

Studies on Possible Modes of Action and Tolerance to Environmental Stress Conditions of Different Biocontrol Agents of Foliar Diseases in Maize

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Abstract

The present study evaluates the possible modes of action of antagonistic bacteria and their tolerance to UV radiation, temperature and osmotic stress. The partial 16S-23S rRNA gene sequencing of eight antagonistic bacteria had a high match with three bacterial genera: *Curtobacterium*, *Pantoea* and *Bacillus*. In this study, the three *Bacillus* isolates showed the most relevant production of enzymes, volatile organic compounds and antibiosis against *Exserohilum turcicum*. Respect to UV radiation and temperature *Pantoea* and *Bacillus* isolates were more tolerant; whereas the eight isolates were tolerant to osmotic stress in varying degree. The three *Bacillus* isolates have the greatest potential as biocontrol agents for foliar diseases in maize. The antagonistic action could be explained through different modes of action such as enzymes, volatile organic compounds and/or direct antibiosis by other secondary metabolites. *Bacillus* isolates tolerance to environmental stresses including UV radiation, temperature and osmotic stress is relevant for survival and persistence on the leaf surface. This work provides new information about the mode of action of antagonistic bacteria with proven efficacy against maize leaf pathogens. In addition, it provides information about the tolerance of antagonistic bacteria against different stress conditions. The data of the present study could contribute to the development of a successful foliar biofungicide.

Keywords

Biocontrol, Maize, Antagonistic Bacteria, *Bacillus*, *Exserohilum turcicum*

1. Introduction

Maize (*Zea mays* L.) is one of the most important crops in Argentina. During the 2017/2018 growing season the sowing area was increased by 35% reaching 5.4 million hectares [1]. Among the factors that limit yield, two foliar diseases have become important in recent years. Common rust is an endemic disease in maize growing area caused by *Puccinia sorghi*, occurring all seasons with varying degrees of intensity according to hybrid susceptibility, pathogen biotypes and environmental conditions during the crop cycle [2]. On the other hand, Northern Corn Leaf Blight (NCLB) caused by *Exserohilum turcicum*, is a foliar disease in which the fungus destroys foliage causing a decrease in yield that varies from 28% to 91% [3].

Fungicides based on strobilurin + triazoles are the most effective method to control foliar diseases [4]. However, the dependence on chemical control has caused undesirable side effects such as food contamination, environmental dispersal and higher production costs [5]. Consequently, biological control is presented as a viable alternative to achieve the management of pests in various crops. Biological control is defined as an “environmentally-friendly” strategy using microorganisms or their derivatives to reduce a targeted pathogen. Furthermore, the narrow spectrum of biological control agents (BCAs) should provide an optimum efficacy without affecting non-targeted organisms [6]. The action of the microbial antagonists can result from direct (parasitism, antibiosis or competitions) or indirect ecological interactions (induction of resistance). The different mechanisms of action are probably never mutually exclusive. It is known that a successful BCA has several mechanisms of action capable of working synergistically [7].

The development of new BCAs is a stepwise approach which begins with the screening of a large number of potential antagonists. Reservoirs for such microorganisms are broad although the screening of antagonists directly isolated from the host plant and surrounding environment seems more appropriate [8]. Taking into account that both *E. turcicum* and *P. sorghi* are foliar pathogens, previous work in our laboratory was carried out to evaluate the epiphytic bacterial community that can share the niche with pathogens in similar stages. In the pre-selection, antagonistic interactions bacteria/pathogens were evaluated over competition for nutrients and reduction effect on growth parameters [9]. Later, the effectiveness of bacterial antagonists in reducing the disease severity was evaluated in greenhouse assays [10] and field trials [11].

