Life Sciences for Sustainable Development: Issues and Challenges

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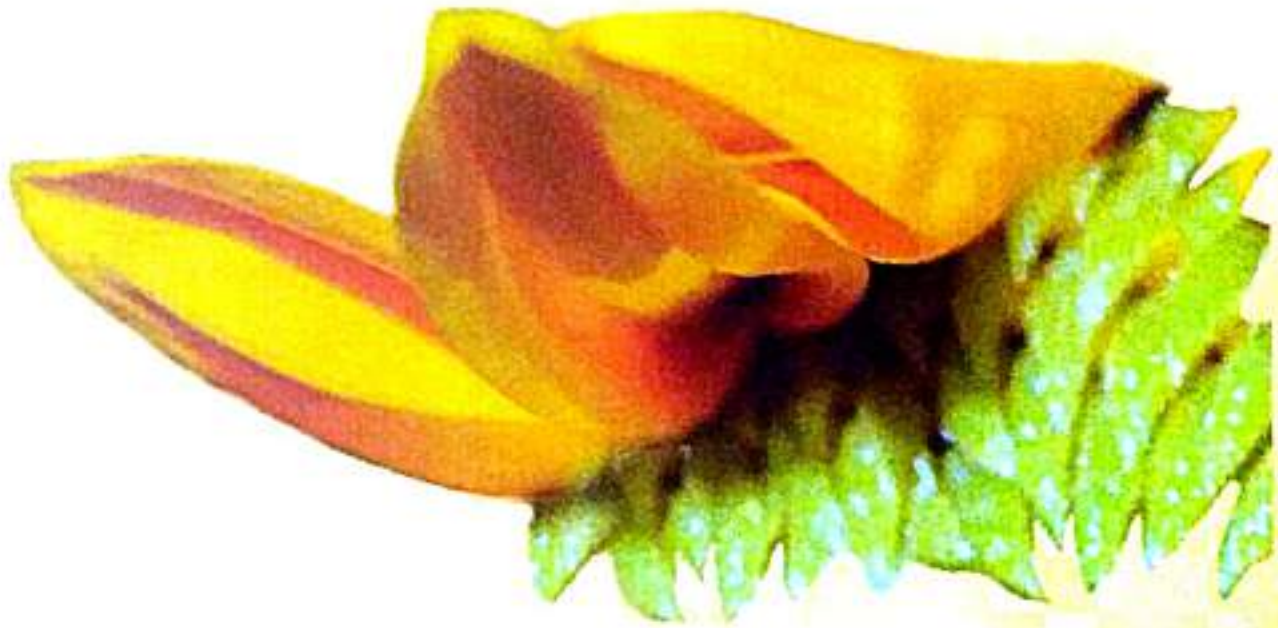

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MICROPROPAGATION AND INDUCTION OF CALLUS IN *CENTELLA ASIATICA* (L.) URBAN

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ABSTRACT

Centella asiatica (L.) Urban is a traditional medicinal herb widely used as a brain tonic, found in the moist shady places as a wild species. This plant has been listed for immediate conservation by the Medicinal Plant Board of the Government of India. Keeping this in mind, we tried to establish *in vitro* cultures of *Centella asiatica* (L.) Urban from nodal explants and to optimize the culture conditions for callus induction and maintenance from leaf explants. Nodal explants were inoculated onto Murashige and Skoog (MS) basal medium without any growth regulators for the successful establishment of in vitro cultures. MS basal medium supplemented with various concentrations of 6- Benzyl Adenine (BA) and Naphthalene Acetic Acid (NAA) were used for callus induction from leaf explants. MS medium fortified with BA (3mg/l) + NAA (1mg/l) produced green, hard, compact and fast growing callus after 4 weeks of inoculation. Callus thus produced can be multiplied and later used for cloning numerous whole plants or for the extraction of secondary metabolites through proper elicitation techniques.

Keywords: *Centella asiatica* (L.) Urban, in vitro culture, Callus.

Medicinal plants play a key role in the world health care systems. The Indian systems of medicine, particularly Siddha, Ayurveda, Unani and Homeopathy, largely use plant-based materials and products of animal origin. The growing demand on natural metabolites is putting a heavy strain on the already available resources, causing a number of species to be either endangered or threatened.

