G. pallida infective second stage juvenile. The rapid preparation of crude nematode DNA samples coupled with a high concentration of biotinylated probe during hybridisation makes the detection process very quick. Non-specific staining of the sample dots is not a problem even when probing impure DNA samples. The significance of this research in the development of simple diagnostic kits for the routine identification of agriculturally important species, races and pathotypes of plant parasitic nematodes is discussed.

&SUMG

Détection rapide et sensible du nématode phytoparasite Globodera pallida *par utilisation d'une sonde de DNA non radio-active marquée à la biotine*

Le présent travail a trait à la première utilisation efficace d'une sonde de DNA non radio-active, marquée à la biotine, pour caractériser la présence du DNA de *Globodera pallida* futé sur un filtre de nitrocellulose. Lors de cette mise au point, la sensibilité de la technique a été améliorée, permettant ainsi la détection d'un seul juvénile de 2e stade de *G. pallida*. La rapidité de la préparation des extraits bruts du DNA du nématode, couplée à la forte concentration de la sonde marquée à la biotine lors de l'hybridation, conduisent à une technique de détection très rapide. Dans le cas d'échantillons relatifs à un DNA non purifié la coloration non spécifique de certaines zones de l'échantillon ne cause aucun problème. Une discussion porte sur les conséquences de la présente recherche pour la mise **au** point d'un système simple d'identification routinière des espèces, races et pathotypes de nématodes d'importance agronomique.

Many species of plant parasitic nematodes pose a significant threat to agriculture worldwide. Accurate and reliable identification of these microscopic pests is fundamental to many aspects of their control and management; Diagnosis of species based on morphology is often difficult as the characters used show considerable intraspecific variation. Moreover, the identification of many morphologically identical nematode races or pathotypes is impossible using physical characteristics alone.

Molecular biology offers a number of exciting new approaches that are revolutionising parasite identification, but with a few notable-exceptions these techniques have been largely neglected by plant nematologists. Digestion of plant parasitic nematode DNA with restriction endonuclease enzymes followed by agarose gel electrophoresis has revealed useful restriction fragment length polymorphisms (RFLPs). WLPs have been shown to differentiate several nematode genera, species and races (Curran, Baillie & Webster, 1985; Burrows & Boffey, 1986; Burrows, 1988). However, it is the use of cloned specific DNA hybridisation probes coupled with dot blot assays that promise to facilitate the greatest advances in parasite diagnosis (Barker *et al.*, 1986; Rollinson, Walker & Simpson, 1986; Ole-Moiyio, 1987; Burrows & Perry, 1988). For routine identification of plant parasitic nematodes, specific DNA probes have an important advantage over RFLPs in that they can give a positive or negative assay for nematode species, race or pathotype without the need for restriction digestion of the DNA or time consuming electrophoresis. The search for diagnostic DNA probes for agriculturally important nematodes is still in its infancy. Even so, it seems that a major impediment to the wider acceptance and use of this technique for routine diagnostics will be the need to detect probes using radioisotopes. Radiolabelled probes are expensive, hazardous to work with and once labelled the activity decays within a few weeks necessitating replacement or frequent relabelling. The full potential of specific DNA probes for the identification of plant parasitic nematodes will only be realised when suitable non-radioactive methods for the detection of bound probes are developed or adopted. Specific DNA probes can be visualised by a number of different non-radioactive methods (Gilliam, 1987) but they are often considered to be too insensitive. Although this is generally correct, one particular group of techniques based on biotin is very promising.

Biotin-11-dUTP is a nucleotide analogue that may be incorporated into a probe by nick translation in the same way as a radionucleotide. Hybridisation of a biotin labelled probe with nematode DNA bound to a nitrocellulose filter can be detected by conjugating a preformed complex of avidin and alkaline phosphatase to the biotin. Enzyme catalysed production of an insoluble blue pigment at the site of hybridisation reveals the presence of the probe.

This work details modifications to a basic biotin technique and its subsequent use to visualise DNA from the potato cyst nematode (PCN) *Globodera pallida* using a *G. pallida* specific DNA probe (Burrows & Perry, 1988) in dot blot assays. The sensitivity of the method in this application has been improved and non-specific background staining has been almost eliminated even when using crude DNA preparations.