The phylloplane is a source of bacterial antagonists much less used than the rhizosphere. In this environment, the bacteria population may be exposed to one or more stresses such as fluctuating temperature and UV radiation [12]. On the other side, changes in water potential may present the most difficult challenge to bacterial survival. Reductions in osmotic potential can eventually lead to bacterial desiccation [13]. Our working hypothesis is that a better knowledge of the

possible modes of action and the tolerance to unfavorable environmental conditions of the antagonists, could give us information about the behavior of BCAs in the maize phyllosphere during critical periods which should be competent against pathogens. Based on this hypothesis the aims of the present work were to: 1) begin to characterize possible modes of action of bacteria proposed as BCA; 2) determine the tolerance and sensitivity to UV radiation, temperature and osmotic stress of epiphytic bacteria native of maize with demonstrated antagonisms against foliar pathogen.

2. Materials and Methods

2.1. Biological Control Agents (BCAs)

Eight potential BCAs isolated from maize leaves from fields located in Córdoba province, Argentina, were selected based on antagonistic ability evaluated in a previous study [9]. The cultures are deposited in the culture collection of the Department of Microbiology and Immunology, Universidad Nacional de Río Cuarto, where the following experience was carried out throughout the year 2018.

2.2. BCA Identification

Bacterial DNA was obtained using the Cell and Tissue Extraction Kit GenomicPrep (Amersham, UK) according to the manufacturer's instructions. Then PCR amplification of 16S-23S sequences with the 785F (5'-GGATTAGATACCCTGGTA-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') primers was performed. PCR experiments were conducted with 10 - 25 ng of DNA in a total volume of 50 μ L of 1X reaction buffer containing 1.5 mM MgCl₂, 200 μ M of each dNTP, 0.6 μ M each of the primers and 1.25 U DNA Taq polymerase and water free of nucleases (Invitrogen, Buenos Aires, Argentina). A negative control, containing all reagents and primers but no DNA was included. PCR was conducted in TECHNE Model TC-512 Thermal Cycler (Bibby Scientific, Staffordshire, UK). The PCR conditions were: 95°C, 3 min then 30 cycles of 95°C, 30 s, 62°C, 30 s, 72°C, 30 s, followed by a final extension step of 10 min, 72°C. The amplified DNA fragments were separated by electrophoresis in 1.5% agarose gel containing 1 μ g·ml⁻¹ ethidium bromide in 1× TBE buffer, using a molecular weight marker (DNA ladder 100 bp, Invitrogen Buenos Aires, Argentina). Fragments were purified by filtration through DNA Wizard Clean-Up Kit (Promega, Madison, WI) and sent for sequencing by Macrogen Korea (Seoul, Rep. of Korea). The sequences were assembled and edited using the program Bioedit version 7.1.9 [14]. Taxonomic identification was performed by aligning with sequences present in GenBank using the Blastn algorithm [15] and deposited in GenBank, National Center for Biotechnology Information (NCBI) under the accession numbers listed in **Table 1**. On the other side, the three *Bacillus* isolates were further identified according to biochemical traits tested by API 50 CH (bioMérieux, Lyon, France).

Table 1. Molecular identification of BCA.

| Isolate | Description | Max score | Total score | Query cover | PMI* | Genbank accession number |
|---------|----------------------------|-----------|-------------|-------------|------|--------------------------|
| EM-A1 | <i>Curtobacterium</i> spp. | 2673 | 2673 | 99% | 100% | LC038166.1 |
| EM-A2 | <i>Curtobacterium</i> spp. | 2689 | 2689 | 99% | 99% | JN689336.1 |
| EM-A3 | <i>Curtobacterium</i> spp. | 1253 | 1253 | 100% | 99% | JX566549.1 |
| EM-A4 | <i>Pantoea</i> spp. | 2669 | 2669 | 100% | 99% | KX500237.1 |
| EM-A5 | <i>Pantoea</i> spp. | 2571 | 2571 | 100% | 99% | KX500238.1 |
| EM-A6 | <i>Bacillus</i> spp. | 1236 | 1236 | 100% | 99% | KX500239.1 |
| EM-A7 | <i>Bacillus</i> spp. | 2723 | 2723 | 99% | 99% | KX500240.1 |
| EM-A8 | <i>Bacillus</i> spp. | 2483 | 2483 | 99% | 100% | KX500241.1 |

*Percentage of maximum identity of partial 16S rRNA sequence according to Blast database.

