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# Osmotic stress adaptation, compatible solutes accumulation and biocontrol efficacy of two potential biocontrol agents on *Fusarium* head blight in wheat

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ABSTRACT

*Fusarium* head blight (FHB) caused by *Gibberella zeae* (anamorph = *Fusarium graminearum*) is a devastating disease that causes extensive yield and quality losses to wheat in humid and semi-humid regions of the world. Biological control has been demonstrated to be effective under laboratory conditions but a few biocontrol products have been effective under field conditions. The improvement in the physiological quality of biocontrol agents may improve survival under field conditions, and therefore, enhance biocontrol activity. *Bacillus subtilis* RC 218 and *Brevibacillus* sp. RC 263 were isolated from wheat anthers and showed significant effect on control of FHB under greenhouse assays. This study showed the effect of water availability measured as water activity ($a_W$) using a growth medium modified with NaCl, glycerol and glucose on: (i) osmotic stress tolerance, (ii) viability in modified liquid medium, (iii) quantitative intracellular accumulation of betaine and ectoine and (iv) the biocontrol efficacy of the physiologically improved agents. Viability of *B. subtilis* RC 218 in NaCl modified media was similar to the control. *Brevibacillus* sp. RC 263 showed a limited adaptation to growth in osmotic stress. Betaine was detected in high levels in modified cells but ectoine accumulation was similar to the control cells. Biocontrol activity was studied in greenhouse assays on wheat inoculated at anthesis period with *F. graminearum* RC 276. Treatments with modified bacteria reduced disease severity from 60% for the control to below 20%. The physiological improvement of biocontrol agents could be an effective strategy to enhance stress tolerance and biocontrol activity under fluctuating environmental conditions.



## 1. Introduction

*Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein) Petch) is the most common causal agent of *Fusarium* head blight (FHB) in humid and semi-humid regions of the world. This devastating disease causes extensive yield and quality losses in wheat grains (Bai and Shaner, 1994; McMullen et al., 1997; Champeil et al., 2004). Besides reducing wheat grain quality through FHB, *F. graminearum* can also produce potent toxins such as the trichotecene deoxynivalenol (DON). During the last 50 years, several epidemics of FHB of varying degrees of severity have occurred in Argentina and *F. graminearum* was isolated as the main pathogen associated with FHB (Lori et al., 1992; Dalcero et al., 1997; Galich, 1997; Ramirez et al., 2007).

Different strategies have been used to reduce the impact of FHB including crop rotation, tillage practices, fungicide application and the planting of less susceptible cultivars. None of these strategies by themselves are able to substantially reduce the impact of the disease (Koehler et al., 1924; Snyder and Nash, 1968; Milus and Parson, 1994; Dill-Macky and Jones, 2000; Hollins et al., 2003). Biological control offers an additional strategy and can be used as part of an integrated management of FHB. *In vitro* assays and trials in greenhouses and under field conditions showed that some bacteria within the genera *Bacillus* and *Pseudomonas* were able to reduce *F. graminearum* growth. Also, yeasts belonging to the genera *Rhodotorula, Sporobolomyces and Cryptococcus* were effective in controlling FHB (da Luz, 2000; Schisler et al., 2000; Wang et al., 2007).

Several bacteria and fungi have been isolated and some are being evaluated for commercial development as biopesticides (da Luz, 2000; Khan et al., 2004). However, one of the main limitations in the use of biopesticides is the limited tolerance to fluctuating environmental conditions and the difficulties in developing a stable formulated product. The level of water stress encountered by microorganisms in natural environments is an important physical parameter that influences their ability to grow and successfully compete for a determined specific habitat. This water stress is directly related with the available water in the environment, one of the best ways to evaluate this water availability for microbial activities is to measure water activity ($a_W$) as was described by

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Scott (1957). Water activity is defined as the ratio between the vapor pressure of water in a substrate (P) and the vapor pressure of pure water (Po) at the same temperature and pressure; thus $a_W$ = P/Po. The $a_W$ of pure water is 1. For example, in solid substrates such as agricultural commodities, water content consists of bound water (water of constitution), which is held in chemical union with the absorbing substrate by very strong forces, and free water, which is weakly bound. Free water is more readily available for microbial growth and metabolism than bound water. Both water activity and relative humidity are fractions of the vapor pressure of pure water. Methods for their measurements are the same. Similar to water activity, water potential ($\Psi$) is a term used in plant, soil and crop science and is measured in pascals (Pa). This is the sum of the osmotic, matric and turgor potentials and is related directly to $a_W$ by the following formula: water potential ($\Psi$) = $RT/V \log_n a_W$ (+$P$), where $R$ is the ideal gas constant, $T$ the absolute temperature, $P$ the atmospheric pressure and $V$ is the volume of 1 mol of water.

Preharvest application of the biocontrol agent to wheat heads may reduce agent survival due to unfavorable conditions in the field and may render biocontrol ineffective. There are few reports on physiological stress responses among biological control agents, most of them describing filamentous fungi or yeasts (Hallsworth and Magan, 1995, 1996; Frey and Magan, 1998; Teixidó et al., 1998; Pascual et al., 2000; Dunlap et al., 2007) and relatively little data related to bacteria (Bochow et al., 2001; Teixidó et al., 2005; Cañamas et al., 2007).

