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Endophytes from wheat as biocontrol agents against tan spot disease

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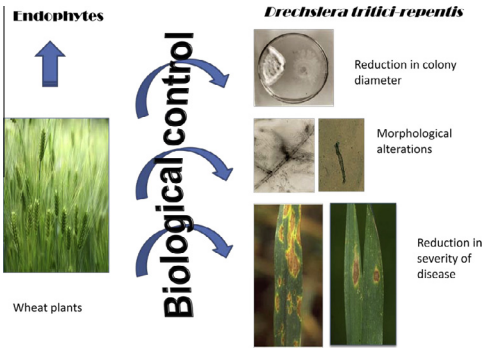
HIGHLIGHTS

- The effect of endophytes from wheat against *Drechslera tritici-repentis* was evaluated.
- Some endophytes reduced mycelial growth and spore germination of the pathogen.
- Hyphae and conidia of *Drechslera tritici-repentis* were alterated by endophytes.
- Endophytes from wheat leaves were effective in reducing the tan spot severity.
- *Bacillus* sp. antagonized the pathogen successfully in all assays.

GRAPHICAL ABSTRACT



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ABSTRACT

Endophytes from wheat cultivars isolated in Buenos Aires province, Argentina, were assessed for their potential as biocontrol agents against *Pyrenophora tritici-repentis* (Died.) Drechsler (anamorph *Drechslera tritici-repentis*) (Died.) Shoem (*Dtr*), the causal agent of tan spot of wheat. Endophytes were screened using dual culture techniques and examining the effect on growth, sporulation and the antifungal activity in greenhouse assays. The most of endophytes tested significantly reduced *Dtr* growth compared to the control except *Rhodotorula rubra* from 11 to 15 days post inoculation. *Trichoderma hamatum*, *Penicillium* sp., *Bacillus* sp. and *Paecilomyces lilacinus* significantly reduced the colony diameter of the pathogen. Most of the endophytes evaluated showed morphological changes in the conidia and/or the mycelia of *D. tritici-repentis*. In addition, two endophytes, *Bacillus* sp. and *Fusarium* sp., reduced significantly the percent spore germination of *Dtr* compared to the control by 82% and 52% respectively. In greenhouse experiment *T. hamatum*, *Chaetomium globosum* and *Fusarium* sp. significantly ($P \leqslant 0.05$) reduced the average disease severity on all three leaves compared to the control. However, the best antagonistic effect was shown with *T. hamatum* as it resulted in the greatest suppression in the greenhouse and in the dual-plate assays. Likewise, *Bacillus* sp. was other highlighted microorganism that antagonized the pathogen in *in vitro* assays. From our promising results, we conclude that endophytes have potential in the biological control of tan spot of wheat caused by *D. tritici-repentis*, particularly *T. hamatum* and *Bacillus* sp.

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1. Introduction

Fungal endophytes, a diverse group of ascomycetous fungi defined functionally by their occurrence within asymptomatic tissues of plants, have been found in all plant families (Arnold, 2007). Likewise, a wide range of bacterial genera can be isolated from surface disinfected plant tissues or extracted from inner plants parts (Chanway, 1988; Hallmann et al., 1997; Quadt-Hallmann et al., 1997).

The role of endophytic community in endophyte/plant associations has been intensively discussed (Porras-Alfaro and Bayman, 2011; Sturtz et al., 2000). Endophytes interact with, and overlap in function with, other core microbial groups that colonize plant tissue, e.g. pathogens, epiphytes, and saprotrophs (Porras-Alfaro and Bayman, 2011). It is shown that the presence of endophytes within plant tissues may confer certain advantages to the host plant (Carroll, 1991). Some fungal endophytes affect plant growth and plant responses to pathogens, herbivores and environmental change; others can provide thermal tolerance for their hosts (Arnold, 2007; Porras-Alfaro and Bayman, 2011; Sturtz et al., 1998). Also endophytic bacterial are known to stimulate host plant growth, either through direct antagonism of microbial pathogens or by inducing systemic resistance to disease-causing organisms (Arnold, 2007; Duijff et al., 1997; Pleban et al., 1995; Sturtz et al., 1998).

Endophytes with biocontrol effect have received attention as an alternative to chemical disease control which also reduces the use of potentially hazardous chemicals (Bacon et al., 2001; Bacon and Hinton, 2007; Porras-Alfaro and Bayman, 2011). For example, the bacterial endophytes isolated from maize as *Bacillus subtilis* and *Bacillus mojavensis* have shown great potential in the biocontrol of *Fusarium moniliforme* in maize and reduced seedling blight of wheat caused by *Fusarium graminearum* and related species respectively (Bacon et al., 2001; Bacon and Hinton, 2007). *Trichoderma* spp. and others endophytic fungi isolated from leaves and pods of *Theobroma cacao* have shown antagonistic effect against the three most common and economically important pathogens of cacao (*Phytophthora palmivora*, *Moniliophthora roreri*, and *Moniliophthora perniciosa*) (Bailey et al., 2006; Mejía et al., 2008). It is demonstrated in several pathosystems that the endophytes are effective biocontrol agents that reduce disease severity of plant diseases (Carroll, 1988; Narisawa et al., 1998; Wicklow et al., 2005).

