ACTA SCIENTIARUM POLONORUM

Acta Sci. Pol. Hortorum Cultus, 18(1) 2019, 39–45

www.acta.media.pl ISSN 1644-0692 e-ISSN 2545-1405 DOI: 10.24326/asphc.2019.1.4

ORIGINAL PAPER

Accepted: 26.05.2018

CLONAL PROPAGATION OF *Gypsophila aretioides*, AN IDEAL ROCK GARDEN PLANT SPECIES

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ABSTRACT

Gypsophila aretioides, a cushion form evergreen plant, is a high potential wild species ideal for the use in rock garden, or as a ground cover in sunny dry areas. This plant has the competence to be developed as a new ornamental species. The purpose of this experiment was to provide an efficient micropropagation protocol for *G. aretioides* in order to facilitate the availability of this species for further studies of domestication. The influence of various concentrations of 6-benzylaminopurine (BAP) and thidiazuron (TDZ) was investigated for multiplication stage. TDZ at low concentration of 0.05 mg dm^{-3} resulted in the maximum shoot (9.7) and leaf (42.3) number. The shoots were best rooted on MS medium containing 0.6 mg dm^{-3} indolebutyric acid (IBA) with 7.8 roots per shoot. Despite achievement of a successful protocol for in vitro multiplication and root induction of Gypsophila, low survival rate was obtained when rooted explants were exposed to ex vitro conditions. This is an important issue, which requires particular consideration and further studies. The possible reasons contributing to the low acclimatization rate of this species are being discussed.

Key words: shoot tip, tissue culture, proliferation, *Gypsophila aretioides*, regeneration, acclimatization

INTRODUCTION

Gypsophila aretioides, an alpine plant from *Caryophillaceae* family, is a low-growing, cushion-forming evergreen plant native to Iran, Turkmenistan and Southern Caucasian areas. Tiny oblong and gray-green leaves form a compact foliage texture that only rises to 5–7.5 cm tall [Melzheimer 1980]. This is a high potential native species that is ideal to be used as container or rock garden plant, however it can be an excellent choice for ground cover in sunny dry areas because of its densely compact leaves and stems. Some species of the genus *Gypsophila*, that are nowadays the commercial species, have been vegetatively propagated with *in vitro* culture techniques. *Gypsophila paniculata*, an ornamental plant with medicinal value, has been propagated successfully with *in vitro* culture techniques using various explant types in Murashige and Skoog medium [1962] (MS) supplemented with benzylaminopurine (BAP) or thidiazuron (TDZ) as efficient cytokinins for shoot regeneration [Ahroni et al. 1997, Zuker et al. 1997, Rady 2006]. Cytokinins are known to be effective in cell division stimulation, release of lateral bud dormancy as well as adventitious bud formation in tissue culture of plant species [Faisal et al. 2012].

Micropropagation is an extensively used technique for rapid multiplication of plant species. This technique has been used successfully for clonal propagation of many cultivated ornamental plants [Han et al. 2004, Gantait et al. 2010, Davoudi Pahnekolayi et al. 2015], however, it is not a common method for vegetative propagation of wild species. Nevertheless, application

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of micropropagation for wild species can be advantageous especially in the situations, where limited plant materials are available or deep dormancy and low germination rate are encountered [Wochok 1981].

Based on available literature, successful micropropagation protocols have been established for wild relatives of *Gypsophila* including *Diaphanoptera khorassanica* [Kiani et al. 2013] *Silene cretacea* [Kritskaya et al. 2016] and *Dianthus grantianopolitanus* [Fraga et al. 2004], however, in some cases, low acclimatization rates were reported. Acclimatization of *in vitro* regenerated plantlets is a critical step in micropropagation of several plant species, which may be a limiting factor in large scale application of this technology [Rai et al. 2011]. Developing *in vitro* culture protocols for wild species for the first time might be difficult since many problems may arise during each step of micropropagation. High rate of explant contamination, limited shoot proliferation, absent or low rooting induction of regenerated shoot along with low acclimatization competence, are some of the main issues in tissue culture process of wild species, which requires extensive optimization studies [Prakash 2009, Kiani et al. 2013, Samiei et al. 2017].

G. aretioides is propagated by seed in the nature, however, seed set and seed germination rate are low in this species (data not shown). There are no reports up to now corresponding to *in vitro* propagation of *G. aretioides* and many works are still devoted to bring this valuable plant species into the market. The purpose of this study was to provide an efficient micropropagation protocol for *G. aretioides* in order to facilitate the availability of this species for further studies on domestication.

