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USE OF ESTERASE AND PROTEIN PATTERNS TO DIFFERENTIATE TWO NEW SPECIES OF *APHELENCHOIDES* ON RICE FROM OTHER SPECIES OF *APHELENCHOIDES* AND FROM *DITYLENCHUS ANGUSTUS* AND *D. MYCELIOPHAGUS*

BY

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Three populations of *Aphelenchoides besseyi*, one population of *A. bicaudatus, A. arachidis, A. fragariae, A. hamatus*, two undescribed species of *Aphelenchoides* from rice and *Ditylenchus angustus* and *D. myceliophagus* were analyzed by native and SDS-PAGE electrophoresis. The highly reproducible banding patterns of all species differed in number, concentration and relative mobility. Certain enzyme bands were common between the species whereas other bands were specific. There were no detectable differences in the protein and esterase banding patterns of nematodes extracted from cultures of *Botrytis cinerea* or *Rhizoctonia cerealis*. The stability and taxonomic usefulness of the α, β esterase banding patterns are discussed.

Keywords: Aphelenchoides, Ditylenchus, electrophoresis, esterase, taxonomy

The nematode genus *Aphelenchoides* contains species parasitic on higher plants, species associated with insects and fungivorous species. The plant parasitic nematode, *Aphelenchoides besseyi*, is primarily a parasite of rice and causes the disease known as 'white tip'. *Aphelenchoides besseyi* is widely distributed and now occurs in most rice growing areas (Ou, 1985). *Ditylenchus angustus* is the cause of 'Ufra' disease in deepwater and lowland rice, and occurs in a wide range of countries (Bridge *et al.*, 1990). Field samples of rice received from Vietnam contained *D. angustus* and an undescribed species of *Aphelenchoides* which appeared morphologically the same as an undescribed species from rice from Sierra Leone.

Identification of plant parasitic nematodes using the classical method of taxonomy based on light microscopic examination of morphological features is of primary importance in characterizing main genera (Pozdol & Noel, 1984). However, within some nematode genera this method may not be sufficient to separate certain closely related species. The close morphological similarities between species of *Aphelenchoides* makes taxonomic study difficult. Gel electrophoresis can be useful in establishing a biochemical basis for defining systematic relationships between nematodes. The analysis of enzymes and nonspecific esterase by polyacrylamide gel electrophoresis is an effective method of distinguishing species and has been used successfully to identify genera and species of nematodes (Dickson *et al.*, 1971; Hussey & Krusberg, 1971; Dalmasso & Bergé,

1978, 1983). Esterase analysis is one of the most effective techniques for comparing organisms and this enzyme has proved of value in the separation of several nematode species of the genera *Meloidogyne* (Hussey *et al.*, 1972; Dalmasso & Bergé, 1978, 1983; Janati *et al.*, 1982; Esbenshade & Triantaphyllou, 1985, 1987, 1990; Ibrahim & Perry, 1993a), *Ditylenchus* (Dickson *et al.*, 1971), *Bursaphelenchus* (De Guiran *et al.*, 1985) and *Heterodera* (Nobbs *et al.*, 1992).

Effective control of nematode pests of rice depends on accurate identification; clearly, it is important to assess whether the undescribed species of *Aphelenchoides* are distinct and to establish definitive biochemical differences to separate them from other species of this genus and from *D. angustus*. The objectives of this study were to use native polyacrylamide gel electrophoresis (PAGE) to compare nonspecific esterase isozymes and SDS-PAGE to compare protein patterns of selected species of *Aphelenchoides* and *Ditylenchus* and to examine the enzymatic and protein relationships of the two new species from Vietnam and Sierra Leone with other species of *Aphelenchoides*. We also determined whether esterase patterns remained stable and characteristic for each species and compared three populations of *A. besseyi* from different geographical regions.

