EUROPEAN ACADEMIC RESEARCH
Vol. IV, Issue 10/ January 2017

Impact Factor: 3.4546 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

Increase *Helianthus annuus* L. tolerance for NaCl *in vitro* and *in vivo*

ZAINAB F. MAHMOOD
Baghdad University, College of Biotechnology
KADHIM M. IBRAHIM
Al-Nahrain University, College of Biotechnology

Abstract:

Several experiments were carried out to study sodium chloride (NaCl) tolerance at the tissue culture and whole plant levels of Helianthus annuus. Callus was induced and maintained on Murashige and Skoog (1962) medium (MS) supplemented with 0.2 mg.L^{-1} Kinetin (KIN), 0.4 mg.L^{-1} Naphthalene acetic acid (NAA) and 0.5 mg.L^{-1} 2,4- dichlorophenoxy acetic acid (2,4-D) using hypocotyl as the source explant for callus induction. Different concentrations of NaCl were added directly to the culture medium as stress agent. Selected tolerant cell lines were subjected to regeneration and whole plants were obtained. The concentration of sodium (Na) and potassium (K) in callus tissues recorded 35 and 17 ppm at 1.5% of NaCl. The study included the effect of the cytokinin Benzyl adenine (BA) and the auxin, NAA on the number of regenerated shoots percentage from NaCl tolerant callus, 80% of callus tolerant to NaCl regenerated shoots at the combination of 1.0 mg.L^{-1} BA and 0.5 mg.L^{-1} NAA supplemented to the medium. The effect of NAA on rooting of shoots showed that the concentration 2.0 mg.L^{-1} of NAA gave rooting percentage mounted to 75%. Shoot and root fresh weights increased in regenerated plantlets despite the significant reduction in plant height. Maximum Na accumulation reached 36 ppm in the roots. Tolerance to NaCl seems to express in the whole plant according to the current study.

Key words: *Helianthus annuus L.,* tolerance for NaCl in vitro and in vivo

INTRODUCTION

Sunflower *(Helianthus annuus* L.) is an annual plant of the genus *Helianthus* grown as a crop for its edible oil and the endosperm of seeds. Sunflower is also used as bird food, as livestock forage (as a meal or a silage plant), and used in some industrial applications. The plant has been reported to tolerate abiotic stresses (1).

Plant survival under adverse environmental conditions relies on integration of stress adaptive metabolic and structural changes in plants. Abiotic environmental factors such as drought and salinity are significant plant stressors with major impact on plant development and productivity thus causing serious agricultural yield losses. Salinity is one of the key factors that limit crop production, high salt concentrations affects negatively on agricultural expansion, particularly in the arid and semi-arid regions. Since salinity induced imbalance of cellular ion homeostasis, it is coped with regulated ion influx and efflux at the plasma membrane and vascular ion sequestration. Salinity has additional major detrimental impacts on the cellular energy supply and redox homeostasis that are balanced by global reprogramming of plant primary metabolism and altered cellular architecture (2).

Tissue culture is a powerful tool that provides the possibility of growing millions of cells under controlled conditions, and to get physiological information about the behavior of the plant cells under stress conditions. Screening and selection at the plant cell level have established plants with increased tolerant to various environmental stresses like salt, drought, diseases, herbicides and heavy metals (3). It seems that a small portion of callus tissues are convenient for selecting tolerance cell lines in a variety of plant species. Selection for salt tolerance at the cellular level was conducted by many researchers (4, 5, 6).

Due to the increasing salinization in agricultural soils as a result of mineral weathering and artificial irrigation, thus this project was designed to investigating the ability of *H. annuus* to tolerate NaCl, screening and selection for NaCl tolerant cell lines and develop a regeneration protocol to whole plants and estimation of Na and K in the vegetative and root systems of the plants raised from tissue culture. This may lead to select sunflower plants exhibit increased tolerance to NaCl.

MATERIALS AND METHODS

Seed sterilization

Seeds of *H. annuus* were washed with tap water and then surface sterilized with 70% ethanol followed by washing with sterile distilled water three times, then they were sterilized with 50% (v/v) chlorex (6.25% sodium hypochlorite) with a drop of tween-20 for 30 minutes. Finally they were washed with sterile distilled water for three times. All steps of sterilization were carried out under aseptic conditions using laminar air flow cabinet. Seeds were transferred to petri dishes containing sterile filter paper to remove excess water (7).

