

Phenols and lignin are involved in the defence response of banana (*Musa*) plants to *Radopholus similis* infection

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Received: 14 October 2013; revised: 26 January 2014

Accepted for publication: 28 January 2014; available online: 28 March 2014

Summary – The role of lignin and phenols in plant defence ranges from preformed characteristic to inducible physical and chemical response against nematode infection. Our study shows the involvement of lignin and phenols in the defence of two newly identified resistant banana (*Musa*) genotypes to burrowing nematode *Radopholus similis* infection. Results were compared with reference resistant and susceptible banana cultivars. Histochemical analysis of root cross sections showed a more extensive secondary cell wall lignification of vascular bundles in *R. similis*-infected plants than in the nematode non-infected plants. Increased extensive lignification was not associated with the cortex cells that are directly attacked by the nematode. This showed that the increased lignification is a general defence response to protect the vascular bundle from damage rather than resisting the nematode development and reproduction at the root cortex. Histochemical localisation showed no preformed phenolic cells in the cortex of the non-infected, *R. similis*-resistant and -susceptible *Musa* genotypes. By contrast, phenolic substances were the major constituents of the nematode-infected necrotic cells. Phenols and lignin contents were also quantitatively assayed. The Folin-Ciocalteu assay confirmed the increase in phenol content of nematode-infected root cells. Phenol content in nematode-infected plants was twice the amount of phenol content in nematode non-infected plants at 3 weeks after infection. This is possibly due to the biosynthesis or accumulation of secondary metabolites such as phenolic phytoalexins in the nematode infection sites of all the banana genotypes. This study clearly demonstrates that phenols and lignin play an important role in the defence mechanisms of *Musa* to *R. similis* infection.

Keywords – burrowing nematodes, histochemistry, resistance, secondary metabolites, susceptibility.

Plants produce more than 100 000 diverse, low molecular weight, secondary metabolites (Dixon, 2001). Secondary metabolites play a major role in plants' interaction with the environment, ensuring successful adaptation and survival (Verpoorte, 2000). Phenolic secondary metabolites such as lignin and phenols were associated with nematode resistance in bananas (Valette *et al.*, 1998; Wuyts *et al.*, 2007). Lignin is the second most abundant natural biopolymer after cellulose, found in all vascular plants (Ferrer *et al.*, 2008) and accounts for approximately 30% of the organic carbon in the biosphere (Boerjan *et al.*,

2003). Lignin deposition strengthens plant cell walls. The plant cell wall is vital for the structural integrity of plants, plant growth, intercellular communication, water movement and pathogen defence (Aquiye *et al.*, 2010).

The resistance of lignin to microbial degradation was expected to provide a hard barrier to invading pathogens (Tronchet *et al.*, 2010). The presence of a higher number of lignified cell walls in the vascular bundles of the *Musa* cv. Pisang Jari Buaya was associated with its resistance to *Radopholus similis* (Fogain & Gowen, 1996). Lower root dry matter was correlated with lower lignin content

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resulting in the susceptibility of the *Musa* cv. Poyo to *R. similis* (Fogain & Gowen, 1996). Higher levels of constitutive lignin contents were observed in *R. similis*-resistant *Musa* cultivars than in the susceptible cv. Grande Naine (Wuyts *et al.*, 2007).

A higher number of preformed phenolic cells was related to the resistance of *Musa* cv. Yangambi km5 to *R. similis* (Fogain & Gowen, 1996). Constitutive presence of lignin, dopamine, flavonoids, caffeic and ferulic acids in cv. Yangambi km5 roots was associated with the resistance to *R. similis*, and Valette *et al.* (1998) hypothesised that they act as chemical barriers for *R. similis* penetration and colonisation.

New banana genotypes were identified as resistant to *R. similis* infection (Dochez *et al.*, 2006). We have confirmed the resistance of four of these newly identified resistant sources in a previous study under glasshouse conditions. Of the four, two genotypes, Long Tavoy (*Musa acuminata* AA, Burmannica subgroup, ITC 0283) and Saba (*Musa* spp. ABB, Saba subgroup, ITC 1138) were the most promising, highly resistant genotypes to *R. similis* infection (Suganthagunthalam *et al.*, 2010). With the aim of characterising the nematode resistance mechanisms in these two new resistant genotypes, we assessed and report the involvement of lignin and phenols against *R. similis* infection. The new, *R. similis*-resistant genotypes were compared with the well-known reference cvs Yangambi km5 (*Musa acuminata* AAA, Ibota subgroup, ITC 1123) (resistant) and Grande Naine (*Musa acuminata* AAA, Cavendish subgroup, ITC 1256) (susceptible) to *R. similis* infection.

Materials and methods

PLANTING MATERIAL

All *Musa* genotypes were initially obtained from the *Musa* germplasm collection maintained at the International Transit Centre (ITC), KU Leuven, Leuven, Belgium. The plant material was proliferated, regenerated and rooted in test tubes on the recommended medium by following standard protocols (Banerjee & De Langhe, 1985). The plantlets were grown in growth chambers at 28°C and 16 h photoperiod.

NEMATODE INOCULUM

A population of *R. similis* originally isolated from banana roots in Uganda was used in the experiments. This

population was maintained and multiplied monoxenically on sterile carrot discs at $25 \pm 1^\circ\text{C}$ in the dark (Speijer & De Waele, 1997). The population from Uganda was characterised by a high reproductive fitness (Fallas *et al.*, 1995). To obtain the inoculum, *R. similis* was extracted from the carrot discs by the maceration-sieving technique (Speijer & De Waele, 1997).

