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EFFECT OF SALT STRESS ON *IN VITRO* PROPAGATION OF *ALHAGI MAURORUM* (MEDIK)

TANVI AGARWAL, AMIT KUMAR GUPTA* and NARPAT SINGH SHEKHAWAT

Biotechnology Unit, Department of Botany,
Jai Narain Vyas University, Jodhpur-342001, Rajasthan (India)
*Department of Botany,
Mohanlal Sukhadia University, Udaipur-313001, Rajasthan (India)
*Corresponding author: E-mail: amitugupta@gmail.com

In present study, effect of salinity stress on *In vitro* cultures of *Alhagi maurorum* (Medik) was evaluated. For this piece of work, *in vitro* cultures were subjected to a range of salt (NaCl) stress conditions and in response to given stress conditions different morphological and biochemical parameters like leaf and root development, survival rate of plantlets and chlorophyll content was investigated. Addition of NaCl to culture medium prompts variation in plant morphology, leaf formation, *ex vitro* rooting and survival rate of plantlets in comparison to cultures, those grown on control medium (NaCl was lacking). Shoots developed on NaCl supplemented (68.37mM) medium were healthier and greener in comparison to control medium. Leaf formation, improved percentage of *ex vitro* root development and increased survival rate was also observed on this medium. Chlorophyll content can be considered as one of the most important parameters that shows a good correlation with salinity tolerance. In present report we found that, plantlets cultured on medium containing 68.37mM NaCl have highest chlorophyll content (both a and b), while lower chlorophyll content was reported in cultures grown on control medium as well as with other NaCl concentration. In overall, salinity positively affects growth and development in *A. maurorum*. These results indicated that *in vitro* cultures exhibited similar growth response, in particular salt adaptation, to that of halophytes and indicate the halophytic nature of this plant species. As good of our knowledge this is the first report related to salt stress on this plant species, which can be useful in large scale propagation of rare plant *A. maurorum*.

Key Words: *Alhagi maurorum* (Medik), Halophyte, *in vitro*, *ex vitro* and Salt stress.

Introduction

Abiotic and biotic stresses are major constraints to plant growth and development. Among, abiotic stress, soil salinity is a major stress that affects the crop production and almost every feature of the plant like morphology, biochemistry, physiology and molecular events [1& 2]. Salt damage to plants has been ascribed to ion toxicity[3], water deficit[4], nutrient imbalance,

alteration of metabolic processes, reduction in photosynthesis[5], oxidative stress[6] and most of them are associated with the changed ultrastructure of plant cells[7]. Due to these deleterious effects crops exhibit slower growth rates, reduced tillering and reproductive development. All these changes ultimately affect the agriculture yield. Global annual losses in agricultural production from salt-affected land are in excess of 12 billion US$ and rising[5]. According to FAO report (2008) more than 800 million hectares of land throughout the world are salt-affected (including both saline and acidic soils), equivalent to more than 6% of the world's total land area[8]. Some of the well-known examples of salinity occur in the arid and semiarid regions. It was estimated that the world is losing at least 3 ha of arable land every minute because of soil salinity[8].

In view of adversarial effects of salt stress on plant growth and productivity, the identification and conservation of plant genetic resources have acknowledged considerable attention in last few decades. The application of plant genetic resources for the breeding of salt tolerant/resistant genotypes is considered as a valuable tool to boost the productivity and to develop sustainable agriculture in salt affected lands[9]. *In vitro* technology offers a meaningful application for characterizing salt tolerant plants and also for quick evaluation of germplasm against salt stress under controlled conditions with limited time and space [10]. Axillary bud/shoot apex culture has been found to be an effective method for testing and selecting salt tolerant genotypes[11]. With respect to the whole plant, a similar response to salt stress could be expected in plantlets grown through *in vitro* shoot apex/Axillary shoot culture. Media supplemented with various salt concentrations have been used for screening genotypes of potato, sugar beet, tobacco, Chinese cabbage and canola[10 & 12]. Recent developments in molecular biology and genetic engineering offers the opportunity to identify the stress tolerant genes and their mechanism in halophytic plants and then introduced into non- halophytic crop species to improve their performance under saline conditions. Extensive research has been done on salinity tolerance of cereals, leguminous crops and field grown vegetable crops[5, 7, 10, 13-16].

