

1
2 **The potential use of ectoine produced by a**
3 **moderately halophilic bacteria**
4 ***Chromohalobacter salexigens* KT989776 for**
5 **enhancing germination and primary seedling**
6 **of flax (*Linum usitatissimum*) under salinity**
7 **conditions**
8
9
10
11
12
13

14 **ABSTRACT**
15

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 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.

16
17 *Keywords: halophilic; Chromohalobacter, compatible solutes, ectoine, flax.*
18
19
20
21

22 **1. INTRODUCTION**

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

28 concentration. Compatible solutes are represented by different biomolecules such as Polyols (e.g. glycerol,
29 sorbitol, and mannitol), nonreducing sugars (e.g. Suc and trehalose), and amino acids (e.g. Glutamine, Prolin,
30 and betaine) (Nakayama et al. 2000).

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

33 On the other hand, microorganisms in a hyper-osmotic environment follow one of the two known strategies to
34 balance the osmotic pressure between cells and the surrounding environment, the salt in- cytoplasm
35 mechanism and accumulation of polar, highly water-soluble, low molecular weight organic osmolytes,
36 compatible solutes (Kraegeloh and Kunte 2002).. which can be found in Methanohalophilus as well as some
37 phototropic and aerobic chemoheterotrophic bacteria (Galinski and Truper 1994; Kai et al. 1991). Types of
38 compatible solutes accumulated by microbial cells resample in most cases that are present in plant cells
39 include amino acids, amino acid derivatives, such as ectoine and sugars. Microorganisms accumulate these
40 molecules through de novo synthesis or a direct uptake from the environment (Bremer and Kramer 2000).

41 The survival of plants in harsh environments depends on many factors including the presence of effective salt-
42 tolerance PGPR and its secondary metabolites (Singh et al. 2019).

43 Ectoine 1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid serves as compatible solute in some
44 halophilic bacteria (Nakayama et al. 2000) ectoine has a stabilizing effect on biomolecules as proteins and
45 nucleic acids, bacteria synthesize and accumulate ectoine in order to protect themselves from drastic
46 conditions especially osmotic stress. the rate of ectoine accumulation inside the bacterial cell is proportionally
47 increased with the increase of outer osmotic pressure (Grammann et al. 2002)

48 *Chromohalobacter salexigens* is a moderately halophilic bacterium adapted at high salt concentration by
49 production and accumulation of ectoine (Oren et al. 2005)

50 Flax seed (*Linum usitatissimum* L.) is a globally important agricultural crop used for its oil (Berti et al. 2010).
51 and stem fiber (El-Nagdy and Nassar 2010). Germination and seedling emergence of flax may be affected by
52 environmental conditions as temperature, moisture and salinity in addition to sowing depth and seedbed
53 conditions (Kurt and Bozkurt 2006). Salinity may cause delayed germination and emergence, low survival,
54 irregular crop stand and lower biomass yield due to biochemical, morphological and physiological changes (
55 Isayenkov and Maathuis 2019 , Muhammad and Husain 2010). NaCl decreased germination percentage, speed
56 of germination and seedling dry matter in different plants (Mondal et al. 2015; Nasri et al. 2011).

57 In the present work, the function of ectoine as a compatible solute in plant cells was investigated in addition
58 to examining the role of ectoine in water stress tolerance in flax. It was found that ectoine conferred increased
59 hyperosmotic tolerance in flax seed germination and primary seedling stage.

60
61

62 2. MATERIAL AND METHODS

63

64 2.1. Microorganism and growth conditions

65 *Chromohalobacter salexigens* KT989776 was isolated previously by one of our team (Husseiny et al. 2015)
66 and cultivated in Sehgal and Gibbons complex broth medium (SGCb medium) (Sehgal and Gibbons 1960)
67 contains (g/L): casmino acids, 7.5, yeast extract, 10, starch, 5, KCl, 2.0, sodium citrate, 3.0 MgSO₄.7H₂O, 20,
68 NaCl, 200, MnCl₂.4H₂O, 0.05 and FeCl₂.nH₂O. 0.01. The medium was adjusted to pH 7.0 by 0.5 M NaOH
69 and HCl before autoclaving at 121°C for 15 min.

70 Flaks (250 ml containing 100 ml SCG medium) was inoculated with 3ml 24h old culture and incubated at 30
71 °C for 48 h on rotary shaker.