EXPERIMENTAL SET-UP

Six-week-old rooted tissue culture plantlets were planted in 1 l pots filled with sand and potting soil (2:1). Eight weeks after planting, two sets of eight plants of each *Musa* genotype were inoculated with approximately 1000 living vermiform nematodes by pipetting 4 ml of the nematode suspension into inoculation holes made in the soil near the plant root zone. Two sets of eight non-infected plants of each *Musa* genotype were included as control plants. The plants were arranged according to a randomised block design.

The plants were grown under glasshouse conditions and fertilised at 10-day intervals throughout the experiment. In the glasshouse, the ambient day and night temperatures were 27 and 20°C, respectively, the relative humidity was 80% and the photoperiod was 12 h.

SAMPLING AND ASSESSMENT OF NEMATODE INFECTION

One set of eight *R. similis*-infected and one set of eight non-infected plants were uprooted at 3 weeks after inoculation. The second set of plants was uprooted at 6 weeks after inoculation. At both sampling times, the root systems were carefully washed free of soil (without destroying the root epidermis) under running tap water. The fresh root and shoot weights of the whole root systems were measured. About half of the fresh roots were used for histochemical staining and nematode extraction. The remaining roots were snap frozen in liquid nitrogen and stored at -80°C for quantitative assays of lignin and total phenols. Necrotic cells from *R. similis*-infected roots (ca 0.5 g) were manually dissected under a stereoscopic microscope for nematode extraction. Nematodes were extracted from the micro-dissected lesions and the nematodes were counted under a stereoscopic microscope.

HISTOCHEMICAL STAINING OF ROOT CROSS SECTIONS

Thin, free hand-cut root cross sections of *R. similis*-infected and non-infected *Musa* genotypes were used for

the histological staining of lignin and total phenols. For the localisation of lignin (syringyl and guaiacyl units), root cross sections were fixed in Wiesner reagent, 2% phloroglucinol in ethanol/water (95:5, v/v) for 10 min and mounted in 37% HCl on glass slides. Wiesner reagent reacts with cinnamaldehyde end groups in the lignin, resulting in the cationic chromophore, which appears as a burgundy-red compound (Vermerris & Nicholson, 2006). For the localisation of the syringyl units of lignin, root cross sections were immersed in Mäule's reagent 1% KMnO_4 for 30 min, rinsed with distilled water and destained using 20% HCl for 2 min. The stained sections were mounted in 10% NH_3 on glass slides. The presence of syringyl units results in the development of a deep red colour while the absence of syringyl units results in the development of a yellow colour (Vermerris & Nicholson, 2006).

For the localisation of total phenols, fresh root cross sections were treated with 0.5% (v/v) toluidine blue in 2.5% Na_2CO_3 at pH 9 for 10 min and rinsed with 70% ethanol. Stained root sections were observed under a bright field transmitted light microscope. Phenolic cells stain as deep blue (Valette *et al.*, 1997).

Fluorescent flavonoids and lignified cell walls were detected by Neu's reagent (Valette *et al.*, 1998). Fresh root cross sections were stained with freshly prepared 0.25% (w/v) diphenylboric acid 2-aminoethyl ester (DPBA) in MilliQ water with 0.02% (v/v) triton-x-100 for 2 min. Sections were observed immediately under an epifluorescence microscope with a DAPI filter (excitation 340–380 nm, suppression LP 430 nm) and a FITC filter (excitation 450–490 nm, suppression LP 520 nm). Photography of the root sections was done using a SPOT RT CCD camera and SPOT RT software version 3.5.5 (Diagnostic Instruments).

EXTRACTION AND QUANTIFICATION OF LIGNIN

Lignin contents of isolated cell wall samples were determined by using the thioglycolic acid derivatised lignin method as described by Lange *et al.* (1995). Approximately 15 mg of the cell wall preparations were suspended in a mixture of 1 ml HCl (2 M) and 200 μl thioglycolic acid and placed in a water bath at $95 \pm 2^\circ\text{C}$ for 4 h. Samples were centrifuged for 10 min at 16 000 g at room temperature. Pellets were washed three times with MilliQ water, suspended in 1 ml NaOH (0.5 M) and vigorously shaken overnight to extract the lignothioglycolic acid (LTGA). Samples were centrifuged for 10 min at 16 000 g and the supernatants were collected. The pellets

were washed with an additional 500 μl of NaOH (0.5 M). The combined supernatants (alkali extracts) were acidified with 300 μl concentrated HCl and incubated for 4 h at 4°C to precipitate the LTGA from the alkali extracts. Samples were centrifuged for 10 min at 16 000 g and pellets were dried in a SpeedVac centrifuge. Dry brown pellets were dissolved in 1 ml NaOH (0.5 M) to measure the absorbance.

Absorbance of the samples was measured against the blank at 280 nm in a NanoDrop spectrophotometer using the UV-Vis high absorbance mode (ND-1000; NanoDrop Technologies). The lignin content (LTGA) was expressed as mg lignin ($\text{g fresh root weight}^{-1}$) using a calibration curve of alkali lignin (Sigma-Aldrich).

