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Research Article

Micropropagation of medicinally important herb *Centella asiatica* L.

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Abstract

The present investigation aimed at developing *in-vitro* propagation protocol, which can be used for conservation of *Centella asiatica.* The MS medium supplemented with Benzyl adenine (BA 0.5, 1.0, 1.5, 2.0 mg/L) kinetin (KIN 0.5, 1.0, 1.5, 2.0 mg/L) with 0.2 mg/L IAA show average shoot proliferation. The combination of BA with kinetin (KIN 0.5 mg/L) and IAA & NAA (0.2 mg/L) in MS media showed maximum shoot elongation. Among the combinations, MS media supplemented with BA: IAA (2:0.2) show maximum number of shoots per explants (11.66±0.56) and maximum shoot length (5.76±0.42). KIN (1.0 mg/L) with IAA (0.2 mg/L) shows highest (13±0.33) number of shoots. MS media supplemented with BA, KIN with IAA and NAA (2:0.5 and 0.2:0.2) resulted in shooting, as well as, rooting simultaneously. Micro propagated plantlets were hardened, acclimatized and transferred to the field. This micro propagation procedure could be useful for mass multiplication of superior plant material for field cultivation as well as research purpose.

INTRODUCTION

Centella asiatica (Brahmi in Hindi; Vallarai in Tamil) is a small herbaceous annual plant of the family *Apiaceae*, and is native to India. It is used as a medicinal herb in Ayurvedic medi-cine for increasing memory power. *Centella asiatica* is a small herbaceous plant belonging to *Apiaceae* (Umbelliferae) family. Centella comprises some 50 species (James and Dubery, 2009), inhabiting tropical and sub-tropical regions. The herb was reported as antidiabetic, antiviral, antiulcer, antibacterial, antitumor and high memory enhancing activity (Chakraborty *et al.*, 1996; Srivasthava *et al.*, 1997; Vasantharuba Seevaratnam *et al.*, 2012). The major bio-active ingredients in the plant are the triterpenes, asiatic acid, madecassic acid and their glycosides such as asiaticosside and madecassoside (Zheng and Qin, 2007). Due to the presence of these active ingredients, it possesses antileprotic, antifilarial, antibacterial, antifeedant, adaptogenic and antiviral properties (Warrier *et al.*, 1994).

In India, *Centella asiatica*, the plant frequently suffers due to growing modern agriculture, in-creasing use of herbicides, drastically depleting water level in river, canals and irrigation channels or adding of sewage water in the river cause rapidly eroding natural habitat. The biodiversity in the species are facing extinction.

Because of the large scale and unrestricted exploitation of this natural resource to meet ever increasing demand by the Indian pharmaceutical industry which is coupled with the limited cultivation and insufficient attempts for its replenishments, the wild stock of this medicinally important plant species has been markedly depleted and it is listed as a threatened species by International Union for Conservation of Nature and National Resources (Pandey *et al.*, 1993)

an endangered species (Singh, 1989) Therefore, it is important to develop an efficient micro propagation technique for Centella asiatica to rapidly disseminate superior clones once they are identified. Tissue culture techniques can play an important role in the clonal propagation of elite clones and germplasm conservation (Tiwari *et al.*, 2000).

MATERIALS AND METHODS

Collection and surface sterilization of explants

The explants were collected from Amrutkund Tq. Basavkalyan, Dist. Bidar near Maharashtra-Karnataka border. The plant material were washed carefully in running tap water for 10 minute and followed by distilled water for 5 minutes. For surface sterilization, chemical such as 70% ethanol and Hgcl2 (0.1 %) were used. Explants kept for 1 minute in 70% ethanol after the 1 minute they are also sterilizing with 0.1% mercuric chloride for 3 minute followed by three subsequent rinses with sterilized double distilled water in a laminar flow. All these explants were dissected very carefully. Explants were cut into small pieces and aseptically inoculated in test tube as well as culture vessels containing MS medium with various concentration of growth hormones.