Regarding bacteria, the osmotic response to environmental conditions is of current and increasing interest due to their potential applications, as, for example, biopesticides. Most of the studies on prokaryotes have been done on genetic regulation and physiological aspects of their adaptation to salt stress (Csonka and Hanson, 1991). Organic osmolytes enable organisms to adapt to environmental conditions by protecting cells or molecules against, e.g., freezing, high temperatures, osmotic or salt stresses. These organic osmolytes are generally "compatible" with the metabolism of the cell without adversely affecting macromolecules or physiological processes and are termed compatible solutes (Wood et al., 2001). Among them, are polyols, heterosides, amino acids (proline, alanine, glutamine and derivatives), *N*-acetylated diamino acids, ectoines, betaines and thetines (dimethylsulfonium compounds). Among compatible solutes, betaine, generally referred as glycine–betaine, is a commonly assayed osmoprotectant, and is produced in large quantities by phototrophic bacteria in hypersaline environments. Ectoine, discovered in the halophilic phototrophic sulfur bacterium *Ectothiorhodospira halochloris* (Galinski et al., 1985), is also the most abundant solute of aerobic heterotrophic eubacteria. *Bacillus* species respond to elevated-ionic-strength media by synthesizing or accumulating a range of osmolytes, including amino acids, various ectoines and glycine–betaine (Boch et al., 1994; Bremer, 2002; Kuhlman and Bremer, 2002). Betaine appears to be the most effective osmolyte to be accumulated in *B. subtilis* cells growing under osmotic stress conditions when its precursor, choline, is present. The high level of betaine allows *B. subtilis* cells to grow over a wide range of salinities (Whatmore et al., 1990; Holtman and Bremer, 2004).

At present, there is limited information on biological control of FHB in Argentina. In a previous study, we have shown that *B. subtilis* RC 218 and *Brevibacillus* sp. RC 263 were effective biocontrol agents for reducing FHB severity and DON accumulation both *in vitro* and in greenhouse assays (Palazzini et al., 2007).

The aims of this study were to determine the effect of $a_W$ of the culture media modified with three types of solutes (NaCl, glycerol and glucose) for *B. subtilis* RC 218 and *Brevibacillus* sp. RC 263 on: (i) osmotic stress tolerance, (ii) viability in modified liquid medium, (iii) intracellular accumulation of betaine and ectoine and (iv) to evaluate the biocontrol efficacy of the physiologically improved agents to control FHB under greenhouse conditions.

## 2. Materials and methods

### 2.1. Bacterial strains and culture media

*Bacillus subtilis* RC 218 and *Brevibacillus* sp. RC 263 were isolated from wheat anthers during the 2004 harvest season. These strains were selected due to their ability to control FHB in greenhouse bioassays. The growth medium (basal medium) consisted of sucrose (10 g $l^{-1}$) and yeast extract (5 g $l^{-1}$) with a pH of 6.7 and $a_W$ of 0.995 (Costa et al., 2001). Bacterial strains were stored at −80 °C in 10% glycerol, thawed at room temperature, streaked for purity in triptic soy agar and incubated at 28 °C for 24 h. From these cultures, single colonies were obtained.

### 2.2. Osmotic stress tolerance of bacterial strains

A single colony of each bacterial strain was used to inoculate 100 ml of the basal medium in 250 ml Erlenmeyer flasks and incubated for 12 h (overnight culture) at 28 ± 0.5 °C in a rotatory shaker (150 rpm) in order to obtain mid-log phase cells (approximately $10^6$ cells $ml^{-1}$). Cells counting was done in a haemocytometer chamber and adjusted to $1 \times 10^3$ cells $ml^{-1}$. An aliquot of 0.1 ml of each bacterial strain was inoculated in Petri dishes using the spread plating technique containing the solid basal medium (agar 2%) modified with the ionic solute NaCl and the non-ionic solutes glycerol or glucose to obtain treatment $a_W$ values of 0.98, 0.97 and 0.96 (Table 1). The $a_W$ of the media was determined using an Aqualab Series 3 (Decagon Devices Inc., Pullman, USA). The inoculated plates of the same $a_W$ were sealed in polyethylene bags in order to maintain the equilibrium relative humidity conditions and were incubated at 28 ± 0.5 °C and then visually examined every 24 h to determine colony growth during 15 days. Colony counting was expressed as percentage of culturability and was calculated comparing the colony forming units (cfu) of different treatments with those of the control (unmodified solid basal medium). The experiments were carried out with three replicates per treatment and repeated once. Data, expressed as percentage (%), were normalized using the arcsin square root transformation before analysis of variance (ANOVA). Means were separated with Holm–Sidak method ($P < 0.001$) using SigmaStat for Windows Version 3.5 (SPSS Inc.).

### 2.3. Viability of bacterial strains under water activity stressed and unstressed conditions

The liquid basal media were modified with NaCl, glycerol and glucose to $a_W$ levels of 0.98, 0.97 and 0.96. For each treatment, an aliquot of 1 ml of the overnight culture previously adjusted to $1 \times 10^6$ cells $ml^{-1}$ was inoculated. These flasks containing 100 ml of modified and unmodified media and an initial concentration of

**Table 1**
Solute concentration of osmotic modified media to achieve the final water activity.

| Solute* | Water activity | | |
|---|---|---|---|
| | 0.98 | 0.97 | 0.96 |
| NaCl | 22.0 | 35.5 | 53.0 |
| Glycerol | 77.0 | 92.0 | 147.2 |
| Glucose | 160.0 | 190.0 | 310.0 |

* Grams of solute added to 1000 ml of water. Final volume was measured and then corresponding sucrose and yeast extract were added to obtain the final concentration of the media.

