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Assessment of Potential for Biological Control of *Botrytis cinerea* by an Indigenous *Trichoderma harzianum* Isolate with a Novel Detached Leaf-droplet Inoculation Bioassay and Correlated Increase in Phytoalexin Production

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ABSTRACT

A simple detached leaf-droplet inoculation bioassay was devised in order to investigate the potential for biological control of *Botrytis cinerea* by indigenous *Trichoderma harzianum* isolates. Following preliminary *in vitro* screening of the antagonistic capacity of four local isolates of *Trichoderma harzianum* (T1-T4) against *B. cinerea*, T3 spore suspensions were then tested for their ability to control infection development of *B. cinerea* on T3 pre-inoculated lettuce and broad bean leaves, using different nutrient concentrations in *B. cinerea* infection inocula. Again, T3 proved to significantly control infection development in all but one of the treatments, as long as T3 spores were introduced in a nutrient solution. This bioassay provides a rapid and simple method of assessing biocontrol efficacy under *in vivo* conditions. T3 was also tested for its ability to induce plant defense mechanisms in the form of phytoalexin production after inoculation of broad bean cotyledons with T3 spore suspension. Thin layer chromatography indicated that T3 lead to the induction of relatively high phytoalexin yields, suggesting that this could be another potential 'indirect' mechanism of protection from pathogens by *T. harzianum* strain T3.

Keywords: broad bean, grey mould, lettuce, thin layer chromatography

INTRODUCTION

Botrytis cinerea Pers. Ex Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetz.) is the causal agent of grey mould disease, parasitising well over 200 hosts including field and glasshouse vegetables, soft fruit, ornamentals, bulb and corm-producing monocotyledons, and forest tree seedlings. *B. cinerea* is a pathogen of major economic importance (Jarvis 1980). Saprophytic soil fungi belonging to *Trichoderma spp*. are potent mycoparasites of several economically important plant pathogenic fungi and especially *B. cinerea*, and have therefore been tested for biocontrol potential in many field and greenhouse trials (Elad 2000). Examples for control of pathogens other than *Botrytis* include *Sclerotinia sclerotiorum* (Li *et al*. 2005), *Fusarium* spp. (Wang *et al*. 2005), *Colletotrichum acutatum* (Freeman *et al*. 2004), and *Bipolaris oryzae* (Abdel-Fattah *et al*. 2007).

Biocontrol of *B. cinerea* by *Trichoderma* spp. can be mediated by mechanisms that either directly or indirectly affect *B. cinerea* development. Direct modes of action include mycoparasitism (Labudova and Gogorova 1988) and production of inhibitory compounds (Ferreira 1990). Examples of indirect mechanisms are competition for nutrients (Zimand *et al*. 1995) and space (Dubos *et al*. 1982) because the presence of *Trichoderma* changes the microenvironment for *B. cinerea* development. For example, it has been shown that *Trichoderma* is a pioneer coloniser of senescing floral caps on developing grapes, thus preventing the saprophytic colonisation by *Botrytis* (Elad 1994). These different strategies are not mutually exclusive since *T. harzianum* T39 competes for nutrients (Zimand *et al*. 1995) and interferes with pathogenicity enzymes to control *B. cinerea* (Zimand *et al*. 1996). Some studies have demonstrated that *Trichoderma* spp. can also affect the host plant. Addition of *T. viride* cellulase to grapevine cell cultures induced plant defense reactions such as the hypersensitive response and phytoalexin production (Calderon *et al*. 1993), while a similar induction of plant defense reactions by *T. longibrachiatum* in tobacco plants was linked to an increased resistance to *Phytophthora parasitica* var. *nicotianae* (Chang *et al*. 1997). de Meyer *et al*. (1998) demonstrated that application of *T. harzianum* T39 at sites spatially separated from *B. cinerea* inoculation resulted in a 25-100% reduction of grey mould symptoms, caused by a delay or suppression of spreading lesion formation. Given the spatial separation of both microorganisms, this effect was attributed to the induction of systemic resistance by *T. harzianum* T39. More recent findings by Howell and Puckhaber (2005) demonstrated the induction of phytoalexins in cotton roots following application of *T. virens*. These findings suggest an indirect biocontrol effect of *Trichoderma* through the induction of plant defense mechanisms.

The aim of the present study was to observe the antagonistic effects of locally isolated strains of a known successful biocontrol agent (*T. harzianum)* against *B. cinerea* under *in vivo* conditions, using a simple detached leaf-based bioassay. This study also demonstrates the direct induction of phytoalexins in plant cotyledons following inoculation with a *T. harzianum* spore suspension.

