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RESEARCH ARTICLE

In vitro biocontrol activity of *Trichoderma harzianum* on *Alternaria alternata* in the presence of growth regulators

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Abstract

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The *in vitro* biocontrol ability of *Trichoderma harzianum* on the phytopathogen *Alternaria alternata* improved in the presence of the growth regulators gibberellic acid (GA_3), or indolacetic acid (IAA) or benzylaminopurine (BAP) or foliar nutrient at concentrations similar or higher than those used at the field level. These plant hormones decreased the secretion of endopolygalacturonase (endo-PG) of *A. alternata* by approximately 20%, did not modify endochitinase (endo-CH) secretion of *T. harzianum* and did not alter germination of conidia or mycelia growth of any of these fungi. The presence of *T. harzianum* decreased endo-PGase secretion of *A. alternata* by about 50%. This inhibitory effect was independent of the presence of growth regulators. The level of secreted endo-PG of *T. harzianum* was not modified by the presence of *A. alternata*, but the presence of this phytopathogen in cultures of *T. harzianum*, increased both the growth of the biocontroller and its secretion of endo-CH.

Article

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Trichoderma harzianum is an efficient biocontrol agent that is commercially produced to prevent development of several soil pathogenic fungi. Different mechanisms have been suggested as being responsible for their biocontrol activity, which include competition for space and nutrients, secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds (Haram et al. 1996; Zimand et al. 1996). Nevertheless, the biocontrol activity of *T. harzianum* could be affected by environmental cues, that include among others, the presence of plant nutrients at the field level, which contain growth regulators as auxins in their formulations (AFIPA, 1993 – 1994). The presence of growth regulators in the soil could come from applications done to the foliar portion of plants or to fruits, where part of applications fall either directly or from plants into the soil. They could also come from treatments of tubers with auxins, which are used to stimulate budding after seeding; or from soil microorganisms. In fact, it has been described that some fungal pathogens are able not only to induce increased levels of IAA in their respective hosts, but are themselves capable of producing IAA which is directly released into the soil (Agrios, 1997). *Agrobacterium tumefaciens*, which causes crown gall on more than one hundred plant species, produces and contains genes coding for IAA and cytokinin production. Other bacteria, such as *Pseudomonas savastanoi*, code for IAA synthesis not only in genes contained in its plasmid but also in its chromosome (Agrios, 1997). In addition, the direct use of cytokinins has been also described to reduce virus multiplication and local lesions in several plant species (Agrios, 1997). Therefore, it seemed important to analyze if commonly used plant growth regulators such as auxins, cytokinins and gibberelic acid, that are applied to different plant species or that are produced by soil microorganisms, could affect the biocontrol activity of *T. harzianum*.

Alternaria diseases are among the most common diseases of many plants throughout the world (Agrios, 1997). They affect primarily the leaves, stems, flowers and fruits of annual plants, especially vegetables and ornamentals (Latorre, 1988). *Alternaria alternata* is a pathogenic fungus that can secrete endo-polygalacturonase (endo-PG) and pectate lyase (PL) activities (Pérez et al. 1991; Aubá et al. 1993). These enzymes are responsible for the hydrolysis of pectic components of the plant cell wall (Collmer and Keen, 1986). Depending on the type of interaction developed between the plant species and the microorganism (compatible or incompatible), these pectinases could represent one of the fungal infection mechanisms, or could be considered within the enzyme systems that trigger an hypersensitive response through the release of oligosaccharides acting as elicitors of the plant response (Roco et al. 1993). The activity of endo-PG and of PL is known to be affected by fungicides, temperature, metal ions or foliar nutrients (Aubá et al. 1993). These latter products may contain growth regulators, or be applied together with them, which could also affect the activity of these enzymes. Due to our knowledge on *A. alternata* we decided to use this fungus as the target microorganism to test the biocontrol activity of *T. harzianum* in the presence or absence of phytohormones. A deepest knowledge of the factors that could affect biocontrol activity of *Trichoderma* would allow us to improve biocontrol conditions for trials at the field level, and to provide information of how hormones could benefit or decrease the biocontrol effect of *T. harzianum* in its specific interaction with *A. alternata.*

Materials and Methods

Chemicals. All chemicals were analytical grade and were purchased from Sigma and from Merck. Commercial plant growth regulators and plant nutrients distributed either by Bayer (Bayfolan^{R}, Bayer^{R} 2T005 SL), BASF (Activol^{R}), Hoechst (liquid Gibberelic acid) or Shell (NAA-800^{R}) were selected from AFIPA (1993 – 1994), and were purchased in the local market.

Fungal isolates. *A. alternata* (strain P_2) and *T. harzianum* (strain N_3) were isolated from sooty molds infecting *Citrus* species in Chile (Pérez et al. 1991) and were maintained on potato dextrose agar (PDA, DIFCO).

