Research Article

Effect of additives on enhanced in-vitro shoot multiplication and their functional group identification of *Chlorophytum borivilianum* Sant. Et Fernand

Poonam Khatri[1] · **J. S. Rana**[1] · **Anil Sindhu**[1] · **Pragati Jamdagni**[1]

Abstract

The present study was commenced with the aim to optimize the proficient in vitro regeneration protocol and phytochemical constituents and their functional groups identification through FT-IR spectrum analysis. As per of our knowledge, very few or no reports are available on the effects of additives on the nodal explant of *C. borivilianum*. Surface sterilized explants viz. nodal segments were inoculated on MS basal medium supplemented with 19 different concentrations of cytokinins (BAP, Kinetin and zeatin). BAP at a concentration of 2 mg ml^{-1} was found superior out of three cytokinins for shoot induction. This study confirmed that the inclusion of additives (Adenine sulphate, casein hydrolysate and putrescine) had a promoting effect on multiple shoot induction and shoot proliferation. Best response was achieved on MS medium supplemented with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA + 20.0 mg l^{-1} $AdSO_4$ from nodal explant on 45th day of culture. For in vitro rooting, the semi solid rooting medium with IBA (2.0 mg l^{-1}) + putrescine (50.0 mg l^{-1}) was found to be best with 90% maximum field survival rate. The proposed protocol can be used for the conservation of germplasm and propagation of these endangered plant species. FT-IR spectroscopic analysis revealed the presence of alcohols, phenols, alkanes, alpha and beta-unsaturated aldehydes, ketones, aromatic amines, alkyl halides, aliphatic amines, ether linkage and alkenes might be responsible for the various medicinal properties of test plant.

Keywords Endangered · Micropropagation · Additives · FTIR · Medicinal plant

Abbreviations

IBA	Indole-3-butyric acid
Kin	Kinetin (6-furfural amino purine)
$AdSO_4$	Adenine sulphate
BAP	6-Benzyl amino purine
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
NAA	Napthalene acetic acid
Put	Putrescine
Zeatin	6-[4-Hydroxy-3-methylbut-2-enylamino]purine

1 Introduction

Since ancient times medicinal plants are recognized as a source of herbal and synthetic medicines for the cure of diseases. Around 20,000 plants are known for therapeutic reasons. Since ancient times, China and India have been on the forefront of the usage of herbal drugs for treatments and preventive medical processes. Herbal drugs have been formulated by a number of renowned and celebrated scientists and owing to their easy accessibility they are still practiced by traditional medicine specialists [36]. Medicinal plants contribute as therapeutic agents and natural resources for production of traditional and modern medicinal products. In addition to exporting medicinal plants,

✉ Poonam Khatri, poonamkhatri2@gmail.com; J. S. Rana, jsrana@outlook.com; Anil Sindhu, sindhu.biotech@gmail.com; Pragati Jamdagni, pragati_318@yahoo.co.in | [1]Department of Biotechnology, Deenbandhu Chhotu Ram University of Science and Technology, Murthal, Sonipat, Haryana 131039, India.

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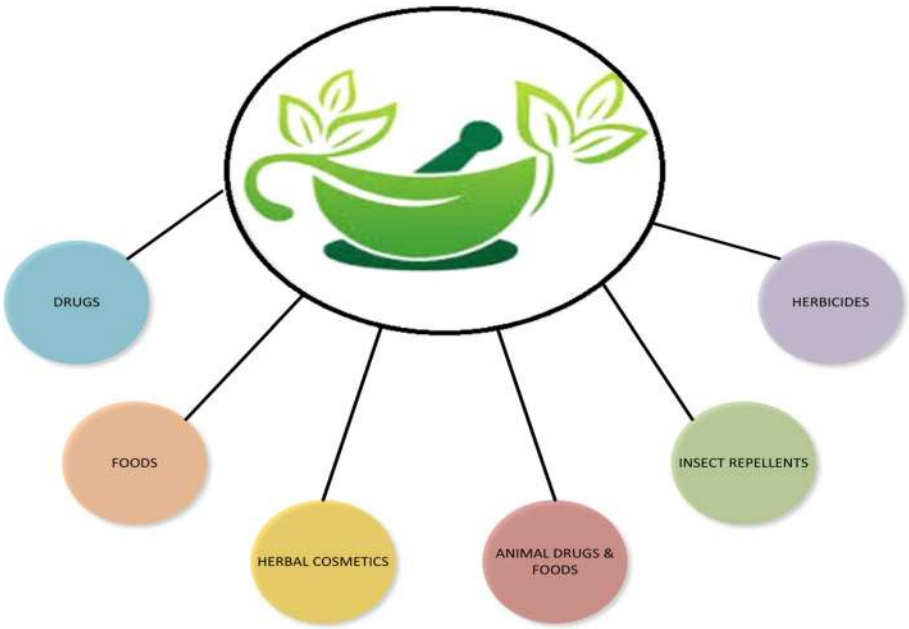

Fig. 1 Various benefits of medicinal plants

monetizing the extraction of medicines from herbal plants can aid to the economy by bringing a sizeable amount of foreign exchange. This then adds an economic outlook to native herbal species thereby promoting conservation and expansion of the plantation areas, not to mention aiding the economic growth of the areas (Fig. 1).

