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Preliminary Evaluation of Biocontrol Agents against Maize Pathogens *Exserohilum turcicum* and *Puccinia sorghi* in Field Assays

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Abstract

Exserohilum turcicum and *Puccinia sorghi* cause foliar fungal diseases that affect maize crop in Argentina. These diseases, northern leaf blight and common rust respectively, are presented each year with different levels of severity affecting significantly the yield in susceptible hybrids. Disease control usually consists in the use of resistant cultivars and chemical control. Biological control as a preventive method is a viable alternative to evaluate. The aims of this study were to evaluate the natural incidence of both foliar diseases in maize after application of two antagonists, to determine the survival of the antagonists in the maize phyllosphere and to evaluate the effect of inoculation on grain yield at harvest. Plants treated with both biological control agents showed significant reductions in the incidence of both foliar diseases. In northern leaf blight the reduction was higher than 50% during 40 days in plants treated with *Bacillus* spp. Moreover, grain yield was significantly higher as compared to control treatments.

Keywords

Maize, Foliar Diseases, Biological Control, Northern Leaf Blight, Common Rust

1. Introduction

Maize grown in tropical and temperate climates has been being affected by two foliar diseases during recent seasons. Northern leaf blight is caused by *Exserohilum turcicum,* and common rust is caused by *Puccinia sorghi* [1] [2]. At the main maize producing area of Argentina, northern leaf blight was the most prevalent disease during 2007/08 to 2009/10, reaching severities higher than 50% in early growth stages and yield losses greater than 40% [3]. Some factors that favour the development of the disease are increasing the areas of direct planting, changes in the date of sowing, intense and frequent rains during the summer months or lots irrigated by sprinkling [4] [5]. On the other hand, common rust is endemic in the maize production area of Argentina [6] [7]. This disease occurs each year with varying degrees of intensity, according to the behaviour of the hybrid and the prevailing environmental conditions [8].

Disease control usually consists of using resistant cultivars and chemical control with mixtures of fungicides like triazole and strobilurins [2] [5] [7]. The application of fungicides to control common rust in Argentina can be of up to1000 kg (1000 - 1500 kg/ha) and of up to 2000 kg (2000 - 3000 kg/ha) for northern leaf blight [9].

Biological control offers advantages over other control methods such as pesticides. This methodology is environmentally friendly, does not cause any type of pollution and it preserves the ecosystem, while chemical control disturbs the ecosystem and provides short term control of harmful organisms [10]. In maize, growth-promoting and antifungal compounds-producing bacteria have been shown to have inhibitory effects on southern leaf blight disease caused by the fungus *Cochliobolus heterostrophus* [11] [12]. Sartori *et al.* [13] [14] reported advances in the biological control of *E. turcicum in vitro* and *in planta* by using epiphytic bacteria of different genera. Those studies allowed us to select potential biocontrol agents (BCA). The aims of the present study were: 1) to evaluate the natural incidence of the foliar diseases northern leaf blight and common rust in maize after the application of two antagonists, 2) to determine the survival of antagonists in the maize phyllosphere and 3) to determine the effect of inoculation on grain yield at harvest.

2. Material and Methods

2.1. Isolates

Antagonistic bacterial strains were isolated from maize leaves with disease lesions from fields of different regions of the Córdoba province, Argentina. The antagonistic ability of isolates was evaluated *in vitro*, and eleven potential biocontrol agents were selected [13]. According to results obtained in greenhouse studies against *E. turcicum*, two bacteria were selected [14]. In the present study, these bacteria were also evaluated against *P. sorghi*. One strain of *Pantoea* spp. (GenBank accession KX500237) and one *Bacillus* spp. (GenBank accession KX500241) were used to assess their effect against *E. turcicum* and *P. sorghi* in the field. These strains were maintained on slants of trypticase soy agar (TSA). Spontaneous mutants resistant to streptomycin 5% and rifampicin 0.5% were obtained. Resistance of the strains to antibiotics as marked was used for monitoring BCAs on maize phylloplane [15].

2.2. Inoculum Preparation

Inoculum for each bacterial antagonist was prepared from cultures grown on nutrient broth (NB) with water activity (a_w) adjusted to 0.97 by the addition of glycerol [16]. Bacterial strains were cultured in NB (0.97 a_w) for 24 h at 140 rpm and 25˚C up to the exponential phase. Total number of viable cells was determined by standard plate count methods. Serial dilutions were performed and plated on nutrient agar (NA) to evaluate cell viability and count of colony forming units per ml (CFU ml^{-1}).

