Investigations into the Biochemical Basis for Nematode Resistance in Roots of Three *Musa* Cultivars in Response to *Radopholus similis* Infection

Frances M. B. Collingborn, Simon R. Gowen, and Irene Mueller-Harvey*

Department of Agriculture, The University of Reading, P.O. Box 236, Reading RG6 6AT, United Kingdom

The *Musa* cultivars, Dwarf Cavendish, Yangambi Km5 and Kunnan, exhibit considerable differences in resistance to *Radopholus similis*. Infection resulted in significant increases in condensed tannins and flavan-3,4-diols in roots ($P < 0.001$). The highly resistant cultivar Kunnan had the highest levels of condensed tannins before and after infection. The preinfection levels were similar to the postinfection levels of the two other cultivars. Tannins had mostly procyanidin character, but Kunnan also contained propelargonidins; these compounds may be involved in the resistance mechanism. It is suggested that the butanol/HCl assay be used as a rapid test in screening for resistance to *R. similis*.

Keywords: *Musa cultivars; Radopholus similis; nematodes; resistance; condensed tannins; flavan-3,4-diols; acid butanol assay*

INTRODUCTION

The burrowing nematode *Radopholus similis* (Cobb) Thorne became a significant pest of commercially grown dessert bananas when the clones of the Cavendish group (*Musa* AAA) were introduced as replacements for Gros Michel (also *Musa* AAA), which is susceptible to *Fusarium oxysporum* fsp *cubense*. To avoid the losses due to nematodes in the Cavendish clones which can be >50% per year (Gowen and Quénéhervé, 1990), commercial banana growers now use a number of oximecarbamate and organophosphate nematicides on a regular basis (Gowen, 1995). None of the commercially grown clones have resistance to *R. similis*. It is an objective of many banana improvement programs to incorporate sources of resistance or tolerance to this major nematode pest. Resistance to *R. similis* has been reported in some diploid bananas (*Musa* AA) (Pinochet and Rowe, 1978), Yangambi Km5 (*Musa* Ibota AAA) (Sarah et al., 1992; Fogain and Gowen, 1997), and in a diploid clone, Kunnan (*Musa* AB) (Collingborn and Gowen, 1997). Kunnan has consistently shown high levels of resistance to both *R. similis* and the lesion nematode, *Pratylenchus coffeae*, in terms of nematode numbers recovered and lesion development in the root. Lesions are commonly arrested at the site of infection. In comparison, Yangambi Km5 always produces well developed root lesions even though nematode numbers remain low (Collingborn, unpublished). Several different classes of compounds have nematicidal properties, e.g., fatty acids, polythienes, terpenoids, alkaloids, isoflavonoids, and phenolics (Chitwood, 1993; Baldridge et al., 1998). Plants synthesize a wide variety of preinfectional and postinfectional compounds which are active against root nematodes (Giebel, 1974; Mace and Howell, 1974; Binks, 1996; Luis, 1998). Only a few *Musa* cultivars have so far been investigated for their biochemical reactions to nematodes and most studies employed nonspecific tests for phenolics. In the resistant cultivar Gros Michel, nematode migration was inhibited by tissue reaction around the nematode. These necrosed areas stained positively for phenols (Mateille, 1994). Valette et al. (1997) found postinfectional production of phenols also in roots of Yangambi Km5 but not in the susceptible Poyo.

