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PLANT REGENERATION THROUGH ORGANOGENESIS FROM YOUNG LEAVES OF *CHLOROPHYTUM BORIVILIANUM*

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SUMMARY

Plant regeneration through organogenesis was standardized from in *vitro* regenerated leaves of safed musli. Callus induced from the leaves within two weeks of culture on MS media with various concentrations of 2,4-D (0.25 to 4.0 mg/l) alone and in combination with other auxins and cytokinins. Among all, 1.5 mg/l 2,4-D resulted in embryogenic callus from the leaves. However, further differentiation of callus was not observed on 2,4-D media. Embryogenic calli were transferred to MS medium with various concentrations of BAP (0.5 to 3.0 mg/l) along with two different concentrations of Kn (0.25 and 0.5 mg/l). The frequency of shoot bud primordia production varied from 31.25 to 58.32% in different concentrations of BAP and Kn. Among all, high frequency of plant regeneration (83.3%) was achieved on MS with 1 mg/l BAP + 0.5 mg/ Kn. Higher concentration of BAP induced more number of shoot buds, but many of them failed to regenerate. Regenerated shoots were rooted on half strength MS media with different concentrations of IAA and IBA. High frequency rooting (73.3%) was observed on half strength MS with 2 mg/l IBA. Rooted plantlets were transferred to pots and established in the field with 90% survival rate.

***Key words*: Callus, leaf explant, organogenesis, plant regeneration, safed musli**

INTRODUCTION

Chlorophytum borivilianum Sant et. Fernand (Family- *Lilliaceae*), commonly known as safed musli, is one of the important medicinal plants, widely used in various traditional medicinal systems of India. It is mainly distributed in the hilly regions of Rajasthan, Madhya Pradesh, Gujarat and Maharastra. Tuberous roots of safed musli contain saponins, steroids, vitamins, amino acids, alkaloids and acts as central nervous system (CNS) stimulant, cardio-vascular corrective agent. The roots are considered as wonder drug and used as aphrodisiac, anti-fatigue, anti-aging agent (Anonymous 1985, Kirtikar 1975). The dried fasciculate roots are exported form India in substantial amount and the present demand is met from the forest. Due to the large-scale trade and indiscriminate collection of roots, the population of safed musli is disappearing from its natural habitat. Because of this, it has been listed in the Red Data Book of Indian Medicinal plants (Nayar *et al*. 1988). The conventional propagation methods are limiting due to low seed set, poor viability, germination and decay of tubers during storage. Thus, in *vitro* method of conservation and multiplication of this species will be much useful to meet the present market demand.

The plant cell and tissue culture has been successfully exploited for *in vitro* production of several important medicinal plants. In safed musli, micropropagation protocols have been standardized from stem discs

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possessing shoot buds and young shoot bases (Purohit *et al*. 1999). Scaling up production by encapsulated shoot buds and field performance of micropropagated plants was also reported (Dave *et al*. 2004, Dave *et al*. 2003). Plant regeneration studies were undertaken from shoot crown explants and genetic fidelity was assessed using RAPD finger printing and karyotype analysis (Lattoo *et al*. 2006). *In vitro* clonal propagation was reported from nodal segments of actively growing immature floral buds along with inflorescence axis (Sharma *et al*. 2006). Embryogenesis and plant regeneration was reported from the long-term cultures of stem disc and seedling derived callus (Arora *et al*. 2006). Studies were carried out on plant regeneration through somatic embryogenesis from stem discs (Sunita *et al*. 1997), seedling explants (Purohit *et al*. 1994) and various factors affecting the somatic embryogenesis (Arora *et al.* 1999). However, the information on plant regeneration through organogenesis with intervening callus is rather meagre. The present study deals with the plant regeneration from *in vitro* grown leaves of safed musli through the intermediate callus phase, which can be exploited for germplasm conservation and genetic manipulation studies.

MATERIALS AND METHODS

Collection of explants: Multiple shoot regeneration was standardized form the stem disc explants of field grown plants of safed musli on MS with 5 mg/l BAP. Leaves were collected from *in vitro* regenerated plants and dissected transversely (4-6 mm) and 5-8 pieces placed in each test tube.

