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Physiological Response to Salinity Stress in Various Populus euphratica Oliv. Ecotypes in Iran

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ABSTRACT

Aims Euphrates poplar (Populus euphratica Oliv.) is a woody species, which is naturally distributed in desert areas of some parts Asia and Africa. This research was conducted to evaluate the physiological response to salinity stress in 12 ecotypes in Iran.

Materials & Methods This study was conducted to evaluate the physiological response to different levels of salinity (75, 150, 225, and 300 mM NaCl) with control and to assess the response physiologic traits such as RWC, EL, MDA, Proline, GB, TSS, plant pigments, SOD, CAT, and GPX.

Findings The analysis of variance showed that there was a significant difference between treatments all traits. Comparing means of ecotypes showed that Hamidieh was the highest group and Mahneshan and Marand were in the lowest group. Comparing means of treatments showed that 75 mM was the highest group in terms of performance. The 75 mM was the highest group in terms of SOD activity; in contrast, 300 mM and control were in the lowest group

Conclusion The result represents that Populus euphratica is a moderate halophyte, which could be suggested to reclamation of saline lands with high water table. This uses multiple mechanisms to overcome salinity stress and there is not a clear path to overcome salinity in this species. Cluster analysis divided the examined ecotypes into five groups based on total traits. The ecotypes grouping was not based on geographical distance, rather it was based on the conditions of the original habitat especially soil salinity

Keywords Antioxidant Enzyme; Populus Euphratica; Resistance Mechanism

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Introduction

Higher plants are not able to escape from environmental stress due to fixed seating in the soil. They are exposed to environmental stresses continuously without any protection. The fixed nature and the need for protection have led to a unique molecular mechanism for dealing with stress in plants. Yet, tolerance mechanisms in plants are diverse; yet, in some plants, morphological features have been able to remain safe from damage stressors partially, but there is no possibility of these changes in all plants. The only option for these plants is reform and change in physical activity, metabolic mechanisms, gene expression, and developmental activities to cope with stress. [1].

Oxidative stress is one of the effects of biotic and abiotic stresses such as salinity, which typically occurs in parts of the plant, where light intensity is high. The first oxygen-free radical is superoxide (O^{2-}), which causes damage to enzymes and membranes associated with photosynthesis. Every condition that disrupts the uniformity of reducing creates oxidative stress in plants that can change the steady-state oxidation potential of cells and accumulation of reactive oxygen species (ROS). Symptoms of this condition are different from membrane damage, physiological and metabolic damage to DNA damage communicating with aging and the aging of plant cells [2].

Effective molecules for salt tolerance in a plant are proteins and metabolites in ion homeostasis, osmotic adjustment and adjustment of water regime, and scavenging toxic radicals (mainly enzyme). Oxidative stress is a side effect of the action on the plants [3]. This secondary impact appears to be a result of high levels of osmosis caused by the exposure to drought or salinity stress conditions and leads to the emergence of reactive oxygen molecules such as O^{-}, OH^{-}, and O^{2-}. Decomposition of ROS associated with antioxidant enzyme activities in cellular processes, especially Superoxide dismutase (SOD), Glutathione peroxidase (GPX), and Catalase (CAT) and osmotic protection compounds such as mannitol and proline [1].

In recent decades, there has been extensive research on the physiology and biochemistry of plant responses to abiotic stresses in poplar (*Populus*) species. They have a wide spread in different climatic regions, while they are of great importance in terms of economic and ecological functions; they are used as a model plant for studying the physiological and molecular mechanisms in stress tolerance. There are considerable differences in resistance to salinity among different species and cultivars of poplars; the Euphrates poplar (*P. euphratica*) is the most significant species.

Mohammadi *et al.* reported that there is a significant difference in the growth and physiological characteristics of *P. euphratica* provenances in terms of proline, chlorophyll a, chlorophyll b, carotenoids, sugar, and total protein [4].

