

Full Length Research Paper

Rapid multiplication of Safed musli (*Chlorophytum borivillianum*) through shoot proliferation

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Accepted 31 May, 2010

Young shoot buds were used as explants for rapid multiplication of Safed musli (*Chlorophytum borivillianum*). The explants were cultured onto medium containing basal salts of Murashige and Skoog (MS) and various concentrations of 6-benzylaminopurine (BAP) and kinetin (KIN) for shoot induction. Treatment containing 3.0 mg/l BAP produced the highest mean number of shoots per explants (18.90) and a mean length of shoots (6.0 cm) after 28 days of culture. Regenerated shoots were successfully rooted on MS medium supplemented with 1.0 mg/l indole-3-butyric acid (IBA) and 30 g/l sucrose. For *ex vitro* establishment, well-rooted plantlets were transferred in potting medium containing vermiculite : organic matters (1:1).

Key words: *Chlorophytum borivillianum*, multiplication, shoot bud, *ex vitro* rooting.

INTRODUCTION

Safed musli (*Chlorophytum borivillianum*) is a traditional medicinal plant which belongs to liliaceae family. This species is valuable for the dried fasciculate storage roots (Nayar and Shastry, 1988) for its aphrodisiac and adaptogenic properties, anti-ageing health restorative effect and use for cure of impotency, sterility and enhance male potency. India has naturally been a huge market for Safed musli due to its medicinal properties. In addition, increasing awareness and appreciation of the goodness on herbal products and the demand for Safed musli has bolstered across the world. Many countries in the Gulf, Europe including USA have been major importers of the dry roots of Safed musli, for its use in the making of various herbal products in pharmaceutical, phytopharmaceutical and nutraceutical industries. At present, according to an

estimate, while the demand for dry Safed musli is in the order of 35,000 tonnes per annum, the supply stands at 5,000 tonnes per year (www.agricare.org, 2005-06). In Malaysia, Safed musli was newly introduced due to its cultivation prospect and medicinal properties. The natural regeneration of this herb is through tuberous roots that have become scarce in nature due to poor seed germination percentage (11 - 24%), low viability and long dormancy period (Rizvi et al., 2007). So to fill the gap of demand and supply and to provide genetically uniform planting material from a known source, tissue culture is one of the most desirable option. Considering that Safed musli is an endangered species and the availability of planting material is scarce, the use of tissue culture technique provides a rapid method to mass produce the plant.

Plant tissue culture system offers a tool for a large scale production of genetically similar plants (Wawrosch et al., 1999) and is used widely for the commercial propagation of a large number of plant species, including many medicinal plants (Rout et al., 2000). *C. borivillianum* has been previously propagated using tissue culture technique. Purohit et al. (1994) used young shoot bases as explants. However, the establishment of cultures free from contamination was difficult because the explants were taken from underground parts like stem disc and

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Abbreviations: BAP, N6-Benzylaminopurine; IBA, indole-3-butyric acid; KIN, kinetin (6-furfurylaminopurine); NAA, α -naphthalene acetic acid; IAA, indoleacetic acid; MS, Murashige and Skoog (1962).

shoot bases. Initial experiments used stem disc which resulted in serious contamination, meanwhile shoot cultures were less contaminated.

The present reports investigated the feasibility of establishing a protocol for *in vitro* propagation of *C. borivilianum* from young shoot bud explants followed by successful *ex vitro* establishment of regenerated plants.

MATERIALS AND METHODS

Establishment of aseptic cultures

Young shoot bud explants of *C. borivilianum* were collected from Lanchang field in Pahang, Malaysia and washed thoroughly under running tap water for 30 min to remove adherent particles, then immersed in 5 g/l (w/v) fungicide for 2 h followed by washing in tap water and rinsed three times with sterilized double-distilled water. Explants were treated with sodium hypochlorite 50% (v/v) in addition with 2 - 3 drops of Tween 20 for 20 min followed by surface disinfecting with 0.1% (w/v) aqueous mercuric chloride solution for 15 min, and finally rinsed three times with sterilized double-distilled water. Shoot buds were inoculated in MS medium supplemented with 30g/l (w/v) sucrose and 3.9 g/l (w/v) gelrite. Shoot buds excised from 28 days old aseptic cultures were used as explants.