Centella asiatica (L.) Urban is an important medicinal plant member of the Apiaceae family, commonly known in India as 'Indian Pennywort'. The plant enjoys considerable reputation in Indian systems of medicine as a brain tonic. It is also a source of bioactive compounds such as isoprenoids and phenyl propanoid[1].

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Centella asiatica (L.) Urban grows in India at an altitude of up to 600m above sea level on moist, sandy or clayey-soils, forming a dense green carpet[2]. In the recent years there is an unrestricted exploitation of *Centella asiatica* (L.) Urban to meet the ever increasing demand by the Indian pharmaceutical industries. It is listed as a threatened species by International Union for Conservation of Nature and National Resources[3]. *In vitro* culture techniques offer a viable tool for germplasm conservation and mass multiplication of rare, endangered and threatened medicinal plants[4]. The aim of the present study was to establish *in vitro* cultures of *Centella asiatica* (L.) Urban and to standardize the medium for callus induction and maintenance.

Materials and methods

Plant material

The plants of *Centella asiatica* (L.) Urban were maintained in the shade net house in the Botanical garden of the Department of Botany, All Saints' College, Thiruvananthapuram (Fig. 1).

Fig.1: *Centella asiatica* (L.) Urban grown in pots in the net house of All Saints' College

Explant selection

The explants were selected from young, healthy and disease free plants. Nodal segments (the third node) were used as explants for establishment of *in vitro* cultures, while leaves were used as explants for callus induction.

Surface sterilization and *in vitro* culture establishment

Healthy shoots of C. *asiatica* (L.) Urban from the *in vivo* established plants were collected and washed gently under running tap water. These were then treated with 2% Teepol solution for 10 minutes and rinsed thrice with sterile distilled water. The shoots were then excised to get the required explants. Surface sterilization of the explants was performed by employing freshly prepared Mercuric Chloride (0.1% w/v) solution for 2 minutes, in aseptic conditions inside the Laminar Air Flow Chamber. This was followed immediately by 5-6 rinses with sterile distilled water, accompanied by vigorous shaking.

The explants were individually inoculated into test-tubes containing MS[5] basal medium (Himedia) supplemented with sucrose and gelled with agar (MS1). Medium pH was adjusted to 5.7.

The cultures were kept under 8 hour light and 16 hour dark period. The light was provided by cool white fluorescent light.

The temperature of incubation was 25 ± 2º C and relative humidity was 50 to 60%. The cultures were monitored daily and the observations were taken periodically.

Callus Induction Studies

MS basal medium (Himedia) fortified with various concentrations of BA and NAA was used for callus induction studies and the medium was supplemented with sucrose and gelled with agar. The media combinations used were as follows:

MS2 – MS + BA (2mg/l)
MS3 – MS + BA (2mg/l) + NAA (0.5mg/l)
MS4 – MS + BA (2mg/l) + NAA (1mg/l)

MS5 – MS + BA (3mg/l) + NAA (1mg/l)

The cultures were kept under the conditions mentioned above and the observations were taken periodically.

RESULTS

Surface Sterilization of the Explants

The primary establishment of the aseptic in vitro culture needs surface sterilization of the explants from in vivo or field grown plants. Most commonly used disinfectants are Calcium hypochlorite (Ca(ClO)2), Sodium hypochlorite (NaOCl), Mercuric chloride (HgCl2) and Hydrogen peroxide (H2O2). In our study HgCl2 solution was used as the surface sterilant.