$1 \times 10^4$ cells ml$^{-1}$ were incubated for 48 h at 28 ± 0.5 °C in a rotary shaker (150 rpm). After 24 and 48 h, samples of each culture were spread plated on unmodified (0.995 $a_W$, control) and on modified media (NaCl $a_W$ 0.96 for *B. subtilis* RC 218 and glycerol $a_W$ 0.96 for *Brevibacillus* sp. RC 263, based on previous experiment on osmotic stress tolerance). In order to avoid an osmotic shock of the cells, the dilutions of each treatment were done using the same solutes and $a_W$ values, as those of the liquid media where the bacteria were cultured. For example, when *B. subtilis* was grown in NaCl 0.98 $a_W$ modified medium, the dilutions were done in liquid media with NaCl at 0.98 $a_W$. The cultures were incubated at 28 ± 0.5 °C and the number of cfu was determined after 24 h and 72 h for unmodified and modified media, respectively. All treatments were carried out with two replicates per treatment and the experiment was repeated once. When needed the data were transformed to $\log_{10}$. Analysis of variance of viability assays was carried out and means were separated with Fisher LSD method ($P \leqslant 0.001$) using SigmaStat for Windows Version 3.5 (SPSS Inc.).

### 2.4. Determination of intracellular betaine and ectoine in cells growing under $a_W$ stressed conditions

Bacterial strains were grown in unmodified (control) and modified liquid media as described in Section 2.3. Solutes and $a_W$ levels used to modify the liquid media were chosen based on the results obtained in the viability assays in liquid cultures. Solutes and $a_W$ selected were: NaCl (0.98, 0.97 and 0.96 $a_W$), glycerol (0.98; 0.97 and 0.96 $a_W$) and glucose (0.98 $a_W$) for *B. subtilis* RC 218 and glycerol (all $a_W$ evaluated) and NaCl 0.98 $a_W$ for *Brevibacillus* sp. RC 263.

One hundred milliliters of modified and unmodified media contained in 250 ml Erlenmeyer flask was inoculated with 1 ml of an overnight culture (approximately $10^6$ cells ml$^{-1}$) and incubated for 48 h at 28 ± 0.5 °C in a rotary shaker (150 rpm). After 24 and 48 h, bacterial suspensions were centrifuged at 7000 rpm for 5 min. Supernatants were discarded and bacterial pellets washed and centrifuged twice with sterile water (HPLC grade) to remove any residual liquid medium. Bacterial pellets were resuspended in 20 ml of sterile water (HPLC grade) and lyophilized. Quantification of betaine and ectoine was determined using a modified version of that originally reported by Kunte et al. (1993). Bacterial pellets (25 mg) were extracted by mixing with 570 µl of a mixture of methanol/chloroform/water (10:4:4 v/v) and vigorously shaking for 5 min. Chloroform and water (170 µl each) were added to the mixture and shaken for 10 min, phase separation was obtained by centrifugation of the mixture at 10,000 rpm for 10 min. The aqueous upper layer was recovered and 800 µl of acetonitrile was added in order to obtain a similar ratio to the mobile phase for HPLC analyses (acetonitrile/water, 85:15).

The HPLC system consisted of a Hewlett–Packard model 1100 pump (Palo Alto, CA) connected to a Hewlett–Packard 1100 Series variable wavelength detector and a data module Hewlett–Packard Kayak XA (HP ChemStation Rev. A.06.01). Chromatographic separations were performed on a Luna Amino™ reversed-phase column (150 × 4.6 mm, 5 µm particle size, Phenomenex). The mobile phase consisted of acetonitrile/water (85:15, v/v), at a flow rate of 1.5 ml min$^{-1}$. The UV detector was set at 200 nm with an attenuation of 0.01 AUFS. The injection volume was 50 µl and the retention time 10.6 min for betaine and 12.5 min for ectoine. Quantification was relative to external standards of 10–400 µg ml$^{-1}$ in acetonitrile/water (85:15). The detection and quantification limits were 1 µg ml$^{-1}$ and 5 µg ml$^{-1}$, respectively. All treatments were carried out with three replicates per treatment and the experiment was repeated once. As needed the data were transformed to $\log_{10}$. Analysis of variance of the data was done and means were separated by Fisher LSD method ($P \leqslant 0.001$) using SigmaStat for Windows Version 3.5 (SPSS Inc.).

### 2.5. Greenhouse evaluation of physiologically improved strains

Five seedlings of wheat (Variety ProINTA Gaucho) were grown in a 24-cm-diameter pot, containing soil, sand and river peat mixed in equal parts, air steam pasteurized at 100 °C for 60 min. The seedlings were maintained under greenhouse conditions for 12 weeks prior to use in the bioassay. *F. graminearum* strain RC276 was used to inoculate wheat spikes during the anthesis period. Three replicates were set up for each treatment and arranged in a randomized block design. Conidial inoculum was produced in Mung bean broth (Rosewich Gale et al., 2002). Approximately 10 days before spikes inoculation, a plug of *F. graminearum* RC 276 was used to inoculate 250 ml Erlenmeyer flasks containing 50 ml of Mung bean broth. The cultures were incubated for 7 days at 25 °C 150 rpm, filtrated and centrifuged at 7000 rpm for 5 min. The pellets were resuspended in sterile distilled water and 0.05% v/v Tween 80. Counting of conidia was done in a haemocytometer chamber and adjusted to $1 \times 10^5$ conidia ml$^{-1}$.

