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Potential of spore-forming bacteria as biocontrol agents of wheat foliar diseases under laboratory and greenhouse conditions

Das Potential der sporenbildenden Bakterien bei der biologischen Bekämpfung von Blattkrankheiten der Weizenpflanzen unter Labor- und Gewächshausbedingungen

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Summary

The potential of *Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, Bacillus pumilus, Brevibacillus laterosporus* and *Paenibacillus polymyxa* as biocontrol agents of four foliar necrotrophic pathogens of wheat in Argentina has been evaluated. The following assays were used: (a) effect of the bacterial antagonists on fungal growth in the central disk test with paired cultures, (b) effect of the antagonists on the germination of fungal spores in the paired suspension assay, and (c) reduction of disease severity in greenhouse experiments. The observed effects were specific for the antagonist-pathogen combination investigated. In the paired culture test, most of the bacterial antagonists inhibited the mycelial growth of the four pathogens tested at 14 days of the evaluation. *B. licheniformis* showed the best results for controlling *Septoria tritici* and *Drechslera tritici-repentis* whereas it was the worst against *Bipolaris sorokiniana.* In the case of *Alternaria triticimaculans,* the best were *B. laterosporus, P. polymyxa* and *B. licheniformis.* Microscopic examination of excised pieces of the fungal colony perimeters showed clear differences among treatments and controls in hyphal morphology (plasmolysis and vacuolation of hyphae, irregular texture in the cytoplasm, formation of chlamydospores). In the central disk test, mycelial necrosis of *A. triticimaculans* was induced by *B. laterosporus, P. polymyxa* and *B. pumilus.* Similar results were obtained with *B. sorokiniana* in the presence of *B. licheniformis* and *B. subtilis* and *D. tritici-repentis* with *B. pumilus.* In some combinations, the presence of inhibition haloes or bacterial chemotaxis were observed. In the paired suspension assay, all the bacteria except *B. cereus,* inhibited the conidial germination of *S. tritici.* Only *P. polymyxa, B. licheniformis* and *B. laterosporus* inhibited the conidial germination of *A. triticimaculans, B. laterosporus* being the most effective. Although all the bacteria inhibited the conidial germination of *B. sorokiniana,* the most efficient were *B. licheniformis, B. cereus* and *B. laterosporus.* In greenhouse experiments, *P. polymyxa* and *B. cereus* were the most efficient to reduce disease severities produced by *S. tritici* and *A. triticimaculans;* on the contrary, these bacteria were not effective against *B. sorokiniana* and *D. tritici-repentis. B. laterosporus* BLA 170 showed a good behaviour against all the fungal pathogens, particularly for *D. tritici-repentis* and *B. sorokiniana.* This is the first report on the efficacy of *B. laterosporus* against fungal wheat pathogens.

Key words: Biocontrol; *Bacillus; Paenibacillus; Brevibacillus;* wheat; antagonism; phylloplane; *Septoria tritici; Alternaria triticimaculans; Drechslera tritici-repentis; Bipolaris sorokiniana*

Zusammenfassung

In der vorliegenden Arbeit wurde das Potential von *Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, Bacillus pumilus, Brevibacillus laterosporus* und *Paenibacillus polymyxa* als Mittel zur biologischen Bekämpfung von vier nekrotrophen Blattpathogenen des Weizens in Argentinien untersucht. Folgende Tests wurden angewandt: a) Wirkung von bakteriellen Antagonisten auf das Wachstum der Pilze mittels Vergleichs der Mikroorganismen in Dualkulturen. b) Wirkung der Antagonisten auf die Keimung der Sporen im fallenden Tropfen und c) Verringerung des Krankheitsbefalls bei im Gewächshaus gehaltenen Weizenpflanzen. Die Ergebnisse dieser Untersuchung zeigten ein verändertes Verhalten jeder Antagonist-Pathogen- Kombination. *P. polymyxa* und *B. cereus* bekämpften am erfolgreichsten die von *Septoria tritici* und *Alternaria triticimaculans* hervorgerufenen Krankheiten, hatten aber wenig Wirkung bei der Kontrolle von *Bipolaris sorokiniana* und *Drechslera tritici-repentis. B. laterosporus* BLA 170 zeigte eine größere Wirksamkeit gegen die vier Pathogene, besonders in Bezug auf *D. tritici-repentis*. Das ist das erste mal, wo die Wirksamkeit von *B. laterosporus* gegen pathogene Pilze des Weizens dokumentiert wird. Es sind jedoch weitere Studien sowohl im Gewächshaus als auch im Freiland notwendig bis eine endgültige Schlussfolgerung gezogen werden kann.

Stichwörter: *Bacillus; Paenibacillus; Brevibacillus;* Biologische Bekämpfung; Phylloplane; Weizen; Antagonismus; *Septoria tritici; Alternaria triticimaculans; Bipolaris sorokiniana; Drechslera tritici-repentis*

1 Introduction

Mycosphaerella graminicola (Fuckel) Schrot. in Cohn [anamorph = *Septoria tritici* Roberge in Desmaz.]; *Pyrenophora tritici-repentis* (Died) Drechs. [anamorph = *Drechslera tritici-repentis* (Died) Shoemaker]; *Cochliobolus sativus* (Ito & Kurybashani) Drechs. ex Dastus [anamorph = *Bipolaris sorokiniana* (Sacc.) Shoemaker] are the most important and widespread necrotrophic pathogens of wheat in Argentina. Frequently, they appear simultaneously causing leaf spots with losses of 50 % or more depending on the environmental conditions (Kohli et al. 1995). *Alternaria triticimaculans* Simmons & Perelló is another pathogen of the complex of foliar diseases of wheat. *A. triticimaculans* is the causal agent of a leaf blight of wheat and it has been reported only on wheat in Argentina (Perelló et al. 1996); the dried holotype was deposited at the BPI Herbarium, U. S. National Fungus Collection, EGS 41-050), being until present the only recorded host (Simmons 1994).

Breeding, chemical treatments and appropriate cultural practices are the main ways for disease control. The possibility of biological control using antagonistic micro-organisms, is added to the former methods as a complementary strategy within the integrated management of these diseases. The use of bacteria as biocontrol agents of foliar diseases of cereals has been reported to be an alternative of great potential (Andrews 1992; Blakeman and Brudie 1976; Hodges et al. 1994; Knudsen and Spurr 1988; Levy et al. 1988, 1989, 1992; Li and Sutton 1995, Reis et al. 1994; Robbs 1991; Rodriguez and Pfender 1997; Rosales et al. 1993; Spurr and Knudsen 1985).

