# Ectoines Mitigate the Reduction of Antagonistic Activity of Bacteria against *Phytophthora drechsleri* Tucker in Saline Conditions

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# ABSTRACT

The objective of the present study was to evaluate antifungal activities of 18 native Bacillus strains against Phytophthora drechsleri Tucker in saline and normal conditions. Besides, the effect of exogenously provided bacterial osmoprotectants (ectoines) on biocontrol activity of three selected Bacillus strains with the highest biocontrol activity and three antagonistic bacteria from two different genera (Pseudomonas fluorescens, Streptomyces rimosus and Streptomyces monomycini) was studied in normal and saline conditions. To reveal the effects of ectoines on the mode of action of antagonism, amylase, protease, lipase, cellulase, chitinase and Hydrogen Cyanide (HCN) activity, biofilm formation and intracellular ectoines of the selected strain (Bacillus amyloliquefaciens UTB96) were investigated in normal and saline conditions. Phylogenetic tree based on the 16S rRNA gene sequences divided Bacillus strains into two groups: one clade included strains that were tolerant up to 5% and the second 13% NaCl. Salt (0.3M NaCl) reduced the antagonistic activity of selected Bacillus strains (10.39-38.34%) and P. fluorescens (25.77%) compared to the control. Exogenously provided ectoines adjusted the biocontrol drop caused by NaCl in Bacillus and Pseudomonas strains and increased biocontrol activity of S. monomycini. Salinity (0.3M NaCl) reduced amylase activity of UTB96 up to 17% and ectoines prevented the reduction. Salt also decreased biofilm formation to about 3-fold and ectoines significantly ameliorated the reduction. The HPLC assay indicated that UTB96 accumulated ectoine and hydroxyectoine 0.16 and 0.10 µg/mg cell dry weight, respectively. Exogenously added ectoine and hydroxyectoine led to a significant increase in UTB96 intracellular ectoines concentrations.

Keywords: Antifungal activity, *Bacillus amyloliquefaciens* UTB96, Hydroxyectoine, biocontrol.

#### **INTRODUCTOIN**

Soil-borne plant pathogens including fungi and *Oomycetes* cause major economic losses for farmers worldwide (Compant *et al.*, 2005). Problems associated with chemical fungicides such as food contamination (Mansour *et al.*, 2008) and environmental pollution (Arias-Estevez *et al.*, 2008) have stimulated interest in biocontrol strategies (Olubukola, 2010). Antagonistic microorganisms, in particular fluorescent Pseudomonads (Vidhysaekaran and Muthamilan, 1999), gram-positive

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*Bacilli* (Asaka and Shoda, 1996; Prashar *et al.* 2013; Bakhshi *et al.* 2018) and *Streptomycetes* (Sabaratnam and Traquair, 2002) have been intensively used in order to control soil-borne fungi.

There are some reports on biocontrol of Phytophthora drechsleri (Maleki et al. 2011; Karimi et al. 2012). P. drechsleri infects a wide range of host plants and causes damping off and root rot in the Cucurbitaceae and Solanaceae, and is recognized difficult to control as in greenhouse and field (Farr and Rossman, 2010).

Salinity is one of the major environmental stresses that cause osmotic stress and reduction in plant growth and crop productivity (Rengasamy, 2010). Because saline conditions influence viability and biocontrol activity of the microorganisms, it is essential that biocontrol agents be adapted to the stress factors (Paulitz and Belanger, 2001). There are several studies about the biological control of plant diseases (Spadaro and Gullino, 2005; Errakhi et al., 2007; Abeysinghe, 2009), but none of them address the problems associated with salinity. The use of salt-tolerant biocontrol agents may be beneficial in order to protect plants against pathogenic fungi in saline soils.

Rangarajan *et al.* (2003) showed that salt tolerant *Pseudomonas* spp. exhibited antibiosis under both natural and saline soil conditions and suppressed bacterial leaf blight and sheath blight diseases in rice. Besides, they found that salt treatment resulted in increased levels of disease suppression by only some of these isolates.

It seems that viability and growth ability at saline conditions (i.e. salt tolerance) are not enough to maintain or increase biocontrol activity of a microorganism in the presence of salt. The results of Karimi *et al.* (2012) indicated that salt has different effects on bacterial traits, which are known as mode of antagonism. They showed that in the presence of salt, production of siderophore and protease and amylase activity of salt tolerant biocontrol isolates were increased. In contrast, chitinase, beta glucanase, amylase and cellulase activity and biosynthesis of salicylic acid were decreased significantly.

