Rev Argent Microbiol. 2015;47(1):62–71

REVISTA ARGENTINA DE MICROBIOLOGÍA

www.elsevier.es/ram

ORIGINAL ARTICLE

Selection of potential biological control of *Exserohilum turcicum* with epiphytic microorganisms from maize

Melina Sartori[a,b], **Andrea Nesci**[a,b], **Ángela Formento**[c], **Miriam Etcheverry**[a,b,*]

[a] *Laboratorio de Ecología Microbiana, Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta 36 km 601 (X5806JRA), Río Cuarto, Córdoba, Argentina*
[b] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina*
[c] *INTA EEA Paraná, Ruta 11 km 12,5 (3101) Oro Verde, Paraná, Entre Ríos, Argentina*

Received 9 June 2014; accepted 5 January 2015
Available online 11 March 2015

KEYWORDS
Exserohilum turcicum;
Biocontrol;
Epiphytic bacteria;
Maize

Abstract The aims of this study were to select microbial isolates from phyllosphere of maize and to examine their antagonistic activity against *Exserohilum turcicum*. Selection was performed through the ability of isolates to compete with the pathogen using an index of dominance and to affect growth parameters of *E. turcicum*. Most of the epiphytic populations obtained for the screening were bacteria. These isolates were found in the order of 6 log CFU/g of leaf fresh weight. According to similar morphological characteristics and staining, 44 out of 111 isolates obtained were selected for testing antagonistic effects. At water potential, ψ, −1.38 MPa and −4.19 MPa, three *Bacillus* isolates showed dominance at a distance (5/0) and a significant reduction of growth rate of the pathogen. Three *Bacillus* isolates only decreased the growth rate of *E. turcicum* at −1.38 MPa. At −4.19 MPa the growth rate decreased with three isolates of *Pantoea* and three *Bacillus*. In this study a negative and significant correlation was observed between the growth rate of *E. turcicum* and the dominance index in the interaction of the pathogen with some bacteria. These results show that with decreasing growth rate of the pathogen the dominance index of the interaction increases. Eleven potential biocontrol agents against *E. turcicum* were selected.

PALABRAS CLAVE
Exserohilum turcicum;
Biocontrol;

Selección de microorganismos epifíticos de maíz como potenciales agentes de biocontrol de *Exserohilum turcicum*

Resumen El objetivo de este estudio fue seleccionar aislamientos microbianos de la filósfera de maíz y examinar su actividad antagonista contra *Exserohilum turcicum*. La selección se

* Corresponding author.
E-mail address: metcheverry@exa.unrc.edu.ar (M. Etcheverry).

http://dx.doi.org/10.1016/j.ram.2015.01.002

Bacterias epifíticas;
Maíz

realizó a través de la capacidad de los aislamientos de competir con el patógeno usando un índice de dominancia y también la capacidad de afectar los parámetros de crecimiento de *E. turcicum*. La mayoría de las poblaciones epifíticas aisladas para la selección fueron bacterias. Estos aislamientos se encontraron en el orden de 6 log de UFC por gramo de peso fresco de hoja de maíz. En base a características morfológicas y tintóreas similares, se seleccionaron 44 de 111 aislamientos obtenidos para evaluar su capacidad antagónica. A los potenciales agua, ψ, −1,38 MPa y −4,19 MPa, tres aislados del género *Bacillus* mostraron dominancia a distancia (5/0) y una reducción significativa de la velocidad de crecimiento del patógeno. Tres aislamientos de *Bacillus* disminuyeron la velocidad de crecimiento de *E. turcicum* a −1,38 MPa. A −4,19 MPa la velocidad de crecimiento disminuyó con tres aislamientos de *Pantoea* y tres de *Bacillus*. En este estudio se observó una correlación negativa y significante entre la velocidad de crecimiento de *E. turcicum* y el índice de dominancia cuando el patógeno interactuó con algunas bacterias. Esto estaría indicando que cuando disminuye la velocidad de crecimiento del patógeno se incrementa el índice de dominancia de la interacción. Se seleccionaron once posibles agentes de biocontrol contra *E. turcicum*.

Introduction

Maize (*Zea mays* L.) is one of the most important cereal crops in Argentina. The national average yield was 7270 kg/ha during growing season 2012–2013[7]. The increase in yield is conditioned by the improvement of several cultural practices. However, a negative factor is the emergence and re-emergence of some foliar diseases[25,59]. The common rust caused by *Puccinia sorghi* (Schwein) and the northern leaf blight caused by *Exserohilum turcicum* (Pass.) Leonard and Suggs (Syn. *Helminthosporium turcicum* Pass.) are two of the diseases that most affect the crop, causing a loss of yield[8,22]. Severe attacks of foliar diseases cause a reduction in the index of green leaf area, number of days with healthy leaf area and radiation interception. Therefore, because the photoassimilates are insufficient to grain filling, the plant begins remobilization of existing reserves in the stem immediately. Mobilization of nutrients leads to weakening of stems. This causes stalk breakage or lodging, favoring the increased occurrence of fungal diseases that cause stalk and root rot[25]. In Argentina, foliar diseases can cause loss up to 40% when these are endemic in the maize core area and occur each year with different levels of severity[9]. De Rossi *et al.*[15] determined that severity values of 60% caused losses close to 40% in yield of susceptible hybrids in Córdoba province, Argentina. The leaf blight becomes important in maize sown in late December and January, after harvest of wheat. The residues on the soil surface, frequent artificial irrigation, and intense rainfall during the summer months and moderate temperatures favor the development of the disease[16,21,24].

The expansion of emerging and reemerging diseases requires the prevention, control and eradication as technological tools necessary for the development of maize crop potential and the achievement of high yields[62]. The most widely used technique to control northern foliar blight is the selection of hybrids that show a better performance. Another alternative is based on cultural practices, avoiding monoculture. It is essential not to sow maize after maize or maize after sorghum, and to perform rotations with other species for one or two years[26]. Chemical control is the most used technique. The chemical fungicides used are mixtures of strobilurins and triazoles *e.g.* (NATIVO, Bayer), pyraclostrobin + epoxiconazole (OPERA, Basf), azoxystrobin + cyproconazole (AMISTAR XTRA, Syngenta) and picoxystrobin + cyproconazole (STINGER, DuPont). In general, these fungicides can reduce the severity and the epidemic rate of disease, showing good yields[11]. The application of these fungicides is performed at critical moments of the disease, depending on the hybrid of maize, climatic conditions and incidence of inoculum in the crop[9,25]. These chemicals are moderately hazardous Class II and to be effective must constantly protect new leaves, which is extremely expensive[5].

