

Nematostatic effects of a leaf extract from *Crotalaria virgulata* subsp. *grantiana* on *Meloidogyne incognita* and its use to protect tomato roots

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Summary – An aqueous extract from leaves of *Crotalaria virgulata* subsp. *grantiana* was assayed for its effects on second-stage juveniles of *Meloidogyne incognita*. The biological activity was nematostatic; nematodes were not killed but were completely paralysed in a 1 mg/ml (w/v) extract: the LD₅₀ equivalent was estimated to be 0.5 mg/ml. The effect was reversible: juveniles previously paralysed by *C. grantiana* extract recovered complete mobility in water and were able to infest a susceptible tomato plant. Freeze-dried aqueous extract from *C. grantiana* leaves added to a sterile sandy substrate at 1 mg/ml protected susceptible tomato plants from *M. incognita* infestation. This suggests a promising use of *C. grantiana* as both a green manure and natural alternative to synthetic chemicals in nematode population control, especially in integrated pest management for vegetable crops in organic agriculture of tropical and temperate areas.

Keywords – biological activity, crop protection, plant extract, root-knot nematode.

Crotalaria virgulata subsp. *grantiana* Harvey is a wild legume (Fabaceae) commonly named *C. grantiana* (Polhill, 1982; Brummit, 1992). *Crotalaria grantiana* has been found in Southern Africa, from the coastal regions of Eastern Cape to Zimbabwe and Botswana (Polhill, 1982). In tropical areas, *Crotalaria* spp., like many other Fabaceae, play a major role maintaining and restoring soil fertility when used as cover or green manure crops, as well as contributing to nematode management (Widner & Dadalto, 1991; Wang *et al.*, 2002). *Crotalaria grantiana* has been investigated particularly for its resistance to plant-parasitic nematodes such as *Meloidogyne javanica*, *Pratylenchus brachyurus*, *P. zaeae* and *Rotylenchulus reniformis* (Antonio & Neumaier, 1986; Silva *et al.*, 1989a, b). However, as far as we know, there is no report of nematocidal effects of *C. grantiana* leaf extracts such as has been investigated for leaf extracts from other species, *i.e.*, *C. juncea* and juveniles of *Radopholus similis* (Jasy &

Koshy, 1992), *C. saharae* and *C. spectabilis* and *M. incognita* (Subramaniyan & Sivagami, 1990; Sellami & Mouffarra, 1994). The aim of this study was to describe accurately the effects of the leaf aqueous extract from *C. grantiana* on the root-knot nematode *M. incognita* as a prerequisite to introducing *C. grantiana* as a nematocidal green manure in African crop systems. We also studied the influence of this biological effect on the capacity of the nematode to infest a crop such as tomato in order to assess how such a plant extract could be used to protect susceptible plants.

Materials and methods

PLANT MATERIAL

Crotalaria grantiana seeds were harvested in Senegal (IRD, Bel-Air, Dakar) from previously introduced and

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cultivated plants, morphologically identified at the Laboratory of Botany (Department of Plant Biology, University Cheikh Anta Diop, Dakar, Senegal). Seeds were preserved at 20°C in the dark and re-hydrated for 48 h in distilled water before sowing in a compost (Kultursubstrat, Frankfurt, Germany). The plants were grown for 4 months in a glasshouse under simulated Mediterranean conditions. Fresh leaves (1 kg) were collected and blended three times in 1 l distilled water for 15 min at room temperature. The three crude extracts were pooled, centrifuged (15 min, 2410 g) and the supernatant filtered on Whatman paper N°1 and 0.22 µm cellulose acetate filter. The filtered extract was freeze-dried and weighed. Aliquots were re-suspended in distilled water at different concentrations (w/v) and used directly to study their effects on nematodes.

