/pot showed stunted growth and developed galls and egg masses on roots. The gall and egg mass indices were the greatest on RK-10 P3 (3.0 and 2.66) and the least on cultivar RK-12 P3 (1.33 and 1.0). In plants concomitantly inoculated with *M. incognita* and *P. aphanidermatum,* the root rot index was significantly greater than in plants inoculated with the fungus alone ($P \leq 0.05$), and the affected roots or parts turned dark brown to black. The cv. RK-12 P3 did not develop measurable root rot in the absence of *M. incognita*, but, in its presence, considerable root rot developed, with root rot indices of 1.0 and 1.33 in the first and second years, respectively (Table 1).

Soil population of root-knot nematode and root rot fungus

Soil population *M. incognita* in pots without *P. aphanidermatum* increased by 11 and 15% (RK-12 P3) to 191 and 198% (RK-10 P3) over the initial population during the 3 months of study in both years, respectively (Table 1). In concomitantly inoculated plants, the nematode population was significantly less than in the absence of *P. aphanidermatum*. The population density of *P. aphanidermatum*, however, increased over time, and this increase was greater in the presence than in absence of *M. incognita* (Table 1). The greatest population of *P. aphanidermatum* was recorded in the rhizosphere of RK-10 P3 and the smallest population in RK-12 P3, during the two years. F values for soil population were statistically significant ($P \leq 0.01$, Table 1).

Plant length and dry weight

Inoculation with *P. aphanidermatum* or *M. incognita* resulted in significant reductions of the plant lengths and dry weights of all cultivars except RK-12 P3, in both years (Table 2). The reductions were the greatest in cultivar RK-10 P3 and the least in RK-12 P3. Concomitant inoculation with fungus and nematode resulted in further suppression of length and dry weight of roots and shoots of cvs RK-10 P3 and RK-18 P8, in comparison to single treatments of the two pathogens in both years (Table 2). RK-12 P3 did not exhibit significant decrease in plant growth and dry weight variables due to concomitant inoculation, except dry weight of roots in the first year. The ANOVA revealed significant F values of root rot fungus for dry weight of shoots and roots at $P \leq 0.05$. For root-knot nematode and the interaction, F-values were significant for all plant growth parameters at $P \leq 0.01$ or $P \leq 0.001$ (Table 2).

Leaf phenol and salicylic acid (SA)

Total phenol content of leaves of all five tobacco cultivars increased gradually and reached a maximum concentration on 15 d after inoculation, and thereafter decreased and became the least at 90 d after inoculation, in both years (Figure 1). In general, the phenol contents were significantly greater in the pathogen-inoculated plants than uninoculated plants. Inoculation with *M. incognita* resulted in a significantly greater increase in phenol contents compared to inoculation with *P. aphanidermatum*. In concomitantly inoculated plants, phenol content increased further, being the greatest on 15 d after inoculation compared to nematode- or fungus-inoculated plants. In single pathogen inoculated plants, phenol concentration was the greatest in cv. RK-12 P3 (root-knot nematode), and RK-10 P3 (root rot fungus) 20 d after inoculation, respectively (Figure 1). The phenol concentrations, however, gradually and significantly decreased after 15 d (concomitant inoculation) and 20 d (single pathogen) from inoculation and reached the lowest level at 90 d (Figure 1). The correlation analysis showed greater phenol concentrations in cultivars that developed lower levels of root rot or root-knot symptoms, and low concentrations in plants with most severe disease (Figures 3 and 4). The concentration of SA in tobacco leaves increased in a similar manner to phenols, and greater SA levels were recorded in inoculated plants than uninoculated plants. Among pathogens, the SA contents were greater in plants inoculated with *M. incognita* than with *P. aphanideramtum.* The SA contents of concomitantly inoculated plants were greater than in single pathogen inoculated plants in both the years of study (Figure 2). The correlation analysis showed greater SA concentration in cultivars that developed low disease severity (Figures 3 and 4).

