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Production of Genetically Modified Grape (Vitis vinifera L.) Plants

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***Abstract*—** *Grape (Vitis vinifera L.) is one of the most economically important fruits in the world. High salinity stress adversely affects plant growth and limits agricultural production worldwide. This study describes a successful method of somatic embryogenesis using in vitro-derived leaf explants and introduction of a vacuolar-type Na^+/H^+ antiporter gene from a halophytic plant, Atriplex gmelini (AgNHX1) confers salt tolerance to grape cv. Superior Seedless using the Agrobacterium-mediated transformation. Callus embryogenic was induced on NN medium 2.0 mgL^{-1} 2,4-D, 0.5 mgL^{-1} BAP and 0.5 mgL^{-1} NAA. Subsequent subculture of callus on NN medium containing 1.5 mgL^{-1} BAP, 0.5 mgL^{-1} kinetin and 0.5 mgL^{-1} NAA induced shoot organogenesis after eight weeks of culture. The leaf explants were co-cultivated with Agrobacterium strain LBA4404 harbouring the binary vector pBI121 which contained the AgNHX1and nptII genes and putative transgenic plants were produced. The presence and stable integration of AgNHX1 gene in transgenic plants was confirmed by PCR and northern blot hybridization. The transgenic grape plants overexpressing the AgNHX1 gene showed a strong tolerance to salt stress under 250 mM NaCl, whereas non-transgenic plants died under the same conditions. Salt tolerance assays followed by salt treatments showed that the transgenic plants overexpressing AgNHX1 could survive under conditions of 250 mM NaCl for 4 weeks while the non-transgenic plants died under the same conditions. These results indicate that overexpression of the Na^+/H^+ antiporter gene in grape plants significantly improves their salt tolerance.*

Keyword—Grape (Vitis vinifera L.), regeneration, transformation, Na^+/H^+ antiporter, PCR and northern blot hybridization.

Abbreviations—2,4-D*- 2,4-di-chloro-phenoxy-acetic-acid;* ***nptII gene****- neomycin phosphotransferase(gene);* ***BAP****- Benzylaminopurine;* ***NAA****- α-Naphthalene Acetic Acid;* ***LB*** *Luria broth medium;* ***Kin****- Kinetin;****CTAB****- Cetyl-trimethyl-ammonium-bromide;* ***NN*** *Nitsch and Nitsch medium;* ***IBA*** *Indole Butyric Acid;* ***OD****- Optical density;* ***PCR****- Polymerase chain reaction;* ***RT-PCR****- Reverse transcriptase polymerase chain reaction.*

I. INTRODUCTION

The grape (*Vitis vinifera* L.) belongs to the family Vitaceae, which comprises of 12 genera and 60 species. It is one of the most commercially grown important fruit crops in the world (Sajid *et al*. 2006). Salinity is one of the major environmental stresses that currently reduce crop growth and productivity worldwide (Zhu, 2001). Soil salinity severely limits agricultural productivity because concentration as low as 25 mM NaCl are not tolerated by many plants and concentrations of 150 mM NaCl are high toxic for most crop plants (Glick *et al*., 2007).

Grapevines (*Vitis vinifera*), by comparison with other crop types, are classified as moderately sensitive to salinity (Maas and Hoffman, 1977). With high salinity, sodium and / or chlorides in the soil may lead to toxicity in the grapevines. Show toxicity as drying and burning tips and edges of leaves, which advance towards the center of the leaves. With increased toxicity, whole leaf die and plants become defoliated (Dorani-Uliaie *et al*., 2012). Recently, many genes encoding Na^+/H^+ transporters of plasma membrane or tonoplast have been isolated from various halophytic and glycophytic plant species including *Oryzasativa* (Fukuda *et al*., 1999), *Atriplex gmelini* (Hamada *et al*., 2001), *Brassica napus* (Wang *et al*., 2003), *Beta vulgaris* (Xia *etal*., 2002), *Hordeum vulgare* (Fukuda*et al*., 2004), *Suaeda salsa* (Ma *et al*., 2004), *Aeluropus littoralis* (Zhang *et al*., 2008) and some other glycophytes and halophytes. The Na^+/H^+ antiporters that are localized in the plasma and the vacuolar membrane catalyse the exchange of $Na^+$for H^+ across membranes using the energy provided by the proton electrochemical potential established by H^+ translocating pumps. Although the capacity for vacuolar compartmentalization of Na^+ and Cl^- is adaptation mechanism conserved in halophytes and glycophytes (Hasegawa *et al*., 2000), the process is more efficient in halophytes (Guan *et al*., 2011). In plants, vacuolar Na^+/H^+ antiporters can pump Na^+ into the vacuoles to reduce Na^+ toxicity and maintain a high K^+/Na^+ratio in the cytosol to alleviate salt stress. Na^+/H^+ antiporters also regulate internal pH, cell volume, and the

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sodium level in the cytoplasm and vacuole (Apse *et al*., 1999).It has been shown that plant vacuolar Na^+/H^+ antiporters play an important role in salt tolerance, ion homeostasis and plant development (Yamaguchi *et al*., 2003).The use of genetic engineering for plant improvement permits introduction of useful agronomic traits without altering the features of the cultivar. The application of tissue culture methods for grape genetic transformation depends on the availability of highly reproducible and efficient in vitro regeneration systems (Gray *et al*. 2014). To date, the regeneration of grape plants has been achieved from different explant types via both embryogenesis and organogenesis (Ehab *et al*., 2016).The objective of this study was to investigate the transgenic alteration of grape cultivar Superior seedless by introduction of the Na^+/H^+antiporter gene from a halophytic plant, *Atriplex gmelini* (AgNHX1) confers salt tolerance to grape (*Vitis vinifera* L.) cv. Superior Seedless.

