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SEM investigation about hyphal relationships between some antagonistic fungi against *Fusarium* spp. foot rot pathogen of wheat

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Summary. Wheat foot rot caused by *Fusarium* species is a worldwide wheat disease against which the use of biocontrol agents is of increasing interest. Mycoparasitic activities of a strain of five antagonistic fungi, *Gliocladium roseum* (GR11), *Penicillium frequentans* (PF), *Trichoderma atroviride* (TA312), *T. longibrachiatum* (TL9) and *T. harzianum* (TH144), against three pathogens, *Fusarium culmorum, F. graminearum* and *F. nivale*, were studied by scanning electron microscopy (SEM). SEM observations suggested that the outcome of interaction between antagonist and pathogen occurred when intimate contact was established between hyphae triggering a series of events in pathogen degradation. The interaction between *Trichoderma* spp. and *Fusarium* spp. revealed that the mycoparasitic hyphae were usually attached longitudinally to the hyphae of the pathogens; hyphal coilings, hooks, pincer-shaped structures, short contact branches and hyphal depressions were also present. GR11 and PF hyphae grew mainly parallel to the pathogen causing its hyphal depression. The parasitic action of the antagonists shown with the formation of pincers, hooks and other structures leading to cell disruption, goes some way towards explaining their mode of action in the biological control of the pathogens studied.

Key words: *Gliocladium roseum*, *Penicillium frequentans*, *Trichoderma* spp., biological control, hyperparasitism.

Introduction

Wheat foot rot caused by *Fusarium* species is a very common and economically important disease in Italy (Balmas and Corazza, 1992; Innocenti, 1996) and in many other wheat-producing areas (Wiese, 1987). The trend towards more intensive crop management to increase productivity may favour the spread of *Fusarium* species. The use of microorganisms for the biological control of wheat foot rot is of interest because up to now resistant cultivars are not available, nor have cultural practices, organic fertilizers or chemical means been really effective. The preference for biological control methods is justified also by the undesirable side effects of pesticides.

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A study done in the Netherlands by Daamen *et al*. (1989) suggests that the extent of *Fusarium* foot rot in clay soil may actually increase as a result of seed treatment with fungicides, which may suppress the antagonists or allow a great number of diseased plants to survive. Al Hashimi and Perry (1986) also stated that an increase of foot rot may be caused by the depletion of antagonistic organisms.

There have been many reports on the successful use of biocontrol agents against plant pathogenic fungi, but only a few studies have dealt with antagonistic fungi applied against *F. graminearum* (Harman *et al*., 1989; Fernandez, 1992), *F. nivale* (Adetuyi, 1992) and *F. culmorum* (Kempf and Wolf, 1989; Tahvonen and Sorri, 1992; Kempf *et al*., 1994; Jensen *et al*., 1995; Knudsen *et al*., 1995; Tahvonen *et al*., 1995). Nyvall and Kommendahl (1973) stated also that *F. culmorum*, when artificially inoculated into the soil, was easily out-competed by naturally occurring saprophytes on dead material and this sensitivity seemed to be an important factor for potential antagonists against this pathogen.

In this paper we have examined by scanning electron microscopy (SEM) the parasitic activity of five antagonistic fungi against three *Fusarium* species. This SEM study follows previous research in which antagonists were tested against *Fusarium* spp. in the greenhouse and in the field (Roberti *et al*., 1996, 1997, 2000).

Materials and methods

Strains of three pathogenic *Fusarium* species, *F. culmorum* (FC17), *F. nivale* (FN40) and *F. graminearum* (FG66) and of five antagonistic fungi, *Gliocladium roseum* (GR11), *Penicillium frequentans* (PF), *Trichoderma atroviride* (TA312), *T. longibrachiatum* (TL9) and *T. harzianum* (TH144) were studied by SEM. FC17, FN40 and FG66 were isolated in Italy from wheat seeds. TL9, TA312, TH144 and GR11 were isolated in Italy from vegetable roots and seeds, loamy soil and wheat crown respectively, while PF was kindly provided by P. Melgarejo (INIA, Madrid, Spain). All numbers refer to our collection.

Two agar disks of 5 mm diameter taken from 1-2-day-old cultures grown on 4.5% potato dextrose agar (PDA) were placed in a Petri dish. Each antagonist mycelium was plated on one end of the agar dish and each pathogen at the other end at 30 mm apart. Pathogen and antagonist disks were also plated singly as controls. Each dual-culture and single control culture was replicated five times and maintained in the dark at 24°C to allow the hyphae of the two fungi to make contact in the middle of the plate.

