

Influence of secondary compounds in the phloem sap of cassava on expression of antibiosis towards the mealybug *Phenacoccus manihoti*

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Accepted: November 18, 1993

Key words: *Phenacoccus manihoti*, Homoptera, Pseudococcidae, *Manihot esculenta*, cassava, antibiosis, phloem sap, honeydew, plant phenolics, rutin, cyanogenic glucosides

Abstract

Identification and assay of cyanogenic and phenolic compounds in phloem sap of cassava (*Manihot esculenta* Crantz, Euphorbiaceae) and in honeydew of the cassava mealybug *Phenacoccus manihoti* Matt. Ferr. (Homoptera, Pseudococcidae) were realised.

Cyanogenic glucosides and three flavonoid glycosides (rutin, kaempferol glycoside-1 and kaempferol glycoside-2) were found to be translocated in cassava phloem sap and consumed by the mealybug. Differences in profiles of secondary compounds of phloem sap and honeydew samples, characterised mainly by the appearance of free cyanide and of a free flavonoid, suggest the metabolic processing of at least some of the ingested compounds.

The relationship between foliar concentrations of these different compounds and expression of the antibiotic resistance of cassava towards *P. manihoti* was also investigated in 7 varieties of cassava and in the 'faux-caoutchouc' hybrid. Infestation by mealybug was followed by a clear increase in levels of both rutin and kaempferol glycoside-2, while no modification in cyanide contents was noted. The best rank correlation between antibiotic resistance (measured by the intrinsic rate of increase r_m) and secondary compounds analyzed is observed with rutin contents of infested plants ($\rho = -0.73$; $p = 0.05$). The possible implication of this compound in the biochemical mechanisms accompanying cassava defence reaction to mealybug attack is discussed.

Introduction

Host-plant resistance of cassava (*Manihot esculenta* Crantz, Euphorbiaceae) against the cassava mealybug (*Phenacoccus manihoti* Matt. Ferr., Homoptera, Pseudococcidae) was investigated in Congo. Following the identification of different categories of resistance in this plant (*sensu* Painter, 1951; Tertuliano *et al.*, 1993), and the demonstration of an aphid-type phloem-feeding behaviour of the cassava mealybug (Calatayud *et al.*, 1994), the present work focused on the biochemical mechanisms involved in antibiosis, defined here as the long term interaction affecting the insect life history by reducing growth, reproduction or survival. For this purpose, the analysis of amino acids

and sugars of foliar extracts in a series of different host plants was undertaken. No relationship could be found between population growth values (r_m) and leaf content of primary compounds (amino acids and sugars). This result suggested that other biochemical factors may be involved in antibiotic resistance (Tertuliano & Le Rü, 1992).

It is well documented that secondary compounds, in addition to nutritional factors, may have important functions in plant resistance to pests (Fraenkel, 1969; Kogan, 1977; Pickett *et al.*, 1992). Until the end of the 70's, most studies on the implication of secondary compounds in plant resistance were based on phytophagous insects (Rhoades, 1983). Since then, numerous data on aphids illustrated the effects, in more spe-

cialised interactions, of alkaloids (Dreyer *et al.*, 1985; Smith, 1966; Wink & Witte, 1991), phenolic acids and flavonoids (Dreyer & Jones, 1981; Leszczynski *et al.*, 1985; Mc Foy & Dabrowski, 1984; Todd *et al.*, 1971) or cyanide compounds (Dreyer & Jones, 1981; Schoonhoven & Derksen-Koppers, 1976). Much less information is available on scale insects (Homoptera: Coccoidea). Newbery *et al.* (1983) showed that the susceptibility of different trees to *Icerya seychellarum* Nestw. (Hom., Margarodidae) was inversely correlated with foliar contents in alkaloids and condensed tannins. Contrary to this, Wargo (1988) could not assign any effect to levels of total phenolics in the resistance of *Fagus grandifolia* against the mealybug *Cryptococcus fagisuga* Lindinger (Hom., Coccidae).

For phloem-feeding insects, the role of such secondary compounds depends highly on their localisation within the plant. The presence of plant compounds strictly located in the mesophyll may only have a deterrent effect during stylet penetration – antixenotic resistance –, while if located in the phloem, they will influence settling or nutrition depending on their behavioural or metabolic effectiveness – antixenotic or antibiotic resistance – (Givovich *et al.*, 1992). Although much studied, the great difficulties in localising precisely these substances in plant tissues have often prevented formal demonstration of their defensive function against aphids (Dreyer & Campbell, 1987; Harrewijn, 1990; Molyneux *et al.*, 1990).

In this context, the potential role of cassava's secondary chemistry in the expression of an antibiotic resistance to *P. manihoti* was investigated as follows:

- i) identification and analysis of secondary compounds present in phloem sap of cassava (alkaloids, phenols and cyanides);
- ii) determination of the secondary compounds that were effectively ingested and transformed by the mealybug, through honeydew analysis;
- iii) correlations between levels of secondary compounds and degree of antibiotic resistance expressed in 7 varieties of cassava and in the 'faux-caoutchouc' (hybrid of *M. esculenta* and *M. glaziovii* Mull. Arg.).

