

Histopathology of the rice root-knot nematode, *Meloidogyne graminicola*, on *Oryza sativa* and *O. glaberrima*

Ma. Teodora Nadong CABASAN^{1,2,3,*}, Arvind KUMAR³,
Stéphane BELLAFFIORE⁵ and Dirk DE WAELE^{1,3,4}

¹ *Laboratory of Tropical Crop Improvement, Department of Biosystems, Faculty of Bioscience Engineering, University of Leuven (KU Leuven), Willem de Croylaan 42, 3001 Heverlee, Belgium*

² *Department of Biological Sciences, College of Arts and Sciences, University of Southern Mindanao, 9407 Kabacan, Cotabato, Philippines*

³ *International Rice Research Institute (IRRI), DAPO Box 7777, Metro Manila, Philippines*

⁴ *School of Environmental Sciences and Development, North-West University, Private Bag X6001, 2520 Potchefstroom, South Africa*

⁵ *IRD Institut de Recherche pour le Développement, UMR-186 Résistance des Plantes aux Bioagresseurs, IRD-Centre de Montpellier, Avenue Agropolis 911, BP 64501, F-34394 Montpellier Cedex 5, France*

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Summary – The root-knot nematode, *Meloidogyne graminicola*, can cause substantial rice yield losses. Understanding the mechanisms of resistance to this nematode species in known resistant rice genotypes may help to improve rice genotypes, aiming at developing and implementing environment-friendly and cost-effective nematode management strategies. Using susceptible and resistant rice genotypes, a comparative analysis of histological response mechanisms was made during two phases of the nematode colonisation: *i*) root penetration; and *ii*) subsequent establishment and development by *M. graminicola* second-stage juveniles (J2). Two types of defence response mechanisms could be distinguished in the resistant rice genotypes. The early defence response consisted of a hypersensitive response (HR)-like reaction in the early stage of infection characterised by necrosis of cells directly affected by nematode feeding. This HR-like reaction was observed only in the *M. graminicola*-resistant *Oryza glaberrima* genotypes and not in the *M. graminicola*-susceptible *O. sativa* genotypes. The late defence response took place after the induction of giant cells by the J2. Giant cells usually collapsed and degenerated before J2 developed into adults. Structural features of the roots of the susceptible *O. sativa* showed greater root and stele diam. and cortex thickness than the resistant *O. glaberrima* genotypes. Desired features of plants with resistance to *M. graminicola* elucidated in this study can be used for selection of plants for breeding programmes.

Keywords – giant cells, hypersensitive response-like reaction, penetration, resistance, root morphology, susceptibility.

The rice root-knot nematode, *Meloidogyne graminicola*, is one of the most important nematode species associated with rice (De Waele & Elsen, 2007). This nematode species is an obligate sedentary endoparasite that can cause extensive damage to plant growth and yield of rice crops (Jain *et al.*, 2012). The motile second-stage juveniles (J2) of root-knot nematodes (*Meloidogyne* species) penetrate the roots usually near the root tips. They migrate intercellularly, establish a feeding site in the zone of differentiation of the vascular cylinder and develop into sessile and swollen females. During feeding site formation some cells become hypertrophied, with intense cellular

multiplication and hyperplasia leading to giant cells and gall formation (Williamson & Hussey, 1996). The post-penetration incompatibility in resistant crops is usually associated with the sub-optimal development of giant cells that fail to develop or develop only partially with limited hyperplasia and hypertrophy (Orion *et al.*, 1980).

Compatibility between a host plant and a nematode during root penetration and feeding site formation is vital for the establishment of a successful host-parasite relationship. Better understanding of the host plant-parasite interactions is important in order to use resistant plants efficiently in breeding programmes. The use of resis-

* Corresponding author, e-mail: teodora.cabasan@gmail.com

tant plants is considered one of the most appropriate strategies for the management of *M. graminicola* in infested rice fields (Dutta *et al.*, 2012). In several host-nematode relationship studies, such as in carrot-*M. javanica*, soybean/pepper/cowpea/chili pepper-*M. incognita*, grapevine/peanut-*M. arenaria* and rice-*M. graminicola*, it has been reported that penetration of J2 is less in resistant plants than in susceptible plants (Huang, 1986; Niblack *et al.*, 1986; Bleve-Zacheo *et al.*, 1998; Anwar & McKenry, 2000; Bendezu & Starr, 2003; Das *et al.*, 2008; Moon *et al.*, 2010; Cabasan *et al.*, 2012). In resistant plants, J2 may penetrate but their development may be delayed in the roots, resulting in limited reproduction. This has been observed in resistant maize and cotton genotypes infected with *M. incognita*, soybean with *M. arenaria*, potato with *M. fallax* and rice with *M. graminicola* (Windham & Williams, 1994; Pedrosa *et al.*, 1996; Kouassi *et al.*, 2004; Faske & Starr, 2009; Cabasan *et al.*, 2012).

