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Evaluation of *Trichoderma harzianum* and *Gliocladium roseum* in controlling leaf blotch of wheat *(Septoria tritici)* under *in vitro* and greenhouse conditions

Auswertung von *Trichoderma harzianum* und *Gliocladium roseum* bei der Bekämpfung der Blattfleckenkrankheit an Weizen, *(Septoria tritici)* unter *in vitro* und Gewächshausbedingungen

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Summary

The suitability of *Trichoderma harzianum* and *Gliocladium roseum* as biocontrol agents on the *in vitro* growing of *Septoria tritici* and their efficiency in reducing disease severity on wheat plants was evaluated under greenhouse conditions. For the *in vitro* tests, the micro-organisms were confronted through dual cultures on PDA. A histopathological clarification and staining technique was used in order to observe, if the penetration and development of the pathogen on wheat leaves was modified in the presence of the antagonistic agents. A highly significant effect of *T. harzianum* and *G. roseum* on the development of *S. tritici* colonies was observed. Both antagonistic agents grew over the *S. tritici* colony and covered it completely. *T. harzianum* was more efficient than *G. roseum* in inhibiting the pathogen. The coiling of the hypha of *T. harzianum* on short sections of *S. tritici* colony was observed with the bioassay on wheat leaves. The spore germination and the superficial mycelial development of *S. tritici* was affected by *T. harzianum* and *G. roseum*. Although, the further stomatic penetration of *S. tritici* was not restrained. The *in vivo* inhibitory capacity of the two antagonists assayed were not expressed on the wheat plants tested. However, there has been a tendency to reduce the percentage of necrotic area with pycnidial coverage when compared with the control. Further studies are needed before unequivocal conclusions can be drawn. Greenhouse experiments are in progress.

Key words: Biological control; phylloplane; wheat; *Septoria tritici; Trichoderma harzianum; Gliocladium roseum;* antagonism

Zusammenfassung

Es wurde die antagonische Wirkung von *Trichoderma harzianum* und *Gliocladium roseum* auf das Wachstum von *Septoria tritici in vitro* und die Befallsstärke an im Gewächshaus kultivierten Weizenpflanzen ausgewertet. In den *in vitro* Tests wurden die Mikroorganismen auf ihre antagonistische Wirkung in Dualkulturen auf Kartoffel-Dextrose-Agar geprüft. In Infektionsversuchen mit Weizen wurden histopathologische Aufhellungs- und Anfärbetechniken angewandt um zu untersuchen, ob Keimung, Penetration und Entwicklung des Pathogens *S. tritici* an Weizenblättern durch die Anwesenheit der Antagonisten beeinflusst werden. Es wurde eine hoch signifikante Wirkung von *T. harzianum* und *G. roseum* auf die Entwicklung von *S. tritici*-Kolonien beobachtet. Beide Antagonisten wuchsen über die *S. tritici*-Kolonien und bedeckten sie vollständig. *T. harzianum* hemmte das Pathogen stärker als *G. roseum*.

T. harzianum-Hyphen wuchsen um Hyphen von *S. tritici*. In den Versuchen an Weizenblättern wurde beobachtet, dass die Sporenkeimung und die Entwicklung des Oberflächenmyzels von *S. tritici* durch *T. harzianum* und *G. roseum* beeinträchtigt wurden. Allerdings konnte im Vergleich zur Kontrolle eine Tendenz hinsichtlich der Reduktion der nekrotischen Bereiche, die mit Pyknidien bedeckt waren, nachgewiesen werden. Weitere Untersuchungen sind erforderlich, bis eindeutige Schlussfolgerungen gezogen werden können. Gewächshausversuche werden durchgeführt.