The presence of endophytes in wheat has been demonstrated by several authors (Sieber et al., 1988; Crous et al., 1995; Istifadah and McGee, 2006; Istifadah et al., 2006) but there are very few studies regarding endophytic associations in wheat in Argentina (Larran et al., 1999, 2002a, 2007). Our previous research has focused on estimating the abundance and/or diversity of fungal endophytes associated with tissues of different agronomical important hosts (Larran et al., 2000, 2001, 2002b). In studies conducted by Larran et al. (1999, 2002a, 2007) in Argentina, *Serratia* sp., *Alternaria alternata*, *Chaetomium globosum*, *Cladosporium herbarum*, *Epicoccum nigrum*, *Cryptococcus* sp., *Rhodotorula rubra*, *Penicillium* sp., *Bacillus* sp., *F. graminearum*, *Bipolaris sorokiniana* and *Trichoderma hamatum* were consistently recovered from leaves and stems of healthy wheat plants.

Tan spot of wheat caused by *Pyrenophora tritici-repentis* (Died.) Drechsler (anamorph *Drechslera tritici-repentis*) (Died.) Shoem (*Dtr*) is one of the most destructive foliar wheat diseases worldwide, including Argentina (Moreno and Perelló, 2010). Biological control using antagonists has been investigated in recent years to minimize the use of chemicals (Perelló and Mónaco, 2007; Perelló and Dal Bello, 2011). The potential of non-pathogenic saprophytic microflora of aerial plant parts for biological control of cereals pathogens has been recognized (Fokkema et al., 1979). Species of *Trichoderma* isolated from the wheat phylloplane have been extensively studied for their biocontrol potential against *Dtr* (Perelló et al., 2003, 2008).

Previous studies on endophytic fungi have shown that *Chaetomium* sp. isolated from healthy wheat leaves reduced the number and development of pustules of leaf rust *Puccinia recondita* f.sp. *tritici* (Dingle and McGee, 2003). Moreover, different species of *Chaetomium* inhibited the growth of *P. tritici-repentis in vitro* (Istifadah and McGee, 2006; Istifadah et al., 2006).

In the present work, experiments were carried out to evaluate the antagonistic effect of ten endophytic microorganisms from healthy wheat plants against *Dtr* for their potential as biocontrol agents of tan spot.

2. Materials and methods

2.1. Isolates and cultures

A monosporic isolate *Dtr* LH 019 of the tan spot pathogen *D. tritici-repentis* was used in all experiments. The isolate was originally obtained from infected wheat plants growing at the experimental field "J. Hirschhorn" of the Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Buenos Aires, Argentina and maintained on 2% potato dextrose agar (PDA) culture media at 5 °C as part of the CIDEFI collection.

The fungal, bacterial, and yeast endophytes used in this work were isolated from wheat in previous studies and maintained on 2% PDA at 5 °C in the CIDEFI collection (Larran et al., 2007). The endophytes evaluated were *A. alternata*, *Bacillus* sp., *C. globosum*, *Cladosporum herbarum*, *E. nigrum*, *Fusarium* sp., *Penicillium* sp., *Paecilomyces lilacinus*, *R. rubra* and *T. hamatum*. The methodology and techniques used for the isolation and identification are described in Larran et al. (2007). Bacteria, fungal and yeast colonies were subcultured in Petri dishes on either nutrient agar (bacteria) or PDA (fungi and yeast). Subcultures were incubated for 4 and 10 days, respectively until used.

2.2. Pathogenicity test

Pathogenicity tests were conducted to ensure that the endophytic microorganisms used in these experiments were non-pathogenic on wheat. This was in connection with the fact that endophytes communities in addition to mutualistic and comensalistic symbionts, could include latent pathogens or strains that are virulent but which are poor competitors relative to others tissues colonizing microorganisms (Arnold, 2007; Porras-Alfaro and Bayman, 2011).