Culture media and conditions

MS medium (Murashige and Skoog, 1962) supplemented with 30 g/l (w/v) sucrose and 3.9 g/l (w/v) gelrite was used for the experiments. The pH of the medium was adjusted to 5.8 by 1 N NaOH. The culture vials containing media were autoclaved at 121°C at 106 kPa for 20 min. All cultures were maintained at 25 ± 2°C under 16 h photoperiod with fluorescence lighting. The light intensity was 13.5 – 18 $\mu\text{molm}^{-2}\text{s}^{-2}$.

Shoot induction and multiplication

For multiple shoot induction, young shoot bud explants were placed on MS medium supplemented with various cytokinins (BAP and KIN) at different concentration levels (0, 1.0, 3.0, 5.0, 10.0 and 15.0 mg/l). Each treatment was replicated 5 times. Each replication contained 3 sub samples. The single factor experiment was arranged in randomized completely block design (RCBD). Cultures were subcultured onto the same fresh medium at every 2 weeks interval. During subculture, shoots were trimmed at the top leaving only 1 cm from the base. Such shoots were subcultured onto fresh medium as the initial explants. The mean number of shoots and mean length of shoots were recorded after 28 days of culture.

Rooting

Regenerated shoots were transferred to MS medium containing various auxins (0.5 mg/l NAA, 1.0 mg/l IBA and 1.0 mg/l IAA) either alone or in combination. Each treatment was replicated 5 times. Each replication contained 3 sub samples. The single factor experiment was arranged in RCBD. The number of roots and root length were recorded after 6 weeks of culture.

Ex vitro root growth and acclimatization

For *ex vitro*, six weeks old rooted plantlets about ± 4.0 cm long

were washed thoroughly with double-distilled water to remove trace of medium and dipped in 3% fungicide (Benlate) solution. The rooted plantlets were then transferred into small pots which contained sterilized media composition of: (1) vermiculite, (2) sand, (3) organic matters, (4) vermiculite: sand (1:1), (5) sand : organic matters (1:1), (6) vermiculite : organic matters (1:1), and (7) vermiculite : sand : organic matters (1:1:1). Each treatment was replicated 12 times. The single factor experiment was arranged in RCBD. Potted plantlets were covered with transparent polythene plastic bags to ensure high humidity and watered with tap water twice a day. These pots were initially maintained in misting chambers (locally fabricated) and were finally transferred to external environment after 3 weeks. Data were recorded on the percentage survival of explants and shoot length after 5 weeks of *ex vitro* transplantations.

RESULTS AND DISCUSSION

The morphogenetic responses of shoot bud explants to BAP and kinetin are summarized in (Table 1). Within two weeks of culture on the shoot induction medium, new shoots started to proliferate from the young shoot bud. All the concentrations of BAP and Kin facilitated shoot bud differentiation. There was significant different between BAP and Kin for a mean number of shoots produced. The shoot proliferation was found to be highest in medium containing BAP. Highest mean number of shoots (18.90) was formed in MS medium supplemented with 3.0 mg/l BAP followed by MS medium containing 5.0 mg/l BAP (13.50) shoots (Table 1). A high response in multiplication rate was also detected from 6 weeks of culture until 8 weeks (Figure 3A). Purohit et al. (1994) reported that the best medium for shoot proliferation of Safed musli was supplemented with 22.2 μM BA which resulted in a mean number of shoots (11.0) after 21 days of inoculation. Similar results were well documented in *Aloe vera* (Davood and Behzad, 2008) in medium supplemented with 0.5 mg/l BA + 0.5 mg/l NAA. Velayutham et al. (2006) demonstrated that the highest number of shoots was obtained on MS medium with 4.0 μM BAP + 1.0 μM IAA for *Chicories intybus* L. Other studies indicated that BA is more efficient than NAA for shoot proliferation in *A. vera* (Velcheva et al., 2005; Debiassi et al., 2007). According to the literature, BA is better than other cytokinins for shoot initiation and proliferation. Velcheva et al. (2005) concluded that efficient shoot initiation in *A. vera* was observed in media supplemented with BA. Nevertheless, there was no significant difference between BAP and Kin on mean length of shoots while high concentration of Kin produced callusing at the base. It was concluded that MS medium with 3.0 mg/l BAP was the best for shoot proliferation.

