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In Vitro Regeneration of Drug-Yielding Tuber Crop *Chlorophytum borivilianum*

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ABSTRACT

Chlorophytum borivilianum Sant *et* Fernard., commonly known as safed musli is an important medicinal herb of commercial importance. Tuberous roots of *C. borivilianum* are used as a health tonic, vitalizer, and aphrodisiac and are used as a herbal substitute for chemical-based sildenafil citrate. The natural regeneration of this herb is through tuberous roots that have become scarce in nature because of indiscriminate collection of wild material. Further, poor seed set and germination associated with this crop has added to the problems of propagation. A shoot bud with a part of the stem disc of *C. borivilianum* were cultured on MS (Murashige and Skoog 1962) medium with different concentrations of BAP (6-benzylaminopurine) at 4.54, 5.11 and 5.68 mg l^{-1}, NAA (α-naphthaleneacetic acid) at 0.93, 1.86 and 2.79 mg l^{-1} and hormone-free medium (control). Shoot proliferation was maximum (17.82) with 5.11 mg l^{-1} BAP alone when compared with other treatments. Among the nutrient media screened viz., MS, Gamborg's (B5), Schenk and Hilderbrandt (SH), and White's (Wh), when supplemented with 5.11 mg l^{-1} BAP, MS medium was found to be best followed by SH for mean number and length of shoots. In separate trials, direct regeneration of shoots from the basal leaf sheath following application of thidiazuron at 0.5 mg l^{-1} was obtained, the first report of its kind. In medium with both half and full strength of both macro and micronutrients the mean number of roots per shoot was quite high (34.2 in half strength and 38 in full strength respectively). Reducing the mineral concentration to half the normal strength of MS media supplemented with 2 mg l^{-1} IBA produced a maximum number (29.2) and length (3.58 cm) of roots. Encouraging results were not obtained when different concentrations of NAA and IAA were used for root induction. However, NAA at 3 mg l^{-1} induced a few roots showing reduced root length (0.5 cm); these roots on prolonged incubation in the dark for 75 days resulted in *in vitro* tuberization. Plantlets obtained *in vitro* were best hardened on a vermicompost: sand (1:1) mixture with 70% survival.

Keywords: aphrodisiac, rooting, hardening, micropropagation, shoot proliferation, thidiazuron
Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **BAP**, 6-benzylaminopurine; **FYM**, farm yard manure; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **NAA**, α-naphthaleneacetic acid

INTRODUCTION

Chlorophytum borivilianum Sant et Fernard. (Lilliaceae) and a few other species (*C. arundinacium*, *C. attenuatum*, *C. tuberosum* and *C. breviscapum*) are collectively called under a common trade name safed musli, an important medicinal herb of commercial importance (Bordia *et al*. 1995). The crop is highly valued for its dried fasciculated tuberous roots, which contribute principal ingredients to Ayurvedic, Sidda, Unani and Allopathic systems of medicines. The dried tubers are used as a health tonic, in anti-ageing, and as an aphrodisiac and are used as a herbal alternative to chemical-based sildenafil. In addition, it is valued as a muscular tonic, supposed to be equivalent to the famous tonic plant *Panax ginseng* (Ajay 2003). Major constituents of Safed musli are carbohydrates (42%), proteins (8-9%), root fibers (3-4%), saponins (2-17%) and alkaloids (15-20%). Saponins and alkaloids are considered to be potent medicinal compounds and are found in tubers (Ajay 2003). Steroidal saponins like stigmasterol, tigogenin, neotigogenin and tokorogenin (Tandon and Shukla 1995), glycosides like stigmosterol-β-D glucoside, benzyl glycosides and acylated glycosides are obtained from the tubers of *C. arundiaceanum* (Manjunatha *et al*. 2005). Current annual demand of safed musli roots in India is estimated to be 3500 t while the supply only amounts to 500-600 t (Manjunatha *et al*. 2005). The natural regeneration of this herb is through tuberous roots that have become scarce in nature because of indiscriminate collection of wild material. Further, poor seed set and germination associated with this crop has added to the problem of propagation (Jat and Bordia 1990). The immediate task is to conserve, multiply and distribute to meet the present commercial demand.

The present study describes direct regeneration of plantlets in *Chlorophytum borivilianum*.

