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Biocontrol mechanisms of *Trichoderma* strains

Summary. The genus *Trichoderma* comprises a great number of fungal strains that act as biological control agents, the antagonistic properties of which are based on the activation of multiple mechanisms. *Trichoderma* strains exert biocontrol against fungal phytopathogens either indirectly, by competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defensive mechanisms and antibiosis, or directly, by mechanisms such as mycoparasitism. These indirect and direct mechanisms may act coordinately and their importance in the biocontrol process depends on the *Trichoderma* strain, the antagonized fungus, the crop plant, and the environmental conditions, including nutrient availability, pH, temperature, and iron concentration. Activation of each mechanism implies the production of specific compounds and metabolites, such as plant growth factors, hydrolytic enzymes, siderophores, antibiotics, and carbon and nitrogen permeases. These metabolites can be either overproduced or combined with appropriate biocontrol strains in order to obtain new formulations for use in more efficient control of plant diseases and postharvest applications. [**Int Microbiol** 2004; 7(4):249-260]

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Introduction

Plant diseases play a direct role in the destruction of natural resources in agriculture. In particular, soil-borne pathogens cause important losses, fungi being the most aggressive. The distribution of several phytopathogenic fungi, such as *Phythium*, *Phytophthora*, *Botrytis*, *Rhizoctonia* and *Fusarium*, has spread during the last few years due to changes introduced in farming, with detrimental effects on crops of economic importance. In addition, not only growing crops but also stored fruits are prey to fungal infections [12].

Chemical compounds have been used to control plant diseases (chemical control), but abuse in their employment has favored the development of pathogens resistant to fungicides. Unfortunately, the more specific the effect of a chemical on an organism, the greater the probability of decreasing the effect through genetic shifts in the population, whereas fungicides of broad spectrum produce undesirable consequences on non-target organisms [52]. By contrast, the use of microorganisms that antagonize plant pathogens (biological control) is risk-free when it results in enhancement of resident antagonists. Moreover, the combination of such biological control agents (BCAs) with reduced levels of fungicide (integrated control) promotes a degree of disease suppression similar to that achieved with full fungicide treatment [42].

Antagonists of phytopathogenic fungi have been used to control plant diseases, and 90% of such applications have been carried out with different strains of the fungus *Trichoderma* (Fig. 1). Most of these strains are classified as imperfect fungi since they have no known sexual stage [42]. However, some *Trichoderma* species are morphologically similar to the anamorph *Hypocrea*, and their internal transcribed spacer (ITS) sequences have revealed their taxonomic

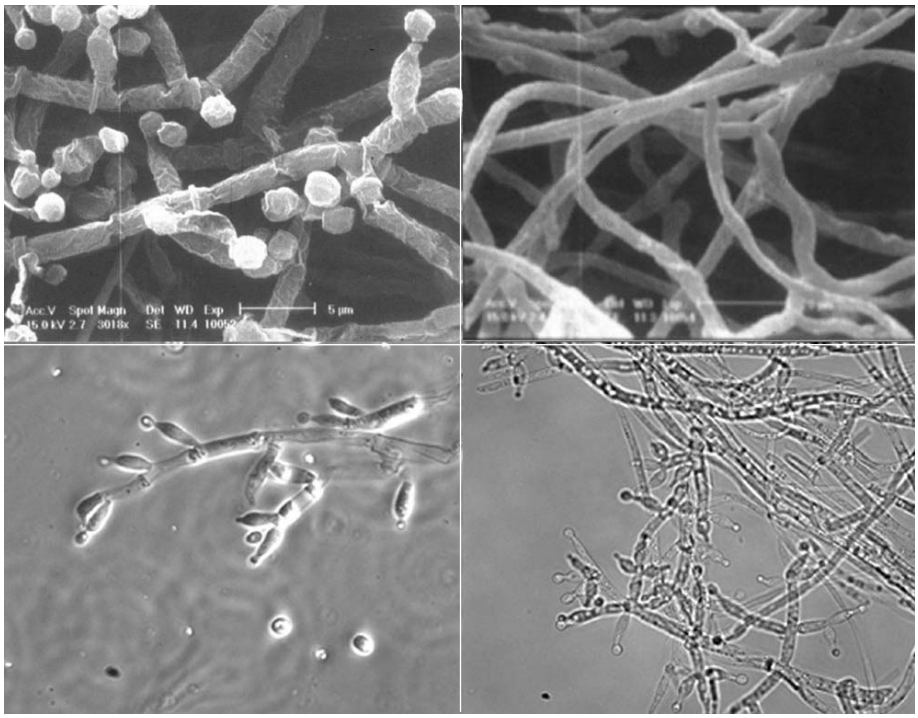

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Fig. 1. *Trichoderma harzianum* CECT 2413 as examined by electron (top) and light (bottom) microscopy.

proximity [30,42]. The most common BCAs of the *Trichoderma* genus are strains of *T. virens*, *T. viride* and, above all, *T. harzianum*, which is a species aggregate that includes different strains used as BCAs of phytopathogenic and viral vector fungi [27]. Molecular characterization and phylogenetic analysis have allowed strains of *T. harzianum* originally identified as the same species to be assigned to different species clustered into distinct sections and groups. No BCA corresponds to biotypes of *T. harzianum* that is pathogenic to mushrooms [30].

The success of *Trichoderma* strains as BCAs is due to their high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi, and efficiency in promoting plant growth and defense mechanisms. These properties have made *Trichoderma* a ubiquitous genus present in any habitat and at high population densities [12]. *Trichoderma* BCAs control ascomycetous, deuteromycetous and basidiomycetous fungi, which are mainly soil-borne but also airborne pathogens [42]. *Trichoderma* is more efficient in acidic than alkaline soils. Excellent results of integrated control have been attained with strains of *T. virens* and metalaxyl against *Pythium ultimum* infecting cotton [12], of *T. harzianum* and captan against *Verticillium dahliae* infecting potato [11], of *T. virens* and thiram against *Rhizoctonia solani* infecting tobacco, and others [12].