72 2.2. Ectoine extraction

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

77 **2.3. Batch fermentations**

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

84 **2.4. Spectroscopic analysis**

85 **2.4.1. HPLC determination**

86 The ectoine was detected in alcoholic extract of plant samples by HPLC with a TSK-GEL reversed-phase
87 column (Tosoh, Japan) the mobile phase was 50 mmol l^{-1} potassium phosphate buffer at 35°C with flow rate
88 1 ml min^{-1} . The UV detector was adjusted to 210 nm. The retention time of ectoine was compared by
89 commercially available ectoine, purity >97%, Biomol, Hamburg, Germany (Zhang et al. 2009).

90 **2.4.2. LC-MS analysis**

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

97 **2.5. Germination experiment**

98 **2.5.1. Plant material and NaCl stress treatment:**

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

106

107

108

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

111

112

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 in 11 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

113

114 **2.5.2. Germination and growth parameters:**

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

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

121 **2.6. Pot experiment**

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

125

Table2: 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
<i>Particle size distribution (%)</i>	
Coarse sand	28.4
Fine sand	13.0
Silt	22.4
Clay	36.2
Texture grade	Sandy clay loam

126

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

133

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

136

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 receive 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 receive 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 receive 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 receive 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 soil

137

138 **2.7.Na⁺, K⁺ and ectoine analysis**

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

142 **2.8.Enzymes assay:**

143 Fresh plant biomass of various treatments was homogenized in liquid nitrogen and suspended in chilled 0.1 M
144 phosphate buffer (pH 7.0). The homogenate was filtered and the filtrate was centrifuged at 4000 rpm for 10
145 min at 4 °C. The final volume of the supernatant was adjusted to 10 mL and served as the enzymes source.
146 Peroxidase (EC 1.11.1.7) and polyphenol oxidase (EC 1.14.18.1) were assayed following the method
147 described by [Kar and Mishra \(1976\)](#). The color intensity was read at 430 nm, and the enzyme activity was
148 expressed as the change in the optical density/gram fresh weight/hour.