Stichwörter: Biologische Bekämpfung; Phylloplane; Weizen; Antagonismus; *Septoria tritici, Trichoderma harzianum Gliocladium roseum*

1 Introduction

Leaf blotch of wheat, caused by *Septoria tritici* Rob. in Desm., is one of the most common diseases of this crop in the world. It is broadly spreaded in Argentine and it can cause significant yield reduction in some years. (Gilchrist et al. 1993). With favourable environmental conditions, yield reductions of 50 % or more have been mentioned (Eyal et al. 1987). Breeding, chemical treatments and appropriate cultural practices are the main ways for disease control (Eyal and Ziv 1974). The possibility of biological control of *S. tritici* using antagonistic micro-organisms is added to the former methods as a complementary strategy within the integrated management of this disease (Eyal 1981).

The biocontrol of foliar pathogens is a relatively new area compared with the biocontrol of soil-borne diseases.

Although scarce, existing information is optimistic, being an important number of antagonist identified (Andrews 1992a; Blakeman and Fokkema 1982; Sutton and Peng 1993; Windels and Lindow 1985, Cook 1993, Sutton et al. 1997; Elad 1994).

The application of bacteria, filamentous fungi and yeast for the control of *Septoria tritici, Septoria nodorum, Pyrenophora tritici-repentis* and *Cochliobolus sativus* on wheat have been mentioned (Levy and Eyal 1988; Fokkema et al. 1979; Li 1991).

The use of bacteria such as *Bacillus* and *Pseudomonas* isolated from soil, inhibited the *in vitro* growing of *S. tritici* and reduced the development of the disease in wheat plants markedly (Mehdizadegan and Gough 1987, Levy and Eyal 1988). Antagonistic fungi were reported to reduce the adverse effect of *Septoria* species. Saprophytic mycoflora of the wheat phyllosphere such as *Sporobolomyces* spp., *Cryptococcus* spp., *Aureobasidium pullulans* and *Cladosporium* spp. antagonized *Septoria nodorum,* the causal agent of *Septoria nodorum* blotch of wheat (Fokkema and van der Meulen 1976). *Sporobolomyces roseus* and *Rhodotorula* sp. from wheat leaves were effective as biocontrol agents in reducing *Cochliobolus sativus* and *Septoria nodorum* development (Luz 1985). Inhibition of spore germination of *Septoria* on barley was detected when spores of the epiphytes *A. pullulans* and *Cladosporium* sp. were added before the pathogen (Dickinson and Skidmore 1976). Interactions of *Septoria* with colonizers of senescent tissue as *Alternaria, Botrytis* and *Stemphylium* were also examined.

A variety of interactions between hyphae of foliar saprophytic fungi and *Septoria* on agar have been grouped by Skidmore and Dickinson (1976). *Phaeotheca dimorphospora,* a *Deuteromycotina* fungus, was found to be antagonistic against *Septoria musiva. P. dimorphospora* significantly reduced the size of the necrotic area caused by *S. musiva* on *Populus tremuloides, P. grandidentata* and *P. berolinensis.* In the greenhouse, both pre and postreatment of *P. berolinensis* with *P. dimorphospora* resulted in a significant reduction in the severity and in the rate of development of *Septoria* leaf spot (Yang et al. 1994; Yang et al. 1993).

The antagonistic agents of foliar pathogens may be chosen from the natural population of the phylloplane or from other native environments (Windels and Lindow 1985); they are, therefore, called foreign introduced organisms. Although they are not as well adapted to the phylloplane conditions as the ones which occur naturally, some of them, such as *Trichoderma* sp. and *Gliocladium* sp. have been succesfully used for biocontrol of pathogens on plant surfaces of cruciferous, solanaceous and gramineous plants (Rai and Singh 1980; Scharen and Bryan 1981; Elad and Kirschner 1993; Sutton and Peng 1993; Tronsmo 1983, 1986; Kumar and Singh 1985; Tapio 1989; Michereff et al. 1995). In this sense, the objective of this work was to determine the antagonistic effect of *Tricho-*

derma harzianum and *Gliocladium roseum* against *Septoria tritici in vitro,* and evaluate the efficacy for control the disease on wheat plants under greenhouse conditions.

