ISSN : 2249-5746

International Journal of Ayurvedicand Herbal Medicine 7:4 (2017) 2643–2650

Journal homepage: http://www.interscience.org.uk
DOI: 10.18535/ijahm/v7i4.02
Impact factor: 4.415

Effect Of Different Culture Medias On Shoot Multiplication And Stigmasterol Content In Accessions Of *Centella Asiatica*

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Abstract

The objective of the study was to assess the effect of three plant tissue culture media (MS, Gamborg's B5 and Nitsch) on the growth of five accessions of Centella asiatica using nodal explant. All the three media supplemented with 1 mg/l BAP resulted in increased growth. Amongst the three Medias, MS media resulted in the highest shoot multiplication in all the five accessions after eight weeks of the incubation period. The highest number of shoots and maximum shoot length were obtained in accession number 342109 (19.6±0.57 shoot number, 4.9±0.53 shoot length) grown in MS media supplemented with 1 mg/l BAP. Estimation of stigmasterol, an important phytosterol present in the leaves (main part for synthesis) was done by using gas chromatography and the highest amount of stigmasterol was obtained in accession number 342109 i.e. 0.0228µg/mg of leaves.

Keywords :*Centella asiatica;* MS media*;* B5 media; Nitsch media; Stigmasterol; Gas Chromatography.

Introduction

Centella asiatica (Gotu Kola: Apiaceae) is an important medicinal plant found in the tropical and subtropical regions and grow well in the high moisture areas. Previous literature has reported the presence of several bioactive compounds in this plant such as alkaloids, triterpenoid and its derivative compounds i.e. madecassoside, asiaticoside (Zhang *et al.* 2008), centelloside D and E (Weng *et al.* 2012), asiatic acid (Krishnamurthy *et al.* 2009), two new flavonoids named castilliferol and castillicetin (Subban *et al.* 2008) and phytosterols such as stigmasterol (Veenrendra *et al.* 2002) etc. Soumyanath *et al.* (2005) reported the use of *Centella asiatica* in the repairing of damaged neurons. Binns *et al.* (2002) reported the use of *Centella asiatica* in treatment of amyloidosis of beta-amyloid peptide in Alzheimer's diseases. *Centella asiatica* also plays an important role in intelligence improvement and cognitive enhancing values (Veenrendra *et al.* 2002), reduction in mitochondrial damage (Gnanapragasam *et al.* 2007), shows antioxidant activity (Heong *et al.* 2011) and helpful in reducing the effect of lead poisoning (Saxena and Flora, 2006).

Due to the presence of important phytocompounds, this plant has been extensively exploited by Indian pharmaceutical industries with limited cultivation and insufficient attempts for its replenishment. This makes the plant categorised under overexploited. Due to the commercial importance of stigmasterol, there is a great interest in growing this plant under *in-vitro* conditions and enhance the production of stigmasterol. Chemical structure of stigmasterol is very close to animal cholesterol. It also possesses acetylcholinesterase inhibitory effect (Sriramya *et al.*, 2017) which shows that it can be a useful substance in the medication of neurodegenerative disease. Vahouny *et al.*, (1983) reported that consumption of β-sitosterol decrease levels of blood cholesterol by preventing its intestinal absorption. Similarly tocopherols play important role in hyperlipidaemia (Qureshi et al., 1991), neurodegenerative disease (Khanna et al., 2006) etc.

Accessions of *Centella asiatica* collected from different locations vary in plant composition. Panichayupakaranant (2011) reported that it is a big challenge to get optimal products from medicinal plants because various factors affect to the chemical constitutes of the plants thereby making the phytomedicine a complex research field as results vary from one location to another. Factors like the genetic composition of the plant, climate variations, the age of plant or the harvesting period and specific part of the plants harvested for processing influence phytocompound concentration. Collin (2001) reported that manipulation of secondary metabolites from the plant can be done by altering the growth regulators type and concentration, composition and pH of the culture medium. Phytosterols have the ability to reduce cholesterol levels and this was first demonstrated in humans in 1953 (Tilvis et al., 1986). Woyengo et al., 2009 reported that phytosterols have potential to inhibit stomach, lunch, breast and ovarian cancers. Phytosterols also have the potential to reduce the elevated triglyceride levels which is a risk factor for cardio vascular diseases (Malloy et al., 2001).

