Short Communication / 10-16

Effective *in vitro* seed germination and direct regeneration from cotyledonary leaf explants of *Nitraria schoberi*

Samaneh Kheirabadi[1], Nasim Zarinpanjeh[2*], Mohammad Ali Ebrahimi[1], Golamreza Bakhshi Khaniki[1], Hamid Reza Naseri[3]

[1]Department of Agricultural Biotechnology, Payame Noor University, Tehran, Iran.
[2]Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, P. O. Box: 31375-1369, Karaj, Iran.
[3]Department of Desert Management, International Desert Research Center (IDRC), University of Tehran, Tehran, Iran.
[*]Corresponding author, Email: zarinpanjeh@imp.ac.ir. Tel: +98-26-34764010. Fax: +98-26-34764021.

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Abstract

Nitraria schoberi (Ghar-e-Daghin in Persian) from *Zygophyllaceae* (*Nitrariaceae*) family is considered as a wonderful exquisite plant in terms of its economical, medicinal, animal feed, significant values and also its unique characteristics of being tolerant to the soil salinity, drought and erosion. On the other hand, this plant is challenging with severe problems such as seed dormancy, plant growth inhibitors and also hard seed coat which lead to retard germination and subsequently to reduce the chance of life and reproduction. The present study was conducted for the first time to cope with its seed germination obstacles and to induce plantlet regeneration in *N. schoberi* through tissue culture method. Effect of cotyledonary leaf and hypocotyl explants and various concentrations of plant growth regulators were studied on shoot and root induction and further development. Based on the obtained results, culturing seeds after removing its seed coat effectively improved germination rate (80%) within a week. Whereas, germination of seeds with seed coats required a longer time (4 weeks) and showed a sharp decline in percentage (10%). At the next step, culturing cotyledonary leaf explants on shoot induction media including MS medium supplemented with BAP (2.0 mgL^{-1}) and IBA (0.5 mgL^{-1}) gave the highest shoot regeneration rate (93.33%), with average shoot number per explant (3.67) and shoot length (4.6 cm) after 60 days. Hypocotyl explants showed no response to shoot induction treatments even after 60 days. Regenerated shoots were rooted on the MS medium supplemented with IAA (1.0 mgL^{-1}) alone or in combination with IBA (2.0 mgL^{-1}). Finally, all regenerated plantlets were acclimatized and transferred to the green house successfully with 70% survival rate.

Key words**:** *Nitraria schoberi*, *In vitro* culture, Seed germination, Direct regeneration, Plant growth regulators.

INTRODUCTION

Nitraria schoberi L. from *Zygophyllaceae* (*Nitrariaceae*) family is widely expanded in sandy and saline areas of central regions of Iran. It is also a drought resistant shrub with the ability to thrive in sandy lands as well as on moving sands and the capability to expand over sand dunes in arid areas. Its high tolerance to salinity and also resistance against high temperature ranges (up to 72 °C) has gained a specific importance and prominence compared to many halophyte plants (Ahkani, 2002; Parida and Das, 2005; Naseri, 2014; karimian *et al.,* 2017).This plant can be used to stabilize moving dunes for combating desertification (Heshmati and Squires, 2013).

N. schoberi is also a medicinal plant with an attractive source of fatty acid components; phenolic compounds which possess effective natural antioxidants and may

help protect cells against the oxidative damage caused by free-radicals (Kahkonen *et al*., 1999; Senejoux *et al*., 2011; Khajeddini *et al*., 2012). Its fruit shows promising use as new pharmaceuticals with antioxidant, antibacterial, antifungal and anti-inflammatory activities to treat diseases and/or as a protective agent against disorders associated with oxidative stress and inflammation (Sharifi-Rad *et al*., 2014; Sharifi-Rad *et al*., 2015). Recent studies have also demonstrated its antiviral properties (Zheleznichenko *et al*., 2018).

Nitraria schoberi is an endangered shrub with potential for rehabilitation of arid and saline lands (Paunescu, 2011). There are many factors such as the low level of autogamy (self-fertilization), seed dormancy, long generative circle, low genetic diversity and sensitivity to some biotic and abiotic stresses which restrict the conventional culturing and breeding techniques and encounter them to certain drawbacks (Bahadori and Javanbakht, 2006). Fresh and matured seeds of *N. schoberi* showed no germination capacity even when the embryo was well developed (Paunescu, 2011). This dormancy retards the germination that might be a result of high adaptation to arid environments.