Conventional methods lag behind as opposed to Plant tissue culture in quick and large-scale multiplication of productive plants under in vitro conditions, regardless of the supplementary factors such as season, with conservation of space and time. Furthermore, upgraded in vitro plant cell culture system provide potential for industrial utilization of secondary metabolites.

Chlorophytum borivilianum Sant. et Fernand (Family: Asparagaceae) is an endangered perennial herb and also commonly known as Safed musli in India; England, USA: Indian spider plant, spider plant, White musale; France: chlorophytum medicinal; Saudia Arabia: Shaqaqule [36]. It is leafy herb with 20.95 ± 4.31 (cm) in height, plane leaf margin, spiral phyllotaxy, fascicles tuber, raceme inflorescence, white colour flower, perianth outwardly recurved, stamen filament longer than perianth and axillary placentation [18].

C. borivilianum (Root tubers) represented a broad variety of pharmacological properties comprising antimicrobial, analgesic, anti-inflammatory, antipyretic, hepatoprotective, antioxidant hypolipidemic, antistress, antiarthritic, antidiabetic, aphrodisiac, immunomodulatory, antiulcer, anticancer, anthelmintic and larvicidal activities [14].

C. borivilianum is widely spread in southern part of Rajasthan, Madhya Pradesh, Gujarat, Khasia hills, Bengal, Assam, and Chennai. Genus Chlorophytum originated in Africa and gained popularity in India owing it from South Africa. [5].

The most variable aspects in tissue culture media are growth hormones namely auxins and cytokinins as these affect the growth and differentiation to a greater extent in comparision to others. Cytokinins are recognized to differentiate shoot bud from shoot tip and nodal explants. BAP is most commonly used cytokinin for shoot bud initiation from nodal explants [27]. The synergistic effect of cytokinin and auxin positively modifies the frequency of shoot regeneration parameters [26, 27].

Growth additives like Polyamines (Pas) are polycations (low molecular weight) found in all living organisms. In plant cells polyamines, specifically the diamine Putrescine (PUT), triamine Spermidine (SPD), Adenine sulphate ($AdSO_4$), Casein hydrolysate (CH) and tetramine Spermine (SPM) plays an important part in cell division

and differentiation, and have different physiological and developmental effects on plants [3, 30, 33]. As per our knowledge, this is the first attempt to study the effects of additives ($AdSO_4$, CH, PUT) on *C. borivilianum*.

2 Materials and methods

2.1 Sterilization of glassware, media and explants

Sterilization is the most vital part in tissue culture. The glassware was subjected to chromic and sulphuric acid mixture (1:3) for 24 h and then washed thoroughly with teepol (10%) detergent solution. It was then cleaned under running tap water and further rinsed with water (distilled). After washing, dried for 3 h in an oven maintained at a temperature of 180 °C. All the media, plugged flasks and test tubes were steam sterilized in an autoclave at a temperature of 121 °C and a pressure of 1.12 kg cm^{-2} (15 lbs $inch^{-2}$) for 20 min. Explants were surface sterilized by soaking in 0.1% $HgCl_2$ solution for 2–3 min depending on the nature of explants and then rinsed with double distilled water (sterilized). In order to avoid contaminations, all protocols were carried out in laminar airflow (Atlantis Clean Air Laminar Flow Systems).

2.2 Media

The Basal media used for in vitro studies during the course of present investigation included MS [19] because of its having balanced composition of macro, micronutrients, vitamins and organics, which are highly suitable for majority of the plant species.

2.3 Stock solutions preparation and storage

All the stock solutions of MS basal medium [19] as major, minor, iron, organic hormones, vitamins and amino acids were prepared separately in double distilled water. Stock solutions were prepared as 10× or 100×. Growth hormones/additives such as cytokinins, auxins, putrescine, adenine sulphate and casein hydrolysate were prepared by dissolving 1 N NaOH or absolute alcohol and then bring to volume with double distilled sterilized water in 1:1 ratio.

2.4 Inoculation

The inoculation was done under laminar airflow clean bench fitted with an ultra-violet (UV) tube and a fluorescent tube. The surface of the chamber was cleaned with 90% alcohol. Both the laminar and UV were kept on for half an hour before starting the inoculation.

