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MOLECULAR METHODS FOR THE IDENTIFICATION
OF LONGIDORID NEMATODES

by

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Summary. Geographically different populations of *Xiphinema index*, *X. santos*, *X. vuittenezi* and *Longidorus latocephalus* were used to develop a method of identification based on inter and intra-specific polymorphism of the enzyme superoxide dismutase (SOD). Samples of a sufficient number of individuals were homogenized and directly loaded for an isoelectrofocusing run carried out with an automated electrophoresis system (Phast System, Pharmacia). After staining the gels for SOD activity, isoform profiles appeared to be species-specific and able to detect intraspecific variability of *X. index* populations. Polymerase Chain Reaction (PRC) amplification products from the Internal Transcribed Spacer (ITS) regions of single *X. index* DNA were also obtained. Modifications to the standard procedures are described.

Many nepoviruses transmitted by longidorid nematodes cause devastating diseases in tree fruits or grapevine (Agrios, 1988). Consequently, the virus-vector *Xiphinema americanum* Cobb *sensu lato* has been included in the EPPO A2 pest quarantine list because of the risk of importing nepoviruses that are currently not reported in the European Community area. Current methods to detect the presence in soil of nematodes which carry and spread plant virus diseases rely mainly on time consuming and sometimes difficult morpho-biometric studies, which can take many weeks and may lead to subjective identification.

In recent years, methods of identification of plant parasitic nematodes based on molecular approaches have been developed which are complementary to traditional methods (Hyman and Powers, 1991; Williamson, 1991; Lamberti *et al.*, 1994).

The main objective of our work is to find suitable genetic molecular markers which can

be used to build up rapid, reliable and standard methods for the routine identification of pathogenically relevant nematodes. Significant progress has already been made in isozyme phenotype discrimination of some species of endoparasitic nematodes (Esbenshade and Triantaphyllou, 1990; Nobbs *et al.*, 1992; Ibrahim and Rowe, 1995). Moreover, several studies have been focused on nucleotide sequence variability of the ITS (Internal Transcribed Spacer) ribosomal regions by using the polymerase chain reaction (PCR) technique (Mullis and Faloona, 1987) which allows amplification of specific sequences starting from minute amounts of DNA (De Giorgi *et al.*, 1992; Wendt *et al.*, 1993; Joyce *et al.*, 1994; Ferris *et al.*, 1994; Zijlstra *et al.*, 1995; Nasmith *et al.*, 1996). Reports of application of such molecular methods to *Xiphinema* spp. are scarce and apparently absent for *Longidorus* spp. (Vrain *et al.*, 1992), mainly because of the difficulty of maintaining longidorids in laboratory or greenhouse

cultures in order to have enough living material for analysis.

In this paper, the first approach for discrimination of longidorid species employs an automated electrophoresis system (Phast System, Pharmacia) and is based on the difference among SOD (Superoxide dismutase) isozyme profiles of morphometrically identified nematode samples. SOD has previously been found to be highly polymorphic in *Heterodera* cysts as well as showing a consistent activity in *X. index* comparable with that of endoparasites (Molinari *et al.*, 1996; Molinari and Miacola, 1997).

The second approach applies the PCR technique for amplification of the ITS DNA region of single individuals, which could not be screened by the former method. This more sensitive method has been already tried by Vrain *et al.* (1992) to distinguish species belonging to the *Xiphinema americanum* group. The application of such a method to *Xiphinema* spp. phylogenetically different from species of the *X. americanum* group or even to *Longidorus* spp. resulted in problems that are discussed later.

Finally, methods that have been described in this paper may represent an aid, if complementarily used and supported by morphological measurements, in detecting inter-and possibly intra-specific variability of *Xiphinema* and *Longidorus* spp. and in rapidly and reliably identifying species, especially those that may be vectors of plant viruses.