Therefore new strategies must be developed to give up the chemical paradigm. Biological control is presented as an alternative aimed to minimize yield losses caused by foliar diseases. This control strategy has the advantage of avoiding the accumulation of xenobiotics in the biosphere, avoiding the application of harmful products for those who manipulate them and reducing the costs of product applications. The use of microorganisms that antagonize foliar pathogens is risk-free when these organisms come from the same ecosystem. The inhabitants of the phyllosphere are termed epiphytes and may consist of a variety of bacteria, yeasts or filamentous fungi[40]. Microorganisms within the phyllosphere can include those that are pathogenic to the plant, but can also include non-pathogenic organisms that prevent the colonization of leaf by pathogens[38,40]. Diverse bacteria and yeast were tested as potential antagonists of different foliar diseases in crops[27,38,58,64,67]. Moreover, the success of biological control of foliar diseases is difficult because microbes of phyllosphere are located in a fluctuating environment. In addition, with global climate change phyllospheric microbes

are also exposed to additional changes in the physical environment[54]. To achieve the selection of a potential biocontrol agent it is important to consider the relationship between biological interactions and environmental stress factors[49]. It is also important to use criteria to determine the result of several interactions. The index of dominance compares the competitiveness of microbial species to dominate under a particular set of environmental conditions. Mostly, water availability, temperature and substrate have been reported influencing several interactions[39]. Numerous changes in environmental factors cause an impact that can be decisive in determining the co-existence level or dominance of species in a particular ecological niche[43,45]. Mainly, it is important to show that any potential biocontrol agent has the ability to decrease the growth of the pathogen.

Our study was carried out to obtain information on the potential of possible antagonists of *E. turcicum* and was aimed to pursue the following objectives: (a) evaluate bacteria from phyllosphere of maize; (b) determine the sensitivity of *E. turcicum* to osmotic stress; (c) evaluate the ability of bacterial antagonistic isolates to compete with *E. turcicum* using an index of dominance and antibiosis, under different water availabilities, and (d) determine the effect of bacteria on growth parameters of *E. turcicum*.

Materials and methods

Collection of samples and microbial isolation from phyllosphere

There have been a large number of studies that report the existence of microbial competition on leaves[1,23,68] between pathogens and possible antagonists[37]. Therefore, the isolation of microorganisms that live in the same ecosystem with the pathogen, allows the selection of potential antagonists. On this basis, the selection of bacteria was performed on leaves of maize with blight lesions from fields of three cultivars in Chucul, Río Cuarto and Vicuña Mackenna, all in Córdoba province, Argentina. Each sample contained fifteen plants and two leaves per plant were chosen for the assays. Leaves fully developed, but not senescent, were picked from the field and transferred to the laboratory. Samples were stored at 4 °C before processing.

To isolate epiphytic microorganisms the samples were subjected to three different techniques. For the first and second techniques, suspensions were prepared as follows. From each plant, ten discs of 1 cm from each leaf were cut with a sterile cork borer. The discs were transferred into tubes containing 10 ml of phosphate buffered saline (PBS: 0.1 M phosphate buffer containing 0.1% Bacto Peptone, pH 7). In the first technique the suspension was vortexed for 2 min. In the second technique another PBS suspension was sonicated at a frequency of 40 KHz for 4 min in an ultrasonic cleaning bath (TESTLAB – TB10TA, Argentina) to displace microorganisms[4,10,47,68]. The third technique consisted of a surface disinfection of the leaf discs in order to reduce inoculum of opportunistic and epiphytic pathogens, which could interfere with the isolation of potential antagonists. Ogliari *et al.*[51] methodology was followed with some modifications. Briefly, disks of infected tissues were placed in a solution of sodium hypochlorite at 2% for 3 min and then rinsed several times with sterile distilled water.

Serial dilutions to 10^{-4} were performed in PBS from all the obtained suspensions. Aliquots of 100 μl suspensions were plated on malt extract agar (MEA: malt extract 20 g, peptone 1 g, glucose 20 g, agar 15 g, distilled water 1000 ml, pH 5) and trypticase soy agar (TSA: tryptone 17 g, soytone 3 g, dextrose 2.5 g, NaCl 5.0 g, K_2HPO_4 2.5 g, agar 15 g, distilled water 1000 ml, pH 7.3 ± 0.2) (TSA, Britania, Argentina). Plates were incubated at 25 °C. Populations observed after 24–48 h were expressed as log CFU per gram of leaf fresh weight.

Colonies were grouped and listed according to their morphology, appearance and bacterial Gram stain. Some of the bacteria that showed consistent antifungal activity were selected for further identification according to Bergey's Manual of Systematic Bacteriology[31]. API Test kit was used to identify Gram-negative bacteria of the *Enterobacteriaceae* family and other Gram-negative bacilli (API®20 E, bioMèrieux, Argentina).

Fungal isolate

The *E. turcicum* fungal strain used was previously isolated from maize (DK 190) growing on Campus Santa Julia of Universidad Nacional de Córdoba (UNC), in Córdoba province, Argentina. The isolate was maintained at 4 °C on slants of potato dextrose agar medium (PDA: dextrose 20 g, potato extract 4 g, agar 15 g, distilled water 1000 ml, pH 5.6 ± 0.2) and in 15% glycerol at −80 °C.

