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Improved attachment and parasitism of *Trichoderma* on *Meloidogyne javanica* in vitro

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Abstract Monoclonal and polyclonal antibodies that bind to eggs and/or second-stage juveniles of the nematode *Meloidogyne javanica* were tested for their effects on the parasitic interactions between this nematode and the fungus *Trichoderma*. Parasitism of *Trichoderma asperellum*-203 and *Trichoderma atroviride* on nematode egg masses, eggs and juveniles was enhanced when antibodies were incorporated into in vitro parasitism bioassays. Parasitism on separated eggs (without gelatinous matrix) and their hatched juveniles was also improved, compared to controls without antibodies that did not attach fungal conidia. Improved parasitism could be due to bilateral binding of the antibodies to the nematodes and conidia, enabling better conidial attachment to the nematodes. Enhanced germination of antibody-bound conidia further improved parasitism. Differences were observed among antibodies in their effects on fungal parasitism and their interaction with *Trichoderma* species. We focused mainly on the egg- and juvenile-binding monoclonal antibody MISC that exhibited a stronger reaction with *T. asperellum*-203 than with *T. atroviride*. Pretreatment of this antibody with fucose inhibited its binding to nematodes and conidial attachment to nematodes, as well as conidial agglutination in the presence of the antibody. Antibody binding to juveniles affected their movement and viability, especially gelatinous matrix-originated juveniles. The fucose-specific lectin *Ulex europaeus*-I enhanced conidial attachment to nematode life-stages, and conidial agglutination occurred in its presence. These phenomena were inhibited by preincubating lectin with fucose. Our results suggest that carbohydrate residues, such as fucose, on the surface of the nematode and fungal conidia are involved in the antibody- and lectin-mediated improved parasitism.

Keywords Antibodies · Biological control · Carbohydrates · Lectin

Abbreviations

gm	gelatinous matrix
J2	second-stage juvenile
MAb	monoclonal antibody
PAb	polyclonal antibody

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Introduction

Root-knot nematodes are sedentary endoparasites that cause great economic losses in agriculture. Polyphagous

species such as *Meloidogyne javanica* and *M. incognita* are among the major limiting factors in crop production (Manzanilla-Lopez et al. 2004). There are very few biocontrol products available for use against nematodes; therefore, it is important to develop a greater understanding of the interactions between nematodes and various other biotic components (Davies 2005), especially in the rhizosphere (Kerry 2000).

Biocontrol activities of *Trichoderma asperellum*-203 and *Trichoderma atroviride*, among other *Trichoderma* species and isolates, against *M. javanica* in soil and their ability to parasitise various nematode life-stages in vitro and in planta have been reported (Sharon et al. 2001, 2007). Mechanisms involved in the attachment and parasitic processes were investigated, with special emphasis on the important role of the nematode's gelatinous matrix (gm) in direct nematode–fungus interactions, and it has been suggested that carbohydrate–lectin-like interactions may be involved in the attachment of conidia to the nematodes (Sharon et al. 2007). The cell surface of *Trichoderma* conidia has been only very scarcely characterised. Puyesky et al. (1999) described a conidial multidomain surface protein, CMP1, of *T. atroviride*; this protein contains N-linked glycosylation sites of *N*-acetylhexosamines.

The nematode's epicuticle—the external cuticular layer, is covered with a fuzzy layer of surface coat (SC) composed mainly of proteins and carbohydrates. This outermost layer is considered to be important in recognition events involving plant hosts and microbial antagonists (Spiegel and McClure 1995; Morton et al. 2004). The nature of *Meloidogyne* species SCs has been studied and the lability of their surface proteins has been demonstrated (Spiegel et al. 1995, 1997; Lin and McClure 1996). Antibodies have been used to characterise surface antigens and to study interactions with plant hosts (Gravato-Nobre and Evans 1998; Lopez de Mendoza et al. 1999). Antibodies that bound to the surface of *M. javanica* second-stage juveniles (J2s) inhibited their movement and therefore reduced root penetration (Sharon et al. 2002). The cuticle SC is involved in the specificity of interactions with microorganisms, such as the obligate bacterial parasite *Pasteuria penetrans* (Davies 2005; Spiegel et al. 1996) and nematophagous fungi (Kerry and Hominick 2001). This study investigated the effects of *M. javanica* surface-binding antibodies on the parasitic interactions of *Trichoderma* with this nematode.

