Acquired virulence in the plant parasitic nematode *Meloidogyne incognita*. 2. Two-dimensional analysis of isogenic isolates[1]

Antoine DALMASSO*, Philippe CASTAGNONE-SERENO*, Michel BONGIOVANNI* and Anke de JONG**

* *INRA, Station de Recherches de Nématologie et de Génétique Moléculaire des Invertébrés, B.P. 2078, 06606 Antibes Cedex, France, and*
** *Department of Nematology, Agricultural University, Binnenhaven 10, 6709 PD Wageningen, The Netherlands.*

SUMMARY

Two lines of *Meloidogyne incognita* virulent to tomato cultivar carrying the Mi gene were selected from avirulent single egg-mass populations. Each line was obtained from a single juvenile belonging to two wild avirulent populations, one coming from France, the other from Ivory Coast. For each population, two lines were established one maintained on the susceptible cv. Saint-Pierre and the other on resistant cv. Piersol at 20-25 °C. Females of the two virulent lines were compared for their soluble proteins to each other and to their non virulent homologous parent. Analyses were made on females. Some differences appeared between the females of the two lines of different geographical origin using two-dimensional gel electrophoresis (2-DGE) and a sensitive silver stain. Approximatively 400 spots were resolved but due to technical difficulties of the technique, only a small number of them could be considered in comparing the virulent and avirulent lines. Small differences could be observed between the two lines of each population of different geographical origins and only one spot enables the homologous virulent and avirulent lines to be distinguished.

RÉSUMÉ

Virulence acquise chez le nématode phytoparasite Meloidogyne incognita. *2. Analyse bidimensionnelle de lignées isogéniques*

Deux isolats de *Meloidogyne incognita* virulents vis-à-vis du gène Mi de la tomate ont été sélectionnés à partir de deux lignées avirulentes, l'une originaire de France « Calissanne » et l'autre de Côte d'Ivoire. Toutes les lignées sont issues d'une larve unique; elles ont été multipliées sur le cultivar hôte Saint-Pierre et sur le cultivar résistant Piersol à 20-25 °C. Les deux isolats virulents ont été comparés pour leurs protéines solubles l'un à l'autre, ainsi qu'à leur parent homologue non virulent. Les analyses ont été effectuées sur les protéines solubles des femelles étudiées sur gel en électrophorèse bidimensionelle (2-DGE), en utilisant la coloration très sensible à l'argent. Environ 400 spots ont pu être séparés, mais, pour des raisons techniques, seule une partie d'entre eux a été prise en considération dans la comparaison entre lignées virulentes et avirulentes : un seul spot sépare distinctement les deux origines étudiées et un autre spot commun aux deux isolats avirulents est très faible, voire absent chez les isolats virulents.

Recently, several studies were made to characterize pathotypes of cyst nematodes by differences in the composition of their proteins (Bakker & Gommers, 1982; Ohms & Heinicke, 1983; Fox & Atkinson, 1984), in the restriction fragment length of their DNA (Curran, McClure & Webster, 1986; Burrows & Boffey, 1986) by using differential DNA probes (Burrows & Perry, 1988).

Biochemical or molecular studies on plant-nematode interactions are often limited by the small size of plant parasitic nematodes, resulting in very small quantities of biological material. Moreover, in the case of heteroderids, allelic forms of proteins complicate the interpretation (Bergé *et al.*, 1981) and isogenic virulent and avirulent lines are more difficult to obtain (Luedders, 1989; Janssen, Bakker & Gommers, 1990).

The root-knot nematode *Meloidogyne incognita* (Kofoid & White) Chitwood reproduces obligatorily by mitotic parthenogenesis (Triantaphyllou, 1971, 1981). This type of reproduction makes it possible to isolate

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populations by rearing the progeny of a single female. The principal individuals of such a population may be considered as genetically identical (clone). Even so, progressive selection for virulence is possible from initially largely avirulent population (Jarquin-Barberena *et al.*, 1991). The absence of biochemical and genetic polymorphism in this group (Dalmasso & Bergé, 1978) confirms that the genus *Meloidogyne* is a good model to study the plant nematode interaction at a molecular level.

Once isogenic avirulent and virulent lines have been established it is possible to isolate the proteins associated with the virulence difference hoping that much information will help to reveal the general resistance process (hypersensitivity) observed in plants. Three approaches are possible, direct analysis of the digestive secretions of the nematode (Hussey, 1989), comparison of soluble proteins, and analysis of nucleic acids extracted from more or less isogenic lines. The objective of the present work was to examine by two-dimensional electrophoresis differences in the general proteins of two pairs of virulent and avirulent *M. incognita* lines, each pair being derived from a single juvenile.