2.3. Detection of Hydrolytic Enzymes *in Vitro*

2.3.1. Detection of Chitinase

Chitinolytic activity was determined on chitin-based agar (4 g colloidal chitin; 0.7 g K₂HPO₄; 0.5 g MgSO₄·7H₂O; 0.3 g KH₂PO₄; 0.01 g FeSO₄·7H₂O; 1.26 g MnSO₄; 15 g agar; 1000 ml of distilled water). A 40 µl suspension of antagonist bacteria in phosphate buffered saline was added to the surface of chitin-based agar plates and incubated at 30°C for 3 to 5 days. Clear zone in the culture medium around the bacterial colony was indicative of hydrolysis of chitin [16].

2.3.2. Detection of Protease

To determine the protease production, 100 µl of suspensions of the antagonist bacteria were plated on LB agar (10 g tryptone, 5 g yeast extract, 10 g NaCl, 12 g agar and 950 ml distilled water) containing 3% skim milk. Plates were incubated at 25°C for 4 days. The formation of a transparent halo around the bacterial colonies was classified as positive [17].

2.3.3. Detection of β-1,3-Glucanase

The quantification of enzymatic activity was evaluated following the microplate method described by Zheng and Wozniak [18] with some modification. Starting from supernatant of an overnight culture from each bacterial isolate, 10 µl was placed on Nunc 96 autoclavable polypropylene plates (PGC Scientific, Gaithersburg, MD, USA) by quadruplicate. Two samples were used for the enzymatic test and two samples as control. Then, 20 µl of the substrate (laminarin 0.4% w·v⁻¹) was added to test wells; the microplates were covered with aluminium foil and shaken lightly. The microplates were incubated at 37°C for 20 min and then the reaction stopped by placing the plates on ice for 20 min. For the development, copper reagent (solution A) and neocuproin (solution B) were used. In each well, 100 µl of each solution was added. In those containing the controls of the samples were added, in addition, the 20 µl of the substrate. The plates wrapped in aluminum foil were placed in a sealed “Ziploc Brand” bag and boiled in a wa-

ter bath, preheated to approximately 99°C for 10 min. Finally, it was cooled on ice and the absorbance at 450 nm was read. The standard glucose curve was prepared using concentrations between 2 - 800 µM in duplicate, placing 30 µl in each well, following the same procedure described above.

From this standard curve, the net OD_{450nm} of an assay sample was converted to the amount of reducing sugar released by β-1,3-glucanase from the substrate laminarin. The net OD_{450nm} was calculated by subtracting the average OD_{450nm} of the duplicate substrate-control wells.

2.4. Determination of Antibiosis

2.4.1. Production of Volatile Compounds

The antagonistic bacterial strains were streaked on trypticase soy agar plates (TSA) (Britania, Argentina) and pathogen mycelial plugs (8 mm diameter) obtained from 10 day-old cultures of *E. turcicum* (ET-RC 5) were placed at the centre of potatoes dextrose agar plates (PDA). The PDA plates with pathogen plugs were inverted over the TSA plates with the bacterial antagonists facing each other but not touching agar surfaces, sealed together using parafilm and incubated at 25°C for 5 days. Four replicates were made for each antagonist. The PDA plates with the pathogen inverted over the TSA plates without bacterial antagonist served as control. The inhibitory effect of volatiles was graded based on the inhibition of pathogen growth compared to control [17].

2.4.2. Characterization of Bacterial Volatile Compounds by Gas Chromatography-Mass Spectrometry

Solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS) were performed using the methodology developed by Khabbaz *et al.* [17]. The fibre Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) was used from Supelco Analytical (Bellefonte, PA, USA). The cultures of the antagonist bacteria were made in flasks containing 50 ml and incubated for 48 h at 28°C. The metabolites were thermally desorbed in the pulsed at 250°C for 15 min and transferred directly to the analytical capillary column. After each extraction and desorption process, the fibre was reconditioned for 15 min at 250°C, according to the manufacturer's recommendations to guarantee the quality of the SPME extractions. The samples were analysed by duplicate, the control was a sample of nutritious broth. The separation and identification of the volatile components were performed using the VARIAN Saturn 2200 GC-MS equipment (SpectraLab Scientific Inc, Markham, Ontario, Canada). The compounds were identified by performing an AMDIS GC-MS Version 2.71 analysis, with the NIST 05 Mass Spectral Search Program (National Institute of Standards and Technology).

