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In vitro induction of tuber formation for the synthesis of secondary metabolites in *Chlorophytum borivilianum* Sant. et Fernand

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Chlorophytum borivilianum **Sant. et Fernand an endangered herb is valued for several medicinal properties in its tuberous roots. An efficient and reproducible method for inducing *in vitro* tubers from stem disc explant has been developed. Stem disc possessing shoot buds were induced to develop multiple shoots in Murashige and Skoog (MS) medium supplemented with vitamins, 3% sucrose, 0.8% agar and 5 mg/L benzylaminopurine (BAP). Healthy regenerated shoots were rooted in MS basal medium containing 3% sucrose (w/v), 0.8% agar supplemented with indole-3-acetic acid (2 mg/L). On further sub culturing, the maximum percentage of tuber formation was obtained in growth hormone free half (½) MS liquid media supplemented with vitamins and 1.5% sucrose after 8 to 9 weeks. The saponin contents of the *in vitro* and *in vivo* raised tubers were qualitatively and quantitatively analyzed by high-performance liquid chromatography (HPLC) and liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). There was a significant similarity in the saponins in both tubers. The *in vitro* raised tubers showed similar high metabolite content than *in vivo* grown tubers which is required for medicinal applications. The rooted plantlets were transferred to peat and sand (2:1) with more than 80% success. This is the 1st report of *in vitro* tuber formation and secondary metabolites screening of *C. borivilianum*. This work will give a strong impetus to the pharmaceutical and neutraceutical sectors.**

Key words: *Chlorophytum borivilianum*, tuber formation, saponin, liquid medium, high-performance liquid chromatography (HPLC).

INTRODUCTION

Chlorophytum borivilianum Sant. et Fernand commonly known as Safed musli is a traditional rare Indian medicinal herb having many therapeutic applications in Ayurvedic, Unani, Homeopathic and Allopathic medicine system. It is an herbaceous plant with fasciculated tuberous root found naturally in forests and its shoots can be seen during the rainy seasons (Kothari and Singh, 2003). Research studies on *Chlorophytum* conducted in India and elsewhere indicate that saponins (viz. neohecogenin, neotigogenin, stigmasterol and tokorogenin) are

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Abbreviations: BAP, 6-Benzylaminopurine; **IAA,** indole-3 acetic acid; **MS,** Murashige and Skoog.

responsible for medicinal properties (Jat and Bordia, 1990). Safed musli is among the few medicinal plants witnessing steady growth in pharmaceutical, phyto-pharmaceutical and nutraceutical products (Debnath et al., 2006, 2007; Thakur et al., 2009). Due to the many therapeutic applications and several bioactive compounds, *C. borivilianum* is also called 'The white gold for biopharmaceuticals and neutraceuticals' (Thakur et al., 2009).

It contains steroidal and triterpenoidal saponins, sapogenins, fructans and flavonone glycosides, which are powerful uterine stimulant. Dried roots of *Chlorophytum* contain 42% carbohydrate, 80 to 89% protein, 3 to 4% fiber and 2 to 17% saponin (Wagle et al*.,* 2000). It is useful in curing impotency with spermatogenic property and is considered as an alternative 'Viagra. It is a rich source of over 25 alkaloids, vitamins, proteins, carbohydrates, steroids, saponins, potassium, calcium, magnesium, phenol, resins, mucilage and polysaccharides with high content of simple sugars mainly sucrose, glucose, fructose, galactose, mannose and xylose (Ramawat et al*.,* 2000; Debnath et al., 2006, 2007; Thakur et al., 2009). Due to their high medicinal value, several medicinal herbs are being indiscriminately collected before they could reach phenological maturity and vegetative regeneration capacity (Biswas et al., 2003). This has led to the depletion of natural source of several valuable plants like Safed musli. The restricted distribution and indiscriminate over exploitation of this plant coupled with low seed set and viability and poor seed germination rates has made its status rare in the wild (Debnath et al., 2006).

