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Nematostatic activity of aqueous extracts of West African *Crotalaria* species

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Summary – The *in vitro* paralysis of second-stage juveniles of *Meloidogyne incognita*, *M. javanica* and *M. mayaguensis* by aqueous extracts of 15 West African *Crotalaria* species was analysed. A multivariate analysis distinguished four groups, based on their nematostatic activity: *i*) *C. glaucoides* extracts were not active; *ii*) the root extracts from *C. goreensis*, *C. lathyroides* and *C. perrottetii* were more active than the shoot extracts; *iii*) the shoot extracts from *C. comosa* and *C. cylindrocarpa* were more active than the root extracts; *iv*) both shoot and root extracts of *C. atrorubens*, *C. barkae*, *C. grantiana*, *C. hyssopifolia*, *C. pallida*, *C. podocarpa*, *C. retusa*, *C. senegalensis* and *C. sphaerocarpa* have nematostatic effects depending on either the *Crotalaria* species or plant tissue from which the extracts were derived. However, considering the greater biomass contribution of the leaves and stems compared to the roots when the plants are used as green manure, *C. barkae*, *C. grantiana*, *C. pallida* and *C. podocarpa* are the most efficient *Crotalaria* species, whatever the *Meloidogyne* species targeted.

Keywords – *Meloidogyne incognita*, *Meloidogyne javanica*, *Meloidogyne mayaguensis*, plant extract.

In tropical areas, natural Fabaceae play an important role in maintaining and restoring soil fertility (Rachie *et al*., 1979). *Crotalaria* is a worldwide genus in various tropical climates ranging from semi-arid to humid forests (Brummitt, 1992). *Crotalaria* species are annual or perennial shrubs and herbs. Only a few species have been described from Asia, Australia and South America (Polhill & Raven, 1981), but Polhill (1982) reported more than 500 species from Africa. *Crotalaria* species have high agronomical and agricultural potentials. Their ability to fix nitrogen by root symbiosis with *Bradyrhizobium* sp. (Samba *et al*., 1999) or *Methylobacterium* sp. (Sy *et al*., 2001) enables these plants to be used for soil fertilisation. Moreover, these leguminous plants develop a high resistance to the main species of root-knot nematodes (Antonio & Neumaier, 1986; Da Silva *et al*., 1989a, b). These nematodes cause severe damage to several crops resulting in annual yield losses ranging from 10 to 50%. The physical, chemical, genetic and biological nematode management methods already applied to fields are not completely effective and may promote resistant nematode biotypes (Barker *et al*., 1998), so the use of *Crotalaria* sp. appears as a very interesting natural alternative to reduce nematode populations in agriculture. Thus, some Asian species have been cultivated as green manure or cover crops in plantations or in inter-cropping systems to restore soil fertility (Kullaya *et al*., 1995; Mwazambi *et al*., 1998) and to control nematode populations in infested soils (Wang *et al.*, 2002, 2003) and, consequently, to enhance crop yields (Sandanam *et al*., 1976; McSorley *et al*., 1994).

The mechanism of the *Crotalaria* resistance to root-knot nematodes has been partially elucidated and involves inhibition of nematode development (Sano *et al*., 1983; Sano, 1986). Moreover, extracts of shoot and root were found active against second-stage juveniles (J2): *C. juncea, C. spectabilis* and *C. saharae* were reported to be nematicidal to juveniles of *Radopholus similis* and *Meloidogyne incognita* (Subramaniyan & Sivagami, 1990; Jasy & Koshy, 1992; Sellami & Mouffarrah, 1994). Recently, an aqueous shoot extract of the West African *C. virgulata*

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subsp. *grantiana* on *M. incognita* was clearly identified as causing paralysis of juveniles and as having nematostatic activity in protecting roots of susceptible cultivated plants from nematode infestation (Jourand *et al*., 2004).

The aim of this study was to analyse the nematostatic activity of aqueous shoot and root extracts from 15 West African *Crotalaria* species on J2 of *M. incognita*, *M. javanica* and *M. mayaguensis*. This investigation screens the potential of these legumes for use as green manure to control root-knot nematode populations in infested soils.

