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RESEARCH ARTICLE

Analysis of phenolic and indole acetic acids in *Meloidogyne graminicola* infected rice plants (*Oryza sativa* L.)

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Abstract

Meloidogyne spp. incite root-knot disease in the roots of Solanaceous and Cereal crop plants inflicting heavy damage to the crops. *M. graminicola*, a root-knot nematode is ubiquitous as a rice pathogen wherever rice is grown. High Performance Liquid Chromatographic (HPLC) analysis of phenolic acids in healthy and root-knot infected plant parts of rice indicated that phenolic acid contents were highly variable in both the cases. Upper leaves of healthy plants had seven phenolic acids in which gallic acid was maximum (140.3 µg/g fresh wt) followed by ferulic, tannic and vanillic acids. However, in root knot-infected plants, upper leaves had six phenolic acids in which gallic acid was maximum (190.68 µg) followed by caffeic, ferulic, o-coumeric, cinnamic and salicylic acids. In healthy leaf sheath gallic acid was the maximum (8.6 µg) followed by tannic, ferulic, vanillic, caffeic acid but o-coumeric, cinnamic and Indole Acetic Acid (IAA) were detected in traces. Root knot-infected leaf sheath had nine phenolic acids, where gallic was the maximum (26.84 µg) followed by vanillic, ferulic, o-coumeric and tannic acids but other phenolic acids, viz., cinnamic, salicylic and IAA were present in traces. Roots of healthy rice plants had seven phenolic acids while infected roots had nine phenolic acids. Moreover, in infected roots without root-knot had eight phenolic acids, in which gallic was the maximum (29.30 µg) followed by ferulic, caffeic, vanillic, tannic and o-coumeric acids but salicylic and IAA were present in traces.

Introduction

Rice is one of the important cereal crops of the world. The crop faces several abiotic and biotic constraints during its cultivation. A number of plant pathogens (fungi, bacteria, viruses, nematodes) are the major biotic constraints. Nematodes use diverse life strategies to proliferate in the intercellular spaces after entering through stomata and hydathodes or gain access via wounds in plants. Nematodes and aphids feed by inserting their stylet directly into the plant cell.

Meloidogyne spp., which incite root-knots, are economically important phytopathogens as they usually attack roots of solanaceous and cereal crop plants. *M. graminicola* is an internationally important pest of the rice crop found throughout the world wherever rice is cultivated (Golden and Birchfield, 1968; Handoo et al., 2004). In India it is a well established pest of upland and well-drained soils and causes enormous losses in yield (about 17%) due to poor filling of kernels (Biswas and Rao, 1971).

Root-knot-infected plants become dwarf and yellow. The roots are galled and deformed and plants lose their vigour (Yik and Birchfield, 1979). The infected plants exhibit characteristic symptoms, viz., root galls, poor growth and eventual death because of vascular dysfunction (Sasser, 1977; Khan and Haider, 1991). Nematodes are sedentary endoparasites which

have complex trophic relationships with their host plant and induce specialized feeding structures known as giant cells required for the nutrition and development of the nematode. Padgham et al. (2004) reported that the negative impact on rice productivity in the rice-wheat cropping system is because wheat roots increase populations of *M. graminicola* in the soil.

Several reports indicate that during interaction of plant pathogens, plants show a variety of biochemical alterations to inhibit infections (Hutcheson, 1998; van Loon et al., 1998; Sticher et al., 1997; Nicholson and Hammerschmidt, 1992). Secondary metabolites, especially phenylpropanoids in rice plants, have been shown to induce resistance against nematodes because of the differential presence of these compounds in the roots of resistant and susceptible cultivars (Bajaj and Mahajan, 1977; Giebel, 1970; Pi and Rhode, 1967; Nicholson and Hammerschmidt, 1992).

Based on such results, the experiments were conducted to see the presence of individual phenolic acids and indole acetic acid (IAA) in healthy and nematode-infected rice plants by High Performance Liquid Chromatography (HPLC).

