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Effect of various carbon sources and gelling agents on *in vitro* shoot multiplication of C*hlorophytum borivilianum*: A medicinal plant with golden roots

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Abstract
Chlorophytum borivilianum (safed musli) is a medicinal plant with golden roots having cure for many health issues. In the present research work, the *in vitro* responses for shoot multiplication employing distinct carbon sources and gelling agents in the media of *Chlorophytum borivilianum* were assessed in two different experiments. The micropropagated plants were used for the experiments. The five different carbon sources like crystal sugar, fructose glucose, maltose and sucrose were employed in the first experiment. In the second experiment, the media was gelled with five different gelling agents like agar, agarose, gelrite, guar gum and isabgol. The shoots cultures were observed at regular time interval for the time period of 28 days for shoot multiplication. Among these carbon sources and gelling agents, sucrose and gelrite showed the maximum shoot multiplication (15.9 and 16.0 mean no. of shoots), respectively. The other carbon sources and gelling agents were not able to evoke better response. So, gelrite would be suggested as most beneficial and economical alternative tested in the experiment helping in the reduction of the cost.

Keywords: *Chlorophytum borivilianum, in vitro,* gelling agents, carbon source, medicinal plant

Introduction
Chlorophytum borivilianum Sant. et Fernand, commonly known as 'Safed Musli', is reported as an endangered aphrodisiac herb with a short life span of 90 to 100 days widespread across the Aravallis of Rajasthan, India. It belongs to the family Asparagaceae, earlier included in the family Liliaceae (Shinde *et al.*, 2016) [19]. It is valued for its thick storage roots possessing aphrodisiac properties and forms an essential ingredient of herbal tonics outlined in ayurvedic system of Indian medicine. It is cited in 'Atharva Veda' as one of the divine herbs and is expressed as "Wonder Drug" because of its natural therapeutic properties for vigor and strength and offer healing for many health disorders. The natural habitats in India include Andhra Pradesh, Assam, Bihar, Eastern Ghats, Himalayas, Gujarat, Madhya Pradesh (Desai *et al.*, 2018) [2]. The thick dried roots of the plant which are meant for storage possess spermatogenic properties making usable as a viagra substitute. The high content of saponin holds the responsibility for the therapeutic capabilities (Desai *et al.*, 2018) [2]. The plant is also used to cure diabetes, arthritis, and for increasing immunity also (Halder and Bhattacharjee, 2018) [7].

The natural regeneration of the herb is by making use of its tuberous roots which have become scanty in nature due to excessive collection of its wild material and ruinous methods of harvesting. The germination through seed is very low (Bharti *et al.*, 2017) [1]. Copius attempts for its cultivation and restoration are accompanied by poor seed set and less vegetative multiplication ratio making the species lie under the endangered category (Halder and Bhattacharjee, 2018) [7]. The sole propagule of the plant is the tuberous roots that can be used for its annual commercial cultivation resulting in the scarcity of planting material on large scale. Micropropagation is the technology which has proven to be most efficient as it can bridge the gap of supply and demand and expedite the production of plants that are pathogen-free in limited time period providing genetically similar planting material. Micropropagation includes rapid production of high ranking clones maintaining their uniformity and producing disease free plants. Different researches have been done on Micropropagation of *Chlorophytum borivilianum* dealing in the effect of different growth regulators and several other factors for development of efficient protocols. Several other factors also require scaling up and optimization for a more efficient protocol.

The present research involves the assessment of carbon sources and gelling agents on *in vitro* multiplication of shoots of *Chlorophytum borivilianum* with special emphasis on attaining high regeneration. The gelling agents are used for imparting semi-solid consistency to the otherwise liquid nutrient medium (Smith and Spomer, 1995) [20]. The most commonly used gelling agent is Agar (Puchooa *et al*., 1999) [17] which is derived from seaweeds called agarophytes (Marinho-Soriano and Bourret, 2003) [11]. The shortcoming lies in its standardization because of the variability in its quality and purity among different brands along with the differences lying in several batches of same brand. Further, the cost of the highly purified grades is a big-budget (Huang *et al*., 1995) [8]. Gelling agents are not the static medium component but can impact the accessibility of nutrients and water to cultured tissues and balance the headspace composition (Ziv, 1991) [22]. Optimization of these factors would contribute in the large scale production of *Chlorophytum borivilianum* through Micropropagation.