Materials and methods

Geographically different populations of *Xiphinema index* Thorne *et* Allen, were used to investigate putative intraspecific variability. The standard population was from the rhizosphere of figs growing in the vicinity of Bari (Italy). Other populations came from Switzerland and Portugal. Comparisons among different *Xiphinema* species were made with populations of *X. index*, *X. vuittenezi* Luc, Lima, Weischer *et*

Flegg, from Slovakia, and *X. santos* Lamberti, Lemos, Agostinelli *et* D'Addabbo, from Portugal. The application of the technique was also tested with *Longidorus latocephalus* Lamberti, Choleva *et* Agostinelli, from Bulgaria. Specimens of the various species were generally obtained from cultures maintained on fig or grapevine in a glasshouse. Nematodes were extracted from the soil by the Cobb wet-sieving technique. Sufficient numbers of nematodes were hand-picked and placed in a small volume of distilled water, rinsed to cleanse them from any soil debris and then 10-20 active specimens of each species were transferred into a plastic, Eppendorf-shaped, miniature homogenizer (Biomedix, UK). The distilled water was replaced with 10 µl of the extraction buffer, consisting of 20% sucrose, 0.1 M Trizma-Base, 0.08 M boric acid, pH 8.4, 2.5 mM EDTA, 5 µg of bromophenol blue, with the inhibitors of proteases PMSF (1 mM), pepstatin (1 µM) and leupeptin (1 µM). Samples were homogenized in the extraction tube, kept in an ice-bath, with a small plastic pestle connected to a rotor and centrifuged at 5,000 rpm for 10 min. The whole of the clarified supernatants were used immediately for electrophoresis.

SOD isozymes were separated using isoelectric focusing using Phast System equipment (Pharmacia Biotech, USA), which permits pre-programming of the chosen separation method. The exact replication of the method in each run is thus ensured by microprocessor control and by the use of precast 0.45 mm thick gels whose separation zone is only 3.8 x 3.3 cm. A 2000 V power supply and a cooled, thermostatically controlled, separation bed are built in to give field strengths up to 500 V/cm, which allows high speed and high resolution separations. The separation method applied has been described in Molinari *et al.* (1996). Samples (4 µl) were applied in the anodal and cathodal positions because of the low protein content and to ensure that the SOD isozymes reached their pIs, which were unknown. Thus, almost the whole homogenate resulting from a sample was load-

ed in a single run. Gels were maintained at 15 °C and runs were stopped at 600 Vh, which corresponded to approx. 30 min. Gels were calibrated by using a broad pI calibration kit (Pharmacia Biotech, USA) containing amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), ß-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.88), human carbonic anhydrase B (6.55), myoglobin-acidic band (6.85), myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65), and trypsinogen (9.30). Relative electrophoretic mobilities (RF in cm) were plotted against pIs and a straight line with a correlation coefficient of -0.994 obtained. SOD activity was determined by incubating the gels in the dark at 28 °C for 30 min in a solution containing 0.12 mM nitrobluetetrazolium (NBT) in 50 mM Tris-HCl, pH 7.6. Thereafter, NBT was removed and the gel immersed in a solution containing 15 mM N, N, N', N' – tetramethylethylenediamine (TEMED) and 0.26 mM riboflavin. Gels were developed on a white light transilluminator until white bands on a dark background appeared as SOD inhibits the reduction of NBT by riboflavin. When bands were visually clear and sharp, the gels were dried and scanned by means of a ScanJet II cx (Hewlett Packard), arranged by computer into negative images and printed. For DNA extraction, single females of *Xiphinema* spp. were hand-picked and placed on a glass slide in 3 μl of the lysis buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 15 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatine) with 90 μg/ml proteinase K and then cut into small pieces. The suspension was recovered from the glass slide which was washed with a further 7 μl of lysis buffer and the washing suspension transferred to a cold 0.5 ml microcentrifuge tube. The 10 μl sample was overlaid with a drop of mineral oil and incubated at 60 °C for 1 h and at 95 °C for 10 min.

Amplification of crude DNA was performed in 100 μl containing 0.2 mM of each dNTP, 15 pmols of each primer and 2.5 units of *Taq* polymerase (Boehringer, Manheim). The PCR conditions were denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and an additional 7 min at 72 °C to allow completion of partially extended amplification products. Purified DNA of *X. index* was the positive control and distilled water the negative control in each run.

