

Accumulation of the betaine and ectoine in osmotic stress adaptation of biocontrol agents against *Fusarium verticillioides* in maize

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ABSTRACT

The aims of this study was to examine the effect of the osmotic (NaCl) water stress on, growth and accumulation of endogenous compatible solutes of *Bacillus amyloliquefaciens* and *Microbacterium oleovorans*, biocontrol agents against *Fusarium verticillioides*. Tryptic soya broth media were modified ionically to 0.99; 0.98; 0.97 and 0.96 a_w with NaCl. The endogenous contents of the intracellular compatible solutes glycine-betaine and ectoine were quantified. Cells grown under ionic solute stress showed accumulation of significant amounts of both amino acids in all treatments. The growth rate of *F. verticillioides* was decreased significantly by interaction with *B. amyloliquefaciens* and *M. oleovorans* physiologically adapted at 0.96 a_w. This study have demonstrated the ability to synthesize betaine and ectoine under high-osmolality conditions of *B. amyloliquefaciens* and *M. oleovorans* cells. Such ecophysiological manipulation, especially to water stress, may increase the potential for biological control of *F. verticillioides* at field under wider a_w conditions.

Keywords: Biological Control; Osmotic Adaptation; Solute Compatible; *Fusarium verticillioides*; Maize

1. INTRODUCTION

Fusarium verticillioides is considered to be a primary phytopathogen of maize, occurring world wide in tropical and subtropical zones. The pathogen life cycle is significantly influenced by environmental factors specially water availability and temperature [1]. *F. verticillioides* produce a large number of microconidia and macroconidia on crop residues, and these asexual spores comprise the

most important inoculum for *Fusarium* ear rot and symptomless kernel infection. *F. verticillioides* can be transmitted from seeds to seedling and can also systemically colonize the entire maize plant without any visible symptoms. This causes transmission from seed to plant to kernels. Another route is for airborne spores to infect through the silks [2]. Kernels can also be infected by *F. verticillioides* strains that originate from the rhizosphere. The relative importance of different pathways may vary in different geographic areas [3]. *F. verticillioides* can produce fusaric acid, fusarins and mainly, fumonisins, clearly the most important toxin produced by the fungus [4,5]. The consumption of fumonisins contaminated maize based foods has been correlated with high rate of human esophageal cancer in Transkei and China [6,7]. Fumonisin B₁ is considered as carcinogen type 2B [8]. There are recommended maximum fumonisin levels adequate to protect human and animal health [9,10]. Thus strategic for minimizing or reducing the infection by these Fusaria and fumonisin contamination are being sought.

One approach is the use of biocontrol micro-organisms which can effectively outcompete the mycotoxigenic strains. Previous studies have shown that the addition of *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* to seeds significantly reduced *F. verticillioides* populations in the root inner tissues of resultant seedlings and also subsequently reduced fumonisin B₁ and B₂ content of maize grain at harvest [11-13]. This type of biocontrol action may be by competitive exclusion of the pathogen. In this way both biocontrol agents are able to colonise the rhizosphere and minimize the opportunity for colonization during the critical early days of plant development. However, such soil microorganisms are exposed to fluctuating and often very dry conditions (reduced water potentials) because of soil matric potential stress and in some cases solute water potential stress in saline soils [14].

Teixidó *et al.* [15,16] suggested that it is possible to

significantly improve environmental stress tolerance of biocontrol agents by physiologically growing them under conditions which facilitate synthesis of useful compatible solutes. Thus there is interest in whether the two bacteria in this study can be grown so that the environmental tolerance can be improved for better efficacy in the rhizosphere during the initial germination and establishment of maize seed. This could also have implications for the effective formulation of such bacteria in the preparation of dry formulations with conserved viability and shelf-life [17].

