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Impact of osmotic/matric stress and heat shock on environmental tolerance induction of bacterial biocontrol agents against *Fusarium verticillioides*

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Abstract

Bacillus amyloliquefaciens and *Microbacterium oleovorans* reduced the *Fusarium verticillioides* count and significantly decreased fumonisin B_1 and B_2 levels in maize grains. The aim of this study was to determine the effect of water stress tolerance and heat shock survival upon cells of the biocontrol agents *B. amyloliquefaciens* and *M. oleovorans*. The a_w of solid and liquid media and tryptic soy medium was modified to 0.99, 0.98, 0.97 and 0.96 by addition of ionic solute NaCl and non-ionic solutes such as glycerol and glucose. The non-ionic solute polyethylene glycol 600 (PEG 600) was used to modify matrically solid media. Bacterial incubation was at 30 °C. After 24, 48 and 72 h of incubation, samples from liquid media were spread-plate on nutrient agar medium and incubated for 24 h to determine the number of viable cells. The bacterial cells were harvested by centrifugation and heat treatment carried out in a water bath at 45 °C for 30 min. The viability of cells from different incubation times in liquid media showed statistically significant differences. Cells of *B. amyloliquefaciens* grown in liquid media amended with glycerol showed better tolerance at low a_w and high survival under heat stress. These results could have important implications for optimizing and improving formulations.
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Keywords: Biological control; Osmotic adaptation; Maize; *Fusarium verticillioides*; *Bacillus amyloliquefaciens*; *Microbacterium oleovorans*

1. Introduction

Maize is the host of a number of fungi that can produce mycotoxins. Among these fungi, *Fusarium verticillioides* produces toxins associated with harmful effects on animal and human health (Marasas et al., 1984). *F. verticillioides* (Sacc.) produces several toxins that have potential toxicity, such as fumonisin B_1 and B_2 related to diseases such as equine leukoencephalomalacia, porcine pulmonary edema and esophageal cancer in humans (Marasas et al., 2000) *F. verticillioides* is responsible for both grain yield and quality losses in maize (Fandohan et al., 2003). Because of this, control of *F. verticillioides* colonization in maize has become a priority in crop safety production. Biological control represents an environmentally friendly alternative to the use of chemical pesticides. A Gram-positive spore-forming bacteria *Bacillus amyloliquefaciens* has been reported to be a potential biological control agent in several plant diseases (Mari et al., 1996; Chen and Wu, 1999; Chiou and Wu, 2001; Yoshida et al., 2001; Abdullah et al., 2008). The treatment of maize seed with *B. amyloliquefaciens* and *Microbacterium oleovorans* not only reduced the *F. verticillioides* count from maize grain, but also significantly decreased the amounts of fumonisin B_1 and B_2 in sampled grains from plants grown from treated seeds (Pereira et al., 2007). The addition of these bacterial biocontrol agents significantly reduced the *F. verticillioides* count from the maize root inner tissues, coupled

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with the fact that bacterial treatment did not affect richness or diversity values of indigenous microbial populations (Pereira et al., 2009). The main goal in development and implementation of biological control products is to improve the ability of biological antagonists to successfully control preharvest diseases under a wider array of conditions and with minimal variability. The success of colonization of the spermosphere and rhizosphere by biocontrol agents added to the seeds is influenced by environmental conditions of these particular microhabitats. Fluctuations in soil water potential is one of the most important factors influencing microbial activity under field conditions. In soil, bacteria mainly develop in water films on the surface of soil particles. Consequently, they are primarily affected by the matric water potential. Soil water potential routinely fluctuates with time, declining gradually with soil drying (percolation, evaporation, evapotranspiration) and then increasing rapidly upon wetting (irrigation, rainfall). Physiological methods for improving tolerance to such environmental stress are considered fundamental to enable development of effective and consistent microbial biocontrol agents (Deacon, 1991). On the other hand, formulation must ensure conditions which maintain viability throughout production, distribution, storage aid handling and product application. Formulation must help to maintain the biological agent in the environment in a viable form for as long as required for its mode of action, and must also enhance the activity of the organisms at the target site (Rhodes, 1993; Fravel et al., 1998). One way of improving survival for microorganisms is by understanding microbial stress response mechanisms and using this knowledge to improve resistance to unfavorable environmental conditions. Culturing the biocontrol agents at above-optimal temperatures produced propagules with improved tolerance to high temperature storage, while modifying water potential in the culture medium can result in cells with increased tolerance to desiccation (Harman et al., 1991; Teixidó et al., 1998). Teixidó et al. (2006) demonstrated that cells of *Pantoea agglomerans* CPA-2 grown in media at low water potential activities (a_w) using NaCl exhibited osmotic adaptation in solid media at low a_w. Osmotic adapted cells also demonstrated thermotolerance and desiccation tolerance after spray drying (Teixidó et al., 2005, 2006). To improve stress response behavior and to stimulate mechanisms of environmental survival, we studied the effect of water stress tolerance, solute stress tolerance Ψs (ionic and non-ionic), matric stress tolerance Ψm and heat shock survival in cells of the biocontrol agents *B. amyloliquefaciens* and *M. oleovorans*.