Following PCR, one tenth of the volume of the amplification reaction was electrophoresed on 1% agarose gel in 1 x TBE, pH 8.3, at 6 V/cm for 1 h. Then the gel was stained with ethidium bromide and visualized by UV light.

The 2000 bp amplified product of *X. index* was cloned into the pMOSBlue T-vector kit (Amersham). The sequencing of double strand recombinant plasmid was carried out by using the Sequenase version 2.0 (USB, Amersham) and vector primers.

Results and discussion

Sensitivity of the identification method, involving isoelectrofocusing SOD isozyme profiles, was tested in relation to the number of longidorid nematodes homogenized per sample (Fig. 1). Fig. 1 shows the printed negative image of the minigel scanned after staining and drying. Three bands of SOD activity are clearly visible with 20 and 40 *X. index* females (samples 4 and 5, respectively). The bands of these samples were also visually detectable as white stains on a dark background directly on the gel. Bands of sample 2 and 3 (4 and 10 females, respectively) were hardly visible on the gel but were revealed by the scanning and the inversion of the image polarity. Samples of single individuals (1) did not show any band of SOD activity.

pIs of these three SOD isoforms of *X. index* females were calculated to be 7.0, 6.2, 5.7 (Fig. 2, samples 4-5). No main difference in the isozyme profile was found between females and juveniles (samples 2). A Swiss population of *X. index* cultured on grapevine in a glasshouse
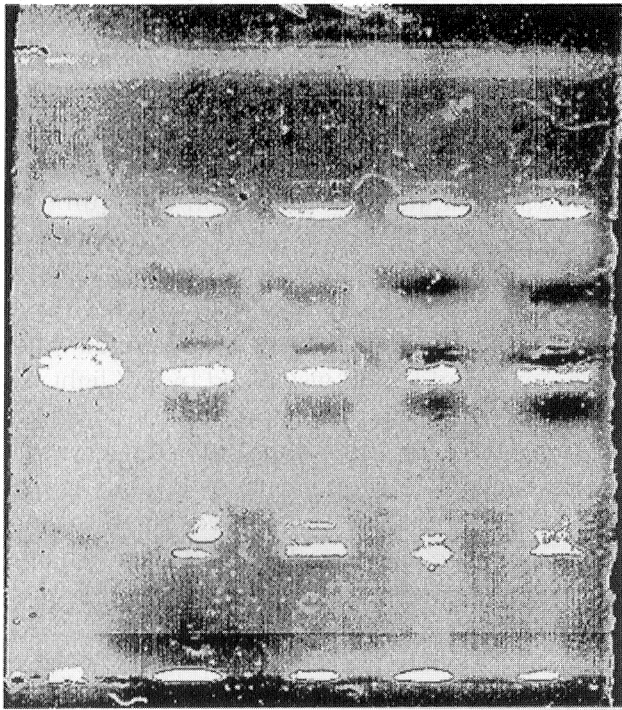
Fig. 1 - Isoelectrofocusing (IEF) SOD isozyme profiles of *Xiphinema index*. The figure is the negative image of the gel directly scanned and printed by a computer system. Bands of SOD activity appear as black spots on a gray background. Samples were obtained by homogenizing different numbers of females in the same volume of extraction buffer, as follows: 1 female (sample 1), 5 females (2), 10 females (3), 20 females (4), 40 females (5).

(sample 3) did not show differences in their profile with respect to the standard population. A population of *X. index*, originally from Portugal, cultured on grapevine in the glasshouse, had the typical 3 bands but it showed an additional slight band with a pI of 6.7 (sample 1). It is, of course, premature to speculate about the possibility of using this method to discriminate the intraspecific variability of longidorids from the figure shown but further analyses being carried out at present are consistent with such feasibility.