Callus induction: For callusing, MS media (Murashige and Skoog 1962) with different concentrations of auxins and cytokinin were employed, i.e. 2,4-D (dichlorophenoxy acetic acid), Kn (kinetin) BAP (6-benzyle amino purine), IAA (indole- 3- butyric acid), NAA (naphthalene acetic acid) etc. After 4 weeks, callus induction quality was scored visually as low (+), moderate (++) and high (+++) based on the growth and nature of the callus. Frequency of callus induction was evaluated by counting the number leaf explants responded by total number of explants inoculated. The pH of the medium was adjusted to 5.8 prior to addition of agar (0.8%: Qualigens). After melting the agar, the medium was dispensed into culture tubes (about 20 ml media / test tube) and autoclaved for 15 minutes at 108 kPa (121^0C). The cultures were maintained at 25 ± 2^0C with 16 h photoperiod. All the treatments were replicated and repeated twice.

Organogenesis: Embryogenic callus was transferred to differentiation media and incubated in the dark, followed by four weeks under continuous light. The differentiation medium consists of MS supplemented with various concentrations of BAP and Kn. The frequency of organogenesis is calculated based on number of calli with green structures or green shoot primordia out of the total number of calli plated on differentiation media. The regeneration frequency is assessed from the number of regenerated plantlets out of total number of calli plated on the differentiation medium.

Rooting from shoots: When the regenerated shoots elongated to 3-4 cm, they were transferred onto rooting medium with half strength MS with different concentration of IAA and IBA. Plantlets with healthy roots were washed free of agar and transferred to protrays filled with vermiculite and soil (1:1) and covered with plastic bags to retain humidity. Gradually, the plastic bags were removed and plantlets were transferred to field.

RESULTS AND DISCUSSION

Transversely sliced leaflets expanded rapidly, turned green within one week of inoculation onto induction medium supplemented with different levels of hormones. The callus initiated within two weeks of inoculation from the both abaxial and adaxial surface of the leaves. Results from the experiments indicates that (Data not shown) the frequency of induction, quality and subsequent differentiation of callus was better from the callus induced in the total darkness, rather than in 16/8 h photoperiod. This indicates the importance of the light on regulation of growth and development. Callus was initiated from the leaf explants in all the concentrations of 2,4-D employed (0.5–3.0 mg/l). Lower concentrations of 2,4-D resulted in high frequency of callus, but it is friable and non-embryogenic (Table 1). At 1.5 and 2.0-mg/l 2,4-D concentration in the induction medium, the quality of the callus was good, proliferate, granular and embryogenic (Fig. 1a). However, the

Table 1. Callus induction from *in vitro* grown leaves of *Chlorophytum borivilianum*

Hormonal con. (mg/l)	Callus induction (%)	Quality of callus	Type of response
1.0 2,4-D	72.1 ± 0.24	+	Slow growth, Friable
1.5 2,4-D	64.7 ± 0.64	++	Fast growth Highly proliferate, granular
2.0 2.4-D	58.0 ± 0.27	+++	Highly proliferate, Embryogenic, granular
2.5 2,4-D	44.5 ± 0.72	+++	Creamy, embryogenic, granular
3.0 2,4-D +	42.2 ± 0.43	++	Friable, little browning
2.5 2 ,4-D +0. 25 Kn	74.3 ± 0.38	+	Creamy, white
2.5 2.4-D +0.50 KN	66.8 ± 0.83	+	Creamy., proliferative
2.5 2.4-D +0.25 IBA	64.1 ± 0.23	+	Brown
2.5 2,4-D+0.5 IBA	70.4 ± 0.67	+	Green
2.5 2,4-D+0.25 BAP	66.9 ± 0.45	+	Dark green
2.5 2,4-D+0.5 BAP	64.36 ± 0.72	+	Green compact
2.5 2,4-D+0.25 NAA	53.8 ± 0.84	+	White, with hairy roots
2.5 2,4-D+0.5 NAA	56.68 ± 3.62	+	White, more hairy roots

*Values are mean ± SE of 6 replicates, **Callus growth after four weeks in culture

frequency of the callusing was low at this concentration, indicating that there is no absolute relationship between the frequency of callus induction and quality of the callus. To further improve the quality and frequency, callus from 1.5 mg/l and 2.0 mg/l 2,4-D was transferred onto MS media with various combination of other hormones. Though the callusing frequency was improved in all the combination of cytokinins and auxins, the callus was not embryogenic. However, in all the concentrations of 2,4-D and other combinations, the further differentiation of callus was not observed. The present study showed that the presence of 2,4-D in the culture medium is critical for induction and differentiation of callus induced from the leaf explants of safed musli.