Materials and methods

Insects. *P. manihoti* reproduces by thelytokous parthenogenesis, and the clone we used in initially collected on cassava from a local garden in Brazzaville (1985). Since then, a culture of *P. manihoti* was

maintained in the laboratory on cassava (*M. esculenta*, variety: M'Pembe) at 22–32°C and L12:D12 photoperiod.

Plants. The first objectives (i and ii) of our work were completed on the variety M'pembe, a control genotype already used for the electrical analysis of feeding behaviour of *P. manihoti* (Calatayud *et al.*, 1994). The third objective was performed on 8 genotypes of *Manihot*, comprising M'pembe. They were characterized previously by different intrinsic rates of increase of cassava mealybug populations (r_c , after Laughlin, 1965). The cassava varieties used were Incoza ($r_c=0.133$), 3M8 ($r_c=0.141$), Moudouma ($r_c=0.143$), M'Pembe and 30M7 ($r_c=0.150$), Zanaga ($r_c=0.155$) and Ganfo ($r_c=0.160$). The 'faux caoutchouc' hybrid ($r_c=0.141$) was also analysed (Tertuliano *et al.*, 1993).

Plants were obtained from cuttings of twenty centimeters, planted vertically at 2/3 of their height in soil embedded in plastic bags (30×22 cm). Cuttings were placed in a shady environment for two weeks to facilitate early development. They were maintained under full light until the 9–10 leaf stage was reached (9 or 10 weeks; approximate height 60 cm). Plants were watered twice a week, and kept in a greenhouse with controlled humidity (mean relative humidity 70% - extremes 60–80%) and semi-controlled temperature (mean 25°C - extremes 21–32°C). Natural photoperiod was used (L12:D12).

Plant infestation. Five plants of each genotype were artificially infested with one hundred neonate larvae of *P. manihoti*; five other plants remained uninfested (control). All plants were examined twice a week, and mealybugs were added or removed when necessary to maintain a constant population. Under these experimental conditions, close to those described by Tertuliano *et al.* (1993), we obtained after two months of experimentation, 4-months old plants with 19–20 leaves and 90–100 cm high.

Under natural conditions in Congo, mealybug outbreaks take place at the beginning of the dry season on 3–8 month old plants coming from cuttings of cassava planted from November to April.

Sample collection. The following extracts were collected, between 8 and 10 a.m., from top, second and third leaves of 4-month old plants:

- 'Petiole exudate': The day before collection, plant foliage of variety M'pembe was washed thoroughly; in the early morning of the next day (22–30°C, satu-

rated humidity), the natural exudate dropping from the petiole (unsevered) was collected in a capillary tube. Natural exudates from cassava collected in this way are thought to be phloem secretions from companion cells, as no structural nectaries were detected at the exudation sites (Pereira & Splittstoesser, 1987).

- Honeydew: Droplets of honeydew were frequently observed on the ovisac of adults. The day before sampling, adult mealybugs without honeydew were selected on M'pembe plants. The next day, droplets of honeydew excreted during the night were sampled with capillary tubes and centrifuged.

- 'Extracellular fluids': Because of difficulties in collecting natural exudates (exudation varies between cassava genotypes, and can only be obtained under saturated humidity), extracellular fluids were retrieved by a centrifugation method modified from Rohringer *et al.* (1983). Leaves were cut without their petiole, washed in distilled water and wiped. They were then enveloped in a nylon muslin (0.05 mm) and centrifuged at 4000 g for 20 min in a Sorvall SS 34 rotor. All extracts were freeze-dried, weighed (± 0.1 mg) and stored at -20°C until used.

Note on sample composition: The chemical analyses in the present study were aimed at representing the closest picture of cassava phloem sap. Although aware of many potential biases, we will consider in the following that 'petiole exudates' represent the better estimate of this compartment; in addition to the referred physiological and histological arguments (Pereira & Splittstoesser, 1987), it may be noted that the measured sugar composition of these samples favour such an assumption (85% sucrose, 7% glucose, 8% fructose). Honeydew samples, are also indicative of the occurrence of plant chemicals in phloem sap, but with a composition potentially altered by insect assimilation, metabolism or excretion. Finally, 'extracellular fluids' are the routine samples available for all plant genotypes for comparative purposes. Although containing exudates from phloem vessels (sugars are 80% sucrose), they are extensively contaminated by material from other tissues and cannot be used as reliable indicators of phloem localisation of phytochemicals.

Chemical analyses. - Alkaloids: extracts were dissolved in 100 μl of distilled water, deposited on a TLC plate of Silicagel 60, developed in pure methanol, and revealed with a Dragendorff reagent (Bounias, 1983). Caffeine and quinine were used as standards.