149 **2.9. Statistical analysis**

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

153

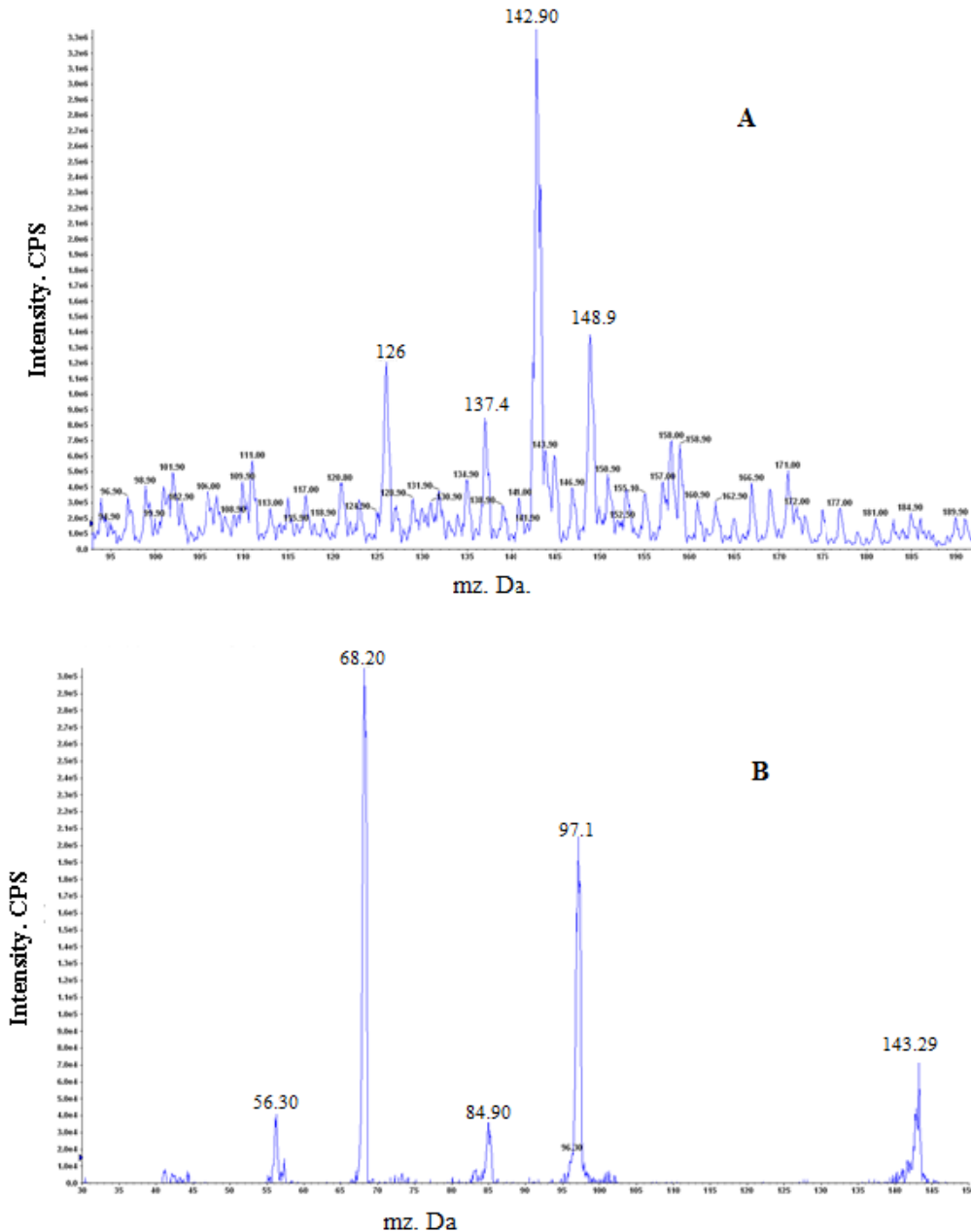
154 **3. RESULTS**

155 **3.1. Confirmation of ectoine production**

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

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

165



166
 167
 168 **Figure 1:** spectra signal of A) LC analysis (pseudo-molecular ion at m/z 143 and B) LC-MS/MS analysis
 169 (product ions at m/z 143.3, 97.0, 68.2, 55.9, and 44.0) of ectoine extracted from *Chromohalobacter salexigens*
 170 cells
 171

172 **3.2. Germination of flax seeds**

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

175 The effect of ectoine addition on MGT recorded in table (4) indicated that the treatment with ectoine lowered
 176 the MGT to about 6.7%. The lower the MGT, the faster a population of seeds has germinated. The greatest
 177 effect of ectoine was recorded for salinity level 0 and the lowest was recorded under salinity level 7 dS.m⁻¹.
 178

179 The CVG gives an indication of the rapidity of germination. It increases when the number of germinated seeds
 180 increases and the time required for germination decreases.
 181 The average increase in CVG, a result of ectoine treatment, was 7.6 %. As in the case of MGT, the highest
 182 effect was recorded at level 0.
 183 The effect of ectoine treatment on first day of germination (FDG) was not significant.
 184 Germination Rate Index (GRI) was significantly affected by ectoine treatment where the average increase was
 185 about 11% over control. Higher GRI values indicate higher and faster germination.
 186 The average increase in hypocotyl length was about 40 % over control when flax seeds were germinated in
 187 presence of ectoine. The effect of ectoine was more obvious in case of radical elongation where the average
 188 increase reached about 62% over control. In addition, the ectoine was more effective as salinity increased
 189 where the radical length of ectoine-treated seeds reached about 2-3 times more than non-treated seeds under
 190 higher salinity levels 7, 9 and 11 dS.m⁻¹.
 191 The effect of ectoine on the fresh and dry weight: Final Germination Percentage (FGP) and Energy of
 192 Emergence (EE) of germinated seeds followed the same trend of radical length where the effect was more
 193 obvious under higher salinity levels.
 194

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)

