Secondary metabolites in roots and implications for nematode resistance in banana (*Musa* spp.)

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Abstract

Secondary metabolites and more specifically phenylpropanoids, in roots of *Musa* have been related to nematode resistance based on the differential presence of these compounds in the roots of resistant and susceptible cultivars. However, a strong biochemical basis for resistance has not yet been elucidated. The current study demonstrated biochemical differences in the phenylpropanoid pathway of secondary metabolism between cultivars resistant to the banana nematode *Radopholus similis*, 'Yangambi km5' and 'Pisang jari buaya', and the susceptible 'Grande naine'. Unlike previously reported results, lignification and preformed phenolic cells were not found to be specific features of the resistant cultivars. Accumulation of sinapic acid in the thickened cell walls of the endodermis and vascular elements was induced by nematode infection in both the resistant and the susceptible cultivars, while flavonoid-containing cells in the vascular cylinder were only found in resistant cultivars after infection. 'Grande naine' roots contained the highest total phenolic content, which could be related to high amounts of proanthocyanidins. In 'Yangambi km5' roots, the proanthocyanidin and total phenolic content was the lowest, but mean amounts of total phenols increased after infection, unlike in the other cultivars.

Resumen - Metabolitos secundarios en raíces y sus implicaciones para la resistencia a nematodos en banano (*Musa* spp.)

Metabolitos secundarios y más específicamente los fenilpropanoides, en sistemas radicales de *Musa*, han sido relacionados a la resistencia a nematodos, basados en la presencia diferencial de estos compuestos en las raíces de cultivares resistentes y susceptibles. Sin embargo, todavía no se cuenta con una fuerte base bioquímica que explique esta resistencia. El presente estudio muestra diferencias bioquímicas en la vía del metabolismo secundario del fenilpropanoide entre cultivares resistentes al nematodo barrenador del banano *Radopholus similis*, 'Yangambi km5' y 'Pisang jari buaya', y el susceptible 'Grande naine'. A diferencia de reportes anteriores, no se encontró que la lignificación y las células fenólicas preformadas estén específicamente relacionadas con los cultivares resistentes. La acumulación de ácido sinapico en el engrosamiento de las paredes celulares de la endodermis y elementos vasculares, fue inducido por la infección de nematodos tanto en los cultivares resistentes como susceptibles, mientras que células conteniendo flavonoides en el cilindro vascular fueron encontradas únicamente en cultivares resistentes después de la infección. Las raíces de 'Grande naine' mostraron el mayor contenido de fenoles, lo que pudo haber estado relacionado con altos niveles de proantocianidinas. En las raíces de 'Yangambi km5', la proantocianidina y el contenido total fenólico fue el mas bajo, pero los niveles totales promedio fenólicos aumentaron después de la inoculación, a diferencia de los otros cultivares.

Introduction

In the past fifteen years, various sources of resistance and tolerance to parasitic nematodes have been identified within banana. However, the underlying mechanisms, which make it possible for these plants to suppress nematode reproduction or suffer little

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injury are, as in many other plant species, poorly understood. Knowledge of these mechanisms is nevertheless of critical importance to develop strategies for the control of nematodes, especially when one wants these strategies to be environmentally-safe and suitable for subsistence banana growers. Such insights can provide 'resistance markers' to facilitate screening of *Musa* germplasm and provide *Musa* breeders and molecular biologists with the necessary principles to create nematode resistant plants.

Plants interact with their environment through secondary metabolism. Within this part of the metabolism, the phenylpropanoid pathway is of critical importance as its products (phenolic compounds) protect the plant against abiotic and biotic factors (reviewed by Dixon and Paiva 1995). To date, resistance against nematodes in *Musa* has been correlated with phenylpropanoids, *i.e.* lignification in 'Pisang jari buaya' (AA, 'Pisang jari buaya' group) (Fogain and Gowen 1996), preformed phenolic cells and infection-induced accumulation of phenolics in 'Yangambi km5' (AAA, Ibota group) (Fogain and Gowen 1996, Valette *et al.* 1998), phenylphenalenone phytoalexins in *Musa acuminata* (Binks *et al.* 1997, Luis 1998) and high levels of proanthocyanidins in 'Kunnan' (AB) (Collingborn *et al.* 2000). However, these metabolites were not characterized to a sufficient biochemical level and/or no reference was made to their physiological relevance in nematode resistance. Research on secondary metabolites in banana roots has been very limited and provides no guidance for crop improvement.