E. turcicum sensitivity to osmotic stress: media, water potential modification and inoculation

Two media were used: potato dextrose agar medium (PDA) and maize leaves agar medium (MLA). MLA medium was made by boiling 30 g fresh maize leaves in 1 l water for 60 min and filtering the suspension through a double layer of muslin. The volume was made up to 1 l with distilled water. This medium was specifically chosen because *E. turcicum* was isolated from fresh maize leaves. All experiments were carried out over a water potential range ψ of −1.38 to −12.9 MPa. The ψ of the unmodified medium was −1.38 MPa, and this was selected as the control treatment. The water potential of PDA medium was adjusted to −2.78, −4.19, −5.62, −7.06, −8.52, −9.99, −11.5 and −12.9 MPa [=0.98, 0.97, 0.96, 0.95, 0.94, 0.93, 0.92 and 0.91 water activity (a_w), respectively] by the addition of known amounts of the non-ionic solute glycerol[12]. According to growth obtained in PDA medium, ψ of −4.19 MPa and −8.52 MPa were chosen to modify MLA medium. The a_w of the media was determined using an equipment AquaLab (Series 4, TE, USA). The media were autoclaved at 121 °C for 20 min before cooling to 50 °C and pouring into 9 cm sterile plastic Petri plates.

Petri plates containing the different media were inoculated aseptically with *E. turcicum* by transferring 4 mm diameter agar plugs of 10-day old culture of the pathogen to the center of PDA and MLA media. Petri plates of the same ψ values were sealed in polyethylene bags. The inoculated plates were incubated at 25 °C for 20 days or until the colony covered the plate. The colony radius was measured

daily. For each colony, two radii, measured at right angles to one another, were averaged to find the mean radius for that colony. All colony radii were determined by using three replicates for each treatment. The radial rate (mm/d) was then calculated by linear regression of the linear phase for growth, and the time at which the line intercepted the *x*-axis was used to calculate the lag phase.

Index of dominance (I_D)

Petri plates containing MLA modified with glycerol to −1.38 MPa, −4.19 MPa and −8.52 MPa[12] were used. A streak of each epiphytic microorganism suspension grown for 24 h in trypticase soy broth (TSB) was inoculated in the middle of each Petri plate. The Petri plates were inoculated with two agar plugs of the pathogen *E. turcicum* at two points equidistant from the center and edge of the plate. Treatments were incubated in polyethylene bags for 15 days at 25 °C[49]. The I_D was developed to measure the ability of a species to dominate under a particular set of environmental conditions[43]. The type of interaction was determined macroscopically. Controls of fungal pathogen and antagonistic bacteria were inoculated in separate plates. The diameter of the fungal colony and the width of the streak of the bacterial colony were measured in controls and compared with the interactions. The methodology used by Magan and Lacey[43] to assign scores to obtain I_D was adapted for interactions between fungus and bacteria[49]. The scores were based on mutual intermingling (1/1), mutual inhibition on contact (2/2), mutual inhibition at a distance (3/3), dominance of one species on contact (4/0) and dominance at a distance (5/0)[43]. This assessment was carried out with at least three separate replicates per treatment.

Antifungal effect of epiphytic microorganisms on *E. turcicum* growth parameters

The MLA medium at −1.38 MPa, −4.19 MPa and −8.52 MPa was prepared following the procedure mentioned above. Before cooling, MLA medium was inoculated with 100 μl of 10^9 CFU/ml suspension of each epiphytic microorganism and poured into Petri plates. An agar plug of *E. turcicum* was inoculated in the center of the plate. Cultures were incubated at 25 °C for 20 days in polyethylene bags[28,49]. The experiments were carried out three times for single and paired cultures. The inhibitory activity on lag phase and growth rate of screened epiphytic microorganisms against *E. turcicum* were evaluated as described previously.

Statistical analysis

The analysis of variance (ANOVA)[19] was used to compare counts of epiphytic microorganisms in different sampling sites, differences between sample processing techniques and differences in growth rate. Means were compared with DGC test ($p < 0.05$) [20]. The Pearson correlation coefficient was used to evaluate correlations between growth rate of *E. turcicum* at different water potentials and dominance index. A significant level of $p < 0.0001$ was used.

Results

Isolates of epiphytic microorganisms

Most of the counts were in the order of 6 log of CFU/g (Table 1), and no significant differences were observed between sampling localities, although there were differences between processing methods. The counts obtained by performing surface disinfection of the samples were significantly lower ($p < 0.001$). A total of 111 epiphytic isolates were obtained. Grouping of total isolates showed the following composition: 46.8% Gram positive, 52.3% Gram-negative and 0.9% yeast (data not shown). According to Gram stain and morphology 9.9% were Gram-positive rods, 11.7% Gram-positive irregular rods, 15.3% Gram-positive spore-forming rods, 9.9% Gram-positive cocci, 20.7% Gram-negative rods and 31.5% Gram-negative irregular rods. According to similar morphological characteristics and staining 44 isolates were selected for antagonistic testing.

Table 1 Total count on maize leaves of epiphytic microorganisms according to sampling localities and processing techniques.

Processing techniques	Counts in sampling localities CFU/g		
	Chucul	Río Cuarto	Vicuña Mackenna
Sonication			
MEA	3.3×10^6 a	2.6×10^5 a	2.4×10^6 a
TSA	5.8×10^6 a	1.5×10^6 a	4.2×10^6 a
Vortexing			
MEA	4.0×10^6 a	2.3×10^6 a	5.1×10^6 a
TSA	1.5×10^6 a	4.7×10^6 a	3.0×10^6 a
Surface disinfection			
MEA	6.0×10^5 b	2.1×10^4 b	ND
TSA	4.5×10^5 b	7.4×10^4 b	ND

ND: not determined; MEA: malt extract agar medium; TSA: trypticase soy agar medium.
Different letters indicate significant differences between processing techniques for each sampling locality, according to the DGC test ($p < 0.001$).

Table 2 Osmotic stress sensitivity of *E. turcicum* in PDA and MLA media.

ψ (a_w)	PDA		MLA	
	Lag phase (h)	Growth rate (mm/h)	Lag phase (h)	Growth rate (mm/h)
−1.38 (0.99)	30.7 a	0.66 a	51.0 a	0.60 a
−2.78 (0.98)	29.8 a	0.60 b	ND	ND
−4.19 (0.97)	40.0 ab	0.46 c	69.1 b	0.42 b
−5.62 (0.96)	46.5 b	0.38 d	ND	ND
−7.06 (0.95)	75.4 c	0.26 e	ND	ND
−8.52 (0.94)	140.2 d	0.11 f	223.7 c	0.11 c
−9.99 (0.93)	>	NG	ND	ND
−11.5 (0.92)	>	NG	ND	ND
−12.9 (0.91)	>	NG	ND	ND

NG: no growth; ND: not determined; PDA: potato dextrose agar medium; MLA: maize leaf agar medium.
>:480 h.
Different letters indicate significant differences between different ψ for growth rate and lag phase in each media, according to the DGC test ($p < 0.05$).