Media Preparation

Throughout the study, different strengths of Murashige and Skoog medium were used for experiments. Full strength MS basal medium and modified MS medium with different concentrations and combinations of BA, KIN, IAA, NAA for studying the in vitro multiplication responses were used as the initiation medium. Sucrose (30%) was used as the carbon source in all the combinations. All media combinations were solidified by adding 1.5gm/L clerigel instead of agar.

The pH of the medium was adjusted between 5.6 and 5.8 using 0.1N HCl or 0.1N NaOH solutions prior to the autoclaving of the medium. Sterilization of the medium was done at a pressure of 15 psi for 20 min and was allowed to cool at room temperature. After inoculation all culture tubes were incubated at 25 ± 2^0 C under cool white fluorescent tubes.

Inoculation of explants

Apical shoot was used as explant. The explants were inoculated on MS (Murashige and Skoog 1962) medium supplemented with different concentration of phytohormones like, BA (Benzyl adenine); KIN (Kinetin), NAA (Naphthalic acetic acid) and IAA (Indol -3-acetic acid) produce maximum percentage of multiple shoots.

Shoot formation

MS medium supplemented with various concentrations of KIN [0.5, 1.0, 1.5, 2.0 mg/L]or BA [0.5, 1.0, 1.5, 2.0 mg/L] individually or in combination [BA 0.5, 1.0, 1.5, 2.0 mg/L & KIN 0.5 mg/L each] were used for shoot formation. Small amount of IAA and NAA were used in the medium.

Root formation

The formed shoots were transferred on MS medium supplemented with various concentrations of IAA [0.5, 1.5, 2.5 mg/L] or NAA [0.5, 1.5, 2.5 mg/L] individually or in combination [IAA 0.5, 1.5, 2.5 mg/L & NAA 0.5mg/L each] gives average percentage of root multiplication.

Hardening

The plantlets with well developed root and shoot systems were transferred to a 500 ml glass jars containing sterilized soil. For humidity polythene bags were used for 1 week. The surviving plants were transferred to pots containing garden soil and maintained.

RESULTS AND DISCUSSION

Shoot induction was achieved in 2-3 weeks of inoculation. Bud break on BA supplemented MS media after two weeks of inoculation followed by shoot proliferation, and further two weeks for root induction (Karthikeyan et al.,2009). Micropropagation protocol of *C. asiatica* through callus proliferation on MS media supplemented with combination of cytokinin and auxins (Thangapandian *et al.*, 2012).

In the present study, BA (Benzyl adenine) was taken in combination with auxins such as Indole 3-acetic acid and Naphthelic acetic acid [IAA & NAA]. The 1st treatment of BA (0.5-2.0 mg/l) along with IAA resulted in shoot as well as root induction. Maximum shoot numbers per explant (11.66±0.56) was recorded, when MS media supplemented with 2 mg/L BA + 0.2 mg/L IAA, followed by 1.5 mg/L BA + 0.2 mg/L IAA (6.66±0.88), which were significantly higher than others, while maximum shoot length (5.76±0.42) was observed on MS media supplemented with 2.0 mg/LBA + 0.2 mg/L of IAA, which was significantly higher than others **(Table 1).** The 2nd treatment of KIN (0.5-2.0 mg/l) in combination with IAA (0.2mg/L) results in maximum

Fig.A

Fig.B

Fig. C

Fig. D

Fig. E

Fig. F

Fig. A-F A. initiation of shoot from the explant. B. multiplication of shoots. C. multiplication of shoot and basal callus formation. D. initiation of roots from regenerated shoots. E&F. induction of rooting and basal callus formation number of shoots than BA.