ROOT-KNOT NEMATODES

Meloidogyne incognita (Kofoid & White, 1919) Chitwood, 1949 populations were reared in glasshouse conditions on susceptible tomato plants (*Lycopersicon esculentum* var. Nainespomor). This tomato stock was specially created for laboratory use to reproduce and multiply nematode populations: seed is available from INRA, Unité de Génétique et Amélioration des Fruits et Légumes (Domaine St-Maurice B.P. 94, 84 143 Monfavet cedex, France). Nematode egg masses were handpicked from the roots and placed in distilled water to hatch. Second-stage juveniles (J2) were concentrated to 100 nematodes per ml by sedimentation in test tubes.

EFFECT OF AQUEOUS LEAF EXTRACT

In 24-well culture polystyrene plates, 100 J2 of *M. incognita* per cell were suspended in 1 ml of distilled water, and 1 ml of *C. grantiana* aqueous leaf extract was added to give final concentrations of 0, 0.001, 0.05, 0.25, 0.5, 1, 2.5, 5 and 10 mg/ml w/v). All concentrations were replicated four times and nematodes kept in the extract at ambient temperature. After 48 and 72 h incubation, all mobile and immobile J2 were counted per cell with the aid of an inverted microscope at magnification 100×. The ratio (number of immobile nematodes/number of total nematodes (mobile + immobile)) directly expresses the percentage of paralysed nematodes per cell. In addition, in order to assess the viability of paralysed J2, Meldola Blue vital stain (Sigma® D8142) was added to each cell as described by Ogiga and Estey (1974). Nematodes stained black were dead while those remaining unstained were

still alive. Immobile, unstained nematodes were considered to be paralysed.

MOBILITY RECOVERY AND ROOT INVASION

One hundred J2 of *M. incognita* were incubated in 24-well culture polystyrene plates for 72 h either in 1 ml of distilled water (control) or 1 ml of *C. grantiana* extract at 1 mg/ml (w/v). After treatment with *C. grantiana* extract, nematodes were washed three times and re-suspended in distilled water. Both mobile and immobile nematodes in both treatments were counted in each cell as described above at 2, 7, 14 and 21 days after the 72 h incubation to determine their ability to recover mobility. Then, to assess their invasion potential, each nematode batch was inoculated onto 2-week-old tomato cv. Nainespomor seedlings transplanted in 50 cm³ polypropylene tubes containing 70 g sterile sand and closed with cotton at the bottom. The plants were kept in growth chambers at 25°C and irrigated daily with 5 ml distilled water and fed weekly with 5 ml of nutrient solution (Bertrand *et al.*, 2000). The plants were uprooted 6 weeks after inoculation, their roots were harvested, washed free of soil under distilled water and stained with acid fuchsin (Byrd *et al.*, 1983). Nematodes that had penetrated roots were stained red and were counted by examination of all tomato roots under a binocular microscope at magnification 20×. Percent invasion potential was estimated from the ratio of stained penetrated nematodes to total nematodes in the inoculum.

INHIBITION OF ROOT INFESTATION

One hundred fresh mobile *M. incognita* J2, suspended in 1.5 ml of distilled water, were inoculated onto 2-week-old tomato seedlings in 50 cm³ tubes prepared as described above. Prior to tomato transplantation, the sand substrate was supplemented once with *C. grantiana* freeze-dried leaf extract at 0, 0.1, 1 and 5 mg/ml w/v). The plants were grown, irrigated and fed as described above, and were uprooted 2, 4, 7, 10, 14, 28 and 42 days after nematode inoculation. Nematode infestation of roots was estimated as described above.

STATISTICAL ANALYSIS

Four replicates were used for each experimental treatment. All data were subjected to a one-way analysis of variance, and means were compared with the Newman-Keuls multiple range test ($P \leq 0.05$) using Super