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Original Research Paper

MATERIALS AND METHODS

Plant material

Lactuca sativa L. cv. 'Diana' and *Vicia faba* L. cv. 'Aquadulce' plants were used. They were obtained from stock plants grown in Shamrock™ Irish moss peat in Imperial College at Wye greenhouses at 22-28°C. Cotyledons were obtained from *V. faba* seeds that had been allowed to imbibe for four days, before emergence of the radicle. Leaves were obtained from approximately two-month-old plants.

Fungal material

An isolate of *Botrytis cinerea* (Bc) was used from stocks originally isolated from lettuce and maintained at Imperial College at Wye. Cultures were grown and maintained on standard potato dextrose agar (PDA; Sigma, St. Louis) medium at 20°C. Cultures were supplied with 16 h photoperiodic ultraviolet and white light to encourage fungal growth.

The four *Trichoderma harzianum* strains (T1, T2, T3 and T4) were provided by Prof. J. Mansfield from colonies isolated from mushroom compost and maintained at Imperial College at Wye. All potential control agents were maintained on PDA medium at 20°C.

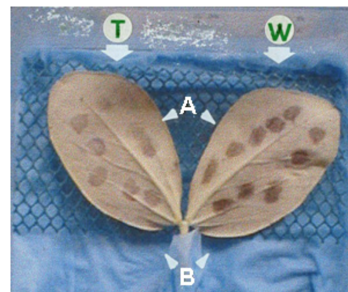

Fig. 2 Broad bean sample representing setup of detached leaf-droplet inoculation bioassay. 'T'= Trichoderma-pretreated leaf, 'W'= SDW-pretreated leaf. 'A' indicates area where Bc inoculum + 0% nutrient solution was introduced, while 'B' indicates area inoculated with Bc + 1% nutrient solution.

In vitro experiments

The four *T. harzianum* strains were assessed as growth antagonists by pairing them with *B. cinerea* on PDA plates. Plugs (0.5 cm diameter) from the growing edge of potential biocontrol agents and *Botrytis* colonies were placed on opposite sides of the Petri dishes. PDA plates inoculated with *Botrytis* only served as controls. Four plates were prepared for each of the pathogen/potential biocontrol agent combinations (see **Fig. 1**). The plates were incubated at 20°C. Growth of *Botrytis* was measured from the centre of the mycelial plug to the edge of inhibited growth formed between the potential biocontrol agent and *Botrytis*. Measurement started the day after inoculation took place (day 2) and proceeded daily until two days after the control plates became fully covered by *Botrytis* mycelial growth (at day 6). The experiment was carried out twice, and the results shown are typical of each repetition.

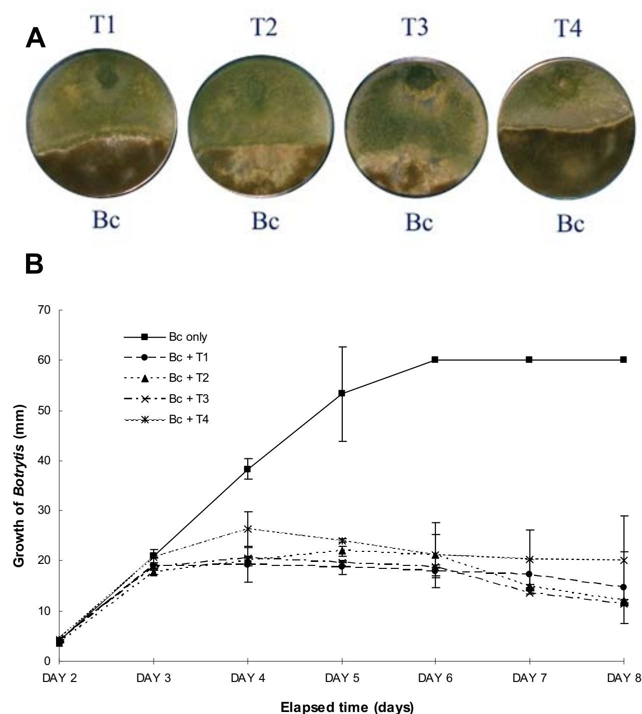

Fig. 1 *In vitro* antagonism assays. (**A**) Agar plates with *T. harzianum* isolates (T1-T4) and *B. cinerea* (Bc) six days after introduction of inocula. (**B**) Distance covered by Bc on agar plates in the presence or absence of potential biocontrol agents. Bc only: control; T1-T4: isolates of *T. harzianum*. Bars = ± SEM (n = 4). N.B.: Growth of *Botrytis* levels after day 6 because the pathogen colonised the whole plate.