The results presented here deal with the biochemical nature of nematode resistance encountered in 'Pisang jari buaya' and 'Yangambi km5', in comparison with the susceptible 'Grande naine' (AAA, Cavendish group). Various techniques were used, including histochemical staining of root sections and spectrophotometric detection of phenols in root extracts. Results obtained so far are not conclusive about the biochemical basis for resistance, but provide guidance for future research into the secondary metabolism of *Musa*.

Materials and methods

Plant material, nematodes and experimental set-up

Plant material was obtained from the International *Musa* germplasm collection at the INIBAP Transit Centre at KULeuven. It included 'Grande naine' (ITC1256) (GN), which was chosen as a susceptible cultivar (Stoffelen *et al.* 2000), and 'Yangambi km5' (ITC1123) (YKm5) and 'Pisang jari buaya' (ITC0312) (PJB), which were chosen as resistant cultivars. Micropropagated banana plants were planted in pots filled with 600 ml of a sterilized 2:1 peat-quartz mixture. To each plant 1 g Osmocote®, a slow release fertilizer, was added.

A highly virulent strain of *R. similis* from banana plants in Uganda was used in the experiment (Fallas *et al.* 1995). After ten weeks of growth in the greenhouse, eight plants of each cultivar were inoculated with 1000 vermiform (female and juvenile) nematodes per plant. Eight uninfected plants were included per cultivar. Histochemical staining of root sections and analysis of root extracts was performed two weeks after infection.

Histochemical staining of root sections

Root sections were hand cut from fresh root samples. For the detection of phenylpropanoids, sections were stained within 2 min with saturated (0.25%, w/v) diphenylboric

acid-2-aminoethyl ester (DPBA) (Sigma-Aldrich Inc., Bornem, Belgium) in MilliQ water containing 0.02% (v/v) Triton-X-100. The sections were visualized immediately with an epifluorescence microscope equipped with a DAPI filter (excitation 340-380 nm, suppression LP 430 nm) and a FITC filter (excitation 450-490 nm, suppression LP 520 nm). Identification was made by comparing colour and intensity to standard references (Peer *et al.* 2001).

For the histochemical staining of lignin in root sections, hand cut sections were fixed in 4% glutaraldehyde for 60 min and rinsed with water. For Mäule staining, fixed sections were immersed in 0.5% $KMnO_4$ for 10 min, rinsed with water and destained with 10% HCl for 5 min. After a final rinse they were mounted in concentrated NH_4OH and examined by bright-field microscopy. For Wiesner staining, fixed sections were stained with 10% phloroglucinol in ethanol/water (95/5, v/v) for 2 min, mounted in concentrated HCl (37%) and examined by bright-field microscopy (Strivastava 1966). No specific staining technique was used for the detection of suberin. However, as suberin has a lignin-like structure in its polyaromatic domain, it fluoresces and is stained by the Wiesner and Mäule reagents. Photographic documentation of root sections was achieved with a SPOT RT CCD camera and SPOT software version 3.3 (Diagnostic Instruments Inc., USA).

Extraction and analysis of phenols

For the extraction of phenols from roots, approximately 1-2 g of freshly harvested roots was ground in liquid nitrogen. Proanthocyanidin (condensed tannins) amounts were determined using the butanol/HCl assay described by Collingborn *et al.* (2000) for banana root samples. Ground samples (100 mg) were treated with 5 ml 1-butanol/HCl (95/5, v/v), mixed and incubated at 95°C. After 1 h, samples were thoroughly mixed and centrifuged at 3000 g for 10 min. The absorbance of the top phase was read at 550 nm against a blank sample using a Novaspec II spectrophotometer (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK).

The total phenolic content of root samples was determined using the Folin-Ciocalteu assay (Singleton and Rossi 1965). Ground samples (1 g) were extracted by shaking continuously in a 2 ml aqueous methanol (50%) solution for 1 h at room temperature. Extracts were filtered through a 0.45 µm pore PTFE syringe filter (Merck, Darmstadt, Germany) and stored at -20°C until analysis. In glass tests tubes, 200 µl of the root extracts was mixed with 5 ml water. To each of the samples 500 µl of the Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) was added. Samples were thoroughly mixed and after 3 min, 1 ml saturated sodium carbonate solution (35% in water, w/v) was added. Samples were further diluted to 10 ml with water and left in the dark at room temperature for 1 h. Absorbance was measured at 727 nm against water using a Novaspec II spectrophotometer. A standard calibration curve of gallic acid was determined and results were expressed as gallic acid equivalents/g fresh roots.