- Phenolics: extracts were dissolved in 250 μl of 50% methanol and centrifuged at 15 000 g for 5 min to

remove solids. Twenty μl were injected on a C18 RP-HPLC column with UV detection at 320 nm (Spherisorb S50DS2, 4.6×250 mm, from Prolabo, FRA). Isocratic elution with a mobile phase of water, acetonitrile and acetic acid (74.6, 23.4, 2% v/v) was performed at a flow rate of 0.8 ml/min. Attribution of each peak to either flavonoid or phenolic acid families was realised by comparison with retention times of standard phenolic compounds, and by comparing isocratic and gradient HPLC profiles, where peak collection was performed if needed for UV spectrum characterisation. Quantification was subsequently expressed either as rutin or as p-coumaric acid equivalent (only comparative values were of interest).

- Identification of flavonoids: Ten mg of 'extracellular fluids' – variety M'pembe – were dissolved in 1 ml of 50% methanol. Compounds were purified by gradient elution HPLC. One hundred μl were injected on a semi-prep RP-HPLC column with UV detection at 320 nm (ODS2 $\mu\text{Bondapak}$, 19×1.5 cm, from Prolabo, FRA), and a mobile phase of aqueous acetonitrile at 0.8 ml/min (A: H_2O , 2% acetic acid; B: 80% acetonitrile, 2% acetic acid in H_2O ; 12–30% B in 20 min, 30–100% B in 15 min, back to 12% B in 5 min). After detection, the main peaks were collected and dried without heating in a speed-vac system (Savant SC 100). Purified peaks, pooled from at least 5 runs, were identified at the Institut de Chimie des Substances Naturelles (ICSN-CNRS, Gif-sur-Yvette, FRA). After acid hydrolysis of flavonoids, aglycone groups were identified by ^1H and ^{13}C NMR, and sugars were reduced by NaBH_4 and acetylated by $(\text{CH}_3\text{CO})_2\text{O}$ before GC-MS identification of derivatives (isobutane vector; EIMS and FABMS).

- Cyanides: dry extracts of phloem sap and honeydew (on variety M'pembe) were dissolved in 2 ml of phosphoric acid 0.1 M. The method described by Monroy-Rivera *et al.* (1990) was used to analyse separately free and bound cyanides in these samples. For extracellular fluids, only free cyanides liberated by hydrolysis of bound forms during the extraction process were analysed. Dry samples ($n=80$) from different genotypes were dissolved in 250 μl of distilled water and cyanides were assayed by spectrophotometry with a commercial kit, using KCN as standard (Spectroquant 14800, Merck, GER). CN values were supposed here to reflect total cyanogenic compounds in extracts, although cyanides are mainly in bound form in the plant.

Toxicity tests on artificial diets. Purified chemicals (KCN, rutin) were tested on artificial diets at 21 °C following the protocol and diets described for an aphid (Rahbé & Febvray, 1993). Three groups of more than 30 neonate larvae hatching from ovisacs (aged 0-24 h) were deposited at day 0 on artificial diets and mortality at day 7 was used to compute LC50s. As aphid diet was not appropriate for long term rearing of *P. manihoti*, control mortality at day 7 was high (60%), and computed mortalities were corrected with this control value. Artificial diets for long term rearing of the cassava mealybug are lacking. The aphid diet used allowed significant long term survival and honeydew production (some individuals survived more than three weeks), indicating sustained feeding of the insects. Such diets proved to be useful for testing the acute toxicity of plant secondary compounds.

Statistical analysis. For analysis of variance, factors 'genotype' and 'infestation' were considered as a fixed model. Contents of identified secondary compounds were found to be homogeneously distributed between factors, as tested with the Kolmogorov-Smirnov test (homoscedasticity hypothesis for ANOVA). Within each significant factor ($p < 0.05$), means were compared using Fisher's PLSD multiple range test (Table 2). Simple or rank (Spearman) correlation analyses were performed to match mealybug performance (r_c) to contents of secondary compounds. These statistics were completed using the Statview software (Abacus Concept, USA).

Contents of secondary compounds in infested and uninfested plants (all genotypes included) were subjected to a multivariate linear discriminant analysis (using all secondary substances detected) to trace effects of mealybug attack on the secondary chemistry of cassava (induced reaction). The AnaMulTM software was used for these calculations and plots (Febvay & Bonnot, 1989).

