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Induction of somatic embryogenesis from leaf explants of safed musli (*Chlorophytum borivilianum*)

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Abstract
Safed musli (*Chlorophytum borivilianum*) is an endangered medicinal plant of liliaceae along with a few other species. It has a very short life cycle (90–100 days). The root of plant is widely used as tonic and aphrodisiac and other important application in Indian system of medicine since ancient time. It contains steroidal saponin viz. neotigogenin, neon hecogenin, stigmasterol and tokorogenin. It is propagated through the fleshy tubers as seeds of plant have only 10-14% germination. Limited success in plant let regeneration from leaf discs and seedling derived calli have been reported via somatic embryogenesis and organogenesis because of heavy contamination (80- 100%) of cultures. Culture contaminations have been controlled to 20-40% level with the leaf and central root disc explants in the present study. Murashige and Skoog's medium proved better for establishment of culture as well as induction of somatic embryogenesis. Induction of somatic embryogenesis was achieved from basal leaf portion while other explants failed to do so. An optimum of 6% sucrose in medium was required for induction and development of somatic embryos. Amongst the different auxins and cytokinins, naphthalene acetic acid alone was effective for induction of somatic embryogenesis. Other growth regulators either alone or in various combinations permutations were inhibitory for induction and development of somatic embryos. The basal leaf portion showed swelling in the first week and development of calli by bursting of leaf surface in the second week of culture initiation. Somatic embryos appeared as globular and developed to scutellar notch stage and finally as well developed scutellum, coleoptile and coleorhiza with simultaneous maturation within 5 to 6 week of culture initiation. Upon subculture of somatic embryo to fresh medium with similar levels of sucrose and NAA in both agar solidified and liquid suspension medium showed germination and converted in to normal plantlets within two weeks. Further growth of the plantlets was achieved in liquid medium with 3% sucrose and plantlets were transplanted in soil compost mix after two weeks. About 80 – 100% of normal and properly matured somatic embryos converted into plantlets and survived well following the acclimatization process.
Abbreviations: BAP- 6- benzylaminopurine, 2,4-D – 2,4- dichlorophenoxyacetic acid, IAA- indoleacetic acid, IBA- indolebutyric acid, NAA- a-naphthaleneacetic acid, TDZ- thidiazuron, MS- Murashige and Skoog.

Keywords: *Chlorophytum borivilianum*, contamination control, embryogenic callus, leaf base, safed musli, somatic embryogenesis.

1. Introduction
Chlorophytum borivilianum is commonly known as safed musli along with a few other species. The species was first described from India in 1954 and reached rare status in nature due to overexploitation. Safed musli is widely distributed in India mainly in Southern Rajasthan, Western Madhya Pradesh, North Gujarat and few parts of Karnataka. Somatic cell chromosome number in *C. borivilianum* is 2n=16, while in other related species it is 2n=28, 32, 40, 84 (Kumar and Subramanium, 1987; Maiti and Geetha, 2004) [12, 13]. The species is diploid with 2*n*=4*x*=28. Thus there are two basic chromosome number in the genus *Chlorophytum,* viz. n=7 and the other being n=8. Hence the genus has a broad biodiversity base (Geetha and Maiti, 2002) [5]. *C. borivilianum* has a very short life cycle (90–100 days). Although the species is cross-pollinated in nature, self-pollination is also feasible artificially. It is among the twenty and odd species of native medicinal plants that enjoys extensive therapeutic applications and expansive global market. It is an endangered species of liliaceae (Nayar and Shastry, 1988) [17] valued for the dried tuberous fasciculated storage roots. These are reputed to have aphrodisiac properties mainly identified as 'Herbal viagra' and form an important ingredient of herbal tonic in ayurvedic system of medicine in India as total rejuvenator, antioxidant and immuno modulator (Kirikar and Basu, 1975) [11].

Chlorophytum borivilianum is a rich source of over 25 alkaloids, vitamins, proteins, carbohydrates, steroids, saponins, potassium, calcium, magnesium, phenol, resins, mucilage, and polysaccharides and also contains high quantity of simple sugars, mainly sucrose, glucose, fructose, galactose, mannose and xylose (Thakur, *et al*., 2009) [31]. It contains steroidal saponins viz. neotigogenin, neon hecogenin, stigmasterol and tokorogenin (Mimaki, *et al*., 1996; Tandon and Shukla, 1992, 1995) [15, 29, 30]. Importance of saponin from *Chlorophytum* species have been studies in detail by Kausik (2005) [8]. Pharmacological investigation of saponin fraction separated from dried roots showed significant inhibition of ^{3}H dopamine uptake in rat striatal synaptosomes thereby directly stimulating the neuronal activity of dopaminergic system in the brain (Ramawat *et al*, 1998) [21].