Microorganisms have evolved different mechanisms in order to cope with saline environments. One of these mechanisms. which is widespread in the domain of bacteria, is accumulation or de novo synthesis of low molecular weight, highly water-soluble organic osmolytes, so-called compatible solutes (Galinski and Truper, 1994; Pastor et al., 2010). In order to balance internal osmolality in hyperosmotic circumstances, microorganisms accumulate these solutes and expel them when exposed to hypoosmotic environment (Ajouz et al., 1998). Ectoines (ectoine and its hydroxyl derivative, hydroxyectoine) are the most abundant studied compatible solutes (da Costa et al., 1998) and distributed in different bacteria including y-proteobacteria like Pseudomonas (Roberts, 2005), several Streptomyces (Sadeghi et al., 2014b) and Bacillus species (Kuhlmann and Bremer, 2002). As well as their osmoprotective function, these compatible solutes affect the stability and correct folding of proteins under stress conditions (Roychoudhury and Haussinger, 2012).

In a previous study, Sadeghi *et al.* 2014b; showed that in *Streptomyces* cells grown with hydroxyectoine, the amount of intracellular free amino acids was increased in salt stressed condition compared to unstressed condition. These results suggest the possibility of a supplementary role of ectoines to improve structure and function of the cells in stressful environments as well as their important role as osmoprotectants. (Sadeghi *et al.*, 2014b)

In the present study, attempts were made to select salt tolerant strains of Bacillus with antifungal activity high against Ρ. drechsleri. In addition, the effect of exogenously provided bacterial osmoprotectants (ectoines) on biocontrol activity of three different genera of bacteria (Bacillus, Pseudomonas and Streptomyces) in normal and saline conditions was

investigated. Further experiments were made to reveal the effect of ectoines on mode of action of antagonism of the selected *Bacillus* strain in normal and saline conditions.

### MATERIALS AND METHODS

# Microorganisms and Growth Conditions

P. drechsleri (strain 44d), from Agricultural Biotechnology Research Institute of Iran Culture Collection (ABRIICC), was originally isolated from naturally infected sugar beet roots and routinely grown on standard potato dextrose agar and stored in a broth medium containing 15% glycerol at -20°C. Eighteen from sugar Bacillus strains beet and Pseudomonas fluorescens UTPF5 isolated from onion [the University of Tehran Microorganisms Collection (UTMC)], with high antagonistic activity against Fusarium culmorum, Neocosmospora solani (Khezri et al., 2011) and Rhizoctonia solani (Bagheri et al., 2016), were used in the present study. These bacteria were routinely cultivated in Nutrient Agar (NA) and stored in broth medium (NB) containing 15% glycerol at -20°C. Streptomyces rimosus C-2012 and S. monomycini 41801 from Agricultural Biotechnology Research Institute of Iran Culture Collection (ABRIICC) (Sadeghi et al., 2014a and b) were propagated and on ISP2 (MYA) medium maintained (containing 10 g  $L^{-1}$  malt extract, 4 g  $L^{-1}$  yeast extract, 4 g  $L^{-1}$  glucose and 18 g  $L^{-1}$  agar, extract, 4 g L adjusted to pH 7.2) at 29 C. Ectoine (Fluka) and hydroxyectoine (Fluka) stocks were prepared and, after passing through a 0.2-micron filter, were added to sterile media. For all the following experiments ectoine, hydroxyectoine (1 mM in liquid medium and 2 mM in solid medium) and sterile water (control) with 0 or 0.3M NaCl were used as treatments.

# Salt Tolerance Assay for Bacillus Strains

The cells of each *Bacillus* strain were suspended in sterile saline solution (0.9%

NaCl) and adjusted to a concentration of  $10^6$  CFU mL<sup>-1</sup>. Fifty µL of this suspension was plated on solid medium supplemented with different NaCl concentrations (0, 5, 7, 10, 11, 12 and 13%) (Karimi *et al.* 2012)

#### **Phylogenetic Studies**

Based on the 16S rRNA gene sequencing of 18 Bacillus strains, a phylogenetic tree was constructed. The used sequences were extracted from genbank database accession numbers HQ234327-32, HQ267752-54, HO267764, HO267755, HO267757-60 and HQ267762-63 (Khezri et al., 2011). The software package MEGA 7 (Kumar et al., 2016) was used after multiple alignments of the sequence data with CLUSTAL W (Thompson et al., 1994). The tree was constructed using the UPGMA method and Sokal, 1973). Bootstrap (Sneath analyses were used to evaluate the stability of relationships based on 500 resamplings (Felsenstein, 1985).

#### **Dual Culture Test**

Fungal inhibition test was performed according to Keel *et al.* (1996) and Yuan and Crawford (1995) as follows:  $5 \mu$ L of the overnight culture of each strain was separately spotted at the edge of the Potato Dextrose Agar (PDA) plate. A 0.6 cm diameter PDA plug of *P. drechsleri* mycelia was placed in the center of each plate. Plates were incubated at 28°C for three days. A positive response was any inhibition zone around the fungal colony, regardless of the size of the zone.