The endophytes *A. alternata*, *Bacillus* sp., *C. globosum*, *C. herbarum*, *E. nigrum*, *Fusarium* sp., *Penicillium* sp., *P. lilacinus*, *R. rubra* and *T. hamatum* were evaluated for pathogenicity using 30-day-old wheat plants of the susceptible cultivar Buck Poncho under greenhouse conditions. Four plants in each of two pots were sprayed till run off with a suspension of 2×10^6 conidia/ml of each fungal endophyte prepared in sterile distilled water containing 0.1% (v/v) of Tween 20 adjusted by a hemocytometer. The yeast, *R. rubra* and the bacterial isolate *Bacillus* sp. were applied at 1×10^8 CFU/ml adjusted using a spectrophotometer (optical density at 600 nm). Control plants were sprayed with sterile distilled water only. All plants were incubated for 48 h in plastic bags and then maintained at 20 ± 2 °C in a greenhouse and were assessed for disease severity every 3 days for 14 days.

2.3. Hyphal interference and inhibition of colony growth

The antagonistic effect of 7 fungi, 1 yeast and 1 bacteria (*A. alternata*, *Bacillus* sp., *C. globosum*, *C. herbarum*, *Fusarium* sp.,

Penicillium sp., *P. lilacinus*, *R. rubra* and *T. hamatum*) was tested using the dual culture technique. Agar discs (6 mm diameter) of the fungal endophyte were placed 3.5 cm away from a *Dtr* on 9 cm Petri dishes containing 2% PDA. The discs of the endophytes and the pathogen were taken from cultures of 10-day-old and 7-day-old respectively. The bacteria and yeast were streaked on the plate with the pathogen plug. A plug with the pathogen alone was the control. All culture plates were incubated at 24 ± 2 °C. The experiment was repeated twice and treatments were replicated four times in each experiment. *Dtr* growth was assessed by measuring the colony diameter (cm) after 3, 7, 11 and 15 days post inoculation in two directions: diameter 1 (D1), perpendicular diameter to the direction of the endophytes, and diameter 2 (D2), diameter in the direction of the endophyte. Measurements were discontinued when the colonies reached the edge of the plate or stopped growing. The average of both diameters was calculated. Growth rate was considered as daily growth (cm) of *Dtr* per interval of time (3–5; 5–7; 7–11, and 11–15 days) among the four evaluation times assayed. Growth rates were calculated as the cm per day of *Dtr* growth for each time interval. Therefore three growth rates (Gr 1, Gr 2 and Gr 3) were determined as follow: Gr 1 = mean colony diameter of ending value − mean colony diameter of starting value/number of days of time interval (4 days). Observations of morphological changes were made on small squares of agar cut from the areas of intermingling growth and from the margin of the colonies for *Dtr*. These pieces were examined using light microscope. Interactions were assessed using a key based on observations of Porter (1924) and Dickinson and Boardman (1971). The mean of diameters (D1 + D2/2) and the growth rates (1–3) were analyzed with GenStat (2008) using generalized linear model (GLM) for repeated measurements with experiments, times and endophytes as factors. Means were compared by LSD test ($P \leqslant 0.05$).

2.4. Inhibition of spore germination

The experiment was designed to evaluate the antagonistic effect of endophytes *Bacillus* sp., *C. globosum*, *C. herbarum*, *E. nigrum*, *Fusarium* sp., *Penicillium* sp., *P. lilacinus*, *R. rubra* and *T. hamatum* against *Dtr* in a paired suspension assay.

Droplets (50 µl) of a 1:1 suspension of conidia from each pathogen–endophyte combination were pipetted into the well of a cavity glass slide. Similar droplets of the pathogen alone were used as a control. The conidial concentration of *Dtr* was 2×10^5 conidia/ml and the fungal endophytes were 2×10^6 conidia/ml. The yeast and the bacteria were used at 1×10^8 CFU/ml. All concentrations were adjusted as described above. The slides were incubated in small moisture chambers at 24 ± 2 °C in darkness for 48 h and then examined using light microscopy. Spore germination was assessed from three replicates of 12 microscopic fields at 100 and 200× magnifications. A conidium was considered to have germinated if the germ-tube was more than one-half of the length of the conidium. The percentage of conidial germination was calculated and germ-tube growth and vesicle formation were also assessed relative to those conidia in the control. Data were arcsin $\sqrt{x}$ transformed to homogenize the residual variance and statistically analyzed using analysis of variance (ANOVA). Means were compared by LSD test ($P \leqslant 0.05$).