Biotechnological methods like *in-vitro* cultures offer the possibility of obtaining desired medicinal compounds from elite accessions of this plant and also ensures the sustainable conservation. It is required to develop an efficient method for identification and propagation of elite accessions of *Centella asiatica*.

Shoot proliferation ability and growth highly depends upon the types of explants, carbohydrate source, culture media (Molassiotis *et al.,* 2003), culture conditions and plant growth hormones (Dj *et al.,* 2008). Regional variation in stigmasterol content can be attributed to a number of factors, biotic and abiotic. Not much literature is available where different medias have been tested for the different accessions of *Centella asiatica,* therefore in the present study we have focused on reproducible and rapid method for *in-vitro* multiplication of different accessions of *Centella asiatica* through high frequency axillary shoot proliferation from nodal explants by growing them in three different plant tissue culture media followed by estimation and comparison of stigmasterol in all the accession of this plant (from the media which showed maximum shoot proliferation).

Experimental

Chemicals

Murashige and Skoog media, Gamborg's B5 (HiMedia Laboratories Pvt. Ltd., India), Nitsch (HiMedia Laboratories Pvt. Ltd., India), Sodium hydroxide, Hydrochloric acid, 6-benzylaminopurine (BAP), potassium hydroxide, stigmasterol (Sigma-Aldrich), hexane(Merck), ethanol (Merck), silylating agent.

Plant Material

Five different accessions (342109, 281347, 331514, 383913 and 347492) of *Centella asiatica* have been collected from NBPGR (National Bureau of Plant Genetic Resources) and grown *in-vitro* in basal Murashige and Skoog (MS) media in photoperiod of 16L: 08D at 25±2^{o}C for 8 weeks. Nodal explants were used as a plant material for axillary shoot multiplication

Effect of different media on multiplication of shoot

Shoot Multiplication

Three different plant tissue culture medium i.e. Murashige and Skoog (MS) (1962) with 3% (w/v) sucrose and 0.8% (w/v) agar, Gamborg's B5 and Nitsch media containing 3% sucrose were utilized for all the experiments. 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. Molten media was dispensed in culture flasks and closed with non absorbent cotton plugs. The media was autoclaved at 121°C temperature, 15psi pressure for 20 minutes. All basal media were supplemented with 1.0 mg/L BAP. 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. All the cultures were transferred to fresh media after 3-4 weeks duration. Mean of the shoot number and shoot length were recorded after eight weeks of inoculation.

Hardening and Acclimatization

Plantlets with well-developed roots were removed carefully from culture medium tubes and washed under running tap water to remove agar, then the plantlets were transferred to pots containing sterilized garden soil and were covered with polythene bags (Figure 2 and Figure 3). The potted plants were maintained inside a culture room at 25± 2◦C for 16 h/day illumination. After a week, the polythene bags were gradually removed over a period of 6 days; the plants were kept in the culture room for another 2 weeks before transferring outside into the field.

Statistical analysis

Experiment was repeated thrice and each treatment had five replicates. The number of cultures per replicate varied for the experiments. Observations were recorded as the number of shoots per explants and length of shoot per nodal explant. The data were analyzed using one-way analysis of variance (ANOVA) ($p<0.05$).

Quantitative estimation of stigmasterol

For stigmasterol analysis, cultures of all the accessions which were grown in MS media supplemented with 1mg/l of BAP was utilized. 200 mg of ground plant material was mixed with the 200µl of internal standard solution (3:1 Hexane: Ethanol) and then vortexed for few minutes. After vortexing 2ml of 2% KOH was added and again vortexed for 1 min and tubes were incubated for 15 min. at 80°C. After incubation samples were allowed to cool at room temperature for 15 min. 1ml hexane and 1.5 ml water were added and then vortexed for 30 sec. After vortexing, tubes were centrifuged at 4000 rpm for 4 min. The upper hexane layer was transferred to a fresh glass tube and left to evaporate at water bath at 50°C. After evaporation, 100µl hexane was further added to solubilise the dried material at walls and transferred to GC vial and then 50 µl silylating agent was added to the GC vial. This final mixture was subjected to Gas Chromatography analysis.
GC conditions: - An Agilent GC (7890B GC system) equipped with flame ionization detector (FID, H2 flow=40 ml/min, air flow=450 ml/min) was used in this study. The analytical column was DB-5 wax capillary column. Temperatures of injection port and detector were 260 °C and 320 °C. Sample injection volume was 1µl and direct injection mode was used.

