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# Ethylene is involved in *Trichoderma*-induced resistance of bean plants against *Pseudomonas syringae*

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**Abstract**

*Trichoderma* strains are effective biocontrol agents against a wide range of fungal plant pathogens. Current findings suggest that, in addition to microbial antagonism, the beneficial effect of *Trichoderma* is related to activation of plant defense responses. The aim of the present investigation was to study the effect of *Trichoderma harzianum* on oxidative enzyme activities and resistance to *Pseudomonas syringae* pv. *phaseolicola* of bean (*Phaseolus vulgaris* L.) plants. Special attention was focused on the role of ethylene as a signal mediating *Trichoderma*-induced responses. Both active and denatured fungal preparations induced sharp and transient increase of ethylene formation in bean leaves. The activity of both peroxidase (EC 1.11.1.7) and polyphenol oxidase (EC 1.10.3.2) increased in bean leaves after the treatment of derooted plants with active or inactivated *Trichoderma* formulations. Treatment with *Trichoderma* resulted in blocking of disease symptoms arising from inoculation with *P. syringae*. To demonstrate if the changes in defense responses and resistance against *Pseudomonas* were ethylene-dependent, bean seedlings were pretreated with 1-methylcyclopropene, a competitive inhibitor of ethylene action. Pretreatment with 1-methylcyclopropene led to a drastic acceleration of disease development both in control and *Trichoderma*-treated plants. The activities of peroxidase and polyphenol oxidase were suppressed by 1-methylcyclopropene treatment, suggesting that ethylene acts as one of the signals in *Trichoderma*-enhanced resistance against *P. syringae*.

**Key words:** ethylene, induced resistance, peroxidase, *Phaseolus*, polyphenol oxidase, *Pseudomonas*, *Trichoderma*.

## Introduction

*Trichoderma* species are among the most widely used biological control agents against soilborne plant pathogens (Hjeljord, Tronsmo 1998). Until recently, research on *Trichoderma*-plant interactions focused mostly on mycoparasitic aspects of plant protection. Only a few studies so far have dealt with plant defense responses induced by *Trichoderma* (Chang et al. 1997; Yedidia et al. 1999; Yedidia et al. 2000; Martinez et al. 2001). *Trichoderma*-derived xylanase and cellulase are well known inducers of ethylene biosynthesis (Avni et al. 1994; Piel et al. 1997). Recently it was shown that cellulase treatment leads to activation of defense mechanisms in melon cotyledons (Martinez et al. 2001). Considering that *Trichoderma* acts in rhizosphere, the effect of native formulations of the mycoparasite on defense responses in plant leaves has been a neglected aspect in plant resistance studies.

Peroxidase and polyphenol oxidase are oxidative enzymes contributing to defense

against pathogens. The peroxidase activity increases in plants during pathogen infection and has been correlated with resistance (Hammerschmidt et al. 1981; Bashan et al. 1987; Bestwick et al. 1998). High activity of peroxidase increased the ability of transgenic tobacco plants to suppress growth of the pathogenic bacterium *Erwinia carotovara* (Elfstrand et al. 2002). Several previous experiments have demonstrated the importance of polyphenol oxidase-mediated phenolic oxidation in restricting plant disease development. For example polyphenol oxidase-overexpressing tomato plants were shown to exhibit a great increase in resistance to *P. syringae* (Li, Steffens 2002). Compared with control plants, these transgenic lines showed less severity of disease symptoms, with over 15-fold fewer lesions, and strong inhibition of bacterial growth, causing more than an 100-fold reduction of bacterial population in the infected leaves. Consequently, high activities of peroxidase and polyphenol oxidase can be components of resistance against pathogens, including bacteria.