Due to large scale and indiscriminate collection and export of wild materials and insufficient attempts either to allow its replenishment or its cultivation, *C. borivilianum* is rapidly disappearing. *C. borivilianum* has been enlisted in the list of National Medicinal Plant Board as one of the prioritized plant species. There is need for commercial cultivation of this species. The natural regeneration of this herb is through tuberous roots that have become scare in nature and seed germination is only 14-16% (Jat and Bordia, 1990) [7]. Maiti and Geetha, (2005) [14] have presented a comprehensive overview of the current state of research in the species with due emphasis on future thrust and possibilities.

Clonal multiplication of *C. borivilianum* from immature floral buds and inflorescence axis has been reported recently (Samantaray and Maiti, 2011; Sharma and Mohan, 2006) [25, 28]. An assessment of genetic fidelity of micropropagated plants of *Chlorophytum borivilianum* Santpau and Fernandes using Random Amplified Polymorphic DNA (RAPD) markers have been performed by Samantaray and Maiti, 2010, 2011) [24, 25]. Limited success in plantlet regeneration from leaf discs and seedling derived calli via embryogenesis as well as organogenesis have been because of heavy contamination (80 - 100%) of cultures (Jain *et al*., 1996, Purohit *et al*, 1994, Arora *et al*, 1999) [6, 20, 3]. Some success plantlet regeneration through organogenesis (Sabitha *et al*., 2009) [23] and through somatic embryogenesis (Prasad *et al*., 2007) [19] from young leaves of *C. borivilianum* have been reported recently. Direct shoot regeneration from immature inflorescence cultures of *Chlorophytum arundinaceum* and *Chlorophytum borivilianum* has been reported by Samantaray *et al*., (2009) [27]. But an efficient *in vitro* method for large scale multiplication and conservation of this species is the need of hour. The present work reports an improved procedure for rapid clonal propagation through somatic embryogenesis of this species.

2. Materials and Methods

Healthy and well developed root fingers were selected from a farmer of Hyderabad. These root fingers were planted in large pots using one part compost two part local red garden soil at JIVAS campus, Bangalore. The pots were kept under partial shade and watered as the need arises. Another stock of root fingers was stored in cool and dry place being used for inoculation later on. Germination of root took place within two weeks of planting. The whole plant was uprooted without making any injury to the leaf base and developing root fingers. The roots and plants were washed to remove the traces of soils. Carefully remove the leaves from the stem crown. The basal 3cm portion of the leaves were cut into three equal parts and sterilized separately as basal, medium and distal portion relative to stem crown. Apical meristem was also collected in a separate flask for sterilization and inoculation.

For explants healthy roots with moderate level of moisture content were selected for surface sterilization. The root fingers were washed under running tap water for 10-15 minutes. The root fingers then cut into 0.6-0.8cm thick discs. These discs were then dissected using a cork borer to separate them into central discs containing meristematic zones and the outer layer with a central hole. These root parts were sterilized separately and used as explants.

All the explants were washed again under running tap water for 5-10 minutes. Explants were surface disinfected for 15 minutes by rigorous shaking in 2% Cetavlon (v/v) a mild antiseptic along with 4-5 drops of Tween-X. Explants were washed again under running tap water for 3-5 minutes to remove surfactants. Further explants sterilization was carried out under complete aseptic condition over a Lamina Air Flow Hood. These explants were rinsed for 60-90 seconds with 80% ethanol followed by sterilization for 15-20 minutes by continuous shaking in the presence of 0.05% $HgCl_2$ solution. The outer root discs with hole were treated for different periods of time ranging from 15-30 minutes, and the apical meristem treated for shortest period of 15 minutes. These explants were washed 4-5 times finally with sterile double distilled water. These explants were inoculated on presterilized medium either directly or after trimming.