Materials and methods

In the present study, two single juvenile populations were obtained from an avirulent population of *M. incognita* collected in France (Calissanne) and one from the Ivory Coast (Adiopodoumé). The populations had been already characterised according to their perineal patterns and isoesterase electrophoregrams (Dalmasso & Bergé, 1978). They were initially largely unable to develop on resistant tomatoes carrying the Mi gene. From each of these two avirulent populations a virulent line was obtained by inoculating juveniles onto the resistant tomato cv. Piersol and rearing their offspring repeatedly for more than 20 generations on the same cultivar. All populations were reared at 20-25 °C in a climatic room for six weeks. Young females of the 20th generation were extracted from roots using pectinase and a blender, as described by Dropkin, Smith and Meyers (1960). Roots were cut into pieces of approximately 1 cm, macerated overnight in pectinase (Fluka 76290) and gently broken for 5 s with a blender. The biggest debris were removed by sieving; small debris and females were recovered on a 250 µm mesh sieve before centrifugation at 1 000 *g* for 10 min in kaolin. The pellet was homogenized in 1.6 g/l sucrose and centrifugated at 1 000 *g* for 2 min. Approximately 3 000 young white females were hand picked from the supernatant for each isolate. Preparation of soluble protein samples was done according to Bakker and Gommers (1982) with slight modifications. A thousand females were dried before immersion in 100 µl of extraction buffer (10 mM tris-HCl, pH 7.4) and then disrupted in a glass potter with a Teflon pestle. The homogenate was centrifugated two times for 1 h at 100 000 *g* to remove lipids and cuticules. Concentration of proteins was estimated according to Bradford (1976). Three µl of 2-mercaptoethanol and 64 mg of urea were added per 100 µl of supernatant. Samples were stored at - 80 °C. Before use, the protein extracts were diluted according to the number of females crushed into the extraction buffer. Approximately 50 µg of proteins were applied to the 2-DGE tubes. Isoelectric focusing was done within the pH range 3-10 strengthened 5-8 respectively 1/4 and 3/4 (2D Pharmalyte, Pharmacia) and using CHAPS (Sigma C 3023), according to two voltage phases (700 V during 1.5 h and 3 000 V during 45 min). The proteins were then separated in the second dimension on 12 % acrylamide gel at a current of 200 mA for 650 V/h. Gels were stained with silver (Sammons, Adams & Nishizawa, 1981). Protein spots were visually analyzed only on gels that showed no evident artifacts, and only proteins which appeared to be consistant on the four replications were considered. The analysis were repeated four times.

Results

Results obtained with the avirulent and virulent isolates of both populations of *M. incognita* from the Ivory Coast and France are shown in Fig. 1. About four hundred spots were obtained on the gels. Only those located in the center of the gels were taken into consideration for the comparative study. Study of four gels (four different electrophoresis) showed that one spot designated " Gil " separated the Calissanne population from that of the Ivory Coast. Another one (" t2 ") differenciated the non virulent isolates from those with acquired virulence, independently of their geographical origin.

Discussion

Two *M. incognita* populations of very different origin (Europe and Africa) were compared for their soluble proteins by means of two-dimensional electrophoresis. Only one half of the 400 spots obtained from young females and revealed by silver staining showed good resolution and were taken into account. Except for one peptide, no obvious differences were observed in spot size, staining intensity and electrophoretic mobility. This large similarity confirms the low rate of biochemical polymorphism reported by Dalmasso and Bergé (1978) who studied the protein and isoenzyme variability. Moreover, this result is not surprising because a major part of the peptides observed on the gels may arise from constitutive proteins less subject to visible electrophoretic variations.

One protein was stained less intensively in both virulent isolates. The lower amount of one protein in both virulent strains might be correlated to the low production (or even the absence) of a nematode se-