2.4.3. Detection of Antibiotic Compounds Production

The technique developed by Bautista-Rosales *et al.* [19] for yeasts was adapted to evaluate mechanisms of antibiosis in the bacterial strains. The selected antago-

nist bacteria were cultured in tripten broth soy (TSB) at 25°C for 48 h, up to a concentration of 1×10^8 cfu·ml⁻¹. Then, the cultures were autoclaved at 121°C for 15 minutes, centrifuged at 10.000 g for 10 min and filtered through a 0.2 µM nitrocellulose membrane to determine the presence of non-peptide antibiotics. An additional treatment was carried out with the cells of antagonist bacteria in order to determine if their presence is necessary for the biological control capacity. Twenty five microliters of filtrates or suspension of bacterial cells were placed on a disc of filter paper (Whatman N°1) deposited on a PDA plate, which was previously inoculated with 500 µl of a suspension of *E. turcicum* ET-RC5 (10^7 conidia·ml⁻¹). The plate was incubated at 25°C for 72 h and then the inhibition halo around the disc was measured. The control was carried out by inoculating a sterile phosphate solution onto the filter paper disk. The treatments were performed by triplicate.

2.5. Determination of Tolerance to UV Radiation, Temperature and Osmotic Stress

2.5.1. Sensitivity to UV Radiation

The microbicide action depends on radiation intensity and the dose applied. The intensity or UV irradiance is the energy per unit area measured in mW/cm². The UV dose corresponds to the product of the intensity by the time applied expressed in mW·seg/cm². The sensitivity to UV of each BCA was assayed determining the dose of radiation which inactivates 90% of the bacterial population (D_{10} value) [20] and the minimal inhibitory dose (MID) of radiation necessary to inhibit the growth of cells spread onto TSB agar plates. Cultures of different antagonists were diluted in TSB to obtain 10^4 cfu·ml⁻¹. One hundred microliter of each cell cultures were spread onto the surface of TSB plates and irradiated to 35 cm with a UV lamp of 15 watt low-pressure (OSRAM HMS germicidal lamp), with a peak of maximum emission at 254 nm (UV-C range) and an intensity of 170 mW·seg/cm². The time of exposure was 5, 15, 40, 80, 160 and 240 s. Unexposed samples (zero seconds) were used for control plates.

2.5.2. Heat Shock Survival of Bacterial Cells

The bacterial cells were obtained from 150 ml of TSB medium inoculated and incubated on a rotary shaker (140 rpm) at 25°C. In order to choose a suitable temperature treatment for heat shock induction of tolerance, when cultures reached the stationary phase of growth, the cells were harvested by centrifugation at 7.000 g for 10 min at 10°C. Then the cells were re-suspended in 2 mL of phosphate-buffered solution (PBS), and cell suspensions incubated in a water bath at 45°C for 30 min. To determine the survival rates after heat shock, the cell paste was serially diluted in PBS. An inoculum of 100 µl of each bacterial suspension was spread onto TSA medium. A new count of viable cells was done after 24 h of incubation at 25°C. Three replicates per treatment were used and repeated twice [21]. Survival at 45°C was expressed as logarithmic values of N and N_0 , where N refers to the bacteria count following exposure to heat shock, and

N_0 represents the initial count prior to heat exposure.