Osmotic stress sensitivity of *E. turcicum*

The effect of osmotic stress in PDA and MLA media on lag phase and growth rate of *E. turcicum* is shown in Table 2. Water potential showed a significant effect ($p < 0.001$) on the lag phase and growth rate of *E. turcicum* in both culture media. When water potential decreased, lag phase increased and growth rate decreased in both media. Lag phase showed significant differences between the culture media PDA and MLA ($p < 0.001$). The lag phases were higher in MLA medium than in PDA medium at −1.38 MPa, −4.19 MPa and −8.52 MPa. However, in PDA medium lag phase was not significantly different at −1.38 MPa, −2.78 MPa and −4.19 MPa ($p < 0.05$). No growth was observed in PDA medium at −9.99 MPa, −11.5 MPa and −12.9 MPa. Growth rate of *E. turcicum* was higher at −1.38 MPa. The values observed were 0.66 and 0.60 mm/h in PDA and MLA media, respectively. The growth rate decreased in both media at −4.19 MPa and −8.52 MPa. According to these results, ψ of −1.38 MPa, −4.19 MPa and −8.52 MPa were selected for the following assays.

Interactions between *E. turcicum* and isolated epiphytic microorganisms

Table 3 shows the effect of biological interaction among the pathogen *E. turcicum* and 44 epiphytic microorganisms selected. The predominant interaction between bacterial antagonists and fungus in dual culture was mutual intermingling ($I_D = 1/1$). At −1.38 MPa three isolates (16, 22 and 23) showed a mutual inhibition on contact (2/2). Other three isolates (12, 13 and 14) showed dominance at a distance (5/0). The isolates 12, 13 and 14 showed a significant reduction of the pathogen growth rate compared with the control. These isolates caused the same effect on the pathogen at −4.19 MPa. At this ψ also the isolate 8 showed an interaction 5/0 and other eight isolates showed an interaction 2/2. Most of the isolates that showed spatial dominance also showed significant increase in the lag phase and reduction of the growth rate. The isolates 15, 34, 35 and 38 showed mutual intermingling at −4.19 MPa and −1.38 MPa; however these isolates reduced growth rate of *E. turcicum*. At −8.52 MPa none of the epiphytic microorganisms were able to grow, so there was no interaction or effect on growth of the pathogen (data not shown).

Epiphytic microorganisms had significant effects on increasing the lag phase. There was a significant increase in the lag phase in treatments where there were spatial interactions or dominance of epiphytic microorganisms on *E. turcicum*. Twenty-seven percent and 43% of the bacterial isolates had significant inhibitory effects on the mycelial growth of *E. turcicum* at ψ −1.38 and −4.19, respectively. However, none of the bacterial isolates inhibited the growth of the pathogen completely. Growth rate of *E. turcicum* was reduced in significant percentages ($p < 0.001$) with the isolates 16 (84%), 12 (89%), 13 (98%) and 14 (84%) at −1.38 MPa. And at −4.19 MPa the growth rate decreased with the isolates 27 (81%), 35 (82%), 38 (83%), 12 (96%), 41 (83%), 13 (95%) and 14 (91%).

Identification of epiphytic microorganisms

Eleven isolates demonstrating significant reducing effect on growth rate or dominance on *E. turcicum* were identified at the genera level. Isolates 27, 34, 35, 38, and 40 showed characteristics of the genus *Pantoea* of the family *Enterobacteriaceae*. Three isolates, 12, 13 and 14 were identified as *Bacillus*, and isolates 3 and 8 were compatible with *Corynebacterium* features. Finally, isolate 15 was identified as *Enterococcus*.

Correlation between biological interactions of *E. turcicum* and epiphytic microorganisms

Table 4 shows the Pearson correlation coefficients' values obtained. Negative and significant correlations were observed between the effect of epiphytic microorganisms on growth rate of *E. turcicum* and index of dominance in the biological interaction at −1.38 MPa and −4.19 MPa.

Table 3 Index of dominance (I_D) and interactions on growth parameters between epiphytic microorganisms and *E. turcicum* in maize leaf agar at different ψ.