2.5 Data analysis

All the experiments were conducted in a completely randomized design and repeated thrice with a minimum of 10 replications per treatment. For statistical analysis the scored data were subjected to analysis of variance to detect significance of difference between means. Means differing significantly were compared using Duncan's multiple range test (DMRT) at 5% probability level. All the statistical analysis was done by using OPSTAT software (one factor analysis) available on CCSHAU, Hisar website.

The following methods have been used for calculating the percentage of direct plant regeneration and rooting [28].

1. $$\text{Percentage Direct Plant Regeneration} = \frac{\text{Number of explants showing direct shoots bud induction}}{\text{Total number of explants cultured}} \times 100$$

2. $$\text{Percentage Rooting} = \frac{\text{Number of shoots responding to rooting}}{\text{Total number of shoots cultured on rooting medium}} \times 100$$

2.6 Extract preparation for Fourier transform infrared (FTIR) analysis

The plant parts (leaves) were weighed (2 g each), washed and meshed in 10 ml of methanol. The plant mixtures were kept at room temperature for 6 h and then further filtered using sterile Whatman filter paper No. 1. The filtrates were centrifuged at 5000 rpm for 5 min and the supernatants were collected in the beaker. The solvents were evaporated to dryness and the residue was stored at 4^0 C in a refrigerator [22].

2.7 FTIR spectroscopic analysis

Fourier Transform Infrared (FTIR) analysis of methanol extract of *C. borivilianum* (leaves) was performed using Perkin Elmer spectrophotometer system, which was used to detect the characteristic peaks with their functional groups using Attenuated Total Reflectance (ATR) accessory. The IR scan was performed in the wave number region of 4000–550 cm^{-1} (mid-infrared range). Data thus obtained was presented in the form of a table in result section.

3 Results and discussion

3.1 Preliminary establishment and shoot induction

For in vitro establishment of endangered medicinal plant (*C. borivilianum)* surface sterilized nodal explants of 2–3 cm were cultured on MS basal medium fortified with 19 different concentrations of plant growth regulators (BAP, Kinetin, BAP + Kinetin and Zeatin) and data scored for per cent regeneration after 15, 30 and 45 days of culture. The nodal explants exhibited swelling after 15th day on the shoot induction medium and later differentiated into shoot buds. There is a linear correlation between the increase in concentration of cytokinins (BAP, Kinetin and Zeatin) up to optimum level (2.0 mg l^{-1}) and number of shoots per explant. Above 2.0 mg l^{-1} concentration of cytokinins the frequency of shoot bud regeneration declined. The combination of BAP and Kinetin in the culture showed almost same response as the other cytokinin responded for the shoot induction. The highest per cent explant responding and mean number of shoots were noticed at the concentration of 2 mg l^{-1} BAP + 0.25 mg l^{-1} of kinetin. Thereafter, there is a decrease in shoot development in terms of both percentage and mean number of shoots. None of the explants responded on MS medium without cytokinins even after 45 days of culture (Table 1). When MS containing singular supplement of 2 mg l^{-1} BAP was used, the highest per cent explants responding for shoot initiation and mean number of shoot buds per explants were recorded after 15 days, 30 days and 45 days. Amongst three cytokinins (BAP, Kinetin, Zeatin and BAP + Kinetin) tested, BAP alone was found superior for shoot induction. The supremacy of BAP over other cytokinin in shoot bud induction from nodal explants has been testified by various researchers in different plants including *C. borivilianum* [23], *Ulmus parvifolia* [39], *Sarcostemma brevistigma* [41], *Clitoria ternatea* [37], *Dalbergia latifolia* [4], *Gymnema sylvestre* [40] and *Bambusa arundinacea* [10].

3.2 Multiple shoots induction and proliferation

Optimum concentration of BAP (2.0 mg l^{-1}) was tested with various concentrations of (0.75–2.0 mg l^{-1}) NAA, IBA and IAA to observe the synergistic effect of different

Table 1 Effect of different growth hormones on in vitro establishment of test medicinal plant