Another common response to root-knot nematode infection in resistant crops is an early hypersensitive response (HR)-like reaction resulting in cell death, which prevents nematode feeding site formation and development, leading to nematode death. In this case, a localised necrosis is visible within a few days of penetration near the anterior end of the nematode (Dropkin *et al.*, 1969; Paulson & Webster, 1972). Evidence of early HR-like reaction upon root-knot nematode infection was observed *inter alia* in tomato, pepper, coffee, chili pepper and myrobalan plum (Williamson & Hussey, 1996; Anthony *et al.*, 2005; Pegard *et al.*, 2005; Albuquerque *et al.*, 2010; Moon *et al.*, 2010; Khallouk *et al.*, 2011).

Resistance to *M. graminicola* has been identified in African rice, *Oryza glaberrima* (Plowright *et al.*, 1999; Soriano *et al.*, 1999), whilst differences in host response upon *M. graminicola* infection were observed among Asian rice, *O. sativa* genotypes (Jena & Rao, 1977a, b; Sharma-Poudyal *et al.*, 2004; Prasad *et al.*, 2006). Cabasan *et al.* (2012) compared soil migration, penetration, development and reproduction of *M. graminicola* on susceptible *O. sativa* and resistant *O. glaberrima* genotypes. Reduced penetration, delayed nematode development and lower reproduction are common useful features of resistant rice genotypes.

The objective of this study was to assess the mechanism(s) of resistance by comparing, at the cellular level, the response of susceptible *O. sativa* genotypes and resistant *O. glaberrima* genotypes upon *M. graminicola* infection. Variations in the morphological structures and fea-

tures of the root tissues of susceptible and resistant rice genotypes were also investigated.

Materials and methods

NEMATODE INOCULUM

An isolate of *M. graminicola* originally collected from an infested rice field in Batangas, Philippines, was cultured on *O. sativa* genotype UPLRi-5 in the glasshouse at the International Rice Research Institute (IRRI), Los Baños, Philippines. J2 were extracted from infected roots using a mistifier (Seinhorst, 1950) and quantified.

RICE GENOTYPES

The four *M. graminicola*-resistant *O. glaberrima* genotypes (TOG5674, TOG5675, CG14, RAM131) and the two *M. graminicola*-susceptible *O. sativa* genotypes (IR64, UPLRi-5) were obtained from IRRI's rice seed collection. Resistance and susceptibility of these rice genotypes have been studied previously (Plowright *et al.*, 1999; Soriano *et al.*, 1999; Cabasan *et al.*, 2012). Pregerminated seeds were sown in a heat-sterilised sandy loam soil in pots (75 cm³ volume) and grown in a controlled growth chamber at 29/26°C (day/night temperature) with a 12 h photoperiod. The soil was kept saturated during the duration of the experiments but not flooded. The pots were arranged in a complete randomised block design.

HISTOPATHOLOGY

Two-week-old seedlings were inoculated with 225 *M. graminicola* J2 plant⁻¹, into 1 cm deep holes around the stem base of the plants, and maintained as referred above. Six plants of each rice genotype were uprooted at 3, 7, 14, 21 and 28 days after inoculation (DAI) and prepared for histological observation. Each root system was first gently washed free of adhering soil. Two to six galled root segments per plant (0.5-1.0 cm long) were selected, excised and fixed in formalin-acetoalcohol (FAA). Then, the root segments were dehydrated in a graded series of ethanol (50-100%, 1 h at each concentration), infiltrated with xylene and embedded in paraffin wax. Serial longitudinal and cross-sections (5 µm) were cut with a Leica RM2235 rotary microtome, transferred to glass slides and left for about 40 h on a hot plate to dry. Paraffin was removed by soaking the sections in xylene and rinsed with ethanol followed by distilled water (de Neergaard *et al.*, 2000; Hoppert, 2003).