In vivo experiments

All leaves used in the detached leaf infection assays were kept inside sterile sandwich boxes. Leaves were placed on a plastic mesh, which in turn was placed on top of sterile distilled water (SDW)-saturated blotting paper. Excess sterile water was removed at initial preparation to avoid waterlogged conditions and consequently reduce rotting risks. Abaxial leaf surfaces were used for all inoculations, so as to facilitate penetration of the pathogen (Verhoeff 1980).

For the purposes of *in vivo* spore suspension assays, approximately 5-10 ml of SDW was added to a one-week-old uncontaminated agar plate of either *B. cinerea* or *T. harzianum* isolate T3, and the surface of the culture was then scraped with a sterile loop to release spores. The water and suspended spores were filtered through fine muslin, and centrifuged at 3000 × g for 3 min. Excess water and nutrients were removed and replaced by clean SDW. Spore concentration was determined with a haemocytometer, and adjusted to the appropriate concentration (5×10^5 spores/ml for *B. cinerea* (Bc), 10^8 spores/ml for *T. harzianum* isolate T3). Two experiments were performed, in which one set of leaves was pre-coated with a T3 spore suspension with no additional nutrients, while the second included 1% (v/v) of 'Tesco' pure grape juice (Tesco Supermarkets, UK) in the T3 inoculum. Control samples were pre-coated with SDW or 1% (v/v) 'Tesco' pure grape juice, respectively. An example of the experimental setup is demonstrated in **Fig. 2**. Pre-coated samples were left on laboratory benches for three days, and subsequently inoculated with Bc spore suspensions containing various concentrations (0, 1, 5 and 10% v/v) of 'Tesco' pure grape juice. A total of twenty lettuce leaves and an equal number of broad bean leaves were inoculated with each of the pathogen/nutrient source combinations in replicate experiments.

Assessment of *B. cinerea* conidial infections

B. cinerea infections were assessed based on a four grade system, adopted as a simplified version of the key described by Rossall (1978): 0 = no lesion, 1 = flecked lesion, 2 = limited lesion, 3 = spreading lesion.

Identification of phytoalexins by Thin Layer Chromatography (TLC)

Vicia faba L. 'Aquadulce' seeds were left to germinate for three days in the dark. The testas from imbibed seeds were removed and the upper surface of the cotyledons was inoculated with 20 µl droplets of spore suspensions of *Bc* (5×10^5 spores/ml) and T3 (10^8 spores/ml), as well as SDW. These were kept in the dark for four days at room temperature, after which the surface layer of the cotyledons (where infection lesions were limited) was scraped off using a razor blade, weighed, and placed in McCartney bottles. Solvent (15 ml) containing 1:1 (v/v) ethanol and ethyl acetate was added to all specimens, which were incubated for five days in the

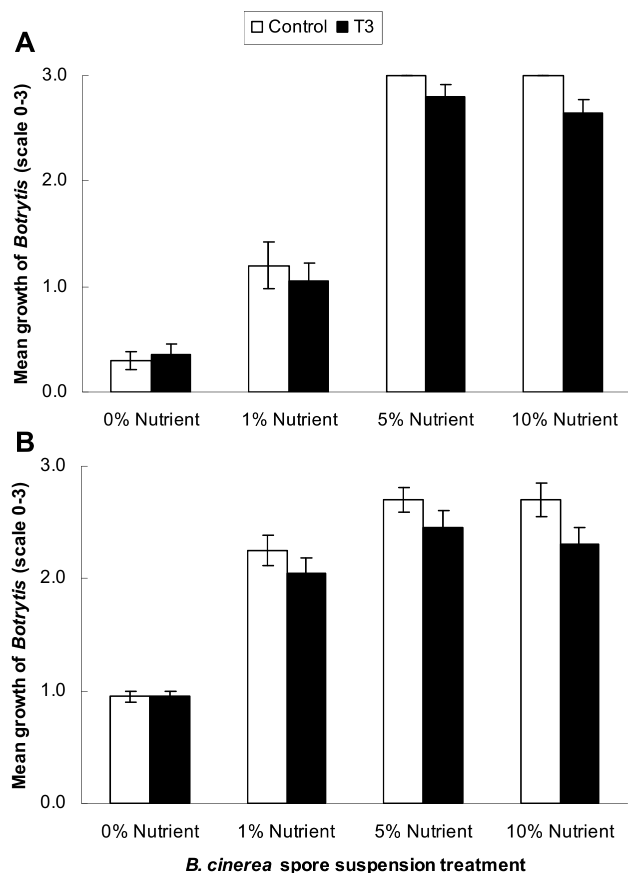

Fig. 3 Infection development by *B. cinerea* on (A) lettuce and (B) broad bean leaves in the presence or absence of *T. harzianum* isolate T3 spores (no nutrients). Clear bars indicate control (SDW-treated) leaves, and solid bars indicate T3-treated leaves. Infection was recorded one week after pathogen inoculation using a scale of 0-3 as adopted by Rossall, (1978). Bars = ± SEM (n = 20). Asterisk indicates significant difference between T3-treated and corresponding control (SDW-treated) leaves according to the Tukey's test (P = 0.05).