FOLIN-CIOCALTEU ASSAY FOR PHENOLIC CONTENT

This method determines the soluble phenolic content based on the reduction of the phospho-molybdene/phospho-tungstate present in the Folin-Ciocalteu reagent. One gram of frozen root samples was ground in liquid nitrogen. Ground root samples were extracted with 5 ml of 50% methanol by shaking at 4°C for 1 h. Extracts were filtered through a 0.45 μm PTFE filter (Merck) on ice. Extracts were diluted five times with distilled water, 250 μl of 1 M Folin-Ciocalteu reagent, and 1250 μl of 20% sodium carbonate solution were added and vortexed. Samples were kept in dark for 40 min. Absorbance was measured at 725 nm against a blank using a Novaspec II spectrophotometer. A standard calibration curve of tannic acid was established and root phenolic content was expressed as tannic acid equivalents g^{-1} of fresh root weight (TAE ($\text{g fresh root weight}^{-1}$)). For nematode-infected plants the infected tissues were sampled. For non-infected plants a random sampling was performed.

STATISTICAL ANALYSIS

Statistical analysis was performed using STATISTICA version 9 (StatSoft). Data on fresh root and shoot weights and lignin content were analysed using analysis of variance (ANOVA). When significant differences ($P \leq 0.05$) were observed, Tukey's HSD test was applied for multiple comparisons of group means. As the nematode numbers and total phenolic content data sets did not meet the basic assumptions of the parametric tests, namely, normal distribution and homogeneous variances, a non-parametric Kruskal-Wallis analysis of variance by ranks was used. Multiple comparisons between treatments were calculated as described by Siegel & Castellan (1988).

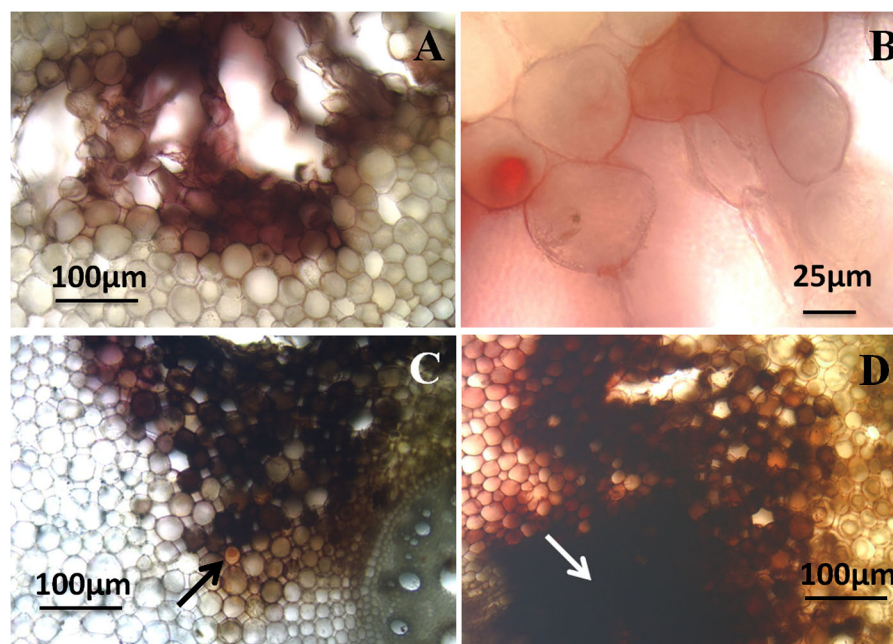


Fig. 1. Necrotic root cross sections of *Radopholus similis*-resistant *Musa* genotypes at 6 weeks after inoculation with *R. similis*. A: Dark brown lesions in the aerenchyma cells of the genotype Long Tavoy; B: A newly developing, reddish brown lesion in Long Tavoy shows increasing phenolic content; C, D: Newly developing (black arrow in C) and developed (white arrow in D) dark brown lesions in cv. Yangambi km5.

Results

ROOT AND SHOOT WEIGHT

No significant differences were observed in the root weights of *R. similis*-infected and non-infected plants at 3 and 6 weeks after infection except in cv. Yangambi km5. In cv. Yangambi km5, the mean root weight was significantly ($P \leq 0.05$) higher with 69.5 g in *R. similis*-infected plants compared to the non-infected ones with only 46.1 g at 6 weeks after infection. The root weights of the genotypes ranged from 17.3 g (cv. Grande Naine) to 44.4 g (Long Tavoy) at 3 weeks, and 34.7 g (cv. Grande Naine) to 69.5 g (cv. Yangambi km5) at 6 weeks after infection. No significant differences were observed in shoot weights of infected and non-infected plants. The fresh root and shoot weights of cv. Grande Naine were always the lowest among the genotypes.

ROOT ANATOMY AND CELLULAR DAMAGE IN INFECTED ROOTS

Thin, hand-cut root cross sections of all *Musa* genotypes were observed under a compound transmitted light

microscope. Images of the *R. similis*-infected root lesions are presented in Figure 1. The necrosis started from the epidermis and extended into the outer cortex, aerenchyma and inner cortex, but it was more extensive in the aerenchyma of all the four *Musa* genotypes (Fig. 1A). Necrosis was also observed in the endodermis, extending into the outer layers of the vascular bundle of cv. Yangambi km5 at 3 weeks after infection. Observation of the lesions showed that the cells were dark brown-coloured in cvs Yangambi km5 and Saba (Fig. 1C, D) and reddish brown coloured in cv. Grande Naine and in Long Tavoy (Fig. 1A, B).