Ethylene is an important factor in the regulation of plant reaction to pathogens. An increase of ethylene formation in pathogen-challenged plants has been related both to defense responses leading to resistance as well as to symptom development during pathogenesis. The role of ethylene in incompatible interactions is mostly contradictory and depends on the pathogen used (Lawton et al. 1995; Hoffman et al. 1999; Thomma et al. 1999). Similarly, regarding to susceptible responses, ethylene insensitivity may lead to both increases and decreases of symptom development (Lund et al. 1998; Hoffman et al. 1999). In addition, recent findings indicate the role of ethylene in systemic acquired resistance (Knoester et al. 2001) and in induced systemic resistance (Pieterse et al. 2001). The fact that ethylene is a known inducer of several pathogen defense-related enzymes, e.g. peroxidase, glucanase, and chitinase, lends support to the regulative role of ethylene in resistance responses (Ishige et al. 1993; Xu et al. 1994; Siefert, Grossmann 1997; Ohme-Takagi et al. 2000).

The aim of the present experiments was to test the hypothesis that treatment of the above-ground parts of *Phaseolus vulgaris* with *Trichoderma* formulations can stimulate resistance against the bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola*. Special attention was focused on investigating the role of ethylene and oxidative enzymes in *Trichoderma*-induced defense mechanisms.

## Materials and methods

### *Plant material*

Seeds of *Phaseolus vulgaris* L. cv. *Saxa* bush bean were surface sterilized with $KMnO_4$ and sown individually into 10-cm plastic pots containing commercial peat-moss (pH 6.0) with addition of mineral nutrients. Plants at a two-leaf stage with fully grown primary leaves were used for experiments.

Plants were grown in a growth chamber with a photoperiod of 16/8 h in a photosynthetic photon flux density of 120 μmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C, 50 to 70 % relative humidity.

### *Treatment and inoculation*

*Trichoderma harzianum* Rifai isolate B-21 (provided by Dr. A. Lielpetere, *Bioefekts*, Riga, Latvia) was grown in darkness on plates with agarized malt extract, containing 50 g $l^{-1}$ malt extract and 15 g $l^{-1}$ agar. For inoculum preparation, the plates were washed with water to

collect spores. The inoculum concentration was determined to be $10^6$ germinated spores $ml^{-1}$. Inactive *Trichoderma* was prepared by autoclaving the inoculum for 40 min at 110 °C, 0.1013 MPa. For treatment, bean plants were derooted and individually incubated in glass flasks containing the appropriate *Trichoderma* inoculum for 4 h. Control plants were incubated in water. After treatment, the plants were further grown in water. Alternatively, for measurement of ethylene production, one leaf of the bean plants was sprayed with the appropriate *Trichoderma* formulation until run-off. Control plants were sprayed with water.

*Pseudomonas syringae* pv. *phaseolicola* was grown on glucose yeast extract agar medium plates for 2 days at 30 °C. The medium consisted of 20 g $l^{-1}$ glucose, 10 g $l^{-1}$ yeast extract, 20 g $l^{-1}$ $CaCO_3$, 17 g $l^{-1}$ agar. The bacterial cells were harvested, washed twice, and resuspended in 10 mM $MgCl_2$. The inoculum concentration was determined to be $10^8$ colony-forming units $ml^{-1}$.

Plants were inoculated 20 h after the start of the *Trichoderma* treatment one leaf per plant with *Pseudomonas syringae* pv. *phaseolicola*, for infiltration with a suspension of bacteria using a syringe without a needle. As a negative control plants were infiltrated with 10 mmol $l^{-1}$ $MgCl_2$.

### *Pretreatment with 1-methylcyclopropene*

For 1-methylcyclopropene (1-MCP) treatment, bean plants were placed within a 5-l polystyrene chamber 12 h before *Trichoderma* treatment was started. To release 1-MCP into a chamber, 10 mg of SmartFresh™ (AgroFresh Inc., USA) was placed in a vial containing 5 ml of water.

### *Measurement of ethylene formation*

For measurement of the basal rate of ethylene production (Kruzmane et al. 2002), detached leaves were rinsed with water, blotted dry, cut in half, rolled and placed individually in 4-ml screw-capped bottles. After 20 min, 1 ml of headspace gas was analyzed for ethylene concentration using a gas chromatograph Chrom 5 (Czech Republic) equipped with a glass column filled with $Al_2O_3$ and a flame ionization detector. Helium was used as a carrier gas.