MATERIALS AND METHODS

Explant and sterilization

The plant material for the experiment was collected from plants grown in pots maintained in a polyhouse (Indo-American, Bangalore, India). Tubers along with intact stem disc were brought to the laboratory and were washed 8-10 times with tap water until adhered soil was completely removed. Shoot buds attached to tubers were removed along with part of the stem disc measuring 1.5-2 cm and were used as the source of explants (**Fig. 1**). Explants were rinsed thoroughly in running tap water for 30 min. The explants were washed in soap water containing 2-3 drops of a wetting agent (Tween-20; Merck, India.) followed by 0.2% Bavistin® (Indofil Pvt. Ltd., India.) for 20 mins and were rinsed with 2-3 changes in distilled water. Explants were taken to a laminar flow bench and treated with sterilents like mercuric chloride (0.1, 0.2 and 0.3%) in combination with sodium hypochlorite (1, 2 and 3%) for a different duration (5 and 10 min).

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Fig. 1 Tubers with stem disc as explant.

Fig. 2 Shoot proliferation observed with 5.11 mg l^{-1} BAP.

Proliferation

Shoots were cultured on MS media (Murashige and Skoog 1962) supplemented with different concentrations of 6-benzylaminopurine (BAP; Extra pure, Himedia Laboratories Pvt. Ltd., Mumbai, India) (4.54, 5.11 and 5.68 mg l^{-1}) and α-naphthaleneacetic acid (NAA; Extra pure, Himedia Laboratories Pvt. Ltd., Mumbai, India) (0.93, 1.86 and 2.79 mg l^{-1}) and medium devoid of growth regulators (i.e. the control). Thidiazuron at 0.25 and 0.50 mg l^{-1} was also tried using basal leaf sheath attached to stem disc as explant on same media.

Screening of different media

The growth regulator treatment that had resulted in maximum shoot proliferation was used to screen different culture media like MS (Murashige and Skoog 1962), Gamborg's (B5; 1976), White's (White 1963) and Schenk and Hildebrandt (SH; Schenk and Hildebrandt 1972). Of these, MS medium was found to be the best and hence was used throughout the study.

Screening different strengths of media for rooting

Full, ¾ and ½ mineral strength MS basal media was used. Different concentrations of indole-3-butyric acid (IBA; Extra pure, Himedia Laboratories Pvt. Ltd., Mumbai, India) at 1, 2, 3 mg l^{-1} were tested.

Screening different rooting hormones

Auxins such as IBA (1, 2, 3 mg l^{-1}), indole-3-acetic acid (IAA; Extra pure, Himedia laboratories Pvt. Ltd., Mumbai, India) (1, 2, 3 mg l^{-1}) and NAA (1, 2, 3 mg l^{-1}) were used.

Acclimatization of plantlets

In vitro rooted plantlets were removed from the media. Roots were washed until free of agar and transferred to different hardening media. The survival percentage at the end of 20^{th} and 40^{th} day of transfer was recorded.

Experimental design

Experiments were laid out according to a Completely Randomized Design (CRD) as it is a laboratory experiment. The observations generated were subjected to transformation at √X+0.5 as required for the CRD. Values in percentages were subjected to arcsine transformation to ensure homogeneity. The means were compared using Critical Difference (CD) obtained from ANOVA table for CRD.

RESULTS AND DISCUSSION

Effect of different sterilents on percent contamination and survival

Among the treatment combinations, a higher percentage of contamination (86.3) and mortality (86.6) was seen in explants treated with lower concentration of mercuric chloride (0.1%) and sodium hypochlorite (1%) for 5 mins (**Table 1**). As the concentration of mercuric chloride and sodium hypochlorite increased there was progressive reduction in contamination and mortality percentage. A treatment combination of 0.3% mercuric chloride + 3.0% sodium hypochlorite for 10 min resulted in the lowest percentage of contamination (8.66) and mortality (17.6) of treated explants. These results are in conformity with the findings of other workers (Purohit *et al*. 1994; Komalavalli and Rao 2000).

Regeneration of plantlets by direct organogenesis

Sharma and Mohan (2006) used immature floral buds of *C. borivillianum* as explants for induction of callus but used shoots buds with stem disc as explants for direct regeneration. Shoots with part of stem disc cultured on media with BAP produced a significantly higher number of shoots than those with NAA. The maximum number of shoots (17.82) was observed to regenerate from explants cultured on medium with 5.11 mg l^{-1} BAP (**Fig. 2**). The number of shoots regenerated per explant showed a slight reduction (14.40) with an increase in the concentration of BAP to 5.68 mg l^{-1}. Maximum length of shoots (4.71 cm) was recorded at a

Fig. 3 Multiple shoot induction on different culture media. From left to right, media are: Control, White's, Gamborg's, Schenk and Hildebrandt, and Murashige and Skoog.

Table 1 Effect of mercuric chloride and sodium hypochlorite on the establishment of shoot tip attached to stem disc.