Three different species of *Xiphinema*, *X. vuittenezi* (1), *X. index* (2) and *X. santos* (3), are clearly distinguished by means of isoelectrofoc-

using SOD isozyme profiles (Fig. 3). Electromorphs of *X. index* are consistent with a dimeric enzyme and a hybrid phenotype (Acquaah, 1992). Accordingly, SOD of *Meloidogyne incognita* and *Heterodera* spp. has been proposed to be a dimer (Vanderspool *et al.*, 1994; Molinari *et al.*, 1996). Interestingly, expression of SOD activity in *X. vuittenezi* seems to be regulated by the same locus of *X. index* but with a homozygotic genotype, thus confirming at molecular level the morphological similarity of these two species (Thorne and Allen, 1950; Luc *et al.*, 1964). Electromorphs of *Xiphinema santos* showed four apparent bands with different intensity, one band having a very basic pI. The marked difference showed by the isozyme profile of *X. santos* with respect to those of *X. index* and *X. vuittenezi* reflects the same difference that can be easily evaluated by morphometrical parameters (Lamberti *et al.*, 1993). As a comparison, the SOD isozyme profile of *Longidorus latocephalus* is shown in Fig. 3 sample 4. The presence of two bands having a marked difference in their pIs suggests the involvement of two different loci rather than a unique locus with a silent allele. Further insights will be accomplished by testing electromorphs
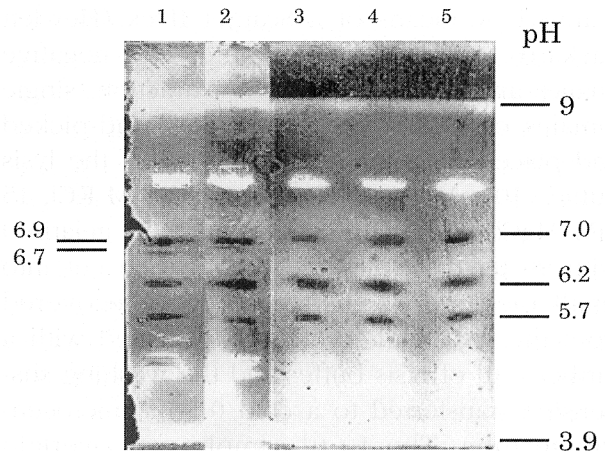

Fig. 2 - IEF SOD isozyme profiles of different populations of *Xiphinema index*. The figure is the negative image of the gels scanned and printed by a computer system. Samples were as follows: females of the standard population (4, 5), a population from Switzerland (3), juveniles of the standard population (2), a population from Portugal (1).
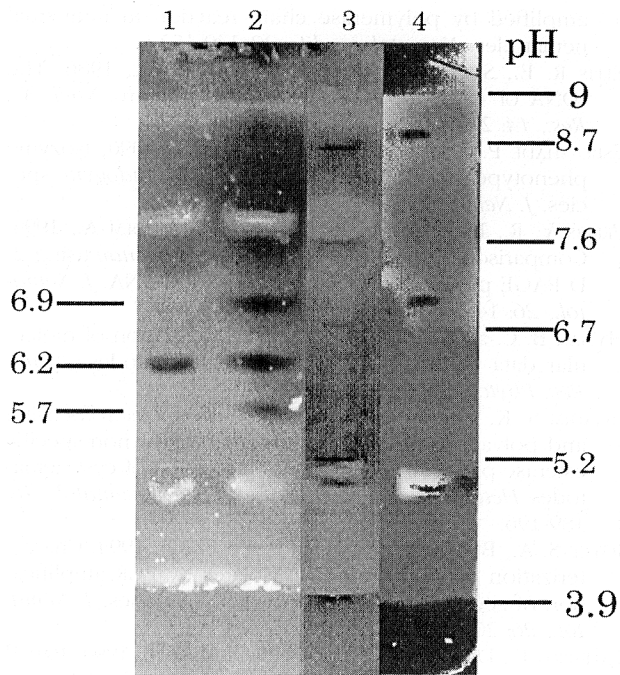
Fig. 3 - IEF SOD isozyme profiles of different species of longidorid nematodes. Samples were as follows: *X. vuittenezi* (1), *X. index* (2), *X. santos* (3), *Longidorus latocephalus* (4).

obtained with native-PAGE which separates isoforms according to their difference in size and molecular weight. Native-PAGE of *X. index* homogenates has confirmed the presence of only three bands with little difference in their R_F values (not shown). Therefore, the method of SOD isozyme profiles described in this paper is able to discriminate among longidorid species and may also detect intraspecific variability. However, at present its sensitivity is limited to samples consisting of more than a single specimen and the smaller the size of the nematode the more of them are required in a sample for band staining. Mixed populations may be recognized as such but the species that comprise them are hardly recognizable unless they had previously been separated.