MATERIAL AND METHODS

Mature plants of *Gypsophila aretioides* were collected in early June from the mountain areas in Kalat district, Razavi Khorassan province, Iran. The plants were tightly cling to the sloppy rocks when collected. In the laboratory, the shoots were separated and placed for one hour under running tap water. Two-centimeter long shoot apices were excised and treated with 70% ethanol for 30 s followed by an immersion in 0.1% mercuric chloride for 4 or 8 min. The shoots were then rinsed for 3 times with sterile distilled water. The lateral leaves were trimmed to produce shoot tip explants with 4 small leaves of 1 cm length.

The explants were inoculated on solidified MS medium without any plant growth regulators. After one month, the contamination free explants were transferred to new MS medium with different concentrations of BAP or TDZ (Tab. 1). Following the two subcultures (30 days each) in the same multiplication medium, the induced shoots with about 2 cm length were transferred to rooting induction medium containing indolebutyric acid (IBA) or naphthaleneacetic acid (NAA) at 0, 0.3, 0.6, 0 mg dm^{-3} concentrations. A combination of 0.3 mg dm^{-3} IBA and 0.3 mg dm^{-3} NAA was also considered in rooting stage for probable positive interaction of these two plant growth regulators. In four weeks, well rooted shoots were transferred to plastic cups containing the mixture of sterilized peat : perlite (1 : 1, v/v) covered with transparent plastic to maintain the humidity. All media were incubated at 24 ±2°C under a 16 h photoperiod with a photon flux density of 40 µmol m^{-2} s^{-1} from a fluorescent lamp. The pH of all media was adjusted to 5.8 prior to adding 0.8% (w/v) agar and autoclaved at 121°C for 20 min. MS medium supplemented with 3% (w/v) sucrose was used in the experiments. Shoot number, shoot length and leaf number were recorded after each stage of subculture in multiplication media, while root number and root length were recorded after 4 weeks in rooting media.

All experiments were performed based on completely randomized design in 3 replications. For each treatment, data of 15 explants were recorded. The data were analyzed using SPSS software version 16.0. Tukey's HSD test was performed revealing differences between the treatments (all p values < 0.05).

RESULTS

The best results regarding sterilization procedure were obtained when $HgCl_2$ (0.1%) was applied for 8 min. In this method, 92% of the explants remained sterile.

Shoot proliferation. The highest shoot numbers were obtained in 0.05 mg dm^{-3} TDZ with the average of 9.75 shoots per explant in proliferation medium, while it was 1.33 in control (Tab. 1, Fig. 1). With increasing the concentration of TDZ, the mean shoot number substantially decreased from 9.75 to 2.33. Regarding BAP, the greatest shoot proliferation was obtained at 2 mg dm^{-3} with 7.75 shoots per explant, however it decreased to 3.50 when BAP concentration

increased to 4 mg dm^{-3}. Shoot length was not extremely influenced by different concentrations of cytokinins as no significant differences were obtained among the treatments and control. However TDZ at 0.1 and 1 mg dm^{-3} resulted in the longest and smallest shoots, respectively (Tab. 1). The maximum leaf number (42.33) was obtained in the medium containing 0.05 mg dm^{-3} TDZ compared to the control (7.85). Leaf number decreased with increasing concentration of TDZ. BAP was less effective in leaf production in comparison with TDZ.