Epiphytic microorganism	−1.38 (a_W 0.99)			−4.19 (a_W 0.97)		
	I_D	Lag phase (h)	Growth rate (mm/h)	I_D	Lag phase (h)	Growth rate (mm/h)
Control of *E. turcicum*	–	53.5	0.610 a	–	69.6	0.459 a
Gram-positive rods						
1	1/1	54.4	0.590 a (3)	2/2	77.8	0.411 a (10)
2	1/1	60.4	0.392 a (36)	1/1	85.0	0.413 a (10)
3	1/1	96.6	0.227 b (63)	2/2	120.0	0.225 b (51)
4	1/1	116.2	0.190 b (69)	1/1	73.4	0.452 a (1)
5	1/1	49.2	0.564 a (7)	1/1	82.1	0.394 a (14)
6	1/1	100.3	0.440 a (28)	1/1	98.4	0.120 b (74)
7	1/1	65.8	0.496 a (19)	1/1	65.0	0.301 a (34)
8	1/1	47.1	0.280 b (54)	5/0	137.5	0.192 b (58)
Gram-positive spore-forming rods						
9	1/1	52.6	0.597 a (2)	1/1	85.9	0.371 a (19)
10	1/1	114.0	0.600 a (2)	1/1	132.9	0.260 a (43)
11	1/1	58.1	0.415 a (32)	2/2	69.1	0.253 b (45)
12	5/0	>	0.065 c (89)	5/0	>	0.019 c (96)
13	5/0	>	0.012 c (98)	5/0	>	0.025 c (95)
14	5/0	288.0	0.100 c (84)	5/0	>	0.040 c (91)
Gram positive cocci						
15	1/1	144.3	0.148 b (76)	1/1	159.0	0.167 b (73)
16	2/2	176.6	0.097 c (84)	1/1	81.6	0.409 a (11)
17	1/1	56.4	0.432 a (29)	1/1	104.0	0.675 a (−)
Gram-positive irregular rods						
18	1/1	66.0	0.629 a (−)	1/1	94.1	0.457 a (−)
19	1/1	61.0	0.650 a (7)	1/1	73.2	0.444 a (3)
Gram-negative rods						
20	1/1	43.6	0.338 a (45)	2/2	81.1	0.266 b (42)
21	1/1	61.3	0.632 a (4)	1/1	63.8	0.400 a (13)
22	2/2	53.3	0.587 a (4)	1/1	80.6	0.411 a (10)
23	2/2	65	0.730 a (−)	1/1	84.5	0.428 a (7)
24	1/1	47.5	0.444 a (27)	1/1	67.7	0.361 a (21)
25	1/1	57.4	0.638 a (−)	1/1	70.9	0.459 a (−)
Gram-negative irregular rods						
26	1/1	91.8	0.490 a (20)	1/1	116.7	0.275 b (44)
27	1/1	42.0	0.315 b (48)	2/2	229.8	0.085 c (81)
28	1/1	63.2	0.475 a (22)	1/1	91.2	0.290 b (37)
29	1/1	67.2	0.556 a (9)	2/2	106.1	0.244 b (47)
30	1/1	60.7	0.642 a (−)	2/2	73.7	0.416 a (9)
31	1/1	66.9	0.401 a (34)	1/1	76.1	0.340 a (26)
32	1/1	107.4	0.451 a (26)	1/1	65.1	0.338 a (26)
33	1/1	68.9	0.307 a (50)	1/1	110.4	0.317 a (31)
34	1/1	116.0	0.540 a (11)	1/1	149.3	0.180 b (61)
35	1/1	103.5	0.413 a (32)	1/1	259.2	0.083 c (82)
36	1/1	66.1	0.450 a (26)	1/1	64.6	0.380 a (17)
37	1/1	61.0	0.710 a (−)	1/1	72.0	0.460 a (−)
38	1/1	129.1	0.103 b (83)	1/1	78.0	0.080 c (83)
39	1/1	77.0	0.425 a (30)	1/1	116.6	0.420 a (8)
40	1/1	112.1	0.300 b (51)	2/2	97.7	0.290 b (37)
41	1/1	97.7	0.420 a (31)	1/1	267.1	0.080 c (83)
42	1/1	71.8	0.512 a (16)	1/1	61.9	0.550 a (−)

Table 3 (*Continued*)

Epiphytic microorganism	−1.38 (a_W 0.99)			−4.19 (a_W 0.97)		
	I_D	Lag phase (h)	Growth rate (mm/h)	I_D	Lag phase (h)	Growth rate (mm/h)
43	1/1	56.9	0.675 a (−)	1/1	71.0	0.470 a (−)
44	1/1	73.2	0.535 a (12)	1/1	62.4	0.397 a (13)

(): percentage of growth rate inhibition.
>:480 h.
Different letters indicate significant differences for the same ψ on growth rate of *E. turcicum* interacting with each epiphytic microorganism isolate, according to the DGC test ($p < 0.05$). Index of Dominance ID: 1/1 mutual intermingling, 2/2 mutual inhibition on contact, 3/3 mutual inhibition at a distance, 4/0 dominance of one species on contact and 5/0 dominance at a distance.

Table 4 Pearson (*r*) correlation coefficients values between growth rate and index of dominance (I_D) of *E. turcicum* and selected bacterial isolates interacting in maize leaf agar at two different ψ.

	Growth rate of *E. turcicum* when interacting with bacterial isolates			
	−1.38 ψ		−4.19 ψ	
	r	*p*-Value	*r*	*p*-Value
I_D	−0.48	<0.0001	–	–
I_D	–	–	−0.53	<0.0001

$p < 0.0001$ indicates a significant relationship between the two variables.

Discussion

This study presents the results of the selection steps of possible biological control agents of *E. turcicum*, by taking into consideration ecological parameters relevant to the agroecosystem. In our study we isolated possible antagonistic epiphytic microorganisms from leaves of maize with blight lesions. Most epiphytic population consisted of bacteria which were found in the order of 6 log CFU per gram of maize leaf, similar to results obtained in other studies[30,35,70]. Therefore, the interaction of phyllosphere microorganisms can play an important role for plant health and protection[3]. Previous studies performed on peanut phyllosphere showed that most of the identified strains were Gram-positive[63], with *Bacillus* that accounted 39% of the total[33]. In our study Gram-positive rods represent 36.9% of the isolates. On the other hand most of the isolates were grouped in Gram-negative bacteria. The microbial ecology of the phyllosphere has been viewed mainly through the biology of Gram-negative bacteria like plant-pathogenic microorganisms[40]. However, some Gram-negative rods were considered antagonistic bacteria of different phyllosphere diseases[42,69]. Antagonistic effect was observed with *Bacillus subtilis* and *Pseudomonas fluorescens* against *E. turcicum* in dual culture *in vitro*[29]. Bacteria, especially the pseudomonads and bacilli have been shown to play a key role in the suppression of plant pathogens in different cropping systems[57]. Other studies showed *in vitro* antagonism of filamentous fungi like *Trichoderma harzianum* and *T. viride* against *E. turcicum*[29,56]. Phyllosphere microbes live in a physical environment of continuous fluctuation[17,30]. Consequently, the leaf surface is considered to be a hostile location for microbial colonization[41]. Fluctuating water availability, incident irradiation and low nutrient availability on leaves are likely selective pressures that influence the composition of epiphytic bacteria[6,41]. Bacteria selected as potential biological control agents must be able to tolerate continuous microclimatic changes. Also these bacteria must demonstrate good growth under similar conditions, such as water potential and temperature. In this study *E. turcicum* was able to grow at a range of water potential of −1.38 MPa to −8.52 MPa in culture media, synthetic potato dextrose agar and maize leaves agar. However, the antagonistic bacteria selected were able to grow at −1.38 MPa and −4.19 MPa. From the selected bacterial isolates, *Bacillus* is known to be more tolerant to environmental changes due to the presence of stress resistant endospores[60]. Gram-positive bacteria are generally resistant to drought stress[61]. A study conducted by Jacobs and Sundin[33] revealed that *Bacillus coagulans* showed a phenotype of tolerance for solar UV radiation in peanut leaves. In our study, three *Bacillus* isolates showed a dominance of the pathogen at a distance, and a reduction of *E. turcicum* growth. Possibly, these isolates have the ability to synthesize a diffusible substance with inhibitory capacity. Kishore *et al.*[38] showed that *Bacillus circulans* GRS 243 was considered a chitinolytic bacterium and inhibited the germination of conidia of *Cercospora arachidicola*, *Phaeoisariopsis personata* and urediniospores of *Puccinia arachidis*. US EPA[65] reported that *Bacillus subtilis* strain QST 713 controlled the growth of certain pathogenic fungi, presumably by competition for nutrients, growth sites in plants and direct colonization and adhesion to fungi.

