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Optimization of Conditions for *in vitro* Regeneration of *Chlorophytum borivilianum* : An Indigenous Medicinal Plant

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ABSTRACT

Chlorophytum borivilianum (Safed Musli) is among the most important medicinal herbs. The present research involves *in vitro* sterilization and regeneration from basal plate explant for direct shoot regeneration. The basal plates were excised from the field from grown matured plants, sterilized with different sterilizing agents with varying concentrations and time intervals followed by inoculation on the Murashige and Skoog's medium fortified with different concentrations of hormones. The sterilization treatment of explants with 0.1% mercuric chloride ($HgCl_2$) for 7 min along with 0.4% streptocyclin and 0.4% bavistin for 120 min was proved to be best with maximum survival of 90% among all the treatments used. The MS medium supplemented with 2.0 mg/l6-benzyl aminopurine (BAP) was found to be the most effective media for shoot induction in safed musli with maximum 80% regeneration after 28 days of inoculation producing 2.9 average shoots per explant.

Key words : *Chlorophytum borivilianum, in vitro,* sterilization, fortified, medicinal

INTRODUCTION

A renewed interest has been observed in herbal medicines during recent years. Herbal pharmaceutical industries depend on the plant parts or the complete plant for secondary metabolites production. *Chlorophytum borivilianum*, commonly called as Safed Musli, is an important medicinal herb belonging to the family Asparagaceae, earlier classified in the family Liliaceae (Shinde *et al.*, 2016). It grows naturally in diverse parts of India, namely, Andhra Pradesh, Assam, Bihar, Eastern Ghats, Himalayas, Gujarat, Madhya Pradesh (Desai *et al.*, 2018). It is known by the name 'Wonder Drug' in the medicinal systems because of the presence of its thick tuberous roots which have natural properties for vigour and strength. The thick dried roots which are meant for storage possess spermatogenic properties resulting in usage as a viagra substitute. The high content of saponin holds the responsibility for the therapeutic capabilities (Desai *et al.*, 2018). The plant is also used to cure diabetes, arthritis, and for increasing immunity also (Halder and Bhattacharjee, 2018). The problems faced in natural habitats are an excessive collection of wild material and ruinous methods of harvesting. Insufficient attempts for its cultivation and replenishment are accompanied by poor seed set and lesser vegetative multiplication ratio which has made the species lie under the endangered category (Halder and Bhattacharjee, 2018). The herb regenerates naturally by its tuberous roots which have become scanty in nature. The germination through seed is very low (Bharti *et al.*, 2017). Therefore, considering the importance of the herb, there is an urgent need for highly productive micropropagation techniques for its cultivation so as to meet the commercial demand as well as to ensure its conservation. Several studies have been reported by various researchers where *in vitro* regeneration has been achieved using shoot buds as explants (Nakasha *et al.*, 2016; Desai *et al.*, 2018). Akhtar and Choudhary (2016) used immature inflorescence as an explant in their research of *Chlorophytum*. The present study was an attempt to optimize the conditions for *in vitro* shoot regeneration of *C. borivilianum* using basal plate as explants for which much work has not been done in this direction.

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MATERIALS AND METHODS

Chlorophytum borivilianum plants were collected from the medicinal section of Haryana Agricultural University, Hisar and were maintained in the greenhouse of the Centre for Plant Biotechnology (now known as the Department of Molecular Biology, Biotechnology and Bioinformatics), Chaudhary Charan Singh Haryana Agricultural University, Hisar. These plants were used as the mother plant for the present study. The basal plate was excised from the mother plant and the attached leaves were removed carefully without harming it. The explants were then washed under tap water followed by washing with the 3-4 drops of detergent (Tween-20) and by washing under the tap of water again 3-4 times. A final wash with sterilized double-distilled water was given three times. The explants were sterilized using antibacterial solution streptocyclin (0.2-0.5%) and antifungal solution bavistin (0.2-0.5%) to lessen the chances of contamination for different time durations. The treated explants were then surface sterilized inside the LAF chamber using $HgCl_2$ solution (0.1-0.3%) followed by their washing with sterilized double-distilled water for 4-5 times.