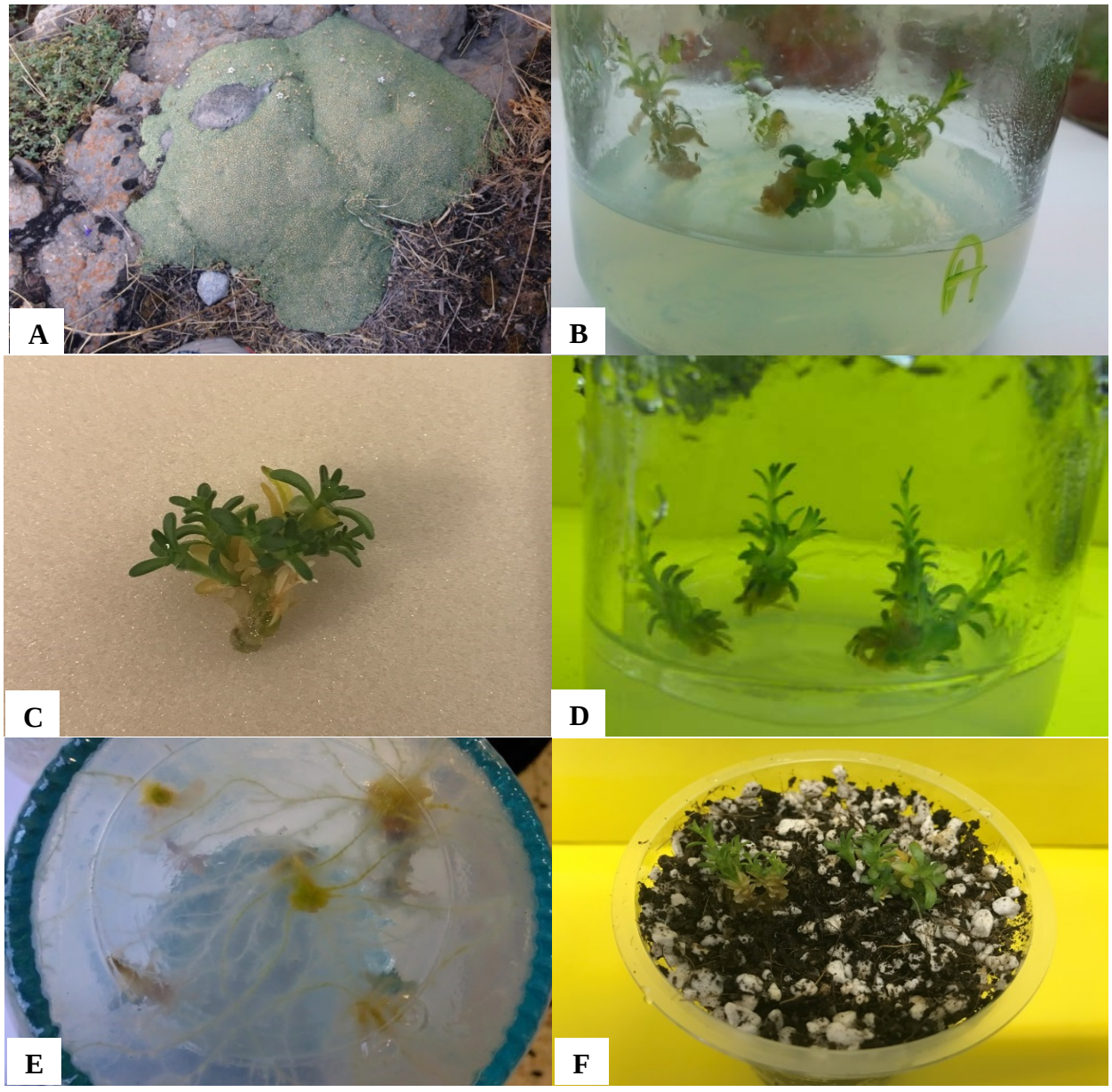

Fig. 1. Plant regeneration from shoot tip explants of *Gypsophila aretioides*; a) mature plant in the nature; b) elongated shoots in control medium; c) shoot proliferation in MS medium containing 0.05 mg dm^{-3} TDZ; d) shoot proliferation in MS medium containing 0.5 mg dm^{-3} BAP; e) root regeneration in medium supplemented with 0.3 mg dm^{-3} IBA; f) slow growth acclimatized plantlets

Table 1. Effects of cytokinins on *in vitro* plant development of *Gypsophila aretioides*

Cytokinins (mg dm^{-3})		Shoot number	Shoot length (cm)	Leaf number
Control		1.33c*	1.45ab	7.84c
BA	0.5	3.58bc	1.10ab	19.16abc
	1	2.08c	0.92ab	9.75c
	2	7.75ab	1.40ab	25.66ab
	4	3.50bc	1.35ab	16.66bc
TDZ	0.05	9.75a	1.43ab	42.33a
	0.1	3.5bc	1.59a	20.41abc
	0.5	3.00c	1.41ab	14.66bc
	1	2.33c	0.71b	11.75c

* Values followed by the same letters in each column are not statistically different (P = 0.05) using Tukey HSD test

Root induction. IBA at 0.6 mg dm^{-3} was the most effective treatment in root induction as the shoots produced the highest root number (7.85) compared to the control that generated 3.6 roots per explant (Fig. 2). IBA at higher concentrations resulted in a pronounced reduction in root number (1 root per explant). Similarly, the highest concentration of NAA (0.9 mg dm^{-3}) led to the reduced root number per explant. Combination of IBA and NAA (0.3 mg dm^{-3} + 0.3 mg dm^{-3}) was not effective in root regeneration compared to each phytohormone solely. As illustrated in Fig. 1, the longest roots (4.711 cm) were achieved in the medium containing 0.3 mg dm^{-3} IBA in comparison with control (1.43).