Taking into account the antagonistic effects on plant pathogens of Gram-negative bacteria, Howell *et al.*[32] reported that volatile compounds such as ammonia produced by *Enterobacter cloacae* were involved in the suppression of cotton seedling damping-off caused by *Pythium ultimum*. In our study, five isolates selected and identified as *Pantoea* showed high percentage of growth inhibition of the pathogen or mutual inhibition on contact. However, in most of the interactions mutual intermingling was observed. This interaction suggests that competition for space and nutrients did not occur between these isolates and *E. turcicum* under the conditions evaluated. For instance, the isolate 38 did not show dominance over the pathogen (I_D = 1/1) but produced 83% of growth inhibition. Therefore synthesis of diffusible inhibitory substances and competition for nutrients and space are not the mechanisms used by this bacterium to inhibit the growth of the pathogen and possibly competitive exclusion is the mechanism used by this

antagonist. The most abundant non-pathogenic microorganisms associated with plants are thought to protect the plant by rapid colonization[36]. The pear and apple disease caused by the bacterium *Erwinia amylovora* was controlled using *Pantoea agglomerans* strain whose mechanism of action is not the synthesis of antibiotics[34,55]. In this study a negative and significant correlation was observed between the growth rate of *E. turcicum* and dominance index when the pathogen interacts with some bacteria (3, 8, 12, 13, 14, 16, 27 and 40). This means that with decreasing growth rate of the pathogen the dominance index of the interaction increases.

On the other hand, it is known that *Pantoea stewartii* subsp. *stewartii* is the causal agent of Stewart's wilt of sweet maize. This phytopathogen is a yellow-pigmented and Gram-negative bacterium[50]. Symptoms of bacterial leaf blight of maize caused by *P. stewartii* in maize fields of central Argentina were observed[2]. Many microorganisms are known to produce pigments[44,48], like members of *Pantoea* genus. Since solar radiation influences the ecology of the phyllosphere, pigmented bacteria dominate the leaf surfaces[33,63]. In the present study, other than *Bacillus*, eight of the eleven isolates selected were pigmented.

Several investigations have demonstrated the antagonistic role of the *Corynebacterium* genus[13,14]. *C. nebraskense* was isolated from maize field and was described as pathogenic species[66]. On the other hand, others species of *Corynebacterium* were used to control different plant fungal diseases like *Pythium* damping-off and some fungi that cause root rot[18,53].

Finally, one of the isolates that showed high inhibition percentage of *E. turcicum* was *Enterococcus*. Mata *et al.*[46] described different *Enterococcus* strains with antagonist bacterial effects against phytopathogenic species *Clavibacter michiganensis*, *Erwinia carotovora* and *Xanthomonas axonopodis in vitro*. Although, *Enterococcus* species have shown a wide distribution in the phyllosphere of plants, many investigations are conducted to evaluate the possible pre-harvest contamination of plants with human pathogens[52]. Further studies on the detrimental effects of the potential antagonists of *E. turcicum* need to be conducted.

At present, studies are carried out to evaluate the eleven potential biocontrol agents obtained against *E. turcicum* in greenhouse conditions.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This work was supported by a grant from the Agencia Nacional de Promoción Científica y Tecnológica de la República Argentina, PICT 2268/12. M. Sartori was provided with a post-doctoral fellowship, A. Nesci and M. Etcheverry are members of Carrera del Investigador Científico from CONICET.

References

1. Adee S, Pfender W, Hartnett D. Competition between *Pyrenophora tritici-repentis* and *Septorianodorum* in thew heat leaf as measured with de Witre placement series. Phytopathology. 1990;80:1177–82.
2. Albarracín Orio A, Brücher E, Plazas M, Sayago P, Guerra F, De Rossi R, Ducasse DA, Guerra GD. First report of Stewart's Wilt of maize in Argentina caused by *Pantoea stewartii*. Plant Dis. 2012;96:1819.
3. Andrews J, Harris R. The ecology and biogeography of microorganisms on plant surfaces. Ann Rev Phytopathol. 2000;38:145–80.
4. Assis S, Mariano R, Michereff S, Silva G, Maranhao E. Antagonism of yeasts to *Xanthomonas campestris* pv campestris on cabbage phylloplane in field. Rev Microbiol. 1999;30:191–5.
5. Bayer (2008) http://www.bayercropscience.com.pe/web/articulo=407 [Online].
6. Beattie G. Water relations in the interaction of foliar bacterial pathogens with plants. Annu Rev Phytopathol. 2011;49:533–55.
7. Bolsa de Cereales (2013) http://infocampo.com.ar/nota/campo/48447/con-una-produccion-de-24-8-m-tn-finalizo-la-campana-2012-2013-de-maiz [Online].
8. Carlos D. Diseases of maize in South-east Asia relevance and management. In: International conference on integrated plant disease management for sustainable agriculture. 1997. p. 22.
9. Carmona M, Melo Reis E, Trezzi Casa R. Identificación y manejo de las principales enfermedades del maíz. Ed. Horizonte A; 2008. p. 44.
10. Correa O, Romero A, Montecchia M, Soria M. Tomato genotype and *Azospirillum* inoculation modulate the changes in bacterial communities associated with roots and leaves. J Appl Microbiol. 2007;102:781–6.
11. Couretot L, Ferraris G, Mousegne F, Russian H. Control químico de roya común del maíz (*Puccinia sorghi*). In: 1° Congreso Argentino de Fitopatología, HM-25. 2008. p. 211.
12. Dallyn H, Fox A. Spoilage of material of reduced water activity by xerophilic fungi. In: Gould G, Corry E, editors. Society of applied bacteriology technical series 15. London, UK: Academic Press; 1980. p. 219–39.
13. Davis M. Taxonomy of plant-pathogenic coryneform bacteria. Annu Rev Phytopathol. 1986;24:115–40.
14. Davis M, Gillaspie A, Vidaver A, Clavibacter HR. A new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and Bermudagrass stunting disease. Int J Syst Bacteriol. 1984;34:107–17.
15. De Rossi R, Plazas M, Brucher E, Ducasse D, Guerra G. El Tizón del Maíz (*Exserohilum turcicum*): presencia e impacto en el centro norte de Córdoba durante tres campañas agrícolas. In: Actas IX Congreso Nacional de Maíz. 2010.
16. de Souza J. Enfermedades del maíz en Entre Ríos Actualización Técnica Maíz, Girasol y Sorgo. INTA EEA Paraná. Ser Ext. 2007;44:80–5.
17. Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R, et al. Community proteogenomics reveals insights

