Biochemical and Morphological Changes in Micropropagated Shoots of GiSeLa6® (*Prunus* spp.) Rootstock Inoculated with *Trichoderma harzianum* Strain T-22

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Abstract
    ***Trichoderma harzianum* strain T-22 (T22) has the ability of enhancing root growth and plant development. The aim of this research is to explain the biochemical basis of the direct plant-growth-promoting activity of T22. Seven days after the transfer to root-inducing medium, in vitro-cultured shoots of GiSeLa6® (*Prunus cerasus* × *P. canescens*), an important *Prunus* rootstock for sweet and sour cherry cultivars, were inoculated with T22. Root and shoot growth were significantly affected by T22 (+76 and +61%, respectively). Indole-3-acetic acid (IAA), *trans*-zeatin riboside (*t*-ZR) and dihydrozeatin riboside (DHZR) were analyzed by HPLC/MS. Acidification of the medium by plant, T22, and plant + T22 were assessed, whereas root morphological changes were observed by light and epifluorescence microscopic analysis. Nine days after inoculation, the levels of indole-3-acetic acid (IAA), *trans*-zeatin riboside (*t*-ZR), dihydrozeatin riboside (DHZR), gibberellic acid (GA$_3$) and abscisic acid (ABA) were analyzed by high performance liquid chromatography coupled with mass spectrometry. The results showed that after T22-inoculation, IAA and GA$_3$ significantly increased in both leaves (+49 and +71%, respectively) and roots (+40 and +143%, respectively) whereas *t*-ZR decreased (-51% in leaves and -37% in roots). Changes in DHZR were observed in T22-inoculated roots (-32%) but not in leaves, whereas the levels of ABA did not differ between the two treatments. Root activity determined a decline of medium acidity, and this effect was more marked in T22-inoculated plants (up to pH 4). Microscopic analysis revealed changes in root cell wall suberification in the exoderm and endoderm, with an increase in suberized cellular layers from 1 to 2-3, and an enhancement of cell wall epifluorescence. All these T22-induced changes promote rooting and shoot growth, and they could increase plant survival during the acclimatisation phase of nursery processes.**

INTRODUCTION

    Trichoderma harzianum has been successfully used for the biological control of many plant pathogens through chemiotropic mycoparasitic interactions with the target fungal organism (Thangavelu et al., 2004; Sahebani and Hadavi, 2008) due to the mycolytic cell wall degrading enzymes active during mycoparasitism (Belén Suárez et al., 2005; Yang et al., 2009). Particularly, *T. harzianum* strain T-22 (T22) is particularly important for agronomic purposes, as it is able to colonize the roots of most plant species across a wide range of soil types (Harman et al., 2004). This strain has the ability to directly enhance root growth and plant development also in the absence of pathogens (Harman, 2000; Sofo et al., 2010a), and it has been suggested that this could be due to the production of some unidentified growth-regulating factors by the fungus or to the induction of the production of these factors in plants (Windham et al., 1986). All these findings indicate the versatility through which *T. harzianum* can directly manifest biological control activity. In spite of their theoretical and practical importance, the mechanisms responsible for the growth response due to the direct action of *T. harzianum*

on agronomic plants have not been investigated extensively.

It is well known that high values of the ratio IAA/CKs in plants promote root formation, whereas low contents induce shoot-bud formation. Thus the growth-promoting action of T22 could be due to changes in phytohormone levels and balance in the plant. Another hypothesized positive direct effect of *T. harzianum* on plants is the solubilisation of some insoluble or sparingly soluble minerals by acidification of the medium, that could determine a better nutrient availability and uptake for the plants (Altomare et al, 1999; Kücük et al., 2008; Singh et al., 2010). For all these reasons, the aim of this research was to investigate about the biochemical basis of the direct plant-growth-promoting activity of T22 on the genotype GiSeLa6® (*Prunus cerasus* × *P. canescens*), one of the most important commercial rootstocks used for sweet and sour cherry cultivars. We hypothesized that the higher shoot and root growth in T22-incoulated plants could be caused by changes in phyto-hormonal balance and root morphology, and/or to the acidification of the medium due to redox fungal activity.