The sterilized explants were inoculated on semi-solid MS media fortified with various concentrations and combinations of plant growth regulators for shoot proliferation. The semi-solid consistency of media was obtained using agar as a gelling agent. The media pH was adjusted to 5.8 prior to autoclaving (at 121°C and 15 lb psi for 20 min) and poured into glass bottles with caps. The inoculated explants were then kept at controlled conditions in a growth chamber at 25±2°C temperature and the intensity of light of 100/µEM^2/sec (1000 lux) under the photoperiod of 8 h dark and 16 h light cycle with the use of fluorescent tubes. The maintenance of cultures was done by sub-culturing them at regular intervals on the media with the same composition.

The keystone in the initiation of tissue culture is the decontamination of the explant keeping in mind that the explant should not lose its biological activity which makes the task highly challenging. The productiveness of the superficial sterilization protocol has an eminent impression on the growth and development of the explants. The explants (basal plate) used in this experiment were given different treatments which is the most essential step for their disinfection. This was done using various sterilizing agents in varied concentrations viz., mercuric chloride i. e. $HgCl_2$ (0.1-0.3%), streptocyclin (0.2-0.5%) and bavistin (0.2-0.5%) for different time intervals for obtaining aseptic cultures. The explant untreated with any sterilizing agent failed to survive. The surface-sterilized explants were inoculated on an MS medium. The data were recorded on 28th day of inoculation for percentage survival and contamination of explants (Table 1).

The data were collected for particular time duration of shoot initiation i. e. on 7th, 14th, 21st and 28th day, shoot initiation per cent and number of shoots regenerated per plant was analyzed statistically for the selection of best establishment medium (Table 2). Usually, nine cultures were used in each treatment and the experiment was repeated three times. All the statistical analysis was carried using OPSTAT software.

RESULTS AND DISCUSSION

The first set of this treatment (from ST_1-ST_9) involved different concentrations of $HgCl_2$ for different durations of time for obtaining the best results. It was observed that the survival percentage of the explants initially increased when treated with $HgCl_2$ (0.1%) for 6-8 min (Table 1). The maximum (40%) survival of explants was recorded on ST_2 when the explants were treated with 0.1% $HgCl_2$ for 7 min. Further increase in $HgCl_2$ concentration resulted in a decrease in survival percentage which may be due to the toxicity of $HgCl_2$. Low degree of explants survival was observed, with high bacterial and fungal contamination, when $HgCl_2$ alone was used for sterilization of explants. The contamination in safed musli explants may be due to endophytic bacterial and fungal problems that caused the subsequent decrease in the survival rate. Therefore, in the present study, more treatments were taken with different sterilizing agents like bactericide as well as fungicide to overcome the systemic contamination in explants. Hence, the explants were further subjected to the next series of treatments for obtaining more survival percentage. The second set of

Table 1. Effect of different concentrations and duration of $HgCl_2$, streptocyclin and bavistin on per cent contamination and survival of the explants of *Chlorophytum borivilianum in vitro*

