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Micropropagation of Safed Musli (*Chlorophytum borivilianum)* - an Endangered Medicinal Herb

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Abstract

The explants were inoculated on different concentration of auxin and cytokinins. The shoot buds were cultured on Murashigae and Skoog (MS) media supplemented with actual concentration of BAP (1.0-4.0 mg/l) and Kinetin (1.0-3.0 mg/l). The better response was achieved in MS medium containing 3.0 mg/l BAP resulting more number of shoots (18.4) as well as high shoot length (6.5 cm). The high and intense response of root proliferation was observed on full strength of MS medium at second and fourth week respectively after inoculation with 2.0 to 3.0 mg/l (IBA) and 2.0 mg/l (BAP). The C. borivilianum plantlets survived better after acclimatization under growth chamber condition and then transfer to field. The plantlets obtained which were similar to the mother plants. These plantlets will be easily provide to farmer and used for plantation in the field.

***Key words**: Chlorophytum borivilianum, Micropropagation.*

Introduction

Low multiplication rate and longer tuber dormancy are major impediments to the introduction of new cultivars and production of high quality safed musli tubers. Micropropagation serves as a means of clonal propagation of economically important species and as a tool for germplasm conservation. Micropropagation technology is used for production of high-quality disease-free, true-to-type plants[1,5]. This technique is independent of seasonal and other environmental conditions in a comparatively smaller space. Hence, introduction of *in-vitro* micropropagation methods considerably enhances the multiplication rate and reduce the cost of production of disease free plantlets. Micropropagation being a clonal method has the potential for large-scale propagation of elite plants, but there is a risk of getting somaclonal variation.

The current investigation has been taken up to identify better explant type and to standardize the growth regulator concentrations to increase the rate of multiplication to produce disease free propagules of *C. borivilianum.*

Material and Methods

The germplasms of safed musli for micro-propagation of *Chlorophytum borivilianum* were procured from NBPGR, New Delhi.

The stock solutions of macronutrients (31.25 ml), vitamins (5.0 ml) were mixed in the required proportion (Table: 1 A, B, C, D) and made up the volume one liter by adding distilled water. The pH of the medium was adjusted to 5.7 by using either 0.1 N HCl or NaOH prior to autoclaving at 121°C on 15 psi for 20 min.

Table 1A Macronutrients of MS media

S. No.	Constituents	Conc. (mg/l)
1	$NH_4 NO_3$	1650 mg
2	KNO_3	1900 mg
3	$CaCl_2.2H_2O$	440 mg
4	$MgSo_4. 7H_2O$	370 mg
5	KH_2PO_4	170 mg

Table 1B Micronutrients of MS media

S. No.	Constituents	Conc. (mg/l)
1	H_3BO_3	6.2 mg
2	$MnSO_4.4H_2O$	22.3 mg
3	$ZnSO_4.4H_2O$	8.6 mg
4	Kl	0.83 mg
5	$Na_2MoO_4.2H_2O$	0.25 mg
6	$CoCl_2.6H_2O$	0.025 mg
7	$CuSO_4.5H_2O$	0.025 mg

Table 1C Table for Vitamins for MS media

S. No.	Constituents	Conc. (mg/l)
1	Thaimine (HCl)	0.4 mg
2	nicotinic acid	0.5 mg
3	Glycine	2.0 mg
4	Pyrodoxine (HCl)	0.5 mg

Table 1D Iron stocks of MS media

S. No.	Constituents	Conc. (mg/l)
1	Na_2EDTA	37.25 mg
2	$FeSO_4.7H_2O$	27.85 mg

Inoculation of explants

In-vitro cultures were initiated from shoot buds possessing young shoot bases and leaf bases obtained from field-grown plants. Explants were treated with sodium hypochlorite (1-2% v/v) and 2-3 drops of Tween-20 for 5-20 min, and rinsed 3 times with autoclaved distilled water, before inoculation. The surface sterilization of explants were carried out and then the shoot base, stem disc and leaf base were cut with the help of a fine sterile stainless steel scalpel blade and placed in culture tubes (25x150 mm) containing solid MS media and supplemented with required phytohormones. Cultures were maintained at 26 ± 2°C under a 16-hr photoperiod (cool-white fluorescent tubes, 45 µmol m^{-2} s^{-1}) and 80-90% humidity. The each treatment was replicated five times and all experiments were repeated three times.