Recently a new group of phenolic phytoalexins, i.e., the phenylphenalenones, was identified in *Musa acuminata* (Luis et al., 1995). These appeared in response to infection with the fungi *Fusarium oxysporum* f sp. *cubense* and *Mycosphaerella fijiensis* and the nematode *R. similis*. Resistant cultivars were found to produce the same family of compounds but in much larger quantities (Luis, 1998; Binks et al., 1997). *Musa* plants also produce a range of other phenolic compounds in seeds and fruits, such as condensed tannins and flavan-3,4-diols (Barnell and Barnell, 1945; Williams and Harborne, 1988; Dhua and Sen, 1989; Ali and Bhutani, 1993). Jambunathan et al. (1986) found that condensed tannins and flavan-4-ols can act as phytoalexins against fungal pathogens. Preliminary studies by Kashaija (1996) suggested that tannins also occur in roots of field grown *Musa* plants and that their concentrations may be related to nematode resistance. However, to our knowledge, *Musa* roots have not been examined for the presence of flavan-3,4-diols or tannins in response to *R. similis* infection. This study was, therefore, undertaken to investigate (a) if flavan-3,4-diols or tannins are present in *Musa* roots and (b) if they are produced in response to the nematode, *R. similis*, and could thus contribute to the resistance mechanism. The aim was to determine these groups of compounds in two resistant cultivars, Kunnan and Yangambi Km5, and a susceptible cultivar, Dwarf Cavendish, before and after infection.

* Corresponding author [telephone (+44) 0118 931 6619; fax (+44) 0118 935 2421; e-mail i.mueller-harvey@reading.ac.uk].

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MATERIALS AND METHODS

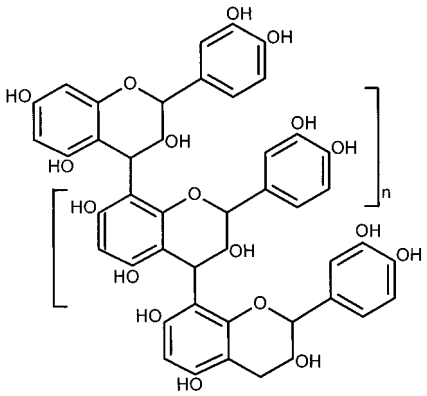
Nematodes. *R. similis* (Cobb) Thorne, population 19 from CABI Biosciences (Egham, U.K.) originally collected from plantain (*Musa* AAB) in Cameroon, was maintained and multiplied on axenic cultures of carrot disks in a laboratory incubator at 28 °C (Huettel, 1990). Nematodes were rinsed from the side of the culture jar with sterile, distilled water into a graduated plastic beaker and counted. The number in the suspension was adjusted to the required amount by addition or removal of water. In all manipulations involving counting and dispensing of nematodes, the suspension was constantly bubbled to ensure an even distribution.

Experiment 1: Testing for Flavan-3,4-diols and Condensed Tannins in *Musa* Roots. In a preliminary experiment, six Kunnan (*Musa* AB) plants were propagated by tissue culture (Vuylsteke, 1989) and transferred to the glasshouse. Five were inoculated with *R. similis* and one plant, the control, was not inoculated. Inoculation took place seven weeks after hardening when plants had a mean height of 6.5 cm and a mean leaf number of 4.5. Four holes, 2−3 cm deep, were made in the soil around the pseudostem of each plant, midway between the plant and the edge of the pot. A total of 500 *R. similis* in 2 mL of water were pipetted into the holes, which were then closed. Three weeks after inoculation roots were carefully washed. At this stage, plants had a mean height of 17 cm and a mean leaf number of 8 and sufficient time had elapsed in order for the hypersensitive reaction to take place. The lesioned areas were marked with Alpha tags, and roots were air-dried for 3 days and then dried at 35 °C overnight. The lesioned areas were removed and ground in a pestle and mortar.

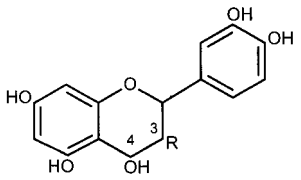
Experiment 2: Response to *R. similis* Infection by Susceptible and Resistant *Musa* Cultivars. Plants of three *Musa* cultivars, Dwarf Cavendish (susceptible), Yangambi Km5 (resistant), and Kunnan (highly resistant), were propagated by using tissue culture (Vuylsteke, 1989) and transferred to the glasshouse at 25−30 °C. Final pot size was 2 L, filled with a proprietary loam based compost (John Innes No. 2). Six weeks after potting, one plant per cultivar, in three replicates, was inoculated as above with about 3000 individuals of *R. similis*. Three replicates of each cultivar were not inoculated and used as controls. Twelve weeks after inoculation, roots were carefully washed and, on the treated root systems, the most heavily lesioned roots were marked with Alpha tags before being dried for 2 weeks in the laboratory at room temperature. Roots were then cut off at the point of attachment to the corm and dried in an oven at 35 ± 1 °C for 1 week until brittle. The marked roots from the treated samples were separated and an equivalent bulk sample from the controls was ground until uniform in a Janke & Kunkel grinder (Fisher Scientific, Loughborough, U.K.).