Because of the widely varying growth of the antagonists and pathogens, plating times were staggered. GR11 and PF grew more slowly than the pathogens and thus were plated 2 days before them, while *Trichoderma* spp. grew faster and were plated 1 day after *Fusarium* spp.

Small pieces of agar (approximately 2 mm^2) were taken from the controls and from the dual-cultures at the interaction zone, when the two fungi were at their early stages of interaction. Excess of agar was removed with a razor blade prior to further preparation. Specimens were immersed in 100% ethanol at room temperature for a few minutes, then dried in a critical point drier apparatus (CPD 030, BAL-TEC, Balzers, Fürstentum, Liechtenstein) using liquid CO_2 as a transitional fluid. Dried samples were mounted on aluminium stubs with silver glue, coated with gold-palladium film using an ion-sputtering unit (Balzers MED010) and observed in a Philips 515 SEM at 7-9 Kv.

Results

Macroscopic examination of the fungal dual-cultures revealed that most of the strains made hyphal contact with *Fusarium* spp. within one-two days after inoculation. *Trichoderma* spp. were the most inhibiting antagonists, since they grew over the pathogens, except for TA312 which acted only as a barrier.

A similar behaviour for each antagonist-pathogen combination was observed by SEM. There were similarities and differences in the ability of various strains to invade the pathogens in dual cultures. Direct contact with the pathogens was always followed by various types of hyphal aggression.

SEM investigations on the interactions between *Trichoderma* spp. and *Fusarium* species revealed that the mycoparasitic hyphae were

usually attached longitudinally to the hyphae of the pathogens. Hyphal coilings, hooks, pincer-shaped structures, short contact branches and hyphal depressions were also observed. GR11 and PF grew mainly parallel to the pathogen causing its hyphal depression.

The results of hyphal interactions as well as their morphologies are described as follows.

TL9 exibited strong parasitic action against all *Fusarium* species growing parallel to them and producing hyphal depressions. Hyphal tips, hooks and pincer-shaped structures were formed against *F. culmorum* (Fig. 1) and *F. nivale* (Fig. 2 and 3), swirls only against *F. graminearum* (Fig. 4).

TA312 displayed its parasitism against *Fusarium* species by growing parallel to them, and against *F. nivale* hyphae by also forming hyphal tips, hooks and swirls accompanied by complete loss of hyphal turgidity, and shrinkage of the pathogen (Fig. 5 and 6). Accumulation and deposition of fibrillar material occurred when TA312 interacted with *F. culmorum* growing abundantly on it (Fig. 7 and 8).

TH144 grew parallel to all the pathogens and formed hooks against *F. nivale* causing hyphal depressions (Fig. 9). It produced swirls and hyphal tips when interacting with *F. culmorum* (Fig. 10) and hyphal tips and pincer-shaped