Treatments	Contamination (%)	Mortality (%)
0.1% $HgCl_2$ 5 min + 1% $NaOCl_2$ 5 min	86.3 (68.284) [a]	86.6 (68.536) [a]
0.1% $HgCl_2$ 10 min + 1% $NaOCl_2$ 10 min	84.0 (66.164) [b]	76.0 (60.669) [b]
0.2% $HgCl_2$ 5 min + 2% $NaOCl_2$ 5 min	67.3 (55.306) [c]	63.3 (52.714) [c]
0.2% $HgCl_2$ 10 min + 2% $NaOCl_2$ 10 min	45.6 (42.475) [d]	52.0 (46.146) [d]
0.3% $HgCl_2$ 5 min + 3% $NaOCl_2$ 5 min	16.6 (23.958) [e]	18.6 (25.543) [e]
0.3% $HgCl_2$ 10 min + 3% $NaOCl_2$ 10 min	8.7 (17.096) [f]	17.6 (24.577) [f]
Mean	45.547	46.363
SE (±)	0.436	0.405
CD (*P*=0.05)	1.346	1.249

Number of shoots / treatment: 15
* Means superscripted by the same letters do not differ significantly at *P*=0.05
Figures in parentheses indicate transformed values

Table 2 Effect of BAP and NAA individually on shoot proliferation.

Growth regulator	Number of shoots	Length of shoots (cm)
BAP 4.54 mg l^{-1}	14.60 (3.884) [b]	4.71 (2.275) [a]
BAP 5.11 mg l^{-1}	17.82 (4.229) [a]	3.05 (1.871) [b]
BAP 5.68 mg l^{-1}	14.40 (3.858) [b]	3.50 (1.989) [b]
NAA 0.93 mg l^{-1}	4.11 (2.138) [c]	2.31 (1.657) [b]
NAA 1.86 mg l^{-1}	4.44 (2.205) [c]	2.90 (1.830) [b]
NAA 2.79 mg l^{-1}	5.18 (2.377) [c]	3.60 (2.014) [a]
Control	1.0 (1.343) [d]	5.30 (2.420) [a]
Mean	2.869	2.005
± SE	0.107	0.147
CD (*P* = 0.05)	0.324	0.447

Number of shoots/treatment: 30
* Means superscripted by the same letters do not differ significantly at *P*=0.05
Figures in parentheses indicate transformed values

Table 3 Effect of different culture media on shoot characteristics of *Chlorophytum borivilianum.*

Medium	Number of shoots	Length of shoots (cm)
MS	16.00 (4.060) [a]	2.95 (1.844) [b]
SH	9.00 (3.079) [b]	2.85 (1.816) [b]
B5	7.00 (2.734) [c]	3.40 (3.400) [b]
White's	6.00 (2.544) [d]	3.65 (2.027) [a]
Mean	3.104	1.912
SE ±	0.097	0.152
CD (*P*=0.05)	0.317	0.495

Number of shoots/treatment: 30
Means superscripted by the same letters do not differ significantly at *P*=0.05
Figures in parentheses indicate transformed values

Table 4 Effect of different strengths of MS medium on number of roots.

Growth regulator	Strengths of media			
	Full	¾	½	Mean
IBA 1 mg l^{-1}	7.0 (2.734) [c]	7.0 (2.734) [c]	7.6 (2.842) [c]	2.770
IBA 2 mg l^{-1}	31.4 (5.647) [b]	27.0 (5.249) [b]	29.2 (5.449) [b]	5.448
IBA 3 mg l^{-1}	38.0 (6.204) [a]	36.0 (6.041) [a]	34.2 (5.890) [a]	6.045
Control	2.0 (1.558) [d]	3.0 (1.857) [d]	3.0 (1.857) [d]	1.758
Mean	19.60(4.036)	18.25(3.970)	18.50(4.009)	4.004
For comparison of:		**SEM ±**	**CD (P=0.05)**	
Growth regulator treatments		0.052	0.153	
Strengths of media		0.060	0.176	
Growth regulator treatments X Strengths of media		0.105	0.306	

Number of shoots / treatment –30
Means superscripted by the same letters do not differ significantly at *P*=0.05
Figures in parentheses indicate transformed values.

Table 5 Effect of different auxins on root characteristics of plantlets regenerated from shoots.

Growth regulator	Number of roots/shoot	Length of roots (cm)
IBA 1 mg l^{-1}	7.00 (2.734) [c]	3.06 (1.889) [c]
IBA 2 mg l^{-1}	29.40 (5.467) [a]	3.40 (1.974) [b]
IBA 3 mg l^{-1}	31.00 (5.612) [a]	2.94 (1.854) [c]
IAA 1 mg l^{-1}	4.60 (2.251) [d]	2.10 (1.612) [d]
IAA 2 mg l^{-1}	7.10 (2.752) [c]	1.95 (1.565) [d]
IAA 3 mg l^{-1}	9.80 (3.206) [b]	1.80 (1.516) [e]
NAA 1 mg l^{-1}	3.00 (1.857) [e]	0.50 (0.999) [g]
NAA 2 mg l^{-1}	3.80 (2.064) [d]	0.70 (1.094) [f]
NAA 3 mg l^{-1}	4.00 (2.195) [d]	0.90 (1.182) [f]
Control	3.00 (1.857) [e]	3.60 (2.024) [a]
Mean	3.000	1.571
SE ±	0.112	0.020
CD (*P*=0.05)	0.331	0.059

Number of shoots/treatment: 15.
* Means superscribed by the same letters do not differ significantly at *P*=0.05.
Figures in parentheses indicate transformed values.

