Journal of Plant Biochemistry & Physiology

Bharadvaja et al., J Plant Biochem Physiol 2018, 6:2
DOI: 10.4172/2329-9029.1000213

Research Article Open Access

Influence of Different Macro and Micro Nutrients on the Shoot Multiplication of *Centella asiatica*

Krishnan ML, Roy A and Bharadvaja N*

Plant Biotechnology Laboratory, Department of Biotechnology, Delhi Technological University, New Delhi, India

***Corresponding author:** Dr. Navneeta Bharadvaja, Plant Biotechnology Laboratory, Department of Biotechnology, Delhi, Technological University, New Delhi, India, Tel: 9013495045; E-mail: navneetab@dce.ac.in

Received date: April 02, 2018; **Accepted date:** April 06, 2018; **Published date:** April 13, 2018

Abstract

The present study deals with the optimization of adventitious shoots cultures of *Centella asiatica* for maximum shoot proliferation under the influence of different nutrient manipulation. The effects of nitrogen source (NH_4NO_3/KNO_3), carbon (sucrose), potassium source (KNO_3), phosphorous source (KH_2PO_4), macronutrient ($MgSO_4$) and micronutrient ($MnSO_4$) of Murashige and Skoog (MS) media on shoot growth were investigated and these experiment treatments were supplemented with 1 mg/l 6-benzylaminopurine (BAP) plant hormones. The shoot growth was influenced by relative ratio of NH_4^+:NO_3^- with maximum shoot number (47.6 ± 1.52) in medium containing 100 mM (40 mM NH_4NO_3:60 mM KNO_3) nitrogen source when NO_3^- concentration is higher than NH_4^+ ions. The MS media with 3% sucrose content showed optimum shoot multiplication (36 ± 1), as with increase in carbon concentration in the media, the shoot proliferation was drastically reduced. In case of media with 30% potassium (KNO_3) highest number of shoots (31.66 ± 1.52) observed and in case of 150% phosphorous (KH_2PO_4) highest shoot number (28 ± 1) obtained respectively. The influence of metal ions like Mg^{2+} and Mn^{2+} showed maximum number of shoots (31 ± 1) in media with 1.5 mM magnesium ($MgSO_4$) and shoot number (43 ± 2) in 200 µM manganese ($MnSO_4$) respectively.

Keywords: *Centella asiatica*; Nutrient manipulation; Shoot multiplication; Murashige and Skoog media

Introduction

Centella asiatica is a small edible, annual herbaceous medicinal plant belonging to Apiaceae family found in moist places up to an altitude of 1800 m [1]. It has orbicular or reniform shaped, yellowish green colored leaves and white or light purple to pink flowers arranged in simple umbels and also bears small oval fruit [1,2]. This plant is native to India, Sri Lanka, Iran, New Guinea, Australia and Indonesia southern and central Africa [3]. It was ranked third position in a priority list of most essential Indian medicinal plants based on their pharmaceutical and economic importance [4]. This plant is listed as threatened plant species by the International Union for Conservation of Nature and Natural Resources (IUCN) [5]. The pharmaceutical companies collecting their raw material from wild populations growing near filthy ditches are sometimes contaminated with heavy metal and chemical pollutants which affects their medicinal efficacy during its formulations, therefore plant tissue culture techniques are an alternative approach to meet the current market demands of the plant biomass as well as its secondary metabolites [6].

The most active secondary metabolites of *Centella asiatica* are triterpene saponin including asiaticoside, madecassoside, asiatic acid, madecassic acids and other valuable terpenoids are brahminoside, thankuniside, sceffoleoside, brahmoside, centellic acid, brahmic acid [7-10]. *Centella asiatica* also contains flavanoids like 3 glucosylkaemferol, 7-glucosylkaemferol [11], fatty acids like palmitic, linoleic, stearic, oleic, linolenic acids [2] and also phytosterols like stigmasterol [12]. It is used as wound healing agent and cures various skin diseases including psoriasis, leprosy, eczema due to its potent anti-inflammatory effects and cell proliferative activity and it also possesses antibacterial, antiviral, antifilarial, anti-stress, anti-ulcer, antioxidative stress and anticancer properties [13-16]. It is known to revitalize the brain and nervous system, thereby increasing the concentration, attention span and delays aging [17]. Centella extracts are commercially exploited in beauty products like skin nourishing creams, anti-ageing serums and in medicines to improve mental abilities, blood circulation, and anxiety [7]. The present work is to study the shoot multiplication of *Centella asiatica* under the influence of different nutrient manipulations including nitrogen, carbon, potassium, phosphorous, magnesium and manganese concentration in the Murashige and Skoog media.