II. MATERIALSANDMETHODS

1. Plasmid construction and bacterial strain

The Na^+/H^+antiporter gene from a halophytic plant, *Atriplex gmelini* (AgNHX1) was amplified by PCR and digested with Sac1 and BamHI restriction enzymes (Hamada *et al*., 2001: Ehab *et al*., 2015).The resulting fragment was inserted between the promoter region and the terminator region of the plasmid vector pBI121to produce p35S/AgNHX1 within the T-DNA region. The promoter region contains the cauliflower mosaicvirus (CaMV) 35S promoter and the terminator region contains the polyadenylation signal of the nopaline synthetase gene (Nos). This binary plasmid contains *nptII* gene which encodes neomycin phosphotransferase as selectable marker under the *nos* promoter and NOS terminator within the TDNA region (**Fig. 1**). *A. tumefaciens* strain LBA4404were used for transforming grape explants using *Agrobacterium* mediated transformation. The bacteria was grown overnight at 28°C in 5 ml Lauria broth (LB) liquid medium supplemented with 50 mgL^{-1}rifampicin and 30 mgL^{-1} kanamycin on a shaker at 150 rpm to mid-logphase (OD600 = 0.8–1). Then, the bacterial cells were collected by centrifugation at 25009g for 5 min and resuspended in MS medium to a final OD600 of 0.2 for use in transformation.

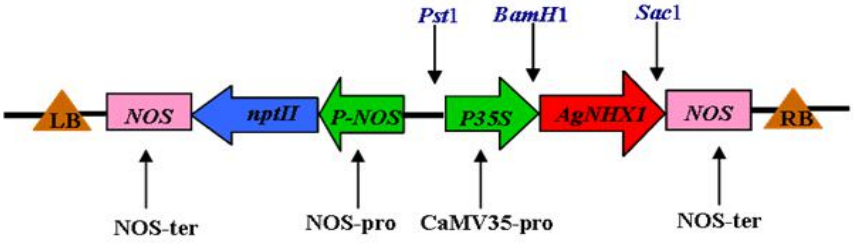

Fig.1: Diagram of T-DNA of the recombinant binary vector pBI121carrying the AgNHX1 gene driven by CaMV 35S promoter. LB; left border, NOS; nopaline synthase gene terminator, nptII; selectable marker gene, P-NOS; nos promoter, p-35S; CaMV 35S promoter, AgNHX1; grape Na^+antiporter gene, NOS; nopaline synthase gene terminator and RB; right border.

2. *In vitro* regeneration of grape (*Vitis vinifera* L.)

Leaves of grape (*Vitis vinifera* L.) cv. Superior Seedless were harvested from *in vitro* grown shoot cultures and leaf segments (one leaf divided into two pieces) were prepared. Leaf explants were placed on Nitsch & Nitsch (1969) media containing various concentrations of 2, 4-dichlorophenoxy acetic acid (2,4-D), 6-Benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA) were used to optimize the culture conditions for callus induction. All the cultures were maintained in the dark at 25±2°C. The frequency of callus induction was estimated as follows: Callus induction frequency (%) =(Number of calluses induced /Number of leaf discs inoculated)× 100. After 4 weeks, the leaf derived calli were transferred for shoot induction on basal NN medium containing different concentration of6-Benzylaminopurine(BAP), Naphthalene acetic acid (NAA) and solidified with 2.5 g L^{-1} phytagel. The cultures were incubated at 25 ± 1°C with a 16-h photoperiod under 3,000 lux light intensity. The frequency of shoot regeneration was calculated as follows: Shoot regeneration frequency (%) = (Number of calluses regenerated into shoots/ Total number of calluses inoculated) × 100. Regenerated small shoots were excised from cultures and transferred to basal NN medium containing 3mg L^{-1}BAP and 0.3 mg L^{-1} IBA to improve shoot length before rooting stage.

2.1. Rooting and acclimatization

Shoots derived from the regeneration stage were cultured on MS medium containing 1.0 mg L^{-1}Indole Butyric Acid (IBA). The plantlets were transplanting in plastic pots filled with a soil mixture of peat and sand (1:1), and covered with polyethylene bags and incubated under

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28±2°C, 16 h light/ 8 h dark photoperiod and 70 umol cm^{-2} s^{-1} light. The plants were hardened by removing the polyethylene bags gradually over a seven to ten day period.

3. *Agrobacterium*-mediated transformation of grape (*Vitis vinifera* L.)

Introduction of the *AtNHX1* gene to grape leaf disc explants was done using *Agrobacterium*-mediated transformation system. The leaf disc explants were dipped in an *Agrobacterium* soluble culture for 10 min and then blotted dry before culturing on co-cultivation medium. The explants were rinsed several times with a sterile water, to remove excess of bacteria and then blotted dry. The infected leaf explants were placed on co-cultivation NN medium free hormones at 25°C in the dark for 2 days and then were transferred on NN medium containing 2 mg L^{-1} 2,4-D, 0.5 mg L^{-1}BAP, 0.5 mg L^{-1} kinetin,300 mg L^{-1}carbinicillin and 100mg L^{-1}kanamycin. The leaf derived calli were transferred to regenerated medium containing 300 mg/l carbinicillin and 100 mg/l kanamycin. All media were supplemented with 3% sucrose and 2.5g/l phytagel (*Sigma*-Aldrich, USA); the pH was adjusted to 5.8. The cultures were incubated under the same *in vitro* conditions for regenerated shoots. The rooted shoots were transferred to pots in greenhouse for acclimatization.

4. Detection of transgenic grape plants

4.1. Genomic DNA isolation and PCR analysis

Genomic DNA was extracted from grape leaves using the acetyl tri-methyl-ammonium bromide (CTAB) method(Gaoge *et al*., 2005). The presence of the AgNHX1 in putative transgenic grape plants was detected by polymerase chain reaction (PCR). The sequence of the specific primers for the AgNHX1 gene was F-5′-TCC CGT GTA CTT GGG AAT GC-3′ and R-5′-CGC CCA CAA TAC CAA ACACC-3′.The PCR reactions were carried out in a 20 µl volume containing 40 ng DNA template, 20 pmole/1ml primers, 200 µl of dNTPs mix, 2 µl 10x amplification buffer, 0.2 mM MgCl2, and one unit Taq DNA polymerase. The volume was completed up to 20 µl with sterilized distilled water. The PCR temperature profile used for the amplification consists of initial denaturation cycle 95^{o}C/2 min followed by followed by 35 cycles with denaturation at 95°C/30 s, annealing at 58°C/30s, extension at 72°C/1 min, and a final extension step at 72°C for 10 min.

