The potential use of ectoine produced by a moderately halophilic bacteria *Chromohalobacter salexigens* KT989776 for enhancing germination and primary seedling of flax "*Linum usitatissimum* L.".under salinity conditions

ABSTRACT

The similarity between plant and microbial cells encourage the use of microbial metabolites of halophilic bacteria for alleviation of salt stress in plants. In the current research work, a compatible solute ectoine extracted from a moderately halophilic bacteria *Chromohalobacter salexigens* KT989776 was used to enhance flax germination and primary seedling under different levels of salinity. Two successive experiments including germination in petri plats under six levels of salinity (0, 3, 5, 7, 9 and 11 dS.m-1) and a pot experiment under three irrigating water salinity levels (2, 3 and 4) with two types of ectoine application (spray and soil addition) were conducted. Germination parameters were recorded for the first experiment while fresh and dry weight of plants and peroxidase activity in addition to sodium-potassium ratio were estimated in the pot experiment. Also, ectoine accumulation in plants was detected using HPLC. Results of LC-MS proved the production of ectoine by *C. salexigens* KT989776 and ectoine enhanced significantly all germination parameters of flax seeds, decreased sodium accumulation in plant, increased potassium content, and lowered peroxidase and phenol oxidase activity. Also, HPLC analysis proved that ectoine was detected in all treated samples while not detected in non-treated control.

Keywords: halophilic; Chromohalobacter; compatible solutes; ectoine; flax; germination.

1. INTRODUCTION

A biotic stress factors such as salinity and drought are the main reasons that limit plant growth and productivity through disturbing the intracellular water balance (**Hernández** 2019, Boyer 1982). To alleviate the effect of these stresses, most plants synthesize and accumulate osmolytes or the so-called compatible solutes (Wani et al. 2016; Brown 1976), which are neutral under physiological pH of the plant cell with low molecular mass, high solubility in water, and are nontoxic to the cell even

when accumulated at a high concentration. Compatible solutes are represented by different biomolecules such as Polyols (e.g. glycerol, sorbitol, and mannitol), nonreducing sugars (e.g. sucrose and trehalose), and amino acids (e.g. glutamine, proline, and betaine) (Nakayama et al. 2000).

The effect of osmoprotectants is generally not species-specific and alien osmoprotectants can be introduced into plants to protect their new host (Kathuria et al. 2009).

On the other hand, microorganisms in a hyper-osmotic environment follow one of the two known strategies to balance the osmotic pressure between cells and the surrounding environment, the salt in- cytoplasm mechanism and accumulation of polar, highly water-soluble, low molecular weight organic osmolytes, compatible solutes (Kraegeloh and Kunte 2002). which can be found in methanohalophilus as well as some phototropic and aerobic chemoheterotrophic bacteria (Galinski and Truper 1994; Kai et al. 1991). Types of compatible solutes accumulated by microbial cells resample in most cases that are present in plant cells include amino acids, amino acid derivatives, such as ectoine and sugars. Microorganisms accumulate these molecules through de novo synthesis or a direct uptake from the environment (Bremer and Kramer 2000).
The survival of plants in harsh environments depends on many factors including the presence of effective salt-tolerance PGPR and its secondary metabolites (Singh et al. 2019).
Ectoine 1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid serves as compatible solute in some halophilic bacteria (Nakayama et al. 2000) ectoine has a stabilizing effect on biomolecules as proteins and nucleic acids, bacteria synthesize and accumulate ectoine in order to protect themselves from drastic conditions especially osmotic stress. the rate of ectoine accumulation inside the bacterial cell is proportionally increased with the increase of outer osmotic pressure (Grammann et al. 2002)
Chromohalobacter salexigens is a moderately halophilic bacterium adapted at high salt concentration by production and accumulation of ectoine (Oren et al. 2005)
Flax seed (*Linum usitatissimum* L.) is a globally important agricultural crop used for its oil (Berti et al. 2010). and stem fiber (El-Nagdy and Nassar 2010). Germination and seedling emergence of flax may be affected by environmental conditions as temperature, moisture and salinity in addition to sowing depth and seedbed conditions (Kurt and Bozkurt 2006). Salinity may cause delayed germination and emergence, low survival, irregular crop stand and lower biomass yield due to biochemical, morphological and physiological changes (Isayenkov and Maathuis 2019 , Muhammad and Husain 2010). NaCl decreased germination percentage, speed of germination and seedling dry matter in different plants (Mondal et al. 2015; Nasri et al. 2011).
In the present work, the function of ectoine as a compatible solute in plant cells was investigated in addition to examining the role of ectoine in water stress tolerance in flax. It was found that ectoine conferred increased hyperosmotic tolerance in flax seed germination and primary seedling stage.

2. MATERIAL AND METHODS

2.1.Microorganism and growth conditions

Chromohalobacter salexigens KT989776 was isolated previously by one of our team (Husseiny et al. 2015) and cultivated in Sehgal and Gibbons complex broth medium (SGCb medium) (Sehgal and Gibbons 1960) contains (g/L): casmino acids, 7.5, yeast extract, 10, starch, 5, KCl, 2.0, sodium citrate, 3.0 $MgSO_4.7H_2O$, 20, NaCl, 200, $MnCl_2.4H_2O$, 0.05 and $FeCl_2.nH_2O$. 0.01. The medium was adjusted to pH 7.0 by 0.5 M NaOH and HCl before autoclaving at 121◦C for 15 min.

Flaks (250 ml containing 100 ml SCG medium) was inoculated with 3ml 24h old culture and incubated at 30 ◦C for 48 h on rotary shaker.