ROOT DAMAGE AND NEMATODE NUMBERS IN NECROTIC LESIONS

The numbers of *R. similis* in the necrotic lesions of the resistant and susceptible *Musa* genotypes at 6 weeks after infection are summarised in Table 1. The mean number of nematodes extracted from the necrotic lesions of the susceptible *Musa* cv. Grande Naine was about four times higher than the number of nematodes extracted from the resistant *Musa* Long Tavoy and cv. Yangambi km5.

Table 1. Mean number of *Radopholus similis* in the necrotic lesions of three resistant genotypes (Saba, Long Tavoy & Yangambi km5) and one susceptible (Grande Naine) *Musa* genotype at 6 weeks after inoculation with 1000 adults and juveniles of *R. similis* ($n = 4$).

Genotype	Nematodes (g necrotic lesion) ⁻¹
Grande Naine	1057 b
Saba	583 a
Long Tavoy	254 a
Yangambi km5	255 a

Means within the same column followed by the same letter are not significantly different from each other ($P \leq 0.05$) according to the Kruskal-Wallis analysis of variance by ranks.

HISTOCHEMICAL STAINING OF *MUSA* ROOT CROSS SECTIONS FOR LIGNIFIED CELL WALLS

Lignified cell walls fluoresce under UV light when stained with DPBA. When a root section is viewed through a FITC filter, the syringyl units fluoresce in green and the guaiacyl units fluoresce in bright yellow colour. The non-infected plants of all *Musa* genotypes examined at 6 weeks after infection showed stronger fluorescence (Fig. 2B) compared with the plants sampled at 3 weeks after infection (Fig. 2A) for the same genotype; *i.e.*, older roots showed more extensive secondary wall thickening due to lignin deposition.

Radopholus similis-infected plants (Fig. 2C, E, F, H, I) showed highly intense fluorescence in the vascular bundles and endodermis compared to the non-infected plants of the same genotype at same age (Fig. 2A, B, D, G). The *R. similis*-infected Long Tavoy plants showed the most extensive secondary wall lignification of vascular parenchyma cells (Fig. 2E, F) compared to other genotypes. Lignification starts at endodermis, extending to the peripheral tissues of vascular system especially the xylem walls, xylem-accompanying parenchyma cells (Fig. 2C, E) and eventually the lignification progress to the vascular parenchyma (sclerenchyma) cells in the centre (Fig. 4F, I).

Cell wall lignifications in the *Musa* genotypes were confirmed by histochemical staining of the root cross sections with lignin specific dyes. Endodermis of all *Musa* genotypes became deep red coloured when treated with Wiesner reagent, indicating endodermal cell wall lignification especially after infection with *R. similis* (Fig. 3A, B). Distinct pink colouration was observed in the endodermis of cv. Yangambi km5 (Fig. 3A). The reactions with Maule's reagents showed the presence of

high syringyl units of lignin in central (Fig. 3C) and peripheral (Fig. 3D) vascular parenchyma (sclerenchyma) of *R. similis*-infected plants.

HISTOCHEMICAL STAINING OF *MUSA* ROOT SECTIONS FOR TOTAL PHENOLS

Observation of toluidine blue-stained fresh root cross-sections of *R. similis*-resistant and -susceptible *Musa* genotypes illustrated the greenish blue-stained lignified cell walls of vascular parenchyma (Fig. 4A). No pre-formed phenolic cells were observed in non-infected plants. In the infected roots, the lesions were well stained with toluidine blue. The nematode-infected necrotic cells were filled with phenolic substances (Fig. 4B).

ROOT CELL-WALL LIGNIN AND ROOT PHENOL CONTENTS

Total lignin contents in the isolated root cell walls of *R. similis*-infected and non-infected *Musa* genotypes are presented in Table 2. The results show that *R. similis* infection significantly ($P \leq 0.05$) increased the lignin content of cv. Yangambi km5 roots at 6 weeks after inoculation. The constitutive root lignin content in cv. Yangambi km5 was lower than the constitutive lignin content in cv. Grande Naine at 6 weeks. However, the post-infection lignin content in cv. Yangambi km5 was significantly ($P \leq 0.05$) higher than the post-infection lignin content in cv. Grande Naine at the same time point. No significant differences were observed in the constitutive and infection-induced lignin content between the genotypes at 3 weeks after inoculation. In the histochemical staining, extensive lignification was observed constitutively in the older roots (6 weeks) compared to the younger (3 weeks) roots. This increase in the constitutive lignin content was observed only in Grande Naine and Long Tavoy during quantification (Table 2).

Root phenol content was determined using the Folin-Ciocalteu method. The phenol content was significantly ($P \leq 0.05$) lower in non-infected roots of all resistant *Musa* genotypes compared to the necrotic tissues of *R. similis*-infected *Musa* roots at 3 and 6 weeks after inoculation. By contrast, in the susceptible genotype Grande Naine, the phenolic contents of non-infected and infected necrotic tissues were comparable at 6 weeks after infection. The constitutive phenol contents of all three resistant genotypes were significantly ($P \leq 0.05$) lower

