Antonie van Leeuwenhoek (2005) 87:179–187
DOI 10.1007/s10482-004-3193-z

Rhizobacteria and their potential to control *Fusarium verticillioides*: effect of maize bacterisation and inoculum density

Lilia Renée Cavaglieri[1,*], Laura Andrés[1], Mercedes Ibáñez[2] and Miriam G. Etcheverry[1]
[1]*Departamento de Microbiología e Inmunología Facultad de Ciencias Exactas Físico Químicas y Naturales Universidad Nacional de Río Cuarto, Ruta 36 Km. 601, (5800) Río Cuarto, Córdoba, Argentina;* [2]*Departamento de Biología Agrícola. Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto, Córdoba, Argentina; *Author for correspondence (e-mail: lcavaglieri@arnet.com.ar)*

Received 14 January 2004; accepted in revised form 13 September 2004

Key words: Antifungal activity, *Arthrobacter globiformis*, *Azotobacter armeniacus*, *Fusarium verticillioides* growth *and* fumonisin B_1 suppression, *Zea mays*

Abstract

Fusarium verticillioides is the most important seed transmitted pathogen that infects maize. It produces fumonisins, toxins that have potential toxicity for humans and animals. Control of *F. verticillioides* colonisation and systemic contamination of maize has become a priority area in food safety research. The aims of this research were (1) to characterise the maize endorhizosphere and rhizoplane inhabitant bacteria and *Fusarium* spp., (2) to select bacterial strains with impact on *F. verticillioides* growth and fumonisin B_1 production *in vitro*, (3) to examine the effects of bacterial inoculum levels on *F. verticillioides* root colonisation under greenhouse conditions. *Arthrobacter* spp. and *Azotobacter* spp. were the predominant genera isolated from maize endorhizosphere and rhizoplane at the first sampling period, whilst *F. verticillioides* strains showed the greatest counts at the same isolation period. All *F. verticillioides* strains were able to produce fumonisin B_1 in maize cultures. *Arthrobacter globiformis RC5* and *Azotobacter armeniacus RC2*, used alone or in a mix, demonstrated important effects on *F. verticillioides* growth and fumonisin B_1 suppression *in vitro*. Only *Azotobacter armeniacus RC2* significantly reduced the *F. verticillioides* root colonisation at 10^6 and 10^7 CFU g^{-1} levels under greenhouse conditions.

Introduction

Fusarium verticillioides (synonym. *Fusarium moniliforme* Sheldon; teleomorph *Gibberella moniliformis* [synonym. *Gibberella fujikuroi* mating population A]) is a seed-transmitted pathogen that infects maize (Nelson 1992; Nelson et al. 1993). *F. verticillioides* produces several toxins that have potential toxicity for humans and animals. The most significant of these toxins are the fumonisins (Nelson et al. 1993; Desjardin et al. 1995). Since the fumonisins have been detected in symptomatic and asymptomatic maize kernels, control of *F. verticillioides* colonisation of maize has become a priority area in food safety research (Brown et al. 2001). Infection of *F. verticillioides* can start through airborne spores infecting the silks. Another infection pathway is systemic through the seed. The fungus moves from the roots to stalk and kernels (Kedera et al. 1992; Munkvold et al. 1997). The systemic infection leads to fumonisin contamination. Control of *F. verticillioides* can

probably be achieved by indigenous bacteria. Many regions of young roots are exposed to microbial colonisation. These root sites give many niches in which a wide range of bacteria belonging to the genera *Arthrobacter*, *Azotobacter*, *Bacillus*, and *Pseudomonas* could develop. These bacteria inhibit deleterious microorganisms. There are numerous reports of rhizobacteria that exert beneficial effects on plant growth that have often been attributed to the displacement or repression of pathogens (Lambert et al. 1987). Colonisation of the rhizosphere and production of antifungal substances are two factors important to this biocontrol.

The aims of the present study were: (1) to characterise the maize endorhizosphere and rhizoplane inhabitant bacteria and *Fusarium* spp., (2) to select bacterial strains with impact on *F. verticillioides* growth and fumonisin B_1 production *in vitro*, (3) to examine the effect of bacteria inoculums levels on *F. verticillioides* root colonisation under greenhouse conditions.

Materials and methods

Sampling

Samples from a commercial maize field (Morgan M401, Argentina SA, Buenos Aires, Argentina) were collected during seedling stage (15 days) and at harvest time (120 days). The field soil was loamy sand. Twenty plants were randomly chosen and removed. Plants were lifted together with adherent soil into plastic bags, transported to the laboratory within 12 h and analysed the same day.