Material and methods

PLANT MATERIAL

Seeds of 15 *Crotalaria* species (Fabaceae, Papilionoideae, Crotalariae) were harvested in Senegal from cultivated plants (IRD, Dakar) and identified morphologically (Laboratory of Botany, Department of Plant Biology, University Cheikh Anta Diop, Dakar, Senegal) according to Polhill (1982): *C. atrorubens* Hochst. *ex* Benth, *C. barkae* Schweinf, *C. comosa* Baker, *C. cylindrocarpa* Don C., *C. glaucoides* Baker, *C. goreensis* Guillemin & Perrottet, *C. grantiana* Harvey, *C. hyssopifolia* Klotzsch, *C. lathyroides* Guillemin & Perrottet, *C. pallida* Aiton, *C. perrottetii* Don C., *C. podocarpa* Don C., *C. retusa* Linneaus, *C. senegalensis* Bacle and *C. sphaerocarpa* Perrottet. The seeds had been stored in a cool, dark room (4°C) and were re-hydrated for 48 h in distilled water before sowing in peat (Kultursubstrat, Frankfurt, Germany). Fifteen plants per *Crotalaria* species were grown for 4 months in a glasshouse under tropical conditions (Laboratoire des Symbioses Tropicales et Méditerranéennes, Montpellier, France). The shoots and roots were either dried for 48 h at 60°C and weighed to determine the plant biomass, or uprooted to prepare fresh extracts.

PLANT EXTRACTS

Fresh shoots (10 g) and fresh roots (10 g) of each *Crotalaria* species were separately blended with 100 ml of distilled water (three times) for 15 min at room temperature. The crude shoot or root extracts were separately pooled and centrifuged (15 min at 2410 *g*). The supernatants were successively filtered on Whatman paper N°1 and 0.22 μm cellulose acetate filter. The filtered extracts were then freeze-dried and weighed. Aliquots were re-suspended in distilled water to obtain aqueous crude extracts at 2 mg ml^{-1} (w/v).

ROOT-KNOT NEMATODES

Meloidogyne incognita (Kofoid & White); Chitwood, *Meloidogyne javanica* (Treub); Chitwood, and *Meloidogyne mayaguensis* Rammah & Hirschmann, populations were reared on susceptible tomato plants (*Lycopersicon esculentum* var. Nainespomor, INRA, Montfavet, France) in a glasshouse (CBGP, Montpellier, France). First generation egg masses of each *Meloidogyne* species were handpicked from the roots and placed in distilled water for hatching. J2 were concentrated to 100 nematodes per ml of distilled water by sedimentation in test tubes.

IN VITRO NEMATOSTATIC SCREENING

In 24 well culture polystyrene plates, 1 ml of nematode suspensions containing 100 J2 of either *M. incognita*, *M. javanica* or *M. mayaguensis* were added to 1 ml of aqueous extract of shoot or root at 2 mg ml^{-1} (w/v, final concentration of the plant extract = 1 mg ml^{-1}). Control treatments were distilled water instead of plant extract. Mobile and immobile J2 were enumerated using an inverted microscope (×40) after 48 h of incubation at room temperature. The proportion of paralysed nematodes ((number of immobile juveniles/total juveniles) × 100) was expressed as described by Jourand *et al.* (2004). As *Crotalaria* shoots and roots are usually incorporated for green manure, a whole plant nematostatic index was evaluated for each *Crotalaria* species based on the respective weights of shoots and roots. The whole nematostatic index was I (%) = ((shoot extract activity × shoot weight) + (root extract activity × root weight))/(shoot weight + root weight).

