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Effect of Different Media and Growth Hormones on Shoot Multiplication of *In Vitro* Grown *Centella asiatica* Accessions

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Abstract

Centella asiatica also known as Gotu Kola is an important medicinal plant which contains several bioactive compounds such as triterpenoid saponins including asiaticoside, madecassoside, centelloside, asiatic acid, etc. In addition, *Centella sp.* contains other components including flavonoids, phytosterols, tannins, amino acids, sugars, etc. Due to its medicinal importance, this plant is being overexploited and it is essential to conserve this plant. In the present investigation, comparative study of different accessions of *Centella asiatica* for shoot multiplication in different media and growth hormones was performed. Shoot multiplication and phytocompounds production from different accessions is required to be assessed to choose an accession giving optimum production of phytocompounds. For this, different media containing different combinations of macro and micro nutrients have to be tested that influence the growth of the plant. Assessment of best culture media and concentrations of plant hormone for shoot culture are some of the critical culture conditions to achieve. So there is a need to optimise such conditions that will enhance the growth of the plant. For shoot culture, explant of different accession of *Centella asiatica* were inoculated in different media i.e. Murashige and Skoog (MS), Gamborg's B5 and Nitsch medium which were supplemented with standard concentrations of plant growth hormones. The cultures were incubated at 25 ± 2°C with photoperiod of 16 hours. After six week of incubation period, highest growth was found in MS media in all accessions. Further MS medium was supplemented with different combinations of growth hormones. After six week of incubation, MS medium supplemented with 1mg/l BAP showed the highest growth of the plant followed by 2mg/l BAP+0.5 NAA.

Keywords: *Centella asiatica;* Murashige and skoog (MS); Gamborg's B5; Nitsch; 6-Benzylaminopurine (BAP); Naphthaleneacetic acid (NAA)

Introduction

Medicinal plants are the traditional source of many pharmaceutically important compounds. In recent times, they are utilized by the pharmaceutical companies for the preparation of several formulations. *Centella asiatica* is one of the important traditional medicinal plant belonging to family Apiaceae and commonly known as Gotu kola in India. It is an important perennial medicinal herb found in the tropical and subtropical countries like India, Sri Lanka and Bangladesh. *C. asiatica* contains several triterpene, saponins like asiaticoside, asiatic acid, sapogenins, madecassic acid, vellarin, adecassoside, glycosides and centelloside [1]. Leaves contains high amount of triterpenoids [2]. It possesses several important properties like antileprotic, anti-stress, anti-feedent, anti-tuberculosis activities, wound-healing properties [3,4], antibacterial, antherosclerosis and fungicidal activity [5]. It is used in the treatment of leprosy, wound, cancer, fever, allergies [6], abscesses, asthma, catarrh, convulsions, dysentery, eczema, gonorrhea, hypertension, bronchitis, headache, jaundice, pleuritis, rheumatism, ulcers, spasms, tuberculosis, urethritis, etc. [7]. Leaves of this plant are rich in Vitamin B, C, and minerals such as magnesium, potassium, calcium, phosphorus and aluminium [8,9]. It is also used as brain tonic and blood purifier [10]. *C. asiatica* contains various flavonoids which include quercetin and kaempferol, rutin and naringin [11]. Roots are rich in amino acids like aspartic, glutamic, serine, alanine, threonine, histidine and lysine [12]. Underground parts contain many polyacetylenic compounds [12]. Due to its medicinal importance, this plant is overexploited and there is a decline in the population of the *Centella asiatica*. International Union for Conservation of Nature and Natural resources (IUCN) listed it as threatened plant and endangered species [13]. Tissue culture techniques can play an important role in the clonal multiplication of elite clones of this plant as well as conservation of its germplasm. For this study, different accessions of *Centella asiatica* have been used. An accession refers to the collection of plant material from a single species which is collected at one time from a specific geographical location. Each accession is an attempt to capture the diversity present in a given plant population. Accession number is given a unique identifier, and it is used to maintain associated information in the database. It exhibits significant variations in morphological parameters like growth of leaf, flowering, stomatal frequency, etc. The purpose of using different accession was to choose the best accession for the phytocompounds production. To the best of our knowledge there is no study which describes the effect of different media on the shoot multiplication of this plant and it provides an opportunity to explore the role of media and growth hormone on the enhancement of *in vitro* culture of different accessions of *C. asiatica.* In this study, effects of different media like MS, Gamborg's B5 and Nitsch and the concentrations of plant growth hormones on the growth of three *Centella asiatica* accessions were observed and reported.