# **Enzyme and Hydrogen Cyanide Activity and Biofilm Formation**

The effect of ectoines on amylase, protease, lipase, cellulase, chitinase and Hydrogen Cyanide (HCN) activity and biofilm formation of UTB96 was investigated in the presence and absence of NaCl. Alpha-amylase activity was determined according to the method of Li *et al.* (2011) with some modifications. Selected strain was cultured onto solid media containing 1% soluble starch, 0.4% glucose, and 0.4% of yeast extract and incubated for 3 days at 28°C. The halo-forming ability was detected by staining with iodine solution (containing 20 g KI and 2 g I<sub>2</sub> per liter) and the ratio of the halo zone diameter to colony diameter was measured.

Extracellular protease activity was determined in Skim Milk Agar (SMA) according to Maurhofer *et al.* (1995). This culture medium contained 15 g L<sup>-1</sup> skim milk powder, 0.5 g L<sup>-1</sup> yeast extract and 13.5 g L<sup>-1</sup> agar. After incubation at 28°C for 48 hours, protease activity was expressed as clearing zone around bacterial colony. The ratio of the clear zone diameter to colony diameter was calculated.

Lipase activity was measured on tween 80 medium containing 10 g  $L^{-1}$  peptone, 5 g  $L^{-1}$  NaCl, 0.2 g  $L^{-1}$  CaCl<sub>2</sub>, 1% v/v tween 80 and 15 g  $L^{-1}$  agar according to Raymond and Crisan (1975).

Carboxymethylcellulase (CMCase) activity was determined by a medium with Carboxymethylcellulose (CMC) as the sole carbon source (Berger and Reynolds, 1958). The bacteria were grown on CMC agar containing 0.4 g  $L^{-1}$  KH<sub>2</sub>PO<sub>4</sub>, 0.02 g  $L^{-1}$ CaCl<sub>2</sub>, 0.02 g  $L^{-1}$  NaCl, 0.02 g  $L^{-1}$ FeSO<sub>4</sub>.7H<sub>2</sub>O, 2.5 g  $L^{-1}$  CMC, 15 g  $L^{-1}$  Agar. The CMC agar plates were incubated at 28°C for 10 days to allow the secretion of cellulase. At the end of the incubation, to visualize the hydrolysis zone, the agar medium was flooded with an aqueous solution of Congo red (1 mg L<sup>-1</sup>) for 20 minutes. The Congo red solution was then poured off, and the plates were further treated by flooding with 1M NaCl for 15 minutes. To indicate the cellulase activity, diameters of clear zone around colonies on CMC agar were measured. The ratio of the clear zone diameter to colony diameter was calculated.

Chitinase activity was determined according to the method of Hsu and

Lockwood (1975) with some modifications. Selected strain spot-seeded on a chitin agar medium containing 0.4% colloidal chitin and 1.5% agar. The colloidal chitin was prepared according to Berger and Reynolds (1958). Plates were incubated for 5 days at 28°C. Chitinase activity was shown by the clear halo around colonies. The ratio of the clear zone diameter to colony diameter was calculated.

HCN activity was assessed on NB medium containing 4.4 g L<sup>-1</sup> of glycine with indicator paper [Whatman filter paper, soaked in 0.5% (w/v) picric acid and 2% (w/v) sodium carbonate] and plates were incubated at 28°C for 48 to 72 hours. Presence of HCN caused the indicator paper to turn from yellow to cream, light brown, dark brown, and brick-red (Principe *et al.*, 2007).

Biofilm formation assay in polystyrene microtiter plates was done by using a modified version of the procedure described by O'Toole and Kolter (1998). Selected strain was grown on solid medium, inoculated into NB for 16 hours, and resuspended in NB to a final cell density of OD (625 nm)= 0.8. The bacterium suspension (20 µL) was inoculated into 180 µL NB in triplicate wells of 96-well polystyrene plates. Additional triplicate wells were not inoculated with bacterium as the negative control. Plates were incubated at 28°C for 24 hours without shaking. Unattached cells were removed by inversion of the plate, followed by washing with sterile physiological serum 3 times (each 300 µL well<sup>-1</sup>). The remaining adherent bacteria were fixed to the plates by adding (250 µL) ethanol 96% for 15 minutes. After removing the ethanol, plate was stained for 5 minutes with 200 µL well<sup>-1</sup> of crystal violet (2%). Excess stain was removed by inversion of the plate followed by two washings (each 300 µL well<sup>-</sup> <sup>1</sup>) with distilled water. Adherent cells were decolorized with a 33% acetic acid (200 µL well<sup>-1</sup>) for 5 minutes to release the dye into solution and the amount of dye (proportional to the density of adherent cells) was quantified with plate reader (OD 492 nm).