Sections were stained with toluidine blue and viewed using a Zeiss Axioplan 2 light microscope equipped with a digital camera. Unstained sections were viewed and photographed using a UV filter set BX53 light microscope with 365 nm excitation and 420 nm emission to detect yellow and orange autofluorescence indicating the presence of phenolic compounds (Pegard *et al.*, 2005).

ROOT MORPHOLOGY

To study the root morphology of the four resistant and two susceptible rice genotypes, six plants per genotype were grown as described above in soil free of nematodes. Twenty-one days after emergence the plants were up-rooted, the root systems gently washed and the length of the three longest roots measured. Fresh root weights, total number of nodal roots and number of lateral roots on three nodal roots were also recorded. Subsamples of three nodal roots per plant were stored in 25% ethanol. Roots stored in ethanol were hand-sectioned at 5-10 mm from the root tip under a stereomicroscope, stained for suberin with a saturated solution of Sudan III (Zeier *et al.*, 1999) and transferred to glass slides. Stained sections were heated for 10 min at 70°C, cleared with glycerol/water (1:1 v/v) and observed using a Zeiss Axioplan 2 light microscope equipped with a digital camera. Root and stele diam., cortex thickness (between epidermis and endodermis), xylem vessel diam. and thickness of the outer part of the root (including sclerenchyma, hypodermal and epidermal layers) were measured with Image J software (Abramoff *et al.*, 2004). Suberisation of the sclerenchyma layer was rated by three individuals based on the intensity of the stain on a scale of 0-5 according to the relative staining intensity observed across all sections (Henry *et al.*, 2012). Sclerenchyma was classified according to types: Type I has a single cell layer in the sclerenchyma region, Type II has a thickened cell wall in the first outer parenchyma cell in the cortex, Type III has a partly doubled cell layer in the sclerenchyma and Type IV has a doubled cell layer in the sclerenchyma (Kondo *et al.*, 2000).

STATISTICAL ANALYSIS

Root data were subjected to analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test for mean comparison. Statistical significance was determined by $P < 0.05$. Statistical analyses were performed using the STATISTICA® software (StatSoft).

Results

HISTOPATHOLOGY OF INFECTED ROOTS OF SUSCEPTIBLE RICE GENOTYPES

Histological observations indicated that at 3 DAI, numerous *M. graminicola* J2 had reached the hypodermis, cortex and stele of the root tip of UPLRi-5 (Fig. 1A) and IR64 inducing slight root swelling. Formation of feeding cells in the perimeter of, and also inside, the vascular cylinder was evident at 7 DAI. Cortical parenchyma proliferation, asymmetry of the stele and formation of healthy giant cells induced by the swollen juveniles were observed (Fig. 1B) resulting in galling of roots. Giant cells had uniformly dense cytoplasm and contained several small nuclei, usually three to five per giant cell. The number of giant cells at each feeding site varied from five to eight. At 14 DAI, clusters of egg-laying females (ELFs) were surrounding the giant cells obliterating and disorganizing the stele (Fig. 1C). At this sampling date, an increase in giant cell wall thickness and presence of secondary cell wall ingrowths were observed (Fig. 1D). At 21-28 DAI, the structure of the feeding sites did not show distinct differences compared with the earlier stages of infection. However, the ELFs had increased in size and laid numerous eggs in the cortex (not shown in the figures). Mechanical damage of the parenchymal and vascular tissues was evident.

HISTOPATHOLOGY OF INFECTED ROOTS OF RESISTANT RICE GENOTYPES

At 3 DAI, few J2 were observed in the sub-apical region of the roots of the four resistant rice genotypes. The J2 that had penetrated the roots migrated towards the vascular cylinder inducing the formation of permanent feeding sites. During histological observations conducted from 3-28 DAI, two types of defence responses could be distinguished. An early defence response consisting of HR-like reaction was observed in some roots at 3 and 7 DAI. The portion of the J2 localised within the hypodermis, cortex and stele showed a dark blue-green (stain) colour indicating necrosis surrounded by collapsed cytoplasm. This HR-like reaction resulted in the death of the invading J2, which appeared distorted in CG14 (Fig. 2A) and in other resistant rice genotypes. A late defence response was observed after some J2 had developed into ELFs, which were feeding upon giant cells. These giant cells had a degraded cytoplasm, high vacuolation and were surrounded by necrotic cells