2. Materials and methods

2.1. Bacterial strains

The strains used in this study were *B. amyloliquefaciens* (GenBank accession EU164542) and *M. oleovorans* (GenBank accession EU164543) (Pereira et al., 2009). Both strains were isolated from commercial maize field and initially identified based on their physiological profiling according to Bergey's manual of Systematic Bacteriology (Holt, 1993).

2.2. Osmotic stress in solid medium

B. amyloliquefaciens and *M. oleovorans* were grown in triptic soy agar (TSA). The water activity (a_w) of the basic medium was modified to 0.99, 0.98, 0.97 and 0.96 by adding different amounts of ionic solute (NaCl) and non-ionic solutes (glycerol and glucose) (Dallyl and Fox, 1980), and the a_w of all media was determined using AquaLab equipment (series 4 TE). Each treatment was inoculated with 1 ml of 1.10^4 CFU ml^{-1} of each bacterial strain. The plate count of bacteria was analyzed after 24 h of incubation. Three replicates per treatment were used and repeated twice.

2.3. Matric stress in solid medium

B. amyloliquefaciens and *M. oleovorans* were grown on petri plate TSA medium modified matrically with polyethylene glycol 600 (PEG 600) which resulted in a matric potential of −1.38, −2.78, −4.19 and −5.62 (= 0.99, 0.98, 0.97, 0.96 a_w, respectively). PEG is known to act predominantly by matric forces (Steuter et al., 1981). Each treatment were inoculated with 1 ml 10^4 CFU ml^{-1} of each bacteria spread onto the media and incubated at 30 °C. Plates of the same osmotic and matric potential were placed in polyethylene bags before incubation (Nesci et al., 2004). The number of viable cells was determined after 24 h of incubation. Three replicates per treatment were used and repeated twice.

2.4. Osmotic stress in liquid medium

B. amyloliquefaciens and *M. oleovorans* were initially grown in TSB medium osmotically modified to 0.99, 0.98, 0.97 and 0.96 a_w by adding different amounts of ionic solute (NaCl) and non-ionic solutes (glycerol and glucose) (Dallyl and Fox, 1980). From each treatment 150 ml of medium were inoculated with 1 ml of 10^4 CFU ml^{-1} and incubated on a rotary shaker (140 rpm) at 30 °C. After 24, 48 and 72 h, samples were spread-plate on TSA medium and incubated at 30 °C. The number of viable cells was determined after 24 h of incubation. Three replicates per treatment were used and repeated twice.

2.5. Heat shock survival of a_w −adapted cells

The bacterial cells from unmodified and treatments osmotically modified by addition of glycerol, glucose and NaCl (0.99, 0.98, 0.97 and 0.96 a_w) were obtained from 150 ml of TSB medium inoculated and incubated on a rotary shaker (140 rpm) at 30 °C. The number of viable cells after 24 h incubation was determined by serial dilution of samples in TSB and then spread onto TSA medium. The cells were harvested by centrifugation (7000 g for 10 min at 10 °C) and the cell paste resuspended in 2 ml of phosphate-buffered solution (PBS). Heat treatments were carried out in water baths at 45 °C for 30 min and later, a new count of viable cells

as done on TSA medium. Three replicates per treatment were used and repeated twice.