In this regard, it has been suggested that implementing an *in vitro* culturing method may provide and facilitate rapid growth as well as maintaining germplasm and genetic enhancement (Nas *et al*., 2012).

Tissue culture technology is an appropriate method for conservation and propagation of plant species from extinction (Gulati, 2018). This approach can introduce the optimized *in vitro* methods for overcoming seed germination obstacles and mass propagation of this plant. The current study was conducted to develop *in vitro* seed germination and plantlet regeneration protocols for mass propagation of *N. schoberi*..

MATERIALS AND METHODS

Plant material

Seeds of *N. schoberi* plants were collected from Myghan desert (coordinates: 39_3401000N, 4900100E), Markazi Province, Iran in July 2017 and stored in the Herbarium of International Desert Research Center, University of Tehran. Seeds were washed under running tap water and stored at 4 °C for 24 h in dark. Then, the pericarp was removed by rubbing with hands and the seeds were kept again at 4 °C for 24 h in dark. In preparation for seed germination, seeds were surface-sterilized by treating with 70% ethanol for 1 min and an aqueous 0.1% (v/v) sodium hypochlorite solution for 15 min. Explants were finally rinsed three times with sterile double-distilled water to remove any traces of the disinfectant.

Seed germination

The surface sterilized seeds with and without seed coat (removed by a forceps) were cultured in test tubes containing10 ml of MS medium (Murashige and Skoog, 1962). The pH of the medium was adjusted to 5.8 and then autoclaved for 15 min at 121 °C. The cultured seeds were incubated at 25±2 °C under the 16/8 h photoperiod and light illumination of 3000 lux. The seed germination (%) was recorded after 21 days.

Shoot induction

Cotyledonary leaf and hypocotyl explants (5-7 mm) aseptically excised from 40-days-old seedlings were cultured on the MS medium supplemented with BAP at 0.0, 1.0 and 2.0 mgL^{-1} and IBA at 0.0, 0.5 and 1.0 mgL^{-1}. The subculture was performed once every month. All cultures were incubated in a growth chamber at 25±2 °C and photoperiod of 16/8 hours for 60 days. The shoot induction (%), the number of shoots per explants and the shoot length were evaluated for shoot regeneration.

Root induction

For rooting, the regenerated shoots were transferred into MS medium supplemented with IBA at 0.0, 1.0 and 2.0 mgL^{-1} or IAA at 0.0, 0.5 and 1.0 mgL^{-1}. These cultures were kept 60 days at the same environmental conditions as mentioned above. The root induction (%) and the number of roots per explant were recorded.

Acclimatization

Rooted plantlets were washed in running water to remove the nutrient media then planted in small plastic pots. The soil mix prepared by mixing cocopeat, peat moss and perlite at a 1:1:1 ratio. The soil was autoclaved at 121 °C for 45 min prior to planting of the rooted plantlets. All the plantlets were acclimatized for 30 days prior to transferring them to the greenhouse. In order to acclimatize the plantlets to the environmental humidity, all plantlets were kept in the plastic cap and every day one hole was made on it to gradually reduce humidity.

Experimental design and statistical analysis

The experiments were setup based on completely randomized design with three replicates per treatment and five explants per replicate. The data expressed as percentage were subjected to arcsine transformation before statistical analysis. Statistical differences were assessed based on analysis of variance using SPSS (Version 15, USA). Differences among means were analyzed using Duncan multiple range test at a probability level of $p<0.05$.

RESULTS AND DISCUSSION

In vitro seed germination

In the first step, seeds were kept under the running tap water for 30 minutes. This helps water soluble germination inhibitors come out of the seed coat. The germination of two kinds of seeds was studied; the seeds with their seed coats and the seeds without seed coats. Only 10% of the seeds with intact seed coat germinated after 4 weeks while the seeds without seed coat started germinating from the 5th day and the germination rate was 80% (data not shown). In this study, the seeds were kept at °C for 24 h twice. Once, after washing seeds under running water and again after removing the seed coat. Keeping seed under certain temperature is one of the suggested ways to stimulate germination in some plants (reference).