Materials and Methods

Effect of various culture conditions on shoot multiplication

The *in vitro* grown cultures of *Centella asiatica*, accession number 347492 was collected from NBPGR (National Bureau of Plant Genetic Resources), New Delhi, India. Experiments on the effect of nitrogen, carbon, potassium, phosphorous, magnesium and manganese were performed in triplicates for a period of 8 weeks. The Murashige and Skoog (MS) media was prepared according to the respective nutrient manipulation while maintaining the other nutrients at a constant level. All the treatments were supplemented with 1 mg/l 6-benzylaminopurine (BAP) hormone. To analyze the effect of relative ratios of NO_3 and NH_4 ions on shoot growth, the amount of ammonium nitrate: potassium nitrate (NH_4NO_3:KNO_3) salts was varied to 20 mM (0:20), 40 mM (20:20), 50 mM (20:30), 60 mM (20:40) (control) and 100 mM (40:60) in MS media respectively. The control (C) media has 20 mM of NH_4NO_3 and 40 mM of KNO_3 which together makes a total nitrogen concentration of 60 mM. To analyze

the effect of carbon content on shoot growth, the sucrose concentration was varied to eight different treatments from 0% to 7% in MS media and the control (C) MS media comprises 3% total sucrose content. To study the effect of potassium, the KNO_3 salt in MS media was varied to 0%, 30%, 60%, 100% (C), 150% respectively of which the control (100%) media is provided with 1900 mg/l KNO_3 salt. In the treatments with 30% and 60% potassium, in order to compensate the total potassium content in the media, among the major salts, we add $(NH_4)_2SO_4$ salt instead of KH_2PO_4 salt. The phosphorous concentration was varied to 0%, 30%, 60%, 100% (C), and 150% respectively of which the control (100%) media is supplemented at 170 mg/l KH_2PO_4 salt. The macronutrient, magnesium in the MS media was varied to 0 mM, 1 mM (C), 2 mM and 3 mM respectively by varying the $MgSO_4$ salt concentration. To study the effect of micronutrient, manganese on shoot growth, the $MnS0_4$ salt in MS media was added at concentrations of 0 µM, 100 µM(C), 200 µM and 300 µM respectively. After solidification of media, sterile shoot nodes of *Centella asiatica* of accession number 347492 were inoculated vertically into all the culture tubes with different treatments. The shoot cultures were incubated at 262°C under 16 h photoperiod and light intensity of 3000 lux with 55-60% relative humidity. Readings and visual data was recorded after 8th week of inoculation in terms of number of shoot and length of shoots for in vitro growth measurement.

Statistical Analysis

The experiments were independently performed in triplicates and shown in terms of mean ± standard error (M ± SE) and the data were compared using one-way ANOVA (Analysis of variance) with $P \geq 0.05$ and Fstat>Fcritical value considered as statistically significant.

Results and Discussion

In general, Nitrogen, phosphorous and potassium is required in large amounts for appropriate growth and development of plants [18]. Several hypothesis has been stated which assume that in high amount of available nutrients, growth predominates and if nutrients are limiting, growth is restricted more than photosynthesis leading to accumulation of fixed carbon which can used up for synthesizing secondary compounds [19-23]. It has been reported that highest shoot multiplication was observed in the MS media compared to Gamborg's B5 media and Nitsch media supplemented with 1 mg/l BAP in five different accession of *Centella asiatica* [10,12]. The results of all the experimental treatments, shown in terms of mean ± standard error (M ± SE) are statistically analyzed using one-way ANOVA and considered as statistically significant when $P \leq 0.05$ and Fstat>Fcritical value. The shoot cultures of *Centella asiatica* with different nutrient treatments were successfully established in MS media supplemented with 1 mg/l BAP hormone as shown in Figure 1.