The embryogenic calli were transferred onto differentiation media, i.e. MS with combination of different concentrations of hormones. Among all the hormones, BAP along with the combination with Kn resulted in morphogenic response within 3 weeks of culture. Various concentrations of BAP (0.5 to 3.0 mg/l BAP) along with two different concentration of Kn (0.25 and 0.5 mg/l) were given in the Table 2. The frequency of green shoot primordia production varied (31.25 to 58.32%) among the different hormonal combinations (Fig. 1b). The shoot bud induction is minimum at lower concentration of BAP and it is increased along with the concentration of BAP. Similarly, the effective role of BAP in shoot induction was earlier reported in many plants (Bhat *et al*. 1995). Among all, maximum shoot bud induction was observed at 2.0 mg/ l BAP with 0.5 mg/l Kn. Further increasing in the concentrations of BAP (3.0 mg/l), the percentage of shoot bud induction was reduced.

The regeneration from shoot primordia is more at lower concentrations of BAP and Kn, whereas, in the higher concentrations of BAP, most of the shoot buds turned brown and failed to regenerate. The frequency of shoot bud regeneration ranged from 45.45 to 83.3% in various concentrations of the BAP and Kn (Fig. 1c). Among all, 1 mg/l BAP + 0.5 mg/l Kn resulted in high frequency of plant regeneration (83.3%). The shoot length of regenerated plantlets was also high at lower concentrations of BAP, whereas, increase in BAP concentration resulted in the stunted growth of the shoots. Similarly, increase in BAP concentration resulted in stunted and abnormal growth of the shoots in groundnut (Vijaya Laxmi *et al*. 2003). Within 4 weeks of culture, the regenerated shoots reached a height of 2 to 3 cms.

Table 2. Green plant regeneration from leaf derived callus of *Chlorophytum borivilianum*

Hormonal con. (mg/l)	Expl Ino.	Expl. Res.	Green shoot primordia (%)	No. plants reg.	Plant regeneration (%)	Shoot length ** (cm)
0.5 BAP+0.25 Kn	32	10	31.25 ± 0.320	6	60.00± 2.13	3.4±1.44
0.5 BAP+0.50 Kn	21	8	38.09 ± 0. 126	5	62.5± 1.71	3.2±1.52
1.0 BAP+0.25 Kn	30	13	43.3 ± 0.117	10	76.9± 2.05	3.0±1.06
1.0 BAP+0.50 Kn	26	12	46.15 ± 0.421	10	83.3 ± 3.08	3.1±1.12
2.0 BA+0.25 Kn	28	15	53.57 ± 0.221	12	80.0± 1.92	3.0±0.81
2.0 BAP+ 0.50 Kn	36	21	58.33 ± 0.182	16	65.82 ± 3.10	2.8±0.63
3.0 BAP+ 0.25 Kn	22	12	54.54 ± 0. 098	7	58.3± 2.62	2.1±1.32
3.0 BAP+ 0.50 Kn	25	11	44.00 ± 0. 562	5	45.45 ± 3.14	1.12±1.62

* Values are mean ± SE of 6 replicates, ** Shoot growth after three weeks in culture

