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Plant Regeneration Via Organogenesis on Petiole of *Centella asiatica* (L.) Urban

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ABSTRACT : An efficient plant regeneration of *C. asiatica* was achieved from organogenesis using petiole explants of *in vitro* plantlet on MS basal medium controled with different plant growth regulators (NAA, 2,4-D, IAA kinetin, and BA). Best results that 50%, efficiency of regeneration per explant for regeneration were obtained with IAA 17.13 μM and BA 8.9 μM. Formation of adventitious shoots via organogenesis from the petiole explant was verified by histological sectioning of plantlets. Regenerated plants were transplanted into soil.

Key words : *Centella asiatica*, adventitious shoot, organogenesis, histological section

INTRODUCTION

Centella asiatica (L.) Urban. which belongs to the Apiaceae is a medicinal plant that has been employed since prehistoric times. The genus Centella comprised of about 33 species of herbs is widely found in tropical and subtropical regions. It is prostrate, faintly aromatic stoloniferous perennial herb, with a glabrous stem and long petiolated fleshy leaves rooting at the nodes, *C. asiatica* grows up to an altitude of 600 m above sea level on moist, sandy or clayey-soils, forming a dense green carpet in India (Anonymous, 1992).

The therapeutic use of this herb remedy has been documented in South East Asia and India for centuries. The plant continues to be used within the framework of folk medicine as an effective remedy (Brinkhaus *et al.*, 2000). *C. asiatica* has many common names including "gotu kola", "hydro cotyle", "indian pennywrot", "marsh penny", "thick-leaved pennywort" and "white rot". *C. asiatica* is the most ubiquitous species of Centella. It is found in Southeast Asia, Sri Lanka, parks of China, the Western South Sea Islands, Madagascar, South Africa, the Southeast of the U.S.A., Mexico, Venezuela, Columbia, and the eastern regions of South America. All the remaining species of Centella grow mainly in South Africa (Kartnig and Hoffmann-Bohn, 1992).

C. asiatica contains about 0.15% essential oils and another volatile constituents. These are derived from the metabolism of phenylpropane and acetate, and belong to the flavonoids and terpenes. The substances of therapeutic interest are the saponincontaining triterpene acids and their sugar esters. The most important being: asiatic acid, madecassic acid and the three asiaticosides, namely asiaticoside, asiaticoside A and asiaticoside B. Among them, asiaticoside showed the most effective antibacterial and fungicidal activity against pathogen and fungal and is its principle ingredient (Mesnard 1975). In case of triterpene saponins, they have differences depending on country of origin. Besides, asiaticoside content of *C. asiatica* varied depending on genetic resources and habitat environments such as levels of shading (Upadhyay *et al.*, 1991; Mathur *et al.*, 2000).

C. asiatica plants have been reported to contain the following glycosides; indocentelloside, brahmoside, brahminoside, asiaticoside, theankuuiside and isothankuniside. Asiaticoside is useful in treatment of leprosy and tuberculosis. Centella leaves are in Carotenoids, vitamins B and C. The plant shows good therapeutic effects on peptic ulcers. It is one of the components of the drug 'Geriforte' which is used for senile pruritns (Anomymeus, 1992). In the traditional system of Indian medicine, Centella is a reputed nervine tonic and is used for the treatment of asthma, bronchitis, dropsy, elephantiasis gastric catarrh, kidney troubles, leprosy, leucokkhoea, skin disease and urethritis (Kakkar, 1988) with antibacteral, antifeedant, antifilarial, antileprotic, antistress, antituberculosis activities and wound-healing properties (Chakraborty *et al.*, 1996; Srivastava *et al.*, 1997).

As reported earlier, Centella micropropagation were experimented using nodal segments with multiplication (Kavindra *et al*, 2000), somatic embryogenesis using leaf segments (Paramageetham, 2004) and regeneration from callus culture (Patra A

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et al, 1998). All these reports described for large scale propagation and conservation of this important neutraceutical herb. Here we report a protocol, for improving organogenesis in *C. asiatica* petiole.

MATERIALS AND METHODS

Plant material and shoots culture

In vitro plantlets of *Centella asiatica,* which was originated in Jeju island, Korea and selected a fast growing line (Kim *et al.*, 2002), were used in this experiment. Mother plant cultures were maintained on liquid MS medium (Murashige and Skoog, 1962) supplemented with sucrose 3% (w/v) at 25℃ for 16 h photoperiod in a 250 mℓ flask. To ensure synchronous conditions, subculture was carried out by inoculation of one node by removing shoot and root of 6 week cultured plantlets of *C. asiatica.*

Petiole culture and plant regeneration

Petioles of 2.0 ㎝ long were excised from the base of 28 day old grown sterile plantlet shoot culture. Explants were placed on MS medium containing 3% sucrose, 0.3% gellan gum and various concentration of cytokins and auxins in different combination.