2 Materials and methods

2.1 Isolates and cultures

T. harzianum isolate T15 and *G. roseum* isolate G10, isolated from soil around the horticultural area of La Plata, were used in this study. The isolate of *S. tritici* (I22) was taken from a collection of cultures of the pathogen with virulence tested on a set of differential hosts (Perelló et al. 1991). All fungi were maintained on potato dextrose agar (PDA) medium, pH 6.5, until used.

2.2 In vitro assays

The antagonistic activity of *T. harzianum* and *G. roseum* was tested *in vitro* using 90 mm Petri plates containing 15 ml of PDA medium, pH 6.5. Agar plugs (diameter 6 mm) taken from colonies of the pathogen and the antagonists were placed onto the plates 3 cm one from each other. *Septoria tritici* was inoculated 4 days before the respective antagonist, taking into consideration its lower growth rate. Three treatments were tested: *S. tritici-T. harzianum; S. tritici-G.roseum* and *S. tritici* control. The plates were incubated at 23 ± 2 °C with a 12 h photoperiod under fluorescent and NUV lighting. Each dual culture (pathogen-antagonist) had eight replications. After 12 days, the plates were evaluated for antagonistic activity, considering the ability of the micro-organisms to reduce pathogen colony expansion. Additionally, microscopic examinations of the area of intermingling growth (pathogen-antagonist) were made.

The inhibitory effect of conidia of *T. harzianum* T15 and *G. roseum* G10 on germination of *S. tritici* I22 was determined on slides containing 0.5 % water agar. Spore suspensions of *T. harzianum* (1×10^8) and *S. tritici* (5×10^6), *G. roseum* (1×10^8) and *S. tritici* (5×10^6) were mixed in a 1 : 1 ratio. A volume of 50 µl of the resulting preparation was pippeted on each slide, which was maintained in a Petri dish containing a wet filter paper. The Petri dishes were maintained at 20 °C under fluorescent light. The germination of spores of the pathogen was observed under light microscopy daily for up to 3 days. Each treatment contained six replicates.

2.3 Wheat leaf bioassay

A histopathological clarification and staining technique (Alippi 1986) in wheat plants was used in order to observe, if the penetration and development of *S. tritici* was modified in the presence of the antagonistic agents. Leaf pieces of 2,5 × 0,6 cm from the wheat cv. "Marcos Juarez INTA" were used for the infection tests. The upper surface of the leaves was sprayed with conidial suspensions of *S. tritici* alone (control) or *S. tritici* plus each antagonist, prepared and used at concentrations of 5×10^6 and 1×10^8 con./ml, respectively. *Septoria tritici* was applied 1 day later than antagonists. The leaves were fixed for 24 h in 2 : 1 (v/v) mixture of glacial acetic acid and absolute alcohol. They were then transferred in chloral hydrate (5 parts) in distilled sterile water (2 parts) for 24 h. Later on, the detached pieces of leaves were placed in a Petri dish with a Whatman N° 1 filter paper until the paper was fully soaked. Clearing was carried out in a 1 : 1 : 2 (v/v/v) solution of acetic acid, phenolic acid and chloral hydrate for 20 h. Trypan blue (0.05 %) in lacto-glycerine was prepared for stainning the leaves for 20–24 h. Treated leaves were removed and placed on glass slides in Petri dishes. Leaves were maintained at laboratory conditions (20 °C) and examined under a light microscope periodically.

2.4 In vivo assays

In order to measure the *in vivo* antagonism, a greenhouse test was carried out. Seedlings of the susceptible wheat cv. "Marcos Juarez INTA" were used. Five seedlings per pot were grown in 12 cm diameter × 15 cm depth plastic pots containing a mix of sand, peat and soil (1 : 1 : 1). At the third expanded leaf stage, a spore suspension of *T. harzianum* or *G. roseum* (each 10^8 con./ml) was sprayed

until runoff. One day later, a *S. tritici* spore suspension (5×10^6 con./ml) was applied. Plants having received *S. tritici*, *T. harzianum* or *G. roseum* spore suspension alone, served as control. After inoculation or application of the antagonists, the plants were covered with plastic bags for 72 h to ensure high humidity. The assay was carried out under the following conditions: Minimum mean temperature, 10 °C, maximum mean temperature, 20 °C. A completely randomized design with six replications was used. Each pot with five plants was considered one replication. After 24 days, the necrotic area with pycnidial coverage (NPA) was analyzed by ANOVA and Tuckey test at 0.05 level of probability.