MATERIALS AND METHODS

Genetically uniform shoots of GiSeLa6® produced by in vitro multiplication (mean heights of 2.0 cm) were cultured in sterile 400-ml transparent glass containers filled with 100 ml of agarized Murashige and Skoog (MS) medium without vitamins (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1.0 mg L^{-1} IBA (indole-3-butyric acid; Sigma-Aldrich) ("rooting medium"). During the rooting phase, micropropagated shoots were maintained under controlled conditions at a constant temperature of 25°C with a 16 h photoperiod and a PAR of 350 µmol m^{-2} s^{-1}. The same conditions were used for micropropagated rooted shoots (successively called 'plants' throughout the text) during the following phases.

A sample of 40-d-old *Trichoderma 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 then filtered through two layers of Whatman No. 1 filter paper (Whatman Ltd., Maidstone, UK) to remove hyphal fragments, and then filtered through 0.20 µm Minisart SFCA sterile filters (Sartorius Stedim Biotech GmbG, Goettingen, Germany).

Phytohormone Determination

Seven days after the shoot transfer to the rooting medium, plants in five containers were inoculated with T22, while the remaining five containers were not inoculated with T22, and were kept as controls. Before the fungal inoculation in the rooting medium, the liquid culture filtrate was suspended in 50 ml sterile water and shaken for 5 min. Fungal inoculation was carried out using a sterile syringe with 5 ml (approximately 50,000 conidiospores) of liquid culture filtrate of T22. Nine days after inoculation, the levels of of indole-3-acetic acid (IAA), trans-zeatin riboside (*t*-ZR), dihydrozeatin riboside (DHZR), gibberellic acid (GA₃) and abscisic acid (ABA) were determined by HPLC/MS (Shimadzu LCMS-2020 equipped with an ESI source, with two LC-2020AD pumps, CBM-20A controller and SIL-20A MS-2020 auto-sampler; Shimadzu Co., Kyoto, Japan) on three groups of plants per container chosen at random (*n*=15), following the method of Sofo et al. (2010b).

Medium pH Changes

Seven days after the shoot transfer to the rooting medium, plants were transferred in agarised MEA (Malt Extract Agar) medium implemented with streptomycin 0.03 mg mL^{-1} and prepared in 1/20 strength Hoagland solution supplemented with 1.0 mg L^{-1} IBA ("pH medium"). Eight 400 ml transparent glass containers were inoculated with T22, whereas the other half were not inoculated. The effect of T22-inoculation on pH changes in the media was measured seven days after the transfer of plants in the pH medium, using a S20 Seveneasy™ pH meter (Mettler-Toledo AG, Switzerland).

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Microscopic Analysis

Root samples, taken at random from T22-inoculated and un-inoculated plants grown in cherry medium, were previous 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). Root fragments were so mounted on slides and observed at different magnifications using a compound optical microscope (Eclipse 80i; Nikon, Tokyo, Japan) under transmitted light and then photographed (Digital Camera DS-Fi1 with NIS-Elements Imaging Software, Nikon). Five fresh apical portions per treatment, at least, were cross-sectioned (≤1 mm), 10 and 20 micrometers thick, at approximately 6 mm from the tip, and observed by an optical microscope with a mercuric vapours lamp (HBO 50 Axiophot, Zeiss, Germany) to check epifluorescence emissions.

Statistical Analysis

Statistical analysis was performed by analysis of variance (ANOVA) with 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

The application of *T. harzianum* strain T-22 (T22) during the rooting phase of GiSeLa6® rootstocks resulted in greater mean root length (+76%) and shoot length (+61%) if compared to un-inoculated plants (Fig. 1).

It is probable that the up-regulation of key genes for hormone biosynthesis or the down-regulation of the genes involved in hormone catabolism was induced by the T22 secretion of elicitors diffused into the medium or directly transferred from the fungal hyphae to the root cells, as suggested by Harman et al. (2004). There is evidence that the change in phytohormone levels is one of the direct mechanism by which *T. harzianum* promoted root and shoot growth.