2.5.3. Sensitivity to Osmotic Stress

The bacterial cells were grown on TSA medium osmotically modified by adding different amount of ionic solute (NaCl) and non-ionic solutes (glycerol and glucose) [22]. The water potential (Ψ) of the unmodified medium was -1.38 MPa, and it was selected as control treatment. The Ψ of TSA medium was adjusted to -2.78 , -4.19 and -5.62 MPa [$=0.98$, 0.97 and 0.96 water activity (a_w), respectively]. The a_w of the media was determined using the equipment AquaLab (Series 4, TE, USA). The media were autoclaved at 121°C for 15 min. Petri plates containing the different modified media were inoculated with $100\ \mu\text{l}$ of each bacterial strain (1×10^4 cfu·ml $^{-1}$). Plates of the same Ψ values were sealed in polyethylene bags and incubated at 25°C for 24 h. Three replicates per treatment were used and repeated twice. Bacterial count was expressed as log cfu·ml $^{-1}$.

2.6. Statistical Analysis

Data analyses were performed by analysis of variance (ANOVA) using InfoStat version 2013 [23]. Mean separation and comparisons for survival to UV and sensitivity to osmotic stress were made by Duncan test at a probability level of $p < 0.05$ and $p < 0.01$, respectively. Mean separation and comparison for heat shock survival were made using a DGC Test ($p < 0.01$).

3. Results

3.1. Identification of BCAs

Analysis of 16S-23S rRNA sequences of antagonistic bacteria had a high match with three bacterial genera: *Curtobacterium*, *Pantoea* and *Bacillus* (Table 1). In the isolates identified molecularly as belonging to the genus *Bacillus*, the identification was complemented with a biochemical characterization. The API system showed that two *Bacillus* isolates EM-A7 and EM-A8 have a high similarity index with *B. subtilis* while the *Bacillus* EM-A6 showed a test with weak discrimination among *Bacillus* species. Given the complexity in the taxonomy of this genus, it is necessary in the future to carry out a phylogenomic analysis that allows us to confirm the identification of these BCAs.

3.2. Detection of Hydrolytic Enzymes

All antagonist bacteria showed no chitinolytic capacity in the chitin-based medium. When determining the proteolytic enzymes, the three *Bacillus* isolates demonstrated a high protease activity on LB agar supplemented with 3% skim milk. *Curtobacterium* isolates showed small haloes around bacterial colonies indicating a slight capacity protease, while the *Pantoea* isolates did not show proteolytic activity (Table 2). On the other side, all bacteria showed ability to digest glucan. Table 2 shows the glucose released using laminarin as a substrate. Among the eight strains evaluated, *Curtobacterium* EM-A2 showed the highest

Table 2. Detection of hydrolytic enzymes and antibiosis by antagonistic bacteria.

| Metabolites/ Enzymes | EM-A1 | EM-A2 | EM-A3 | EM-A4 | EM-A5 | EM-A6 | EM-A7 | EM-A8 |
|-------------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Chitinase | - | - | - | - | - | - | - | - |
| Protease | - | + | + | - | - | +++ | ++ | +++ |
| β -1,3-glucanase* | 115 | 494 | 227 | 156 | 229 | 230 | 300 | 341 |
| Volatile compounds | + | + | + | ++++ | +++ | +++ | ++ | +++ |
| Antibiosis | - | - | - | + | + | +++ | +++ | +++ |

++++ = very strong, +++ = strong, ++ = moderate, + = low, - = no production. *Glucose released (μ M) by β -1,3-glucanase from substrate laminarin.

β -1,3-glucanase activity followed by *Bacillus* EM-A8.

3.3. Determination of Antibiosis

The production of volatile compounds with antibiosis capacity on *E. turcicum* growth in sealed double plate assays determined that all isolates were producers of volatile compounds. *Pantoea* EM-A4 showed the highest production of volatile compounds, followed by *Pantoea* EM-A5 and *Bacillus* EM-A6 and EM-A8 (Table 2). Gas chromatography-mass spectrometry analysis of extracted volatile compounds identified 16 volatile organic compounds. As shown in Table 3, *Pantoea* EM-A4 produced the highest amount of volatile compounds (8 compounds), including the production of indole and tetrahydrofuran-2-propyl. The *Bacillus* EM-A7 and EM-A8 release high levels of silanediol, dimethyl, and cyclopentasiloxane, decamethyl, respectively. On the other hand, during evaluating the production of antibiotic compounds, it was determined that all antagonists were not producers of non-peptidic antibiotics (filtrated and sterilized extracts). However, when the inoculation was performed using viable cells, the three *Bacillus* isolates showed a total inhibition of *E. turcicum* growth. The two *Pantoea* isolates allowed a slight growth of the pathogen and the three *Curtobacterium* isolates did not produce antibiotic compounds (Table 2).

