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Effects of *Trichoderma harzianum* strain T-22 on the growth of two *Prunus* rootstocks during the rooting phase

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SUMMARY
Trichoderma harzianum strain T-22 (T22) is one of the most effective strains of this fungus that is able to colonise the roots of most plant species across a wide range of soil types. This fungus is used as a biocontrol agent during crop production, and for the improvement of the rooting and acclimatisation phases in plant nurseries. *In vitro*-cultured shoots of GiSeLa6® (*Prunus cerasus* × *P. canescens*) and of GF677 (*P. amygdalus* × *P. persica*), two important *Prunus* varieties used as commercial rootstocks, were inoculated with T22. The results showed that early inoculation of the fungus (at the stage of shoot transfer to root-inducing medium) seriously damaged both GiSeLa6® and GF677 plants; whereas, following later inoculation (7 d after shoot transfer to root-inducing medium), the plants survived and showed significant increases in shoot growth and root development. In particular, root lengths in GiSeLa6® and GF677 plants increased by 180% and 136%, respectively, compared to non-inoculated controls. Microscopic analysis revealed T22 hyphae spreading on the root surface in GiSeLa6® (fungus colonisation frequency = 20%), but not in GF677 roots. Our results demonstrate that the application of T22 during the rooting phase resulted in greater shoot lengths, as well as increased numbers of leaves, roots, and stem diameters. These morphological characteristics could increase the quality and viability of nursery planting material and provide advantages during the plant acclimatisation phase.

Trichoderma spp. are among the most abundant, culturable fungi found in many soil types. They are able to colonise plant roots and plant debris. Fungi in this genus are genetically diverse and show a number of different activities between strains (Harman *et al*., 2004). Species of *Trichoderma* are rarely associated with diseases of living plants (Gams and Bissett, 2002). On the contrary, many *Trichoderma* species (e.g., *T. harzianum* or *T. viride*) have been used to antagonise the growth of plant pathogenic fungi, and thus act as biocontrol agents. Fungal antagonists restrict the growth of plant pathogens by one or more of three mechanisms: antibiosis, competition, and/or parasitism. They also induce defense responses in host plants (e.g., 'induced systemic resistance'; SAR; Mathivanan *et al*., 2008). Several research studies and commercial trials have shown that *T. harzianum* strain T-22 is one of the most effective strains, and is able to colonise the roots of most plant species across a wide range of soil types (Harman *et al*., 2004).

The biocontrol mechanism exhibited by *Trichoderma* spp., could be attributed to competition for nutrients, the release of extracellular hydrolytic enzymes, and/or the production of secondary metabolites that are toxic to plant pathogens at low concentrations (Mathivanan *et al*., 2008). In particular, *T. harzianum* produces a variety of antibiotic peptides, called 'peptaibols', that interact with the cell membranes of fungal plant pathogens, inhibiting their growth (Rebuffat *et al*., 1995). Furthermore, *T. harzianum* has been shown to inhibit wood rots and other fungal pathogens by ≤ 60%, through the production of such antibiotics (Morrell, 1990). An analysis of the expressed sequence tag (EST) database developed by Liu and Yang (2005) from a cDNA library constructed from the mycelial DNA of *T. harzianum*, elucidated this integrated biocontrol mechanism and indicated sequences similar to a broad range of genes encoding enzymes, structural proteins, and regulatory factors.

T. harzianum is used as an inoculant for crop production, and to improve the rooting and acclimatisation phases in plant nurseries (Ellouze *et al*., 2008). Commercial fruit scions are often grafted onto rootstocks. Rootstocks have different genetic backgrounds compared to commercial varieties, and are used to confer positive agronomic features such as tolerance of biotic stresses. Rootstocks used for the cultivation of stone fruit species are micropropagated, but a significant number of plants die during the period of acclimatisation when they are particularly susceptible to pathogen attack. To avoid such problems, it is necessary to improve the quality of nursery planting material in terms of achieving more extensive and rapid root and shoot development. For this purpose, the substrate (e.g., peat) is usually inoculated with *T. harzianum* at approx. 1.0 kg m^{-3} of substrate 1 – 4 d before the rootstocks are transplanted (Harman *et al*., 2004). Inoculation with *T. harzianum* during the rooting phase, when plants have been cultured under sterile conditions *in vitro*, could be

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another way to minimise plant losses. This method could avoid competition between *T. harzianum* and other micro-organisms usually found in soils, so allowing improved interactions with the new plant roots, and more effective induction of plant growth.