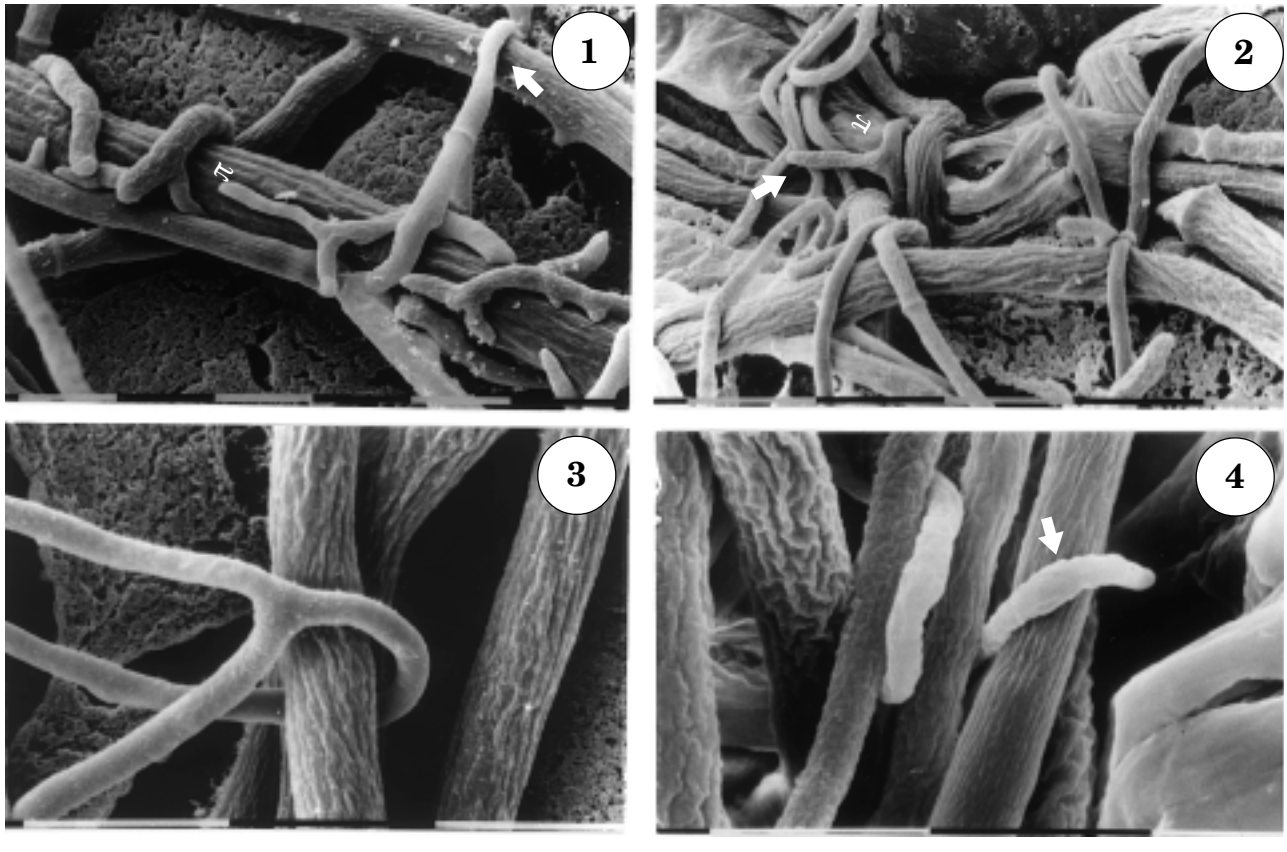

Fig. 1-4. Parasitic action of *Trichoderma longibrachiatum* (TL9) against *Fusarium culmorum* (FC), *F. graminearum* (FG) and *F. nivale* (FN). 1. TL9 hyphal tips, hook (arrow) and pincer (head of arrow) formed against FC. 2. TL9 hyphal tip (arrow), hooks and pincer (head of arrow) formed against FN causing its hyphal depression and 3. a detail of TL9 pincer-shaped structure. 4. TL9 swirl (arrow) around FG inducing its hyphal depression. Bars = 10 μm.

structures in its interaction with *F. graminearum* (Fig. 11 and 12).

GR11 hyperparasitised *Fusarium* spp. by growing parallel to their hyphae. When GR11 reached the host, its hyphae formed short contact branches against *F. nivale* and produced depression on hyphal walls of *F. culmorum* (Fig. 13), *F. nivale* and *F. graminearum* (Fig. 14).

PF, which is easily detectable by its phialides, caused hyphal depression in all *Fusarium* species. It grew parallel to the pathogens, produced hyphal tips and pincer-shaped structures in the presence of *F. nivale* (Fig. 15), and coiled around itself and *F. graminearum* hyphae adhering to them by short contact branches (Fig. 16).

No penetration or lysed sites were found in any of the samples.