Biocontrol mechanisms and biocontrol improvement

BCAs are living organisms whose activities depend mainly on the different physicochemical environmental conditions to which they are subjected. For this reason, biocontrol exerted by *Trichoderma* strains is sometimes unpredictable. Understanding both the genetic diversity of strains within *Trichoderma* species and their mechanisms of biocontrol will lead to improved application of the different strains as BCAs. These mechanisms are complex, and what has been defined as biocontrol is the final result of different mechanisms acting synergistically to achieve disease control [32]. Biocontrol results either from competition for nutrients and space or as a result of the ability of *Trichoderma* BCAs to produce and/or resist metabolites that either impede spore germination (fungistasis), kill the cells (antibiosis) or modify the rhizosphere, e.g. by acidifying the soil, so that pathogens cannot grow. Biocontrol may also result from a direct interaction between the pathogen itself and the BCA, as in mycoparasitism, which involves physical contact and synthesis of hydrolytic enzymes, toxic compounds and/or antibiotics that act synergistically with the enzymes. *Trichoderma* BCAs can even exert positive effects on plants with an increase in plant growth (biofertilization) and the stimulation of plant-defense mechanisms.

This article is a general overview of the different reported mechanisms of biocontrol. In addition, some specific mechanisms and/or strategies used—or of potential use—to improve biocontrol are discussed. Additional and extensive information can be obtained from recent reviews listed in the References, such as those of Howell [31,32] and Harman et al. [29].

Biocontrol by competition

Fungistasis. Good antagonists are usually able to overcome the fungistatic effect of soil that results from the presence of metabolites produced by other species, including plants, and to survive under very extreme competitive conditions. *Trichoderma* strains grow rapidly when inoculated in the soil, because they are naturally resistant to many toxic compounds, including herbicides, fungicides and pesticides such as DDT, and phenolic compounds [12], and because the strains recover very rapidly after the addition of sublethal doses of some of these compounds. Resistance to toxic compounds may be associated with the presence in *Trichoderma* strains of ABC transport systems [29], as will be further discussed. For this reason, preparations of *Trichoderma* strains are very efficient in controlling several phytopathogens, such as *R. solani*, *P. ultimum* or *Sclerotium rolfsii*, when alternated with methyl bromide, benomyl, captan or other chemicals [57].

Competition for nutrients. Starvation is the most common cause of death for microorganisms, so that competition for limiting nutrients results in biological control of fungal phytopathogens [12]. For instance, in most filamentous fungi, iron uptake is essential for viability [22], and under iron starvation, most fungi excrete low-molecular-weight ferric-iron-specific chelators, termed siderophores, to mobilize environmental iron [22]. Subsequently, iron from the ferrisiderophore complexes is recovered via specific uptake mechanisms. In *Aspergillus fumigatus* and *Aspergillus nidulans*, siderophore biosynthesis is negatively regulated by carbon source [22]. In *Ustilago maydis*, gene products related to iron uptake affect the development of plant disease [41]. Some *Trichoderma* BCAs produce highly efficient siderophores that chelate iron and stop the growth of other fungi [11]. For this reason, soil composition influences the biocontrol effectiveness of *Pythium* by *Trichoderma* according to iron availability. In addition, *T. harzianum* T35 controls *Fusarium oxysporum* by competing for both rhizosphere colonization and nutrients, with biocontrol becoming more effective as the nutrient concentration decreases [52]. Competition has proved to be particularly important for the biocontrol of phytopathogens such as *Botrytis cinerea*, the main pathogenic

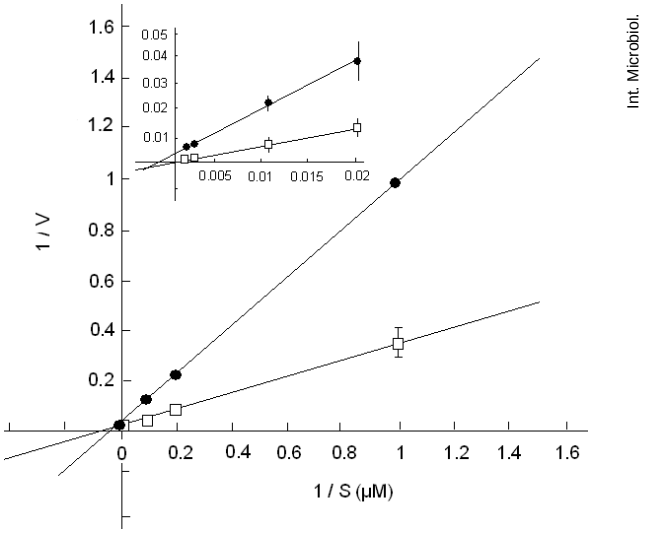

Fig. 2. Double-reciprocal plots used to obtain the K_m and V_{max} values for Gtt1, a high-affinity D-glucose transport of *Trichoderma harzianum* CECT 2413. Inset, amplification of the 1/S values from 0 to 0.02 µM. V, velocity; black circles, CECT 2413; white squares, T1. Error bars indicate standard deviations. Modified from [18].

agent during the pre- and post-harvest in many countries [36]. The extraordinary genetic variability of this fungus makes it possible for new strains to become resistant to essentially any novel chemical fungicide it is exposed to [36]. The advantage of using *Trichoderma* to control *B. cinerea* is the coordination of several mechanisms at the same time, thus making it practically impossible for resistant strains to appear. Among these mechanisms, the most important is nutrient competition, since *B. cinerea* is particularly sensitive to the lack of nutrients.

Trichoderma has a superior capacity to mobilize and take up soil nutrients compared to other organisms. The efficient use of available nutrients is based on the ability of *Trichoderma* to obtain ATP from the metabolism of different sugars, such as those derived from polymers wide-spread in fungal environments: cellulose, glucan and chitin among others, all of them rendering glucose [12]. The key components of glucose metabolism include assimilation enzymes and permeases, together with proteins involved in membrane and cell-wall modifications. While the role of the glucose transport system remains to be discovered, its efficiency may be crucial in competition [18], as supported by the isolation of a high-affinity glucose transporter, Gtt1, in *Trichoderma harzianum* CECT 2413 (referred to here as strain 2413). This strain is present in environments very poor in nutrients, and it relies on extracellular hydrolases for survival. Gtt1 is only expressed at very low glucose concentrations, i.e. when sugar transport is expected to be limiting in nutrient competition

[18]. In fact, glucose uptake is increased three- to four-fold in a transformant derivative that carried an additional copy of the transporter gene (Fig. 2). Only two other genes encoding glucose transporters have been described in filamentous fungi [25,56], one of them in *Uromyces fabae*. This basidiomycete has an ATPase and a proton-coupled glucose transport system that is expressed during infection of *Vicia faba*. This suggests an additional, antagonistic role for Gtt1, allowing the fungus to obtain energy from hydrolyzed polymers and to transport sugar rapidly into the cells. As a consequence, transformants able to transport glucose more rapidly than the wild-type [18] should be more efficient BCAs. This would serve as a very useful mechanism of nutrient competition during mycoparasitism interactions.

