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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 as difficult to control 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 γ-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 high antifungal activity against *P. 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

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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 *Bacillus* strains from sugar 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 maintained on ISP2 (MYA) medium (containing 10 g L^{-1} malt extract, 4 g L^{-1} yeast extract, 4 g L^{-1} glucose and 18 g L^{-1} agar, 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^{-1}. 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, HQ267764, HQ267755, HQ267757-60 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 (Sneath and Sokal, 1973). Bootstrap 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 µL 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

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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_2 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^{-1} skim milk powder, 0.5 g L^{-1} yeast extract and 13.5 g L^{-1} 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} $CaCl_2$, 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_2PO_4, 0.02 g L^{-1} $CaCl_2$, 0.02 g L^{-1} NaCl, 0.02 g L^{-1} $FeSO_4.7H_2O$, 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^{-1}) 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^{-1} 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 $well^{-1}$). 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 $well^{-1}$ of crystal violet (2%). Excess stain was removed by inversion of the plate followed by two washings (each 300 µL $well^{-1}$) with distilled water. Adherent cells were decolorized with a 33% acetic acid (200 µL $well^{-1}$) 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 were harvested by centrifugation and were lyophilized. The dry weight of the cells was determined, and 10 mg lyophilized cells were extracted with 570 µL of an extraction 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^{-1} 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

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

strains	Maximum salt tolerance (%)	Bacteria growth[a]	
		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

[a] 0 means no growth and (1) represent low, (2) moderate and (3) high growth, respectively. All data are mean values of three independent experiments

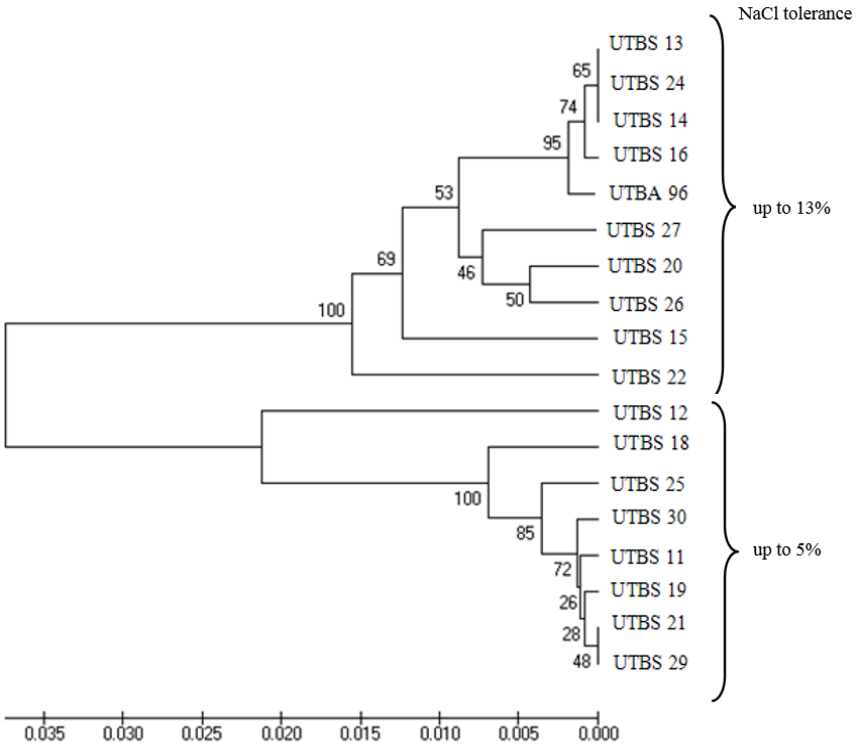

Figure 1. Phylogenetic relationship between *Bacillus* strains, based on *16S rRNA* gene sequences developed with the Clustal W program in MEGA 7 and constructed using the UPMGA method with 500 bootstrap replicates

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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).

Table 2. Antagonistic activity of *Bacillus* strains against *P. drechsleri.*[a]

Bacillus strains	Inhibition (%)
BS11	59.5 ±0.0
BS12	39.1 ± 6.0
BS13	0.0
BS14	61.9 ±4.1
BS15	6.0 ±1.0
BS16	47.5 ±6.9
BS18	0.0
BS19	0.0
BS20	40.9 ±6.0
BS21	7.0 ±1.5
BS22	45.4 ±5.0
BS24	10.0 ±1.0
BS25	0.0
UBS26	5.0 ±0.2
BS27	52.5 ± 8.6
BS29	5.0 ±0.3
BS30	0.0
UTB96	61.2 ±0.2