Materials and methods

Nematodes

Monoxenic cultures of the nematode were grown aseptically on excised tomato roots. The roots were grown on Petri dishes on Gamborge-B5 medium (Duchefa, Haarlem, The Netherlands) which contained sucrose (20 g l^{-1}) and 0.75% (*w*/*v*) Gelrite (an agar substitute; Duchefa), and kept in an incubator at 25±1°C. Egg masses were crushed to obtain gm-originated eggs. Eggs free of gm were extracted by shaking the roots with a 0.5% sodium hypochlorite (NaOCl) solution for 1 min. Eggs were collected on a 30-μm sieve and washed thoroughly with sterile water. Preinfective J2s were hatched either from gm-free eggs (designated gm-free J2s), or directly from egg masses (designated gm-J2s).

Trichoderma

Fungal cultures were grown on potato dextrose agar (PDA; Difco™, Becton Dickinson, Sparks, MD, USA) in 9-cm diam Petri plates. Conidia were collected from the plates in water. The following species and isolates of *Trichoderma* were used: (1) *T. atroviride* IMI 206040 (previously defined as *T. harzianum*), provided by Prof. A. Herrera-Estrella, Mexico. (2) *T. asperellum*-203 (previously defined as *T. harzianum*-203).

Antibodies

Monoclonal (MAb Misec3F.4) and polyclonal (PAb MiPC373) antibodies were raised against *M. incognita* (race 1) by Dr. Rosane Curtis, IACR-Rothamsted, UK. PAb MjE2 was raised against *M. javanica* J2s (Sharon et al. 2002). MAb MISC was raised against *M. incognita* (race 3) and provided by Prof. M.A. McClure, USA (Gravato-Nobre et al. 1999; Hu et al. 2000). Immunofluorescent labelling of nematodes was carried out according to Sharon et al. (2002). MAb MISC (1:50) was used to label fungal conidia by a procedure similar to that used for the nematodes. Negative controls were treated with rabbit nonimmune serum for the PAbs and with Dulbecco's Modified Eagle's medium containing 20% (*v*/*v*) fetal calf serum (20D medium) for the MAbs. Controls with

the secondary antisera alone were also tested. The effect of carbohydrates was tested after preincubation of MAb MISC with 0.1 M L-fucose (Sigma) or α-methyl-mannoside (Sigma).

In vitro attachment and parasitism bioassays and effects of antibodies and lectin on nematode-fungus interactions

Attachment and parasitism of the *Trichoderma* species were tested on various life-stages of *M. javanica* in 96-well plates as described by Sharon et al. (2007). The plates contained 80 µl of diluted medium [20-fold diluted potato dextrose broth (PDB; Difco™); 0.05% *w/v* KCl; 0.05% *w/v* $MgSO_4.7H_2O$; 1 mM $CaCl_2$], 10 µl of an aqueous suspension of 10^5 fungal conidia ml^{-1}, and about 100 J2s or eggs, or two egg masses. There were five replicates for each treatment. Antibodies and their appropriate controls (nonimmune serum for PAbs and 20D medium for MAbs), at the same final concentrations used for nematode labelling, were added to the diluted growth medium to evaluate their effect on fungal attachment and parasitism. Attachment of fungal conidia to various nematode life-stages was qualitatively estimated. Percentages of parasitised nematode eggs and egg masses were assessed after 1 day by comparing the proportions of hatched J2s in treated vs. untreated nematodes. Parasitism on J2s was observed directly using an inverted microscope.

The fluorescein isothiocyanate (FITC)-conjugated fucose-specific lectin *Ulex europaeus agglutinin* (UEA-I; Sigma, 0.5 mg ml^{-1}) was incubated with *T. asperellum*-203 conidia or nematodes (egg masses and the eggs and J2s originated from them) in phosphate-buffered saline (PBS; 0.15 M, pH 7.2) containing 1 mM Ca^{2+} and Mg^{2+}, for 45 min at room temperature and then washed with the buffer. In parallel control treatments, the reaction mixture was incubated with 0.1 M L-fucose (Sigma), a lectin-specific carbohydrate.