The pH of the media was adjusted to 5.7-5.8 with 1 N NaOH or 1 N HCl before dispensed into culture vessels and autoclaved at 121℃ for 20 min.

Histological studies

For histological studies, material was fixed in formalin:acetic:alcohol (5 : 5 : 90) for 48 h, dehydrated in tertiary-butyl alcohol for 30 min per stage, and embedded in paraplast (Lu and Vasil, 1981). Sections of 10-12 ㎛ were obtained with a microtome (Microm, Walldorf, Germany). After deparaffination, the sections were stained with haematoxyline (Sigma), and examined using a light microscope (Labophot Nikon, Tokyo, Japan)

Acclimatization

C. asiatica plantlets with well developed roots were removed from culture medium and after washing the roots were washed under running tap water to remove gellan gum. Plantlets were transferred to plastic cups containning sterilized garden soil and were covered with polyethylene bags.

The potted plants were maintained inside a culture room at 25 ± 2℃ under a 16h photoperiod (20 μmol m^{-2} s^{-1}) provided by cool white fluorescent lamps and cooling the plants with cold tap water. After 2 weeks, the polyethylene bags were gradually removed over period of 6 days and the plants were kept in the culture room for another 2 weeks before being transferred into the outside field.

RESULTS AND DISCUSSION

In vitro propagation

Liquid cultures of *C. asiatica* plantlet for propagation were established by Kim *et al.*, (2002). They used nodes excised from leaf and petiole as inoculants. For obtaining the synchronous status of inoculating tissues for this experiment, whole plants or plantlet were cultured using "node- inoculating" subculture method. In order to obtain a number of petiole, plant cultured by airlift type bioreactor were used as experimental materials for regeneration. Shoot multiplication achieved from the outgrowth of lateral basal buds stock plant cultures were increased by repeatedly subdividing cultures at 28-day intervals. Plant cultured in liquid medium produced leaves with elongated petioles.

Effects of Plant growth regulators (PGRs) on organogenesis

Minimal shoot was observed in petiole explant cultured on phytagel solidified basal medium. Shoot organogenesis was promoted only in the presence of both exogenous auxin and cytokinin. Of the three cytokinins screened, BA was the most effective in stimulating adventitious shoot formation when added in combination with IAA. In general, shoot organogenesis *in vitro* was weakly promoted in the presence of kinetin, regardless of auxin supplementation. Addition of NAA to media was generally inhibitory to shoot organogenesis (Matthew. *et al.*, 2000).

Petiole explants were inoculated at various hormone concentration of the size 10×10 ㎟ and then number of adventitious shoots were counted. Optimum shoot regeneration occurred on explants cultured on medium supplemented with both 8.9 μM BA and 17.13 μM IAA with the exception of stolons produced *in vitro Centella* (Table 1, Fig. 1). As a result, regeneration showed 50% efficiency. Regeneration medium was experimented by adding of and TDZ to the highest regeneration efficiency, but both callus and adventitious shoots were not occurred. (Data not shown)

Kothari and Chandra (1986) found that the highest bud formation (69.4%) was achieved at 17.1μM IAA and 13.3 μM BA. These authors reported 12% as the highest value of shoot formation with very high BA and IAA concentrations. Regeneration shoots were transferred to MS basal and MS medium supplemented with 8.9 μM BA in combination with 17.13 μM IAA for elongation (Fig. 2). Shoots organogenesis did not induce in the presence of BA alone. Similar to *Centella,* shoot

Table 1. Effect of Plant growth regulators (PGRs) on organogenesis in *C. asicatica*.

PGRs (μM)				Efficiency of regeneration (%)
BA	0	NAA	2.68	–
	2.2		5.37	30
	4.4		10.74	–
	8.9		16.12	–
	13.3		26.85	–
	0	KIN	2.32	–
	2.2		4.65	–
	4.4		9.29	–
	8.9		13.94	–
	13.3		23.23	–
	0	IAA	2.68	–
	2.2		5.71	–
	4.4		11.42	10
	8.9		17.13	50
	13.3		28.54	–
	0	2,4-D	2.26	–
	2.2		4.52	–
	4.4		9.05	–
	8.9		13.57	–
	13.3		22.62	–

