Salinity Effects on Direct Shoot Regeneration of Two Male Populus Clones

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Abstract

Plant regeneration from leaf and root of two Populus clones, P. tremulaL. and hybrid P.tremulaL. x P. tremuloides"Michx", under different salinity levels (control, 8, 12 and 14 dS/m) were investigated. The exposure of salinity stress to both leaves and roots explants during regeneration stage decreased the number of shoots/explant, the height of regenerated plantlets and explant weight. In addition, increased salinity level also decreased the mean values of thickness of midrib, mesophyll tissue, and the diameter of vascular bundle and biggest xylem vessels of both clones. Consistent with the decrease in regeneration and growth, the chlorophyll a and b, as well as leaf carotenoid content of the regenerated explants were reduced with increasing sodium ion and the ratio of Na^+/K^+ and Na^+/Ca^{2+} ions.

Key words - *In vitro, Populus tremula, hybrid poplar, salinity*

I. INTRODUCTION

Soil salinity is a major abiotic stresses worldwide (Schwabeet. al.,2006; Safarnejad, 2004). It is mainly due to the presence of predominantly Na^+ and Cl^- ions, which reduces the soil water potential, and disturbs the ions uptake and translocation processes, leading to nutritional imbalance. Subsequently, the accumulation of high Na^+ and Cl^- ions in cell cytoplasm will result in cell toxicity (Kafkafi and Bernstein, 1996). Salt stress affects plants at all stages, from seed germination to plant growth and development by altering different cellular processes such as photosynthesis, energy metabolism, gene expression and protein synthesis (Gupta and Huang, 2014; Yadavet. al., 2011; Parida and Das, 2005). In addition, high salinity also causes anatomical changes in plants, which some of these changes on root and leaf had been reported (Bizet et. al., 2015; Atabayeva et. al., 2013; Dolatabadian et. al., 2011;Kiliçetet. al., 2007;Junghans et al., 2006;Parida et. al., 2004; An et. al., 2003; Hu and Schmidhalter, 2001)

In-vitro selection has been widely used to select for desired phenotypes such as resistance or tolerance to both abiotic and biotic stresses. During the selection process, plant cells or tissues will be exposed to specific stress in order to generate "mutants" (variants) lines, which can overcome the corresponding stress (Gandonou et. al., 2005; Scandalios, 1993). Woody trees are important components of ecosystems, and also resources for bioenergy. Increase salinity in arable lands has led to many studies on salt responses and the screening for salt-tolerant woody trees (Melger et. al., 2008; Cha-um et. al., 2004; Nguyen et. al., 2004; Khasa et. al., 2002; Tewaryet. al., 2000).

Populus is a diocious plant genus with 25 to 35 species, which are native to Northern hemisphere. It is a rapid growing tree commonly used for wood and fuel, as well as a model organism for study of biological functions of trees (Taylor, 2002; Xioa et. al., 2009). Various studies have reported that male and female clones of the same poplar species respond differently under stress conditions (Yang et. al., 2015; Jiang et. al., 2012; Zhang et. al., 2010a, 2010b, 2012). Among these, Zhang et. al. (2012) and Yang et. al. (2015) have found that male clones perform better than female clones under potassium deficiency and drought stress.

Populus species show a wide range of sensitivity towards salinity stress as an adaptation to their native habitat (Polle and Chen, 2015; Sixto et. al., 2005). *P. euphratica* Oliver, which is commonly found in saline arid and semi-arid habitat is the most salt tolerant species (Polle and Chen, 2015), while *P. tremula* (European aspen) is moderately sensitive to salt (Jouve et. al., 2004), and *P. tremuloides* (American quaking aspen) is highly sensitive to salt stress (Polle and Chen, 2015). In this study, we have compared the ability of leaf and root explants of two poplar clones, the *P. tremula*L. and the hybrid aspen *P.Tremula*L. x *P. tremuloides*"Michx" to regenerate on media with different seawater concentrations as well as leaf histological features of the regenerated plantlets under salinity were studied.