Microbial population studies

Bacterial isolation and identification

To obtain bacterial populations from the maize rhizoplane, roots were gently washed in phosphate buffered saline (PBS, Oxoid, Ltd., London, UK) for 2 min to remove the adhering soil, and shaken for 5 min in PBS containing 0.025% Tween 20. Three-fold dilution of the homogenates was plated on tryptic soy broth plus 2% agar (TSBA) and King's medium B (King et al. 1954). Plates were incubated for 24–48 h at 28 °C. Total counts and counts per colony type were made. One colony per colony type was isolated and purified on TSBA. To quantify the endorhizosphere populations, maize roots were washed and dried between sheets of tissue, weighed and surface sterilised by gently shaking in 70% ethanol (1 min), 20% household bleach (5 min) and thiosulphate Ringer solution (5 min) (Oxoid Ltd., London, UK). Samples were macerated in 90 ml of PBS with a mortar pestle. Three-fold dilution was plated on TSBA. Petri plates were incubated for 24–48 h at 28 °C. Total counts and counts per colony type were made. One colony per colony type was isolated and purified on TSBA.

Bacterial identification was performed according to Bergey's Manual of Systematic Bacteriology (Krieg 1984; Sneath 1986).

Fusarium species isolation and identification

Fusarium strains were isolated from the same roots as used for bacterial recovery. Three-fold dilution of the homogenates was plated on Nash–Snyder agar. Plates were incubated for 7 days at 28 °C. Total counts and counts per colony type were made. One colony per colony type was isolated and purified on carnation leaf agar (CLA). *Fusarium* strains were classified according to Nelson et al. (1983).

Toxigenic ability of endorhizospheric F. verticillioides strains

Sterile Petri dishes (120×20 mm) containing 50 g of sterilised maize seeds (gamma irradiation at 25 °C, 1200 krad) and adjusted with sterile distilled water to achieve a 0.97 water activity were inoculated with 1 ml of 10^6 spore ml^{-1} of each strain of *F. verticillioides* separately. Cultures were incubated for 25 days at 25 °C in a chamber with controlled relative humidity of 97% with K_2SO_4 saturated solution. To avoid changes in the equilibrium relative humidity, the saline solution was replaced daily with fresh solution. Maize kernels (25 g) were extracted with 50 ml of methanol and water (3:1, v/v) and shaken for 30 min on an orbital shaker. The extracts were filtered through filter paper (n°4, Whatman, Inc., Clifton, NJ, USA). Aliquots of the extracts (10–50 μl) were diluted (1/100 or 1/50, respectively), with acetonitrile–water (1:1, v/v). The diluted extracts were quantitatively determined by a modified HPLC method proposed by Shephard et al. (1990) and modified by Doko et al. (1995). An aliquot (50 μl)

of each extract was derivatised with 200 μl of *o*-phthaldialdehyde (OPA). The OPA solution was obtained by adding 5 ml of 0.1 M sodium tetraborate and 50 μl of 2-mercaptoethanol to 1 ml of methanol containing 40 mg of OPA. The fumonisin OPA derivatives (20 μl solution) were analysed using a reversed-phase high pressure liquid chromatography/fluorescence detection system (HPLC). The HPLC system consisted of a Hewlett Packard 1050 pump (Palo Alto, CA, USA) connected to a Hewlett Packard 3395 integrator. Chromatographic separations were performed on a stainless steel Supelcosil LC-ABZ, C 18 reversed-phase column (150×4.6 mm i.d., 5 μm particle size; Supelco). A methanol 0.1 M sodium dihydrogen phosphate (75:25) solution, adjusted to pH 3.35 with orthophosphoric acid was used as the mobile phase at a flow rate of 1.5 ml min^{-1}. The fluorescence of fumonisin OPA derivatives was recorded at excitation and emission wavelengths of 335 and 440 nm, respectively. Fumonisins quantification was performed by peak height measurements and comparison with reference standard solutions. The standard solution was obtained by dissolving pore fumonisin FB_1 (Division of Food Science and Technology, Pretoria, South Africa) in acetonitrile–water (1:1) at concentrations of 100 μg ml^{-1} for FB_1. The detection limit of the analytical method was 1 μg g^{-1}.

Microorganisms

From all bacteria identified, six bacterial strains, *Arthrobacter globiformis RC1*, *Azotobacter armeniacus RC2*, *Azotobacter armeniacus RC3*, *Arthrobacter globiformis RC4*, *Arthrobacter globiformis RC5*, *Azotobacter armeniacus RC6* were selected. They belong to the predominant genera able to colonise the maize endorhizosphere. Thirteen toxigenic strains of *F. verticillioides* from endorhizosphere were used.