MATERIALS AND METHODS

Nematode Material

The designations and sources of nematode species were: three populations of *A. besseyi* from rice plants (*Oryza sativa*) from the Philippines, India and Sierra Leone; *A. bicaudatus* from a Pit-Pit plant (*Setaria palmaefolia*) originally from Papua New Guinea; *A. arachidis* from ground nuts (*Arachis hypogaea*) from Nigeria; *A. fragariae* from fern (*Asplenium nidus*) from California and *A. hamatus* from strawberries (*Fragaria* × *ananassa*) from Bristol, England. The two undescribed species of *Aphelenchoides* from rice were from Vietnam (V) and Sierra Leone (S).

To determine whether the fungus substrate affected the esterase banding patterns of the nematodes, populations of the undescribed species of *Aphelenchoides* were cultured on the fungi, *Botrytis cinerea* or *Rhizoctonia cerealis*, growing on potato dextrose agar Petri dish cultures at 20-23°C. All other species of *Aphelenchoides* were routinely maintained on *Botrytis cinerea* cultures. *Ditylenchus myceliophagus* was cultured on mushroom (*Agaricus bisporus*) compost. *Ditylenchus angustus* was originally received from Hau Giang Province, Vietnam and cultured on rice (cv. IR-36) and extracted as previously described (Ibrahim & Perry, 1993b).

Enzyme Extraction

Suspensions of *D. angustus* were filtered through a cotton wool milk filter to remove dead or inactive nematodes and then thoroughly washed with several

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changes of distilled water. *Aphelenchoides* and *D. myceliophagus* were collected from culture plate lids and rinsed twice with distilled water. Mixed stages (ca. 90% J4 and adults; approximately 10,000 to 15,000 nematodes in 50 µl distilled water) were used. Each species was transferred to separate centrifuge tubes. A 40 µl solution of extraction buffer, containing 20% glycerol, 2% Triton X-100 and 0.01 g bromphenol blue, was added to each tube. During subsequent processing, the nematodes remained in an ice bath when possible. Nematodes were homogenized in the extraction tube with a small plastic pestle and centrifuged at 14,000 r.p.m. for 10 min at 4°C. The clarified supernatant was introduced immediately into the electrophoresis cell.

Polyacrylamide Gel Electrophoresis

The procedures used for gel electrophoresis and enzyme detection have been detailed previously (Ibrahim & Perry, 1993a). In brief, the esterase isoenzymes present in the nematode samples were determined by native polyacrylamide gel electrophoresis in either a mini-slab gel apparatus (Atto, Japan) using gel size 90 mmW, 80 mmL × 1 mm thick or a standard gel apparatus (Atto, Japan) using gel size 138 mmW, 130 mmL × 1 mm thick. A 3% acrylamide stacking gel, and 7% acrylamide separation gel were used. Samples of 20-25µl were injected into the electrophoresis cell. The buffer system was essentially that of Laemmli (1970), except that Triton X-100 was substituted for sodium dodecyl sulfate (SDS). For protein separations, polyacrylamide gel electrophoresis (Laemmli, 1970) in the presence of SDS was performed using a 10% (w/v) separating gel (pH 8.8) and a 4% (w/v) stacking gel (pH 6.8). With each electrophoretogram, the following commercially available proteins (Sigma) were used as standard markers for molecular weight estimations: β-galactosidase (MW 116 kD); fructose-6-phosphate kinase (84 kD); pyruvate kinase (58 kD); ovalbumin (45 kD); lactic dehydrogenase (36.5 kD); triosephosphate isomerase (26.6 kD).

Enzyme Staining

Nonspecific esterase activity was determined by incubating the gels at 37°C in the dark for 15-30 min in a solution of 100 mg Fast Blue RR Salt, 50 µg α, β-naphthyl acetate and 50 µg α-naphthyl butyrate dissolved in 5 ml acetone made up to 100 ml with 0.2M Tris-Chloride buffer pH 6.6. The substrates were filtered through filter paper and used immediately. The reaction was stopped by adding 10% acetic acid. Relative electrophoretic mobility (Rm) for each enzyme band was calculated as the ratio of its movement to that of the marker dye.