One of the most important germination retarding elements in some seed coat is abscisic acid (ABA) whose deterring effect can be reduced by soaking or rinsing practices with water (Linkies and Leubner-Metzger, 2012). For example, long-term post-maturation (12 month at 20 °C) followed by cold stratification (6 month at 4 °C) combined with dry heat treatments (50 °C for 20 days) were proved to overcome seed dormancy of *N. schoberi* and a good germination percentage was obtained (73.4% within 12 days) (Paunescu, 2011).

Seeds of *N. sphaerocarpa*, *N. tangutorum*, *Peganum nigella strum* and *Zygophyllum fabago* were stored in soil during winter and also stored at 10, 15, 20, 25 °C for 80 days before their germination tested. The seeds of *N. sphaerocarpa* stored in soils gave the highest germination of 40-50% in the seeds stored at 15 and 20 °C. The seeds of *P. nigella strum* and *Z. fabago* stored in soils in winter had the highest germination of 80% and 90-100%, respectively (Wang and Zhang, 2009). Another study was carried out to standardize the techniques for mass propagation of *N. retusa*. Results indicated that exposure of *N. retusa* seeds to dry heat (50 °C) for 20 days followed by treatment with 500, 750 and 1000 ppm GA_3 was effective in enhancing the germination to 94, 91 and 90%, respectively, compared to the control (79%) (Suleiman *et al*., 2008).

Scraping and removing the seed coat can ease and facilitate the process of germination by reducing the mechanical resistance as well as increasing the water and gas permeability. The seed coat can play a significant negative role in dormancy in many different plant species not only by hindering water penetration but also by affecting other factors like the amount of oxygen needed for respiration or CO_2 emitted from embryo (Borisjuk and Rolletschek, 2009).

Direct regeneration

Direct regeneration method is an effective way to maintain strict clonal fidelity and true-to-type nature of regenerants to the mother plants (Chaudhury *et al*., 2010). Different response was observed in terms of type of explants, the hypocotyls explants had no sign of shoot induction even after 8 weeks whereas, the cotyledonary leaf explants started to swell up after 2 weeks. They showed different regeneration responses to the various concentrations and combinations of BAP and IBA (Figure 1).The results showed that the response to shoot regeneration depended on explant type as well as concentration and type of plant growth regulators used. The variance analysis of the effect of plant growth regulators on shoot regeneration was considered significant at 5% probability level (Table 1).The highest shoot induction (93.33%), shoot numbers per explant (3.67) and shoot length (4.6 cm) were obtained by culturing cotyledonary leaf explants on the MS supplemented with 2.0 mgL^{-1} BAP and 0.5 mgL^{-1} IBA (Figure 2).

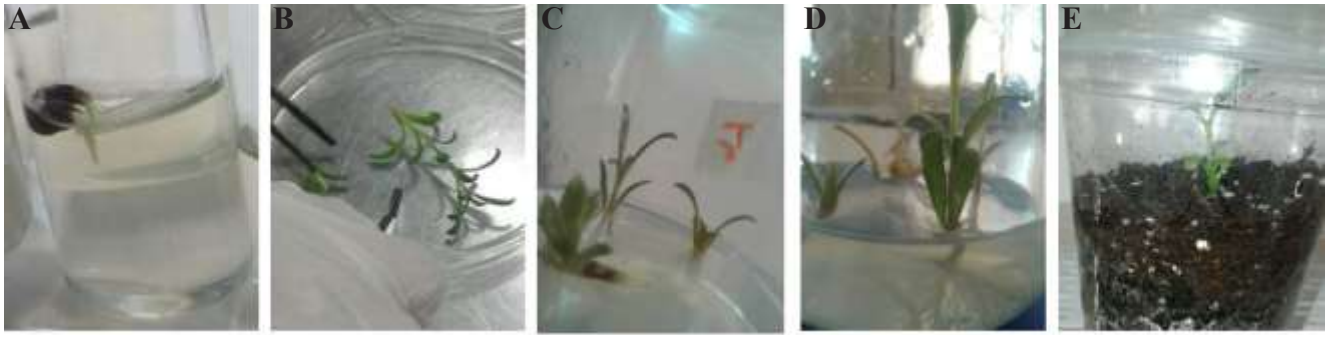

Figure 1. A: Seed germination of *N. schoberi* at 5th day culturing in *in vitro* conditions on MS cultural medium, **B:** aseptically excised cotyledonary leaf explants, **C:** shoot regeneration after 60 days of inoculation, **D:** Root regeneration after 60 days of inoculation, **E:** plantlet acclimatization.