2.3. Field Assays

Seeds of maize cultivar LT 621 MGRR2 (La Tijereta, Monsanto, Argentina) were used for the field assay. This cultivar is resistant to insects and to the herbicide glyphosate. The field experiment was performed in a commercial field in Río Cuarto, Córdoba (33˚2'S latitude, 64˚14'O longitude, 562 m altitude) during the growing season 2014-2015. Treatments were distributed using a complete randomized block design with three replications per treatment. Individual plots consisted of two rows with 50 plants. The design was performed twice. Sowing was carried out during mid-January, while harvest was performed during mid-May. One application of the herbicide glyphosate was performed 1 week after sowing according to common agricultural practices used for RR cultivars.

The treatments used were: 1) Control; 2) *Pantoea* spp.; 2* two applications of *Pantoea* spp; 3) *Bacillus* spp; 3* two applications of *Bacillus* spp. Plants in VT phenological stage [17], were inoculated by foliar spraying with antagonists, using an atomiser. Cultures of antagonists were diluted in NB to obtain inocula of 10^9 CFU ml^{-1} for *Pantoea* spp. and 10^7 CFU ml^{-1} for *Bacillus* spp. These inocula concentrations were effective in reducing leaf blight in a previous greenhouse study [14]. Before applying the inocula on maize leaves, a commercial surfactant based on organ-silicones and refined vegetable oil was added in a dilution of 1 ml in 1000 ml of inoculum. Monitoring of antagonists on phyllosphere was performed at time of inoculation (T0) and 20 days post-inoculation (T1). A leaf from each plot was weighed and suspended in phosphate buffer to obtain a 10^{-1} dilution and incubated for one hour at 180 rpm and 30°C. After that, serial dilutions were performed in NB and plated on nutrient agar (NA) amended with antibiotics, streptomycin 5% and rifampicin 0.5%. Plates were incubated for 48 h at 30˚C, and then CFU counts were performed. A second application of antagonists was performed in plants during the R3 (milk stage) phenological stage.

2.4. Disease Evaluation

Pustules of common rusts by *P. sorghi* and lesions of leaf blight by *E. turcicum* were evaluated during the phenological stages R2 (blister stage), R3 (milk stage) and R4 (dough stage) for the first application, and R4 for the second application of antagonists. The assessment was carried out from three leaves per plant (ear leaf, one below and one lower). To estimate the level of common rust, a rule of spaces was used twice per leaf [18], then the number of spaces measured by the rule were related to the level of disease (1 and 2: very low; 3 and 4: low, alarm threshold; 5 and 6: moderate, control threshold; 7 and 8: high; 9 and 10: very high). Leaf blight was determined by the percentage of leaf tissue infected using the scale developed by Bleicher [19]. In this scale four levels were measured (0: undeveloped, 1: early development with lesions smaller than 5 cm, 2: average development with lesions larger than 5 cm, 3: advanced development on the leaves).

2.5. Influence of Treatments on Grain Yield

Physiologically mature cobs were collected 150 days after sowing, when samples had reached the R6 phenological stage [17]. All cobs present in each individual plot were removed from the plants. After that, grains were separated from cobs with a static threshing machine (Forti MA, Buenos Aires, Argentina). After threshing, kernels were weighed to determine total yield ($kg \cdot ha^{-1}$). Moisture contents of grains were determined by using a hygrometer (Delver HD1000D). Total grain yields (Kg grain ha^{-1}) were calculated for each treatment according to current regulations for maize commercialization in Argentina [20], after adjusting humidity to 14.5%.

2.6. Statistical Analysis

The analysis of variance (ANOVA) with InfoStat 2012 was used to compare effects of both diseases and grain yields differences in different treatments. Means were compared according to DGC test with $p > 0.10$. PCA (principal components analysis) with treatments (classification variable) and severity of both diseases in three evaluations was performed [21].

3. Results

Table 1 shows the effect of different treatments on disease incidence in three phenological stages. In general, BCAs showed a significant reduction of common rust and northern leaf blight incidences in the first and second evaluation (R2 and R3). However, application of *Bacillus* spp in R2 did not reduce the incidence of common rust. The number of rust pustules showed a significant decrease in evaluations performed in R3. Plants treated with both biological control agents reported minor disease incidence than control treatment. In R4, the lowest incidence of pustules was obtained with treatment 3* (4.98). This treatment was the only significantly different to the control. On the other hand, T2 (*Pantoea* spp.) caused a significant increase of the disease (10.96) with a single application. With respect to northern leaf blight during phenological stages R2 and R3, a significant decrease of lesions on leaves treated with both biocontrol agents was observed. In the third evaluation (R4), the highest percentage of lesions was observed in plants with double application of *Pantoea* spp., with a percentage of lesions equivalent to medium development with lesions larger than 5 cm. A lower

Table 1. Effect of different treatments on diseases incidence analysed in three phenological stages.