Plant growth regulators (mg l^{-1})	15th day of inoculation		30th day of inoculation		45th day of inoculation	
	% Explant responding	No. of shootbuds/explant	% Explant responding	No. of shoot-buds/explant	% Explant responding	No. of shoot-buds/explant
BAP						
0 (control)	0.000	0.10 ± 0.10	00.00	0.00 ± 0.00	30.00	0.00 ± 0.00
1	50.00	1.86 ± 0.29	50.00	2.40 ± 0.10	70.00	4.40 ± 0.10
2	**53.33**	**2.20 ± 0.10**	**53.33**	**2.86 ± 0.13**	**80.33**	**6.53 ± 0.39**
3	40.00	0.63 ± 0.20	43.33	1.30 ± 0.17	63.33	3.30 ± 0.17
4	30.00	0.10 ± 0.10	40.00	0.53 ± 0.23	50.00	2.30 ± 0.17
5	0.000	0.00 ± 0.00	40.00	0.40 ± 0.10	50.00	1.26 ± 0.33
Kinetin						
1	40.00	0.40 ± 0.10	53.33	2.86 ± 0.13	63.33	3.00 ± 0.00
2	40.00	0.96 ± 0.20	63.33	2.96 ± 0.33	63.33	3.20 ± 0.10
3	40.00	0.53 ± 0.29	43.33	1.40 ± 0.10	53.33	2.96 ± 0.20
4	33.33	0.20 ± 0.10	40.00	0.40 ± 0.10	50.00	2.16 ± 0.29
5	0.000	0.00 ± 0.00	40.00	0.73 ± 0.13	50.00	1.90 ± 0.20
BAP (2 mg l^{-1}) + *Kinetin*						
0.25	50.00	1.83 ± 0.23	53.33	2.63 ± 0.20	66.67	3.83 ± 0.39
0.50	43.33	0.86 ± 0.26	43.33	1.40 ± 0.10	63.33	3.30 ± 0.17
1.00	43.33	0.60 ± 0.00	43.33	1.40 ± 0.20	50.00	2.30 ± 0.17
Zeatin						
1	50.00	1.53 ± 0.23	50.00	2.30 ± 0.17	66.67	3.83 ± 0.23
2	53.33	2.40 ± 0.20	63.33	2.93 ± 0.33	63.33	3.40 ± 0.10
3	33.33	0.30 ± 0.00	43.33	0.63 ± 0.20	50.00	1.00 ± 0.00
4	30.00	0.10 ± 0.10	33.00	0.20 ± 0.10	43.33	0.63 ± 0.20
5	30.00	0.00 ± 0.00	30.00	0.10 ± 0.10	43.33	0.63 ± 0.20

Table 2 Effect of different plant growth regulators along with auxins on in vitro shoot proliferation of test medicinal plant

Plant growth regulators (mg l^{-1})	30th day		45th day	
	% Explant responding	No. of shootbuds/explant	% Explant responding	No. of shootbuds/explant
BAP (2 mg l^{-1}) + *NAA*				
0 (control)	00.00	0.00 ± 0.00	00.00	0.00 ± 0.00
0.75	80.00	6.93 ± 0.30	80.00	6.63 ± 0.30
1.00	**83.33**	**7.30 ± 0.00**	**83.33**	**7.73 ± 0.29**
2.00	80.00	7.06 ± 0.20	80.00	7.40 ± 0.10
BAP (2 mg l^{-1}) + *IBA*				
0.75	60.00	3.16 ± 0.29	70.00	4.06 ± 0.23
1.00	73.33	5.16 ± 0.29	76.67	5.40 ± 0.10
2.00	63.33	3.40 ± 0.10	66.67	3.83 ± 0.23
BAP (2 mg l^{-1}) + *IAA*				
0.75	50.00	2.20 ± 0.20	53.33	2.76 ± 0.23
1.00	53.33	2.63 ± 0.20	53.33	2.86 ± 0.13
2.00	50.00	2.40 ± 0.10	50.00	2.50 ± 0.10

growth hormones on multiple shoot induction and shoot proliferation in *C. borivilianum* using nodal explants. Results for in vitro shoot induction and multiplication from explants (nodal) in test plants are shown in Table 2. Addition of auxin (NAA) to MS medium containing optimal concentration of BAP (2.0 mg l^{-1}) significantly improved the frequency of shoot regeneration and multiplication. NAA (1.00 mg l^{-1}) resulted in better response over IBA and IAA in terms of per cent shoot induction and mean number of shoots per explant. Amongst the array of media used MS basal medium supplemented with the combination BAP (2.0 mg l^{-1}) and NAA (1.0 mg l^{-1}) was found to be the most suitable for multiple shoot induction. Stimulatory effect of these growth hormones is well supported by previous studies performed on *Dianthus chinensis* [12], *Adenium obesum* [11] and *Vitex trifolia* [1]. On the contrary, Badoni and Chauhan [2] reported shoots with 9.4 nodes in medium (0.25 mg l^{-1} GA3 and 0.01 mg l^{-1} NAA) attained 8.28 cm and 11.9 cm root length. Park et al. [21] also studied the synergistic response of BAP and NAA in MS medium that resulted in the highest proficiency in shoot regeneration per explant and maximum shoot growth in *Singonia speciosa*.

Hence the results obtained in the present study revealed that it is better to use MS medium supplemented with BAP (2.0 mg l^{-1}) and NAA (1.0 mg l^{-1}) for in vitro multiplication of *C. borivilianum*.