The first steps in understanding the interactions between a plant and *T. harzianum* are to define and optimise the most appropriate method and time of inoculation, in order to verify the effects of this fungal symbiont on the micropropagated plant material, in a way that will fit with existing production processes. So far, such information is scarce and fragmentary, especially with regard to commercial fruit rootstocks. The aim of this work was to conduct trials on the presence and development of *T. harzianum* T-22, and to verify its effects on the growth of GiSeLa6® (*Prunus cerasus* × *P. canescens*) and GF677 (*P. amygdalus* × *P. persica*), two of the most important commercial rootstocks used for stone fruit production.

MATERIALS AND METHODS

Preparation of a liquid culture of Trichoderma harzianum

A sample of 40-d-old *T. harzianum* strain T-22 (T22) was cultured in liquid potato dextrose broth (PDB; Oxoid Ltd., Cambridge, UK) for 20 d at 25°C on a rotary shaker at 150 rpm. The culture was filtered through two layers of Whatman No. 1 filter paper (Whatman Ltd., Maidstone, UK) to remove hyphal fragments, then filtered through a sterile 0.20 µm Minisart SFCA filter (Sartorius Stedim Biotech GmbG., Goettingen, Germany). A 40-d period of fungus growth was chosen after microscopic analyses to assess the abundance of conidiospores (approx. 10^4 ml^{-1}) according to Klein and Eveleigh (2002).

Experiment 1: early inoculation

Genetically uniform, micropropagated shoots of GiSeLa6® (*Prunus cerasus* × *P. canescens*) and GF677 (*P. amygdalus* × *P. persica*), produced by *in vitro* multiplication (with mean shot heights of 2.3 cm and 2.1 cm, and mean numbers of leaves of 3.7 and 7.6 for GiSeLa6® and GF677, respectively) were cultured in sterile 400 ml transparent glass containers. Each container was filled with 100 ml of agarised Murashige and Skoog (MS) medium (Sigma-Aldrich, St. Louis, MO, USA) without vitamins, but supplemented with 1.0 mg l^{-1} indole-3-butyric acid (IBA; Sigma-Aldrich) for GiSeLa6® ('Cherry rooting medium'), or with 0.6 mg l^{-1} IBA for GF677 ('GF rooting medium'). The optimum chemical compositions of these media were based on previous trials carried out in our laboratory. Each container contained 20 shoots and 20 containers were used (n = 400 shoots).

Plants were maintained under controlled conditions at a constant temperature (25°C) with a 16 h photoperiod (PAR = 1,000 µmol m^{-2} s^{-1}). At the stage of transferring the shoots to root-inducing medium (day-0), half of the shoots (ten containers) were inoculated using a sterile syringe with 5 ml (approx. 5×10^4 conidiospores) of the liquid culture filtrate of T22 (R0 + T22). Prior to inoculation of the plant medium, the liquid culture filtrate was suspended in 50 ml sterile water and shaken for 5 min. The remaining shoots (ten containers) were not inoculated with T22, and were kept as controls (R0). The effects of the T22 inoculum on various growth characteristics (e.g., shoot lengths, numbers of roots, numbers of leaves, and basal stem diameters) of R0 and of R0 + T22 plants were evaluated 6 d after fungal inoculation on each of ten plants per container, chosen at random (n = 100).