2.5. Greenhouse experiment

This experiment was designed to determine if pre-inoculation of leaves with wheat endophytes, *Bacillus* sp., *C. globosum*, *C. herbarum*, *E. nigrum*, *Fusarium* sp., *Penicillium* sp., *P. lilacinus*, *R. rubra*, and *T. hamatum* have an antagonistic effect against *Dtr* on wheat seedlings. Two experiments in a completely randomized design were conducted in a greenhouse. Five seeds of wheat cultivar Buck Poncho were sown in 12 cm diameter plastic pots at the greenhouse at temperatures ranging from 15 to 25 °C with a 14 h photoperiod. One of the experiments has 4 replications, whereas a variable number of replications (3–10) was scored in the other experiment. Plants were inoculated at growth stage 15 according Zadoks et al. (1974) until runoff with suspensions of each one of the endophytes using a hand-held atomizer. The endophytes were applied to the leaves in distilled water with 0.02% Tween 80, 1 day before the pathogen. Suspensions of the pathogen and the endophytes were prepared by flooding the 4 and 10-day-old cultures (bacteria, yeast and fungi, respectively) with sterile distilled water and then rubbing the cultures surfaces with a sterile glass rod. The suspension was vortexed and filtrated through two layers of cheesecloth. The conidial concentration used for *Dtr* was 2×10^5 conidia/ml, whereas for the fungal endophytes a 2×10^6 conidia/ml was prepared. The yeast and the bacteria were used at 1×10^8 CFU/ml. The concentrations were adjusted with the aid of a hemocytometer and a spectrophotometer for fungi and yeast, and for the bacteria respectively as described above. Control plants were inoculated with the pathogen only until runoff using a hand-held atomizer. All plants were incubated for 48 h in plastic bags and then maintained at 20 ± 2 °C in a greenhouse. The development of tan spot disease was assessed by estimating the percentage necrotic leaf area on the three youngest leaves of each plant per treatment after 12 days. Data were arcsin $\sqrt{x}$ transformed to fit a normal distribution and normalize the residual variance. The effect of each endophyte was evaluated by means of an ANOVA for an unbalanced completely randomized design with experiments and endophytes as factors. Means were compared by LSD test ($P \leqslant 0.05$).

3. Results

3.1. Pathogenicity test

The pathogenicity test showed that the endophytes did not caused symptoms in the inoculated plants. These results lead us

Table 1
ANOVA for the effect of endophytes against the growth of *Drechslera tritici-repentis* at four evaluation times under *in vitro* test.

Source of variation	DF	Means square	F^a	*P* value
Experiments	1	0.274	0.28	0.595
Endophytes	9	144.411	150.30	$P \leqslant 0.001$
Experiments × endophytes	9	0.459	0.49	0.884
Error	60	0.961		
Evaluation times	3	159.217	1956.70	$P \leqslant 0.001$
Evaluation times × experiments	3	0.096	1.18	0.307
Evaluation times × endophytes	27	9.574	117.66	$P \leqslant 0.001$
Evaluation times × experiments × endophytes	27	0.165	2.03	0.015
Error	180	0.081		

[a] Fisher test.

Table 2
ANOVA for growth rates of *Drechslera tritici-repentis* against endophytes at four evaluation times under *in vitro* test.

Source of variation	DF	Means square	F^{a}	*P* value
Experiments	1	0.002	0.45	0.504
Endophytes	9	0.841	176.49	$P \leqslant 0.001$
Experiments × endophytes	9	0.006	1.38	0.218
Error	57	0.005		
Evaluation times	2	8.602	935.01	$P \leqslant 0.001$
Evaluation times × experiments	2	0.019	2.03	0.147
Evaluation times × endophytes	18	0.578	62.88	$P \leqslant 0.001$
Evaluation times × experiments × endophytes	18	0.020	2.17	0.014
Error	120	0.009		

[a] Fisher test.

to consider evaluating their potential as biocontrol agents against *Dtr*.

3.2. In vitro antagonistic activity assays

The results obtained in the dual culture experiment to evaluate the antagonistic activity of endophytes against *Dtr* showed that there was a significant ($P \leqslant 0.001$) effect of endophytes, evaluation times and endophytes × evaluation times interaction with respect to *Dtr* colony size (Tables 1 and 2). The most of endophytes tested significantly reduced *Dtr* growth compared to the control except *R. rubra* from 11 to 15 days post inoculation (Table 3). As it is shown in the Table 3, the suppressive effect was best demonstrated in the first 3–7 days (Gr1). Growth rate 2 and 3 resulted in lower values due to the *Dtr* colony reaching the Petri dish edge after 7 days or stopped growing.

When the colony interaction in all possible paired combinations between endophytes and *Dtr* on PDA were evaluated we observed different types of interactions. The interactions of *Dtr* with endophytes are shown in Fig. 1 and the following types were assigned: Type A: mutually intermingling growth, e.g. *A. alternata* and *Fusarium* sp. (Fig. 1A); Type Bi: mutually intermingling growth where *Penicillium* sp., *P. lilacinus* and *R. rubra* were growing above *Dtr* (Fig. 1B); Type Bii: intermingling growth, where *Dtr* has ceased growth and is overgrown by *T. hamatum*, *C. herbarum* and *C. globosum* (Fig. 1C); Type D: mutual inhibition at distance, where *Dtr* opposed to *Bacillus* sp. (Fig. 1D); and the control (*Dtr* alone) (Fig. 1E).