3.3 Effect of adenine sulphate ($AdSO_4$) on in vitro shoot morphogenesis

$AdSO_4$ acts as a booster source of nitrogen to cells, which can be spontaneous consumed as opposed to inorganic nitrogen available in culture medium [25]. Of different media combinations used, the best response was found in media supplemented with 20 mg l^{-1} $AdSO_4$ + (MS + 2 mg l^{-1} BAP + 1.0 mg l^{-1} NAA) in *C. borivilianum* after 30–45 days of culture (Table 3). It was examined that $AdSO_4$ had a promoting effect on multiple shoot induction and shoot proliferation. The frequency of shoot regeneration and mean number of shoots per explant increased with increased in concentration up to an optimum concentration (20 mg l^{-1}) $AdSO_4$ but there after decreased as the concentration increased. In the study, adenine sulphate at 20 mg l^{-1} concentration with BAP was best for multiple shoot bud induction. Adenine sulphate also used for shoot multiplication of *Carica papaya* and Musa sp. [32] and *Phaseolus vulgaris* [8]. There is very few literature precedence, which indicates that adenine sulphate in combination BAP improves shoot bud induction

Table 3 Effect of additives (Adenine sulphate and casein hydrolysate) with optimal combination of PGRS on in vitro shoot morphogenesis of test medicinal plant

Plant growth regulators (mg l^{-1} MS + 2 mg l^{-1} BAP + 1 mg l^{-1} NAA + additives (mg l^{-1}))	30th day		45th day	
	% Explant responding	No. of shoot-buds/explant	% Explant responding	No. of shoot-buds/explant
Adenine sulphate				
0 (control)	00.00	00.00 ± 0.00	00.00	00.00 ± 0.00
10	76.67	15.63 ± 0.20	76.67	15.86 ± 0.13
20	**96.67**	**24.30 ± 0.17**	**96.67**	**24.86 ± 0.29**
30	70.00	10.63 ± 0.49	80.00	12.30 ± 0.17
Casein hydrolysate				
10	**90.00**	**19.87 ± 0.57**	**93.33**	**21.00 ± 0.00**
20	90.00	17.76 ± 0.62	90.00	18.53 ± 0.62
30	70.00	10.63 ± 0.52	80.00	12.86 ± 0.80

in *Jatropha curcas* [34], *Ficus religiosa* [38], *Balanites aegyptiaca* [6] and *Stevia rebaudiana* [13].

3.4 Effect of casein hydrolysate on in vitro shoot morphogenesis

Casein hydrolysate (10–30 mg l^{-1}) to the optimal combination selected (MS basal + BAP 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}) was evaluated with a view to increase in vitro shoot morphogenesis *C. borivilianum* and the data were recorded for per cent regeneration response and shoots number per culture after 30th and 45th day of cultures (Table 3). Casein hydrolysate can be a good source of amino acids, calcium, phosphate, vitamins and microelements [9]. Shoot initiation was effectively induced in a timeframe of 4–5 days along with successive multiplication from nodal bud explants by introduction of Casein hydrolysate to the culture medium. Similar results also reported with CH in *A. latifolia and A. pendula* [31]. Healthy shoot formation was also induced in *Crataeva nurvala* [42] using CH as medium supplement. *Asclepias* regeneration studies haven resulted in analogous research conclusions, where significant volumes of callus were yielded at the cut ends upon addition of Casein hydrolysate in growth medium in addition to cut end readily turning brown and decelerated the strong development of shoots [29].

3.5 Effect of putrescine (put) on in vitro shoot morphogenesis

The effect of inclusion of ethylene inhibitor like putrescine (10–100 mg l^{-1}) to the optimal combination selected (MS basal + 2 mg l^{-1} BAP + 0.25 mg l^{-1} NAA) was tested to improve in vitro shoot morphogenesis in *C. borivilianum* and the data were recorded in terms of per cent response and shoots number per culture after 30th and 45th day.

Table 5 Effect of different auxins concentration on in vitro root induction of *C. borivilianum* on MS, ½ MS and ¼ MS medium after 30th day of culture

Plant growth regulators (mg l^{-1})	MS	½ MS	¼ MS
NAA			
–	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1	0.30 ± 0.00	1.16 ± 0.72	0.86 ± 0.29
2	1.50 ± 0.10	1.33 ± 0.66	1.43 ± 0.29
3	1.63 ± 0.20	**2.00 ± 0.00**	2.96 ± 0.33
4	0.00 ± 0.00	1.10 ± 0.10	0.43 ± 0.29
IBA			
1	2.00 ± 0.00	2.86 ± 0.13	0.50 ± 0.10
2	2.76 ± 0.39	**4.73 ± 0.29**	2.63 ± 0.37
3	0.30 ± 0.17	2.76 ± 0.23	1.20 ± 0.20
4	0.20 ± 0.10	1.73 ± 0.43	0.63 ± 0.33
IAA			
1	0.40 ± 0.10	1.00 ± 0.00	1.30 ± 0.17
2	0.53 ± 0.39	2.86 ± 0.13	1.76 ± 0.39
3	1.06 ± 0.53	**4.00 ± 0.00**	0.73 ± 0.29
4	0.30 ± 0.17	2.10 ± 0.10	0.40 ± 0.10

