Trichoderma harzianum *strain T-22 induces changes in phytohormone levels in cherry rootstocks (*Prunus cerasus × P. canescens*)*

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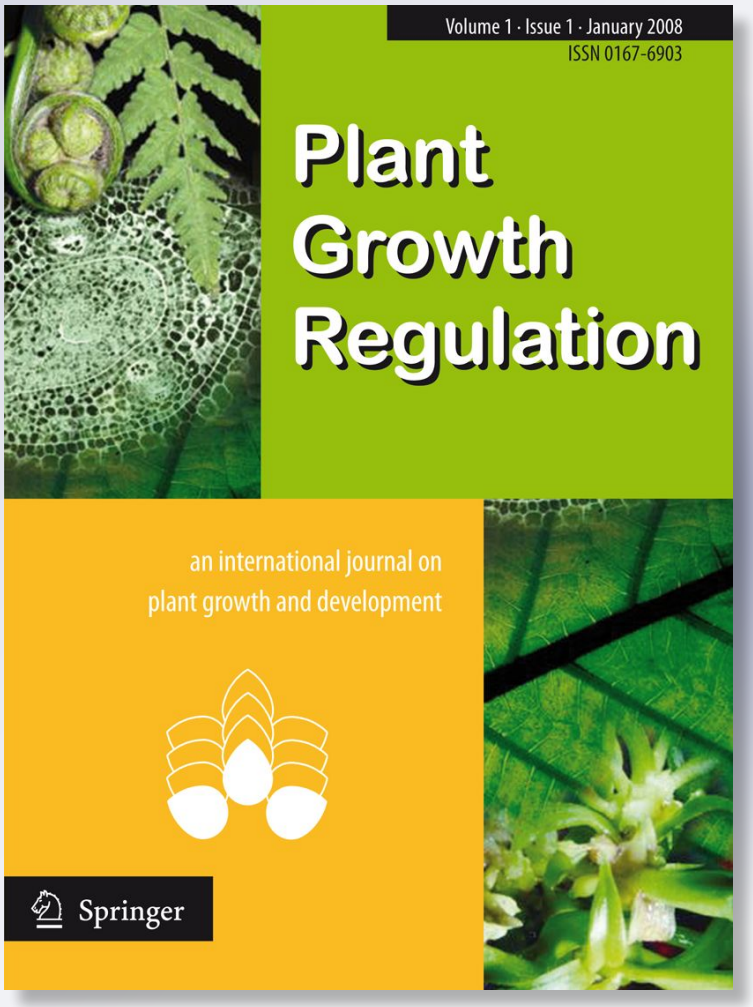

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BRIEF COMMUNICATION

Trichoderma harzianum strain T-22 induces changes in phytohormone levels in cherry rootstocks (*Prunus cerasus* × *P. canescens*)

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Abstract The aim of this research was to explain the direct plant growth-promoting activity of *Trichoderma harzianum* strain T-22 (T22), hypothesizing the involvement of different classes of plant growth regulators. Seven days after the transfer to root-inducing medium, in vitro-cultured shoots of GiSeLa6® (*Prunus cerasus* × *P. canescens*) were inoculated with T22. Root and shoot growth were significantly affected by T22 (+76 and +61%, respectively). Ten days after inoculation, the levels of indole-3-acetic acid (IAA), *trans*-zeatin riboside (*t*-ZR), dihydrozeatin riboside (DHZR), gibberellic acid (GA3) and abscisic acid (ABA) were analyzed by high performance liquid chromatography coupled with mass spectrometry. The results showed that after T22-inoculation, IAA and GA3 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. The extraction method allowed the simultaneous extraction of phytohormones. There is evidence that the change in phytohormone levels is one of the direct mechanism by which T22 promotes rooting and shoot growth, with notable advantages for rootstock production during nursery processes.