Results

The unit used throughout this work (mg/g dry weight), although not representing actual concentrations in plant or insect fluids, is a good comparative index as it reflects the relative 'investment' in secondary chemistry as compared to the total solutes present in a sample. In some instances, it may be a better estimate than true concentration data, which may vary due to

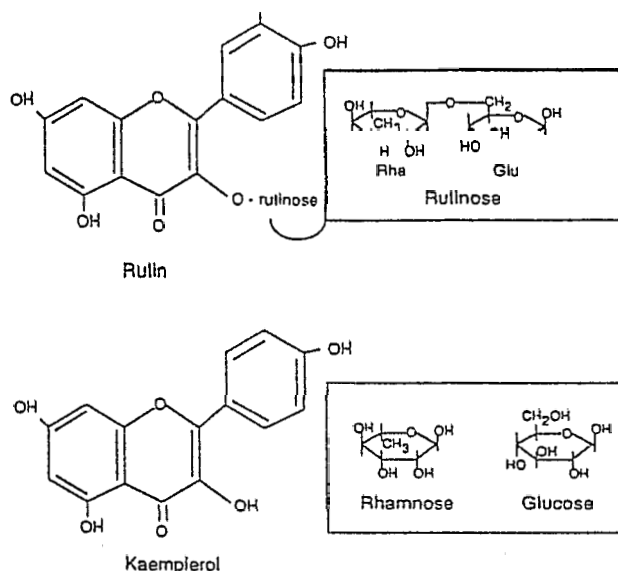


Fig. 1. Chemical structures of flavonoid glycosides identified in cassava 'phloem sap'.

dilution effects caused by artefacts or due to natural physiological responses.

Secondary compounds in 'petiole exudate' and honeydew (Table 1). The absence of alkaloids in samples from all cassava varieties suggested that these substances were not involved in the defence mechanisms against *P. manihoti*.

'Petiole exudate' contained substantial levels of bound cyanide (1.6 mg/g of dry weight), traces of phenolic acids and three flavonoid glycosides (Table 1). These flavonoid glycosides were identified as rutin and two kaempferol derivatives (termed KG1 and KG2), both containing glucose and rhamnose in undetermined order and configuration, but differing from rutinose (Fig. 1). Free cyanides or flavonoid aglycones were not detected from 'petiole exudate' samples.

Honeydew contained cyanogenic glucosides (0.6 mg/g of dry weight), free cyanides (0.8 mg/g of dry weight) and phenolic compounds (8.1 $\mu\text{g/g}$). HPLC analysis of phenolic compounds showed major differences between profiles from honeydew and 'petiole exudate' (Fig. 2a and b). Only KG1 and KG2 were present in both samples, although in different relative amounts. Rutin (retention time 5.26') was not found in honeydew, while another flavonoid glycoside appeared at 5.06'. We observed one phenolic acid at 4.65' and one free flavonoid at 12.88', both lacking in 'petiole exudate'. It should be noted that all HPLC runs were

Table 1. Secondary compounds detected in natural exudate ('phloem sap') of cassava and in honeydew of cassava mealybug

Compounds	Natural exudate	Honeydew
Alkaloids	nd	nd
Phenolic compounds (n=3)		
phenolic acids	trace	1.0±0.3 µg/g
flavonoid glycosides	19.0±2.3 µg/g	7.0±3.0 µg/g
free flavonoids	nd	0.1±0.03 µg/g
Cyanide compounds (n=4)		
Cyanogenic glucosides	1.6±0.3 mg/g	0.6±0.1 mg/g
Free cyanides	nd	0.8±0.2 mg/g

* Mean ±standard error, expressed as µg or mg/g of dry weight
n=replicate number; nd=not detected