Promoter analysis of genes related to antagonism in *Trichoderma* strains revealed the presence of consensus sequences for transcription factors responsible for carbon (CreaA), nitrogen (AreA), Stress (Msn2/Msn4), pH (PacC) and mycoparasitism (MYC) regulation, among others [40]. Thus, appropriate manipulation of these regulators would provide an alternative to the isolation of more competitive BCAs. Extensive information on these regulators may be found in Mach and Zeilinger [40].

Biofertilization and stimulation of plant defense mechanisms

Trichoderma strains are always associated with plant roots and root ecosystems. Some authors have defined *Trichoderma* strains as plant symbiont opportunistic avirulent organisms, able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi and to produce compounds that stimulate growth and plant defense mechanisms [29].

Plant root colonization. *Trichoderma* strains must colonize plant roots prior to stimulation of plant growth and protection against infections. Colonization implies the ability to adhere and recognize plant roots, penetrate the plant, and withstand toxic metabolites produced by the plants in response to invasion by a foreign organism, whether pathogen or not. There are no data in the literature concerning *Trichoderma* genes specifically expressed during the interaction between fungus and plant roots, but there are several reports on altered gene expression during mycorrhizal development [25]. Mycorrhizal fungi interaction is modulated by plant flavonoids and fungal auxins, followed by morphogenetic events that include appressorium development [25]. In addition, genes that encode hydrophobins and other cell-wall structural proteins are specifically expressed, or their expression is up-regulated [25]. Hydrophobins and repellents are small, functionally similar hydrophobic proteins, that play fundamental roles in fungal morphogenesis, including infection structures, hyphal aggregation, cell to cell communication, and attachment of hyphae to hydrophobic surfaces and adhesion [33]. Strain 2413 stimulates growth of at least tobacco, tomato and cotton plants, and also protects them against several fungal plant pathogens (Fig. 3). Preliminary results have indicated the specific up-regulation of hydrophobins during colonization of *Trichoderma* strains and tomato plant roots (M.R. Chacón, Department of Genetics, University of Sevilla, personal communication).

Some *Trichoderma* strains establish long-lasting colonization of plant roots and penetrate into the epidermis. There, they produce or release compounds that induce localized or systemic plant resistance responses [29]. Plants react against fungal invasion by synthesizing and accumulating phytoalexins, flavonoids and terpenoids, phenolic derivatives, aglycones and other antimicrobial compounds. *Trichoderma* strains are generally more resistant to these compounds than most fungi; nonetheless, their ability to colonize plant roots strongly depends on the capacity of each strain to tolerate them [29]. This resistance, considered an essential requirement for plant colonization, has been associated with the presence of ABC transport systems in *Trichoderma* strains [29]. Furthermore, spontaneous mutants of *Aspergillus oryzae* that had high resistance to azoles overexpressed ABC transporter genes that were barely detectable in the wild-type [41]. Colonization of plant roots would thus be favored by the isolation of strains that, along with hydrophobins and repellents, also overexpress ABC transporters. Alternatively, the isolation of strains highly resistant to toxic compounds, such as fungicides and/or herbicides would also increase colonization, since such strains frequently display cross-resistance to antimicrobial compounds synthesized by plants.

Biofertilization. Root colonization by *Trichoderma* strains frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients [3]. Crop productivity in fields can increase up to 300% after the addition of *Trichoderma hamatum* or *Trichoderma koningii*. In experiments carried out in greenhouses, there was also a considerable yield increase when plant seeds were previously treated with spores from *Trichoderma* [12]. The same increase was observed when seeds were separated from *Trichoderma* by a cellophane membrane, which indicates that *Trichoderma* produces growth factors that increased the rate of seed germination [7]. However, there are very few reports on strains that produce

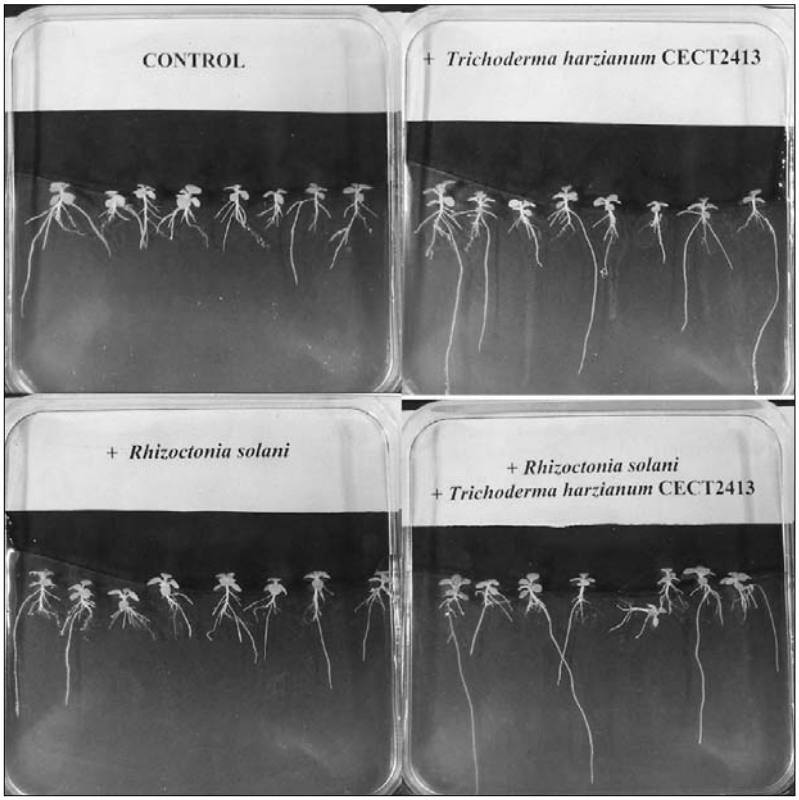

Fig. 3. *Rhizoctonia solani* protection and stimulation of tobacco root growth by *Trichoderma harzianum* CECT 2413.