3 Results

3.1 In vitro assays

The ANOVA for the diameter of the colonies in the three treatments showed a highly significant effect of both antagonists on the development of *S. tritici* colonies (Fig. 1). After 12 days, both antagonistic agents grew over the *S. tritici* colony and covered it completely (Figs. 3 and 4). *T. harzianum* was more efficient than *G. roseum* in inhibiting the pathogen. Microscopic examination of cultures of *T. harzia-*

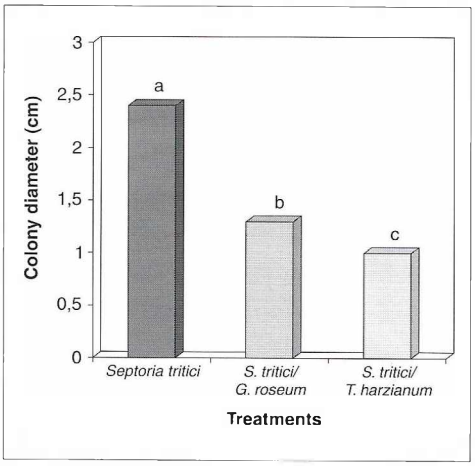


Fig. 1. Effect of *Gliocladium roseum* and *Trichoderma harzianum* on growth of *Septoria tritici* in dual cultures on PDA. Different letters indicate significant difference between treatments according to Tukey's test ($P < 0.05$).
Abb. 1. Durchmesser der Kolonie (cm) von *S. tritici* allein und in Anwesenheit von *G. roseum* und *T. harzianum* in Dualkultur auf Kartoffel-Dextrose-Agar.

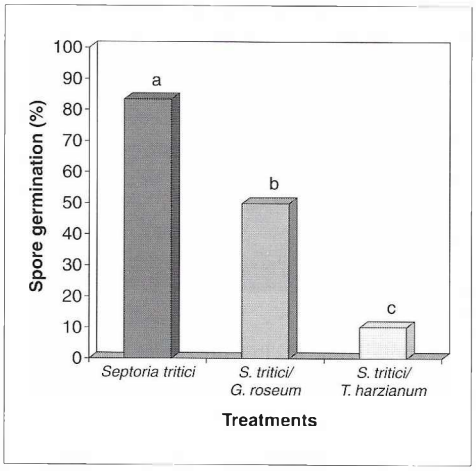


Fig. 2. Effect of *Gliocladium roseum* and *Trichoderma harzianum* on spore germination of *Septoria tritici*. Different letters indicate significant difference between treatments according to Tukey's test ($P < 0.05$).
Abb. 2. Einfluss von *G. roseum* und *T. harzianum* auf die Sporen-Keimung von *S. tritici*. Verschiedene Buchstaben bedeuten signifikante Unterschiede zwischen den Behandlungen nach dem Tukey's Test ($P < 0.05$).