The results of a study conducted by Janz *et al.* showed that the *P. euphratica* did not have a general pathway for salt tolerance, but it had preserved the activity with a series of mechanisms such as control of osmotic adjustment (sugars and sugar alcohol), compartment of ions (Na^{+} and K^{+}), and detoxifies ROS [5]. Bogeat-Triboulot *et al.* reported the increased compatible solutions such as inositol, salicin, fructose, sucrose, and galactose in water shortage; Chlorophyll and carotenoid content in leaves were not affected, but there was an increase in the chlorophyll a/b ratio in the lack of water conditions and water shortage had led to the peroxidation of lipids [6]. Watanabe *et al.* reported that salinity and drought stress significantly increased proline and sugars in leaves of the *P. euphratica* [7].

Hypotheses and Objective: The research hypothesis was that "there is no difference between ecotypes of *P. euphratica* in terms of salt tolerance". The objective of this research was to study the physiological responses of *P. euphratica* seedlings from different ecotypes in different levels of salinity in greenhouse conditions and to determine the factors related to the distinction more tolerant ecotypes.

Materials and Methods

Study Area: In mid-February, 1-year-old cuttings of *P. euphratica* were collected from 12 regions of Iran. Table 1 shows the locations and properties of the collection areas [8].

Sampling: The cuttings were planted in individual pots containing Sandy-Loam soil in the nursery at the University of Tehran and placed in a greenhouse; the cuttings were rooted in April. The plants in pots were irrigated 2-3 times per week, depending on the evaporative demand and received 1 l of full-

strength Hoagland's nutrient solution every 2 weeks. Rooted cuttings were maintained in the greenhouse for hardening and acclimation for 6 months prior to the initiation of the salt treatments (October). 180 uniform plants in height and number of leaves were used in the following experiment.

Stress Treatments: Plants were subjected to increase salinity for 2 months, and the saline treatments were imposed by top watering with 1 l of 75, 150, 225, and 300 mM NaCl solution twice a week. When salt treatments were initiated, plants received 1 l of full-strength Hoagland's solution weekly. Control plants were kept well-watered with distilled water and fertilized with no addition of NaCl. Destructive harvests were made after 2 months of exposure to salt treatments. 6 replicated samplings (pot) per treatment were harvested at each sampling time.

Table1) Collection areas' information [8]

Name of region	Province	Symbol	Longitude	Latitude	Elevation (m)	Mean annual temperature (°C)	Mean annual precipitation (mm)
Jolfa	Azerbaijan	E1	38 57 N	45 41 E	0703	14.4	179.8
Marand	Azerbaijan	E2	38 31 N	45 24 E	1077	12.3	342.2
Maranjab	Esfahan	E3	34 13 N	51 40 E	0930	18.8	138.4
Manjil	Gilan	E4	36 15 N	49 26 E	0330	17.3	196.4
Dashlibrun	Golestan	E5	37 46 N	54 54 E	0037	17.1	201.9
Sarakhs	Khorasan	E6	36 18 N	61 09 E	0303	17.6	203.3
Dezful	Khuzestan	E7	32 14 N	48 20 E	0063	24.0	444.3
Hamidieh	Khuzestan	E8	31 31 N	48 28 E	0023	24.2	194.5
Mahalat	Markazi	E9	34 00 N	50 33 E	1850	12.8	294.2
Masumieh	Qom	E10	34 43 N	50 52 E	0910	18.7	146.1
Gilvan	Zanjan	E11	36 46 N	49 26 E	0376	17.3	196.4
Mahneshan	Zanjan	E12	36 46 N	47 43 E	1706	14.6	207.0

Traits Assessment: In order to study on physiological responses to salinity traits such as Relative Water Content (RWC), Electrical Leakage (EL), Malondialdehyde (MDA), Proline, Glycine betaine (GB) and Total Soluble Sugar (TSS), plant pigments, SOD, CAT, and GPX were measured in different treatments and ecotypes.

RWC (%) was measured by Ritchie *et al.* method [9]; In order to assess the damage to cell membranes, EL (%) was measured by Zhao *et al.* method [10]. Lipid peroxidation was measured according to MDA production; MDA (µmol/mg protein) was calculated by Stewart and Bewley method [11]; Proline (µmol/g fresh weight) content in fresh weight of plant was measured by Bates *et al.* method [12]; GB (µg/g dry weight) content in dry weight of plant was measured by Grieve and Grattan method [13]; TSS (mg/g dry weight) in dry weight of plant was measured by Irigoyen *et al.* method [14]; Plant Pigments (mg/g fresh weight) was measured by Arnon and Kopeika method [15].