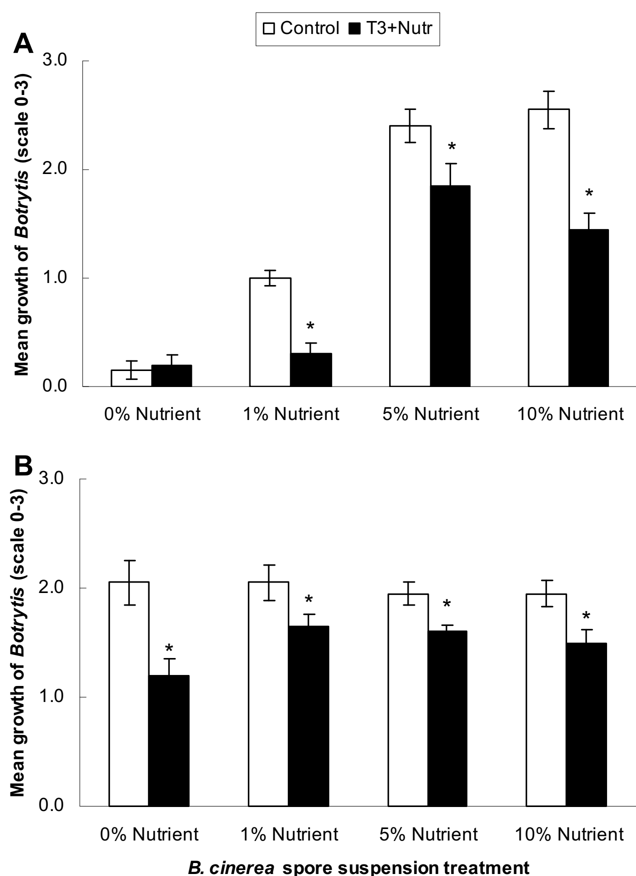

Fig. 4 Infection development by *B. cinerea* on (A) lettuce and (B) broad bean leaves in the presence or absence of *T. harzianum* isolate T3 spores (T3 spore suspension including 1% nutrient source). Clear bars indicate control (1% grape juice-treated) leaves, and solid bars indicate T3-treated leaves. Infection was recorded one week after pathogen inoculation using a scale of 0-3 as adopted by Rossall, (1978). Bars = ± SEM (n=20). Asterisk indicates significant difference between T3-treated and corresponding control leaves according to the Tukey's test (P = 0.05).

dark. After homogenisation of the tissue, extracts were recovered by centrifugation at 3000 × g, evaporated, and the residues of evaporation were dissolved with 5 ml of absolute ethanol.

The extracts were subsequently applied on 'Whatman' silica gel pre-coated TLC plates (20 × 20 cm, 250 mm thick; silica gel K6F, 60 Å). Thin layer chromatography was carried out, in order to isolate phytoalexins from the solution (as described by Hargreaves *et al*. 1977). Samples were analyzed under a UV absorption spectrophotometer using a Pye Unicam SP8-100 UV/VIS Spectrophotometer.

Identification of phytoalexins (PA) was achieved through a combination of maximum wavelength (λ) absorbance values recorded by UV spectrophotometry and calculation of retention factor (R_F) values for each compound. R_F equals to the fraction of the distance moved on the TLC plate by the compound and the distance moved by the solvent front. The total yield of each identified phytoalexin was calculated using the following formula (adapted from Hargreaves 1976):

Total yield (μg) = Absorbance at λ max. × Conversion factor × Volume of solution (ml). Conversion factor values were also taken from Hargreaves (1976). Finally, yield per g of bean tissue was calculated by dividing total yield by weight of bean tissue extracted.