2.6. Statistical analysis

Analysis of variance (ANOVA) was made for viable counts in unstressed and water-stressed media and survival after heat shock using an SAS program (SAS *System for Windows* 6.11. SAS Institute, Cary, NC, USA). To establish the significant differences, Duncan's multiple range test ($P < 0{,}05$) was performed.

3. Results

3.1. Effects of osmotic and matric potential on bacterial growth on solid medium

Changes in cell growth of *B. amyloliquefaciens* and *M. oleovorans* in relation to osmotic and matric treatments are compared in Fig. 1. Viable counts of *B. amyloliquefaciens* and *M. oleovorans* at different a_w levels for each solute tested were statistically significant. The CFU count decreased as the water activity level decreased in all treatments.

In treatments with *B. amyloliquefaciens*, significant differences between all media with osmotic stress (ionic and non-ionic stress) and matric stress were observed. PEG treatment was more stressful at low a_w, while high growth (4.12 CFU log) was observed at 0.99 a_w. However, no growth was observed at 0.97 and 0.96 a_w. Significant differences between NaCl medium (osmotic stress) and PEG 600 medium (matric stress) were observed in *M. oleovorans* growth. The CFU count on NaCl medium was 4.11 CFU log and on PEG 600 3.86 CFU log at 0.99 a_w. Between NaCl and glucose media (ionic and non-ionic osmotic stress), and between glucose and glycerol treatments (non-ionic osmotic stress), significant differences were observed in *M. oleovorans* growth. No significant differences between NaCl (ionic) and glycerol (non-ionic) treatments were observed, on NaCl medium. The count was 4.11 CFU log and on glycerol medium the count was 4.03 CFU log at 0.99 a_w. PEG treatment was more stressful, as also observed with *B. amyloliquefaciens* growth.

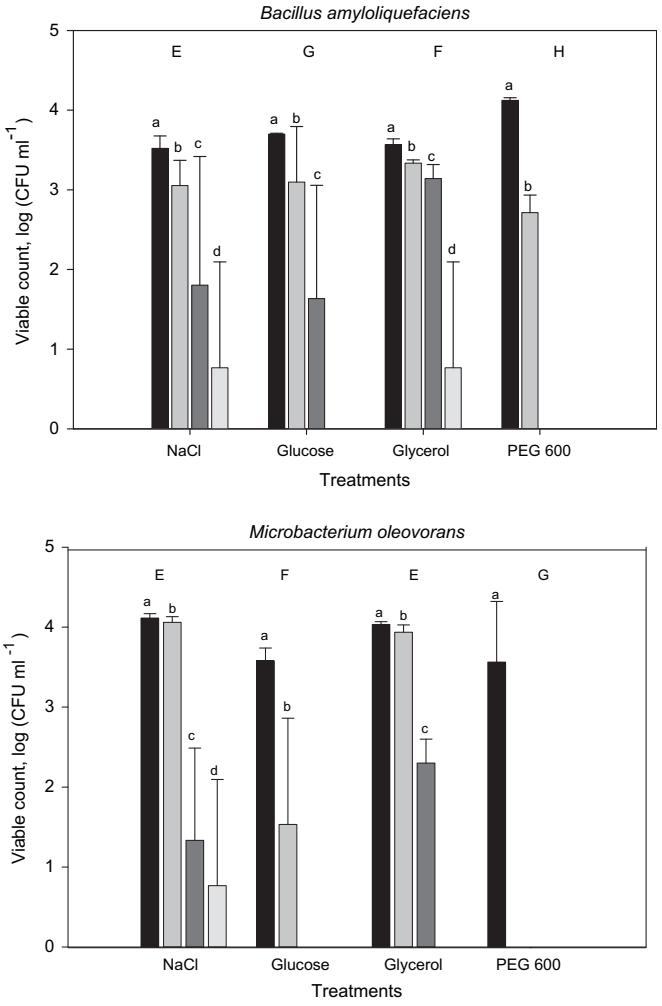

Fig. 1. Viability of *B. amyloliquefaciens* and *M. oleovorans* cell growth in different stressed solid media (0.99 a_w ■, 0.98 a_w ■, 0.97 a_w ■ and 0.96 a_w □), after incubation at 30 °C for 24 h. Statistical analysis was performed on different water activities of the same treatment and between different solutes. Data with the same letter (small) for different water activities in the same solute are not significantly different, and data with the same letter (capital) for different solutes are not significantly different according to Duncan's multiple range test ($P < 0{,}05$).