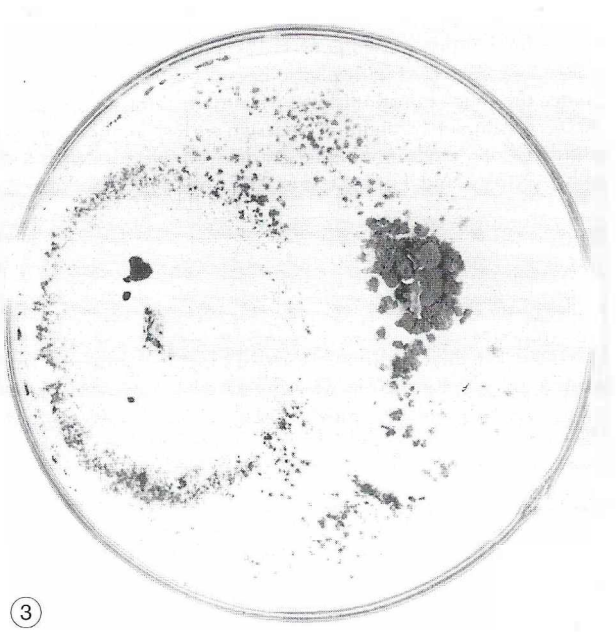


Fig. 3. Dual culture of *Trichoderma harzianum* and *Septoria tritici* on PDA 12 days after incubation.
Abb. 3. Dualkultur von *T. harzianum* und *S. tritici* auf Kartoffel-Dextrose-Agar nach 12 Tagen Inkubation.

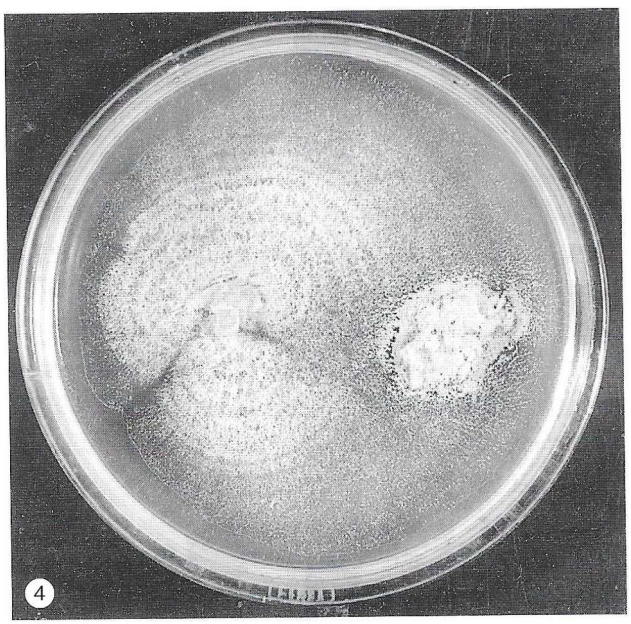


Fig. 4. Dual culture of *Gliocladium roseum* and *Septoria tritici* on PDA 12 days after incubation.
Abb. 4. Dualkultur von *G. roseum* und *S. tritici* auf Kartoffel-Dextrose-Agar nach 12 Tagen Inkubation.

num and *S. tritici* in close proximity showed hyphae of *T. harzianum* coiled around hyphal segments of *S. tritici*. However, the frequency of appearance of coiling was quite low.

Germination of conidia of *S. tritici* in the presence of *T. harzianum* and *G. roseum* was reduced significantly after 72 h (Fig. 2). Paired suspensions indicated that *T. harzianum* was the greatest antagonist of conidial germination, inhibiting germination by 87,91 %.

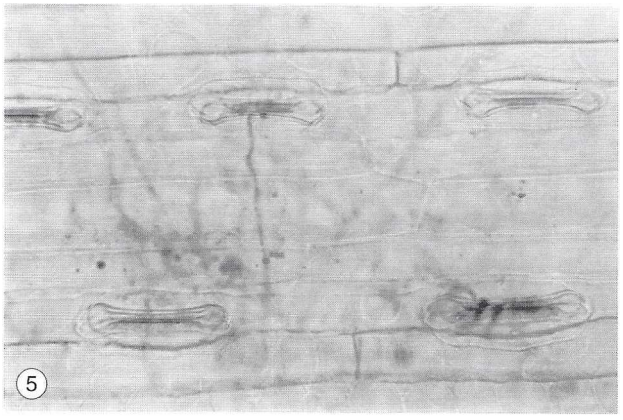
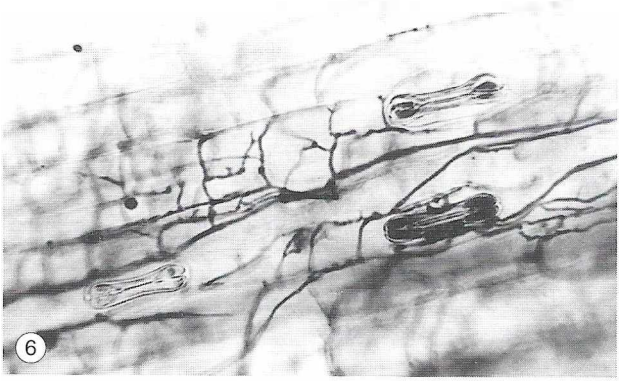