In vitro *studies*

Antifungal activity

Culture medium was 2% maize meal extract agar (MMEA). The water activity (a_W) of the basic medium was adjusted to 0.982, 0.955 and 0.937 a_W with glycerol (Dallyn and Fox 1980). About 10 μl with 10^9 spores ml^{-1} of each *F. verticillioides* strain were separately inoculated in the centre of the plates. About 10 μl with 10^8 CFU ml^{-1} of the bacterial suspensions were placed around the fungal inoculum. Plates were incubated for up to 10 days at 25 °C in polyethylene bags. The antibiosis was determined by measurements of the size (mm) of the zone of inhibition formed between the edge of the colony of *F. verticillioides* to the edge of a bacterial colony on the plate. Control plates consisted of *F. verticillioides* placed alone.

Influence on growth rate

Before cooling, MMEA (10 ml) adjusted to different water activities (0.982, 0.955 and 0.937 a_W) was combined with 100 μl (10^8 cells ml^{-1}) of each antagonist and poured into the Petri dishes (60×10 mm). Each strain of *F. verticillioides* was inoculated in the centre of the plates with a spore from monosporic culture. Cultures were incubated for 20 days at 25 °C in polyethylene bags. The radius of *F. verticillioides* colony was measured daily with a ruler. The growing radius of the cultures containing both microorganisms was compared with the control cultures. For each colony two radii, measured at right angles to one another, were averaged to find the mean radius. All colony radii were determined by using three replicates for each test. The radial growth rate (mm h^{-1}) was subsequently calculated by linear regression of linear phase for growth and the time at which the line intercepts the *x*-axis was used to calculate the lag phase in relation to bacterial antagonists and water activity (González et al. 1987). The experiments were carried out three times. After growth was evaluated, all samples were frozen for later extraction and FB_1 quantification.

Determination of inhibition of fumonisin B_1 production

Toxins were extracted with acetonitrile–water (1:1, v/v) by shaking the cultures media and mycelia from co-inoculated and control cultures with the solvent for 30 min on an orbital shaker (150 rev min^{-1}) and then filtering the extracts through filter paper (no. 4; Whatman) (Etcheverry et al. 2002). The extracts were frozen and stored at −20 °C until analysed. An aliquot of the extract (1000 μl) was taken and diluted with acetonitrile–water as necessary for high performance liquid chromatography. The fumonisin content of the OPA derivative was evaluated by HPLC, as described above.

Greenhouse studies

Arthrobacter globiformis RC5 inoculums and *Azotobacter armeniacus RC2* inoculums were prepared separately from 25 °C overnight culture in tryptic soy broth medium (TSB) and harvested by centrifugation. Cells were re-suspended in spore suspension (sodium laurylsulphate 0.01%, w/v). Serial decimal dilutions were done to obtain 10^6 and 10^7 cells ml^{-1}. The concentration of cell suspensions was determined by using a haemocytometer. Viability was confirmed by standard plate count method using TSBA. Antagonistic treatments were prepared: (1) *Az. armeniacus RC2* at 10^6 cells ml^{-1}, (2) *Az. armeniacus RC2* at 10^7 cells ml^{-1}, (3) *Ar. globiformis RC5* at 10^6 cells ml^{-1}, (4) *Ar. globiformis RC5* at 10^7 cells ml^{-1}, (5) *Ar. globiformis RC5–Az. armeniacus RC2* in a mixture, at 10^6 cells ml^{-1}, (6) *Ar. globiformis RC5–Az. armeniacus RC2* in a mixture, at 10^7 cells ml^{-1}, (7) control without antagonists.

Seventy-five maize seeds were submerged in 100 ml TSB in 250 ml Erlenmeyer flasks for each bacterial treatment. Control seeds were submerged in 100 ml of TSB. Flasks were incubated at 25 °C on a rotatory shaker for 2 h at 70 rpm, to allow bacterial cells to adhere to seeds. After incubation, excess inoculum was removed and seeds were immediately planted in the plastic tubes.

Ar. globiformis RC5 and *Az. armeniacus RC2*, alone and in a mixture, were tested for their ability to inhibit the *F. verticillioides* root colonisation with a modification of the tube assay described by Weller and Cook (1986). Plastic tubes 2.5 mm in diameter $\times$16.5 cm long were filled with 2 cm of field maize soil. One treated seed was placed on top of the soil and covered with 2 cm of sand-vermiculite (1:1, v/v). Non-treated seeds were planted as control in each assay. After planting, racks of tubes were covered with aluminum foil, kept in dark for 5 days at 20 °C, then uncovered and kept at 20 °C with a 14 h photoperiod. Seventy-five seeds were used for each treatment (with and without antagonists). Five replications for each treatment were made. This assay was done twice.