3.2. Osmotic potential and incubation time effects on bacterial growth in liquid medium

The viability of *B. amyloliquefaciens* and *M. oleovorans* cells at 24 h incubation time showed statistically significant differences in four water activities for each treatment (Fig. 2). At 48 and 72 h of incubation, bacterial growth was in stationary phase for all treatments (data not shown).

Statistical analyses of osmotic potential, water activity, bacterial strains and two and three–way interactions for 24 h of incubation time are shown in Table 1. Statistically significant differences were observed for osmotic potential, a_w, bacterial strains and osmotic potential − bacterial strains/ interaction. The major effect was produced by osmotic potential ($F = 213{,}28$; $p > 0{,}0001$). No significative differences in the CFU count were observed between 0.99, 0.98 and 0.97 a_w. The CFU count of *B. amyloliquefaciens* and *M. oleovorans* decreased at 0.96 a_w.

3.3. Heat shock survival of osmotic adapted cells

Cells of *B. amyloliquefaciens* grown in media modified by NaCl and glucose showed significant differences before and after heat shock treatment. Results are shown in Fig. 3. No differences in viable count were observed before or after heat shock in media modified with glycerol. Better tolerance toward low water activity and better survival after heat shock were observed. The CFU count was 1.05×10^{11} before shock and 1.25×10^{11} CFU ml^{-1} after heat shock at 0.99 a_w.

In all osmotically modified media, water activity had no influence on *B. amyloliquefaciens* viability after heat shock. No significant differences were observed among the four a_w

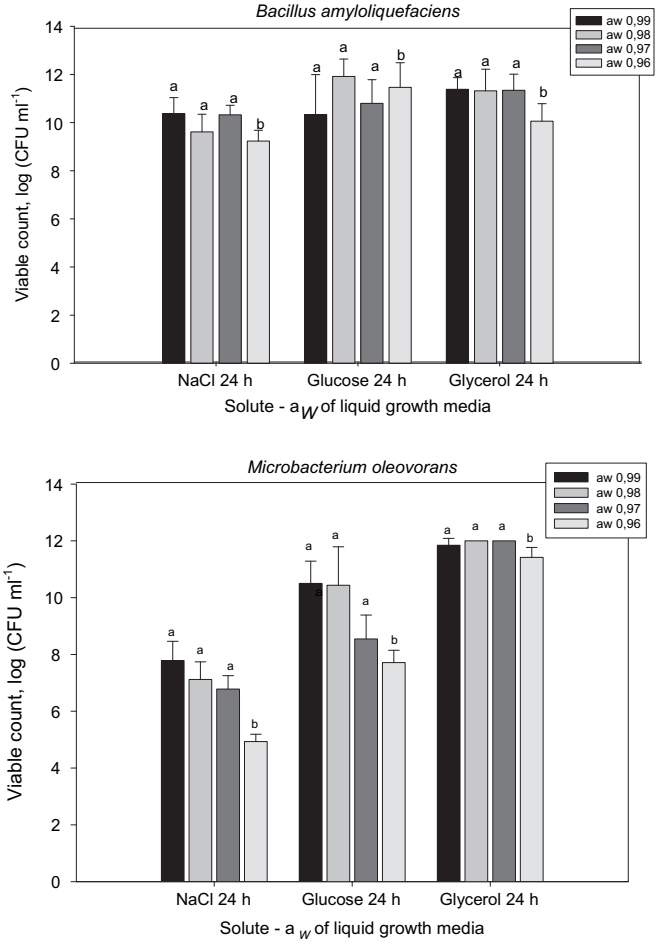

Fig. 2. Viability of *B. amyloliquefaciens* and *M. oleovorans* cell growth in different stressed liquid media (0.99 a_w ■, 0.98 a_w ▒, 0.97 a_w ▓ and 0.96 a_w □), after incubation at 30 °C for 24 h. Statistical analyses were performed on different water activities of the same treatment between different solutes. Data with the same letter for different water activities in the same solute are not significantly different according to Duncan's multiple range test ($P < 0,05$).