Novel propagation techniques like tissue culture can play an important role in the rapid multiplication of elite clones and germplasm conservation of *C. borivilianum.* Rapid micropropagation procedure for this endemic medicinal plant have been developed from various explant in solid (Purohit et al., 1994a, 1994b*,* 1994c; Dave and Purohit, 2002; Dave et al., 2004; Debnath et al., 2006, 2007) and liquid media (Rizvi et al., 2007). The transfer of *in vitro* raised plantlets into *ex vitro* conditions are most critical factors for higher production cost of the micropropagation process (Mathur et al., 2008). Tissue culture-based propagation technique of *C. borivilianum* have limited success because of high mortality at trans-plantation stage (Purohit et al., 1994a, 1994b*,* 1994c; Rhizvi et al., 2007; Arora et al., 1999; Dave et al., 2003, 2004). Micro-propagation of the Chlorophytum via vegetative buds or somatic embryogenesis, suffered from poor multiplication rate, low establishment in soil, cytological instability and early loss of regeneration potential of the *in vitro* cultures (Dave et al., 2003). To ameliorate this problem, a successful attempt has been made by the authors to induce the *in vitro* tuber of this valuable medicinal and nutritional herb. Previous tissue culture reports on this plant showed normal root development from the regenerated shoot buds. In the present study, shoot proliferation, growth and multiplication as well as root formation was studied with special attention towards for-mation of *in vitro* tubers and their secondary metabolites screening.

MATERIALS AND METHODS

Induction of *in vitro* tuber formation

Actively growing plants and certified tubers of *C. borivilianum* were collected from Jawaharlal Nehru Krishi Vishwavidyalaya (JNKVV), Jabalpur and maintained in the botanical garden. *In vitro* cultures were established from the stem disc explants. These explants were excised from the growing shoots. Sterilized stem discs of *C. borivilianum* were cultured on MS medium (Murashige and Skoog, 1962) with vitamins, 3% sucrose, 0.8% agar supplemented with different concentration of BAP (0.5 to 5.0 mg/L) and Kinetin (0.5 to 5.0 mg/L). All cultures were incubated at 27 ± 2°C under light provided by white fluorescent tubes giving the intensity of about 2000 to 2500 lux for 16 h/day. All the experiments were repeated twice and had 12 replicates with single explants. Regenerated and elongated shoots were excised and cultured on MS medium (0 MS, ½ MS, ¾ MS) with vitamins, 3% sucrose, 0.8% agar fortified with auxins viz. IAA and indole-3-butyric (IBA) in various concentration (0.5 to 5.0 mg/ L) for rooting. The rooted plantlets were then transferred on filter paper wick in growth hormones free ½ MS liquid medium with vitamins, and 1.5% sucrose for root hair development and tuber induction. The effects of half strength of liquid medium on rooted plantlets of *C. borivilianum* were also studied by subculturing the explants after an interval of three weeks for successive three times on same medium. Plantlets with well-developed roots were transferred to green house and cultured in a mixture of peat and sand (2:1). Plantlets were washed thoroughly with distilled water and transferred to pots. Initially, high humidity was maintained by covering with white plastic bags. Plastic bags were removed after 10 days and survival percentage was recorded one month later.

Qualitative and quantitative determination of saponins from *in vitro* and *in vivo* grown tubers

Extraction and separation of saponins

Saponins were extracted from vegetative propagated and *in vitro* raised tubers and analyzed qualitatively and quantitatively (Thakur et al., 2009). Samples (crude saponins) of *in vivo* and *in vitro* raised tubers with the standard (Saponin from Sigmaaldrich - 47036) were loaded on the pre-coated TLC plates Silica gel 60 F_{254} (Merck). Mobile phase chloroform: methanol: water (60:30:10 v/v/v) was used for chromatographic separation. 50 µl of standard and samples were loaded and the plates were developed with the spraying reagent (5%, H_2SO_4) to locate the spots.

HPLC analysis of saponins

Spots were scraped from TLC plates and eluted in deionized water to analyze further using Binary gradient HPLC (Dionex) with PDA Detector (Ultimate 3000). A mixture of Millipore filtered (0.2 µl), degassed, solvent system Acetonitrile: water (50:50 v/v) was used for the elution of saponins. The flow rate of 0.2 ml/min was maintained throughout the run time of 30 min and the column used was Acclaim 120®C18 column (4.6 x 250 mm, 5 µm). Saponins were scanned at 190 nm and the peak area was calculated according to the formula ½ bh, where b = breath of peaks, h = height of peaks. Saponins of *C. borivilianum* were detected with the help of retention time compared with the standard (Saponin from Sigmaaldrich - 47036).