Fig. 5. *Septoria tritici* appressoria on "Marcos Juarez INTA" wheat leaves 24 h after incubation.
ap: appresoria gc: guard cell.
Abb. 5. Appressorien von *S. tritici* 24 h nach Inkubation auf Blättern von Weizen cv „Marcos Juarez INTA" (× 280)
ap: Apressorien cg: Schliesszelle.



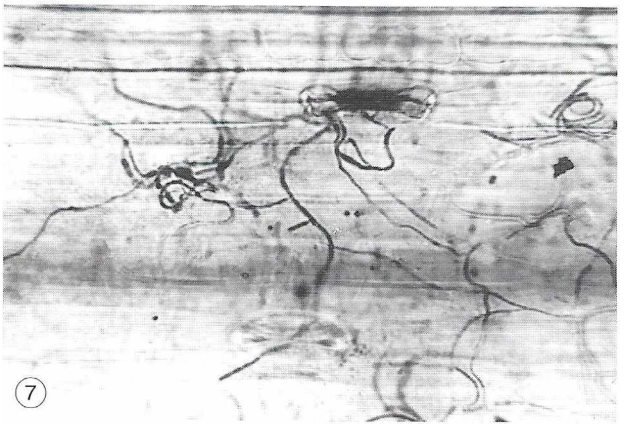


Fig. 6 and 7. Pycnidiospores germinated and mycelial development of *Septoria tritici* over the surface of wheat leaves (× 280).
Abb. 6 und 7. Keimung von Pyknosporen und Myzelentwicklung von *S. tritici* auf der Oberfläche von Weizenblättern (× 280).

3.2 Wheat leaf bioassay

At 24 h after inoculation, 70–80 % of the conidia of *S. tritici* had germinated and structures like appressoria (Fig. 5) were detectable occurring over stomata. At 48 h post-inoculation, germination rate increased and hyphal ramification was observed (Figs. 6 and 7). Only stomatic penetration of

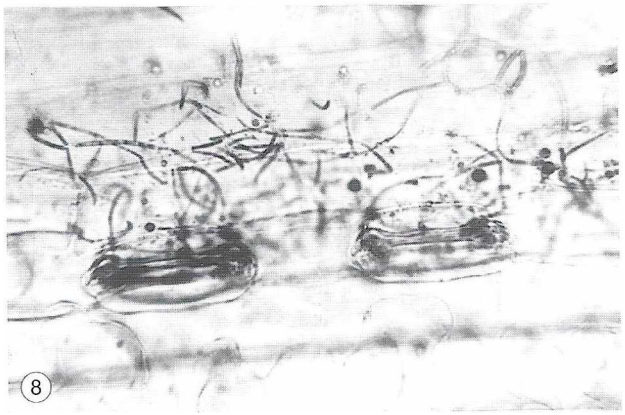
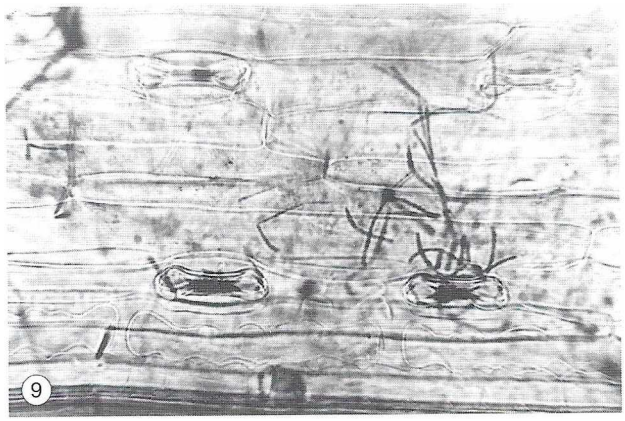