growth factors which have been detected and identified in the laboratory (auxins, citokinins and ethylene) [3], despite the identification of many filamentous fungi that produce phytohormones, such as indol acetic acid (IAA) and ethylene, whose metabolic pathways have been identified [3,44]. *Trichoderma* strains that produce cytokinin-like molecules, e.g. zeatyn and gibberellin GA3 or GA3-related, have been recently detected. The controlled production of these compounds could improve biofertilization [44]. Together with the synthesis or stimulation of phytohormone production, most *Trichoderma* strains acidify their surrounding environment by secreting organic acids, such as gluconic, citric or fumaric acid [26]. These organic acids result from the metabolism of other carbon sources, mainly glucose, and, in turn, are able to solubilize phosphates, micronutrients and mineral cations including iron, manganese and magnesium [29]. Therefore, the addition of *Trichoderma* to soils where these cations are scarce results in biofertilization by metal solubilization and an increase in crop productivity; the poorer the soil, the more significant the subsequent yield increase is.

Stimulation of plant resistance and plant defense mechanisms. The ability of *Trichoderma* strains to protect plants against root pathogens has long been attributed to an antagonistic effect against the invasive pathogen [12]. However, these root-fungus associations also stimulate plant defensive mechanisms. Strains of *Trichoderma* added to the rhizosphere protect plants against numerous classes of pathogens, e.g. those that produce aerial infections, including viral, bacterial and fungal pathogens, which points to the induction of resistance mechanisms similar to the hypersensitive response (HR), systemic acquired resistance (SAR), and induced systemic resistance (ISR) in plants [29]. At a molecular level, resistance results in an increase in the concentration of metabolites and enzymes related to defensive mechanisms, such as the enzymes phenyl-alanine ammonio-lyase (PAL) and chalcone synthase (CHS), involved in the biosynthesis of phytoalexins (HR response), chitinases and glucanases. These comprise pathogenesis-related proteins (PR) (SAR response) and enzymes involved in the response to oxidative stress [49].

Plant genes respond to pathogens and elicitors. For this reason, plant defense mechanisms do not necessarily require stimulation by the living organism. The addition of *Trichoderma* metabolites that may act as elicitors of plant resistance, or the expression in transgenic plants of genes whose products act as elicitors, also results in the synthesis of phytoalexins, PR proteins and other compounds, and in an increase in resistance against several plant pathogens, including fungi and bacteria [14,24], as well as resistance to hostile abiotic conditions [29]. Barley expressing *Trichoderma atro-*

viride endochitinase Ech42 showed increased resistance towards *Fusarium* infection [41]. Expression of the chitinase Chit42 from *T. harzianum* in tobacco and potato plants resulted in transgenic lines highly tolerant or completely resistant to the foliar pathogens *Alternaria alternata*, *Alternaria solani* and *Botrytis cinerea* and to the soil-borne pathogen *Rhizoctonia solani* [32]. Similar results have been obtained with the heterologous expression of Chit42 in strawberry infected with *Colletotrichum* and with Chit42 and a β-1,6-glucanase in melon and tomato plants. In other cases, heterologous expression of pectinases and glucanases in plants resulted in a reinforcement of the enzymatic pool against pathogens but not in specific stimulation of plant resistance mechanisms [A.M. Rincón, PhD Thesis, Univ. of Sevilla, 2004]. Thus, plant protection seems to result exclusively from an increase in enzymatic activities.

Activation of defense responses using elicitors could be a valuable strategy—as an alternative to the use of transgenic plants—to protect plants against pathogens. Addition of laminarin, a β-1,3-glucan, to grapevines induced several defense genes and reduced infection by *B. cinerea* and *Plasmopara viticola* [5]. *Phytophthora parasitica* produces a cell-wall glycoprotein (CBEL) that has a cellulose-binding domain (CBD) and elicits defense responses in the host plant. Infiltration of CBEL into non-host plants also induced defense reactions [41]. Expression in plants of fungal chitinases with CBDs, such as Chit42CBD, which already has increased antifungal activity [38], may result in greater resistance against phytopathogens. It would be of interest to test the effect on plants of the addition of chitinases or glucanases produced by *Trichoderma* strains with respect to long-term increases in defense mechanisms, that is, a SAR-like response. Some authors have described that, when such plants have been in the presence of *Trichoderma*, their resistance persisted for long periods, at least several months [29]. A similar effect could take place after the addition of *Trichoderma* elicitors, which is particularly important in protecting fruits against post-harvest infections.

Rhizosphere modification

The soil environment influences spore germination, chlamydospore formation and the production of secondary metabolites, such as siderophores [22], antibiotics [12] and enzymes [4]. There are abundant data in the literature describing rhizosphere modifications by BCAs that impede colonization by pathogens; for instance, antibiotics and toxic metabolites produced by enthomopathogenic, mycoparasitic or mycoherbicide fungi [53]. Environmental pH is one of the major factors affecting the activity of both *Trichoderma* and pathogenicity factors secreted by different microorganisms. Some antibiotics are degraded at high pH; air drying and low pH may induce enzyme degradation by acidic proteases [16,17]; and the growth of many fungi is inhibited by weak acids, such as sorbic acid, due to a rapid decline in cytosolic and vacuolar pH [4]. Therefore, the ability to thrive over a wide range of external pH conditions is an important component of the complex set of characteristics that *Trichoderma*, best adapted to acidic soil, encounters during its interaction with other organisms. One of the mechanisms of *Trichoderma* strains for achieving colonization and pathogen control in a dynamic pH environment is an appropriate response to each given pH condition. Some strains of *T. harzianum* control external pH strictly, ensuring optimal values for their own secreted enzymes [41]. Different extracellular proteins are synthesized at different pHs. In addition, at the transcriptional level, several proteases, glucanases, cell-wall proteins and a glucose transporter are pH-controlled, which suggests a pH-dependent transcriptionally controlled response of different enzymes. External pH is also important to pathogens because their pathogenicity factors are produced only within a very narrow range of pHs [46] (Fig. 4A), so that pH modification determines the pathogen's ability to successfully colonize and invade the targeted host. *Trichoderma* strains able to modify external pH and to adapt their own metabolism to the surrounding growth conditions would consequently reduce the virulence of phytopathogens because most pathogenicity factors could not be synthesized.