Effect of monoclonal antibody MISC on the viability of juveniles

Antibody-treated J2s (1 and 16 h) were washed and placed on agar plates (100 J2s in a 10 µl drop) according to the methods described by Sharon et al. (2002). Live and immobile J2s that remained in the centre (1 cm) after 8 h were counted. The treated J2s were gm-free J2s or gm-originated J2s (gm-J2s).

Results

Effect of antibodies on parasitism of *Meloidogyne javanica* by *Trichoderma*

Fungal parasitism on all of the nematode life-stages tested, i.e., gm-free eggs and J2s, egg masses and gm-originated eggs and J2s, was strongly enhanced when antibodies were added to the in vitro parasitism bioassays. This is demonstrated in Fig. 1 for the egg- and J2-binding PAb MiPC373. Antibodies enhanced both the attachment of fungal conidia to the nematode's various life-stages and fungal germination. A detailed description of these processes is presented in the following sections.

Effect of antibodies on attachment of conidia to *Meloidogyne javanica*

Eggs and J2s (gm-free), which normally experience almost no attachment by *Trichoderma* conidia, were

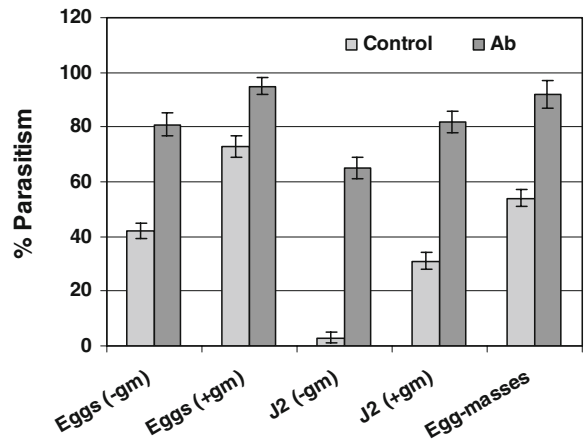

Fig. 1 Effect of polyclonal antibody (*Ab*) MiPC373 on parasitism of *Trichoderma asperellum*-203 on *Meloidogyne javanica* gelatinous matrix (*gm*)-free (*−gm*) or gm-originated (*+gm*) eggs or second-stage juveniles (*J2s*), and egg masses. Assays were performed in 96-well plates, with five replicates per treatment. Controls contained nonimmune serum. Parasitism percentages on eggs and egg masses after 1 day were assessed by comparing the proportions of hatched J2s in treated vs. untreated nematodes. Parasitism on J2s was observed directly. Fungal parasitism in the presence of antibody was significantly different from that of the control, in each treatment

Table 1 Attachment of *Trichoderma* conidia to *Meloidogyne javanica* in the presence of antibodies

Antibody	*T. atroviride*				*T. asperellum*-203			
	Eggs (−gm)	J2 (−gm)	Egg masses	E+J2 (+gm)	Eggs (−gm)	J2 (−gm)	Egg masses	E+J2 (+gm)
PAb MjPCE2	−	+	nt	nt	−	++	nt	nt
PAb MiPC373	±	++	nt	nt	+	++	nt	nt
MAb MISC	+	+	++	++	++	++	+++	+++
MAb Misec3F.4	+	++	nt	nt	±	+	nt	nt
Control serum (for PAbs)	−	−	+	+	−	−	++	++
Control medium (for MAbs)	−	−	+	+	−	−	++	++

Conidial attachment to nematodes was tested in 96-well plates containing diluted medium after 1 h incubation. Attachment intensity was qualitatively assessed as high (+++), medium (++), low (+) and very low (±); (−) designates no attachment; (nt) not tested; (E) egg; (J2) second-stage juvenile; (−gm) eggs or their hatched J2s without gelatinous matrix (gm); (+gm) eggs and J2 from egg mass.