### *Determination of enzyme activity*

For measurement of enzyme activitiy, leaves were rinsed with deionized water, frozen in liquid nitrogen and stored at -80 °C until analysis. For extraction of enzymes, leaf tissues were ground in liquid nitrogen and extracted with 25 mmol $l^{-1}$ HEPES (pH 7.2) for 15 min at 4 °C. The homogenate was filtered through nylon cloth and centrifuged at 15,000 $g_n$ for 15 min. Guaiacol peroxidase (EC 1.11.1.7) activity and diphenolase (EC 1.10.3.2) activity of polyphenol oxidase was measured spectrophotometrically in the supernatant as described previously (Kruzmane et al. 2002).

Standard Bradford assay was used to test the protein concentration in leaf extracts in each sample.

All experiments were repeated two or three times. All calculations (means and standard error) were performed using MS Excel. The Student's *t*-test was used to determine the levels of significance. All chemicals were purchased from Sigma-Aldrich Chemie, Germany.

## Results

### *Effect of* Trichoderma *on ethylene formation*

Two treatments were used for analyzing the effect of *Trichoderma* on ethylene formation in bean leaves: (i) one leaf of an intact bean plant was sprayed with an appropriate *Trichoderma* formulation and (ii) bean plants were derooted and individually incubated in *Trichoderma* inoculum. In general, treatment of bean plants with active or inactivated *Trichoderma* formulations resulted in an increase of the rate of basal ethylene production from the leaves (Fig. 1 and 2). However, when derooted seedlings were incubated in *Trichoderma* formulations, ethylene production intensity increased by ten times over that induced in intact seedlings. When *Trichoderma* was applied only to one leaf of intact bean plants, stimulation of ethylene production intensity was observed both in the treated and untreated systemic leaves (Fig. 1). Maximum ethylene production was reached at 4 h and 2 h after treatment with *Trichoderma* for intact and derooted bean plants respectively. In contrast to intact plants, where active formulation was more effective in eliciting an increase in ethylene production (Fig. 1), induction of ethylene production by active *Trichoderma* was less pronounced in leaves of derooted bean plants (Fig. 2). In addition, the dose response experiment revealed that a ten-times diluted *Trichoderma* formulation was more active in eliciting enhanced ethylene production from leaves of derooted seedlings than the undiluted solution (data not shown).

As treatment of derooted plants resulted in an earlier and more pronounced increase

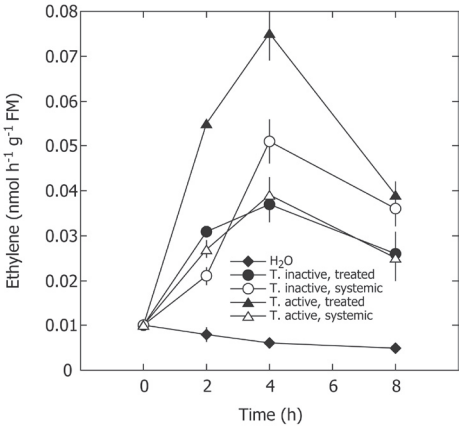


**Fig. 1.** Time course of ethylene formation in bean leaves treated with heat-inactivated or active culture of *Trichoderma harzianum*. One leaf of bean plants was sprayed with $H_2O$ or appropriate *Trichoderma* formulation. After the indicated intervals of time, both treated and non-treated leaves were detached and placed in bottles to accumulate ethylene during the subsequent 20 min. Values are the means ± SE of two independent experiments with four replicates each.