Statistical analysis

Data analysis were performed by analysis of variance. To compare means of antibiosis and growth rate treatments, Duncan multiple range test was made. Bacterial and *Fusarium* spp. counts were transformed to $\log_{10}(x + 1)$ to obtain the homogeneity of variance. Means of populations count treatments were compared using Fisher's protected LSD test. The fumonisin B_1 data obtained were transformed using a logarithmical function $\log_{10}(x + 1)$ before applying the analysis of variance. The Scheffé test was used to determine the significant differences between the control and co-inoculated cultures (Quinn and Keough 2002). The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC).

Results

Bacterial populations on maize roots

Seven bacterial genera were isolated from rhizoplane and endorhizosphere (Table 1). Bacterial populations ranged between 10^5 and 10^9 colony forming units per gram of root tissue (CFU g^{-1}) throughout the first sampling period (15 days) with *Arthrobacter* and *Azotobacter* as predominant genera. During the second sampling period (120 days), the population counts ranged between 10^9 and 10^{10} CFU g^{-1} of root tissue in the rhizoplane and between 10^5 and 10^{10} CFU g^{-1} of root tissue in the endorhizosphere. *Arthrobacter*, *Agromyces* and *Pseudomonas* were the predominant genera in the endorhizosphere samples.

Table 1. Rhizobacterial plate counts from maize roots at different sampling periods

Rhizobacterial genera	Rhizobacterial counts ($\log_{10}$ CFU g^{-1} of root tissue)[a]			
	Rhizoplane		Endorhizophere	
	15 days	120 days	15 days	120 days
Arthrobacter	9	10	9	10
Azotobacter	8	1	7	1
Agromyces	1	9	6	9
Bacillus	6	9	6	5
Micrococcus	5	1	5	1
Pseudomonas	5	9	6	8
Listeria	1	10	1	1

[a]Values are the means ± SD (standard deviation) of three repetitions.

Table 2. Fusarium species plate counts from maize roots at different sampling periods

Fusarium species	*Fusarium* species counts (log_{10} CFU g^{-1} of root tissue)[a]			
	Rhizoplane		Endorhizophere	
	15 days	120 days	15 days	120 days
F. verticillioides	3	3	3	1
F. proliferatum	1	1	3	1
F. subglutinans	1	1	3	1
F. nygamai	1	1	3	1
F. oxysporum	1	3	2	1
F. solani	1	3	1	1
Other *Fusarium* spp.	3	3	1	4

[a]Values are the means ± SD (standard deviation) of three repetitions.

Table 3. Fumonisin B_1 concentrations in maize cultures of *F. verticillioides* strains isolated from endorhizosphere of maize

F. verticillioides strains	Fumonisin B_1 (ng g^{-1})
1	1210
2	1335
3	708
4	792
5	20,897
6	3132
7	1410
8	1108
9	1899
10	1573
11	927
12	7377
13	14,380

Fusarium species populations on maize roots and toxigenic ability of endorhizospheric F. verticillioides strains

Table 2 shows the *Fusarium* species populations obtained from rhizoplane and endorhizosphere at the two sampling periods previously mentioned. *F. verticillioides* showed the highest counts on rhizoplane and endorhizosphere at the first sampling period (15 days). This species was not isolated in significant counts in the endorhizosphere at day 120. *Fusarium proliferatum*, *Fusarium subglutinans*, and *Fusarium nygamai* were isolated at high counts from the endorhizosphere at the first sampling period while significant counts of *Fusarium oxysporum* and *F. solani* were obtained in the rhizoplane at the second sampling time (120 days).

Table 3 shows the fumonisin B_1 levels produced by 13 *F. verticillioides* strains in maize cultures. The strains were isolated from maize endorhizosphere at the first sampling period. They showed a great potential to produce fumonisin B_1. The values ranged between 708 and 20,897 ng g^{-1}.

In vitro *studies*

We studied the percentage of *F. verticillioides* strains inhibited by antifungal activity, growth rate, fumonisin B_1 production and fumonisin B_1 accumulation and percentage of fumonisin B_1 reduction in co-cultures. Each of 13 *F. verticillioides* strains was paired with each bacterial strain (three *Ar. globiformis* and three *Az. armeniacus*) in dual cultures.