Table 1. The variance analysis of the effect of plant growth regulators on shoot regeneration.

Source of variation	df	Mean of square		
		Shoot induction	Shoot number	Shoot length
BAP	2	10414.815**	9.593**	26.037**
IBA	2	2325.926**	4.148**	5.815**
BAP×IBA	4	437.037*	1.426*	1.093*
Error	18	148.148	0.333	0.370

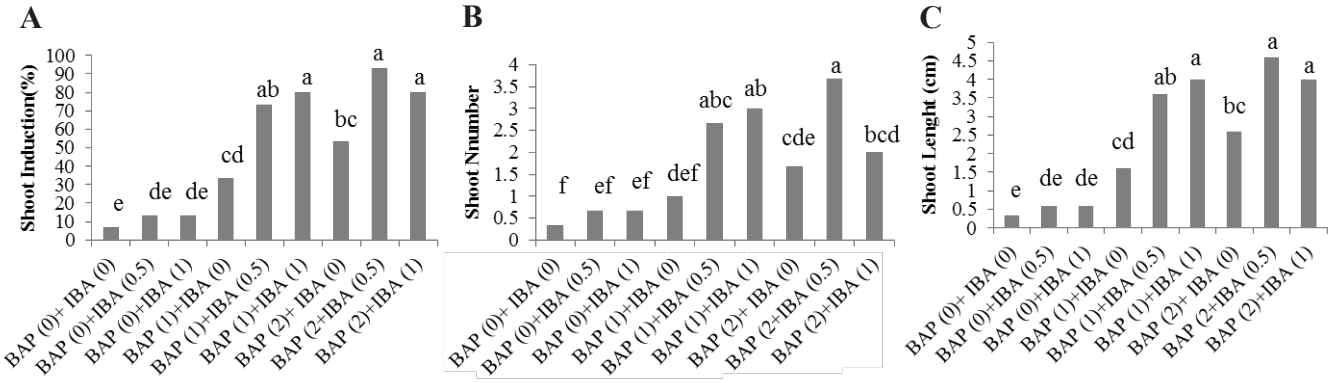

Figure 2. Effect of different concentrations and combinations of plant growth regulators on **A:** Shoot induction (%), **B:** shoot number per explant, **C:** regenerated shoot length (cm) in *N. schoberi*.

Similar results showing variations among explant types with respect to shoot induction have been reported in many other plants like *Paeonia lactiflora* Pall (Tian *et al*., 2009); *Chrysanthemum morifolium* (Somg *et al*., 2011) and *Salvia sclarea* L. (Ghanbar *et al.,*2016). Applying BAP and IBA alone or in combination with other plant growth regulators for shoot induction has been reported in various species (Sasidharan and Jayachitra, 2017; Sagina Rency *et al*., 2018; Hesami *et al*., 2019) including *Nitrariaceae*. The best shoot multiplication medium for the infancy plants of *N. tangutorum* as explants was the MS medium supplemented with 0.5 mgL^{-1} BAP, 1.0 mgL^{-1} NAA and 2.0 mgL^{-1} GA3 (Hongxiao and Xiangyang, 2004). N6 culture medium supplemented with 2 mgL^{-1} BAP and 0.1 mgL^{-1} IBA was the optimal medium for shoot proliferation in *N. sibirica* (Qichang *et al*., 2008). The optimum shoot multiplication for *N. tangutorum* was achieved by culturing immature stem in MS medium supplemented with 2 mgL^{-1} BAP and 1 mgL^{-1} NAA (Guo *et al*., 2009).

In the next step, for root induction the regenerated shoots were subcultured on the MS medium containing different concentrations and combinations of IBA and IAA. The variance analysis of the effect of plant growth regulators on root regeneration was considered significant at 5% probability level (Table 2). They rooted successfully with 80% efficiency after 60 days of culturing on the MS medium supplemented with 2.0 mgL^{-1} IBA and 1.0 mgL^{-1} IAA. The maximum number of root per explant (3.8) and root length (6.2 cm) were observed on the medium containing only IAA at 1.0 mgL^{-1} (Figure 3).