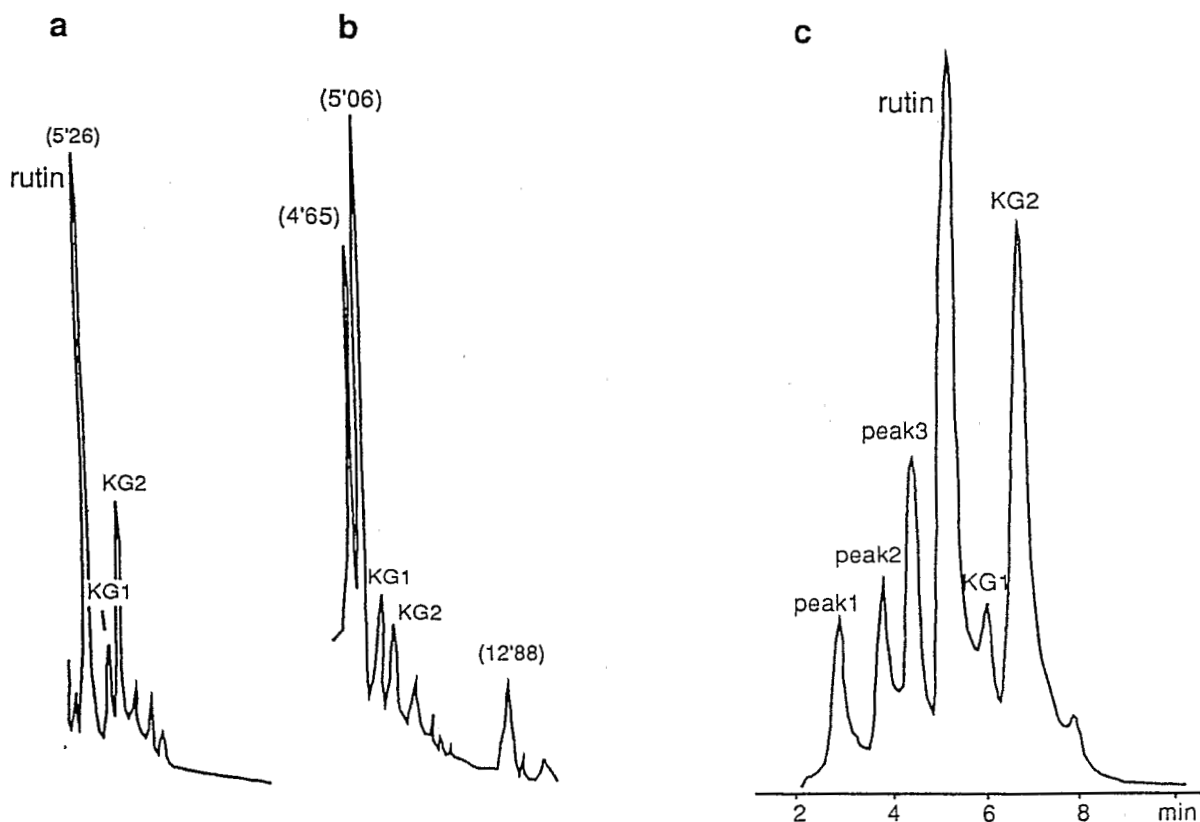


Fig. 2. Isocratic RP-HPLC chromatograms of different cassava extracts. (a) natural exudate ('phloem sap'); (b) honeydew extract; (c) typical chromatogram obtained with 'extracellular fluids' (expanded scale).

Table 2. Means of secondary compounds analysed in leaf 'extracellular fluids' of eight host plants, infested and uninfested (mean±standard error*, n=5, in mg/g of dry weight) and results of 2-way ANOVA (genotype and infestation)

Plants	R _c	Rutin		KG1		KG2		Free cyanide	
		uninfested	infested	uninfested	infested	uninfested	infested	uninfested	infested
Ganfo	0.16	7.8±0.9 c.u	7.8±1.4 a.u	1.8±0.3	1.8±0.4	5.1±0.6 c.u	5.6±0.9 ab.u	0.7±0.2 a	1.3±0.2
Zanaga	0.155	1.8±0.7 ab.u	8.1±1.4 a.v	0.9±0.1	2.1±0.4	1.7±0.5 a.u	6.3±1.1 b.v	3.0±0.3 c	1.6±0.3
30 M 7	0.15	1.9±0.9 ab.u	8.1±1.3 a.v	0.6±0.3	0.4±0.1	2.4±0.9 ab.u	8.1±2.1 b.v	3.3±0.6 c	2.6±0.4
M' pembe	0.15	0.9±0.1 a.u	6.5±0.4 a.v	1.4±0.1	3.2±0.2	1.4±0.1 a.u	8.6±0.5 bc.v	1.4±0.2 b	0.8±0.1
Moudouma	0.143	2.3±1.2 ab.u	9.8±0.6 a.v	0.5±0.3	3.3±0.4	1.3±0.6 a.u	6.7±0.8 abc.v	2.0±0.3 abc	1.7±0.4
3 M 8	0.141	4.7±4.1 bc.u	15.6±4.1 b.v	0.4±0.1	0.7±0.1	3.1±1.9 abc.u	10.0±2.2 c.v	3.3±1.5 c	1.9±0.8
'Faux coutchouc'	0.141	7.8±0.2 c.u	8.9±1.1 a.u	1.7±0.4	8.3±6.7	2.1±0.4 ab.u	3.3±0.6 a.u	0.7±0.1 a	0.9±0.2
Incoza	0.133	4.5±1.3 bc.u	9.1±0.1 a.v	0.4±0.1	0.5±0.1	3.9±1.2 bc.u	7.6±0.7 bc.v	0.6±0.3 ab	0.8±0.3
Factors of ANOVA									
Genotype (A)			0.0039		0.2047		0.0225		0.0001
Infestation (B)			0.0001		0.0853		0.0001		0.0809
(A×B)			0.037		0.57		0.0214		0.3132

* Means followed by the same letter are not different at the 5% level (Fisher's PLSD test following ANOVA).
a,b,...: Column comparison (genotype factor); u,v,...: Line comparison (infestation factor).

performed in a single session, in isocratic conditions, so that retention times differing by 0.2 min unambiguously represented different compounds (as judged by t-tests on retention times, clearly discriminating rutin and the unknown '5.06' peak, but not KG1 and KG2, in the two samples; n=3).

