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RESEARCH ARTICLE

In vitro NaCl tolerances of *Artemisia dracunculus*

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Abstract: *In vitro* evaluation of salinity effects on *Artemisia dracunculus* was investigated using five NaCI concentrations (0, 25, 50, 100 and 150 mM). The exposure to NaCl in multiplication and root stages affects, all the vegetative parameters which showed significant decrease with increasing NaCl concentrations in the both stage. Biochemical and chemical parameters such as pigments, Na^+, K^+ and Cl^- in plant were tested in order to put forward the relative tolerance of plant to salinity. Additionally, electrophoretic analysis of total soluble protein (SDS-PAGE) has revealed that plant subjected to NaCl showed induction in the synthesis of new polypeptides. This finding suggest that, the response of *Artemisia dracunculus* to salt stress may be accomplished by synthesis of new protein which could be in turn contribute to select a salt resistant lines. The highest values of estragol were obtained under non-salinity condition (control) using HPLC analysis .Salinity stress significantly decreased estragol.

Keywords: *In vitro*; *Artemisia dracunculus*; salinity; NaCl; SDS-PAGE; estragol**;** HPLC.

Introduction

Soil salinity is one of the major environmental abiotic stresses especially in arid and semi-arid regions and can severely limit plant growth and yield (Safarnejad, 2004). Its prevalence throughout the world is increasing regularly in extent (Schwabe *et al*., 2006). According to the most recent statistics, over 800 million hectares of land throughout the world are salt-affected, either by salinity (397 million ha) or the associated condition of sodicity (434 million ha) (FAO, 2005). The increase in salinization of arable land is expected to have disturbing global effects, resulting in 30% land loss within the next 25 years and up to 50% by the middle of 21st century (Hasegawa *et al*., 2000). Nearly 20% of the world's cultivated areas are affected by salinity (Zhu, 2001).

Soil salinity imposes two types of stresses on plants. The first one is nutritional imbalance caused by saline ions and low soil water potential in both uptake and translocation process. The second one is toxicity due to the high accumulation of Na^+ and Cl^- ions in the cytoplasm (Kafkafi *et al*., 1996).

It is possible to use cell and tissue culture techniques for the assessment of salt tolerance competence in plants since it allows for relatively fast responses, short generation time, controlled environment, obtaining salt tolerance plants and may offer potential for quick evaluation of germplasm against salt stress (Scandalios, 1993, Gandonou *et al*., 2005). Shoot apex culture has been found to be an effective method for isolating salt-tolerant genotypes from a large population within a short period of time (Martinez *et al*., 1996).

Salt stress affects all the major processes such as growth, photosynthesis, protein synthesis, and energy and lipid metabolism, in which there are much research information about response to salt stress (Parida and Das, 2005). Plant responses to salinity and other water deficit stresses such as drought have been investigated using proteomic/genomic based approaches (Wang *et al*., 2003, Vinocur and Altman 2005; Yildiz, 2007). There are many reports showing that the protein pattern changes are accompanied by the biological changes in the adaptation process, which makes the organism more fit in the altered environment (Singh *et al*., 1985, Hurkman *et al*., 1988, Mohamed *et al*., 2010).

Medicinal and aromatic plants have received much attention in several fields such as agroalimentary, perfumes, pharmaceutical in-

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dustries and natural cosmetic products (Baatour *et al*., 2009). The use of medicinal plants is increasing worldwide. According to the world health organization (WHO), approximately 80% of the world's population currently uses herbal medicines directly as teas, decocts or extracts with easily accessible liquids such as water and milk (Julsing *et al*.,2007). Russian tarragon (Asteraceae) is a bitter warming aromatic herb that stimulates the digestive system and uterus, lowers fevers and destroys intestinal worms. The essential oil is used in aromatherapy to treat digestive and menstrual problems. An ethanolic extract of *A. dracunculus* was recently shown to reduce blood glucose concentrations in STZ induced diabetic mice (Bown *et al*., 1995, Ribnicky *et al*., 2004 Ribnicky *et al*., 2006). Estragole (4-allyl-1-methoxybenzene) is a naturally occurring food flavoring agent found in tarragon, basil, fennel, bay leaves, and other spices. Estragole and its metabolite, 1_-hydroxyestragole (1_-HE), are found to be hepatocarcinogens in rodent models (Ribnicky *et al*., 1997). Occurrence of estragole in the essential oil fractions from different aromatic plants has been reported (Iyer *et al*., 2003), its concentration in tarragon has been estimated to be from 60%-75% which was considered the highst amongst the other tested plants. The study about changes of secondary metabolites under different environmental conditions such as salinity is necessary (Bandaranayake, 2002).