Ready-made MS medium

Murashiage and Skoog, 1962 (MS) medium components (8) were used according to the manufacture instructions. A quantity of 4.99g of the powder medium was dissolved in one liter of distilled water supplemented with 30 g/L sucrose and growth regulators at different concentrations. The pH of the medium was adjusted to 5.8, then 7g/L agar was added to the medium. The medium was dispensed in universal tubes (8×2.5) cm 10 ml/tube. The culture medium was autoclaved at a pressure of 1.04 $Kg.cm^{-2}$, 121^{o}C for 15 min., and then left at room temperature until use.

Raising of seedlings

Surface sterilized seeds were germinated in universal tubes (8×2.5) cm/tube on hormone free MS medium, and then incubated in a growth chamber under total darkness at 25 ºC for 5-7 days.

Callus induction and maintenance

The seedlings were transferred to sterilized petri dishes under sterile conditions, the hypocotyls were cut into 0.5 cm explants and inoculated into callus induction medium containing 0.5 $mg.L^{-1}$ 2,4-D, 0.4 $mg.L^{-1}$ NAA and 0.2 $mg.L^{-1}$ KIN. Explants were cultured with 10 replicates for each treatment; cultures were incubated at 25±2ºC at 1000 lux light intensity for 16/8 hrs light/darkness. Results were recorded after four weeks of incubation (9). Small pieces of calli weighting 50 mg were transferred into fresh MS medium supplemented with the same hormonal combination used for callus induction after four weeks. This medium was suitable for maintaining calli for the next experiments.

Inoculation of callus cultures to a medium containing NaCl

Different concentrations (0.0, 0.5, 1.0, 1.5 or 2.0)% of NaCl were added to the maintenance medium. About 100 mg of fresh weight calli were subcultured directly onto the prepared medium. Callus fresh weights were recorded after four weeks and recultured three times on the same medium. Each treatment was carried out with 10 replicates.

Determination of Na/K ratio in NaCl callus cultures

A quantity of 0.2 g of dried callus at 60 ºC was placed in 15 ml test tube, 5 ml of HNO_3 and 1ml of 70% $HClO_3$ were added and mixed with 0.5 ml of H_2SO_4. The sample was left for 2 hrs till complete digestion. The Na and K values were estimated using flame atomic absorption spectrophotometer (10).

Selection of NaCl tolerant cell lines

NaCl at 1.5% was considered under the lethal dose hence tolerant cell lines can be selected. These NaCl tolerant cell lines were recultured on MS free medium then transferred to MS medium containing the same NaCl concentration.

Determination of callus relative fresh weight

Relative fresh weight of the callus initiated from hypocotyl explants was recorded at different stress levels of NaCl and calculated according to the following formula (11).

$$RFW=\frac{FWF-FWI}{FWI}$$

where FWI = initial callus fresh weight; FWF= final callus fresh weight.

REGENERATION

Shoot regeneration from NaCl tolerant cell lines

Tolerant calli were selected and transferred into the regeneration medium under aseptic conditions. The regeneration medium consisted of full strength MS medium supplemented with BA (0.0, 0.5, 1.0, 1.5 or 2.0) $mg.L^{-1}$ and NAA (0.0, 0.3, 0.5, 0.7 or 1.0) $mg.L^{-1}$. All cultures were maintained at 25±2 ºC for 16/8 hrs (light/dark) photoperiod with a light intensity of 1000 lux.

Root regeneration on NaCl tolerant shoots

Shoots were transferred into the same regeneration medium except the replacement of plant growth regulators with 2 mg/L NAA only (12).

Determination of Na and K in plantlets (ppm)

Na and K were determined in the vegetative and root tissues separately after drying in an oven depending on the method described by (13).

RESULTS AND DISCUSSION

Callus induction, maintenance and dry weights

Callus was initiated and maintained on MS medium supplemented with 0.5 mg.L^{-1} of 2,4-D, 0.4 mg.L^{-1} of NAA plus 0.2 mg.L^{-1} of KIN as has been described by (9). This combination was reexamined by the researcher to confirm that the above medium is convenient for callus initiation and maintenance under the experimental conditions in our laboratories; results are summarized in tables 1 and 2.