Induction of shooting and multiplication

The cultures were multiplied by sub culturing on fresh medium every three weeks. For root induction the four week old micro shoot were separated from the mother cultures and transferred to MS media supplemented with different concentrations of BAP (1.0,, 2.0, 3.0, 5.0 mg/l) and Kinetin (1.0, 2.0, 3.0, 5.0 mg/l). There were five replication of each treatment and each experiment was repeated three times. The single factor experiment was arranged in randomized completely block design (RCBD). After every two weeks interval the cultures were sub cultured onto the same fresh media. During subculture, shoots were trimmed at the top leaving only 1.0 cm from the base. These shoots were sub cultured onto fresh medium as the initial explants. The mean number of shoots and mean length of shoots were recorded after three weeks of culture.

Rooting

Multiple shoot generated through the culturing on MS media supplemented with a concentration of cytokines were subjected to rooting on different strength of MS media (MS and ¾ MS) supplemented with IBA (1.0, 2.0, 3.0, 4.0 mg/l). There were twelve replication of each treatment and each experiment was repeated three times. The single factor experiment was arranged in randomized completely block design (RCBD). The number of roots and root length were recorded after four weeks of culture.

Hardening and acclimatization

Four-weeks-old rooted plantlets were taken out of the culture vessel and washed thoroughly with double-distilled water to remove trace of medium without damaging the delicate root system. Rooted shoots were transferred to plastic cups containing autoclaved Perlite and moistened with ¼ MS salt solution and covered with polybags. The

plastic cups were initially kept in culture room for three weeks and subsequently shifted to hardening chamber. After initial stabilization phase, the plastic cups were gradually opened. The plantlets were exposed to reducing humidity gradient (40-50%) under temperature regime of 28±2°C. The plantlets were subsequently transferred to polybags containing soil, farmyard manure and vermicompost in 1:1:1 ratio. Data were recorded on the percentage survival of explants after 15, 30 and 45 days of transplantation.

There were five replicates for each treatment and each experiment was repeated three times. The results were recorded in the form of Mean ± Standard Error (SE) [13 & 14], which was calculated with the help of Standard Deviation (SD).

Results and Discussion

Effect of cytokinins on shoot multiplication and elongation

Shoot buds stayed fresh in BAP supplemented medium till the next subculture and Kn supported the elongation of the explants. Stem disc were inoculated on MS medium supplemented with 3% vitamins, 3% sucrose, 0.8% agar and different concentration of BAP. After three days of inoculation, the effects of cytokinin on various parameters were studied. The effect of different concentrations of BAP was found to be not significant for all the parameters as shown in Table 1. The shoot proliferation was found to be highest in medium containing BAP. Highest mean number of shoots (18.40) and mean shoot length was 6.50 cm was observed in MS medium supplemented with 3.0 mg/l BAP followed by MS medium containing 5.0 mg/l BAP (15.50) shoots. A high response in multiplication rate was also detected from six weeks of culture until eight weeks (Fig. 1). It was reported that the best medium for shoot proliferation of Safed musli was supplemented with 3.0 mg/l BAP which resulted in a mean number of shoots (18.90) after 28 days of inoculation[8,9]. Similar findings were also reported by many others[2,3,6]. According to the previous reports, BAP is better than other cytokinins for shoot initiation and proliferation. In the present study, BAP was also superior to Kn, the mean number of shoots was and mean shoot length (cm) was 12.5 and 5.54 cm, respectively. However, there was no significant difference between BAP and Kn on mean length of shoots while high concentration of Kn produced callusing at the base. It was concluded that MS medium containing 3.0 mg/l BAP was the best for shoot multiplication and elongation[7,10,11].

Effect of Auxin on rooting

Regenerated shoots were transferred to various strengths of MS medium (MS and ¾ MS), supplemented with different concentration of IBA (1.0 mg/l, 2.0 mg/l, 3.0 mg/l and 4.0 mg/l). It was observed that 90% rooting was obtained with all the treatments at all IBA concentration, including the control treatment. IBA showed a significant effect on supplementation in MS medium on the root initiation, proliferation and length (Table 2). IBA @ 3.0 mg/l with ¾ MS strength was found to be the best combination for rooting. For MS medium supplemented with IBA @ 3.0 mg/l, number of roots and root length (2.62 and 3.67 cm) was less as compared to ¾ MS supplemented with 3.0 mg/l IBA, 4.20 and 3.98 cm, respectively. It was reported that, for most species, auxin is required to induce rooting. IAA, NAA and IBA are most commonly used for root induction[4,8]. It was reported that IBA at 1.0 mg/l was the best rooting hormone either alone or in combination with NAA and IAA[8].

Table 2 Effect of cytokinins on multiple shoot proliferation from stem disc explant of *C. borivilianum* after 21 days of inoculation.