2.2.Ectoine extraction

Cells of *C. salexigens* were collected by centrifugation at 6000 rpm under cooling and the pellets were washed twice by phosphate buffer containing the same NaCl concentration (200gl^{-1}) of SCG medium. Washed cells were resuspended overnight in 80%, v/v ethanol. The suspension was centrifuged under cooling and the supernatant was used for further investigations (Zhang et al. 2009)

2.3. Batch fermentations

The working volume of the fermentor was 10 L. The fermentor was filled with 6 L of SGC fermentation medium which was set according to experimental conditions of shake flask and inoculated with 300 mL *C. salexigens* KT989776 shake flask cultures. The temperature was set at 30 ◦C and the pH was 7.0. The dissolved oxygen level was never less than 40%. After 48 h cells were harvested by centrifugation using CEPA® Z 41 High-Speed Tubular Centrifuge, then ectoine was extracted as mentioned before. The pellets were extracted as mentioned above.

2.4. Spectroscopic analysis

2.4.1. HPLC determination

The ectoine was detected in alcoholic extract of plant samples by HPLC with a TSK-GEL reversed-phase column (Tosoh, Japan) the mobile phase was 50 mmol l^{-1} potassium phosphate buffer at 35°C with flow rate 1 ml min^{-1}. The UV detector was adjusted to 210 nm. The retention time of ectoine was compared by commercially available ectoine, purity >97%, Biomol, Hamburg, Germany (Zhang et al. 2009).

2.4.2. LC–MS analysis

HPLC (Waters 2695 separation module) and a mass spectrometer (Quattro Micro Waters Co., USA) were used to identify and quantify ectoine. HPLC conditions: A 2.1 × 150 mm Xterra MS C18 reversed-phase column was used. 5µl samples were eluted with (80%, v/v) methanol and the flow rate was adjusted at 0.2 ml min^{-1} at 35°C and UV detector at 210 nm. The effluent from the LC column was passed to mass spectrometer (Waters, USA). Mass spectrometer was conditioned a follow: source temperature, 120°C; electrospray ionization (ionization mode ES+); detector, Waters 2996 photodiode array.

2.5. Germination experiment

2.5.1. Plant material and NaCl stress treatment:

The seeds of flax (*L. usitatissimum* L.) variety, ‘‘evian 1’’ were kindly supplied by the “Egyptian Company for Flax & Its Products’’. For germination, seeds were divided in two groups, the first one considered as control and soaked for 2 h in distilled water, while the second was soaked in 500 ppm ectoine solution for 2h also. The seeds were then placed in Petri dishes with double layer filter paper initially moistened with a solution of the respective salt concentration 0, 3, 5, 7, 9 and 11 dS.m^{-1}. (Table1). The Petri dishes were incubated for 10 days in the dark at room temperature (25 ± 2°C). Each treatment consisted of 20 seeds per Petri dish in three replicates. Seeds with emerged radicle were counted daily.

Table 1: Description of used treatments in the germination experiments within the current study.

Treatment	Details
	Ectoine
S_E	Seeds soaked in 500 ppm ectoine solution
S_d	Seeds soaked in distilled Water
	Salinity
g_0	Seeds germinated in 0 $dS.m^{-1}$ solution
g_3	Seeds germinated in 3 $dS.m^{-1}$ solution
g_5	Seeds germinated in 5 $dS.m^{-1}$ solution
g_7	Seeds germinated in 7 $dS.m^{-1}$ solution
g_9	Seeds germinated in 9 $dS.m^{-1}$ solution
g_{11}	Seeds germinated in11 $dS.m^{-1}$ solution
	Interaction between salinity and ectoine
T0 (S_E + g_0)	Seeds soaked in dis. Water and germinated in 0 $dS.m^{-1}$ solution
T1 (S_d + g_0)	Seeds soaked in dis. Water and germinated in 3 $dS.m^{-1}$ solution
T2 (S_E + g_3)	Seeds soaked in dis. Water and germinated in 5 $dS.m^{-1}$ solution
T3 (S_d + g_3)	Seeds soaked in dis. Water and germinated in 7 $dS.m^{-1}$ solution
T4(S_E + g_5)	Seeds soaked in dis. Water and germinated in 9 $dS.m^{-1}$ solution
Ts (S_d + g_5)	Seeds soaked in dis. Water and germinated in 11$dS.m^{-1}$ solution
T6 (S_E + g_7)	Seeds soaked in 500 ppm ectoine solution and germinated in 0 $dS.m^{-1}$ solution
T7 (S_d + g_7)	Seeds soaked in 500 ppm ectoine solution and germinated in 3 $dS.m^{-1}$ solution
T8 (S_E + g_9)	Seeds soaked in 500 ppm ectoine solution and germinated in 5 $dS.m^{-1}$ solution
T9 (S_d + g_9)	Seeds soaked in 500 ppm ectoine solution and germinated in 7 $dS.m^{-1}$ solution
T10 (S_E + g_{11})	Seeds soaked in 500 ppm ectoine solution and germinated in 9 $dS.m^{-1}$ solution
T11 (S_d + g_{11})	Seeds soaked in 500 ppm ectoine solution and germinated in 11 $dS.m^{-1}$ solution

2.5.2. Germination and growth parameters:

Germination parameters: Mean Germination Time **(MGT)**, Coefficient of Velocity of Germination **(CVG)**, First Day of Germination**(FDG)**, Germination Rate Index **(GRI)**, Final Germination Percentage **(FGP %)**, Vigor Index (**VI**), Energy of Emergence (**EE**) and Germination Speed(**GS**) were calculated according to (Kader 2005)

Dry weights (DW) were measured from 6 seedlings on 10th day after sowing. Plant material was dried at 60°C for 2 days and dry weights (DW) were measured.

2.6. Pot experiment

A pot experiment was carried out at Sakha Research Experimental Station, Kafer El SheiKh Govern., Agri. Res Cent., Egypt during the winter season of 2017. The phsico–chemical properties of the experimental soil were estimated according to Black et al. 1965 table (2).