STATISTICAL ANALYSIS

Four replicates were used for each of the combinations 'nematode species × extract × *Crotalaria* species'. A principal component analysis (PCA) was performed to study the relationship between each combination and nematode paralysis. This PCA analysis gave a loading plot for the six percentages of paralysis analysed (effect of the shoot and root extracts on *M. incognita* (SI and RI, respectively), *M. javanica* (SJ and RJ) and *M. mayaguensis* (SM and RM) defined by the first four eigenvalues), and a score plot for the four replicates of the 15 *Crotalaria* plants. The PCA was performed with the ADE-4 multivariate analysis and graphical display software (Thioulouse *et al*., 1997). The significant data were subjected to a one-way analysis of variance. Means were compared with the

Newman-Keuls test ($P \leqslant 0.05$) using software SuperANOVA (Gagnon *et al.*, 1989). Arcsine ($\sqrt{}$) transformed percentages were used for analysis.

Results

The fraction of variance accounted by the first four PCA eigenvalues was 96%. As shown by the loading plots, the first axis PC1 was related mainly to the percentages of paralysis due to all shoot and root extracts with its positive values (Fig. 1A). The second axis PC2 was related to the percentages of paralysis due to the root extracts with its positive values and to those due to the shoot extracts with its negative values (Fig. 1A). The third axis PC3 distinguished the effects of the root extracts on *M. incognita*, *M. javanica* and *M. mayaguensis* related mainly to its positive, negative and neutral values, respectively (Fig. 1C). The effects of the shoot extracts on the three nematode species were differentiated by the fourth axis PC4 (Fig. 1E).

The score plots of the samples showed that the first PC1 and the second PC2 axes distinguished the effects of different groups of *Crotalaria* species (Fig. 1B; Table 1): *i*) a nematostatic activity only in shoot extracts of *C. comosa* and *C. cylindrocarpa*; *ii*) a nematostatic activity only in root extracts of *C. goreensis*, *C. lathyroides*, *C. perrottetii*; *iii*) an activity in both shoot and root extracts of *C. atrorubens*, *C. hyssopifolia*, *C. pallida*, *C. podocarpa*, *C. retusa*, *C. senegalensis* and *C. sphaerocarpa* with more root than shoot activity, and for *C. barkae* and *C. grantiana* with more shoot than root activity. Both shoot and root extracts from *C. glaucoides* had a very weak nematostatic activity. The third axis PC3 distinguished the effects of the root extracts on the three nematode species (Fig. 1D; Table 1): *i*) *C. goreensis* extracts were more effective on *M. incognita*; *ii*) *C. lathyroides*, *C. perrottetii* and *C. hyssopifolia* extracts were more effective on *M. javanica*; *iii*) *C. atrorubens* and *C. lathyroides* extracts were more effective on *M. mayaguensis*. The fourth axis PC4 distinguished the effects of the shoot extracts on the three nematode species (Fig. 1F; Table 1): *i*) *C. cylindrocarpa* extracts were more effective on both *M. incognita* and *M. javanica*; *ii*) *C. barkae* and *C. comosa* extracts were less active on *M. incognita*; *iii*) *C. grantiana* extracts were effective on all three nematode species.

Taking into account the differences observed on the shoot and root weights between the *Crotalaria* species (Table 2), the whole plant nematostatic indexes vary according to the *Meloidogyne* species. *Crotalaria glaucoides* and *C. lathyroides* provided a very low activity on the three nematode species. *Crotalaria cylindrocarpa*, *C. goreensis* and *C. perrottetii* controlled only from 30-62% of the J2 of the *Meloidogyne* species tested. The other *Crotalaria* species exhibited excellent nematostatic activities: most of them were very effective either on both *M. incognita* and *M. javanica* (*i.e.*, *C. atrorubens*, *C. retusa* and *C. senegalensis*) or on both *M. javanica* and *M. mayaguensis* (*i.e., C. barkae*, *C. comosa*, *C. grantiana*, *C. hyssopifolia*, *C. podocarpa* and *C. sphaerocarpa*). *Crotalaria pallida* was highly efficient irrespective of the *Meloidogyne* species.

