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In-vitro Mass Propagation of *Limonium sinuatum* L. Mill. (Statice)

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Abstract

Limonium sinuatum* L. Mill. (Statice), a rosulate plant with showy inflorescences is characterized by its high ornamental value as a cut flower for both fresh and dry-flower arrangements with its increasing demand in the international markets. Nepalese farmers are dependent on Indian market for ornamental plants with a huge investment but are not able to get quality plants. This research work is focused to develop the protocol for *in vitro* mass propagation of Statice so as to provide the farmers with quality plants. Shoot tips were excised from the mother plant and were surface sterilized with freshly prepared 0.1% w/v aqueous solution of $HgCl_2$ for 5 minutes. The explants were cultured in Murashige & Shook (1962) medium supplemented with different concentrations and combinations of BAP, KIN, NAA and IBA. During *in vitro* establishment of explants, concentration of BAP at 1.0 mg/L and NAA at 0.1 mg/L gave the best results of shoot induction and proliferation. About 94% of explants were established on MS medium enriched with BAP and NAA. Rooting was best induced on MS medium containing NAA 1mg/L. Thick and fibrous roots were developed within 30-35 days. Regenerated plantlets were acclimatized for 10 to 15 days in greenhouse at 20±5ºC. Plantlets were successfully established in sterile sand and were transferred into poly bag containing a mixture of garden soil, organic matter and sand in 1:1:1 ratio. Plantlets survival percentage was 100% in open field condition.

Keywords: *Acclimatization, Explants, Micropropagation, Murashige & Skoog (MS), Tissue culture*

Introduction

Limonium sinuatum L. Mill. (Statice), a beautiful ornamental plant commonly known as "sea lavender" which belongs to Plumbaginaceae family (Kunitake et al., 1995), comprises 150 wild species (Morgan et al., 1998; Aly et al., 2002; Lledo et al., 2003). *L. sinuatum,* a perennial herb is native to the eastern Mediterranean are grown commercially around the world as a cut flower for both fresh and dry-flower arrangements (Cohen et al., 1995). There are about 15 to 20 horticulturally cultivated species, involving *L. sinuatum*, *L. bonduelli*, *L. dregeanum*, *L. sinense*, *L. latifolium*, *L. psylliostachys*, *L. bellidifolium*, *L. gmelinii* and *L. perezii* (Jeong et al., 2001). These species were grown in borders and rock gardens in European countries, a decade ago. Gradually, they have been produced as a cut flower in Japan and the Netherlands with an advancement of mass-propagation techniques using plant tissue culture (Kunitake, et al., 1995; Rout et al., 2006; Bose et al., 2017). Statice has become a popular ornamental flower crop in recent years and are highly stress-tolerant angiosperm (Aly et al., 2002). The excellent agronomic character such as flower color, vigor and long-lasting quality makes it an ideal flower in floriculture industry. Growers are involved in hybridization with breeding efforts, extending the variations in color and shape of flower (Henny & Chen, 2004). Among the cut flower grown in the country, Statice is currently in high demand by new investors for a large scale production owing to its easy plant care requirement and good selling price in the auction market (Mellesse et al., 2013). Ethiopian Statice flower exported to the global market have increased five-fold between 2006 and 2008. In 2008 alone, Ethiopia earned 114 million dollars from the floriculture industry (Mellesse et al., 2013). In context of Nepal, with the increasing number of nurseries, the number of floriculture shops/retailers are also increasing. The floriculture businesses are growing by 10-15% per year. The floriculture sector has already fetched investment over NRs. 375 million in infrastructure and planting materials. Nepal has already started exporting floral products just a few years back such as cut flowers