Among the several approaches to solving the problem of saline soils, the biological approach aims to identify and grow salt tolerant plants under these conditions. It is essential to test important medicinal plants for their salinity tolerance as research efforts aim to derive economic benefits under saline soil conditions. This study was design to study the effect of different sodium chloride (NaCl) concentrations, *in vitro*, on the growth and shoot multiplication derived from explants of *A. dracunculus*.

Results and Discussion

Effect of salinity on shoot and root formation

Salinity has been adversely affecting quality and quantity of vegetative characters of multiplying plantlets (Table 1), it is obvious that the fresh weight, numbers of shoots and leaves/explants decreased with increasing NaCl concentrations. In addition, increasing NaCl level changed color of leaves from green to greenish yellow Figure 1. Survival of explants was 100% in all NaCl concentration (data did not shown). NaCl at the level 150 mM gave the lowest result for all parameter under investigation (Table 1). The data suggested that the *Artemisia dracunculus* could tolerate a NaCl concentration of 150 mM.

Effect of NaCl concentrations on root formation after four weeks was observed in Table 2. The highest plant height, fresh weight, dry weight, root length, number of roots and number of leaves of plant were produced at 25 mM NaCl and then decreased with increasing NaCl concentrations (Table 2). The lowest results for all root stage parameter were obtained when the cultures were subjected to higher level 150 mM NaCl. In the present study, the acclimatization procedures applied was successful. *In vitro* regenerated plantlets showed 80% survival when transferred to soil (data did not shown).

Table 1: Tolerance of *Artemisia dracunculus* to different NaCl levels after eight weeks at multiplication stage.

NaCl mM	Explant fresh weight (g)	Longest shoot (cm)	No. of shoots/ explant	No. of leaves/ explant
0.0	2.8a*	3.7b	30.0a	113.4a
25	1.6b	4.1b	15.9b	74.1b
50	1.9ab	7.1a	8.5bc	68.8b
100	1.6b	5.4ab	4.3c	55.1b
150	1.5b	4.9b	4.7c	45.7b

*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

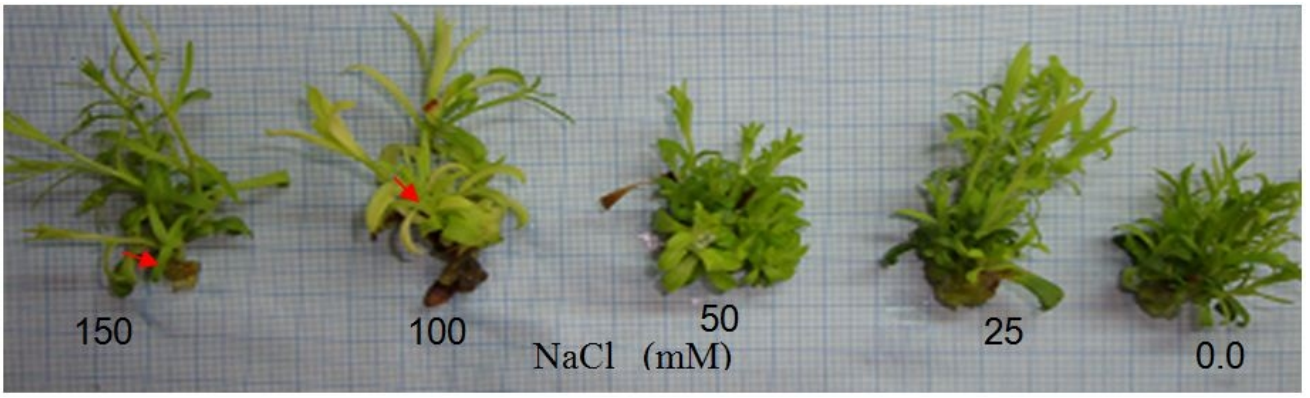

Figure 1: Effect of different levels of NaCl on multiplication stage of *Artemisia dracunculus* after eight weeks growth on MS medium supplemented with 1.0 mg l^{-1}BA (greenish and yellow leaves is arrowed).