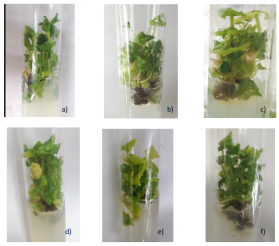

Figure 1: Shoot cultures of *Centella asiatica* grown in MS media under different nutrient concentrations like a) 100 mM nitrogen, b) 3% sucrose, c) 30% potassium, d) 150% phosphorous, e) 1.5 mM magnesium, f) 200 µM manganese after 8 weeks of culture period.

Total nitrogen concentration

Nitrogen is vital for overall growth of the plant and protein synthesis and it is added as inorganic salts, the nitrate ion and ammonium ion. The MS media with different nitrogen treatments showed constant increase in shoot growth throughout the culture period with maximum shoot number (47.6 ± 1.52) and length (4.6 ± 0.52 cm) in media with 100 mM (40:60) nitrogen concentration followed by 60 mM (20:40 mM) (C) and 40 mM (20:20) treatment after 8 weeks as shown in Table 1. There was an overall increase in shoot development observed with enhanced nitrogen levels in the media, therefore reduction in NH_4 ions and enrichment of NO_3 ions favored better growth. Studies indicate that high NH_4 level in media has depressed calcium, potassium and magnesium uptake thereby restricting the nutrient flow into the tissues and lowers the biomass yield [24-26]. There is a need for a proper balance between the two nitrogen sources (NH_4 and NO_3) and neither of the two sources can replace each other in the media [6,27]. Prasad et al. [6] reported that biomass production did not have much difference in media with total nitrogen content of 60, 40 and 20 mM.

S. No	Total nitrogen concentration (NH4NO3:KNO3)	Shoot number(M ± SE)	Shoot length (cm) (M ± SE)
1	20 mM (0:20)	25 ± 2.64*	2.76 ± 0.25*
2	40 mM (20:20)	44.33 ± 1.52*	3.5 ± 0.057*
3	50 mM (20: 30)	40 ± 2.64*	3.33 ± 0.15*
4	60 mM (20:40) (C)	45 ± 1.52*	3.9 ± 0.36*
5	100 mM (40:60)	47.6 ± 1.52*	4.6 ± 0.52*

Table 1: Effect of varying the total nitrogen concentration on shoot growth of *C. asiatica* after 8 weeks of culture period. *Statistically significant at $P \leq 0.05$ and Fstat>Fcritical value.

Total sucrose concentration

The carbon source is usually sugars like sucrose, fructose which maintains the osmotic potential of the plants. The 0% and 1% sucrose treatment failed to support any shoot growth since the nodal explants dried up after the 4th week. The 3% (C) sucrose treatment, showed maximum number of shoots (36 ± 1) and shoot length (4.2 ± 0.3 cm), observed to be the most optimum carbon composition for efficient plant growth as shown in Table 2. The beneficial effect of sucrose on

shoot growth was assumed to be associated with better carbon availability and higher osmotic stress [28]. With increase in sucrose level from 4 to 6% there was a drastic decrease in shoot induction and 7% sucrose treatment showed least growth as the leaves were turning from green to yellow color by the end of 6th week, may be due to higher sucrose levels that induce stress condition to the plant development. Among the carbon sources, 3% sucrose showed highest shoot generation compared to fructose in *Centella asiatica* of accession no. 347492 compared to other accessions [8]. It was reported by Prasad et al. [6] that maximum growth rate with 3 times drier biomass observed in media with 5% sucrose than the control followed by 7% sucrose which showed better growth and biomass yield.

S. No	Total Sucrose concentration	Shoot Number (M ± SE)	Shoot length (cm) (M ± SE)
1	0%	-	-
2	1%	-	-
3	2%	14.3 ± 2.08*	2.2 ± 1.1*
4	3%(C)	36 ± 1*	4.2 ± 0.3*
5	4%	28.66± 2.08*	3.3 ± 0.43*
6	5%	22 ± 2.64*	3.6 ± 0.4*
7	6%	14 ± 1*	3.13 ± 0.41*
8	7%	9.33 ± 1.52*	2.7 ± 0.26*

Table 2: Effect of varying the total sucrose concentration on shoot growth of *C. asiatica* after 8 weeks of culture period. *Statistically significant at P ≤ 0.05 and Fstat>Fcritical value.