MS, full strength; ½ MS, Half strength; ¼ MS, One forth strength

Putrescine at a concentration of 50 mg l^{-1} had maximum promoting influence on shoot morphogenesis (Table 4). There is a linear correlation among the increase in concentration of putrescine up to the optimum level (50 mg l^{-1}) and percentage shoot development. The shoots number per culture also increased with increments in putrescine concentration up to the optimum level (2.0 mg l^{-1}). Putrescine has already been used in other plant species for enhancing shoot regeneration frequency [24, 33]. To best of our information, this is the first report on effect of additives viz. adenine sulphate, casein hydrolysate and putrescine on in vitro shoot morphogenesis in *C. borivilianum*.

Table 4 Effect of putrescine as ethylene inhibitor with optimal combination of PGRs on in vitro shoot morphogenesis of test medicinal plant

Plant growth regulators (mg l^{-1}) MS + 2 mg l^{-1} BAP + 1.00 mg l^{-1} NAA + Putrescine (mg l^{-1})	30th day		45th day	
	% Explant responding	No. of shoot-buds/explant	% Explant responding	No. of shoot-buds/explant
0 (control)	0.00	0.00 ± 0.00	00.00	0.00 ± 0.00
10	66.67	4.06 ± 0.230	73.33	5.83 ± 0.390
30	86.67	10.83 ± 0.10	90.00	11.06 ± 0.39
50	**90.00**	**10.20 ± 0.49**	**96.67**	**13.53 ± 0.30**
70	50.00	3.06 ± 0.390	66.67	3.73 ± 0.290
100	43.33	1.96 ± 0.370	50.00	2.83 ± 0.230

Table 6 Effect of putrescine as root promoters on in vitro rooting in test medicinal plant on different salt concentration of MS medium and optimal concentrations of auxins after 30th day of culture

Auxins (mg l^{-1})	Root promoters (mg l^{-1})	*C. borivilianum*	
		Per cent root induction (%)	Mean no. of roots/shoot
IBA	Putrescine		
2	0	60.00	2.06 ± 0.62
2	3	66.67	2.73 ± 0.29
2	6	70.00	3.40 ± 0.49
2	**9**	**83.33**	**5.16 ± 0.43**
2	12	70.00	3.83 ± 0.39
2	18	66.67	2.63 ± 0.37
IAA			
3	0	60.00	1.30 ± 0.17
3	3	63.33	1.73 ± 0.56
3	6	66.67	2.26 ± 0.66
3	**9**	**76.67**	**4.83 ± 0.23**
3	12	70.00	3.00 ± 0.00
3	18	60.00	1.63 ± 0.72
NAA			
4	0	40.00	1.20 ± 0.49
4	3	46.67	1.30 ± 0.51
4	6	50.00	2.40 ± 0.10
4	**9**	**60.00**	**2.63 ± 0.20**
4	12	53.33	2.23 ± 0.20
4	18	46.67	1.40 ± 0.10

3.6 In vitro rooting

3.6.1 Effect of auxins and putrescine on in vitro root formation

Isolated shoots were cultured on ¼, ½ and full strengths of MS medium fortified with different concentrations of IAA, IBA and NAA individually for rooting (Table 5). Maximum roots number per shoot was produced on IBA supplemented medium as compared to medium supplemented with IAA and NAA. IBA was found to be more efficient at concentration of 2 mg l^{-1} in medicinal plant as compared to IAA and NAA in terms of roots number per shoot. The effectiveness of IBA for in vitro root induction was earlier investigated in *S. chinensis* by [16, 17, 35], the report of whom are similar to our results. Similarly, half strength MS medium containing IBA was determined to be best for in vitro rooting in *Jatropha curcas* [15], *Bixa orellana* [20] and *Gymnema sylvestre* [40]. When the media was supplemented with auxins (IBA, IAA and NAA) and putrescine (Table 6, Fig. 2), highest root promotion was observed with putrescine (9.0 mg l^{-1}) containing IBA (2.0 mg l^{-1}) followed by IAA (2.0 mg l^{-1}) +PUT (9.0 mg l^{-1}) and NAA (2.0 mg l^{-1}) +PG (9.0 mg l^{-1}).