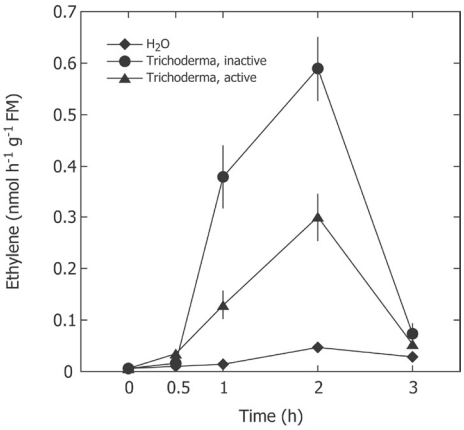


**Fig. 2.** Time course of ethylene formation in leaves of bean plants incubated in heat-inactivated or active cultures of *Trichoderma harzianum*. Bean plants were cut and immersed with the end of the stems in $H_2O$ or appropriate *Trichoderma* formulation. After the indicated intervals of time, leaves were detached and placed in bottles to accumulate ethylene during the subsequent 20 min. Values are the means ± SE of three independent experiments with three replicates each.

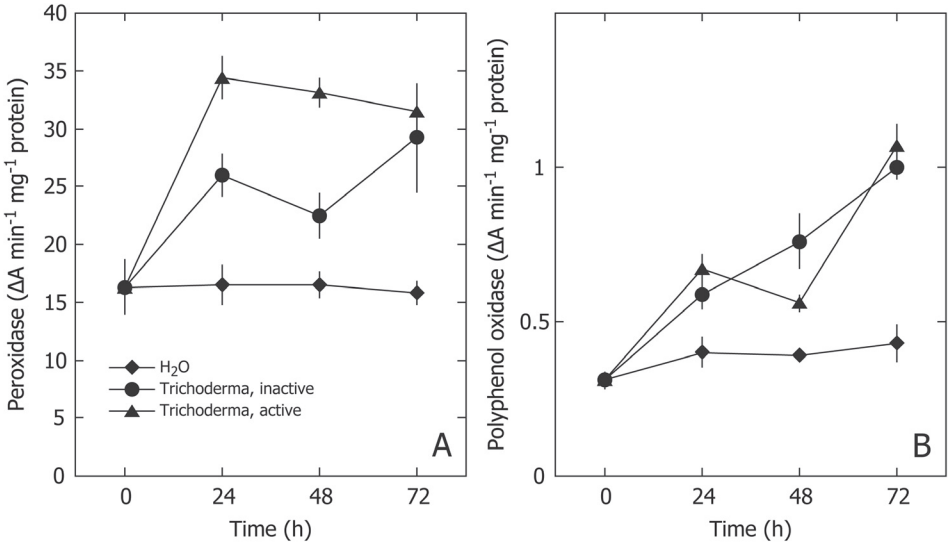


**Fig. 3.** Time course of peroxidase activity (A) and polyphenol oxidase activity (B) in leaves of bean plants incubated in heat-inactivated or active culture of *Trichoderma harzianum*. Bean plants were cut and immersed with the end of the stems in $H_2O$ or appropriate *Trichoderma* formulation for 4 h. After treatment, plants were further grown in water. After appropriate intervals of time, leaves were detached and used as material for enzyme analysis. Values are the means ± SE of two independent experiments with three replicates each.

of ethylene production than treatment of intact plants, for further experiments only derooted bean plants were used for treatment with *Trichoderma*.

### *Effect of* Trichoderma *on enzyme activity*

The activity of both peroxidase and polyphenol oxidase increased in bean leaves after the treatment of derooted plants for 4 h with active or inactivated *Trichoderma* formulations (Fig. 3). Treatment of plants with active *Trichoderma* resulted in a higher peroxidase activity 24 and 48 h after treatment than that with the inactivated mycoparasite formulation (Fig. 3A). In contrast, the polyphenol oxidase activity did not differ significantly between the active and inactivated formulations (Fig. 3B).