into the physiology of phyllosphere bacteria. Proc Natl Acad Sci U S A. 2009;106:16428–33.
18. Dhinakaran A, Rajasekaran R, Jayalakshmi S. Antiphytopathogenic activity of bacterial protein of a marine *Corynebacterium* sp. isolated from Mandapam, Gulf of Mannar. J Biopest. 2012;5:17–22.
19. Di Rienzo J, Casanoves F, Balzarini M, Gonzalez L, Tablada M, Robledo C. InfoStat versión 2012. Argentina: Grupo InfoStat, FCA, Universidad Nacional de Córdoba; 2012. http://www.infostat.com.ar [Online].
20. Di Rienzo J, Guzmán A, Casanoves F. A multiple comparisons method based on the distribution of the root node distance of a binary tree. J Agric Biol Environ Stat. 2002;7:1–14.
21. Díaz C. Evolución e impacto de enfermedades foliares en el cultivo de maíz: *Cercospora* y Tizones. In: Actas IX Congreso Nacional de Maíz. 2010. p. 200–4.
22. Ferraris G, Couretot L. Caracterización y evaluación comparativa de cultivares de maíz en la localidad de Colón (Bs. As.) Campaña 2009/10. Available from: http://www.inta.gov.ar/pergamino/info/documentos/ext10/Maiz_hibridos200910_Colon.pdf
23. Fokkema N, Riphagen I, Poot R, deJong C. Aphid honey dew, a potential stimulant of *Cochliobolus sativus* and *Septoria nodorum* and the competitive role of saprophytic mycoflora. Trans Br Mycol Soc. 1983;81:355–63.
24. Formento A. El Tizón Foliar del Maíz en Siembras de Segunda. INTA Paraná; 2001. http://www.inta.gov.ar/parana/info/documentos/producciónvegetal [Online].
25. Formento A. Enfermedades foliares reemergentes del cultivo de maíz: Royas (*Puccinia soghi* y *Puccinia polysora*) Tizón foliar (*Exserohilum turcicum*) y Mancha ocular (Kabatiellazeae). INTA Paraná; 2010. http://www.inta.gov.ar/parana/info/documentos/producciónvegetal/maiz/enfermedad [Online].
26. Formento A, Vicentin I. Mancha ocular em maíz (Aureobasidium zeae Syn *Kabatiella zeae*); 2005. http://www.inta.gov.ar/parana/info/documentos/producciónvegetal/maiz/enfermedad/20314 [Online].
27. Gomes R, Semedo L, Soares R, Linhares L, Ulhoa C, Alviano C, et al. Purification of a termostable endochitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. J Appl Microbiol. 2001;90:653–61.
28. Harlapur S. Epidemiology and management of turcicum leaf blight of maize caused by *Exserohilum turcicum* (Pass.) Leonard and Suggs; 2005 [Doctoral thesis] http://etd.uasd.edu/ft/th8538.pdf [Online].
29. Harlapur S, Kulkarni M, Wali M, Kulkarni S. Evaluation of plant extract, bio-agents and fungicides against *Exserohilum turcicum* (Pass.) Leonard and Suggs causing turcicum leaf bligh to maize. Karnataka J Agric Sci. 2007;20:541–4.
30. Hirano S, Upper C. Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* a pathogen, ice nucleus, and epiphyte. Microb Mol Biol Rev. 2000;64:624–53.
31. Holt JG, Krieg NR, Sneath PHA, Staley J, Williams ST. (Eds). Bergey's Manual of Determinative Bacteriology, Group 17: Gram positive cocci and Group 18: Endospore-forming Gram-positive rods and cocci, Williams and Wilkins, Baltimore, Md, USA, 9th edition, 1994, p. 527–64.
32. Howell C, Beier R, Stipanovic R. Production of ammonia by *Enterobacter cloacae* and its possible role in the biological control of *Phythium* pre-emergence damping-off by the bacterium. Phytopathology. 1988;8:1075–8.
33. Jacobs J, Sundin G. Effect of solar UV-B radiation on a phyllosphere bacterial community. Appl Environ Microbiol. 2001;67:5488–96.
34. Johnson K, Stockwell V. Management of fire blight: a case study in microbial ecology. Annu Rev Phytopathol. 1998;36:227–48.
35. Jurkevitch E, Shapira G. Structure and colonization dynamics of epiphytic bacterial communities and of selected component strains on tomato (*Lycopersicon esculentum*) leaves. Microb Ecol. 2000;40:300–8.
36. Kagayama K, Nelson E. Differential inactivation of seed exudates stimulation of *Phythium ultimum* sporangium germination by *Enterobacter cloacae* influences biological control efficacy on different plant species. Appl Environ Microbiol. 2003;69:1114–20.
37. Kinkel L, Lindow S. Microbial competition and plant disease biocontrol. In: Andow D, Ragsdale D, Nyvall R, editors. Ecological interactions and biological control. Boulder, CO, Westview Press; 1996.
38. Kishore G, Pande S, Podile A. Biological control of late leaf spot of peanut (*Arachishypogaea*) with chitinolytic bacteria. Biol Control. 2005;95:1157–65.
39. Lacey J, Magan N. Fungi in cereal grains: their occurrence and water and temperature relationships. In: Chelkowski J, editor. Cereal grain. Mycotoxins, fungi and quality in drying and storage. Amsterdam, Elsevier, 1991. p. 77–118.
40. Lindow S, Brandl M. Microbiology of the phyllosphere. Appl Environ Microb. 2003;69:1875–83.
41. Lindow S, Leveau J. Phyllosphere microbiology. Curr Opin Biotechnol. 2002;13:238–43.
42. Lindow S, McGourty G, Elkins R. Interactions of antibiotics with *Pseudomonas fluorescens* strain A506 in the control of fire blight and frost injury to pear. Phytopathology. 1996;86:841–8.
43. Magan N, Lacey J. Effect of water activity, temperature and substrate on interactions between field and storage fungi. Trans Br Mycol Soc. 1984;82:305–14.
44. Margalith P. Pigment Microbiology. London, UK: Chapman and Hall; 1992.
45. Marín S, Sanchis V, Vinas I, Canela R, Magan N. Effect of water activity and temperature on growth and fumonisin B_1 and B_2 production by *Fusarium proliferatum* and *F. moniliforme* on maize grain. Lett Appl Microbiol. 1995;21:298–301.
46. Mata L, Chavez C, Rodríguez-Herrera R, Hernández-Castillo D, Aguilar C. Growth inhibition of some phytopathogenic bacteria by cell-free extracts from *Enterococcus* sp. Br Biotech J. 2013;3:359–66.
47. Melo R, Mariano R, Michereff S. Controle biologico da podridao mole do pimentao (*Capsicumannuun*) causada por *Erwinia carotovora* subsp. *carotovora*. Summa Phytopathol. 1995;21:206–12.
48. Moss M. Bacterial pigments. Microbiologist. 2002;3:10–2.
49. Nesci A, Bluma R, Etcheverry M. *In vitro* selection of maize rhizobacteria to study potential biological control of *Aspergillus* section *Flavi* and aflatoxin production. Eur J Plant Pathol. 2005;113:159–71.
50. OEPP/EPPO. *Pantoea stewartii* subsp. *stewartii* diagnostic bulletin. OEPP/EPPO Bull. 2006;36:111.
51. Ogliari J, Guimaraes M, Geraldi I, Camargo L. New resistance genes in the *Zea mays-Exserohilum turcicum* pathosystem. Genet Mol Biol. 2005;28:435–9.
52. Ott E, Muller T, Muller M, Franz C, Ulrich A, Gabel M, Seyfarth W. Population dynamics and antagonistic potential of enterococci colonizing the phyllosphere of grasses. J Appl Microbiol. 2001;91:54–66.
53. Parke J, Rand R, Foy A, King E. Biological control of *Pythium* damping-off and *Aphanomyces* root rot of peas by application of *Pseudomonas cepacia* or *Pseudomonas fluorescens* to seed. Plant Dis. 1991;75:987–92.
54. Peñuelas J, Rico L, Ogaya R, Jump AS, Terradas J. Summer season and long-term drought increase the richness of bacteria and fungi in the foliar phyllosphere of *Quercusilex* in a mixed Mediterranean forest. Plant Biol. 2012;14:565–75.