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# HPLC Analysis of Ectoine and Hydroxyectoine from Cell Extracts

Bacterial cells harvested were by centrifugation and were lyophilized. The dry weight of the cells was determined, and 10 mg lyophilized cells were extracted with 570 µL extraction of an mixture [methanol/chloroform/water, 10:5:4 (v/v/v)] by vigorous shaking for 5 min. Equal volumes (170 µL) of chloroform and water were then added. The mixture was again shaken for 10 min; phase separation was enhanced by centrifugation in an Eppendorf table-top centrifuge at 13,000 rpm for 30 minutes. The hydrophilic top layer containing compatible solutes was recovered, and then dried via speed vacuum concentration in an Eppendorf Concentrator plus at 37°C. The pellet was suspended in 100 µL of water and 400 µL of Acetonitrile (ACN); if necessary, this suspension was diluted to suitable concentrations in 80% (v/v) ACN and was used for HPLC analysis. Twenty microliters of each sample were analyzed on a GROM-SIL 100 Amino-1PR, 125- by 4-mm (3 µm) column (GROM, Herrenberg, Germany), then, ectoine and hydroxyectoine were monitored by their absorbance at 210 nm by using a UV/VIS detector (SYKAM. Gilching. Germany). The solvent employed for compatible solute separation was 80% (v/v) ACN. Chromatography was carried out isocratically at a flow rate of 1 mL min<sup>-1</sup> and at 20°C (Kuhlmann and Bremer, 2002).

#### **Statistical Analyses**

Data were analyzed for significance by analysis of variance (ANOVA), followed by Least Significant Difference (LSD) (P< 0.05), with the SAS general linear model procedure (SAS Institute, Cary, NC). Normal distribution and homogeneity of variances were checked beforehand. All experimental design was set as factorial based on Complete Randomized Design (CRD). There were three replicates for each treatment.

#### RESULTS

# Salt Tolerance Assay for Bacillus Strains

The maximum salt tolerances of the 18 strains of *Bacillus* were different and they were divided into two main groups. Nine strains (BS13, 15, 16, 20, 22, 24, 26, 27 and UTB96) tolerated up to 13% NaCl, while the maximum tolerance of others (BS11, 12, 14, 18, 19, 21, 25, 29 and 30) was 5% and had no growth in 10% NaCl. NaCl (10%) led to a growth reduction, even in strains that tolerated up to 13% (Table 1). Phylogenetic tree based on the *16S rRNA* gene sequences divided these strains into two groups, high and moderate salt tolerant (Figure 1).

# Antagonistic Activity against P. drechsleri

Eight strains had the ability to control *P. drechsleri* more than 30% in the dual culture assay (Table 1). Two strains (BS11, BS14) and UTB96 with highest antagonistic activity were chosen for further experiments.

# Effect of Ectoines and NaCl on Bacterial Antagonistic Activity against *P. drechsleri*

NaCl (0.3M) led to a reduction in antagonistic activity of BS11, BS14, UTB96 and UTPF5 compared to the control (0M NaCl). Inhibition percentages caused by BS11, BS14, UTB96, and UTPF5 decreased from 59.46 to 33.69%, from 61.90 to 23.56%, from 61.18 to 50.79%, and from 35 to 10.50%, respectively. In the case of *Streptomyces* strains, NaCl had positive effect on antagonistic activity: inhibition percentage caused by strain 41801 and

strains	Maximum salt tolerance (%)	Bacteria growth <sup>a</sup>			
		10% NaCl	0% NaCl		
BS11	5	0	3		
BS12	5	0	3		
BS13	13	2	3		
BS14	5	0	3		
BS15	13	2	3		
BS16	13	2	3		
BS18	5	0	3		
BS19	5	0	3		
BS20	13	2	3		
BS21	5	0	3		
BS22	13	2	3		
BS24	13	2	3		
BS25	5	0	3		
BS26	13	2	3		
BS27	13	2	3		
BS29	5	0	3		
BS30	5	0	3		
UTB96	13	2	3		

Table 1. Maximum NaCl concentration tolerance and effect of NaCl on growth of Bacillus strains.

<sup>*a*</sup> 0 means no growth and (1) represent low, (2) moderate and (3) high growth, respectively. All data are mean values of three independent experiments



**Figure 1.** Phylogenetic relationship between *Bacillus* strains, based on *16S rRNA* gene sequences developed with the Clustal W program in MEGA 7 and constructed using the UPMGA method with 500 bootstrap replicates

C-2012 increased from 58.58 to 70.34% and from 27.15 to 80.57%, respectively.