Alhagi maurorum is a rare legume[17] native to arid region of western Rajasthan and associated with low rainfall with high salinity and alkalinity[18]. *A. maurorum* shows extremophile characteristics manifested by extreme tolerance to a variety of abiotic stresses; low humidity and high salinity are amongst them. *Alhagi maurorum* is a halophyte as determined by its ability to grow and reproduce in saline soil of Thar Desert. The genetic or biochemical basis that confers such high tolerance is unknown. Cell and tissue culture are useful tools for studying salt tolerance mechanisms as they allow use of controlled environments, relatively rapid responses & short generation time. Such types of studies have been done in various plant species including *Hordeum vulgare* [7], *Ipomoea batatas*[10], *Thellungiella*[19], *Salicornica* [20], *Zea mays* [21], etc.

In present study, we have evaluated different morphological and biochemical parameters of *Alhagi maurorum* cultures (grown under *in vitro* conditions) to a range of NaCl concentrations. Among Morphological parameters plant health, leaf formation, *ex vitro* root development and survival rate of plantlets were evaluated. While among the biochemical parameters both chlorophyll a and b content were estimated.

Material and Methods

Plant material

In vitro proliferating cultures of *Alhagi maurorum* were used as the source material. *In vitro* cultures were maintained on Modified MS medium solidified with 0.8% agar and containing BAP (0.5 mg l^{-1}), IAA (0.1 mg l^{-1}) and additives, according to protocol given by Agarwal et al., 2015[22].

Effect of Salt stress on shoot growth

For salt stress experiments, *in vitro* raised shoots were transferred on MMS medium solidified with 0.8% agar, containing BAP (0.5 mg l^{-1}), IAA (0.1 mg l^{-1}), additives and different concentrations of NaCl. Various concentrations (8.54 mM, 17.09 mM, 57.28 mM, 68.37 mM and 102.56 mM) of NaCl were added to the medium before the adjustment of pH to 5.8 ± 0.02. Nutrient medium without NaCl used as control in all experiments. The cultures were maintained at 26 ± 2°C with 40-50 µmol $m^{-2}s^{-1}$ PFD, light intensity provided by cool and white fluorescent tubes (Philips, India) and 55–60% RH.

Effect of Salt stress on *ex vitro* rooting

For *ex vitro* root induction, *in vitro* raised shoot were excised individually and thereafter, the basal cut ends of individual shoot were dipped in auxin solution (IBA 250mgl^{-1} + NOA 250mgl^{-1}) for 4 min [22]. The auxin treated shoots were transferred to bottles containing sterile Soilrite® (a combination of perlite with peat moss and exfoliated vermiculite procured from Kel Perlite, Bangalore (India). To study the effect of salt stress on *ex vitro* root induction, NaCl in different concentrations (8.54 mM, 17.09 mM, 57.28 mM, 68.37 mM and 102.56 mM) added to sterile solirite before the inoculation of shoots. Soilrite was moistened with one-fourth strength of MS salts. The bottles were kept near the pad section (80–90% RH and 28 ± 2°C) of green house. Once the root formation initiated in auxin treated shoots, these were subjected to hardening and acclimatization[22].

Acclimatization and survival of plantlets

For acclimatization of plantlets developed from *ex vitro* rooting (developed under salt stressed conditions), polycarbonate-caps of bottles were gradually loosened over a period of 15 days and finally removed. The bottles (kept near the pad section) were gradually shifted towards the fan section providing comparatively higher temperature and low humidity (RH 40-50% and 32±2°C) for further acclimatization. After 30-35 days, the hardened plantlets were transferred to black poly-bags containing sand, farm yard manure (FYM), soil-rite and vermiculite in 1:1:1:1 ratio.

Effect of Salt stress on chlorophyll content

Fresh shoots harvested from *in vitro* cultures for chlorophyll estimation. Prior to extraction fresh samples were cleaned with sterile water to remove traces of culture medium from surface. For chlorophyll extraction 100mg plant sample was grounded in 10 ml of 80% acetone using a pestle mortar and mixed well. After that extract was centrifuged at 5000rpm and supernatant was separate out to record the absorbance. The optical density was measured at 645nm and 663nm using UV-Visible Elico-Spectrophotometer and chlorophyll concentration was calculated using equation proposed by Arnon (1949)[23].