3.4. Tolerance to UV Radiation, Temperature and Osmotic Stress in the BCAs

Table 4 shows D-values to UV radiation of the eight BCAs. The values showed that *Pantoea* and *Bacillus* isolates were the most resistant bacteria examined. Isolates EM-A4, EM-A6 and EM-A7 showed a MIDc higher than 500 J·m⁻² and isolates EM-A5 and EM-A8 showed a MIDc > 400 J·m⁻². *Curtobacterium* isolates were the most sensitive to UV radiation and a dose 26 J·m⁻² was sufficient to inactivate 90% of the bacterial population.

Bacterial growth of BCAs before and after heat shock treatment is shown in Table 5. *Pantoea* and *Bacillus* isolates showed better tolerance and survival after heat shock. All *Curtobacterium* isolates showed significant reductions in the

cells viability after the heat shock treatment. For three *Bacillus* isolates, growth occurred over an osmotic potential range -1.38 to -5.62 MPa in ionic and non-ionic modified media. The *Curtobacterium* and *Pantoea* isolates showed no

Table 3. GC-MS characterization of organic volatile compounds produced by antagonistic bacteria on nutrient broth.

| RT (min)* | Library Search Component | Peak Area (%)** | | | | | | | |
|-----------|---|-----------------|-------------|-------------|--------------|--------------|-------------|-------------|-------------|
| | | EM-A1 | EM-A2 | EM-A3 | EM-A4 | EM-A5 | EM-A6 | EM-A7 | EM-A8 |
| 3612 | 2(3H)-Furanone, dihydro-3,5-dimethyl | - | 0.46 ± 0.17 | - | - | 7.24 ± 2.53 | - | 0.15 ± 0.09 | 0.38 ± 0.02 |
| 3787 | Tetrahydrofuran,2-propyl | - | - | - | 9.10 ± 4.10 | - | - | - | - |
| 4254 | Silanediol, dimethyl | 1.33 ± 1.10 | - | - | - | - | - | 3.77 ± 2.43 | 1.31 ± 0.16 |
| 9652 | Dimethyl trisulfide | - | 0.62 ± 0.43 | - | 0.53 ± 0.24 | - | - | 0.82 ± 0.05 | - |
| 10,496 | Heptene,2,2,6,6-tetramethyl-4-methylene | 0.36 ± 0.08 | - | 0.39 ± 0.22 | 0.21 ± 0.08 | - | - | 0.30 ± 0.01 | 1.47 ± 0.26 |
| 11,467 | D-Limonene | - | - | 0.20 ± 0.91 | 0.11 ± 0.01 | - | 0.44 ± 0.32 | 0.85 ± 0.04 | - |
| 11,954 | 2-Pentene, 2,4,4-trimethyl | 0.27 ± 0.09 | - | - | - | - | - | - | - |
| 12,302 | 1-Pentene,2,4,4-trimethyl | 0.46 ± 0.22 | - | 0.44 ± 0.09 | 0.32 ± 0.03 | 0.44 ± 0.27 | - | 0.26 ± 0.04 | 1.11 ± 0.42 |
| 15,331 | Cyclopentasiloxane, decamethyl | - | - | - | - | - | - | - | 3.85 ± 1.98 |
| 19,232 | Indole | - | - | - | 16.67 ± 4.53 | 16.55 ± 7.56 | - | - | - |
| 23,847 | 2-Undecanethiol, 2-methyl | - | - | - | - | - | 0.28 ± 0.07 | - | - |
| 24,364 | 2-Tridecanone | - | - | - | 1.68 ± 0.44 | - | - | - | - |
| 27,072 | Cyclododecanol | - | - | - | - | 0.380 ± .11 | 0.54 ± 0.11 | - | - |
| 28,507 | Pyrrolidine-2,5-dione, 3-(2-morpholin-4-ylethylamino)-1-4 | - | - | - | - | - | 1.58 ± 0.13 | - | - |
| 28,995 | 2-Nonadecanone | 0.13 ± 0.06 | - | - | 1.15 ± 0.42 | 0.45 ± 0.12 | 0.22 ± 0.02 | - | - |
| 30,549 | 2,15-Hexadecanedione | - | - | - | - | - | - | 0.35 ± 0.21 | - |