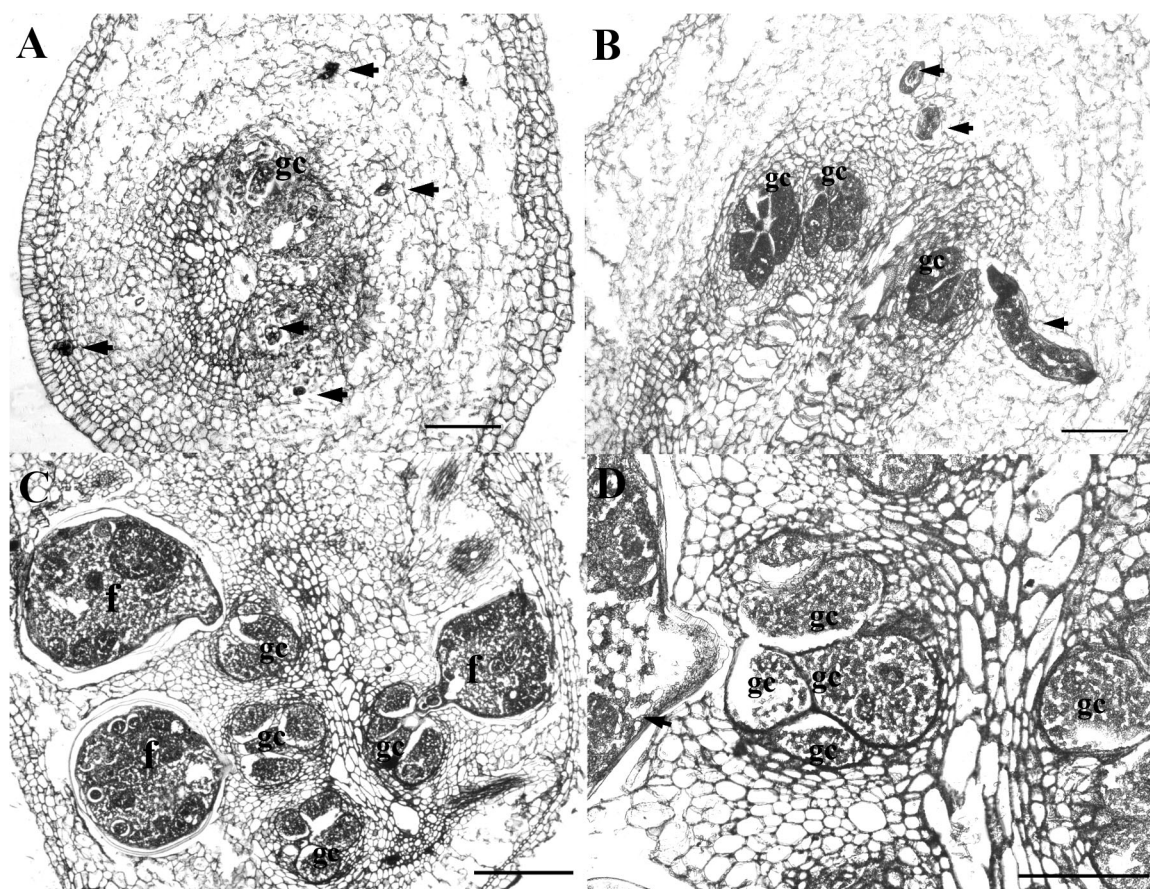


Fig. 1. Anatomical changes induced by *Meloidogyne graminicola* on rice (*Oryza sativa*) UPLRi-5. A: Cross-section of a root tip showing numerous second-stage juveniles (arrowheads) at 3 days after inoculation (DAI); B: Root cross-section showing formation of giant cells (gc) and nematode juvenile (arrowhead) with the head localised among the giant cells at 7 DAI; C: Root cross-section showing the sectioned body of swollen females (f) feeding upon giant cells rich in granules and densely stained cytoplasm at 14 DAI; D: Egg-laying female (arrowhead) with the head surrounded by giant cells with thickened cell walls at 14 DAI. (Scale bars, A, B, C = 100 μm ; D = 200 μm .)