Previously, we have demonstrated that *B. amyloliquefaciens* and *M. oleovorans* were more tolerant to ionic and non-ionic potential stress than matric potential stress and showed tolerance to heat-shock of the 45°C in a physiological adaptation assays [18]. Previous studies have also shown that growth on MSB medium (molasses soy powder broth) and subsequent freeze drying of *B. amyloliquefaciens* and *M. oleovorans* cells had good viability, regardless of the water stress treatment used [19]. This suggests that growth under ecophysiological stress conditions can result in the synthesis of compatible solutes to help overcome water stress and to enable the cell functioning to continue under environmental stress [20]. Compatible solutes can make important contributions to the restoration of turgor under conditions of low water activity by counteracting the efflux of water from the cell. Important representatives of this class of molecules are the amino acid proline, the trimethylammonium compound glycine-betaine and tetrahydropyrimidine ectoine [21]. The accumulation of all or some of these compounds enables cell proliferation under unfavourable environmental stress conditions [22]. An additional role for compatible solutes is that they can also mitigate deleterious effects of heat stress, freezing, drying, high salinity, oxygen radicals, radiation, urea and other denaturing agents on the integrity of proteins, nucleic acid, biomembranes and even whole cells [23,24].

The objective of this work were to determine the effects of water potential stress on bacterial growth in liquid medium, to quantify the accumulation of glycine-betaine and ectoine in cells of the potential biocontrol agents *B. amyloliquefaciens* and *M. oleovorans*, and to determine the effect of physiological adaptation of both bacteria in antifungal activity.

2. MATERIALS AND METHODS

2.1. Bacterial Strain

The strains used in this study were *Bacillus amyloliquefaciens* (GenBank accession EU164542 and BNM 0531) and *Microbacterium oleovorans* (GenBank accession EU164543 and BNM 0532) [12]. These strains were isolated from a commercial maize field and identified on

the basis of 16S DNA gene sequence similarity and additionally identified based on their physiological profiling according to Bergey's Manual of Systematic Bacteriology [25]. These strains are deposited in the National Bank of Microorganisms, dependent of Biosciences Research Institute in Agricultural and Environmental (INBA) and National Council of Scientific and Technical Research (CONICET), National University of Buenos Aires, Buenos Aires, Argentina.

2.2. Growth under Osmotic Stress in Liquid Medium

B. amyloliquefaciens and *M. oleovorans* were initially grown in TSB medium (tryptic soy broth), and osmotically modified to 0.99, 0.98, 0.97 and 0.96 a_w by addition of the ionic solute (NaCl) [26], the a_w of control treatments was 0.99. For each treatment, 150 mL medium in conical flasks (250 mL) were inoculated with 1 mL of 10^4 CFU·mL⁻¹ and cultured on a rotatory shaker (140 rpm) at 30°C for 24 h. After incubation samples were spread-plate on TSA medium and incubated at 30°C and the number of viable cells was determined after 24 h. All cell cultures of *B. amyloliquefaciens* and *M. oleovorans* were centrifuged for 10 min at 7000 g and 10°C. Cells were harvested in sterile tubes. Three replicates per treatment were used and the experiments repeated twice.

2.3. Determination of Endogenous Glycine-Betaine and Ectoine Accumulation

The bacterial pellet of *B. amyloliquefaciens* and *M. oleovorans* cell treatments were resuspended in 2 mL of skim milk and frozen at -20°C for 24 h. Then samples were connected to the freeze-drier operating at chamber pressure <0.05 mbar and -45°C for 24 h. Extraction was done using the method described by Kunte *et al.* [27]. Firstly, to obtain endogenous compatible solutes, 20 mg of freeze-dried cells were added to 570 µl of extraction mixture (methanol/chloroform/water 10:5:4 by vol.) by vigorous shaking for 5 min. Immediately, equal volumes (170 µl) of chloroform and water were added and the mixture shaken for another 10 min. Phase separation was enhanced by centrifugation at 9720 xg and 10 min. The hydrophilic top layer containing the compatible solutes was recovered and diluted to suitable concentrations (low µM range) prior to analyses by high performance liquid chromatography (HPLC).