Regenerated shoots were transferred to MS medium containing various auxins (0.5 mg/l NAA, 1.0 mg/l IBA and 1.0 mg/l IAA) either alone or in combination. It was observed that 100% rooting was obtained with all auxins treatments at all concentrations, including the control treatment. IBA at 1.0 mg/l was found to be the best rooting hormone either alone or in combination with NAA

Table 1. Effect of cytokinins on multiple shoot proliferation from shoot bud explants of *C. borivilianum* after 28 days of inoculation.

Cytokinin concentration (mg/L)		Mean number of shoots	Mean length of shoots (cm)
BAP	KIN		
Control		6.9±0.34 ^g	2.68±0.28 ^g
1.0		12.5±0.46 ^c	3.28±0.19 ^f
3.0		18.9±0.29 ^a	6.0±0.25 ^a
5.0		13.5±0.42 ^b	4.51±0.32 ^c
10.0		11.8±0.10 ^{de}	4.06±0.32 ^{de}
15.0		11.6±0.14 ^e	3.89±0.59 ^e
	1.0	12.1±0.32 ^{cde}	5.04±0.32 ^{bc}
	3.0	12.3±0.20 ^{cd}	5.54±0.76 ^{ab}
	5.0	11.9±0.11 ^{cde}	4.93±0.50 ^c
	10.0	9.5±0.55 ^f	4.55±0.23 ^{cd}
	15.0	9.2±0.99 ^f	4.06±0.13 ^{de}

Value represent means ± SE. Means followed by same letter within columns are not significantly different ($p = 0.05$) using Duncan multiple range test.

Table 2. Effect of different auxins either alone or in combination on root induction of regenerated shoots of *C. borivilianum* after 6 (weeks) of culture.

Concentration of PGRs (mg/l)			Number of roots	Root length (cm)
NAA	IBA	IAA		
--	--	--	26.20±4.82 ^f	3.90±0.39 ^g
0.5	--	--	47.80±4.71 ^b	5.76±0.26 ^b
--	1.0	--	52.40±4.61 ^a	6.60±0.25 ^a
--	--	1.0	30.60±1.95 ^{ef}	4.76±0.21 ^{de}
0.5	1.0	--	45.60±3.79 ^{bc}	5.20±0.16 ^c
--	1.0	1.0	37.20±1.92 ^d	4.40±0.31 ^{ef}
0.5	1.0	1.0	32.80±3.07 ^{de}	4.14±0.31 ^{fg}
0.5	--	1.0	42.40±2.61 ^c	4.98±0.33 ^{cd}

Value represent means ± SE. Means followed by same letter within columns are not significantly different ($p = 0.05$) using Duncan multiple range test.

and IAA. For most species, auxin is required to induce rooting. NAA and IBA are most commonly used for root induction (Bhojwani and Razdan, 1992). Saurabh et al. (2005) reported that good rooting response was observed when individual regenerated *Asparagus* shoots were inoculated onto MS with 1.48 μM IBA, 3.90 μM ancymidol and 3% sucrose. Hence, similar results are well documented in *Cichorium* (Rehman et al., 2003). The maximum of (52.40) roots were obtained in 1.0 mg/l IBA with an average length of 6.60 cm (Table 2). MS medium supplemented with IBA (1.0 mg/l) produced thick and hair-like secondary roots (Figures 3B and C). Roots in NAA or IAA treatment alone or in combination with IBA were short and produced abundant hair-like secondary roots.

Hardening of tissue culture plant is the most crucial step in micropropagation. The plants produced are very soft to face ambient environmental conditions during acclimatization (Bhojwani and Razdan, 1992). The rooted plantlets were carefully taken from culture flask and transferred to pots containing sterilized vermiculite, sand and organic matters. Sterilized soil minimized the cost of transplantation as documented by several authors (Agretious et al., 1996; Anand et al., 1997). Out of 84 plantlets, 56 successfully survived after 8 weeks of transfer to pots (Figures 3D - F). Among all the media composition treatments, the highest percentage of plantlets survival was observed in vermiculite: organic matters (1:1) with the 83% percentage of survival plants (Figure 1) and 18.69 cm mean number of shoot length (Figure 2).