3.6.2 Hardening

In the present investigation, the micropropagated shoots with well-developed root were carefully removed from the culture bottles, washed with sterile water to eradicate agar media. After washing, plantlets were transferred to transparent punctured polythene bags to prevent desiccation and provide high humidity. These plantlets were maintained in the culture room at 24 ± 2 °C and 16 h photoperiod with a light intensity of 50 µmol m^{-2} s^{-1} PPFD. The relative humidity was reduced progressively. After 4 weeks, the plantlets were taken out from the polybags and separately planted in small pots containing a mixture of soil, sand and vermicompost (1:1:1) with about 80% relative humidity under polyethylene hoods (moistened) in the green house for acclimatization. These plantlets were watered with ½ strength MS inorganic salt solution at 3 days interval. The plants were hardened for 2 months and finally established in the field. The per cent survival rate of rooted plantlets was recorded after 30 days. Field survival rate of in vitro rooted plantlets showed a positive correlation with percentage root induction and mean number of roots per shoot which clearly indicates that healthy and profuse rooting supported the higher survivability of the in vitro regenerated plantlets during their acclimatization and field transfer.

3.7 Phytochemical constituents and their functional groups identification through FT-IR spectrum analysis

The results of FT-IR spectroscopic analysis of *C. borivilianum* revealed the presence of alcohols, phenols, alkanes, alpha and beta-unsaturated aldehydes, ketones, aromatic amines, alkyl halides, aliphatic amines, ether linkage and alkenes (Table 7, Fig. 3). The absorption at 3329 cm^{-1} is due to the H-bond and O–H stretch, which showed the presence of alcohols and phenols in the extract. The band at 2972.2 cm^{-1}, 2880.6 cm^{-1}, 1381.4 cm^{-1} and 694.02 cm^{-1} is due to C–H stretching showed alkanes, band at 1653 cm^{-1} showed alpha and beta-unsaturated aldehydes and ketones (C=O stretch), the band at 1322.5 cm^{-1} showed aromatic amines, the band at 1273.4 cm^{-1} showed alkyl halides C–H wag (–CH_2 X), the band at 1086.8 cm^{-1} showed aliphatic amines compounds (C–N stretch); the band at 1044.3 cm^{-1} showed ether linkage (C–O–C stretch) and the band at 880.6 cm^{-1} showed alkenes (=C–H stretch).

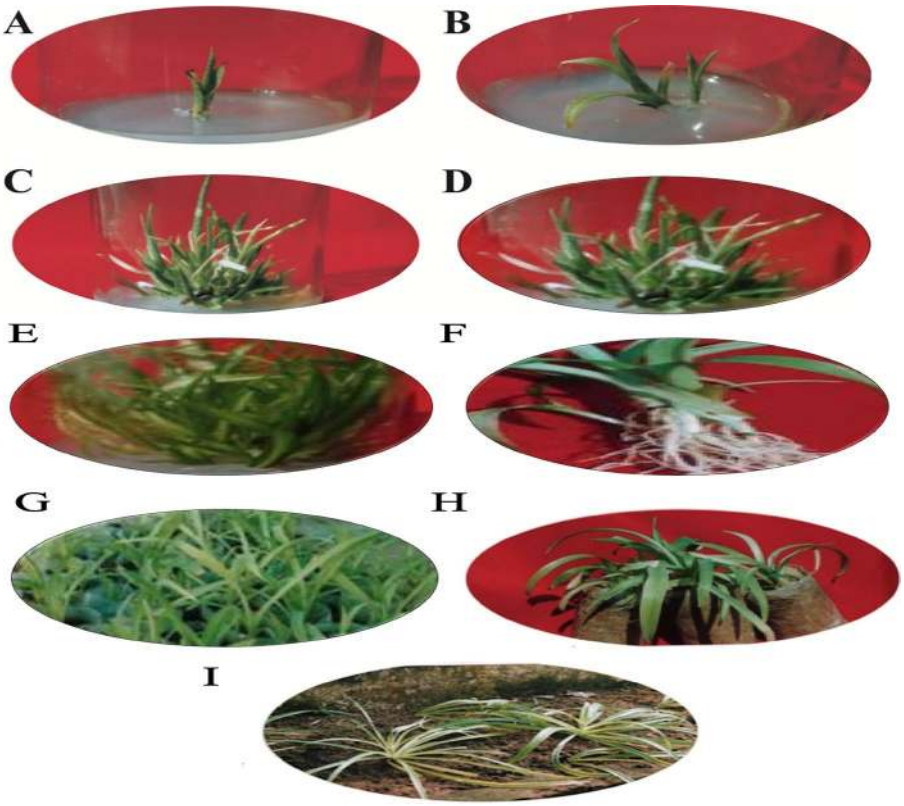

Fig. 2 Multiple shoot induction from nodal explants of *C. borivilianum*. **a** Nodal segments regeneration after 2 weeks, **b** Axillary bud proliferation from nodal segments after 3 weeks of culture; **c** Shoot multiplication on MS medium supplemented with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA; **d**, **e** In vitro shoot multiplication using additives MS medium supplemented with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA + 20 mg l^{-1} $AdSO_4$. **f**, **g** In vitro rooting of shoots on half-strength MS medium with IBA (2.0 mg l^{-1}) after 4 weeks; **h** rooted plant growing in the plastic cup with soil, sand, vermicompost in the ratio of 1:1:1.; **i** field transferred plants