Spore-forming bacteria are appealing candidates for biocontrol because they produce endospores that are tolerant to heat and desiccation (Nielsen and Sørensen 1997; Podile and Prakash 1996) which is an advantage over other epiphytic bacteria of the phylloplane like *Pseudomonas* species.

Bacteria of the genera *Bacillus, Paenibacillus* and *Brevibacillus* have been reported to produce antibiotics, antibiotic-like compounds and antifungal metabolites (Aramayo and Castro 1986; Fiddaman and Rossal 1993; Gordon et al. 1973; Lebbadi et al. 1994; Leifter et al. 1995; Thimon et al. 1992; Podile and Prakash 1996; Pusey 1989; Robbs 1991; Shimanuki et al. 1992; Rosales et al. 1993; Stabb et al. 1994). Considering the ability of different of these genera to reduce fungal growth and to resist desiccation, the purpose of the present work has been to evaluate the potential of *Bacillus subtilis* (Ehrenberg) Cohn; *Bacillus cereus* Frankland & Frankland; *Bacillus licheniformis* (Weigmann) Chester; *Bacillus pumilus* Gottheil; *Brevibacillus laterosporus* (Laubach) Shida et al. and *Paenibacillus polymyxa* (Prazmowski) Ash, Priest & Collins as biocontrol agents of *S. tritici, B. sorokiniana, D. tritici-repentis* and *A. triticimaculans.*

2 Materials and methods

2.1 Bacterial strains

Six aerobic spore-forming bacteria were selected according to previous studies (Alippi and Mónaco 1994) and reported as producers of antifungal compounds or antibiotics. The collection includes: *Bacillus subtilis* (ATCC 10783); *Bacillus cereus* (ATCC 11778); *Bacillus licheniformis* (NRRL B-1001), *Bacillus pumilus* (ATCC 7061); *Brevibacillus laterosporus* (BLA170) and *Paenibacillus polymyxa* (NRRL B-510). *Brevibacillus laterosporus* BLA170 was isolated from larvae affected by European Foulbrood disease as previously described (Alippi 1991) and the rest of the strains were obtained from the American Type Culture Collection, Rockville, Maryland, USA (ATCC) and the Northern Utilization Research and Development Division, Peoria, Illinois, USA (NRRL).

These species are capable to sporulate at the optimum growth temperature of the tested fungi. Bacterial strains were preserved in nutrient broth containing 20 % (vol./vol.) glycerol and stored at –20 °C until used.

2.2 Fungal isolates

S. tritici, B. sorokiniana, D. tritici-repentis and *A. triticimaculans* were isolated from wheat leaves in the field that were naturally infected. The pathogens were maintained in 2 % PDA at 4 °C until used.

2.3 Laboratory experiments

The effect of the bacterial antagonists on the fungal growth was evaluated using two methods: central disk and paired cultures. In both experiments, the tested fungi were cultured on PDA for 5 days at 24 ± 1 °C and the bacterial strains were cultured on nutrient agar (NA) for 24 h at 35 ± 1 °C.

In addition, the effect of the bacterial antagonists on the germination of fungal spores was registered.

2.3.1 Central disk test

In the central disk test, each bacterial antagonist was adjusted to a concentration of about 10^6 cells/ml in sterile distilled water using a photometer at a wavelength of 620 nm. Two hundred microliters of the suspension were added to individual Petri plates containing 15 ml of NA and spread by means of sterile cotton swabs (Britania®). When the plates were perfectly dried, a 5-mm diameter agar disk of each isolate of *S. tritici, A. triticimaculans, D. tritici-repentis* and *B. sorokiniana* was transferred to the center of the plates. For the control, 200 µl of sterile distilled water were previously spread over each plate. There were five replications for each treatment and five for the controls. After 4, 7 and 14 days of incubation at 25 °C ± 1, the plates were evaluated by measuring the diameter of the fungal colony.

2.3.2 Paired culture test

In the paired culture test, a 5 mm-diameter disk of each fungus was placed at a distance of 40 mm apart from a drop of 5 µl of a bacterial suspension of each bacterial strain on a Petri dish containing 15 ml of NA (Alippi and Mónaco 1995). Bacterial suspensions in sterile distilled water of each antagonist were adjusted to a concentration of 10^6 cells/ml using a photometer at a wavelength of 620 nm. For controls, 5 µl of sterile distilled water were spotted instead of bacteria. There were five replicate plates for each combination bacterium-fungus and 5 for each fungus as controls. Plates were incubated at 25 °C ± 1 and the evaluation was performed by measuring the diameter of the fungal colony at 4, 7 and 14 days. The analysis was conducted by two way ANOVA, with F-test for effects due to treatment and time. Means were compared by Tukey test. The percentage of mycelial growth inhibition (MGI) was calculated according to the following formula:

MGI (%) = [(MGC – MGT) / MGC] × 100, where MGC = mean length of mycelial growth of the control, and MGT = mean length of mycelial growth of the treatment (Michereff et al. 1994).

In all cases, the fungal colony was observed microscopically after 14 days in order to determine abnormal characteristics of the mycelia.

2.3.3 Paired suspensions (effect of the antagonists on the germination of fungal spores)

The effects of the antagonists on the germination of fungal spores were evaluated using the paired suspension technique (Reis et al. 1994).

The fungal cultures were incubated in a growth chamber at 18 ± 2 °C for 10 days with alternating light (3,500 lx-dark cycles of 12 h plus the addition of near UV light (365 nm) to induce sporulation. Spore suspensions were prepared from 10-day-old cultures of *S. tritici, B. sorokiniana* and *A. triticimaculans* on 2 % PDA slants and obtained by flooding each one with 5 ml sterile distilled water and gently scrapping with a flamed loop. The suspensions were filtered through a single layer of cheesecloth and spore concentration were determined with a hemacytometer.

Six droplets of 50 µl of sterile distilled water containing *S. tritici* (6×10^6 spores/ml), *B. sorokiniana* (2×10^5 spores/ml) and *A. triticimaculans* (4×10^5 spores/ml) and each test bacterium (50 µl of a suspension containing 10^6 spores/ml) were placed inside the cavity of sterile glass slides. Controls contained 50 µl of each fungal suspension plus 50 µl of sterile distilled water. In all cases, the final volumes of 100 µl were homogenized by mixing it with sterile tips and a micropipette. As *D. tritici-repentis* sporulates poorly, different methods were tested in order to obtain a good level of conidia (Odvody and Boosalis 1978; Raymond et al. 1985). Nevertheless, the minimum number of 10 spores per field was not reached, so the statistical analysis for the *D. tritici-repentis* combinations was not performed.