exposed to *T. atroviride and T. asperellum*-203 conidia in the presence of the antibodies. Attachment of the conidia to nematode J2s increased (Table 1) as compared to controls without antibodies, i.e., nonimmune rabbit serum (for PAbs) or 20D medium (for MAbs). J2s with attached conidia sometimes formed aggregates in the presence of the antibodies. PAb MiPC373, MAb MISC and MAb Misec3F.4 enhanced conidial attachment to the eggs (Fig. 2). Although they all reacted with each of the *Trichoderma* species tested, differences among the antibodies were observed in their ability to enhance conidial attachment to the various nematode life-stages (Table 1). In general, the reaction with the MAbs was more intense; therefore, subsequent investigations focused on the MAb MISC, which binds to both nematode eggs and J2s. This MAb strongly enhanced the attachment of fungal conidia to the egg masses and gm-eggs (Fig. 2) and J2s; the reaction with *T. asperellum*-203 was more intense than that with *T. atroviride* (Table 1). The effect of MISC on the interaction of *T. asperellum*-203 with gm-free J2s was tested by means of serial double dilutions (×20, ×40, ×80) of the antibody: the enhancement of conidial attachment decreased successively, with 72±5, 32±4 and 12±3 conidia per J2, respectively, compared with the average conidial attachment of 3±2 conidia per J2 in the controls.

Effect of antibodies on conidial agglutination and growth

Fungal conidia were agglutinated by the four antibodies listed in Table 1 (Fig. 3a,c). The nonimmune serum (for PAbs) and 20D medium (for MAbs) did not exhibit this agglutination capability (Fig. 3b,d). Binding of antibodies to the conidia was visualised by immunofluorescent labelling of MISC on *T. asperellum*-203 conidia (Fig. 3a). The antibody-treated conidia exhibited enhanced germination and hyphal growth, as compared with those in the control treatments with nonimmune serum or medium, or those of nontreated conidia (Fig. 3c,d). The MAb MISC reacted differently with *T. asperellum*-203 and with *T. atroviride*. *Tricho-*

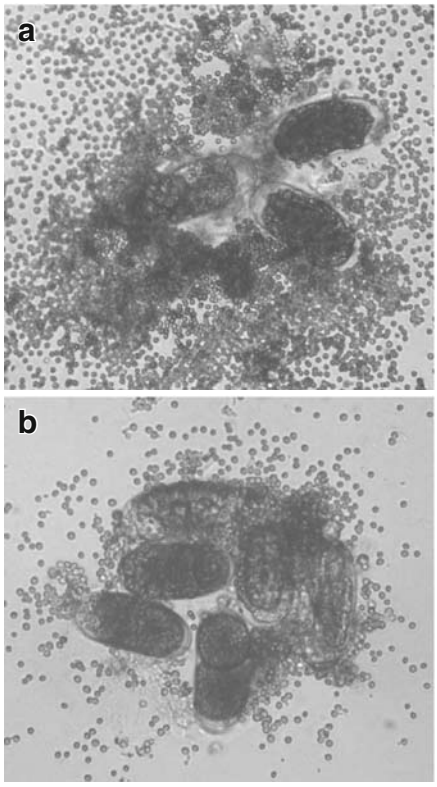

Fig. 2 Effect of monoclonal antibody MISC on attachment of *Trichoderma asperellum*-203 to **a** egg-mass-originated eggs and **b** 20D medium, serving as a control