Table 7 FTIR peak values of methanolic extract of *C. borivilianum* leaves

Characteristic absorption (s) cm^{-1}	Bond	Functional group
3329	O–H stretch, H-bonded	Alcohols, phenols
2972.2	C–H stretch	Alkanes
2880.6	C–H stretch	Alkanes
1653	C=O stretching	σ, β-unsaturated aldehydes, Ketone
1381.4	C–N stretch	Alkanes
1322.5	C–N stretch	Aromatic amines
1273.4	C–H wag (–CH_2 X)	Alkyl halides
1086.8	C–N stretch	Aliphatic amines
1044.3	C–O–C stretch	Ether linkage
880.6	=C–H stretch	Alkenes
694.02	C–H stretch	Alkanes

4 Conclusion

Factors like revival of public interest in plant-based medicines, in addition to hasty expansion of pharmaceutical industries compelled the demand for medicinal plants to overshoot as a result leading towards over-exploitation. The plant species of important medicinal and ornamental properties have been markedly diminished due to destruction of its natural habitat, excessive over exploitation, unresolved problems of seed viability and poor germination. As per of our knowledge, very few or no reports are available on the effects of additives on *C. borivilianum*. Keeping this in mind, the present investigation was carried out with the aim to optimize the efficient in vitro regeneration protocol and phytochemical constituents and their functional groups identification through FT-IR spectrum analysis. Amongst, the three cytokinins, BAP was found superior to kinetin and zeatin for shoot induction. The shoot regeneration frequency increased with increase in concentration of cytokinin up to an optimal concentration (2.0 mg l^{-1}) above, which the frequency of shoot bud regeneration declined drastically. None of the explants showed any response on MS medium without cytokinins even after 45 days of culture. The highest proliferation of shoots was recorded on MS medium added with BAP (2.0 mg l^{-1}) + NAA (1.0 mg l^{-1}) with maximum shoots number per explant. Inclusion of additives (20.0 mg l^{-1} $AdSO_4$, 10.0 mg l^{-1} CH and 50.0 mg l^{-1} putrescine) had a promoting effect on multiple shoot induction and shoot proliferation. IBA (2.0 mg l^{-1}) + putrescine (9.0 mg l^{-1}) was found to be finest for in vitro rooting. The rooted plantlets obtained from above treatments exhibited maximum field survival rate (90%) after proper hardening. The results of

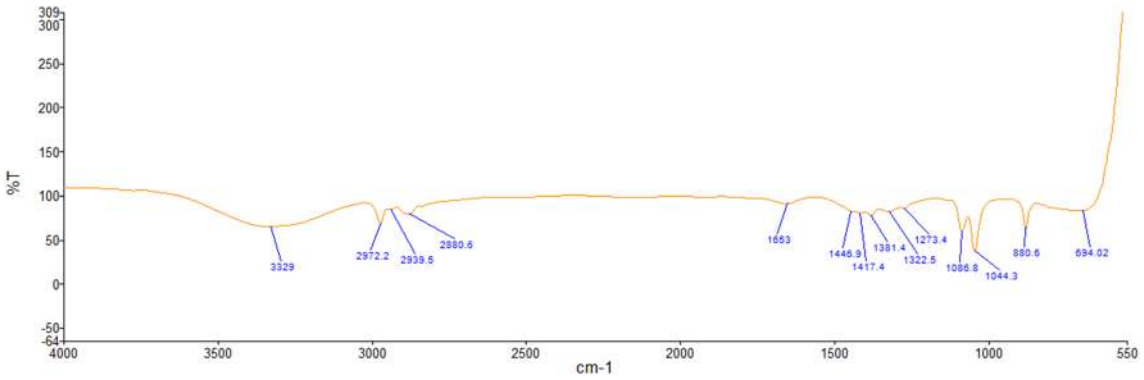

Fig. 3 FTIR spectra of *C. borivilianum* leaves extracted in methanol

FT-IR spectroscopic analysis of in vitro grown medicinal plant revealed the presence of alcohols, phenols, alkanes, alpha and beta-unsaturated aldehydes, ketones, aromatic amines, alkyl halides, aliphatic amines, ether linkage and alkenes. The proposed in vitro regeneration protocols can be used for conservation of germplasm and propagation of these species. It is expected that further qualitative and quantitative study of bioactive compounds can be done for the development of effective drugs.

Authors' contribution Dr. PK has written the manuscript and carried out the experimental work. Dr. PJ helped in data analysis. Prof. AS has designed the experimental work. Prof. JSR has reviewed and finalized the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Competing interests The authors declare that they have no competing interests.

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