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Immunological detection of nematode antigens on the surface of a wood section

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Summary – Antigens of *Bursaphelenchus xylophilus* and *B. mucronatus* were detected on a debarked and kiln-dried *Pinus sylvestris* section. The antigens were examined using a polyclonal antibody developed against *B. xylophilus*. The antibody could detect the nematode proteins on the surface of the wood at a lower limit of 0.4 µg but could not discriminate between the two species examined.

Résumé – ***Détection immunologique d'antigènes provenant de nématodes à la surface du bois*** – Des antigènes provenant de *Bursaphelenchus xylophilus* et *B. mucronatus* ont été détectés à la surface d'une tranche de *Pinus sylvestris* écorcée et séchée au four. Ces antigènes ont été examinés à l'aide d'un anticorps polyclonal dirigé contre *B. xylophilus*. L'anticorps peut détecter les protéines du nématode à la surface du bois jusqu'à un taux inférieur à 0,4 µg, mais ne peut différencier les deux espèces en cause.

Key-words : Immunodiagnosis, wood section, *Bursaphelenchus xylophilus, Bursaphelenchus mucronatus.*

A variety of matrices are available for the immunological detection of plant pathogens. These include nitrocellulose or other similar membranes used in immunoblotting (Towbin *et al.*, 1979) and polystyrene or other suitable plastics in microtitre plates used in the Enzyme Linked Immunoabsorbent Assay or ELISA (Harlow & Lane, 1988). These synthetic matrices have been used in the immunodiagnosis of plant pathogenic nematodes such as *Globodera rostochiensis* and *Globodera pallida* (Schots *et al.*, 1990).

Immunological assays on natural plant surfaces such as wood may have the potential to detect certain plant pathogens. Tests performed on plant surfaces may however suffer interference from the presence of endogenous compounds such as peroxidases which may cause similar enzyme-linked colorimetric reactions. The surface itself may have a contrasting or unsuitable background colour which would hinder the use of colorimetric detection methods. If an immunological assay is to be performed on a plant surface then the conditions that apply to synthetic matrices must equally apply to this natural matrix. These include the capacity to absorb or retain antigen, minimal background reaction and the use of a substrate which can facilitate quantitative or qualitative analysis.

In this paper we describe the immunological detection of nematode antigens applied on a debarked *Pinus sylvestris* wood section. The tests combine aspects of ELISA and immunoblotting and involved the use of microwaving to fix the antigen on the wood surface. The antigens examined were present in homogenates of mixed life cycle stages of various populations of the nematodes *Bursaphelenchus xylophilus* (see : Steiner & Buhrer, 1934; Mamiya & Kiyohara; 1972; Nickle *et al.*, 1981) and *Bursaphelenchus mucronatus* Mamiya & Enda, 1979.

Materials and methods

EXTRACTION OF NEMATODE PROTEIN

B. xylophilus and *B. mucronatus* populations were raised on 2 week-old cultures of a sterile strain of *Botrytis cinerea*. The nematodes, containing mixed life stages, were harvested using sterile distilled water (SDW) and were stored at – 20 °C. Nematode homogenates were prepared by grinding the samples in liquid nitrogen, followed by suspension in SDW. The protein concentration was determined, using the Bradford assay (Bradford, 1976).

Table 1. Nematode populations used in this study.

Code	Country	Host
Bursaphelenchus xylophilus		
US4	USA-Florida	*Pinus elliotti*
US9	USA-Arizona	*P. halapensis*
US15	USA-Illinois	*P. sylvestris*
J3	Japan-Nishiaizu	*P. densiflora*
J10	Japan-Ueki	*P. densiflora*
Bursaphelenchus mucronatus		
J13	Japan-Yachiyo	*P. thunbergii*

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PREPARATION OF POLYCLONAL SERUM

One mg/one ml homogenates of *B. xylophilus* (J10) of mixed life cycle stages were prepared using the methods already described. These suspensions were mixed with equal volumes of Freund's complete adjuvant (Harlow & Lane, 1988) and injected subcutaneously into two New Zealand White rabbits. This was repeated twice more over a 4 week-period at 2 week-intervals. The antigen concentration remained the same, but an equal volume of Freunds adjuvant minus the *Mycobacterium tuberculosis* bacteria or also known as Freund's incomplete adjuvant was used in this case. After 6 weeks the rabbits were bled and the antibody titre was determined using an indirect ELISA system.