An aliquot of 0.75 ml of acetonitrile (ACN) was added to each sample to obtain the same ratio of acetonitrile/water (80:20) as mobile phase for component separation. The samples were centrifuged at 9720 xg during 10 min to discard impurities and before HPLC analysis and the liquid filtered through a 0.22 µm (What-

man) sterile filter unit. The samples (50 μl) were injected and analysed using a RP8-NH₂ column (125 \times 4 mm filled with 3 μm Gromsil amino 100 RP-8; Grom Analytic, Rottenburg-Hailfingen, Germany) using an auto-sampler. The flow rate of the mobile phase was 1.5 $\text{ml}\cdot\text{min}^{-1}$ at room temperature. Glycine-betaine and ectoine were detected with an Agilent Refractive index (RI) Detector (Agilent G1362A module) and an autosampler connected to the Agilent Millennium software package for data analyses. The peak areas were integrated and compared with calibration curves constructed with standards (50 - 800 $\mu\text{mol}\cdot\text{g}^{-1}$) of each component analysed. Results were expressed as μmol compatible solute g^{-1} (dry weight bacterial cells). All results are the mean of three replicate freeze-dried bacterium samples per treatment condition.

2.4. Influence of Physiological Adaptation of Antagonistic Agents on Growth Rate of *F. verticillioides*

The inhibitory activity on lag phase and growth rate of *B. amyloliquefaciens* and *M. oleovorans* against *F. verticillioides* were tested. Culture medium for bacteria, at different water activity levels and control unmodified was prepared following the procedure used for growth under osmotic stress assessment. The basic medium used was maize meal extract agar (MMEA) containing 3% maize meal and 1.5% agar. The water activity of the basic medium was adjusted to 0.99, 0.98, 0.97 and 0.96 by the addition of NaCl, according to Dallyn and Fox [26]. Each bacterial suspension with 10^8 cells $\cdot\text{ml}^{-1}$ was poured in 20 mL of MMEA and after solidification, MMEA were inoculated with 10^4 spores $\cdot\text{mL}^{-1}$, obtained from 7 days sporulated media Capellini-Peterson [28], in the centre of each plate. Treatments were incubated at 25°C for 12 days in polyethylene bags. This assessment was carried out with three separate replicates per treatment. The colony radius was measured daily. For each colony, two radii, measured at right angles to one another, were averaged to find the mean radius for that colony. The radial growth rate ($\text{mm}\cdot\text{d}^{-1}$) was subsequently calculated by linear regression of the linear phase for growth and the time at which the line intercepted the x-axis was used to calculate the lag phase in relation to bacteria and water activity.

2.5. Statistical Analysis

Analysis of variance (ANOVA) was made for viable counts in unstressed and water-stressed media, endogenous glycine-betaine and ectoine accumulation and lag phase and growth rate using a SAS program (SAS System for Windows 6.11. SAS Institute, Cary, NC, USA). To establish the significant differences, Duncan's multi-

ple range test ($P < 0.05$) was performed.

3. RESULTS

3.1. Osmotic Potential Effects on Bacterial Growth in Liquid Medium

The viability of *B. amyloliquefaciens* and *M. oleovorans*, in physiological adaptation assays to water stress in broth media under ionic stress, after 24 h of incubation showed statistically significant differences between different water activities (see **Figure 1**). No significant differences in the CFU count was observed between 0.99, 0.98 and 0.97 a_w , the highest count of *B. amyloliquefaciens* was $\log 10.37$ CFUs $\cdot\text{ml}^{-1}$ at 0.99 a_w , and the lowest at $\log 9.23$ CFUs $\cdot\text{ml}^{-1}$ at 0.96 a_w . For *M. oleovorans* the highest cell count was observed at 0.99 a_w , with $\log 7.78$ CFUs $\cdot\text{ml}^{-1}$ and the lowest was $\log 4.93$ CFUs $\cdot\text{ml}^{-1}$ at 0.96 a_w . The number of cells obtained for both bacteria was significantly ($P = 0.05$) decreased at 0.96 a_w .