Table 1. Mean indices of root rot, nematode galling , egg mass production and soil populations of *Pythium aphanidermatum* and *Meloidogyne incognita* in after single and concomitant inoculations of different tobacco cultivars[a].

Tobacco cultivars	Inoculation/pot		Root rot index (0-5) I yr/ II yr	Gall index (0–5) I yr/ II yr	Egg mass index (0–5) I yr/ II yr	Soil population of nematode (J_2 kg^{-1}) I yr/ II yr	Soil population of fungus (10^3 CFU kg^{-1}) I yr/ II yr
	Pa (g)[b]	Mi (J_2)c[b]					
RK-10 P3	0.0	0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
	2.0	0.0	2.66/2.33	-	-	-	5600/5242
	0.0	2000	-	3.0/2.66	2.66/2.33	5814/5962	-
	2.0	2000	3.00/2.66	3.66/3.66	3.00/3.0	6019/6124	7100/68465
RK-18 P8	0.0	0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
	2.0	0.0	2.33/2.33	-	-	-	5400/5248
	0.0	2000	-	2.66 /2.33	2.0/2.0	5421/5196	-
	2.0	2000	2.66/2.33	3.33/3.0	2.66/2.33	5817/5741	6920/
RK-26 P3	0.0	0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
	2.0	0.0	1.33/1.66	-	-	-	2264/2200
	0.0	2000	-	2.33/2.33	1.66/1.33	4354/4215	-
	2.0	2000	2.00/2.00	3.00/2.66	2.00/2.00	4860/4754	3147/2965
RK-13 P4	0.0	0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
	2.0	0.0	1.00/1.33	-	-	-	2018/1984
	0.0	2000	-	2.0 /2.0	1.33/1.33	3842/3614	-
	2.0	2000	1.33/1.66	2.33/2.33	2.00/1.66	4008/4102	2834/2675
RK-12 P3	0.0	0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
	2.0	0.0	0.33/0.66	-	-	-	510/494
	0.0	2000	-	1.33/1.0	1.0/1.0	2213/2302	-
	2.0	2000	1.0/1.33	1.66/1.66	1.0/1.0	2548/2542	689/622
LSD	$P \leq 0.05$	-	0.459/0.545	0.239/0.254	0.267/0.274	6.28/7.11	0.595/0.502
F-value[c]	Fungus (F)	-	12.77 b/11.5 b	-	-	8.22 b/7.85 b	181.31 c/174.5 c
	Nematode (N)	-	-	10. 90 b/9.58 b	10.9 b/ 8.85 b	22.18 c/21.4 c	227.23 c/ 254 c
	N X F	-	18.43 c/17.25 c	20.4 c/19.84 c	8.22 b/8.52 b	42.70 c/ 40.15 c	419.56 c/475.2 c

[a] Each value is the mean of three replicates.
[b] Pa, *Pythium aphanidermatum*; Mi, *Meloidogyne incognita*; J_2, Juveniles.
[c] F-values followed by a ($P \leq 0.05$), b ($P \leq 0.01$) and c ($P \leq 0.001$) are significant.

Table 2. Mean plant parameters for different tobacco cultivars inoculated with *Pythium aphanidermatum* or *Meloidogyne incognita* as single or concomitant inoculations.

