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In vitro Response of Nine Different Genotypes of 'Safed musli' (*Chlorophytum borivilianum*) using Crown Shoot Bud as an Explant

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ABSTRACT

Chlorophytum borivilianum, commonly known as 'Safed musli', is a historic medicinal herb. Explants were surface sterilized using sequential application of carbendazim-50%, streptocycline, kanamycin and 0.1% mercuric chloride. Sprouts were induced on Murashige and Skoog (MS) medium supplemented with 1 mgl^{-1} Benzylaminopurine (BAP) and 1 mgl^{-1} Kinetin. The shoot multiplication was carried out on MS supplemented with 2 mgl^{-1} BAP and 3 mgl^{-1} Kinetin. In vitro rooting was achieved on half strength MS medium supplemented with 2 mgl^{-1} Indole-3-butyric acid (IBA). Maximum number of shoots (12.75 ± 1.32) as well as roots (15.75 ± 1.26) was recorded for Vireshwar genotype while minimum number of shoots (3.63 ± 0.56) and roots (2.88 ± 0.40) was recorded for Pipalakhunt. Hardening of the in vitro rooted plants was achieved in sand, soil and cocopeat mixtures.

Key words: *Chlorophytum borivilianum, Crown shoot bud, Nine genotypes.*

INTRODUCTION

Safed musli is a monocotyledonous plant belongs to family *Liliaceae*. The genus includes more than 300 species, which are distributed throughout the tropical and subtropical parts of the world[4]. The center of origin is in Africa which comprises about 85% of the total species reported in the world. *C. borivilianum* is an endogenous medicinal herb and distributed in various parts of India *viz.* Assam, Eastern Ghats, Himalaya, Bihar, Madhya Pradesh, Rajasthan, Gujarat and Andhra Pradesh[12].

C. borivilianum holds a unique place in Ayurvedic, Unani and Allopathic formulations[22]. Major phytochemical components reported from the roots of *C. borivilianum* includes steroidal saponins, fructans, fructooligosaccharides (FOS), acetylated mannans, phenolic compounds and proteins[17, 23]. Steroidal saponins are considered to be the principal bioactive components responsible for the pharmacological properties in various formulations[13]. Borivilianosides, furostane type steroidal saponins further enrich its medicinal value[22,2,5].

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As per the list proclaimed by the Indian Medicinal Plant Board, *C. borivillanum* has been ranked 26th position among the highly promoted and protected medicinal plants of India[21]. Safed Musli is propagated both by vegetative means using tubers and sexually through seeds. Propagation through vegetative methods has a very slow growth rate and produces a limited number of plants[3], while propagation through seeds poses the problem of low seed viability, poor seed germination (11-24%) and long dormancy period[8]. Variation may also arise due to preferential outcrossing in nature[11]. The annual demand of safed musli roots in India is estimated to be 3500 tonnes as against the supply of 500 – 600 tonnes[10]. Further, due to overexploitation and extensive harvesting of the forest based wild strands, International Union for Conservation of Nature and Natural Resources (IUCN) has declared its status as 'endangered' species[3]. In India safed musli is commercially propagated through tissue culture and the technique offers the advantage of large scale production of disease free high quality planting material. Micropropagation also serves as an important method of *ex situ* conservation for endangered medicinal plant species. Genotype specific response of *in vitro* cultures is the major obstacle in micropropagation and has not been studied so far[9]. The present investigation was mainly focused to study the variation in *in vitro* response of nine different genotypes (Anand Safed Musli-1, Vireshwar, Ambapada, Namangir, Ahwa Dang, Atarsumba, Pipalakhunt, RC-5, MCB-414) collected from different regions of the India (Table 1). The data generated through this study will be helpful in large scale production and conservation of various genotypes of Safed Musli.

MATERIAL AND METHODS

Preparation of the culture medium

MS medium was prepared and dispensed into 80 ×150 mm glass bottles (Borosil, India) using a Masterflex Digi-Static auto dispenser and autoclaved at 121˚C and 15 psi pressure for 15 min. The pH of the medium was adjusted to 5.7 ± 0.01 prior to autoclaving.