In Fig. 4 the amplification products of single specimens of *X. index* and *X. vuittenezi* are shown. The amplified product of *X. index* was approximately 2000 base pairs (bp), the same size as that obtained when *X. index* purified total DNA was amplified of the same figure. The amplification product of *X. vuittenezi* is shown in lane 8. It is approximately 1800 bp, 200 bp smaller than that obtained with *X. index*. Therefore, if the sizes of PCR products obtained with *X. index* and *X. vuittenezi* are compared with that obtained with *X. americanum* group (about 1500 bp) it can be concluded that the length of ITS regions can be used to discriminate *X. index* and *X. vuittenezi* from the *X. americanum* group.

The experiments described in this paper were carried out by using the same primers as designed by Vrain *et al.* (1992) but with a different lysis buffer. The results reported clearly demonstrate that the modifications introduced into the classical procedure described by Vrain *et al.* (1992) and mainly used afterwards, are still compatible with a successful amplification. However, the use of a lysis buffer of appropriate composition enables adaptation to possible different requirements of different *Xiphinema* species.
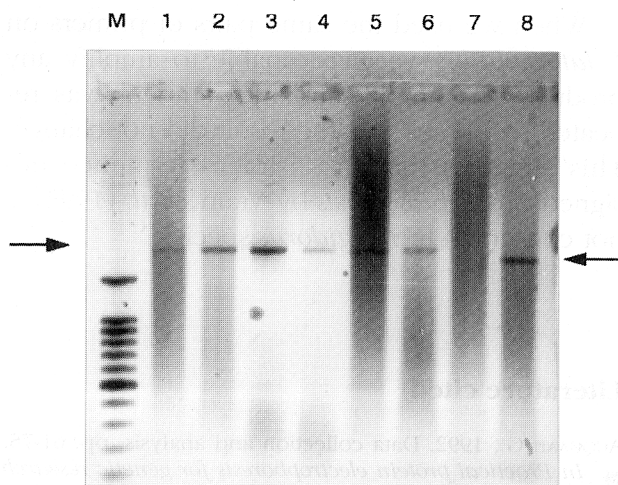

Fig. 4 - Electrophoresis of the PCR amplified Internal Transcribed Spacer Regions from DNA of single individuals of *Xiphinema index* and *X. vuittenezi* (ethidium bromide stained 1% agarose gel). DNA marker (M); single nematodes of *X. index* (1 to 7); single *X. vuittenezi* (8).

The relative amount of amplification product on both purified DNA and a single nematode was low but always clearly detectable, even when *Taq* enzymes from different companies were used. A possible explanation of the reduced efficiency of amplified product may be, as Joyce *et al.* (1994) observed, the changing to different batches of *Taq* enzyme and of PCR primers.

Amplification was unsuccessful with nematodes stored at 4 °C for at least two weeks. Neverthless, a dilution of the material to be amplified gave rise to the 2000 bp product. The lack of amplification could be due to the presence of spurious residues as contaminants that inhibit DNA amplification. In fact, we could discern only a smear on the gel.

The 2000 bp amplified fragment was cloned and the sequence of both ends was determined showing that the 2000 bp fragment is the expected ribosomal region by comparison with the *Caenorhabdtis elegans* corresponding regions (Ellis *et al.*, 1986). The sequence, obtained with an upstream primer to that used in the amplification, revealed that the annealing region of the 26S primer also is conserved in *X. index*.

When we used the same pairs of primers on *L. latocephalus* we were unable to amplify any product. The amplification experiment was repeated few times, but the result did not change. This suggests that the primers sequence designed on *X. bricolensis* by Vrain *et al.* (1992) is not conserved in *Longidorus* species.

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