(Fig. 2B). At 14 DAI, the giant cells deteriorated further and the ELF's were smaller than those observed at the same time in the roots of the susceptible rice genotypes. A fast senescent process of the giant cells began resulting in loss of their granular cytoplasm (Fig. 2C). At 21 and 28 DAI, most of the giant cells were on the verge of collapse. They were devoid of cytoplasm and the cell walls between the giant cells were also thin (Fig. 2D). J2 that reached maturity developed into ELF's that were smaller in size compared to those observed in susceptible rice genotypes. Degradation of the giant cells was the most common defence response observed in the roots of the resistant rice genotypes examined. Some roots showed both HR-like reaction and degradation of the giant cells.

Histological changes in resistant rice genotypes inoculated with *M. graminicola* were examined microscopically under UV light in comparison with the susceptible rice genotypes. Root sections of the resistant rice genotypes exhibited a yellow autofluorescence, induced by the presence of aromatic compounds, observed in the cells surrounding the nematodes at 7 DAI (Fig. 3B). Accumulations of aromatic compounds have been associated with resistance to nematodes. This autofluorescence was not observed in root sections of the susceptible rice genotypes (Fig. 3A). Autofluorescence in root sections of the resistant rice genotypes was also observed at the late infection stage when the nematodes had reached maturity. At 14 DAI, the autofluorescence became intense yellow