II. MATERIALS AND METHODS

A. Establishment of in-vitro Populus plantlets

In-vitro plantlets of two male poplar clones, *P. tremula*L., clone W52 and hybrid *P. tremula* L. x *P.tremuloides* "Michx", clone T89 were kind gift from Dr. Matthias Fladung, University of Hamburg (Fladung, pers. communication). These plantlets were then acclimatized and maintained in the greenhouse at Experimental Farm of Ismailia Agriculture Research Station, Ismailia Governorate, Egypt. The tissue culture experiment was conducted from June 1, 2015 until November 1, 2015 at the Tissue Culture Laboratory, Department of Horticulture, Suez Canal University, Egypt.

Shoot tips were excised from both poplar clones W52 and T89 grown in the greenhouse. Shoot tips were sequentially surface sterilized with 70% ethanol solution for 30 sec, 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) for 5 min, and 15% (v/v) sodium hypochlorite solution for 5 min. The traces of sodium hypochlorite was removed by performing the rinsing of the shoot tips with sterile tap water for 3 times in a laminar air-flow hood. Surface sterilized shoot tips were then cultured in a 40 mL tissue culture jar containing 10 mL of medium, which consists of half strength MSbasic salts and vitamins medium, 2% (w/v) sucrose and 6.0 gL^{-1} agar. The pH of the medium was adjusted to 5.7, prior to autoclaving at 121°C and 1.2-1.3 kg/cm^2 pressure for 20 min. The tissue culture jars were incubated in a growth room with controlled temperature at 22 ± 2°C. A 16 h photoperiod is provided by florescent lamps (Phillips TLM 40W/33RS) with light intensity of 4000 Lux.

B. Effects of different seawater salinity levels on shoot regeneration from leaf and root explants

Leaf and root of eight months old (10-15 mm in length) were excised from the established *in-vitro* plantlets of both clones W52 and T89 and used as initial explants. The explants were cultured in 92 x 16mm Petri dishes containing 35mL medium, which consists of full strength MS basic salts and vitamins medium, 2.0% (w/v) sucrose, 6.0 gL^{-1} agar, and 0.01 µMthidiazuron (TDZ) (El Sherif and Khattab, 2011). Salinity of the medium was adjusted by adding appropriate amount of seawater (54.69dS/m) to achieve the final salinity level of 8, 12 and 14 dS/m. Control medium was medium without the supplement of seawater. The pH of all media was adjusted to 5.7, before autoclaving at 121°C for 20 min. The thidiazuron (TDZ) was added to the autoclaved medium after it was cooled down to 47°C. The leaf explants were placed on Petri dishes with abaxial surface facing up. Each treatment contained 10 Petri dishes (replicates) with 4 explants discs. After eight weeks, the explant weight, number of shoots regenerated from each explant, and length of the longest shoot from both leaf and root explants of poplar clones W52 and T89 were recorded.

C. Chlorophyll and carotenoid pigments determination

After 8 weeks of culturing, the content of chlorophyll a (Chl-a), chlorophyll b (Chl-b) and carotenoid in the leaves of the regenerated plantlets of poplar clones W52 and T89 were determined calorimetrically according to A.O.A.C. (1980).

D. Mineral composition determination

After eight weeks of culturing, whole regenerated plantlets from poplar clones W52 and T89 leaves and roots explants were collected and dried at 70°C for 24 h. Analyses of sodium, calcium and potassium were performed by grinding the dried plantlets followed by digestion with H_2SO_4 as described by Piper (1947).The sodium, calcium and potassium contents were then measured by using Atomic Absorption flame photometric (3300) according to Wilde et. al. (1985).