4.2. RNA and AgNHX1 gene expression in transgenic grape plants

Total RNA was extracted from leaves as described previously (Fukuda *et al*. 1991). AgNHX1 mRNA was detected using full-length cDNA as a probe. Northern blot analysis was performed according to the method described by Hayakawa *et al*. (1992). RT-PCR was performed using total RNA pre-treated with primers forward 5′-CAT CAG TGT CAA TTCGAG AAA CAA CAG-3′and reverse 5′-CTA TGT TCT GTC TACCAA ATT GTT GTT GCT-3′ and cDNA was synthesized under conditions of 94°C for 5 min; then 35 cycles of 94°C for 30s, 60°C for 30 s, and 72°C for 45 s; with a final step of 72°C for 7 min. DNA probes were made by a multiprime labelling system (Amersham Pharmacia Biotech, Cleveland, OH) with a portion of the cloned cDNA fragment and 32P-dCTP. For assessing the relative quantities, the loaded RNAs were stained with ethidium bromide after electrophoresis.

5. Plant growth and salt stress treatment.

The expression of *AgNHX1*gene was analyzed in transformed grape plantlets for their salt tolerance in growth room conditions. A number of 100 transformed grape plants were cultured on MS medium supplemented with 100–300 mM NaCl in rooting stage. These transgenic grape plants were then transferred in pots containing a mixture of peatmoss and sandy soil (1:1/v: v) for three months and then treated with 100–300 mM NaCl under greenhouse conditions. The control plants (non-transformed) of similar age and height were also analyzed. The salt treatments were conducted in an incremental manner for two weeks each period i.e., starting with 50 mM, followed by 100 mM, 150 mM, 200 mM, 250 mM, and finally 300 mM NaCl according to (Dorani-Uliaie *et al*., 2012). Leaves from the growing plants were excised carefully to determine the concentration of Na^{+} content. In addition, the dry weight was measured.

5.1. Measurement of Na^{+}content in grape leaves

Leaves were cut from the grape plants before and after three months of salt treatment and washed with deionized water. The leaves were dried in an oven at 120°C and their dry weights were measured. To extract the salts, the leaves were burned to ashes at 450°C in the presence ofHNO3. The ashes from the leaves were then dissolved in 0.1 NHCl, and the sodium content was determined by atomic absorption spectrophotometry.

6. Statistical analysis

Differences between individual means were estimated according to Snedecor and Cochran (1982).All values are reported as means ± should be standard error.

III. RESULTSANDDISCUSSION

1. Callus induction and *in vitro* shoot regeneration

Leaf explants taken from *in vitro* shoot cultures were used for callus induction. Leaf explants were placed on Nitsch & Nitsch (1969) medium supplemented with the different concentrations of plant growth regulators (2,4-D, BAP and NAA). NAA was used at a constant rate of 0.2 and 0.5 mg

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L^{-1} and BAP was used at a constant rate of 0.3 and 0.5 mg L^{-1}. The concentration of 2,4-D (2.5 mgL^{-1}) combined with the higher concentration of BAP (0.5 mg L^{-1}) and NAA (0.5 mg L^{-1}) induced the highest number of calluses (83.33 %), followed by the concentration of 2.5 mgL^{-1}2,4-D + 0.5 mgL^{-1} BAP and 0.5 mgL^{-1} NAA, which induced 80.00 % callus induction frequency. Moreover, the lower level of 2,4-D (1.0 and 1.5 mg L^{-1}) combined with the higher level of BAP (0.5 mg L^{-1}) resulted in lower callus induction frequency (63.0 and 66.67 %) respectively (**Table 1**). Thus, in the present study, The higher concentration of 2,4-D (2 mgL^{-1}) combined with the lower concentrations of BAP and NAA (0.5 mgL^{-1}) favored callus formation (**Fig. 2 B**).Hence, NAA and BAP did stimulate explants to initiate growth and form callus as compared to 2,4-D. However, the small amount of BAP (0.5 mgL^{-1}) had a positive effect on callus induction. BAP promotes RNA and protein synthesis which activates enzyme activity for cell division and cell wall loosening (Kulaeva, 1980). The present results are supported by Nadra *et al*. (2015), who stated that 2.5 mg L^{-1} 2,4-D with a small concentration of NAA and BAP produced callus in grape when subcultured at 4- week intervals. This study suggests that 2, 4- D with the lower concentrations of NAA and BAP constitute the most favorable combination of growth regulators for the production of callus in grapevine cv. King's Ruby. The light yellow friable callus was transferred to shoot induction media containing different concentrations (1.0, 1.5 and 2 mgL^{-1}) of BA and 0.2-0.5 mgL^{-1} NAA or combinated with 0.5 mgL^{-1}kinetin (**Table 2**). The calli became more greenish and appeared highly competent for shoot bud initiation (**Fig. 2C and D**) particularly on a NN medium containing 1.5 mgL^{-1} BA, 0.5 mgL^{-1} and 0.5mgL^{-1} where about 83% percentage of callus induction was recorded. BAP induced the development of greenish nodular callus with shoot buds which later developed into shoots, a similar response to that reported by other authors (Gharyal and Maheshwari, 1990; Maity *et al*., 2005). BAP with kinetin were found to be superior than BAP only as it induced (83.4**)** shoots/unit callus with (93.4%) regeneration frequency at 1.5 mgL^{-1} BAP with 0.5 mgL^{-1} Kinetin, while BAP only at the same concentration without kinetin gave a maximum of (68.5) shoots/unit callus and maximum regeneration frequency (38.5%) while, BA at 2.0 mgL^{-1}, Kinetin at 0.5 mgL^{-1} in combination with 0.5 mgL^{-1} NAA resulted in the highest recorded shoot length (29 mm) after 8 weeks of incubation (**Fig.2E and F**). Previously, many studies have indicated that the ideal composition of grapevine culture medium depends on the species and cultivars, so the results obtained with one genotype in a given medium may differ from those obtained with other genotypes (Reisch 1986; Botti *et al*.,1993; Ehab *et al*., 2016).

Table.1: Effect of different concentrations of plant growth regulators on callus induction of grape (Vitis vinifera L.) cv. Superior Seedless after 4 weeks.