A pH-sensing response system that most likely evolved in order to enable fungi to tailor their arsenal to best fit their environment is modulated by PacC, a transcriptional activator of alkaline-responsive genes and a repressor of acidic ones under alkaline conditions [4]. Regulation by PacC has been found in many fungi, including *T. harzianum* [41]. Loss of function mutations in *pacC* (pacC$^-$) cause expression of a phenotype that mimics the response to acidic conditions and results in the increased expression of acid-responsive genes and the reduced expression of alkaline ones [22]. Gain of function mutations in *pacC* (pacC^c) mimicking the response to alkaline conditions result in the opposite phenotype. PacC controls virulence in *Fusarium oxysporum*, so that PacC$^-$ mutants were more virulent than the wild-type in tomato root infections whereas pacC^c were significantly reduced in virulence [9,41] (Fig. 4B). *Trichoderma*'s ability to change environmental pH may be affected by the surrounding conditions such that a given strain may exert a different kind of control in response to different environmental pHs. PacC$^-$ and PacC^c *Trichoderma* mutants of strain 2413 have been isolated, and the mutants display phenotypes similar to those of *F. oxyspo-*

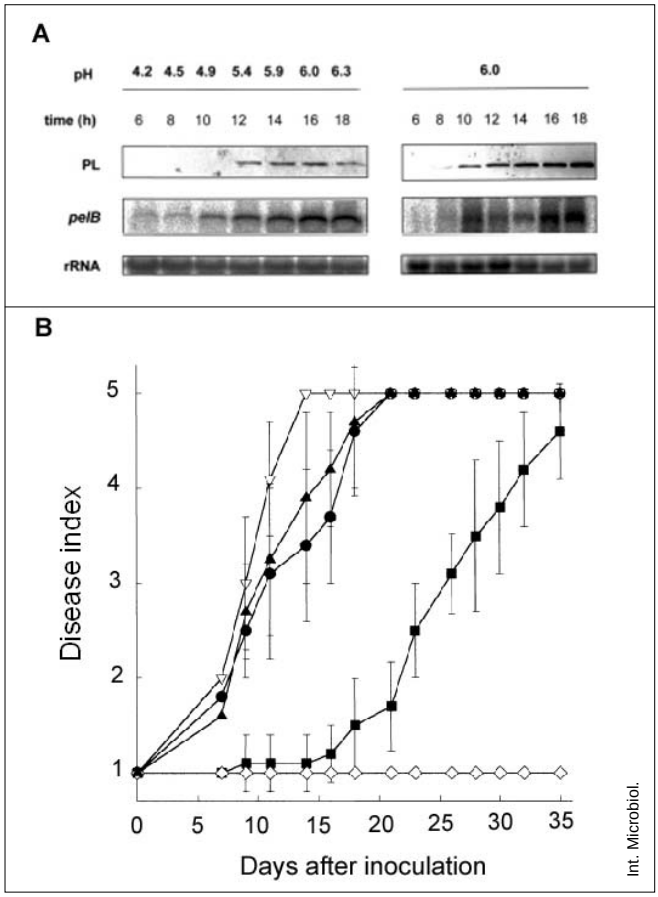

Fig. 4. Virulence is affected by pH. (**A**) Transcriptional activation of the pectinase genes *pelB* and *pl* and pectinase secretion in buffered and non-buffered secondary media. The growth medium for the phytopathogenic fungus *Colletotrichum gloeosporioides* was either not buffered or buffered to pH 6.0. Pectate lyase (PL) from the culture medium was analyzed by Western blotting. (Modified from [20]). (**B**) PacC negatively regulates virulence in *Fusarium oxysporum*. Incidence of *Fusarium* wilt caused by different *F. oxysporum* strains on tomato plants (cultivar Vemar). Severity of disease symptoms was recorded at different times after inoculation, using an index ranging from 1 (healthy plant) to 5 (dead plant). Symbols refer to plants inoculated with the wild-type strain 4287 (filled circles), loss-of-function mutant *pacC* +/ 12 (open inverted triangles), complemented strain *pacC* +/ 12 +*pacC* (filled triangles), dominant activating mutant *pacC*°9 (filled squares) and the uninoculated control (open diamonds). Error bars indicate the standard deviations from 20 plants for each treatment. Modified from [9].

rum with regard to their behavior at different pHs [41]; these could be introduced into environments characterized by pHs that are non-permissive for the wild-type.

Antibiosis

Antibiosis occurs during interactions involving low-molecular-weight diffusible compounds or antibiotics produced by *Trichoderma* strains that inhibit the growth of other microorganisms. Most *Trichoderma* strains produce volatile and non-volatile toxic metabolites that impede colonization by antagonized microorganisms; among these metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described [53]. In some cases, antibiotic production correlates with biocontrol ability, and purified antibiotics mimic the effect of the whole agent. However, there are also examples of antibiotic-overproducing strains, such as gliovirin-overproducing mutants of *T. virens*, which provide control similar to that of the wild-type, and of gliovirin-deficient mutants which failed to protect cotton seedlings from *Phytium ultimum*, whereas the parental strain did [12]. In general, strains of *T. virens* with the best efficiency as biocontrol agents are able to produce gliovirin [31]. Also, the most effective isolates of *T. harzianum* against *Gaeumannomyces graminis var. tritici* produce pyrone antibiotics, and the success of the strains was clearly related to the pyrones they produced.