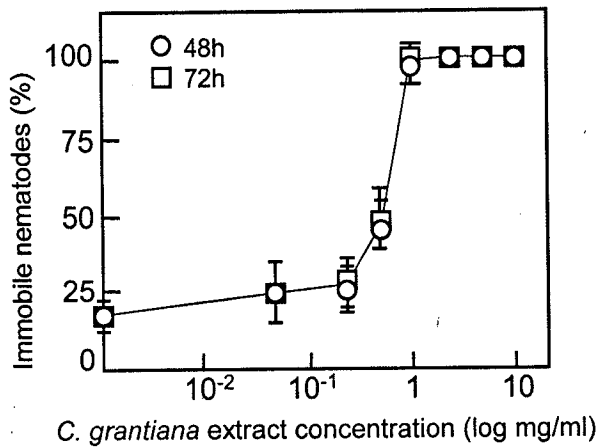


Fig. 1. In vitro nematostatic effect of *Crotalaria grantiana* extract on *Meloidogyne incognita* second-stage juveniles (% immobile) after 48 and 72 h incubation (bars represent the standard error, $P \leq 0.05$).

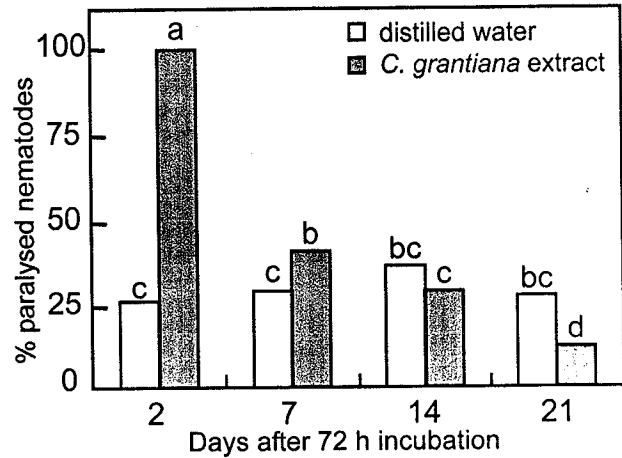


Fig. 2. In vitro reversibility of paralysis of *Meloidogyne incognita* second-stage juveniles transferred to distilled water after 72 h incubation in 1 mg/ml *Crotalaria grantiana* extract (data with the same letters are not significantly different, $P \leq 0.05$).

ANOVA computer software (Gagnon *et al.*, 1989). Percentages were arcsine (sqrt) transformed for analysis.

Results

IN VITRO NEMATOSTATIC EFFECT

Compared to mobile *M. incognita* J2 placed in distilled water, J2 *C. grantiana* extract at 1 mg/ml were immobile, irrespective of incubation period (48 and 72 h). Immobile nematodes were never stained by Meldola Blue and therefore were alive while J2 killed by heat (5 min at 100°C) were stained black. The biological effect of *C. grantiana* aqueous leaf extract on nematodes resulted in juvenile paralysis, correlated with extract concentration (Fig. 1). At 10⁻³ mg/ml, only 18% of nematodes were paralysed and paralysis increased to 100% at concentrations ≥ 1 mg/ml. The LD₅₀ equivalent (50% nematodes paralysed) was estimated graphically as 0.5 mg/ml of aqueous leaf extract.

REVERSIBILITY OF EXTRACT ACTIVITY

All J2 incubated in *C. grantiana* leaf extract remained immobile at 2 days after transfer to distilled water (Fig. 2). After then, percentage immobile individuals significantly decreased when the juveniles were maintained in distilled water and mobility returned to levels comparable to the control in which, after 72 h incubation, there was also a small percentage paralysed nematodes. After a treatment with *C. grantiana* extract and washing with distilled