Table 2: Tolerance of *Artemisia dracunculus* to different NaCl levels after four weeks at rooting stage.

NaCl mM	Plant height (cm)	Plant fresh weight (g)	Plant dry weight (g)	Length of the Longest root (cm)	No. of roots/ explant	No. of leaves/ explant
0.0	4.5c*	0.65ab	0.16a	2.6b	3.3ab	24.3a
25	9.4a	0.97a	0.12ab	8.8a	4.3a	28.9a
50	8.8ab	0.86a	0.10b	6.9a	3.8ab	25.5a
100	7.3b	0.69ab	0.10b	3.1b	2.2bc	18.2b
150	2.6c	0.38b	0.08b	1.1b	0.67c	14.8b

*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

Reduced tissue growth in stressful medium is a typical phenomenon that has been interpreted as a change in metabolism initiated to resist stress. This reduction could be caused by toxicity associated with excessive uptake of Na^+ and nutrition imbalance (Cabanero *et al*., 2004, Yong *et al*., 2004). Similar results were obtained by (Shiyab *et al*., 2003; Aziz *et al*., 2008; Khorasaninejad *et al*., 2010).

Chemical analysis

Salinity causes decrease in photosynthetic pigments (Chl a and b) in plants (Figure 2). Chlorophyll content decreased as NaCl concentration increased in the media. Maximum values for chlorophyll a and b were 70.7 and 44.9 mg\100g F.W. respectively at 0.0 NaCl, whereas the lowest values 15.1 and 8.5 mg\100g F.W. were obtained at 150 mM NaCl. Decrease in chlorophyll content in response to salt stress is a general phenomenon (Parida and Das 2005; Chen and Yu 2007; Erturk *et al.*, 2007).

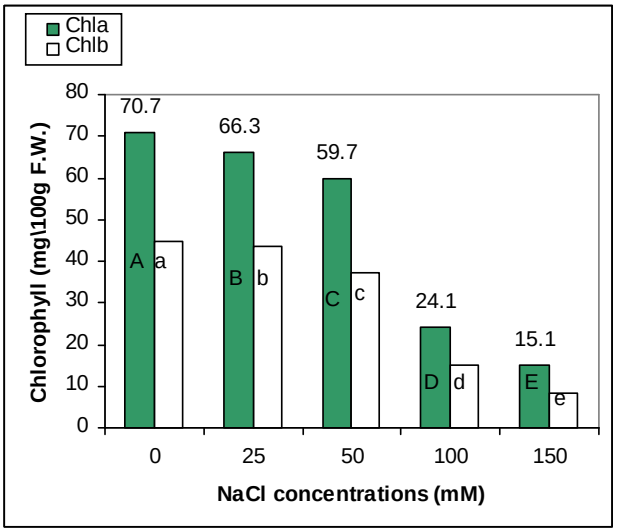

Figure 2: Effect of different levels of NaCl on chlorophyll a and b contents of *in vitro* grown *Artemisia dracunculus* after four weeks of growth on MS medium supplemented with 0.1 mg l^{-1}IBA.