Acclimatization. Regenerated plantlets showed limited growth during acclimatization. After 3 weeks of transfer to *ex vitro* conditions, the majority of plantlets died irrespective of the auxin treatments in rooting stage. Only three percent of plants survived, which still showed a slow growth compared to *in vitro* conditions.

DISCUSSION

In our study, TDZ exhibited the maximum shoot induction in shoot tip explants of *G. aretioides* compared to BA. This finding supports previous researches on micropropagation of other species of *Gypsophila* [Ahroni et al 1997, Zuker et al 1997]. In contrast, Kiani et al [2013] found TDZ less effective compared to BAP during *in vitro* clonal propagation of *Diaphanoptera khorassanica*, an endemic potential ornamental species from *Caryophyllaceae* family. TDZ has been widely reported to be effective in shoot proliferation in several woody plant species [Ahmad and Anis 2007, Meiners et al. 2007, Singh et al. 2016], however, there are many reports indicating the effectiveness of TDZ in herbaceous plants, as well [Shaik et al. 2009, Nhut et al. 2010, Ahmad et al. 2013, Padmanabhan et al 2015]. As an effective bioregulant in tissue culture studies, TDZ influences the number of morphological, physiological and biochemical processes in plants [Huetteman and Preece 1993]. It has a cytokinin-like activity, which may influence the endogenous cytokinins by suppressing the cytokinin oxidase and this process is highly correspondent to TDZ concentration [Hare and Van Staden 1994].

Relatively low concentrations of TDZ compared to BAP were applied in the current experiment. Huettemman and Preece [1993] proposed that the use of lower TDZ levels compared to amino purine cytokinins (like BAP) were favorable in micropropagation studies as excessive callus formation and inhibition of shoot growth might be the result of exposure of explants to higher TDZ concentrations in medium. Correspondingly, our findings supported this statement as the reduced shoot length obtained in the presence of elevated TDZ. Similar results were also indicated in several studies that reported the stunted shoot in media containing high TDZ concentrations. [Huetteman and Preece 1993, Parveen and Shahzad 2010, Ahmad et al. 2013].