and flower buds suitable for bouquets or for ornamental purposes, dried, dyed, bleached, impregnated to India, USA, Japan, the Netherlands, Norway, Australia, Taiwan, Italy, Germany and some of the Gulf countries (Gauchen et al., 2009). Nepal is giving hand on bulb, tubers, tuberous roots, corms, crowns and rhimzomes, in growth or in flower, chicory plants and roots, unrooted cuttings and slips (Thapa & Dhimal, 2017). Nepal is mainly dealing with cut flower crops including Roses (*Rosa hybrida*), Gypsophila or Baby's Breath (*Gypsophila paniculata*), Carnations (*Dianthus caryophyllus*) and Chrysanthemum (*Chrysanthemum* spp.) (Gauchen et al., 2009). Among them, Statice (*Limonium* spp.) is widely commercially produced cut flower grown in high altitude (Getu, 2009). It is one of the most popular cut flowers used both as cut flower and potted plant. With the increasing number of horticulture business in Nepal, the demand for Statice plants has also increased (Gauchan et al., 2009, Thapa & Dhimal, 2017). Farmers are dependent on Indian market for plants with heavy investment but are not able to get quality plants. This research is focused in developing the protocol for *in vitro* mass propagation of Statice that could overcome the problem by producing quality plants.

Materials and Methods

Plant material collection

The plant materials were collected from Bhaisipati, Kathmandu on April, 2018, cultivated in green house of local farmer.

Surface Sterilization

Shoot tips were excised from the mother plant and were washed under running tap water for about 1 hour with few drops of liquid detergent Tween 20. The explants were thoroughly rinsed with distilled water for 4-5 times The explants were surface sterilized with freshly prepared 0.1% w/v aqueous solution of $HgCl_2$ for 4, 4.5, 5, 5.5 and 6 minutes respectively to standardize the appropriate time and were thoroughly rinsed for 4-5 times with sterilized water.

Culture media and inoculation of explants

Shoots (clumps each having two to three leafy microshoots) were inoculated in MS basal medium supplemented with different concentration of plant growth regulator (BAP+NAA, KIN+NAA, BAP+IBA and KIN+IBA) in various combination and concentration. Sucrose 3% were used as a carbon sources and media was adjusted to pH 5.8 using sodium hydroxide before autoclaving. Agar, plant tissue culture grade, Merck (0.8%) were used to solidify the media and was autoclaved at 121°C for 15 minutes. The cultures were incubated at 16 h photoperiod with light intensity of 3000 lux using fluorescent tube lights and temperature of 25± 2°C for 4 weeks (Rana et al., 2018).

In-vitro shoots proliferation

After successful initiation of the shoot, newly formed shoots were excised and sub-cultured on the MS medium supplemented with different hormonal concentration: BAP (0.5mg/L, 1.0mg/L, 2.0mg/L, 2.5mg/L, 3.0mg/L) and Kinetin (0.5mg/L, 1.0mg/L, 2.0mg/L, 2.5mg/L, 3.0mg/L) with 0.1 mg/L NAA as well as BAP (0.5mg/L, 1.0mg/L, 2.0mg/L, 2.5mg/L, 3.0mg/L), Kinetin (0.5mg/L, 1.0mg/L, 2.0mg/L, 2.5mg/L, 3.0mg/L) with 0.1 mg/L IBA. For each treatment, 15 explants were used and each experiment were repeated three times.

In-vitro rooting of microshoots of Statice

MS medium with either NAA or IBA or IAA had profound effect on inducing early rooting. *In-vitro* rooting of micro shoots of Statice were observed on MS medium supplemented with different concentration of auxins: IBA (1.0 mg/L and 0.5 mg/L), NAA (1.0 mg/L and 0.5mg/L), IAA (1.0mg/L and 0.5mg/L), according to Echeverrigaray et al., 2005. Among MS medium supplemented with auxins IBA, IAA or NAA, the prominent root growth was recorded on MS medium supplemented with 1 mg/L NAA.

Sand rooting and Hardening

After 4 to 5 successive *in vitro* shoot proliferation of explants, the cultured bottles were moved to green

house for 10 to 15 days for acclimatization. The plantlets were removed from bottles with the help of sterile forceps and washed with distilled water to remove media from the plantlets. Then, the plantlets were inserted into sand trays and covered with polythene hood to maintain moisture. The temperature and humidity of the greenhouse was maintained at 20±5°C and 80% respectively. Plants were assessed for rooting at 3-4 weeks. After six weeks of transplantation, rooted plantlets were transferred to nursery polybags containing garden soil, organic matter and sand (1:1:1).