In the experiment to evaluate the ability of the endophytes to inhibit spore germination, results indicate that two endophytes, *Bacillus* sp. and *Fusarium* sp., reduced significantly the percent spore germination of *Dtr* compared to the control by 82% and 52% respectively (Fig. 2).

Microscopic examinations of the pathogen excised from the perimeters of colonies and from the paired suspension assay showed clear differences in hyphae and conidia morphology among treatments and the control. In combinations with *Bacillus* sp., *Fusarium* sp., *C. globosum*, *Penicillium* sp. and *R. rubra*, conidia of *Dtr* presented plasmolysis and shorter and swollen germ-tubes evaluated by visually. *P. lilacinus* also resulted in conidial plasmolysis of *Dtr*. Conidium germ-tube with vesicle formation and hyphal vacuolation were observed in the presence of *Bacillus* sp. *Penicillium* sp. induced chlamydospores formation. *T. hamatum*, *Bacillus* sp., *C. globosum*, *P. lilacinum* and *Penicillium* sp. treatments exhibited production of pigmented compounds inside the hyphae or in media. Melanization zones of hyphae-wall and mycelial plasmolysis was observed when *Dtr* was confronted with *T. hamatum* (Fig. 3). All these alterations were not observed in the media or in the cultures with the pathogen alone.

Table 3
Means of diameters and growth rates of *Drechslera tritici-repentis* at four evaluation times under *in vitro* test.

	Mean diameter of growth of *D. tritici-repentis* (cm)				Growth rates (Gr)*** (cm/day)		
	3**	7	11	15	Gr 1	Gr 2	Gr 3
D. tritici- repentis (control)	3.06d*	8.80e	9.00e	9.00e	1.43e	0.05a	0.00a
Alternaria alternata	2.90cd	6.08c	6.22c	6.41c	0.79c	0.03a	0.05a
Bacillus sp.	1.39a	2.49a	2.72b	2.86b	0.25b	0.08a	0.03a
Chaetomium globosum	3.05d	6.22c	6.28c	6.44c	0.79c	0.01a	0.04a
Cladosporium herbarum	2.95cd	6.00c	7.64d	8.03d	0.76c	0.41b	0.01a
Fusarium sp.	2.89cd	6.19c	6.24c	6.32c	0.83c	0.01a	0.02a
Penicillium sp.	1.91ab	2.10a	2.11a	2.11a	0.04a	0.00a	0.00a
Paecilomyces lilacinum	2.44bc	3.22b	3.22b	3.22b	0.20b	0.00a	0.00a
Rhodotorula rubra	3.07d	7.50d	9.00e	9.00e	1.10d	0.38b	0.00a
Trichoderma hamatum	1.73ab	1.90a	1.90a	1.90a	0.04a	0.00a	0.00a

* Means followed by the same letter in the same column are not statistically different ($P \leqslant 0.05$), LSD test.
** Evaluation times: 3, 7, 11 and 15 days.
*** Growth rate (Gr): daily growth (cm) of *Drechslera tritici-repentis* for each of the three interval times among the four evaluation times: 3–7; 7–11 and 11–15. Gr was calculated as follow: Gr = mean colony diameter of ending value – mean colony diameter of starting value/number of days of time interval (4 days).

3.3. Greenhouse experiment

The results of ANOVA showed that there were significant differences ($P \leqslant 0.001$) between endophytes in severity of tan spot on the first, second and third leaves and for the mean of the three leaves of wheat inoculated with *Dtr* (Table 4). In addition, experiments showed significant differences for the first leaf and the average, but there were not interactions between experiments and endophytes. *T. hamatum*, *C. globosum*, and *Fusarium* sp. significantly ($P \leqslant 0.05$) reduced the average disease severity on all three leaves compared to the control (Table 5). A clear effect of *T. hamatum* in reducing the percentage leaf area diseased is particularly highlighted.

4. Discussion

In Argentina, biological control is considered an attractive option for management of some plant disease. In previous studies, the microflora of the wheat phylloplane showed potential for biocontrol fungal wheat pathogens and against *Dtr* (Perelló et al., 2001, 2002, 2003, 2008; Perelló and Mónaco, 2007). Another additional strategy could be the use of antagonistic endophytes as biocontrol agents (Bailey et al., 2006; Dingle and McGee, 2003; Istifadah and McGee, 2006; Arnold, 2007).