Fig. 8 and 9. Pycnidiospores germinated and mycelial development of *Septoria tritici* over the surface of wheat leaves sprayed with *Trichoderma harzianum* and *Gliocladium roseum* (× 280).
Abb. 8 und 9. Keimung von Pyknosporen und Myzelentwicklung von *S. tritici* auf der Oberfläche von Weizenblättern in Anwesenheit von *T. harzianum* und *G. roseum* (× 280).



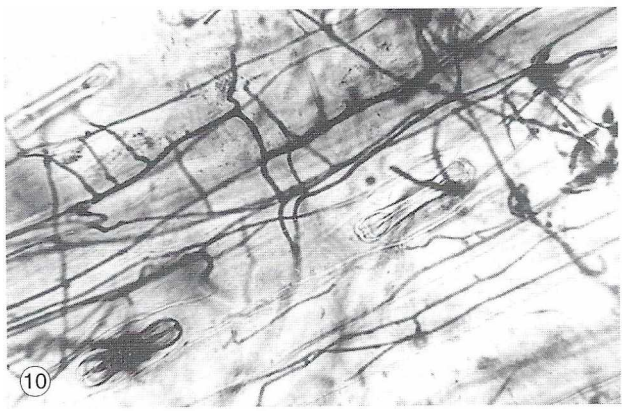


Fig. 10. *Septoria tritici* hyphae emerging through the stomatas of wheat leaves (× 280).
Abb. 10. Hyphen von *S. tritici*, die durch die Stomata von Weizenblättern penetrieren (× 280).

S. tritici was observed. On wheat leaves previously sprayed with *T. harzianum* and *G. roseum*, the spore germination and the mycelium development of the pathogen was lower than the control (Figs. 8 and 9). It has been observed, that hyphae emerging through stomata were thicker than those coming from germinated spores. This has also been previously observed in *S. apiicola*, *S. musiva* (Alippi 1986) and *S. lycopersici* (Perelló et al. 1991). It is thought that such mechanism might be typical of the

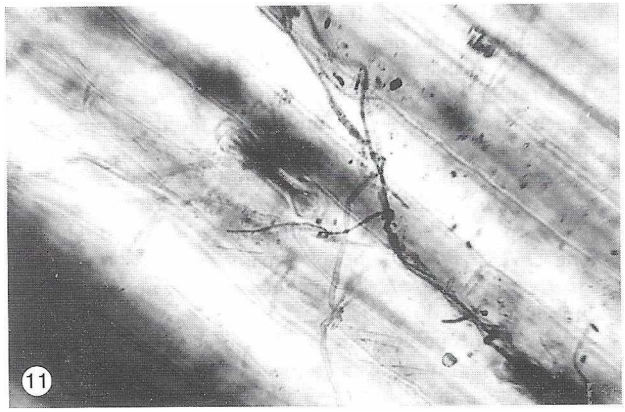


Fig. 11. Coiling of *Trichoderma harzianum* hyphae around *Septoria tritici* hyphae and mycelial plasmolysis (× 280).
Abb. 11. Wachstum der Hyphen von *T. harzianum* um die Hyphen von *S. tritici* und Plasmolyse des Myzels (× 280).