Growth regulator (mgL-1)			Number of explants cultured	Number of callus induced	Frequency (%) of callus induction
2,4-D	**BAP**	**NAA**			
1.0	0.3	0.2	30	16	53.33±1.03j
1.0	0.3	0.5	30	17	56.67±1.59i
1.0	0.5	0.5	30	18	60.00±2.07h
1.5	0.5	0.2	30	19	63.33±2.18g
1.5	0.5	0.5	30	20	66.67±1.95f
2.0	0.2	0.2	30	22	73.33±1.22d
2.0	0.2	0.5	30	21	70.00±1.45e
2.0	**0.5**	**0.5**	30	**25**	**83.33±2.43a**
2.5	0.5	0.5	30	24	80.00±2.15b
2.5	0.2	0.2	30	23	76.67±1.37c

Values are presented by mean ± SE Same letters represent non-significant differences between means level. Bold value indicated the highest value obtained for the treatment compared to other treatment.

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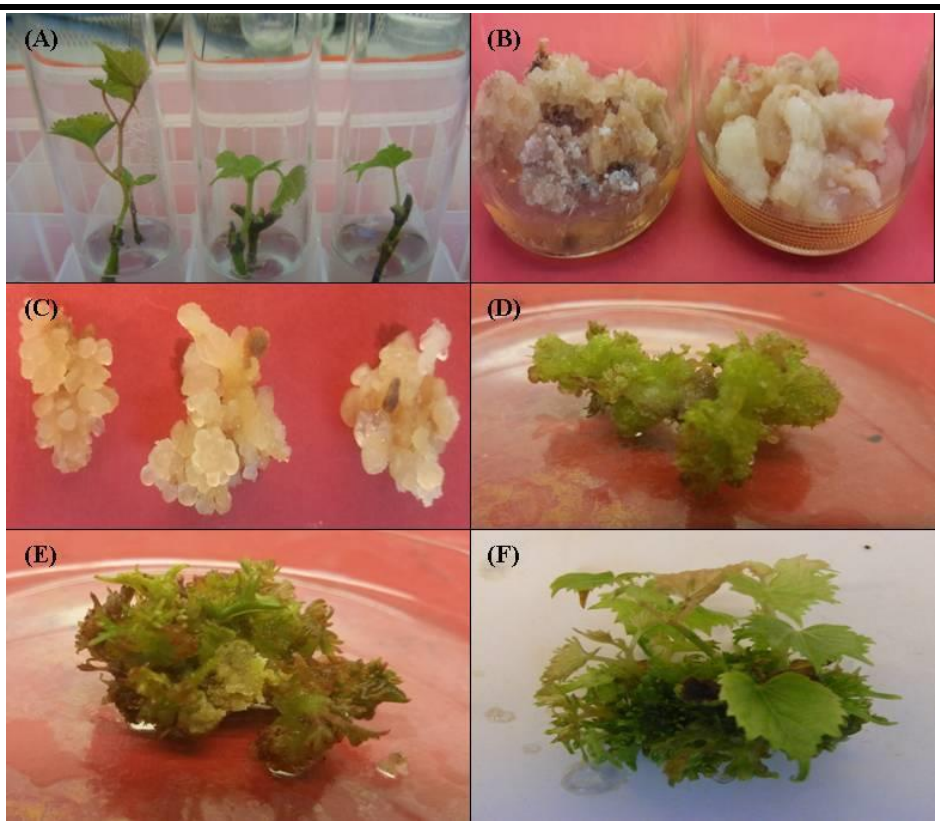

Fig 2:Callus induction and regeneration of plantlets from leaf sections in grape (Vitis vinifera) cv. Superior Seedless. (A) Leaf explants of grape were taken from plantlets generated previously on MS medium supplied with 0.3 mg L^{-1}IBA and 3.00 mg L^{-1}BA. (B) Callus formation from leaf explants on NN medium supplemented with 2.0 mg L^{-1}2,4-D, 0.5 mg L^{-1}BAP and 0.5 mg L^{-1}NAA. (C) Matured somatic embryos derived from callus cultured on NN medium containing with 1.5 mg L^{-1}BAP, 0.5 mg L^{-1}kin and 0.5 mg L^{-1}NAA. (D and E) Green shoots from callus in shooting medium (1.5 mg L^{-1}BAP, 0.5 mg L^{-1}kin and 0.5 mg L^{-1}NAA)after 8 weeks and (F) development shoot elongation from small shoots were cultured on *NN medium containing0.3 mg L^{-1}IBA with 3.0 mg L^{-1}BAP.*

Table.2: Effect the best growth regulators concentrations of embryogenic callus of grape (Vitis vinifera L.) cv. Superior Seedless on % callus response, mean no. of shoots/ unit callus and mean shoot length after 8 weeks.

Growth regulator (mgL^{-1})			**% Callus response**	**Mean no. of shoots/ unit callus**	**Mean shoot length (mm)**
BAP	**Kin**	**NAA**			
1.0	0.0	0.2	29.0±1.24^{j}	18.5±1.04^{i}	13±2.6^{j}
1.0	0.0	0.5	38.0±2.24^{i}	12.8±1.72^{j}	19±3.2^{i}
1.0	0.5	0.2	42.5±1.58^{h}	23.5±1.25^{h}	15±2.8^{h}
1.0	0.5	0.5	53.0±2.35^{g}	27.9±1.58^{g}	23±3.4^{e}
1.5	0.0	0.3	68.5±1.78^{e}	38.5±1.82^{f}	20±2.9^{g}
1.5	0.5	0.3	72.3±2.65^{d}	75.5±1.69^{d}	22±3.1^{f}
1.5	**0.5**	**0.5**	**83.4±2.04^{a}**	**93.4±1.38^{a}**	27±3.8b
2.0	0.0	0.3	65.4±1.85^{f}	68.3±1.08^{e}	24±2.5^{d}
2.0	0.5	0.3	79.8±2.36^{c}	81.3±2.26^{c}	26±3.7^{c}
2.0	0.5	0.5	81.5±1.89^{b}	84.5±2.41^{b}	**29±3.6^{a}**

Values are presented by mean ± SE Same letters represent non-significant differences between means level. Bold value indicated the highest value obtained for the treatment compared to other treatments.