Total protein was extracted by Bradford method [16]. The activity of SOD (U/minute/mg protein) was measured by Dhindsa *et al.* method, as a photometric method [17]. The activity of CAT (H_2O_2/minute/mg protein) was measured by Aebi method, as the kinetic method [18]. The activity of GPX (nmol composed Tetraguaiacol/minute/mg protein) was measured by Chance and Maehly method, as the kinetic method [19].

Statistical Analysis: The experiment was conducted in a completely randomized design (CRD) with a double factorial and 6 replications (pot). In different treatments due to effects of salinity levels, test data and statistical analysis, including two-way analysis of variance, Duncan's multiple range test, and Pearson correlation coefficient were performed, using the statistical software SAS 9.2 and Microsoft Excel 2010 was used for drawing the diagrams.

Data homogeneity was tested, using Kolmogorov–Smirnov test. Statistical analysis was performed, using the Statistical Analysis System (9.1. SAS Institute Inc.). Analysis of variance was performed to identify statistically significant differences between ecotypes, treatments (levels of salinity), and interaction between ecotypes × treatments. The significant differences between the means were determined by Duncan's multiple range test. Pearson Correlation Coefficients analysis was performed to identify the statistically

significant correlation between different traits. Cluster analysis was performed to group and determine the distance of ecotypes, using all traits.

Findings

Analysis of variance indicated that the effects of different levels of salinity treatments were significantly different in all traits at 0.1%. The effect of ecotype was significantly different in EL, MDA, chlorophyll, and enzymes at 0.1%; the RWC and carotenoids were significantly different in level of 5%. While ecotype effect on Proline content and GB had no significant difference in the level of 5%. The interaction between treatment × ecotypes was significantly different in EL, MDA, chlorophyll, and enzymes at p=0.1%, but they were not significantly different in RWC, Proline, GB, TSS, and carotenoids in the level of 5%. Overall, the coefficient of variation ranged between 1.35% and 9.43%; this range is optimal due to the experimental nature and type traits (Table 2).

Comparison of the *Duncan's* multiple range *test* classified the ecotypes into separate or joint groups in terms of physiological traits (Table 3). Overall, ecotype 8 was higher than other groups in terms of physiological traits associated with vitality and performance (RWC and plant pigments content) alone or together with ecotypes (4). Also, ecotype 10 was higher than other groups in terms of physiological traits associated with stress tolerance (Proline content, GB, TSS, carotenoids, and enzymes) alone or together with ecotype 4. In contrast, ecotypes 2 and 12 were grouped at the lowest of these traits.

Duncan's multiple range *test* classified the treatments into 4 or 5 groups in Table 4. Overall, control was higher than other groups in terms of physiological associated with vitality and performance (RWC and plant pigments content) alone or together with 75 mM. In contrast, 300 mM was grouped at the lowest of these traits. Also, 300 mM was higher than other groups in terms of physiological traits associated with stress tolerance (EL, MDA, Proline content, GB, TSS) and control was grouped at the lowest of these traits. Treatment 225 mM was grouped at the highest in terms of SOD, CAT, and GPX; in contrast, control was grouped at the lowest in terms of SOD and CAT, while 300 mM was grouped at the lowest in terms of GPX.

Pearson correlation coefficient showed that there was a significant correlation (positive or negative) between the most studied traits (Table 5). There was a positive correlation between growth and performance together; and there was a positive correlation between traits, indicating symptoms damage and stress together. While there was a negative correlation between growth and performance with traits, indicating symptoms damage and stress.

Cluster analysis by Ward method in 0.49 Euclidean distance separated ecotypes in 5 clusters according to the average traits (Figure 1). Clustering placed ecotypes Jolfa with Gonbad and Sarakhs in the first, Maranjab and Masumiyeh with Mamjil and Hamidieh in the second, Gilvan alone in the third, Marand and Mahneshan in the fourth, and Dezful and Mahalt in the fifth cluster.