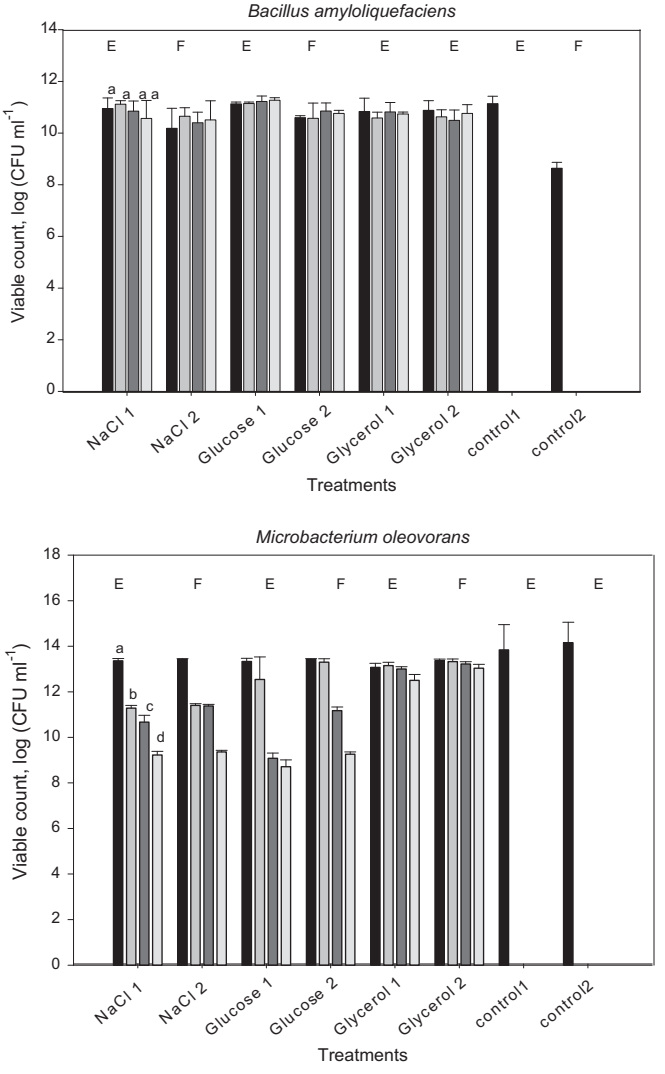

Fig. 3. Survival after 30 min at 45 °C of *B. amyloliquefaciens* and *M. oleovorans* growth for 24 h in liquid media stressed (0.99 a_w ■; 0. 98 a_w ▒; 0.97 a_w ▓ and 0.96 a_w □) with different solutes and control (unmodified). 1, before heat shock; 2, after heat shock. Statistical analyses were performed on different water activities of the same treatment and on different solutes before and after heat shock. Data with the same letter (small) for different water activities in the same solute are not significantly different, and data with the same letter (capital) for different solutes before and after heat shock are not significantly different according to Duncan's multiple range test ($P < 0,05$).

tested in each treatment. The biggest difference in cell viability was obtained in the control treatment, which was 11.2 before and 8.69 CFU log after heat shock.

Significant differences were observed in *M. oleovorans* cell survival in media modified by glucose, NaCl and glycerol at the four a_w tested. The effect of a_w on CFU count was minimum in medium modified by glycerol: values were 13.1 at 0.99 a_w and 12.55 CFU log at 0.96 a_w before heat shock. The growth in three ionically modified media showed significant differences in *M. oleovorans* survival before and after heat shock, but not for the control treatment (unmodified media).

M. oleovorans showed more resistance to heat shock under control treatment than *B. amyloliquefaciens*.

Table 1
Significance of osmotic potential (Ψs), water activity (a_w) and bacterial strains (B) and their interactions in terms of potential osmotic effects on bacterial growth in liquid medium at 24 h of incubation.