Materials and Method

Plant material

Three different accessions of *in vitro* grown plantlets of *Centella asiatica* were collected from the NBPGR (National Board of Plant

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Genome Research), New Delhi. Nodes of *C. asiatica* were utilized as explant for this study.

Culture medium and conditions

Effect of different media on shoot multiplication and shoot length: Three different plant tissue culture medium i.e. Murashige and Skoog (MS) (1962) [14] with 3% (w/v) sucrose and 0.8% (w/v) agar, Gamborg's B5 (HiMedia Laboratories Pvt. Ltd., India) [15] and Nitsch (HiMedia Laboratories Pvt. Ltd., India) [16] media were used. The composition of each medium is mentioned in Table 1. These media were supplemented with standard concentration of plant growth hormone i.e. 4.0 mg/L BAP+0.4 mg/L NAA. The pH of media was adjusted to 5.8 with 1N NaOH or 1N HCl and media were autoclaved at 121°C for 20 minutes at 15 psi. The explants were then inoculated in the medium under aseptic conditions and incubated at 25 ± 2°C with photoperiod of 16 hours under cool-white fluorescent tubes for six weeks.

Effect of growth hormones on shoot multiplication and shoot length: MS medium was augmented with different concentrations of plant growth hormones i.e. 1.0 mg/l BAP, 2.0 mg/l BAP, 1.0 mg/l BAP+0.5 NAA and 2.0 mg/l BAP+0.5 mg/l NAA. Explants were inoculated and cultures were incubated at 25 ± 2°C with photoperiod of 16 hours under cool-white fluorescent tubes for six weeks. Regenerated shoots were subcultured every three weeks in the same media (Table 1).

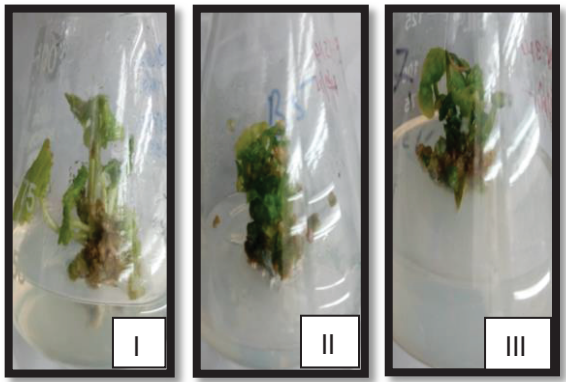

Figure 1: *In vitro* culture of *Centella asiatica* after six weeks of inoculation. I) MS, II) B5 and III) Nitsch Medium.

Data analysis

Visual observations were recorded in terms of number of shoots per explant and the length of each shoot. Experiments were done in triplicates and means of each experiment was carried out to detect the significant differences.

Results and Discussion

Effect of different media on shoot multiplication and shoot length

This study was an attempt to correlate the effect of different media and plant hormone concentration on the shoot multiplication of *Centella asiatica* accessions. To initiate the study, nodal explant were taken from *in vitro* grown plants. Shoot multiplication of *Centella asiatica* nodal explants cultured on MS, Gamborg's B5 and Nitsch media supplemented with 4.0 mg/L BAP+0.4 mg/L NAA. After two weeks of incubation explants showed the growth response in different media. After six weeks of incubation period it was found that MS media showed highest shoot multiplication as compared to Gamborg's B5 and Nitsch media in all the three accessions. In case of MS media, the highest shoot multiplication was observed as follow, 5.5 ± 0.22 in accession no.-342109, 5.0 ± 0.22 in accession no.-347492 and 4.5 ± 0.22 in accession no.-331514. In case of Nitsch media highest shoot multiplication was observed as follow 4.3 ± 0.37 in accession no.-342109, 4 ± 0.33 in accession no.-331514 and 2 ± 0.31 in accession no.-347492. Whereas Gamborg's B5 media showed lowest shoot multiplication in all the three accessions. Similar results were also reported where MS media was supplemented with 4.0 mg/l BAP+0.1mg/l NAA [17]. The details of each accession are mentioned in Table 2 (Figure 1).