Code	Bavistin		Streptocyclin		$HgCl_2$		Contamination (%)	Survival (%)
	Conc. (%)	Time (min.)	Conc. (%)	Time (min.)	Conc. (%)	Time (min.)		
ST_0	-	-	-	-	-	-	100	0
ST_1	-	-	-	-	0.1	6	80	20
ST_2	-	-	-	-	0.1	7	60	40
ST_3	-	-	-	-	0.1	8	70	30
ST_4	-	-	-	-	0.2	6	80	20
ST_5	-	-	-	-	0.2	7	90	10
ST_6	-	-	-	-	0.2	8	100	0
ST_7	-	-	-	-	0.3	6	90	10
ST_8	-	-	-	-	0.3	7	100	0
ST_9	-	-	-	-	0.3	8	100	0
ST_{10}	-	-	0.2	60	0.1	7	60	40
ST_{11}	-	-	0.2	90	0.1	7	60	40
ST_{12}	-	-	0.2	120	0.1	7	60	40
ST_{13}	-	-	0.2	150	0.1	7	50	50
ST_{14}	-	-	0.3	60	0.1	7	60	40
ST_{15}	-	-	0.3	90	0.1	7	60	40
ST_{16}	-	-	0.3	120	0.1	7	50	50
ST_{17}	-	-	0.3	150	0.1	7	60	40
ST_{18}	-	-	0.4	60	0.1	7	60	40
ST_{19}	-	-	0.4	90	0.1	7	50	50
ST_{20}	-	-	0.4	120	0.1	7	40	60
ST_{21}	-	-	0.4	150	0.1	7	50	50
ST_{22}	-	-	0.5	60	0.1	7	60	40
ST_{23}	-	-	0.5	90	0.1	7	50	50
ST_{24}	-	-	0.5	120	0.1	7	60	40
ST_{25}	-	-	0.5	150	0.1	7	60	40
ST_{26}	0.2	60	0.4	120	0.1	7	40	60
ST_{27}	0.2	90	0.4	120	0.1	7	40	60
ST_{28}	0.2	120	0.4	120	0.1	7	30	70
ST_{29}	0.2	150	0.4	120	0.1	7	30	70
ST_{30}	0.3	60	0.4	120	0.1	7	40	60
ST_{31}	0.3	90	0.4	120	0.1	7	30	70
ST_{32}	0.3	120	0.4	120	0.1	7	30	70
ST_{33}	0.3	150	0.4	120	0.1	7	40	60
ST_{34}	0.4	60	0.4	120	0.1	7	40	60
ST_{35}	0.4	90	0.4	120	0.1	7	30	70
ST_{36}	0.4	120	0.4	120	0.1	7	10	90
ST_{37}	0.4	150	0.4	120	0.1	7	30	70
ST_{38}	0.5	60	0.4	120	0.1	7	40	60
ST_{39}	0.5	90	0.4	120	0.1	7	30	70
ST_{40}	0.5	120	0.4	120	0.1	7	40	60
ST_{41}	0.5	150	0.4	120	0.1	7	40	60

sterilization treatments from ST_{10}-ST_{25} involved the treatment of explants with streptocyclin to overcome bacterial contamination in different concentrations i. e. 0.2-0.5% for different duration of time along with 0.1% $HgCl_2$ (Table 1). The maximum survival (60%) was observed on ST_{20} when the explants were treated with 0.4% streptocyclin for 120 min along with 0.1% $HgCl_2$ for 7 min. As the survival rate was not satisfactory and fungal contamination continued, hence the explants were subjected to the next series of treatments for better survival percentage. The third set of treatments for sterilization from ST_{26}-ST_{41} involved fungicidal treatment of explants using a fungicide named bavistin in different concentrations (0.2-0.5%) along with 0.1% $HgCl_2$ and 0.4% streptocyclin to overcome systemic fungal contamination (Table 1). The maximum survival (90%) was obtained on ST_{36} when the explants were treated with 0.4% bavistin for 120 min with 0.1% of $HgCl_2$ and 0.4% streptocyclin.

The above treatments were performed by varying the concentration of one chemical and keeping the others at constant concentration.

Table 2. Effect of different growth regulators on the establishment of *Chlorophytum borivilianum in vitro*

