PRELIMINARY ATTEMPT TO DIFFERENTIATE PINEWOOD NEMATODES (*BURSAPHELENCHUS* SPP.) BY ENZYME ELECTROPHORESIS.

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The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 (syn. *B. lignicolus* Mamiya & Kiyohara, 1972) kills several million trees each year in Japan. It has also been reported from many parts of the United States of America (Dropkin *et al.*, 1981) from where it probably originates. *B. mucronatus* Mamiya & Enda, 1979 which also reproduces on pine, but is not pathogenic to it, has been described from Japan and differentiated from *B. xylophilus* by its female having a " mucro... conspicuously longer than " *B. xylophilus*. The validity of *B. mucronatus* was questioned by Baujard (1980) who synonymized it with *B. xylophilus* on the basis of the variability of the tail tip profile in the genus.

Among the three characters given by Nickle *et al.* (1981) to differentiate *B. xylophilus* from other species in the genus, only the lack of a digitate female tail separates it from *B. mucronatus* which, according to the original description and to personnal observations of one of us (G. de G.), shows the other characters : a distinct vulval flap and a disc-like distal expansion of the spicules. These authors, on the basis of this single character supported by unsuccessfull mating experiments, reestablished the validity of *B. mucronatus*. They mention that *B. fraudulentus* Rühm, 1956 is " similar to *B. xylophilus* " but with " female [having] a tail tip similar to *B. mucronatus* " and do not express any opinion on the identity of this species.

Although the original measurements of these three species are very similar, Tarjan (1982) separated *B. mucronatus* from *B. fraudulentus* by small differences in the length of the stylet and spicules.

Baujard *et al.* (1979) reported the presence of *B. xylophilus* in France. The study of this French strain shows that it falls within the common measurements of these three species but has the stylet length of *B. fraudulentus* and the spicule length of *B. mucronatus*. Furthermore, it has the digitate female tail of *B. mucronatus*, but can hybridize with both *B. xylophilus and B. mucronatus* and is more virulent on young pine trees than *B. mucronatus* (de Guiran, unpublished).

Morphobiometry being obviously insufficient to identify this French strain, electrophoresis of some enzymes has been performed on it and on *B. xylophilus* and *B. mucronatus* from Japan, and compared in order to find any other discriminating characters.

MATERIALS AND METHODS

The French strain of *Bursaphelenchus* was isolated from *Pinus pinaster* from Campet forest, 47700 Casteljaloux. *B. mucronatus* and *B. xylophilus*, from *Pinus densiflora*, Mito (Ibaraki) Japan, were kindly supplied by Y. Mamiya, Forest Research Institute, Tsukuba. The three strains were cultured on *Botrytis cinerea* growing on crushed corn grains.

About one million nematodes of various developmental stages, concentrated on 3 µm filters, were homogenized in a Ernst Schütt tissue homogenizer in an equal volume of 20 % sucrose solution. After one centrifugation at 20 000 g, the supernatant was divided into 10 µl samples, each corresponding to about 100 000 individuals.

Electrophoresis was conducted vertically in polyacrylamide gel plates 0.4 mm thick. Samples were positioned in spacer gel above a homogeneous 7 % gel at *p*H 8.9 by Tris-HCl. Tank buffer : Tris-HCl glycerine *p*H 8.3 (Ornstein & Davis, 1964).

Enzymatic activity was revealed by the following methods :

Esterase : 15 mg fast blue RR and 20 mg naphthyl acetate in 25 ml phosphate buffer 0.1 M at *p*H 7.2.

Malate dehydrogenase : Na_2CO_3 and malic acid + 12.5 mg NAD; 7.5 mg MTT; 0.5 mg PMS in 25 ml Tris-HCl buffer 0.5 M at *p*H 7.1.

Total proteins and phosphatases were also analyzed but gave less clear results and must be further investigated.

RESULTS

Figure 1 shows that the three populations are easily distinguishable on the basis of isoesterases as well as malate dehydrogenase bands since they exhibit a different pattern for each enzyme.

Isoesterases

The French strain showed a large esterase band migrating about the middle of the gel. This is not the case for *B. mucronatus* and *B. xylophilus* populations in which the main bands are the fastest ones.

Some bands appear double, especially at the bottom level.

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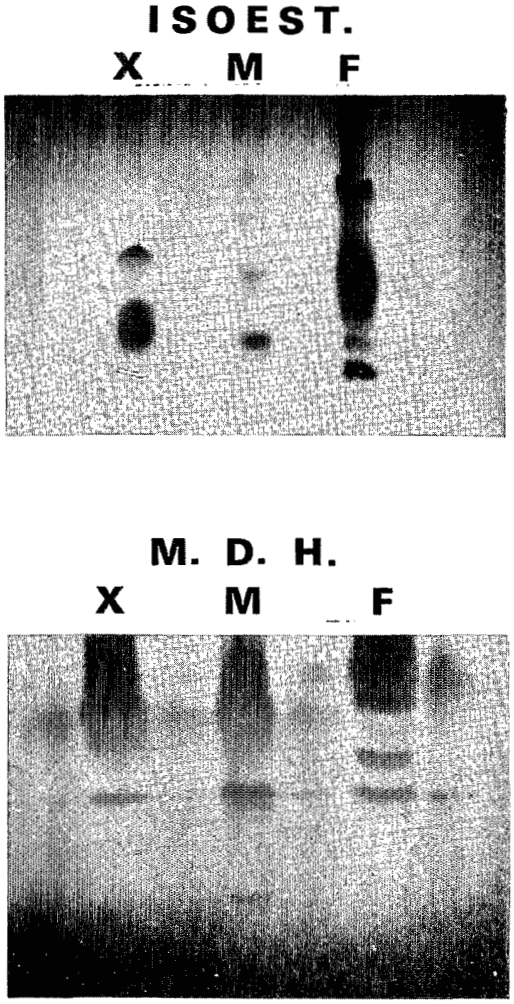

Fig. 1. Isoesterase (Isoest.) and malate dehydrogenase (M.D.H.) in *Bursaphelenchus xylophilus* (X), *B. mucronatus* (M), both from Japan, and *Bursaphelenchus* sp. from Campet, France (F).