Table 2) Analysis of variance for salt levels and ecotype

Source of variance	Symbol	MS				CV%
		Treatment df=4	Ecotype df=11	Treatment*Ecotype df=44	Error	
Relative Water Content	RWC	7786***	10.29*	3.32^{ns}	5.26	2.92
Electrical Leakage	EL	7757***	23.39***	12.97***	2.36	6.64
Malondialdehyde	MDA	231.9***	1.64***	0.35***	0.09	5.97
Proline	Proline	7151***	1.02^{ns}	0.73^{ns}	4.37	9.43
Glycine betaine	GB	178382***	14.60^{ns}	23.07^{ns}	27.83	3.58
Total Soluble Sugar	TSS	0.562***	0.002*	0.0006^{ns}	0.0009	2.31
chlorophyll ^{a}	Chl a	0.305***	0.055***	0.004**	0.0010	2.86
chlorophyll ^{b}	Chl b	0.121***	0.001***	0.0007***	0.0003	7.40
carotenoids	Caro	0.144***	0.0002***	0.0001^{ns}	0.0001	6.22
Superoxide dismutase	SOD	81901***	23.30***	19.53***	2.19	1.35
Catalase	CAT	1094***	0.334***	0.115***	0.047	2.18
Glutathione peroxidase	GPX	25433***	248.67***	50.34***	20.79	3.02

***, **, * are significantly different at p<0.001, p<0.01, and p<0.05, respectively, and ns is not significantly different at p<0.05.

Table 3. Duncan's multiple range test for variables in ecotypes

Variable	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
RWC	77.9^{bac}	77.1^{c}	78.5^{bac}	79.8^{a}	79.3^{ba}	78.8^{bac}	77.7^{bc}	79.7^{a}	78.0^{bac}	78.8^{bac}	79.0^{bac}	78.1^{bac}
EL	23.6^{dc}	25.5^{ba}	21.6^{e}	23.5^{dc}	24.6^{bc}	24.3^{c}	25.6^{ba}	22.6^{de}	24.1^{c}	21.7^{e}	22.6^{de}	25.9^{a}
MDA	4.8^{cdc}	5.4^{a}	4.6^{e}	4.8^{cd}	5.0^{cb}	4.9^{cb}	5.1^{b}	4.9^{cd}	5.1^{b}	4.3^{f}	4.7d^{e}	5.4^{a}
Proline	22.6^{a}	22.3^{a}	21.7^{a}	22.4^{a}	22.1^{a}	22.2^{a}	22.1^{a}	22.0^{a}	22.4^{a}	22.1^{a}	22.4^{a}	22.0^{a}
GB	146^{a}	146^{a}	148^{a}	149^{a}	147^{a}	148^{a}	146^{a}	148^{a}	148^{a}	149^{a}	147^{a}	147^{a}
TSS	1.30^{bc}	1.31^{bac}	1.33b^{a}	1.32^{bac}	1.32^{bac}	1.31^{bac}	1.30^{c}	1.34^{a}	1.32^{bac}	1.34^{a}	1.33^{a}	1.31^{bac}
Chl ^{a}	1.13^{b}	1.02^{f}	1.07d^{e}	1.17^{a}	1.09^{dc}	1.15^{a}	1.10^{c}	1.18^{a}	1.18^{a}	1.09^{dc}	1.05^{e}	0.99^{g}
Chl ^{b}	0.23^{bc}	0.21^{ed}	0.22^{bdc}	0.23^{bac}	0.22^{bdc}	0.23^{bac}	0.23^{bac}	0.23^{bac}	0.23^{bac}	0.24^{a}	0.22^{bdc}	0.21^{e}
Caro	0.178^{ba}	0.171^{b}	0.183^{a}	0.182^{a}	0.183^{a}	0.183^{a}	0.180^{a}	0.183^{a}	0.178^{ba}	1.181^{a}	0.181^{a}	0.180^{a}
SOD	110^{dc}	108^{e}	111b^{a}	110b^{dc}	110^{bdc}	109^{d}	108^{e}	110^{bdc}	108^{e}	112^{a}	111^{bac}	107^{f}
CAT	10.1^{ba}	9.6^{e}	10.0^{bac}	9.9^{dc}	9.9^{bc}	10.0^{bac}	10.1^{a}	9.9^{dc}	10.1^{a}	9.8^{ed}	9.9^{bc}	9.9^{dc}
GPX	142^{c}	137^{d}	146^{bac}	146b^{ac}	145^{bac}	143^{bc}	138^{d}	147^{a}	138^{d}	146^{ba}	146^{ba}	137^{d}