Bacterial treatments were chosen based on the results obtained in the viability assays and betaine accumulation. Solutes and $a_W$ selected were: NaCl (0.98 and 0.97 $a_W$), glycerol and glucose 0.98 $a_W$ for *B. subtilis* RC 218 and glycerol (0.97 and 0.96 $a_W$) and NaCl 0.98 $a_W$ for *Brevibacillus* sp. RC 263. For each treatment, an aliquot of 1 ml of an overnight culture was inoculated in 100 ml of liquid media (modified and unmodified) and incubated for 48 h at 28 ± 0.5 °C in a rotary shaker (150 rpm). Grown cultures were centrifuged at 10,000 rpm for 10 min and resuspended in sterile distilled water and 0.05% v/v Tween 80. Bacterial suspension counting was done in a haemocytometer chamber and adjusted to $1 \times 10^8$ cells ml$^{-1}$.

At anthesis, 5 ml of bacterial suspension and, immediately after, 5 ml of *F. graminearum* RC 276 conidial suspension were sprayed over wheat spikes. Spikes inoculated either with *F. graminearum* conidia alone or sterile distilled water and 0.05% v/v Tween 80 were the controls. Inoculated spikes were maintained at 90% relative humidity (RH) for 6 days to ensure *F. graminearum* colonization, and then the humidity was reduced to 70% for 10 days. Relative humidity inside the greenhouse was maintained with an automatic system consisting of an universal controller system (Novus N480D, Porto Alegre, Brazil), a humidity detector, water sprinklers, a water pump and an air compressor. Inoculated wheat spikes were evaluated after sixteen days. *Fusarium* head blight disease severity was visually estimated using a 0-to-100% scale (Stack and McMullen, 1995). The bioassay was repeated once. Data from repeated experiments were pooled because statistical analysis demonstrated that experiment interactions were not significant. Disease severity data as percentage (%) were normalized as needed using the arcsin square root transformation before analysis of variance (ANOVA). Means were separated with Fisher LSD method ($P \leqslant 0.001$) using SigmaStat for Windows Version 3.5 (SPSS Inc.).

## 3. Results

### 3.1. Osmotic stress tolerance of bacterial strains

*Bacillus subtilis* RC 218 and *Brevibacillus* sp. RC 263 showed variability under different $a_W$ conditions generated by ionic and non-ionic solutes. *B. subtilis* RC 218 reached similar cfu ml$^{-1}$ relative to the control culture in NaCl (0.98 and 0.96 $a_W$) and glycerol (at all $a_W$ evaluated) media at 24 h (Data not shown). At 48 h, *B. subtilis* RC 218 reached similar growth relative to their control in both NaCl 0.97 $a_W$ and glucose (0.98 and 0.97 $a_W$) media. Maximum growth of 42.8% with respect to the control was obtained in 0.96 $a_W$ glucose medium at 120 h (5 days) (Fig. 1).

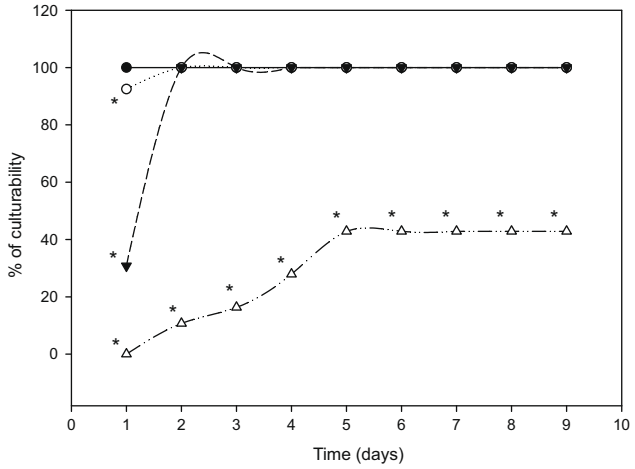


**Fig. 1.** Culturability of *Bacillus subtilis* RC 218 growing on glucose modified medium and unmodified media (control). $a_W$ evaluated: (●) (control) 0.995; (○) 0.98; (▼) 0.97 and (△) 0.96. Values are mean of two experiments with three replicates each. Statistical analysis was performed comparing the control versus each treatment over time. On each time (days), lines with an asterisk indicates significant differences with respect to the control ($P \leqslant 0.001$) according to Holm–Sidak test.

*Brevibacillus* sp. RC 263 showed a limited adaptation to the stressed conditions, reaching a 89% growth in glycerol (0.98 $a_W$) modified medium with respect to the control at 48 h. Lower growth percentage was observed in glycerol (0.97 and 0.96 $a_W$) modified media at 48 h, reaching a growth of 53.6% and 40.5%, respectively, of the control growth (Fig. 2). In NaCl 0.98 $a_W$ medium, *Brevibacillus* sp. RC 263 only reached 11.6% of growth with respect to the control. This strain was not able to grow in NaCl (0.97 and 0.96 $a_W$) and glucose modified media (at all $a_W$ evaluated) during the evaluated period (15 days).