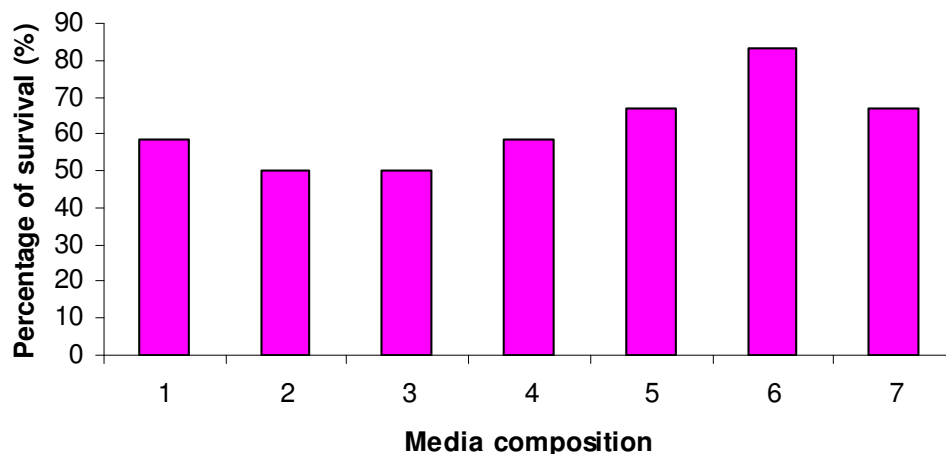


Figure 1. Effect of media composition on percentage of survival (%) of regenerated plantlets. (1) Vermiculite, (2) sand, (3) organic matters, (4) vermiculite : sand (1:1), (5) sand : organic matters (1:1), (6) vermiculite : organic matters (1:1); and (7) vermiculite : sand : organic matters (1:1:1).

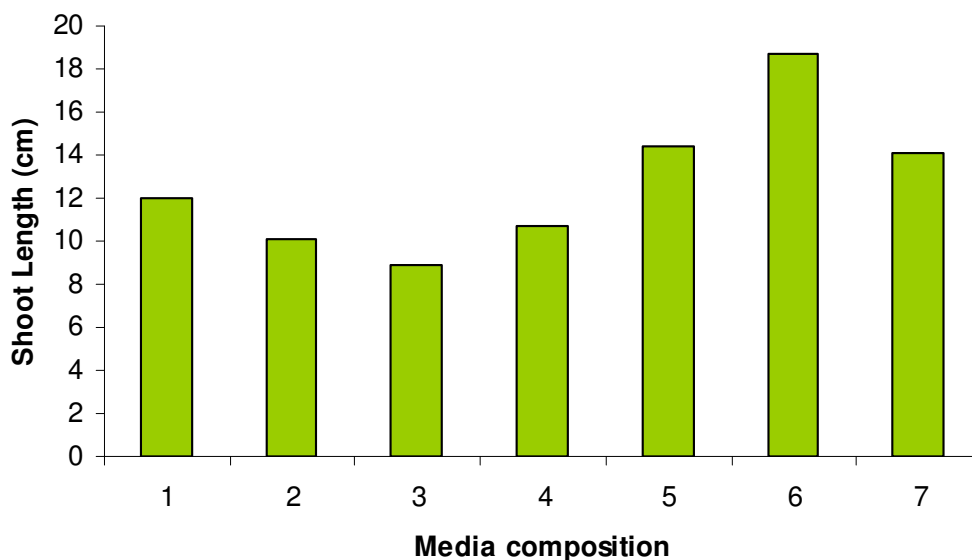


Figure 2. Effect of media composition on shoot length (cm) of regenerated plantlets. (1) vermiculite, (2) sand, (3) organic matters, (4) vermiculite : sand (1:1), (5) sand : organic matters (1:1), (6) vermiculite : organic matters (1:1) and (7) vermiculite : sand : organic matters (1:1:1).

The response may be due to the ability of the media to provide enough moisture for root growth and development. The *in vitro* plants obtained were similar in respect to morphology and growth characteristics as compared to their wild parents.

Conclusion

The present study has proven that young shoot bud explants are potential for *in vitro* mass propagation of Safed musli (*C. borivillianum*). BAP at the concentration of 3 mg/l was more effective on enhancing shoot prolife-

ration and elongation. Regenerated plants survived and successfully grew normally in natural environment. The mass propagation method developed in this study, is therefore suggested to be used for rapid and efficient *in vitro* mass propagation of *C. borivillianum*.