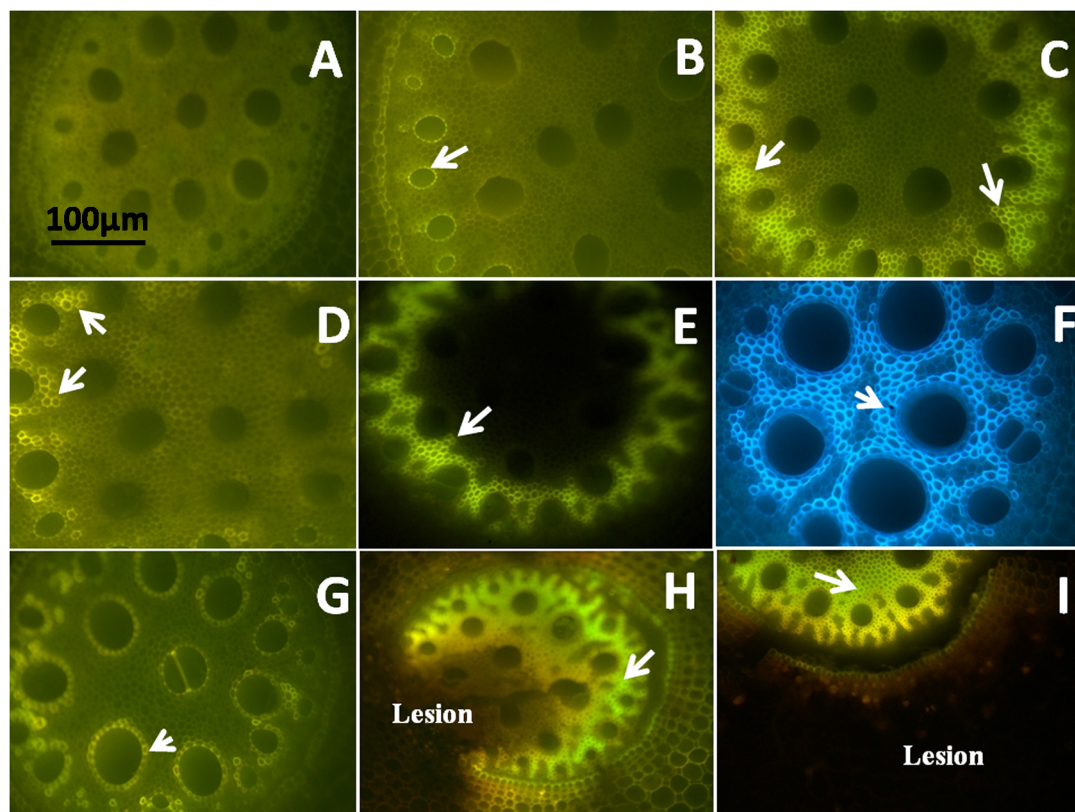


Fig. 2. Lignifications in non-infected and *Radopholus similis*-infected roots at 3 and 6 weeks after inoculation with 1000 adults and juveniles of *R. similis*. Root cross sections were stained with DPBA, observed under epifluorescence microscope using FITC (except F) and DAPI filter (F). A, B: Lignifications in non-infected susceptible (cv. Grande Naine) plants at 3 and 6 week time points, respectively; C: Extensive lignifications (arrow) in *R. similis*-infected cv. Grande Naine at 6 weeks; D, G: Lower levels of lignification in non-infected resistant plants, Long Tavoy and Yangambi km5, respectively, at 3 weeks; E, F: Extensive lignification in *R. similis*-infected Long Tavoy plants fluorescing peripheral vascular cells at 3 weeks (E) and vascular sclerenchyma cells (F) at 6 weeks; H, I: Extensive lignification in *R. similis*-infected cv. Yangambi km5 plants fluorescing vascular sclerenchyma at 3 weeks (H) and intense vascular deposition of syringyl (greenish) and guaiacyl (yellowish fluorescence) at 6 weeks (I). (Scale bars = 100 μ m.)

than the constitutive phenol content in cv. Grande Naine at 6 weeks after inoculation. No significant differences were observed between the constitutive root phenol contents of the resistant and susceptible genotypes at 3 weeks after inoculation (Table 2).

LOCALISATION OF FLAVONOIDS

Observations of DPBA-treated root sections under an epifluorescence microscope with a FITC filter showed various fluorescing compounds. Green, bright yellow, yellow-gold, and orange fluorescence were observed in cells bordering the necrotic cells in cortex and aerenchyma of the root sections (Fig. 5).

Discussion

Lignin and phenols were tissue-localised by histochemical analysis and quantitatively assessed in two newly identified *R. similis*-resistant *Musa* roots to understand their involvement in the resistance to *R. similis*. Results are compared with the well known reference susceptible (cv. Grande Naine) and resistant (cv. Yangambi km5) genotypes. The effect of *R. similis* infection on lignin and phenols accumulation was studied by comparing their constitutive levels in non-infected plants. Our study presents comprehensive results on lignin and phenol contents, as well as histochemical localisation to map their cellular localisation, in newly identified *R. similis*-