The combination of hydrolytic enzymes and antibiotics results in a higher level of antagonism than that obtained by either mechanism alone [31, 42]. Synergetic effects between an endochitinase from *T. harzianum* and gliotoxin, and between hydrolytic enzymes and peptaibols on conidial germination of *B. cinerea* is well known [32]. A mutant from strain 2413 that had higher levels of extracellular enzymes and of α-pyrone performed better than the wild-type in in-vitro confrontation experiments against *R. solani* and in assays of grape protection against *B. cinerea*, both under repression (only pyrones were produced) and derepression conditions (enzymes and pyrones were produced) [47] (Table 1). Similarly, in transformants of strain 2413 that overexpressed Chit42 chitinase, biocontrol activity correlated with chitinase production, except for one transformant which was unable to completely overgrow *R. solani* and did not produce α-pyrone, so that synergism did not occur [38] (Fig. 5).

Sequential roles of antibiosis and hydrolytic enzymes during fungal interactions have also been described [32]. When combinations of antibiotics and several kinds of hydrolytic enzymes were applied to propagules of *B. cinerea* and *F. oxysporum*, synergism occurred, but it was lower when the enzymes were added after the antibiotics, indicating that cell-wall degradation was needed to establish the interaction [32].

Peptaibols—a class of linear peptides that generally have strong antimicrobial activity against gram-positive bacteria and fungi—act synergistically with cell-wall-degrading enzymes (CWDEs) to inhibit the growth of fungal pathogens and elicit plant resistance to pathogens [58]. In tobacco plants, exogenous applications of peptaibols trigger a defense

Table 1. Viability of *Rhizoctonia solani* after incubation under repression and derepression conditions with *Trichoderma harzianum* CECT 2413

Time (h)	Viability (%) of *R. solani* cocultivated with *T. harzianum*			
	WT (RC)	WT (DC)	PF1 (RC)	PF1 (DC)
0	100	100	100	100
10	80	83	84	65
20	89	15	65	0
36	80	0	23	0
60	75	0	8	0
90	58	0	0	0

WT, Wild-type; PF1, PFI mutant; DC, derepression conditions; RC, repression conditions. Viability was calculated as the ratio between growth of the dual-culture samples and that of the control without *T. harzianum*. Modified from [47].

response and reduce susceptibility to tobacco mosaic virus [58]. A peptaibol synthetase from *T. virens* has recently been purified, and the corresponding gene, which has been cloned, will facilitate studies of this compound and its contribution to biocontrol. An extensive review on antibiosis and production of *Trichoderma* secondary metabolites is provided in Howell [31,32].

Mycoparasitism

Mycoparasitism, the direct attack of one fungus on another, is a very complex process that involves sequential events, including recognition, attack and subsequent penetration and killing of the host. *Trichoderma* spp. may exert direct biocontrol by parasitizing a range of fungi, detecting other fungi and growing towards them. The remote sensing is partially due to the sequential expression of CWDEs, mostly chitinases, glucanases and proteases [29]. The pattern of induction differs from one *Trichoderma* strain to another. It is believed that fungi secrete exochitinases constitutively at low levels. When chitinases degrade fungal cell walls, they release oligomers that induce exochitinases, and attack begins.

Morphological changes. Mycoparasitism involves morphological changes, such as coiling and formation of appressorium-like structures, which serve to penetrate the host and contain high concentrations of osmotic solutes such as glycerol [41]. *Trichoderma* attaches to the pathogen with cell-wall carbohydrates that bind to pathogen lectins. Once *Trichoderma* is attached, it coils around the pathogen and forms the appresoria. The following step consists of the production of CWDEs and peptaibols [32], which facilitate both the entry of *Trichoderma* hypha into the lumen of the parasitized fungus and the assimilation of the cell-wall content. The significance of lytic enzymes, reviewed by Viterbo et al. [55], has been demonstrated by overexpression and deletion of the respective genes. Investigation on the responsible signal transduction pathways of *T. atroviride* during mycoparasitism have led to the isolation of key components of the cAMP and MAP kinase signaling pathways, such as α-subunits of G proteins (G-α), which control extracellular enzyme, antibiotic production and coiling around host hypha [41]. In *Trichoderma*, there is biochemical evidence for the participation of G-α in coiling, since an increase in coiling around nylon fibers was detected after the addition of activators of G-protein (mastoparan and fluoroaluminate) [43]. G-α gene (*tga1*) has been expressed either under the control of its own promoter or under the control of the promoter of the basic proteinase *prb1* in *T. atroviride* [48]. Both types of transformants showed an increase in coiling. Moreover, the capacity of *T. viride* overexpressing *tga1* to overgrow *Rhizoctonia* was also enhanced.

Cell-wall-degrading enzymes: chitinases. The chitinolytic system of *Trichoderma* comprises many enzymes and the list of its components is rapidly being updated as new enzymes and genes are reported. Chitinases are divided into 1,4-β-acetylglucosaminidases (GlcNAcases), endochitinases and

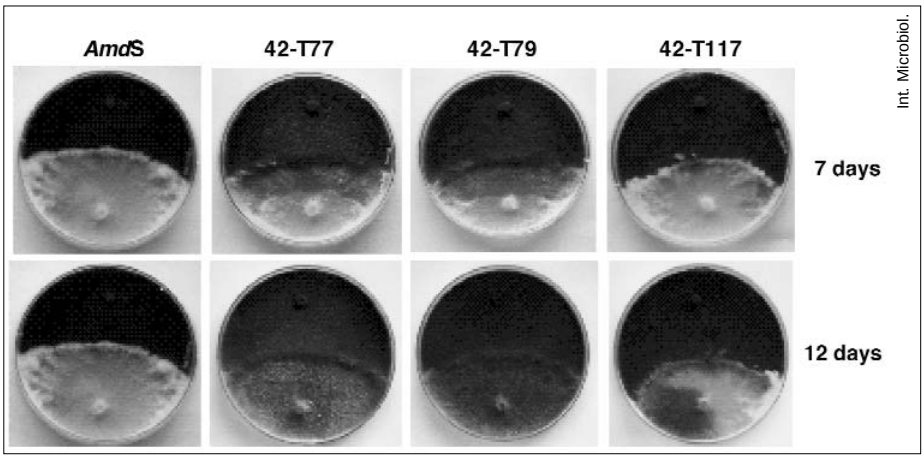

Fig. 5. Overgrowth and growth inhibition of *Rhizoctonia solani* by Chit42 transformants and the *AmdS* control. Mycelial discs of *Trichoderma harzianum* transformants and *R. solani* were place at the opposite sides of potato-glucose-agar (PPG) Petri dishes. Plates were incubated and photographs taken after 7 (top) and 12 (bottom) days incubation. Each Petri dish has *T. harzianum* at the top and *R. solani* at the bottom. Modified from [38].