Code	Growth regulators (mg/l)					Mean no. of shoots (days)				Renewal after 28 days (%)
	BAP	KIN	2,4-D	IAA	NAA	7th	14th	21st	28th	
E_0	-	-	-	-	-	-	-	-	-	0
E_1	0.5	-	-	-	-	0.0±0.00	0.3±0.19	1.1±0.11	1.8±0.22	40
E_2	1.0	-	-	-	-	0.3±0.19	1.0±0.00	1.6±0.11	2.1±0.11	40
E_3	1.5	-	-	-	-	0.6±0.11	1.1±0.11	2.0±0.19	2.3±0.00	50
E_4	2.0	-	-	-	-	0.9±0.11	1.2±0.11	1.9±0.11	2.9±0.11	80
E_5	2.5	-	-	-	-	0.6±0.11	1.1±0.11	1.6±0.11	2.2±0.11	75
E_6	3.0	-	-	-	-	0.2±0.11	1.0±0.19	1.4±0.22	1.9±0.29	50
E_7	-	0.5	-	-	-	0.2±0.11	0.9±0.11	1.2±0.11	1.2±0.11	25
E_8	-	1.0	-	-	-	0.4±0.11	0.9±0.11	1.4±0.11	1.8±0.29	40
E_9	-	1.5	-	-	-	0.8±0.22	1.1±0.11	1.6±0.11	2.2±0.11	40
E_{10}	-	2.0	-	-	-	0.7±0.00	1.0±0.00	1.6±0.11	2.1±0.11	50
E_{11}	-	2.5	-	-	-	0.4±0.22	0.9±0.11	1.3±0.19	1.9±0.11	50
E_{12}	-	3.0	-	-	-	0.1±0.11	0.8±0.11	1.2±0.11	1.3±0.00	40
E_{13}	-	-	0.5	-	-	0.3±0.19	0.8±0.22	1.2±0.29	1.7±0.19	20
E_{14}	-	-	1.0	-	-	0.7±0.00	1.0±0.00	1.6±0.11	2.0±0.00	25
E_{15}	-	-	1.5	-	-	0.8±0.22	1.1±0.11	1.4±0.22	2.3±0.19	50
E_{16}	-	-	2.0	-	-	0.7±0.00	1.0±0.00	1.6±0.11	2.1±0.11	40
E_{17}	2.0	-	-	0.1	-	0.9±0.11	1.4±0.11	1.9±0.11	2.9±0.11	50
E_{18}	2.0	-	-	0.2	-	0.6±0.11	1.1±0.11	1.7±0.00	2.4±0.11	50
E_{19}	2.0	-	-	0.3	-	0.9±0.11	1.3±0.19	1.9±0.11	2.8±0.11	75
E_{20}	2.0	-	-	0.4	-	0.6±0.11	1.3±0.19	1.7±0.00	2.3±0.00	75
E_{21}	2.0	-	-	-	0.1	0.8±0.11	1.3±0.00	1.8±0.11	2.8±0.11	40
E_{22}	2.0	-	-	-	0.2	0.6±0.11	1.1±0.11	1.6±0.11	2.3±0.00	50
E_{23}	2.0	-	-	-	0.3	0.7±0.19	1.1±0.11	1.7±0.00	2.3±0.19	75
E_{24}	2.0	-	-	-	0.4	0.6±0.22	1.0±0.00	1.7±0.19	2.2±0.11	75
E_{25}	-	1.5	-	-	0.1	0.7±0.00	1.1±0.11	1.8±0.11	2.2±0.11	25
E_{26}	-	1.5	-	-	0.2	0.6±0.11	1.1±0.11	1.8±0.11	2.3±0.19	40
E_{27}	-	1.5	-	-	0.3	0.7±0.00	1.2±0.11	1.8±0.11	2.4±0.11	50
E_{28}	-	1.5	-	-	0.4	0.7±0.19	1.2±0.11	1.9±0.11	2.6±0.11	75
E_{29}	-	1.0	1.0	-	-	0.2±0.11	0.8±0.11	1.1±0.11	1.2±0.11	25
E_{30}	-	1.5	1.0	-	-	0.8±0.11	1.1±0.11	1.8±0.11	2.3±0.00	50
E_{31}	-	2.0	1.0	-	-	0.7±0.00	1.0±0.00	1.6±0.11	2.1±0.11	40
E_{32}	-	2.5	1.0	-	-	0.7±0.19	1.0±0.00	1.6±0.11	2.1±0.11	50

± represents standard error.