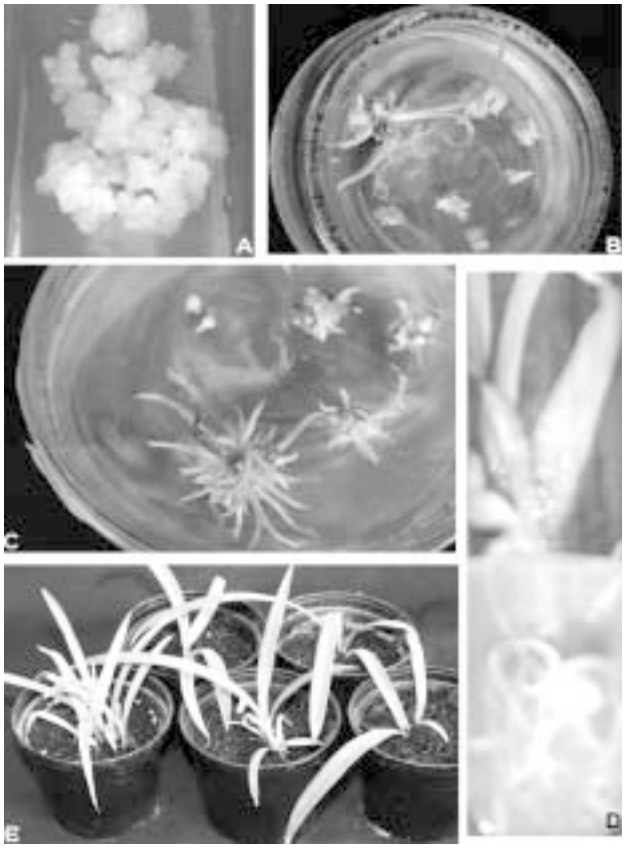

Fig. 1. (a) Induction of callus from leaf explants on MS with 1.5-mg/l 2,4-D, (b) Induction of shoot primordia from the callus from MS with BAP + Kn, (c) Plant regeneration from the shoot bud of leaf callus on MS + 1 mg/l BAP + 0.5 mg/l Kn, (d) Rooting from regenerated shoots on ½ MS + 2 mg/IBA, (e) Regenerates transferred to pots.

Regenerated shoots were transferred to half strength MS medium supplemented with different concentration of IAA and IBA (Table 3). Adventitious roots were developed from the base of the shoot in all the concentrations of auxins (Fig. 1d). In IBA supplemented media, root initiation started within one week of initiation and number of roots/ shoots was more. However, in IAA supplemented media, the root initiation is very slow (23 to 30 days) and number of roots was also less. The frequency of rooting varied from 42.8 to 73.3% among different concentrations of IAA and IBA. Long slender roots with 73.3% frequency were observed at 2mg/l IBA concentration. Between the two auxins, IBA was found to be better than IAA. In all the auxin supplemented media, especially at high concentrations, callus induction was high at basal region which may be due to accumulation action of auxins at the basal ends (Marks *et al*. 1994). Regenerated shoots kept in the dark showed faster root growth when compared to the culture at 16 h photoperiod. The effect of light on the growth of rooting is a significance observation in the present study. All the regenerates developed very good root system within three weeks duration in rooting media. The regenerated plantlets were transferred to protrays (Fig. 1e) filled with vermiculite and soil (1:1) and covered with plastic bags to retain 80 to 90 % humidity. Gradually, the plastic bags were removed and transferred to field with 90% survival rate.

Table 3. Rooting from regenerated shoots of *Chlorophytum borivilianum.*

Hormones (mg/l)		No. of plants for	No. of plants	Rooting (%)	No. of roots /	Root length (cm)
IAA	IBA	rooting	rooted		shoot	
0.5	-	14	6	42.8	3.2 ± 1.4	1.2 ± 1.4
1.0	-	15	7	46.6	3.8 ± 0.8	1.4 ± 1.6
2.0	-	17	9	52.9	4.2 ± 1.0	1.4 ± 1.5
3.0	-	17	11	64.7	4.5 ± 1.1	1.6 ± 1.2
-	0.5	16	9	56.2	4.6 ± 0.6	1.8 ± 1.8
-	1.0	14	9	64.2	5.2 ± 1.2	2.0 ± 2.2
-	2.0	15	11	**73.3**	6.8 ± 1.4	2.2 ± 2.1
-	3.0	16	11	68.7	5.8 ± 0.9	2.1 ± 2.3

* Values are mean ± SE of 6 replicates, ** Root growth after four weeks in culture

The present study of plant regeneration from leaves of *safed musli* through callus phase offers a great potential in genetic transformation of this important species. The protocols can be exploited for *in vitro* generating new genetic variability and production of bioactive constituents from the callus.

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