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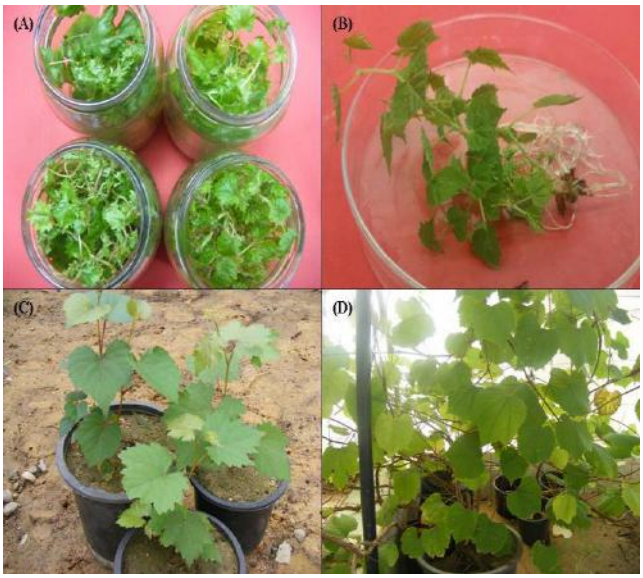
Fig.3:Multiple shoots developments from small shoots were cultured on NN medium containing 3 mg L^{-1}BAP and 0.3 mg L^{-1}IBA.

2. Transformation of grape (*Vitis vinifera* L.) cv. Superior Seedless

The transformation of grape plants was achieved using *Agrobacterium* –mediated transformation using the binary vector pBI121 contains the *AgNHX1*and *nptII* gene which encodes neomycin phosphotransferase as selectable marker with inoculation time of 10 minutes. In this study, the leaf segment explants were co-cultivated with *Agrobacterium* solution. Explants were co-cultivated with *Agrobacterium* for 2 days on MS medium-free hormones. After co-cultivation, leaf segment explants were washed at least three times by using different concentrations of cefotaxime in MS liquid medium to kill the bacteria from surface of the leaf segment explants. Callus was produced from leaf segment explants after one month and leaf derived calli were transferred to regenerated medium containing 1.5 mg L^{-1} BAP, 0.5 mg L^{-1} Kinetin, 0.5 mg L^{-1} NAA and 100 mg L^{-1}kanamycin. New shoots were regenerated from callus tissue after 4-week and transferred to rooting medium and then the rooted plantlets (5-6 cm in length) were ready for acclimatization in the soil. About 200 putative transgenic plants were obtained after *Agrobacterium* co cultivation and these plants were used for molecular analysis and functional test.

Kanamycin at the level 100 mg L^{-1} was standardized as optimum because when concentration of kanamycin exceeded from this dose ,high necrosis rate was resulted in progressive necrosis of the tissue. It was also noticed that high levels of kanamycin damaged the non-transformed leaf. Also, grapevine calli were proved to be very sensitive to kanamycin, which led to questions concerning efficiency of kanamycin as a selection agent. These results were in confirmation with the findings of both Motioike *et al*. (2002) and Das *et al*. (2002) have used kanamycin to recover viable transformed calli of *V. labruscana* and *V. vinifera*, respectively; but none of them observed the kanamycin insensitivity problem as reported by Perl *et al*. (1996). However, transformation in grapevine explants was achieved by using kanamycin for selection and the cultures were effectively transformed using the *nptII* resistance gene (Gray *et al*., 2005). In grapevine (*Vitis spp*.), somatic embryogenesis is the most frequently used regeneration system adopted for genetic engineering (Gambino *et al*., 2005). However, transformation procedure of grapes depends on the induction of embryos and their selection (Iocco *et al*., 2001), *in vitro* regeneration system (Xiaoqing *et al*., 2016).

Fig.4: In vitro rooting and acclimatization of transgenic grape plantlets. (A and B); root development from shoots on MS medium containing 1.0 mg L^{-1}IBA. (C) ;acclimatization and establishment of ex vitro plantlets of grape (Vitis vinifera L.) cv. Superior Seedless. (D); potted plants four months after transfer to a greenhouse.



2.1. Rooting and acclimatization of transgenic plants.

Small shoots were excised from regenerated callus tissues and transferred to basal NN medium containing 3 mg L^{-1}BAP and 0.3 mg L^{-1}IBA to improve shoot length (**Fig.3**)

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and then transferred to MS medium containing 1.0 mg L^{-1}IBA with constant concentration of cefotaxime (200 mg L^{-1}) and kanamycin (100 mg L^{-1}) were used to observe the rooting response of transgenic shoots (**Fig.4A and B**).Plantlets were transplanting in plastic pots filled with a soil mixture of peat and sand (1:1), and covered with polyethylene bags and incubated under 28±2°C, 16 h light/ 8 h dark photoperiod and 70 umol $cm^{-2}s^{-1}$light. The plants were hardened by removing the polyethylene bags gradually over a seven to ten day period (**Fig. 4C and D**).

3. Molecular analysis of transgenic grape plants

3.1. PCR detection of transformed grape plants

To confirm the presence of *AgNHX1*gene in kanamycin resistant plants, PCR analyses were conducted on these putative transgenic grape plants. Recombinant binary vector pBI121containings the *AgNHX1* gene was identified by double digestion with with *Sac1* and *BamHI*. DNA fragments corresponding to the *AgNHX1* gene (2600 bp) was amplified for all kanamycin resistant plantlets as well as for the plasmid DNA, whereas the corresponding band was not detected in the untransformed control, indicating that the T-DNA of the binary plasmid vector was present in the genome of the transgenic plants (**Fig. 5 A and B**).