Results and Discussion

***In-vitro* multiplication of accessions**

Demand for the *in-vitro* grown cultures increases due to the production of phytocompounds and their application in industrial process. In this study, an attempt has been made to see the effect of different media on the shoot multiplication of *Centella asiatica* accessions. The composition of media plays an important role in morphogenesis and culture medium strongly affects the proliferation rate of cultures.
In the present study, an attempt has been made to test the different media on the five different accessions of *Centella asiatica*. Nodal explants of all the five accessions were cultured on MS, B5 and Nitsch medium which were supplemented with 1.0 mg/L BAP. All the three media showed shoot induction after two weeks of incubation period further multiplication of shoots were observed in all the three media. After eight weeks of incubation period, MS media resulted in the highest shoot multiplication in all the five accessions of *C. asiatica* (Table 1 and Figure 1). The role of BAP in internal phytohormone regulation levels for production of multiple shoots has been established in various plants such as *Solanum hainanense* (Nguyen and Huynh, 2011). Ghanti *et al.*, (2004) reported that shoot multiplication occurs due to the cytokinine activity and BAP

showed an increase in activity as compared to the Kinetin. Sivakumar et al., (2006) reported that *C. asiatica* showed maximum multiplication of shoots at 17.76 µM concentration of BAP in MS media.

Estimation of Stigmasterol using Gas Chromatography

Generally phytosterols amount in plant species is constant (Benveniste, 2004). Synthesis of sterol begins with the conversion of farnesyl diphosphate into squalene (Susana et al., 2006). Stigmasterol is one of the important phytocompounds in *Centella asiatica*. In the present study *in-vitro* grown cultures in MS media were used as experimental material for the estimation of stigmasterol so as to identify the best accessions for the production of stigmasterol. Estimation of stigmasterol was carried out with the help of Gas Chromatography. The amount of stigmasterol variation is due to the number of factors i.e. genetic variations, location, etc. On GC analysis of hexane extract, chromatogram showed three peaks at different retention time. Retention time of tocopherol was found at 8.18 min, β-sitosterol was at 10.57min and stigmasterol was at 9.901 min. Stigmasterol was quantified by calculating the peak area at retention time corresponding to standard stigmasterol (Sigma-Aldrich). National Institute of Standard and technology library source (NIST) were used for the matching the identified compounds from the extract. Results showed that out of the five accessions, accession 342109 contains highest amount of stigmasterol i.e. 0.0228±0.70 µg/mg of leaves followed by accession 281374 (0.0098±0.72 µg/mg of leaves), accession 331514 (0.0076±0.51µg/mg of leaves), accession 383913 (0.0066±0.61µg/mg of leaves) accession 347492 (0.0018±0.75µg/mg of leaves) (Table 2 and Figure 4). Phytosterol analysis also revealed that the accessions also contain tocopherol, beta-sitosterol along with stigmasterol. It was also observed that stigmasterol concentration increases with the growth of the *C. aisatica* which shows that there is a direct relationship between growth and stigmasterol production. Zainol *et al.,* 2003 reported that different accessions affect composition of phenolic compounds in petiole, leaf, and roots of *Centella asiatica*. Sethiya and Mishra (2015) reported the presence of stigmasterol content in wild type plants of *Evolvulus alsinoides* (92.75 mg/gram), *Convolvulus pluricaulis* (154.95 mg/gram), *Clitorea ternatea* (31.947 mg/gram) and *Canscora decussate* (39.21 mg/gram). Chowdhary et al, 2014 reported that panvel source contains highest amount of stigmasterol i.e. 0.0582 %.

