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Characterization of resistance breaking *Meloidogyne incognita* - like populations using lectins, monoclonal antibodies and spores of *Pasteuria penetrans*

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Summary – Six single eggmass lines of *Meloidogyne,* originating from populations collected in the Ivory Coast which showed a particular esterase phenotype (coded pVI) and an ability to overcome the resistance conferred by the *Mi* gene, were compared with *M. incognita,* using different probes. Labeling with different lectins gave similar results for the pVI lines and *M. incognita.* Strains of *Pasteuria penetrans* differentially recognized some of the populations, supporting the view that the pVI populations are different from *M. incognita* but also showing some variability among the resistance breaking group of populations. Two monoclonal antibodies, raised to *M. incognita* and specifically reacting to this species, indicated that the pVI populations were related to *M. incognita* but also demonstrated some differences.

Résumé – ***Caractérisation par des lectines, des anticorps monoclonaux et des souches de* Pasteuria penetrans, *de populations de* Meloidogyne *proches de* M. incognita *et capables de se développer sur cultivars résistants*** – Six clones de *Meloidogyne,* provenant de populations récoltées en Côte d'Ivoire et qui possèdent un phénotype estérasique particulier (codé pVI) ainsi que la faculté de briser la résistance conférée par le gène *Mi,* ont été comparés à *M. incognita* à l'aide de différentes sondes. Le marquage à l'aide de lectines des exsudats amphidiaux de juvéniles de second stade ne permet pas de distinguer ces lignées de *M. incognita.* Des souches de *Pasteuria penetrans* permettent de les distinguer et mettent aussi en évidence une certaine variabilité au sein de ce groupe. Deux anticorps monoclonaux, spécifiques de *M. incognita* indiquent que les clones de phénotype pVI partagent certains caractères avec *M. incognita* mais aussi qu'ils s'en distinguent.

Key-words : *Meloidogyne,* resistance breaking populations, characterization, surface components.

Meloidogyne spp. are major pests of many tropical crops. Resistant cultivars potentially provide the most effective means of controlling them. The alternatives, such as chemicals and biological control agents generally are either environmentally unacceptable or too expensive and technically demanding. Unfortunately, populations of *Meloidogyne* spp. with virulence against many sources of resistance have been identified (Riggs & Winstead, 1959; Netscher, 1976; Prot, 1984; Fargette & Braaksma, 1990). Methods for readily identifying such populations, e.g. isozyme phenotypes (Fargette & Braaksma, 1990), need to be developed. Also, it is desirable to determine from which species and how frequently such virulence has arisen. Answers of these questions would help clarify the potential sources of resistance-breaking biotypes.

Early studies (Fargette & Braaksma, 1990) focused on populations of *Meloidogyne* from the Ivory Coast that were able to overcome the resistance of a number of cultivars including tomato cv. Rossol, the resistance of which is conferred by the *Mi* gene. On the basis of morphological characters and host-range (Sasser, 1979), these populations were classified as *M. incognita.* Also their chromosome disposition during the prophase of the first division of the mitotic parthenogenetic process, as described by Triantaphyllou (1985), was characteristic of *M. incognita* (Fargette & Braaksma, 1990). In contrast, the esterase phenotype of these populations, coded pVI, was very different from that of *M. incognita* which, in previous studies, had been shown to be very consistent (Janati *et al.,* 1982; Esbenshade & Triantaphyllou, 1985; Fargette, 1987). Rather, the pVI phenotype was identical to that coded VS-S by Esbenshade and Triantaphyllou (1985) and exhibited by *M. mayaguensis* (Rammah & Hirschmann, 1988). These results distinguish the pVI lines from *M. incognita.*

The aim of the present study was to further characterize the pVI lines and their relationships with other species, especially *M. incognita.* With that objective, we used the following range of techniques and probes :

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– lectins as probes for specific sugar moieties on the surface of the second stage juveniles (in the exudates present at the amphidial apertures and on the cuticular surface);

– populations of *Pasteuria penetrans,* the spores of which adhere differentially to the cuticle of different species and populations of *Meloidogyne* (Stirling, 1985; Davies *et al.,* 1988 *a*);

– monoclonal antibodies, specific to *M. incognita* and selected for their identification usefulness (Jones *et al.,* 1988).