3.2. Accumulation of Betaine and Ectoine in Bacterial Cells

Endogenous accumulation of ectoine and glycine-betaine in *B. amyloliquefaciens* and *M. oleovorans* cells grown under ionic stress are shown in **Figures 2** and **3**. All cells grown under ionic solute stress (0.99, 0.98, 0.97 and 0.96 a_w) showed an accumulation of both compatible solutes. The highest glycine-betaine accumulation in cells of *B. amyloliquefaciens* was 511.37 $\mu\text{mol}\cdot\text{g}^{-1}$ at 0.98 a_w . At 0.97 a_w accumulation of glycine-betaine was significantly lower with about 232 $\mu\text{mol}\cdot\text{g}^{-1}$ being present (see **Figure 2**). For the second bacterium examined,

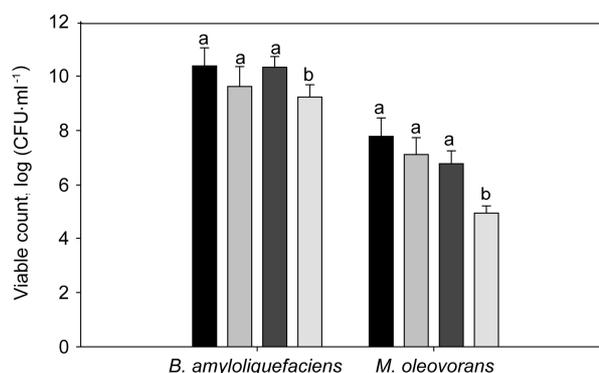


Figure 1. Viability of *B. amyloliquefaciens* and *M. oleovorans* cell growth in stressed liquid media (0.99 a_w ■, 0.98 a_w ■, 0.97 a_w ■ and 0.96 a_w □), after incubation at 30°C for 24 h. Statistical analysis was performed on different water activities. Bars represent means and standard deviation of treatment replications. Data with the same letter for different water activities are not significantly different according to Duncan's multiple range test ($P < 0.05$).

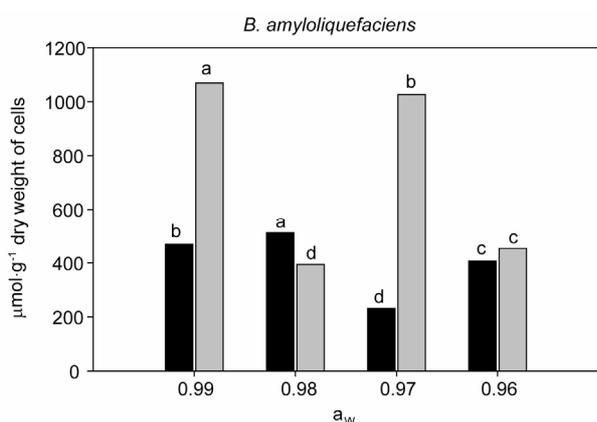


Figure 2. Accumulation of intracellular betaine (■) and ectoine (▒) in *B. amyloliquefaciens* growth for 24 h at 30°C in TSB modified with NaCl at 0.99; 0.98; 0.97 and 0.96 a_w . Results are mean values of three replicates per treatment. Data with the same letter for different water activities are not significantly different for each compatible solute, according to Duncan's multiple range test ($P < 0.05$).

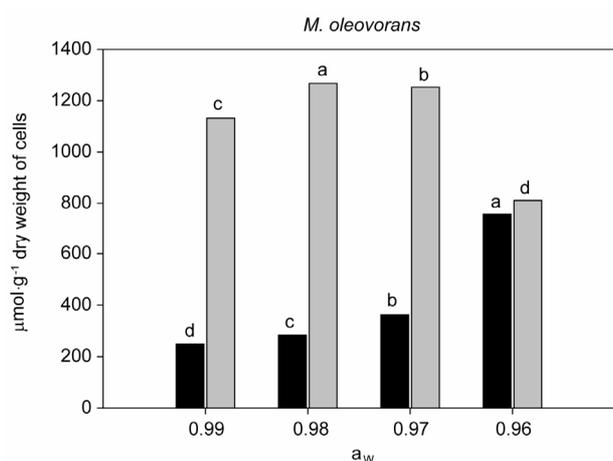


Figure 3. Accumulation of intracellular betaine (■) and ectoine (▒) in *M. oleovorans* growth for 24 h at 30°C in TSB modified with NaCl at 0.99; 0.98; 0.97 and 0.96 a_w . Results are mean values of three replicates per treatment. Data with the same letter for different water activities are not significantly different for each compatible solute, according to Duncan's multiple range test ($P < 0.05$).