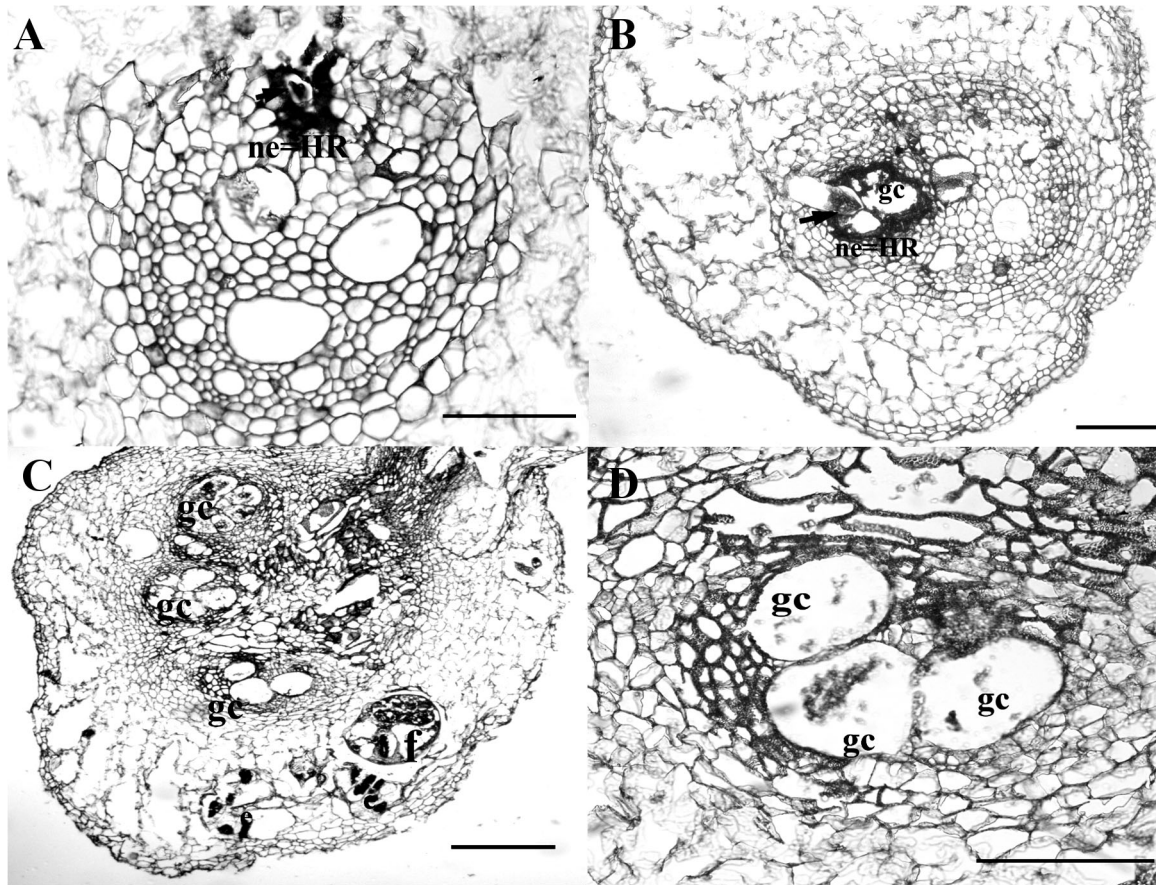


Fig. 2. Anatomical changes induced by *Meloidogyne graminicola* on rice (*Oryza glaberrima*) CG14. A: Root cross-section showing necrotic tissues (ne) at the periphery of the stele surrounding the sectioned dead body of a nematode juvenile. Note accumulation of dark-stained material, possibly aromatic compounds (dark blue-green colouration) at 7 DAI; B: Root cross-section showing necrotic areas surrounding small giant cells at 14 DAI; C: Root cross-section showing egg-laying female and giant cells devoid of cytoplasm at 14 DAI. Note disorganisation of the stele induced by the nematode parasitisation; D: Giant cells with thin cell walls and devoid of cytoplasm at 14 DAI. (Scale bars, A, B, C = 100 μm ; D = 200 μm .)

and orange, and substantial necrosis was observed around the giant cells (Fig. 3D). No yellow or orange autofluorescence was observed in root sections of the susceptible rice genotypes, indicating the absence of necrosis (Fig. 3C).

ROOT MORPHOLOGY OF SUSCEPTIBLE AND RESISTANT RICE GENOTYPES

No significant differences in mean root length and weight, and the average number of nodal and lateral roots were observed between the non-inoculated susceptible and resistant rice genotypes (data not shown). The susceptible rice genotypes had on average a significantly larger root diam. than the resistant rice genotypes ($P < 0.05$;

724 vs 561 μm , respectively). The cortex thickness of the susceptible genotype UPLRi-5 was significantly higher than all the other genotypes ($P < 0.05$) and the susceptible genotypes IR64 was not statistically different from resistant genotype RAM131 ($P > 0.05$). Although stele diam. of susceptible genotypes was not statistically different from resistant genotype TOG5675 ($P > 0.05$), there was a trend in susceptible rice genotype to show greater values. No significant differences ($P > 0.05$) were observed on the thickness of the outer part of the root, xylem vessel diam. and suberisation rate of the sclerenchyma layer (Table 1). All rice genotypes examined had Type I sclerenchyma or a single cell layer in the sclerenchyma region.