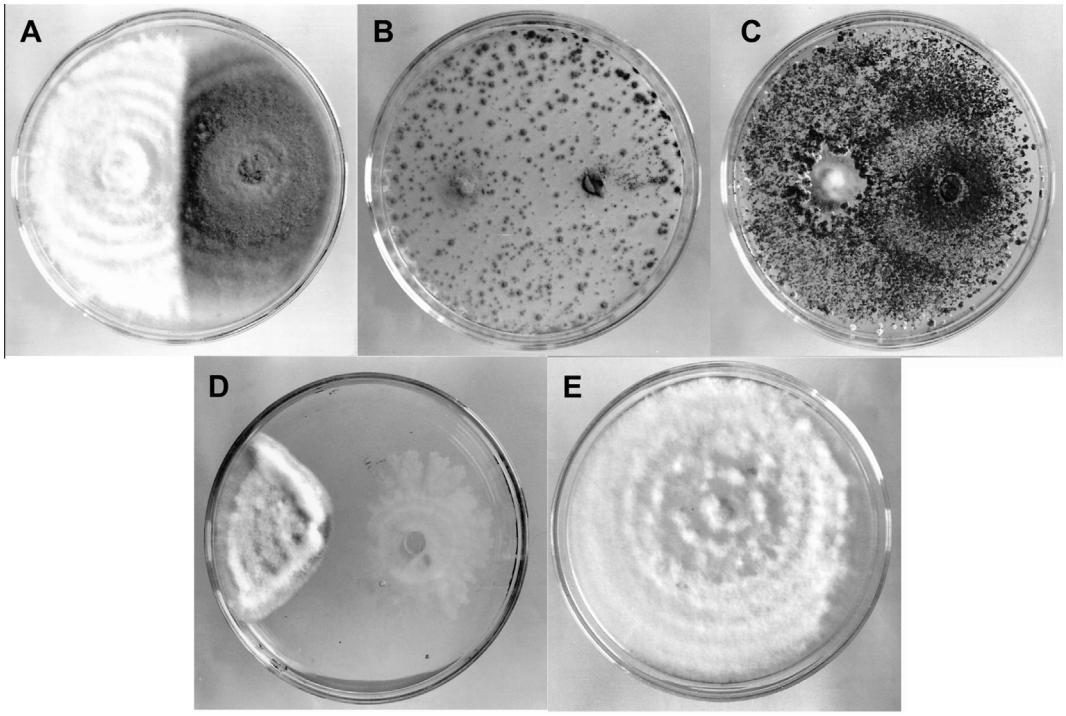

Fig. 1. Interaction types between *Drechslera tritici-repentis* (*Dtr*) and endophytes in dual cultures on 2% PDA after fifteen days incubation: (A) type A: mutually intermingling growth *A. alternata*/*Dtr*; (B) type Bi: mutually intermingling growth *Penicillium* sp. growing above *D. tritici-repentis*; (C) type Bii: intermingling growth *Dtr* has ceased growth and is overgrown by *T. hamatum*; (D) type D: mutual inhibition at distance. *D. tritici-repentis* opposed to *Bacillus* sp.; (E) control (*Dtr*).

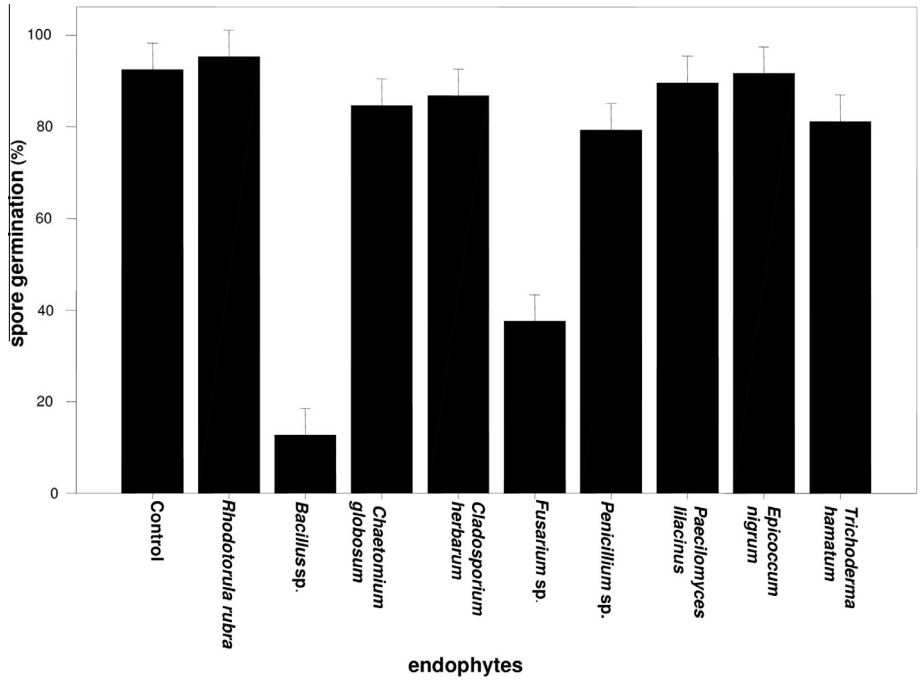

Fig. 2. Spore germination of *Drechslera tritici-repentis* in the presence of each of nine endophytes.