Treatment s	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

197

198 3.3. Pot experiment

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

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

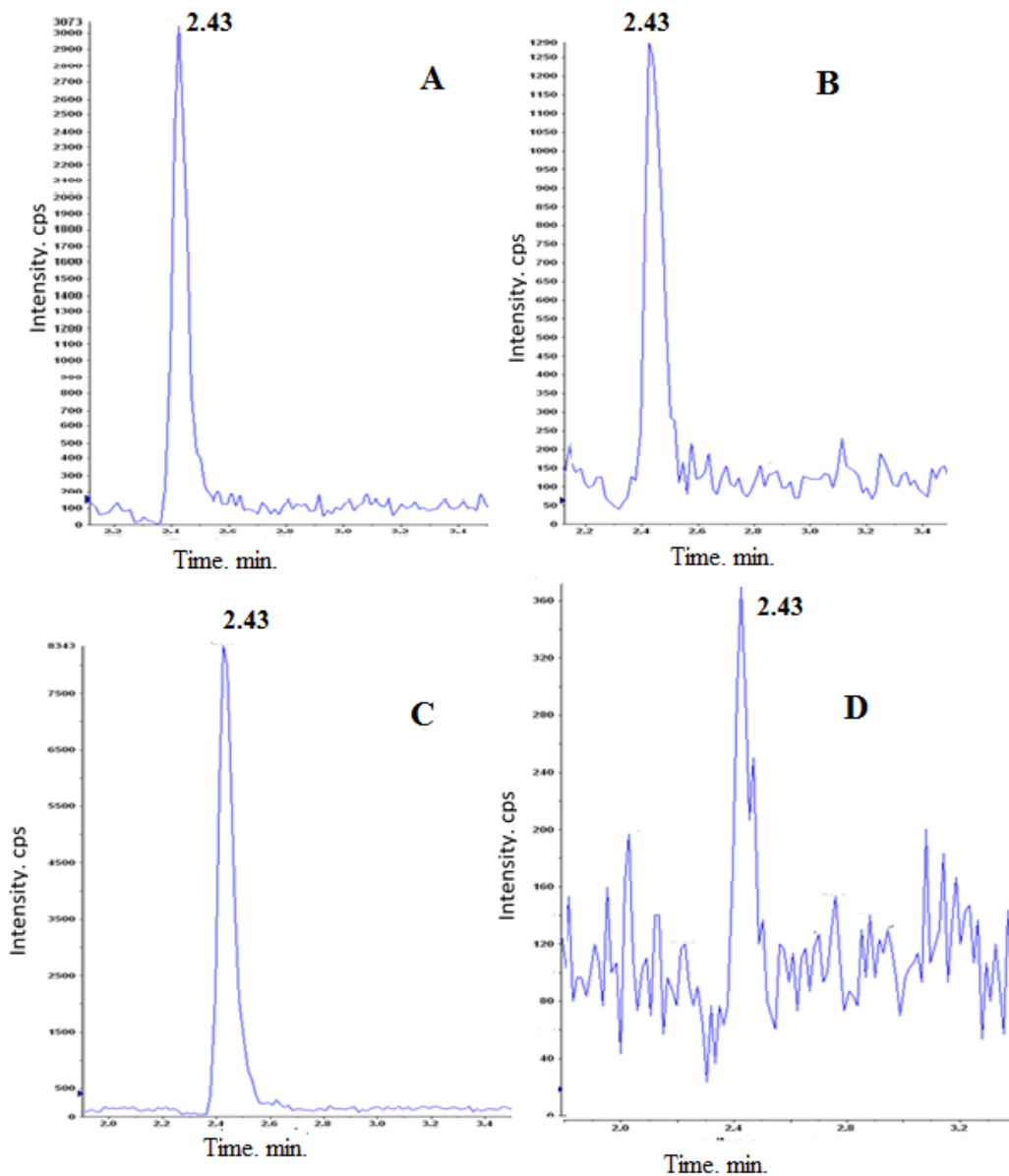
Treatment	Na ppm	Kppm	Na/K	POD Δ 430 m ⁻¹ g ⁻¹ fresh wighet	PPO Δ 490 m ⁻¹ g ⁻¹ fresh wighet	Dry weight (mg plant ⁻¹)
Salinity						
W ₂	8233.3	27466.7	0.30	0.198	0.100	426.667
W ₃	8483.3	21861.1	0.40	0.236	0.102	284.833
W ₄	9622.2	17922.2	0.55	0.271	0.102	184.333
LSD 0.01	496.2**	837.6**	0.034**	0.014**	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**

208

209 3.4. Ectoine detection in plant

210 The results of ectoine detection in alcoholic extract of plants by HPLC revealed that ectoine was successfully
 211 absorbed and accumulated by plant cells even when treated by soil addition or by foliar spray. Intense peaks at
 212 retention time 2.43 min were detected in treated samples. Plants treated with ectoine through foliar spray (Fig
 213 2 a) accumulated higher concentration of ectoine compared with soil addition treatments (Fig 2b). This may be
 214 due to the loose of part of ectoine in soil by microbial uptake and leakage in irrigation water. The growth
 215 parameters represented by dry weight of plants and sodium/potassium ratio were proportionally increased with
 216 ectoine concentration in plant tissue.