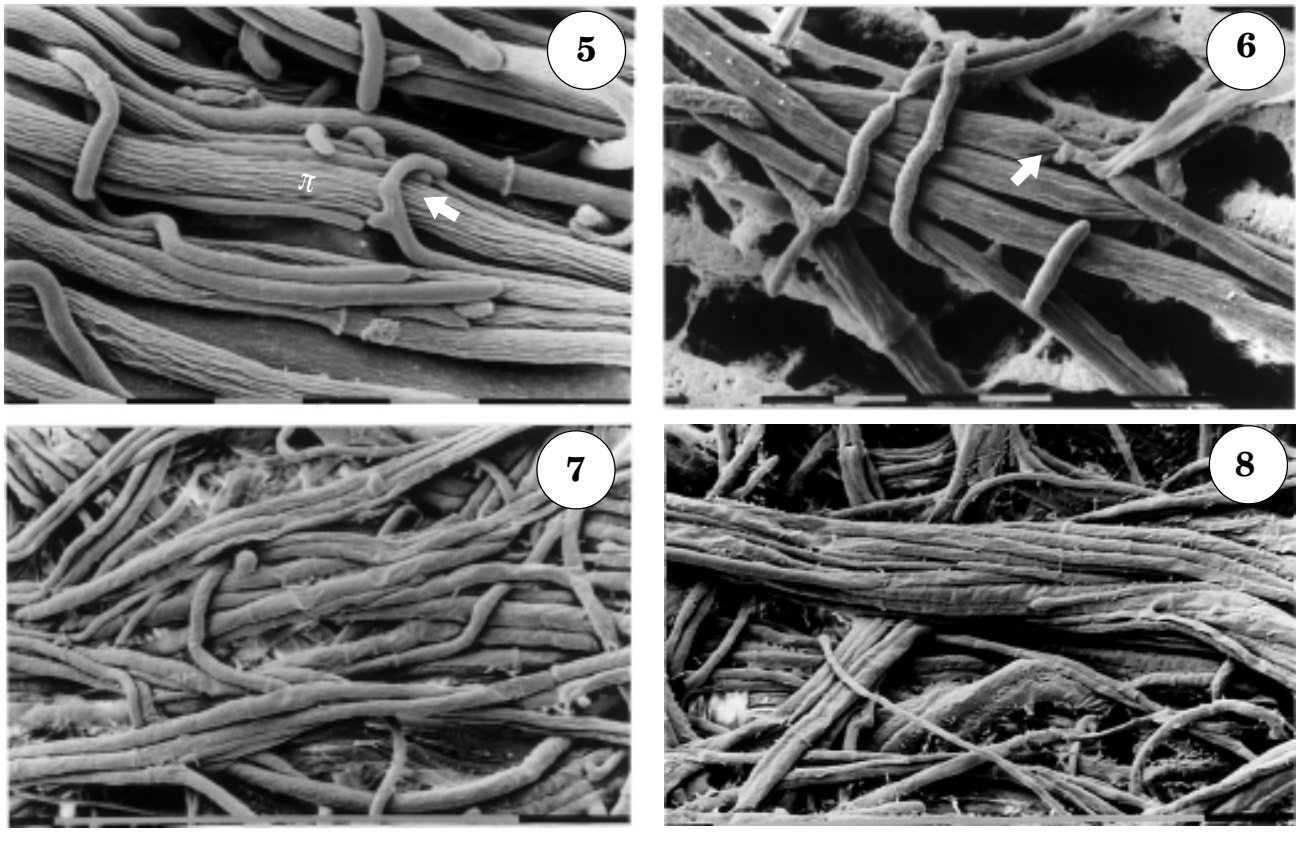

Fig. 5-8. Parasitic action of *Trichoderma atroviride* (TA312) against *Fusarium culmorum* (FC) and *F. nivale* (FN). 5. TA312 hook (arrow), hyphal tip (head of arrow) and parallel growth formed against FN. 6. Loss of turgidity and shrinkage (arrow) of FN hyphae. 7 and 8. Presence of fibrillar-like material, TA312 parallel growth and FC hyphal depression. Bar = 10 μm, Fig. 5 and 6; Bar = 0.1 mm, Fig. 7 and 8.