Experiment 2: late inoculation

Genetically uniform shoots of GiSeLa6® and GF677 produced by *in vitro* multiplication (with mean shoot heights of 2.2 cm and 2.1 cm, and mean numbers of leaves of 3.1 and 7.0 for GiSeLa6® and GF677, respectively) were cultured in sterile 400 ml transparent glass containers filled with 100 ml of 'Cherry rooting medium' or 'GF rooting medium', respectively. All containers were maintained under controlled conditions, as decribed above. To estimate the possible effects of the different media on changes in the growth parameters of the plants, a reciprocal control was carried out by culturing GiSeLa6® on 'GF rooting medium', and GF677 on 'Cherry rooting medium'. Therefore, there were four treatments with 20 containers per treatment: GiSeLa6® cultured on 'GF medium', GiSeLa6® on 'Cherry medium', GF677 on 'GF medium', or GF677 on 'Cherry medium'. Seven d after transferring the shoots to root-inducing medium (day-7), shoots in ten containers from each treatment were inoculated with T22 (R7 + T22), while the remaining ten containers were not inoculated with T22 and were kept as controls (R7). The effects of T22 inoculum on the growth characteristics (e.g., shoot lengths, mean root lengths, numbers of roots, numbers of leaves, and basal stem diameters) were evaluated 6 d and 9 d after inoculation in GiSeLa6®, and 19 d and 23 d after inoculation in GF677, respectively. Measurements were carried out on each of ten plants per container, chosen at random (n = 100). GiSeLa6® and GF677 plants were monitored during the acclimitisation phase in a greenhouse to evaluate the percentages of survival.

Microscopic analysis

Roots from GiSeLa6® and GF677 plants from Experiment 2 were analysed microscopically in order to observe the rates of colonisation by T22. Root samples taken at random from *in vitro* plants were cleaned by heating in 10% (w/v) KOH for 45 min at 80°C, then treated with 2.5% (v/v) HCl for 30 min, and stained with 0.05 (w/v) Trypan Blue (Sigma-Aldrich), according to Phillips and Hayman (1970). The presence of T22 fungal hyphae and the frequency of colonisation, were estimated on 50 root fragments (length = 1 cm) per treatment, according to Trouvelot *et al.* (1986). Root fragments were mounted on slides and observed at different magnifications using a compound optical microscope (Eclipse 80i; Nikon, Tokyo, Japan) under transmitted light, then photographed (Digital Camera DS-Fi1 with NIS-Elements Imaging Software, Nikon).

Statistical analysis

The data obtained were represented as the means of ten separate measurements on ten different plants, each with ten true replicates (ten containers). Statistical analysis was performed by analysis of variance

TABLE I
Shoot heights, numbers of roots, basal stem diameters, and numbers of leaves (± SE) in in vitro-*cultured GiSeLa6® and GF677* Prunus *plants inoculated with* Trichoderma harzianum *strain T-22 (R0 + T22) or non-inoculated controls (R0) in Experiment 1 (early inoculation)*

Rootstock	Sample	Time after inoculation (d)	Shoot height (mm)	Number of roots	Basal stem diameter (mm)	Number of leaves
GiSeLa6®	R0	6	38.76 ± 7.22 a†	1.2 ± 0.2	1.73 ± 0.34 a	5.36 ± 1.41 a
	R0 + T22‡	6	25.74 ± 2.89 b	0	1.56 ± 0.59 a	2.28 ± 0.16 b
GF677	R0	6	31.86 ± 6.48 a	2.5 ± 0.4	2.47 ± 0.54 a	10.38 ± 2.94
	R0 + T22	6	18.18 ± 4.62 b	0	1.75 ± 0.26 b	0

‡R0 + T22 indicates that T22 inoculation was carried out at the time of shoot transfer to root-inducing medium.
†Values are the means of ten measurements on ten different plants (± SE), with ten independent replicates (n = 100). For each column and scion, mean values followed by a different lower-case letter are significantly different at $P \leq 0.05$, according to Fisher's LSD test.

(ANOVA) using SAS software (SAS Institute, Cary, NC, USA). Significant differences were determined at $P \leq 0.05$, according to Fisher's LSD test.

RESULTS AND DISCUSSION

T. harzianum and soil bacteria are in direct competition in soils when scarcity of resources is a severe limiting factor; thus, reducing the effectiveness of any potential competitor to access resources provides a distinct advantage to the growth of T22 (Ellouze *et al.*, 2008). The effectiveness of a range of soil bacteria in limiting the growth of *T. harzianum* has been reported (Mackie and Wheatley, 1999). Therefore, *T. harzianum* cultured *in vitro* under controlled and stable conditions, without any nutrient limitations or other competing micro-organisms, should display maximum growth and interact optimally with plants in order to promote plant growth and rooting.