*RT: Retention time, **Values ± standard errors are the mean percentage peak area from three replicates

Table 4. D-values ($J \cdot m^{-2}$) of bacterial isolates to UV-C (254 nm) radiation treatments.

| Isolates | MIDc ($J \cdot m^{-2}$)* |
|----------|----------------------------|
| EM-A1 | 272 |
| EM-A2 | 68 |
| EM-A3 | 68 |
| EM-A4 | 544 |
| EM-A5 | 408 |
| EM-A6 | 544 |
| EM-A7 | 544 |
| EM-A8 | 408 |

*MIDc (minimal inhibitory dose): Radiation necessary to inhibit the cell growth in Petri plate.

Table 5. Survival of antagonistic bacteria exposed to heat shock.

| Isolate | N ₀ * | N** | p-value*** |
|---------|------------------|-------|------------|
| EM-A1 | 12.4a | 9.4b | <0.0001 |
| EM-A2 | 11.7a | 9.9b | <0.0001 |
| EM-A3 | 11.4a | 10.3b | <0.0001 |
| EM-A4 | 10.6b | 12.5a | <0.0001 |
| EM-A5 | 11.5b | 12.4a | <0.0001 |
| EM-A6 | 8.1b | 9.1a | 0.0004 |
| EM-A7 | 7.9b | 9.9a | <0.0001 |
| EM-A8 | 8.3a | 8.6a | 0.0348 |

*N₀ initial count prior to heat shock exposure, **N bacterial count following heat shock exposure. ***Data with the same letter for N₀ and N are not significantly different according to DGC test (p < 0.01).

differences in growth in water potential modified media with non-ionic solutes, but they did not grow in media with NaCl to -5.62 MPa. The *Curtobacterium* isolate EM-A2 was the most sensitive to osmotic stress. For all BCAs, NaCl treatment (ionic osmotic stress) was more stressful in all osmotic potential, while glycerol treatments (non-ionic osmotic stress) favoured growth at all water potentials, even at -5.62 MPa not showed statistical differences with -1.38 MPa.

4. Discussion

Phyllosphere microbioma could contribute to the health of plant species through surface protection against pathogens [12]. In the present study, eight epiphytic bacteria previously evaluated in greenhouse and field trials experiments against foliar fungal pathogens [10] [11], were characterized for hydrolytic enzymes production, volatile compounds and antibiosis and tolerance to UV radiation, temperature and osmotic stress.

The three *Curtobacterium* isolates showed the lowest production of metabolites and enzymes, with a slight production of volatile compounds with antibiosis action. These isolates were selected in previous studies because they showed to decrease the growth rate of *E. turcicum* and one of them had shown inhibition at distance in dominance tests [9]. Regarding *Pantoea* EM-A4 and EM-A5, both isolates showed significant inhibition of *E. turcicum* in sealed double plate assays suggesting the action of volatile compounds that could play a vital role in pathogen inhibition [24]. The GC-MS profile indicated that *Pantoea* EM-A4 produced eight compounds while *Pantoea* spp. EM-A5 produced five detectable compounds, being in both isolates the indole the most relevant compound. Indole controls diverse aspects of bacterial physiology, such as spore formation, plasmid stability, drug resistance, biofilm formation and virulence in indole-producing bacteria [25] [26]. By the other hand, *Pantoea* isolates did not produce proteolytic and chitinolytic enzymes, and a low production of β -1,3-glucanase was detected. Also antibiotics production was slight. These results suggest that the possible mode of

action of *Pantoea* isolates against *E. turcicum* is due to volatile compounds. Several studies have shown that this contact-independent antagonism can be used to inhibit fungal mycelia growth, conidia viability, pigment production and virulence [27] [28] [29].