Statistical analysis

Data on the phenolic content of roots were analysed with the STATISTICA® package (Anonymous 2001). Since the number of samples was small (five per cultivar for each treatment) and variances not homogeneous (Levene's test for homogeneity of varian-

ces), the non-parametric equivalent of ANOVA, Kruskal-Wallis analysis of variance by ranks, was applied. If Kruskal-Wallis was significant, the significance of the differences between treatments and cultivars was pair wise calculated as described by Siegel and Castellan (1988).

Results and discussion

Histochemical staining of root sections

Lignified root cell walls

The DPBA-staining technique was used for the localization and identification of phenolic compounds in root sections. Syringyl lignin units (derived from sinapic acid) and guaiacyl lignin units (derived from caffeic acid) appeared blue and greenish-white respectively when viewed through a DAPI filter (Figure. 1A, F) and green and bright yellow respectively through a FITC filter (Figure 1B, C, D).

Various studies on resistance mechanisms in banana roots deal with the hypothesis that PJB constitutively contains higher amounts of lignin (Pinochet 1988, Binks 1996, Fogain and Gowen 1996, Elsen *et al. in press*). Among the cultivars tested earlier, PJB and 'Calcutta 4' had the highest lignification of the central cylinder, while in the susceptible cultivars (including GN) and YKm5, no or very few cell walls were lignified (Fogain and Gowen 1996, Elsen *et al. in press*). In the present experiment however, the level of lignification did not differ between the cultivars. In roots of the susceptible GN, cells of the endodermis and central cylinder showed the same degree of thickening of their walls as those of the resistant PJB and YKm5 (Figure 1A-D). In individual roots of the three cultivars, lignification of cell walls increased with the age of the root and the development of laterals. Roots that had recently emerged from the corm were not lignified (Figure 1E, F), while older roots showed the strongest cell wall thickening (Figure 1A-D). This observation might explain the contradictory reports on the occurrence of nematodes in the central cylinder of banana roots (Valette *et al.* 1997 and 1998, Elsen *et al. in press*). The lignified anticlinal walls of the endodermis prevent nematodes from penetrating the central cylinder and affecting the vascular elements (Blake 1961). But in young roots, nematodes can be found in the vascular tissue (Mateille 1994, Sarah *et al.* 1996) because the barrier – the lignified walls of the endodermis – is not developed yet.

In YKm5, the thickened endodermis was predominantly composed of guaiacyl units (yellow fluorescence) (Figure 1D), while in PJB and GN syringyl units (green fluorescence) prevailed (Figure 1A-C). This observation was confirmed by staining with the Wiesner and Mäule reagents which are specific for total lignin (guaiacyl and syringyl units) and syringyl units respectively. In the three cultivars, guaiacyl units were the dominant constituents of lignified cell walls inside the central cylinder. The suberized walls of the outer cortical cells contained predominantly sinapic acid in the case of PJB and GN and caffeic acid in the case of YKm5.

In response to nematode infection, syringyl lignin concentrations increased in the endodermis of YKm5 and in the central cylinder of GN, YKm5 and PJB. This reaction seems to be part of the general defence response of banana, as it occurred in both resistant and susceptible cultivars and in response to both nematodes and fungi