Further, increase in the duration of bavistin and streptocyclin treatments resulted in browning of explants and decrease in survival percentage. Hence, from Table 1, it was concluded that the treatment of explant with 0.1% $HgCl_2$ for 7 min along with 0.4% streptocyclin for 120 min and 0.4% bavistin for 120 min was proved best as there was maximum survival (90%) among all the treatments given in this experiment.

These results are in accordance with Nakasha *et al.* (2016) and Desai *et al.* (2018). These researchers also used fungicides and antibiotics and $HgCl_2$ for the sterilization resulting in effective reduction of contamination in the explants. Desai *et al.* (2018) used 0.1% $HgCl_2$ for 8 min which was in accordance with present study. Savlon germicide with bavistin and streptomycin for shoot bases in safed musli. Kaushal *et al.* (2021) also used 0.1% $HgCl_2$ for surface sterilization of nodal segment explants taken from flower stalk. Acheampong *et al.* (2015) reported the higher effectiveness of $HgCl_2$ as compared to sodium hypochlorite (NaOCl) in resisting bacterial and fungal endogenous infections. This was one of the reasons of using $HgCl_2$ as a sterilant in the present study. On the other side, some researchers stated that when there was an adverse high exposure to agents like $HgCl_2$ it led to death of explants because of the mercury that was phytotoxic (Desai *et al.*, 2018). The findings outlined that the explants taken from underground parts are highly contaminated and gave poor response and failed to survive. The reports also say that making underground parts free from contamination is very laborious and a demanding task. But in the present findings, in making the explant contamination free with 90% rate was succeeded. The different studies carried by various researchers included

different sterilizing agents for the purpose of disinfection of explants of diverse plants.

Similar results were reported in other plants as Lakshmi *et al.* (2021) found 0.4% bavistin more effective for the sterilization of *Ruellia tuberosa*. Similarly, Khanchana *et al.* (2019) reported 0.5% bavistin for 2 h and 5% NaOCl for 10 min to disinfect *Polianthes tuberosa*. Sterilizing agent like sodium hypochlorite solution was also outlined to be used by many researchers like Khanchana *et al.* (2019) who reported effective disinfection of *Polianthes tuberose* explants with 5% NaOCl for 10 min, while Zinabu *et al.* (2018) used lower concentration of sodium hypochlorite solution (1.5%) for 15 min for surface sterilization *in vitro* propagation of elite *Ensete ventricosum*.

All the explants used in the present study were having the potential for induction of shoots on the medium used. There was no sign of shoot initiation in the absence of growth regulators. The explants, after surface sterilization, were cultured on MS basal medium fortified with different concentrations and combinations of plant growth regulators viz., 6-benzyl amino purine (BAP), kinetin (KIN), 2,4-D, IAA and NAA. Data were recorded for percentage response of explants and standard error for a specific time interval was calculated (Table 2).