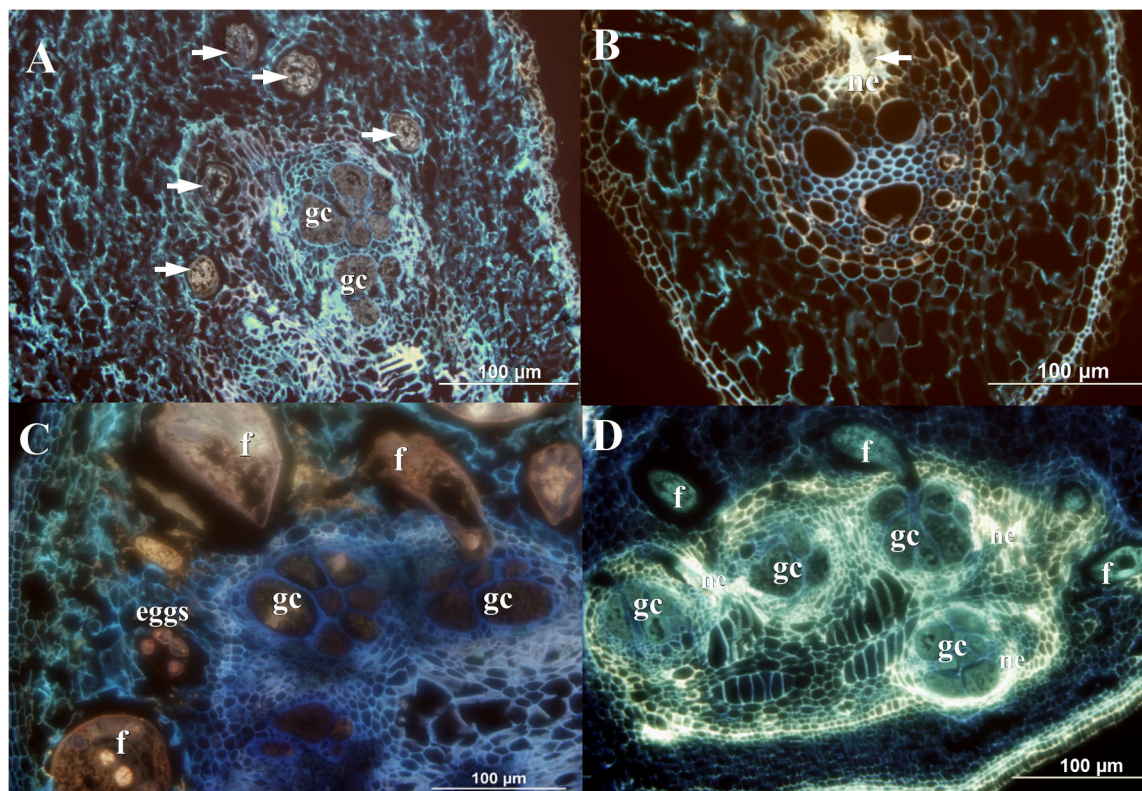


Fig. 3. Cross-sections of the roots of susceptible *Oryza sativa* genotype UPLRi-5 (A, C) and resistant *O. glaberrima* genotype CG14 (B, D) after inoculation with *Meloidogyne graminicola*, observed under UV light. Note the fluorescence in the cross-sections of the resistant *O. glaberrima* roots and the lack of fluorescence in the cross-sections of susceptible *O. sativa* roots at 7 (A, B) and 14 (C, D) DAI. Abbreviations: ne = necrosis, gc = giant cells, f = female, arrowheads = nematode. This figure is published in colour in the online edition of this journal, which can be accessed via <http://booksandjournals.brillonline.com/content/15685411>.

Table 1. Morphological parameters of the roots of uninfected *Meloidogyne graminicola*-susceptible and -resistant rice genotypes, 21 days after emergence in a water-saturated sandy-loam soil in pots grown in an indoor growth chamber.

Rice genotype	Root diam. (μm)	Cortex thickness (μm)	Stele diam. (μm)	Outer portion of the root (μm)	Xylem diam. (μm)	Suberisation (scale 1-5)
TOG5674 ^R	556 \pm 87 ab	166 \pm 33 a	152 \pm 28 a	42 \pm 7 a	27 \pm 5 a	3.7 ab
TOG5675 ^R	579 \pm 68 a	165 \pm 28 a	164 \pm 19 ac	50 \pm 9 b	41 \pm 8 c	3.4 b
CG14 ^R	503 \pm 105 b	154 \pm 46 a	123 \pm 19 b	40 \pm 5 a	36 \pm 6 b	4.1 a
RAM131 ^R	607 \pm 151 a	202 \pm 61 b	136 \pm 33 b	42 \pm 13 a	25 \pm 6 a	3.7 ab
IR64 ^S	679 \pm 164 c	210 \pm 55 b	168 \pm 45 c	48 \pm 9 b	29 \pm 7 a	4.0 a
UPLRi-5 ^S	769 \pm 305 d	261 \pm 108 c	174 \pm 76 c	42 \pm 11 a	32 \pm 13 a	3.8 ab

Values are the mean \pm standard deviation. In a column, means followed by the same letter are not significantly different according to Tukey's honestly significant difference test ($P > 0.05$). R = resistant rice genotype; S = susceptible rice genotype.

Discussion

In the resistant rice genotypes in our study, some J2 that had penetrated the roots at 3 DAI were already in contact

with the stele and the formation of giant cells had begun. In the *M. graminicola*-resistant rice genotypes studied by Jena & Rao (1977b), J2 in contact with the stele were observed later at 12 DAI while the formation of giant

cells was observed at 16-18 DAI. Several factors could be at the origin of this difference, including differences in the pathogenicity of the *M. graminicola* populations used or rice genotype-dependent differences. In our study, the resistant rice genotypes were *O. glaberrima* while *O. sativa* was used in the study of Jena & Rao (1977b). In the susceptible rice genotypes in both studies, J2 were observed in contact with the stele within 3 DAI.