Means with the same letter in the rows are not significantly different at p<0.05

Table 4) Duncan's multiple range test for variables in treatments of salt levels

Variable	Treatments				
	Control	75 mM	150 mM	225 mM	300 mM
RWC	90^{a}	89^{a}	85^{b}	73^{c}	55^{d}
EL	10^{e}	11^{d}	21^{c}	34^{b}	44^{a}
MDA	2.0^{e}	3.3^{d}	4.3^{c}	6.5^{b}	8.4^{a}
Proline	6^{e}	13^{d}	30^{b}	42^{a}	20^{c}
GB	62^{e}	99^{d}	180^{b}	243^{a}	153^{c}
TSS	1.18^{e}	1.23^{d}	1.29^{c}	1.41^{b}	1.48^{a}
Chl ^{a}	1.13^{b}	1.22^{a}	1.10^{c}	1.10^{c}	0.96^{d}
Chl ^{b}	0.29^{a}	0.27^{b}	0.23^{c}	0.19^{d}	0.15^{e}
Caro	0.24^{a}	0.25^{a}	0.17^{b}	0.14^{c}	0.10^{d}
SOD	46^{e}	97^{d}	130^{b}	176^{a}	99^{c}
CAT	3.7^{e}	8.7^{c}	14.1^{b}	17.0^{a}	6.1^{d}
GPX	129^{d}	138^{c}	145^{b}	181^{a}	111^{e}

Means with the same letter in the rows are not significantly different at p<0.05.

Table 5) Pearson correlation coefficients analysis between variables

Variable	Yield	RWC	EL	MDA	Proline	GB	TSS	Chl a	Chl b	Caro	SOD	CAT	GPX
Yield	1.00												
RWC	0.54	1.00											
EL	0.64	0.97	1.00										
MDA	0.94	0.60	0.68	1.00									
Proline	-0.64	-0.18	-0.24	-0.59	1.00								
GB	-0.92	-0.55	-0.62	-0.90	0.72	1.00							
TSS	-0.93	-.061	-0.68	-0.92	0.68	0.92	1.00						
Chl ^{a}	0.50	0.96	0.97	0.55	-0.06	-0.47	-0.51	1.00					
Chl ^{b}	0.26	0.92	0.88	0.31	0.09	-0.25	-0.31	0.95	1.00				
Caro	-0.80	0.75	0.66	0.05	0.31	0.04	0.01	0.78	0.78	1.00			
SOD	-0.55	-0.38	-0.45	-0.49	0.51	0.58	0.55	-0.34	-0.23	0.02	1.00		
CAT	-0.70	-0.53	-0.60	-0.70	0.43	0.69	0.70	-0.49	-0.32	-0.10	0.80	1.00	
GPX	0.70	-0.64	0.23	0.69	-0.41	-0.68	-0.71	0.61	0.47	-0.23	-0.80	-0.95	1.00

The correlation coefficient above 0.22 are significantly different at p<0.05.

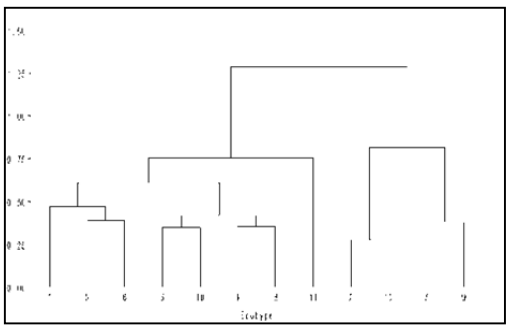

Figure 1) Clustering of ecotype dendrogram by cluster analysis

Discussion

Evaluation of salinity on Euphrates poplar ecotypes in this study showed that although this species is not a halophyte species, it had shown some characteristics of plants tolerant to salinity; it is similar to the results of a study carried out by Ma *et al.* [20]. Analysis of variance showed a significant difference in the effect of salinity on all of the traits; differences in treatment show different responses to treatments in different levels of salinity.

