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Pyrrolizidine alkaloids of *Chromolaena odorata* act as nematicidal agents and reduce infection of lettuce roots by *Meloidogyne incognita*

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Summary – 1,2-dehydropyrrolizidine alkaloids (PAs) represent a class of secondary plant compounds that are active in defence against herbivory. They are present in *Chromolaena odorata*, one of the most invasive weeds of Asia and Africa. *In vitro* studies demonstrate that pure PAs from *C. odorata* roots have nematicidal effects on the root-knot nematode *Meloidogyne incognita*, even at concentrations of 70-350 ppm. *In vivo* experiments show that mulch or aqueous crude extracts from *C. odorata* roots reduce the infection of lettuce by *M. incognita*. Thus, the use of PA-containing plants appears to be a valuable element for integrated nematode management.

Keywords – biological control, botanicals, host finding, integrated pest management (IPM), mulch, root-knot nematode, secondary plant metabolites.

The neotropical *Chromolaena odorata* (King & Robinson, 1970) (=*Eupatorium odoratum* L.) (Asteraceae) is a widespread, vigorous invasive weed of plantations, fields, pastures and roadsides in Asia and Africa (M'Boob, 1991). *Chromolaena odorata* has also been described as a non-host or a poor host for plant-parasitic nematodes (Atu & Ogbuji, 1982; Mateille *et al.*, 1994; Müller & Sturhan, 1994; Goly & Téhé, 1997). Furthermore, it was reported that either fallow dominated by *C. odorata* or the direct application of plant material, *e.g.*, as mulch, reduced the population density of plant-parasitic nematodes (Litzenberger & Lip, 1961; M'Boob, 1991; Mateille *et al.*, 1992, 1994; Matondo *et al.*, 1993; Ajith & Sheela, 1996; Adekunle & Fawole, 2002, 2003; Adediran *et al.*, 2005). In addition, aqueous crude extracts of *C. odorata* have been shown to have nematicidal properties (Subramaniyan, 1985; Jasy & Koshy, 1992).

The mechanism underlying these nematicidal effects was unknown. However, roots of *C. odorata* contain large amounts of 1,2-dehydropyrrolizidine alkaloids (PAs) (Biller *et al.*, 1994), a class of secondary plant compounds that play an important role in chemoecological relationships (Boppré, 1990; Boppré & Fischer, 1999; Hartmann, 1999). In plants PAs occur as *N*-oxides and as free bases and serve as defences against herbivores (Hartmann, 1999; Narberhaus *et al.*, 2005).

We hypothesise that PAs have a negative impact on plant-parasitic nematodes as well. Therefore, the objectives of the present study are to determine whether mulch or aqueous crude extracts from *C. odorata* roots can reduce the infection of lettuce seedlings by the root-knot nematode *Meloidogyne incognita* and whether the nematicidal effects are due to PAs.

Materials and methods

PLANT MATERIAL/NEMATODES

Roots (0.1-2 cm diam.) of *C. odorata* were collected near Kumasi, Ghana, West Africa, chopped into 2-5 cm pieces, air-dried, and stored at room temperature (20-25°C; humidity *ca* 50%).

Lettuce seeds (*Lactuca sativa* cv. Viktoria) were allowed to germinate on moist filter paper within covered transparent plastic boxes (7.5 cm diam.). After 5 days of

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growth at room temperature the seedlings were used for the experiments.

Meloidogyne incognita (Kofoid & White, 1919) Chitwood, 1949 was reared under glasshouse conditions (20-25°C; humidity *ca* 60%) on tomato (*Lycopersicon esculentum* cv. Moneymaker). For inoculum production heavily galled tomato roots were extracted by the mistifier technique described by Hooper *et al.* (2005).

Preparation of mulch, aqueous crude extract and pure PAs

Mulch of *C. odorata* was prepared by grinding the dried roots with a coffee mill into pieces of about 0.01-1.0 cm. Aqueous crude extract of *C. odorata* roots was prepared from 10 g of the mulch material soaked in 100 ml tap water (pH 6.8), shaken for 10-15 s and 24 h later filtered through a 200 μm aperture sieve. Aqueous crude extracts were always prepared fresh and used immediately.