### 3.2. Viability of bacterial strains under water activity stressed and unstressed conditions

Differences were observed in the viability of both strains when they were grown in liquid modified media. Considering each strain, the viability was similar at 24 and 48 h. A viability of $2.5 \times 10^8$ cfu ml$^{-1}$ was observed in *B. subtilis* RC 218 (control cells) at 24 h on unmodified media and onefold viability decrease was observed when cells were grown in modified media (NaCl 0.96 $a_W$). At 24 h, on unmodified media, all cultures showed lower viability (<$7.5 \times 10^7$ cfu ml$^{-1}$) than control cells except for glycerol 0.98 $a_W$ cells ($2.3 \times 10^8$ cfu ml$^{-1}$). However, increased viability in the cultures growing on NaCl modified media was observed at 24 and 48 h, compared to the control cells on modified media. Cells cultured in glycerol and glucose modified media were not able to grow on NaCl modified media at 24 h and only a limited viability ($3–5 \times 10^6$ cfu ml$^{-1}$) was observed in glycerol and glucose at 0.98 $a_W$ and 48 h of incubation. The best viability under water stressed conditions was observed in NaCl 0.98 and 0.97 $a_W$ treatments with $1.1 \times 10^9$ and $5.5 \times 10^8$ cfu ml$^{-1}$, respectively (Fig. 3).

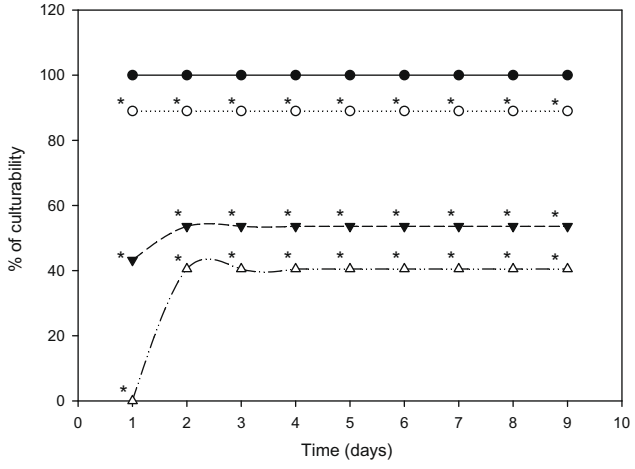


**Fig. 2.** Culturability of *Brevibacillus* sp. RC 263 growing on glycerol modified medium and unmodified media (control). $a_W$ evaluated: (●) (control) 0.995; (○) 0.98; (▼) 0.97 and (△) 0.96. Values are mean of two experiments with three replicates each. Statistical analysis was performed comparing the control versus each treatment over time. On each time (days), lines with an asterisk indicates significant differences with respect to the control ($P \leqslant 0.001$) according to Holm–Sidak test.

*Brevibacillus* sp. RC 263 control cells reached a viability of $4 \times 10^8$ cfu ml$^{-1}$ at 24 h. In glycerol (0.98 $a_W$) modified liquid media, a maximum viability of $3.1 \times 10^7$ cfu ml$^{-1}$ was observed on unmodified solid medium at 48 h, but on modified medium (glycerol 0.96 $a_W$) the viability decreased to $2.5 \times 10^5$ cfu ml$^{-1}$. Poor viability (<$10^5$ cfu ml$^{-1}$) was observed in NaCl (0.98 $a_W$) and glycerol (0.97 and 0.96 $a_W$) modified media. On NaCl (0.97 and 0.96 $a_W$) and glucose (all $a_W$ evaluated) modified media, the bacterium was not able to grow (Fig. 4).

### 3.3. Determination of intracellular betaine and ectoine in growing cells under modified conditions

Data on endogenous accumulation of betaine in *B. subtilis* RC 218 and *Brevibacillus* sp. RC 263 are shown in Fig. 5. Control cells of *B. subtilis* RC 218 accumulated small amounts of betaine after 24 and 48 h at levels of 3615 and 4404 ng of solute per mg dry cell, respectively. In contrast, cells growing in NaCl modified media accumulated significantly higher levels of betaine at 24 h. At 48 h, an accumulation up to three times in treatments modified with NaCl (all $a_W$), glycerol (all $a_W$) and glucose (0.98 $a_W$) was observed when compared to control cells.

In *Brevibacillus* sp. RC 263 control cells, betaine was not detected while in glycerol modified treatments (at all $a_W$) significant amounts of betaine were detected at levels of 4035, 6655 and 4542 ng solute mg cell$^{-1}$ after 48 h of incubation, respectively. In NaCl 0.98 $a_W$, no betaine was detected.

Ectoine was detected in both control cells (unmodified medium) at levels of 320–860 ng of solute per mg cell, but there was no significant accumulation of ectoine in cells growing in modified media after 24 h and 48 h (data not shown).