Chlorophyll a (µg ml^{-1}) = 12.7 (A 663) – 2.69 (A 643)

Chlorophyll b (µg ml^{-1}) = 22.9 (A 645) – 4.68 (A 663)

Total Chlorophyll (a + b) (µg ml^{-1}) = 20.2 (A 645) + 8.02 (A 663)

Note:

- A 645- Absorbance at 645nm

- A 663- Absorbance at 663nm

Three replicates were used for the study of chlorophyll content.

Data analysis

The experiments were set up in complete randomized block design (RBD) and repeated thrice. All the experiments were conducted with a minimum of 10 replicates per treatment. One replicate means one culture vessel. The significance of differences among means was carried out using Duncan's multiple range tests (DMRT) at $P < 0.05$. The results expressed are means ± SD of three independent experiments and subjected to one-way analysis of variance (ANOVA) using SPSS v.17 (SPSS, Chicago, USA).

Three replicates were used for the chlorophyll estimation. MS- Office was used for the statistical analysis and graph formation.

Data was recorded (on morphological basis) after 18-25 days of inoculations for study of morphological parameters like leaf formation, shoot quality, root number, root length and survival percentage.

Results and Discussion

Halophytes are defined as plants that naturally inhabit saline environment and benefit from having substantial amounts of salt in the growth media. Optimal halophyte growth is achieved at a concentration of around 50 mM NaCl for monocots and between 50 to 200 mM for dicots[5 & 24]. It is necessary to understand the integration and expression of tolerant mechanisms from the cellular level to the whole-plant level, *in vitro* culture provides an excellent means to study the physiological and genetic processes of the plant at cell and tissue levels [25]. In our study, addition of NaCl to culture medium led to significant variation in plant morphology, leaf formation, root development, survival rate of plantlets and chlorophyll content. Culture medium supplemented with 68.37mM NaCl was found optimum for survival of shoots under *in vitro* conditions, whereas higher concentrations of NaCl inhibited growth. Shoots developed on this medium were healthier and greener as compared to control medium in which NaCl was lacking and medium supplemented with other concentration of NaCl. Besides that, leaf formation was also observed on NaCl supplemented medium which were not formed on control medium (Fig. 1 A & B). Significant effect of NaCl incorporation was observed on *ex vitro* rooting, optimum response was observed when soilrite was fortified with 68.37mM NaCl. On this treatment 79.99±6.66% response was observed with 6.4±1.24roots/shoot. While in control medium 51% response was observed with 2.33± 0.72 roots/ shoot (Fig.1 C& D; Table-1). Plantlets developed on medium containing 68.37mM NaCl showed higher percentage of survival as compared to plantlets grown on NaCl free medium. These results indicated that *Alhagi* cultures exhibited similar growth response, in particular to salt adaptation, as that of halophytes. Similar type of response of NaCl treatment on morphology and growth of cultures has been reported in *Thellungiella* [19] and *P. euphratica* [26]. Chlorophyll content can be considered as one of the most important parameters that shows a good correlation with salinity tolerance[27 & 28]. Incorporation of NaCl led to enhancement of total chlorophyll content in our study. Higher chlorophyll content (both a and b) was observed in plantlets treated with 68.37mM NaCl and lower chlorophyll content was observed in control as well as plantlets treated with other NaCl concentration (Fig. 2). The results regarding the ''increase'' in chlorophyll content with

the increase in salt concentrations agree with results reported by Mishra et al. (1997)[29]. Mishra et al. (1997) concluded that increase in the chlorophyll content under salt stress could be due to an increase in the number of chloroplasts in stressed leaves. Our study supports this opinion, as we have discussed, salt stress in present study induce the leaf formation and due to the leaf formation chlorophyll content increase in this study. Our results are in accordance with the findings of other species such as in rice[28] and maize[30]. The present data clearly support the general correlation between the chlorophyll content and leaf area, which is in agreement with the hypothesis of Reich et al. (1999)[31] that no species can improve photosynthetic capacity without increasing leaf area because of biophysical limitations. Some authors also believe that photosynthetic capacity of *in vitro* cultured plants is sufficiently high to support autotrophic growth during periods of acclimatization[32].