Statistical analysis

Statistical analysis, when required, was completed using SPSS v.11 for Windows (SPSS Inc., Chicago, USA). Results of experiments were subjected to one-way analyses of variance (ANOVA) and where the F-value showed significant difference, Tukey's pairwise comparison test was applied to treatment means (at P=0.05). All graphs were displayed showing standard error mean (SEM) bars. It should be noted that as the dependent variable scale in **Figs. 3** and **4** has only four distinct values, the assumption of normal distribution in groups of ANOVA may likely be violated and any conclusions from ANOVA should therefore be treated as approximate.

RESULTS

In vitro antagonism assays of potential biocontrol agents against *B. cinerea*

The four potential biocontrol agents (T1-T4) were tested for their antagonistic properties against *B. cinerea* on PDA plates. All *T. harzianum* strains lead to inhibition of *Botrytis* growth, while strains T3 and T2 appeared to be most effective (**Fig. 1**). Subsequent calculation of the mean daily growth rate (DGR) of *Botrytis* confirmed the superiority of T3 and T2 in inhibiting *Botrytis* growth when compared to the other treatments (data not shown). Furthermore, T3 was the only agent that showed potential antibiotic production, as inhibition bands were produced (data not shown).

In vivo spore suspension assays of *T. harzianum* strain T3 against *B. cinerea* on lettuce and broad bean leaves

Having established the increased biocontrol efficiency of agent T3 under *in vitro* conditions, more detailed assays were carried out in order to further elucidate its biocontrol potential. For the purposes of these experiments, spore

Table 1 Yield/g of bean tissue (μg/g) of phytoalexins detected in SDW, *B. cinerea* (Bc) and T3-treated broad bean cotyledons. 'n.d.' indicates yield below detection levels.

Compound	Treatment		
	SDW	Bc	T3
Wyerone	21.3	6532.2	1941.4
Wyerone epoxide	-	165.1	52.46
Dihydrodihydroxyketowyerone	-	n.d.	n.d.

suspension inocula of T3 were used, since these are easier to produce in large quantities and have better potential of mass commercial production. *B. cinerea* infection inocula were supplemented with various concentrations of grape juice as nutrient source, as indicated in materials and methods.

No nutrients on T3 spore suspension inocula

Examination of the results in **Fig. 3** indicates that T3 does not significantly control *B. cinerea* on either lettuce or broad bean leaves, although it did actually slightly reduce the growth of the pathogen, when the latter was introduced with a source of nutrients. This inability of T3 to control *B. cinerea* is supported by the fact that T3-treated leaves did not differ significantly compared to SDW-treated (control) leaves in both lettuce and broad beans.

Use of nutrient source on T3 spore suspension inocula

The same experiment was repeated using a nutrient source (1% v/v grape juice) in the T3 spore suspension as well. The purpose of this experiment was to examine whether the presence of nutrients would enhance the survival of T3 on the phylloplane, as well as its overall biocontrol ability. Examination of the results in **Fig. 4** shows that the presence of a nutrient source in T3-treated leaves lead to significant suppression (at P=0.05) of the pathogen in both lettuce and broad bean leaves, with the exception of the 0% nutrient treatment on lettuce.

Phytoalexin production in *Vicia faba* L. following inoculation with *T. harzianum* T3

Four phytoalexins (PAs) were recognised following inoculation of *V. faba* cotyledons with *T. harzianum* and *B. cinerea*: wyerone, wyerone epoxide, dihydrodihydroxyketowyerone and a fourth PA which could not be identified. Only wyerone was produced in the case of SDW treatment. Dihydrodihydroxyketowyerone and the unknown PA did not produce quantitative data. Results obtained are summarised in **Table 1**.

DISCUSSION

Successful inhibition of *B. cinerea* growth both *in vitro* and on detached lettuce and broad bean leaves was achieved with a locally isolated *T. harzianum* strain (T3). Induction of relatively high phytoalexin yields was also recorded following inoculation of broad bean cotyledons with a T3 spore suspension, therefore concluding that this could be another potential 'indirect' mechanism of protection from pathogens by *T. harzianum* strain T3.

Initial screening of the four potential biocontrol agents for their antagonistic properties against *B. cinerea* under *in vitro* conditions showed that all agents had a suppressive effect on the pathogen, leading to inhibition of mycelial growth (**Fig. 1**). In all treatments apart from the control, daily growth rates for *B. cinerea* produced negative values from as early as 4d post-inoculation (data not shown), indicating that the potential biocontrol agents were starting to outgrow the pathogen, rapidly colonising and utilising the available medium. Strain T3 demonstrated the greatest capacity for competition for nutrients and space, since they lead to highest *B. cinerea* growth inhibition levels. In addition, T3 was the only agent that showed signs of potential antibiotic production, as inhibition bands were produced. Such a finding would be in general accordance with the conclusions of Prokkola (1992), who reported that the most promising isolates of *T. harzianum* for biocontrol purposes are those producing non-volatile antibiotics.