The slides were placed over moist cotton inside sterile Petri plates (130 mm diameter) and incubated in dark at 25 ± 1 °C. After 24 h and 48 h, the number of germinated conidia was counted under a light microscope (× 200 or 400 magnification according to the fungal species) evaluating three microscopic fields in each combination and in each control. Only those fields containing at least 10 fungal spores were used. Differences in the proportions of germinated conidia (arcsin transformed) were analyzed by one way ANOVA and means were compared by Tukey test. The percentage of conidia germination inhibition (CGI) was calculated according to the following formula:
CGI (%) = [(CGC – CGT) / CGC] × 100, where CGC = conidia germination in the control, and CGT = conidia germination in the treatment (Michereff et al 1994). Also, the abnormalities of the germ tubes, if any, were registered.

2.4 Greenhouse experiments

Each bacterial strain was cultured in two screw-capped flasks with 150 ml nutrient broth each and incubated at 30 ± 1 °C with constant agitation at 30 rpm for 7 days.

Bacterial cells were concentrated by centrifugation at 3,700 rpm, the resulting pellet washed twice and resuspended in 100 ml of sterile distilled water and adjusted to a final concentration of about 6×10^8 cells/ml (A_{620nm} = 0.700). Broth cultures were examined microscopically under phase contrast before centrifugation to confirm that they contained spores only.

The fungal isolates were cultured in Erlenmeyer flasks with PDA for 10 days at 20 ± 2 °C in the growth chamber. Inoculum was prepared in sterile distilled water at the following concentrations: *S. tritici* 6×10^6 spores/ml, *B. sorokiniana* 2×10^5 spores/ml and *A. triticimaculans* 4×10^5 spores/ml. In the case of *D. tritici-repentis,* poor sporulation was observed, so as the mycelium is infective, colony forming units were used as inoculum at a concentration of 4×10^5 cfu/ml.

All suspensions were amended with 0.05 % Tween 80 in distilled water.

The assay was carried out in a greenhouse at temperatures ranging from 15 to 25 °C. Five-week-old wheat seedlings at stage 15 (Zadocks et al. 1974) of cv. 'ProInta Isla Verde' were first sprayed with the bacterial suspensions until runoff using a manually operated sprayer. Afterwards, each fungal pathogen was inoculated in the same way. Control plants were treated with the pathogens only. All treatments were kept in humid chambers for 48 h, except for those treatments with *S. tritici* controls and *S. tritici* plus bacterial treatments that were maintained for 72 h.

The experimental design was a completely randomized with five replications. Each experimental unit consisted of a plastic pot (12 cm in diameter) with five wheat seedlings. For the evaluation, the 4th leaf of each plant was selected. For each disease the percentage necrotic leaf area was estimated using specific scales (see Table 2). Differences between treatments were analyzed by Kruskal-Wallis test and Mann-Whitney U-test for each pair of samples was computed for multiple comparisons (Sokal and Rohlf 1995).

3 Results

The results of the present paper showed a behaviour differing according to each antagonist-pathogen combination.

3.1 Laboratory experiments

3.1.1 Central disk test

In the central disk test, all the bacterial antagonists inhibited the growth of the four pathogens, which was limited to the size of the initial disk (6 mm). The mycelial growth of controls was normal according to the species tested. In the combinations *B. laterosporus/A. triticimaculans; P. polymyxa/A. triticimaculans; B. pumilus/A. triticimaculans; B. licheniformis/B. sorokiniana; B. subtilis/B. sorokiniana; B. pumilus/ D. tritici-repentis,* a mycelial necrosis was observed while in the rest of combinations, the mycelia were normal. Other patterns of interaction observed were the presence of an inhibition halo between the bacterial growth and the fungal disk in the combinations of *B. pumilus/B. sorokiniana* and *B. laterosporus/ B. sorokiniana* or a bacterial chemotaxis, consisting of a strong tendency to swarm towards the fungal disk, in the combination *B. subtilis/A. triticimaculans.*

3.1.2 Paired culture test

The results for each combination of pathogen and antagonist are summarized in Table 1 for *S. tritici, A. triticimaculans, D. tritici-repentis* and *B. sorokiniana* respectively.

In the case of *S. tritici* (Table 1), the inhibitory effect of bacterial antagonists did not appear until 7 days of the inoculation. At 14 days, the percentage of inhibition of mycelial growth was between 25 and 65 %, *B. licheniformis* and *B. laterosporus* being most effective. All bacterial antagonists inhibited the mycelial growth of *A. triticimaculans, B. laterosporus, P. polymyxa* and *B. licheniformis* being most effective (Table 1). Mycelial growth of *D. tritici-repentis* was reduced by *B. laterosporus, B. licheniformis* and *P. polymyxa* as early as 4 days, with *B. cereus, B. pumilus* and *B. subtilis* showing activity against the fungus after 7 and 14 days, and *B. licheniformis* and *B. laterosporus* being the most effective throughout the experiment (Table 1). *B. licheniformis* did not inhibit the growth of *B. sorokiniana,* while the rest of the antagonists exhibited mycelial growth inhibition between 18 and 27 % only after 14 days (Table 1).

The different patterns of inhibition observed in relation to the control are shown in Figure 1. In the first pattern, the fungus presented a defined edge of growth opposite to the bacterium (Fig. 1b), this front can or cannot be darker than the color of the normal mycelium. The second pattern (Fig. 1c) showed an irregular development of the fungal colony growing towards the opposite side of the bacterium; with melanization in the contact zone of the two micro-organisms. Finally (Fig. 1d), the fungal growth was totally inhibited and overgrown by the bacterium.