Tobacco cultivars	Inoculation/pot		Plant length (yr I/ yr II)[a]		Dry weight (yr I/ yr II)[a]	
	Pa (g)[b]	Mi (J_2)[b]	Shoot (cm)	Root (cm)	Shoot (g)	Root (g)
RK-10 P3	0.0	0.0	60.8/57.5	23.9/24.9	10.0/10.3	3.15/3.25
	2.0	0.0	56.5/54.8	20.6 a/21.7	8.9 a/8.4 a	2.50 a/2.7 a
	0.0	2000	45.7 a/47.2 a	18.3 c/17.4 a	8.31 a/7.87 a	2.53 a/2.20 a
	2.0	2000	36.5 a/37.3 a	16.6 c/15.8 a	7.6 a/7.1 a	2.0 a/2.30 a
RK-18 P8	0.0	0.0	55.3/56.2	18.1/17.5	10.7/10.1	3.24/3.31
	2.0	0.0	50.6/51.3	15.2 a/14.7 a	9.9 a/10.1 a	2.6 a/2.40
	0.0	2000	47.1 a/45.9 a	14.6 a/15.3 a	8.2 a/7.8 a	2.4 a/2.3 a
	2.0	2000	35.7 a/28.5 a	13.9 a/12.8 a	8.25 a/7.9 a	2.25 a/2.11 a
RK-26 P3	0.0	0.0	32.6/31.8	18.1/17.8	5.5/5.3	3.45/3.25
	2.0	0.0	26.1 a/25.4 a	16.4/15.8	4.9 a/5.2	3.15 a/3.00a
	0.0	2000	26.4 a/25.8	15.3 a/16.7	4.8 a/4.8 a	2.9 a/2.84 a
	2.0	2000	23.2 a/22.5 a	11.7 a/11.2 a	4.1 a/4.5 a	2.6 a/2.70 a
RK-13 P4	0.0	0.0	41.8/40.2	20.0/19.3	8.1/7.6	2.60/2.71
	2.0	0.0	38.9/37.8	17.9/18.6	7.9/7.6	2.45/2.32
	0.0	2000	38.5/37.5	17.8/17.1	7.5 a/6.9	2.3 a/2.45 a
	2.0	2000	34.9 a/35.5	16.8 a/16.9 a	7.3 a/7.8	2.2 a/2.35 a
RK-12 P3	0.0	0.0	46.8/44.7	16.4/17.3	10.1/10.6	2.80/2.65
	2.0	0.0	46.3/45.2	16.1/17.2	10.2/10.5	2.75/2.80
	0.0	2000	45.3/43.9	15.9/16.8	10.0/9.7	2.6/2.74
	2.0	2000	42.8/43.6	14.40/14.9	9.60 a/10	2.47 /2.45
LSD	$P \leq 0.05$	-	6.700/6.810	2.829/2.791	0.505/0.524	0.348/0.312
F-value[c]	Fungus (F)	-	NS/NS	6.31 a/7.12 a	4.74 a/5.32 a	12.09 b/ 11.9 a
	Nematode (N)	-	8.09 a/8.21 a	185.9 c/165.4 c	41.11 c/ 43.54 c	128.5 c/143.2 c
	N X F	-	20.36 c/21.3 c	367.8 c/354.4 c	101.0 c/98.78 c	c/334.5 c

[a] Each value is the mean of three replicates. Values followed by a ($P \leq 0.05$) are significantly different from the control (uninoculated), otherwise not significant at $P \leq 0.05$.
[b] Pa, *Pythium aphanidermatum*; Mi, *Meloidogyne incognita*; J_2, Juveniles.
[c] F-values followed by a ($P \leq 0.05$), b ($P \leq 0.01$) and c ($P \leq 0.001$) are significant otherwise not significant (NS) at $P \leq 0.05$.

Chlorophylls and carotenoids

Total chlorophyll content of leaves decreased in response of inoculation with *M. incognita* in cultivars RK-10 P3, RK-18 P8 and RK-26 P3 (Table 3). Significant decreases in the chlorophyll contents due to inoculation with *P. aphanidermatum* were recorded for the cultivars RK-10 P3 and RK-18 P8 ($P \leq 0.05$). Response of carotenoids to the pathogens was similar to chlorophyll in both years of study (Table 3).