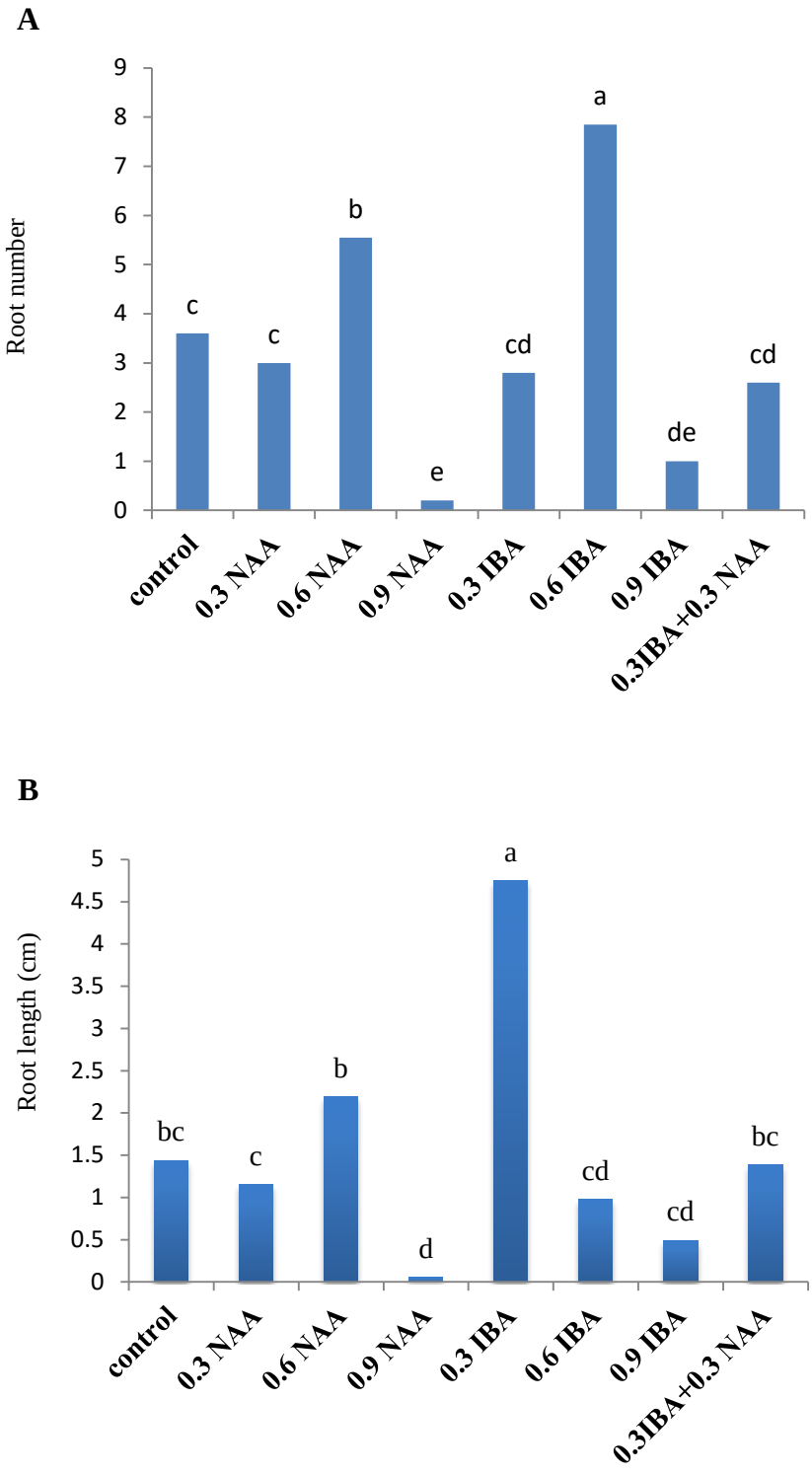

Fig. 2. Effect of auxins on root induction in *Gypsophila aretioides*: a) root number b) root length

IBA at relatively low concentration (0.3 mg dm^{-3}) resulted in the longest root in *G. aretioides*. This finding is in line with reports of Zuker et al. [1997], who found the benefit of lower IBA concentrations for root elongation of *G. paniculata*. In contrary, Rady [2006] demonstrated that IBA at much higher concentrations (3 mg dm^{-3}) were effective in generating the longest root in the same species.

G. aretioides showed low survival rate when the regenerated plantlets were transferred to *ex vitro* conditions. Low *ex vitro* acclimatization is reported frequently as one of the main issues that hinder the success of plant micropropagation [Desjardin et al. 2009, Chandra et al. 2010, Goncalves et al. 2017]. This is associated with the inability of plants to cope with the stress conditions, namely low relative humidity and high irradiance after transplantation, which contribute to water stress and photo-inhibition [Hazarika 2003]. Incomplete development of stomata and cuticular wax on leaves, high stomatal conductance, low water potential of plants [Posposilova et al. 1999], frequent water loss, low photosynthetic potential of the *in vitro* formed leaves [Dewir et al. 2014] and the inability to establish the vascular connection on root to shoot interface [Smith et al. 1991], are the main reasons involved in low acclimatization rate of the *in vitro* plantlets. In our experiment, the new root growth was observed approximately 10 days after transplantation, which facilitated the absorbance of water and nutrient elements for plants. This indicates that other factors, rather than low root-shoot vascular connection, might be responsible for low survival rate of *G. aretioides* plantlets *ex vitro*. Hence, frequent water loss through high stomatal conductance or absence of cuticle layers could be the possible causes of low acclimatization of *G. aretioides* plantlets. This is consistent with findings of Daniels [1994], who indicated that lack of stomatal functionality is accounted for the low survival rate of *in vitro* regenerated plantlets of some *Dionysia* species (*Caryophyllaceae*) in *ex vitro* condition.

CONCLUSIONS

In the present study, we were able to propose a suitable method for sterilization of *G. aretioides* explants that were directly collected from the nature, then a high *in vitro* shoot multiplication and root regeneration rates were obtained. However, we were not succeeded in *ex vitro* adaptation of *G. aretioides*. This step requires particular consideration for *G. aretioides* and complimentary studies are performing to surmount the problem.

ACKNOWLEDGEMENTS

This work was financially supported by a grant from Ferdowsi University of Mashhad (No. 42949).

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