Total potassium concentration

Potassium being positively charged, balances the negative ions in plants. The media with 30% potassium content showed highest shoot number (31.66 ± 1.52) followed by 100%(C) and 60% potassium treatments and maximum shoot length (3.9 ± 0.1 cm) in 100% potassium treatment after 8 weeks as shown in Table 3. Muller et al. [18] reported that potassium supply induced no significant difference among treatment in the leaf yield until 2 weeks but after 8 weeks highest yield obtained in 30% followed by 60% and 100%, and lowest yield in 0% treatment.

S. No	Total Potassium concentration	Shoot number (M ± SE)	Shoot length(cm) (M ± SE)
1	0%	14 ± 1*	2.63 ± 0.15*
2	30%	31.66 ± 1.52*	3.56 ± 0.15*
3	60%	22.6 ± 1.52*	3.2 ± 0.88*
4	100%(C)	27 ± 1.73*	3.9 ± 0.1*
5	150%	17.6 ± 0.57*	2.86 ± 0.23*

Table 3: Effect of varying total potassium concentration on shoot growth of *C. asiatica* after 8 weeks of culture period. *Statistically significant at P ≤ 0.05 and Fstat>Fcritical value.

Total phosphorous concentration

Phosphorous is a part of nucleic acids and structural compounds of plant. After 8 weeks, the highest shoot number (28 ± 1) in 150% phosphorous treatment followed by 100% (C) and 60% treatment and shoot length (3.83 ± 0.15 cm) in media treated with 100% phosphorous as shown in Table 4. From previous study, highest herb and leaf yield were achieved in 150% phosphorous treatment after 8 weeks and lowest yield in 0% treatment [18]. The herb yield and leaf yield were augmented with increase in Phosphorous supply [18].

S. No	Total Phosphorous concentration (M ± SE)	Shoot number	Shoot length (cm)
1	0%	13.66 ± 1.52*	2.4 ± 0.17*
2	30%	20.3 ± 0.57*	3.2 ± 0.17*
3	60%	24 ± 1*	3.4 ± 0.1*
4	100%(C)	26.3 ± 2*	3.83 ± 0.15*
5	150%	28 ± 1*	3.6 ± 0.1*

Table 4: Effect of varying total phosphorous concentration on shoot growth of *C. asiatica* after 8 weeks of culture period. *Statistically significant at P ≤ 0.05 and Fstat>Fcritical value.

Total magnesium concentration

The macronutrient, magnesium (Mg^{2+}) helps in balancing the negative ions, critical for enzymatic functioning and basic component of chlorophyll molecule. After 8 weeks, maximum shoot number (31 ± 1) and shoot length (3.83 ± 0.25 cm) in 1.5 mM (C) magnesium concentration followed by 1 mM and 2 mM magnesium treatment as shown in Table 5. Prasad et al. [6] stated that lowering magnesium was associated with lowering asiaticoside content but did not have significant effect on growth. The beneficial influence of Mg results in reduction of Mn uptake, and a higher concentration of Mg in plant tissues confer tolerance to high concentrations of Mn [29].

S. No	Total Magnesium concentration	Shoot number (M ± SE)	Shoot length (cm)
1	0 mM	11.33 ± 1.5*	1.76 ± 0.25*
2	1 mM	29.6 ± 1.52*	2.5 ± 0.3*
3	1.5 mM (C)	31 ± 1*	3.8 ± 0.25*
4	2 mM	26.6 ± 1.52*	2.2 ± 0.4*
5	3 mM	14.6 ± 1.52*	1.63 ± 0.15*

Table 5: Effect of varying total magnesium concentration on shoot growth of *C. asiatica* after 8 weeks of culture period. *Statistically significant at P ≤ 0.05 and Fstat>Fcritical value.