Materials and Methods

Planting material

The mother plants of *Chlorophytum borivilianum* were collected from the medicinal section of Chaudhary Charan Singh Haryana Agricultural University, Hisar Haryana. and were maintained in the greenhouse of the Centre for Plant Biotechnology (now known as the Department of Molecular Biology, Biotechnology, and Bioinformatics),. These plants were employed as explants and cultured *in vitro* on MS medium (Murashige and Skoog, 1962) [12]. The maintenance and multiplication of cultures was done by sub-culturing at regular intervals on the fresh MS medium with the same composition.The *in vitro* produced cultures were used for experiments in the present study.

The cultures were incubated in controlled conditions in growth chamber at 25 ± 2 ℃ temperature and light intensity of 100 µEM-2 sec-1 (1000 lux) under a photoperiod regime of 16 hours light and 8 hours dark cycle with the use of florescent tubes. The shoots from *in vitro* grown plants were employed as the explants in the present experiment work.

Glassware and Chemicals

The glassware used in the present study was from Borosil India Limited. The chemicals used in the research experiments were highly purified with analytical grade and bought from Hi-Media Limited, Imperial Bioscience and Merck.

Sterilization of glassware

The glassware like beakers, flasks, measuring cylinders, petridishes, test tubes, pipettes, jam bottles etc. were initially washed thoroughly with a liquid detergent (Teepol) followed by washing under running tap water to remove traces of detergent, finally rinsing with double distilled water prior each use. The culture bottles used in the study were dried in oven at 60-80oC temperature before use for 3-4 hours. The forceps, scalpel, petridishes and other glassware were autoclaved for 20 min. at 121°C (15 lbs./inch^2) before every use for sterile handling and transfer of cultures. The forceps, scalpels, scissors were further sterilized by flaming till red hot and were kept in spirit inside laminar air flow chamber for further usage.

Media Preparation and sterilization

The micropropagation experiments of all the recent research investigation were carried using Murashige and Skoog's medium (1962) [12]. The 1N HCl or 1N NaOH were used or added dropwise to adjust the pH of the medium to 5.8. After preparation, medium was dispensed in the culture bottles and the screw caps of culture bottles were tightly closed. The culture bottles containing medium were then autoclaved at 121 °C temperature and pressure of 15 lbs./inch2 for 20 min. After sterilization of the medium in autoclave, the media containing vessels were stored at the room temperature for further usage.

Inoculation of explants with the essential culture conditions

The inoculation of explants is carried inside the Laminar air flow cabinet. The UV light of the laminar airflow was switched on before each usage for 15-20 min. followed by disinfection of the floor of the cabinet with spirit or 70% ethanol dipped cotton. All the equipment (forceps, scalpel and scissor) used during inoculation were flame sterilized for decontamination with the use of spirit and they were kept immersed in spirit while working in the laminar with a lightened burner with occasional sterilization of the equipment till red hot. The cabinet floor was wiped properly with 70% ethyl alcohol repeatedly during the working. The explants were trimmed off and then were carefully inoculated on the prepared nutrient medium in the culture vessels with the help of sterilized equipment like forceps, scalpel, scissor etc. under aseptic conditions in laminar air flow cabinet.

Experimental design

In the first experiment, five forms of carbon sources were compared: crystal sugar, fructose glucose, maltose and sucrose (30g/l each). The MS medium was supplemented with agar (8g/l) and BAP (2.0mg/l). In the second experiment, five different gelling agents i.e. agar (8g/l), agarose (6g/l), gelrite (2g,l), guar gum (40g/l) and isabgol (24g/l). The MS medium was supplemented with sugar (30g/l) and BAP (2.0mg/l).