Results and Discussion

Surface sterilization

As we know tissue culture techniques are often considered to be free from microorganisms, bacterial contamination has been a problem for both research and commercial production of plant tissue cultures (Idowu et al., 2009). Endophytic bacteria are especially troublesome because these microorganisms cannot be eliminated by external sterilization (Pierik, 1988), and their presence in cultured plantlets frequently can be recognized only after prolonged subculture (Leifert et al., 1991; Reed et al., 1995). Since internal infection by bacteria may cause poor growth and a decrease in the proliferation rate of cultured plantlets (Pierik, 1988), it was considered that leaf-tip necrosis of statice plantlets might be associated with endophytic bacteria (Liu et al., 2005). Therefore, in order to reduce such problems, in this study, we have described the micropropagation of the statice plant using tissue culture technique in four stages. Firstly, shoot tips explants are taken from a mother plant, sterilized and aseptically cultured in vitro to establish culture. Secondly, the microshoots are grown to form a clump of shoots which are further subdivided into several small clumps and is propagated for several generations in a multiplying medium at the ''multiplication stage.'' Thirdly, an individual shoot from the clump is placed in a rooting medium. The rooted plantlets are transferred to greenhouse for acclimatization and finally, were transplanted into the field.

Surface sterilization of explants were done using 0.1% concentration of $HgCl_2$. Treatment time varied from 4 to 6 minutes. Surface sterilization by 0.1% $HgCl_2$ at 5 minutes was found to be suitable for Statice.

The effect of growth hormone on the shoot multiplication

The addition of sugar in the medium as a carbon source is a requisite for plant growth. Cytokinins such as 6-benzylaminopurine (BA), kinetin (KN) are a class of plant hormones which plays an essential role in plant morphogenesis and influences on the formation of shoots and their relative growth rate (Debi et al., 2005). This study is also concern to investigate the effects of BA concentration, the type of supporting material as well as other hormones on growth and multiplication of Statice plantlets cultured and to assess the possibility of shoot multiplication of Statice plantlets grown on MS medium. The effect of the different concentration of BAP and NAA is shown in table 2. The efficacy of combination of growth hormones was assessed based on number of shoots and the height of the shoot induced after inoculation. Shoot formation on explants cultured on MS medium supplemented with BAP 1.0 mg/mL + NAA 0.1 mg/L gave rise to luxuriantly growing shoots within 2-3 weeks. Number of shoots/explant was 21.8±0.663 and

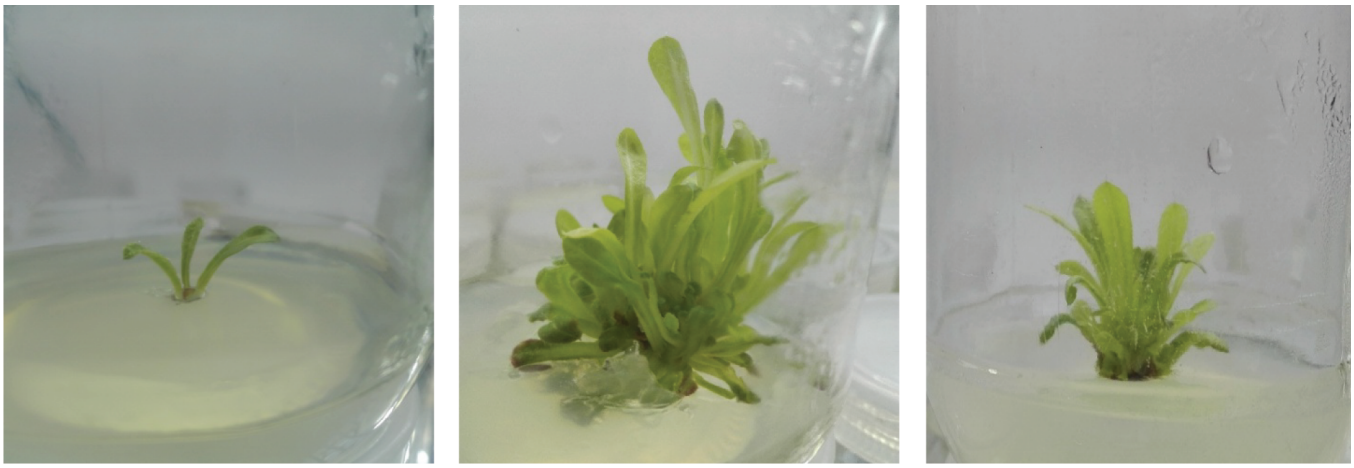
Table 1: Results of sterilization of Statice shoots using $HgCl_2$