Total manganese concentration

The micronutrient, manganese is required for respiratory and photosynthetic processes. Nutrients such as Fe, Ca and Mg in the growth medium can modify the uptake of Mn from media [30,31]. After 8 weeks, the maximum shoot number (43 ± 2) and shoot length (3.86 ± 0.15 cm) in 200 µM manganese followed by 300 µM and 100

µM (C) manganese treatments as shown in Table 6. Manganese did not show any significant effect on growth as reported by Prasad et al. [6].

S. No	Total Manganese Concentration in MS media	Shoot Number (M ± SE)	Shoot length(cm) (M ± SE)
1	0 µM	24 ± 1.73*	2.3 ± 0.43*
2	100 µM (C)	30.33 ± 1.5*	2.93 ± 0.3*
3	200 µM	43 ± 2*	3.86 ± 0.15*
4	300 µM	40.66 ± 1.5*	3.26 ± 0.25*

Table 6: Effect of varying total manganese concentration on shoot growth of *C. asiatica* after 8 weeks of culture period. *Statistically significant at $P \leq 0.05$ and Fstat>Fcritical value.

Conclusion

From the investigation, it can be concluded that the nutrient composition of MS media has a strong impact on shoot multiplication of *Centella asiatica*. The different experimental treatments in MS media showed that maximum shoot proliferation was observed in MS media supplemented 100 mM (40 mM NH_4NO_3:60 mM KNO_3) nitrogen source when NO_3^- concentration is higher than NH_4^+ ions, 3% sucrose as carbon content, 30% potassium, 150% phosphorous, 1.5 mM magnesium and 200 µM manganese respectively. The proper balance of these nutrients in the MS media contributes to proper growth and metabolism of shoot cultures. These findings may be useful to optimize the media for mass propagation of this potential medicinal plant, *Centella asiatica* both in terms of its biomass as well as for its secondary metabolites. Future perspective is to analyze the important centellosides present in *Centella asiatica* and to enhance it through biotechnological interventions.

Acknowledgement

The authors are highly grateful to the Department of Biotechnology, Delhi Technological University, New Delhi, India for providing the research facilities and financial support to carry out this project. We thank National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India for providing us with the required plant species.