For concomitantly inoculated plants, decreases in the chlorophyll contents were significantly great-

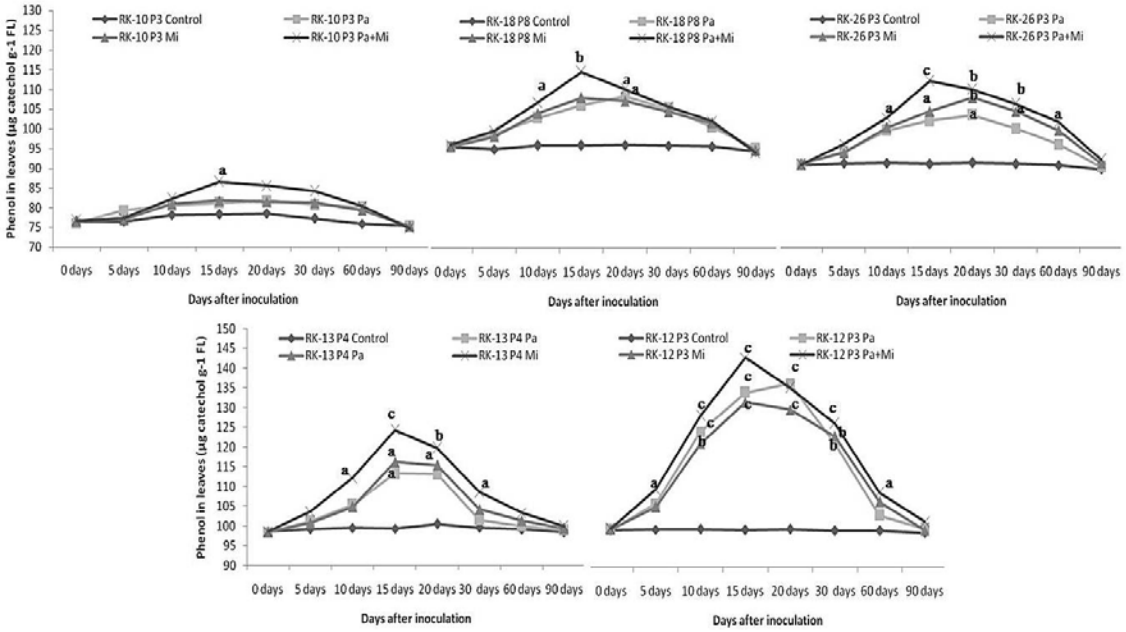

Figure 1. Mean total amounts of phenols in leaves of five tobacco cultivars 0–90 days after inoculation singly and concomitantly with *Meloidogyne incognita* (Mi) or *Pythium aphanidertmatum* (Pa).. Values followed by a ($P \leq 0.05$), b ($P \leq 0.01$) and c ($P \leq 0.001$) are significantly different from uninoculate controls. FL, Fresh leaf.