exochitinases. Many GlcNAcases and their genes—*exc1* (=*nag1*), *exc2*, *tvnag1*, and *tvnag2* from *T. harzianum* T25-1, *T. atroviride* P1 and *T. virens* Tv29-8 —have been described [29,34]. The 73-kDa Nag1 represents the main GlcNAcase in *T. atroviride*. Nag1-disruption strain lacks chitinase activity, and the endochitinase *chit42* mRNA is absent [29]. This indicates that *nag1* is essential for triggering chitinase gene expression. The pathogen cell wall and chitin induce *nag1*, but it is only triggered when there is contact with the pathogen [10,29,32,39]. GlcNAcases CHIT73 and CHIT102 were detected in *T. harzianum* TM and *Trichoderma asperellum* [28]. CHIT102 triggers the expression of other chitinolytic enzymes [28]. In addition, strain 2413 produces three extracellular endochitinases whose genes, *chit33*, *chit37* and *chit42*, have been cloned from this strain. Other genes coding for Chit42 chitinase—*ech42*, *cht42* and *ThEn4*—have also been cloned from *T. atroviride* IMI206040 [10], Gv2908 [32] and *T. atroviride* P1 [32], respectively. Chit37 shows 89% similarity to Chit36 from *T. harzianum* TM at the amino-acid level [29]. Chit36 inhibits *B. cinerea* spore germination and the growth of both *Sclerotium rolfsii* and *Fusarium oxysporum* [54]. Other genes homologous to *chit36* have been cloned from *T. harzianum* TM, *T. atroviride* P1 and *T. asperellum* T–203. Endochitinases are regulated by a variety of mechanisms but induction by stress has been reported for *chit33*, *chit36* and *chit42*. However, the induction under mycoparasitic conditions is not clear. *ech42* is induced prior to any physical contact with *R. solani* [35]. *chit33* is expressed only during the contact phase and not before overgrowing *R. solani* [14]; and *chit36Y* does not need the direct contact of the pathogen to be expressed. *chit33*, *chit42* and *chit36* have been overexpressed in *Trichoderma* spp. in order to test the role of these chitinases in mycoparasitism, and the 42-kDa chitinase is believed to be a key enzyme [32]. *T. virens* transformants overexpressing Chit42 showed significantly enhanced biocontrol activity compared with the wild-type when assayed against *R. solani* in cotton seedlings experiments [32]. Other *Trichoderma* ssp. transformants overexpressing *chit42* resulted in better antagonism than obtained with the wild-type (see Fig. 5) [10,38]. In greenhouse biocontrol tests, however, the activity of chit42 disruptants did not differ from that of the wild-type [10,29]. *T. harzianum* transformants overexpressing Chit33 chitinase constitutively inhibited the growth of *R. solani* under both repressing and derepressing conditions; the antagonist tests demonstrated that this chitinase also has an important role in mycoparasitism [37]. *T. harzianum* Rifai TM transformants overexpressing Chit36 chitinase inhibited *F. oxysporum* and *S. rolfsii* more strongly than the wild-type. Moreover, culture filtrates inhibited the germination of *B. cinerea* almost completely [54]. The antagonism of Chit33 and Chit42 transformants has been improved by the addition of a cellulose-binding domain to the chitinase genes. As a result, the strains producing the chimeric enzymes increased their specific chitinase activity [38].

Cell-wall-degrading enzymes: glucanases. It has been shown that β-1,3-glucanases inhibit spore germination or the growth of pathogens in synergistic cooperation with chitinases [7,23] and antibiotics [29,32]. Many β-1,3-glucanases have been isolated, but only a few genes have been cloned, e.g. *bgn13.1* [7] and *lam1.3* [13] from *T. harzianum*, *glu78* [19] from *T. atroviride*, and *Tv-bgn1* and *Tv-bgn2* [34] from *T. virens*. However, only strains overexpressing *bgn13.1* from *T. harzianum* have been constructed [A.M. Rincón, PhD Thesis].

Transformants overexpressing BGN13.1 have been reported to inhibit the growth of *B. cinerea*, *R. solani* and *Phytophthora citrophthora*. Transformant T28, which had the highest BGN13.1 glucanase activity under both repressing and inducing conditions, showed the highest inhibition of pathogens. Antagonism was higher against *P. citrophthora*—oomycete with cellulose and glucans as the main cell wall components [7]—than against *Botrytis* or *Rhizoctonia* [A.M. Rincón, PhD Thesis], which have chitin and glucan as their main cell-wall components [7]. In addition, three β-1,6-glucanases have been purified from strain 2413 [7,15,24]. BGN16.2 exhibited antifungal properties alone or in combination with chitinases [7] and reduced the growth of *B. cinerea* and *Gibberella fujikuroi* [15]. Transformants producing BGN16.2 controlled *R. solani* and *B. cinerea* growth [7]. Cellulases (β-1,4-glucanases), comprising cellobiohydrolases, endoglucanases (*egl1*, *egl2*) and β-glucosidases, have not been widely studied for biocontrol purposes, although cellulose is abundant in oomycetes [6]. Migheli et al. [32] obtained transformants with greater biocontrol activity than the wild-type against *P. ultimun* on cucumber seedling. *T. harzianum* T3 produces a variety of cellulases, which make this isolate very effective in the control of *P. ultimun*. Other hydrolases, such as α-1,3-glucanases, have been purified from strain 2413, and their genes isolated and overexpressed, which resulted in increased biocontrol activity of the transformant strains [1].

