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SHORT COMMUNICATION

Selective recovery of the virus-vector trichodorid nematode *Paratrichodorus anemones* from soil samples by immunomagnetic capture

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Abstract Lectins and polyclonal antiserum that bind specifically and reproducibly to the overall surface of *Paratrichodorus anemones* were identified and bound to monodisperse superparamagnetic particles (Dynabeads) to assess their efficiency as probes for capturing target nematodes from test suspensions. In recovery experiments, while both types of probe isolated nematodes, antibody-coated beads recovered them more efficiently than beads coated with lectins. When immunomagnetic capture was used to isolate *P. anemones* from mixtures of naturally occurring populations of nematodes extracted in bulk, from soil samples, 80% of the target nematodes were recovered.

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Isolating target phytosanitary nematodes that occur in low abundance from the total soil nematode community is a laborious task (Powers 2004), especially for physically small nematodes such as trichodorids. Of more than 90 species of trichodorids (Decraemer and Baujard 1998), 13 species are known to vector tobraviruses which cause economically important diseases to potatoes and ornamental bulbs (Taylor and Brown 1997; MacFarlane et al. 2002). Thus, isolation and subsequent identification of trichodorids to species is crucial for crop management strategies as they typically co-exist as mixed field populations of vector and non-vector species (Boag 1981; Decraemer 1995), the former often in low abundance.

To overcome limitations of conventional methods (Decraemer 1995), novel techniques that will facilitate detection of trichodorid nematodes in a sensitive and specific way are increasingly being sought. New methodologies employing magnetic capture to isolate plant-parasitic nematode species (Chen et al. 2001; Chen et al. 2003) have been reported with isolation efficiencies of target nematodes in mixed populations ranging from 37 to 80%, depending upon nematode species and probe (lectin or antiserum) used. Lectins are carbohydrate-binding (glyco)proteins which are highly specific for their sugar moieties. Compared with recovery of nematodes using lectin-coated

magnetic beads, indirect immuno-magnetic capture of target nematodes using antibody-coated beads was more efficient (up to 80% from mixed nematode populations; Chen et al. 2001; Chen et al. 2003).

Rapid, simple and robust identification and quantification of trichodorid nematodes is a prerequisite for effective integrated TRV disease management strategies. The objective of this work was to reduce reliance on morphological identification and to assess the utility of magnetic separation in a phytosanitary context as an enrichment approach for the recovery of trichodorid nematodes directly from soil samples.

In order to identify suitable probes for magnetic capture, a panel of fluorescent-labelled lectins was screened for binding to the surface of *Paratrichodorus anemones*, a virus-vector trichodorid species widely distributed and prevalent within Europe (Alphey and Taylor 1986). A polyclonal antiserum against *P. anemones* was also produced and its potential use in the diagnostic procedure was assessed. The most effective recovery method was selected to test the efficiency of the system in isolating target nematodes from populations containing mixed species of nematodes.

Soil samples containing *P. anemones* were collected from Yorkshire (England). Free-living, active adult *P. anemones*, were extracted using a modified decanting and sieving technique, with final separation over Baermann funnels (Brown and Boag 1988). *P. anemones* individuals were hand-picked into groups of 50 for use in assays. Trichodorid-free soil samples were also collected from a field at the Scottish Crop Research Institute, Dundee. Soil nematode communities were extracted, as described above and enumerated.

Twelve fluorescein isothiocyanate (FITC)-conjugated lectins (E.Y Laboratories, USA; Table 1) were screened for their ability to bind to surface glycoconjugates of *P. anemones*. All lectins were used at concentrations of 200 μg ml^{-1} in the buffers recommended by the manufacturer. Nematodes were prepared as described by Chen et al. (2001) and used within 24 h of collection. Comparison of the fluorescence intensity between nematodes incubated for 2 h in lectin solution and those subjected to pre-bound (1 h incubation in the dark) lectin with the appropriate inhibitory carbohydrate (E.Y. Laboratories, USA; Table 1) tested the binding specificity. Target nematodes incubated in phosphate-buffered saline (PBS; 137 mM NaCl, 1.4 mM KH_2P0_4, 2.6 mM KCl, 8.1 mM Na_2HP0_4, pH 7.4) were used as a negative control. Specimens from each treatment were immediately mounted on glass microscope slides and observed under a Nikon fluorescence microscope equipped with excitation and emission filters specific to FITC (Absorbance, 492 nm/emission, 520 nm). All experiments were repeated four times, with a minimum of 50 nematodes per treatment.