References

1. Gohil KJ, Patel JA, Gajjar AK (2010) Pharmacological review on Centella asiatica: a potential herbal cure-all. Indian J Pharm Sci 72: 546-456.
2. Chopra RN, Nayar SL, Chopra IC (1956) Glossary of Indian Medicinal Plants. Council for Scientific and Industrial Research, CSIR, New Delhi.
3. Hoang N, Nguyen LT (2010) Asiaticoside production from Centella (Centella asiatica L. Urban) Cell Culture. Biotechnol Bioprocess Eng 15: 1065-1070.
4. Sivakumar G, Alagumanian S, Rao MV (2006) High Frequency in vitro Multiplication of Centella asiatica: An Important Industrial Medicinal Herb. Eng Life Sci 6: 597-601.
5. Pandey NK, Tewari KC, Tewari RN, Joshi GC, Pande VN, et al. (1993) Medicinal plants of Kumaon Himalaya. Strategies for conservation of Himalayas, Himavikas Publication, Nanital, India.
6. Prasad A, Mathur A, Singh M, Gupta M, Uniyal GC, et al. (2012) Growth and asiaticoside production in multiple shoot cultures of a medicinal herb, Centella asiatica (L.) Urban, under the influence of nutrient manipulations. J Nat Med 66: 383-387.
7. Singh S, Gautam A, Sharma A, Batra A (2010) Centella asiatica (l.): a plant with immense medicinal potential but threatened. Int J Phar Sci Rev Res 4: 9.
8. Kundu K, Roy A, Saxena G, Kumar L, Bharadvaja N (2016) Effect of Different Carbon Sources and Elicitors on Shoot Multiplication in Accessions of Centella asiatica. Med Aromat Plants 5: 251.
9. Roy A, Kundu K, Saxena G, Bharadvaja N (2017) Estimation of Asiaticoside by Using RP-HPLC and FAME Analysis of Medicinally Important Plant Centella asiatica. J Plant Biochem Physiol 5: 198.
10. Roy A, Kundu K, Saxena G, Kumar L, Bharadvaja N (2016) Effect of different media and growth hormones on shoot multiplication of in vitro grown Centella asiatica accessions. Adv Tech Biol Med 4: 172.
11. Rastogi RP, Mehrotra BN (1969) Compedium of Indian Medicinal Plants, Central Drug Institute, Lucknow and Publication and Information Directorate, CSIR, New Delhi.
12. Roy A, Bharadvaja N (2017) Effect of Different Culture Medias on Shoot Multiplication and Stigmasterol Content in Accessions of Centella Asiatica. Int J Ayurvedic & Herbal Medicine 7: 2643-2650.
13. Bonfill M, Mangas S, Moyano E, Cusido MR, Palazon J (2011) Production of centellosides and phytosterols in cell suspension cultures of Centella asiatica. Plant cell Tissue Organ Cult 104: 61-67.
14. James J, Dubery IA (2011) Identification and Quantification of Triterpenoid Centelloids in Centella asiatica (L.) Urban by Densitometric TLC. J Planar Chromatogr 24: 82-87.
15. Roy A, Attre T, Bharadvaj N (2017) Anticancer agent from medicinal plants: a review. New aspects in medicinal plants and pharmacognosy. JB Books, Poland.
16. Roy A, Bharadvaja N (2017) Medicinal Plants in the Management of Cancer: A Review. Int J Complement Alt Med 9: 00291.
17. Brinkhaus B, Linder M, Schuppan D, Hahn EG (2000) Chemical, pharmacological and clinical profile of the East Asian medical plant Centella asiatica. Phytomedicine 7: 427-448.
18. Muller V, Lankes C, Benno Zimmermann F, Nogaa G, Hunschea M (2013) Centelloside accumulation in leaves of Centella asiatica is determined by resource partitioning between primary and secondary metabolism while influenced by supply levels of either nitrogen, phosphorus or potassium. J Plant Physiol 170: 1165-1175.
19. Watson DJ (1963) Some features of crop nutrition. The growth of the potato. Butterworth, London.
20. Epstein E (1972) Mineral Nutrition of Plants: Principles and Perspectives. Wiley, New York.
21. Smith D (1973) The non-structural carbohydrates. Chemistry and biochemistry of herbage. London, Academic Press Inc.
22. McKey D (1979) The distribution of secondary compounds within plants, Herbivores: their interaction with secondary plant metabolites. Academic Press, New York.
23. Bryant JP, Chapin FS, Klein DR (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40: 357-368.
24. Zhong, JJ, Wang SJ (1998) Effects of nitrogen source on the production of ginseng saponin and polysaccharide by cell cultures of Panax quinquefolium. Process Biochem 33: 671-675.
25. Pilbeam DJ, Kirkby EA (1992) Nitrate and ammonium utilization by plants, Nitrogen metabolism of plants. Oxford Science Publications, Oxford.
26. Liu S, Zhong JJ (1998) Simultaneous production of ginseng saponin and polysaccharide by suspension cultures of Panax ginseng: nitrogen effects. Enzyme Microb Technol 21: 518-524.
27. Omar R, Abdullah MA, Hasan MA, Marziah M, Mazlina MKS (2005) Optimization and elucidation of interaction between ammonium, nitrate and phosphate in Centella asiatica cell culture using response surface methodology. Biotechnol Bioprocess Eng 10: 192-197.
28. Liu M, Dai Y, Li Y, Luo Y, Huang F, et al. (2008) Madecassoside isolated from Centella asiatica herbs facilitates burn wound healing in mice. Planta Medica 74: 809-815.

29. LeBot J, Goss MJ, Carvalho PR, Beusichem ML, Kirkby EA (1990) The significance of the magnesium to manganese ratio in plant tissues for growth and alleviation of manganese toxicity in tomato (Lycopersicon esculentum) and wheat (Triticum aestivum) plants. Plant and Soil 124: 205-210.

30. Chinnery LE, Harding CP (1980) The effect of ferrous ion on the uptake of manganese by Juncus effuses L. Ann Bot 46: 409-412.

31. Maas EV, Moore DP, Mason BJ (1969) Influence of calcium and magnesium on manganese absorption. Plant Physiol 44: 796-800.