Localised necrosis close to the nematodes, suggesting a HR-like reaction, was observed within a few days after nematode penetration in the resistant rice genotypes in this study. An HR-like reaction upon infection with root-knot nematodes has also been observed in other cereals such as oat and barley (Siddiqui, 1971; Balhadere & Evans, 1995). In *M. graminicola*-resistant rice genotype Hamsa, cell necrosis was observed in the cortex near the head of the J2 (Jena & Rao, 1977b). According to these authors, J2 gained access to the protophloem by damaging the cells of the pericycle and this further delayed the establishment of the feeding sites. However, in our study, pericycle rupture was caused by the proliferation and enlargement of cells, and nematode growth. Giant cells were induced in the resistant rice genotypes after successful establishment of feeding sites by some J2. These giant cells were poorly developed, highly vacuolated and with thin cell walls unlike those in the susceptible rice genotypes. Similar observations have been made in cotton (McClure *et al.*, 1974), coffee (Anthony *et al.*, 2005) and cowpea (Das *et al.*, 2008). Vacuolisation of giant cells in resistant crops has been associated with the accumulation of hydrolases and toxins (Jones, 2001), which leads to cell degradation. In our study, vacuolisation of the giant cells started at 14 DAI, when the fourth-stage juveniles (J4) moulted into ELF's and a reduction in the size of the giant cells results in a lower nutritional status of the feeding sites, which in turn leads to a delayed development of the juveniles, smaller size and lower fecundity of the ELF's (Pedrosa *et al.*, 1996; Cabasan *et al.*, 2012).

The dark blue coloration of material surrounding the nematode in the roots of resistant rice genotypes stained with toluidine blue suggests the accumulation of phenolic compounds. Similar colouration was observed in coffee resistant to *M. exigua* (Silva *et al.*, 2013). Fluorescence observation in root tissues of *M. graminicola*-resistant *O. glaberrima* genotypes under UV light confirmed the presence of aromatic compounds. In related studies, high intensity of fluorescence in root tissues of prunus and pepper genotypes resistant to *Meloidogyne* spp. indicates high contents of phenolic compounds such as chlorogenic

acid (Pegard *et al.*, 2005; Khallouk *et al.*, 2011). The high levels of phenolic compounds in tomato confer resistance to nematode infections (Bajaj & Mahajan, 1977).

Based upon previous observations (Jena & Rao, 1977a), it was hypothesised that differences in root morphology may also explain differences in host response upon *M. graminicola* infection between susceptible and resistant rice genotypes. In our study, significant differences in some root morphology parameters among the rice genotypes were noticed. In the susceptible rice genotypes, root and stele diameters, and thickness of the cortex were greater than in the resistant rice genotypes as also observed by Jena & Rao (1977a). A greater root diam. may facilitate the penetration and migration of a larger number of nematodes. Furthermore, roots with a wider stele diam. will have more conditions for the establishment of more feeding sites and formation of giant cells near the vascular cylinder. The cell wall ingrowths of the giant cells usually come in contact with the xylem vessels to transport water and nutrients (Abad *et al.*, 2009) but no difference was observed in xylem diam. between susceptible and resistant rice genotypes. The outer portion of the root is the first obstacle for the invading J2 but, in our study, the thickness of the outer part of the root was similar in the susceptible and resistant rice genotypes. Suberin and structural proteins in maize root cells were considered a good indication of the presence of a mechanical obstacle to the penetration of pathogens (Schreiber *et al.*, 1999) but differences in suberisation between susceptible and resistant rice genotypes were not observed.

Our results suggest that resistant rice genotypes display at least two kinds of post-penetration mechanisms of resistance to *M. graminicola* infection: first, an early defence response consisting of a HR-like reaction that was observed at 3 and 7 DAI in some roots, and second, a late defence response consisting of poorly developed giant cells after some J2 had developed into ELF's. The latter was the most common mechanism of resistance observed in all *O. glaberrima*-resistant genotypes in this study. Similarly, coffee genotypes resistant to *M. exigua* exhibit early HR while some nematodes that did not induce HR were able to bypass early defence mechanisms and the host cell defence response was activated later (Anthony *et al.*, 2005). Factors involved in differential timing of expression of resistance genes remain to be determined. The expression of HR-like reaction and/or late defence response in infected roots could indicate that multiple genes are likely to be involved in the resistance of *O. glaberrima* genotypes to *M.*

graminicola such as in pepper with *Me3* gene controlling HR-like response and *Me1* gene responsible for blocking nematode development (Castagnone-Sereno *et al.*, 1996). The role of root morphology in the resistance to *M. graminicola* in *O. glaberrima* genotypes is less clear and needs further study.

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