NaCl Conc. (mM)	Response (%)	Root number per shoot	Root length (Cm)
00.00	51.10±3.85^{c}	2.33±0.72^{d}	3.40±0.42^{d}
08.54	55.55±7.70^{c}	2.53±0.63^{d}	3.67±0.27^{cd}
17.09	57.77±3.85^{c}	3.06±0.88^{c}	3.91±0.56^{c}
57.28	66.66±6.66^{b}	4.4±0.98^{b}	5.86±0.79^{b}
68.37	79.99±6.66^{a}	6.4±1.24^{a}	7.46±0.91^{a}
102.56	33.33±6.67^{d}	1.53±0.63^{e}	3.08±0.38^{e}

Table-1: Effect of NaCl on *ex vitro* rooting of *in vitro* regenerated shoots of *A. maurorum*.
Data having the same *letter* in a column were not significantly differed by Duncan's multiple comparison test (P<0.05).

Conclusion

This is first report, which validates that salt stress possess positive effect on growth and development of *A. maurorum* (Medik), under *in vitro* as well as *ex vitro* conditions. These results indicated that *Alhagi* cultures exhibit similar

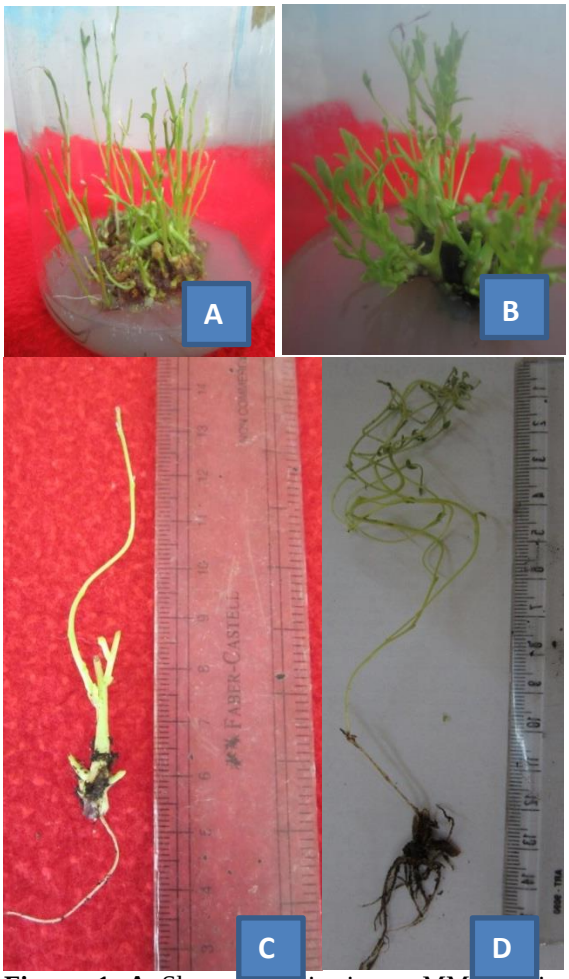

Figure- 1: A: Shoot multiplication on MMS medium containing 0.5 mg l^{-1} of BAP and 0.1 mg l^{-1} of IAA. **B**: Shoot multiplication on MMS medium containing 0.5 mg l^{-1} of BAP and 0.1 mg l^{-1} of IAA + NaCl (68.37mM). **C**: Ex vitro rooted shoot after treatment with 250 mg l^{-1} IBA + 250 mg l^{-1} NOA. **D**: Ex vitro rooted shoot after treatment with 250 mg l^{-1} IBA + 250 mg l^{-1} NOA + NaCl (68.37mM)

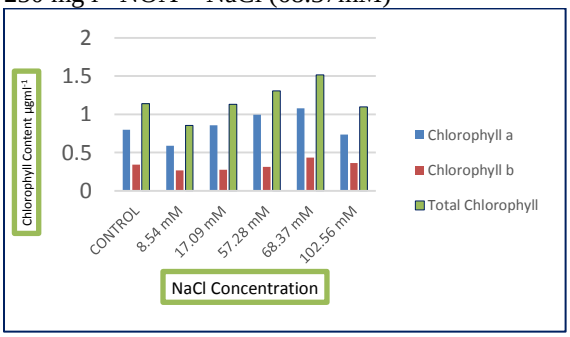

Figure-2: Effect of NaCl on Chlorophyll (a, b & total) Content of *A. maurorum*

growth response, in particular salt adaptation, as that of halophytes. Therefore, this method can be used for large scale propagation and conservation of elite genotype of *A. maurorum*.

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