Table 2: Physicochemical characteristics of experimental soil

Character	Value
pH (1: 2.5 soil:water suspension)	8.4
Electrical conductivity (dS m^{-1})	4.0
Soil organic matter (%)	1.2
Soluble cations *(*meq L^{-1}*)*	
Na^+	23.1
K^+	0.4
Mg^{2+}	5.3
Ca^{2+}	11.7

Soluble anions *(*meq L^{-1}*)*	
SO_4^{2+}	19.8
Cl^-	15.0
HCO_3^-	5.8
CO_3^{2-}	0.0
Available macronutrients (mg kg^{-1})	
N	24.3
P	18.7
K	93.8
Particle size distribution (%)	
Coarse sand	28.4
Fine sand	13.0
Silt	22.4
Clay	36.2
Texture grade	Sandy clay loam

The experiment was conducted under three levels of irrigating water salinity 2, 3 and 4 $dS.m^{-1}$. To detect the effect of ectoine, two treatments (soil addition and spray of plants after 1 weeks and 3 weeks of planting with 5ml of 500 ppm ectoine solution for each pot) in addition to control were conducted under the three levels of salinity (Table 3). After 40 days the following parameters were measured: fresh and dry weight of plants, K^+ and Na^+ content, peroxidase and phenol oxidase enzymes. In addition, ectoine uptake and accumulation in plant cells was detected.

Table 3: Description of used treatments in the germination experiments within the current study.

Treatment	Details
	Salinity
W_2	Plants irrigated with 2 $dS.m^{-1}$ water
W_3	Plants irrigated with 3 $dS.m^{-1}$ water
W_4	Plants irrigated with 4 $dS.m^{-1}$ water
	Ectoine
E_c	Pots did not recive ectoine
E_s	5ml of 500 ppm ectoine solution was sprayed for each pot
E_d	5ml of 500 ppm ectoine solution was added to the soil for each pot
	Interaction between salinity and ectoine
T1 (W_2 + E_c)	Plants irrigated with 2 $dS.m^{-1}$ water and didn't recive ectoine
T2 (W_2 + E_s)	Plants irrigated with 2 $dS.m^{-1}$ water and sprayed with 5ml of 500 ppm ectoine
T3 (W_2 + E_d)	Plants irrigated with 2 $dS.m^{-1}$ water and 5ml of 500 ppm ectoine was added to soil
T4(W_3 + E_c)	Plants irrigated with 3 $dS.m^{-1}$ water and didn't recive ectoine
Ts (W_3 + E_s)	Plants irrigated with 3 $dS.m^{-1}$ water and sprayed with 5ml of 500 ppm ectoine
T6 (W_3 + E_d)	Plants irrigated with 3 $dS.m^{-1}$ water and 5ml of 500 ppm ectoine was added to soil
T7 (W_4 + E_c)	Plants irrigated with 4 $dS.m^{-1}$ water and didn't recive ectoine
T8 (W_4 + E_s)	Plants irrigated with 4 $dS.m^{-1}$ water and sprayed with 5ml of 500 ppm ectoine
T9 (W_4 + E_d)	Plants irrigated with 4 $dS.m^{-1}$ water and 5ml of 500 ppm ectoine was added to

2.7. Na+, K+ and ectoine analysis

The plant samples were dried at 60 °C then grounded into a fine powder. Samples were extracted with 10 ml of 1 N HCl for 24 h at room temperature. The Na^+ and K^+ concentrations of the extracts were determined using a flame photometer (Moghaieb et al. 2007).

2.8. Enzymes assay:

Fresh plant biomass of various treatments was homogenized in liquid nitrogen and suspended in chilled 0.1 M phosphate buffer (pH 7.0). The homogenate was filtered and the filtrate was centrifuged at 4000 rpm for 10 min at 4 °C. The final volume of the supernatant was adjusted to 10 mL and served as the enzymes source.

Peroxidase (EC 1.11.1.7) and polyphenol oxidase (EC 1.14.18.1) were assayed following the method described by Kar and Mishra (1976). The color intensity was read at 430 nm, and the enzyme activity was expressed as the change in the optical density/gram fresh weight/hour.

2.9. Statistical analysis

Data analysis was performed using Microsoft Excel 2010 (mean values), and the statistical analysis was conducted in two way complete randomized block design with three replicates using co state software program.

3. RESULTS

3.1. Confirmation of ectoine production

To confirm that ectoine was really synthesized and excreted into the conversion solution, LC-MS and LC-MS/MS analyses were performed Fig (1 A.B)

The same HPLC retention time was observed for authentic ectoine and the compound present in the alcoholic extract of *C. salexigens* cells (2.43 min) and the spectra obtained by tandem mass spectrometry were also consistent (Fig. 1); a signal was detected at 143 (*m*/*z*), which is in good agreement with the molecular weight of ectoine (142). Signals of ectoine and its induced dissociation in the spectra obtained by tandem mass spectrometry occurred at 143, 97, 68, 56, and 42 (*m*/*z*) (Figs. 1b) (Galinski et al. 1985). Retention time and tandem MS fragmentation patterns in comparison with the standards confirmed the identities of the detected compound as ectoine.