For general protein patterns, gels were stained with 0.1% Coomassie brilliant blue R 250 in 50% methanol and 10% acetic acid at 45°C for 1 h. Coomassie-stained gels were subsequently destained with several changes of 50% methanol and 7% acetic acid solution. Since silver staining is far more sensitive than

Coomassie blue, gels were also stained with silver using the procedure of Oakley *et al.* (1980).

RESULTS

Separation of multiple forms of nonspecific α, β esterase isozymes by native gel electrophoresis was consistent and highly reproducible for all species tested (Fig. 1). Each nematode species investigated had a characteristic esterase pattern. In total, 26 bands were resolved for all species of *Aphelenchoides* and *Ditylenchus* tested.

The three populations of *A. besseyi* exhibited similar banding patterns: two strong bands at Rm = 0.38 and 0.53, and a weak band at Rm = 0.40. However, an inconsistent extra weak band, observed in the two populations from the

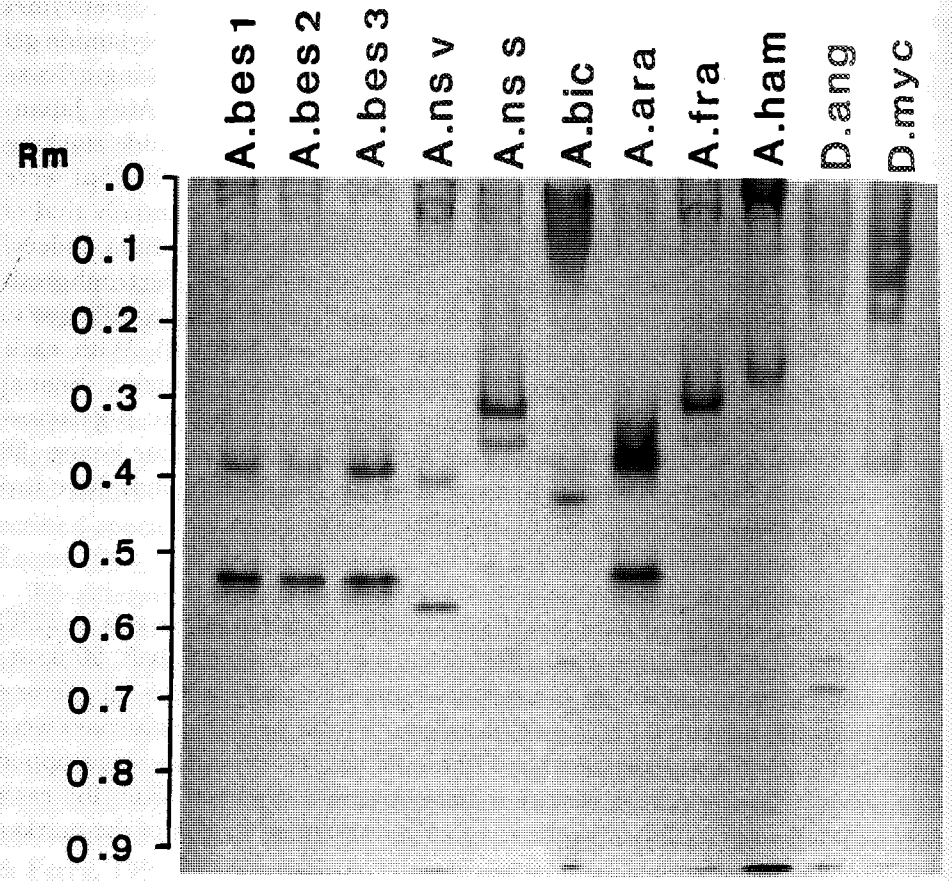

Fig. 1. Comparative nonspecific esterase banding patterns separated by slab gel electrophoresis: *Aphelenchoides besseyi* (A. bes. 1)-Philippines, (A. bes 2)-India, (A. bes 3)-Sierra Leone; undescribed species of *Aphelenchoides* (A.ns V)-Vietnam, (A.ns S)-Sierra Leone; *A. bicaudatus* (A.bic); *A. arachidis* (A.ara); *A. fragariae* (A.fra); *A. hamatus* (A.ham); *Ditylenchus angustus* (D.ang); *D. myceliophagus* (D.myc).