217

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

220 **4. DISCUSSION**

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

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

235 The retention time: the spectra signal of LC-MS analysis (pseudo-molecular ion at m/z 143) (Fig1a) and of
236 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
237 with the data of the authentic ectoine. Therefore, the compound in the cells alcoholic extract was identified as
238 ectoine (He et al. 2015)

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

247 As far as our knowledge is concerned, there is no previous studies describing the potential effect of ectoine
248 addition on the growth of plants under salinity. However, there is a few studies describing the ability of
249 transgenic plants receiving gens of ectoine synthesis to alleviate salinity stress. Genetically engineered tomato
250 plants expressing the three *H. elongata* genes (*ectA*, *ectB* and *ectC*) generated showed no phenotypic
251 abnormality. Expression of the ectoine biosynthetic genes was detected in the T3 transgenic plants by northern
252 blot analysis. The ectoine accumulating T3 plants were evaluated for salt tolerance by examining their
253 photosynthetic activity, osmotic adjustment and carbon
254 partitioning. Nuclear magnetic resonance (NMR) detected the accumulation of ectoine. The concentration of
255 ectoine increased in proportion to increasing salinity. The transgenic lines showed higher activities of
256 peroxidase, while the malondialdehyde (MDA) concentration was decreased under salinity stress condition. In
257 addition, preservation of higher rates of photosynthesis and turgor values as compared to control was evident.
258 Within a week of ¹³C₂ feeding, salt application led to increases in the partitioning of ¹³C into roots at the
259 expense of ¹³C in the other plant parts. These results suggest that under saline conditions ectoine synthesis is
260 promoted in the roots of transgenic plants, leading to an acceleration of sink activity for photosynthate in the
261 roots. Subsequently, root function such as water uptake is improved, compared with wild-type plants. In this
262 way, the photosynthetic rate is increased through enhancement of cell membrane stability in oxidative
263 conditions under salt stress (Moghaieb et al. 2011).

264 ability of ectoine to protect plants against salinity was also proved previously by (Nakayama et al. 2000)
265 which investigates the function of ectoine as a compatible solute in plant cells, the three
266 genes responsible for ectoine synthesis in *Halomonas elongata* OUT30018 were individually cloned in
267 cauliflower
268 mosaic virus 35S promoter and introduced together into cultured
269 tobacco (*Nicotiana tabacum* L.) cv Bright Yellow 2 (BY2) cells. The
270 transgenic BY2 cells accumulated a small quantity of ectoine (14–79
271 nmol g⁻¹ fresh weight) and showed increased tolerance to hyperosmotic shock (900 mOsm). Furthermore, the
272 transgenic BY2 cells

273 showed a healthy growth even under hyperosmotic conditions (up to 530 mOsm), in which the growth of the
274 untransformed BY2 (wild type) cells was obviously delayed (Nakayama et al. 2000).

275 5.CONCLUSION

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

280
281
282
283
284
285
286

COMPETING INTERESTS

No competing interests is exist.