IMMUNOLOGICAL TEST ON WOOD SECTION

The wood section used was $10 \times 5 \times 1$ cm in dimension. The section was obtained from a debarked and kiln-dried *P. sylvestris* log collected at Dublin port. Two µg of each nematode protein were added to the wood surface and five and ten fold dilutions of the respective suspensions were added to the surface. The negative control consisted of 2 µl of 1X phosphate buffered saline (PBS), (10X PBS; 1.38 M NaCl, 0.038 M KCl, 0.021 M KH_2PO_4, 0.092 M $Na_2HPO_412H_2O$, pH 7.3-7.5). The suspensions were allowed to dry onto the surface after which the dry wood section was then microwaved at 650 watts for 1.5 min to fix the antigen onto the wood surface. This was followed by blocking in PBS/0.3 %. Tween 20 for 1 h at room temperature. The section was then incubated in 1 : 1000 dilution in PBS/0.05 %. Tween 20 (Wash Buffer) of the polyclonal antibody raised against *B. xylophilus* antigen for 2 h at room temperature. This was followed by five one minute washes with wash buffer to remove any unbound antibody. This was followed by incubation in a 1 : 1000 dilution of porcine anti-rabbit antibody conjugated to horse radish peroxidase or HRPO (Dako-Paks), in wash buffer for 2 h at room temperature. The section was then washed as before and the added substrate containing 3'3'-diaminobenzidine (DAB), H_2O_2, 4-chloronaphtol, all in PBS, as described by Young (1989), was used to detect the reaction. The wood section was washed in distilled water to stop the reaction and immediately photographed.

For nematode populations used in this study, see Table 1.