Materials and Methods

Seed Bed Preparation

The seed bed was prepared by ploughing the field thrice. The seeds (30 kg/ha) pretreated with 3 g thiram / 2 g carbendazim + 1.5 g planotomicine were sown in 500 m^2 area of the field in the month of May-June. Seedlings (20-25 days old) were transplanted in one hectare of land which was kept ready by applying 120-150 kg nitrogen (N), 60 kg phosphorus (P) and 40 kg potash (K). The P and K were applied as basal application while N as top dressing in two splits. After 2-3 days of transplanting, the field was irrigated to maintain water level 5 cm in the field. The field was irrigated to avoid crack development. Weeds were managed by using Butachlor/thiobencarb @ 1.0-1.5 kg/ha. Timely weeding was done twice 20-40 days after transplanting. Nitrogen was applied @ 30 kg / ha 20 days after transplanting as top dressing. Rotary weeder was used in rows for weeding the field. Second top dressing of N 30 kg/ha was done after 40 days of transplanting.

Three healthy and infected plants of rice (cultivar MUT-7029) from five plots of rice grown on Agriculture Research Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India were randomly selected. Similar sampling was also done for infected plant roots after removing the nematode galls. The samples were pooled together to make one sample, each of upper leaves, lower leaves, sheath, roots and root galls for extraction of phenolic acids. One gram of each freshly harvested sample was macerated in a pestle and mortar and finally crushed samples were suspended in 5 ml of 80% ethanol. The samples were collected in screw-capped sample tubes and suspension was subjected to ultrasonication (Branson Sonifier, USA) for 15 minutes at 4^0 C followed by centrifugation at 7500 rpm for 15 minutes. The clear greenish supernatant was subjected to charcoal treatment to remove the pigments from each sample and then the clear supernatant was transferred to sample tubes. The residue was re-extracted twice and supernatant was pooled prior to evaporation under vacuum (Buchhi Rotavaper Re-Type). The dried samples were re-suspended in 1.0 ml HPLC grade methanol by vortexing and filtered through membrane filter (pore size 0.45 µm millipore) before HPLC analysis.

High Performance Liquid Chromatographic (HPLC) analysis

Prepared samples were analyzed through High Performance Liquid Chromatography (HPLC) as per Singh et al. (2002) with HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with two Shimadzu LC-10 ATVP reciprocating pumps and an UV-VIS detector (Shimadzue SPD-10 AVP) and integrator and class VP software for data processing and analysis. Reverse phase chromatographic analysis was carried out in an isocratic mode by using a C-18 reverse phase HPLC column (250 x 4.6 mm id, particle size 5 µm), Luna 5 µ C-18 (2), Phenomenex, USA at 25^0 C. Running conditions included mobile phase methanol with 0.4 % acetic acid (66%), flow rate 1.0 ml / min, injection volume 5 µl and detection at 290 nm. Samples were injected thrice in the sample loop and the means of the peak areas of individual compounds were taken for quantification. Tannic, gallic, caffeic, ferulic, vanillic, o-coumeric, cinnamic, salicylic and indole acetic acid (IAA) were used as internal and external standards. Phenolic compounds and IAA and salicylic acid present in the samples were identified by comparing retention time (Rt) of standard as well as by co-injection. Concentrations were calculated by comparing peak areas of the reference compounds with those in the samples run under same eluting conditions.

Table 1. Phenolic and Salycilic acid content (µg/g fresh weight) in healthy and root-knot nematode (*Meloidogyne graminicola*)-infected transplanted rice (*Oryza sativa*) plants

Plant parts	Healthy Plant Parts									Root Knot-Infected Plant Parts								
	TA	GA	Caf-	VA	FA	OcouA	IAA	CA	SA	TA	GA	Caf-A	VA	FA	OCou	IAA	CA	S
Upper Leaf	3.85	140.3	UDL	1.70	16.4	0.03	UDL	0.58	0.44	UDL	190.68	10.77	UDL	10.04	1.30	UDL	0.13	0.
Lower Leaf	14.91	73.94	UDL	UDL	10.99	0.57	0.46	0.24	0.40	21.96	112.62	11.11	11.79	9.77	6.16	3.41	0.70	3.
Leaf Sheath	3.98	8.6	2.36	3.43	3.54	0.12	0.16	0.02	UDL	1.52	26.84	1.93	7.92	4.16	2.15	0.61	0.13	0.
Root	1.86	3.46	UDL	1.09	3.39	0.40	0.02	UDL	0.08	1.23	36.95	6.48	8.66	11.14	0.25	0.79	0.03	0.
Root without knot	-	-	-	-	-	-	-	-	-	1.16	29.30	6.48	3.54	11.64	1.13	0.09	UDL	0.