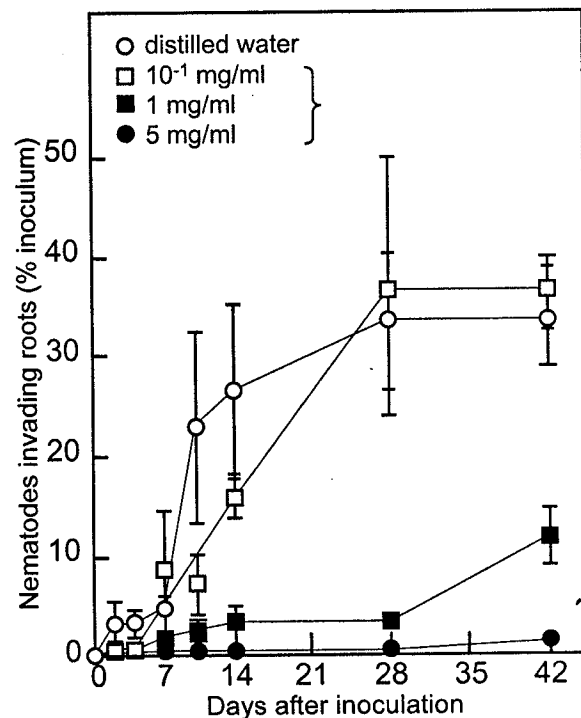


Fig. 3. Invasion of tomato roots by *Meloidogyne incognita* second stage juveniles inoculated in sandy soil supplemented with different concentrations of *Crotalaria grantiana* extract (bars represent the standard error, $P \leq 0.05$).

water, J2 having recovered mobility were able to penetrate into tomato roots at a comparable level to untreated juveniles (data not shown).

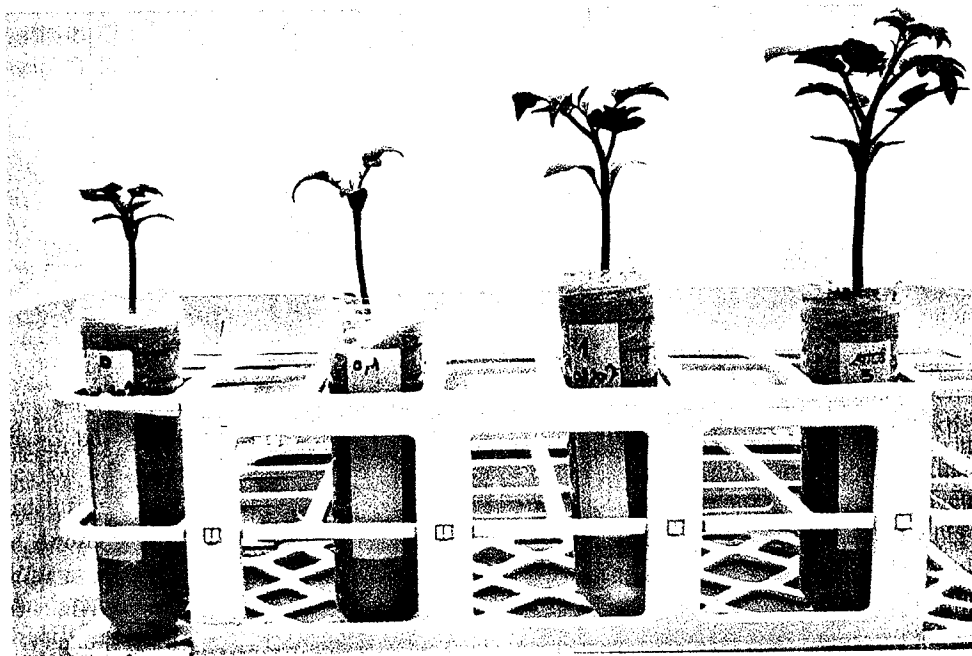


Fig. 4. Growth of tomato plants in sand amended before planting with *Crotalaria grantiana* extract (from left to right: 0; 0.1; 1; 5 mg/ml) at 6 weeks after inoculation of 2-week-old seedlings with 100 second stage juvenile *Meloidogyne incognita*.