Table 1. *Nematostatic activity (% of paralysed second-stage juveniles) of aqueous shoot and root extracts of the 15 West African* Crotalaria *species on* Meloidogyne *species. Data followed with the same letter are not significantly different (*P $\leqslant$ *0.05).*

Plant species	*M. incognita*		*M. javanica*		*M. mayaguensis*	
Crotalaria species with very weak activity						
C. glaucoides	Shoot and root activity < 25					
Crotalaria species with only shoot extract activity						
C. comosa	65^b		100^a		97^a	
C. cylindrocarpa	99^a		100^a		79^a	
Crotalaria species with only root extract activity						
C. goreensis	100^a		100^a		100^a	
C. lathyroides	41^b		20^c		90^b	
C. perrottetii	42^b		90^b		91^b	
Crotalaria species with both shoot and root extracts activities						
	aerial	root	aerial	root	aerial	root
C. atrorubens	78^c	96^a	99^a	96^a	52^d	100^a
C. barkae	90^b	70^c	100^a	94^b	100^a	78^c
C. grantiana	100^a	54^d	100^a	95^b	100^a	66^d
C. hyssopifolia	79^c	72^c	100^a	100^a	81^b	93^b
C. pallida	100^a	100^a	100^a	87^c	97^a	96^a
C. podocarpa	89^b	69^c	92^b	91^c	96^a	100^a
C. retusa	98^a	96^a	100^a	97^a	75^c	93^b
C. senegalensis	100^a	94^b	100^a	98^b	71^c	90^b
C. sphaerocarpa	80^c	74^c	100^a	69^c	78^b	100^a

Discussion

In order to improve sustainable integrated pest management, the use of plants as green manure in crop rotations to control nematodes is of a great interest. Thirteen of the 15 West African *Crotalaria* species studied in