### 3.4. Greenhouse evaluation of physiologically improved strains

*Bacillus subtilis* RC 218 and *Brevibacillus* sp. RC 263 modified and unmodified cells were inoculated on flowering wheat heads before pathogen inoculation. In spite of the bacterial strain and solute used, all physiologically improved cultures showed significant reductions of FHB disease severity below 20% ($P \leqslant 0.001$) (Fig. 6). *F. graminearum* RC 276 inoculated alone showed a disease severity of 60%. *B. subtilis* RC 218 growing in NaCl 0.97 $a_W$ medium was the best inocula to control FHB, reducing the disease severity by 96% and showing an improved biocontrol efficacy and significantly different in comparison with the strain without physiological improvement.

## 4. Discussion

Biological control has been demonstrated to be effective under laboratory conditions for many fungal diseases, but only a few biocontrol products have been successfully commercialized. One reason for this lack of commercial success is that biocontrol agents

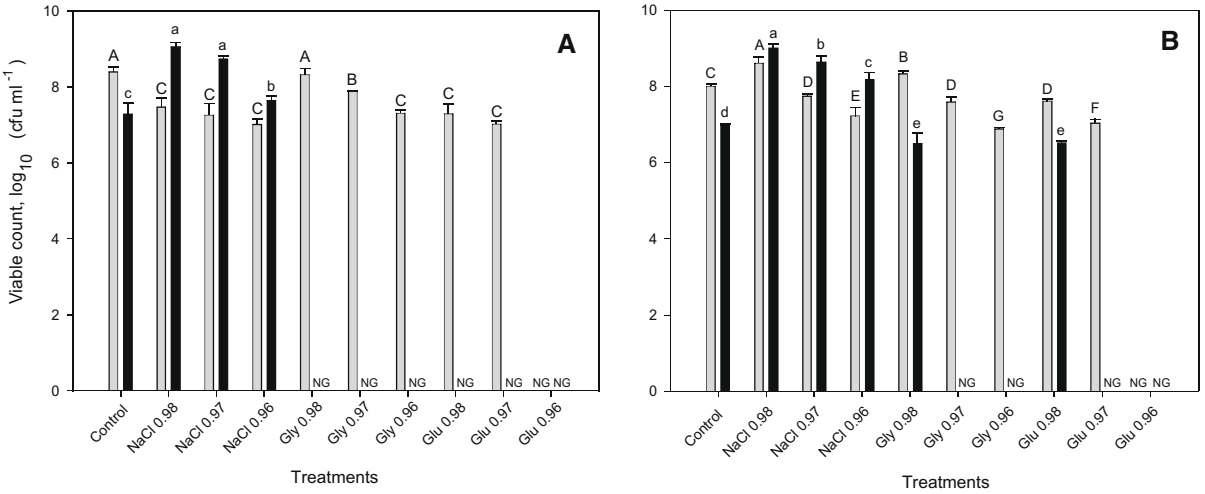


**Fig. 3.** Viability of *Bacillus subtilis* RC 218 cells grown for 24 h (A) and 48 h (B) in liquid media with different solutes and $a_W$ on unmodified (0.995 $a_W$, ▭) after 24 h and modified (NaCl 0.96 $a_W$, ■) after 72 h. Gly, glycerol; Glu, glucose; NG, no growth. Values are mean of two experiments with three replicates each. The separation of mean values is based on Fisher's LSD method. On each graph, statistical analysis was performed within modified or unmodified solid media. Columns with different letters indicate significant differences ($P \leqslant 0.001$).

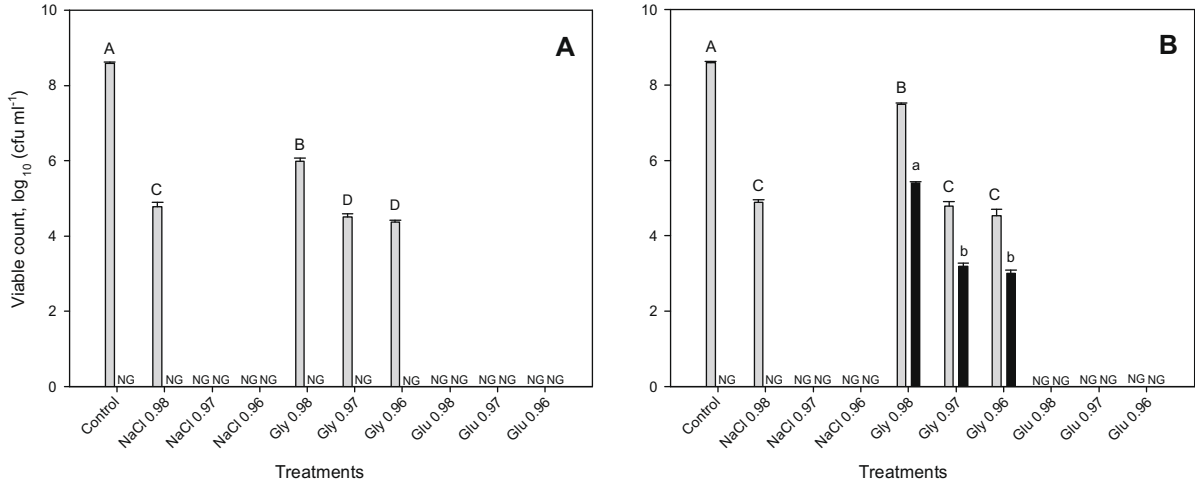


**Fig. 4.** Viability of *Brevibacillus* sp. RC 263 cells grown for 24 h (A) and 48 h (B) in liquid media with different solutes and $a_W$ on unmodified (0.995 $a_W$, ▭) after 24 h and modified (Gly 0.96 $a_W$, ■) after 72 h. Gly, glycerol; Glu, glucose; NG, no growth. Values are mean of two experiments with three replicates each. The separation of mean values is based on Fisher's LSD method. On each graph, statistical analysis was performed within modified or unmodified solid media. Columns with different letters indicate significant differences ($P \leqslant 0.001$).

are living organisms affected by variable environmental conditions. Preadaptation to one particular stress condition can also render cells resistant to other stresses, a phenomenon known as cross-protection (Sanders et al., 1999).