Both auxin and cytokinins are involved in shoot and root growth, and morphology. Indole-3-acetic acid (IAA) is the most widely naturally-occurring auxin in vascular plants, and it is involved in lateral and adventitious roots initiation and emergence, as well as in shoot development by changes in cell division, expansion and differentiation (Hedden and Thomas, 2006). In this work, the levels of IAA in both leaves and roots of T22-treated plants increased significantly by 49 and 40%, respectively, if compared to un-inoculated controls (Table 1). Among cytokinins, *trans*-zeatin (*t*-ZR) and dihydrozeatin (DHZR), two of the most active in plants, control cell division in plants, and they are involved in reducing apical dominance, inhibiting xylem formation and root growth, promoting leaf expansion and chloroplast development, and delaying senescence (Srivastava, 2002). The results shows that T22 application significantly decreased *t*-ZR levels in both leaves and roots by 51 and 37%, respectively, if compared to the inoculated plants (Table 1), whereas in DHRZ was significantly lower only in roots (-32%). As root induction and growth are stimulated by auxins and inhibited by cytokinins, the observed increase in IAA and decreases in *t*-ZR and DHZR could explain the higher root growth observed in T22-treated plants (Fig. 1).

The morphological changes induced by T22 also reflect the different levels of GA_3, that significantly increased both in leaves and roots (+71 and +143%, respectively) of inoculated plants (Table 1). This hormone is involved in the promotion of elongation in axial organs in combination with auxins, and induces mitotic division in leaf buds and leaves (Blake et al., 2000; Srivastava, 2002). This could explain the higher shoot elongation here observed (Fig. 1) and the results of Sofo et al. (2010a), that found increases in the number of leaves and in stem diameter of T22-treated GiSeLa6® rootstocks. Generally, abscisic acid (ABA) acts as a general inhibitor of growth and metabolism, and negatively affects the synthesis of proteins and nucleic acids, even though these effects vary with tissue and developmental stage (Kobashi et al., 2001; Srivastava, 2002). Notwithstanding the significant differences in ABA levels between leaves and roots, T22 did not induce a higher ABA accumulation in both the tissues and

thus did not determine growth inhibition (Table 1 and Fig. 1).

Altomare et al. (1999) emphasised that the plant-growth-promoting capacity of *T. harzianum* was associated with in vitro solubilisation of certain insoluble minerals accomplished via production of chelating metabolites and fungus redox activity. In our experiment, we clearly demonstrated that a strong acidification in the medium inoculated with T22 occurred (Table 2). This acidification could determine the solubilisation of some salts and their higher availability for plants (Kücük et al., 2008; Singh et al., 2010). It is noteworthy that the values of pH showed a marked decrease also in presence of T22 alone (Table 2), but it appears that the synergistic action by plant and T22 caused a greater acidification of the medium. This can depend on the fact that T22 enhances the acidifying capacity of the root due to the proton extrusion of the root cells through the plasmalemma (Kücük et al., 2008). The pH decrease could allow the solubilisation of MnO_2, Fe_2O_3, metallic zinc, and calcium phosphate, and the reduction of Fe(III) and Cu(II), with evident benefits for plant nutrient uptake (Kücük et al., 2008; Singh et al., 2010). The minimum pH values found by Altomare et al. (1999) in sucrose-yeast extract liquid cultures plus *T. harzianum* but without plants were approximately 5.0, so similar to our values (Table 2). In our experiment, we observed a further pH decrease of approximately 1 unit of pH during T22-plants interaction (Table 2). The low pH values here observed could be probably due to the fact that the media used did not contain redox buffers and that the volume within the test tubes and containers was limited. We hypothesize that in natural soils, that usually have a pronounced buffering capacity, the acidification due to T22 could be less pronounced, as recently suggested by Singh et al. (2010).

Microscopical observations were carried out to compare root systems of T22-inoculated and un-inoculated plants. Plant overall morphology of T22-inoculated and un-inoculated plants grown in cherry medium differed significantly (Fig. 1). Root tissues contain abundant alkaloids: berberine, chelerythrine, sanguinarine and chelidonine (along with other isoquinoline alkaloids), and some of them act as fluorochromes for suberin and lignin, providing numerous potential natural dye sources for fluorescence microscopic techniques (O'Brien and McCully, 1981; Brundrett et al., 1988). In our case, the observed cell wall epifluorescence (Fig. 2A) indicated that T22 seems to induce the synthesis ex novo, of phenolic compounds in the plants, likely by the secretion of elicitors and the following induction of defense responses, as suggested by Mathivanan et al. (2008). Among the compounds induced and involved in plant defence there are the enzymes of the phenylpropanoid pathway, involved in lignin biosynthesis (Gianinazzi-Pearson et al., 1994). The microscopic analysis of T22-inoculated plants highlighted a higher wall suberification and the increase in the number of suberized cellular layers from 1 to 2-3 both at the level of exoderm and endoderm (Fig. 2B). The increased lignification of root endodermal cells induced by mycorrhiza was already found by Dehne (1982) but it was never demonstrated for T22. We suggest that the accumulation of protective molecules, such as lignin and suberin, in plants inoculated with T22 could accelerate their hardening. The accumulation of structural substances may be of key importance in the resistance process, increasing the mechanical strength of the host cell walls (Dalisay and Kuc, 1995).