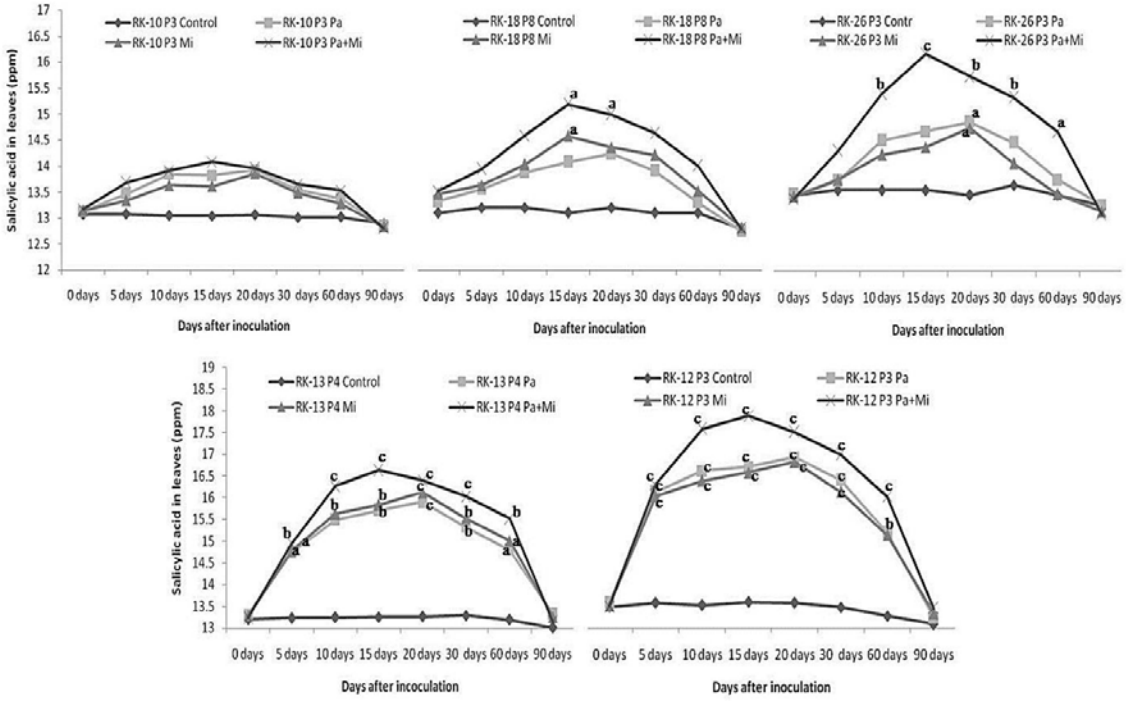

Figure 2. Mean amounts of salicylic acid in leaves of five tobacco cultivars 0–90 days after inoculation singly or concomitantly with *Meloidogyne incognita* (Mi) or *Pythium aphanidertmatum* (Pa). Values followed by a ($P \leq 0.05$), b ($P \leq 0.01$) and c ($P \leq 0.001$) are significantly different from the control.