Ectoine and hydroxyectoine increased antagonistic activity at salt conditions (0.3M NaCl) in all tested bacteria except C-2012. In 0.3M NaCl, inhibition percentages caused by BS11, BS14, UTB96, UTPF5, and 41801 were 33.69, 23.56, 50.79, 10.50, and 70.34% respectively, which increased to 34, 29, 56.14, 20.60, and 90% in presence of ectoine and increased to 53.60, 29, 59.25, 22.85%, and 90% in presence of hydroxyectoine. In the case of C-2012, ectoines had no effect on biocontrol activity in presence of 0.3M NaCl. Strain UTB96 showed the highest antagonistic activity and ectoines had significant effects on this activity in the normal and saline conditions compared to other Bacillus strains. UTB96 was selected for further studies to assess the mode of action of antagonism in the presence of ectoines (Table 2).

# Effect of Ectoines and NaCl on Population, Enzyme, and Hydrogen Cyanide Activity and Biofilm Formation of UTB96

In normal condition (0M NaCl), ectoine increased bacterial population up to 13% and hydroxyectoine had no significant effect on it. Salt (0.3M NaCl) had no effect on bacterial population, while ectoine and hydroxyectoine in saline conditions led to 14.5 and 9% increase, respectively (Figure 2).

Ectoine and hydroxyectoine had no effect on amylase activity in normal condition (0 M NaCl). Salinity reduced amylase activity up to 17% and ectoines prevented this reduction in presence of 0.3M NaCl, such that amylase activity in the presence of ectoines and NaCl was as much as the control.

There was a positive relation between salinity and protease activity in all three treatments (water, ectoine and hydroxyectoine). The salt-caused increase of the protease activity in water, ectoine and

**BS15**  $6.0 \pm 1.0$  $47.5 \pm 6.9$ **BS16 BS18** 0.0 **BS19** 0.0 **BS20**  $40.9 \pm 6.0$ **BS21**  $7.0 \pm 1.5$ **BS22** 45.4 ±5.0 **BS24**  $10.0 \pm 1.0$ **BS25** 0.0 UBS26  $5.0 \pm 0.2$ **BS27**  $52.5 \pm 8.6$ **BS29**  $5.0\pm0.3$ **BS30** 0.0 UTB96  $61.2 \pm 0.2$ 

Bacillus strains

**BS11** 

**BS12** 

**BS13** 

**BS14** 

<sup>*a*</sup> Growth inhibition percentage of *P*. *drechsleri* by *Bacillus* strains was measured based on dual culture assay. All data are mean values of four independent experiments±Standard Deviation



Figure 2. The effect of ectoine, hydroxyectoine, and NaCl on population of *B. amyloliquefaciens* UTB96. All data are mean values of three independent experiments, error bars indicate standard deviation. Bars with different letters are statistically different at 5% (LSD, P < 0.05)

Table	2.	Antagonistic	activity	of	Bacillus
strains					

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Inhibition (%)

 $59.5 \pm 0.0$ 

 $39.1 \pm 6.0$ 

0.0

 $61.9 \pm 4.1$ 

1089

hydroxyectoine was 17, 11, and 19%, respectively.

There was a negative relation between salinity and lipase activity, the rate of reduction was 40%. Hydroxyectoine and ectoine increased lipase activity at the normal and in saline conditions, respectively.

In normal conditions (OM NaCl), ectoine hydroxyectoine decreased biofilm and formation approximately 3-fold. Salinity decreased biofilm formation about 3-fold, as conditions, ectoines well. In salt significantly ameliorated salt-caused reduction in biofilm formation (Figure 3).

UTB96 was not able to produce cellulase and chitinase, neither in 0 nor in 0.3M NaCl, and ectoines did not stimulate its activity. UTB96 was able to produce a trace of HCN, but ectoines and NaCl did not affect its activity (Data are not shown).

# Effect of Ectoines and NaCl on Ectoine and Hydroxyectoine Accumulation

Presence of NaCl (0.3M) led to a significant growth in ectoine accumulation in water treatment. Presence of ectoine increased accumulation significantly, ectoine but presence of hydroxyectoine did not affect ectoine accumulation. Combination of ectoine and salt resulted in an increase in ectoine accumulation compared to ectoine alone. Hydroxyectoine accumulation increased in the presence of NaCl, but this increase was not significant. Presence of hydroxyectoine resulted significant increase in in a hydroxyectoine accumulation, but presence of did not affect hydroxyectoine ectoine accumulation. Hydroxyectoine accumulation increased using the hydroxyectoine and salt combination compared to hydroxyectoine alone (Figure 3).