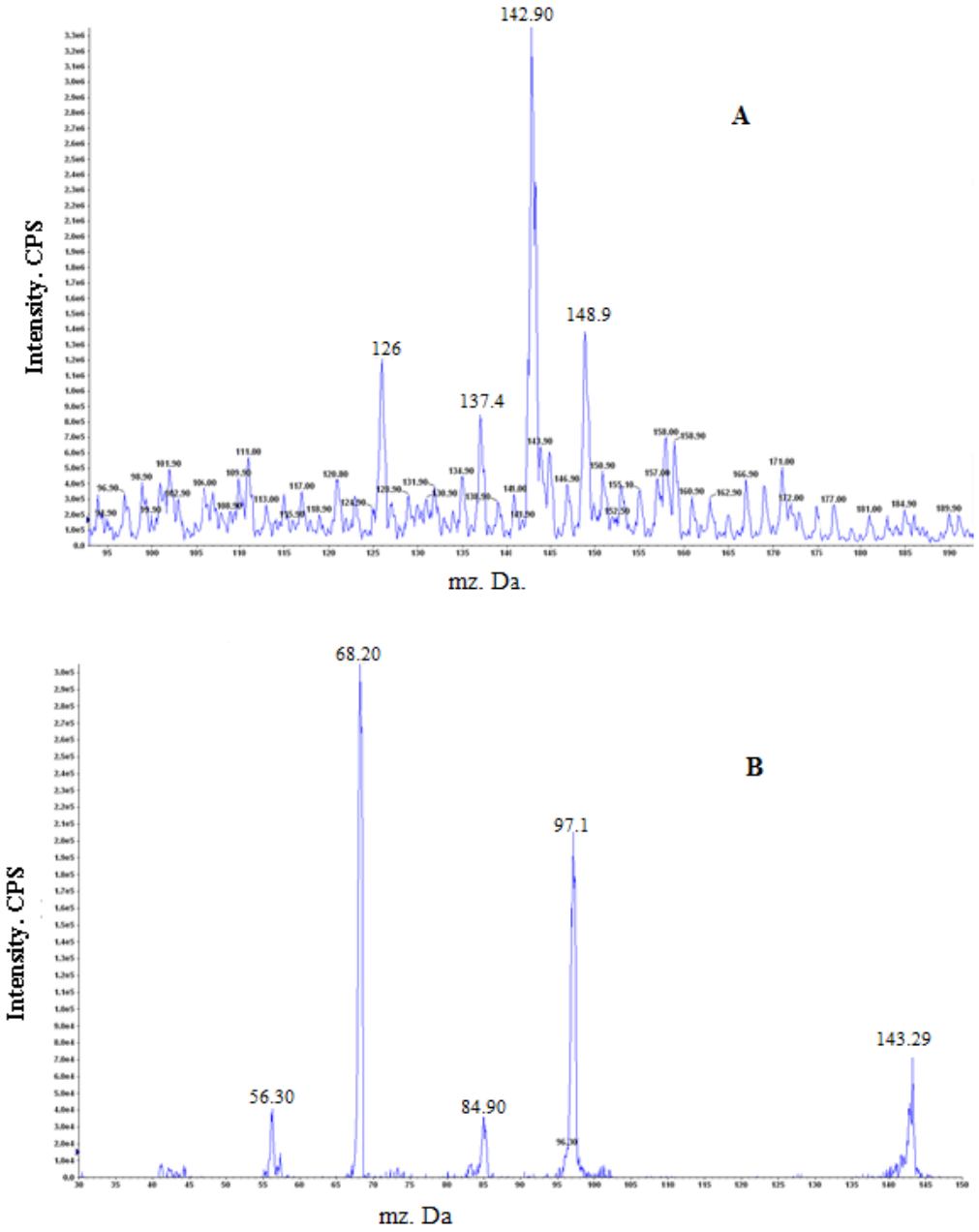

Figure 1: Spectra signal of A) LC analysis (pseudo-molecular ion at m/z 143 and B) LC-MS/MS analysis (product ions at m/z 143.3, 97.0, 68.2, 55.9, and 44.0) of ectoine extracted from *Chromohalobacter salexigens* cells

3.2. Germination of flax seeds

In general, the increased salinity lead to a negative effect on all germination parameters. The addition of ectoine enhanced germination under all salinity levels.

The effect of ectoine addition on MGT recorded in table (4) indicated that the treatment with ectoine lowered the MGT to about 6.7%. The lower the MGT, the faster a population of seeds has germinated. The greatest effect of ectoine was recorded for salinity level 0 and the lowest was recorded under salinity level 7 $dS.m^{-1}$.

The CVG gives an indication of the rapidity of germination. It increases when the number of germinated seeds increases and the time required for germination decreases.

The average increase in CVG, a result of ectoine treatment, was 7.6 %. As in the case of MGT, the highest effect was recorded at level 0.

The effect of ectoine treatment on first day of germination (FDG) was not significant.

Germination Rate Index (GRI) was significantly affected by ectoine treatment where the average increase was about 11% over control. Higher GRI values indicate higher and faster germination.

The average increase in hypocotyl length was about 40 % over control when flax seeds were germinated in presence of ectoine. The effect of ectoine was more obvious in case of radical elongation where the average increase reached about 62% over control. In addition, the ectoine was more effective as salinity increased where the radical length of ectoine-treated seeds reached about 2-3 times more than non-treated seeds under higher salinity levels 7, 9 and 11 $dS.m^{-1}$.

The effect of ectoine on the fresh and dry weight: Final Germination Percentage (FGP) and Energy of Emergence (EE) of germinated seeds followed the same trend of radical length where the effect was more obvious under higher salinity levels.

Table 4: Effect of ectoine solution 500 ppm on flax germination parameters under different levels of salinity (0, 3, 5, 7, 9 and 11 $dS.m^{-1}$)