– : not detected

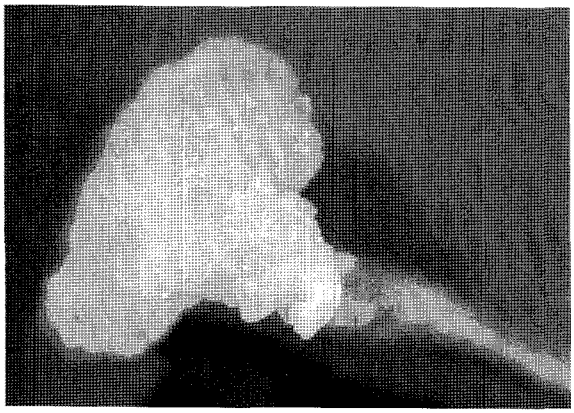

Fig. 1. Formation of adventitious shoots via callogenesis on petiole bases of *C. asiatica*.

organogenesis from internodal stem explants of *Myriophyllum aquaticum*, was completely inhibited in the presence of 10 μM BA alone (Kane *et al.*, 1991). Elongation of regeneration shoots were also subcultured to regeneration medium, for

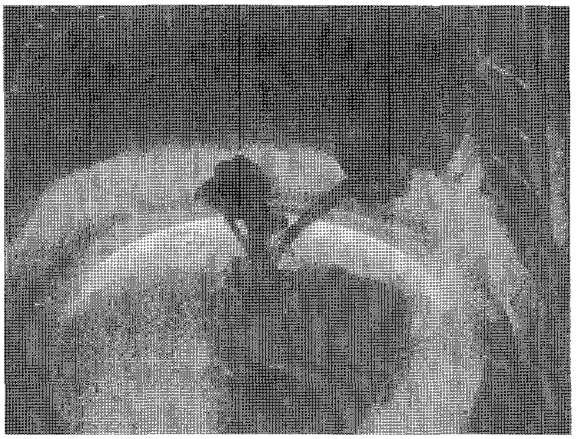

Fig. 2. Proliferation of adventitious shoots in MS medium.

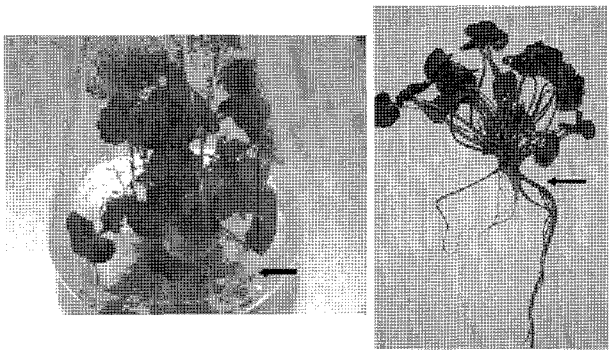

Fig. 3. Roots formed from regenerated shoot on PGRs free MS medium after 35 days culture; Arrow indicates adventitious root formed from regenerated plantlet.

about 4 weeks. Rooting of elongated shoots was assessed by subculturing on MS media (Fig. 3). Kavindra *et al.* (2000) reported root development was not occurred in only MS basal medium but, occurred in MS basal medium supplemented with 2.46 μM IBA. However, we induced roots in MS basal medium (Fig. 3).

Adventitious shoots produced *in vitro* consisted of a small basal meristem with multiple leaves but no defined stem axis. Enhanced callus production was observed on petiole explants, cultured on various concentrations (Data not shown).

However, leaf explants inoculated in various hormone concentration subsequently induced callus but regeneration were not occurred. The origin of leaf explant showed no significant effect on shoot organ formation capacity. *Nymphoides indica* exhibits an intermediate capacity for shoot organogenesis *in vitro*. (Kakkar and Mohan Ram, 1986; Kane and Albert, 1989; Kane *et al.*, 1991, 1993).

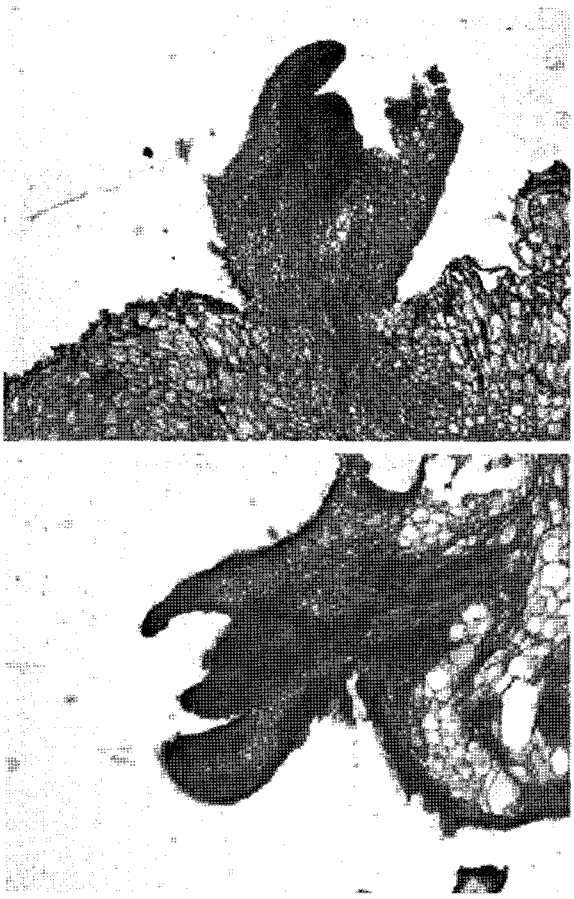

Fig. 4. Histological studies of organogenesis. Adventitious shoots developed from surface of callus after 20 days culture.