The results of Experiment 1 showed that early inoculation with T22 (at the time of the setting up the *in vitro* cultures) damaged the plants severely (Table I). After 6 d, T22-inoculated plants of both varieties showed root and shoot darkening and damage, and leaf abscission. Furthermore, shoot heights, stem basal diameters, and leaf numbers in any surviving plants decreased significantly compared to the non-inoculated controls. Therefore, early inoculation, when the shoots were too young, was not successful, as the fungus did not establish a symbiotic relationship with the plants, but damaged them by acting first as a competitor for nutrients in the agar medium, then as a saprophyte. In fact, in Experiment 1, no roots were observed in GiSeLa6® or GF677 plants (Table I).

The results from Experiment 2, using a different time of inoculation (7 d after transferring shoots to root-inducing medium) were completely different (Figure 1; Table II) and both GiSeLa6® and GF677 shoots survived fungal inoculation. In addition, T22 caused significant increases in shoot growth and root length (Table II). In GiSeLa6®, these effects were visible starting 6 d after inoculation, and were more marked after 9 d, with increases of 123%, 180%, 133%, 136%, and 161% in mean shoot height, mean root length, the numbers of roots, basal stem diameters, and numbers of leaves, respectively, compared to non-inoculated plants (R7).

In GF677, these effects were slower and less marked, starting 19 d after inoculation and were more evident after 23 d, with increases of 118%, 136%, 105%, 110%, and 126% in mean shoot height, mean root length, the numbers of lateral roots, basal stem diameters, and numbers of leaves, respectively, compared to non-inoculated plants (R7).

In a different, aseptic hydroponic system, *Trichoderma*-treated cucumber plants showed a similar physiological behaviour (Yedidia *et al.*, 1999). Similar effects of *T. virens* or *T. atroviride*, producing characteristic auxin-related phenotypes such as increased biomass production and stimulated production of lateral roots, were observed in *Arabidopsis* seedlings by Contreras-Cornejo *et al.* (2009). In our case, in Experiment 2 (late inoculation), a symbiotic system of mutual benefit to the plant and the fungus had been established.

The different behaviour of the two *Prunus* rootstock varieties was not due to the different compositions of the substrates, as GiSeLa6® plants cultured on 'GF medium', and GF677 plants cultured on 'Cherry medium' showed similar growth patterns compared to GiSeLa6® plants cultured on 'Cherry medium', and GF677 plants cultured on 'GF medium', respectively (Table II). Different strains of *Trichoderma* spp. were capable of enhancing plant growth and bio-controlling a range of wood-rot fungi when grown on a low-nutrient medium (Ellouze

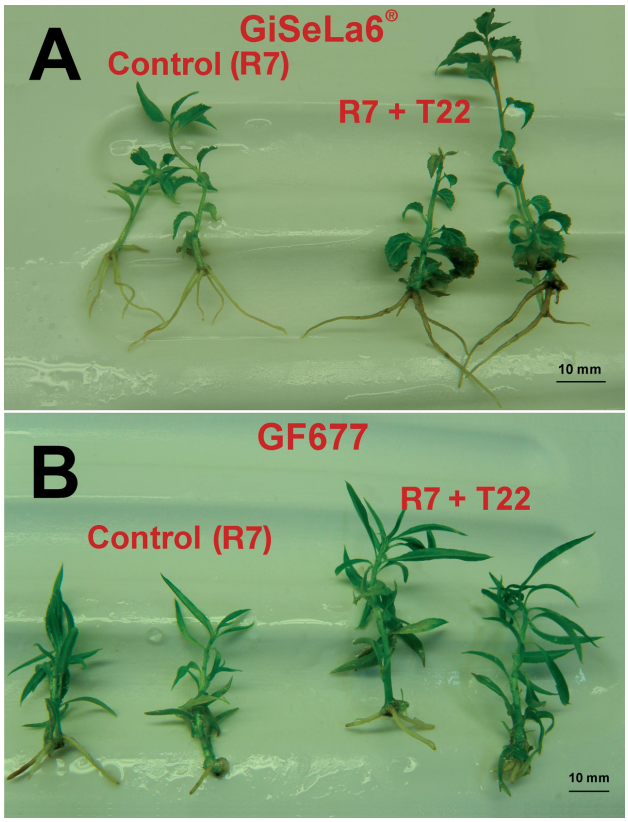

FIG. 1
Morphological comparisons between GiSeLa6® (Panel A) and GF677 (Panel B) plants treated with *Trichoderma harzianum* strain T-22 (R7 + T22), and the respective non-inoculated control plants (R7). Plants were obtained from Experiment 2 (late inoculation). Scale bars = 10 mm.