E. Histological analysis of leaf segments

Leaves from regenerated plantlets produced from leaf explants of both poplar clones W52 and T89 were excised, and fixed in FAA (Formalin-Acetic-Alcohol), followed by dehydration with a series of ethyl alcohol. The leaves were then embedded in paraffin wax, and sectioned with microtome to a thickness of 15µm. The leaf sections are double-stained with Safranin and Light green, followed by clearing with Xylene and mounted in Canada balsam (Willey, 1971). Histological examination and measurements were performed using a Leica light Research Microscope model DM500/13613210, which was supplied with a digital camera. Cuticle measurements were performed using the eyepiece micrometer.

III. RESULT AND DISCUSSION

A.Effect of different seawater salinity levels on regeneration and growth of poplar clones

Incorporation of seawater in the regeneration medium enabled us to study the effects of salt stress at the regeneration stages. In addition, *in-vitro* regeneration under salinity condition has also been used for the selection of salt-tolerant phenotype (Sajid and Aftab, 2014; Priya et. al., 2011; Akram et al. 2010; Kumar et. al., 2008;Shankhdhar et. al., 2000;Ochatt et. al., 1999; Winicov, 1993; Beloualy and Bouharmont, 1992;Reddy and Vaidyanath, 1986). In this study, we obtained hundred percent of shoot regeneration in both W52 and T89 leaf and root explants cultured in the control medium without addition of seawater, as well as

those cultured on medium with moderate salinity (8 dS/m). Increased salinity caused gradual decrease in the percentage of shoot regeneration from leaf and root explants of both W52 and T89 clones, with the lowest regeneration rate (20%) as observed for explants cultured in medium at 14 dS/m salinity level (data not shown). The effects of salinity level on shoot regeneration from shoot and root explants of poplar clones T89 and W52 are shown in Fig 1 and Table-1.The number of regenerated shoots per explant decreased significantly with increasing salinity levels as compared with control. In clone T89, the number of regenerated shoots reduced remarkable at salinity level as low as 8 dS/m as compared to control. On the other hand, similar effect was only observed at higher salinity level (12 dS/m) for clone W52. In addition, clone W52 shoot and root explants cultured at different salinity levels also produced more shoots as compared to the corresponding explants of clone T89 cultured at similar salinity level. Root explants from both T89 and W52 clones cultured on medium with or without different salinity level consistently produced more shoots as compared to the leaf explants cultured on medium with similar salinity level. In addition, remarkable reduction in the number of regenerated shoots per root explants was only observed at high salinity (14 dS/m) as compared to that of the shoot explants. Taken together, we thus concluded that root explants is a better choice than shoot explants for shoot induction under control and salinity conditions. Furthermore, the clone W52 is found to regenerate more shoots as compared to clone T89 when cultured under salinity conditions.

The whole explant fresh weight and shoot height of regenerated shoots from both shoot and root explants of poplar clones T89 and W52 are shown in Table-1. Regenerated plantlets from shoot and root explants of both clones T89 and W52 that were cultured in medium with moderate salinity (8 dS/m) showed the highest average plant height and whole explant weight as compared to the control. This implies that moderate salinity promotes cell proliferation without causing any morphological abnormality to the regenerated plantlets. Increased salinity, above 8 dS/m resulted in significant decrease in plantlets height and whole explants weight from both W52 and T89 clones. However, it is interesting to note that regenerated plantlets from leaf explants of clone W52 cultured on medium at salinity level of 12 dS/m also showed higher plant height and fresh weight as compared to control. This observation may implies that clone W52 is more tolerant to salinity, which is consistent with the observation that clone W52 explants consistently regenerated more shoots under salinity conditions as compared to clone T89. Under salinity stress, which causes nutritional imbalance, osmotic and metabolic interruptions, higher plants in general slowdown their metabolism and growth (Shahid et. al., 2013; Zhu, 2001), in order to manage and utilize the available resources more efficiently (Zahoor and Faheem, 2014).