All lectins, with the exception of DBA, GS-I, PNA and UEA-I bound to the head region of *P. anemones* (Table 1). Lectins LEA and STA also produced a strong fluorescence over the entire cuticle surface of *P. anemones* (Table 1) and were selected to be used as

Table 1 Fluorescein isothiocyanate (FITC)-conjugated lectins screened for binding to *Paratrichodorus anemones* and their carbohydrate specificity at room temperature (15–20°C)

Code	Latin name of lectin source	Carbohydrate specificity (200 μM)	Binding
DBA	*Dolichos biflorus*	Methyl-2-acetamido-2-deoxy-D-galactose	No binding
GS-I	*Griffonia simplicifolia*	melibiose, α-D-galactose	″
PNA	*Arachis hypogaea*	Terminal β-galactose	″
UEA-I	*Ulex europaeus*	α-L-fucose	″
BPA	*Bauhinia purpurea*	*N*-acetylgalactosamine	Binding to head region
ConA	*Canavalia ensiformis*	α-D-mannose, α-D-glucose	″
GS-II	*Griffonia simplicifolia*	Terminal α- or β-*N*-acetylglucosamine	″
MPA	*Maclura pomifera*	*N*-acetylgalactosamine>galactose	″
SBA	*Glycine max*	α- and β-*N*-acetylgalactosamine>α- and β-galactose	″
WGA	*Triticum vulgare*	(GlcNAc β (1,4) $GlcNAc)_{1-4}$>β GlcNAc>Neu5Ac (sialic acid)	″
LEA	*Lycopersicon esculentum*	β (1,4) *N*-acetylglucosamine	Cuticle surface
STA	*Solanum tuberosum*	Oligomers of *N*-acetylglucosamine β (1,4) *N*-acetylglucosamine	(including head)

Where GlcNAc: *N*-acetylglucosamine

probes for magnetic capture. There was no autofluorescence of *P. anemones* at the excitation wavelength of FITC. Labelling on the surface cuticle of *P. anemones* was still visible but was significantly decreased with carbohydrate-pre-treated LEA, MPA, STA and WGA. No fluorescence was detected when nematodes were incubated with appropriate carbohydrate pre-treated BPA, ConA, GS-II and SBA lectins, confirming the specificity of the lectin binding reaction.

Dynabeads M-280 Tosylactivated (Dynal, Norway) coated with lectin and *P. anemones* were recovered using the method described by Chen et al. (2001). Microscopic examination revealed attachment of LEA- and STA-coated Dynabeads to target nematodes (results not shown) and no binding of uncoated beads to the nematode surface. However, only a few (5–30) coated beads were bound to the external surface and the efficiency of recovery by magnetic capture of *P. anemones* from suspension was low (<5%).

A polyclonal antiserum IACR–Pan was generated against whole body homogenates of hand-picked adult *P. anemones*, following standard protocols (Galfré and Milstein 1981). A Balb/c mouse was immunised 4 times intraperitoneally at three-week intervals. For each injection, 1,000 *P. anemones* in water were emulsified in an equal volume of Freund's adjuvant; complete Freund's adjuvant was used for the first injection only and incomplete Freund's adjuvant for the subsequent injections to protect the antigen from degradation. Blood was collected and incubated for 2 h at 37°C and left overnight at 4°C. Serum was separated by centrifugation at 500 g and stored at −20°C.