Phenological stage	Treatment	Common rust	Northern leaf blight
R2	1	3.78 A	1.53 A
	2	2.86 B	0.73 B
	3	4.05 A	0.60 B
		p-value 0.0043	*p*-value 0.0001
R3	1	5.56 A	1.20 A
	2	3.92 B	0.60 B
	3	3.10 B	0.40 B
		p-value 0.0996	*p*-value 0.0745
R4	1	7.17 B	1.60 A
	2	10.96 A	1.40 A
	2*	6.98 B	1.60 A
	3	6.07 B	0.80 B
	3*	4.98 C	0.80 B
		p-value < 0.0001	*p*-value 0.0013

Treatment: 1 control; 2 *Pantoea* spp.; 2* two applications of *Pantoea* spp; 3 *Bacillus* spp.; 3* two applications of *Bacillus* spp. Rust pustules: mean lesions estimated with ruler of spaces (1 - 2: very low, 3 - 4: low, threshold alarm, 5 - 6: moderate, control threshold; 7 - 8: high; 9 - 10: very high). Northern leaf blight: percentage of lesions (Scale: 0 undeveloped; 1 incipient; 2 medium; 3 advanced). Values followed by different letters for a disease between treatments in each phenological stage, indicate significant differences according to DGC test ($p > 0.10$).

incidence of disease was observed in plants treated with one application of *Bacillus* spp. (T3), with a value of 0.8 interpreted as an intermediate between the absence of disease and an incipient development. Therefore, after 27 days post-application (R3), plants treated with *Bacillus* spp. showed disease reduction higher than 65% and a negative correlation. Treatment 2 showed a lower reduction effect (−50%). Populations of both BCAs on phyllosphere were maintained above 10^4 CFU ml^{-1}, after 20 days of application (data not shown).

The sum of two principal components (CP1 and CP2) explained 100% of total data variability (Figure 1). It was observed that treatment 3 caused the major effect in reducing incidence of both diseases showing a negative correlation (angles between vectors over 90˚) with respect to control treatment. Two applications of treatment 3 reduced both diseases significantly. Treatment 2 showed a lower reduction effect, while a double application of this treatment resulted in stimulation of disease incidence or without differences with respect to control. This was observed as a positive correlation.

Figure 2 shows that grain yield at harvest was significantly higher in treatments with both BCA (F: 35.7; *P*: 0.0001) according to DGC test. The control treatment showed an average yield of 8001 + 134 Kg ha^{-1}. Treatments 2 and 3 showed yields of 9155 + 104 Kg ha^{-1} and 9140 + 105 Kg ha^{-1}, respectively. No

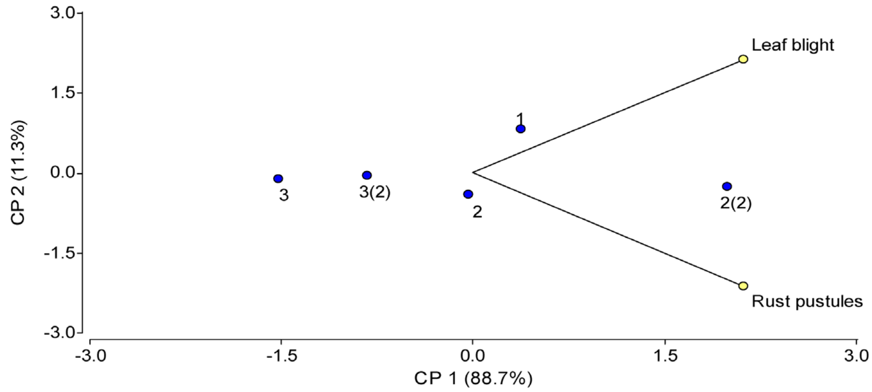

Figure 1. Principal component analysis of different treatments and effect on incidence of leaf blight and rust pustules. Variables analysed: treatments and incidence of both diseases in three evaluations from R2 to R4.