Variability of flavonoid glycoside and free cyanide contents in 'extracellular fluids' with genotype and infestation status (Table 2). Only major phenolic peaks were considered for this analysis (chromatogram in Fig. 2c). Phenolic acid contents (peaks 1-3 on the chromatogram) did not increase significantly with infestation for most genotypes and no correlation could be found between their levels in 'extracellular fluids' and r_c values. These peaks were thus not identified. Furthermore, the 3 flavonoid glycosides identified so far (rutin, KG1 and KG2) were the only compounds clearly detected in 'petiole exudate' considered as phloem secretion. Therefore, our interest focused on these 3 glycosides. Infestation by *P. manihoti* induced significant increases in flavonoid glycoside contents in 'extracellular fluids' (from 2 to 8 times, depending on the flavonoid and the genotype; Table 2). This increase is more important with rutin and KG2 (for variety M' pembe, in the present experiment, rutin content increased 7.2 times and KG2 6.6 times).

Our results did not show any influence of mealybug infestation on cyanide levels. The linear discriminant analysis indicated that, when the relative levels of all secondary compounds were considered together (and for all genotypes), most plants (83%) could be correctly assigned chemically regarding their infested or uninfested status (ANOVA significant between groups, p<0.001). The discriminator is a linear combination of the analysed compounds (data and figure not shown), and its correlation coefficients with initial variables showed that the major coefficients were attributed to KG2 and rutin (0.95 and 0.82 respectively), confirming ANOVA results.

Finally, when comparing chemical analyses of infested plants and scores of the different genotypes for antibiotic resistance (indexes both related to a long-term interaction), the intrinsic rate of increase (r_c) was shown to be significantly correlated (Spearman's rank test) with rutin contents (ρ=-0.73; p=0.05), but not to KG1 (ρ=-0.02; p=0.95), nor to free cyanides (ρ=0.18; p=0.62) or even to KG2 (ρ=-0.24; p=0.52).

Toxicity assays. We obtained a corrected LC50 on day 7 of 274 µg/ml for KCN on *P. manihoti*, when tested on a range of 100 to 1600 µg/ml. Below 100 µg/ml, no additional mortality was observed as compared with controls. For comparison, the pea aphid displayed an

LC50 of 112 $\mu\text{g/ml}$ (a surprisingly high value). It must be noted that LC50 is very probably underestimated for *P. manihoti* due to the use of non-optimal artificial diets, a statement corroborated by the fact that even at the highest concentration used (1600 ppm), 5–10% of the mealbugs survive after 7 days on all replicates.

For rutin, no additional mortality was observed up to 250 $\mu\text{g/ml}$, but effects on development or growth impairments could not be measured because of the poor performance of *P. manihoti* on the aphid diet used (on control diet in an independent experiment, mortality was scored at 58.5% on day 8 and 96% on day 17). Solubility problems occurred with rutin at higher concentrations, preventing estimation of acute toxicity levels.

Discussion and conclusion

Direct demonstration of translocation of secondary compounds in phloem sap is rarely provided (Harrewijn, 1990; Molyneux *et al.*, 1990; Dreyer & Campbell, 1987). Before 1986, only two studies showed the translocation of alkaloids: Dreyer *et al.* (1985) on *Astragalus lentiginosus* and Wink & Witte (1984) on *Lupinus* spp. More recently, phloem transport was also demonstrated in wheat for hydroxamic acid glucosides (Givovich *et al.*, 1992) and in *Castanospermum australe*, *Senecio vulgaris* and *Nerium oleander* for alkaloids or cardenolides (Molyneux *et al.*, 1990). The presence of phenolics in phloem was reported by Pend & Miles (1991) on *Rosa*, and by Mullin (1986) on *Gossypium*.

In the present work, we considered that 'petiole exudates' of cassava were phloem secretions, on the basis of histological arguments (Pereira & Splittstoesser, 1987), chemical data (low levels of reducing sugars) and physiological observations (exudation rate increased in case of water stress; unpublished results). Of course, chemical filtering may occur, and the true demonstration of identity with phloem will only be possible when a Homopteran adequate for stylectomy is found. This technique is widely used on aphids (Fisher & Frame, 1984; Rahbé *et al.*, 1990), but is almost impossible on pseudococcids, and all wounding techniques are unsuitable due to latex contamination in Euphorbiaceae.

The cyanogenic glucosides of cassava are the L-valine and L-isoleucine derived linamarin and lotustralin (Conn, 1980). Although not chemically identified in this study, they are assumed to represent