The role of ethylene as an inducer of enzyme activities resulting from *Trichoderma* treatment was tested by pretreatment of bean plants with gaseous 1-MCP, a competitive inhibitor of ethylene action. For active *Trichoderma*-treated plants, 1-MCP pretreatment resulted in a statistically significant decrease of *Trichoderma*-induced enzyme activities in the leaves, with a more pronounced inhibitory effect for polyphenol oxidase (Table 1). In contrast, when bean plants were pretreated with 1-MCP and subsequently treated with inactivated *Trichoderma*, both activities were further stimulated. 1-MCP pretreatment alone did not affect peroxidase activity in $H_2O$-incubated plants, however, polyphenol oxidase activity was slightly inhibited by the pretreatment.

### *Effect of* Trichoderma *on disease development in* P. syringae*-inoculated leaves*

Disease development in leaves of bean plants was analyzed 20 days after inoculation with

**Table 1.** Effect of 1-methylcyclopropene (1-MCP) pretreatment on the activity of peroxidase and polyphenol oxidase in bean leaves. The data represent means of two independent experiments performed in triplicate. Mean values of activity with the same letter within each column are not significantly different at P = 0.05

| Treatment | Enzyme activity (%) | |
|---|---|---|
| | Peroxidase | Polyphenol oxidase |
| $H_2O$ | 100[a] | 100[a] |
| 1-MCP + $H_2O$ | 99[a] | 87[a] |
| *Trichoderma* (active) | 175[c] | 168[c] |
| 1-MCP + *Trichoderma* (active) | 145[b] | 103[a] |
| *Trichoderma* (inactive) | 149[b] | 148[b] |
| 1-MCP + *Trichoderma* (inactive) | 207[d] | 206[d] |

*P. syringae*. Leaves inoculated with $MgCl_2$ were generally symptomless with an occasional wound at the site of inoculation (Fig. 4A). Leaves inoculated with *P. syringae* produced characteristic necrotic lesions at the sites of infiltration. Leaves of water-treated bean seedlings inoculated with *P. syringae* became diseased, with large spreading chlorotic areas developing both on inoculated leaves as well as on non-inoculated leaves (Fig. 4B). Treatment of bean plants with heat-inactivated *Trichoderma* resulted in smaller chlorotic areas on *P. syringae*-inoculated leaves with complete blocking of disease symptoms on non-inoculated leaves (Fig. 4C). Development of disease symptoms was blocked in active *Trichoderma*-treated plants inoculated with *P. syringae* (Fig. 4D). In comparison with control or inactive *Trichoderma*-treated bean plants development of broad, red-coloured zones around lesions at the sites of bacterial inoculation were evident on leaves of active *Trichoderma*-treated plants.

To test for possible involvement of ethylene in *Trichoderma*-induced limitation of disease development, bean plants were pretreated with gaseous 1-MCP 12 h before *Trichoderma* treatment. Blocking ethylene receptors with 1-MCP led to a drastic acceleration of disease development both in control and *Trichoderma*-treated plants. Ten days after inoculation with *P. syringae*, 1-MCP-pretreated plants had already developed intensive disease symptoms on their leaves with large chlorotic areas (Fig. 5C,D). In comparison, at that time 1-MCP non-treated control plants showed only the first signs of disease development (Fig. 5A). Further, 1-MCP-treated plants showed extremely fast disease development, with complete abscission of *P. syringae*-inoculated leaves only a few days later (data not shown).

## Discussion

Biocontrol mechanisms of *Trichoderma* have been studied mostly as mycoparasitic interactions, with focus on antibiosis and hydrolytic enzyme activity (Grondona et al. 1997). Here for the first time we report on *Trichoderma*-induced inhibiting effect on the development of bacterial disease symptoms in plant leaves. Based on our results, it is difficult to identify the precise means by which *Trichoderma* formulations act to restrict the pathogenicity of *P. syringae* in bean leaves. It can not be excluded that *Trichoderma*-produced