Having established the increased antagonism efficiency of agent T3 under *in vitro* conditions, more detailed assays were carried out in order to further elucidate its biocontrol potential. An *in vivo* spore suspension assay was devised and carried out, in which various concentrations of grape juice were included in the inocula as a nutrient source. Spore suspension inocula of T3 were preferred, since these are easier to produce in large quantities and have better potential of mass commercial production.

Observations made in control leaves (treated with SDW and grape juice; **Figs. 3** and **4**, respectively) suggest a correlation between grape juice concentration and disease development, thus indicating the importance of a nutrient source for successful establishment and colonisation by the pathogen (Blakeman 1980). However, a more interesting finding was that of the inability of T3 to efficiently inhibit pathogen growth when introduced without a nutrient source, while the presence of a nutrient source in the *Trichoderma* spore suspension greatly improved its level of protection against *Botrytis*, achieving statistically significant control of infection development in all but one of the treatments. This is likely the result of better survival of *Trichoderma* on the phylloplane (see Elad and Zimand 1991). According to work carried out by Tronsmo (1986) and Gullino *et al*. (1989) on strawberry and grape respectively, *Trichoderma* was more efficient against grey mould if the conidial suspension sprayed contained soluble cellulose. Furthermore, high relative humidity levels (which are easily reached within the sandwich boxes) proved to have a negative effect on disease control levels achieved by *Trichoderma* (see Elad *et al*. 1992). In general, poor activity often shown by *Trichoderma* against grey mould under severe disease pressure can be partly explained by its poor survival on the phylloplane under *in vivo* conditions (McKenzie *et al*. 1991). Future experiments could be carried out focusing on the evaluation of alternate nutrient sources (at various concentrations) for optimal performance.

T3 was then tested for its ability to induce plant defense mechanisms in the form of phytoalexin production following inoculation with a spore suspension on cotyledons of *Vicia faba*. Previous reports have demonstrated phytoalexin production in grapevine cells in response to inoculation with a *T. viride* elicitor (Calderon *et al*. 1993), in cucumber plants following application of *T. asperellum* to the root system (Yedidia *et al*. 2003), as well as in stems of pepper plants following similar root drenching with *T. harzianum* spores (Ahmed *et al*. 2000) Examination of the results presented in **Table 1** shows that all three treatments induced production of phytoalexins, including treatment with SDW. Mansfield *et al*. (1980) showed that traces of wyerone, wyerol and their dihydro analogues were even found in uninoculated broad bean cotyledons. High levels of phytoalexin production and accumulation after inoculation with *Botrytis* were also expected (Mansfield 1980). However, T3 also induced a noticeable although less significant yield of phytoalexins. Such an induction might act as one of the contributing factors, but not necessarily the main factor, in delaying lesion development in the leaves of infected plants, by rendering the plant more sensitive to future pathogenic infections, leading to faster and greater production and accumulation of PAs. In every case, the accumulation of phytoalexins demonstrates the potential of using T3 as a 'cross protection' agent against *Botrytis*, combining *Trichoderma*'s modes of antagonism with induced resistance.

However, it should be noted that the same induction of phytoalexin production might be one of the factors that lead to very low survival rates of *Trichoderma* on the phyllo-

plane for a prolonged period, since phytoalexins are toxic to microorganisms (Hahlbrock and Scheel 1987). Additional tests could be carried out following phytoalexin production after inoculation with T3, so as to prove whether or not these compounds have an actual antifungal effect on T3 and to which degree; such an investigation could lead to a possible explanation of one of the reasons causing low survival rates of *Trichoderma* on the phylloplane. Pacher *et al.* (2001) carried out a similar study, in which he identified various phytoalexins in leaves of *Glycosmis parviflora* and *G. pentaphylla* following infection with *B. cinerea*, and went on to verify their strong antifungal activity by bioautographic tests with *Cladosporium herbarum*.

There is a great potential for the future of biocontrol of *Botrytis cinerea*, with continuing research (e.g. the production of a genetically marked strain of *T. harzianum* to study soil population dynamics, Thrane *et al*. 1995; GUS transformants of *T. harzianum* isolate T39 for studying interactions on leaf surfaces, Freeman *et al*. 2002) allowing a greater understanding of the differences between *in vitro* and *in vivo* conditions and consequently producing more efficient antagonists in the field. Advances in molecular biology and biotechnology could also lead to the bioengineering of agents with enhanced biocontrol activity (e.g. Mendoza-Mendoza *et al*. 2003). Several 'biofungicides' have already been registered for commercial use in a number of countries (for examples see Batta 2004), and a number of other biopreparations should obtain registration for the control of *B. cinerea* in the near future, demonstrating the increasing interest towards biological control as people are becoming more aware of the need for an agriculture system which is economical and more respectful to the environment.