Pure PAs of *C. odorata* roots were obtained from 100 g of pulverised roots soaked in 500-600 ml methanol and stirred vigorously for 24 h on a magnetic stirrer at room temperature. This extract was then passed through a filter paper (pore size 4.5 μm; Schleicher & Schuell No 595 1/2, Dassel, Germany) and the methanol removed by evaporation in a rotary evaporator at 45-50°C. The residue was dissolved in 350 ml aqueous sulphuric acid (2 M) to which 20 g zinc dust was added (leading to a reduction of PA *N*-oxides into their free bases). After being stirred for 4 h at room temperature, the solution was again passed through filter paper, washed three times with ether and basidified with ammonia (pH 11-12). Extraction with dichloromethane finally yielded the PA free bases.

One part of the sample of free bases was oxidised with hydrogen peroxide to form the corresponding PA *N*-oxides: therefore, 100 mg of the free bases was dissolved in 20 ml methanol and 2.5 ml hydrogen peroxide. This solution was stirred for 4 h in a water bath at 40°C and finally the methanol evaporated as described above.

The obtained pure PA *N*-oxides and free bases were used to prepare an aqueous PA stock solution for the *in vitro* experiments. Its total PA concentration was 700 ppm, consisting of equal shares of free bases and *N*-oxides. This proportion was equivalent to the proportion of *N*-oxides and free bases detected in the aqueous crude extract. To prevent hydrolysis and, thus, degradation of the PAs, the aqueous stock solutions were always prepared fresh and used immediately.

To confirm the presence and nature of PAs, aqueous crude extracts and solutions of pure PAs were subjected to TLC analyses (Mattocks, 1967; Molyneux & Roitman, 1980). These were done on Alugram® silica gel TLC plates (0.20 mm silica gel 60, Macherey-Nagel, Düren, Germany). Ten to 20 μl of the samples, aqueous crude extracts or purified PAs from *C. odorata* roots at a concentration of 1000 ppm dissolved in water or methanol, were spotted on the plates and these spots were run with a mixture of methanol-dichloromethane-ammonia (15:82:3, v/v). For detecting PA *N*-oxides the plates were sprayed with acetic anhydride, heated for 10 min at 70°C and finally resprayed with Ehrlich reagent (10 g 4-dimethylaminobenzaldehyde in 90 ml hydrochloric acid). For detection of the PA free bases the plates were sprayed with *o*-chloranil (1 g in 70 ml chloroform), heated for 3 min at 75°C and finally resprayed with Ehrlich reagent.

In vivo experiments

For the *in vivo* experiments, 100 g washed sand (moisture 20%) was placed into a series of plastic pots (6 × 6 × 6 cm), each of which was inoculated with 400 second-stage juveniles (J2) of *M. incognita* in 2 ml tap water. The following day the pots were treated with either *C. odorata* mulch, the corresponding aqueous crude extract, or tap water.

Mulching

The root mulch was applied at rates of 0.1%, 0.5%, 1.0% and 5.0% (w/w), mixed into the top 1.5 cm of the sand and each pot watered with 10 ml tap water. The next day one lettuce seedling was planted into each pot and given 10 ml water every 48 h.

Application of aqueous crude extract

The aqueous crude extract (10%, w/v) was diluted with water to obtain final crude extract concentrations of 0.1%, 0.5%, 1.0% and 5.0% (w/v) and 10 ml of these dilutions was applied 24 h before and every 48 h after planting the lettuce seedlings.

Controls were treated with equivalent amounts of tap water. Each treatment was repeated eight times. Pots were kept in a glasshouse (25-35°C) and arranged in randomised complete blocks.

Seven days after planting, the lettuce seeds were uprooted and roots were stained with acid fuchsin (Byrd *et al.*, 1983). The number of stained juveniles inside the roots was counted under a stereomicroscope (20-50×) and root lengths were measured from digital photographs with

ImageJ (freeware: http://rsb.info.nih.gov/ij/) in order to calculate the number of juveniles per cm root.