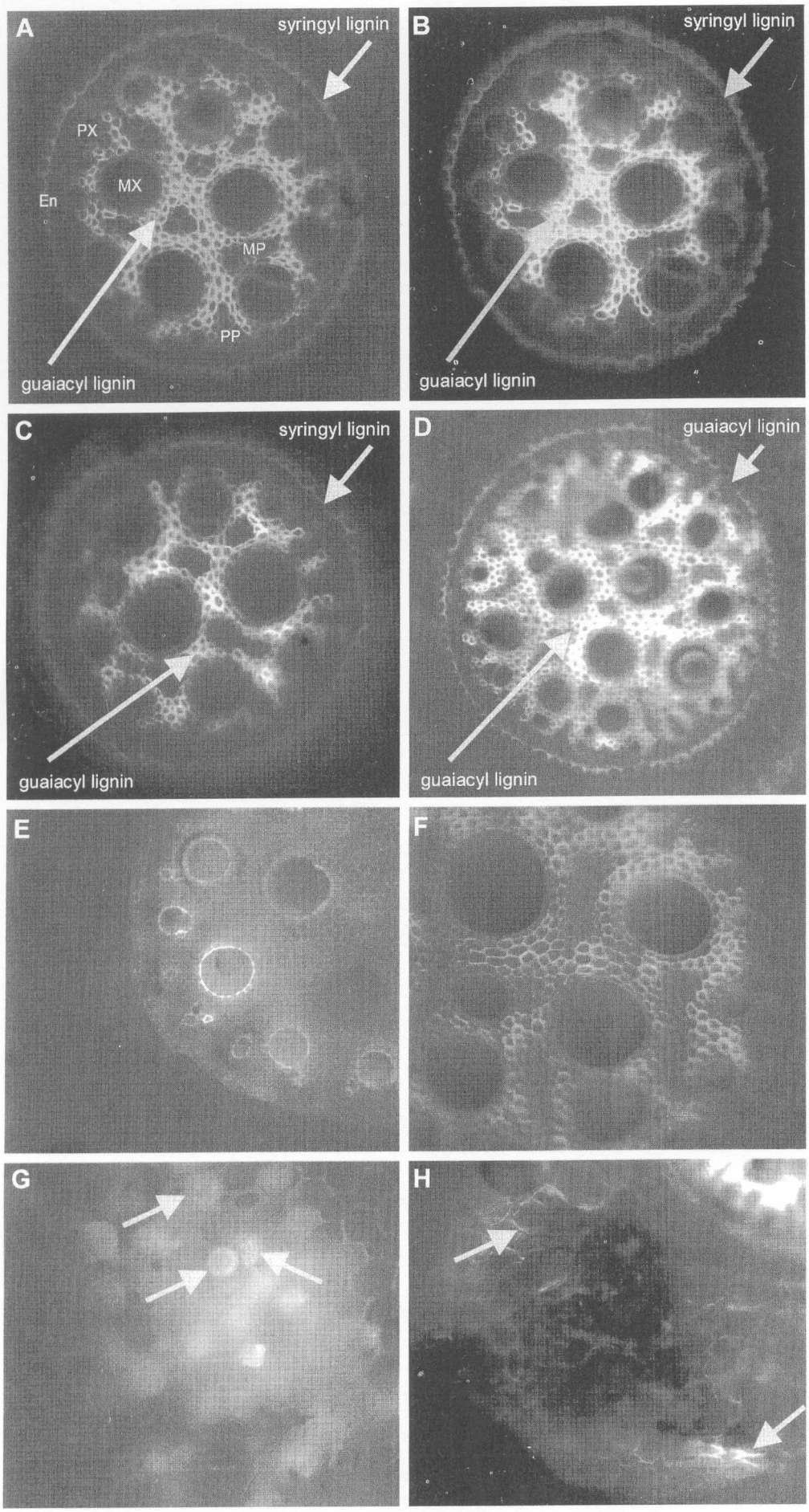

Figure 1. A-D - Cross sections of an older root of 'Grande naine' (A, B), 'Pisang jari buaya' (PJB) (C) and 'Yangambi km5' (D) showing the strong lignification of the endodermis and central cylinder (100x). E, F - Cross sections of young PJB roots showing the absence of, or weak lignification of, the endodermis and central cylinder (100x). G - Flavonol-containing cells (arrows) in the cortex of a PJB root (100x). H - Cross section through a nematode-damaged PJB root showing the presence of flavonols (arrows) in the tissue bordering the necrotic area (100x). Sections were stained with DPBA and viewed under a DAPI filter (A, F) or a FITC filter (B-E, G, H).
En: endodermis, PP: protophloem, MP: metaphloem, PX: protoxylem, MX: metaxylem.

(De Ascencao and Dubery 2000). However, the rate of increase and the total amount could be the decisive factor for resistance/tolerance, as was observed in Fusarium wilt-tolerant *Musa* (De Ascencao and Dubery 2000). Syringyl lignin could be more resistant to enzymatic degradation by pathogens; or compounds released after wall degradation could be toxic to the invaders. In *in vitro* bioassays, sinapic acid had a low toxicity and did not repel or inhibit egg hatch of *R. similis* (Wuyts, *unpubl. data*).

Phenol-containing root cells

Phenolic compounds were detected in cells as granules or as an amorphous mass (Figure 1G), as was described by Mueller and Beckman (1974). They were identified as the flavonols quercetin and kaempferol by their characteristic fluorescence, which was golden yellow and bright green when viewed with a FITC-filter. The lowest number of phenolic cells was found in the cortex of YKm5, while PJB contained the most. This is not in agreement with the proposed mechanism of constitutive resistance in YKm5 (Fogain and Gowen 1996). In the cortex of infected roots, the number of flavonol-containing cells did not increase in the three cultivars, which corresponds with the findings of Elsen *et al.* (*in press*) for YKm5 in *in vitro* screenings for resistance. In the central cylinder of YKm5 and PJB, however, flavonoid-containing cells were present when necrosis had developed in the cortex. Preliminary results of HPLC-analysis of root extracts, confirmed that infected roots of YKm5 and PJB contained higher concentrations of quercetin and kaempferol, while in GN a decrease was found. Flavonols could function in the central cylinder as protective agents or as regulators of physiological processes related to infection, or defence against infection.