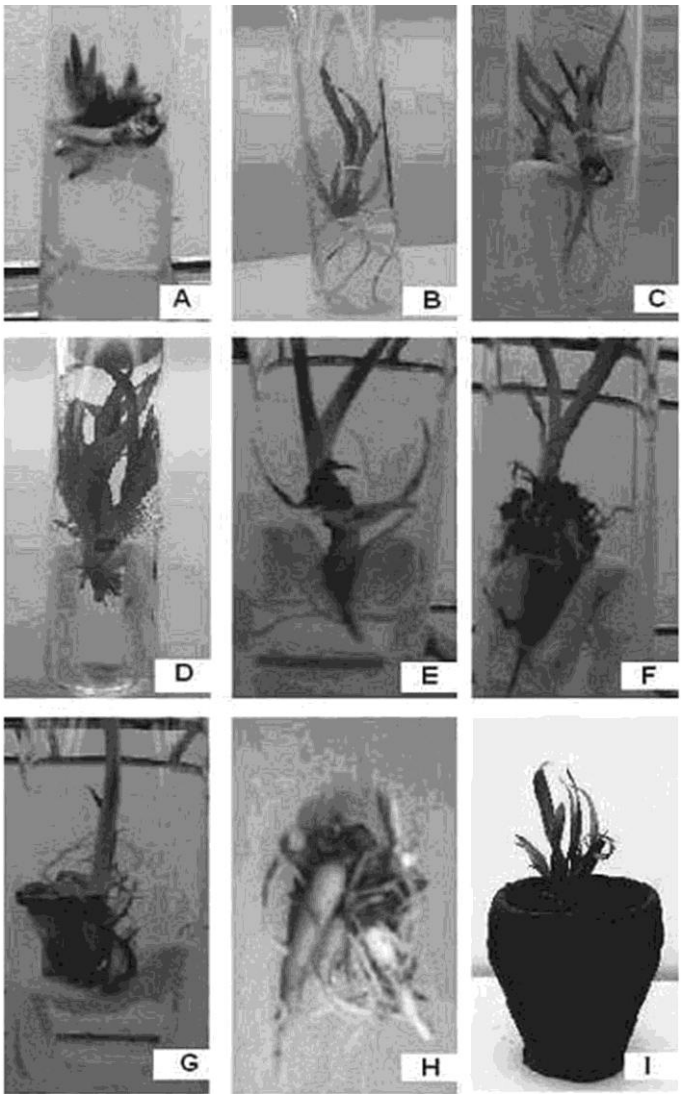

Figure 1. *In vitro* induction of tubers of *C. borivilianum* **A.** Shoot bud initiation on MS medium supplemented with 5 mg/L BAP from stem disc explant after 21 days of incubation; **B.** Rooting observed in MS medium supplemented with IBA at 2.0 mg/L; **C.** Root thickening and root elongation on ½ MS medium; **D.** The rooted plantlets on subculturing on ½ MS liquid medium showing the appearance of small and thick root hairs after three weeks; **E.** Rooted plantlets with root hairs and tuber formation after 2 weeks of growth; **F.** Plantlets after three subculturing with 4 tubers after 3 weeks of growth; **G.** The tubers with root hairs after 4 weeks of growth in 3rd subculture; **H.** The tubers after 6 weeks of *in vitro* growth; **I.** 1 month old plant after transferring in pots.

LC-ESI-MS analysis of saponins

LC-ESI-MS analyses were carried out for both samples with standard. The samples were dissolved in methanol: chloroform mixture (60:30 v/v) and filter through 0.2 µm MILLEX-GV syringe driven filter. Mass spectrums were obtained by the Liquid Chromatography Electrospray Mass Spectrometry (Thermo Finnigan LCO Advantage max ion trap mass spectrometer). The column was thermo-ODS-2 50 x 4.6, 5 µm and solvent was eluted as given gradient program at 500 µl/min. The 25 µl sample was introduced into the ESI source through Finnigan surveyor autosampler. The mass spectra were scanned in the range 150 to 1000 Da and the maximum ion injection time was set at 200 ns. Ion spray voltage was set at 5.3 KV and capillary voltage 40 V. The MS scan run up to 35 min. The data reduction was performed by using Xcalibur 1.4 SRI. The qualitative analysis was done on the basis of common ion peak available in the spectrum of both the samples and standard.