Cell-wall-degrading enzymes: proteases. Biocontrol of *B. cinerea* by *T. harzianum* has been attributed in part to the action of proteases produced by the BCA that inactivate hydrolytic enzymes produced by this pathogen on bean leaves [32]. Proteases involved in the degradation of heterologously produced proteins have been characterized [16]. For

example, alkaline protease Prb1 from *T. harzianum* IMI 206040 has been demonstrated to play an important role in biological control [7], and *prb1* transformants showed an increase of up to five-fold in the biocontrol efficiency of *Trichoderma* strains against *R. solani*. Protease Pra1 from *T. harzianum* has affinity for fungal cell walls [24]. The gene for an extracellular serine protease (*tvsp1*) has been cloned from *T. virens* [45] and its overexpression significantly increased protection of cotton seedlings against *R. solani*. This gene shows great potential in improving biocontrol ability, as serine proteases are effective against oomycetes [21] and nematodes [8,32]. A serine protease of 28-kDa with trypsin activity isolated from strain 2413 also reduced the number of hatched eggs of root-knot nematodes and showed synergistic effects with other proteins produced during antagonistic activity of the strain [50]. Antal et al. [2] screened cold-tolerant strains and found that most of them antagonized phytopathogens and produced chitinases, β-glucosidases and trypsin-like, and chymotrypsin-like proteases, active at low temperatures. The role of proteases in mycoparasitism has been reinforced with the isolation of new protease-overproducing strains of *T. harzianum* [51]. Mutants with new profiles and higher quantities of secreted proteases were obtained by UV-irradiation. Some of these mutants have proved to be effective antagonists against plant pathogenic fungi such as *Fusarium culmurum* and *R. solani*.

Cell-wall-degrading enzymes: synergism. Synergism among lytic enzymes and between enzymes and antibiotics suggests formulations to test mixtures of *Trichoderma* transformants that produce different enzymes, in order to improve the antagonistic effects of BCAs on phytopathogenic fungi. For instance, in experiments carried out using cellophane, which show the effect of enzymes and secondary metabolites secreted by BCAs, *T. harzianum* wild-type inhibited the growth rate of *B. cinerea* by 30% and transformants expressing either a β-1,3-glucanase, a chitinase, or a β-1,6-glucanase inhibited the growth rate of *B. cinerea* by 60%. Transformants were differently combined in order to test synergism among the enzymes secreted against several phytopathogens. The combination that overproduced chitinase and β-1,3-glucanase was more effective than the individual transformants in inhibiting *Rhizoctonia meloni*, whereas using other combinations the inhibition was not improved [A.M. Rincón, PhD Thesis]. Southern experiments showed that none of the transformants replaced the other transformant when grown together or had any advantage in growth or enzyme production. On the whole, results seemed to be strain-specific.

Concluding remarks

Research on the mechanisms responsible for the biocontrol exerted by *Trichoderma* spp. on phytopathogenic fungi have led to a better understanding of such mechanisms, as well as to the isolation of several genes encoding either enzymes and structural or regulatory proteins, or components of signaling pathways that are involved in processes such as the specific recognition of hosts by *Trichoderma* strains. These tools will allow the isolation of improved strains and thus of more efficient formulations to control fungal pathogens in pre- and post-harvest periods.

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Mecanismos de biocontrol de cepas de *Trichoderma*

Resumen. El género *Trichoderma* está integrado por un gran número de cepas fúngicas que actúan como agentes de control biológico y cuyas propiedades antagónicas se basan en la activación de mecanismos muy diversos. Las cepas de *Trichoderma* pueden ejercer el biocontrol de hongos fitopatógenos indirectamente, compitiendo por el espacio y los nutrientes, modificando las condiciones ambientales, estimulando el crecimiento de las plantas y sus mecanismos de defensa o produciendo antibióticos. También pueden realizar ese biocontrol directamente, mediante micoparasitismo. Estos mecanismos pueden actuar de forma coordinada y su importancia en los procesos de biocontrol depende de la cepa de *Trichoderma*, del hongo al que antagoniza, del tipo de cultivo y de condiciones ambientales tales como la disponibilidad de nutrientes, el pH, la temperatura o la concentración de hierro. La activación de cada uno de los mecanismos implica la producción de metabolitos y compuestos específicos tales como factores de crecimiento de plantas, enzimas hidrolíticas, sideróforos, antibióticos y permeasas de carbono y nitrógeno. Estos metabolitos pueden sobreexpresarse o combinarse con cepas de biocontrol apropiadas, a fin de obtener nuevas formulaciones que puedan ser más eficaces en el control de enfermedades de plantas y en la protección de frutos post-cosecha. [**Int Microbiol** 2004; 7(4):249-260]

Palabras clave: *Trichoderma* · hongos fitopatógenos · antibiosis · biofertilización · enzimas hidrolíticas · protección de plantas

Mecanismos de biocontrole de cepas de *Trichoderma*

Resumo. O gênero *Trichoderma* é formado por um grande número de cepas fúngicas que atuam como agentes de controle biológico (BCAs) e cujas propriedades antagônicas se baseiam na ativação de mecanismos muito diversos. As cepas de *Trichoderma* podem exercer o biocontrole de fungos fitopatógenos indiretamente, competindo por espaço e nutrientes, modificando as condições ambientais, estimulando o crescimento das plantas e seus mecanismos de defesa ou produzindo antibióticos, ou diretamente mediante o micoparasitismo. Estes mecanismos podem, além do mais, atuar de forma coordenada e sua importância nos processos de biocontrole depende da cepa de *Trichoderma*, do fungo a que antagoniza, do tipo de cultivo e das condições ambientais tais como disponibilidade de nutrientes, pH, temperatura ou concentração de ferro. A ativação de cada um dos mecanismos implica na produção de metabólitos e compostos específicos tais como fatores de crescimentos de plantas, enzimas hidrolíticas, sideróforos, antibióticos e permeases de carbono e de nitrogênio. Estes metabólitos podem sobreexpressar-se ou combinar-se com cepas de biocontrole apropriadas, a fim de se obter novas formulações que possam ser mais eficientes no controle das enfermidades de plantas e na proteção de frutos pós-colheita. [**Int Microbiol** 2004; 7(4):249-260]

Palavras chave: *Trichoderma* · fungos fitopatógenos · antibiose · biofertilização · enzimas hidrolíticas · proteção de plantas