Surface sterilization

Nine different genotypes of *C. borivilianum* were collected from different regions of India (Table 1, Figure 1). Roots with crown shoot buds were used as explants. Size of the explants was reduced to 1.5 - 2 cm. The explants were then washed with Tween-20 detergent and surface sterilized with 200 ppm carbendizim-50% for 12 min, 200 ppm streptocycline for 10 min, 200 ppm kanamycin for 8 min and 0.1% mercuric chloride for 8 min.

Initiation and growth condition

Explants of all the genotypes were inoculated on MS medium supplemented with 1 mgl^{-1} BAP, 1 mgl^{-1} kinetin, 3% Sucrose and 0.8% Agar (The media composition for initiation was adapted from the work of Purohit *et. al*[19]. All the cultures were maintained at 25 ± 1 ºC with a relative humidity of 60-70 percentage. The source of illumination consisted of 2.5 feet wide fluroscent tube (40 watt) with the illumination intensity of 3500 lux at the level of cultures. Cultures were subjected to alternate 16 hour photo and 8 hour dark period.

Multiple shoot induction, subculture and rooting

Sprouted cultures of each genotypes were transferred to MS supplemented with 2 mgl^{-1} BAP, 3 mgl^{-1} kinetin, 3% Sucrose and 0.8% Agar for shoot multiplication (The media composition for the multiplication was adapted from the work of Minakshi *et. al*[14]. All the cultures were subcultured and transferred to a fresh medium after every 21 days interval. Explants containing four to five shoots were subcultured on half strength MS supplemented with 2 mgl^{-1} IBA, 3% sucrose and 0.9% agar for *in vitro* root induction (The media composition for root induction was adapted from the work of Purohit *et. al*[19]. Observations on number of sprouts, number of roots and length of roots were recorded for each genotype.

Hardening of plants

The *in vitro* rooted shoots were carefully taken out from the culture vessels and gently washed with sterile distilled water to remove the agar adhered to it. The shoots were then treated with 200 ppm carbendazim-50%, 200 ppm rhidomil and 200 ppm blytox each for 2 minutes followed by rinsing with distilled water. The plantlets were primary hardened into 10 × 9 cm plastic cups containig moist and autoclaved substrates (pot mix-sand: soil: cocopeat,1:1:1). The plants were covered with polythene to maintain high humidity and watered daily to maintain high relative humidity (95%). The plants were gradually weaned to natural conditions and transferred to polyhouse for secondary hardening.

Statistical Analysis

The data obtained from the observations recorded for shoot proliferation and root induction for the nine genotypes were subjected to one way analysis of variance in the excel sheet using completely randomized design. The means were separated by Duncan's Multiple Range Test using DSAASTAT software[18].

RESULTS AND DISCUSSION

The preliminary objective of this study was to establish axenic cultures of various genotypes of *C. Borivillianum*. The major contaminants in the cultures were found to be of fungal origin. A pretreatment with an antifungal agent Carbendazim-50% was found to be effective in reducing overall fungal contamination. Highest and lowest percentage of the axenic culture establishment recorded were 53% and 31% in case of Vireshwar and ASM-1, respectively (Table 2). Seabrook[20] and Purohit *et al*[19] reported that decontamination of underground parts is very difficult and a challenging task. Use of 0.1% mercuric chloride for surface sterilization of explants has no adverse effect on initiation of sprouting in the present investigation. These results are in contradiction with the findings of Mishra *et al*[15]., where they reported death of the leafy explants due to phytotoxicity upon exposure to sterilants like Mercuric chloride.