M. oleovorans, the glycine-betaine accumulation was higher at 0.96 a_w with 758 $\mu\text{mol}\cdot\text{g}^{-1}$ and this was again significantly higher than other treatments at 0.99, 0.98 and 0.97 a_w . The lower concentrations were found in the 0.99 a_w treatment with 249 $\mu\text{mol}\cdot\text{g}^{-1}$ (see **Figure 3**).

The ectoine accumulation in cells of *B. amyloliquefaciens* was 1070 $\mu\text{mol}\cdot\text{g}^{-1}$ at 0.99 a_w and this was statistically different from the other a_w levels tested (0.98, 0.97 and 0.96), less ectoine was accumulated in these cells at 0.98 a_w . In contrast *M. oleovorans* cells accumulated very high concentrations of ectoine at this a_w level (0.98) with

1268 $\mu\text{mol}\cdot\text{g}^{-1}$. This was shown to be statistically significant from the other a_w levels tested. The lowest ectoine concentration was at 811 $\mu\text{mol}\cdot\text{g}^{-1}$ at 0.96 a_w (see **Figure 3**).

3.3. Effects of Antagonistic Agents on Growth of *F. verticillioides*

The lag phase and growth rate of *F. verticillioides* of paired cultures at different water activities are shown in **Table 1**. Antagonistic agents produced a significant increase in the lag phase and decrease in the growth rate of *F. verticillioides* ($P < 0.001$). The lag phase of control cultures of *F. verticillioides* increased when water activities of the medium decreased in all treatments. Both antagonistic agents caused increase of lag phase of *F. verticillioides*. The lag phase of *F. verticillioides* was increased significantly by interaction with *B. amyloliquefaciens* physiologically adapted at 0.97 and 0.96 a_w , the values ranged between 7.26 h and 8.56 h respectively. *M. oleovorans* also showed significant differences in lag phase between control and interaction with bacteria physiologically adapted at 0.98 and 0.96 a_w with values ranged between 7.42 h and 8.47 h respectively. In addition, the effect of *B. amyloliquefaciens* physiologically adapted on lag phase was higher at the lower water activities assayed.

Changes in a_w alone of control treatments reduced growth by more than 35% between 0.99 and 0.96 a_w . Growth of bacteria *F. verticillioides* interactions was always more inhibited at lower a_w values. *B. amyloliquefaciens* and *M. oleovorans* without physiological adaptation produced the greatest reduction of growth rate of *F. verticillioides* at 0.97 and 0.98 a_w respectively, with values of 0.19 and 0.37 $\text{mm}\cdot\text{h}^{-1}$ respectively. *B. amyloliquefaciens* physiologically adapted gave greater than 55% control at 0.96 a_w , while *M. oleovorans* gave more than 50% control of the pathogen at 0.98 and 0.96 a_w .

4. DISCUSSION

Several previous studies have demonstrated the effectiveness of these biocontrol agents on toxigenic *F. verticillioides* *in vitro*, *in situ* [29-32] and at field level [11-13, 33]. For a biofungicide agent can be successfully marketed, the stages of formulate preparation are important to ensure the survival of biocontrol agents against environmental fluctuations and survival in the stage of storage [34]. Dehydration of a microbial culture and maintenance in low water availability is one of the best ways to formulate microbial agents and enhance survival of biofungicide to facilitate its handling at points of sale and distribution of pesticides. Not all the biological control agents are likely to be subjected to dehydration and can lose viability conditions. In this sense, to improve seed

Table 1. Effect of bacteria—*F. verticillioides* interactions on the lag phase and growth rate at different water activities.