Treatments	MGT	CVG	FDG	GS	EE	FGP	GRI	VI	Hypocotyl length(cm)	Radical length (cm)	Fresh weight (g)	Dry weight (g/seed)
	Salinity											
g_0	1.89	53.10	31.67	36.36	94.17	99.16	63.19	1081.5	5.08	5.82	0.054	0.0070
g_3	1.93	52.07	25.00	34.64	94.17	97.50	59.18	979.66	4.77	5.27	0.037	0.0063
g_5	2.08	48.27	24.17	32.27	89.17	94.17	55.31	625.75	2.68	3.95	0.033	0.0053
g_7	2.17	46.50	25.83	31.61	85.83	93.33	54.79	564.83	2.75	3.23	0.030	0.0047
g_9	2.74	36.77	22.50	24.71	80.00	87.50	42.94	414.83	2.01	2.73	0.023	0.0040
g_{11}	2.87	34.89	20.83	21.27	73.33	79.16	41.92	297.91	1.73	1.88	0.020	0.0030
L.S.D 0.01	**0.28****	**5.89****	**N.S**	**3.15****	**9.14****	**8.13****	**7.04****	**68.69****	**0.58****	**0.48****	**0.005****	**0.001****
	Ectoine											
S_E	2.36	43.61	21.67	28.54	82.78	89.44	50.16	520.03	2.64	2.928	0.029	0.0041
S_d	2.20	46.93	28.33	31.74	89.44	94.16	55.62	801.47	3.70	4.700	0.037	0.0060
L.S.D 0.01	**0.16****	**3.4****	**5.94****	**5.28****	**5.28****	**4.8***	**4.06***	**39.66****	**0.32****	**0.28****	**0.003****	**0.001****
	Interaction between ectoine and salinity											
T0	2.00	50.11	26.67	34.62	93.33	98.33	59.56	976.33	4.70	5.23	0.052	0.0060
T1	1.78	56.08	36.67	38.11	95.00	100.0	66.83	1186.7	5.47	6.40	0.056	0.0080
T2	2.00	50.13	21.67	33.53	91.67	96.67	56.92	835.17	4.07	4.57	0.033	0.0053
T3	1.86	54.00	28.33	35.74	96.67	98.33	61.44	1124.2	5.47	5.97	0.041	0.0073
T4	2.17	46.34	21.67	31.11	86.67	93.33	53.08	482.00	2.27	2.90	0.029	0.0047
Ts	2.00	50.21	26.67	33.42	91.67	95.00	57.56	769.50	3.10	5.00	0.037	0.0060

T6	2.17	46.33	20.00	29.82	83.33	90.00	50.81	400.67	2.10	2.33	0.025	0.0033
T7	2.17	46.66	31.67	33.41	88.33	96.67	58.78	729.00	3.40	4.13	0.035	0.0060
T8	2.89	34.79	20.00	23.31	76.67	86.67	40.50	279.83	1.50	1.73	0.018	0.0033
T9	2.58	38.76	25.00	26.10	83.33	88.33	45.39	549.83	2.53	3.73	0.027	0.0047
T10	2.95	33.93	20.00	18.86	65.00	71.67	40.11	146.17	1.23	0.80	0.015	0.0020
T11	2.79	35.84	21.67	23.68	81.67	86.67	43.72	449.67	2.23	2.97	0.025	0.0040
L.S.D 0.01	**0.39****	**8.33****	**N.s**	**3.94****	**14.98****	**13.12****	**9.95****	**97.14****	**0.79****	**0.68****	**0.0064****	**0.002****

(MGT) Mean Germination Time, **(CVG)** Coefficient of Velocity of Germination, **(FDG**) First Day of Germination , (**GS**) Germination speed , (**EE**) Energy of emergence, **(FGP %)** Final Germination Percentage **(GRI)** Germination Rate Index, (**VI**) vigor index

3.3. Pot experiment

Pot experiment was conducted to evaluate the potential effect of ectoine addition on the growth and survival of flax under different levels of salinity. Results (table 5) show that sodium was less accumulated in ectoine-treated plants compared to control which accumulated higher concentrations. However, potassium was detected with high concentrations in ectoine-treated plants. The uptake of both sodium and potassium was proportional to salinity levels. The role of ectoine in alleviation of salt stress on flax was further proved by measuring peroxidase and phenol oxidase activity which was higher in control than treated plants. All the above findings were reflected on the morphological characters of the plants where the dry weight of treated plant was higher compared to control.

Table 5: Effect of ectoine treatment on biomass yield and stress markers of flax after 40 days of planting.

Treatment	Na ppm	Kppm	Na/K	POD Δ 430 m^{-1} g^{-1} fresh wighet	PPO Δ 490 m^{-1} g^{-1} fresh wighet	Dry weight (mg $plant^{-1}$)
	Salinity					
W_2	8233.3	27466.7	0.30	0.198	0.100	426.667
W_3	8483.3	21861.1	0.40	0.236	0.102	284.833
W_4	9622.2	17922.2	0.55	0.271	0.102	184.333
LSD 0.01	**496.2****	**837.6****	**0.034****	**0.014****	**0.001****	**29.82****
	Ectoine					
E_c	10583.3	20388.9	0.55	0.370	0.110	220.333
E_s	8150.0	24800.0	0.34	0.174	0.097	360.333
E_d	7605.6	22061.1	0.36	0.160	0.098	315.167
LSD 0.01	**496.2****	**837.6****	**0.034****	**0.013****	**0.001****	**29.82****
	Interaction between salinity and ectoine					
T1 (W_2 + E_c)	9333.3	25850.0	0.36	0.280	0.103	349.000
T2 (W_2 + E_s)	8250.0	29283.3	0.28	0.173	0.098	490.500

T3 (W_2 + E_d)	7116.7	27266.7	0.26	0.140	0.101	440.500
T4(W_3 + E_c)	12433.3	20216.7	0.62	0.400	0.115	157.000
Ts (W_3 + E_s)	6533.3	24066.7	0.27	0.143	0.095	365.500
T6 (W_3 + E_d)	6483.3	21300.0	0.30	0.163	0.095	332.000
T7 (W_4 + E_c)	9983.3	15100.0	0.66	0.430	0.111	155.000
T8 (W_4 + E_s)	9666.7	21050.0	0.46	0.207	0.098	225.000
T9 (W_4 + E_d)	9216.7	17616.7	0.52	0.177	0.099	173.000
LSD 0.01	**859.4****	**1450.8***	**0.058****	**0.024****	**0.0018****	**51.65****

220

221 **3.4. Ectoine detection in plant**

222 The results of ectoine detection in alcoholic extract of plants by HPLC revealed that ectoine was
223 successfully absorbed and accumulated by plant cells even when treated by soil addition or by foliar
224 spray. Intense peaks at retention time 2.43 min were detected in treated samples. Plants treated
225 with ectoine through foliar spray (Fig 2 a) accumulated higher concentration of ectoine compared
226 with soil addition treatments (Fig 2b). This may be due to the loose of part of ectoine in soil by
227 microbial uptake and leakage in irrigation water. The growth parameters represented by dry weight
228 of plants and sodium/potassium ratio were proportionally increased with ectoine concentration in
229 plant tissue.