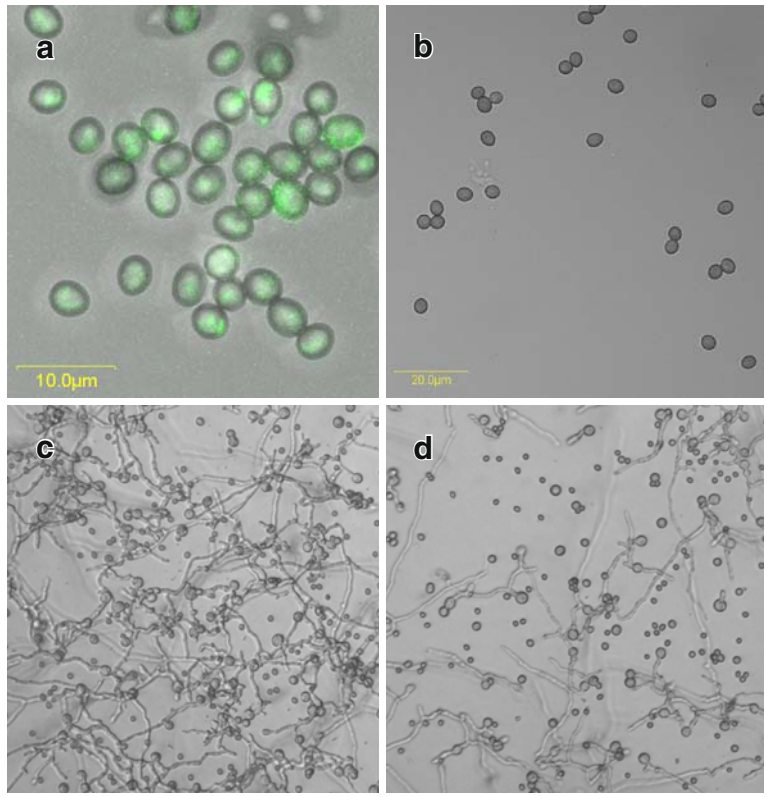

Fig. 3 **a** FITC-immunolabelling of *Trichoderma asperellum*-203 conidia with monoclonal antibody MISC. **b** Nonlabelled, medium (20D)-treated spores served as a control. **c** Conidial agglutination and germination after 24 h in diluted growth medium. **d** 20D medium served as a control

derma asperellum-203 conidia exhibited 95% more germination than controls after 24 h of incubation in a diluted medium with MISC, whereas *T. atroviride* germination was increased by 285%.

Binding of monoclonal antibody MISC to conidia and nematodes

The MAb MISC bound to *M. javanica* eggs: in contrast to the smooth labelling pattern on separated eggs, binding to gm-originated eggs revealed a rough labelling pattern, which was attributed to the matrix surrounding the eggs (Fig. 4a). Both gm-free and gm-originated J2s were labelled by this MAb; however, gm-free J2s were not labelled on the head region (Fig. 4b), whereas gm-J2s did bind the MAb along the head region (Fig. 4c). Labelling intensity of the gm-J2s was higher than that of gm-free J2s. Pretreatment of the MAb with fucose inhibited its binding to both gm-free and gm-J2s. Conidial agglutination by the MAb and the MAb-enhanced conidial attachment to nematodes were also inhibited by fucose, whereas α-methyl-mannoside did not inhibit the MAb's effects.

Binding of the MAb to J2s, with or without gm, affected their movement and viability differently, with gm-originated J2s being more affected: incubation of these J2s for 16 h with the MAb caused immobilisation of all the J2s on the plate, whereas the gm-free J2s were less affected by this treatment, with only 58% of them staying in the plate centre and of these, 30% were still alive but could not move normally. Treatment of the J2s with the antibody for only 1 h did not significantly affect their viability, regardless of their origin. Controls comprising untreated and medium-treated J2s exhibited no effects on J2 viability, even when the incubation lasted 16 h.

Effects of *Ulex europaeus* I lectin on *Meloidogyne-Trichoderma* interactions

The presence of the fucose-specific lectin UEA-I enhanced conidial attachment to nematode egg masses and to the eggs and J2s originating from them. Conidial binding to gm-free eggs and J2s that had exhibited very low conidial attachment was also enhanced. Fungal conidia were agglutinated in the presence of the lectin. All of these processes were inhibited by preincubation of the lectin with fucose.