Evaluation of mycelium growth and of biocontrol activity. Disks (0.5 cm diameter) from pure cultures of *A. alternata* or *T. harzianum* were seeded in a Petri dish containing either PDA or Mandels pectin agar MPA, (Mandels et al. 1974), with or without the addition of 15, 30 or 40 ppm of gibberellic acid (GA_3) or indolacetic acid (IAA) or benzylamino purine (BAP), or of 15, 30 or 40 ppm of the commercial products mentioned in chemicals. Fungi were grown up to one week at 28°C. Colony diameter was recorded every two days. When biocontrol activity was tested, both *A. alternata* and *T. harzianum* were seeded in the same dish at opposite sides (dual cultures), and their growth was evaluated as above. Controls were performed seeding each fungus against itself. Results correspond to the mean of six different experiments run in duplicates. Data was analyzed through the test of Student at $p<0.05$.

Evaluation of conidia germination. Conidia from *A. alternata* or *T. harzianum* were obtained as recommended by AOAC (1980). One hundred conidia from each fungus were grown on 2% (w/v) water agar (WA) for 18 hours in the absence or presence of 15, 30 or 40 ppm of GA_3 or IAA or BAP or of the commercial products mentioned in chemicals. Germination was evaluated by light microcopy. Results correspond to the mean of five different experiments run in triplicates. Data was analyzed through the test of Student at $p<0.05$.

Production of endo-polygalacturonase (endo-PG) and of endo-chitinase (endo-CH) in submerged cultures. The secretion of the enzymes was evaluated in submerged cultures using the Mandels mineral salt medium (Mandels et al. 1974) with the addition of 4 g/l of Citrus pectin or glycol chitin for production of endo-PG or endo-CH, respectively. Flasks, containing 200 ml of any of the above media were inoculated with 1×10^6 conidia of either *A. alternata* or *T. harzianum* in the absence or presence of 15, 30 or 40 ppm of GA_3 or IAA or BAP or the commercial formulations mentioned in chemicals.

To test the effect of *T. harzianum* on the ability of *A. alternata* to secrete endo-PG, 0.5×10^6 or 1×10^6 conidia of each fungus were inoculated in the pectin-containing medium (200 mL), to give a final concentration of 1×10^6 or 2×10^6 conidia per flask.

To test the effect of *A. alternata* on the ability of *T. harzianum* to secrete endo-CH, 0.5×10^6 or 1×10^6 conidia of each fungus were inoculated in the glycol chitin-containing medium (200 mL), to give a final concentration of 1×10^6 or 2×10^6 conidia per flask. Flasks were incubated at 28°C up to fourteen days with shaking at 150 rpm. The whole medium was then centrifuged at 9,000 x *g* for 10 minutes to remove mycelia, and the supernatant was used to test endo-PG or endo-CH activity. Results correspond to the mean of three independent experiments run in triplicates. Controls were performed with heat inactivated conidia. Data was analyzed through the test of Student at $p<0.05$.

Endo-polygalacturonase (endo-PG) activity. It was tested by a modified Nelson-Somogyi assay (Nelson, 1944). The reaction mixture contained 0.5% (w/v) polygalacturonic acid in 100-mM sodium acetate pH 5.2 (Pérez et al. 1991). One unit was defined as the amount of enzyme that released 1 mM of reducing sugars per minute. The direct effect of GA_3 or IAA or BAP on endo-PG activity was tested adding to the assay media of submerged cultures without additions, 15, 30 or 40 ppm of the corresponding growth regulator or of the commercial products mentioned in chemicals. Controls were performed with boiled enzyme (20 minutes). Results correspond to the mean of three different experiments run in triplicates. Data was analyzed through the test of Student at $p<0.05$.

Endo-chitinase (endo-CH) activity. Endochitinase activity was tested by the method of Pan et al. (1991) modified as follows: multi-well plates (20 mm diameter per well) were filled with 2% agarose containing 1% glycol chitin (with or without the addition of 40 ppm of GA_3 or IAA or BAP or of the

commercial products mentioned in chemicals) and were seeded with *A. alternata*, with *T. harzianum* or with the two fungi. Plates were incubated at 28°C for 72 hours, and total endo-CH activity was developed as described (Pan et al. 1991). Hydrolysis diameters were measured with a millimeter ruler. Results correspond to the mean of three experiments run in triplicates. Data was analyzed through the test of Student at $p<0.05$.