CONCLUSIONS

Considering the positive effect on shoot and root growth in plants inoculated with T22, we hypothesise that changes in auxin/cytokinins ratios could be involved in this process. Several lines of evidence indicate that while auxins stimulate root formation, cytokinins inhibit it (Srivastava, 2002). In GiSeLa6® plants, the herein observed alterations in hormonal balance could be an adaptative response induced by T22, that could benefit from a greater root surface area for colonisation, so reinforcing symbiotic behaviors with the plants. Furthermore, increased absorptive surface by branched roots may increase water and nutrient uptake capacity of plants.

The acidification of the medium could be one of the causes of the direct benefits of T22 on plants, as this implies a reduction in the electrical potential within the cells that

could favour the diffusion of cations from the medium ('soil' in the natural systems) to the root against the concentration gradient. Finally, infection of plants with T22 accelerated root suberification and lignifications, so promoting a quicker and stronger reaction against the pathogens.

During the acclimatisation phase of nursery processes, all these observed biochemical and morphological changes induced by T22 constitute an advantage, as inoculated GiSeLa6® plants could acclimatise better to new and hostile environments, so increasing plant survival in the absence of pesticides.

ACKNOWLEDGEMENTS

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Tables

Table 1. Levels of indole-3-acetic acid (IAA), *trans*-zeatin riboside (*t*-ZR), dihydrozeatin riboside (DHZR), gibberellic acid (GA$_3$) and abscisic acid (ABA) ($\pm$SD; *n*=25) in un-inoculated and T22-inoculated plants. Values followed by a different letter are significantly different at *P*≤0.05, according to Fisher's LSD test.

Phytohormone (ng g^{-1} fresh weight)	Without T22		With T22	
	Shoots	Roots	Shoots	Roots
IAA	36.0±2.2 b	32.8±2.4 b	53.6±3.2 a	46.0±4.9 a
t-ZR	7.6±1.1 a	5.1±0.6 b	3.7±1.3 c	3.2±0.5 c
DHZR	8.4±1.0 a	7.6±0.4 a	7.8±2.4 a	5.2±0.6 b
GA$_3$	2.1±0.4 b	1.4±0.6 b	3.6±0.5 a	3.4±0.5 a
ABA	2.0±0.4 a	1.0±0.3 b	2.5±0.6 a	1.4.±0.4 b
IAA	36.0±2.2 b	32.8±2.4 b	53.6±3.2 a	46.0±4.9 a

Table 2. Values of pH of the media in three treatments (±SD; *n*=8). Statistics as in Table 1.

	Bromothymol blue	Methyl red	Bromocresol green
		pH	
Only plant	6.2±0.1 a	6.3±0.1 a	6.2±0.1 a
Plant + T22	4.0±0.4 c	4.2±0.5 c	4.0±0.1 c
Only T22	5.0±0.1 b	5.0±0.4 b	4.9±0.1 b