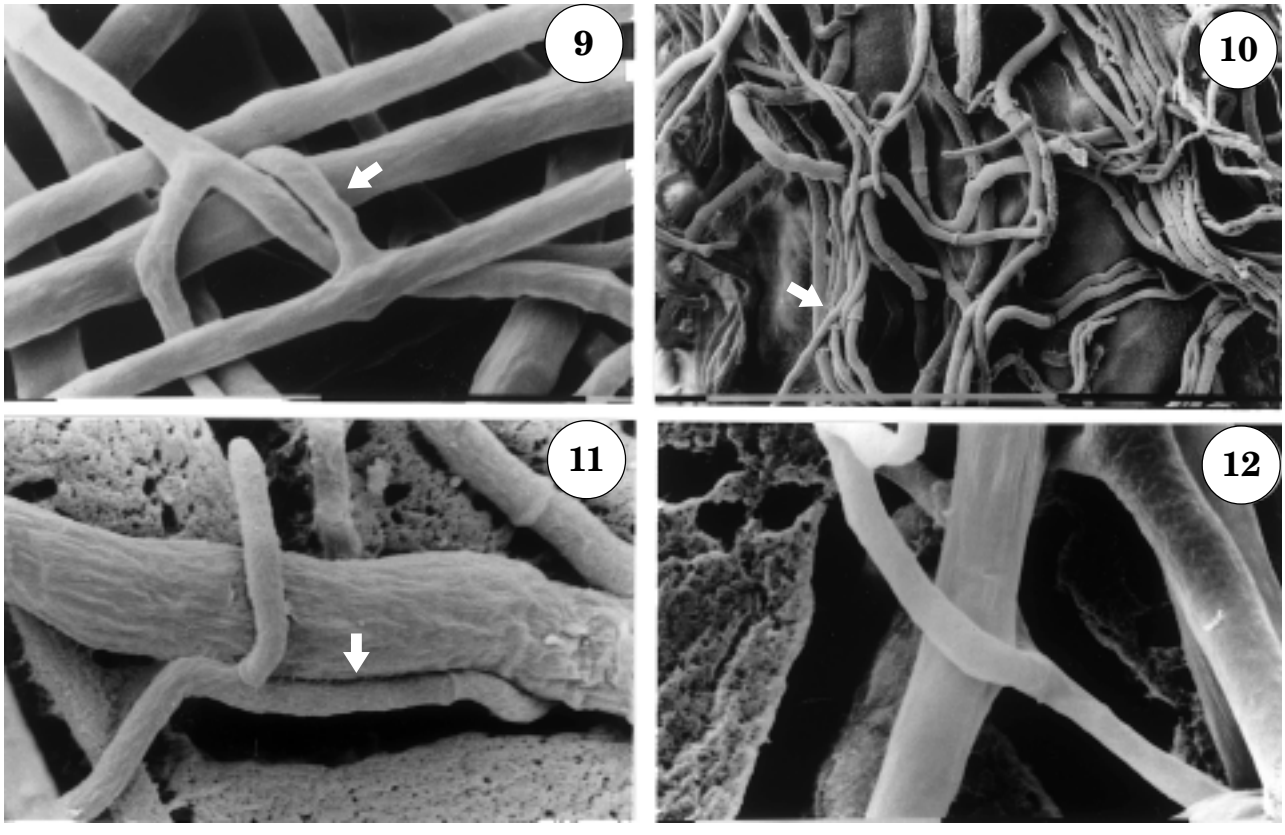

Fig. 9-12. Parasitic action of *Trichoderma harzianum* (TH144) against *Fusarium culmorum* (FC), *F. graminearum* (FG) and *F. nivale* (FN). 9. Hook (arrow) formed by TH144 hyphae interacting with FN. 10. TH144 hyphal tips and swirl (arrow) around FC that shows hyphal depression. 11. Hyphal tips and parallel growth (arrow) of TH144 against FG. 12. A detail of TH144 pincer. Bar = 0.1 mm, Fig. 10; Bar = 10 μm, Fig. 9, 11 and 12.

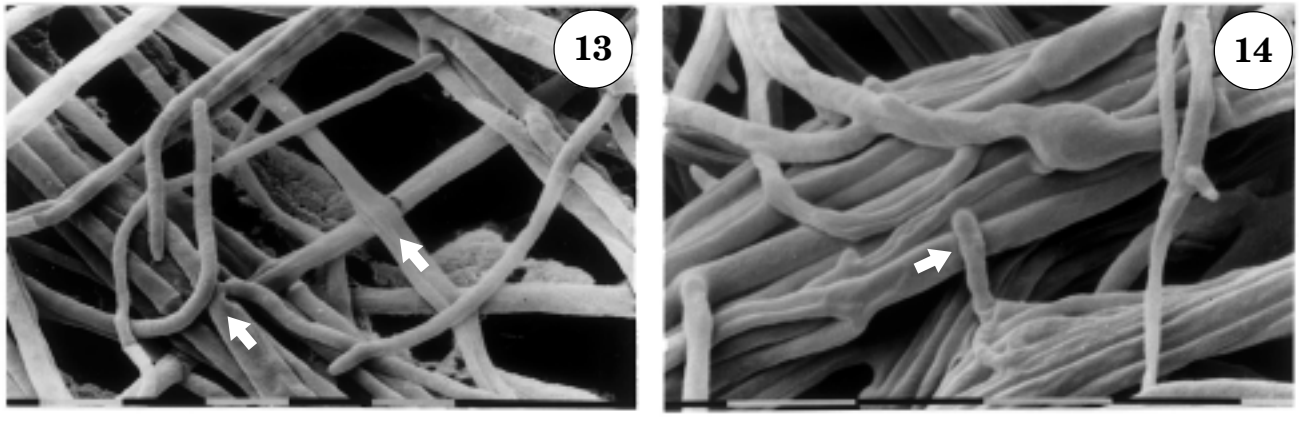

Fig. 13 and 14. Parasitic action of *Gliocladium roseum* (GR11) with *Fusarium culmorum* (FC) and *F. graminearum* (FG). 13. FC hyphal wall depression (arrows). 14. GR11 hyphal tip (arrow) formed against FG. Bar = 10 μm.