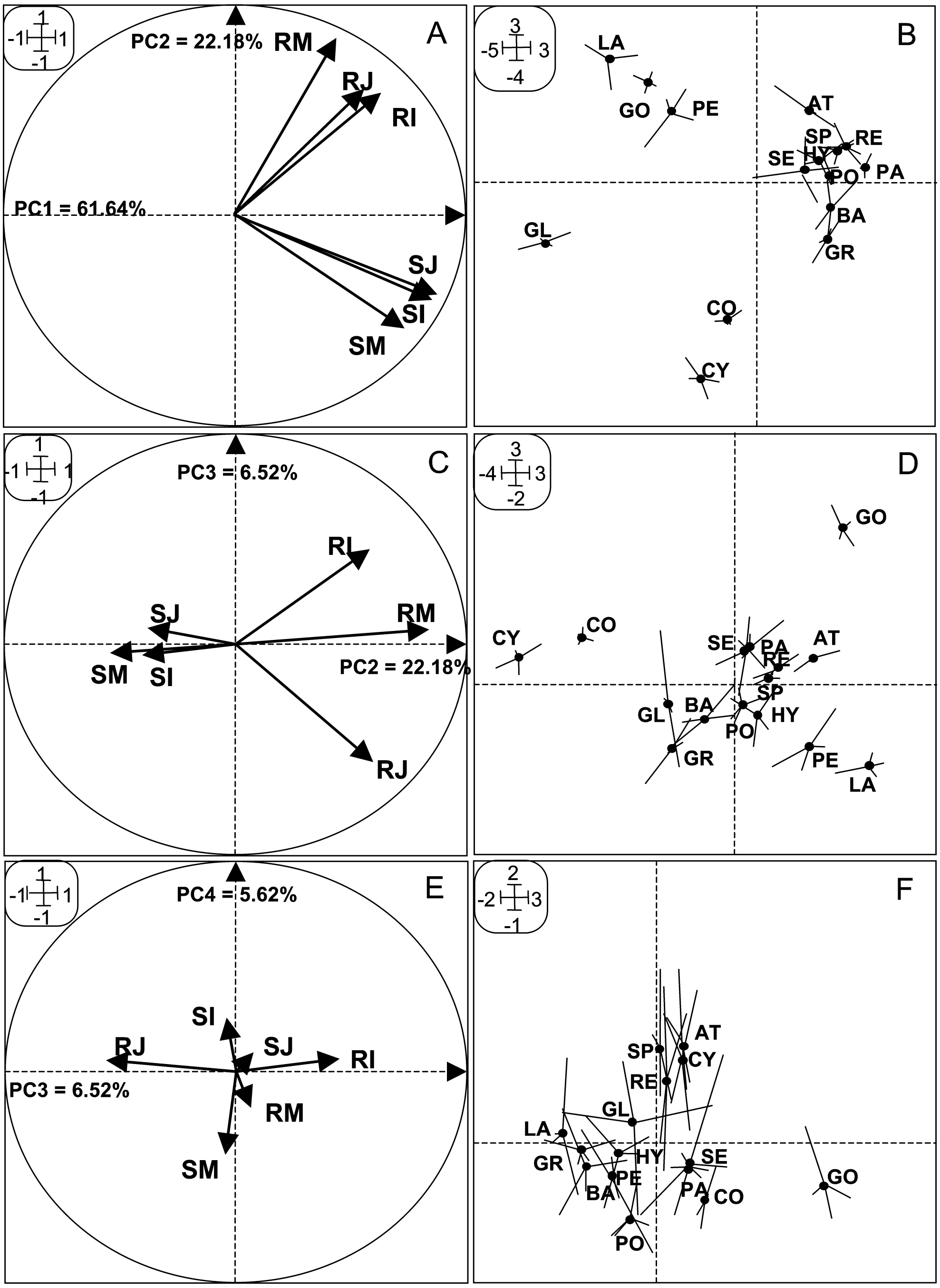

Fig. 1. *PCA diagram loading plots for the shoot and root extracts from 15 West African* Crotalaria *species (A, C, E) and score plots for the samples (B, D, F) according to their nematostatic activity on second-stage juveniles of* Meloidogyne incognita*,* M. javanica *and* M. mayaguensis *(A and B = PC1 × PC2; C and D = PC2 × PC3; E and F = PC3 × PC4). Labels used for PCA combined shoot extracts activities: SI for* M. incognita*; SJ for* M. javanica*; SM for* M. mayaguensis*. Labels used for the PCA combined root extracts activities: RI for* M. incognita*; RJ: for* M. javanica*; RM for* M. mayaguensis*. Labels used for* Crotalaria *species:* C. atrorubens*: AT,* C. barkae*: BA,* C. comosa*: CO,* C. cylindrocarpa*: CY,* C. glaucoides*: GL,* C. goreensis*: GO,* C. grantiana*: GR,* C. hyssopifolia*: HY,* C. lathyroides*: LA,* C. pallida*: PA,* C. perrottetii*: PE,* C. podocarpa*: PO,* C. retusa*: RE,* C. senegalensis*: SE,* C. sphaerocarpa*: SP.*

Table 2. *Dry weight of shoots and roots from* Crotalaria *species and whole plant nematostatic index* on* Meloidogyne *species. Data followed with the same letters are not significantly different (P ⩽ 0.05).*

Plant species	Dry weight (g)		Nematostatic index (%)		
	shoot	roots	*M. incognita*	*M. javanica*	*M. mayaguensis*
C. atrorubens	1.78[d]	1.15[e]	85.1[b]	97.6[a]	71.1[d]
C. barkae	0.85[b]	0.28[b]	85.0[b]	98.6[a]	94.6[a]
C. comosa	1.23[c]	0.52[c]	65.7[d]	88.5[c]	78.9[c]
C. cylindrocarpa	0.55[b]	0.43[b]	59.5[e]	58.6[d]	55.0[e]
C. glaucoides	1.90[d]	0.82[d]	22.7[h]	18.3[g]	38.3[f]
C. goreensis	0.94[c]	0.59[c]	50.4[f]	30.4[e]	61.9[e]
C. grantiana	0.39[a]	0.17[a]	86.4[b]	98.6[a]	89.9[b]
C. hyssopifolia	0.64[b]	0.30[b]	76.9[c]	100.0[a]	85.2[b]
C. lathyroides	1.25[c]	0.52[c]	21.4[h]	15.8[f]	42.5[f]
C. pallida	0.37[a]	0.37[b]	100.0[a]	93.8[b]	96.4[a]
C. perrottetii	0.33[a]	0.14[a]	37.0[g]	59.1[d]	60.8[e]
C. podocarpa	3.94[f]	1.69[f]	82.7[b]	91.9[b]	97.5[a]
C. retusa	2.40[e]	0.54[c]	97.7[a]	99.5[a]	78.8[c]
C. senegalensis	0.51[b]	0.40[b]	98.3[a]	99.5[a]	76.6[c]
C. sphaerocarpa	2.28[e]	0.98[d]	77.4[c]	86.6[c]	87.8[b]