#### DISCUSSION

The ability of *Bacillus* species to tolerate high concentrations of NaCl has been

reported previously (Principe et al., 2007; Kumar et al., 2014). In this study, 18 strains of Bacillus were analyzed based on their salt tolerance. Fifty percent of tested strains showed high salt tolerance (up to 13% NaCl) and others were sensitive to NaCl concentrations of over 5%. The phylogenetic tree based on the 16S rRNA gene sequences divided these strains into two groups, highly and moderately salt tolerant. These data suggest that the ability of *Bacillus* strains to tolerate high concentrations of NaCl has been the result of a vertical transfer event. Classification of **Streptomyces** and Cyanobacteria based on salt tolerance reported by Tresner et al. (1968) and Nubel et al. (2000), respectively. It was reported that Cyanobacteria strains that were tolerant to similar cocentrations of salt were placed in a tight monophyletic cluster apart from all other Cyanobacteria. Understanding the lineal inheritance of salt tolerance could screening microorganisms facilitate of producing valuable materials that are involved in salt tolerance or have an important activity such as biocontrol at salt conditions.

Although the growth ability of all *Bacillus* strains decreased in high concentrations of NaCl in accordance with other reports (Palomino *et al.*, 2009; Brill *et al.*, 2011), low NaCl concentrations (0.3M) did not reduce the bacterial population in the selected strain. Palazzini *et al.* (2009) showed that the viability of *B. subtilis* RC 218 in NaCl modified media was similar to the unmodified control. According to Sartori *et al.* (2012), there was no significant difference in CFU (Colony Forming Units) count of *Bacillus* strain UTB96 at the presence of 0 or 0.3M NaCl.

Forty-four percent of the tested strains had antagonistic ability against P. *drechsleri*. Antagonistic ability of these strains against F. *culmorum* was reported by Khezri *et al.* (2011), previously. Several strains belonging to genus *Bacillus* were reported effective for the biocontrol of multiple plant disease caused by soil borne pathogens (Asaka and Shoda, 1996).

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**Figure 3.** The effect of ectoine, hydroxyectoine, and NaCl on enzyme activity, biofilm formation, and ectoines accumulation of *B. amyloliquefaciens* UTB96. All data are mean values of three independent experiments. Error bars indicate standard deviation and bars with different letters are statistically different at 5% (LSD, P<0.05)

Understanding the impact of salt on biocontrol mechanisms of plant diseasesuppressive microorganisms is an essential step towards improving the level and reliability of their activity in natural conditions. Although there are several reports on osmotic stress adaptation in biocontrol bacteria by accumulation of compatible solutes (Canamas et al., 2007; Paul and Neir, 2008; Palazzini et al., 2009; Khare et al., 2011; Sartori et al., 2012), there are few reports on direct effects of salt on biocontrol ability. Positive effects of salt on antagonistic activity in Pseudomonas (Rangarajan et al., 2003) and Streptomyces (Karimi et al., 2012; Sadeghi et al., 2017) was reported previously (Table 3). Khare et al. (2011) showed that P. fluorescens EKi inhibited growth of Macrophomina phaseolina in the presence of up to 0.4M NaCl due to production of biocontrol metabolites in these conditions. In current study, 0.3M NaCl decreased inhibition percentage of P. drechsleri by Bacillus strains. Negative influence of NaCl on biocontrol ability of tested strains was not identical. Range of this negative effect was 17 to 62%. In the presence of 0.3M NaCl, inhibition percentage of the fungus by strain UTB96 decreased to 17%, while bacterial growth in this condition did not decrease significantly.(Table 4).

Exogenously added ectoines adjusted biocontrol drop caused by 0.3M NaCl in all tested Bacillus strains. (Table 4) Besides Bacillus strains, we tested P. fluorescens UTPF5 and the same result was obtained. In 0.3M NaCl, presence of ectoines led to an increase in the inhibition percentage of P. drechsleri by this strain 2-times more than the control. We also tested S. rimosus C-2012 and S. monomycini 41801 whose biocontrol activity was increased in the presence of NaCl. In the case of S. monomycini, ectoines increased this activity in both the absence and presence of NaCl. Biocontrol ability of S. rimosus did not change when it was exposed to exogenous ectoines (Table 4). Sadeghi et al. (2014b) showed ectoine accumulation in these strains

previously Strain C-2012 accumulated ectoines approximately 1,000-fold more than UTB96, which may guide us to the hypothesis that there is a threshold for ectoine and hydroxyectoine accumulation to adjust salinity-caused reduction of antagonistic activity. Therefore. exogenously added ectoines did not improve antagonistic activity of S. rimosus C-2012 because it naturally has the ability to produce appropriate amounts of ectoines (more than the probable threshold). In the cases of B. amyloliquefaciens UTB96, S. monomycini 41801 and P. fluorescens UTPF5, ectoines accumulation would not reach the threshold, so, exogenously added ectoines led to an increase in biocontrol activity, which may be due to ectoines accumulation.

We previously showed the effects of ectoines on fungal growth inhibition by *P. fluorescens* UTPF5 in the salt stress conditions (Kamaly *et al.*, 2016). In the present study, we found that ectoines mitigated salinity-caused reduction in antagonistic activity of three different genera of bacteria. The reason for this adjustment may be the effect of ectoines on one or more antagonism mechanisms.