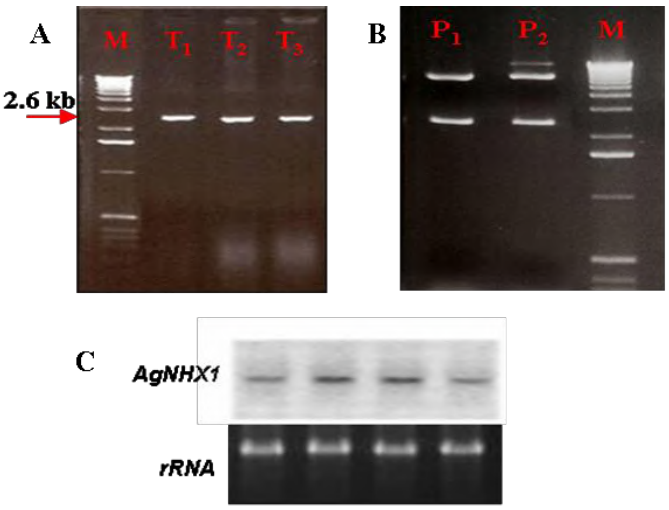

Fig.5: PCR analysis and Expression of the AgNHX1 gene in grape (Vitis vinifera L.) cv. Superior Seedless. A: PCR detection of AgNHX1gene in putative transgenic grape plants, amplifying about 2.6 kb with transgenic plants (Lanes T1-T3). B: Restriction digestion of the cloned fragment of AgNHX1gene in the binary vectorpBI121 under the control of the P35S promoter with Sac1 and BamHI (Lanes P1 and P2). C: Northern blot analysis of total RNA from leaves of transgenic grape plants. Lane M: DNA marker (1kb plus DNA ladder).

Polymerase chain reaction as indicator for the presence of *AgNHX1* gene into transformed fig plants cv. Black Mission grown on selective media by obtaining the expected product size (2.6 kbp) was detected by Ehab *et al*. (2015). Also, PCR was used to confirm the integration of the *P5CS* gene into the genomic DNA of the putative transgenic bialaphos resistant grape tissues (Ehab *et al*., 2016).

3.2. Expression of the AgNHX1 gene in transgenic grape plants

The Na^+/H^+ antiporter gene (AgNHX1)from *Atriplex gmelini*, a halophytic plant was introduce in a salt-sensitive grape (*Vitis vinifera* L.) cv. Superior Seedless under control of the cauliflower mosaic virus (CaMV) 35S promoter. Overexpression of the AgNHX1 gene in the transgenic plants was confirmed by mRNA detection. In examining the mRNA for the Na^+/H^+ antiporter gene (AgNHX1) was observed by RNA gel blot analysis (**Fig. 5C**).

4. Salt tolerance in the transgenic grape plants

Transgenic plants were tested for the expression of *AgNHX1* gene by salt stress tolerance treatment in a growth room. *In vitro* grape plants were cultured on MS medium supplemented with 50–300 mM NaCl in rooting stage. It started with 50 mM, followed by 100 mM, 150 mM, 200 mM, 250 mM, and finally 300 mM NaCl. Growth of non transformed plants was severely inhibited by 150 mM NaCl treatment. However, the growth of *AgNHX1*-expressed transformed grape plants was more tolerant to the same concentration of NaCl and to the other remaining tested concentrations as well. After 4 weeks, the transformed plants under all other tested NaCl concentrations were significantly vigorous than those of the non- transformed plants. To test the maximum tolerance to NaCl by grape transformed plants, the NaCl concentration was increased up to 250 mM. Transgenic grape plants could not expand in size during the salt treatment, indicating that 300 mM NaCl completely inhibited their growth compared to control (non-transgenic grape plants) that can not tolerate NaCl concentration more than 100 mM. The results indicated that the transgenic grape plants could not grow up at more than 250 mM NaCl. Similar results were reported by Masaru *et al*. (2002) in salt-sensitive rice cultivar (*Oryza sativa* cv. Kinuhikari). They reported that the transgenic plants showed over expression of *AgNHX1 gene* in soil irrigated with 300 mM NaCl. While, Singh *et al*. (2000)reported that *in vitro* screening of eight grapes genotypes for NaCl tolerance. Thompson Seedless was found to be the most tolerant among the *Vitis vinifera* genotypes as it tolerated NaCl up to 125 mM. Among *V. champini*, 'Dogridge' was more tolerant and the cultures survived NaCl up to 200 mM. After salt-stress treatments, the surviving transgenic grape plants were transferred to soil conditions without salt

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stress and grown in the green house, following NaCl exposure for three months, 100% of the grape plants survived in soil containing NaCl up to 250 mM, and new leaves and roots appeared on the transgenic plants in this treatment which is considered the maximum level of *AgNHX1* gene expression under salt condition. However, it was found that non transgenic plants could only grow in soil containing NaCl up to 100 mM.

The sodium content in the leaves of transgenic grape plants was increased 6-fold after three months of 250 mM NaCl treatment, indicating that the transgenic plants absorbed and translocated Na into leaves (**Fig. 6**). These experiments were conducted under conditions whereby grape plants also survived following the salt treatments. In contrast, we have shown that transgenic grape plants overexpressing AgNHX1 gene, survive under conditions that generally kill grape plants (250 mM NaCl for three days).These results demonstrate that transgenic grape plants containing the AgNHX1 gene are suitable for practical applications and are capable of producing crops even if they are exposed for high concentration of saline.

High-salt stress is one of the major adverse environmental conditions that affect plant growth, development and crop yield. To avoid salt damage, plants have evolved different mechanisms to limit Na^+ uptake or compartmentalize Na^+ into vacuoles, and Na^+/H^+ antiporters play a key role in the maintenance of osmotic balance (Bartels and Sunkar, 2005). The high concentration of Na^+in the 100, 200 and 250 mM NaCl treatment compared with the control (**Fig. 6**) may indicate an effective sequestration of Na^+ in the vacuoles, confirming tolerance to high cytoplasmic Na^+concentrations. Although to avoid disorder of ion homeostasis under saline conditions plant cells have to maintain a low Na^+concentration and a concurrent high K^+concentration in the cytosol where enzymes for metabolism are, uptake of $K^+$is difficult under saline conditions due to direct competition from $Na^+$for K^+-binding sites on transport systems and also because of a smaller electrochemical potential difference for passive K^+uptake. Overexpression of vacuolar type Na^+/H^+ antiporter gene, *AgNHX1* from *A. gmelini*, in some species, including fig and rice, enable transgenic plants to grow in high concentration of salt, demonstrating the feasibility of producing salt-tolerant crop plants via introducing plant vacuolar Na^+/H^+ antiporter into aimed plants (Masaru *etal*., 2002: Ehab *et al*., 2015). We obtained similar results in grape (*Vitis vinifera* L.) cv. Superior Seedless, further verifying the importance of *AgNHX1* in salt tolerance.