[a] 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

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

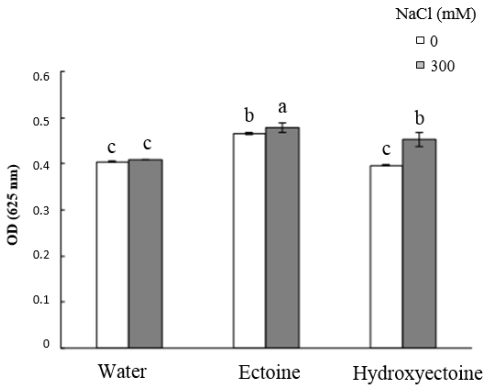

Figure 2. The effect of ectoine, hydroxyectoine, and NaCl on population of *B. amyloliquefaciens* UTB96. All data are mean values of three independent experiments, error bars indicate standard deviation. Bars with different letters are statistically different at 5% (LSD, P< 0.05)

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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 (0M NaCl), ectoine and hydroxyectoine decreased biofilm formation approximately 3-fold. Salinity decreased biofilm formation about 3-fold, as well. In salt conditions, ectoines 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 ectoine accumulation significantly, 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 in a significant increase in hydroxyectoine accumulation, but presence of ectoine did not affect hydroxyectoine 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 facilitate screening of microorganisms 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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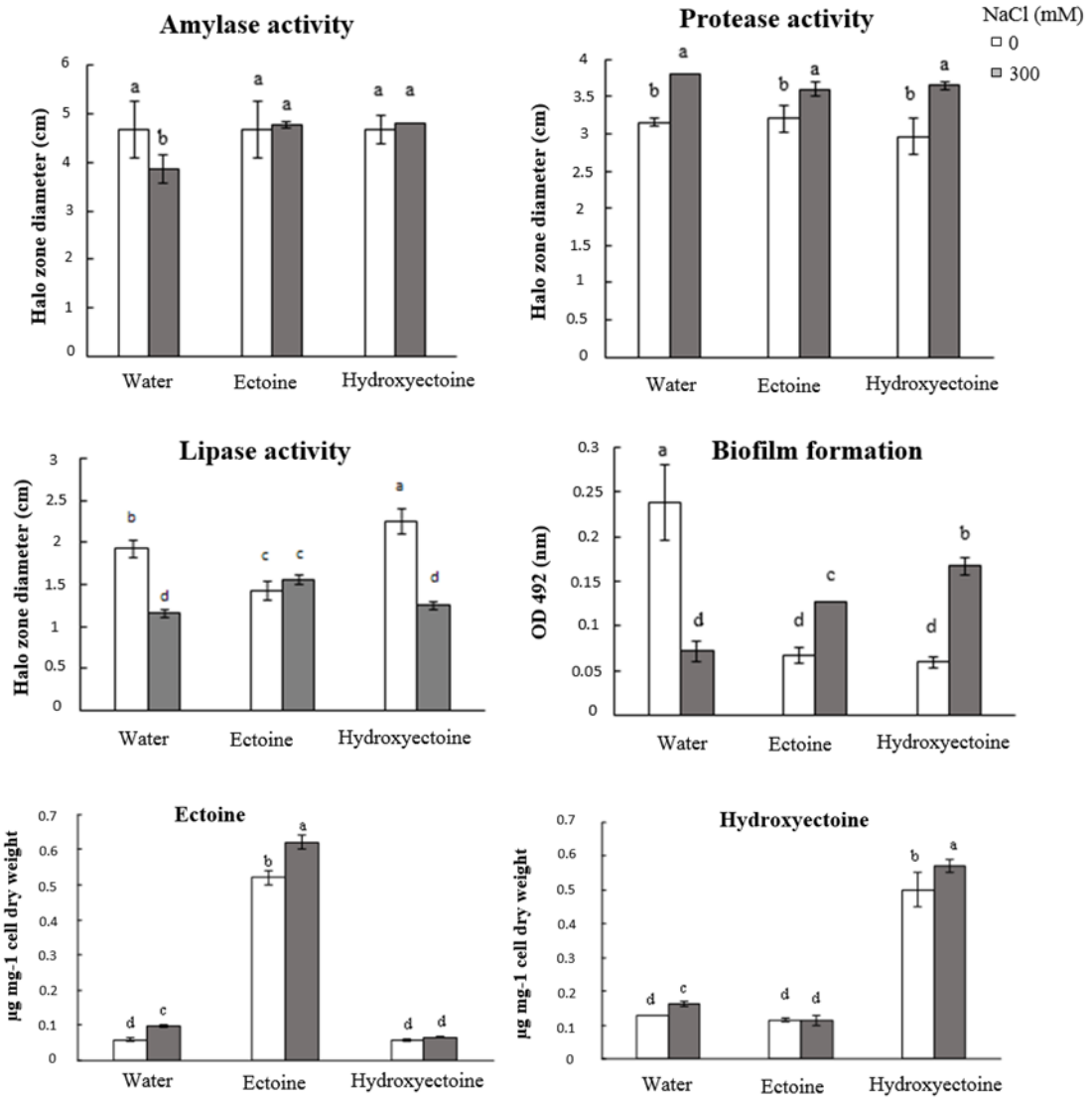