the bound cyanides measured in our natural phloem exudates. They were previously detected from cassava exudates, as reported by Fahn (1979), though not by Pereira & Splittstoesser (1987). These conflicting results might be explained either by varietal differences or by a potential loss of volatile cyanides during the preparative step of the Warburg method, used by the latter authors. Cyanogenic glucosides were also present in honeydew of mealybug, indicating that they were translocated in the insect's food, which is essentially phloem sap. Following our EPG analysis of *P. manihoti* (Calatayud *et al.*, 1994), we are convinced that sustained ingestion occurs strictly in phloem sap. Although access to the apoplast or to the intracellular compartment of non-phloem tissues has been observed, it is highly probable that honeydew originates quantitatively from phloem sap and not from other tissue fluids. This is not a general statement valid for all Homoptera (see EPG on *Nilaparvata lugens*, Kimmins, 1989) but should hold for the aphids, whiteflies and mealybugs analysed so far. Therefore, the lower content of cyanogenic glucosides in honeydew, compared with phloem sap, and the appearance of free cyanides in this honeydew suggest that the digestive metabolism of mealybug is responsible for the hydrolysis of part of the ingested cyanogenic glucosides (linamarase activity). This phenomenon indicates the existence of efficient excretion or detoxification mechanisms for HCN in *P. manihoti*, as found in many microorganisms and in some insects (Meyers & Ahmad, 1991; Segretain & Bories, 1986). Actually, a linamarase activity distinct from that of cassava linamarase was found in *P. manihoti* (Calatayud, 1993), confirming potential cyanide liberation in the insect. Our toxicity assays on *P. manihoti* indicated that free cyanides were not very toxic to this insect (LC50 around 300 ppm). They appear to be much less harmful on a weight basis than many proteins towards another sucking insect, the pea aphid *Acyrtosiphon pisum* (some LC50s were around 15 ppm; Rahbé & Febvay, 1993). The effect of linamarin on *P. manihoti* has not yet been investigated in artificial diets, but assuming that free cyanides could occur in the insect's gut at concentrations found in the honeydew (0.8 mg/g dry weight, resulting in not more than 80 $\mu\text{g/ml}$ of sap), this molecule would be ineffective on mealybug physiology: in the range of 1–100 $\mu\text{g/ml}$ of KCN in the diet, no acute toxicity was reported. It is therefore probable that *P. manihoti* has evolved physiological mechanisms turning cyanogenic compounds from toxic to non-toxic, or even to nutrients. Another indication

of such a possibility is the very high level of bound cyanides in the insect's food as compared with other components (approx. 100× the levels in flavonoid glycosides on a weight basis). Free amino acid levels in 'petiole exudate' were found to be very low in cassava (data not shown), and cyanides amounted to more than 2.4× the total free amino acid levels on a molar basis, thus representing a major nitrogen source in phloem. Together with their possible phagostimulatory function, as deduced from behaviour/chemistry correlations (Calatayud *et al.*, 1994), these features fit well with the scheme of evolution from a toxin/allomone to a nutrient/kairomone system in the specialised herbivory of *P. manihoti* on cassava, as illustrated in many insect-plant models. Of course, such a statement needs unambiguous demonstration of cyanide assimilation (especially for the nitrogen atom) and feeding stimulation by cyanogenic glucosides, which could easily be achieved on appropriate artificial diets. Feeding stimulation by cyanogenic glucosides has been reported, for example, with amygdalin in a Lasiocampidae/Rosaceae interaction (Kogan, 1977).

In plants, phenolic compounds are stored in vacuoles as glycosides, less toxic than the free forms (Harborne, 1967; Leszczynski, 1985). These storage compounds may be hydrolysed into the corresponding aglycones by glycosidases of plant or pest origin (Hosel, 1981). In spite of the amount of published work on the links of secondary compounds with plant resistance, few of them deal with compartmentalisation of these substances in plants, which is essential for Homoptera. In many reports on this order, the accessibility of secondary substances and actual ingested amounts are known (Dreyer & Jones, 1981). In cassava, phenolic compounds translocated in phloem sap and consumed by *P. manihoti* are mainly flavonoid glycosides (rutin, KG1 and KG2). The differences in phenolic profiles-of-exudates-and honeydew indicate metabolisation of these substances in the insect. The unknown peaks observed in honeydew of *P. manihoti* might correspond to transformation of rutin, a flavonoid not found in honeydew. Such modification of the flavonoid pattern was previously reported for aphids (Dreyer & Jones, 1981; Peng & Miles, 1991). As regards rutin in this present work, Peng & Miles (1991) detected the presence of catechin in phloem sap of Rosaceae, but not in honeydew of *Macrosiphum rosae*. They also showed a differential composition in phenols between haemolymph, gut and honeydew. Although little documented, metabolism of alimentary phenols by phloem-feeding insects may be a common

phenomenon (Mullin, 1986). Its physiological and ecological significance is still obscure.