Various treatments (from E_1-E_{32}) were used for establishment of sterilized explants using varied plant hormones which were either used alone or in combinations with other hormones. Firstly, the hormone which was used alone in MS medium in this experiment was BAP (E_1-E_6) whose concentration range was 0.5-3.0 mg/l for shoot initiation and subsequent proliferation of shoots. The MS medium supplemented with 2.0 mg/l BAP alone was found best among various concentrations of BAP alone used in this experiment of shoot initiation. The maximum (80%) response was observed on 2.0 mg/l BAP with mean number of shoots initiated as 2.9. The decrease and increase in BAP concentrations resulted in shoot number reduction. KIN was the hormone that was used alone for initiation of shoots in different concentrations ranging from 0.5-3 mg/l in the MS media (E_{15}-E_{20}). The shooting response obtained by addition of KIN (1.5 mg/l) alone reduced to 50% (E_{17}), whereas there was not much change in the number of shoots initiated. Thirdly, 2,4-D was the hormone used alone in the concentration of 0.5-2.0 mg/l (E_{25}-E_{28}). The best-found concentration among those used was 2.0 mg/l on which the per cent response was 75% which was better than KIN but the number of shoots initiated was less (E_{28}). The above three cytokinins thereafter were used in combination with two auxins, namely, IAA and NAA, both in the concentration of 0.1-0.4 mg/l separately. When BAP was used in constant concentration of 2.0 mg/l in combination with varying concentrations of IAA (E_7-E_{10}), the per cent response was 50% maximum on E_{10}. When BAP was combined with NAA (E_{11}-E_{14}), the maximum response was 50% on $E_{11.}$ When KIN was used as 1.5 mg/l with NAA in varying concentrations (E_{21}-E_{24}), the response was 75% on E_{23} and E_{24}. When 2,4-D (1.0 mg/l) was kept constant and used in combination with KIN (1.0-2.5 mg/l) in varying concentrations in the MS media (E_{29}-E_{32}), the per cent response was 50%. Hence, from Table 2, it was outlined that among all the hormones used either alone or in combination in different concentrations, the concentration of 2.0 mg/l of BAP was found to be best (E_4) with a maximum 80% response and a maximum number of shoots (2.9) was also initiated on the same concentration of BAP for the establishment of *in vitro* cultures of safed musli using basal plate as explant. The present results are in accordance with the findings of Khatri *et al.* (2019) who stated that among all the growth hormones tested, the only hormone which was reported to be superior for inducing shoot was BAP alone. BAP was also tested by other researchers in various plants including *Bambusa arundinacea, Clitoria ternatea, Dalbergia latifolia, Gymnema sylvestre, Sarcostemma brevistre* and *Ulmus parvifolia*.

Many other studies done by various scientists were also in support of results obtained in the present study. It was stated that the hormone responsible for shoot formation was BAP as BAP released lateral buds from dormancy. Kaushal *et al.* (2021) supported the effectiveness of BAP over KIN for shoot induction in safed musli. In many reports on micropropagation of safed musli various researchers used high concentration of BAP (5 mg/l) alone or in combination for best results. Kaushal *et al.* (2021) in safed musli and Patil *et al.* (2021) in *Lilium candilum* (belonging to the family of safed musli) used BAP in combination with NAA for better results. The present study focused on using less concentration of hormones for

effective results. The results obtained in this experiment were comparable with those obtained by Desai *et al.* (2018) who initiated shoot buds on 2.0 mg/l BAP and found it more effective as compared to KIN. El-Shafey *et al.* (2019) also reported 2.0 mg/l BAP as best for shoot induction in *Rumex pictus*. So, it can be concluded that the media enriched with 2.0 mg/l of BAP can be effectively used for optimization of media combination for shoot induction using basal plate as an explant (Fig. 1).

CONCLUSION

The pre-requisite for successful *in vitro* cultures is the efficient sterilization protocol that supports the appropriate development of the explants in an aseptic environment. To overcome contamination of underground parts of *Chlorophytum borivilianum*, the antibacterial and antifungal agents should be used in combination for obtaining aseptic cultures. The treatments used in the present study resulted in the evolution of efficient protocol for