The normal strength Murashige and Skoog's (MS) (1962) [16] as well as formulation were used in combination with sucrose and growth regulators. The pH of the medium was maintained at 5.8±0.2 before autoclaving and was gelled with 1% agar-agar. The medium was supplemented with 3%, 6% and 9% sucrose and modified with 0.01, 0.1, 0.5, 1.0, 2.0, 4.0, 8.0 mg/l of various growth regulators. Two different auxins viz. 2,4-dicholophenoxy acetic acid (2,4-D) and Naphthalene acetic acid (NAA) as well as two cytokinins viz. 6-benzyl amino purine (BAP) and kinetin were used for establishment of various explants either alone or in different combination permutations. Pretreatment of explants with gibberellic acid and cold pretreatment was also tried in order to break the dormancy of explants.

All the media were sterilized by autoclaving for 15-20 minutes of times at 121 ^{0}C temperature and 15 psi pressure. A sample size of 12 was maintained constantly for all the treatments and each treatment was repeated 3-5 times. All the cultures were incubated in a culture room uniformly maintained at 25±2 ^{0}C temperature and 65% relative humidity maintained with an air condition. The cultures were provided with 16 hours photoperiod using white fluorescent tube light (Phillips Cool TL 40W/220V) receiving an intensity of 30-50 $\mu molm^{-2}s^{-1}$. For dark incubation culture racks were covered with double layered thick black cloth in the same culture room. Cultures were observed routinely at regular intervals of 2-4 days and changes in shape, size and colour of explants were recorded weekly. *In vitro* response of various explants sources under different treatments are described and discussed in the following sections.

2.1. Statistical analyses

In order to validate the results of leaf base somatic embryogenesis experiments, in which the main parameters were sucrose and NAA concentrations in the induction media, statistical analyses were performed using one ways ANOVA. Completely randomized design was used. A sample size of 16 was maintained for every treatments and each experiment was repeated thrice. Statistical analysis was performed using SPSS (version 10.0) software for means, standard deviation and one-way ANOVA and significance was determined at $P<0.05$. The

results have been presented as average of mean value of three independent experiments ± standard deviations and means were compared using least significant difference (LSD) procedures.

3. Results and Discussion

3.1. Control of contamination

One of the bottleneck in the regeneration of *Chlorophytum borivilianum* is the very high rate of culture contamination (80 -100%) (Arora *et al*., 1999; Purohit *et al*., 1994) [3, 20]. In the present study different explants were treated with $HgCl_2$ ranging from 0.01-0.1% for 5-60 minutes. There was no control over the contaminations from whole root, and outer ring explants, while the central root discs with meristematic portions showed best established explants with only 5% contamination frequency. A concentration of 0.05% of $HgCl_2$ was sufficient to sterilize various explants. The central discs, and different leaf portions treated for 15-20 minutes were found optimum for establishment of cultures with 5-40% contamination frequency (Table1). Arora *et al*., 1999 have reported higher frequency of contamination, moreover, explants type was not clearly defined for the in vitro morphogenesis. Purohit *et al*., 1994 have reported 80% and 40% of culture contamination established from root and stem base explants respectively.

(One squire cm leaf base is being cultured on MS medium supplemented with 6% sucrose for four weeks and subcultured to MS basal medium with 3% sucrose. Average number of somatic embryos and frequency of convertible somatic embryo is counted after four weeks of subculture to MS basal medium)

Table 1: Induction of somatic embryogenesis from leaf base explants of safed musli (*Chlorophytum borivilianum*).

Sl. No.	Concentration of NAA	Frequency of Contamination	Frequency of Callogenesis	Relative callus growth	Frequency of Embyogenesis	Average no. of embryos per culture	Frequency of convertible somatic embryos
	(mg/l)	(%)	(%)	(Relative)	(%)		(%)
1	0.00	28.97 ± 6.37	0.00	--	0.00	0.00	0.00
2	0.01	29.13 ± 3.96	24.45 ± 5.83	+	0.00	0.00	0.00
3	0.1	28.32 ± 4.33	28.57 ± 4.14	++	16.67 ± 8.21	8.32 ± 7.16	78.95 ± 6.77
4	0.5	28.88 ± 4.27	42.85 ± 5.13	++++	28.57 ± 4.48	68.59 ± 23.33	76.43 ± 8.29
5	1.0	19.75 ± 3.33	96.57 ± 4.83	+++++	77.76 ± 6.73	276.32 ± 34.67	62.53 ± 5.66
6	2.0	28.26 ± 4.68	71.43 ± 8.76	+++	42.86 ± 6.89	119.12 ± 18.99	44.43 ± 6.78
7	4.0	18.75 ± 5.65	62.56 ± 6.89	+++	16.97 ± 8.83	38.52 ± 17.77	42.56 ± 4.19
8	8.0	42.44 ± 13.49	16.66 ± 6.38	+	0.00	0.00	0.00
F(7, 24); P<0.01		1.34	141.76	--	122.29	19.80	13.215
LSD (P<0.05)		13.81	11.37	--	8.45	43.63	9.76

Value represents average of mean value of three independent experiments ± standard deviations. + Low callusing; + + low moderate callusing; +++ moderate callusing; + + + + high moderate callusing; + ++ + + profuse callusing. (Observations based on amount of callus in 16 cultures).