Flavonoid glycosides present in phloem sap, and especially rutin, are ingested by mealybug. Only the rutin contents of infected plants is somehow linked to antibiosis, but the relationship seems too weak to reflect direct effects of rutin levels on the insect (rank correlation without linear dose effect; Table 2). However, the previous relationship still holds on widening the host range: flavonoid glycosides were measured in leaves of Poinsettia (*Euphorbia pulcherrina* Wild., Euphorbiaceae) and *Talinum* (*Talinum triangulare* Jacq., Portulacaceae). These two plants are optional hosts that are rarely infested by *P. manihoti* in natural conditions (Calatayud *et al.*, 1994), but display a marked difference in antibiosis when tested in the laboratory (Tertuliano *et al.*, 1993). Rutin is present in extracellular fluids from both plants, at levels 13 times greater in 'resistant' poinsettia (rutin at 16 mg/g; $r_c=0.038$) than in 'susceptible' *Talinum* (rutin at 1.2 mg/g; $r_c=0.150$). In addition, rutin levels are sensitive to the presence of the mealybug, regardless of plant genotype (together with KG2). This observation has been confirmed in independent experiments involving either higher levels of infestation or other plant growth conditions, such as experimental water stress or open-field experiments (unpublished results). These indirect arguments support the hypothesis that rutin may participate in an antibiotic resistance of cassava to *P. manihoti*, or at least be linked to an induced (defensive?) reaction of cassava towards *P. manihoti*.

However, other arguments are in favour of the null hypothesis, i.e. rutin has no effects at all. The cassava cultivar with the highest r_c value (Ganfo) displayed high levels of rutin in non-infested plants. Moreover, there was no significant correlation whatsoever between r_c -values over all cultivars and either rutin concentrations in uninfested plants or increased concentration following infestation (ρ -values are -0.273 and -0.156 respectively). In addition, rutin ingested by the mealybug was apparently extensively transformed in the insect (in contrast to the other glycosides KG1 and KG2). This suggests an apparent ability of *P. manihoti* to metabolise rutin, an indication that could be related either to toxicity or to detoxification mechanisms. Because of solubility problems with rutin in our artificial diets, combined with mediocre performance of *P. manihoti* on our standard aphid diet, we were not able to show any toxicity of this flavonoid in artificial diets (no acute toxicity noted at the concentrations tested and no possibility to test effects on

growth or reproduction). Overcoming these problems is crucial to the understanding of rutin function in our insect-plant system. Involvement of flavonoids in plant resistance to phloem feeders has already been claimed in many instances, although never completely demonstrated (Dreyer & Jones, 1981; McFoy & Dabrowski, 1984; Todd *et al.*, 1971). Rutin is reported as an allelochemical affecting growth, development, reproduction and metabolism of certain insects (Beck & Reese, 1976). It is possible that in phloem- or xylem-feeding Homoptera secondary compounds not only affect the insects themselves but also their symbiotes, as suggested by Dryer & Jones (1981) for *Schizaphis graminum*. It is similarly plausible to hypothesise that the symbiotic physiology of *P. manihoti*, which also harbours endosymbiotic bacteria, may be altered by ingested and metabolised rutin. Recently, Mbaye (1989) has shown that quercetin, the rutin aglycone, might be involved in the resistance of cassava against the pathogenic bacteria *Xanthomonas campestris* pv *manihoti*.

In conclusion, cyanogenic glucosides and flavonoid glycosides were translocated in phloem sap of cassava, consumed by *P. manihoti*, and therefore able to interact with its physiology. The involvement of these substances in mealybug/cassava interactions seems probable. Nevertheless, additional data are needed, such as conclusive demonstration of their strict phloem origin, and confirmation of their biological activities (positive or negative) towards cassava mealybug on artificial diets.

Acknowledgements

We thank A. Kiakouama and A. Agossou for their technical assistance. This work was partially supported by the French Ministry of Research and Technology (PhD grant P.A.C.). Thanks are given to V. James for her english corrections.

Résumé

Ce travail présente l'identification et le dosage des substances secondaires (composés cyanés et phénoliques) présentes dans la sève phloémienne du manioc (*Manihot esculenta* Crantz, Euphobiaceae) et dans le miellat de la cochenille farineuse *Phenacoccus manihoti* Matt. Ferr. (Homoptera, Pseudococcidae).

Des glycosides cyanogéniques et trois flavonoïdes glycosylés majeurs (rutine, kaempferol glycoside-1 et

kaempferol glycoside-2) sont transportés par la sève phloémienne du manioc et consommés par la cochenille. Le profil sensiblement différent des composés secondaires du miellat de la cochenille, en particulier l'apparition d'un flavonoïde libre et de cyanure, suggère une modification de certains composés lors du transit intestinal.

La relation entre les teneurs de ces différentes substances dans le liquide foliaire de 7 variétés de manioc et du 'faux-caoutchouc' (hybride de *M. esculenta* et de *M. glaziovii*) et l'expression de leur résistance antibiotique vis-à-vis de *P. manihoti* a également été étudiée. L'infestation par la cochenille se traduit par une importante augmentation des teneurs en rutine et en kaempferol glycoside-2, alors qu'aucune modification des teneurs en cyanure libre n'est enregistrée. La meilleure corrélation entre la résistance par antibiose, exprimée par la capacité intrinsèque d'accroissement r_c , et les teneurs en substances secondaires analysées est observée avec les teneurs en rutine des plants infestés ($\rho = -0.73$; $p = 0.05$). L'association éventuelle de ce glycoside aux mécanismes biochimiques de défense du manioc à la cochenille farineuse est discutée.

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