All endophytes assessed in the present work were previously isolated at high frequencies from symptomless wheat leaves (Larran et al., 1999, 2002b, 2007). Likewise, because foliar endophytes occupy the same ecological niche as foliar pathogens, it can be hypothesized that the foliar endophytes might to have a best suited for biocontrol of foliar pathogens as *Dtr*.

As it was shown in the results of our work, *Bacillus* antagonized the pathogen in all *in vitro* assays. Interestingly, *Bacillus* spp. have been reported as successful biocontrol agents by several authors (Nielsen and Sorensen, 1997; Alippi et al., 2000; Kloepper et al., 2004).

A reduction in the spore germination rate and alterations in the morphology of *Dtr* in addition to a significant reduction in severity of tan spot in the greenhouse was shown by *Fusarium* sp and *C. globosum*. Likewise, pigmented compounds were observed inside the hyphae or in media in treatments with *T. hamatum*, *Bacillus* sp.,

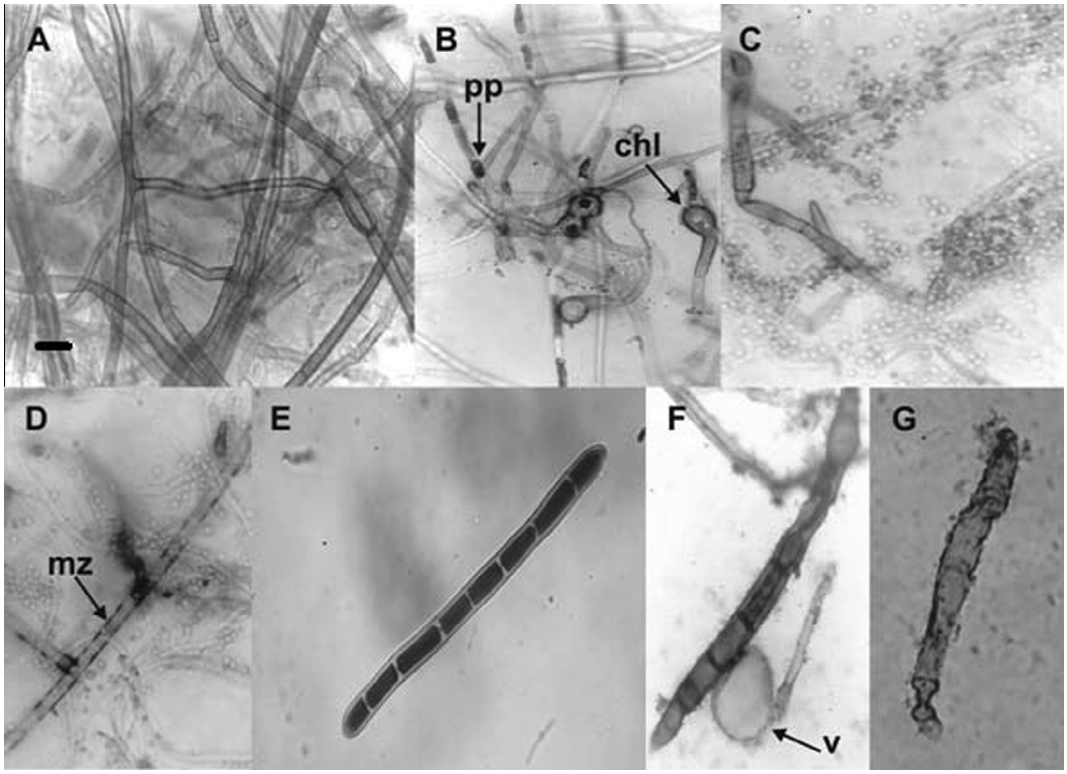

Fig. 3. Light micrographs (400×) of mycelium and conidia alterations of *D. tritici-repentis* (*Dtr*) in dual culture: (A) normal mycelium of *Dtr* (control); (B) chlamydospores (chl) and pigmented precipitate compounds inside the mycelium (pp) (*Dtr/Penicillium* sp.); (C) mycelial plasmolysis (*Dtr/Trichoderma hamatum*); (D) melanization zones of the hyphal cell wall (mz) (*Dtr/T. hamatum*); (E) normal conidia of *Dtr*; (F) shorter and swollen germ-tubes and vesicle formation (v) (*Dtr/Bacillus* sp.) and (G) conidium plasmolysis (*Dtr*/*Penicillium* sp.). Scale bars = approximately 7 μm.

Table 4
ANOVA for the effect of endophytes inoculated on three wheat leaves to reduce tan spot disease severity.