Effect of salinity on sodium, chloride and potassium ions concentrations is presented in

Figure 3. As NaCl in the medium increased, Na^+ and Cl^- contents increased in plant tissue of *Artemisia dracunculus* after eight weeks of growth Figure 3. Na^+ and Cl^- reached maximum values (53.1 and 50.9 meq m^{-1} respectively) at NaCl 50 mM with significantly different as compared to control (27.4 and 25.9 meq m^{-1} respectively) Mineral nutrients and uptake is adversely affected by high salinity levels (Al-Karaki *et al*., 1995). In this study, Cl^- and Na^+ responded in a similar pattern. As salinity increased Cl^- and Na^+ contents were increase until reached 50 mM NaCl and then decreased. Potassium content in plant tissues of *Artemisia dracunculus* was significantly decreased as NaCl level increased in the medium (Figure 3).There are significant differences among all values (Figure 3). Reduction of K^+ content in plant leaves adversely affect metabolic function and eventually reduce plant growth (Greenway and Munns 1980). Potassium ions are known to be a major component of osmotic adjustment under NaCl stress (Shannon, 1992; Ottow *et al*., 2005). The presence of a high concentration of K^+ in control, is supposed to be acting as a natural inorganic osmoregulator (Watad *et al*., 1991; Hasegawa *et al*., 2000; Chen *et al*., 2003). Maybe, it is allowing Na^+ to enter in tissues, situation of lowering of K^+ content is because of Na^+ shock (Figure 3). So, optimum K^+ concentrations narrow with increasing Na^+ (Lutts *et al*., 1999; Lacerda *et al*., 2001). A similar result was obtained by (Tarakcioglu and Inal, 2002; Shiyab *et al*., 2003; Abu-Romman and Suwwan, 2008).

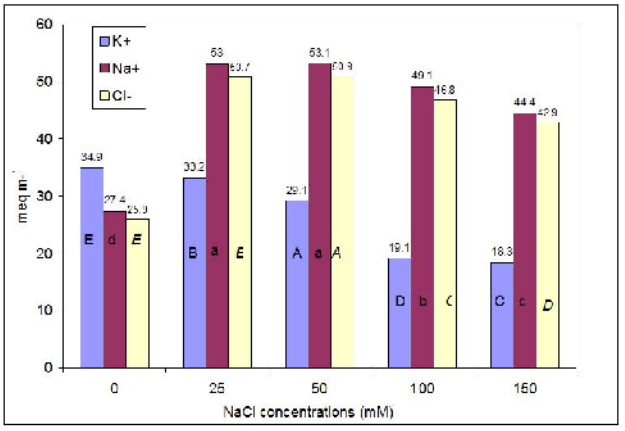

Figure 3: Effect of different levels of NaCl on K^+, Na^+ and Cl^- contents of *in vitro* grown *Artemisia dracunculus* after four weeks of growth on MS medium supplemented with 0.1 mg l^{-1}IBA.

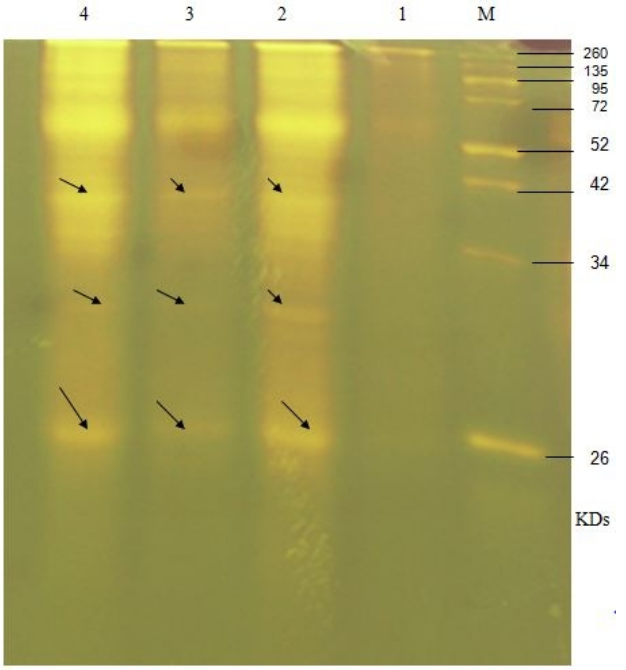

Figure 4: Protein pattern *of Artemisia dracunculus* extract. Lanes 1, 2, 3 and 4 from right to left represent proteins extracted from control, 50,100 and 150 mM NaCl respectively. Lane M represents the molecular weight marker (KDs).