Immunofluorescence assays were performed as described by Duncan et al. (1997). The polyclonal antibody was screened for strength of binding to the nematode surface and for specificity. Ten groups of 50 hand-picked *P. anemones* were washed three times in PBS. Four of the groups were incubated in 500 µl of the primary polyclonal IACR–Pan antibody diluted 1:100, 1:250, 1:500 and 1:1,000 in PBS. Four other groups were incubated in 500 µl of pre-immune mouse serum at the same dilutions as the test serum in PBS. The remaining two groups of nematodes (negative controls) were kept in either PBS or the secondary antibody (goat anti-rabbit IgG, Cy3 conjugate, Sigma, UK). All incubations were performed for 1 h at room temperature. Nematodes were then washed three times in PBS and incubated in 500 µl of the secondary antibody diluted 1:1,000 in PBS for 1 h, at room temperature. Following three washes in PBS, specimens were resuspended in 20 µl of PBS, transferred onto glass microscope slides and examined using an Olympus fluorescence microscope (Cy3: absorbance, 552 nm/emission, 570 nm). The antiserum IACR–Pan diluted 1:100 in PBS labelled the overall cuticle surface of *P. anemones*. Antiserum dilution 1:250 gave a reduced binding intensity to the nematode surface and further dilutions, 1:500 and 1:1,000, bound only to the head and tail of the specimens. No labelling was observed on specimens treated with pre-immune mouse serum, PBS or directly the secondary antibody. The specificity of the antiserum was assessed by testing its reactivity to other commonly occurring plant-parasitic nematode species belonging to the following genera: *Globodera*, *Helicotylenchus*, *Longidorus*, *Pratylenchus* and *Xiphinema*. Cross-reactivity was only observed with *Globodera* and *Helicotylenchus* species.

Dynabeads M-450 covalently linked with secondary, rat anti-mouse IgM antibodies, attached to the cuticle surface of *P. anemones* pre-treated with the IACR–Pan antiserum (Fig. 1). Nematodes collected within 24 h were washed three times in PBS and pre-treated with the primary polyclonal antibody diluted 1:100 in PBS for 1 h at room temperature. Nematodes were then rinsed three times in PBS to remove the unbound antibody and incubated with Dynabeads M-450 conjugated with rat anti-mouse IgM (Dynal), diluted 1:20 in PBS for 1 h. When the IACR–Pan antiserum was replaced with pre-immune mouse serum, no bead attachment was present on the external surface of the nematodes.

To assess the efficiency of the immunomagnetic separation (IMS) recovery, ten replicates of 50 hand-picked *P. anemones* per treatment were prepared. Nematodes bound to Dynabeads were recovered using a Dynal Magnetic Particle Concentrator (MPC) and washed three times with PBS. Captured and uncaptured nematodes were enumerated. Furthermore, to assess the efficiency of recovery from a soil community, 50 hand-picked *P. anemones* (12 replicates) were added to the total nematode fauna extracted from a 200 g soil sample, known not to contain trichodorids. Nematodes were recovered using the immunomagnetic capture protocol and

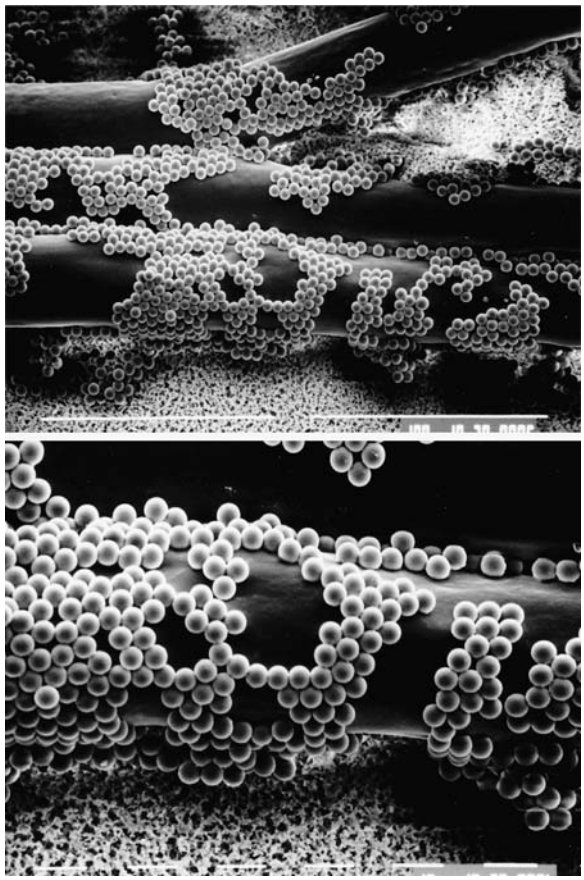

Fig. 1 Low temperature scanning electron microscopy (*LTSEM*) micrograph showing attachment of Dynabeads to *P. anemones* treated with the polyclonal antiserum IACR–Pan (dilution 1:100)