Amylase activity decreased with salinity in accordance with Karimi *et al.* (2012) who reported a negative effect of NaCl on amylase activity by *Streptomyces* isolates. Our results showed that ectoines stimulated amylase activity in 0.3M NaCl and, therefore, stimulation of amylase activity by ectoines in salinity may be one of the reasons for ameliorating the fungal growth inhibition.

Protease activity in *S. caespitosus* increased exponentially with increasing NaCl (Inouye *et al.*, 2007). Karimi *et al.* (2012) reported that protease activity in *Streptomyces* isolates increased after adding salt. Our results also showed that salinity induced protease activity in UTB96 and there was no significant difference between ectoine, hydroxyectoine, and water treatments in either presence or absence of 0.3M NaCl.

**Table 3.** Maximum salt tolerance and amounts of ectoine and hydroxyectoine accumulation ( $\mu$ g mg<sup>-1</sup> dw) in *Bacillus amyloliquefaciens* UTB96, *P. fluorescens* UTPF5 and 2 *Streptomyces* strains (41801 and C-2012) at salt conditions (0.3 M).

Bacterial strains	Maximum tolerance (%)	salt	Ectoine (µg mg <sup>-1</sup> dw)	Hydroxyectoine ( µg mg <sup>-1</sup> dw)	Reference
UTB96	13		0.16	0.10	Present study
UTPF5	5		0.20	0.50	Present study
C-2012	13		95.33	99.60	Sadeghi et al., 2014b
41801	13		35.47	95.49	Sadeghi et al., 2014b

**Table 4.** Antagonistic activity (based on dual culture assay) of 2 *Bacillus* strains (BS11, BS14), strain UTB96, *P. fluorescens* UTPF5 and 2 *Streptomyces* strains (41801 and C-2012) against *P. drechsleri* in absence (0M) or presence (0.3M) of NaCl.<sup>*a*</sup>

	Inhibition (%)							
strains		0M NaCl			0.3M NaCl			
	W	Е	HE	W	Е	HE		
BS11	59.4 ±0.0	59.4 ±1.0	59.68 ±3.4	$33.6 \pm 0.2$	$34.0 \pm 6.0$	$53.6 \pm 1.0$		
BS14	$61.9 \pm 4.1$	$71.7 \pm 4.0$	$68.0 \pm 2.0$	$23.5 \pm 2.0$	$29.0 \pm 3.0$	$29.0 \pm 1.5$		
UTBA96	61.1 ±0.1	$57.6 \pm 1.4$	$71.9 \pm 1.0$	$50.7 \pm 5.0$	$56.1 \pm 1.0$	$59.2 \pm 1.5$		
UTPF5	$35.0 \pm 0.5$	$40.8 \pm 1.5$	45.7 ±2.0	$10.5 \pm 0.2$	$20.6\pm2.0$	$22.8 \pm 2.2$		
41801	$58.58 \pm 4.0$	$60.0 \pm 5.0$	$60.0 \pm 0.2$	$70.3 \pm 0.2$	$90.0 \pm 5.0$	$90.0 \pm 4.0$		
C-2012	$27.1 \pm 3.2$	$27.4 \pm 4.0$	$27.5 \pm 0.20$	$80.5 \pm 3.0$	$80.5 \ \pm 0.6$	$80.6 \pm 5.0$		

<sup>*a*</sup> *P. drechsleri* was cultured on potato dextrose agar plates at 25°C for seven days, then, a mycelial disc was cut and placed 1.5 cm from the edge of a fresh plate. Bacteria grown in 2mM of Ectoine (E), Hydroxyectoine (HE) or Water (W) as control were toothpick inoculated 1.5 cm from the opposite edge of the same plate, then the inhibition percentages were measured.  $\pm$  is represented for standard deviation.

The salt and ectoine led to a decrease in lipase activity and this trend was unlike Karimi *et al.* (2012) study, in which salt increased lipase activity in *Streptomyces* isolates. According to our results, ectoines could not inhibit salinity-caused reduction in lipase activity.

Salinity led to a noticeable drop in biofilm formation similar to O'Toole and Kolter (1998) results. They showed that biofilm formation of *P. fluorescens* WCS365 decreased up to four-fold at concentrations of NaCl at or above 0.2M. In current study, ectoine and hydroxyectoine treatments significantly adjusted this drop. Therefore, another probable reason for antagonistic activity adjustment in the presence of NaCl would be the positive effect of ectoines on biofilm formation.