Data collection and statistical analysis

The data was collected for particular time duration of shoot multiplication i.e., on 7th, 14th, 21st and 28th day, number of shoots regenerated per plant was analyzed statistically for the selection of best medium (Table 1& 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 Discussions

Sugar is known to be an essential component of medium contributing to the high production cost followed by the gelling agent. The sole source of carbon used in the media of plant tissue culture is sugar. The explants were cultured on MS basal medium fortified with 6-benzyl aminopurine (BAP). In this experiment, five different types of sugars were used as shown in the table 1. All the five carbon sources were added to the MS media in quantity of 30g/l. Among the various carbon sources tested in the present study of micropropagation of safed musli, the maximum shoot multiplication was observed on sucrose followed by crystal sugar, glucose, maltose and fructose. The present results are in accordance with the findings of Dave *et al*. (2003) [3], Nhut

et al. (2011) [14], Rizvi *et al*. (2010) [18] and Gerdakaneh *et al*. (2009) [5]. The maximum length of the shoot was observed on maltose followed by glucose, fructose, sucrose and crystal sugar. Hence, from the table 1, it was outlined that among all the carbon sources tested sucrose was found to be best with a maximum number of shoots (15.9) initiated on the same concentration of BAP for the multiplication of *in vitro* cultures of safed musli.

Table 1: Effect of different types of carbon sources on average number of shoot proliferation after a fixed time interval

Different carbon sources (30gm/l)	Average number of shoots ± standard error on			
	7th day	14th day	21th day	28th day
Crystal Sugar	5.1 ± 0.11	9.6 ± 0.11	12.2 ± 0.22	14.9 ± 0.56
Glucose	4.4 ± 0.11	7.8 ± 0.11	8.7 ± 0.33	9.8 ± 0.29
Fructose	3.6 ± 0.11	4.4 ± 0.29	5.1 ± 0.68	8.4 ± 0.29
Maltose	4.8 ± 0.22	7.7 ± 0.19	8.6 ± 0.11	9.3 ± 0.33
Sucrose	6.1 ± 0.11	10.0± 0.69	12.4 ± 0.11	15.9 ± 0.59

± represents standard error

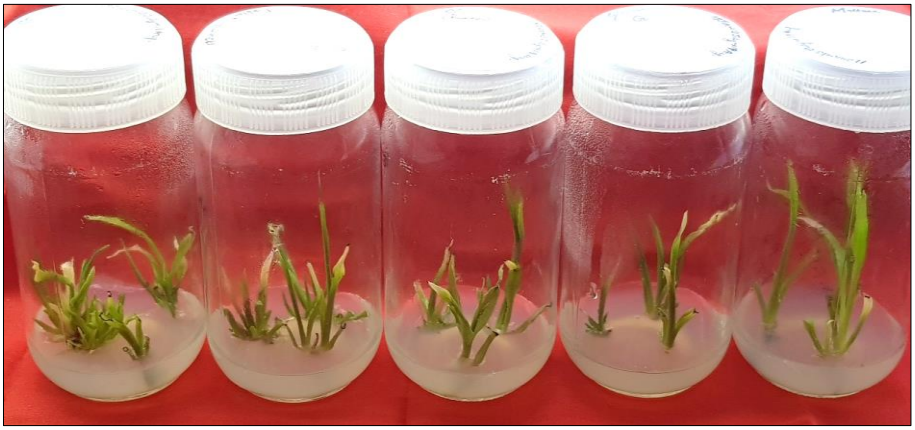

Fig 1: The effect of different types of energy sources on average number of shoots of *Chlorophytum borivilianum* (1) Sucrose (2) Crystal sugar (3) Glucose (4) Maltose (5) Fructose

The search for novel substitutes for decreasing the cost have been practiced from time to time for protocols for micropropagation of various species of plants. One of the most commonly used gelling agent in media is Agar -agar which was introduced for the first time by Robert Koch, Peltzar *et al*. (1986) for culturing bacteria and was later on employed by White (1939) [21] in plant tissue culture. Agar contributes about 70% to the total cost of the media (Prakash, 1993) [16]. Gelrite derived by microbial synthesis is the second most common and high price gelling agent used after agar. It is transparent and provides better *in vitro* regeneration and growth.