Material and methods

The nematodes

Eight populations of *Meloidogyne* were compared in each test. These were six single eggmass lines (corresponding to I1, I2, I3, I5, I7 and I8 in Fargette & Braaksma, 1990) showing the pVI esterase phenotype and originating from the Ivory Coast, and *M. incognita* and *M. arenaria* (both single eggmass lines), also originating from the Ivory Coast. The study involving *P. penetrans* also included single eggmass lines from populations of *M. incognita,* races 1 to 4 and of *M. mayaguensis,* provided by North Carolina State University. The antibody test included an additional population of *M. incognita* and of *M. arenaria* cultured at Rothamsted Experimental Station as controls.

The nematodes were grown on tomato cv. Money Maker, in pots (15 cm diam.), in a glasshouse kept at 25-27 °C. Five weeks after inoculation, the roots were washed free of soil and the tomato plants replanted in pots filled with small (2-4 mm) gravel (Lahtinen *et al.,* 1988). The pots were watered twice a day and the water which percolated through the gravel was collected, sieved and the second-stage juveniles (J2) were concentrated. These juveniles were used for the lectin binding tests. In addition, eggmasses were hand-picked from roots of infected plants, placed for a few days in 0.3M NaCl (Dropkin *et al.,* 1958), then put in a small dish of water. Freshly hatched J2 obtained from the eggmasses were used for the attachment tests with *P. penetrans.* Additional J2 and hand-picked females, were kept in a deep freezer (– 80 °C) until they were used for tests with monoclonal antibodies.

Lectin labelling

Four rhodamine conjugated lectins (Peanut, Soybean, Concanavalin and *Ulex europaeus* agglutinins, respectively PNA, SBA, ConA and UEA) (Sigma Ltd), were used with or without their respective specific sugar ligand (Table 1), according to the technique of Forrest and Robertson (1986). Small volumes of water containing 500 J2 were transferred to Eppendorf tubes and the nematodes were washed three times in Phosphate Buffered Saline (PBS) pH 7.2, before they were added to a solution of lectin in PBS (final concentration of lectin = 0.1 mg/ml), to both the lectin and its complementary sugar (final concentration of lectin = 0.1 mg/ml, final concentration of sugar = 200 mM) or to PBS only as a control. The tubes were incubated for one and a half hours at 4 °C, after which the nematodes were washed twice in PBS and placed on a slide under a sealed coverslip. At least 50 J2 were observed under a fluorescence microscope at a wave-length of 546 nm.

Table 1. *The lectins used and their respective competitive sugars*

Symbol	Lectins	Specific sugar ligands
PNA	Peanut Agg.	galactose
SBA	Soybean Agg.	N-acetyl, D-galactosamine
ConA	Concanavalin Agg. (jack bean)	methyl-mannopyranose
UEA	*Ulex europaeus* Agg.	fucose

Pasteuria penetrans attachment tests

Three populations of *P. penetrans,* PP1, PCal and PNG, all isolated from *M. javanica* and all able to attach to a greater or lesser extent on *M. incognita, M. arenaria* and *M. javanica* (Davies *et al.,* 1988 *b*), were used for these tests. PP1 was obtained from S. Gowen, University of Reading, UK; PNG was isolated from a soil sample from Papua New Guinea, supplied by J. Bridge, CAB International Institute of Parasitology, St Albans, UK; PCal was obtained from R. Mankau, University of California, Riverside, USA. Populations of endospores of *P. penetrans* were routinely increased in the laboratory using the method of Stirling and Wachtel (1980) and the number of spores in a suspension of tap water were estimated with a haemocytometer slide. Freshly hatched J2 (approximately 50) were placed for 24 hours in a 0.1 ml suspension of spores (spore concentration = 1×10^6/ml) in a 96 well microtitre plate at room temperature. Nematodes were transferred to glass slides and observed using a high power (× 400) light microscope. Spores attached to the surface of ten randomly chosen J2 were counted. Mean numbers of spores attached and standard errors were calculated and an attachment index assigned a scale.

Serological tests

Two monoclonal antibodies (MAbs) raised to *M. incognita* and believed to be specific to this species (Jones *et al.,* 1988) were tested : one, P2 MAb had been raised to females and the other, F133/1.9 MAb, to J2.

Samples of J2 kept at – 80 °C were thawed and resuspended in 2500 J2 aliquots in 50 µl of water. An equal volume of 1 % NaDoc, 10 mM Tris, pH 8.3 was added and the juveniles were carefully ground up using an Eppendorf homogenizer. Coating buffer (0.1 M Na_2CO_3, pH 9.6) was added and 50 µl subsamples were tested with the P2 and F133/1.9 MAbs.