B. Chemical analysis of regenerated shoots

Increased salinity caused decrease in both chlorophyll a and b contents in regenerated plantlets from both shoot and root explants of clones T89 and W52 (Table-2). Decrease in chlorophyll contents resulted from salinity stress is a general observationin many plant species as associated with the toxicity effects of Na^+ or Cl^- on photosynthesis (El-Sherif, 2013; Erturk et. al., 2007; Parida and Das, 2005,). However, our result also indicated a surge in chlorophyll a and b contents in regenerated plantlets from shoot and root explants of clone T89 cultured in medium with 8 dS/m salinity (Table-2), which correlate with the observed higher growth rate of these clone in the corresponding media (Table-1). On the other hand, the contents of chlorophyll a and b in the regenerated plantlets from shoot and root explants of clone W52 cultured in similar salinity level (8.0 dS/m) was lower than that of the control, despite the observed higher growth rate as compared to the control treatment.

Carotenoids, an antioxidant, is reported to be associated with salt tolerance in crop plants (Hernandez et. al., 1995). Elevated level of carotenoid in plants under salinity stress has been reported to protect the leaves tissues against oxidative stress induced damages (Singh et. al., 2008; Verma and Mishra, 2005). Nevertheless, decrease carotenoids contents have also been reported in respond to salinity treatment in some plant species (Agastian et. al., 2000; Gadallah, 1999; Mitra and Banerjee, 2010). Our results have indicated that the carotenoid contents followed the same trends as that of the chlorophyll a and b contents in all corresponding plantlets (Table-3).

The effects of seawater salinity on the contents of sodium (Na^+), calcium (Ca^{2+}) and potassium (K^+) ions in the regenerated plantlets of clones T89 and W52 shoot and root explants are presented in Table-3. As seawater salinity level in the medium increased, the level of Na^+ and Ca^{2+} ions in the regenerated shoots of both clones significantly increased. On the other hand, the K^+ ion concentrations significantly decreased with the increased of salinity level in the growth media. Our results agreed with that observed by Sixtoet. al. (2005), in which the concentration of Na^+ ion, as well as the ratio of Na^+/K^+ and Na^+/Ca^{2+} increased with increase of salinity level in the treatment. In the same report, Sixtoet. al. (2005) has found that salt tolerant *Populus* species such as *P. euphratica* and *P. alba*showed less changes as compared to salt sensitive species such as *P x euramericana*. However, in our study, we found that

clone W52, which showed better growth and regeneration than clone T89 under saline conditions, surprisingly showed more prominent changes in concentration of Na^+ ion and the ratio of Na^+/K^+ and Na^+/Ca^{2+} as compared to clone T89.

Previous reports from other poplar species have confirmed that the concentration of Na^+ ion in leaf correlate negatively with the contents of chlorophyll a, b and carotenoid as well as plant growth (Beritognolo et. al., 2007; Chen et. al., 2001, 2002, 2003). Our results have also indicated that the increase of Na^+ ion in leaves (Table-3) correlates with the decrease of chlorophyll a, b and carotenoid contents (Table-2) for both clone W52 and T89, with only the exception in regenerated shoots from shoot and root explants of clone T89 that were cultured in medium at 8 dS/m. In these plantlets, higher chlorophyll and carotenoid contents as well as growth as compared to control plantlets were observed. This observation may be due to the ability of T89 to compartmentalize the Na^+ ion in vacuole or apoplast, which avoid the Na^+ ion to cause any negative effects on photosynthesis and plantlets growth. As for clone W52, despite the increase of Na^+ ion in leaf and the corresponding decrease in chlorophyll and carotenoid contents, the plantlets from shoot explants cultured at 8 and 12 dS/m were showing higher growth as compared to that of the plantlets in control media.