Butanol/HCl Assay. Ground samples (50 mg, experiment 1; 25 mg, experiment 2) were treated with 5 mL (experiment 1) and 10 mL (experiment 2) of butan-1-ol/HCl (95:5, v/v) at room temperature for 1 h to detect flavan-3,4-diols **3** and at 95 °C for 1 h to detect condensed tannins **1**, respectively (Figure 1; Watterson and Butler, 1983). [It can be assumed that the leucoanthocyanidins detected in *Musa* roots by this assay are flavan-3,4-diols **3**, rather than flavan-4-ols **2**, as the related proanthocyanidins **1** are flavan-3-ol derivatives as shown below (Stafford, 1990).] Extracts were thoroughly mixed with a Whirlimixer and centrifuged at 3000 rpm for 10 min. The absorbance at 550 nm was read against butan-1-ol/HCl using a Cecil CE2040 spectrophotometer (Cecil Instruments Ltd, Cambridge, U.K.).

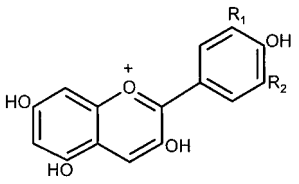
Thin-Layer Chromatography (TLC). Samples (50 mg) were extracted with acetone/water (7:3, v/v) in an ultrasonic bath for 10 min. The extracts (5−10 μl) were applied to cellulose MN300 plates (5 × 5 cm) (Camlab, Cambridge, U.K.) (Mueller-Harvey et al., 1987). Condensed tannins were developed in the first direction with acetic acid/water (2:98, v/v) and in the second direction with butan-1-ol/acetic acid/water (60:15:25, v/v/v) and detected as red spots after spraying with vanillin/HCl (1:10 g/mL). Anthocyanidins released by the butanol/HCl reagent were developed in the first direction in formic acid/acetic acid/water (10:1:3, v/v/v) and in the second direction in pentan-1-ol/acetic acid/water (2:1:1, v/v/v). They were detected by their characteristic colors and R_f values. UV/vis spectroscopy, using the HPLC system described below and coupled to a ProStar 330 diodearray detector (Varian Ltd, Walton on Thames, U.K.), also confirmed their presence in the extract.



1, a condensed tannin



2, R = H: a flavan-4-ol
3, R = OH: a flavan-3,4-diol



4 R_1, R_2 = H: pelargonidin
5 R_1 = OH, R_2 = H: cyanidin
6 R_1, R_2 = OH: delphinidin

Figure 1. Examples of a (**1**) condensed tannin, (**2**) flavan-4-ol, (**3**) favan-3,4-diol, and structures of (**4**) pelargonidin, (**5**) cyanidin, and (**6**) delphinidin.

Characterization of Condensed Tannins by HPLC. *Musa* root samples (50 mg) were treated with 3 mL of butan-1-ol/HCl (95:5, v/v) at 100 °C for 1 h. The supernatant was separated from the residue and placed at 50 °C under a stream of nitrogen until dry. Samples were stored at −25 °C overnight. The residue was dissolved in 1 mL of methanol/HCl (99:1, v/v) and centrifuged at 2500 rpm for 10 min.