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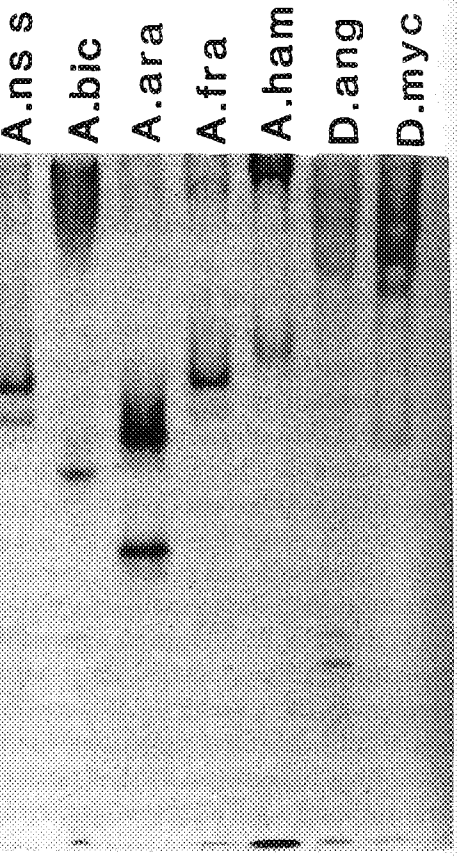

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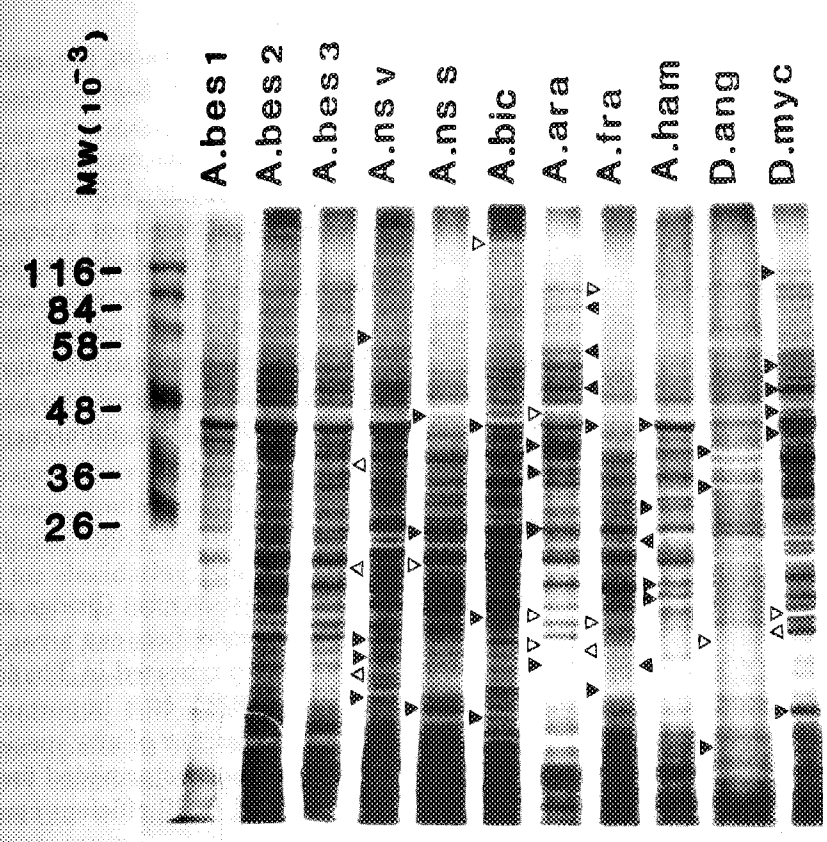

Fig. 2. SDS-PAGE (30% T, 2.67% C) of proteins from populations of *Aphelenchoides* and *Ditylenchus*; key to species as for Fig. 1. Arrows between tracks indicate protein bands which are present (◀) or absent (◁) when adjacent species are compared.