Effect of growth hormones on shoot multiplication and shoot length

Nodal explants were cultured in MS medium supplemented with different concentrations and combination of auxin (NAA) and cytokinine (BAP) to assess their effect on shoot multiplication of *C. asiatica* accessions. The highest number of shoots as well as length of

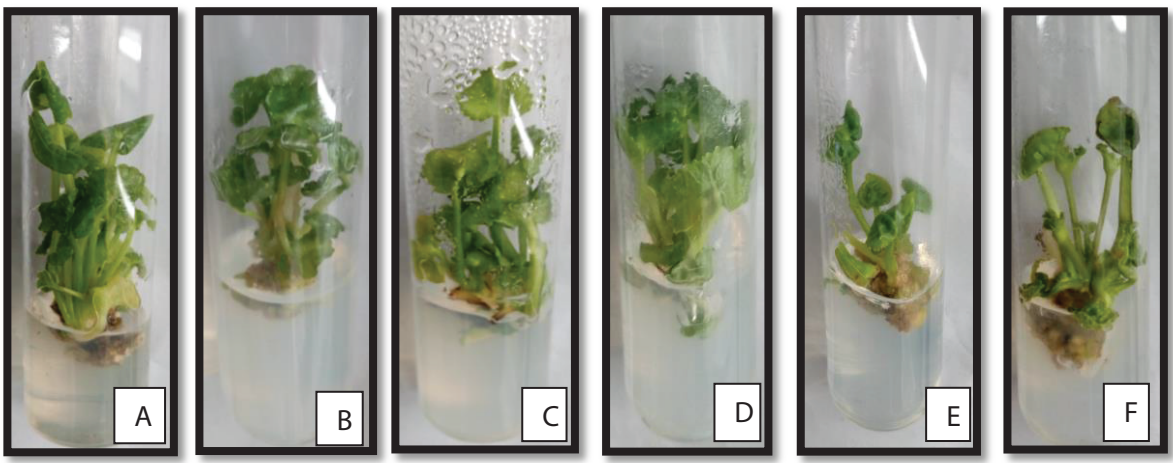

Figure 2: Figure showing effect of MS+1mg/l BAP on the growth in accession no-342109 (A), accession no-347492 (B) accession no-331514 (C) and also effect of MS+2mg/l BAP+0.5NAA on the growth in accession no-342109 (D), accession no-347492 (E) accession no-331514 (F).

MS Medium [14]		B5 Medium [15]		Nitsch Medium [16]	
Components	**mg/L**	**Components**	**mg/L**	**Components**	**mg/L**
Macronutrients		**Macronutrients**		**Macronutrients**	
Ammonium nitrate	1,650.00	Potassium nitrate	2500	Potassium nitrate	950
Potassium nitrate	1,900.00	Ammonium Sulphate	134	Ammonium nitrate	720
Calcium chloride (anhydrous)	332.2	Calcium chloride.$2H_2O$	150	Magnesium sulphate anhydrous	90.34
Magnesium sulfate (anhydrous)	180.7	Magnesium sulphate	122.1	Potassium phosphate monobasic	68
Potassium phosphate monobasic	170	Sodium phosphate monobasic	130.4	**Micronutrients**	
Micronutrients		**Micronutrients**		Manganese sulphate.H_2O	18.94
Manganese sulfate monohydrate	16.9	Manganese sulphate. H_2O	10	Boric acid	10
Ferrous sulfate heptahydrate	27.8	Boric acid	3	Molybdic acid (sodium salt).$2H_2O$	0.25
Zinc sulfate heptahydrate	8.6	Potassium iodide	0.75	Zinc sulphate.$7H_2O$	10
Boric acid	6.2	Molybdic acid (sodium salt).$2H_2O$	0.25	Copper sulphate.$5H_2O$	0.025
Potassium iodide	0.83	Zinc sulphate.$7H_2O$	2	Ferrous sulphate.$7H_2O$	27.85
Sodium molybdate dehydrate	0.25	Copper sulphate.$5H_2O$	0.025	EDTA disodium salt.$2H_2O$	37.25
Cobalt chloride hexahydrate	0.025	Cobalt chloride.$6H_2O$	0.025	**Vitamins**	
Cupric sulfate pentahydrate	0.025	Ferrous sulphate.$7H_2O$	27.8	Myo-Inositol	100
Disodium EDTA dehydrate	37.26	EDTA disodium salt.$2H_2O$	37.3	Thiamine hydrochloride	0.5
Vitamins		**Vitamins**		Pyridoxine hydrochloride	0.5
myo-Inositol	100	Myo-Inositol	100	Nicotinic acid	5
Nicotinic acid	0.5	Thiamine hydrochloride	10	Folic acid	0.5
Pyridoxine hydrochloride	0.5	Pyridoxine hydrochloride	1	Biotin	0.05
Thiamine hydrochloride	0.1	Nicotinic acid	1	Glycine	2
Sugar		**Sugar**		**Sugar**	
Sucrose	30000	Sucrose	20000	Sucrose	20000

Table 1: Composition of MS, B5 and Nitsch medium investigated in this study.