3.2. Culture Establishment

There had been a varying degree of success 60% from leaf basal segments, 60-70% from middle portion and more than 70% from distal leaf segments in establishment of culture. The central root discs portion have shown up to 95% of culture establishment but showed very little morphogenetic response in different combination permutations of growth regulators and sucrose concentrations. The central root discs explants treated with cold treatments and gibberellic acid in order to break the dormancy as well as incubated in complete dark or varying dark/light cycles did not improve the morphogenetic response.

3.3. The Morphogenesis

Induction of callus had been the first morphogenetic response from different segments of leaf explants as a function of sucrose concentrations. MS medium supported the best callus growth compared to B5 nutrient formulation as they need to be subcultured again on MS medium similar to the other report (Arora *et al*., 1999) [3]. Among the different leaf portions, the basal region (3cm from the stem discs) had proved best responsive explants for callus development (Figure 1A) and somatic embryogenesis (Table 1). The middle leaf portion was found slightly less responsive while, distal tip region showed least callus development. Young leaf and leaf base explants have also been used for somatic regenerants of *Chlorophytum borivilianum* (Arora *et al*., 2006; Prasad *et al*., 2007) [3, 19].

The normal strength MS medium supplemented with 6% sucrose and modified with 1.0 mg/l of NAA showed the best production from basal leaf explants. The 3% and 9% sucrose containing medium showed very little callus development with more browning of callus in the presence of 9% sucrose. Arora *et al*., (1999) [3] have reported induction of callus on B5 medium modified with 2.25µM 2,4-D and 1.15 µM kinetin with a subculture frequency of every 4 weeks, but have not mentioned the level of carbon source. They also found that callus induced on B5 medium in the presence of thidiazuron (TDZ) need to be subcultured on MS-9 medium supplemented with 400mg/l KNO_3, 100 mg/l $(NH4)_2SO_4$, 60-400 mg/l of glutamine or praline. This MS-9 medium also supported the induction of callus in the presence of other growth regulators 2,4-D, 2ip, BAP, kinetin and TDZ. However, in the present study we were able to induce callus in the presence of NAA without any additives in to normal MS medium but supplemented with higher concentration of sucrose (Figure 1B). Apical meristem and stem discs explants were mostly contaminated while remaining cultures showed shoot bud development and plantlet regeneration in the presence of 3% sucrose with auxin and cytokinin containing medium confirming the results of Purohit *et al*., (1994) [20].

3.4. Induction of Somatic Embryogenesis

Among the different explants, the central root discs and the basal region of leaf were found responsive to embryogenic treatments. Two types of callus viz. embryogenic and non-embryogenic produced from these two explants sources in confirmation with Arora *et al*., (1999) [3]. Embryogenesis had been the most frequent process in the case of leaf base (Figure 1C-F) compared to central root discs explants. Both callus production and embryogenic response were less frequent from central root discs explants. Induction of somatic embryogenesis was poor in B5 basal salt concentrations compared to MS medium supplemented with similar levels of sucrose and growth regulators.

3.5. Effect of Sucrose Concentration

The leaf explants inoculated in the presence of different concentration of BAP showed necrosis starting from the cut edges in the first week and finally become black in longer incubation period irrespective of sucrose concentrations. Necrosis was less in 3% sucrose added medium. In NAA containing medium leaf base showed swelling from the edges in 3% to entire surface in 6% sucrose in first week of culture. The swelled leaf surfaces showed bursting of callus mostly in 6% sucrose in the second week of culture. In 9% sucrose added medium leaf base also showed swelling but most of them turned brown and showed necrosis on the entire surface.

3.6. Effect of Growth Regulators

The two cytokinins i.e. kinetin and BAP tried for in vitro response from leaf and root explants showed negative effect on culture establishment. Both the cytokinins showed browning and complete necrosis of the explanted tissues at all concentration of sucrose. Among the different auxins (2,4-D and NAA), only NAA was found effective for induction of somatic embryogenesis from basal leaf explants. All the concentration of 2,4-D (0.01 – 8.0 mg/l) induced browning and complete necrosis of explants at all levels of sucrose in the medium.