MS media supplemented with KIN (1 mg/L & 2.0mg/L) with combination of (0.2 mg/L) IAA resulted in maximum shoot numbers per explant (13±0.33) & (12±0.57) respectively **(Table 1).** In the 3 rd treatment BA (0.5-2.0 mg/l) and KIN (0.5mg/L) along with IAA & NAA (0.2mg/L each) were used. The maximum number of shoots i.e. (21.66±3.57) was recorded when MS medium

supplemented with 2 mg/L BA + 0.5 mg/L KIN +0.2 mg/L IAA &NAA. The maximum shoot length (6.1±0.057) was recorded **(Table 1).**

Root initiation was achieved from the bases of excised shoots in the presence of IAA (0.5-2.5 mg/L) or NAA (0.5-2.5mg/L) or both (i.e IAA 0.5-2.5mg/L and NAA 0.5 mg/L) after 2 weeks of transfer. Among all concentrations tested, maximum root proliferation was recorded on MS medium with IAA (2.5 mg/L) with maximum mean number of roots (**42.66±1.76**) and mean root length (**6.36±0.21**) (**Table 2**).

Acclimatization and hardening

Well developed rooted plants were carefully removed from culture tubes and washed to remove the remnants of agar. These healthy shoots were transferred to tray containing soil and vermiculate in 1:1 ratio for acclimatization. For a period of week the plants were kept in polythene membrane. After that the surviving plants were transferred to pots and maintained under greenhouse for hardening

Table 1. Effect of different cytokinin on *in vitro* shooting using MS medium

BENZYL ADENINE	KINETIN	INDOLE 3-ACETIC ACID	NAPHTHALIC ACETIC ACID	NO. OF SHOOTS	LENGTH OF SHOOTS in cm	CALLUS FORMATION
0.5mg/L	-	0.2 mg/L	-	2.33±0.32	1.33±0.32	+
1.0 mg/L	-	0.2 mg/L	-	4±0.57	5.1±0.66	+
1.5 mg/L	-	0.2 mg/L	-	6.66±0.88	5±0.28	++
2.0 mg/L	-	0.2 mg/L	-	11.66±0.56	5.76±0.42	+++
-	0.5 mg/L	0.2 mg/L	-	4± 0.57	3.1±0.057	+
-	1.0 mg/L	0.2 mg/L	-	13±0.33	4.16±0.11	++
-	1.5 mg/L	0.2 mg/L	-	9.66±0.66	8.63±0.32	+++
-	2.0 mg/L	0.2 mg/L	-	12±0.57	7.06±0.52	+
0.5 mg/L	0.5 mg/L	0.2 mg/L	0.2 mg/L	13±0.33	3.06±0.06	++
1.0 mg/L	0.5 mg/L	0.2 mg/L	0.2 mg/L	6.66±0.88	5.46±0.20	++
1.5 mg/L	0.5 mg/L	0.2 mg/L	0.2 mg/L	11±1,52	6.1±0.057	+++
2.0 mg/L	0.5 mg/L	0.2 mg/L	0.2 mg/L	21.66±3.57	5.76±0.14	+++

Table 2. Effect of different auxins on *in vitro* rooting using MS medium

INDOLE 3-ACETIC ACID	NAPHTHALIC ACETIC ACID	LENGTHOF SHOOTS	NO.OF ROOTS	LENGTH OF ROOTS (cm)
0.5mg/L	-	9.66±0.32	1.33±0.32	4±0.28
1.5 mg/L	-	13.1±0.20	14.33±1.20	5.73±0.11
2.5mg/L	-	16.16±0.60	31±2.08	6.26±0.14
	0.5mg/L	5.33±1.187	2.66±0.88	2.1±0.057
	1.5 mg/L	8.66±0.66	25.66±1.115	6.2±0.15
	2.5mg/L	12.36±0.23	37.66±1.45	5.16±0.08
0.5mg/L	0.5mg/L	8.66±1.20	18.33±0.32	5.03±0.03
1.5 mg/L	0.5mg/L	19.33±2.33	33.33±2.02	5.66±0.087
2.5mg/L	0.5mg/L	19.33±0.66	42.66±1.76	6.36±0.21

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