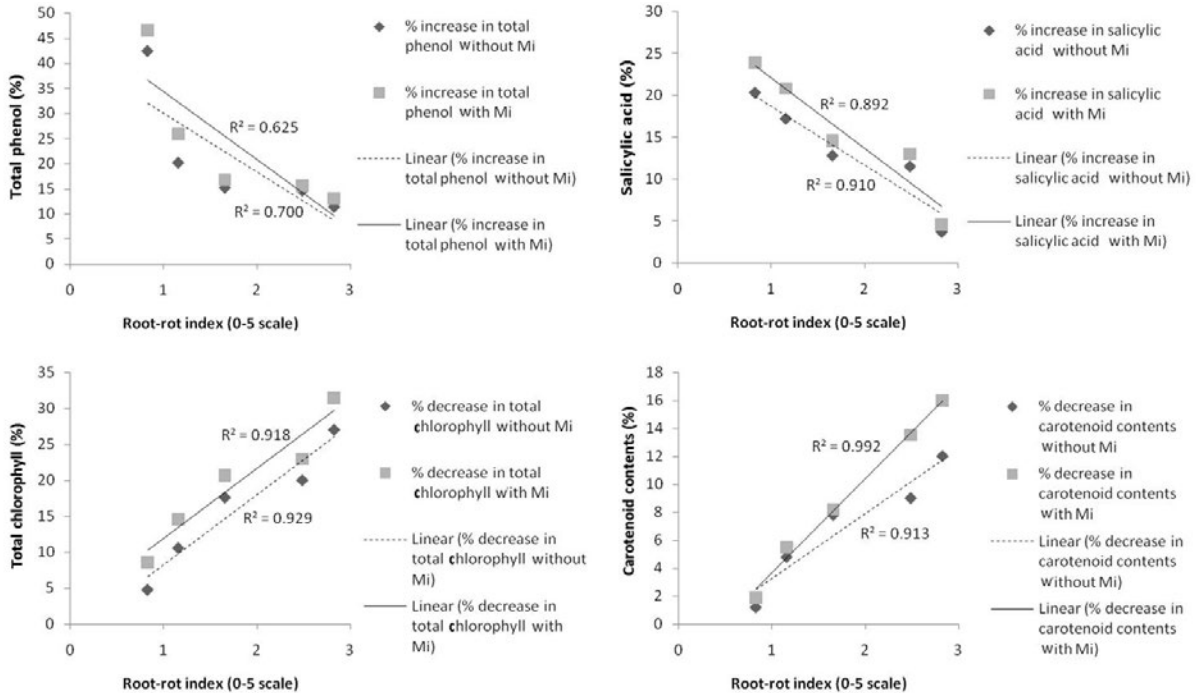

Figure 3. Correlations between root rot indices and percent variation in phenolic content, salicylic acid, total chlorophylls and carotenoids in tobacco cultivars inoculated with *Pythium aphanidertmatum* (Pa).

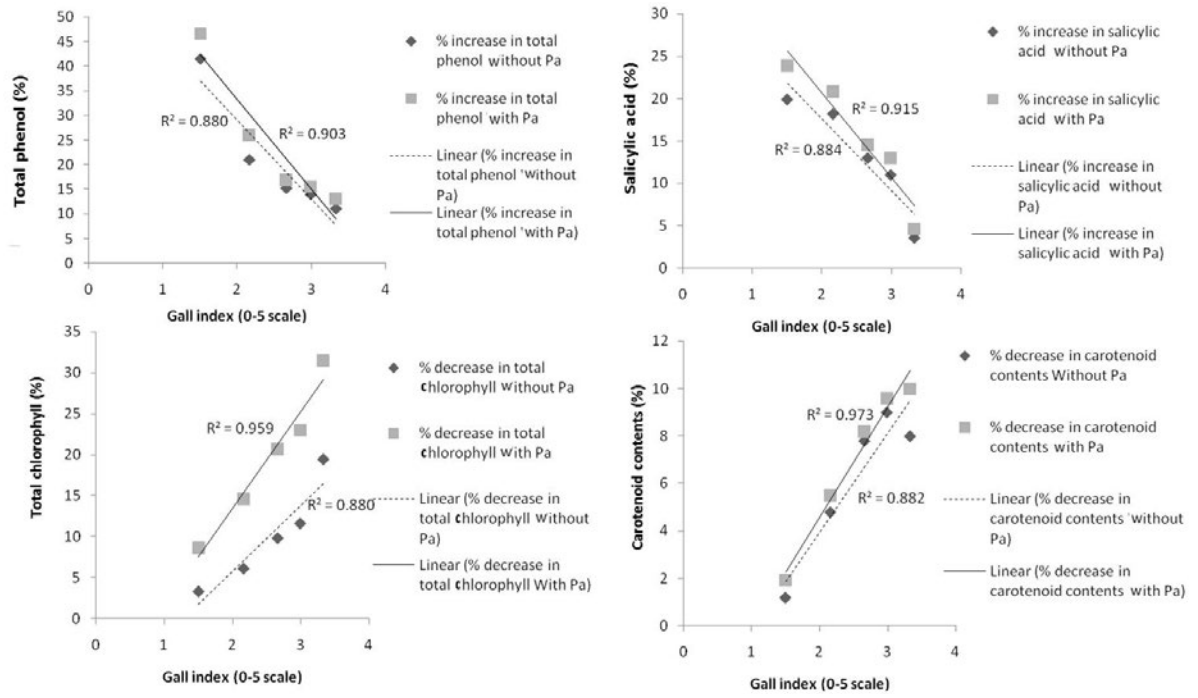

Figure 4. Correlations between nematode gall indices and percent variation in phenolic content, salicylic acid, total chlorophylls and carotenoids in tobacco cultivars inoculated with *Meloidogyne incognita* (Mi).

Table 3. Mean amounts of leaf pigments in different tobacco cultivars inoculated with *Pythium aphanidermatum* or *Meloidogyne incognita* as single or concomitant inoculations.