Samples containing 30-50 females from lines I5 and

l8 were prepared in the same way. The tests were made using routine ELISA procedures (Jones *et al.,* 1988) in which the solutions containing the antibodies were allowed to react with the antigen for 1 h at 37 °C. The intensity of the reaction was recorded (absorbance) and scores grouped in four classes.

Results

Lectin binding

Where lectin binding occurred, it was mainly to the amphids (Table 2). ConA and UEA also sparingly labelled the cuticle of some lines.

Peanut agglutinin (PNA) which binds to galactose or galactosamine (Goldstein & Poretz, 1986) did not bind to any of the lines of *Meloidogyne* tested. Soybean agglutinin (SBA) bound specifically but weakly to the amphid apertures of *M. arenaria* only. The competitive sugar (N-acetyl, D-galactosamine; Goldstein & Poretz, 1986) blocked binding, indicating the presence of N-acetyl-galactosamine in the amphidial exudate of *M. arenaria* but its absence from the *M. incognita* and the pVI lines.

Concanavalin agglutinin (ConA) and *Ulex europaeus* agglutinin (UEA) bound to all lines, although binding by ConA on the amphids was stronger in *M. arenaria* than in *M. incognita* and the pVI lines. The binding was specific as it was blocked by the specific sugars (respectively methyl-mannopyranose and fucose) on *M. arenaria* and *M. incognita.* On the pVI lines, binding of ConA was totally specific (except on l8, where some non-specific binding persisted with the competitive sugar on 20 to 60 % of the juveniles observed; however, this non-specific fluorescence was diffuse and confined to the head region; quantitatively it was less important than the binding observed with the lectin only, showing that the lectin binding is at least partly specific with this line). With UEA, binding was specific on the pVI lines except for l5 where some 40 % of the juveniles observed showed some non-specific binding. On the cuticle, some specific but weak binding of ConA was observed on l1, l2, l3 and *M. incognita,* while UEA sparingly, but still specifically, bound to some juveniles (without any clear pattern). Hence, glucose/mannose and fucose are present in amphidial exudates of all lines and variably present on their cuticle.

Table 2. *Labelling of the amphidial exudates of the different lines of* Meloidogyne *by the four rhodamine conjugated lectins; the occurence or absence of fluorescence is given for each lectin in absence (L) or presence (L + S) of the corresponding competitive sugar.*
Scale : – = no recognition; + = recognition; ++ = strong recognition.

	PNA	SBA		ConA		UEA	
	L	L	L+S	L	L+S	L	L+S
M. incognita	–	–		+	–	++	–
pVI lines	–	–		+	–*	+/++	–**
M. arenaria	–	+	–	++	–	++	–

* : except for l8 (see text)
** : except for l5 (see text)

Pasteuria penetrans attachment

Attachment rates were similar within each line/species (standard error usually < five spores per nematode) except with *M. arenaria* (standard error > 30 spores per nematode with PP1 and PNG). The variability observed in the numbers of spores attached to *M. arenaria* must be intrinsic to this line/species (Table 3). There were consistent differences in spore attachment between the races/populations of *M. incognita;* races 1 and 2 were heavily infested (> 50 spores/J2) by all three *P. penetrans* populations whereas races 3 and 4 generally carried fewer spores. Few spores of PP1 and PNG attached to the *M. incognita* line from the Ivory Coast; however more spores of PCal attached. Few or no spores of all three *Pasteuria* populations attached to the pVI lines l2, l3, l7 and l8 and to *M. mayaguensis.* However, spores of *P. penetrans* PP1 adhered to the pVI lines l1 and l5 in similar numbers to those observed with *M. incognita* races 3 and 4 and *M. arenaria* whereas attachment of PCal and PNG were lower.

Table 3. *Attachment of three populations of* Pasteuria penetrans *to the different lines and species of* Meloidogyne *tested.*
Scale : 0 = 0 spore; A = 1-5 spores; 2 = 6-10 spores; 3 = 11-50 spores; 4 = > 50 spores.