Different media with standard hormones	Accessions					
	342109		347492		331514	
	Number of Shoots (M ± SE)	Length of Shoots (in cm) (M ± SE)	Number of Shoots (M ± SE)	Length of Shoots (in cm) (M ± SE)	Number of Shoots (M ± SE)	Length of Shoots (in cm) (M ± SE)
MS + 4 mg/l BAP + 0.4 mg/l NAA	5.5 ± 0.22	2.6 ± 0.11	5.0 ± 0.22	2.9 ± 0.31	4.5 ± 0.22	2.1 ± 0.22
B5 + 4 mg/l BAP + 0.4 mg/l NAA	1.0 ± 0.38	1.4 ± 0.22	1.0 ± 0.33	0.6 ± 0.22	2.0 ± 0.38	0.35 ± 0.24
Nitsch + 4 mg/l BAP + 0.4 mg/l NAA	4.3 ± 0.37	1.25 ± 0.31	2.0 ± 0.31	0.8 ± 0.33	4.0 ± 0.33	0.48 ± 0.21

Table 2: Effect of different media on the number and length of shoots of three different accessions of *Centella asiatica* after six week of inoculation. Values are expressed as mean ± Standard Error (M ± SE). MS: Murashige and Skoog medium; B5: Gamborg's B5 medium; BAP: 6-Benzyl amino purine; NAA: Naphthaleneacetic acid.

MS Media with different hormonal concentrations	342109		347492		331514	
	Number of Shoots (M ± SE)	Length of Shoots (in cm) (M ± SE)	Number of Shoots (M ± SE)	Length of Shoots (in cm) (M ± SE)	Number of Shoots (M ± SE)	Length of Shoots (in cm) (M ± SE)
MS+1 mg/l BAP	12.5 ± 0.22	3.5 ± 0.32	7.0 ± 0.22	2.7 ± 0.31	11.5 ± 0.22	2.9 ± 0.24
MS+2 mg/l BAP	3.0 ± 0.22	2.5 ± 0.22	3.0 ± 0.37	1.6 ± 0.24	4.0 ± 0.4	1.7 ± 0.31
MS+1 mg/l BAP+0.5 NAA	2.7 ± 0.37	1.0 ± 0.31	2.0 ± 0.22	1.3 ± 0.32	3.0 ± 0.38	1.2 ± 0.21
MS+2 mg/l BAP+0.5 NAA	5.3 ± 0.38	2.0 ± 0.24	6.2 ± 0.22	2.7 ± 0.24	8.2 ± 0.33	1.5 ± 0.22

Table 3: Effect of different hormone concentrations on number and length of shoots of three accessions of *Centella asiatica* after six week of inoculation. Values are expressed as mean ± Standard Error (M ± SE). MS: Murashige and Skoog medium; BAP: 6-Benzyl amino purine; NAA: Naphthaleneacetic acid.

shoots was observed in MS medium with hormonal concentration of 1mg/l BAP followed by 2 mg/l BAP+0.5 mg/l NAA. Multiplication of shoots was observed after 14 days. After six weeks of incubation MS medium supplemented with 1mg/l BAP showed shoot multiplication as follow, 12.5 ± 0.22 in accession no.- 342109, 7 ± 0.22 in accession no.- 347492 and 11.5 ± 0.22 in accession no.- 331514. While MS medium supplemented with 2 mg/l BAP+0.5 mg/l NAA showed shoot multiplication of 5.3 ± 0.38 in accession no.- 342109, 6.2 ± 0.22 in accession no.- 347492 and 8.2 ± 0.33 in accession no.- 331514. Similar results were reported where BAP alone showed the good shoot

induction. In general, BAP is the most efficient growth hormone for the shoot proliferation [18]. It mimics as an inhibitor agent and function against apical dominance of shoot induction and shoot bud formation [19]. Several studies reported that media supplemented with BAP and NAA have also useful for the shoot multiplication. One article reported that MS media supplemented with 22.2 µM BA+2.68 µM NAA showed highest growth [20] where as another article reported that maximum shoot multiplication was observed at 2 mg/l BAP [21]. Results of this study indicate that large scale propagation of this plant is feasible and several plantlets can be regenerated from a single nodal explant. Details of this experiment are mentioned in Table 3 (Figure 2).

Conclusion

In this investigation, it was concluded that the MS medium with concentration of BAP 1 mg/l supports the maximum shoot multiplication and length of shoots for all the three accessions of *Centella asiatica*. It was also observed that the maximum number of shoots and length of shoots obtained for Accession-342109 in comparison to the other two accessions. Further analysis of phytocompounds will be done with all these cultures. Findings in this investigation have proven an efficient media and plant growth hormone concentration for the mass propagation of this plant. These findings would be useful in conservation and micropropagation of this plant. Future efforts are in progress to evaluate the phytocompound present in this plant.

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