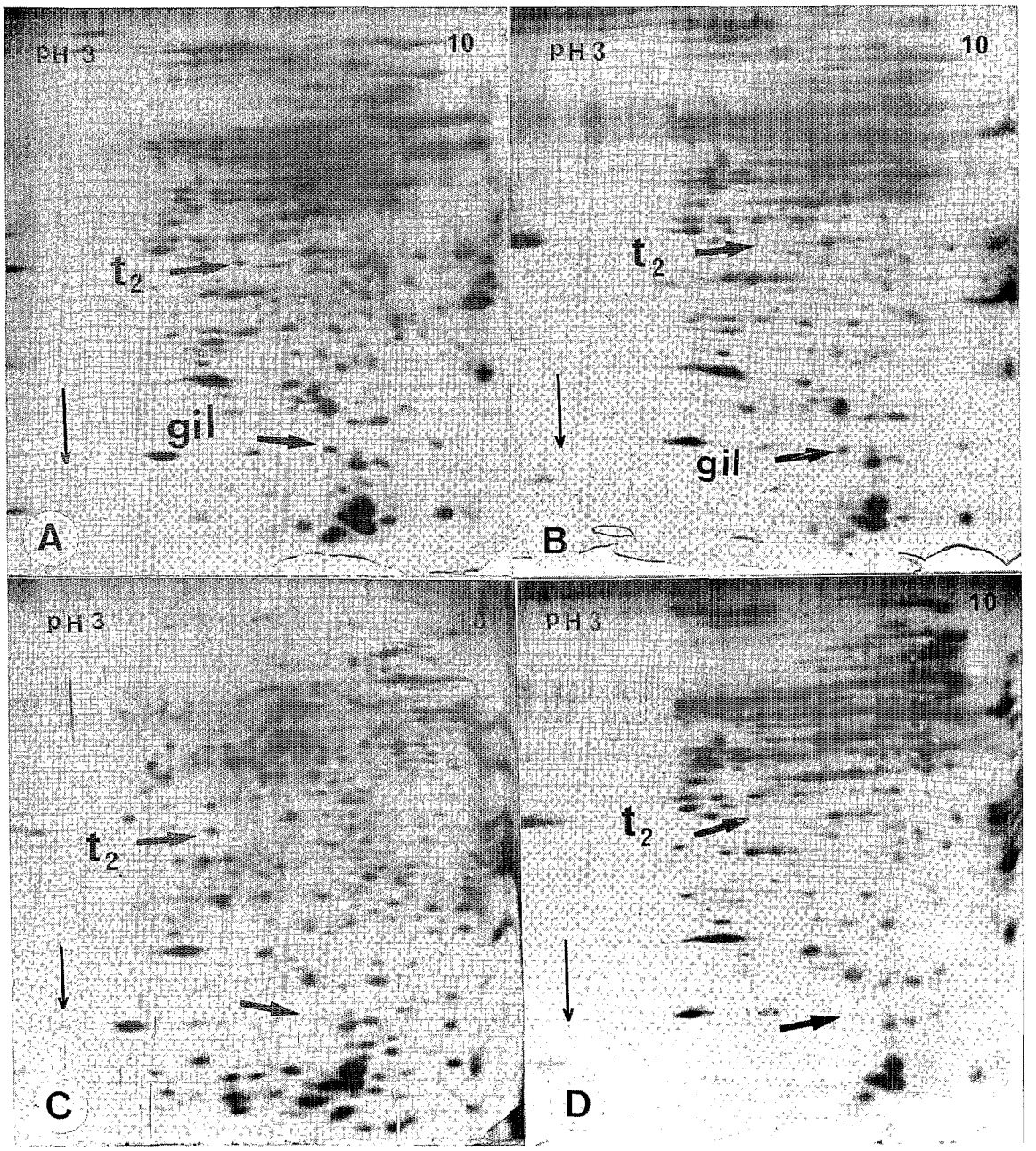

Fig. 1. Silver stained polyacrylamide gels after two-dimensional electrophoresis of total proteins from *M. incognita* females. Arrows indicate : the spot " Gil ", differential for the geographic origin of the strains and the spot " t2 ", present in the avirulent but not in the virulent isolates. A : Ivory Coast avirulent; B : Ivory Coast virulent; C : France avirulent; D : France virulent.

cretion involved in the initiating steps of the plant defense mechanisms. A larger amount of the protein in the avirulent strains might contribute to the hypersensitive reaction of the resistant tomatoes. But further studies are needed on other strains of *M. incognita*, even those which are naturally virulent, in order to confirm the importance of this protein in the plant nematode interaction. It would also be interesting to analyse the virulent isolate having been multiplied on susceptible tomatoes in order to know whether the differenciating protein is host inducible. Piersol cv. was bred from cv. Saint-Pierre, so they are close from a genetic point of view. Moreover, as the digestion of *Meloidogyne* is known to be external, the possibility for the nematode to ingest, in a sufficient amount to be detected, a plant protein that would differenciate the two tomatoes is very unlikely. Wilski and Giebel (1966) observed that biotypes of *Globodera rostochiensis* virulent to *Solanum andigenum* secreted less β-glucosidase, an enzyme possibly involved in the mechanism of potato resistance (Giebel, 1979), than the non resistance-breaking biotype. The larger size of the t2 spot of avirulent lines of *M. incognita* as compared with that of virulent ones may be similarly explained.

The smaller amount of a protein in both virulent isolates might be related to a reverted phenomenon of gene amplification under the selective pressure of the resistant cultivar (Jarquin-Barberena *et al.*, 1991). Moreover, it would be possible to confirm by western blots, using antibodies like those obtained by Hussey (1989) whether the virulence differenciating proteins are related to the digestive secretions of *Meloidogyne*.

More investigations are needed, nevertheless bidimensional electrophoresis allows interesting approaches to study the general process of plant-*Meloidogyne* interactions.

These results seem slightly paradoxical : although the biochemical polymorphism of strictly parthenogenetically reproducing species of *Meloidogyne* is very low compared to other nematodes under a selection pressure, they show an astounding capacity to adapt their physiology and host-parasite relations, as demonstrated in a previous study (Jarquin-Barberena *et al.*, 1991).

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