Bacillus isolates showed a relevant production of enzymes, volatile organic compounds and antibiosis. The three *Bacillus* isolates showed a relevant protease production, standing out *Bacillus* EM-A6 and EM-A8 and β -1,3-glucanase activity, one of the enzymes responsible for lysis and degradation of cell wall and sclerotium wall in fungi [30]. Production of extracellular lytic enzymes such proteases and β -1,3-glucanase by bacteria might contribute to their antagonistic mechanism, which in many situations may partially explain the biological control of plant disease [31]. Also a significant production of volatile compounds such as silanediol, dimethyl cyclopentasiloxane and decamethyl was determined in *Bacillus* isolates. Diverse authors have also reported the volatiles compound production by different *Bacillus* species and their effect on mycelial growth, spore germination and tube elongation in different fungal pathogens [28] [32]. In addition the three *Bacillus* isolates also showed *in vitro* antibiosis against *E. turcicum*, generating in many cases a total growth inhibition. Members of this genus produce several biologically active molecules with antimicrobial activity such as polyketides and cyclic lipopeptides [33] [34]. Further studies are necessary to analyse the gene clusters encoding for the biosynthesis of secondary metabolites with antifungal activity in these BCAs. This is relevant in the *Bacillus* EM-A8 that caused the greatest effect on reducing the severity of northern leaf blight in previous field studies [10] [11].

The leaf surface is a continuously fluctuating physical environment and their resident microbes are subjected to changes in the microclimate. Adaptations conferring tolerance to stress may be critical to survival of epiphytic bacteria used as BCA on phyllosphere conditions. A tolerance strategy requires the ability to tolerate direct exposure to environmental stresses on leaf surfaces, including UV radiation and low water availability [13]. In our study, *Curtobacterium* isolates with a strong pigmentation were found to be the most sensitive to UV radiation. This is in contrast with previous studies which showed that organisms resistant to radiation are usually intensively pigmented; containing UV-absorbing compounds [20]. By the other side, *Pantoea* and *Bacillus* isolates showed higher resistance to UV radiation than *Curtobacterium* isolates in spite of differences in pigmentation. In the present study epiphytic bacteria were exposed to heat shock under temperature condition that can occur on phyllosphere in summer, during the period that maize is susceptible to be infected by the pathogen. Once again *Pantoea* and *Bacillus* isolates were more tolerant to heat shock than *Curtobacterium* isolates. In a previous study we demonstrated that osmotic tolerance of *B. amyloliquefaciens* helps to maintain survival population after heat shock [21]. The reduction in free water on leaf surface may cause increases in the concentration of solutes, resulting in osmolarities that may be sufficient high to damage bacte-

ria and modify the concentration of plant-derived antimicrobial compounds [13]. Our results indicate that epiphytic bacteria evaluated have shown a significant tolerance to osmotic stress. Most of isolates, especially *Bacillus*, modified their growth in media with different solutes. The media modified with glycerol generated abundant and gelatinous colonies of BCAs, whereas media modified with glucose and NaCl generated flat and small colonies. In this sense, the acclimatization of BCAs using glycerol could be help to improve the protection on leaf surface because it stimulates the production of extracellular polysaccharides (EPS).

5. Conclusion

The present work complements previous studies performed in the greenhouse and field trials and shows that the three *Bacillus* isolates have the greatest potential as BCAs for foliar diseases in maize. *Bacillus* isolates showed a good enzymatic production, synthesis of volatile organic compounds and direct antibiosis towards *E. turcicum*. Further studies are necessary to analyse the gene clusters encoding for the biosynthesis of secondary metabolites associated with the antifungal activity. In addition, these isolates showed tolerance to environmental stresses including UV radiation, temperature and osmotic stress which are important factors for survival on the leaf surface and their persistence in future foliar applications. Furthermore, the information obtained about tolerance to different stress conditions will be useful to select the type and formulation process in order to obtain an effective product for biocontrol of foliar disease.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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