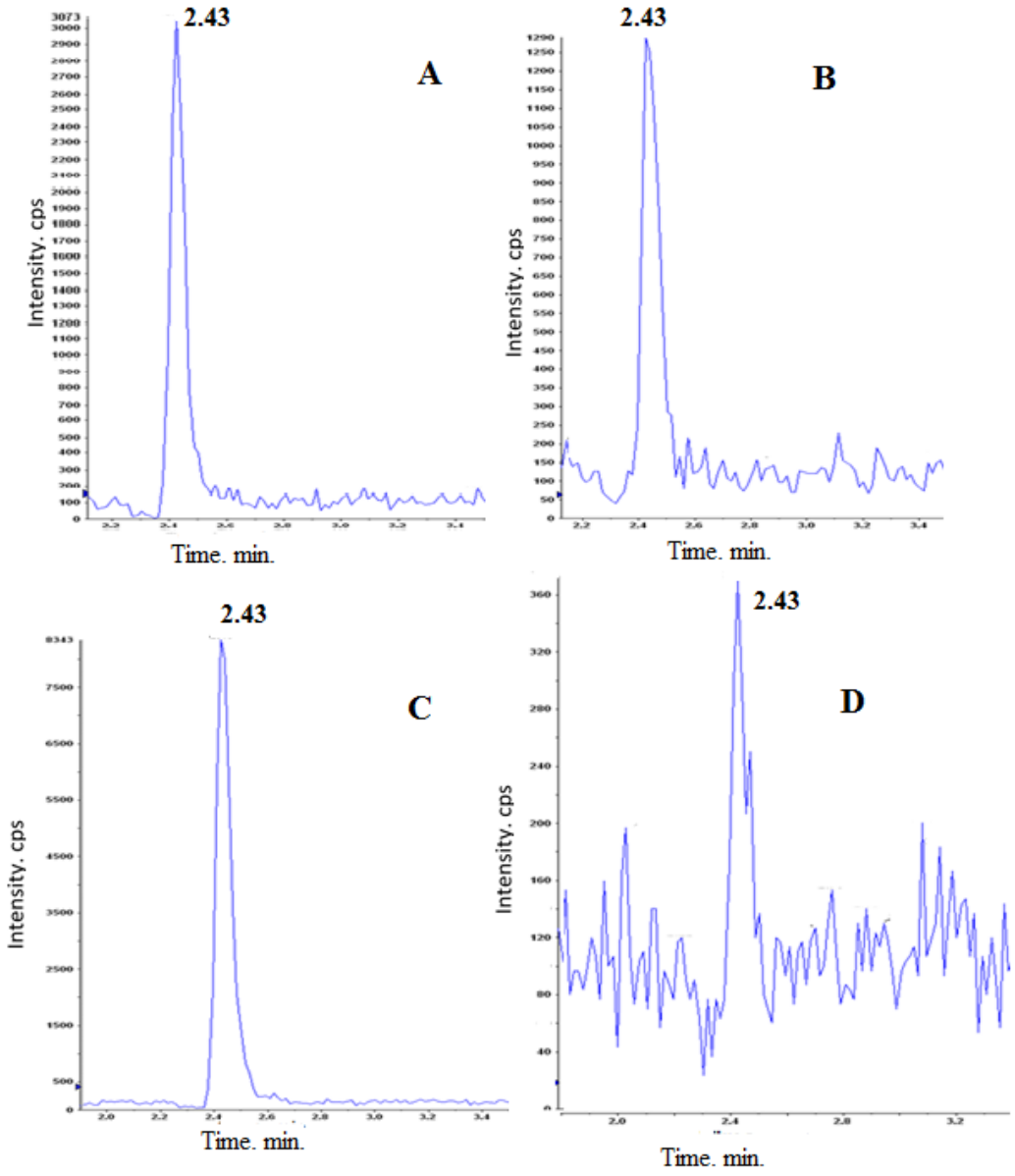

Fig. 2 Detection of ectoine by HPLC analysis of alcoholic extract of flax tissue (a) foliar spray (b), soil application C. authentic sample explain, D. control

4. DISCUSSION

Microbial metabolites were long used in plant growth promotion and alleviation of unfavorable conditions (Bradáčová et al. 2016; Krasilnikov 1961; Mehta et al. 2015; Singh et al. 2017). The ability to accumulate compatible solutes, such as betaines, proline and sugar alcohols, is a common response in plants that protect it against drastic environmental conditions (Chen and Murata 2002). In general, the obvious role of compatible solutes is that they can alleviate deleterious effects of environmental stresses such as heat stress, freezing, drought, high salinity, free radicals, radiation, urea and other denaturing agents affecting the integrity of macromolecules such as proteins, nucleic acids, biomembranes and even

whole cells (da Costa et al. 1998; Lentzen and Schwarz 2006). Among the different compatible solutes investigated, ectoines have shown to possess the most powerful stabilizing properties (Lippert and Galinski 1992). Microorganisms produce and accumulate ectoine to protect themselves from environmental stresses. ectoines are attracting eyes of the scientific community because of their multiple applications (Pastor et al. 2010).