55. Pusey P. Crab apple blossoms as a model for research on biological control of fire blight. Phytopathology. 1997;87:1096–102.
56. Ramachandra C [M.Sc. (Agri.) thesis] Studies on leaf blight of dicoccum wheat caused by *Exserohilum hawaiienesis* (Bugnicourt). Dharwad, India: University of Agricultural Sciences; 2000.
57. Ramarathnam R, Dilantha Fernando W. Preliminary phenotypic and molecular screening for potential bacterial biocontrol agentes of *Leptosphaeria maculans*, the black leg pathogen of canola. Biocontrol Sci Tech. 2006;16:567–82.
58. Reis A, Azevedo S, Assis S, Mariano R. Screening yeasts isolates for biological control of *Bipolaris zeicola* leaf spot on corn. In: Wenhua T, Cook R, Rovira A, editors. Advances in biological control of plant diseases. China, Beijing, China Agricultural University Press; 1996. p. 367–73.
59. Romagnoli J. (2005) El maíz carga con su roya. http://edant.clarin.com/suplementos/rural/2005/10/22/r-01211.htm [Online].
60. Sadoff H. Sporulation antibiotics of *Bacillus* species. In: Halvorson HO, Hanson R, Campbell LL, editors. Spores, vol. V. Bethesda, MD, American Society of Microbiology; 1972. p. 157–66.
61. Schimel J, Balser T, Wallenstein M. Microbial stress-response physiology and its implications for ecosystem function. Ecology. 2007;88:1386–94.
62. Sillon M, Berardo M, Mandrile M, Albrecht J, Fontanetto H, Marinone D. Las enfermedades fúngicas del cultivo de maíz en Santa Fe durante el ciclo agrícola 2008/2009. Agromercado Clásico, 2009; N° 157, Maíz.
63. Sundin G, Jacobs J. Ultraviolet radiation (UVR) sensitivity analysis and UVE survival strategies of a bacterial community from the phyllosphere of field-grown peanut (*Arachishypogeae* L.). Microb Ecol. 1999;38:27–38.
64. Urquhart E, Punja Z. Hydrolytic enzymes and antifungal compounds produced by *Tilletiopsis* species, phyllosphere yeasts that are antagonists of powdery mildew fungi. Can J Microbiol. 2002;48:219–29.
65. US Environmental Protection Agency. *Bacillus subtilis* QST713 (006479); 2003. http://www.epa.gov [Online].
66. Vidaver A, Mandel M. *Corynebacterium nebraskense*, a new, orange-pigmented phytopathogenic species. Int J Syst Bacteriol. 1974;24:482–5.
67. Williamson M, Fokkema N. Phytosphere yeasts antagonize penetration from appresoria and subsequent infection of maize leaves by *Colletotrichum grammicola*. Neth J Plant Pathol. 1991;91:265–76.
68. Wilson M, Lindow S. Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. Appl Environ Microbiol. 1994;60:4468–77.
69. Wilson M, Lindow S. Interactions between the biological control agent *Pseudomonas fluorescens* A506 and *Erwinia amylovora* in pear blossoms. Phytopathology. 1993;83:117–23.
70. Yadav R, Karamanoli K, Vokou D. Bacterial colonization of the phyllosphere of Mediterranean perennial species as influenced by leaf structural and chemical features. Microb Ecol. 2005;50:185–96.