Table (1) Percentage of callus induction on *H. annuus* hypocotyl explants cultured on MS medium supplemented with 0.5 mg.L^{-1} of 2,4-D, 0.4 mg.L^{-1} of NAA and 0.2 mg.L^{-1} of KIN after four weeks, n=10.

Treatment	Callus induction (%)
MS free hormones	0.0
MS + plant hormones	100

The addition of auxins and cytokinins are important for callus induction, since cytokinins work as a key for cellular division in the presences of auxins. Inclusive of 2,4-D, NAA and KIN at certain combinations to MS medium could be the key for good callusing on hypocotyl explants. In order to maintain and increase callus mass, portions of calli obtained from previous experiment were transferred to MS medium supplemented with the same combination of plant growth regulators. Harvested callus gave 650 mg of callus fresh weight and 122 mg dry weight (table 2).

Table (2) Callus fresh and dry weights (mg) grown on MS medium supplemented with 0.5 mg.L^{-1} of 2,4-D, 0.4 mg.L^{-1} of NAA and 0.2 mg.L^{-1} of KIN after four weeks. Initial callus fresh weight was 35 mg. n=10.

Treatment	Fresh weight (mg)	Dry weight (mg)
MS free hormones	0.0	0.0
MS + plant hormones	650	122

Effect of NaCl on callus RFW

A steady decline in callus RFW occurred with the increasing of NaCl concentrations in the medium (table 3). In the current study callus RFW values were very low at high concentration (2.0)% of NaCl reached 0.55 g. while callus RFW at 0.0% NaCl concentration was the highest recording (3.61) g. Increasing salt concentrations resulted in a decrease in callus RFW at the concentrations 0.5, 1.0 and 1.5% recording 2.44 g, 1.85 g and 1.60 g respectively. The mean reduction in callus RFW was significant at all NaCl levels compared with the control.

Table (3) The Effect of different NaCl concentrations on callus RFW. The initial callus weight is 100 mg. n=10. ± represents standard error for mean values.

NaCl (%)	Callus RFW (g)
0.0	3.61 ± 0.08
0.5	2.44 ± 0.06
1.0	1.85 ± 0.06
1.5	1.60± 0.06
2.0	0.55 ± 0.009
L.S.D: 0.05	0.644

Results are similar to (14) who demonstrated that *H. annuus* calli directly exposed to high concentrations of NaCl exhibited a decrease in callus growth rate and even caused death at high concentration in some *H. annuus* species. This reduction in callus fresh weight may due to induced osmotic stress associated with more ionic imbalance causing a reduction in cell division.

Quantitation of Na and K in callus tissues

Callus of *H. annuus* showed a moderate tolerance to salt stress (table 4) represented Na and K accumulation in callus tissues. The highest Na value recorded 35 ppm at 1.5% NaCl while K value was 17 ppm. Na accumulation in callus tissues increased with increasing concentration of NaCl recording 21 and 29 ppm

at 0.5 and 1.0% of NaCl while K levels decreased to 16 and 15 ppm respectively. All Na mean values significantly reduced when compared to the control treatment except Na at 2.0%.

Table (4) Na and K accumulation (ppm) in *H. annuus* callus tissues cultured on MS medium supplemented with different concentrations of NaCl after four weeks of culture.

NaCl (%)	Na	K	Na/K
0.0	14	12	1.16
0.5	21	16	1.31
1.0	29	15	1.93
1.5	35	17	2.05
2.0	17	7	2.42
L.S.D:0.05	5.039	3.108	

A gradual increase in the Na/K ratio with the increase of NaCl concentrations was recorded as a result of increasing Na accumulation and a decrease in K exclusion. The accumulation of Na ion in plant tissues and exclusion of K ion is due to the competition between both for comparatmentation in vacuoles resulting in enzymatic defect causing loss of plasmalemma permeability; this will negatively affect K accumulation in the plant tissues.

Shoot regeneration

Results shown in table 5 exhibited that MS medium supplemented with BA at 1.0 and NAA at 0.5 $mg.l^{-1}$ gave the highest shoot formation percentage reached 90% for control. No shoots formed neither in the absence of BA and NAA nor at high concentrations of BA (2.0) and (1.0) $mg.l^{-1}$ NAA. Results showed a synergistic relation between auxins and cytokinins promoting tissues differentiation indicating that the preference of higher cytokinin level over the auxin (15).