The retention time: the spectra signal of LC-MS analysis (pseudo-molecular ion at m/z 143) (Fig1a) and of LC-MS/MS analysis (product ions at m/z 143.3, 97.0, 68.2, 55.9, and 44.0) (Fig 1b) were all in agreement with the data of the authentic ectoine. Therefore, the compound in the cells alcoholic extract was identified as ectoine (He et al. 2015)

Seed germination is the most critical stage during life span of most plants, a lot of plants can survive and tolerate abiotic stresses when safely pass this stage. The addition of compounds aid plant seeds to germinate under unfavorable conditions like high salinity being more effective if it applied before sawing or during primary stages of plant life. In the current study, the addition of ectoine to the flax seeds before germination enhanced all germination parameters as mentioned in details in the previous section. This may be attributed to the stabilizing properties of ectoine for biological macromolecules as proteins and nucleic acids leading it to perform well under salinity conditions. Also, ectoine may serve as its original function in halophilic bacteria where it accumulate in the cell to equilibrate the external osmotic pressure

As far as our knowledge is concerned, there is no previous studies describing the potential effect of ectoine addition on the growth of plants under salinity. However, there is a few studies describing the ability of transgenic plants receiving gens of ectoine synthesis to alleviate salinity stress. Genetically engineered tomato plants expressing the three *H. elongata* genes (*ectA*, *ectB* and *ectC*) generated showed no phenotypic abnormality. Expression of the ectoine biosynthetic genes was detected in the T3 transgenic plants by northern blot analysis. The ectoine accumulating T3 plants were evaluated for salt tolerance by examining their photosynthestic activity, osmotic adjustment and carbon partitioning. Nuclear magnetic resonance (NMR) detected the accumulation of ectoine. The concentration of ectoine increased in proportion to increasing salinity. The transgenic lines showed higher activities of peroxidase, while the malondialdehyde (MDA) concentration was decreased under salinity stress condition. In addition, preservation of higher rates of photosynthesis and turgor values as compared to control was evident. Within a week of $^{13}CO_2$ feeding, salt application led to increases in the partitioning of ^{13}C into roots at the expense of ^{13}C in the other plant parts. These results suggest that under saline conditions ectoine synthesis is promoted in the roots of transgenic plants, leading to an acceleration of sink activity for photosynthate in the roots. Subsequently, root function such as water uptake is improved, compared with wild-type plants. In this way, the photosynthetic rate is increased through enhancement of cell membrane stability in oxidative conditions under salt stress (Moghaieb et al. 2011).

The ability of ectoine to protect plants against salinity was also proved previously by (Nakayama et al. 2000) which investigates the function of ectoine as a compatible solute in plant cells, the three genes responsible for ectoine synthesis in *Halomonas elongata* OUT30018 were individually cloned in cauliflower mosaic virus 35S promoter and introduced together into cultured tobacco (*Nicotiana tabacum* L.) cv Bright Yellow 2 (BY2) cells. The

transgenic BY2 cells accumulated a small quantity of ectoine (14–79 nmol g^{-1} fresh weight) and showed increased tolerance to hyperosmotic shock (900 mOsm). Furthermore, the transgenic BY2 cells showed a healthy growth even under hyperosmotic conditions (up to 530 mOsm), in which the growth of the untransformed BY2 (wild type) cells was obviously delayed (Nakayama et al. 2000).

5. CONCLUSION

According to the previous study, the use of ectoine in alleviation of salt stress in plants are promising but there is a demand for more studies on different plant species and determination of suitable concentration, plant age and application technique in addition to development of fermentation process of ectoine production and extraction to be economically valuable.

COMPETING INTERESTS

No competing interests is exist.

REFERENCES

Berti M, Fischer R, Wilckens F, Hevia B J. Adaptation and genotype × environment interaction of flax seed (*Linum usitatissimum* L.) genotypes in South Central Chile. Chil J Agri Res. 2010; 70: 345–356.

Black A C, Evans D D, White J L, Ensminyer E L, Clark E F. Methods of soil analysis Amer. Soc Agro Inc Madison Wisconsin, USA; 1965.

Boyer J S. Plant productivity and Environment. Science. 1982; 218: 443–448

Bradáčová K, Weber N F, Morad-Talab N, Asim M, Imran M, Weinmann M, Neumann G. Micronutrients (Zn/Mn), seaweed extracts, and plant growth-promoting bacteria as cold-stress protectants in maize. Chem Biol Technol Agric. 2016;3: 1–10. https://doi.org/10.1186/s40538-016-0069-1

Bremer E, Kramer R. Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in bacteria. Storz, G. Hengge-Aronis, R. (Eds.), Bact. Stress responses, ASM Press Washington D C. 2000; 79–97.

Brown A D. Microbial water stress. Bacteriol Rev. 1976; 40: 803–846

Chen T H H, Murata N. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Curr Opin Plant Biol. 2002; 5: 250–257. https://doi.org/10.1016/S1369-5266(02)00255-8

da Costa M S, Santos H, Galinski E A. An overview of the role and diversity of compatible solutes in bacteria and archaea. Adv Biochem Eng Biotechnol. 1998; 61: 117–153.

El-Nagdy G A, Nassar E A. et al. Response of flax plant (*Linum usitatissimum* L.) to treatments with mineral and bio-fertilizers from nitrogen and phosphorus. Ame. Sci. 2010; 6: 207–217.

Galinski E A, Truper H G. Microbial behaviour in salt-stressed ecosystems. FEMS Microbiol. Rev. 1994; 15: 95–108.

Galinski E A, Pfeiffer H P, Trüper H G. 1,4,5,6,-Tetrahydro-2-methyl-4- pyridinecarboxylic acid. A novel cyclic amino acid from halophilic phototrophic bacteria of the genus Ectothiorhodospira. Eur J Biochem. 1985; 149: 135–139.

Grammann K, Volke A, Kunte H.J. New type of osmoregulated solute transporter identified in halophilic members of the bacteria domain: TRAP transporter TeaABC mediates uptake of ectoine and hydroxyectoine in Halomonas elongata DSM2581T. Bacteriol. 2002; 184: 3078–3085.