RESULTS AND DISCUSSION

Induction of *In vitro* tuber formation

Stem disc explant of *C. borivilianum* transferred to MS medium supplemented with 5 mg/L BAP gave the maximum shoot proliferation (Figure 1A) and shoot bud initiation (14.83 shoots) as compared to different concentration of BAP (Figure 2). BAP at 5 mg/L after sub-culturing produced highest significant value of shoot numbers per explant (14.83) with no callusing in the cultures (Purohit et al., 1994b). Thus, there was no chance of genetic variability. Kn showed no increment in shoot number. The observations revealed that different concentration of cytokinins influenced the shoot length of the *in vitro* growth of *C. borivilianum*. It was further observed that Kn at 5 mg/L gave the optimum shoot length (4.11 cm) and BAP at 5 mg/L gave the maximum (4.51 cm) shoot length (Figure 2). Contrary to the reports of Purohit et al. (1994a), the interaction of BAP and Kn showed no significant results in shoot proliferation and shoot elongation. On sub-culturing, shoot proliferation and shoot elongation was not affected by different strengths of MS media (Figure 3) and maximum shoots (4.33) were observed in the full strength MS media but ½ MS medium showed significant shoot length (7.51 cm) elongation.

Auxins showed a significant effect on supplementation in MS medium on the root initiation, proliferation and growth. For MS medium supplemented with IBA at 2 mg/L, number of roots/explant were less (3.58) compared to IAA at 2 mg/L (3.67) (Figure 1B). The results obtained with the studies on combination of IBA and IAA supplementation in MS medium were not significant (Figure 4). Observations on the effect of different strengths of medium concentration (Full MS, 1/2 MS, ¾ MS) on root number/explant and root length/explant revealed that maximum number of roots were obtained in ½ MS medium (6.42) (Figure 1C), optimum response in full MS (3.67) and minimum in ¾ MS (3.08). The maximum lengths of roots were also obtained in ½ MS medium (7.24 cm). Handling of liquid medium is easier in comparison to solid medium (Rhizvi et al., 2007) and so the response of rooting was observed using liquid media.

The rooted *Chlorophytum* plantlets on subculturing on ½ MS liquid medium with the help of filter wick showed the appearance of small and thick root hairs after three weeks (Figure 1D). Successive sub culturing on same (½ MS liquid) medium after three weeks time intervals