enumerated as described above. Capture rates were expressed as a percentage of the original trichodorid inoculum. Rat anti-mouse IgM coated Dynabeads were as efficient at recovering *P. anemones* from suspensions (80.0±2.7%) of this species only from mixed soil nematode populations (79.8±5.9%). Of the total nematode community, 10.0±0.7% was also extracted, indicating a degree of non-specificity. This value was less than that reported for antiserum IACR–PXa (raised against *X. americanum*) but greater than that reported for antisera E157 (raised against *Meloidogyne arenaria*), Phy IACR–8A8.3.2 and 20D(–C; both raised against *X. americanum*); however the latter two had significantly poorer recovery efficiencies (Chen et al. 2001; Chen et al. 2003). Of those non-target species, *Globodera* spp. was prevalent. Compared to previous studies, recovery of *P. anemones* was lower than that achieved for *M. arenaria* (97.0±1.0%) but higher than that for both *G. rostochiensis* (51.3±1.1%) and *X. americanum* group (36.7–73.3%; Chen et al. 2001; Chen et al. 2003).

From the screened lectins, LEA and STA are derived from tomato and potato, respectively, both of which are hosts for *P. anemones*. In contrast, the other lectins tested which bound poorly to the nematode cuticle are derived from plants known to be non-hosts for *P. anemones*. No previous reports on lectin binding patterns and distribution of carbohydrate residues or carbohydrate-recognition domains (CRD) on the surface of trichodorid nematodes are available for comparison. Notwithstanding the strong fluorescence produced by LEA and STA, the recovery rate of *P. anemones* was poor compared with that of the polyclonal antiserum used (<5.0 cf. 79.8%). Also, the recovery efficiency was poor compared to the 44% achieved for *M. arenaria* with lectin UEA-I (Chen et al. 2001). It is unclear why the recovery efficiency for *P. anemones* was so low, but contributory effects could include the possibility that 'free' lectins might find their receptors more easily than lectins bound to Dynabeads, or that non-specific binding of bacteria to the Dynabeads block the sites available for nematode binding (Lund et al. 1988; Morgan et al. 1991).

Lectins constitute readily available and cost-effective probes in comparison with antibodies. However, cross-reactivity due to the nature of ligand recognition (i.e. carbohydrate moieties rather than specific epitopes) could complicate recovery of target nematodes from mixed samples (Van Damme et al. 1998). Moreover, the carbohydrate binding characteristics of lectins suggest that they may have the same nominal monosaccharide specificity, but differ in the precise carbohydrate structure to which they selectively bind (Brooks et al. 1996). Thus, lectins classified as binding to the same ligand can exhibit dissimilar binding patterns to the same tissue (Brooks et al. 1996). Nevertheless, the lectin-magnetic capture technique remains a potential enrichment step for downstream diagnostic procedures. Nematode trapping efficiency could be improved by using magnetic beads coated with streptavidin. The streptavidin-biotin affinity is the strongest non-covalent interaction known in nature (Stryer 1995) and biotin is easily incorporated into lectins, proteins or nucleic acids, giving streptavidin-coated beads wide utility.

The results obtained in the present study show that immunomagnetic capture has the potential to provide a serological-based target nematode enrichment process to recover and concentrate trichodorids occurring in mixed nematode populations in soil samples. Removal of the non-target nematodes substantially reduces 'interference' and increases sensitivity in subsequent downstream applications (e.g. PCR tests; Subbotin et al. 2001) when target nematodes, usually occurring only in small numbers, are present in large soil nematode populations. For quarantine surveys where it is essential to have a high probability of detection, the use of polyclonal antiserum as a probe is likely to be of significant utility considering the high recovery rates obtained in immunomagnetic capture experiments. Consequently, the application of an immunomagnetic nematode enrichment approach in conjunction with species-specific primers (Boutsika et al. 2004) and real-time PCR (Holeva et al. 2006) could form an integral part of a routine diagnostic system for trichodorids.

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