Concentration	Treatment of time (minute)	Number of shoot treated	Number of browning shoot	Number of aseptic shoot	Number of contaminated shoot
0.1%	4	7	-	-	7
	4.5	7	-	1	6
	5	7	-	7	-
	5.5	7	2	5	-
	6	7	7	-	-

Table 2: The effect of growth hormones (BAP+NAA) on the shoot multiplication

S.N.	BAP+NAA mg/L	No. of culture bottle	No. of weeks for shoot proliferation	No. of shoot/ explants*	Average height of shoot (cm)*
1	0.5+0.1	5	6-8	17 ±1.224	6.74 ±0.132
2	1.0+0.1	5	6-8	21.8±0.663	7.4 ±0.040
3	1.5+0.1	5	6-8	15.8 ±1.157	6.36 ±0.128
4	2.0+0.1	5	6-8	13.4 ±1.886	7.04 ±0.050
5	2.5+0.1	5	6-8	15 ±1.581	6.7 ±0.126

* Average number of shoot and average height of shoots were given as mean±standard error



(A) (B) (C)

Figure 1: (A) Explant establishment on MS medium, (B) & (C) Induction of Statice shoots on MS medium supplemented with 1mg/L concentration of BAP and 0.1 mg/L NAA at 16 hrs. photoperiod with light intensity of 3000 lux and temperature of 25± 2°C for 4 weeks (A) and 8 weeks (B)

maximum average height of shoots was 7.4±0.040 cm as shown in table 2. MS medium supplemented with BAP 2.5mg/L+0.1mg/L IBA gave rise to alternatively high number of shoots 21.2 ±0.374 but considerably shorter height of shoots 5.7±0.141 as shown in table 4.

Data represented in table 2, 3, 4 and 5 showed that MS media with different concentration of BAP (0.5mg/L, 1.0mg/L, 2.0mg/L, 2.5mg/L, 3.0mg/L) and Kinetin (0.5mg/L, 1.0mg/L, 2.0mg/L, 2.5mg/L, 3.0mg/L) with 0.1 mg/L NAA as well as BAP (0.5mg/L, 1.0mg/L, 2.0mg/L, 2.5mg/L, 3.0mg/L), Kinetin (0.5mg/L, 1.0mg/L, 2.0mg/L, 2.5mg/L, 3.0mg/L) with 0.1 mg/L IBA. This variation may have resulted from the different requirements of shoot proliferation in lab such as light intensity and temperature given, pH measurement of media etc. Moreover, differences in stimulation of shoot proliferation may be related to differences in macronutrients (Matt & Jehle, 2005, Liu & Pijut, 2008, Ruzic &Vujovic, 2008). Increase and decrease in the concentration of BAP and Kinetin along with variation of IBA and NAA on MS medium is essence to observe.

Table 3: The effect of growth hormones (KIN+NAA) on the shoot multiplication

S.N.	KIN+NAA mg/L	No. of culture bottle	No. of weeks for shoot proliferation	No. of shoot/ explants*	Average height of shoot (cm)*
1	0.5+0.1	5	6-8	15.4 ±0.400	5.84 ±0.074
2	1.0+0.1	5	6-8	16.8 ±0.583	5.64 ±0.146
3	1.5+0.1	5	6-8	19.2±0.489	6.38 ±0.058
4	2.0+0.1	5	6-8	19 ± 0.547	6.28 ±0.058
5	2.5+0.1	5	6-8	20 ±0.547	6.36 ±0.092

* Average number of shoot and average height of shoots were given as mean±standard error

* Culture condition: 16 hrs. photoperiod with light intensity of 3000 lux and temperature of 25± 2°C for 8 weeks.

Table 4: The effect of growth hormones (BAP+IBA) on the shoot multiplication

S.N.	BAP+IBA mg/L	No. of culture bottle	No. of weeks for shoot proliferation	No. of shoot/ explants*	Average height of shoot (cm)*
1	0.5+0.1	5	6-8	16.4±0.812	5.2±0.070
2	1.0+0.1	5	6-8	17.4±0.400	5.62±0.162
3	1.5+0.1	5	6-8	19.4±0.400	5.82±0.111
4	2.0+0.1	5	6-8	19.4±0.400	5.98±0.66
5	2.5+0.1	5	6-8	21.2 ±0.374	5.7±0.141

* Average number of shoot and average height of shoots were given as mean±standard error
* Culture condition: 16 hrs photoperiod with light intensity of 3000 lux and temperature of 25± 2°C for 8 weeks.