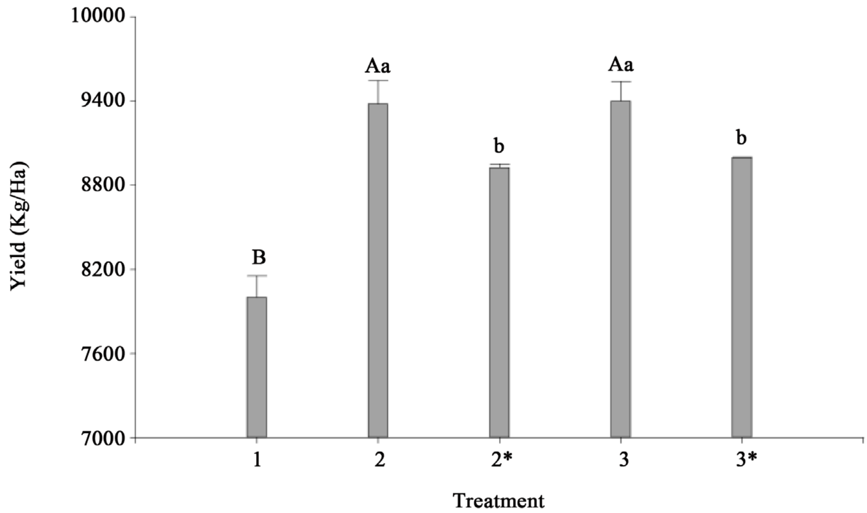

Figure 2. Effect of treatments against foliar diseases northern leaf blight and common rust on grain yield at harvest. Treatment: 1 control; 2 *Pantoea* spp.; 2*two applications of *Pantoea* spp; 3 *Bacillus* spp.; 3*two applications of *Bacillus* spp. Different capital letters indicate significant differences between treatments and different small letters indicate significant differences between the number of applications according to DGC test ($p > 0.05$).

significant differences (F: 9.18; *P*: 0.0143) were observed between treatments with BCAs. Yields were significantly higher in treatments of BCAs with a single application.

4. Discussion

The evaluation of incidence of both diseases was conducted during the critical period of the crop (R2 - R4). The control treatment showed an incidence of common rust corresponding to threshold alarm (3.78) in R2, while in R3 the incidence of the disease was higher (5.56) corresponding to threshold control [22]. When the leaf blight was evaluated in R2, a level between incipient and medium

(1.53) was determined. This value corresponds to the threshold control developed by experts to apply the chemical fungicides [9]. BCAs caused a significant reduction of common rust and northern leaf blight after 27 days post-application (R3). The most effective treatment was T3, which showed reductions of diseases higher than 65%. Treatment 2 showed a lower reduction effect. Also, a double application of this treatment resulted in a stimulation of disease incidence or in lack of differences with respect to control. A study conducted by Cary *et al.* [23] showed that, under some environmental conditions, one strain of *Bacillus* spp (JC12GB43) from potato phyllosphere with potential for biocontrol stimulated proliferation of fungal pathogens. These authors concurred with others that suggested that fungi can use metabolites derived from bacterial cells [24]. We suggest that stimulation mechanisms similar to those shown in other studies could be occurring in the interaction between *Pantoea* spp and the foliar pathogens *E. turcicum* and *P. sorghi*.

Campbell [25] reported on the role of the genus *Bacillus* as a source of antagonists for many plant pathogens. Species of *Bacillus* are endowed with added ecological advantage due to their endospores which are resistant to extreme environments. Harlapur *et al.* [26] showed that *B. subtilis* caused a growth reduction of *E. turcicum* of 49% *in vitro*. However, an antagonist is considered as an efficient BCA when it is able to replicate the promising results obtained in the laboratory by reducing the disease intensity under field conditions. In this study, reduction of northern leaf blight was higher than 50% during 40 days (from R2 to R4) in plants treated with *Bacillus* spp., under field conditions. These results agree with a previous screening *in vitro* where isolates belonging to *Bacillus* caused reductions in the growth rate of *E. turcicum* between of 84 to 98% [13]. Moreover, these antagonistic isolates were the most effective against *E. turcicum* under greenhouse conditions, since they efficiently controlled northern leaf blight [14]. On the other hand, *Pantoea* spp. was unable to control the symptoms of both diseases in R4. With one or two applications, similar or higher values of symptoms (stimulation) were observed, as compared to control treatment. Some *Pantoea* species have both antibacterial and antifungal activity *in vitro* and *in vivo*, thus protecting host plants against infection by pathogenic fungi and other bacteria [27]. Isolates of *P. ananatis* from buck weed seed have been shown to have strong antifungal activity against *Rhizopus* spp. *in vitro* [28]. However, *P. ananatis* is regarded as an emerging pathogen based on the increasing number of reports of diseases occurring in different parts of the world [29], such as Mexico [30], Brazil [31] and South Africa [32]. In Argentina, there was a first report of leaf spot disease of maize caused by *P. ananatis* [33]. In a previous greenhouse trial, we did not observe symptoms by *Pantoea* spp. in maize leaves [17]. For this reason, we continued the evaluation with this possible antagonist at the field level.

In this field assay, grain yield was significantly higher in plants treated with both BCAs (approximately 14%) at harvest. Our results are consistent with a study where maize treated with biocontrol agents such as *B. subtilis* showed an effective inoculum reduction of the pathogen *Fusarium* spp, as well as promising increases in vegetative biomass and reproductive yield of the maize plants [34] Moreover, grain yield was significantly higher when a single application of BCAs was carried out. This may be due to the fact that the second application of the biocontrol agents was performed in R3. In this phenological stage, the maximum rate of filling of the grain begins [35]. That is, the biocontrol agents did not have enough time to significantly influence the maximum rate of grain filling.

To summarize, these results encourage us to evaluate the antagonist efficacy of *Bacillus* spp. in different growing seasons, in several maize hybrids and in a number of agro-ecological zones.

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