Conclusions

In this study, it was concluded that media plays any important role in the growth of cultures and phytocompound concentration. MS media showed maximum shoot multiplication as compared to the other two medias. Further estimation of stigmasterol was done in all the accession and it was found that accession number 342109 showed the high amount of stigmasterol concentration i.e. 0.0228µg/mg of leaves. This information can be utilized for the further studies to scale up the stigmasterol production in this accession. Gas chromatography analysis reveals the presence of tocopherol and β-sitosterol along with the stigmasterol.

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Table 1. Effect of different media on the shoot multiplication of different accessions of *Centella asiatica*

Accession No.	Media with standard hormones					
	MS + 1 mg/l BAP		B5 + 1 mg/l BAP		Nitsch + 1 mg/l BAP	
	Number of Shoot (M±SE)	Length of Shoot (M±SE)	Number of Shoot (M±SE)	Length of Shoot (M±SE)	Number of Shoot (M±SE)	Length of Shoot (M±SE)
342109	19.6±0.57	4.9±0.53	16.3±0.57	2.5±0.32	8.6±0.44	2.0±0.53
347492	16.6±0.50	2.8±0.32	12.3±0.44	2.7±0.32	7.6±0.57	1.5±0.55
331514	18.3±0.57	5.1±0.55	15.6±0.50	2.8±0.55	8.3±0.57	2.3±0.55
383913	16.3±0.57	3.5±0.32	13.6±0.50	2.2±0.53	7.6±0.44	2.0±0.53
281374	17.3±0.57	5.6±0.55	13.6±0.44	2.7±0.32	8.6±0.57	2.9±0.32

Table 2. Stigmasterol content of *in- vitro* grown *Centella asiatica* accessions leaves ± Standard deviation of three replicates

Accession no.	Stigmasterol Peak (in minutes)	Stigmasterol content (µg/mg of leaves)
342109	9.901	0.0228±0.70
281374	9.893	0.0098±0.72
331514	9.907	0.0076±0.51
383913	9.896	0.0066±0.61
347492	9.866	0.0018±0.75