	Pasteuria penetrans		
	PNG	*PCal*	*PP1*
M. incognita			
race 1	4	4	4
race 2	4	4	4
race 3	1	2	3
race 4	1	2	3
Ivory Coast	1	3	1
pVI lines			
1	0	0	3
2	0	1	1
3	0	0	0
5	1	0	3
7	0	1	0
8	1	1	1
M. mayaguensis	1	1	1
M. arenaria	3	1	3

ANTIGENIC REACTIONS

P2 MAb, raised to females of *M. incognita,* reacted strongly with juveniles from the two *M. incognita* populations, including that from the Ivory coast (Table 4). It did not react with *M. javanica* and reacted only slightly with *M. arenaria* from the Ivory Coast. There results, therefore, supported previous results (Jones *et al.,* 1988) showing strong specificity for *M. incognita.* However, the P2 Mab reacted with both females and juveniles of the pVI lines as strongly as with *M. incognita.*

F133/1.9 MAb, raised to juveniles of *M. incognita,* also reacted strongly with juveniles from both populations of *M. incognita* and very slightly with *M. arenaria* and *M. javanica.* In striking contrast to the results obtained with the P2 MAb, the pVI juveniles did not react at all to this antibody and pVI females reacted only slightly.

Table 4. *Antigenic reaction of the different lines and species of* Meloidogyne *against two monoclonal antibodies specific to* M. incognita. Scale : – = no reaction; + = weak reaction; ++ = strong reaction; +++ = very strong reaction.

	pVI lines		*M. inc.*	*M. are.*	*M. jav.*
	J2	Fem.	J2	J2	J2
P2	+++	+++	+++	+	–
F133/1.9	–	+	++	+	+

Discussion

The surface of nematodes and their exudates are fundamental to their interaction with external environmental factors in chemoreception, orientation, recognition of suitable hosts and recognition and adhesion by fungal or bacterial parasites. This largely justifies current research interest in them (Zuckerman, 1983; Zuckerman & Jansson, 1984; Bird *et al.,* 1988; Forrest *et al.,* 1988). Probes such as lectins, *P. penetrans* and antibodies, which are targeted at particular compounds or structures present on the surface or in exudates, facilitate a rapid comparison of many populations. Lectins bind to specific sugar moieties, present on the surface or in the exudates as polysaccharides, glycoproteins or glycolipids. Recent work also suggests that, in relation to the binding of *P. penetrans* to *Meloidogyne* cuticles, carbohydrate moieties, principally N-acetylglucosamine on the surface of the spore (Bird *et al.,* 1989; Persidis *et al.,* 1991) may interact with carbohydrate recognition domains on the nematode cuticle (Davies & Danks, 1993). Equally, antigenic differences between populations of *P. penetrans* spores (Davies *et al.,* 1992) and root-knot nematode cuticles (Davies & Danks, 1992) are likely to relate to differences observed in host specificity between the bacterium and the nematode cuticle. Consequently, binding studies involving lectins, antibodies and *Pasteuria* spores can provide an understanding of the biochemical variation present on the cuticle of different populations of root-knot nematodes.

Other authors have also used lectin probes to investigate the surface and exudates of J2 belonging to different species of *Meloidogyne* (Spiegel *et al.,* 1982; McClure & Zuckerman, 1982; Davis *et al.,* 1988; McClure & Stynes, 1988; Robertson *et al.,* 1989; Davis & Kaplan, 1992). Variation in observations by different authors may be due partly to differences in methodology (mainly pH, lectin and competitive sugar concentrations) or in commercial origin of the lectins. However, our results are in good agreement with those of McClure and Stynes (1988) who also were able to distinguish *M. incognita* and *M. arenaria.* Similarly, the monoclonal antibodies used in this study also allowed the distinction between *M. incognita* and *M. arenaria.*

The lectin binding assays did not clearly distinguish the pVI lines from *M. incognita.* Differences in the specificity of the *P. penetrans* isolates between populations and lines indicate, however, that the pVI lines are distinct from both *M. incognita* and *M. arenaria.* The binding pattern of the monoclonal antibody raised to juveniles of *M. incognita* tends to confirm the data provided by *Pasteuria* in that the pVI lines are different from *M. incognita.* However, the lack of difference between *M. incognita* and the pVI lines when using the antibody prepared to *M. incognita* females indicates some similarity between the pVI lines and *M. incognita.*

The tests involving *P. penetrans* indicate variability between races of *M. incognita* and also between some of the pVI lines. Overall, rates of attachment of *P. penetrans* populations to the pVI lines were generally low and similar to those for *M. mayaguensis.* However the identity of the pVI lines still needs to be confirmed. If, as seems possible, the pVI lines are *M. mayaguensis* it should be stressed in grand that it is the pVI phenotype which appears to be the reliable characteristic.

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