Initiation

Average sprouting response of all the genotypes was more than 25% in the medium supplemented with 1.0 mgl^{-1} BAP and 1.0 mgl^{-1} kinetin. Purohit *et al*[19] also reported highest mean number of shoots (14) and length of shoots (6.5 cm) on MS medium supplemented with 1 mgl^{-1} BAP and 1 mgl^{-1} kinetin. Maximum sprouting percentage (100.0 ± 0.00) and number of sprouts per explant (5.50 ± 1.16) was observed in case of Vireshwar genotype while least percentage of sprouting (25.0 ± 0.17) and number of sprouts per explant (2.00 ± 0.33) was observed in case of Pipalakhunt. Second highest percentage of sprouting was recorded in case of MCB-414 (87.5 ± 0.19) while maximum number of sprouts per explant was recorded in case of Ahwa Dang (4.25 ± 0.65) and Atarsumba (4.25 ± 0.62). Second lowest sprouting percentage was recorded in case of Atarsumba (50.0 ± 0.33) and Ambapada (50.0 ± 0.19) while second lowest number of sprouts per explant (2.50 ± 0.33) were recorded in case of cultivated genotype ASM-1 (Table 2, Figure 1).

Multiplication

Vireshwar reported with highest number of shoots (12.75 ± 1.32) while lowest (3.63 ± 0.56) was recorded in case of Pipalakhunt. Increase in the concentration of kinetin showed gradual increase in the number of shoots. Minakshi *et al*[14] also recorded maximum number of safed musli shoots (21.67) with 2 mgl^{-1} BAP and 3 mgl^{-1} kinetin in the media. A marked difference was observed in growth pattern of all the genotypes studied. The crown shoot buds of Vireshwar gave maximum multiplication with a multiplication rate of 11.44 while minimal multiplication rate (2.07) was observed in Pipalakhunt (Table 2, Figure 1). The multiplication rate ranged from 2 to 11 fold (Table 2). These observations clearly suggest that the multiplication media for each genotype requires refinement and also the tissue culture protocol is genotype specific even for clonally propagated plants. Abe and Futsuhara[1] tested 66 indica and japonica cultivars and reported large differences in their

culturability. Hartke and Lorz[7] tested 15 indica rice lines and found that seven of them could produce embryogenic calli but only four could regenerate into plants.

Root induction

Different root induction responses generated in terms of number and length of roots when *in vitro* developed shoots of all the genotypes were transferred to half strength MS supplemented with 2 mgl^{-1} IBA. All the genotypes showed 100% rooting. Haque *et. al*[6] reported 92.2% rooting frequency with 1mgl^{-1} IBA. Purohit *et al.*(1994) also reported 16 roots per shoots with 2 mgl^{-1} IBA. Maximum number (16.88 ± 1.42) and length (1.66 ± 0.06) of roots was obtained in case of Vireshwar while minimum number of roots (2.88 ± 0.40) and root length (0.20 ± 0.04) was obtained in the cultures of Pipalakhunt (Table 3, Figure 1).

Hardening

Hardening of tissue culture raised plants is the most crucial step in micropropagation. Four week old rooted plants were successfully transplanted to soil:sand:cocopeat mixtures (Figure 2). The water holding capacity of soil is an important parameter to be considered for successful hardening of plants. Although, all the genotypes were hardened using same hardening mixture, differential responses were generated during acclimatization. The average survival rate during the primary and secondary hardening of all the genotypes was 65%. The highest survival rate recorded was 74% in case of Vireshwar and the lowest 54% in case of Pipalakhunt. The plants produced were susceptible to face the change in environmental conditions during acclimatization. The leaves of the plants developed cuticle and its photosynthetic system started functioning gradually under green house conditions. Further acclimatization was carried out in poly house and maintained till their field transplantation.

Table 1: Details of the various Safed musli genotypes collected from the different regions of India

Sr. no.	Genotypes	Genotype Code	Location	Remarks
1	Anand Safed Musli-1	ASM-1	Anand, Gujarat	Cultivated
2	Vireshwar	VR	Anand, Gujarat	Wild
3	Ambapada	AM	Dang, Gujarat	Wild
4	Namangir	N	Dang, Gujarat	Wild
5	Ahwa Dang	AH	Dang, Gujarat	Wild
8	Atarsumba	AT	Sardar Krishinagar, Gujarat	Wild
6	Pipalakhunt	P	Rajasthan	Wild
7	RC-5	R	Rajasthan	Cultivated
9	MCB-414	M	Madhya Pradesh	Cultivated

Table 2: *In vitro* response of Safed musli genotypes on initiation and multiplication media