IN VITRO EXPERIMENTS

Water test

Meloidogyne incognita J2 were exposed to pure PAs from *C. odorata* roots in Falcon® 24-well cell-culture plates (BD Biosciences, San José, CA, USA) (Jourand *et al.*, 2004a). Each well received approximately 100 J2 of *M. incognita* suspended in 500 μl water and 500 μl of dilutions from the aqueous PA stock solution. Thus, final PA concentrations were 0 ppm, 7 ppm, 70 ppm and 350 ppm, simulating the PA concentrations of the *in vivo* mulch experiment. These were calculated on the basis of the known PA concentration of the root material and the average amount of water in a pot. To prevent desiccation the plates were covered and stored in the dark at room temperature. Each concentration was repeated six times and the experiment conducted twice.

Every 24 h the activity of J2 was recorded under a stereomicroscope (15-20×) and expressed as the ratio of inactive (long-stretched) J2 to total number of J2 per well. The experiment was terminated after 7 days. To see if there was any recovery, all J2 were then transferred into fresh water for 24 h and re-evaluated for their activity.

Sephadex® test

As well as directly affecting nematode activity, PAs might also influence their ability to find and invade a host plant. This was tested in Sephadex® (Sigma-Aldrich, Munich, Germany) following the procedure described by Moltmann (1982) and Lung (1989). Once water is added, Sephadex® becomes transparent and has a granular sand-like structure in which lettuce seedlings grow readily. Therefore, it is ideal for studying the impact of PAs on the movement of nematodes, their host finding ability and root invasion.

Sephadex® 100 and Sephadex® 150 were mixed 3:1 to achieve a medium with similar particle size as sand (bead size 40-300 μm). Then 125 mg of this mixture was placed into each of a series of plastic Petri dishes (6 cm diam.) and moistened with 1 ml of tap water plus 1 ml aqueous PA solution of a given concentration. The resulting PA concentrations were 0 ppm, 7 ppm, 70 ppm and 350 ppm, respectively. Thereafter, two lettuce seedlings were placed into the Sephadex® at one side of each Petri dish and approximately 100 *M. incognita* J2 in 10 μl water were inoculated on the opposite side. Petri dishes were covered, sealed with Parafilm® and stored in transparent plastic boxes at room temperature and 10 h daylight. Each of the four concentrations was tested seven times and dishes were arranged in randomised complete blocks.

Every 24 h, the number of *M. incognita* that had located and were attached to a root tip were counted under a stereomicroscope (20×). After 7 days the seedlings were removed from the Sephadex®, juveniles inside the roots were stained with acid fuchsin and their number counted under a stereomicroscope (20-50×).

STATISTICAL ANALYSIS

Data were subjected to analysis of variance using SPSS® 14.0 and means were separated by Duncan's multiple range test. Percentage values were transformed to arcsin values before analysis.

Results

THIN-LAYER CHROMATOGRAPHY (TLC)

TLC of the various PA preparations from *C. odorata* roots showed the presence of three or four different PAs (Fig. 1, lanes 1-3). In the aqueous crude extract, PA *N*-oxides and free bases were both present (Fig. 1, lanes 1, 2). The free bases detected in the aqueous crude extract were identical to the two major compounds found in the purified methanolic extract (Fig. 1, lanes 2, 3).