An aliquot (20 μL) of the supernatant was injected onto a μ-Bondapak C_{18} column (8 mm i.d. x 100 mm; Waters, Watford, U.K.), which was protected by a cartridge guard column packed with 5 μm Spherisorb ODS (Hichrom, Reading, U.K.). Water/acetic acid (96:4, v/v; solvent A) and methanol (solvent B) were used for gradient elution at 2 mL/min. The gradient profile was 5−40% B (0−5 min), 40−50% B (5−10 min), 50−100% B (10−15 min), and 100−5% B (15−20 min) (Stewart et al., 2000). Absorbance at 525 nm was recorded on a Gilson HPLC system with Unipoint software (Gilson, Villiers le Bel, France) and a Kratos SF769 variable wavelength detector. Retention times of eluting peaks were compared to authentic chlorides of pelargonidin **4**, cyanidin **5**, and delphinidin **6** (Extrasynthèse, Genay, France).

Table 1. Production of Flavan-3,4-diols and Condensed Tannins in Kunnan in Response to Root Infection with *R. similis* 3 Weeks after Inoculation with 500 Individuals (Absorption at 550 nm)

treatment	flavan-3,4 diols	condensed tannins
− *R. similis*	0.017	0.438
+ *R. similis*	0.143	1.786

Lignin. Acid detergent lignin was determined according to Goering and van Soest (1970).

Statistical Analysis. Data were subjected to analysis of variance using the Genstat 5.3 statistical package (Genstat 5 Committee, 1993).

RESULTS AND DISCUSSION

Condensed Tannins and Flavan-3,4-diols. The presence and possible role of condensed tannins **1** (CTs) and flavan-3,4-diols **3** (FLs) (Figure 1) as phytoalexins was indicated in a preliminary study as both groups of compounds were detectable in roots of 10 week old Kunnan plants pre- and postinfection. Furthermore, levels of CTs and FLs increased within 3 weeks in response to *R. similis* infection (Table 1). Therefore, a more detailed study was undertaken with the above cultivars of known resistance differences.

All three cultivars responded to nematode infection with highly significant increases in phenolics: average CT values increased from 0.754 to 1.512 absorbance units ($P < 0.001$) and FLs from 0.038 to 0.212 absorbance units ($P < 0.001$) (Table 2). Differences were highly significant between cultivars with CT means averaged over the nematode treatments being 1.65 for Kunnan, 0.82 for Yangambi, and 0.94 for Dwarf Cavendish ($P < 0.001$). Kunnan had significantly higher CT concentrations *pre-* and *post*infection than the other two cultivars. In fact, Kunnan *pre*infection levels were similar to Yangambi and Dwarf Cavendish *post*infection levels (interaction effect was $P < 0.05$).

Monomeric flavan-4-ols and flavan-3,4-diols (*syn*. leucoanthocyanidins) generally function as precursors of polymeric CTs (*syn*. proanthocyanidins) (Stafford, 1990). FLs tend to be highly reactive and therefore unstable. As a result there are only a few reports of their natural occurrence (Watterson and Butler, 1983; Jambunathan et al., 1986; Stafford, 1990). As CTs were produced in response to *R. similis* infection, it is, therefore, not surprising that levels of FLs also increased significantly in response to infection ($P < 0.001$). These were higher in Kunnan, although not significantly so (Table 2).

The proportional increases of tannins decreased with increasing resistance: 145% for Dwarf Cavendish, 94% for Yangambi, and 83% for Kunnan. This indicated a stronger response to infection by the less resistant variety in terms of CT synthesis. The results suggest, therefore, that CTs are produced in *Musa* roots in response to *R. similis* infection and contribute to resistance. All cultivars had considerable amounts of CTs before infection, but the resistant Kunnan cultivar had the highest levels. As a result, Kunnan required a relatively smaller increase in CTs to arrest nematode development. The literature provides other examples of the pre- and postinfectional occurrence of the same compounds with pesticidal properties (Luis, 1998; Binks, 1996). The phytoalexin, anigofurone, was produced in the roots of the resistant cultivar Pisang Sipulu after infection with *R. similis* and it has also been found in the roots of the susceptible cultivar Grande Naine (Cavendish, AAA) in response to fungal attack (Binks et al., 1997).