Figure 3. The effect of ectoine, hydroxyectoine, and NaCl on enzyme activity, biofilm formation, and ectoines accumulation of *B. amyloliquefaciens* UTB96. All data are mean values of three independent experiments. Error bars indicate standard deviation and bars with different letters are statistically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

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Understanding the impact of salt on biocontrol mechanisms of plant disease-suppressive 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.

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Table 3. Maximum salt tolerance and amounts of ectoine and hydroxyectoine accumulation (µg mg^{-1} 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 salt tolerance (%)	Ectoine (µg mg^{-1} dw)	Hydroxyectoine (µg mg^{-1} 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.[a]

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

[a] *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. ± 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 accumulation and salinity in *B. 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* (Bursy *et al*., 2008). In addition, exogenously added ectoine and hydroxyectoine led to an increase in ectoine and hydroxyectoine 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

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tested strains and salinity-caused reduction in antagonistic activity because this 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 on antagonistic activity of *Bacillus*, *Streptomyces* and *Pseudomonas* strains under salt stress. Ectoine and hydroxyectoine probably adjust salinity-caused 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 می‌یابد

ا. کمالی، ا. صادقی، ز. ارقند، و م. احمد زاده

چکیده

هدف این مقاله ارزیابی فعالیت ضدقارچی ۱۸ استرین بومی از جنس باسیلوس (۱۷ عدد از گونه *Bacillus subtilis*و یک عدد از گونه *Bacillus amyloliquefaciens*) در برابر *Phytophthora drechsleri* Tucker در شرایط شور و معمولی است. افزون‌براین، اثر حفاظت‌کننده‌های اسمزی (اکتوئین‌ها) با منبع خارجی، بر فعالیت کنترل زیستی سه استرین منتخب جنس باسیلوس با توان بیشینه فعالیت کنترل زیستی و سه باکتری آنتاگونیست از دو جنس دیگر (*Pseudomonas fluorescens* ، *Streptomyces rimosus* و *Streptomyces monomycini*) در شرایط شور و معمولی بررسی شد. به منظور آشکارسازی اثر اکتوئین‌ها بر طرز عمل آنتاگونیستی، آمیلاز، پروتئاز، لیپاز، سلولاز، کیتیناز و سیانید هیدروژن، تشکیل بیوفیلم و اکتوئین‌های خارج سلولی در استرین منتخب (*Bacillus amyloliquefaciens* UTB96) در شرایط شور و معمولی بررسی شد. درخت فیلوژنیکی بر اساس توالی‌های ژن 16S rRNA، استرین‌های باسیلوس را به دو گروه تقسیم کرد: یک کلاد شامل استرین‌هایی که تا ۵ درصد و کلاد دیگر استرین‌هایی که تا ۱۳ درصد NaCl را تحمل می‌کردند. نمک (NaCl ۰/۳ مولار) فعالیت آنتاگونیستی استرین‌های منتخب باسیلوس (۱۰/۳۹-۳۸/۳۴ درصد) و *P. fluorescens* (۲۵/۷۷ درصد) را در مقایسه با شاهد کاهش داد. اکتوئین‌های با منبع خارجی کاهش، کنترل زیستی کاهش‌یافته توسط NaCl در استرین‌های باسیلوس و سودوموناس را تخفیف و فعالیت کنترل زیستی *S.*

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monomycini را افزایش دادند. شوری (NaCl ۰/۳ مولار)، فعالیت آمیلاز در UTB96را تا ۱۷ درصد کاهش داد و اکتوئین‌ها از این کاهش جلوگیری کردند. نمک همچنین تشکیل بیوفیلم را در حدود یک سوم کاهش داد و اکتوئین‌ها به طور قابل توجهی این کاهش را تخفیف دادند. آزمون HPLC نشان داد که UTB96، اکتوئین و هیدروکسی اکتوئین را به ترتیب به میزان ۰/۱۶ و ۰/۱۰ میکروگرم در میلی‌گرم وزن خشک سلول تجمع می‌دهد. منابع خارجی اکتوئین و هیدروکسی اکتوئین، منجر به افزایش غلظت‌های داخل سلولی اکتوئین‌ها در UTB96 شدند.

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