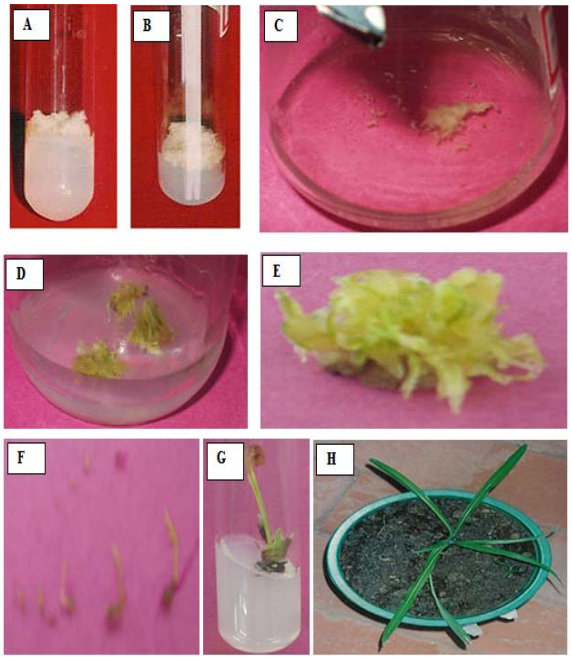

Fig 1: Somatic embryogenesis and plant let regeneration from leaf base explants of safed musli (*Chlorophytum borivilianum*). A- Callus developing from leaf base explants in 3rd week of incubation. B– Same as A in 4th week of incubation. C– Somatic embryos developing in suspension culture in fourth and longer period of incubation. D– Germinating somatic embryos on agar solidified medium after fourth week of subculture. E– An enlarged view of the D showing germinating somatic embryos with green shoots and root with numerous root hairs. F- Different stages of somatic embryos separated from germination medium during fifth and sixth weeks of culture. G– A somatic plantlet growing in agar solidified medium. H– A four week old acclimatized somatic plantlets.

3.7. Effects of NAA

Different morphogenetic response from basal leaf explants portions in 6% sucrose containing medium is shown in table 1. The basal leaf explants showed swelling in the presence of 0.5-4.0 mg/l of NAA at the end of first week of culture initiation. Small amount of friable callus exploded out of the swelled leaf portion in the second week of culture. The callus masses continued to grow in third and fourth week of culture. By the end of third week about 30-40% of explants showed necrosis up till 4.0 mg/l of NAA while higher concentrations were found inhibitory for callogenesis, rhizogenesis and embryogenesis. The amount of callus increases between 0.1-1.0 mg/l NAA with mostly white or yellowish calli. Slightly reduced amount of callus were induced in 2.0 and 4.0 mg/l and mostly dark yellow to light brown in colour while, in 8.0 mg/l of NAA significantly reduced amount of dark brown callus produced after the third week of culture initiation.

Somatic embryos appeared as globular protruding mass from yellowish to yellow calli in the fourth week of culture initiation. These somatic embryos developed into scutellar notch following a short but not so visible heart stage in the fifth week of culture initiation. Somatic embryos further grown to well define scutellum and coleoptiles reached to final stage of development in the sixth week of culture initiation. During these periods several new globular stage embryos appeared and followed the similar developmental trajectory. There was no further growth in size of somatic embryos after six weeks of culture. The effect of various concentration of NAA on induction of somatic embryogenesis is shown in table 1. It is clear from the result that induction of somatic embryogenesis from leaf basal portion was best achieved in the presence of 1.0 mg/l of NAA in 6% sucrose added MS medium. Different combinations permutations of auxins and cytokinins along with varying sucrose concentration in the medium were found inhibitory for induction of somatic embryogenesis irrespective of sucrose concentrations.