Source of variation	DF	First leaf			Second leaf			Third leaf			Overall mean		
		MS[a]	*F*[b]	*P* value	MS	*F*	*P* value	MS	*F*	*P* value	MS	*F*	*P* value
Experiments	1	0.116	5.62	0.020	0.002	0.17	0.680	0.00011	0.04	0.846	0.015	4.43	0.039
Endophytes	9	0.142	6.92	$P < 0.001$	0.072	7.31	$P < 0.001$	0.016	6.83	$P < 0.001$	0.044	12.81	$P < 0.001$
Experiments × endophytes	9	0.017	0.81	0.607	0.013	1.30	0.253	0.003	1.40	0.203	0.003	1.00	0.449
Error	74	0.021			0.009			0.002			0.008		

[a] MS: mean square.
[b] Fisher test.

Table 5
Effect of endophytes in the severity means of tan spot caused by *Drechslera tritici-repentis* evaluated on three wheat leaves in greenhouse.

Endophytes	First leaf	Second leaf	Third leaf	Overall mean
	Mean percentage leaf area diseased			
Bacillus sp.	74.01c*	14.58bcd	0.76abcd	29.78c
Chaetomium globosum	54.32b	10.93ab	0.36abc	21.87b
Cladosporium herbarum	69.34bc	19.10cde	1.61def	30.02c
Epicoccum nigrum	69.29bc	22.87e	0.99bcde	31.05c
Fusarium sp.	59.74b	10.94ab	0.34ab	23.67b
Penicillium sp.	70.36cd	24.59e	1.67ef	32.21c
Paecilomyces lilacinus	68.60bc	11.88abc	0.25a	26.91bc
Rhodotorula rubra	74.92c	23.90e	1.07bcde	33.30c
Trichoderma hamatum	38.89a	7.27a	0.20a	15.46a
Control. (*D. trititi-repentis only*)	74.45c	21.55de	2.67f	32.89c

* Means followed by the same letter in the same column within the same treatment are not statistically different according to LSD ($P \leqslant 0.05$). Data are back-transformed means from the arcsin transformation.

C. globosum, *P. lilacinum* and *Penicillim* sp. By other hand, *Bacillus* sp., *Penicillium* sp. and *P. lilacinum* have shown to produce plasmolysis, chlamidospores formation and conidial alteration while *T. hamatum* produced melanisation and hyphal plasmolysis.

Regarding *Trichoderma*, different species have been successfully proved for biocontrol of plant pathogens (Sutton and Peng, 1993; Elad, 1994; Michereff et al., 1995), among them endophytic isolates that have demonstrated antagonistic effect against diseases of *Theobroma cacao* (cacao) (Bailey et al., 2006) and against *Fusarium* wilt of lentil (Dolatabadi et al., 2012). Likewise, *Trichoderma* spp. isolated from wheat phylloplane showed inhibitions of mycelial growth and both conidial and hyphal plasmolysis of *Dtr* (Perelló et al., 2003, 2006) and the reduction in the severity of tan spot under field conditions (Perelló and Dal Bello, 2011). According the results obtained in our work *T. harmatun* is a good candidate for further study as it resulted in the greatest suppression in the greenhouse assay and in the dual-plate assay.

Previous reports showed that species of *Chaetomium* inhibited the *P. tritici-repentis* growth in *in vitro* and *in planta* assays (Istifadah et al., 2006; Istifadah and McGee, 2006). During our

in vitro test the endophytic *C. globosum* produced conidial alterations of *Dtr*. Additionally, in this work *Penicillium* sp. and *P. lilacinus* which demonstrated a great potential in *in vitro* assays as biocontrol agents were cited previously as efficient antagonists against leaf spot diseases of wheat in Argentina (Perelló et al., 2002).

In addition to the filamentous fungi and bacteria the effect of *R. rubra*, a cosmopolite yeast and common contaminant (air, soil, and plants) causing opportunistic mycoses (Gyaurgieva et al., 1996) was tested in this work. This yeast was previously isolated from the wheat phylloplane (Perelló and Mónaco, 2007) and it's antagonistic effect has been demonstrated against fungi in the wheat foliar disease complex *Septoria tritici*, *B. sorokiniana*, *Dtr* and *Alternaria triticimaculans*. Interestingly, our isolate of *R. rubra* produced conidial plasmolysis, shorter germ-tubes of the pathogen, and a significant reduction of the severity of tan spot in youngest wheat leaves.

The antagonistic effect of endophytes tested here against *Dtr* in dual culture was confirmed clearly by results obtained in greenhouse when they were pre-inoculated with the pathogen on wheat leaves. From our promising results, we conclude that *T. hamatum* and *Bacillus* sp. are particularly highlighted and could be an important component of integrated wheat diseases management.

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