The first pattern was observed in *D. tritici-repentis/ B. subtilis, S. tritici/B. pumilus, S. tritici/P. polymyxa, B. sorokiniana/B. laterosporus* and *B. sorokiniana/P. polymyxa* combinations. The second pattern was found in the *D. tritici-repentis/P. polymyxa, D. tritici-repentis/B. licheniformis, S. tritici/P. polymyxa* and *S. tritici/B. pumilus* combinations and the third pattern was observed in the combinations of *D. tritici-repentis/B. licheniformis, S. tritici/B. licheniformis* and *S. tritici/B. laterosporus.* In the case of *B. sorokiniana,* an abundant formation of tufts was registered in the combinations with *B. pumilus, P. polymyxa* and *B. subtilis;* the same situation was observed in the case of *S. tritici* with *B. cereus* and *B. pumilus.*

Microscopic examination of excised pieces of the fungal colony perimeters showed clear differences in hyphal morphology among treatments and controls. In the combinations *D. tritici-repentis/B. pumilus* (Fig. 2b); *D. tritici-repentis/B. cereus* and *D. tritici-repentis/ B. subtilis* vacuolation of hyphae, plasmolysis of mycelium and a great number of chlamydospores in relation with each control was observed. In the combinations *S. tritici/B. laterosporus, S. tritici/B. subtilis* and *S. tritici/B. licheniformis,* plasmolysis of mycelium and irregular texture of the cytoplasm were observed, while in the combinations *A. triticimaculans/P. polymyxa, A. triticimaculans/B. licheniformis* and *A. triticimaculans/B. laterosporus* plasmolysis of mycelium and formation of vacuole inside the hyphae was noticed. In addition, *B. laterosporus, B. subtilis* and *B. licheniformis* induced the plasmolysis of mycelium and chlamydospore formation on *S. tritici.*

Table 1. Mycelial growth (MG) and percentage of inhibition of mycelial growth (IMG) of four wheat pathogens induced by bacterial antagonists in the paired culture test

Tab. 1. Myzelwachstum (MG) und Prozentsatz der Myzelwachstumshemmung (IMG) von Blattpathogenen auf Weizen durch Antagonisten-Bakterien beim Para-Kultur Test

	4 days		7 days		14 days	
	MG (mm)	IMG (%)	MG (mm)	IMG (%)	MG (mm)	IMG (%)
S. tritici						
Control	6.2 a	–	9.0 a	–	14.2 a	–
B. licheniformis	5.0 a	19.35	5.0 c	44.44	5.0 d	64.79
B. laterosporus	5.0 a	19.35	5.0 c	44.44	5.0 d	64.79
B. subtilis	5.6 a	9.68	6.4 bc	28.89	7.8 c	45.07
B. pumilus	6.3 a	–1.61	7.0 b	22.22	9.8 b	30.98
B. cereus	7.1 a	–14.52	7.1 b	21.11	10.6 b	25.35
P. polymyxa	6.6 a	–6.45	8.7 ab	3.33	10.7 b	24.64
A. triticimaculans						
Control	22.6 a	–	42.8 a	–	52.6 a	–
B. laterosporus	6.2 c	72.57	6.2 c	85.51	6.2 c	88.21
P. polymyxa	7.0 c	69.03	8.8 c	79.44	10.2 c	80.61
B. licheniformis	11.2 b	50.44	11.5 c	73.13	11.5 c	78.14
B. subtilis	25.2 a	–11.50	29.8 b	30.37	31.2 b	40.68
B. pumilus	24.0 a	–6.19	34.2 b	20.09	34.2 b	34.98
B. cereus	25.2 a	–11.50	31.4 b	36.63	34.4 b	34.60
D. tritici-repentis						
Control	38.4 a	–	48.8 a	–	49.8 a	–
B. laterosporus	5.0 c	86.98	5.0 d	89.75	5.0 d	89.96
B. licheniformis	5.4 c	85.94	5.4 d	88.93	5.4 d	89.16
P. polymyxa	15.4 b	59.89	27.8 c	43.03	27.8 c	44.18
B. cereus	31.2 a	18.75	32.6 b	33.20	34.2 b	31.32
B. pumilus	33.0 a	14.06	34.8 b	28.69	34.2 b	30.12
B. subtilis	33.8 a	11.98	34.8 b	28.69	34.8 b	28.91
B. sorokiniana						
Control	33.8 a	–	43.2 a	–	47.6 a	–
B. cereus	28.2 a	16.56	34.6 b	19.91	34.6 b	26.89
B. pumilus	32.4 a	4.14	36.2 ab	16.20	36.2 b	23.95
P. polymyxa	31.2 a	7.70	35.6 b	17.59	36.6 b	23.11
B. subtilis	32.8 a	2.90	37.0 ab	14.35	37.6 b	21.01
B. laterosporus	32.4 a	4.14	37.2 ab	13.89	39.0 b	18.07
B. licheniformis	31.8 a	5.90	41.4 ab	4.17	41.4 ab	13.02

Means followed by the same letter in each column for each pathogen do not differ ($P < 0.01$) according to TUKEY-test.
Die mit gleichen Buchstaben gekennzeichneten Mittelwerte innerhalb der Spalte unterscheiden sich nicht signifikant nach dem TUKEY-Test ($P < 0.01$).

3.1.3 Paired suspensions

All the bacteria inhibited the conidial germination of *S. tritici* more than 70 % ($P < 0.01$), except for *B. cereus* which did not show significant differences in relation to controls (Fig. 3).

P. polymyxa, B. licheniformis and *B. laterosporus* inhibited the conidial germination of *A. triticimaculans* (Fig. 4), *B. laterosporus* being the most effective (80 % of inhibition, Fig. 4b) while *B. subtilis, B. cereus* and *B. pumilus* did not show any significant effect (Fig. 3). Nevertheless, a reduction of conidial size was observed in the case of *B. cereus* (Fig. 4c) and formation of abnormal vesicles in the case of *B. subtilis* (Fig. 4d).

All the bacterial antagonists tested inhibited the conidial germination of *B. sorokiniana* for 60 % or more, *B. licheniformis, B. cereus* and *B. laterosporus* being the most efficient (Fig. 3).