Antifungal activity

At 0.982 a_w, *Az. armeniacus RC2* and *Az. armeniacus RC3* application showed inhibition of the highest percentage of *F. verticillioides* strains (80 and 100%, respectively). At 0.955 a_w, *Az. armeniacus RC2* and *Ar. globiformis RC5* application inhibited between 71 and 80% of *F. verticillioides* strains, respectively (Table 4).

Influence on growth rate

The percentage of *F. verticillioides* strains inhibited on growth rate ranged between 54 and 100% at 0.982 a_w. This percentage was higher than 0.955 a_w. It ranged between 31 and 69%. None of the rhizobacteria were able to inhibit the *F. verticillioides* growth rate at 0.937 a_w (Table 4).

Percentage of F. verticillioides strains inhibited on fumonisin B_1 production and fumonisin B_1 accumulation reduction

At 0.982 a_w level, the fumonisin B_1 accumulation by *F. verticillioides* strains ranged between 1166 and 7382 ng g^{-1}. At 0.955 a_w, these values ranged between 2702 and 10,810 ng g^{-1}. At 0.937 a_w, the fumonisin B_1 accumulation was not detected.

In the presence of bacterial antagonists, at 0.982 a_w, 7–36% of *F. verticillioides* strains showed reduction on fumonisin B_1 ability production. The fumonisin B_1 accumulation ranged between 933 and 10,484 ng g^{-1}. The fumonisin B_1 reduction

Table 4. Bacterial antagonism on *F. verticillioides* strains *in vitro*

Rhizobacteria	a_w	Percentage of *F. verticillioides* strains inhibited (%)			Fumonisin B_1 accumulation (ng g^{-1})	Reduction of fumonisin B_1 accumulation (%)
		Antifungal activity	Growth rate	Fumonisin B_1 production		
F. verticillioides strains	0.982				1166–7382	
(control)	0.955	–	–	–	2702–10,810	
	0.937				ND	
Ar. globiformis RC1	0.982	35	69	7	933–7181	6–39
	0.955	60	54	29	2780–7061	29
	0.937	11	NG	ND	ND	0
Az. armeniacus RC2	0.982	80	89	36	933–4365	50
	0.955	71	54	43	1351–6756	56–75
	0.937	14	NG	ND	ND	0
Az. armeniacus RC3	0.982	100	100	21	1483–7270	14
	0.955	60	54	29	1450–9459	20–63
	0.937	20	NG	ND	ND	0
Ar. globiformis RC4	0.982	71	69	7	1826–10,484	22–96
	0.955	57	64	29	2702–9865	20–63
	0.937	7	NG	ND	ND	0
Ar. globiformis RC5	0.982	50	61	14	1255–4490	8–22
	0.955	80	69	35	2831–6756	43–75
	0.937	21	NG	ND	ND	0
Az. armeniacus RC6	0.982	64	54	14	1940–7203	13–23
	0.955	40	31	14	2830–9459	38–43
	0.937	21	NG	ND	ND	0

NG: no growth; ND: not detected.

percentage ranged between 6–96%. *Az. armeniacus RC2* and *Ar. globiformis RC5* produced the lowest fumonisin B_1 accumulation levels that ranged between 933–4365 and 1255–4490 ng g^{-1}, respectively. At 0.955 a_w, the percentage of strains reduced on fumonisin B_1 production was higher than at 0.982 a_w level (14–43%). The fumonisin B_1 accumulation ranged between 1351–9865 ng g^{-1}. Percentage of fumonisin B_1 reduction ranged between 20 and 75%. *Az. armeniacus RC2* and *Ar. globiformis RC5* were reliable microorganisms that exerted this effect. These antagonists reduced the fumonisin B_1 accumulation levels that ranged between 1351–6756 ng g^{-1} and 2831–6756 ng g^{-1}, respectively (Table 4). At 0.937 a_w, fumonisin B_1 was not detected. The bacterial antagonists did not stimulate fumonisin B_1 accumulation.

Greenhouse studies

According to the results obtained with the *in vitro* assays, *Az. armeniacus RC2* and *Arthrobacter globiformis RC5* were selected as the best antagonists to test the maize root colonisation of *F. verticillioides* at two inoculum sizes under greenhouse experiments.