	DF	MS	F. value [a]	P > F
Ψs	2	149.39	213.28	0.0001
a_w	3	17.16	24.51	0.0001
B	1	71.55	102.16	0.0001
$a_w \times$ Ψs	6	2.47	3.53	0.0030
Ψs × B	2	71.64	102.28	0.0001
$a_w \times$ B	3	2.28	3.26	0.0241
$a_w \times$ B × Ψs	6	1.43	2.05	0.0763
Error	–	115	80.55	–

DF: degrees of freedom. MS: mean square.
[a] Significant at $P > 0.0001$.

4. Discussion

This study demonstrates improved response and better in vitro stress tolerance of *B. amyloliquefaciens* and *M.*

oleovorans. Such a manipulation could enhance the yield of tolerant cells, a significant event when producing a formula that can support environmental water potential stress of the maize rhizosphere. It is known that the environment surrounding the root has high water potential, and for this reason inoculated bacteria must be tolerant to high solute concentrations. To improve the response to stress and trigger mechanisms to stimulate their effectiveness and suitability for practical conditions, physiological methods were used which are essential for increasing the performance of microbial biological agents (Deacon, 1991; Teixidó et al., 2005). In the present study, cells of *B. amyloliquefaciens* grown in ionic osmotic medium showed better survival against heat stress. The osmotic strength of the environment is an important physical parameter that influences the ability of microorganisms to proliferate and successfully compete for a given habitat. Gram-positive bacteria such as *Bacillus subtilis* maintain high turgor, estimated at approximately −1.9 MPa = 0.989 a_w (Whatmore and Reed, 1990). *Microbacterium* comprises more than 30 physiologically versatile species isolated from various environments. One of their physiological characteristics is their capacity to grow between 2% and 6.5% NaCl (equivalent to 0.995 and 0.965 a_w respectively) (Schippers et al., 2005). Culturability increased when the Incubation time increased. The culturability of bacteria in a high osmolarity environment triggers rapid fluxes of cell water, causing dehydration of the cytoplasm. To avoid the outflow of water microorganisms increase organic osmolytes, called compatible solutes (Kempf and Bremer, 1998). Studies demonstrating the quality and quantity of such osmolyte accumulation under osmotic and heat stress conditions are ongoing. The significant increase in viability of stressed media is important in the yield of biocontrol agents during mass production. Matric potential was more stressful than osmotic potential (ionic and non-ionic) for cells of *B. amyloliquefaciens* and *M. oleovorans*. Time of incubation plays a significant role in the count of harvest cells at low water activity; in glycerol treatments at 48 h incubation, high production levels were obtained. The results obtained showed that osmotic tolerance is linked to thermotolerance. The behavior of *B. amyloliquefaciens* showed that osmotic tolerance helps to maintain survival after thermal shock. The thermotolerance of biocontrol agents is advantageous for the formulation process and shelf life. Ours results are in agreement with those of Teixidó et al., (2005), whose research showed that *P. agglomerans* grown in low a_w media was modified ionically with NaCl and improved a_w stress tolerance and cross-protection against heat stress in comparison with cells grown in unmodified basal medium. Teixidó et al. (2006) suggested that it is possible to improve stress tolerance of the microorganism, and thus its behavior under non-controlled environmental conditions and/or during its formulation process, without affecting its biocontrol potential.

M. oleovorans showed significant differences before and after heat shock, and green on all osmotically modified media. Practically no differences were observed between NaCl and glucose. The cells of *B. amyloliquefaciens* grown in liquid media modified by glycerol were shown to have better tolerance to low a_w and improved survival in heat stress of 45 °C. For *M. oleovorans*, the medium modified by glycerol produced an increase in viability at 48 h incubation, better tolerance to low a_w and less effect against heat shock.

Field experiments are studying the success of physiologically modified *B. amyloliquefaciens* and *M. oleovorans* at colonizing the rhizosphere of maize and surviving in the rhizosphere ecosystem. Moreover, studies on loss of cell culturability by formulation of improved biocontrol agents are in progress.

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