The CT levels of the susceptible Dwarf Cavendish, however, were not as expected: postinfection it contained significantly higher CT concentrations than the partially resistant Yangambi cultivar. Although the rate of tannin synthesis was not measured in this study, it would be worth investigating if the CT synthesis was more rapid in Yangambi than in Dwarf Cavendish. Evidence in the literature indeed suggests that the rate of phytoalexin production can be an important resistance mechanism (Veech, 1979; Deverall, 1982). If phytoalexin production is slow at infection sites, the pathogen may take hold in susceptible cultivars (Deverall, 1982). For example, the resistance to *Verticillium albo-atrum* in cotton appeared to depend on the speed at which phytoalexin was accumulated (Coxton, 1982). Terpenoid aldehyde synthesis in a nematode-resistant cultivar of cotton was more rapid than in a susceptible cultivar (Veech, 1979). Resistance may also be compromised if phytoalexin accumulation is not only slow but is not anatomically localized to contain pathogen development (Veech, 1982). Although further work is required, we postulate that the accumulation of CTs may be rapid and/or highly localized in Kunnan, as the migration of *R. similis* is completely inhibited (Collingborn, unpublished).

Lignin. It has been suggested that lignin is implicated in resistance to nematodes (Giebel, 1974; Pinochet, 1988). There were no significant differences in lignin content ($P < 1.411$, SED = 0.84; Table 3). However, Yangambi Km5 had slightly more lignin postinfection (10.03% vs 8.63%) while Dwarf Cavendish had higher lignin content in the control (no nematode). However, lignin is a complex secondary product and its analysis can be problematic, especially in the presence of tannins (Reed, 1986). It was, therefore not too surprising to find no relationship between lignin content and resistance among the three *Musa* cultivars in this study (Table 3).

Tannin Composition. TLC indicated the presence of CTs with a high degree of polymerization as the R_f values were zero in both solvents. Only traces of anthocyanidins were detected by TLC and HPLC in the acetone/water extracts. This, therefore, indicated that the butanol/HCl reaction produced cyanidin **5** and pelargonidin **4** (Figure 1) from CTs. The HPLC separations (Figure 2) show that the CTs in *Musa* roots were

Table 2. Production of Flavan-3,4-diols and Condensed Tannins by Three *Musa* Cultivars 12 Weeks after Inoculation with 3000 *R. similis* (Absorption at 550 nm)

	flavan-3,4-diols			condensed tannins		
cultivar	− *R. similis*	+ *R. similis*	mean	−*R. similis*	+*R. similis*	mean
Dwarf Cavendish	0.036	0.217	0.127	0.543	1.330	0.937
Yangambi Km5	0.035	0.164	0.099	0.557	1.078	0.818
Kunnan	0.043	0.253	0.148	1.162	2.128	1.645
SED	0.026		0.019	0.104		0.073
mean	0.038	0.212		0.754	1.512	
SED		0.015			0.060	

Table 3. Lignin Content (g/100 g on DM Basis) in *Musa* Roots from Three Cultivars Pre- and Postinfection with 3000 *R. similis*[a]

cultivar	− *R. similis*	+ *R. similis*
Dwarf Cavendish	12.87	10.40
Yangambi Km5	8.63	10.03
Kunnan	10.87	10.23

[a] Means are not significantly different.