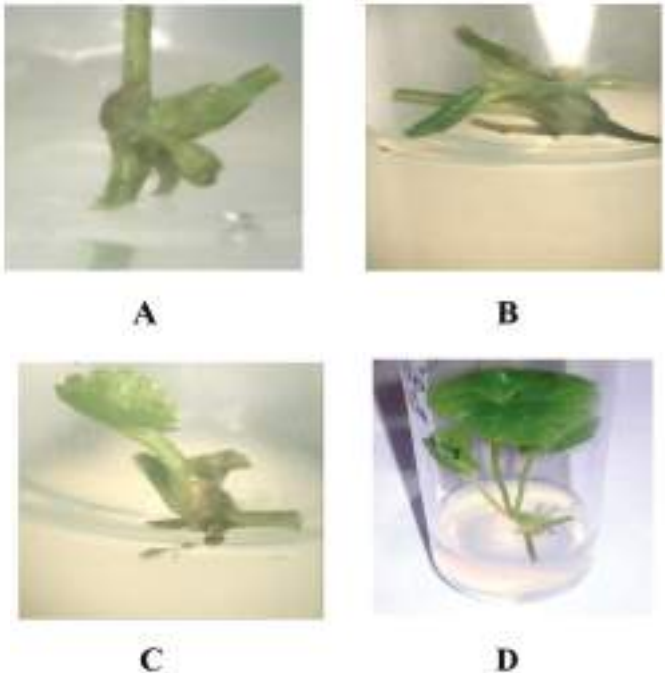

Fig.2: Establishment of *in vitro* cultures of *Centella asiatica* (L.) Urban

Establishment of *in vitro* cultures

It was observed that explants were very susceptible to high concentrations of $HgCl_2$ and majority of them turned black and died. Surface sterilization of nodal explants with 0.1% $HgCl_2$ for 2 minutes gave 90% successful establishment response, which was found to be the best among all the treatments for *Centella asiatica* (L.) Urban.

Single nodes, after removing leaves, were inoculated into MS basal medium without any growth hormones. After one week slight swelling was observed at the nodes and they exhibited the emergence of minute shoot buds at the end of second week (Fig. 2A). At the end of the fourth week, 93±2% nodal explants produced single shoot per explant (Fig. 2B-D).

We attempted the induction of callus from leaf explants using varying concentrations of BA and NAA. The leaf explants inoculated on MS5 medium (fortified with 3mg/l BA + 1mg/l NAA) produced green, hard, compact and fast growing callus after 4 weeks of inoculations (Fig 3E & F).

The explants inoculated into the medium with BA alone (MS2) and BA and low concentration of NAA (MS3) did not induce callusing, instead they browned and died (Fig 3B & C). MS4 medium fortified with BA (2mg/l) + NAA (1mg/l) produced friable callus (Fig 3D).