Keywords Abscisic acid · Cytokinins · Gibberellic acid · Indole-3-acetic acid · Plant-fungus symbiosis · Root growth

Abbreviations

DHZR	Dihydrozeatin riboside
GA3	Gibberellic acid
IAA	Indole-3-acetic acid
t-ZR	*trans*-zeatin riboside
T22	*Trichoderma harzianum* strain T-22

Introduction

Trichoderma harzianum has been successfully used in biological control of many plant pathogens through chemiotropic mycoparasitic interactions with the target fungal organism. Its mechanism of action includes the excretion of mycolytic cell wall degrading enzymes (Yang et al. 2009) and the production and release of atpenins, potent and specific inhibitors mitochondria metabolism in the parasite (Miyadera et al. 2003). From another point of view, *T. harzianum* has the ability to directly enhance root growth and plant development in the absence of pathogens, and it has been suggested that this could be due to the production of some unidentified growth-regulating factors by the fungus (Windham et al. 1986; Harman et al. 2004).

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Plant hormones play a crucial role in controlling plant growth, development, and defensive responses, as they are able to transduce signals among plant organs and integrate them to produce adequate and concerted responses to environmental stimuli. Contreras-Cornejo et al. (2009) recently discovered that wild-type Arabidopsis seedlings inoculated with *Trichoderma* spp. show characteristic auxin-related phenotypes, including increased biomass production and stimulated lateral root development. It was also confirmed that the phytohormones indole-3-acetic acid (IAA), abscisic acid (ABA), and several cytokinins have important functions in developmental stages and growth of *Prunus* spp. (Blake et al. 2000; Kobashi et al. 2001; Moncaleán et al. 2001; Tworkoski et al. 2006; Sorce et al. 2007).

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. We hypothesize that the biochemical basis of the direct plant-growth-promoting activity of this fungus could be due to the release of phytohormone-like compounds or, alternatively, to the induction of phytohormone synthesis in plants. On the basis of the results of previous experiments focused on the interaction *T. harzianum*-plant (Kleifeld and Chet 1992; Yedidia et al. 2001; Singh et al. 2010; Sofo et al. 2010), we believe that the higher shoot and root growth in the plants inoculated with the fungus, could be due to changes in phytohormonal balance. Considering the economical importance of *Prunus* spp. and the lack of research on the interactions between *T. harzianum* and tree species, we used the plant genotype GiSeLa6® (*Prunus cerasus* × *P. canescens*), one of the most important commercial rootstock used for sweet and sour cherry varieties, and the fungus *T. harzianum* strain T-22, particularly important for agronomic purposes due to its ability to colonize the roots of most plant species across a wide range of soil types (Harman et al. 2004).

Materials and methods

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 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).

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). 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 400 µmol m^{-2} s^{-1}. The same conditions were used for micropropagated rooted shoots (successively called 'plants' throughout the text) during the following phases.

Seven days after the shoot transfer to the medium, plants in five containers were inoculated with T22, while the remaining five containers were not inoculated with T22, and were kept as controls. Prior the fungal inoculation in the 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. Ten days after inoculation, the levels of indole-3-acetic acid (IAA), *trans*-zeatin riboside (*t*-ZR), dihydrozeatin riboside (DHZR), gibberellic acid (GA3), and abscisic acid (ABA) (molecular structures in Fig. 1) were determined on a group of five plants chosen at random per each of five containers ($n = 25$). Samples of the medium, both from un-inoculated and inoculated containers ($n = 5$), were also analysed. The effects of the T22 inoculum on mean root length and shoot height was evaluated 9 d after inoculation, and measurements were carried out on each of ten plants per container chosen at random ($n = 50$).

Both for leaves and root tissues, an aliquot of 250 mg of shoot or root tissue was ground into powder with liquid nitrogen with a mortar and pestle, and put in a tube. To each tube, 2.5 mL extraction solvent (2-propanol/H_2O/HCl 37%; 2:1:0.002, v/v/v) was added. The tubes were shaken at a speed of 100 rpm for 30 min at 4°C. To each tube, 2.5 mL of dichloromethane was added, and then the samples were shaken for 30 min at 4°C and centrifuged at 13,000*g* for 5 min. After centrifugation, two phases were formed, with plant debris between the two layers, so 1.0 mL of the solvent from the lower phase was transferred using a Pasteur pipette into a screw-cap vial, and the solvent mixture was concentrated using a evaporator with nitrogen flow. Finally, the samples were re-dissolved in 0.1 mL methanol and stored at −20°C before quantitative analysis.