This study showed that physiological modifications by osmotic stress treatments and accumulation of compatible solutes was a good strategy to improve *B. subtilis* RC 218 and *Brevibacillus* sp. RC 263 as biocontrol agents against *Fusarium* head blight in wheat. B. subtilis RC 218 cells could grow in media with low $a_W$ and different solutes, simulating environmental stress conditions. It is important to remark that significant improvements were observed in the viable counts of *B. subtilis* RC 218 cells. The yield of cells was increased two times when the bacteria were grown in NaCl modified treatments. Similar results also were found in *Pantoea agglomerans* CPA-2 cells growing in NaCl, glycerol and glucose modified media. Also, viable counts of *P. agglomerans* CPA-2 cells growing in modified media were higher than control cells (Teixidó et al., 2006; Costa et al., 2002). In addition, it was demonstrated that *B. subtilis* FZB24 promotes growth of both eggplant and pepper plants and increased salt-stress tolerance when the bacteria are applied to plant roots and when plants are irrigated with saline water (Bochow et al., 2001). Thus, some *B. subtilis* strains can both grow in water salt-stress conditions and also increase plant growth parameters.

*Brevibacillus* sp. RC 263 cells could grow only in media modified with NaCl (0.98 $a_W$) and glycerol (at all $a_W$ tested) and the total viable counts of cells was not increased.

To compete and survive under fluctuating environmental conditions, *B. subtilis* achieves a considerable degree of osmoprotection by synthesizing betaine, but the precursor choline must be acquired from exogenous sources (Boch et al., 1994). In our study, the basal medium used included yeast extract, which is a source of choline (Teixidó et al., 2005). Although betaine and ectoine accumulate when *B. subtilis* RC 218 cells are grown in unmodified medium, the levels of betaine accumulated were higher when the strain was grown in NaCl (at all $a_W$) and glucose (0.98 $a_W$) modified

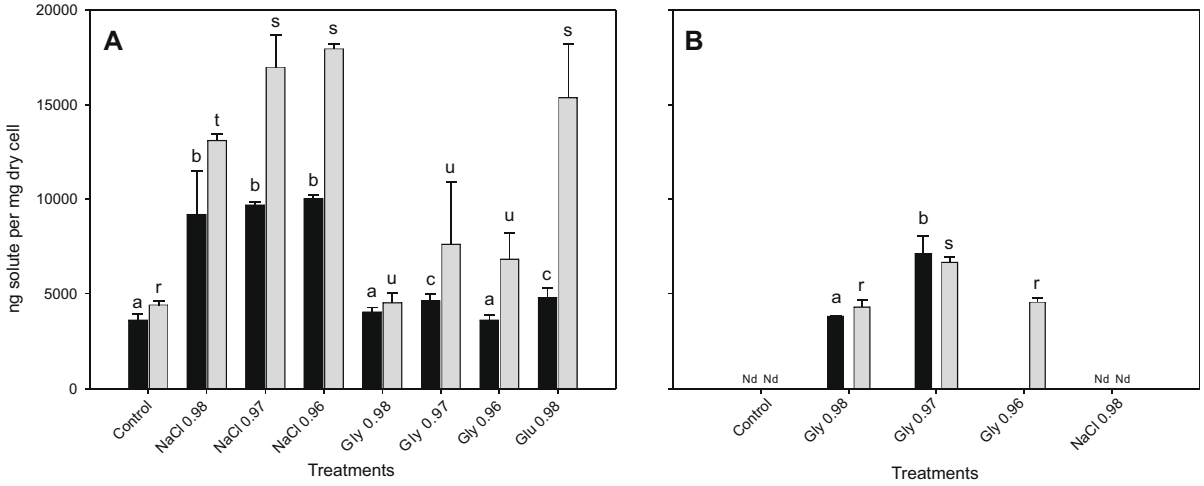


**Fig. 5.** Accumulation of betaine in *Bacillus subtilis* RC 218 (A) and *Brevibacillus* sp. RC 263 (B) grown in modified liquid media for 24 h (■) and 48 h (□) at 28 °C. Gly, glycerol; Glu, glucose; Nd, not detected. Results are mean of three replicates per treatment. The separation of mean values is based on Fisher's LSD method. Statistical analysis was performed comparing 24 or 48 h. On each graph (A and B), columns with different letters indicate significant differences at 24 or 48 h ($P \leqslant 0.001$) Nd: not detected. Detection limit, 1 μg ml$^{-1}$.