Water activity	Treatments	Lag phase (h)	Growth rate (mm·h ⁻¹)
0.99	Fv-C	1.87 cC	0.59 aA
	Ba-W	4.39 a	0.40 c
	Ba-PA	3.06 b	0.50 b
	Mo-W	2.59 B	0.56 B
	Mo-PA	2.90 A	0.53 C
0.98	Fv-C	3.03 cC	0.54 aA
	Ba-W	4.24 a	0.53 a
	Ba-PA	4.11 b	0.30 b
	Mo-W	4.11 B	0.37 B
	Mo-PA	7.42 A	0.27 C
0.97	Fv-C	3.94 cB	0.55 aA
	Ba-W	4.09 b	0.19 c
	Ba-PA	7.26 a	0.45 b
	Mo-W	4.30 A	0.49 B
	Mo-PA	3.84 C	0.44 C
0.96	Fv-C	4.75 bB	0.38 aA
	Ba-W	5.20 b	0.25 b
	Ba-PA	8.56 a	0.16 c
	Mo-W	4.64 C	0.39 A
	Mo-PA	8.47 A	0.17 B

Fv-C: *F. verticillioides* control, growth without antagonistic agents at different water activities; Ba-W: *B. amyloliquefaciens* without physiological adaptation; Ba-PA: *B. amyloliquefaciens* physiologically adapted; Mo-W: *M. oleovorans* without physiological adaptation; Mo-PA: *M. oleovorans* physiologically adapted. Data with the same letter (small) for different treatment with *B. amyloliquefaciens* are not significantly different, and data with the same letter (capital) for different treatment with *M. oleovorans* are not significantly different according to Duncan's multiple range test ($P < 0.05$).

quality and improve survival during storage biofungicide trade and greater efficiency in the rhizosphere ecosystem, we proceeded to the physiological improvements of *B. amyloliquefaciens* and *M. oleovorans* [18].

This study has demonstrated the ability to synthesize betaine and ectoine under high-osmolality conditions of *B. amyloliquefaciens* and *M. oleovorans* cells, both accumulated large amounts of ectoine, maybe like ectoine synthesis in *Bacillus pasteurii* is a true osmotic effect rather than a salt-specific response, since both ionic and non-ionic osmolytes trigger the production of ectoine [21,35]. Compatible solutes are operationally defined as osmolytes that can be amassed by the cell in exceedingly high concentrations (up to several moles per liter) without disturbing vital cellular functions and the correct folding of proteins [36], the level of compatible solute accumulation is set by the environmental osmolarity [37]. Upon a hypo-osmotic shock, cells can restore the osmotic balance by releasing osmolytes via specific efflux

systems, which are mechanosensitive channels different from the uptake systems [38]. Therefore, this strategy enables organisms for rapid adaptation to an osmotically fluctuating environment by simply adjusting the internal solute pool to counteract the osmolarity of the surrounding environment, these solutes serve as stabilisers of proteins and cell components against the denaturing effects of high ionic strength [39]. Compatible solutes can be accumulated by bacteria by novo synthesis or by transport from the culture medium [20], and is possible to significantly improve environmental stress tolerance of bacterial biocontrol agents by physiologically growing them under conditions which facilitate synthesis of useful compatible solutes such as glycine-betaine and ectoine. This can give cross-protection during formulation against heat stress as well [15]. Studies by Louis *et al.* [40] showed that ectoine and hydroxyectoine stabilized air-dried and freeze-dried *E. coli* cells during drying process. Teixidó *et al.* [15] showed the improvement in

osmotic and thermal tolerance of cells of the biocontrol agent *Pantoea agglomerans* CPA-2. The authors showed that compatible solutes glycine-betaine and ectoine play a critical role in environmental stress tolerance improvement. Our results showed that *M. oleovorans* accumulated the highest content of ectoine when the water activity was reduced to 0.96 a_w . The accumulation of betaine and ectoine in cells of *B. amyloliquefaciens* and *M. oleovorans* can improve the tolerance of both bacteria to water potential modifications and desiccation in the process of freeze drying and production of formulated.

Etcheverry *et al.* [41] demonstrated the potential biocontrol agents *M. oleovorans* and *B. amyloliquefaciens* influenced the growth rate and increased the lag phase of *F. verticillioides* strains assayed *in vitro* at different water activities. In this study we obtained a similar result, consequently we can infer that the physiological adaptation leaving a clear unaffected biocontrol potential, by contrary the reduction effect on the growth rate of *F. verticillioides* was higher with cells previously adapted to low water activities.

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