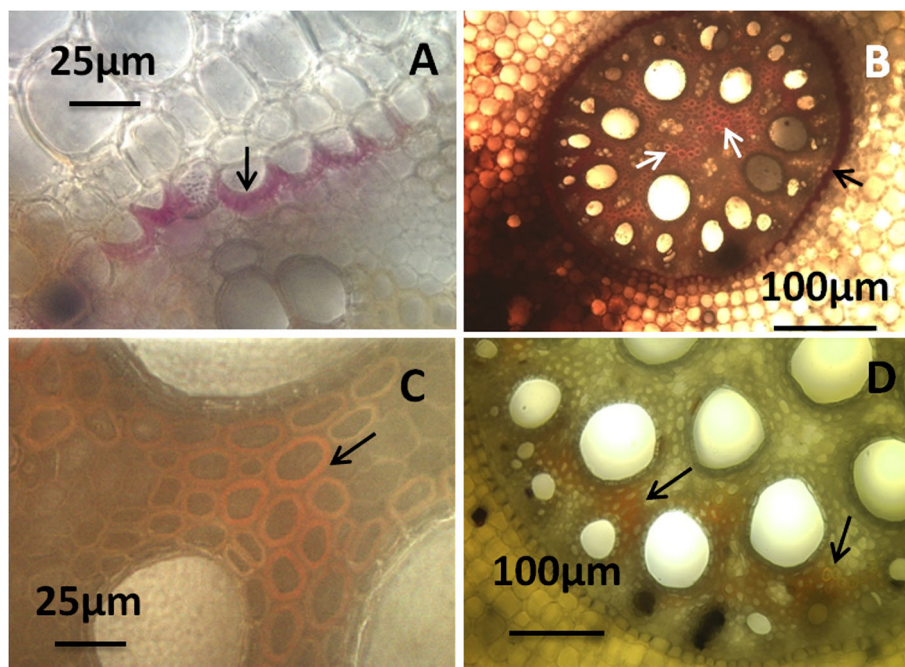


Fig. 3. Histochemical localisation of lignin in root cross sections of *Radopholus similis*-resistant *Musa* genotypes at 6 weeks after inoculation with 1000 adults and juveniles of *R. similis*. A, B: In cv. Yangambi km5, lignified endodermis cells (arrow) (A) and lignified central vascular sclerenchyma cells (white arrows) and endodermis (black arrow) (B) were stained 'red' with Wiesner reagent; C, D: In Long Tavoy, Mäule's staining shows secondary cell wall lignification with syringyl lignin units in central vascular sclerenchyma (arrow) (C) and peripheral vascular sclerenchyma cells (arrows) (D). All images were made under bright field transmitted light microscope.

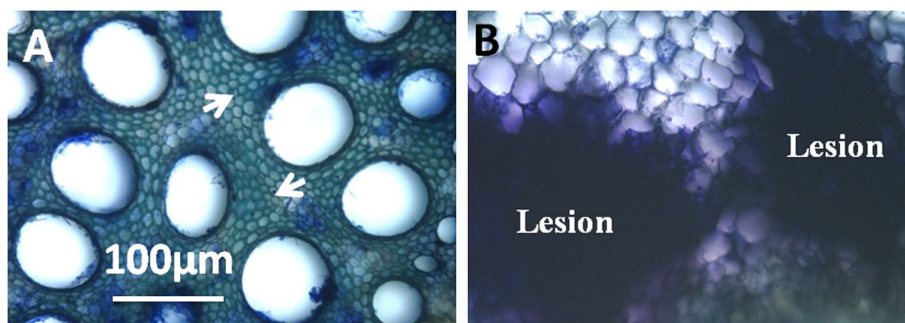


Fig. 4. Tissue localisation of total phenols by toluidine blue staining in root cross sections of *Musa* genotypes at 6 weeks after inoculation with 1000 adults and juveniles of *Radopholus similis*. A: Blue-green staining of lignified vascular cell walls of non-infected cv. Grande Naine root; B: Phenols in the necrotic cells of *R. similis*-infected cv. Grande Naine root.

resistant genotypes compared with a resistant and susceptible genotype. All observations were made on two time points, providing a time-course comparison. Uniquely, necrotic tissues were sampled for all analysis to enhance the understanding of the changes occurring in nematode-infected tissues. Only lesion tissues were sampled for extraction of nematodes to assess the nematode numbers in

the tissues where higher levels of infection-induced chemical responses occurred.

In our study, nematode infection was associated with a significant increase in root weight of the resistant reference genotype cv. Yangambi km5. Talwana *et al.* (2006) observed a higher number of roots in the *R. similis*-infected banana genotypes cvs Nabusa, Pisang Awak and

Table 2. Lignin content in root cell walls and root phenol content (mg Tannic Acid Equivalents (g fresh root weight)⁻¹) of *Radopholus similis*-infected and non-infected plants of resistant and susceptible (cv. Grande Naine) *Musa* genotypes at 3 and 6 weeks after inoculation with 1000 adults and juveniles of *R. similis* ($n = 8$).

Genotype/time point	Lignin content (mg (g fresh roots) ⁻¹)		Phenol content (mg TAE (g fresh roots) ⁻¹)	
	Non-infected	Infected with <i>R. similis</i>	Non-infected	Infected with <i>R. similis</i>
Grande Naine/3 weeks	0.31	0.26	0.49*	0.98*
Grande Naine/6 weeks	0.53	0.50	1.16	1.13
Saba/3 weeks	0.44	0.49	0.54*	0.94*
Saba/6 weeks	0.44	0.57	0.69*,**	1.07*
Long Tavoy/3 weeks	0.71	0.46	0.51*	1.05*
Long Tavoy/6 weeks	0.74	1.09	0.62*,**	0.92*, **
Ykm5/3 weeks	0.39	0.52	0.50*	0.96*
Ykm5/6 weeks	0.23	1.67	0.67*,**	0.92*, **

Means followed by * indicate a significant difference ($P \leq 0.05$) between the *R. similis*-infected and non-infected plant of the same genotype at the same time point. Means followed by ** indicate a significant difference ($P \leq 0.05$) compared to the susceptible reference genotype cv. Grande Naine. Ykm5 = Yangambi km5.