Malate dehydrogenase

Due to technical difficulties, the upper part of the gel was not well stained. A M.D.H. band was clearly observed at the bottom of this coloured zone, especially in the French strain. This strain showed three other bands : one double and a third which migrated equidistantly in the three populations. *B. mucronatus* and *B. xylophilus* showed the same M.D.H. pattern but in *B. mucronatus* there was a fast band, not observed in *B. xylophilus*.

MDH isozymes discriminated very well the French strain.

Discussion

The taxonomic situation of *B. xylophilus* and related species is rather puzzling. The French strain studied here cannot be placed definitively in one or the other species. Another strain which has been isolated from *Abies balsamea* in Minnesota, U.S.A. (Wingfield, Blanchette & Kondo, 1983) is in the same situation. It shows a mucronate tail and is not pathogenic on scotch pine, *i.e.* it resembles *B. mucronatus* in both these characters. However this strain mates with *B. xylophilus* but not with *B. mucronatus*. This is not the only case in zoology where the separation of species is not complete (Bernardi, 1980). The populations studied or referred to in this paper, and others, to be discovered, could be intermediate between the two species and are separated by genetic factors governing : *i)* their ability to hybridize with each other; *ii)* their morphological features (mucro); *iii)* their pathogenicity on pine. These factors are not necessarily linked. The only link appearing, but likely to be contradicted by the discovery of other strains, is that the pathogenic populations have females with more or less rounded tail and the nonpathogenic populations have females with strongly digitate tails.

All these strains could belong to a *supraspecies* (Genermont & Lamotte, 1980) but more information is nedded before a clearer view of their affinities and relationships is established.

Esterases and MDH which have shown phenotypic differences among the three strains examined here, and perhaps other enzymes, could help clarify the taxonomic status of these nematodes. The present study must be extended to include several strains of each species in order to clarify whether intraspecific variation does not overlap the specific differences observed here.

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A MICROPLOT METHOD FOR RECOVERY OF ENTIRE PLANT ROOT SYSTEMS AND THEIR ASSOCIATED ENDOPARASITIC AND SEMI-ENDOPARASITIC NEMATODES (1)

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Microplots are enclosures that allow plant growth in limited volumes of field soil which is physically isolated from ambient soil. By definition, microplot systems are implemented under field or near-field conditions. Dimensions should be large enough to allow root growth similar (not identical) to that which occurs under natural conditions. They facilitate study of interactions between soil-borne pathogens and their plant hosts under biotic and environmental conditions which are more realistic than greenhouse conditions (Jones, 1956a, 1956b; Olthof & Potter, 1972; Barker, Shoemaker & Nelson, 1976; Benson, Barker & Aycock, 1976; Abawi & Mai, 1980; Martin, Riedel & Rowe, 1982; Weingartner, Shumaker & Smart, 1983). Greenhouse studies using small pots permit the recovery of entire plant root systems, but do not allow accurate determination of the effect of pathogens on root growth due to the confining nature of small pots.

Although several methods for establishing microplots in the field are published (Jones, 1956 b; Olthof & Potter, 1972; Barker, Daughtry & Corbett, 1979; Johnson, Rich & Boatright, 1981; Martin, Riedel & Rowe, 1982), we have several criticisms of these. Our first concerns the shallow depth to which the microplots are placed in the soil, 30-60 cm. Even the deepest of these, 60 cm, is not adequate to contain the root system of many crop plants, such as maize *(Zea mays)*, sugarbeet *(Beta vulgaris)*, tomato *(Lycopersicon esculentum)*, and potato *(Solanum tuberosum)* (Weaver, 1926; Weaver & Bruner, 1927). This is important because the soil bed into which the microplots are placed is often fumigated prior to use (Benson, Barker & Aycock, 1976; Abawi & Mai, 1980; Martin, Riedel & Rowe, 1982) and inoculated soil is subsequently added to the microplot. Using this technique the inoculum potential is confined to only a portion of the soil volume eventually occupied by the root system. Because the distal end of the microplot is open, growing roots leave the microplot and move into nonfumigated soil, effectively escaping further infection. It is possible that nematodes from deeper soil strata moving upwards towards the plant root system will contaminate the microplot (Weingartner, Shumaker & Smart, 1983). This situation is not desirable in experiments putatively designed to assess the impact of nematode population density on crop growth. Another criticism of these methods is that they do not allow recovery of the entire plant root system with its associated nematodes. A microplot system which allows such recovery is necessary for quantitative studies addressing the biological interactions between plant growth and the population dynamics of endoparasitic or semi-endoparasitic nematodes within the root system.

In this paper we describe a microplot technique for establishing individual plants under near-field conditions using a limited soil volume which allows recovery of entire plant root systems and the endoparasitic or semi-endoparasitic nematodes associated with them. This system allows us to obtain quantitative information

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