Cytokinin concentration (mg/l)		Mean number of shoots*	Mean length of shoots (cm)*
BAP	Kn		
Control		6.50±0.34	2.58±0.25
1.0		11.9±0.46	3.25±0.24
2.0		13.8±0.47	4.09±0.25
3.0		18.4±0.42	6.50±0.19
5.0		15.5±0.14	4.80±0.35
	1.0	12.2±0.35	5.05±0.37
	2.0	12.3±0.25	5.25±0.19
	3.0	12.5±0.55	5.54±0.76
	5.0	11.7±0.13	4.56±0.25

*Values represent means ± SE

Table 3 Effect of various strength of MS and different concentration of auxin (IBA) on root induction of regenerated shoots of *C. borivilianum* after 28 days of culture

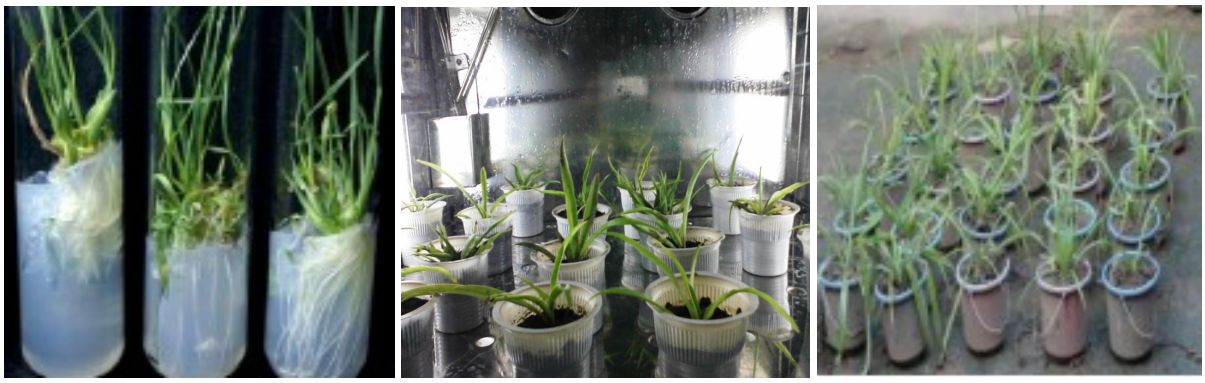
MS strength and IBA concentration (mg/l)	Number of roots*	Root length (cm)*
MS + no IBA	1.26±0.63	1.15±0.06
MS+ IBA 1.0 mg/l	2.10±0.03	2.92±0.04
MS + IBA 2.0 mg/l	2.62±0.01	3.12±0.07
MS + IBA 3.0 mg/l	2.62±0.02	3.67±0.06
MS + IBA 4.0 mg/l	2.52±0.01	3.41±0.05
¾ MS + IBA 1.0 mg/l	2.80±0.02	2.95±0.05
¾ MS + IBA 2.0 mg/l	3.65±o.04	3.52±0.06
¾ MS + IBA 3.0 mg/l	4.20±0.01	3.98±0.02

*Values represent mean ± SE

Hardening and acclimatization

Hardening of tissue culture plants is the most crucial step in micropropagation. The plants produced are very soft to face ambient environmental conditions during acclimatization[4]. Plantlets subjected to *in-vitro* hardening grew vigorously, had bright green and healthy leaves and attained an average height of 10 cm in 15 days in culture room conditions. More than 80 % of the plants survived in the greenhouse and continued to grow either under agro-shadenet or in the open field conditions. Plants with short and thick roots could not sustain growth during hardening (Fig. 1).

A **B** **C**



D E F

Figure 1. *In vitro* micropropagation and plant establishment of *C. borivilianum* (A-B) Shoot induction in sprouted shoot tips of tubers of *C. borivilianum* (C) Rapid proliferation and elongation of *C. borivilianum* shoots (D) Rooted plantlets of C. borivilianum (E-F) Hardening of in vitro raised *C. borivilianum* (after 15 days E and after 3 months F).

Conclusion

In the present investigation, an attempt was made that young stem disc explants are potential for rapid clonal propagation of Safed musli (*C. borivilianum*). The BAP at concentration of 3.0 mg/l was more effective on enhancing shoot proliferation and elongation, whereas IBA at the concentration of 3.0 mg/l with ¾ MS strength was found more effective for rooting. Regenerated plants survived and successfully grew normally in natural environment. This *in-vitro* protocol would provide an effective strategy for the conservation populations of this widely exploited plant species. This protocol also provides disease free and true to type plnats.

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