Philippines and India at Rm = 0.25, was absent in the Sierra Leone population. The two new species from rice had distinct esterase banding patterns; the population from Vietnam showed a strong band at Rm = 0.57 and a weak band at Rm = 0.39, while the Sierra Leone population had two strong bands at Rm = 0.32 and 0.35 and two faint bands at Rm = 0.51 and 0.62. A faint band at Rm = 0.57 was present in both species.

Aphelenchoides bicaudatus also had distinctive banding patterns with one strong band at Rm = 0.42 and two weak bands at Rm = 0.65 and 0.72. The *A. arachidis* population had two strong bands at Rm = 0.38 and 0.52. *Aphelenchoides fragariae* exhibited different banding patterns with one strong band at Rm = 0.30 and one weak band at Rm = 0.62. *Aphelenchoides hamatus* had two bands at Rm = 0.2 and 0.27. *Ditylenchus angustus* showed two moderately strong bands at Rm = 0.63 and 0.65. Three strong bands at Rm = 0.10, 0.14 and 0.20 and a moderately strong band at Rm = 0.38 were observed in the extract collected

from *D. myceliophagus*. There were no detectable differences in the esterase patterns of *Aphelenchoides* spp. (V and S) cultured on either *Botrytis cinerea* or *Rhizoctonia cerealis* (data not shown).

Protein banding patterns of all species and populations examined were highly reproducible. Protein analysis using SDS-PAGE indicated distinct banding differences between species (Fig. 2), although the presence or absence of bands was more distinct in silver stained gels where over 50 bands per species could be distinguished. However, the numerous differences between species means that protein profiles are less easy to use for diagnostic purposes than the esterase banding patterns. SDS-PAGE banding patterns demonstrated distinct differences between the two new species of *Aphelenchoides* by at least four bands, and between other species tested. There were no detectable differences in protein profiles between the three populations of *A. besseyi*.

DISCUSSION

Many examples of the economic importance of *A. besseyi* exist (Bridge *et al.*, 1990) but reports are inconsistent, with marked differences between areas. For example, no symptoms of *A. besseyi* infection have been reported in Thailand, despite widespread infestations (Buangsuwon *et al.*, 1971). It is important to ensure that *A. besseyi* is being correctly identified; the presence of new species of *Aphelenchoides* makes it imperative that information should be provided for the accurate separation and identification as this is central to all efficient control measures. It is also important to investigate the biology of the two new species, and this is the subject of future work to determine whether they are parasites of rice or if they are exclusively fungivorous.

We report the separation of two undescribed species of *Aphelenchoides* from Vietnam and Sierra Leone. Although the morphological studies (Hooper & Ibrahim, 1994) of these new species showed that they are very closely related, isozyme analysis and protein banding patterns resolved by SDS-PAGE indicate that they are genetically different. Esterase patterns showed that the two species had one common band at Rm = 0.57; the number of bands detected in the species from Sierra Leone was greater than in the species from Vietnam. Similarly, the only morphological difference between *Caenorhabditis elegans* and *C. briggsae* is in the tail bursal ray pattern of the males (Friedman *et al.*, 1977) but biochemically they differ in 22 out of 24 enzyme-encoding loci (Butler *et al.*, 1981). Two races of *Radopholus similis* were eventually considered as different species, based on enzyme analysis, including α and β esterase patterns (Huettel *et al.*, 1983).