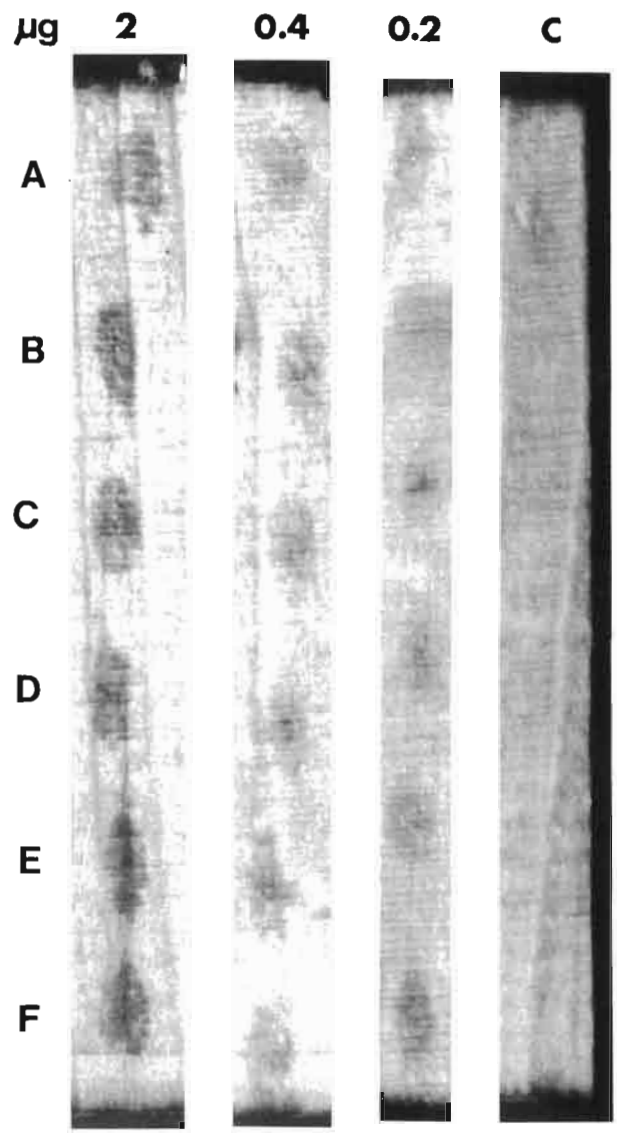

Fig. 1. Immunological test on *Bursaphelenchus* antigens applied onto a *Pinus sylvestris* wood section. The antigens were applied in quantities of 2, 0.4 and 0.2 µg and the control (C) consisted of 10 µl of 1X PBS. The *B. xylophilus* populations examined were US4, US9, US15, J3 and J10 (rows A-E, respectively). The *B. mucronatus* population examined was J13 (row F.).

Results

Nematode proteins at a range of concentrations were spotted onto the wood surface and were immobilised using microwaves. These proteins were subsequently examined using a polyclonal antibody raised against *B. xylophilus* (Fig. 1). Positive areas appeared as black dots on the pale brown surface of the wood as seen in the reactions involving 2 µg of nematode protein. The black colour changed to a dark brown after 24 h but the colour persisted for up to a number of weeks. The use of microwaving to fix the antigen onto the wood surface should inactivate any peroxidase activity. However the activity of unknown interfering compounds present on the wood surface resulted in a similar background reaction to that involving the nematode protein. The background had varying degrees of intensity as seen in Fig. 1. The reaction involving 2 µg of nematode protein corresponds to approximately 130 nematodes (unpubl.). The lower

limit of detection was 0.4 μg which represents approximately 25 nematodes.

There is no difference in the reaction involving the *B. xylophilus* populations (Fig. 1, rows A, B, C, D, E) and the *B. mucronatus* population (Fig. 1, row F) examined using the polyclonal antibody. This is seen in the similar intensity of the reaction in Fig. 1. In this case it was not possible to differentiate the two *Bursaphelenchus* spp. using the polyclonal antibody.

Discussion

As part of an attempt to develop an immunological test to detect *B. xylophilus* in timber sections the possibility of performing the test directly on the timber surface was investigated. Homogenised nematodes were used as antigen because the use of microwaving causes intact nematodes to be lysed and therefore internal antigens as well as cuticular antigens will be exposed to subsequent antibody detection. However any nematodes which may be detected must be on or near the wood surface. Therefore the test would not have the capacity to detect sub-surface nematodes present in the wood section. The application of such a test for the detection of *B. xylophilus* directly on the timber would be limited for a number of reasons. These include the fact that the nematode is often present beneath the surface and is also dispersed within the timber section in aggregates of one to ten, depending on the size and type of section examined (Yik & Birchfield, 1981). Therefore the lower limit of detection of approximately 25 nematodes on the wood surface using the polyclonal antibody would not detect the numbers which are representative of a natural infestation.

However the use of natural plant material as the antigen absorbing matrix in an immunological test, may be exploited in the detection of certain surface pathogens. The use of the antibodies directly on the wood surface has a number of positive aspects and include the ability to detect the presence of surface pathogens without the requirement of antigen extraction. Applications of such a technique would include a decrease in the time and a reduction in costs needed to perform such a test. Therefore after the surface pathogen has been identified by immunological tests on synthetic matrices it could be further confirmed by similar tests on the plant surface which could yield information on pathogen distribution.

In the case of the detection of *B. xylophilus* the nematode must be present on the surface and exist in reasonable quantities to allow detection. The positive identification of *B. xylophilus* will depend on the specificity of the antibodies involved. In the case of *B. xylophilus* the use of monoclonal antibodies offers the potential to detect certain life stages of the various populations examined. Finally the choice of substrate is important and must yield a colour reaction of sufficient strength and/or contrast to be used in an enzyme linked assay. In this case the combination of 4-chloronaphtol and 3'3-diaminobenzidine along with H_2O_2 in 1X PBS yields such a result.

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