287 REFERENCES

- 288 Berti M, Fischer R, Wilckens F, Hevia B J. Adaptation and genotype × environment interaction of flax seed
289 (*Linum usitatissimum* L.) genotypes in South Central Chile. *Chil J Agri Res.* 2010; 70: 345–356.
- 290 Black A C, Evans D D, White J L, Ensminger E L, Clark E F.
291 *Methods of soil analysis* Amer. Soc Agro Inc Madison Wisconsin, USA; 1965.
- 292 Boyer J S. Plant productivity and environment. *Science.* 1982; 218: 443–448
- 293 Bradáčová K, Weber N F, Morad-Talab N, Asim M, Imran M, Weinmann M, Neumann G. Micronutrients
294 (Zn/Mn), seaweed extracts, and plant growth-promoting bacteria as cold-stress protectants in maize. *Chem*
295 *Biol Technol Agric.* 2016;3: 1–10. <https://doi.org/10.1186/s40538-016-0069-1>
- 296 Bremer E, Kramer R. Coping with osmotic challenges: osmoregulation through accumulation and release of
297 compatible solutes in bacteria. Storz, G. Hengge-Aronis, R. (Eds.), *Bact. Stress responses*, ASM Press
298 Washington D C. 2000; 79–97.
- 299 Brown A D. Microbial water stress. *Bacteriol Rev.* 1976; 40: 803–846
- 300 Chen T H H, Murata N. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and
301 other compatible solutes. *Curr Opin Plant Biol.* 2002; 5: 250–257. [https://doi.org/10.1016/S1369-](https://doi.org/10.1016/S1369-5266(02)00255-8)
302 [5266\(02\)00255-8](https://doi.org/10.1016/S1369-5266(02)00255-8)
- 303 da Costa M S, Santos H, Galinski E A. An overview of the role and diversity of compatible solutes in bacteria
304 and archaea. *Adv Biochem Eng Biotechnol.* 1998; 61: 117–153.
- 305 El-Nagdy G A, Nassar E A. et al. Response of flax plant (*Linum usitatissimum* L.) to treatments with mineral
306 and bio-fertilizers from nitrogen and phosphorus. *Ame. Sci.* 2010; 6: 207–217.
- 307 Galinski E A, Truper H G. Microbial behaviour in salt-stressed ecosystems. *FEMS Microbiol. Rev.* 1994; 15:
308 95–108.
- 309 Galinski E A, Pfeiffer H P, Trüper H G. 1,4,5,6,-Tetrahydro-2-methyl-4- pyridinecarboxylic acid. A novel
310 cyclic amino acid from halophilic phototrophic bacteria of the genus *Ectothiorhodospira*. *Eur J Biochem.*
311 1985; 149: 135–139.
- 312 Grammann K, Volke A, Kunte H.J. New type of osmoregulated solute transporter identified in halophilic

313 members of the bacteria domain: TRAP transporter TeaABC mediates uptake of ectoine and hydroxyectoine
314 in *Halomonas elongata* DSM2581T. *Bacteriol.* 2002; 184: 3078–3085.

315 [Hernández J A.](#) Salinity Tolerance in Plants: Trends and Perspectives. *Int. J. Mol. Sci.* **2019**, *20*, 2408;
316 doi:10.3390/ijms20102408

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

320 Husseiny S M, Sheref F, Amer H, Elakhawy T A. Biological Activity of Chemically Modified Levan
321 Produced by Moderately Halophilic *Chromohalobacter salexigens* KT989777. *Middle East J Appl Sci.* 2015;
322 5: 812–822.

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

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

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

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

331

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

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

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

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

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

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

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

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

351 Mondal P, Remme R N, Das D, Ali Y, Kabir E. Germination and seedling growth of indigenous Aman rice
352 under NaCl salinity. *Int J Multidiscip Res Dev.* 2015; 2:251–257.

353 Muhammad Z, Husain F. Effect of NaCl salinity on the germination and seedling growth of some medicinal

354 plants. *Pakistan J Bot.* 2010; 42: 889–897.

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

358 Nasri N R, Kaddour H, Mahmoudi O, Baâtour N B, Lachaâl M. The effect of osmopriming on germination,
359 seedling growth and phosphatase activities of lettuce under saline condition. *Afr J Biotech.* 2011; 10: 14366–
360 14372.

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

363 Pastor J M, Salvador M, Argandoña M, Bernal V, Reina-Bueno M, Csonka L N, Iborra J L, Vargas C, Nieto J
364 J, Cánovas M. Ectoines in cell stress protection: Uses and biotechnological production. *Biotechnol Adv.*
365 2010; 28: 782–801. <https://doi.org/10.1016/j.biotechadv.2010.06.005>

366 Sehgal S N, Gibbons N E. Effect of metal ions on the growth of *Halobacterium cutirubrum*. *Can J Microbiol.*
367 1960; 5, 165–169.

368 Singh B P, Rateb M E, Rodriguez-Couto S, Polizeli M d LTdM, Li W-J. Microbial Secondary Metabolites:
369 Recent Developments and Technological Challenges. *Front. Microbiol.* 2019; 10:914. doi:
370 10.3389/fmicb.2019.00914

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

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

376 Zhang L H, Lang Y J, Nagata S. Efficient production of ectoine using ectoine-excreting strain. *Extremophiles.*
377 2009; 13: 717–724. <https://doi.org/10.1007/s00792-009-0262-2>

378