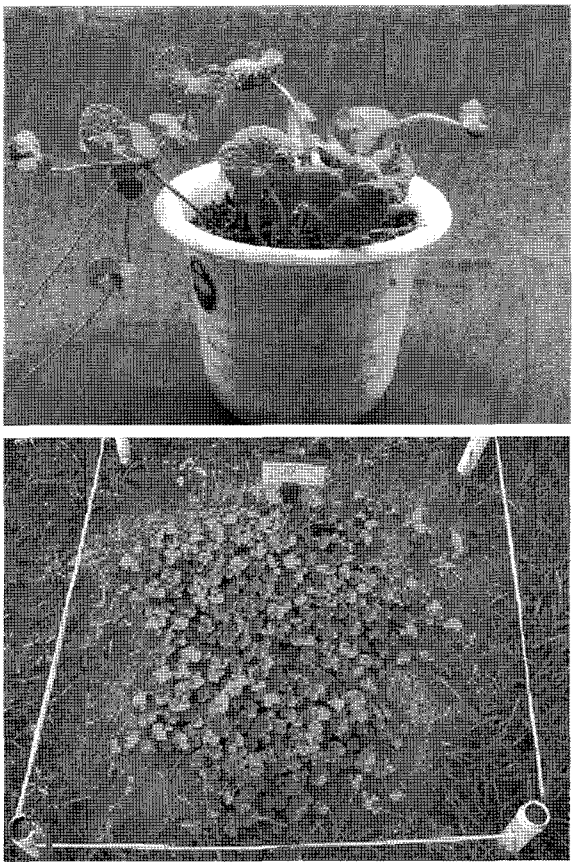

Fig. 5. Acclimatization and field test. Acclimatized plants were transplanted to soil.

Histological studies of shoot organogenesis

Adventitious shoots were developed on petiole explants by indirect organogenesis. After 18th day of explants inoculations, adventitious shoots derived from these meristems were consisted of well defined shoot apices and several subtending leaf primodia (Fig. 4). Adventitious shoots from callus were formed mostly toward the tip than toward the basal part of petiole explants. Shoots organogenesis was also occurred well in surface of callus in contact with regeneration medium. Mostly, shoots organogenesis was occurred on both white and browny color callus of petiole explants. Adventitious shoots were developed from callus after 20 days culture. This shoots organogenesis were observed histologicaly through paraffin section.

Applications of effective *in vitro* mutation breeding techniques require induction of genetic change in individual cells and subsequent regeneration of solid whole plant mutants via direct and/or indirect organogenesis (Broertjes and Van Harten, 1978).

This study clearly indicates that optimum adventitious shoot formation can be readily induced from petiole explants cultured on gellan gum solidified basal medium supplemented with 8.9 μM BA and 17.13 μM IAA.

Acclimatization

The acclimatization of plantlets were successfully established in the field (Fig. 5). These findings may be helpful in micropropagation and ex situ conservation of *C. asiatica.* All the acclimated plants completed their cycle normally; Kothari and Chandra (1984) reported plant acclimation, but only 20% of their transplanted plants survived. In this work, the frequency of survival was up to 100% and no problem was found in the plant adaptation.

Up to now, *Centella* has been experimented using various explants. Shoot regeneration has been reported from leaf derived callus (Patra *et al.*, 1998; Banerjee *et al.*, 1999), stem (Patra *et al.*, 1998) and nodal (Tiwari *et al.*, 2000) segments of *C. asiatica.* Although the efficiency of regeneration was different in with more than 95% but various reports, all these experiments were very successful in *Centella* acclimatization. This study also observed successful acclimatization more than 97%

via organogenesis. The *Centella* leaves withered up at the initial stage of acclimatization. However, it was not that wrong. It was adapted *Centella* slowly into new circumstance that new leaves developed in node a part.

In summary, a whole plant regeneration system was developed for *C. asiatica* utilizing petiole explants using MS media containing BA(8.9 μM) and IAA(17.13 μM) incubating for 28 days. The regenerated shoots were then transplanted to hormone free MS medium for elongation and rooting. Approximately after four weeks, the plantlets having roots were able to transferred to sterile soil for acclimatization and fully developed into plant in the greenhouse.

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