Hernández J A. Salinity Tolerance in Plants: Trends and Perspectives. Int. J. Mol. Sci. **2019**, *20*, 2408; doi:10.3390/ijms20102408

He Y Z, Gong J, Yu HY, Tao Y, Zhang S, Dong ZY. High production of ectoine from aspartate and glycerol by use of whole-cell biocatalysis in recombinant Escherichia coli. Microb Cell Fact. 2015;14: 1–10. https://doi.org/10.1186/s12934-015-0238-0

Husseiny S M, Sheref F, Amer H, Elakhawy T A. Biological Activity of Chemically Modified Levan Produced by Moderately Halophilic Chromohalobacter salexigens KT989777. Middle East J Appl Sci. 2015; 5: 812–822.

Isayenkov SV , Maathuis FJM. Plant Salinity Stress: Many Unanswered Questions Remain. Front.2019, Plant Sci. 10:80. doi: 10.3389/fpls.2019.00080

Kader M A. A Comparison of Seed Germination Calculation Formulae and the Associated Interpretation of Resulting Data. R Soc New South Wales. 2005; 138: 65–75.

Kai M C, Sowers K R, Robertson D E, Roberts M F, Gunsalus R P. Distribution of compatible solutes in the halophilic methanogenic archaebacteria. Bacteriol. 1991; 173: 5352–5358.

Kar M. Mishra D. Catalase, Peroxidase, and Polyphenoloxidase Activities during Rice Leaf Senescence. Plant Physiol. 1976;57(2):315-9

Kraegeloh A, Kunte H J. Novel insights into the role of potassium for osmoregulation in Halomonas elongate. Extremophiles. 2002; 6: 453–462.

KRASILNIKOV N. The Role of Soil Bacteria in Plant Nutrition. J Gen Appl. Microbiol. 1961; 7: 128–144. https://doi.org/10.2323/jgam.7.128

Kurt O, Bozkurt D. Effect of temperature and photoperiod on seedling emergence of flax (*Linum usitatissimum* L.). Agro. 2006; 5: 541–545.

Lentzen G, Schwarz T. Extremolytes: natural compounds from extremophiles for versatile applications. Appl Microbiol Bio- technol. 2006; 72: 623–634

Lippert K, Galinski E A. Enzyme stabilization by ectoine type compatible solutes: protection against heating, freez- ing and drying. Appl Microbiol Biotechnol. 1992; 37: 61-65.

Mehta P, Walia A, Kulshrestha S, Chauhan A, Shirkot C K. Efficiency of plant growth-promoting P-solubilizing *Bacillus circulans* CB7 for enhancement of tomato growth under net house conditions. J Basic Microbiol. 2015; 55: 33–44. https://doi.org/10.1002/jobm.201300562

Moghaieb R E, Saneoka H, Yossef S S, El-sharkawy A M, Fujita K. Improvement of Salt Tolerance in Tomato Plant (*Lycopersicon esculentum*) by Transformation with Ectoine Biosynthetic Genes. Transgenic Plant J. 2007; 1: 228–232.

Moghaieb, R E, Nakamura A, Saneoka H, Fujita K. Evaluation of salt tolerance in ectoine-transgenic tomato plants (*Lycopersicon esculentum*) in terms of photosynthesis, osmotic adjustment, and carbon partitioning. GM Crops. 2011; 2: 58–65. https://doi.org/10.4161/gmcr.2.1.15831

Mondal P, Remme R N, Das D, Ali Y, Kabir E. Germination and seedling growth of indigenous Aman

rice under NaCl salinity. Int J Multidiscip Res Dev. 2015; 2:251–257.

Muhammad Z, Husain F. Effect of NaCl salinity on the germination and seedling growth of some medicinal plants. Pakistan J Bot. 2010; 42: 889–897.

Nakayama H, Yoshida K, Ono H, Murooka Y, Shinmyo A. Ectoine, the Compatible Solute of *Halomonas elongata* , Confers Hyperosmotic Tolerance in Cultured Tobacco Cells. Plant Physiol. 2000; 122: 1239–1248. https://doi.org/10.1104/pp.122.4.1239

Nasri N R, Kaddour H, Mahmoudi O, Baâtour N B, Lachaâl M. The effect of osmopriming on germination, seedling growth and phosphatase activities of lettuce under saline condition. Afr J Biotech. 2011; 10: 14366–14372.

Oren A, Larimer F, Richardson P, Lapidus A, Csonka LN. How to be moderately halophilic with broad salt tolerance: Clues from the genome of *Chromohalobacter salexigens*. Extremophiles. 2005; 9: 275–279.

Pastor J M, Salvador M, Argandoña M, Bernal V, Reina-Bueno M, Csonka L N, Iborra J L, Vargas C, Nieto J J, Cánovas M. Ectoines in cell stress protection: Uses and biotechnological production. Biotechnol Adv. 2010; 28: 782–801. https://doi.org/10.1016/j.biotechadv.2010.06.005

Sehgal S N, Gibbons N E. Effect of metal ions on the growth of Halobacter iumcutirubrum. Can J Microbiol. 1960; 5, 165–169.

Singh B P, Rateb M E, Rodriguez-Couto S, Polizeli M d LTdM, Li W-J. Microbial Secondary Metabolites: Recent Developments and Technological Challenges. Front. Microbiol.2019; 10:914. doi: 10.3389/fmicb.2019.00914

Singh R, Kumar M, Mittal A, Mehta P K. Microbial metabolites in nutrition, healthcare and agriculture. Biotech. 2017; 7: 1–14. https://doi.org/10.1007/s13205-016-0586-4

Wani SH, Kumar V, Shriram V, Sah SK. Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. Crop J. 2016; 4(3):162–176. doi:10.1016/j. cj.2016.01.010.

Zhang L H, Lang Y J, Nagata S. Efficient production of ectoine using ectoine-excreting strain. Extremophiles. 2009; 13: 717–724. https://doi.org/10.1007/s00792-009-0262-2