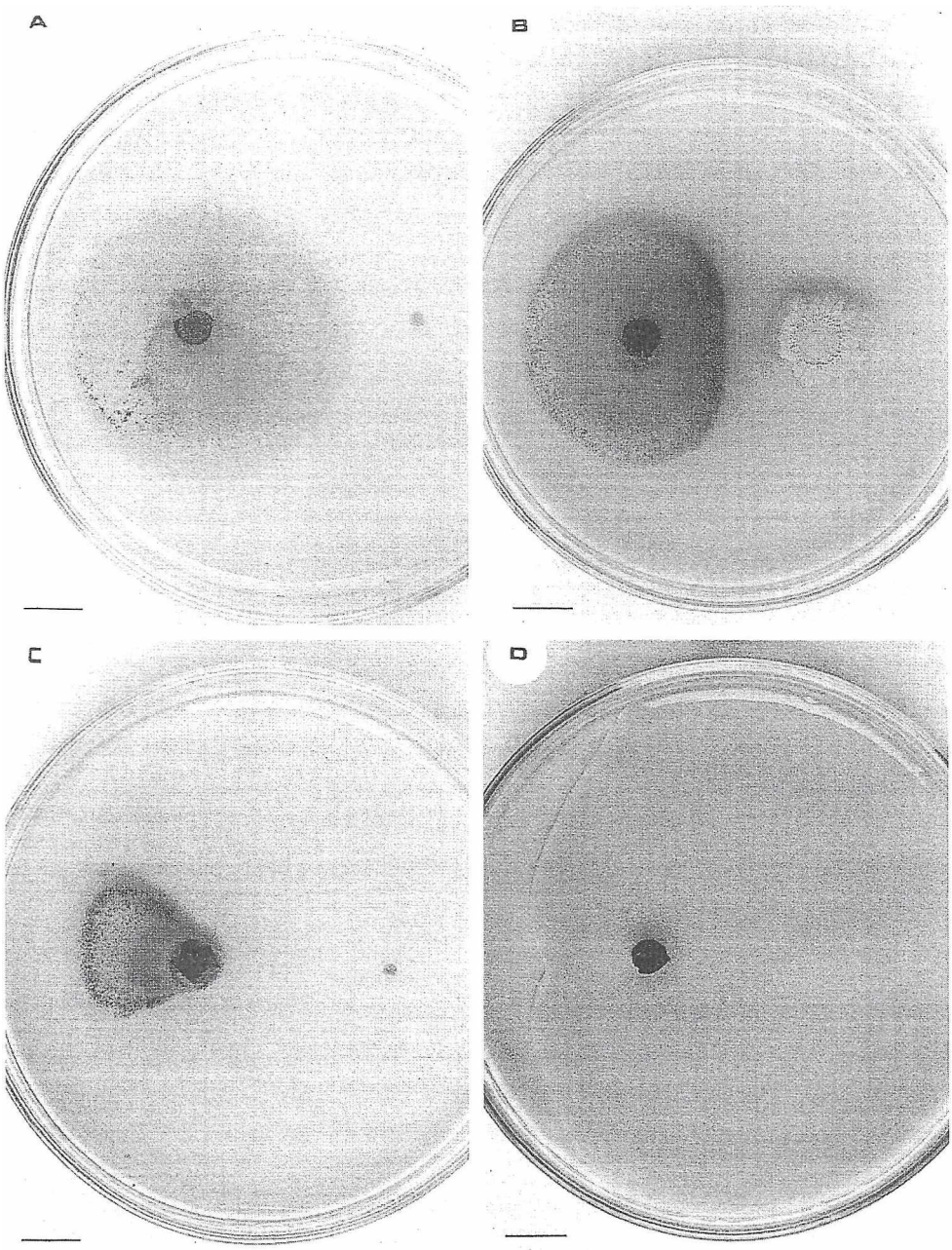

Fig. 1. Patterns of inhibition observed in the paired culture test for different *Drechslera tritici-repentis*/antagonist combinations growing on nutrient agar after 14 days of incubation at 25 ± 1 °C. a) Control (*D. tritici-repentis*/sterile distilled water); b) *D. tritici-repentis* vs. *Bacillus subtilis;* c) *D. tritici-repentis* vs. *Paenibacillus polymyxa;* d) *D. tritici-repentis* vs. *Brevibacillus laterosporus.* Bars = 1 cm.

Abb. 1. Hemmungsmuster für *D. tritici-repentis* durch bakterielle Antagonisten beim Paar-Kultur Test auf Nähr-Agar nach 14 tägiger Inkubationszeit bei 25 ± 1 °C. a) Kontrolle *D. tritici-repentis*/steriles destilliertes Wasser, b) *D. tritici-repentis* vs. *B. subtilis;* c) *D. tritici-repentis* vs. *P. polymyxa;* d) *D. tritici-repentis* vs. *B. laterosporus*. Balken = 1 cm.

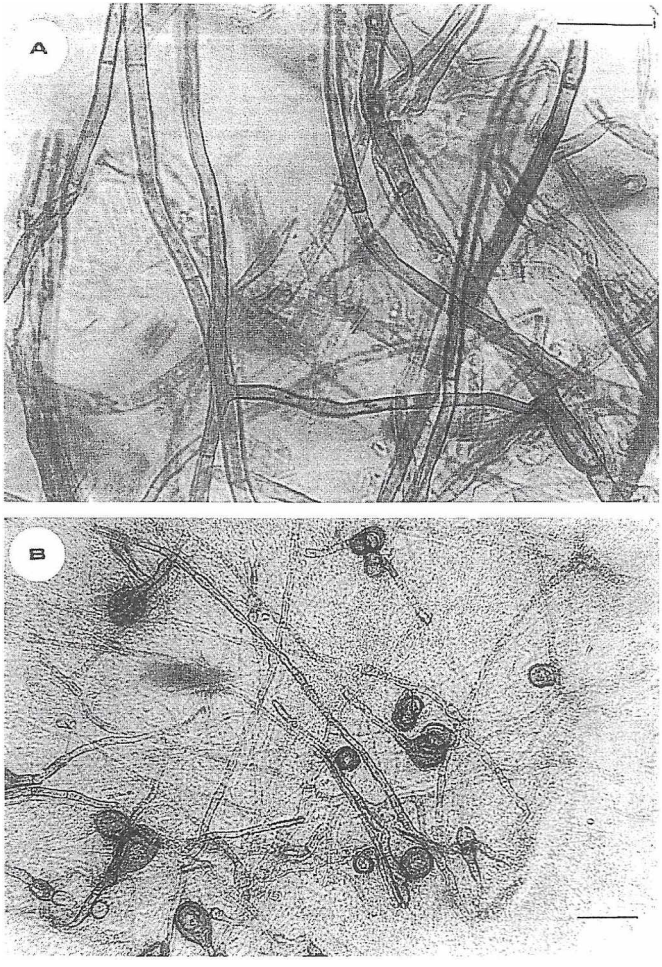

Fig. 2. Mycelium of *Drechslera tritici-repentis* after 14 days of incubation at 25 ± 1 °C in nutrient agar in the paired culture test. a) Normal mycelium in the control. Bar = 50 µm. b) Mycelium showing chlamydospore formation in the combination with *Bacillus pumilus*. Bar = 100 µm.
Abb. 2. Myzel von *D. tritici-repentis* mit Bildung von Chlamydosporen in Kombination mit *B. pumilus* nach 14 Tagen Inkubationszeit bei 25 ± 1 °C in Paar-Kulturen auf angereichertem Agar. Balken = 100 µm.