ACKNOWLEDGEMENTS

The authors are grateful to Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra, Malaysia for the facilities and Yayasan Felda, Malaysia for the financial support through research grant.

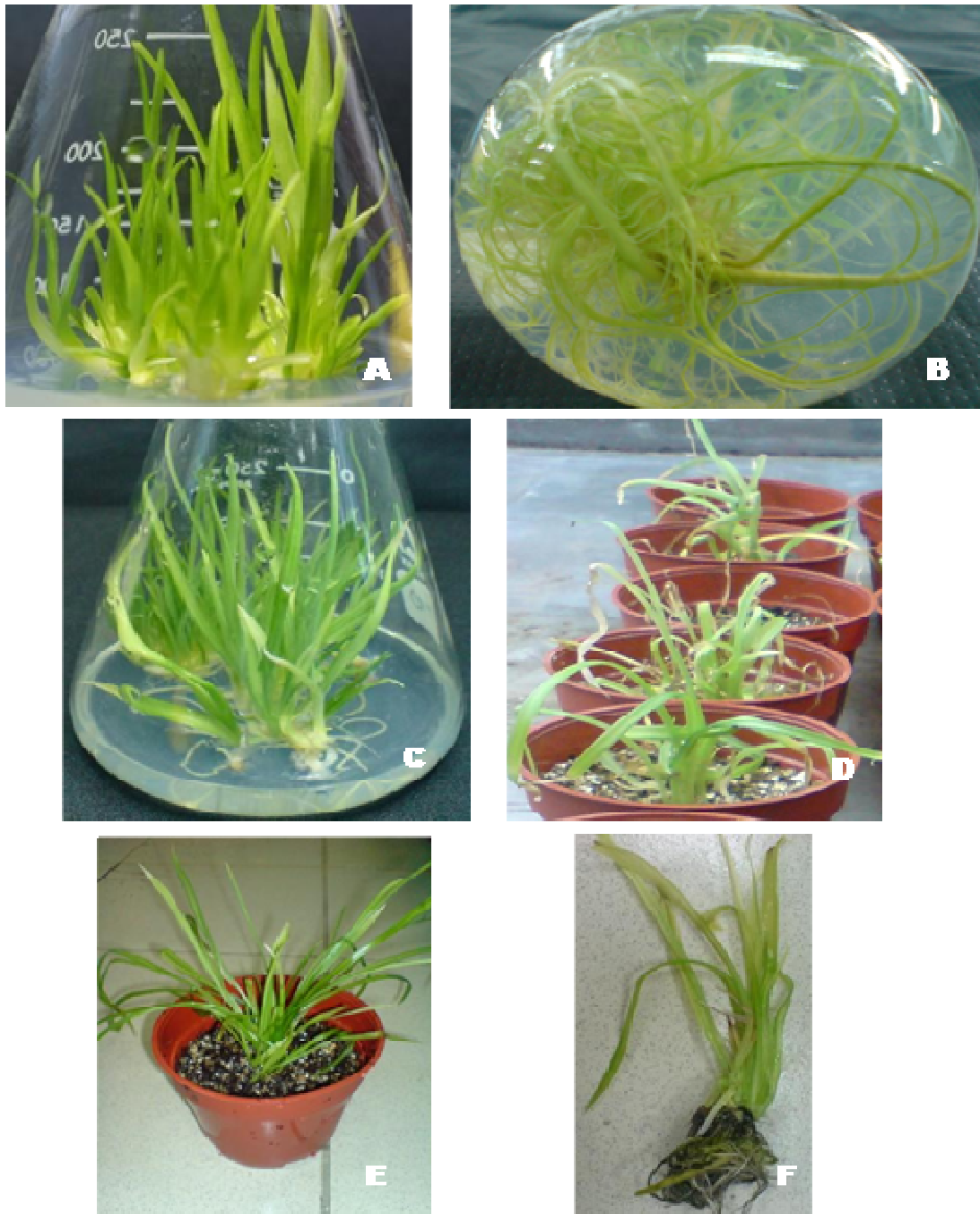


Figure 3. *In vitro* regeneration and plant establishment of *C. borivilianum*. (A) High frequency shoot proliferation and elongation after 56 days (including 4 subculture every 14 days). (B - C) Rooting in regenerated shoots on MS + 1.0 mg/l IBA. (D - E) Establishment plants in soils. (F) *Ex-vitro* rooted plantlets.

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