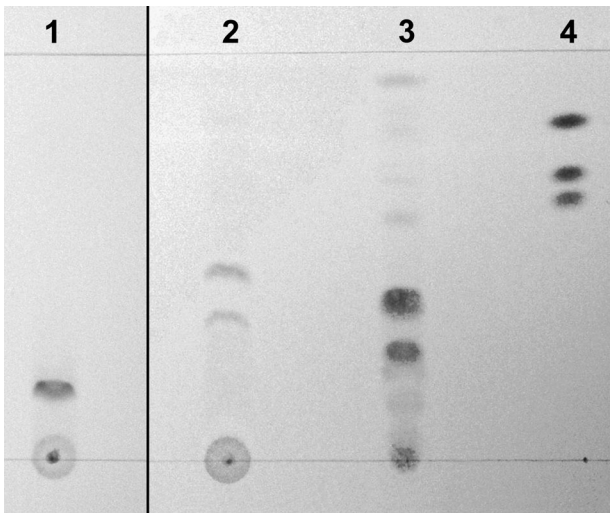

Fig. 1. *TLC tracks of an aqueous crude extract (lanes 1, 2) and the purified methanolic extract from* Chromolaena odorata *roots (3) showing PA* N*-oxides (1) and PA free bases (2, 3). For comparison a mixture of the PA free bases of senecionine, heliotrine and monocrotaline is shown (4).*

Table 1. *The effect of mulch or aqueous crude extracts from* Chromolaena odorata *roots on the number of* Meloidogyne incognita *penetrating lettuce seedlings.*

Treatment	E 0.1%	E 0.5%	E 1.0%	E 5.0%	C	M 0.1%	M 0.5%	M 1.0%	M 5.0%
Nematodes per cm root	14.33b	4.83c	4.02c	0.06c	33.10a	3.23c	1.33c	0.31c	0.00c
Nematodes per plant	74.17b	33.75c	27.00c	0.43d	113.75a	25.00c	12.75d	2.88d	0.00d
Root length (cm)	5.16b	6.99c	6.72c	6.87c	3.48a	7.74d	9.71e	9.30e	8.53e

Means with same letter within a line are not significantly different according to Duncan's multiple range test ($P \leqslant 0.05, n = 8$). E = extract, C = control, M = mulch.

In the course of the experiments no degradation or transformation of the tested compounds occurred.

IN VIVO EXPERIMENTS

Mulching with *C. odorata* roots as well as the application of aqueous crude extracts of *C. odorata* led to a significant reduction in the number of juveniles able to invade lettuce roots (Table 1). Overall, mulch had a stronger effect on nematode invasion than the aqueous crude extracts. Higher concentrations of mulch (0.5-5.0%) or extracts (5.0%) led to an almost complete protection of the lettuce seedlings against nematode invasion.

It was notable that roots of control plants and plants treated with the lowest extract concentration were so heavily infected that it was difficult to count the exact number of J2. Furthermore, high nematode numbers caused deterioration of the root tip tissue and, therefore, the numbers of nematodes given in Table 1 might be underestimates.

Table 1 also indicates remarkable differences in root length. Roots of plants treated with any form of *C. odorata* preparation were much longer than roots from untreated plants. This could be due either to the lower numbers of invaded nematodes or to a fertilising effect of the mulch or aqueous extract.

IN VITRO EXPERIMENTS

Water test

Exposure of *M. incognita* juveniles to different amounts of pure PAs from *C. odorata* caused a significant decrease in their activity (Fig. 2). Even at a PA concentration of 7 ppm the percentage of inactive J2 was significantly higher than in the controls. Increasing PA concentrations resulted in higher J2 inactivity with the strongest effect observed at 350 ppm.

The main reduction in juvenile activity occurred within 24 h and further exposure to PAs did not result in a pronounced additional decrease of activity, except in the 350 ppm treatments (Fig. 2). At the end of the study the proportion of inactive J2 ranged from 21% in the control to 86% at PA concentrations of 350 ppm. In addition, at higher PA concentrations (70 and 350 ppm) the J2 that were still active appeared visually to be in a poorer condition compared with the controls. Their movements were much slower and many of them seemed to be cramped. Transferring the J2 into fresh water did not lead to recovery of inactive J2, indicating that they were dead and not just paralysed.

Sephadex® test

Exposure to pure PAs from *C. odorata* led to a significant reduction of the number of *M. incognita* J2 attracted and attached to the root tips of the lettuce seedlings (Fig. 3). This was particularly so at higher PA concentrations (70 and 350 ppm), where, over a period of 168 h, hardly any J2 were observed on the lettuce roots (Fig. 3). However, even at 7 ppm there was a significant reduction in J2 attraction compared with the control (Fig. 3). For all treatments, the highest numbers of J2 attached to roots were recorded after 72 h (Fig. 3). Over the subsequent days J2 numbers decreased, indicating that some of them might have invaded the roots. While at PA concentrations of 350 ppm J2 remained close to the inoculation point and seemed to be dead or paralysed, in all other treatments the nematodes were spread all over the Petri dish.