C. Effect of seawater salinity on some histological features of leaf section

Table-4 and Figure 2 present the measurements of several leaf ultra-structures from regenerated plantlets of *P. tremula* clone W52 and hybrid clone T89 leaf explants, which were cultured on MS media with different seawater salinity levels (control, 8 and 12 dS/m). The leaf samples were collected after eight weeks from the date of explants culturing. We observed that the midrib thickness, and diameter of vascular bundle, as well as the number of xylem vessels/ transverse section of clone W52 clone were significantly higher than that of clone T89; while the thickness of mesophyll layer of clone T89 was superior. However, besides these genotypic difference, the histological changes resulted from the salinity treatments was similar between clones W52 and T89. We found that the average diameter of vascular bundle and biggest xylem vessels were significantly decreased, while the number of xylem vessels/transverse section increased as the result of increase salinity in the culture medium. The xylem anatomy of Poplar species show high plasticity, in which the number of xylems and diameter of xylem varies according to water availability (Beniwal et. al., 2010). Junghanset. al. (2006) and Janzet. al. (2012) have reported that salinity treatment reduced vessel lumen area and increased cell wall thickness in salt sensitive poplar species such as *P. canescens.* Reduced width or diameter of vascular bundles as the result of salinity treatment were also observed in barley, rice and mung beans (Atabayeva et. al, 2013; Rashid et. al., 2004). In addition, we also observed that the average thickness of midrib and mesophyll tissue layer decreased with increase salinity level in the media. These observations were in agreement with that observed in wheat, kallar grass and mangrove (Hu and Schmindhaltar, 2001; Ola et. al., 2012, Parida et. al., 2004).

IV. CONCLUSION

In terms of regeneration and growth traits (shoot height and whole explants weight), we have found that *Populustremula*L., clone W52 is more superior as compared with hybrid *Populustremula* L. x *Populustremuloides* "Michx, clone T89. Root explants from both W52 and T89 clones consistently regenerated more shoots as compared to the shoot explants; therefore it is deemed to be a better source for shoots induction and could be further explored for micro-propagation of poplar. Increase salinity level in the medium resulted in increase in Na^+ ion contents in leave of both clone W52 and T89.

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Explant type	**Seawater levels (dS/m)**	**Longest shoot (cm)**		**Explant fresh weight (g)**		**No. of shoots/ explant (n)**	
		Clone T89	**Clone W52**	**Clone T89**	**Clone W52**	**Clone T89**	**Clone W52**
Leaf segment	Control	0.83 bcd*	0.65 bcd	0.17 c	0.24 c	9.33 cd	8.25 cdef
	8	2.00 ab	2.13 ab	0.28 c	0.48 abc	5.00 fg	7.50 cdefg
	12	0.67 bcd	1.32 bc	0.19 c	0.50 abc	4.13 efg	4.83 fg
	14	0.25 cd	0.10 d	0.12 c	0.22 c	2.50 g	6.00 defg
Root segment	Control	0.63 cd	2.10 ab	0.24 c	0.68 ab	11.25 bc	16.00 a
	8	3.33 a	2.67 a	0.29 c	0.75 a	9.33 cd	15.00 ab
	12	0.50 cd	0.88 bcd	0.22 c	0.36 bc	8.75 cde	14.75 ab

	14	0.23 cd	0.10 d	0.20 c	0.29 c	4.67 fg	10.00 c

Table 1.Effect of seawater salinity levels on adventitious shoot regeneration from leaf and root explants of *Populustremula*L., clone W52 and hybrid *Populu1s. tremula*L. x *Populustremuloides*Michx., clone T89. *Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test.