Sukali Ndizi compared with the non-infected plants. This stimulation in root growth is a mechanism to compensate for the nematode damage by the dormant root primordia (Talwana *et al.*, 2006), especially at the initial infection stage. However, root damage by nematodes increases with explosion of the nematode population densities causing a reduced root system, root weight and anchorage upon completion of the many nematode life cycles in the host roots.

No difference in the general structural root anatomy was found between the *R. similis*-resistant and -susceptible *Musa* genotypes. The anatomical structures were similar to the previous descriptions of *Musa* roots (Acquarone, 1930; Wuyts, 2006). The nematode damage and necrosis were extensive in the outer cortex and aerenchyma. The necrosis has been extended to the endodermis and vascular bundle of a cv. Yangambi km5 plant at an early stage. Invasion of the vascular bundles by *R. similis* in *Musa* has been occasionally reported (Mateille, 1992; Sarah *et al.*, 1996; Valette *et al.*, 1998). Poor lignification in the young roots might be the reason for this vascular invasion by the nematode.

Histochemical localisation of total phenols by staining with toluidine blue showed that the phenolic substances are more abundant in nematode-infected tissues than in other root areas. No preformed phenolic cells were observed in the *R. similis*-resistant and -susceptible *Musa* genotypes as previously reported in the *R. similis*-resistant *Musa* genotypes cvs Yangambi km5 and Gros Michel (Fogain & Gowen, 1996).

The results of the quantitative assay of total phenols by the Folin-Ciocalteu assay corroborate the histochemical localisation. The nematode infection has almost doubled the total phenol contents in all *Musa* genotypes at 3 weeks after infection. This drastic increase in total phenols was observed in the *R. similis*-resistant *Musa* genotypes also at 6 weeks after infection. Infected tissues were sampled for this phenolic assay. Hence, the increase in total phenolic contents is due to the phenols accumulation in the lesions. The enhanced synthesis of phenols could be due to the biosynthesis or accumulation of phytoalexins in the nematode feeding cells with anti-nematode properties. Phenolic compounds play major roles in providing protection against pest, diseases and herbivores (Ajila *et al.*, 2011). However, our results also showed a similar increase in phenol content in the susceptible genotype cv. Grande Naine. Further studies using analytical techniques such as chromatography, NMR spectroscopy and mass spectrometry can facilitate the structural identification of the individual phenolic compounds present in the lesions. A recent study (Hoelscher *et al.*, 2014) reports that the phenylphenalenone group of phytoalexins mediates *R. similis* resistance in cv. Yangambi km5 based on the structural identification of phenolic compounds in necrotic tissues. Same study shows that the cellular concentration of individual phytoalexin such as anigorufone plays a major role in plant resistance to nematodes rather than the presence or absence of it. Extending this structural identification of phenolic compounds in necrotic tissues of newly identified resistant genotypes such as Long

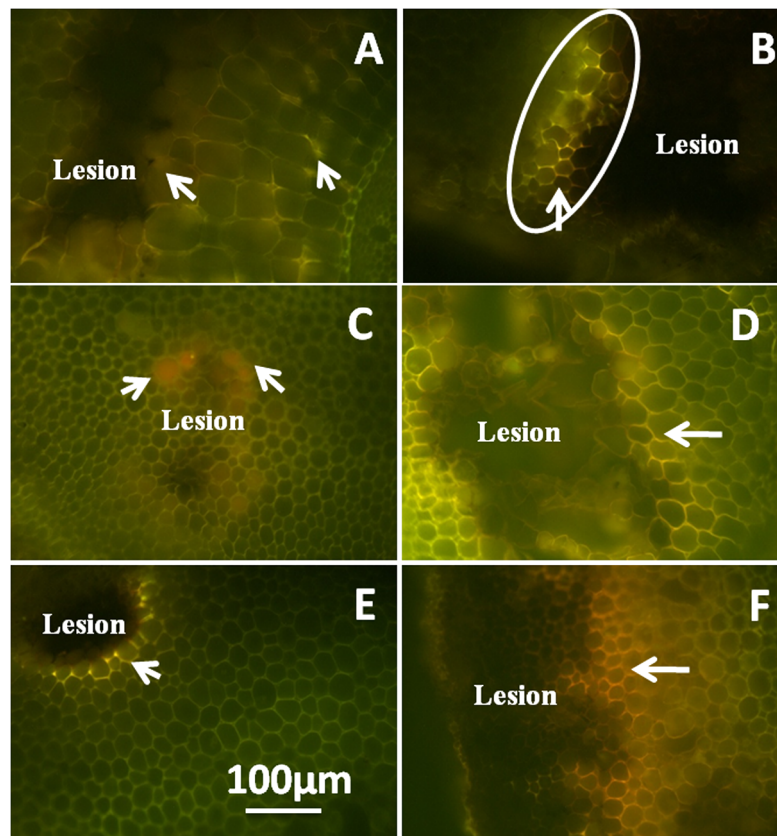


Fig. 5. Fluorescence of phenolic compounds in root cross sections of *Radopholus similis*-infected susceptible and resistant *Musa* genotypes at 3 and 6 weeks after inoculation. Root cross sections were stained with DPBA and observed under an epifluorescence microscope with a FITC filter. A, B: Bright and golden yellow fluorescing compounds in cell walls and intercellular spaces of lesion-adjacent cells in cv. Grande Naine at 3 (A) and 6 (B) weeks after inoculation; C, D: Orange fluorescing compounds inside the cells adjacent to lesions in cv. Long Tavoy at 3 weeks (C) and bright and golden yellow compounds (D) in the cell walls at 6 weeks. E: Bright yellow fluorescing compounds are seen in cell walls of only lesion-bordering cells not in other cells in cv. Yangambi km5 at 3 weeks. F: Orange fluorescing compounds in cell walls and intercellular spaces adjacent to lesions at 6 weeks in cv. Yangambi km5. (Scale bars = 100 μ m.)