Protein profile

The protein patterns of *Artemisia dracunculus* at different concentration of NaCl were analyzed. The separated bands of protein subunits were photographed and presented in (Figure 4).When comparison between control and salt treated samples, two new bands of low molecular weight appeared in sample prepared from salt stressed. Arrows in (Figure 4) marked a couplet of peptides in lanes 2, 3 and 4 but missed in lane 1. The pattern of low molecular weight band indicated that, there was a particular strong induction of biosynthesis of (39.8, 31.1 and 26.2 KDs) proteins in plant under different salt treatments. The expression of proteins, which induced under salt stressed plants was genetically regulated, depending on the salt concentrations as well as the genetic differences (El-Farash *et al*., 1993; Ozalp *et al*., 2000; Naqvi *et al*., 2009; Mohamed *et al*., 2010). In general, these patterns may give a remarkable marker to relay the discrimination between treated and untreated plant since one could consider the presence of new bands as an adaptive band for stress treatment. Identification of in-

duced proteins necessitates exploring their function, which is not an easy task. Transport of ions to various compartments is a function of biomembranes; hence peptides induced during salinity may play some role in transport across these barriers by acting as components of ion exchange pumps/channels embedded in these membranes. Finding sub-cellular localization is therefore an important aspect (Naqvi *et al*., 2009).

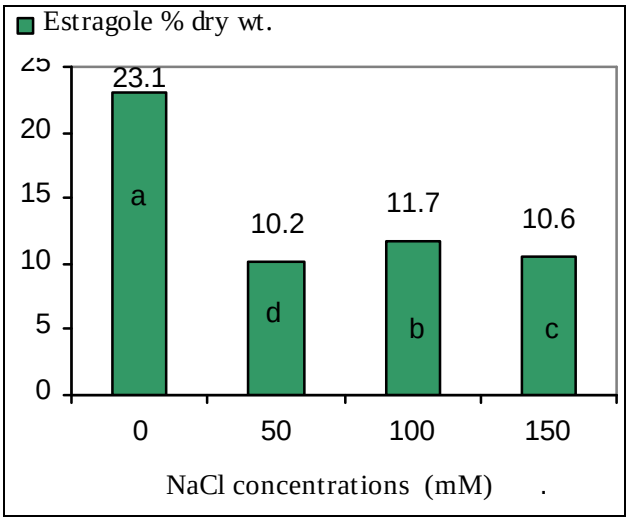

Figure 5: Effect of different level of NaCl on estragole contents of *in vitro* grown *Artemisia dracunculus* after four weeks of growth on MS medium supplemented with 0.1 mg l^{-1}IBA.

Quantitative determination of estragole

Estragole concentrations in correlation to different external NaCl concentrations as shown in Figure 5. Estragole concentration was decreased as salinity increased (Figure 5). The highest and lowest proportion of estragole was observed in control and 50 mM NaCl treatments, respectively. Significantly higher levels of estragole were observed in the control (Figure 5). Salinity stress significantly decreased essential oil yield and essential oil percent. Khorasaninejad *et al*. (2010) and Baatour *et al.* (2009) also showed that oil content in Marjoram and Peppermint were decreased consistently with increase in external salt levels

Material and methods

Plant materials and salt treatments

Shoot tip (0.5 cm height) explants of *A. dracunculus* produced from *in vitro* germinated seed (Flora-Frey Solingen, Germany) was cultured into 200 ml capacity jars containing 60 ml MS medium (Murashige and Skoog, 1962) containing basic salts and vitamins, supplemented with 3% (w/v) sucrose, 7.0 g/L agar, 1.0 mg l^{-1} benzyl amino purine (BAP) according to Khattab and El Sherif (2010) and different concentrations of NaCl (0.0, 25, 50,100 and150 mM). Medium was adjusted to pH 5.7, prior to autoclaving at 121°C and 1.2–1.3 kg/cm^2 pressure for 20 min. The cultures were grown for eight weeks before data were recorded based on number of shoots, plant height, plant weight as well as the number of leaves.