Table 5: The effect of growth hormones (KIN+IBA) on the shoot multiplication

S.N.	KIN+IBA mg/L	No. of culture bottle	No. of weeks for shoot proliferation	No. of shoot/ explants*	Average height of shoot (cm)*
1	0.5+0.1	5	6-8	11.8±0.800	5.44±0.169
2	1.0+0.1	5	6-8	14.4±0.400	5.64±0.146
3	1.5+0.1	5	6-8	16.6±0.509	6.16±0.120
4	2.0+0.1	5	6-8	19.2±0.583	5.86±0.120
5	2.5+0.1	5	6-8	20±0.547	6.14±0.150

* Average number of shoot and average height of shoots were given as mean±standard error
* Culture condition: 16 hrs. photoperiod with light intensity of 3000 lux and temperature of 25± 2°C for 8s weeks.

Rooting of microshoots of Statice and survival of plantlets in polybag

Induction of healthy root system from the regenerated shoots is an essential part of successful development of plantlets. The concentration and source of auxin (IBA/IAA/NAA) also has a significant influence on root initiation and development. Here, for root induction, regenerated shoots were cultured on MS medium supplemented with different concentration of IBA, IAA and NAA according to Echeverrigaray et al., 2005. MS medium enriched with NAA 1mg/L shows 100% of shoot forming roots. Roots were initiated at 15-20 days and were developed into thick and fibrous within 30-35 days as shown in figure 2 (A). Among MS medium supplemented with auxins IBA, IAA or NAA, the good root growth was recorded on MS medium supplemented with 1 mg/L NAA in table 6.

Plantlets were acclimatized for 10 to 15 days in greenhouse at 20±5ºC. Plantlets with established roots were successfully established in sterile sand (98%) in figure 2 (B & C) and were transferred in poly bag containing a mixture of garden soil, organic matter and sand in 1:1:1 ratio in figure 3 (A & B). After 2 months, field trial of plantlets was done in the garden of Department of Plant Resources in figure 3 (C). Plantlets survival percentage was 100% in open field condition. For recommendation, routine observation of the environmental condition should be noted until inflorescence.

Table 6: Effect of different auxins on root formation in Statice

Growth regulators (mg/L)	% of shoots forming roots	Days to root initiation	Days to root development	Nature of the roots
IBA(0.5)	55	20-25	40-45	Thick &fibrous
IBA0(1)	65	20-25	40-45	Thick &fibrous
IAA(0.5)	35	20-25	40-45	Thick &fibrous
IAA(1)	45	20-25	40-45	Thick &fibrous
NAA(0.5)	65	15-20	30-35	Thick &fibrous
NAA(1)	100	15-20	30-35	Thick &fibrous

(A) (B) (C)

Figure 2: Hardening, acclimatization and sand rooting of plantlets (A) The roots of statice shoots cultured on MS medium supplemented with NAA (1mg/L), (B) Statice plantlets transferred to the sand, (C) Statice plantlets with developed roots.

(A) (B) (C)

Figure 3: Statice plantlets transferred on polybags after 2 weeks (A) Plantlets of Statice after 4 weeks at 20±5°C temp and 80% humidity (B) Plantlets of Statice after 2 months at 20±5°C temp and 80% humidity, (C) Open field trial on the garden of department of plant resources.

Conclusion

The sterilization of shoot tips with 0.1% concentration of $HgCl_2$ for 5 minutes was the ideal condition for the surface sterilization of the explant. MS medium supplemented with 1.0 mg/L BAP plus 0.1 mg/L NAA, was suitable condition for maximum number of shoot proliferation. Tissue culture technique of the Statice was established (94%) on MS medium enriched with BAP and NAA. Thick and fibrous roots were observed on MS medium enriched with NAA 1mg/L within 30-35 days. Plantlets were successfully established in sterile sand and were adapted ex vitro with surviving rate up to 98% in greenhouse at 20±5ºC. Plantlets were successfully grown in the garden of Department of Plant Resources and the survival percentage was 100%.

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