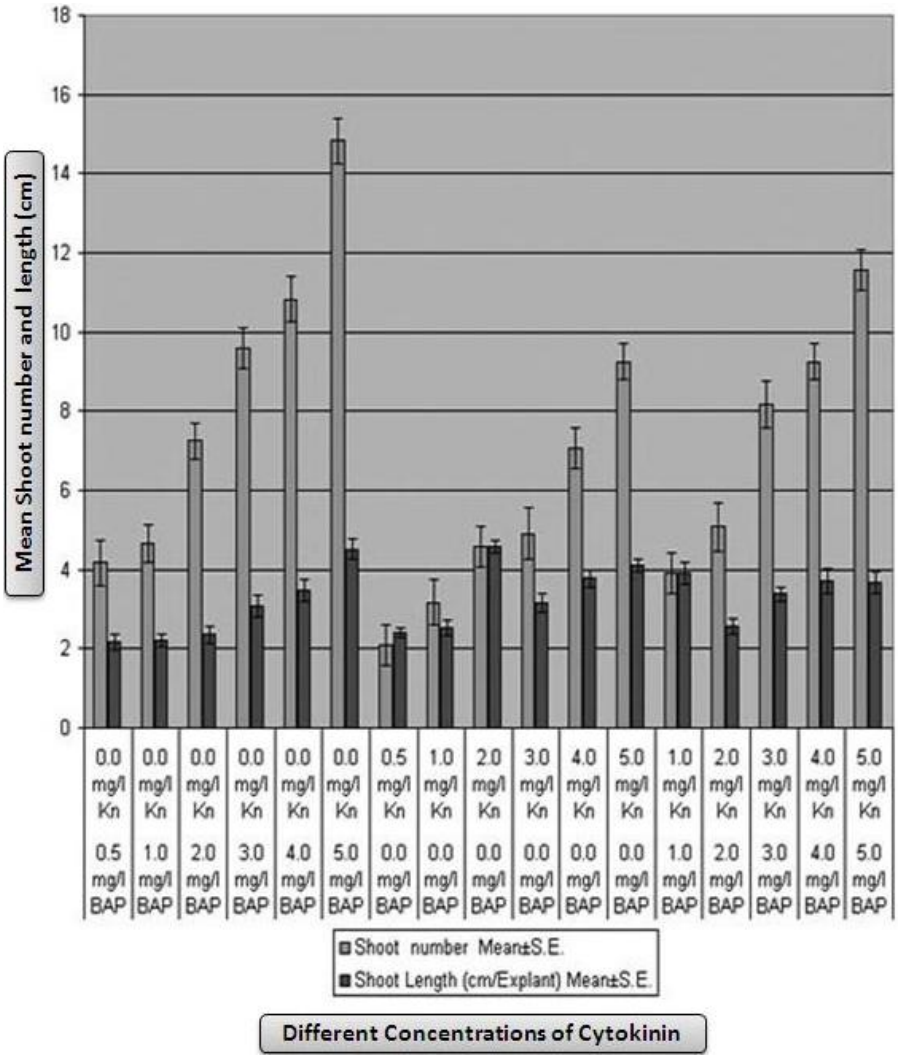

Figure 2. Effect of different concentration of BAP and Kn on average shoot number and shoot length/explant of *C. borivilianum.*

showed induction of more vigorously growing root hairs and on further subculture, the rooted plantlets generate tubers (Figure 1E). No growth regulators were added to avoid the potential modification of the response to the environmental stimuli. Each plantlet after three sub culturing (Table 1) generated 3 to 4 tubers of length ranging from 3 to 4 cm (Figure 1F, G). The tubers with root hairs were observed after 3rd subculture only. The fresh weight of the *in vitro* induced tubers ranged from 0.4342 to 0.9725 g and multiple root hairs were found on them (Figure 1H). In the whole plantlet, most of the tubers were formed on the basal nodes; however, once correlative inhibition was eliminated by the dissection of the shoot to single node sections, tubers were formed on every axillary bud. The plantlets were separated and adopted individually in a pot (Figure 1I) that contained mixture of peat and sand (2:1). The survival percentage was found to be 80%.

In vitro tuber formation was reported in potato (Mándi and Dobránszki, 1993; Veramendi et al., 2000). The intensity of "tuberization stimulus" was reported to be influenced by exposure to photoperiods less than the critical photoperiod (Ewing and Wareing, 1978) in the case of potato shoots. Mandi and Dobranszki (1993) observed tuberization *in vitro* in potato using 8% sucrose solution. In the present study, tuber production was found after 8 to 9 weeks to be triggered by lowering the sucrose concentration along with nutrient stress and stimulated by subculturing in hormone free ½ MS liquid media. One more factor leading to tuber induction is explants' maturity; as a plant only initiates tuberization at its maturation stage. Hussey and Stacey (1984) also emphasized that tubers were induced within 6 to 8 weeks on subculturing but on medium containing 2 mg/L benzylaminopurine (BAP) and 6% sucrose in 24 days. Xu et al. (1998) reported that sucrose regulates tuber