These results are in agreement with (16) who reported good shoot formation resulted from sunflower callus cultures when 1 mg/l BA and 0.5 mg/l NAA were added to the medium.

Presence of cytokinins at relatively high level accompanied with low level of auxins promotes shoots initiation and multiplication. Results also showed no shoot formation at high levels of BA and NAA. This may due to the negative effect of NAA on callus response to regenerate shoots.

Table (5) Shoot formation percentage of NaCl tolerant calli grown on MS medium supplemented with different concentrations of BA and NAA, after four weeks. n=10.

BA ($mg.l^{-1}$)	NAA ($mg.l^{-1}$)	Non-treated	shoot formation % from NaCl tolerant callus
0.0	0.0	0.0	0.0
0.5	0.3	81	76
1.0	0.5	90	80
1.5	0.7	68	42
2.0	1.0	0.0	0.0
Mean		48.0	33.4

Rooting of regenerated shoots

Elongated and healthy shoots were transferred to a rooting medium consisted of full strength MS medium supplemented with 2.0 $mg.l^{-1}$ NAA (table 6) as has been described by (12). The highest rate of rooting (90%) occurred in the non-stressed callus, 75% in shoots derived from NaCl tolerant calli.

Table (6) Root formation percentage of NaCl tolerated callus and untreated callus grown on MS medium supplemented with 2 $mg.l^{-1}$ NAA after six weeks.

Treatment	Root formation %
Untreated callus	90
NaCl tolerated	75

Effect of NaCl on *H. annuus* plantlets shoot and root fresh and dry weights

A negative effect of NaCl on the fresh and dry weight of shoots and roots was recorded. Selection of NaCl tolerant plantlets had

improved all the studied parameters significantly except plant height. Plantlets fresh and dry weight recorded 6.6 and 10.4 g for control and plantlets derived from NaCl tolerant callus shoots fresh weight, while the roots fresh weight reached 9.1 and 14.1 g for control and 1.5% NaCl in plantlets derived from NaCl tolerant callus respectively (table 7). There was a significant increase in shoots dry weight reaching its upmost (3.1) g in control and 4.1 g at 1.5% of NaCl. Dry weights of roots were 4.2 and 5.2 g for the control and the plantlets derived from NaCl tolerant callus. In contrast, plantlets height decreased at 1.5% NaCl. The plant capacity to withstand salinity is linked to its ability to get rid of accumulated salts and ions; this is accomplished through rapid growth and increase in biomass with increasing cell content of water (17). The current study revealed that selection at the cellular level for NaCl tolerance has been expressed at the whole plant level.

Table (7) Effect of NaCl on the fresh, dry weight and height of *H. annuus* plantlets derived from NaCl tolerant and untreated callus after 6 weeks of rooting.

NaCl Conc. (%)	fresh wt. (g)		Dry wt. (g)		Height (cm)
	shoot	Root	shoot	Root	
0.0	6.6	9.1	3.1	4.2	13.9
1.5	10.4	14.1	4.1	5.2	11.5
L.S.D: 0.05	1.77	2.05	0.67	0.59	1.04

Accumulation of Na and K in *H. annuus* plantlets

Table 8 shows that NaCl treatments led to an increased in Na concentrations in the shoots reaching 34 ppm at 1.5% NaCl compared with the control treatment which recorded 16 ppm. K recorded 22 ppm at the control in the shoots but decreased at 1.5% of NaCl. Na in the roots of the control recorded 21 ppm while Na accumulation at 1.5% of NaCl reached 36 ppm. All treatments were significantly different except in K accumulation at 1.5% in plantlets derived from NaCl tolerant

callus recording 15 ppm when compared to the control treatment. (18) reported that the increase in Na can be attributed to the increase in Na ions absorbed by cells due to the increased NaCl concentration in the growth medium, the accumulation of ions is a dominant characteristics of salt tolerance mechanism. The control mechanism relies on the preservation bloating effort in plant cell, when Na enters into the cell through the plasma membrane channels leading to K flow out because of the competitive impact of Na and K on the active sites in the plasma membrane.

Table (8) Accumulation of Na and K (ppm) in the shoots and roots of *H. annuus* plantlets derived from callus after 6 weeks of rooting.