Figures

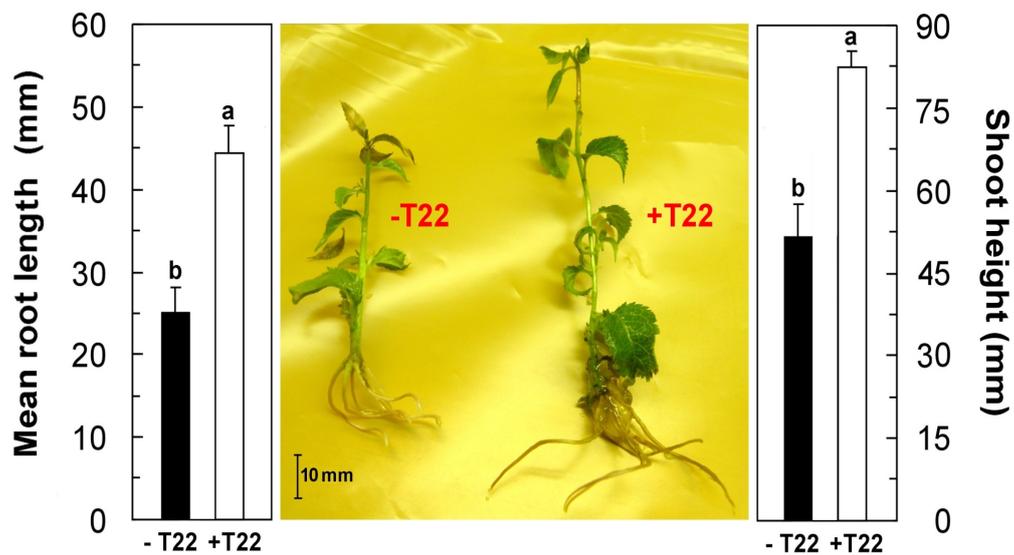Fig. 1. Mean root length and shoot height (±SD; *n*=50) in un-inoculated and T22-inoculated plants. Values followed by a different letter are significantly different at *P*≤0.05, according to Fisher's LSD test.
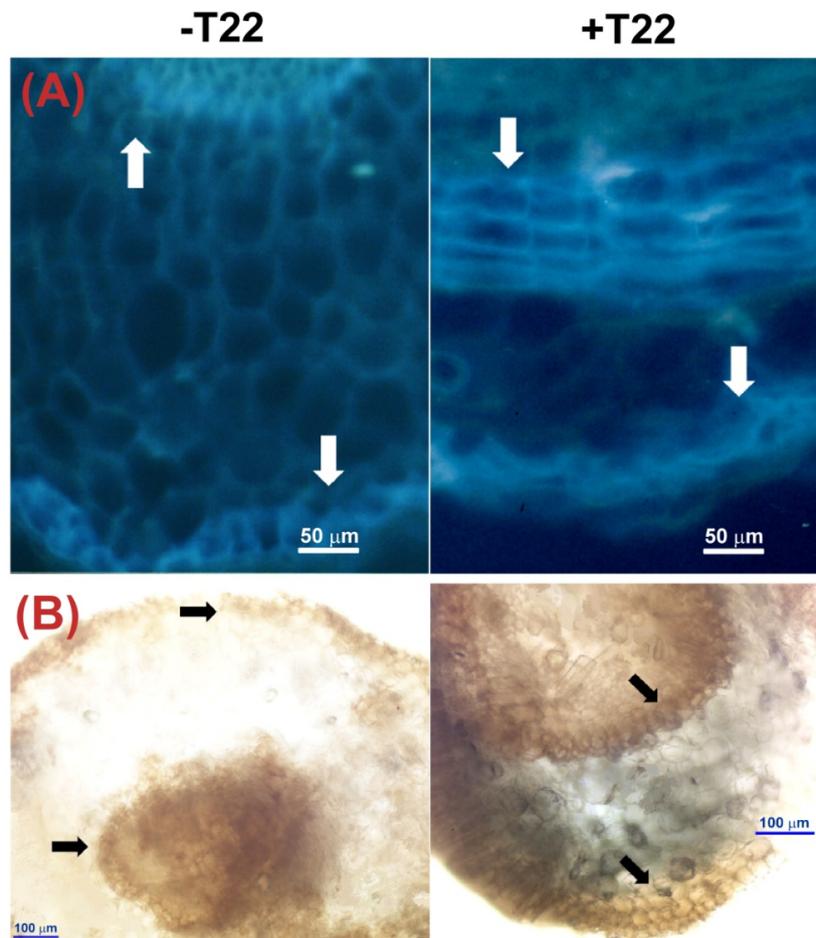
Fig. 2. (A) Root cross sections (diameter ≤1 mm; 6 mm from the root tip) of plants inoculated with T22 (right) and un-inoculated plants (left) observed at 10× magnification with a mercuric lamp. The arrows indicate the endodermic (above) and exodermic (below) layers. (B) Root cross sections (diameter ≤1 mm; 6 mm from the root tip) of plants inoculated with T22 (right) and un-inoculated plants (left) under transmitted light at 10× magnification. Wall suberification (arrows) of inoculated roots increased from 1 to 2-3 layers both in exoderm and endoderm (right).