Cells bordering the nematode-damaged tissue in the cortex contained flavonols in their walls and in the extracellular spaces in GN, PJB and YKm5 (Figure 1H). The accumulation of phenolic compounds, including flavonols, at the site of infection or wounding is a general feature of the plant response to biotic factors (Dixon and Paiva 1995). In *in vitro* bioassays quercetin and kaempferol were not toxic to *R. similis*, but they did repel the nematode at concentrations as low as 50 ppm (Wuyts N., *unpubl. data*). Flavonols may also act as phytoalexins against secondary infection by pathogenic soil organisms which use the damaged tissue as an 'easy' access to the root.

Analysis of phenol content in root extracts

Proanthocyanidins were analysed in roots of susceptible and resistant cultivars to determine their role in the response to nematode infection (Table 1). Amounts were not significantly different between cultivars, but the highest values were obtained for GN and the lowest for YKm5. Nematode infection had no significant effect on proanthocyanidin accumulation, although the increase was the highest in PJB. Total phenolic content of roots did not differ significantly between cultivars or between treatments. The high concentration in GN roots could be related to the high concentrations of anthocyanidin-related compounds. Only in YKm5 roots (with the lowest constitutive amounts of total phenolics) did the mean phenolic concentration increase in nematode infected roots, which cannot be related to the proanthocyanidins. HPLC-analysis of root extracts is necessary to determine the absolute amounts of the different phenolic compounds in roots.

Table 1. Amount of proanthocyanidins and total phenolic compounds determined by spectrophotometry in roots of three banana cultivars either uninfected or infected by *R. similis* (two weeks after inoculation).

	Proanthocyanidins[a] (A550 nm)			Total phenolic content[a] (µg GAE[b]/g FW)		
Cultivar	- *R. similis*	+ *R. similis*		- *R. similis*	+ *R. similis*	
Grande naine	0.471	0.486	(+ 3%)	230	224	(- 3%)
Yangambi km5	0.279	0.311	(+ 11%)	157	205	(+ 31%)
Pisang jari buaya[c]	0.335	0.446	(+ 33%)	246	240	(- 2%)

[a] results were not significantly different ($P < 0.05$) between cultivars and treatments (GN and YKm5) ($n = 5$) as calculated by the Kruskal-Wallis analysis of variance by ranks; [b] gallic acid equivalents; [c] $n = 1$, not included in the statistical analysis.

Collingborn *et al.* (2000) have suggested the butanol/HCl-assay as a rapid test in screening for resistance against *R. similis*, based on their observation that the resistant 'Kunnan' contained significantly higher amounts of proanthocyanidins pre- and post-infection compared with the susceptible 'Dwarf Cavendish'. However, in the current experiment as well as in the findings of Binks (1996), no correlation existed between resistance and proanthocyanidin amounts. Moreover, the amounts were not significantly higher in roots of infected plants compared with uninfected plants in either the resistant or the susceptible cultivar. However, in the experiment, a random sample of roots was taken from infected and uninfected plants, while Collingborn *et al.* (2000) specifically selected areas with lesions for extraction. In general, proanthocyanidins accumulate in response to wounding by the action of polyphenoloxidases (browning reaction). As a result, higher amounts of proanthocyanidins can be expected in roots with lesions than in undamaged ones. Furthermore, in susceptible cultivars the relative accumulation of proanthocyanidins is higher after nematode infection than in resistant ones (data of Collingborn *et al.* 2000). Proanthocyanidins have strong negative effects on herbivorous insects through their protein-binding capacity, which results in reduced efficiency of nutrient absorption in gut and midgut lesions (Hagerman and Butler 1991). To determine whether proanthocyanidins can be considered as phytoalexins against nematodes in banana roots, they need to be tested in bioassays with nematodes.

Conclusions

The aim of the study was to elucidate the biochemical basis for nematode resistance. Constitutive lignification of root cell walls, and induced cell wall strengthening upon nematode infection, were similar in susceptible and resistant cultivars. In resistant cultivars, the number of cells containing flavonols in the central cylinder was higher when the cortex was damaged by nematodes. The absolute amount of proanthocyanidins in roots cannot be considered as a 'biochemical' marker for nematode resistance, as the highest amounts were found in the susceptible cultivar. Future research includes the analysis of root extracts by HPLC and a search for the physiological relevance of flavonoids in nematode-infected roots.

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