The microscopic examination of conidia of *B. sorokiniana,* in the presence of *B. laterosporus, B. cereus* and *P. polymyxa,* showed swollen and shorter germ tubes (Fig. 5b and c), in some cases with torulose aspect. In *D. tritici-repentis* combinations, conidial germ tubes were shorter and swollen and appeared torulose. Conidial tip burst and plasmollysis were also observed.

3.2 Greenhouse experiments

The results showed that in the case of *S. tritici,* all tested bacteria decreased the DI with the exception of *B. pumilus* which did not differ significantly from the control, the most effective being *B. cereus* and *P. polymyxa* (Table 2). The infection of *A. triticimaculans* was reduced by all the bacterial antagonists, the most effective being *B. cereus* and *P. polymyxa* in relation to the control (Table 2). The foliar area affected by *D. tritici-repentis* was reduced by all bacterial antagonists with the exception of *B. cereus; B. laterosporus* and *B. subtilis* being most efficient (Table 2). In the case of *B. sorokiniana,* only *B. laterosporus* and *B. subtilis* were effective (Table 2).

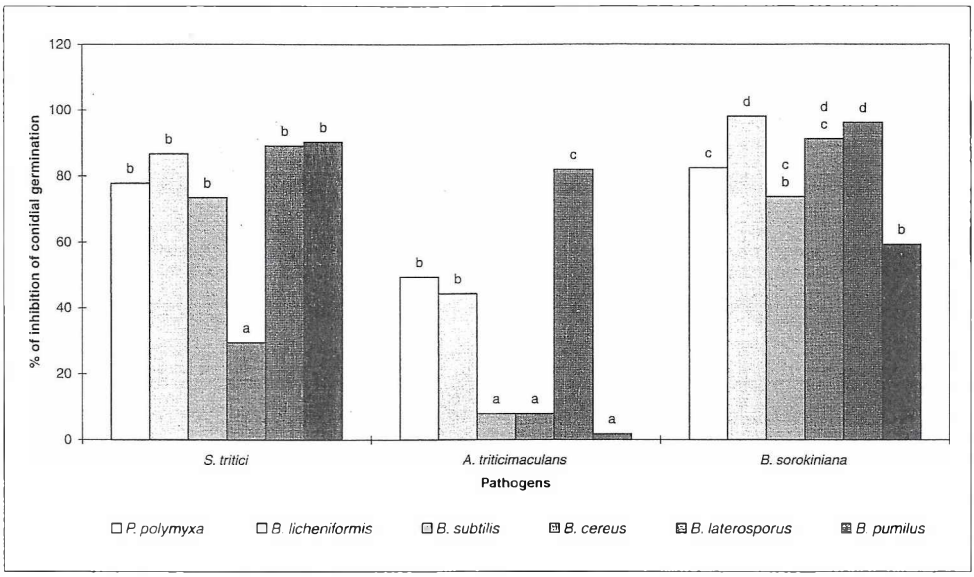

Fig. 3. Inhibition of conidial germination of *Septoria tritici, Alternaria triticimaculans* and *Bipolaris sorokiniana* by the bacterial antagonists. Data represent six replicates. Bars with same letters do not differ significantly at P = 0.01 according to Tukey's mean separate test.

Abb. 3. Hemmung der Sporenkeimung von *S. tritici, A. triticimaculans* und *B. sorokiniana* durch bakterielle Antagonisten. Die Daten repräsentieren Mittelwerte von sechs Wiederholungen. Balken mit gleichen Buchstaben unterscheiden sich nicht signifikant nach dem Tukey Test (P = 0.01).

4 Discussion

During the last decade, biological control of foliar diseases including those affecting Gramineae by means of bacteria has become increasingly promising (Li and Sutton 1995).

Bacillus species and related genera are known to be effective antagonists against several post-harvest and rhizosphere pathogens (Alippi and Monaco 1991; Hwang et al. 1996; Mari et al. 1996; Nielsen and Sørensen 1997; Utkede and Smith 1993). On the other hand, in the phylloplane, the effectiveness of *Bacillus subtilis* strains in controlling different diseases has been established (Berger et al. 1996, Douville and Boland 1992; Michereff et al. 1994; Mitzubuti et al. 1995a, Mitzubuti et al. 1995b; Montesinos et al. 1996).

The ability of spore-forming bacteria to inhibit fungal plant pathogens by secreting antibiotics with antifungal properties like iturins, bacitracins, bacillomycins, zwittermicins, polymyxins, subtilins, mycosubtilins, chlorotetain, bacilysin, rhizocticin and surfactins (Galvez et al. 1993; Lebbadi et al. 1994; Stabb et al. 1994; Thimon et al. 1992) and cell wall degrading enzymes (Nielsen and Sørensen 1997; Podile and Pakash 1996) has been well studied, particularly within the genera *Bacillus* and *Paenibacillus*. Furthermore, these genera are attractive candidates as biocontrol agents because of their abundance in soil and aerial parts of plants and their ability to produce drought-resistant endospores providing a long survival and resistance against physical stress (Nielsen and Sørensen 1997).

Some other authors observed a mycolytic activity of *B. subtilis* against *Rhizoctonia solani, Pythium ultimum* (Fidamann and Rossal 1993), *Aspergillus niger* (Podile and Prakash 1996) and *Alternaria helianthi* (Kong et al. 1997) expressed as swelling of hyphal tips, disintegration of mycelium, cell wall dissolution, germ tube lysis and vesicle formation. There are several reports regarding the activity of *B. subtilis* isolates on the germination of fungal conidia, expressed as different deformations of the germ tubes (Douville and Boland 1992; Kong et al. 1997; Michereff et al. 1994; Mizubutti et al. 1995a, 1995b; Reis et al. 1994) probably related to the production of extracellular non-volatile metabolites (Michereff et al. 1994). Similar results have been reported for *B. cereus* on *Phytophtora*