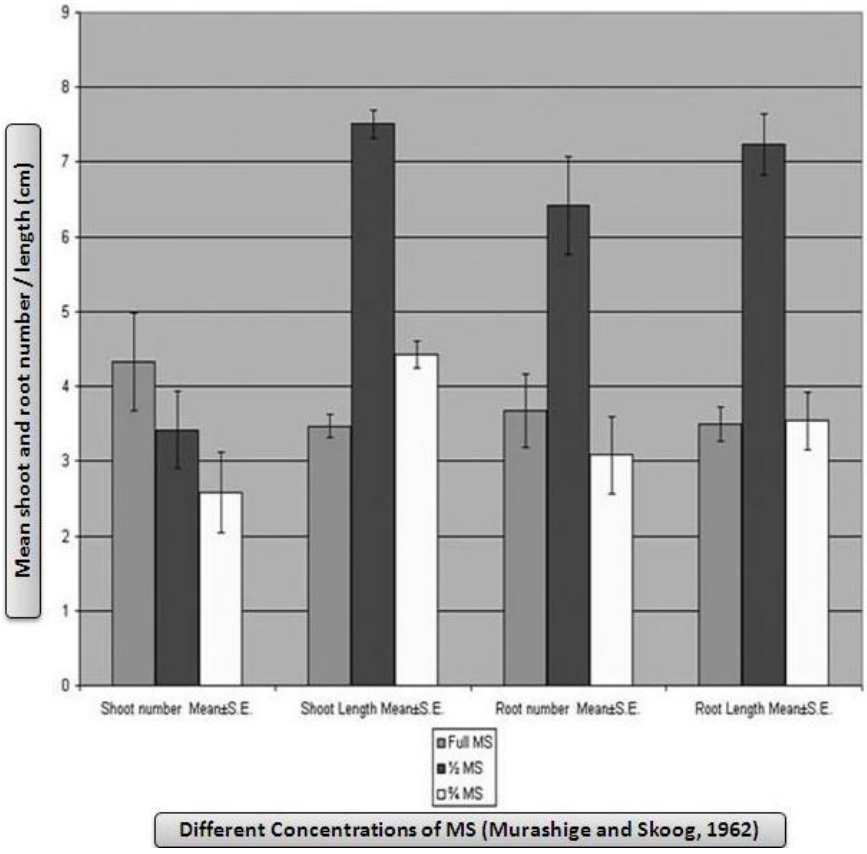

Figure 3. Effect of different strength of medium on average shoot number, shoot length/explant, average root number and root length /explant of *C. borivilianum.*

formation in potato (*Solanum tuberosum* L.).

Qualitative and quantitative determination of Saponins from *in vitro* and *in vivo* grown tubers

Saponin contents were extracted (Thakur et al., 2009) and finally whitish–brown powder as 'crude saponins' were obtained and characterized by the surfactant properties with stable soap-like foam in aqueous solutions. Similarly, four steroidal saponins were isolated from methanolic extract of *C. malayense* rhizome by following n-butanol partitioning (Li et al., 1990). Thin layer chromatography of crude saponins of *in vitro* and *in vivo* tubers, with standard saponins showed the active band of black colour with $R_{f\ value}$ 0.95 cm. The active band (black spot) was scraped from the TLC plates and eluted with deionized water and saponins were further substantiated. The TLC technique was reported for the separation of sapogenins and saponins with chloroform: diethylether mobile phase using ß-sitosterol as standard (Govindarajan et al., 2005).

HPLC analysis of TLC eluted samples of saponins showed retention time of 8.25 min and 10.36 min; similar to the peak and retention time was observed with the standard (Figure 5). Linearity was determined by calibration curve obtained by HPLC analysis of the standard solution of saponin. Thus for the *in vivo* tubers I^{st} peak, 40 pg and the 2^{nd} peak 0.56 pg and *in vitro* tubers I^{st} peak, 47.26 and the 2^{nd} peak, 1.03 pg per 20 µl loaded samples were calculated. It was therefore apparent that in both the samples, saponin was present with slight increase in the saponin content in the *in vitro* raised tubers. Determination of saponin through HPLC using the mobile phase consisted of a mixture of acetonitrile: water (70:30, v/v) was reported (Simone et al., 2005). LC-ESI-MS spectrum in all the cases showed a common ion peak at *m/z* 352, 680 and 681 $[M]^+$ conforming the similarity in the nature of saponin.

Conclusion

The present study envisages an alternative method for conservation of germplasm. This also opens the opportunity for obtaining high yielding tubers of *C. borivilianum* all round the year. These propagules can serve as a direct propagule for plantation, germplasm preservation