Seed bacterisation with *Az. armeniacus RC2* at 10^6 and 10^7 cells ml^{-1} and *Ar. globiformis RC5* at 10^6 cells ml^{-1} inhibited *F. verticillioides* counts at rhizoplane and endorhizosphere levels (treatments 1–3). The *F. verticillioides* CFU counts did not show differences from the control when treatment 4 was applied (*A. globiformis RC5* at 10^7 cells ml^{-1}) at the rhizoplane level. On the contrary, this treatment significantly reduced the *F. verticillioides* CFU counts at the endorhizosphere level. The bacterial mixture treatments showed a significant impact on *F. verticillioides* CFU counts at endorhizosphere level. There was a total inhibition of *F. verticillioides* colonisation when the bacterial mixture at 10^6 cells ml^{-1} was applied (Table 5).

To analyse the behaviour of *Az. armeniacus RC2* at 10^6 cells ml^{-1} inoculum level between *in vitro* experiments (antifungal activity; 0.982 a_w) and greenhouse studies (*F. verticillioides* CFU root counts), the Pearson correlation coefficient index

Table 5. F. verticillioides roots counts from rhizoplane and endorhizosphere at 20 maize growing days

Seed treatments	*F. verticillioides* counts (log_{10} CFU g^{-1} of root tissue)[a]			
	Rhizoplane	LSD test	Endorhizosphere	LSD test
Untreated control	4.7103 ± 0.5271	ab	3.6028 ± 0.0062	a
1. *Az. armeniacus RC2* 10^6 cells ml^{-1}	0	c	0	c
2. *Az. armeniacus RC2* 10^7 cells ml^{-1}	0	c	0	c
3. *Ar. globiformis RC5* 10^6 cells ml^{-1}	0	c	0	c
4. *Ar. globiformis RC5* 10^6 cells ml^{-1}	4.3904 ± 0.5204	ab	2.0275 ± 0.0480	b
5. *Az. armeniacus RC2* 10^6 cells ml^{-1} + *Ar. globiformis RC5* 10^6 cells ml^{-1}	4.2466 ± 0.5503	a	0	c
6. *Az. armeniacus RC2* 10^7 cells ml^{-1} + *Ar. globiformis RC5* 10^7 cells ml^{-1}	5.0088 ± 0.5368	a	2.0916 ± 0.0088	b

[a]Values are the mean ± SD (standard deviation) of five replicates. The letters in common are not significantly different according to Fisher's protected LSD test. Values were transformed to $log_{10}(x + 1)$.

was applied. Both experiments showed a positive correlation ($r = 0.9364$; $p < 0.001$). There was an important antifungal activity *in vitro* assay (50–60%). Moreover, a similar behaviour in greenhouse trials, where the colonisation inhibition reached high values (90–98%), could be observed. These results showed that *in vitro* antagonistic ability was maintained in the greenhouse study (Figure 1).

Discussion

The selection of indigenous bacterial communities naturally associated with maize root systems to study the effect of individual rhizosphere isolates on native maize *F. verticillioides* was carried out.

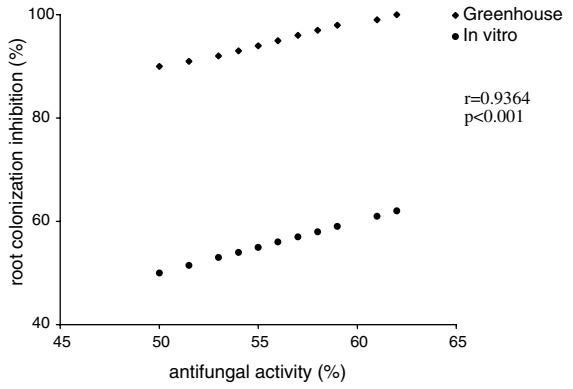

Figure 1. Correlation coefficient between *in vitro* and greenhouse experiments on antifungal activity on *F. verticillioides* strains.

In this study, *Fusarium* species from maize rhizosphere were isolated. *F. verticillioides* was the most prevalent *Fusarium* species present in the maize rhizoplane and endorhizosphere. Different studies have identified the same *Fusarium* species isolated here (Ocamb and Khommedahl 1994; Windham and King 1983) and demonstrated their ability to colonise the maize endorhizosphere.

All rhizosphere *F. verticillioides* strains isolated in this study were able to produce fumonisin B_1 *in vitro*. This result indicates their potential to produce fumonisin B_1 in maize kernels.

Additionally, the diversity and size of maize rhizosphere bacterial populations were determined. They were in the same order as those reported for other crops (Hagedorn et al. 1989; Chiarini et al. 1998). Among these organisms, *Arthrobacter* and *Azotobacter* genera represent the predominant genera at the early growing period of maize at the rhizoplane and endorhizosphere levels. Our studies demonstrated that *Arthrobacter globiformis RC5* and *Azotobacter armeniacus RC2* were able to exert an effective control on *in vitro* and fumonisin B1 production under harsh environmental conditions given by different water activities. This is one of the most important characteristics necessary for biocontrol agents that must survive and exert its biocontrol activity at field level.