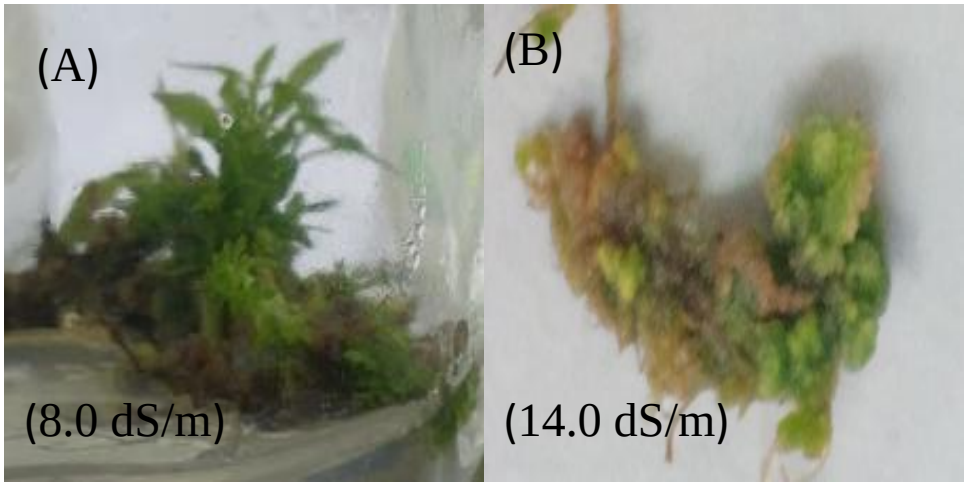
Explant type	**Seawater levels (dS/m)**	**Chl a (mg/100g F.W.)**		**Chl b (mg/100g F.W.)**		**Carotenoids (mg/100g F.W.)**	
		Clone T89	**Clone W52**	**Clone T89**	**Clone W52**	**Clone T89**	**Clone W52**
Leaves segments	Control	0.14 c*	0.13 d	0.042 e	0.044 e	0.18 de	0.17 def
	8	0.32 a	0.11 g	0.10 b	0.032 f	0.34 c	0.13 efg
	12	0.09 h	0.04 l	0.03 h	0.014 i	0.11 fgh	0.08 gh
	14	0.07 j	0.03 m	0.02 fg	0.011 i	0.08 gh	0.06 h
Root segments	Control	0.11 f	0.15 b	0.06 c	0.044 e	0.57 b	0.21 d
	8	0.15 b	0.14 e	0.13 a	0.043 e	1.15 a	0.11 fgh
	12	0.04 kl	0.08 i	0.05 d	0.02 g	0.36 c	0.10 gh
	14	0.03 n	0.04 k	0.01 i	0.01 i	0.06 h	0.07 h

Table 2.Effect of seawater salinity levels on the contents of chlorophyll a (mg/100g F.W.), chlorophyll b (mg/100g F.W.) and carotenoids (mg/100g F.W.)in the leaves of regenerated shoots from leaf and root explants of *Populustremula*L., clone W52 and hybrid *Populus. tremula*L. x *Populustremuloides*Michx., clone T89. *Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test.

Explant type	**Seawater levels (dS/m)**	**K%**		**Na%**		**Ca%**	
		Clone T89	**Clone W52**	**Clone T89**	**Clone W52**	**Clone T89**	**Clone W52**
Leaf segments	Control	2.01 a*	0.92 e	0.31 h	0.34 h	0.099 ef	0.110 ef
	8	1.12 c	0.60 h	0.95 ef	2.10 b	0.279 b	0.262 b
	12	0.97 d	0.59 h	1.90 c	2.81 a	0.140 d	0.211 c
	14	0.91 e	0.30 j	1.60 d	1.05 ef	0.161 d	0.211 c
Root segments	Control	0.82 f	1.22 b	0.30 h	0.55 g	0.023 g	0.083 f
	8	0.72 g	0.71 g	0.40 gh	1.10 e	0.110 e	0.151 d
	12	0.71 g	0.61 h	1.70 d	1.60 d	0.151 d	0.299 a
	14	0.18 k	0.39 i	1.10 e	0.90 f	0.112 e	0.141 d

Table (3). Effect of seawater salinity levels on the contents of potassium (%) , sodium (%) and calcium (%) in the leaves of regenerated shoots from leaf and root explants of *Populustremula*L., clone W52 and hybrid *Populus. tremula*L. x *Populustremuloides*Michx., clone T89. *Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test

Table (4). Effect of seawater salinity levels on some histological measurements of leaf structureof regenerated plantlets from leaf explants of *Populustremula*L., clone W52 and hybrid *Populus. tremula*L. x *Populustremuloides*Michx., clone T89. *Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test.