TABLE II

Shoot heights,mean root lengths, numbers of roots, basal stem diameters, and numbers of leaves (± SE) in in vitro *cultured GiSeLa6® and GF677* Prunus *plants inoculated with* Trichoderma harzianum *strain T-22 (R7 + T22) or non-inoculated controls (R7) in Experiment 2 (late inoculation)*

Rootstock	Name	Time after inoculation (d)	Shoot height (mm)	Mean root length (mm)	Number of roots	Basal stem diameter (mm)	Number of leaves
GiSeLa6® on 'Cherry medium'	R7	6	55.82 ± 11.20 a†	14.20 ± 7.97 b	3.3 ± 1.2 b	1.37 ± 0.37 b	11.20 ± 2.14 a
	R7 + T22‡	6	57.43 ± 8.51 a	29.91 ± 6.80 a	4.4 ± 1.1 a	1.72 ± 0.38 a	14.80 ± 2.86 a
	R7	9	53.54 ± 14.31 b	26.88 ± 7.61 b	3.7 ± 1.2 b	1.45 ± 0.30 b	12.31 ± 3.22 b
	R7 + T22	9	65.92 ± 8.64 a	47.95 ± 7.08 a	4.9 ± 1.9 a	1.97 ± 0.44 a	19.87 ± 4.00 a
GiSeLa6® on 'GF medium'	R7	6	46.50 ± 4.75 a	19.74 ± 6.47 a	2.9 ± 1.9 a	1.55 ± 0.40 b	11.80 ± 2.18 a
	R7 + T22	6	47.40 ± 5.78 a	18.64 ± 5.97 a	2.8 ± 1.2 a	1.87 ± 0.29 a	11.07 ± 1.62 a
	R7	9	50.56 ± 7.10 b	22.54 ± 7.26 b	3.5 ± 1.1 b	1.51 ± 0.29 b	13.47 ± 1.81 a
	R7 + T22	9	57.13 ± 5.23 a	34.38 ± 6.62 a	4.3 ± 1.7 a	1.92 ± 0.19 a	13.40 ± 2.44 a
GF677 on 'GF medium'	R7	19	35.68 ± 5.71 b	16.30 ± 4.72 b	3.3 ± 1.4 a	1.91 ± 0.20 a	22.60 ± 4.15 a
	R7 + T22	19	41.92 ± 3.05 a	28.56 ± 7.52 a	3.5 ± 1.1 a	1.83 ± 0.26 a	20.40 ± 4.26 a
	R7	23	35.01 ± 3.95 b	17.59 ± 4.63 b	3.9 ± 1.4 a	1.81 ± 0.28 b	9.60 ± 3.09 b
	R7 + T22	23	41.39 ± 2.88 a	23.93 ± 4.54 a	4.1 ± 2.8 a	2.00 ± 0.44 a	12.13 ± 3.81 a
GF677 on 'Cherry medium'	R7	19	37.96 ± 5.55 a	33.61 ± 2.48 a	2.9 ± 0.2 b	1.77 ± 0.79 a	11.80 ± 2.48 a
	R7 + T22	19	36.53 ± 5.31 a	31.95 ± 3.77 a	3.1 ± 0.7 a	1.99 ± 0.76 a	13.93 ± 3.77 a
	R7	23	32.06 ± 5.11 b	27.01 ± 2.48 a	3.3 ± 0.8 b	2.07 ± 0.79 a	16.13 ± 2.48 b
	R7 + T22	23	40.58 ± 8.32 a	26.75 ± 3.77 a	4.7 ± 1.8 a	2.43 ± 0.76 a	22.47 ± 3.77 a

‡R7 + T22 indicates that T22 inoculation was carried out 7 d after shoot transfer to root-inducing medium.
†Values are the means of ten measurements on ten different plants (± SE), with ten independent replicates (n = 100). For each treatment, mean values followed by a different lower-case letter are significantly different at $P \leq 0.05$, according to Fisher's LSD test.

et al., 2008). We used a simple MS medium that was representative of fresh softwood, with a C:N ratio of 410:1, and amino-acid and glucose levels analogous to those found in the sap of growing peach and cherry trees (Harman *et al.*, 2004).