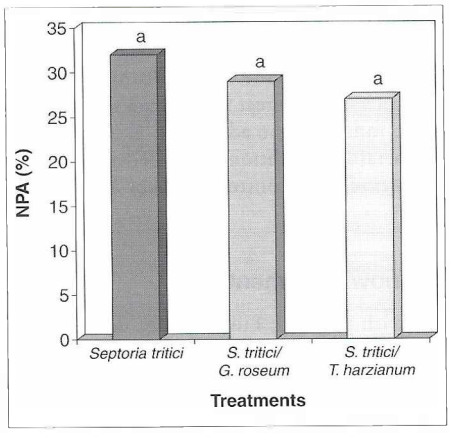


Fig. 12. Necrotic area with pycnidial coverage (%) on wheat plants in greenhouse. The same letter indicate no significant difference between treatments according to Tukey's test (p = 0.05).
Abb. 12. Mit Pyknidien bedeckter nekrotischer Bereich (%) von Weizenpflanzen im Gewächshaus. Die gleichen Buchstaben bedeuten keinen signifikanten Unterschied zwischen den Behandlungen nach dem Tukey's Test (P = 0.05).

Septoria genus and could act as another alternative of the pathogen spreading. Plasmolysis of *S. tritici* hyphae and the coiling of the hyphae of *T. harzianum* around hyphal segments of *S. tritici* has been observed (Fig. 11).

3.3 In vivo assays

The ANOVA results for the greenhouse tests showed that there were no significant differences between wheat plants treated with the antagonistic agents and the control, as regards the intensity of the symptoms. However, there has been a tendency to reduce the percentage of NPA when compared with the control (Fig. 12).

4 Discussion

Micro-organisms that are not natural residents of leaf surfaces but perform a well-known antagonistic ability, are commonly employed for the biological control of phylloplane diseases (Bettiol 1991; Sutton and Peng 1993).

The introduction of the soil fungus *Trichoderma* and *Gliocladium* has been pointed out (Fokkema 1993; Sutton and Peng 1993). These organisms present several advantages for its use as biocontrol agents because of rapid growth rates, different antagonistic mechanisms and scarce nutritional require-

ments (Tronsmo 1986; Melo 1991; Barnett and Lilly 1962; Papavizas 1985). *Trichoderma* spp. was eventually found on wheat phylloplane (Mangiarotti et al. 1987), even though it was demonstrated that it is able to survive in the aerial part of the plants for long periods (Tronsmo 1986; Melo 1991; Vitti and Ghini 1990). In our assay, *T. harzianum* T15 and *G. roseum* G10 isolated from soils in Argentina were capable of suppressing the development of *S. tritici in vitro* and the number of germinated spores and mycelial development on wheat leaves under laboratory conditions.

T. harzianum and *G. roseum* behaved in a very similar manner, being both active against a slow growing fungus as *S. tritici*. The protective characteristic of *T. harzianum* may be associated with its parasitic ability against the plant pathogens in the pre-penetration period. This fact was already observed in the interaction among *Trichoderma* and other plant pathogens (Papavizas 1985; Melo 1991).

However, it was observed that antagonists did not affect the development of *Septoria tritici*, that had been well controlled *in vitro*.

Several authors pointed out the low correlation between the antagonism performed *in vitro* and the effectiveness to control diseases *in vivo* (Bettiol 1991; Tronsmo 1986; Andrews 1985, 1992a, 1992b; Lopes 1986; Elad 1990). This phenomenon may be associated, in part, with the poor ability of these biocontrol agents to survive under environmental conditions that were highly conducive for the disease but not for the antagonists. The low level of control on wheat plants of the isolates of *T. harzianum* and *G. roseum* suggests their weak ability to colonize the phylloplane and to form a biologically active population whose metabolites remain active in concentrations sufficient to inhibit the pathogen. Additional studies are necessary to quantify the surviving population and determine if it is sufficient to protect leaves from subsequent infection by *S. tritici*. Preliminary results obtained in this work indicate that additional greenhouse studies using other isolates, and under a wide range of temperature conditions, are needed to fully assess the potential and limitations of *T. harzianum* and *G. roseum* as biocontrol agents of *S. tritici*.

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