Similar results were observed on the effective role of IBA and IAA alone or in combination with other plant growth regulators on root formation in different species (Saurabh *et al*., 2017; Hesami and Daneshvar, 2018; Salari *et al*., 2018). For example, ½MS with IBA at 0.5mgL^{-1} and Kin at 1.0 mgL^{-1} (Hongxiao and Xiangyang, 2004; Guo *et al*., 2009) for root formation in *N. tangutorum,* MS with IBA at 0.5 mgL^{-1} for root induction in *N. sibirica* (Qi *et al*., 2007; Qichang *et al.*, 2008) have been reported. There are also many other reports showing that basal medium without any hormone or with other kinds of hormones are effective on *in vitro* root induction in different plants (Arulananthu *et al*., 2019; Raveesha and Vasudha, 2019).

Table 2. The variance analysis of the effect of plant growth regulators on root regeneration.

Source of variation	df	Mean of square		
		Root induction	Root number	Root length
IBA	2	3214.815**	6.778^{ns}	19.704^{ns}
IAA	2	2503.704**	32.444**	81.037**
IBA×IAA	4	992.593*	2.722^{ns}	6.593^{ns}
Error	18	207.407	1.963	6.519

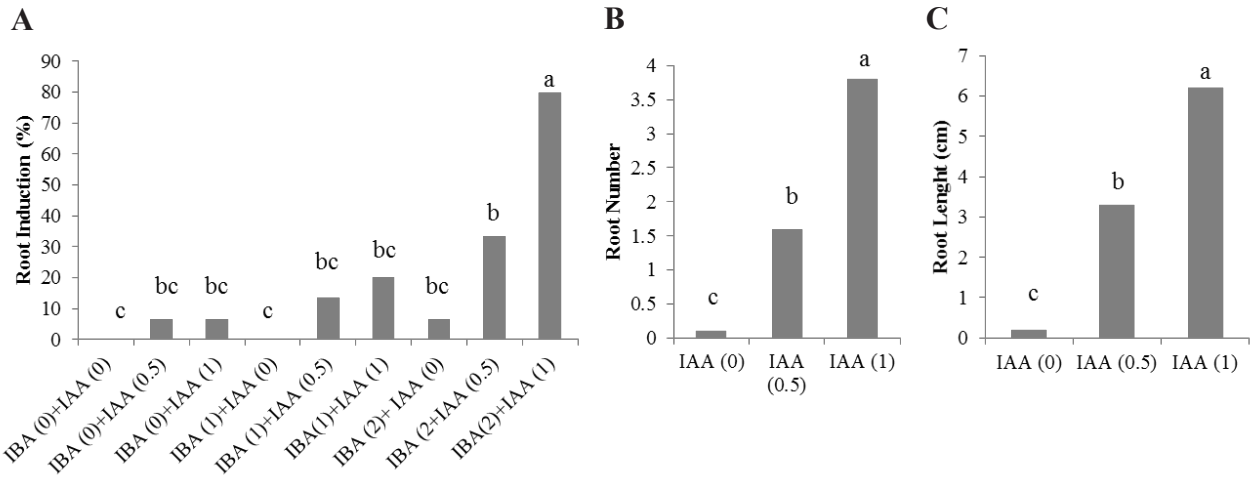

Figure 3. Effect of different concentrations and combinations of plant growth regulators on **A:** Root induction (%), **B:** root number per explant, **C:** root length (cm) in *N. schoberi*.

After 60 days, when the number and the length of regenerated roots seemed sufficient, the plantlets were transferred form *in vitro* conditions to the soil. The survival rate during the acclimatization procedure reached 70%. Previous reports in many plants confirmed that the gradual decrease in environmental humidity of plantlets after transferring from *in vitro* conditions increased the survival rate. Hosseinpour *et al*. (2015) placed cherry rootstocks in plastic containers with transparent covers and after one week, the covers were opened gradually over a 48 h period. The survival rate obtained *ex vitro* was 95%. Mittal *et al*. (2017) covered *Tinospora cordifolia* plantlets with inverted glass beakers to maintain humidity and kept them in a plant growth chamber for hardening.

In summary, it seems that the present study is the first report on *in vitro* seed germination and shoot regeneration of *N. schoberi* which may help to develop a protocol for its conservation and possibly large scale propagation for industrial, medicinal and environmental purposes in the future.

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