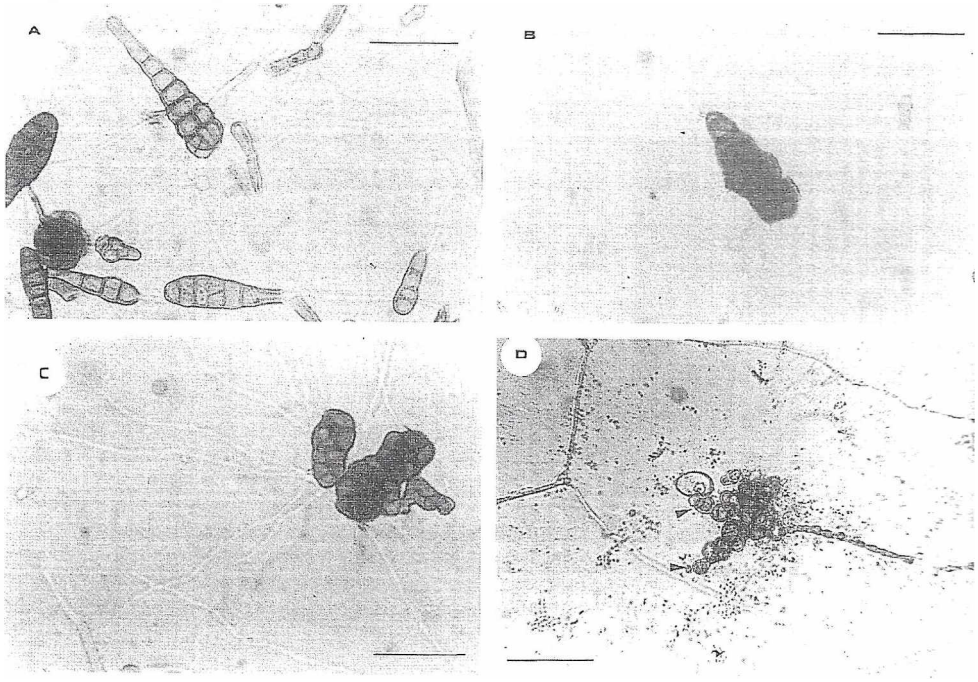

Fig. 4. *Alternaria triticimaculans* inhibition of conidia in the paired suspension test after 48 h: a) Normal germination of conidia in the water control, b) Complete inhibition of conidial germination by *Brevibacillus laterosporus,* c) Reduction of conidial size without significant reduction of germination by *Bacillus cereus.* d) Vesicles (shown by arrowheads) caused by *Bacillus subtilis.* Bars = 50 µm.
Abb. 4. Keimhemmung der Konidien von *A. triticimaculans* im Paar-Kultur-Test nach 48 Std. a) Normale Keimung der Konidien in Wasser (Kontrolle), b) Keimhemmung der Konidien durch *B. laterosporus,* c) Verringerung der Größe der Konidien ohne wesentlichen Einfluss auf die Keimung durch *B. cereus,* d) Vesikelbildung verursacht durch *B. subtilis.* Balken = 50 µm.

medicaginis (Silo-Suh et al. 1994) and on *Alternaria helianthi* (Kong et al. 1997). In addition, *B. cereus* inhibited the vegetative growth and ascospore germination of *Sclerotinia sclerotiorum* in *in vitro* tests; and also significantly reduced the severity of basal pod rot by decreasing lesions size in *in vivo* studies (Huang et al. 1993). Nielsen and Sørensen (1997) reported the inhibitory effect of *P. polymyxa* and *B. pumilus* against *Pythium ultimun, Pythium irregulare, Rhizoctonia solani,* and *Botrytis cinerea.* According to these authors, bacterial enzymatic activity enhanced the decomposition of fungal cell walls and their antibiotic production affected the membrane permeability. Hwang et al. (1996) reported that *P. polymyxa* and *B. subtilis* were effective for the control of *Pythium* root rot of field pea suggesting that the suppression of *Pythium* growth involved the production of antifungal compounds as shown *in vitro.*

In our paired suspension tests, *B. laterosporus, B. cereus* and *P. polymyxa* induced the formation of swollen germ tubes with torulose aspect in *B. sorokiniana* and *D. tritici-repentis* while *B. subtilis* induced the formation of abnormal vesicles in *A. triticimaculans.* In our greenhouse studies, *B. cereus* and *P. polymyxa* highly reduced the disease severity induced by *S. tritici* and *A. triticimaculans.* In the case of *P. polymyxa,* it was a positive correlation between reduction of disease severity and inhibition of spore germination, suggesting that the mechanism for protection may be linked to the inhibition of germ tubes formation. Under greenhouse conditions, *B. cereus* provided a good protection against *S. tritici,* in spite that in *in vitro* studies failed to provide a good inhibition of spore germination or a significant inhibition of mycelial growth. This difference in results suggests that *B. cereus* could give protection by excluding *S. tritici* from the stomata and subestomatic chambers rather than by antibiotic production.

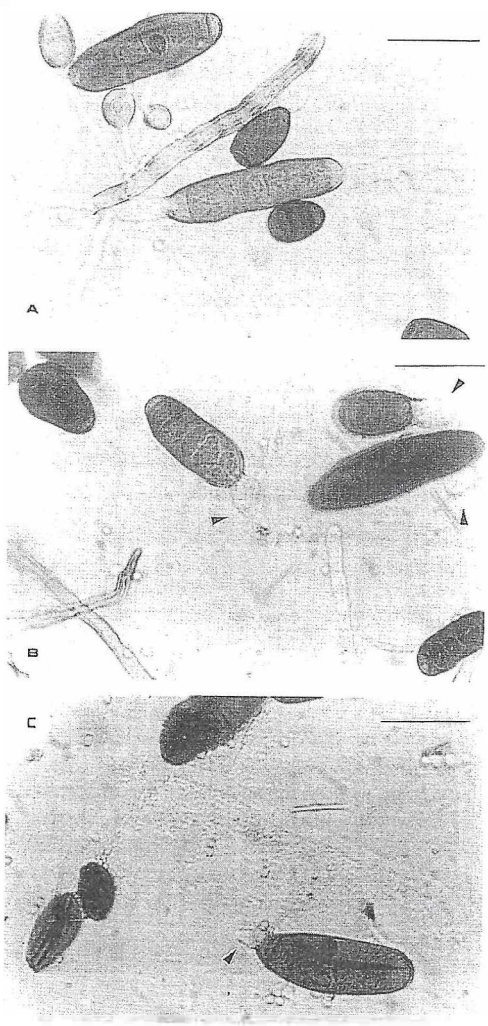

Fig. 5. *Bipolaris sorokiniana* inhibition of conidia in the paired suspension test after 48 h: a) Normal germination in the water control, b) and c) Conidia with swollen and shorted germ tubes by *Paenibacillus polymyxa* and *Brevibacillus laterosporus*, respectively. Abnormal germ tubes are marked by arrowheads. Bars = 50 µm.