The quantitative determinations of IAA, *t*-ZR, DHZR, GA3 and ABA were carried out in both un-inoculated and inoculated plants by high performance liquid chromatography coupled with mass spectrometry (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).

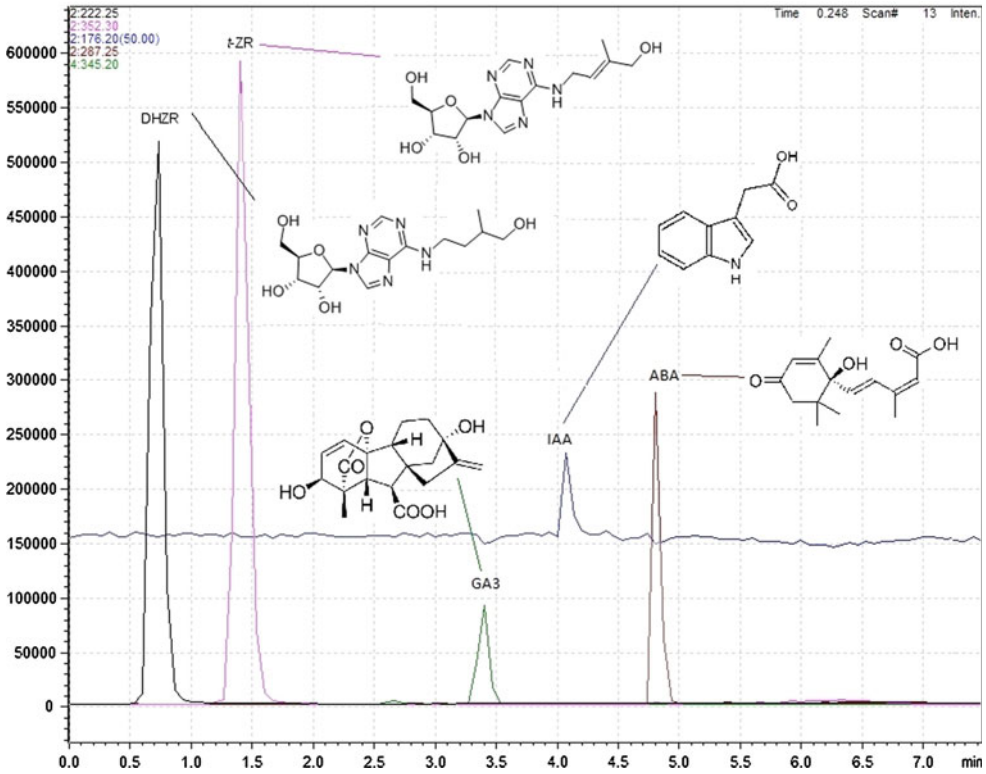

Fig. 1 HPLC chromatogram of root extracts of T22-inoculated GiSeLa6® plants recorded after 8 min (*m/z* values: IAA = 176.20, *t*-ZR = 352.30, DHZR = 222.25, GA3 = 345.2, ABA = 287.25). The *peak labels* corresponds to the phytohormones reported in Table 1