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REFERENCES

Abdel-Fattah GM, Shabana YM, Ismail AE, Rashad YM (2007) *Trichoderma harzianum*: a biocontrol agent against *Bipolaris oryzae*. *Mycopathologia* **164**, 81-89

Ahmed AS, Sánchez CP, Candela ME (2000) Evaluation of induction of systemic resistance in pepper plants (*Capsicum annuum*) to *Phytophthora capsici* using *Trichoderma harzianum* and its relation with capsidiol accumulation. *European Journal of Plant Pathology* **106**, 817-824

Batta YA (2004) Postharvest biological control of apple gray mold by *Trichoderma harzianum* Rifai formulated in an invert emulsion. *Crop Protection* **23**, 19-26

Blakeman JP (1980) Behaviour of conidia on aerial plant surfaces. In: Colley-Smith JR, Verhoeff K, Jarvis WR (Eds) *The Biology of Botrytis*, Academic Press, pp 115-152

Calderon AA, Zapata JM, Munoz R, Pedreno MA, Ros-Barcelo A (1993) Resveratrol production as a part of the hypersensitive-like response of grapevine cells to an elicitor from *Trichoderma viride*. *New Phytologist* **124**, 455-463

Chang P-FL, Xu Y, Narasimhan ML, Cheah KT, D'Urzo MP, Damsz B, Kononowicz AK, Abad L, Hasegawa PM, Bressan RA (1997) Induction of pathogen resistance and pathogenesis-related genes in tobacco by a heat-stable *Trichoderma* mycelial extract and plant signal messengers. *Physiologia Plantarum* **100**, 341-352

De Meyer G, Bigirimana J, Elad Y, Höfte M (1998) Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. *European Journal of Plant Pathology* **104**, 279-286

Dubos B, Jailloux F, Bulit J (1982) Protection du vignoble contre la pourriture grise: les propriétés antagonistes du *Trichoderma* a l'égard du *Botrytis cinerea*. *Colloq. INRA* **11**, 205-219

Elad Y (1994) Biological control of grape grey mould by *Trichoderma harzianum*. *Crop Protection* **13**, 35-38

Elad Y (2000) Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Protection* **19**, 709-714

Elad Y, Shtienberg D, Yunis H, Mahrer Y (1992) Epidemiology of grey mould, caused by *Botrytis cinerea* in vegetable glasshouses. In: Verhoeff K, Malathrakis NE, Williamson B (Eds) *Recent Advances in Botrytis Research, Proceedings of the 10th International Botrytis Symposium*, Heraklion, Crete, Greece, Pudoc Scientific Publishers, Wageningen, pp 147-158

Elad Y, Zimand G (1991) Experience integrated chemical-biological control of grey mould (*Botrytis cinerea*). *WPRS Bulletin* **14**, 195-199

Ferreira JHS (1990) *In vitro* evaluation of epiphytic bacteria from table grapes for the suppression of *Botrytis cinerea*. *South African Journal of Enology and Viticulture* **11**, 38-41

Freeman S, Maymon M, Kirshner B, Rav-David D, Elad Y (2002) Use of GUS transformants of *Trichoderma harzianum* isolate T39 (TRICHODEX) for studying interactions on leaf surfaces. *Biocontrol Science and Technology* **12**, 401-407

Freeman S, Minz O, Kolesnik I, Barbul O, Zveibil A, Maymon M, Nitzani Y, Kirshner B, Rav-David D, Bilu A, Dag A, Shafir S, Elad Y (2004) *Trichoderma* biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* and survival in strawberry. *European Journal of Plant Pathology* **110**, 361-370

Gullino ML, Aloi C, Garibaldi A (1989) Evaluation of the influence of different temperatures, relative humidities and nutritional supports on the antagonistic activity of *Trichoderma* spp. against grey mould of grape. In: *Influence of Environmental Factors on the Control of Grape Pests, Diseases and Weeds*, Proceedings of the EC Expert's Group, Thessaloniki, Greece, pp 100-104

Hahlbrock K, Scheel D (1987) Biochemical responses of plants to pathogens. In: Chet I (Ed) *Innovate Approaches to Plant Disease Control*, John Wiley and Sons, New York, pp 229-254