Sr. no.	Genotypes	% Axenic culture established	Sprouting percentage (%)	No. of Sprouts/ Explants (Initiation)	No. of new Shoots/explant (Multiplication)	Multiplication Rate*
1	Anand Safed Musli-1	31	75.0 ± 0.17	2.50 ± 0.33^{f}	4.5 ± 0.71^{ef}	3.21
2	Vireshwar	53	100.0 ± 0.00	5.50 ± 1.16^{a}	12.75 ± 1.32^{a}	11.44
3	Ambapada	39	50.0 ± 0.19	3.50 ± 0.57^{bcde}	10.50 ± 0.73^{b}	9.19
4	Namangir	47	75.0 ± 0.17	2.88 ± 0.44^{ef}	4.63 ± 0.50^{ef}	4.13
5	Ahwa Dang	42	75.0 ± 0.17	4.25 ± 0.65^{b}	9.25 ± 1.11^{bc}	8.37
6	Atarsumba	52	50.0 ± 0.33	4.25 ± 0.62^{bc}	8.63 ± 0.98^{c}	7.29
7	Pipalakhunt	33	25.0 ± 0.17	2.00 ± 0.33^{f}	3.63 ± 0.56^{f}	2.07
8	RC-5	50	75.0 ± 0.17	3.38 ± 0.56^{bde}	6.00 ± 1.04^{de}	5.28
9	MCB-414	48	87.5 ± 0.19	3.75 ± 0.49^{bcd}	6.63 ± 0.73^{d}	6.19

Parameters have been recorded after 4 weeks and 16 weeks of transfer in initiation and multiplication media, respectively. Data are in the form of mean ± SE Means with the same letter along the column are not significantly different at p=0.05

Table 3: *In vitro* response of Safed musli genotypes on root induction media

Sr. No.	Genotypes	Rooting Percentage	No. of roots/ Explant	Length of root
1	ASM 1	100.0 ± 0.00	7.38 ± 0.68^{c}	0.99 ± 0.09^{c}
2	Vireshwar	100.0 ± 0.00	16.88 ± 1.42^{a}	1.66 ± 0.06^{a}
3	Ambapada	100.0 ± 0.00	9.25 ± 1.90^{b}	1.08 ± 0.08^{c}
4	Namangir	100.0 ± 0.00	4.13 ± 0.44^{d}	0.30 ± 0.05^{de}
5	Ahwa Dang	100.0 ± 0.00	9.38 ± 1.44^{b}	1.40 ± 0.06^{b}
6	Atarsumba	100.0 ± 0.00	10.13 ± 1.66^{b}	1.18 ± 0.18^{bc}
7	Pipalakhunt	100.0 ± 0.00	2.88 ± 0.40^{e}	0.20 ± 0.04^{e}
8	RC - 5	100.0 ± 0.00	6.25 ± 0.75^{c}	1.04 ± 0.11^{c}
9	MCB-414	100.0 ± 0.00	6.88 ± 0.88^{c}	0.48 ± 0.05^{d}

Parameters have been recorded after 4 weeks of transfer in rooting media. Data are in the form of mean ± SE
Means with the same letter along the column are not significantly different at p=0.05

Anand Safed Musli-1	Vireshwar	Ambapada	Namangir	Ahwa Dang	Atarsumba	Pipalakhunt	RC-5	MCB-414