The data on nematode penetration of the lettuce roots showed that the highest numbers of J2 were at PA concentrations of 7 ppm (Table 2). A total of 24 J2 were recorded, which was significantly higher than in the control with 11 J2. However, at PA concentrations of 70 and 350 ppm, no J2 invaded the lettuce roots.

While in the *in vivo* experiments root length of seedlings treated with *C. odorata* mulch or aqueous crude extract was higher and roots were visually better developed than in the control, results of this experiment were biased

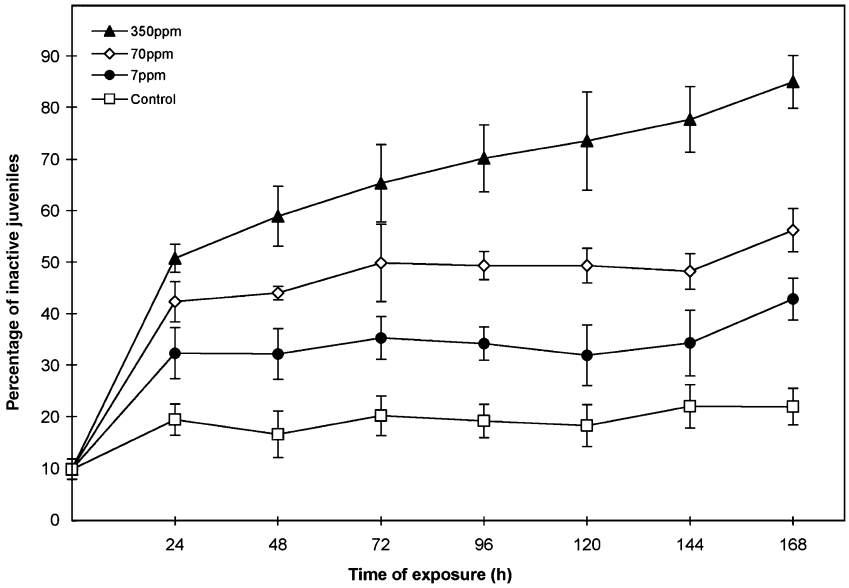

Fig. 2. *The effect of pure PAs from* Chromoalena odorata *roots on the ratio of inactive* Meloidogyne incognita*. Bars show standard deviations.*

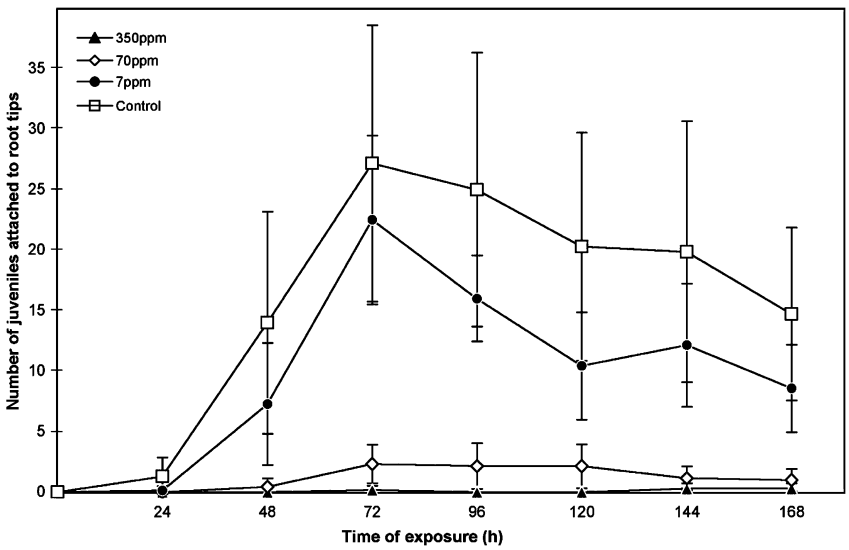

Fig. 3. *The effect of pure PAs from* Chromolaena odorata *roots on the number of* Meloidogyne incognita *attracted and attached to lettuce roots tips. Bars show standard deviations.*

(Table 2): pure PAs, at higher concentrations, had a negative influence on root growth and root vitality. This was most obvious at 350 ppm where the root hairs of the lettuce seedlings were lost and root length was significantly reduced compared with the other treatments (Table 2). Phytotoxic effects of PAs were apparent (Thoden & Boppré, unpubl.).