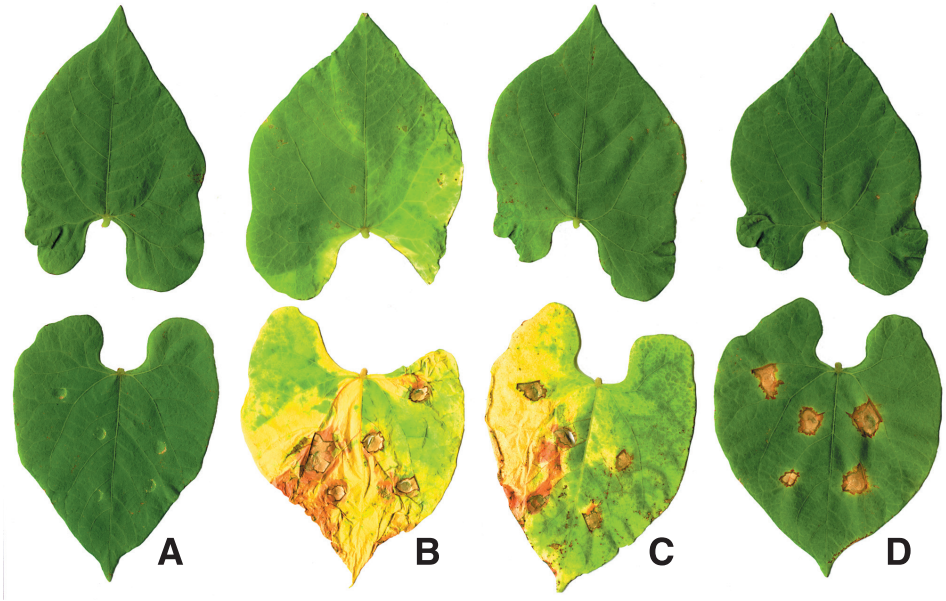


**Fig. 4.** Effect of heat-inactivated and active *Trichoderma harzianum* on disease development in leaves of bean plants inoculated with *Pseudomonas syringae* pv. *phaseolicola*. A, control plants, 10 mmol $l^{-1}$ $MgCl_2$; B, control plants, *P. syringae*; C, inactivated *Trichoderma*, *P. syringae*; D, active *Trichoderma*, *P. syringae*. Lower row, inoculated leaves; upper row, systemic leaves of the same plants. Bean plants were cut and immersed with the end of the stems in $H_2O$ or appropriate *Trichoderma* formulation for 4 h. After treatment, plants were further grown in water. Inoculation of bean plants with *P. syringae* was performed 20 h after the start of the *Trichoderma* treatment. Photographs were taken 20 days after inoculation. This experiment was repeated three times with ten plants per treatment and the representative symptoms are shown.

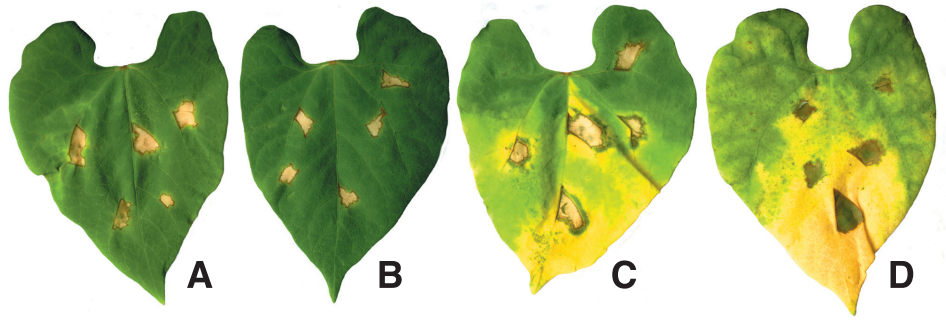


**Fig. 5.** Effect of 1-methylcyclopropene (1-MCP) pretreatment on disease development in leaves of bean plants treated with active *Trichoderma harzianum* and inoculated with *Pseudomonas syringae* pv. *phaseolicola*. A, no 1-MCP, control plants; B, no 1-MCP, *Trichoderma*-treated plants; C, 1-MCP pretreatment, control plants; D, 1-MCP pretreatment, *Trichoderma*-treated plants. Photographs were taken 10 days after inoculation. This experiment was repeated two times with ten plants per treatment and the representative symptoms are shown.