For rooting, shoot tips (0.5-1.0 cm in length) were cut and cultured on the MS medium containing 3% (w/v) sucrose, 7.0 g/L agar, 0.1 mg l^{-1} IBA according to Khattab and El Sherif (2010) and different concentrations of NaCl (0.0, 25,50,100 and150 mM) (each treatment was recultured on the same NaCl concentration for rooting). After four weeks data were recorded on number of roots, root length, fresh and dry weight of explants and plant height. The dry weight of the plants cultured on various concentration of NaCl in rooting stage was measured after drying the samples at 70°C for 24 h.

The rooted plantlets were transferred to greenhouse for acclimatization in pots with a moist mixture of (1:1) sand and perlite and maintained inside a plant growth chamber and irrigated with a fine mist of water for three weeks. The percentage of survival plant was determined after four weeks.

Chlorophyll pigments determination

The chlorophyll a (Chl-a) and chlorophyll b (Chl-b) in leaves developed from rooting stage explants were determined calorimetrically according to A.O.A.C. (1980).

Mineral composition

Plant samples (from rooting stage) were dried at 70 ºC for 24 h, the obtained dry matter was ground and digested according to (Piper, 1947) methods to analyzed chloride, sodium and

potassium contents. Sodium and potassium were determined by using Atomic Absoption flame photometric (3300). Chloride was measured with chloride meter according to Wilde *et al*. (1985) and Black *et al*. (1965).

Protein analysis

SDS-polyacrylamide gel electrophesis (SDS-PAGE) was used to study the pattern of total soluble proteins**.** Protein content was extracted from leaf tissues of rooted plantlets on NaCl (50,100 and150 mM) as well as control plants according to Laemmli (1970).

Electrophoresis

Thirty µg protein were loaded on polyacrylamide gels 12% (Laemmli 1970). A wide range of standard proteins of known molecular weights (260, 135,95,72,52,42,34,26,17 and 9 KDa) were run on a corresponding gel and used for characterization and determination of molecular mass of *A. dracunculus* polypeptides. The protein of each individual clones was extracted separately and applied to the electrophoresis unit in a separate lane, a consistent protein pattern (molecular weight and concentration) was found for all individual clones.

Following electrophoresis gel was stained with a solution containing 0.002% Commassie Blue-R-250 (National Diagnostics), and then destained with a mixture of glacial acetic acid, methanol and water. Once the position and matches of fingerprint bands had been scored, the data were ready for scanning using a LKB Recording Laser Densitometer equipped with LKB Recording Integrator (El-Manar Co., Cairo, Egypt).

Quantitative determination of estragole

Estragole content was extracted from the dried samples of rooted plantlets per treatment of NaCl (0.0, 50,100 and 150 mM) according to Ibrahim *et al.* (2011).

Statistical design and analysis

Experiment was set up in randomized complete block design with ten replicates per treatment. Data were statistically analyzed using ANOVA\MANOVA of Statistica 6 software (Statsoft, 2001), the significance of differences among means were carried out using the Least Significant Test (L.S.D) at $p = 0.05$.

Conclusion

After exposure of plantlets to different concentrations of NaCl (0, 25, 50,100 and 150 mM) for three months, morphological changes were observed. The growth of plant on both multiplication and rooting stages were gradually decreased by increasing NaCl. Both Na^+ and Cl^- in the plant tissues increased with increasing in salt concentration of the culture medium. Growth reductions and salt damage appear to be associated with ions toxicity. NaCl stress caused significant reduction in the content of chlorophyll a and b. This is in addition to the occurrence of a new protein band with molecular weight of (30 and 35 KDa) which was unique to the salt exposed cells. The results of the present study clearly showed that screening of *Artemisia dracunculus* simulate the *in vivo* conditions which might provide a high efficacy *in vitro* screening method for abiotic stresses. This might identify promising cultivars recommended for growers in salt-affected areas of the world. Further physiological and molecular studies are still needed to understand many physiological issues of *Artemisia dracunculus*, to salinity tolerance**.**

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