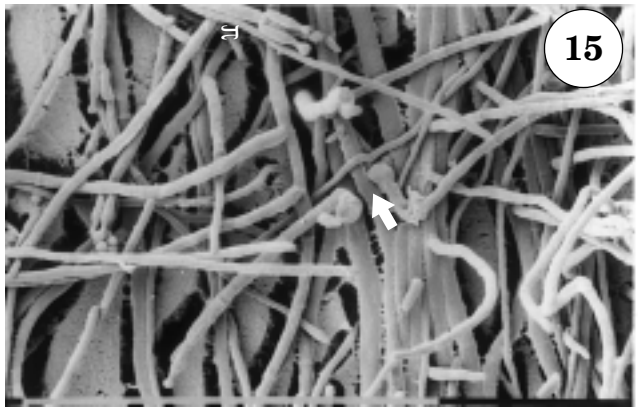

Fig. 15 and 16. Parasitic action of *Penicillium frequentans* (PF) against *F. nivale* (FN) and *F. graminearum* (FG). 15. PF phialides (head of arrow), hyphal tips and pincer (arrow) formed against FN. 16. PF parallel growth (head of arrow) and hyphal tip (arrow) adhering to FG hyphae. Bar = 0.1 mm, Fig. 15; Bar = 10 μm, Fig. 16.

Discussion

Wheat foot rot is one of the greatest challenges now facing agriculture. Research on the forms of antagonism *in vitro* are important to understand the problems involved in the control of the pathogens responsible for the disease. Our observations at SEM confirmed the complexity of the interactions between the pathogens under investigation.

The exclusive growth of the antagonists towards their target was probably a chemotropic response to substances leaking out or released from *Fusarium* hyphae. Active chemotropic growth, once the initial contact between the interacting fungi has taken place, is described by Elad *et al*. (1983). These authors also detected an extracellular mucilaginous substance, apparently consisting of polysaccharides, produced by *T. harzianum* in the early stages of hyperparasitism (Elad *et al.*, 1987). A similar material linking the hyphae of TA312 when interacting with *F. culmorum* was observed in our SEM study (Fig. 7, 8 and 9). Presumably this substance has adhesive properties and facilitates contact between the antagonist and pathogen hyphae since it is produced only when TA312 is in a dual culture with *F. culmorum*.

The overlapping of the characteristic structures observed, such as short contact branches, hooks, pincers, hyphal coils and depressions, may be due to the lesser aggressiveness of the antagonists. Initially they probably weaken the pathogen by growing parallel and adhering closely to its hyphae; later, they also damage the pathogen's cell wall. The parasitic action of the antagonists, shown by the formation of pincers, hooks and other structures, eventually leading to cell disruption, goes some way toward explaining how they exercise biological control of the pathogens, although other modes of action, such as antibiosis, competition or induced resistance, cannot be excluded.

The antagonists studied produced considerable alterations of the pathogen cell wall with rapid collapse and loss of cell turgor, even though antagonist hyphae did not interact physically by coiling around or penetrating the pathogen hyphae. Nevertheless, they probably excreted wall lytic enzymes or antifungal substances, causing wrinkling and collapse of *Fusarium* spp. mycelium.

Enzymatic decomposition of the host cell wall is brought about by several mycoparasites, such as *T. harzianum* against *Sclerotium rolfsii* (Elad *et al*., 1982) and *Botrytis cinerea* (Elad and Kapat, 1999), *Gliocladium roseum* against *B. allii*

(Pachenari and Dix, 1980) and *G. virens* against *Sclerotinia sclerotiorum* (Tu, 1980).

An enzymatic activity of GR11, PF, TA312, TH144 and TL9 interacting with *F. culmorum*, *F. graminearum* and *F. nivale* has been detected in some experiments that are still in progress. This activity may induce cell wall damage and degradation along with hyphal penetration; however, the evidence of hyphal penetration was not as clear by SEM as the other pathogen alterations above mentioned.

We therefore suppose that the hyphal morphologies observed at SEM along with the *in vitro* enzyme activities detected (unpublished data), may play a role in the biological control of wheat foot rot.

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