antibiotics directly suppress the growth of the bacteria (Faull et al. 1994). However, several observations suggest activation of plant defense responses resulting from the activity of the components of the *Trichoderma* formulations. Several cell wall-degrading enzymes in *Trichoderma* culture, e.g. xylanase and cellulase, can induce the formation of plant cell wall-derived oligogalactournoides, which further act as endogenous elicitors (Boudart et al. 1998; Enkerli et al. 1999). The increased activities of peroxidase and polyphenol oxidase can limit disease development through the formation of polymerized phenolic barriers around the sites of infection (Smit, Dubery 1997; Li, Steffens 2002). In this respect, in the present experiments *Trichoderma*-treated plants had noticeably intense red-colored zones around the sites of bacterial inoculation (Fig. 4). In addition, peroxidase and polyphenol oxidase can contribute to synthesis of anti-nutritive, antibiotic, and cytotoxic compounds leading to enhanced resistance against pathogens (Hammerschmidt, Nicholson 1999). We also found that *Trichoderma* increased ethylene formation in leaves of bean seedlings (Fig. 2). Ethylene, in turn, was essential for a delay of disease development (Fig. 5) and was partially necessary for the increase of oxidative enzyme activity (Table 1), suggesting that ethylene acts as one of the signals leading to enhanced resistance against *P. syringae*.

It is evident that *Trichoderma* can affect plant resistance to a pathogen either by inducing the basal level of defense reactions immediately after treatment or by enhancing a capacity for rapid and effective activation of cellular defense responses, which are induced only after contact with a challenging pathogen, a process known as "sensitization" or "priming" (Conrath et al. 2002). In the present experiments, we focused on the direct effect of active and heat-inactivated *Trichoderma* on defense responses.

One of the well-known responses of *Trichoderma*-derived xylanase and cellulase in plant tissues is induction of ethylene biosynthesis (Avni et al. 1994; Piel et al. 1997; Martinez et al. 2001). Our data on increased ethylene production due to *Trichoderma* treatment are in accordance with those reported earlier. Xylanase treatment in tobacco leaves caused an increase of ethylene biosynthesis with a maximum within 3 to 4 h (Avni et al. 1994). Similarly, cellulase from *Trichoderma viride* induced ethylene production in *Phaseolus lunatus* leaves with a maximum at 3.5 h after the treatment (Piel et al. 1997). In contrast, ethylene production from melon cotyledons infiltrated with cellulase from *Trichoderma longibrachiatum* peaked at 24 h after the treatment (Martinez et al. 2001). It is evident that, in comparison with purified cell wall-degrading enzymes, native *Trichoderma* formulations contain several substances that may affect ethylene biosynthesis. The more effective stimulation of ethylene production by diluted *Trichoderma* inoculum compared to undiluted suggests the presence of inhibitors of ethylene formation in the formulation. These inhibitors might be heat sensitive, as inactivated *Trichoderma* was more effective in eliciting ethylene production than active *Trichoderma*.

In our experiments, 1-MCP accelerated the development of bacterial infection both in control and *Trichoderma*-treated bean plants (Fig. 5). Consequently, ethylene is involved in mechanisms leading to delay of the development of infection in bean plants both in natural conditions as well as under the effect of *Trichoderma*. As 1-MCP completely blocked *Trichoderma*-induced polyphenol oxidase activity and partially blocked *Trichoderma*-induced peroxidase activity (Table 1), possibly ethylene-induced enzyme activities after *Trichoderma* treatment are among the putative mechanisms leading to increased resistance against *P. syringae*. However, 1-MCP-increased activities of peroxidase and polyphenol oxidase in heat-inactivated *Trichoderma*-treated bean plants suggests different effects