4. Maturation and Germination of Somatic Embryos

Somatic embryos in the present study represents globular, scutellar notch and fully developed scutellar and coleoptile stages. These somatic embryos remained in the same stages of development if continued to be there on the same medium for longer period of culture. Upon subculture to fresh medium with similar or reduced of levels of sucrose with or without NAA showed germination of these somatic embryos. All somatic embryos turned green irrespective of stages of development in 3% sucrose whereas, in 6% sucrose medium yellowish green and shorter height plant lets were produced. Subculture of somatic embryos to fresh MS medium in the presence of both 3% and 6% sucrose along with NAA showed decreased conversion frequency. Moreover, plantlets were produced with profused hairy roots from 3 to 6 cm long in 3% sucrose and 0.5-2.0cm long in 6% sucrose containing medium with 0.1-2.0mg/l of NAA. However, Prasad *et al*. (2007) [19] have reported 100% maturation of somatic embryos on plain MS and on MS medium with 0.1 mg l^{-1} abscisic acid (ABA) after 60 days of culture in 2,4- D containing induction media. Embryos derived from treatments with 2,4-D did not show 100% embryo maturation, which could, however be achieved if these were added to cytokinin-supplemented medium. Sixty two per cent of matured embryos formed shoots on media devoid of plant growth regulators but these took nine days to germinate unlike the precocious germination (5 days) demonstrated by matured embryos treated with 0.5 mg l^{-1} gibberellic acid (GA_3). Shoot induction formed in 60% of embryos treated with 0.5 mg l^{-1} TDZ and 0.5 mg l^{-1} Kn but most shoots showed malformation during growth.

5. Cell Suspension Culture

The callus from induction medium when transferred after four weeks to MS liquid medium supplemented with 6% sucrose and modified with 0.5-4.0mg/l of NAA induces the conversion

of somatic embryos to yellowish green to green plantlets within two weeks of subculture (Figure 1C). Most of the plantlets in the above liquid medium were having 1-2cm shoot length with little or no root development depending upon NAA concentration. The somatic embryos at early stage of development were swelled and turned yellowish to yellowish green but without shoot or root formation in the same medium, indicating germination but no plantlet formation. However the same callus subcultured to MS basal medium with 3% and 6% sucrose without any growth hormone showed mostly dark green normal and robust plantlet developments. These plantlets from 3% sucrose medium survived well upon soil transfer and acclimatization in contrast to slightly yellowish green and short plantlets with swelled stem and roots in 6% sucrose containing MS basal medium.

6. Plantlet Growth and Acclimatization

Upon subculture of somatic embryo to fresh medium with similar levels of sucrose and NAA in both agar solidified and liquid suspension medium showed germination and converted in to normal plantlets within two weeks (Figure 1D-F). Further growth of the plantlets was achieved in liquid medium with 3% sucrose and plantlets were transplanted in soil compost mix after two weeks. About 80-100% of normal and properly matured somatic embryos converted into plantlets and survived well following the acclimatization process (Figure 1G-H). Advantages of liquid media for enhancing shoot propagation have also been reported for *Allium sativum* (Kim *et al*., 2003) [9]. Further, handling of liquid medium is comparatively easier than solid medium, which saves labour and energy. Due to higher response of shoot growth and multiplication, liquid culture medium is being suggested for large-scale multiplication of *C. borivilianum* (Dave *et al*., 2003; Rizvi *et al*., 2007) [4, 22]. For scale-up cultures using bioreactors, the use of liquid culture medium has been recommended *Allium sativum* (Kim *et al*., 2004) [10], *Stevia rebaudiana* (Akita *et al*., 1994) [1] and other horticultural and medicinal plants (Paek *et al*., 2005) [18]. Well grown plantlets from MS liquid basal medium showed a high degree of survival frequency (96.85%) upon soil transfer and acclimatization similar to the earlier report (Dave *et al*., 2003) [4]. More than one thousand plantlets from 1.0 mg/l NAA induction medium and liquid MS basal germination medium were acclimatized and transferred to large pots under the green house condition, produced normal fasciculated storage roots comparable to their wild parents within 6-8 weeks of transfer. It was difficult to find out variations in these somatic plantlets during the green house condition. A more detailed study is required in order to confirm the stability of somatic plantlets in terms of their medicinally important active principle. Cytological preparations of root tip squash of germinating somatic plantlets showed a stable diploid status with very low frequency of variable results in contrast to the Arora *et al*., (1999) [3]. This signifies the clonal fidelity of somatic plantlets developed from leaf base explants in the present study. Similarly Arora *et al*. (2006) [2] have reported very little variations (only 0.62% in RAPD fingerprinting) among the somatic plantlets developed from leaf base explants.

7. Conclusion

In the present study we have developed a good control of culture contamination leading to high rate of culture establishment. This has lead to high success in induction of somatic embryogenesis from the leaf base explants of *C. borivilianum*. There has been cent per cent plantlet survival that forms the basis for high volume clonal propagation for commercial exploitation of this species.

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