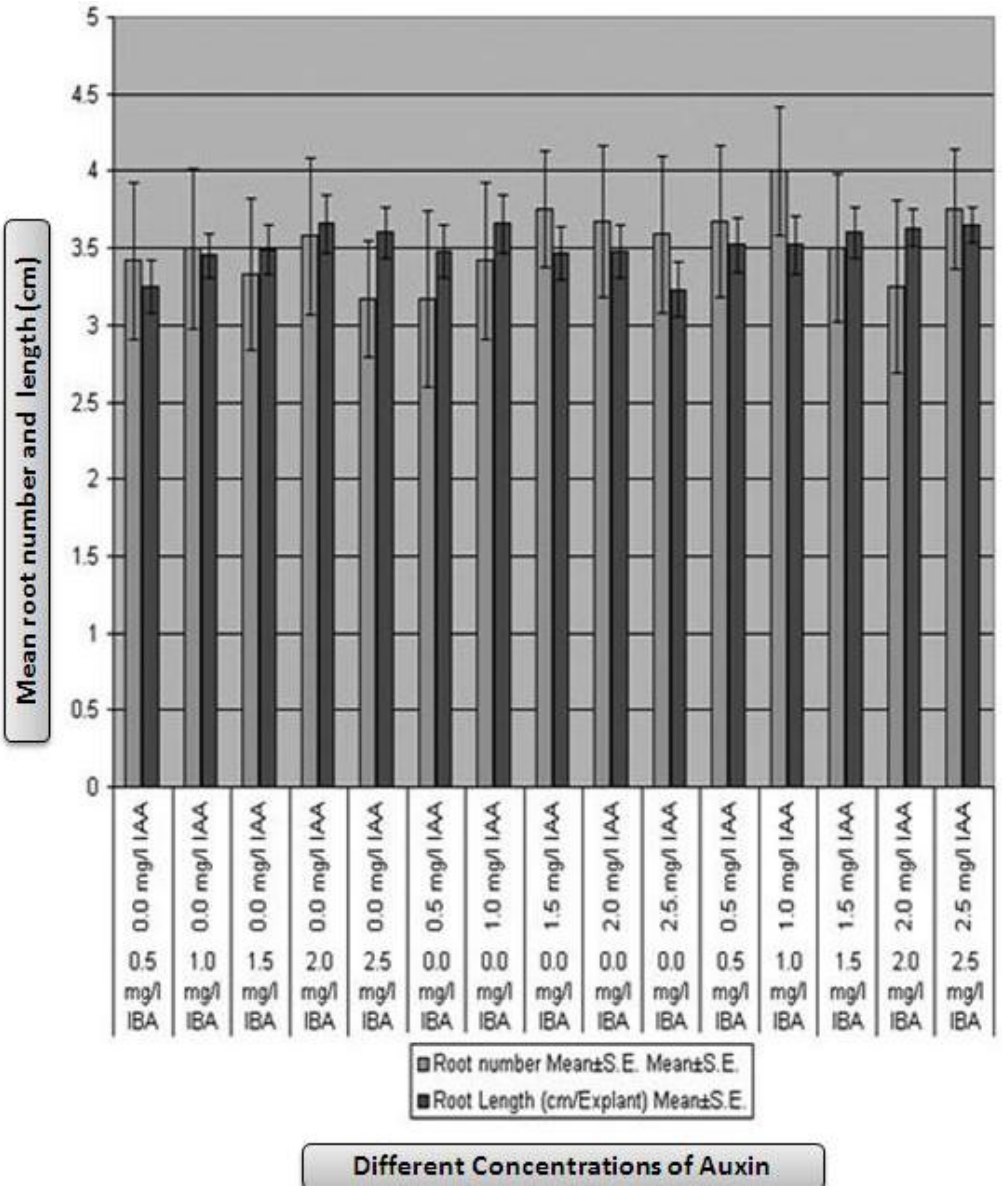

Figure 4. Effect of different concentration of auxins on the rooting of *C. borivilianum*.

Table 1. Effect of sub culturing on rooted plantlets of *C. borivilianum* (Medium: ½ MS; Type: liquid medium; subculturing interval: 3 weeks).

Sub culturing	Root hair	Tuber generation	Tuber number/explant	Tuber length/explant (cm)	Fresh weight
I	no response	no response	no response	no response	no response
II	80-95	no response	no response	no response	no response
III	90-120	positive response	3.08± 0.793	4.23± 0.4181	0.6925 ±0.1946

Values are Mean ± S.E.

as well as source of phytopharmaceuticals. The saponin contents of the *in vitro* and *in vivo* raised tubers were analyzed using HPLC and LC-ESI-MS. There was a significant similarity in the saponin in both the tubers. The *in vitro* tuberization method can be regarded as a novel technique to raise the tubers in short time showing similar high metabolite content required for medicinal and industrial applications. So, it is necessary to study and observe