Abb. 5. Hemmung der Konidienkeimung von *B. sorokiniana* im Paar-Suspensions-Test nach 48 Std. a) Normale Keimung in Wasser (Kontrolle), b) und c) Konidien mit breiteren und verkürzten Keimschläuchen, hervorgerufen durch *P. polymyxa* und *B. laterosporus*. (siehe Pfeile). Balken = 50 µm.

Epiphytic bacterial colonization in relation to leaf topography of bean leaves had been studied, showing that bacterial cells were more concentrated on anticlinal walls along the veins, in stomatal pores and hair bases (Windels and Lindow 1985).

B. laterosporus (BLA 170) was the best species in decreasing the disease severity induced by *D. tritici-repentis* and *B. sorokiniana* and also showed a good behaviour against *S. tritici*. The mechanisms of protection may be associated with the production of antibiotics and other antifungal compounds that strongly inhibited the spore germination *in vivo* and *in vitro*, producing alterations in the germ tubes in the pair suspension assay and also inhibiting mycelial growth in the paired culture test.

Despite certain variability observed in our trials (paired culture tests, paired suspensions and greenhouse experiments), there is enough evidence for the effectiveness of the *Bacillus, Brevibacillus* and *Paenibacillus* strains tested as biocontrol agents. Some lack of correlation between the antifungal activity *in vitro* and the disease reduction *in vivo* has been reported by several authors (Andrews 1992; Blackeman and Brudie 1976; Montesinos et al. 1996).

It is important to point out that *B. laterosporus* BLA 170 was efficient against all the fungal pathogens tested, particularly *D. tritici-repentis* and *B. sorokiniana*. This bacterial species has been reported as a

Table 2. Effects of bacterial antagonists applied on wheat seedlings on the disease index (DI) induced by four fungal leaf pathogens

Tab. 2. Wirkung bakterieller Antagonisten auf den Krankheitsindex (DI) hervorgerufen durch vier pilzliche Blattpathogene auf Weizen

	DI	Sum of ranks (b)
S. tritici		
Control	3.9	165.0 *
B. pumilus	2.8	124.5 * *
B. subtilis	2.4	116.5 * *
B. laterosporus	1.6	65.0 * *
B. licheniformis	1.6	71.0 * *
B. cereus	1.1	44.5 *
P. polymyxa	1.1	43.5 *
A. triticimaculans		
Control	2.0	162.5 *
B. pumilus	1.4	116.5 *
B. subtilis	1.3	109.0 *
B. laterosporus	1.2	100.0 *
B. licheniformis	1.1	87.0 *
P. polymyxa	0.4	32.5 *
B. cereus	0.2	22.5 *
D. tritici-repentis		
Control	2.7	154.5 *
B. cereus	2.2	134.5 * *
B. pumilus	1.9	119.0 *
P. polymyxa	1.8	93.0 * *
B. licheniformis	1.3	72.0 * *
B. subtilis	0.7	41.5 * *
B. laterosporus	0.4	15.5 *
B. sorokiniana		
Control	3.3	145.5 *
P. polymyxa	3.1	129.5 *
B. pumilus	2.7	104.0 * *
B. licheniformis	2.6	88.5 * * *
B. cereus	2.4	86.0 * * *
B. subtilis	1.8	46.0 * *
B. laterosporus	1.4	30.5 *

(b) Sum of ranks from the Kruskal-Wallis test

* Homogeneous groups (P > 0.05) from multiple comparisons (Mann-Whitney U-test)

Disease index (DI) induced by *Septoria tritici:* 0 = no visible infection; 1 = 1–20 %; 2 = 21–40 %; 3 = 41–60 %; 4 = 61–80 % and 5 = more than 80 % of affected foliar area

Disease index (DI) induced by *Alternaria triticimaculans:* 0 = no visible infection; 1 = 1–20 %; 2 = 21–40 % and 3 = more than 40 % of affected foliar area

Disease index (DI) induced by *Drechslera tritici-repentis:* 0 = no visible infection; 1 = 1–20 %; 2 = 21–40 %; 3 = 41–60 % and 4 = more than 40 % of affected foliar area

Disease index (DI) induced by *Bipolaris sorokiniana:* 0 = no visible infection; 1 = 1–20 %; 2 = 21–50 %; 3 = 51–75 % and 4 = more than 75 % of affected foliar area

In all cases, numbers are means from five replicates

(b) Summe der Ränge des Kruskal-Wallis Test

* Homogene Gruppen (P > 0.05) von mehrfachen Vergleichen (Mann-Whitney U-Test)

Krankheitsindex (DI) hervorgerufen durch *S. tritici:* 0 = keine Symptome; 1 = 1–20 %; 2 = 21–40 %; 3 = 41–60 %; 4 = 61–80 % und 5 = mehr als 80 % der Blattfläche befallen

Krankheitsindex (DI) hervorgerufen durch *A. triticimaculans:* 0 = keine Symptome; 1 = 1–20 %; 2 = 21–40 %, und 3 = mehr als 40 % der Blattfläche befallen

Krankheitsindex (DI) hervorgerufen durch *D. tritici-repentis:* 0 = keine Symptome; 1 = 1–20 %; 2 = 21–40 %, 3 = 41–60 % und 4 = mehr als 60 % der Blattfläche befallen

Krankheitsindex (DI) hervorgerufen durch *B. sorokiniana:* 0 = keine Symptome; 1 = 1–20 %; 2 = 21–50 %; 3 = 51–75 %, und 4 = mehr als 75 % der Blattfläche befallen

Zahlenwerte repräsentieren Mittelwerte von fünf Wiederholungen

producer of two antibiotics called laterosporin A and laterosporin B (Barnes 1949; Shimanuki et al. 1992). It is a common colonizer of apiaries and has also been found on soil, in water, on flower surfaces (Gordon et al. 1973) and roots of healthy plants (Rosales et al. 1993).

To our knowledge, this is the first report case on the efficacy of *B. laterosporus* against wheat fungal pathogens. Further studies are needed in order to determine not only the toxic metabolites produced but also the potential control of other diseases by this promising micro-organism.

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