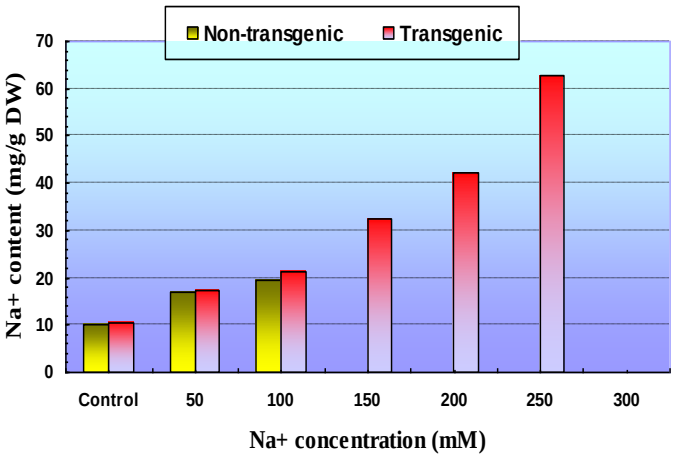

Fig.6. Comparison of sodium content in grape leaves. Non transgenic and transgenic plantlets were treated with culture media containing 0.0, 50,100, 150, 200, 250 and 300 mM NaCl for 4 weeks and Na^+ content was determined by atomic absorption spectrometry.

IV. CONCLUSION

Efficient transformation and regeneration methods are a priority for successful application of genetic engineering to vegetative propagated plants such as grape. In the current work, we describe somatic embryogenesis and plant regeneration of grape (*Vitis vinifera* L.) cv. Superior Seedless and the production of transgenic grape plants are based on *Agrobacterium*-mediated transformation by introduction of the Na^+/H^+antiporter gene from a halophytic plant, *A. gmelini* (AgNHX1). Our experiments showed that callus formation can be induced from leaf explants taken from in vitro grown shoots using NN medium containing 2 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA , and then callus were transferred to NN medium containing 1.5 mg L^{-1} BAP, 0.5 mg L^{-1} kinetin and 0.5 mg L^{-1} NAA for maximum shoot regeneration after eight weeks. Also, our results showed that the production of salt tolerant transgenic grape plants expressing *AgNHX1*demonstrated that *AgNHX1* gene might play a role in the protection of plants under salt stress conditions. Therefore, the *AgNHX1* gene can be successfully used for genetic improvement of grape cv. Superior Seedless for salt tolerance.

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REFERENCES

[1] Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999). Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in arabidopsis. *Science*, 285: 1256–1258.

[2] Bartels, D., Sunkar, R. (2005). Drought and salt tolerance in plants. *Critical Reviews in Plant Sciences*, 24, 23-58.

[3] Botti, C., Garay, L. and Reginato, G. (1993). The influence of culture dates, genotype and size and type of shoot apices on *in vitro* shoot proliferation of *Vitis vinifera* cvs. Thompson Seedless, Ribier and Black Seedless. *Vitis*, 32:125–126.

[4] Das, D.K., Reddy, M.K., Upadhyaya, K.C. and Sopory, S.K. (2002). An efficient leaf disc culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). *Plant Cell Reports*, 20: 999-1005.

[5] Dorani-Uliaie, E., Ghareyazie, B., Farsi, M., Kogel, K. and Imani, J. (2012). Improved salt tolerance in canola (*Brasica napus*) plants by overexpression of arabidopsis Na+/H+ antiporter gene AtNHX1. *J Plant Mol Breed*., 1:34–42.

[6] Ehab, M. R. Metwali, Hemaid, I. A. Soliman, Fuller, M. P., Al-Zahrani, H. S. and Howladar, S. M. (2015). Molecular cloning and expression of a vacuolar Na^+/H^+ antiporter gene (*AgNHX1*) in fig (*Ficus carica* L.) under salt stress. *Plant Cell, Tissue and Organ Culture*, 123:377–387.

[7] Ehab, M.R. Metwali, Hemaid, .I.A. Soliman, Omar .A. Almaghrabi and Naif M. Kadasa (2016). Producing Transgenic Thompson Seedless' Grape (*Vitis vinifera* L.) plants using *Agrobacterium tumefaciens.International Journal of Agriculture and Biology*, 18-3-661-670.

[8] Fukuda, Y., Ohme, M. and Shinshi, H. (1991). Gene structure and expression of a tobacco endochitinase gene in suspension-cultured cells. *Plant Mol. Biol*., 16:1–10.

[9] Fukuda, A., Nakamura, A. and Tanaka, Y. (1999). Molecular cloning and expression of the Na+/H+exchanger gene in *Oryza sativa*.*Biochem. Biophys. Acta*., 1446, 149-155.

[10] Fukuda, A., Chiba, K., Maeda, M., Nakamura, A., Maeshima, M. and Tanaka, Y. (2004). Effect of salt and osmotic stresses on the expression of genes for the vacuolar H+ pyrophosphatase H+-ATPase subunit A, and Na+/H+ antiporter from barley. *J. Exp. Bot*., 55: 585–594.

[11] Gambino, G., Grebaudo, I., Leopold, S., Schartl, A. and Laimer, M. (2005). Molecular Characterization of grapevine plants transformed with GPLV resistance genes: I. *Plant Cell Reports*, 24: 655-662.

[12] Gaoge ,W., Yuhui, L., Peng, X. and Delin, D.(2005). A simple method for DNAextraction from sporophyte in the brown alga *Laminaria ponica*. *Applied Phycology*, 8:1573-5176.

[13] Gharyal, P.K., Maheshwari, S.C. (1990). Differentiation in explants from mature leguminous tree. *Plant Cell Reports*, 8, 550.

[14] Glick, B.R., Cheng, Z., Czarny J. and Duan, J. (2007). Promotion of plant growth by ACC deaminase-producing soil bacteria. *Eur.J. Plant Pathol*., 119:329–339.

[15] Gray, D.J., Jayasankar, S. and Li, Z. (2005). Vitaceae (Vitis spp.). *In*: Litz RE (ed.). Biotechnology of Fruit and Nut Crops. *CAB International Wallingford*, Oxford, UK. Pp. 672-706.