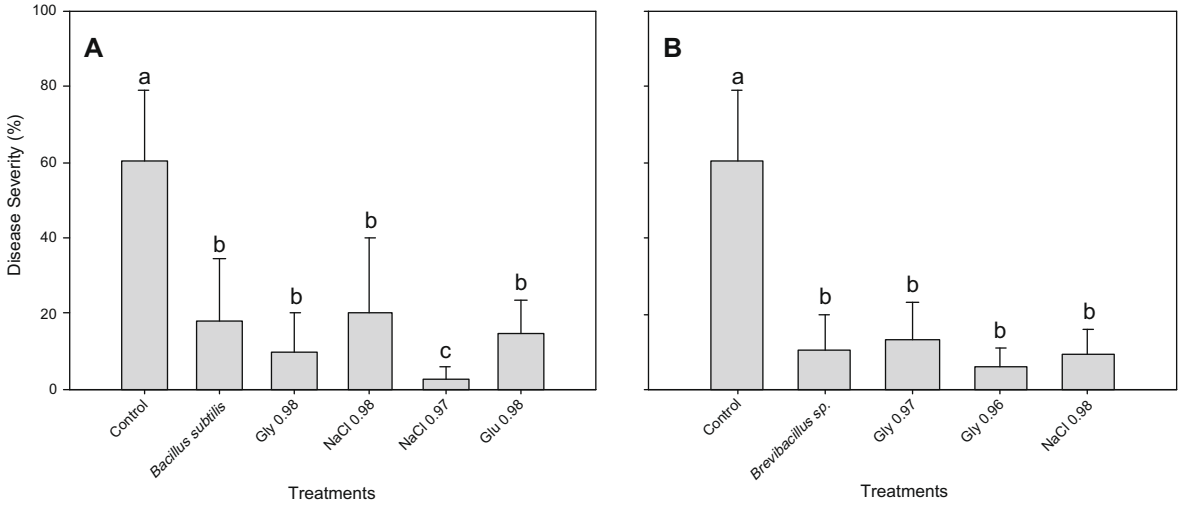


**Fig. 6.** Effect of physiologically modified and unmodified bacteria on FHB severity in wheat inoculated with *F. graminearum* RC 276 at anthesis. (A) *Bacillus subtilis* RC 218; (B) *Brevibacillus* sp. RC 263. Control, *F. graminearum* RC 276 inoculated alone; Gly, glycerol; Glu, glucose. Water activities tested on growing liquid media: 0.98; 0.97 and 0.96 $a_W$. The separation of mean values is based on Fisher's LSD method. On each graph, columns with different letters indicate significant differences ($P \leqslant 0.001$).

media. Betaine was not detected in *Brevibacillus* sp. RC 263 control cells, but significant quantities were detected when the strain was grown in liquid media containing glycerol. By manipulating growth conditions, both *B. subtilis* RC 218 and *Brevibacillus* sp. RC 263 cells increased intracellular concentration of betaine up to threefold in some treatments. Loshon et al. (2006) also found higher levels of glycine–betaine in *B. subtilis* cells growing in media containing NaCl. Also, the physiological quality of *P. agglomerans* cells as biocontrol agents improved when the growth medium contained NaCl and, the accumulation of glycine–betaine and ectoine, played a critical role in stress tolerance improvement and heat shock protection (Teixidó et al., 2005). A strong correlation was observed between the usage of glycine betaine in the growing media of *Lactococcus lactis* strains and the tolerance to sodium chloride (ÓCallaghan and Condon, 2000).

The age of the culture affects the accumulation of betaine in *B. subtilis* RC 218, since betaine content increased in NaCl (0.98, 0.97 and 0.96 $a_W$) and glucose (0.96 $a_W$) treatments after 48 h of incubation. Similar results were showed in *P. agglomerans*, since the maximum accumulation of glycine betaine occurred at 48 h in media containing NaCl (0.98, 0.97 and 0.96 $a_W$) (Cañamas et al., 2007).

*Bacillus subtilis* AS 43.3 was less effective against FHB disease in the field than in the greenhouse (Schisler et al., 2002). One explanation for biocontrol failure under field conditions is that biocontrol agents are not adapted to competition for nutrients, solar radiation, reduced water availability, etc. These stresses could reduce the production of antibiotics or other active compounds responsible for biocontrol. Thus, modified stress-tolerant cells of *B. subtilis* RC 218 or *Brevibacillus* sp. RC 263 could survive and become effectively established on wheat spikes in a fluctuating environment and could have a competitive advantage over cells grown in basal medium.

Furthermore, preparation of biocontrol agents, e.g. spray drying and storage, as well as field application protocols, may reduce the viability of the biocontrol agent (Costa et al., 2001; Teixidó et al., 2006; Yuen et al., 2007). Teixidó et al. (2006) showed that NaCl stressed *P. agglomerans* cells survive the spray-drying process bet-

ter than do the control cells. Thus, physiological improvement by osmotic treatments and the accumulation of compatible solutes could increase bacterial survival during the formulation process, storage and application stages.

We demonstrated that physiologically modified strains of *B. subtilis* RC 218 and *Brevibacillus* sp. RC 263 were effective biocontrol agents against FHB in greenhouse trials. The best condition to control FHB under greenhouse was *B. subtilis* RC 218 on NaCl (0.97 $a_W$) modified media. Field trials of the selected biocontrol agents are in progress to evaluate their efficacy in reducing FHB disease severity and DON levels.

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