Materials and methods

NEMATODE POPULATIONS

Two populations of potato cyst nematodes *Globodera rostochiensis* Rol (Woburn) and *G. pallida* Pa 4 (New Leake), were multiplied on the susceptible potato cv. Arran Banner in glasshouse pot cultures. Eggs and second stage juveniles (J2) were collected and prepared for DNA extraction as detailed (Burrows & Boffey, 1986).

EXTRACTION AND PREPARATION OF DNA

Nematode DNA was extracted and purified as previously reported (Burrows & Perry, 1988). A 100 µl pellet of packed *Globodera* eggs and J2 yielded 50-100 µg of clean DNA. DNA prepared in this way was a good substrate for many of the enzymes such as restriction endonucleases, ligases, kinases and polymerases commonly used in molecular biology.

CRUDE DNA PREPARATION

This method results in comparatively impure nematode DNA. However, samples may be prepared from very few nematodes in as little as 30 min.

Globodera eggs and J2 (10 µl packed pellet or a single cyst) were washed twice with DNA extraction buffer (EB) (0.1 M Tris·Cl pH 8.5, 0.1 M NaCl, 0.05 M EDTA) and transferred to a centrifugal micro-homogeniser (Biomedix®) (Hearse, 1984). The nematodes were pelleted in the homogeniser tube in a microcentrifuge, resuspended in 20 µl EB, and homogenised on ice for 1 min.

The homogeniser was centrifuged briefly (1 sec) to clear the polypropylene pestle of cell debris, and 180 µl EB, 30 µl SDS (20 % W/V in EB) and 20 µl proteinase K (25 mg/ml, Sigma) were added. The tube was capped, the contents gently but thoroughly mixed and then incubated at 55 °C for 25 min.

The nematode homogenate was extracted once with an equal volume of phenol chloroform 1 : 1, and the aqueous phase adjusted to 2 × SSC (SSC : 0.15 M NaCl, 0.015 M Na_3 citrate. 2 H_2O, pH 7.0) with approximately 12 µl 20 × SSC stock. DNA samples prepared in this way could be used directly in dot blot assays.

DOT BLOT

Aliquots of purified *G. pallida* DNA or crude DNA preparations from either *G. pallida* or *G. rostochiensis* were diluted to 500 µl with 2 × SSC, denatured by boiling for 7 min in a waterbath and cooled on ice.

A 9 × 12 cm sheet of nitrocellulose paper was washed in distilled water, until it was uniformly hydrated, incubated in 20 × SSC for 10 min and then assembled into a 96 well dot-blot microfiltration manifold (Bio-rad). The denatured *Globodera* DNA samples (500 µl) were applied and allowed to filter passively through the nitrocellulose membrane. The filter was removed from the dot blot manifold, air dried and baked for 1 h at 80 °C. At this stage filters could either be used immediately or stored desiccated at 4 °C for up to 6 months.

INCORPORATION OF BIOTIN OR RADIO-LABEL INTO THE *G. PALLIDA* SPECIFIC DNA PROBE

A *G. pallida* specific DNA probe (Burrows & Perry, 1988) incorporated into the plasmid cloning vector pUC-9 was labelled by nick-translation with either the nucleotide analogue Biotin-11-dUTP (GIBCO BRL) or [α-^{32}P] dATP (Amersham). Unincorporated radionucleotide was separated from the probe by chromatography through a sephadex G 50 column; however, excess Biotin-11-dUTP was not removed.

DETECTION OF *G. PALLIDA* DNA WITH A BIOTINYLATED DNA PROBE

Maximum economy of solutions and reagents was achieved by performing the steps of prehybridisation, hybridisation, filter blocking and staining in sealed plastic bags only slightly larger than the filters. Washing the filters required larger volumes of solution and could be carried out satisfactorily in plastic trays.

Nitrocellulose filters or filter strips were placed in a plastic bag containing prehybridisation fluid (50 % V/V

Formamide, 5 × SSPE, [SSPE : 0.18 M NaCl, 0.01 M $NaHPO_3$ pH 8.3, 1 mM EDTA], 5 × Denhardt's (without the BSA), 0.1 % SDS, 100 µg ml^{-1} sheared and

[redacted]

avidin-AP complex (avidin-AP 2 µg ml^{-1}, 1 % BSA, in incubation buffer : use 40 µl per cm^2 of filter) was conjugated to the biotin at 10 °C for 20 min and the

[redacted]

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