Tobacco cultivars	Inoculation/pot		Total chlorophyll (mg g^{-1} FL) I yr/ II yr[a]	Carotenoids (mg g^{-1} FL) I yr/ II yr[a]
	Pa (g)[b]	Mi (J_2)[b]		
RK-10 P3	0.0	0.0	1.991/1.974	0.144/0.138
	2.0	0.0	1.461 a/1.485 a	0.121 a/0.119 a
	0.0	2000	1.791 a/1.770 a	0.125 a/0.121 a
	2.0	2000	1.363 a/1.391 a	0.111 a/ 0.110 a
RK-18 P8	0.0	0.0	1.594/1.570	0.139/0.141
	2.0	0.0	1.227 a/1.205 a	0.127 a/0.126 a
	0.0	2000	1.398 a/1.369 a	0.124 a/0.125 a
	2.0	2000	1.226 a/1.240 a	0.113 a/0.115 a
RK-26 P3	0.0	0.0	1.530/1.515	0.128/0.130
	2.0	0.0	1.369 a/1.370 a	0.120/0.123
	0.0	2000	1.404/1.450	0.118/0.120
	2.0	2000	1.213 a/1.223 a	0.110/0.113
RK -13 P4	0.0	0.0	2.027/2.051	0.131/0.130
	2.0	0.0	1.876/1.856	0.125/0.124
	0.0	2000	1.896/1.872	0.120/0.126
	2.0	2000	1.722 a/1.740 a	0.125/0.122
RK-12 P3	0.0	0.0	1.710/1.732	0.123/0.125
	2.0	0.0	1.640/1.659	0.122/0.121
	0.0	2000	1.653/1.632	0.121/0.121
	2.0	2000	1.562/1.539	0.122/0.120
LSD	$P \leq 0.05$	-	0.164/0.126	0.015/0.013
F-value	Fungus (F)	-	70.99 c/67.94 c	6.30 a/6,54 a
	Nematode (N)	-	34.16 c/29.06 c	6.00 a/5.67 a
	N X F	-	103.24 c/ 109.76 c	13.67 b/12.43 b

[a] See Table 2. FL, Fresh leaf.
[b] See Table 1.
[c] See Table 1

er than in the plants inoculated with single pathogens. This difference was significant for the cultivars RK-10 P3, RK-18 P8, RK-26 P3 and RK-13 P4 (Table 3). F-values for single and interactive effects of the fungus and nematode were significant for chlorophylls and carotenoids ($P \leq 0.05$, Table 3). Correlation analysis showed linear negative relationships between leaf pigments and disease severity (root rot and gall indices), where increases in the root rot and gall indices resulted to corresponding decreases in the chlorophyll and carotenoid contents of leaves (Figures 3 and 4).

Discussion

Stunted growth, mild yellowing of leaves and rotting of roots with brown to black lesions are characteristic symptoms of *P. aphanidermatum* infection on susceptible tobacco plants (Devaki *et al.*, 2008). Blackened discolorations of roots that did not appear on cv. RK-12 P3 indicated that this cultivar expressed tolerance to infection by *P. aphanidermatum*. This cultivar also did not exhibit significant declines in the plant growth parameters due to fungal inoculation. Other research has shown tolerance/resistance in tobacco germplasm in India (Grover and Gowthaman, 2003). The tolerance expressed in RK12 P3 against *P. aphanidermatum* is, however, a new report. Formation of nematode galls and egg masses on the roots of all cultivars tested is an alarming observation, because root-knot nematodes possess capability to predispose plants for greater colonization/infection by pathogenic fungi and/or alter host reaction of resistant/tolerant cultivars (Khan and Dasgupta, 1993). Tobacco is a highly susceptible crop to *M. incognita* and develops discrete galls (Khan *et al.*, 2010). The root rot symptoms were considerably greater on plants concomitantly inoculated with *P. aphanidermatum* and *M. incognita*, than on single inoculated plants, and also appeared on the cv. RK-12 P3 which was without the symptoms when inoculated only with the fungus. Several researchers have reported that root-knot nematodes can break down fungus resistance/tolerance responses of cultivars (Evans and Haydock, 1993).