Ecotype effect was a significant difference in El, MDA, plant pigments, and some enzymes. Significant differences in the effects of ecotype showed the ecotypes reaction and no significant differences showed a similar response. Ecotype × treatment interaction was the significant difference in EL, MDA content, plant pigments, and enzymes and there was no significant difference in the RWC, Proline, carotenoids, GB and TSS. The significant difference between treatments × ecotype illustrates the effect of these two factors (salinity and ecotypes); so, ecotype has been dependent on the effect of changes in traits for different levels of salinity. In contrast, no significant difference in the interaction treatment × ecotype illustrates that the effect of salinity and ecotype is independent; while the interaction is zero, so the effects are additive.

Compared means of the ecotypes showed that Hamidieh was the highest in terms of vitality and performance and Mahneshan was in the lowest group. Since Hamidieh is an almost high salt and Mahneshan is the low salt habitats, it can be concluded that one of the factors affecting salt tolerance in *P. euphratica* is probably present in saline conditions.

Compared means of treatments showed that 75 mM was the highest in terms of vitality and performance (RWC and Chlorophylls) and in contrast, 300 mM was in the lowest group; while 300 mM in terms of traits was in the highest, indicating symptoms damage and stress (EL, MDA, Proline, GB, and TSS). Antioxidant enzymes, including SOD and CAT were in the highest group in 225 mM; in contrast, these two enzymes were in the lowest group in control and 300 mM. High levels of antioxidant enzymes in high concentration of salt indicated that antioxidant response is one way for tolerance to salinity in *P. euphratica*; however, reducing the level of this enzymes in 300 mM shows that high levels of salinity impairs the enzymatic reaction and this plant cannot regulate its physiological conditions at this level of salinity.

A correlation coefficient showed a significant correlation (positive or negative) between more traits. There was a positive correlation between growth and performance (RWC and plants pigments) together and there was a positive correlation between traits indicating symptoms damage and stress (EL, MDA, Proline, GB, and TSS) together. While there was a negative correlation between growth and performance with traits indicating symptoms damage and stress; in the other words, there was an antagonistic effect among them.

There was no difference between chlorophyll a and carotenoids in different levels of salinity or there was no significant trend by increasing salinity levels, but increasing salinity decreased the amount of chlorophyll b and showed an increase in chlorophyll a/b ration in high salinity levels (Figure 1). This agrees to the results of a study conducted by Bogeat-Triboulot *et al.* They reported an increase of chlorophyll a/b in terms of water scarcity [6]; on the other hand, these results showed similar responses in salinity and drought in *P. euphratica* [21, 5].

Cluster analysis distinguished ecotypes into 5 groups in terms of studied traits; cluster analysis revealed that clustering *P. euphratica* ecotypes was based on the physiological traits and not associated to geographical distance, namely clustering the ecotypes was based on habitat conditions, particularly soil salinity.

Conclusion

Overall, this result represents that *Populus euphratica* is a moderate halophyte, which could be suggested to reclamation of saline lands with high water table. This uses multiple mechanisms to overcome salinity stress and there is not a clear path to overcome salinity in this species. The presence of antioxidant enzymes such as SOD and CAT at 225 mM salinity was greater and less at control (without salt). Also, the enzyme GPX was higher at this level and it was less at 300 mM. This evidence suggests that antioxidant regulation is also one of the ways to cope with stress. The ecotypes grouping was not based on geographical distance, rather it was based on the conditions of the original habitat, especially soil salinity.

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Ethical Permissions: This study was not carried out in a protected or private area and no specific permission was required. We confirmed that the field study did not involve

endangered or protected species. In this study, no animal was tested, therefore, not subject to regulation.

Conflict of Interests: There is no conflict of interest in this research.

Authors' Contribution: Hossein Tavakoli Neko (First author), Introduction author/ Original researcher (40%); Anoushirvan Shirvani (Second author), Methodologist/ Statistical analyst (30%); Mohammad Hassan Assareh (Third author), Discussion author (20%); Mohammad Reza Morshedloo (Fourth author), Assistant (10%).

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