It is assumed that many biocontrol agents first colonise the spermosphere and rhizosphere to protect the plant. A pathogen may germinate and colonises the root within a few days of planting; rhizobacteria can be metabolically active during

that time period. In view of their potential application as biocontrol agents, *Ar. globiformis RC5* and *Az. armeniacus RC2*, alone or in a mix, could compete and exclude the highly competitive indigenous *F. verticillioides* strains, and colonise the plant roots during this critical period when the protection against pathogens is required. This fact may be critical in order to inhibit the colonisation or to suppress the plant pathogen before it enters the roots.

Application of bacteria to seeds has been used for the biological control of soil-borne plant pathogens that affect many host plants (Hebbar et al. 1992; Reddy and Hynes 1994). There are few data about inhibition of *F. verticillioides* by bacterial isolates. Hinton and Bacon (1995) reported that one isolate of *Enterobacter cloacae* associated as endophyte with corn roots, stems and leaves is antagonistic to *F. moniliforme* and other toxic fungi associated with corn. Motomura et al. (1997) obtained Gram positive bacilli with anti-*Fusarium moniliforme* activity from soil and corn. More recently studies involving *F. verticillioides* and *Bacillus subtilis* showed promising reduction for mycotoxin accumulation during the endophytic growth phase (Bacon et al. 2001). In this study we demonstrated that *Ar. globiformis RC5* and *Az. armeniacus RC2* were able to totally inhibit the endorhizosphere colonisation at 10^6 cells ml^{-1} whereas a bacterial mixture, at the same inoculum size, exerted the same effect in the endorhizosphere. Only *Az. armeniacus RC2* totally reduced the *F. verticillioides* rhizosphere colonisation under greenhouse experiments, at the two inoculum and root levels tested. The expression of antagonism by a microorganism towards a pathogen in culture media cannot be regarded as evidence that the microorganism will have the same role in controlling the pathogen in the field (Reddy and Hynes 1994). In this research, a high correlation between antagonisms recorded *in vitro* and under greenhouse trials was obtained. In contrast, other researchers (Hagedorn et al. 1989; Bevivino et al. 1998) found a very poor interaction when antagonism *in vitro* and in greenhouse studies was correlated.

In conclusion, *Az. armeniacus RC2* has a potential as maize inoculant to inhibit the *F. verticillioides* root colonisation. Research continues to develop this bacterium as a biocontrol agent in field trials.

Acknowledgements

This work was carried out thanks to grants from Secretaría de Ciencia y Tecnica de la Universidad Nacional de Río Cuarto (Res. 241/99) and Agencia Nacional de Promocion Cientifica y Tecnologica (PICT99/08-07197) throughout the years 2002–2003.

References

Bacon C.W., Yates I.E., Hinton D.M. and Meredith F. 2001. Biological control of *Fusarium moniliforme* in maize. Environ. Health Persp. 109(2): 325–332.

Bevivino A., Sarrocco S., Dalmastri C., Tabacchioni S., Cantale C. and Chiarini L. 1998. Characterization of a free living maize-rhizosphere population of *Burkholderia cepacia*: effect of seed treatment on disease suppression and growth promotion of maize. FEMS Microbiol. Ecol. 27: 225–237.

Brown R.T., Cleveland C., Woloslmk C., Payne G.A. and Bhatnagar D. 2001. Growth inhibition of a *Fusarium verticillioides* GUS strain in corn kernels of aflatoxin-resistant genotypes. Appl. Biotechnol. Microbiol. 57: 708–711.

Chiarini L., Bevivino A., Tabbachini S. and Dalmastri C. 1998. Inoculation of *Burkholderia cepacia, Pseudomonas fluorescens* and *Enterobacter* sp. on *Sorghum bicolor*: root colonization and plant growth promotion on dual strain inocula. Soil Biol. Biochem. 30: 81–87.

Dallyn H. and Fox A. 1980. Spoilage of material of reduced water activity by xerophilic fungi. In: Gould G.H. and Carry J.E.L. (eds), Society of Applied Bacteriology Technical Series 15. Academic Press, London, pp. 129–139.

Desjardin A., Plattner R.D., Nelson T.C. and Leslie J.F. 1995. Genetic analysis of fumonisin production and virulence of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*) on maize (*Zea mays*) seedlings. Appl. Environ. Microbiol. 61: 79–86.