The different gelling alternatives were used in different quantities for the substitution of Agar in the MS medium for the micropropagation of safed musli in present experiment as shown in table 2. Among the five different gelling agents used in the present study, the medium gelled with gelrite was observed to show better results than agar followed by agarose, isabgol and guar gum. These results are in harmony with Dave *et al*. (2003) [3] and Kumar *et al*. (2010) [10]. In *Rosa damascena*, Kumar *et al*. (2003) [9] also observed the beneficial effects of gelrite over agar on multiplication of shoots and their growth. Gopal *et al*. (2008) [6] reported that agar caused water stress in potato cultures. In some previous studies, inhibitory effect of agar has been indicated on the growth in some cultures by Debergh and Maene (1984) [4] and Nairn (1988) [13]. The maximum length was observed in case of agar followed by gelrite, isabgol, agarose and guar gum.

Table 2: Effect of different types of gelling agents on average number of shoot(s) multiplication after a fixed time interval

Different Gelling agents (gm/l)	Average number of shoots ± standard error on			
	7th day	14th day	21th day	28th day
Gelrite (2)	6.9 ± 0.29	11.2 ± 0.40	13.2 ± 0.22	16.0 ± 0.51
Agar-agar (8)	6.3 ± 0.19	9.3 ± 0.51	11.3 ± 0.19	13.0 ± 0.19
Agarose (6)	5.7 ± 0.19	8.1 ± 0.95	10.0 ± 0.51	11.6 ± 0.29
Guar gum(40)	3.2 ± 0.11	4.0 ± 0.51	5.2 ± 0.48	6.6 ± 0.40
Isabgol (24)	3.8 ± 0.11	5.9 ± 0.73	7.2 ± 0.59	8.4 ± 0.22

± represents standard error

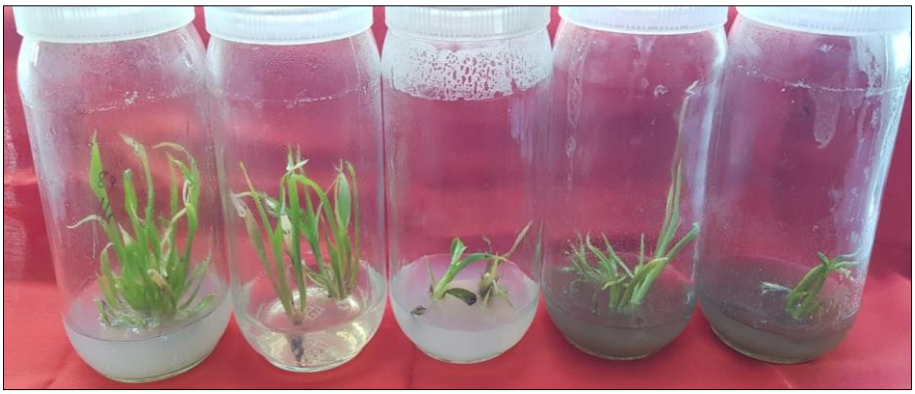

Fig 2: The effect of different types of gelling agents on average number of shoots of *Chlorophytum borivilianum* (1) agar-agar (2) Gelrite (3) Agarose (4) Isabgol (5) Guar gum

Conclusion

The precondition for any *in vitro* experiment is to maintain an aseptic environment throughout the process. It involves sterilization of explant to be inoculated on the prepared medium and standardizing an efficient protocol for disinfecting the explant using various sterilizing agents. The explants used in the present study are taken from the *in vitro* cultures which were produced in aseptic environment. The experiments conducted in the present study resulted in finding of different suitable and cost effective alternatives of carbon sources and gelling agents for their appropriate use in *in vitro* studies. A successful attempt was made in achieving *in vitro* multiplication of safed musli using sucrose as a carbon source and gelrite as a gelling agent with maximum shoot multiplication with mean no. of shoots as 15.9 and 16 respectively among all others tested in the experiments. This finding in this medicinally important plant would aid in the cost effective and appropriate use of carbon sources and gelling agents during *in vitro* research studies.

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