INHIBITION OF ROOT INFESTATION BY *C. GRANTIANA*

The addition of either 1 or 5 mg/ml freeze-dried *C. grantiana* extract around the roots of tomato plants, induced a very significant decrease in nematode penetration (Fig. 3). The proportion of nematodes in roots did not exceed 10 and 4% at 1 and 5 mg/ml extract, respectively, at 42 days after inoculation. In addition, penetration was much delayed in the presence of *C. grantiana* extract at 1 and 5 mg/ml, by 10 days compared to 10^{-1} mg/ml or distilled water. Regardless of the concentration of *C. grantiana* extract added to the sand, J2 that penetrated roots were able to complete development. In the control as well as in 10^{-1} mg/ml *C. grantiana* leaf extract (Fig. 3), penetration rates increased up to 36% of nematodes at day 28 after inoculation. Second-stage juveniles were able to mature to third and fourth stages and achieved their full development into mature females. Nevertheless, *C. grantiana* extract used as an amendment at 1 and 5 mg/ml significantly decreased the proportion of nematodes that penetrated susceptible tomato plant roots. This inhibition by *C. grantiana* extract of *M. incognita* penetration contributed to improved plant growth compared to infested tomato (Fig. 4).

Discussion

Crotalaria grantiana aqueous leaf extract had an effect *in vitro* on J2 of the root-knot nematode *M. incognita*, as previously reported for *C. saharae* (Sellami & Mouffarah, 1994) and *C. spectabilis* (Subramaniyan & Sivagami, 1990) on *Meloidogyne* species. However, in those studies, immobile juveniles were described as dead nematodes but without testing this conclusion. This study on *C. grantiana* leaf extract demonstrated paralysis of J2 of *M. incognita* and from the vital staining technique (Ogiga & Estey, 1974), we conclude that *C. grantiana* aqueous leaf extract does not act as a nematicide *sensu stricto*: the juveniles are not killed by the plant extract but only paralysed. Consequently, the biological activity of *C. grantiana* leaf extract can be considered to be a nematostatic effect: the LD₅₀ equivalent, corresponding to a concentration that paralysed 50% of the juveniles, was determined to be 0.5 mg/ml.

With regard to secondary metabolites responsible for this biological activity, the literature on Fabaceae, like the Sophorae, linked alkaloids to nematicidal properties (Zhao, 1999). *Crotalaria* species also contain alkaloids described as pyrrolizidine alkaloids (Kinghorn & Smolenski, 1981) and well known as chemosystematic mark-

ers (Polhill, 1982). According to Fassuliotis and Skucas (1969), monocrotaline, a pyrrolizidine alkaloid isolated from *C. spectabilis*, altered the movement of juveniles *M. incognita* *in vitro* to a jerking motion and significantly reduced infestation of tomato plants. As *C. grantiana* also contains pyrrolizidine alkaloids such as grantianine and grantaline (Smith & Culvenor, 1984), their presence could explain the paralysis induced by *C. grantiana* leaf extract as well as the reduction and delay of penetration of susceptible tomato roots. The protection of tomato plants from *M. incognita* infection by *C. grantiana* extract added to the substrate may be due to either *i*) loss of mobility due to paralysis as revealed here by *in vitro* experiments, or *ii*) disturbed orientation and host finding (linked to disturbance of chemoreception or injuries of sensory organs) disrupting nematode co-ordination in stimuli gradients and affecting response to root attractants. Such consequences of behavioural disturbance were suggested by Green (1971).

Therefore, these observations on the nematostatic activity of *C. grantiana* leaf extract on *M. incognita* clearly indicate another mechanism for the control of root-knot nematode populations by *Crotalaria* green manure. Usually, green manures cause reduction of nematodes by increasing the release of ammonia (Rodriguez-Kabana, 1986) and enhancing antagonists to nematodes (Sikora, 1992). Our data emphasise the significance of the protection of susceptible tomato plants by addition to the substrate of a sterile aqueous leaf extract of *C. grantiana*.

The observation that leaf extracts of *C. grantiana* reversibly paralyse *M. incognita* provides new insight into the mechanism by which green manures of this and possibly other *Crotalaria* species reduce nematode populations (Silva *et al.*, 1989a, b; Widner & Dadalto, 1991; Jourand *et al.*, 2002). Although the specific components in *C. grantiana* leaves responsible for this paralysis remain to be determined, their identification should assist in the selection and development of germplasm with enhanced nematode antagonism. Such germplasm would have value for farmers in developing countries who need an effective method for reducing nematode populations in low-value crop systems.

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