of ethylene in plants treated with active or inactivated *Trichoderma* extracts. It might be suggested that ethylene-suppressed enzyme activity in inactive *Trichoderma*-treated bean plants gives a lower degree of protection by inactivated *Trichoderma* against *P. syringae*. Our data contradicted those by Martinez et al. (2001), who reported that ethylene was responsible for the induction of peroxidase and chitinase activities in melon cotyledons by heat-denatured cellulase from *Trichoderma longibrachiatum*. The increase of activities of peroxidase and chitinase by active cellulase appeared to be independent of ethylene synthesis in these experiments. This contradiction may been ascribed to the fact that the *Trichoderma* cultures used in our experiments probably contained several substances besides cellulase with a potential effect on defense responses in general and on ethylene formation in particular.

As ethylene is involved in regulating the delay of disease symptoms in control plants (as suggested by the 1-MCP-induced acceleration of disease development, Fig. 5) without a statistically significant effect on peroxidase and polyphenol oxidase activities (Table 1), ethylene may be involved in the regulation of *P. syringae*-induced defense reactions. It is possible that ethylene is necessary for the regulation of the timing of induced defenses after pathogen challenge. Similarly, it has been shown that ethylene is involved in the generation or translocation of the systemically transported signal for rhizobacteria-mediated induced systemic resistance (Pieterse et al. 2001). A faster production of ethylene during the initial phase of infection might contribute to enhanced resistance. Therefore, we suggest ethylene not only as an inducer of basal defense responses, but also as one of the signals regulating priming during *Trichoderma*-induced resistance in bean plants. However, for a more complete understanding of the role of ethylene during natural and *Trichoderma*-induced resistance it is necessary to characterize the defense-related responses induced by *P. syringae* treatment.

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## Etilēns piedalās *Trichoderma*-inducētās izturības nodrošināšanā dārza pupiņām pret *Pseudomonas syringae*

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**Kopsavilkums**

*Trichoderma* sugas ir efektīvi biokontroles aģenti pret dažādiem sēņu dabas augu patogēniem. Jaunākie pētījumi liecina, ka *Trichoderma* labvēlīgā ietekme uz augiem ir saistīta ne tikai ar mikrobiālo antagonismu, bet arī ar augu aizsardzības reakciju aktivāciju. Dotā darba mērķis bija pētīt *Trichoderma harzianum* ietekmi uz dārza pupiņu (*Phaseolus vulgaris*) oksidatīvo fermentu aktivitāti un augu izturību pret *Pseudomonas syringae* pv. *phaseolicola*. Īpašu uzmanību pievērsa etilēna kā signāla nozīmei *Trichoderma*-inducētajās atbildes reakcijās. Gan aktīvs, gan denaturēts sēnes preparāts izsauca krasu un īslaicīgu etilēna veidošanās pieaugumu pupiņu lapās. Nogrieztu pupiņu augu inkubēšana aktīvā vai neaktīvā *Trichoderma* preparātā izraisīja peroksidāzes (EC 1.11.1.7) un polifenolu oksidāzes (EC 1.10.3.2) aktivitātes pieaugumu. Pupiņu augu apstrāde ar *Trichoderma* bloķēja *P. syringae* izsauktās slimības simptomus. Lai noteiktu, vai aizsargreakciju izmaiņas un izturība pret *Pseudomonas* bija atkarīga no etilēna, veica pupiņu augu priekšapstrādi ar 1-metilciklopropēnu, konkurējošu etilēna darbības inhibitoru. Šāda priekšapstrāde izsauca dramatisku slimības simptomu attīstību gan kontroles, gan ar *Trichoderma* apstrādātiem augiem. Inhibitora ietekmē samazinājās arī *Triochoderma* izsauktais peroksidāzes un polifenolu oksidāzes aktivitātes pieaugums, kas apliecināja etilēna kā signāla nozīmi *Trichoderma* izraisītās izturības pret *P. syringae* nodrošināšanā.