The chromatographic separation was conducted using a Shim-Pak XR-ODS column, 2 mm × 50 mm (Shimadzu) and a mobile phase that consisted of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in methanol (Solvent B) delivered in gradient elution mode at a flow rate of 0.3 mL min^{-1}. The elution programme used was as follows: 0–1 min, 30% B; 1–5 min, 80% B; 5–5.5 min, 100% B; 5.5–10 min, 100% B; 10–11 min, 30% B; 11–15 min, 30% B. Mass scans were measured from *m/z* 150 up to *m/z* 400, at 350°C interface temperature, 230°C DL temperature, ±4,500 V interface voltage, neutral DL/Qarray, using N_2 as nebulizing gas. Mass spectrometry data for IAA, *t*-ZR, DHZR and ABA were acquired in the positive ionization mode, whereas for GA3 they were acquired in the negative ionization mode. Pure standards of each phytohormone (Duchefa Biochemie B.V., Haarlem, The Netherlands) were used for quantification analysis. The amounts of plant hormones in the samples were determined by calculating the correction factor of each authentic plant hormone in comparison with its corresponding internal standard. Correction factors were calculated as the ratio of the signal intensity ratio of the internal standard to the corresponding plant hormone. The internal standards used were the following (OlChemIm Ltd., Olomouc, Czech Republic): [$^2H_5$] indole-3-acetic acid (cat. no. 0311533); [$^2H_5$] *trans*-zeatin riboside (cat. no. 0300313); [$^2H_3$] dihydrozeatin riboside (cat. no. 0300613); [$^2H_2$] gibberellic acid (cat. no. 0322503); [$^2H_6$] (+)-*cis,trans*-abscisic acid (cat. no. 0342723).

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. 2).

None of the phytohormones was detected in the medium of both the treatments, so indicating that they have not been released by T22 and that the fungus induced their ex novo synthesis in the plants. It is probable that the up-regulation of key genes for hormone biosynthesis or the down-regulation of the genes involved in hormone catabolism

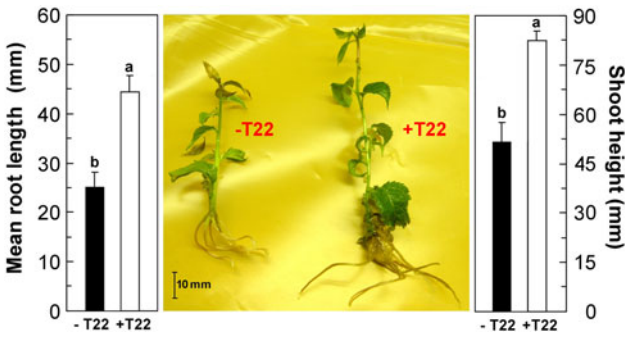

Fig. 2 Mean root length, shoot height and morphological comparison of un-inoculated and T22-inoculated GiSeLa6® plants. Values (±standard deviation) are means of 50 replicates ($n = 50$). Mean values followed by a *different lower-case letter* are significantly different at $P \leq 0.05$, according to Fisher's LSD test

Table 1 Levels of indole-3-acetic acid (IAA), *trans*-zeatin riboside (*t*-ZR), dihydrozeatin riboside (DHZR), gibberellic acid (GA3), abscisic acid (ABA), and auxin/cytokinins ratio (IAA/CKs) in GiSeLa6® plants inoculated with T22 and in un-inoculated plants

Phytohormone (ng g^{-1} fresh weight)	Plants without T22		Plants with T22	
	Leaves	Roots	Leaves	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
GA3	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

Values (±standard deviation) are means of 25 replicates ($n = 25$). For each row, values followed by a different lower-case letter are significantly different at $P \leq 0.05$, according to Fisher's LSD test

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).

Both auxin and cytokinins are involved in shoot and root growth, and morphology. Indole-3-acetic acid 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. 2). Our data are also in accordance with those of Tworkoski et al. (2006) and Sorce et al. (2007), that found in peach (*Prunus persica*) trees and rootstocks positive relationships between IAA content and tree vigour in terms of enhanced shoot development.

The morphological changes induced by T22 (Fig. 2) also reflect the different levels of GA3, 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. 2) and the results of Sofo et al. (2010), 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. 2).

The results obtained confirmed that the method used for the simultaneous extraction of different phytohormones was effective and reliable, and that photo-oxidation and oxidation, two critical steps for this kind of analyses, did not occur (Fig. 1). There is evidence that the change in phytohormone levels is one of the direct mechanism by which *T. harzianum* promotes plant growth. In GiSeLa6® plants, the simultaneous alteration in hormone levels, accompanied by a higher root length, 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, a higher root absorptive surface can enhance water and nutrient uptake, increasing plant survival. The observed higher shoot development could allow T22-inoculated plants to accelerate leaf production and stem lignifications, so facilitating plant hardening, with notable advantages for rootstock production during nursery processes.

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