Discussion

Our results of the *in vivo* experiments support findings of other studies, indicating that the use of *C. odorata* plant material, either as mulch or as aqueous crude extract, appears to be a valuable alternative to other ways of nematode management (Atu & Ogbuji, 1982; Matondo

Table 2. *Numbers of* Meloidogyne incognita *second-stage juveniles infecting lettuce seedlings at different concentrations of pure PAs from* Chromolaena odorata.

Treatment	Control	7 ppm	70 ppm	350 ppm
Mean number of penetrated juveniles	11a	24b	0c	0c
Root length (cm)	3.70a	3.31a	3.10a	2.20b

Means with same letter in a line are not significantly different according to Duncan's multiple range test ($P \leqslant 0.05$; $n = 7$).

et al., 1993; Müller & Sturhan, 1994; Adekunle & Fawole, 2003). Even low concentrations of *C. odorata* root mulch (0.5%, w/w) led to a complete protection of lettuce seedlings from *M. incognita* infection. *Chromolaena odorata* might, thus, be an inexpensive, as well as efficacious tool for nematode management, most likely but not necessarily limited to subsistence farming, especially in regions where this plant occurs naturally as a weed.

For determination of the necessary amounts of *C. odorata* roots to be applied, it is important which part of the roots is used and how the material is processed. As in other PA-plants, the PAs of *C. odorata* are located in the root bark and/or epidermis (Hartmann *et al*., 1989; Boppré & Thoden, unpubl.). Consequently, fine roots have a higher proportion of PAs than the woody main roots. Furthermore, because the PAs are stored in the vacuoles (Hartmann & Witte, 1995) the plant material has to be ground as finely as possible to release as much of the PAs as possible. This might also explain why *C. odorata* did not prevent nematode infection of tomato roots when *C. odorata* was grown in the same pots as the tomato plants and not used as mulch (Müller & Sturhan, 1994).

The results of the *in vitro* experiments showed that PAs play a major if not the only role in the nematicidal effect of *C. odorata* roots. Besides being nematicidal, the Sephadex® test also showed that the presence of PAs interferes with the host finding of *M. incognita* J2. In general, the importance of secondary plant compounds affecting plant-parasitic nematodes is widely known (Chitwood, 2002). Some of the best studied examples are the thiophenes and isothyocianates from *Tagetes* and *Brassica* spp., respectively, two crops already being used for nematode management (Topp *et al.*, 1998; Chitwood, 2002; Riga *et al.*, 2005). Nematicidal effects of PAs have so far not been demonstrated although PA-containing plants, such as *Crotalaria* spp., are already used successfully to manage plant-parasitic nematodes (Fassuliotis & Skucas, 1969; Wang *et al.*, 2002; Jourand *et al.*, 2004a, b). Our results demonstrate for the first time that the nematicidal effect of a PA-containing plant is due to its PAs. In consequence, several PA-containing plant species are promising candidates for nematode management. This hypothesis is supported by data on several other PA-plants that negatively affected *M. incognita* infestation (Thoden & Boppré, unpubl.). However, before becoming a practical management tool for plant-parasitic nematodes, further experiments with different PA-plants, as well as susceptible host plants are required, as are those with individual pure PAs of different types.

Finally, this study has revealed another unlooked for point of interest: a supression and visual deterioration of rootlets growth, especially at high PA concentrations. This PA-induced reduction in root vitality may also be an explanation for the obscure finding that, in the Sephadex® test, the infection of lettuce roots at a PA concentration of 7 ppm was significantly higher than in the control.

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