TA: Tannic acid, GA: Gallic acid, Caf-A; Caffeic acid, VA: Vanilic acid, FA: Ferulic acid, O-cou-A: O-Coumeric acid, IAA: Indole acetic acid, CA: Cinnamic acid and SA: Salicylic acid, UDL: Under detectable level

Results

HPLC analysis of phenolic acids in healthy and root-knot-infected plant parts of rice indicated that phenolic acids were highly variable in both the samples. Upper leaves of healthy plants had seven phenolic acids in which gallic acid was maximum (140 µg/g fresh wt) followed by ferulic (16.4 µg/g), tannic (3.85 µg/g) and vanillic (1.70 µg/g) acids. O-coumeric, cinnamic and salicylic acids were in traces. However, upper leaves of root-knot-infected plants had six phenolic acids in which gallic acid was maximum (190.68 µg/g) followed by caffeic (10.77 µg/g), ferulic (10.04 µg/g) and o-coumeric (1.30 µg/g) acids. Salicylic and cinnamic acids were in traces.

Lower leaves of healthy plants also had seven phenolic acids but their amounts were variable as compared to healthy upper leaves. The gallic acid was maximum (73.94 µg/g fresh wt) followed by ferulic (10.99 µg/g) and tannic (14.93 µg/g) acids. o-coumeric, cinnamic and salicylic acids and IAA were in traces. However, the lower leaves of root-knot-infected plants had nine phenolic acids, in which gallic acid was maximum (112.62 µg/g) followed by tannic (21.96 µg/g), vanillic (11.79 µg/g), caffeic (11.11 µg/g), ferulic (9.77 µg/g), o-coumeric (6.16 µg/g) and salicylic (3.78 µg/g) acids and IAA (3.41 µg/g). Cinnamic acid was in trace.

Leaf sheath of healthy plants had eight phenolic acids where gallic acid was maximum (8.6 µg/g) followed by tannic (3.98 µg/g), ferulic (3.54 µg/g), vanillic (3.43 µg/g) and caffeic (2.86 µg/g) acids but o-coumeric and cinnamic acids and IAA were detected in traces. The root-knot-infected leaf sheath had nine phenolic acids where gallic acid was maximum (26.84 µg/g) followed by vanillic (7.92 µg/g), ferulic (4.16 µg/g), o-coumeric (2.15 µg/g) and tannic (1.52 µg/g) acids but other phenolic acids like cinnamic, salicylic and IAA were present in traces.

Roots of healthy plants had seven phenolic acids where gallic acid was detected maximum (3.46 µg/g) followed by ferulic (3.39 µg/g), tannic (1.86 µg/g) and vanillic (1.09 µg/g) acids. o-coumeric, cinnamic and IAA were found in traces. Root-knot-infected roots had nine phenolic acids. Gallic acid was detected maximum (36.95 µg/g) followed by ferulic (11.14 µg/g), vanillic (8.66 µg/g), caffeic (6.84 µg/g) and tannic (1.23 µg/g) acids. Cinnamic, o-coumeric, salicylic acids and IAA were in traces. Phenolic acids in infected root without root-knot had eight phenolic acids where gallic and was maximum (29.30 µg/g) followed by ferulic (11.64 µg/g), caffeic (6.48 µg/g), vanillic (3.54 µg/g), tannic (1.16 µg/g) and o-coumeric (1.13 µg/g) acids. Salicylic and IAA were present in traces (Table 1).