T. harzianum strain T-22 promoted plant growth by both indirect and direct mechanisms (Herrera-Estrella and Chet, 2004). Indirectly, the fungus protects the root system of the plant by acting as a physical barrier against pathogen attack, removing nutrients from pathogens, releasing hydrolytic enzymes that degrade the cell walls of pathogens, and parasitising pathogenic micro-organisms. Directly, T22 promotes enhanced root growth, root development, and root function by means of hormonal and biochemical signals. In this way, the plants can explore the soil more efficiently, and higher amounts of water and nutrients are directed to the leaves, flowers, and fruit. As the *in vitro*-cultured plants were aseptic, with the exception of those inoculated with T22, we suggest that the effect of the fungus was to increase the overall health and development of the plants by means of direct, hormone-mediated mechanisms. The improved growth parameters of T22-inoculated plants increased the percentage survival rate during the acclimatisation phase to 92.2 ± 3.4% (SE) for both GiSeLa6® and GF677, compared to plants that had been conventionally treated with fungicides, that showed percentage survival rates of approx. 80.5 ± 3.8% (SE).

T. harzianum is capable of invading roots, but is typically restricted to the outer layers of the cortex (Yedidia *et al.*, 1999). Infection is accompanied by the production of several classes of signal compounds from the fungus that activate plant resistance responses (Harman *et al.*, 2004). Under natural conditions, T22

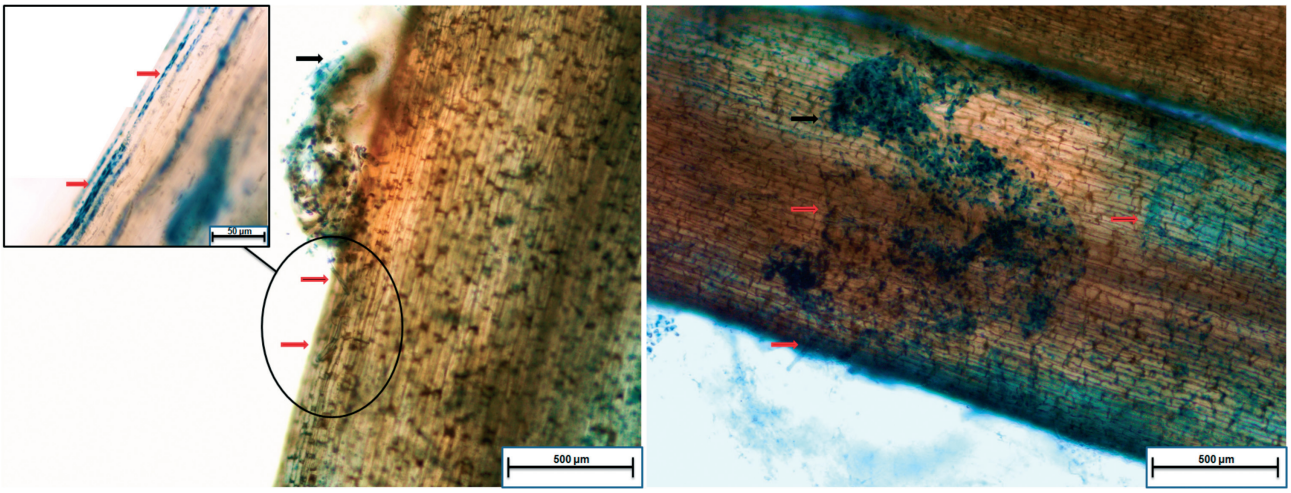

FIG. 2

Inoculum material (black arrows) and hyphae (red arrows) of *Trichoderma harzianum* strain T-22 on the roots of *in vitro*-micropropagated GiSeLa6® *Prunus* rootstock, 9 d after T22 inoculation (Experiment 2; late inoculation). Roots were stained with 0.05 (w/v) Trypan Blue and observed using a compound optical microscope under transmitted light at 10× magnification. Scale bars = 500 µm. Insert shows details at 40× magnification. Scale bar = 50 µm