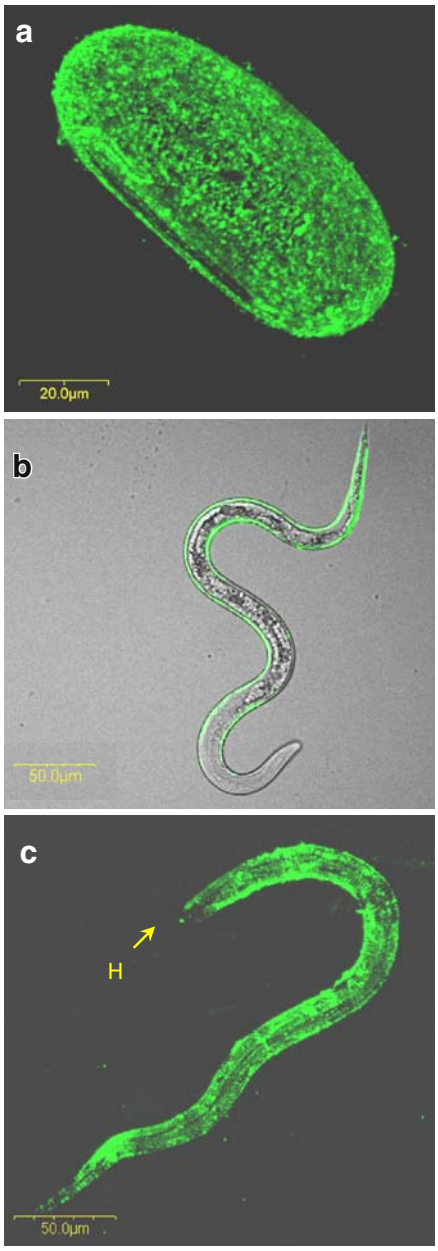

Fig. 4 FITC-immunolabelling of *Meloidogyne javanica* with monoclonal antibody MISC. **a** Egg-mass-originated egg. **b** A gelatinous matrix (gm)-free second-stage juvenile (J2) not labelled on the head region. **c** A gm-originated J2 labelled on the head region (H)

Nematodes and conidia were exposed to a fluorescence (FITC)-conjugated UEA-I. Fluorescent labelling was observed on the egg masses: the lectin bound to the gm surrounding the eggs and to individual eggs that were covered with gm (Fig. 5a, 1–3). This binding was specifically inhibited by fucose (Fig. 5b, 1–3). Labelling could not be observed on the J2s using this method.

Labelling of *T. atroviride* and *T. asperellum*-203 germinated conidia with FITC-UEA-I resulted in higher labelling intensity of *T. asperellum* conidia and hyphae (Fig. 5a, 4) relative to those of *T. atroviride*. Preincubation of the lectin with fucose increased the lectin binding (Fig. 5b, 4).

Discussion

Parasitism is one of the modes of action of *Trichoderma* species against *M. javanica*. Fungal conidia can attach to nematode egg masses and to eggs and J2s that had contact with the gm, whereas gm-free J2s and eggs are almost unattached by fungal conidia (Sharon et al. 2007). Four *M. javanica* surface-binding antibodies—two MAbs and two PAbs—were tested for their effects on the nematode–*Trichoderma* interactions: PAb MjPCE2 and MAb Misec3F.4 are J2-binding antibodies; PAb MiPC373 is an egg- and J2-binding antibody (Sharon et al. 2002). MAb MISC was also observed in this work to be an egg- and J2-binding antibody. Interestingly, the presence of nematode surface-binding antibodies enabled fungal conidium attachment to the various *M. javanica* life-stages, with or without gm, and improved the in vitro parasitism. Facilitation of these processes was due to the binding of the antibodies to the fungal conidia as well as to the nematodes to form a connection between the two, even with gm-free nematodes, to which conidial attachment was originally very low. The egg-binding antibodies enhanced conidial attachment to the eggs. Although MAb Misec 3F.4 is not considered to be an egg-binding antibody (Sharon et al. 2002), it enhanced some conidial attachment to the eggs; this may have been due to antibody binding that could not be observed by the FITC immunolabelling. The antibody-bound conidia were agglutinated and exhibited an enhanced germination rate, which further increased the parasitic activity. To the best of our knowledge, such antibody-triggered conidial germination has never been reported. This phenomenon probably involves signalling pathways that affect swelling and germination. Interestingly, similar stimulation of conidial germination and growth was observed with gm-treated conidia that were also