The HPLC analysis indicated that the strain UTB96 is capable of ectoine and

hydroxyectoine accumulation. Sartori et al. (2012) reported ectoine accumulation in B. amyloliquefaciens. Our results represented a positive relation between ectoine salinity in В. accumulation and amyloliquefaciens UTB96. There was a 50% increase in ectoine in the presence of 0.3M NaCl. A linear relationship between the ectoine content of the cells and the salinity of the growth medium was observed in B. pasteurii (Kuhlmann and Bremer, 2002), Virgibacillus pantothenticus (Kuhlmann et al., 2008), and Salibacillus salexigens 2008). addition, (Bursy et al., In exogenously added ectoine and hydroxyectoine led to an increase in ectoine hydroxyectoine and accumulation, respectively, which is in agreement with Sadeghi et al. (2014b).

As a conclusion, there is no relationship between the amounts of salt tolerance in

tested strains and salinity-caused reduction antagonistic activity because this in reduction was observed both in highly and moderately salt tolerant strains. In addition, both ectoine and hydroxyectoine, and especially the latter, have a positive effect activity on antagonistic of Bacillus. Streptomyces and Pseudomonas strains Ectoine under salt stress. and hydroxyectoine probably adjust salinitycaused reduction in antagonistic activity by affecting the amylase activity and biofilm formation in B. amyloliquefaciens UTB96, but proving how ectoines and these mechanisms are related needs more research. Based on the results obtained in this study, accumulation of ectoines reduces the negative effects of salt on bacterial antifungal activity.

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# کاهش فعالیت آنتاگونیستی باکتریها در برابر Phytophthora drechsleri Tucker در شرایط شوری، توسط اکتوئینها تخفیف می یابد

ا. كمالي، ا. صادقي، ز. ارقند، و م. احمد زاده

چکیدہ

هدف این مقاله ارزیابی فعالیت ضدقارچی ۱۸ استرین بومی از جنس باسیلوس (۱۷ عدد از گونه Bacillus amyloliquefaciens در شرایط شور و معمولی است. افزون براین، اثر Phytophthora drechsleri Tucker در شرایط شور و معمولی است. افزون براین، اثر حفاظت کننده های اسمزی (اکتوئین ها) با منبع خارجی، بر فعالیت کنترل زیستی سه استرین منتخب جنس حفاظت کننده های اسمزی (اکتوئین ها) با منبع خارجی، بر فعالیت کنترل زیستی سه استرین منتخب جنس حفاظت کننده های اسمزی (اکتوئین ها) با منبع خارجی، بر فعالیت کنترل زیستی سه استرین منتخب جنس معاطر با توان بیشینه فعالیت کنترل زیستی و سه باکتری آنتا گونیست از دو جنس دیگر Streptomyces rimosus ، Pseudomonas fluorescens و Streptomycini و معمولی بررسی شد. به منظور آشکارسازی اثر اکتوئین ها بر طرز عمل آنتا گونیستی، آمیلاز، پروتئاز، لیپاز، سلولاز، کیتیناز و سیانید هیدروژن، تشکیل بیوفیلم و شرایط شور و معمولی بررسی شد. درخت فیلوژنیکی بر اساس توالیهای ژن AS rRNA)، در شرایط شور و معمولی بررسی شد. درخت فیلوژنیکی بر اساس توالیهای ژن ماده درصد و کلاد شریزهای باسیلوس را به دو گروه تقسیم کرد: یک کلاد شامل استرین هایی که تا ۵ درصد و کلاد آنتا گونیستی استرین های منتخب باسیلوس (۱۰/۳۹ مولار) فعالیت آنتا گونیستی استرین های منتخب باسیلوس (۱۰/۳۹ پر مولار) فعالیت آنتا گونیستی استرین های منتخب باسیلوس و معرو ی و معالی درصد و کند. مولار) و معایت کنترل زیستی کاهش، کنترل زیستی کاهش یافته آنتا گونیستی استرین های با شاهد کاهش داد. اکتوئین های با منبع خارجی کاهش، کنترل زیستی کاهش یافته توسط NaCl در استرین های باسیلوس و سودوموناس را تخفیف و فعالیت کنترل زیستی . . *monomycini* را افزایش دادند. شوری (NaCl ، مولار)، فعالیت آمیلاز در JUTB96 را تا ۱۷ درصد کاهش داد و اکتوئین ها از این کاهش جلوگیری کردند. نمک همچنین تشکیل بیوفیلم را در حدود یک سوم کاهش داد و اکتوئین ها به طور قابل توجهی این کاهش را تخفیف دادند. آزمون HPLC نشان داد که UTB96، اکتوئین و هیدروکسی اکتوئین را به ترتیب به میزان ۱۶/۰ و ۰/۰ میکرو گرم در میلی گرم وزن خشک سلول تجمع میدهد. منابع خارجی اکتوئین و هیدروکسی اکتوئین، منجر به افزایش غلظتهای داخل سلولی اکتوئین ها در UTB96 شدند.