Results and Discussion

Effect of plant growth regulators on the germination of conidia, on the growth of *A. alternata* and of *T. harzianum*, and on the biocontrol of *A. alternata* by *T. harzianum*

GA_3 or IAA or BAP or commercial formulations of hormones, at concentrations of 15 or 40 or 80 ppm did not affect germination of conidia or fungal growth. In fact, 10 $\pm$ 1% of conidia from *A. alternata* and 90 $\pm$ 10% of conidia from *T. harzianum* were germinated after 18 hours at 25°C in the presence or absence of the mentioned growth regulators. The presence of the foliar nutrient Bayfolan^R did not affect *T. harzianum* germination but slightly increased (12 $\pm$ 1%) that of *A. alternata* probably due to the presence of macro and micro elements in the formulation. On the other hand, the 2.4 $\pm$ 0.2 cm growth of *A. alternata* and 8.8 $\pm$ 0.2 cm growth of *T. harzianum* on PDA or MPA, after five days at 28°C, was not altered by the presence of any of the growth regulators or the foliar nutrient at the concentrations tested (Table 1). These results suggest that these plant growth regulators, either alone or included in the foliar nutrient formulation, may not affect germination and/or growth of the phytopathogen *A. alternata* or of the biocontrol agent *T. harzianum* at the field level, as a consequence of their use for the improvement of crop growth and productivity.

The *in vitro* biocontrol activity of *T. harzianum* was not affected by the presence of the growth regulators (either analytical grade or commercial products) or the foliar nutrient. In fact, a 20% decrease in *A. alternata* development was observed in dual cultures both in the absence and in the presence of the hormones, suggesting that the inhibition of growth of *A. alternata* was due to the presence of *T. harzianum* (Table 2). Also, an increase in the growth of *T. harzianum* was observed in these dual cultures, probably induced by the presence *of A. alternata* because none of the growth regulators or the foliar nutrient at the concentration used, altered this growth. Therefore, it could be expected that the use of plant growth regulators or formulations that contain any of these hormones at the field level would not affect the ability of *T. harzianum* to antagonize this fungal pathogen. Also, it appears that the presence of components added to commercial formulations of growth regulators or foliar nutrient do not alter the behavior of these fungi or of the phytohormones.

Effect of plant growth regulators and of *T. harzianum* on the secretion of endo-PG from *A. alternata*

The maximal secretion of endo-PG from *A. alternata* (Pérez et al. 1991) into submerged cultures was reduced in 20 - 25% when this phytopathogen was grown in the presence of 40 ppm of either GA_3, IAA, or BAP (Table 3). These results suggest that the hormones may interfere the secretion process of the enzyme or its levels, because none of the growth regulators tested decreased endo-PG activity of control. A stronger inhibitory effect was observed in the presence of active *T. harzianum*, which may be explained as a consequence of the growth inhibitory effect of the biocontrol agent on *A. alternata.* Due to the fact that *T. harzianum* also secretes endo-PG, although the enzymatic levels are much smaller than those secreted by *A. alternata* (Pérez et al. 1991), it was necessary to determine if endo-PG secretion by *T. harzianum* was altered by the presence of the phytopathogen. No changes were observed in enzyme activity at day of maximal secretion of endo-PG by *T. harzianum* as a consequence of the presence of *A. alternata*. On the other hand, and opposite to the effect on this phytopathogen, the sole presence of growth regulators in culture media resulted in an increase of endo-PG secretion by *T. harzianum* (Table 4). Endo-PG activity was not modified by the addition of the growth regulators at the assay medium of control, confirming the effect of these hormones on endo-PG secretion. It has been demonstrated that endo-PG from the biocontrol agent *T. harzianum*

are participating in the release of oligogalacturonide elicitors from *Citrus limon* cell walls during the development of the hypersensitive response (Fanta et al. 1992; Roco et al. 1993). These may be concentrated into elicitor-active molecules through the formation of complexes between endo-PG and PGIPs (Hahn et al. 1989). Therefore, the presence of growth regulators would stimulate one of the mechanisms for plant defense along with the ability to control fungal pathogens. Then, it may be suggested that during antagonism *T. harzianum* affects cellular mechanisms in *A. alternata* that result in a slow down in its development and in a decrease of its infectious ability based on the levels of secreted endo-PG.

Effect of plant growth regulators and of *A. alternata* on the secretion of endo-CH from *T. harzianum*

The presence of 40 ppm of GA_3, IAA or BAP did not affect the ability of *T. harzianum* to secrete endo-CH (Table 5). *A. alternata* slightly increased the secretion of total endo-CH activity of the biocontrol agent, suggesting that the presence of the pathogen could serve as an additional inducer of this fungal cell wall degrading enzymes. Then, the presence of this pathogen would stimulate one of the mechanisms *T. harzianum* uses for its biocontroller activity. The differential expression of isoenzymes of endo-CH by *Trichoderma* has been described during the mycoparasitism of this fungus on pathogens (Haram et al. 1996), thus accounting for its antagonism against several fungal pathogens.

It may be concluded that the presence of growth regulators does not affect the ability of *T. harzianum* to control *A. alternata*. Also, their presence could benefit plant from the elicitor activity of this *Trichoderma* that induces plant defense mechanisms against invading phytopathogens.

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