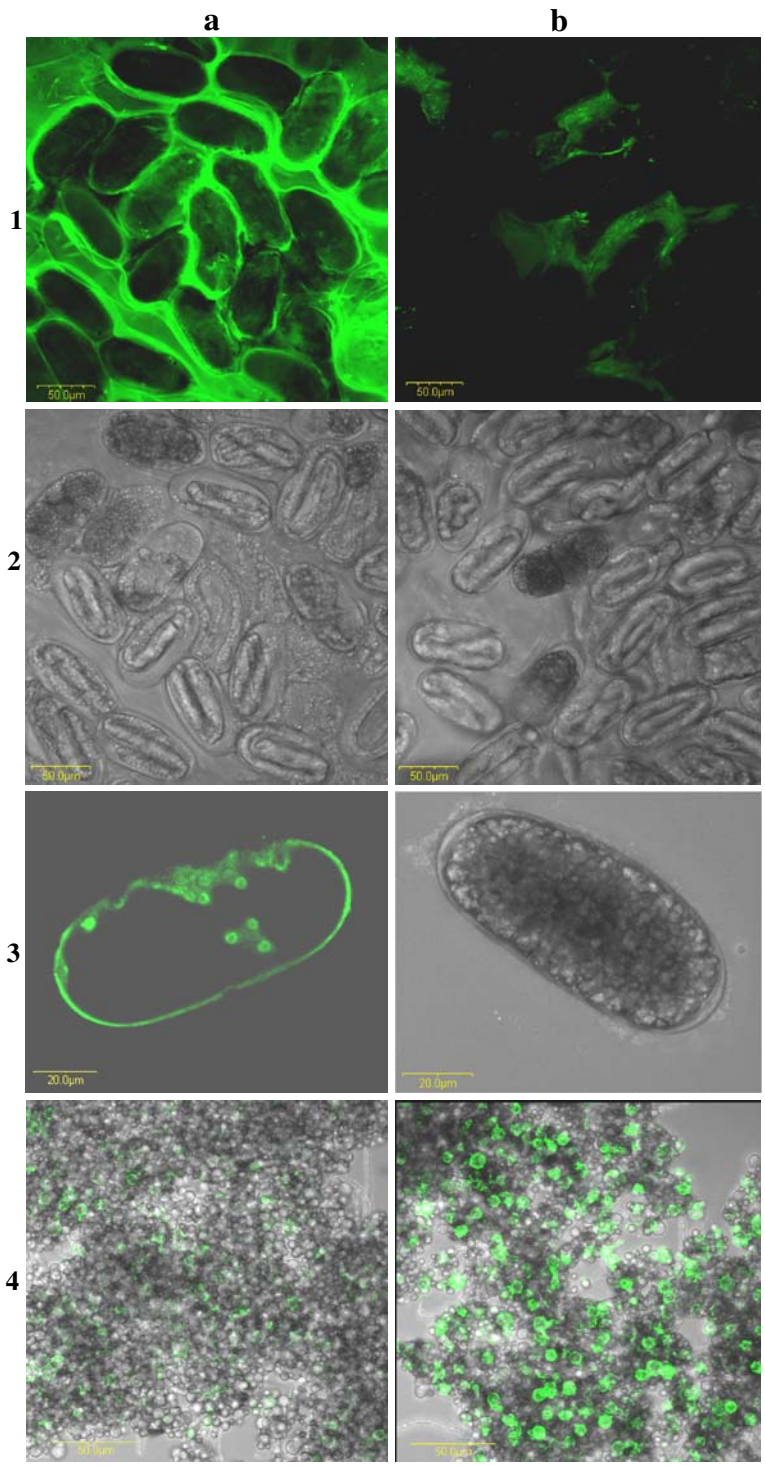

Fig. 5 **a** FITC-conjugated *Ulex europaeus*-I (UEA-I) lectin labelling of (*1*) *Meloidogyne javanica* egg mass (*2*) bright field of the above; (*3*) egg-mass-originated egg with attached conidia; (*4*) *Trichoderma asperellum*-203 germinating conidia. **b** Fucose-treated UEA-I: (*1*, *3*) inhibition of labelling on egg mass and egg, respectively; (*4*) enhancement of conidial labelling by fucose-treated lectin

agglutinated by the gm, a process that was inhibited by carbohydrates (Sharon et al. 2007).