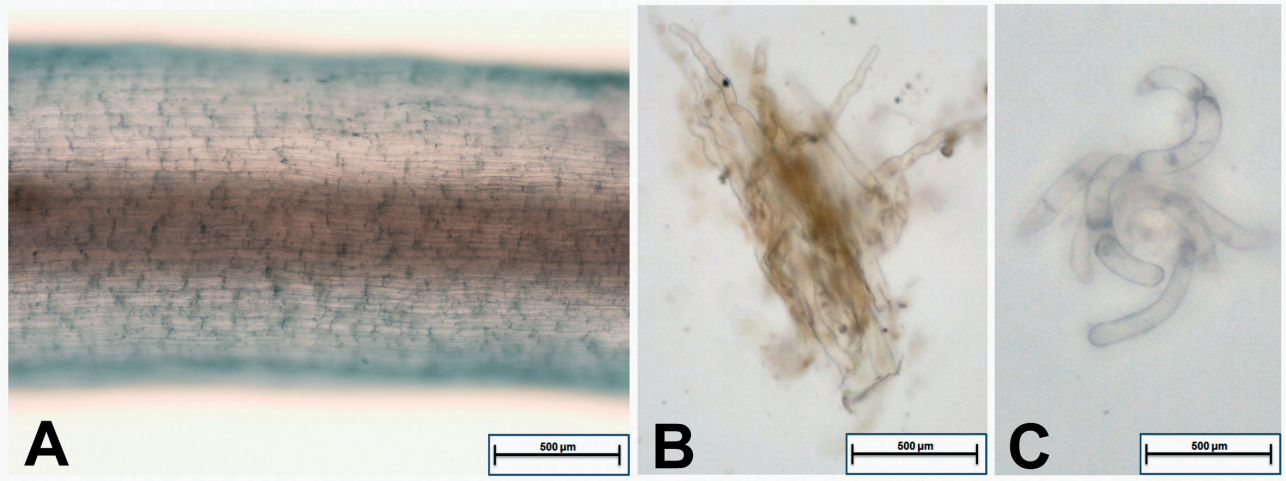

Fig 3
Roots of *in vitro*-micropropagated GF677 rootstock (Panel A) and inoculum material and hyphae of *Trichoderma harzianum* strain T-22 in the root-inducing medium (Panels B, C) 9 d after T22 inoculation (Experiment 2; late inoculation). Roots were stained with 0.05 (w/v) Trypan Blue and observed using a compound optical microscope under transmitted light at 10× magnification. Scale bars = 500 µm.

grows and develops using resources from the infected plant, forming a robust and stable, self-organising, symbiotic root-microbial system (Harman, 2000). In this investigation, the frequency of colonisation (i.e., the number of colonised root fragments/the total numbers of root fragments × 100) in the *in vitro* system, GiSeLa6®-T22, was only 20% in Experiment 2 (late inoculation). This was probably due to the difficulty of *T. harzianum* hyphae adhering to young, rapidly growing roots under *in vitro* conditions. Furthermore, the technique used to stain the root samples involved clearing and washing steps that could have caused weakly attached hyphae to detach from the root surface. Although the fungal colonisation frequency measured here was not high, the morphological effects of fungal colonisation on GiSeLa6® plants were evident (Figure 1A; Table II), and hyphae of the T22 inoculum were clearly observed on the roots of GiSeLa6® (Figure 2).

Hyphal colonisation of GF677 plant roots was not detected microscopically (Figure 3A), despite the presence of hyphae in the medium (Figure 3B,C). The effects of T22 on GF677 shoots and roots were less rapid and less evident (Figure 1B; Table II). The different responses of GiSeLa6® and GF677 plants to T22 could be due to different patterns of gene expression during the interaction of T22 with the different varieties, or to the particular signal transduction pathways that the symbioses induced. Thickening of the epidermal and cortical cell walls, and the deposition of newly-formed barriers, were observed in the roots of cucumber seedlings inoculated with *T. harzianum* under aseptic, hydroponic conditions (Yedidia *et al*., 1999). We hypothesise that these kinds of host-specific reactions may have been present in GF677 plants that reacted strongly to T22 penetration.

In conclusion, our results have shown that the application of *T. harzianum* strain T-22, 7 d after the start of the pre-acclimitisation rooting phase, caused improved shoot and root development. In particular, the fungus significantly enhanced root lengths and the basal diameters of the stems, thereby providing notable advantages during nursery processes. Better quality nursery plants could promote faster growth of micropropagated plants *in vitro* during the rooting phase, increasing their rate of survival during the following acclimitasation phase.

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