[16] Gray, D.J., Li, Z.T. and Dhekney, S.A. (2014). Precision breeding of grapevine (*Vitisvinifera* L.) for improved traits. *Plant Science*, 228:3–10.

[17] Guan, B., Hu, Y., Zeng, Y., Wang, Y. and Zhang, F. (2011). Molecular characterization and Functional analysis of a vacuolar Na+/H+ antiporter gene (HcNHX1) from *Halostachys caspica*. *Molecular Biology Rep*., 38:1889-1899.

[18] Hamada, A., Shono, M., Xia, T., Ohta, M., Hayashi, Y., Tanaka, A. and Hayakawa, T. (2001). Isolation and characterization of a Na+/H+ antiporter gene from the halophyte *Atriplex gmelini*. *Plant Mol. Biol*., 46: 35–42.

[19] Hasegawa, P.M., Bressan, R.A., Zhu, J.K. and Bohnert, H.J. 2000. Plant cellular and molecular Responses to high salinity. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol*., 51: 463–499.

[20] Hayakawa, T., Zhu, Y., Itoh, K., Kimura, Y., Izawa, T., Shimamoto, K. and Toriyama, S. (1992). Genetically engineered rice resistant to rice stripe virus, an insect-transmitted virus. *Proc, Natl Acad Sci*., USA 89:9865–9869.

[21] Iocco, P., Franks, T.M. and Thomas, R. (2001). Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. *Transgenic Res*., 10: 105-112.

[22] Kulaeva, O. N. (1980). Cytokinin action on enzyme activities in plants, pp. 119-128. *In: Plant GrowthSubstances 1979 - Proceedings of the 10th International Conference on Plant GrowthSubstances*, Skoog F. (Ed.), Springer.

[23] Ma, X.L., Zhang, Q., Shi, H.Z., Zhu, J.K., Zhao, Y.X., Ma, C.L. and Zhang, H. (2004). Molecular cloning and different expression of a vacuolar Na+/H+ antiporter gene in *Suaeda salsa* under salt stress. *Biologia Plantarum*, 48 (2): 219–225.

https://dx.doi.org/10.22161/ijhaf.2.4.1 *ISSN: 2456-8635*

[24] Maas, E.V., Hoffman, G.J. (1977). Crop salt tolerance - current assessment. J.Irrig. and Drainage Div., ASCE 103 (IR2): 115-134.

[25] Maity, S., Ray, S. and Banerjee, N. (2005). The role of plant growth regulators on direct and indirect plant regeneration from various organs of *Leucaena leucocephala*. *Acta Physiologia Plantarum*, 27: 473–840.

[26] Masaru, O., Yasuyuki, H., Asae, N., Akira, H., Akira, T., Tatsunosuke, N. and Takahiko, H. (2002). Introduction of a Na+/H+antiporter gene from *Atriplex gmelini*confers salt tolerance to rice.*FEBS Letters*, 532; 279-282.

[27] Motioike, S.Y.R., Skirvin, R.M., Norton, M.A. and Otferbacher, A.G. (2002). Development of methods to genetically transform American grape (*Vitislabruscana* L.H. Bailey). *J. Hort. Sci. Biotech*., 77: 691-696.

[28] Nadra, K., Maqsood, A., Ishfaq, H., Nadeem, A., Shaghef E. and Muhammad, A. (2015). Optimizing the concentrations of plant growth regulators for *in vitro* shoot culture, callus induction and shoot regeneration fro calluses of grapes *J. Int. Sci. Vigne Vin*., 49, 37-45.

[29] Nitsch, J.P., Nitsch, C. (1969). Haploid plants from pollen grains. *Science*.163: 85–87.

[30] Perl, A., Lotan, O., Abu-Abied, M. and Holland, D. (1996). Establishment of an *Agrobacterium* mediated transformation system for grape (*Vitis vinifera* L.): the role of antioxidants during grape-*Agrobacterium* interactions. *Nat Biotech*., 14: 624628.

[31] Reisch, B.I. 1986. Influence of genotype and cytokinins on *in vitro* shoot proliferation of grapes. *J Am Soc Hortic Sci*., 111:138–141.

[32] Sajid, M.G., Ilyas, M.K. and Anwar, R. (2006). Effect of diverse hormonal regimes on *in vitro* growth of grape germplasm. *Pak. J. Bot*., 38: 385-391.

[33] Singh, S.K., Sharma, H C., Goswami, A.M. and Singh, S. P. (2000). *In vitro* screening of some grape genotypes (*Vitis* spp.) for NaCl tolerance. *Physiology and Molecular Biology of Plants*, 6 (2):175-178.

[34] Snedecor, G.W., Cochran, W.G. (1982). Statistical Methods. 7th Edition, Iowa State University Press, Towa, 511.

[35] Wang, J., Zuo, K., Wu, W., Song, J., Sun, X., Lin, J., Li, X. and Tang, K. (2003). Molecular cloning and characterization of a new Na+/H+ antiporter gene from *Brassica napus*. *DNA Seq*., 14: 351–358.

[36] Xia, T., Apse, M.P., Aharon, G.S. and Blumwald, E. (2002). Identification and characterization of a NaCl inducible vacuolar Na+/H+ antiporter in *Beta vulgaris*. *Physiologia Plantarum*.116: 206–212.

[37] Xiaoqing, X., Cecilia, B., Agüero, Y. and Andrew Walker, M. 2016. Genetic transformation of grape varieties and rootstocks via organogenesis. *Plant Cell, Tissue and Organ Culture*, 126:541–552.

[38] Yamaguchi, T., Apse, M.P., Shi, H.Z. and Blumwald, E. (2003). Topological analysis of a Plant vacuolar Na+/H+ antiporter reveals a luminal C terminus that regulates antiporter cation selectivity. *Proc. Natl. Acad. Sci*., 100: 12510–12515.

[39] Zhang, G.H., Su, Q., An, L.J. and Song, W. (2008). Characterization and expression of a vacuolar Na+/H+ antiporter gene from the monocot halophyte *Aeluropus littoralis*. *Plant Physiol. Biochem*., 46: 117–126.

[40] Zhu, J.K. (2001). Plant salt tolerance. *Trends in Plant Science*, 6: 66-71.