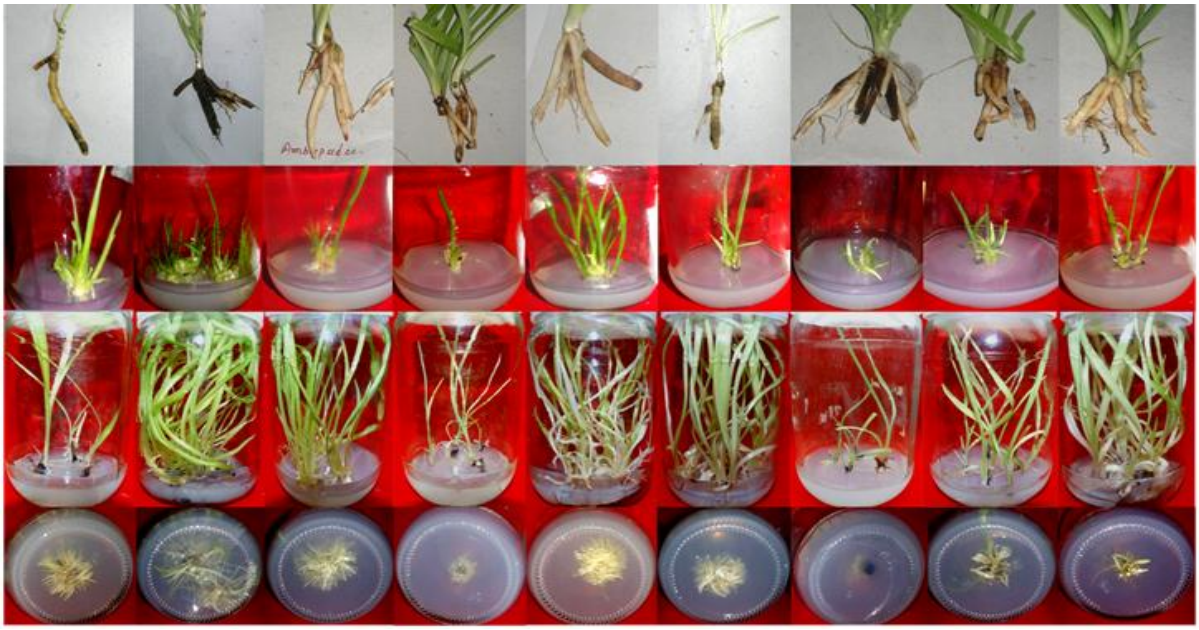

Fig. 1: Nine different genotypes of Safed musli collected from different regions of India
1. Explants **2.** Shoot induction **3.** Shoot Multiplication **4.** Root induction

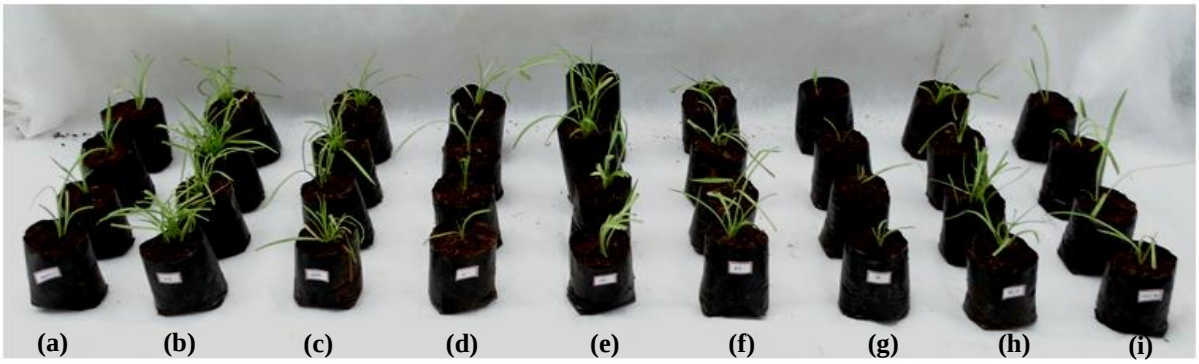

Fig. 2: Ex vitro hardening of various Safed musli genotypes in Green house a. ASM-1
a. ASM-1 **b.** Vireshwar **c.** Ambapada **d.** Namangir **e.** Ahwa dang **f.** Atarsumba **g.** Pipalakhunt **h.** RC-5 **i.** MCB-414

CONCLUSIONS

As the Safed musli explants are the underground parts, combinational use of antibacterial, antifungal and heavy metal for surface sterilization is suggested for establishment of higher percentage of axenic cultures. Further, it is concluded that a genotype specific protocols need to be developed for each nine genotypes as they respond variedly with a single medium formulation for each stage. Hence, *in vitro* response of nine different genotypes of 'Safed musli' (*Chlorophytum borivilianum*) using crown shoot bud as an explant has been checked.

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