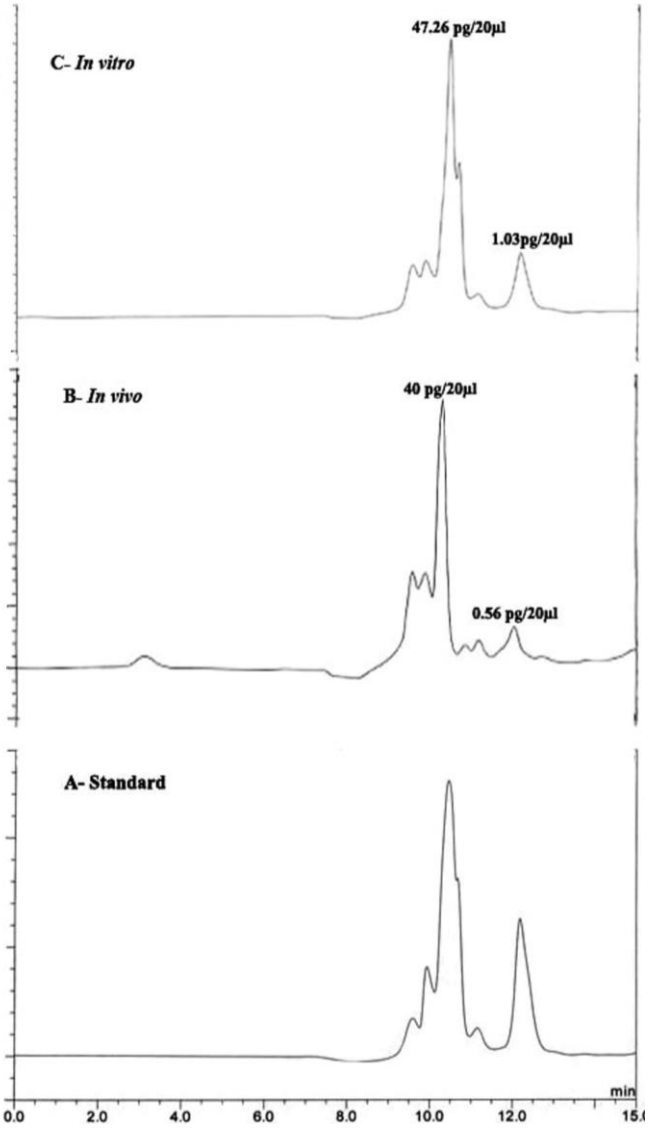

Figure 5. HPLC analysis of *In vivo* and *in vitro* grown tubers with standard.

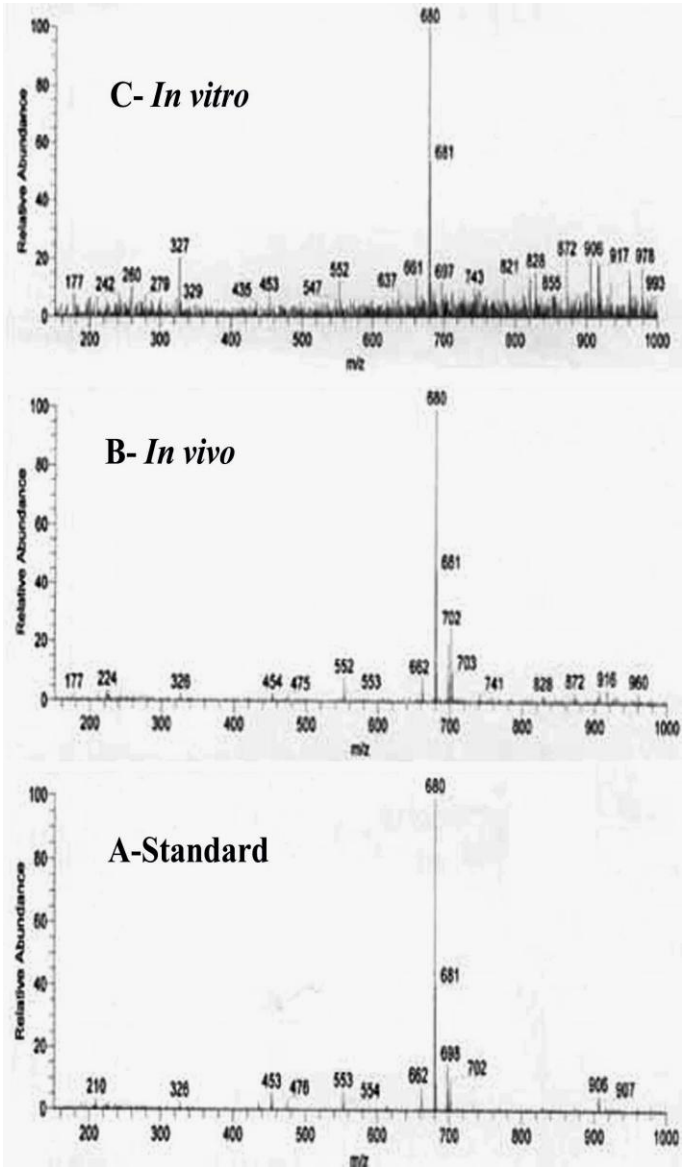

Figure 6. LC-ESI-MS analysis of *in vivo* and *in vitro* grown tubers with standard. A, Standard; B, *In vitro;* C, *in vivo.*

the physiological and pharmacological aspects including nutrient stress along with the study to molecular level. The work will give a strong impetus to the pharmaceutical and neutraceutical sectors.

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