Root rot fungus in concomitantly inoculated plants increased the severity of root-knot nematode infestation, as evidenced by significantly less galling and egg mass production in all five tobacco cultivars in comparision to the nematode alone. *Pythium* spp. and other root rot and wilt causing fungi invade the nematode feeding sites (giant cells) of root-knot nematodes, and this that leads to reduced galling and egg mass production (Wallace, 1983). However, presence of *M. incognita* accelerated the pathogenesis of *P. aphanidermatum,* and, as a result, plants underwent severe damage and rotting of root tissue within a few days after inoculation (Khan and Dasgupta, 1993). The internal tissues of the lateral roots disintegrate due to root rotting and root galling leading to impairment in the absorption of water and minerals, and subsequently progressive decline of the plant growth takes place (Oluma and Oladiran, 1993). Chlorophylls and chloroplasts are basic units for photosynthesis as these pigments absorb light and transfer energy for CO_2 fixation (Wallace, 1987). The contents of chlorophyll pigments were found to be highly sensitive to infection by *P. aphanidermatum* and *M. incognita*. The water stress, as evidenced by wilting, resulted due to root rot, root-knot, and/or the toxic metabolites produced by the pathogens, may denature the chlorophyll and carotenoid molecules, consequently the leaf contents of these pigments were reduced. The amount of chlorophylls and carotenoids in the leaves has direct relationship with photosynthetic activity of plants and is reflected in corresponding biomass production (Khan and Khan, 1987).

Increases in the phenol and SA contents of leaves were greater with the treatments that caused greater symptoms and decreases the length and dry weight of the tobacco plants. For example, in cultivar RK-10 P3, concomitantly inoculated plants exhibited 14 and 12% greater decreases, respectively, in the length and dry weight of shoot compared with plants only inoculated with nematodes. The concentration of phenols was also greater in concomitantly inoculated plants than plants only inoculated with nematodes. However, cultivar response with regard to TP and SA contents, was different. Relatively small increases in the TP and SA were recorded in cv. RK-10 P3, which exhibited the greatest suppression in the plant growth parameters, leaf pigments and other parameters measured. Whereas the greatest concentrations were recorded in cv. RK-12 P3, which expressed tolerance and exhibited the smallest decrease in the plant growth variables. This indicates that phenols (Nicholson and Hammerschmidt, 1992; Meena *et al.*, 2000) and SA (Ryals *et al.*, 1996) contribute in the self-defence of plants against pathogen attack. Higher accumulation of SA in tobacco leaves contributed to a significant reduction of symptoms caused by the *Phytophthora parasitica*, *Cercospora nicotianae* and *Peronospora tabacina* (Ryals *et al.*, 1996) and *M. incognita* (Nandi *et al.*, 2000; Molinari and Baser, 2010). SA-mediated resistance of cultivars was also evident from the correlation analysis between SA and root rot index. Greater increase in SA and TP concentrations in concomitantly inoculated plants has indicated that plants, through self defense mechanisms, reacted to resist the concomitant invasion of the two pathogens. Invasion and infection of plant roots by soilborne pathogens primarily takes place during first 2 weeks after inoculation. The tobacco cultivars

reacted to avert pathogen attack by synthesizing TP and SA as evidenced by highest concentrations of these chemicals recorded at 15 d after inoculation.

This study has revealed that among the five tobacco cultivars assessed, RK-12 P3 showed a considerable degree of tolerance to *P. aphanidermatum*, which can be exploited commercially. This tolerance can be considered as a reliable character as it was based on the greater synthesis of phenols and SA in response of fungus infection. However, the cultivars became susceptible to the fungus in the presence of *M. incogmita*, so the cultivar is unlikely to be useful for root-knot infested areas.

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