Discussion

The rhizosphere of plants is a microbially dense area where the roots must compete with the root systems of the neighboring plant species for space, water and mineral nutrients as well as soil-borne microorganisms, viz., bacteria, fungi and nematodes that feed on abundant source of organic material (Ryan and Delhaize, 2001). Plants defend themselves from pathogen using a variety of mechanisms including rapid induction of localized necrosis at the site of infection, e.g., hypersensitive response (HR), increased expression of defense-related proteins or pathogenesis related proteins (PRs), production of antimicrobial compounds, lignin formation and the oxidative burst (van Loon et al., 1998; Nicholson and Hammerschmidt, 1992; Sticher et al., 1997). Yamada (2006) reported that during nematode infection plants show biochemical changes leading to wilting and death of pine trees due to blockage of water conduction but in resistant pine trees plants produced inhibitins / or phytoalexin including histological changes. Mohamed and Hasabo (2005) evaluated five different cotton (*Gossypium barbadense*) cultivars against root-knot nematode (*M. incognita*) under greenhouse conditions and found that only Giza 86 cultivar showed resistance because of induced biochemical changes after nematode infection and sequential development of chitinase, peroxidase and acid phosphatase in both cultivars (Giza 86 and Giza 89 race 2000) but the level of these compounds was much higher in roots of Giza 86 as compared to susceptible Giza 89 race 2000 which supported lower population of *M. incognita*. These results indicate the role of these enzymes in the resistance mechanism of cotton against nematode infection. Devrajan and Srenivasan (2002) reported the synthesis of peroxidase and polyphenol oxidase (catechol oxidase) in the roots of banana (Musa sp. cv. Robusta) due to infection with *M. incognita* and *Paecilomyces lilacinus*. Sundararaju and Suba (2006) also concluded that the biochemical and molecular changes associated with resistance reaction of banana against root-lesion incited by *Pratylenchus coffeae* and root-knot nematodes (*Meloidogyne incognita*) on five varieties of banana, viz., Nendran, Robusta, Pisang Jari Buaya, Karthobiumtham and *Musa balbisiana*. The activity of polyphenol oxidase (catechol oxidase) was reduced in infected cultivars. Nendran and Robusta cultivar showed higher protein concentration, mRNA and peroxidase as compared to cultivars *M. balbisiana*, Karthobiumtham and Pisang Jari Buaya. The accumulation of phenolic acids was also increased up to 56% in cv. Nendran after nematode infection as compared to Karthobiumtham (2%). Patel et al. (2001) reported that the

Meloidogyne spp. have ability to induce synthesis of peroxidase, polyphenol oxidase and total phenol in roots of chickpea (*Cicer arietinum*) but the leaf chlorophyll content was decreased. Borah and Phukan (2006) reported that the plants inoculated with VAM + nematode significantly decreased the number of galls, egg masses and final nematode population in soil; increased nitrogen, crude protein, phosphorus, potassium, total sugar and total phenol, total free amino acids in the roots of brinjal as compared to nematode alone. Rao et al. (1984, 1988) also reported that the rice plants showed nutritional deficiencies such as total sugar, protein, IAA, cytokinin and thyamine and phenol due to infection by *Heterodera oryzicola* and *M. graminicola*. According to Mishra and Mohanty (2007) maximum phenolic acids were observed in resistant cultivar Ramakrishna by 104.2%. Even in susceptible (var. Annapurna) and moderately resistant (Monika) varieties the total phenol was higher than their healthy plants. In the present study it is quite interesting to note that the amount of most of the phenolic acids increased in infected plant parts by several folds. While the healthy roots had several phenolic acids and the roots without galls also showed several fold high amount of phenolic acids than healthy ones. The high amount of phenolic acids in infected plant parts is because of stress induced by the pathogen. The roots after removing the galls showing high amount of these acids is also because of the stress already induced by the pathogen in the host.

Review of literature reveals that there is no study on the individual phenolic acids as well as IAA changes in different plant parts of rice infected with *M. graminicola*. Phenolic acids as well as indole acetic acid (IAA) were increased in most cases after nematode infection in different plant parts of rice as compared to control. HPLC analysis of rice plant parts indicated that the IAA is synthesized in lower leaves and then after it is translocated to the site of infection.

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