Hargreaves JA (1976) The role of phytoalexins in the disease resistance of *Vicia faba* L. to infection by *Botrytis*. PhD thesis, University of Stirling

Hargreaves JA, Mansfield JW, Rossall S (1977) Changes in phytoalexin concentration in tissues of broad bean plant (*Vicia faba* L.) following inoculation with species of *Botrytis*. *Physiological Plant Pathology* **11**, 227-242

Howell CR, Puckhaber LS (2005) A study of the characteristics of "P" and "Q" strains of *Trichoderma virens* to account for differences in biological control efficacy against cotton seedling diseases. *Biological Control* **33**, 217-222

Jarvis WR (1980) Taxonomy. In: Colley-Smith JR, Verhoeff K, Jarvis WR (Eds) *The Biology of Botrytis*, Academic Press, pp 1-18

Labudova I, Gogorova I (1988) Biological control of phytopathogenic fungi through lytic action of *Trichoderma* species. *FEMS Microbiology Letters* **52**, 193-198

Li GQ, Huang HC, Acharya SN, Erickson RS (2005) Effectiveness of *Coniothyrium minitans* and *Trichoderma atroviride* in suppression of sclerotinia blossom blight of alfalfa. *Plant Pathology* **54**, 204-211

McKenzie KL, Benzin D, Dellavalle D, Gullino ML (1991) Survival on the phylloplane of strains of *Trichoderma* spp. antagonistic to *Botrytis cinerea*. *Petria* **1**, 133-134

Mansfield JW (1980) Mechanisms of resistance to *Botrytis*. In: Colley-Smith JR, Verhoeff K, Jarvis WR (Eds) *The Biology of Botrytis*, Academic Press, pp 181-218

Mansfield JW, Porter AEA, Smallman RV (1980) Dihydrowyerone derivatives as components of the furanoacetylenic phytoalexin response of tissues of *Vicia faba*. *Phytochemistry* **19**, 1057-1061

Mendoza-Mendoza A, Pozo MJ, Grzegorski D, Martinez P, Garcia JM, Olmedo-Monfil V, Cortés C, Kenerley C, Herrera-Estrella A (2003) Enhanced biocontrol activity of *Trichoderma* through inactivation of a mitogen-activated protein kinase. *Proceedings of the National Academy of Sciences USA* **100**, 15965-15970

Pacher T, Bacher M, Hoferb O, Gregera H (2001) Stress induced carbazole phytoalexins in *Glycosmis* species. *Phytochemistry* **58**, 129-135

Prokkola S (1992) Antagonistic properties of *Trichoderma* species against *Mycocentrospora acerina*. In: Jensen DF, Hockenhull J, Fokkema N (Eds) *New Approaches in Biological Control of Soil-Borne Diseases*, IOBC/WPRS Bulletin, pp 76-79

Rossall S (1978) The resistance of *Vicia faba* L. to infection by *Botrytis*. PhD thesis, University of Stirling

Thrane C, Lübeck M, Green H, Degefu Y, Allerup S, Thrane U, Jensen DF (1995) A tool for monitoring *Trichoderma harzianum*: I. Transformation with the GUS gene by protoplast technology. *Phytopathology* **85**, 1428-1435

Tronsmo A (1986) *Trichoderma* used as a biocontrol agent against *Botrytis cinerea* rots of strawberry and apple. *Meldinger Fra Norges Landbrukshogskole* **65**, 1-22

Verhoeff K (1980) Infections and host-pathogen interactions. In: Colley-Smith JR, Verhoeff K, Jarvis WR (Eds) *The Biology of Botrytis*, Academic Press, pp 153-180

Wang H, Chang KF, Hwang SF, Turnbull GD, Howard RJ, Blade SF, Callan NW (2005) Fusarium root rot of coneflower seedlings and integrated control using *Trichoderma* and fungicides. *BioControl* **50**, 317-329

Yedidia I, Shoresh M, Kerem Z, Benhamou N, Kapulnik Y, Chet I (2003) Concomitant induction of systemic resistance to *Pseudomonas syringae* pv. *lachrymans* in cucumber by *Trichoderma asperellum* (T-203) and accumulation of phytoalexins. *Applied and Environmental Microbiology* **69**, 7343-7353

Zimand G, Elad Y, Chet I (1996) Effect of *Trichoderma harzianum* on *Botrytis cinerea* pathogenicity. *Phytopathology* **86**, 1255-1260

Zimand G, Elad Y, Gagulashvily N, Chet I (1995) Effect of the biocontrol agent *Trichoderma harzianum* T39 on the pathogenicity of *Botrytis cinerea*. *Phytoparasitica* **23**, 241-242