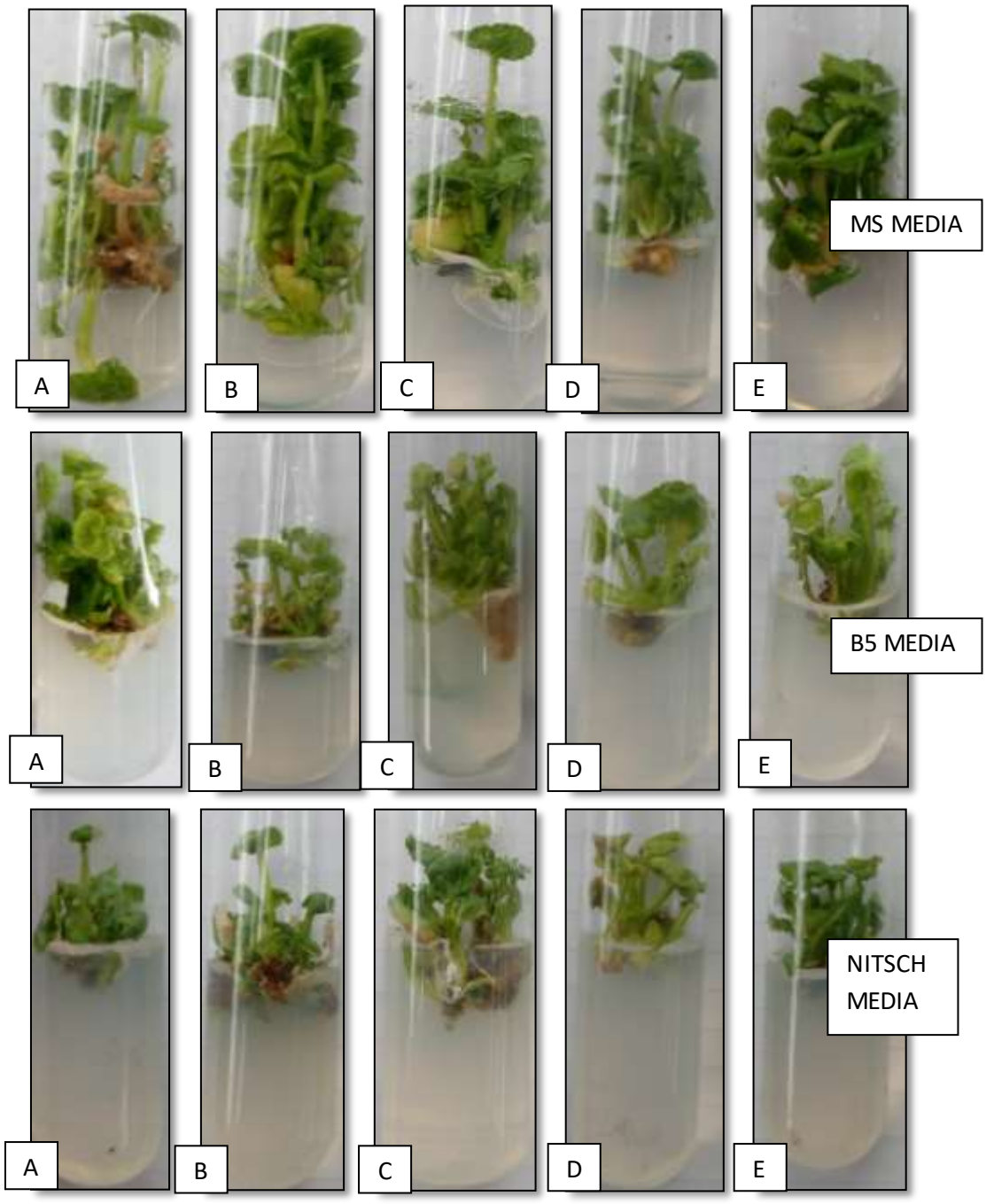

Figure 1 Effect of different media with 1mg/l BAP on the growth in A) Accession no. - 342109, B) Accession no.- 281374, C) Accession no.- 331514, D) Accession no.- 383913, E) Accession no.- 347492

Figure 2 Rooted plant of *Centella asiatica*

Figure S3 Hardening of plant

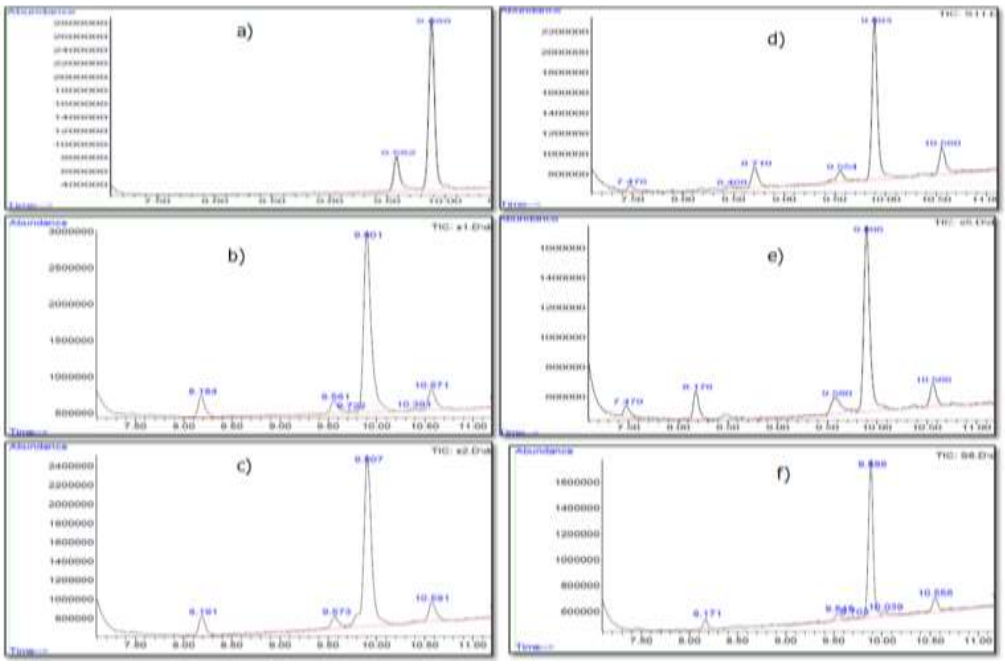

Figure 4 GC Chromatogram of a) stigmasterol standard b) accession no.-342109, c) 281347 d) 331514 e) 383913 and f) 347492.