Binding of the antibodies to the J2s has been shown to cause a reduction in their mobility and viability, with MAb Misec3F.4 showing the most prominent effect (Sharon et al. 2002). In this study, these phenomena were particularly salient with the MAb MISC, which even had a lethal effect following continuous binding for several hours, especially with gm-originated J2s. These effects of the antibodies on nematode behaviour probably contributed to their enhancement of fungal parasitism. Although all of the antibodies affected nematode movement, the drastic effect of this MAb on nematode viability was unique, and its combination with *T. asperellum* was the most effective at improving nematode parasitism; therefore, this study focused mainly on MISC.

One of the most interesting features of the nematode SC is its dynamic nature: there is a continuous turnover that involves shedding and replacement of the surface antigens (Blaxter and Robertson 1998). In plant–parasitic nematodes, this has been demonstrated for the pre-parasitic J2s of *Meloidogyne* species (Lin and McClure 1996; Spiegel et al. 1997). However, surface-characterisation studies have been performed mainly on gm-free J2s and no attention has been paid to the role of the gm and its effect on interactions between nematodes and microorganisms. Labelling of J2s with MISC demonstrated that gm-free J2s differ in their outermost layer from gm-originated J2s, the latter binding the antibody more intensively; this might explain the higher lethality rate of antibody-bound gm-originated J2s. The MAb MISC has also been observed to label the gm of *M. incognita* (race 3) and the rectal glands, where the gm originates (Hu et al. 2000). Nevertheless, the fate of gm-originated components on the surface of *Meloidogyne* J2s during the surface antigen turnover process remains unclear.

The MAbs, including Misec3F.4, which reacted with the *M. javanica* SC, have been shown to recognise carbohydrate epitopes of *M. incognita* (race 1), as reflected by the inhibition of antibody binding following periodate treatment (Lopez de Mendoza et al. 1999). The MAb MISC, raised against *M. incognita* (race 3), exhibits cross-reactivity with other *Meloidogyne* species—*M. javanica*, *M. hapla* and *M. arenaria*, but not with other plant-parasitic or free-living nematodes, and specificity to fucosyl-bearing epitopes has been suggested, since in situ antibody labelling of host tissues is blocked by fucose (Gravato-Nobre et al. 1999). In this study, binding of MISC to *M. javanica* egg masses, eggs and J2s could be inhibited by pretreatment of the MAb with fucose; therefore, the fucose-specific lectin, UEA-I, was used, and it also resulted in specific enhancement of conidial binding to nematodes and conidial agglutination, similar to the effect of the antibody. The labelling of gm and gm-originated eggs with UEA-I and its specific inhibition by the carbohydrate fucose indicate that the gm contains fucose residues. Conidia of *T. asperellum*-203 that were attached to the eggs were also specifically labelled by the lectin, suggesting the presence of fucose residues also on the conidia, as previously suggested by Elad et al. (1983). If this fungus presents more fucose residues on its surface than *T. atroviride*, it might explain the higher efficacy of MISC in enhancing *T. asperellum*-203's attachment to the nematodes, though *T. atroviride* exhibited a higher conidial germination rate following MISC treatment. Preincubation of UEA-I with fucose prior to its binding to the conidial surface caused enhanced lectin labelling, especially with *T. asperellum*-203, probably due to the binding of fucose to the conidia; this suggests the presence of fucose-binding domains (FBDs) on the conidial surface.

The results support the mechanism of attachment of *Trichoderma* to *M. javanica* suggested by Sharon et al. (2007). During hatching from egg masses, binding of gm components to the nematodes could alter their ability to attach fungal conidia by possible carbohydrate–lectin interactions, such as fucose residues on the nematodes that bind to FBDs on the fungus. The enhanced attachment mediated by the fucose-specific antibody and lectin, which bind to fucose residues on both nematodes and conidia, was more efficient with gm-originated nematodes.

Parasitic interactions between *Trichoderma* and nematodes may take place in the soil, on root surfaces (Sharon et al. 2007) and in the rhizosphere, sites that can be colonised by these opportunistic avirulent plant symbionts (Harman et al. 2004). The improved attachment and parasitism observed in vitro could facilitate the development of new strategies to affect the tritrophic interactions between the nematode, plant and fungus, for successful nematode biocontrol.

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