NaCl Conc. (%)	Shoot		Root	
	Na	K	Na	K
0.0	16	22	21	16
1.5	34	18	36	15
L.S.D: 0.05	5.682	3.266	4.861	2.25

It has been concluded from the present work that *H. annuus* callus can be manipulated *in vitro* to increase tolerance to NaCl in plantlets derived from selected cell lines. This gain in NaCl tolerance can be expressed at the whole plants regenerated from callus cultures and thus, it is possible to enhance NaCl tolerance in *H. annuus* plants grown in the field.

REFERENCES

1. Kenneth, D.; Rebecca, A. and Loren, H. (2010). Adaptive introgression of abiotic tolerance traits in the sunflower *Helianthus annuus*. J. of New Phytol. 187(1): 230–239.
2. Agarwal, P.; Shukla, P.; Gupta, K. and Jha, B. (2013). Bioengineering for salinity tolerance in plants: state of the art. Mol. Biotechnol. 54:102–123.

3. Tal, M. (1983). Selection for stress tolerance. In Handbook of Plant Cell Culture: Techniques for Propagation and Breeding. Macmillan Publishing Company. New York. 1: 461-488.
4. Collins, J. C.; Ibrahim, K. M. and Collins, H. A. (1990). *In vitro* selection for salt tolerance in *Coleus blumei*. Acta. Hort. (ISHS). 280:431-434.
5. Ibrahim, K. M.; Collins, H. A. and Collins, C. (1990). Selection for salt tolerance in regenerating cultures of *Coleus bluemei*. Acta. Hort. 280:425-430.
6. Ibrahim, K. M.; Collins, H. A. and Collins, C. (1992). Characterization of progeny of Coleus bluemei following in vitro selection for salt tolerance. Plant Cell, Tissue and Organ Cultures. 28:139-145.
7. Akbar, S. and Azra, Q. (1994). Tissue culture of sunflower. Pak. J. Agric. 15(1): 153-160.
8. Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant**.** 15:473-497.
9. Aurelia, B. (2000). Callus initiation and morphogenesis induction in somatic tissue culture of *Helianthus annuus* L. Trcu. Mus. His. 22: 33-41.
10. Cresser, M. and Persons, J. (1979). Sulfuric – perchloric acid digestion of plant materials for the determination of nitrogen, phosphorus, potassium, calcium, sodium and magnesium. Anal. Chimica. Acta. 109: 431-436.
11. Lateef, Z. M. (2015). Investigating heavy metals tolerance in *Ruta graveolens in vivo* and *in vitro*. MSc. Thesis. Biotechnology Dept. College of Sci. Al-Nahrain Univ. Iraq.
12. Pandurang, C.; Devindra, S. and Srinath, R. (2012). Induction of callus from various explants and regeneration of plantlets in sunflower (*Helianthus annuus* L.) var. APSH-H. J. of Crop Sci. 3(3): 87-89.

13. Achakzi, A.; Mojeed, O. and Oladele, J. (2012). Effect of mycorrhizal inoculation on the growth and phytoextraction of heavy metals by maize grown in oil contaminated soil. Pak. J. Bot. 44: 221-230.
14. Vieiria, D. and Gustavo, C. (2012). Comparative responses of *Helianthus annuus* plants and calli exposed to NaCl, growth rate and osmotic regulation in intact plants and calli. J. of Plant Physiol. 155 (6):769-777.
15. Dalila, Z.; Jaadar, H. and Manaf, A. (2013). Effect of 2,4-D and KIN on callus induction of *Barringtonia racemoso* leaf and endosperm explants in different types basal media. Asian J. of Plant Sci. 12(1):21 – 27.
16. Ozyigit, I.; Nermin, G. and Belma, D. (2007). Geno type dependent callus induction and shoot regeneration in sunflower (*Helianthus annuus*). Afri. J. of Biotech. 6 (13):1498-1502.
17. AL-Mashhadani, I.; Izz-AL Din, M. and Atia, H. (2001). Salt tolerance mechanism in some genetic compositions produced from wheat. Ebaa J. of Agric. Res. 11 (1):56-74.
18. Al-Obaidy, H. K.; Hassan, S. and Ibrahim, K. M. (2008). Selection for salt tolerance in cell suspension cultures of two soy bean synthetic varieties. J. of Mustansyria Sci.19(6): 121-132.