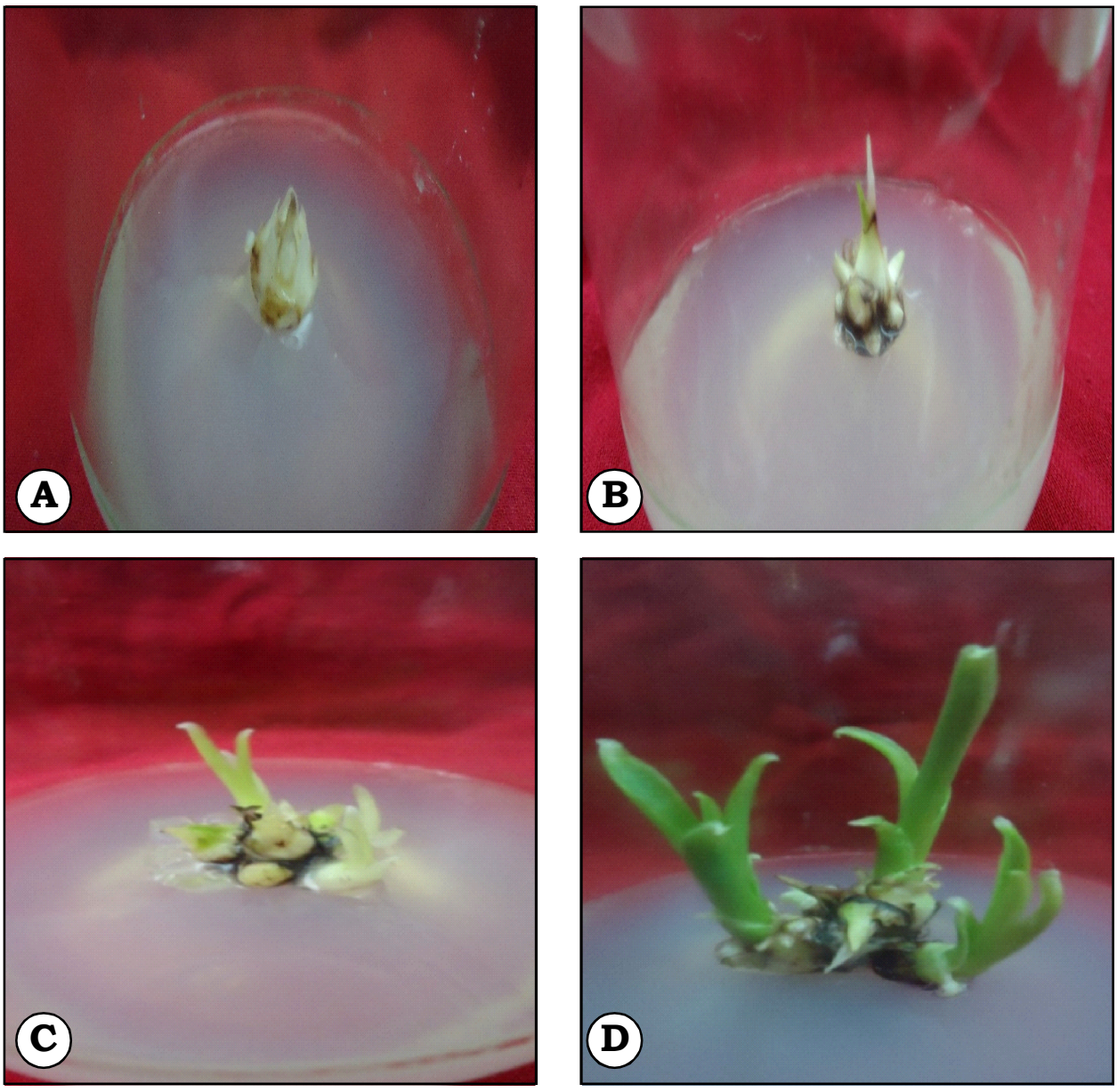

Fig. 1. *In vitro* establishment in *Chlorophytum borivilianum* using basal plate after fix interval of time of culture. (A) Basal plate explant just inoculation, (B) after 14 days, (C) after 21days and (D) after 28 days.

initiation of safed musli cultures with basal plate explants. A successful attempt was made in achieving *in vitro* sterilization with maximum survival of 90% and minimum contamination of 10% when the explants were treated with 0.1% $HgCl_2$ for 7 min along with 0.4% streptocyclin for 120 min and 0.4% bavistin for 120 min among all the treatments. Also, a high-frequency initiation of shoots from basal plate explant of *C. borivilianum* was obtained by manipulating the growth regulators. The concentration of 2.0 mg/l of BAP was found to be best with maximum 80% regeneration response and maximum number of shoots (2.9) for establishment of *in vitro* cultures of safed musli using basal plate as explant. Efficient regeneration protocol of this medicinally important plant would aid in the development of protocols for genetic manipulation as well as selection and propagation of the genotypes that are acceptable for use in pharmaceuticals.

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