Doko B., Rapior S., Visconti A. and Schroth J. 1995. Incidence and levels of fumonisin contamination in maize genotypes grown in Europe and Africa. J. Agric. Food Chem. 43: 429–434.

Etcheverry M., Torres A., Ramírez M.L., Chulze S. and Magan N. 2002. *In vitro* control of growth and fumonisin production by *F. verticillioides* and *F. proliferatum* using antioxidants under different water availability and temperature regimes. J. Appl. Microbiol. 92: 624–632.

González H.H.L., Resnik S.L. and Vaamonde G. 1987. Influence of inoculum size on growth rate and lag phase of fungi isolated from Argentine corn. Int. J. Food Microbiol. 4: 111–117.

Hagedorn C., Gould W.D. and Bardinelli T.R. 1989. Rhizobacteria of cotton and their repression of seedling disease pathogens. Appl. Environ. Microbiol. 55(11): 2793–2797.

Hebbar K.P., Davey A.G., Merrin J. and Dart P.J. 1992. Rhizobacteria of maize antagonistic to *F. moniliforme*, a soil-borne fungal pathogen: isolation and identification. Soil Biol. Biochem. 24(10): 989–997.

Hinton D.M. and Bacon C.W. 1995. *Enterobacter cloacae* is an endophitic symbiont of corn. Mycopathologia 129: 117–125.

Kedera C.J., Leslie J.F. and Clattin L.E. 1992. Systemic infection of corn by *Fusarium moniliforme*. Phytopathology 84: 603–607.

King E.O., Ward M.K. and Ranney D.E. 1954. Two simple media for the demonstration of pyocianin and fluorescin. J. Lab. Clin. Med. 44: 301–307.

Krieg N.R. 1984. In: Holt J.G. (ed.), Bergey's Manual of Determinative Bacteriology, Vol. 1. Williams and Wilkins, Baltimore, USA, pp. 141–199; 220–230.

Lambert B., Leyns F., Van Rooyen L., Gosselé F., Papon I. and Swings J. 1987. Rhizobacteria of maize and their antifungal activities. Appl. Environ. Microbiol. 53(8): 1866–1871.

Motomura M., Lourenço C.E., Venturini D., Ueno Y. and Hirooka E.Y. 1996. Screening and isolation of anti-*Fusarium moniliforme* compounds producing microorganisms from soil and corn. Rev. Microbiol. 27: 213–217.

Munkvold G.P., McGee D.C. and Showers W.E. 1997. Importance of different pathways for maize kernel infection by *Fusarium verticilliodes*. Phytopathology 97: 209–217.

Nelson P.E. 1992. Taxonomy and biology of *Fusarium moniliforme*. Mycopathologia 117: 29–36.

Nelson P.E., Desjardins A.E. and Plattner R.D. 1993. Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry and significance. Ann. Rev. Phytopathol. 31: 233–252.

Nelson P.E., Toussoun T.A. and Marasas W.F.O. 1983. *Fusarium species*. An Illustrated Manual for Identification. The Pennsylvania State University Press, University Park and London.

Ocamb C.M. and Kommedahl T. 1994. Rhizosphere competence of *Fusarium* species colonizing corn roots. Phytopathology 84(2): 166–172.

Quinn G.P. and Keough M.J. 2002. Experimental Design Data Analysis for Biologists. Cambridge University Press, Cambridge, United Kingdom, pp. 72–76.

Reddy M.S. and Hynes R.K. 1994. Relationship between *in vitro* growth inhibition of pathogens and suppression of pre-emergence damping-off and post-emergence root rot of white beans seedlings in the greenhouse by bacteria. Can. J. Microbiol. 40: 113–119.

Shephard G.S., Sydenham E.W., Thiel P.G. and Gelderblom W.C.A. 1990. Quantitative determination of fumonisin B_1 and B_2 by high-performance liquid chromatography with fluorescence detection. J. Liq. Chromatogr. 13: 2077–2087.

Sneath P.H.A. 1986. In: Holt J.G. (ed.), Bergey's Manual of Determinative Bacteriology, Vol. 1. Williams and Wilkins, Baltimore, USA, pp. 1105–1139; 1288–1301.

Weller D.M. and Cook R.J. 1986. Increased growth of wheat by seed treatment with fluorescent pseudomonads and implications of *Pythium* control. Can. J. Plant Pathol. 8: 328–334.

Windham M.T. and King S.B. 1983. Micoflora of roots of maize at seedling and silking stages in Mississipi. Plant Dis. 67: 1366–1368.