Tavoy and Saba will broaden our understanding of nematode resistance in bananas. The high constitutive phenols content in cv. Grande Naine at 6 weeks is most likely caused by the high polyphenolic content in older cv. Grande Naine roots due to tissue damage caused during sampling and handling. The degree of browning after cutting in banana was correlated with the polyphenol oxidase activity and the concentration of free phenolic substrates (Chaisakdanugull & Theerakulkait, 2009). A previous report shows that the constitutive levels of phenolic enzymes such as peroxidase polyphenol oxidase are much higher in cv. Grande Naine than cv. Yangambi km5 (Wuyts *et al.*, 2006). Moreover, the same study reported that wounding caused a more significant increase in per-

oxidase activity in cv. Grande Naine than nematode infection. This was not observed in the resistant genotype cv. Yangambi km5. The older cv. Grande Naine roots in tissue cultures also show intense browning and secrete dark brown compounds in the medium without any infection.

Histochemical staining in our study shows that lignin was a major constituent of the cell walls of vascular bundles and endodermis. Moreover, we observed highly extensive secondary cell wall lignification in the endodermis, xylem-accompanying cells and the vascular parenchyma of *R. similis*-infected plants than in the nematode non-infected plants. Increased extensive lignification was not associated with the cortex cells that are directly attacked by the nematode. This showed that the increased

lignification is a general defence response to protect the vascular bundle from damage, providing an overall tolerance to plants rather than limiting nematode development and reproduction in root cortex. Lower levels of cortical cell-wall lignification were previously observed in *Musa* roots (Fogain & Gowen, 1996; Wuyts *et al.*, 2007). Stronger lignification in the endodermis and vascular bundle is considered as a general defence response to nematode infection creating a physical and chemical barrier to the vascular invasion (Keen, 1992; Zacheo *et al.*, 1997; Valette *et al.*, 1998; Wuyts *et al.*, 2007). Our new *R. similis*-resistant genotypes also follow the same response as the resistant reference genotype cv. Yangambi km5. Hence the role of infection-induced lignin synthesis appears to be a common response of most *Musa* genotypes, including the wild type Long Tavoy.

Histochemical studies also indicated more extensive lignification of vascular bundles in older plants than in younger plants. Increased cell wall lignification with increase in age is documented in *Musa* (Wuyts *et al.*, 2007) and in other crops. For example, the proximal roots of wheat contain more lignin than the distal younger roots (Rengel *et al.*, 1994). The higher levels of indole acetic acid (IAA) in younger root tissues inhibit phenylalanine ammonia lyase activity and lignin deposition (Burnell, 1988).

The quantitative assessment of lignin content based on the thioglycolic acid assay of cv. Yangambi km5 plants confirmed the *R. similis* infection induced intense lignification observed in our histochemical staining. The increase in the lignin content of cv. Yangambi km5 was seven times higher than in the non-infected plants at 6 weeks after infection. However, the quantitative assay did not confirm the increased lignification observed in histochemical staining in the other genotypes. In cv. Grande Naine, the extended cellular damage due to nematode infection might have caused a reduction in total lignin content compared to the non-infected plants. A prominent increase in lignin contents due to *R. similis*-infection was reported in *R. similis*-resistant *Musa* genotypes cvs Pisang Jari Buaya and Calcutta 4, as well as in the susceptible genotype cv. Grande Naine. By contrast, a reduction in lignin content was previously reported in cv. Yangambi km5 after *R. similis* infection (Wuyts *et al.*, 2007).

Histochemical staining of *R. similis*-infected roots with Neu's reagent showed the presence of fluorescing compounds bordering the lesions in all the *Musa* genotypes. These fluorescing compounds could be lignin accumu-

lation induced by nematode infection to protect the adjacent cells from nematode invasion. Similar fluorescing compounds were also reported as candidates of certain flavonoids (Valette *et al.*, 1998; Wuyts *et al.*, 2007). However, it is ambiguous to identify the secondary metabolites based on the fluorescence. These compounds were mainly present in the cell walls and intercellular spaces, suggesting their role as defence-related signalling molecules. Phenolic compounds were suggested to play a key role in internal signalling in plants (Ferrer *et al.*, 2008). No striking differences were observed between the susceptible and resistant cultivars in the presence of constitutively fluorescing compounds as previously reported (Valette *et al.*, 1998). The phenols in the lesions were non-fluorescent.

Our study provides strong supportive evidence on the involvement of phenolic compounds in the resistance of *Musa* genotypes to *R. similis* infection. This response was the same in all the three resistant genotypes. The post-infectional increase in lignification is associated with the plant's tolerance to nematode infection as a general response to pathogenic infection by protecting the vascular bundle. This response was shown by resistant as well as susceptible genotypes. Future studies using high throughput analytical techniques are necessary to identify structurally and to quantify the individual phenolic compounds that are biosynthesised or accumulated in the nematode infection site.

Acknowledgements

This study was supported by a Ph.D. fellowship of the Interfaculty Council for Development Co-operation (IRO), from the University of Leuven to S. Dhakshinamoorthy and a VLIR-UDC grant from the Belgian Government to K.M.

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