* Expressed as the plant weight ratio [(shoot extract activity × shoot weight) + (root activity × root weight)] / (shoot weight + root weight).

these experiments have nematostatic activity as previously demonstrated for *C. grantiana* by Jourand *et al.* (2004). Consequently, these 13 *Crotalaria* should be useful as a nematostatic green manure to control tropical *Meloidogyne* species.

The PCA analysis revealed that both shoot and root aqueous crude extracts paralyse J2 of *M. incognita*, *M. javanica* and *M. mayaguensis*. These results are especially important for the management of *M. mayaguensis* which is spreading in many African countries. *Meloidogyne mayaguensis* is aggressive even on plants resistant to the other *Meloidogyne* species (Rammah & Hirschmann, 1988; Mateille *et al.*, 1995; Fargette *et al.*, 1996; Willers, 1997).

The range of the nematostatic activity observed for *Crotalaria* species could be the effect of secondary metabolites as reported by Polhill (1982). In some cases, the active compounds are alkaloids as demonstrated for Leguminosae such as Sophorae (Zhao, 1999) and Crotalariae (Fassuliotis & Skucas, 1969). Within the West African *Crotalaria* genus, many pyrrolizidine alkaloids have been identified: *C. grantiana* (section *Crotalaria*, sub-section *Crotalaria*) contains grantaline and grantianine (Smith & Culvenor, 1984); *C. pallida* (section Hedriocarpae, sub-section Macrostachyae) contains crotastriatine, integerrimine, mucronatinine, nilgerine and usaramine (Saxton, 1971; Atal & Sawhney, 1973; Batra *et al.*, 1975); *C. retusa* (section *Crotalaria*, sub-section *Longirostres*) exhibits monocrotaline, retrocenine, retusamine and retusine (Atal & Sawhney, 1973). The pyrrolizidine alkaloid monocrotaline, isolated from *C. spectabilis*, inhibits root infestation of susceptible plants (Fassuliotis & Skucas, 1969). Consequently, the broad spectrum of activity on nematodes from shoots and root aqueous extracts of 15 West African *Crotalaria* species could be due to significant differences in alkaloid composition and content of plant tissues.

The PCA analysis also revealed that the paralysis of each of the three *Meloidogyne* species varied with plant parts. Root extracts seem to be more efficient than shoot extracts. However, according to the weight ratios observed between the shoots and the roots of each *Crotalaria* species (Table 2), the shoot input may be a major contributor to the green manure nematostatic potential. *Crotalaria barkae*, *C. grantiana*, *C. pallida* and *C. podocarpa* were the most efficient species whatever the *Meloidogyne* species targeted. *Crotalaria atrorubens*, *C. hyssopifolia*, *C. retusa* and *C. senegalensis* were also effective in control of *M. javanica* and *M. incognita*. In addition, considering the plant weight ratio, *C. hyssopifolia* and *C. sphaerocarpa* showed promise for control of *M. mayaguensis*.

Crotalaria grantiana, *C. pallida* and *C. retusa* have been already reported as resistant to the most important plant-parasitic nematodes when used as cover crops, rotations and green manure (Wang *et al.*, 2002). So, considering the great potential of West African *Crotalaria* species such as *C. atrorubens*, *C. barkae*, *C. comosa*, *C. hyssopifolia*, *C. podocarpa, C. senegalensis* and *C. sphaerocarpa* as nematostatic green manure, these leguminous could be cultivated in West Africa as inter-crops and ploughed into soils rather than being considered as weeds (Le Bourgeois *et al.*, 1997). These leguminous plants have considerable potential for use by farmers in developing countries to control nematode populations in low-value crop systems.

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