Seawater levels (dS/m)	**Thickness of midrib (µm)**		**Thickness of mesophyll tissue layer (µm)**		**Diameter of vascular bundle (µm)**		**Diameter of biggest xylem vessels (µm)**		**Number of xylem vessels/ transverse section**	
	Clone T89	Clone W52	Clone T89	Clone W52	Clone T89	Clone W52	Clone T89	Clone W52	Clone T89	Clone W52
Control	4.0 $_{b}$*	4.3 $_{a}$	1.80 $_{a}$	1.20$_{a}$	1.7$_{b}$	2.6 $_{a}$	0.17 $_{a}$	0.19 $_{a}$	32.0 $_{c}$	33.0 $_{c}$
8.0	1.8 $_{e}$	3.0$_{c}$	1.00 $_{b}$	0.77 $_{c}$	0.71 $_{d}$	1.3 $_{c}$	0.14 $_{b}$	0.16 $_{b}$	34.0 $_{b}$	35.3 $_{b}$
12.0	1.1$_{f}$	2.6$_{d}$	0.85 $_{bc}$	0.26$_{d}$	0.37 $_{d}$	1.3 $_{c}$	0.12 $_{c}$	0.14 $_{c}$	42.7 $_{a}$	43.0 $_{a}$

Figure 1. Effect of seawater salinity levels on shoot regeneration from root explant of Populus clones W52 (A and B) after eight weeks growth on MS medium supplemented with 0.01 µM TDZ .

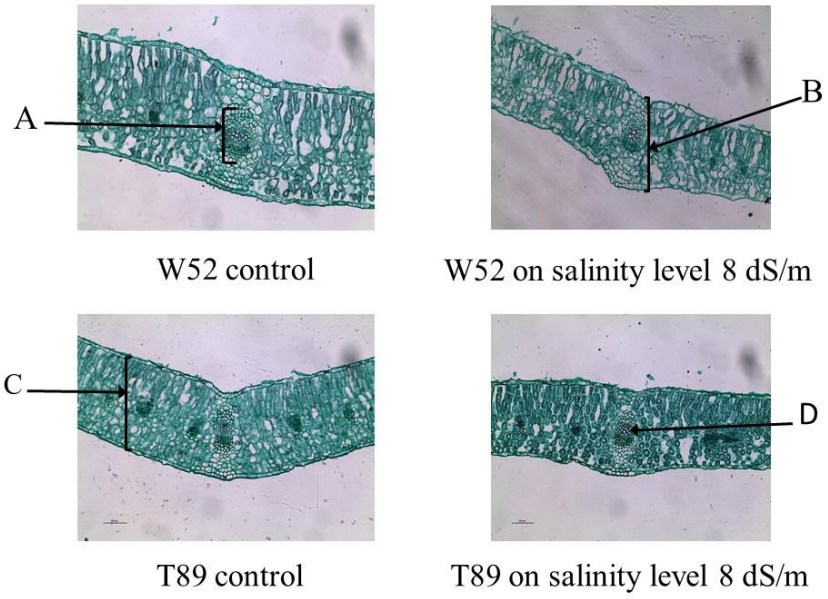


Figure 2. Transverse sections on some leaf anatomical characters of two poplar clones (T89 and W52) after eight weeks of in vitro cultured on MS medium supplemented with different concentration of salinity levels. X = 18x40 A(vascular bundle), B(midrib), C (mesophyll tissue) and D (xylem tissue).