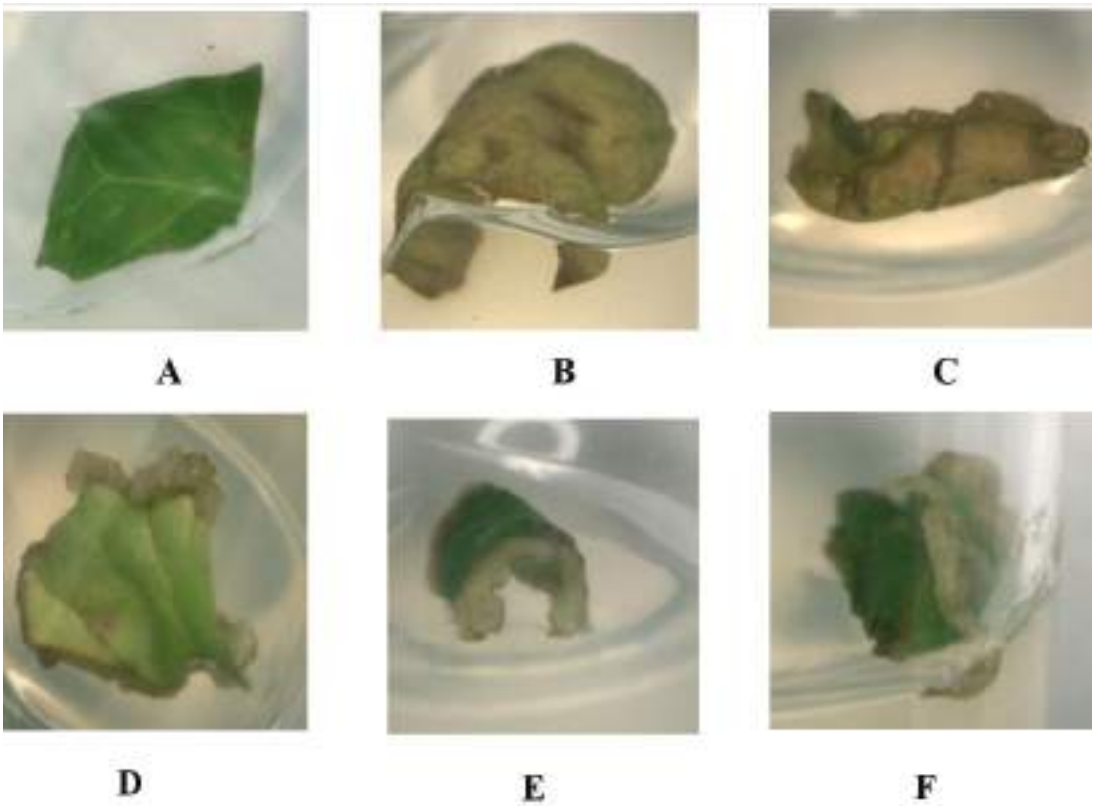

Fig. 3 Callus induction studies using leaf explants. 3A – Leaf explants inoculated into the medium; 3B-F – Callus induction after 4 weeks of incubation in varying media combinations. 3B – MS2; 3C – MS3; 3D – MS4; 3E & 3F – MS5

DISCUSSION

In vitro culture of nodal explants resulted in single shoot per explant. Multiple shoot induction through axillary shoot bud has proved to be a reliable method for the micropropagation of a large number of plant species. Das *et al.*,[6] reported multiple shoot induction in *Centella asiatica* (L.) Urban using shoot tip explant in MS medium augmented with 4.0 mg /l BA +0.1 mg/l NAA. Biradar[7] also successfully induced multiple shoots in *Centella asiatica* (L.) Urban using MS media supplemented with 2 mg/l BA + 0.2 mg/l IAA. Failure of multiple shoot induction in our study may be due to the lack of any growth hormones in the medium.

The differential application of growth regulators and the control conditions in the culture medium induces the development of callus and subsequent organogenesis from the explant. The best combination which gave moderate compact growth of callus was in the medium supplimented with 3mg/l BA + 1 mg/l NAA. Patra *et al.*,[8] successfully established *in vitro* plant regeneration from callus derived from stem and leaf explants of *Centella asiatica* (L.) Urban on semisolid modified MS medium supplemented with 2.0 mg/l kinetin and 4.0 mg/l NAA.

Arumugam *et al.*, [9] effectively established callus culture from leaf explants of *C. asiatica* (L.) Urban on MS medium supplemented with different concentration of plant growth regulators for callus initiation, with maximum percentage of callusing achieved in medium supplemented with BA 4.0 mg/l and 2,4-dichlorophenoxyacetic acid 2.0 mg/l. In our studies we found that MS5 medium containing basal MS medium fortified with BA (3mg/l) + NAA (1mg/l) produced green, hard, compact and fast growing callus after 4 weeks of inoculations and was found to be the best medium for callus induction.

CONCLUSION

In the present study we attempted the establishment of in vitro cultures through nodal culture and tried to standardize the medium for callus induction from leaf explants. Nodal explants were cultured on MS basal medium without any growth regulators and shoot formation was observed after 4 weeks of inoculation. 93±2% nodal explants exhibited shoot formation with single shoot per explant. The failure of induction of multiple shoots per explant may be due to the absence of growth regulators in the medium.

Callus induction studies were conducted using varying combinations of BA and NAA in the medium. MS5 medium containing basal MS medium fortified with BA (3mg/l) + NAA (1mg/l) produced green, hard, compact and fast growing callus after 4 weeks of inoculations and was found to be the best medium for callus induction. The explants inoculated onto the medium with BA alone (MS2) and BA and low concentrations of NAA (MS3) did not induce callusing, instead they browned and died.

Thus we have successfully established a protocol for *in vitro* culture of *Centella asiatica* (L.) Urban using nodal explants in MS basal medium without any growth hormones. We have also standardized the medium for induction and propagation of callus from leaf explants. Callus cultures are good sources of secondary metabolites, if properly elicited. The successful establishment of callus cultures will result in preventing the destruction of plants for medicinal purpose and can act as a method of conservation of this endangered medicinal plant. Thus the study aimed at conserving the threatened plant using *in vitro* techniques and callus establishment for isolation of important medicinal compounds in future.

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