The esterase patterns indicated a minor difference between the populations of *A. besseyi* collected from different locations. Fargette (1987) found that esterase phenotypes of some *Meloidogyne* species collected from different locations were affected by the climate. The difference detected in this study was

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restricted to a weak band; however, there was no clear difference in the protein profiles detected by SDS-PAGE. Intraspecific differences were observed within species of *Meloidogyne* collected from various geographical regions around the world (Dickson *et al.*, 1971). Slight differences of Rm values between laboratories may require the co-electrophoresis of reference populations. In the future, it may be necessary to establish a database of biochemical information of reference populations, including Rm activity, 1- and 2-D gel maps and DNA analysis data, all obtained under carefully defined, fully described experimental conditions.

The techniques used in this study were also able to differentiate between species of two different genera, *D. angustus* and *A. besseyi*, and the undescribed species of *Aphelenchoides*, all of which are associated with rice. Although the damage to rice and their economic importance differs, morphologically they are very similar; as they occur together on the same crop (Plowright & Akehurst, 1992), accurate identification is very important for control strategies and for elucidating aspects of the host-parasite interactions.

There was no difference in the esterase patterns of nematodes cultured on *Botrytis* or *Rhizoctonia*. These results are in agreement with those of Dickson *et al.* (1971), Fargette (1987), Pais & Abrantes (1989) and Ibrahim & Perry (1993a). By contrast, Ishibashi (1970) and Bergé & Dalmasso (1975) reported that esterase patterns of species of *Meloidogyne* were influenced by the host. Fargette (1987) examined 57 populations belonging to three *Meloidogyne* species and found that the host had no effect on the esterase phenotype of the nematodes.

The taxonomic approach based on morphology is useful in identifying well defined species but may prove to be difficult with hermaphroditic species (Friedman *et al.*, 1977). Supporting evidence from electrophoresis techniques based on biochemical analysis can provide information for species separation. The data presented in this study show that non-specific esterase patterns resolved by native gel polyacrylamide electrophoresis are very reliable and useful in making taxonomic differentiations between different genera and, especially importantly closely related species found on the same host.

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ZUSAMMENFASSUNG

Die Verwendung von Esterase- und Proteinmustern zur Unterscheidung von zwei neuen Aphelenchoides-*Arten an Reis von anderen* Aphelenchoides-*Arten, von* Ditylenchus angustus *und von* D. myceliophagus

Drei Populationen von *Aphelenchoides besseyi*, je eine Population von *A. bicaudatus, A. arachidis, A. fragariae, A. hamatus*, zwei unbeschriebene *Aphelenchoides*-Arten von Reis sowie *Ditylenchus angustus* und *D. myceliophagus* wurden mit Hilfe nativer und SDS-PAGE Elektrophorese untersucht. Die leicht reproduzierbaren Bindungsmuster aller Arten unterschieden sich in der Anzahl, der

Konzentration und der relativen Beweglichkeit. Gewisse Enzymbanden waren den Arten gemeinsam, andere waren sehr spezifisch. Es konnten keine Unterschiede im Bindungsmuster der Proteine und Esterasen von Nematoden aus Kulturen von *Botrytis cinerea* oder *Rhizoctonia cerealis* festgestellt werden. Die Stabilität und Nützlichkeit der Bindungsmuster von α, β Esterasen werden diskutiert.

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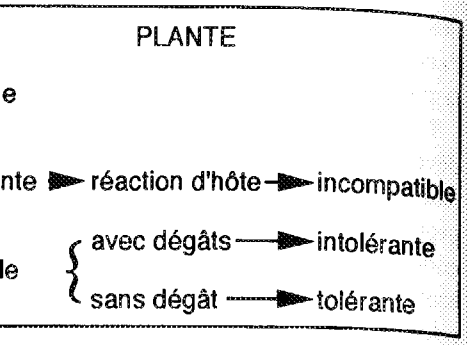

hôte en fonction du parasitisme.