Table 6 *Ex vitro* survival of plantlets as influenced by hardening media at 20th and 40th day after transfer.

Hardening media	Percentage survival	
	20th day	40th day
Coir pith	40.00 (39.147) [bc]	20.00 (26.070) [c]
Vermi compost	50.00 (45.000) [b]	50.00 (45.000) [a]
Coir pith + sand (1:1)	50.00 (45.000) [b]	30.00 (33.002) [b]
Vermicompost + sand (1:1)	70.00 (56.997) [a]	70.00 (56.997) [a]
FYM + sand+ red earth (1:1:1)	20.00 (26.070) [d]	10.00 (15.000) [c]
Mean	46.00(42.443)	36.00 (35.214)
SE ±	3.616	4.855
CD (*P*=0.05)	11.395	15.298

Number of microcuttings/treatment: 30.
* Means superscribed by the same letters do not differ significantly at *P*=0.05.
FYM = Farm Yard Manure.
Figures in parentheses indicate transformed values.

lower concentration (4.54 mg l^{-1}) of BAP followed by 2.79 mg l^{-1} NAA (3.60 cm) (**Table 2**). An increase in BAP concentration resulted in a drastic reduction in shoot length but gave an increased number of shoots. Shoots were shorter in BAP treatments than in control and NAA treatment. This might be due to suppression of apical dominance of shoots by BAP (Arora *et al*. 1999).

Direct regeneration of shoots was also observed when leaf sheaths attached to the stem disc explants were cultured on medium supplemented with 0.5 mg l^{-1} TDZ. This might be due to close proximity of meristematic cells in the stem disc to the leaf sheath.

Differential response was noticed with respect to the number and length of shoots when different culture media were screened. The number of shoots were maximum (16) on MS with BAP (5.11 mg l^{-1}) followed by SH (9). The least number of shoots (6) was recorded on White's medium (**Fig. 3**). All the media differed significantly with respect to their influence on number of shoots produced (**Table 3**). Reduced shoot numbers (6) and longer shoots (3.65 cm) developed on White's medium. This could be attributed to lankiness exhibited by shoots as a result of reduced nutrient level in White's medium. Since White's medium has a diluted mineral component in the majority of cases this medium is used for rooting studies. The superiority of MS medium for *in vitro* culture of *C. borivilianum* had been reported by several authors (Purohit *et al*. 1994; Arora *et al*. 1999; Pudake and Dhumale 2003; Sharma and Mohan 2006).

In general, media supplemented with full strength mineral nutrients with 3 mg l^{-1} IBA produced more roots (38) per shoot than ¾- (36) and ½-strength (34.2). Reduction in mineral concentration in the media resulted in fewer but longer roots (**Table 4**).

Among the auxins screened for rooting, in general, IBA resulted in maximum number and length of roots compared

Fig. 4 Effect of different concentrations of IBA on rooting. From left to right, in mg l^{-1} IBA: 1, 2, 3.

Fig. 5 *In vitro* tuberization with 3 mg l^{-1} NAA upon prolonged incubation in dark for 75 days.

Fig. 6 Rooted microcuttings being hardened in polyhouse.

Fig. 7 Hardened plants at various stages of development.

to IAA and NAA (**Fig. 4; Table 5**). In contrast, NAA at all concentrations resulted in fewest and shortest roots. The number of roots per shoot (31) was maximum with 3 mg l^{-1} IBA followed by 2 mg l^{-1} IBA (29.4), differing significantly from the remaining treatments, indicating that IBA is a strong rooting hormone. Roots produced with NAA at 3 mg l^{-1} when incubated in the dark for 75 days resulted in *in vitro* tuberization (**Fig. 5**).

Maximum (70%) survival of *in vitro*-derived acclimatized plantlets was recorded on a vermicompost + sand combination hardening medium (**Figs. 6, 7; Table 6**). This may be due to favorable effects of vermicompost as an easily available source of nitrogen, phosphorous and potash (Bano and Suseela Devi 1996). When vermicompost alone and combination of coir pith + sand was used only a 50% survival was noticed.

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