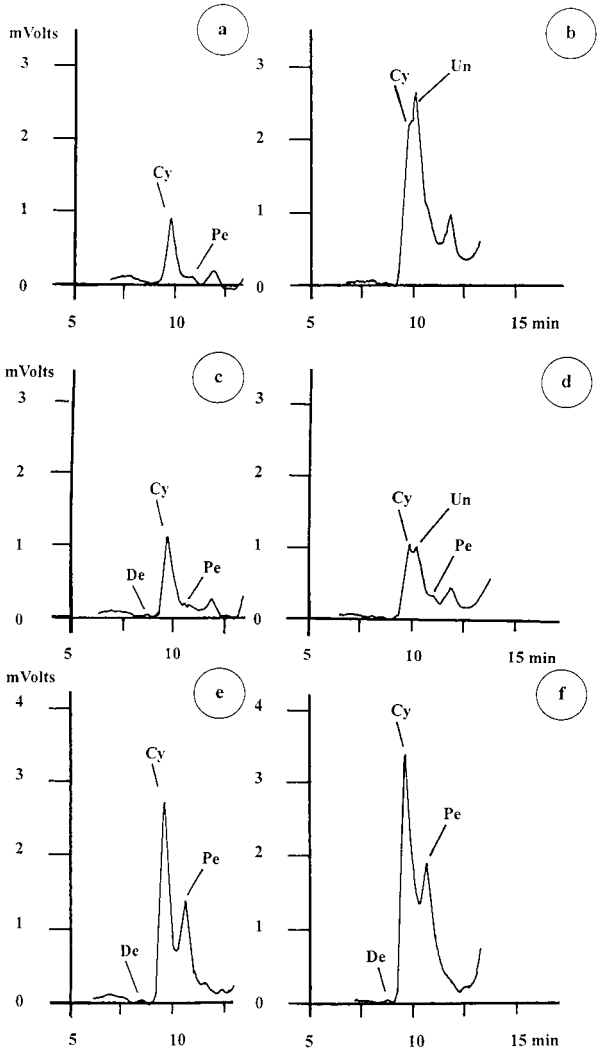


Figure 2. HPLC separations of anthocyanidins released from condensed tannins in *Musa* roots by butanol/HCl: (a and b) Dwarf Cavendish, (c and d) Yangambi Km5, (e and f) Kunnan; (a, c, e) before and (b, d, f) after infection with *Radopholus similis*. De = delphinidin, Cy = cyanidin, Pe = pelargonidin, Un = unknown anthocyanidin.

essentially procyanidins **1**. However, there were some distinct differences between cultivars: Kunnan CTs consisted of procyanidin and propelargonidin units, whereas Dwarf Cavendish and Yangambi Km5 CTs contained mainly procyanidins and neglible amounts of propelargonidins. The relative composition of procyanidins vs propelargonidins was the same before and after infection in Kunnan roots. No evidence was found for prodelphinidins, although they have been reported from other parts of *Musa* sp. (Williams and Harborne, 1988). In addition, an unidentified anthocyanidin (R_T = 10.1 min) was detectable in infected Dwarf Cavendish and Yangambi roots, whereas Kunnan roots appeared to have just a trace of this constituent.

The results from this study suggest that *Musa* roots produce CTs and flavan-3,4-diols in response to *R. similis* in addition to the phenylphenalenones reported previously by Luis et al. (1995). Further work is required to investigate rates of tannin synthesis and differences in tannin composition in relation to resistance and to test the nematicidal properties of these compounds in vitro. It would also be interesting to challenge Kunnan and Yangambi Km5 with *P. coffeae* and investigate the biochemical responses, as their responses to this nematode are similar to those found with *R. similis* (Collingborn and Gowen, 1997). Furthermore, once clarified, the resistance of Kunnan could be genetically engineered into agronomically suitable cultivars of *Musa*, for both commercial and subsistence production.

CONCLUSION

Concentrations of condensed tannins and flavan-3,4-diols were significantly higher after *R. similis* infection in all *Musa* cultivars. The cultivar Kunnan consistently had higher levels of condensed tannins pre- and postinfection than the other two cultivars, which indicated that they could be involved in the resistance mechanism. Tannin composition differed between the cultivars: propelargonidins were present only in the resistant Kunnan. It is suggested that preliminary screening for *R. similis* resistance of *Musa* cultivars could include the butanol/HCl assay as a rapid test for both flavan-3,4-diols and condensed tannins.

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