ons superficielles (Wehunt *et al.*, 1965; 81; Davide & Marasigan, 1985; Ravi- ier dénombrement est ramené à l'unité dice de lésions à cinq niveaux. Or le l'origine des racines (Quénéhervé & ysico-chimiques des sols (Quénéhervé, tif, parce que trop qualitatif, car d'une peuvent être divers, d'autre part il est é récemment démontré par Hugon & nstant donné, sur trois types de taches seul était représentatif de l'infestation rstma (1991), qui ont prouvé qu'il n'y re les espèces de nématodes contenues nunautés de nématodes extraits de ces

les nématodes

s variables puisqu'il a été remarqué de ématodes (Rebois *et al.*, 1970), ou à un mez *et al.*, 1983) dont les nématodes. post-infectieux.

éfinissent une immunité, concourent à ans les racines. Celle-ci peut être due à rmes racinaires (Rohde, 1965) ou à la ires non attractifs, répulsifs ou même nt des substances allélochimiques.

la plante hôte répond à la pénétration natodes. Deux réponses typiques sont, nces toxiques (phytoalexines par exem-

ple), d'autre part la réaction d'hypersensibilité qui consiste en la mort des cellules végétales contiguës au parasite, et qui peut être une conséquence de la réaction précédente.

Immunité liée à l'attraction des nématodes

Nous savons que les nématodes phytoparasites sont capables d'effectuer de longues distances dans le sol (Harrison & Smart, 1975; Prot, 1978a) et que les racines participent à ces migrations par attraction des nématodes (Bird, 1959; Chen & Rich, 1963; Prot, 1980) essentiellement au niveau des régions d'élongation cellulaire (Bird, 1962; Lavallee & Rohde, 1962; Pitcher, 1967) et des primordia secondaires (Kämpfe, 1960). Mais cette attraction dépend de la nature de l'hôte (Viglierchio, 1961) et de son état physiologique (Lownsbery & Viglierchio, 1961). Des preuves de cette attraction ont été fournies par l'utilisation, d'une part de sols ayant porté des cultures de pomme de terre ou de canne à sucre (Weischer, 1959), de mil (Luc *et al.*, 1969) ou de tomate (Prot, 1975), d'autre part d'eau de trempage de racines de riz (Lee & Evans, 1973). Lownsbery & Viglierchio (1961) montrèrent que les agents attractifs des exsudats étaient dialysables, et Bird (1962) précisa que les acides ascorbique, gibérellique et glutamique étaient attractifs. Enfin, le gaz carbonique diffusé par les racines, et plus précisément un gradient de CO_2 (Klingler, 1972), représente aussi un stimulus d'attraction.

Or, la pénétration des nématodes dans une racine induit une modification qualitative et quantitative des exsudats racinaires. Ainsi, l'analyse chimique d'exsudats racinaires de tomate infestée par *Meloidogyne incognita* indique une diminution globale de la concentration en acides aminés et une augmentation de la concentration en sucres, plus spécialement en polysaccharides (Wang & Bergeson, 1974). Mais l'analyse chromatographique a révélé la disparition de glucose, de thréonine, sérine et histidine, et d'acide citrique. Des résultats semblables ont été obtenus sur la sève brute.

Altérations physiologiques de la plante

La nutrition minérale et l'eau

Les éléments minéraux sont absorbés en même temps que l'eau et suivent le même trajet racinaire (poils absorbants, parenchyme cortical, endoderme et vaisseaux) grâce à des mécanismes de perméabilité cellulaire par diffusion, osmose, translocation, etc. Ils entrent dans la constitution des éléments plastiques, agissent à l'état de sels dans les phénomènes d'osmose, de turgescence et de maintien du pH, et jouent le rôle de catalyseurs au niveau de nombreuses réactions enzymatiques.

L'influence des nématodes sur les mouvements hydriques a été étudiée avec beaucoup de difficultés car les techniques directes de mesure des flux