

 <p>ISSN NO. 2320-5407</p>	<p>Journal Homepage: - www.journalijar.com</p> <h2 style="text-align: center;">INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)</h2> <p style="text-align: center;">Article DOI: 10.21474/IJAR01/4206 DOI URL: http://dx.doi.org/10.21474/IJAR01/4206</p>	 <p>INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR) ISSN 2320-5407 Journal homepage: http://www.journalijar.com Journal DOI: 10.21474/IJAR01</p>
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RESEARCH ARTICLE

FOLIAR REGENERATION IN *CENTELLA ASIATICA* (L.) URBAN (APIACEAE): AN IMPORTANT THREATENED MEDICINAL HERB.

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Manuscript Info

Manuscript History

Received: 17 March 2017
Final Accepted: 15 April 2017
Published: May 2017

Key words:-

Centella asiatica, regeneration,
callogenesis, shoot multiplication,
tryptophan.

Abstract

A tissue culture protocol for mass propagation of *Centella asiatica* from leaves was worked out. Better callus induction was observed from the young pale green leaves on MS medium supplemented with 1mg/l 2,4-D, 0.5mg/l KIN and 4mg/l TRY. Calli were non-embryogenic, granular and friable. No callus induction was observed on MS medium without hormones. Maximum shoot regeneration from callus was observed on MS medium supplemented with 1mg/l KIN and 0.1mg/l BAP while the supply of NAA and BAP reduced the regeneration of multiple shoots. Present study prospects the *in vitro* conservation of *C. asiatica* and provides a novel source for the extraction of medicinally important secondary metabolites.

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Abbreviations:-

BAP: 6-Benzylaminopurine
NAA: 1-Naphthaleneacetic acid
2,4-D: 2,4-Dichlorophenoxyacetic acid
KIN: Kinetin
TRY: Tryptophan

Introduction:-

Centella is a diverse genus encompassing approximately 50 species which include the medicinally eminent plant *Centella asiatica* (L.) Urban (James and Dubery, 2009). The plant is otherwise called Indian pennywort and belongs to Apiaceae family. It is a stoloniferous, perennial, slightly aromatic herb flourishes plentifully in moist localities and distributed all over the world including India, China, Nepal, Madagascar, Srilanka, Indonesia and Southern America (Schaneberg et al., 2003).

Traditionally, the plant is used as nervine tonic and for the treatment of asthma, hypertension, bronchitis, dropsy, skin diseases and urethritis. The most eminent bioactive components present in *C. asiatica* are triterpenoid glycosides, saponin glycosides, indocentelloside, brahmoside, brahminoside, asiaticoside, theankuniside and flavonoids which are known to possess antileprotic, antifilarial, antibacterial, adaptogenic, antifeedant and antiviral properties (Warrier et al., 1994; Aziz et al., 2007; James and Dubery, 2009). Extract of whole plant is reported to have anticancerous activity (Yu et al., 2006) and the methanol extract of aerial parts of the *C. asiatica* inhibits the growth of human uterine carcinoma, human gastric carcinoma and murine melanoma cells *in vitro* (Yoshida et al., 2005).

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C. asiatica is a threatened medicinal herb and endemic to Western Ghats of South India (Nayar and Satry, 1987; Singh et al., 2015). Thus, it has become imperative to develop alternative approaches for its conservation, in order to protect it from the verge of extinction. *In vitro* culture technique offers a viable tool for the mass propagation of plant and the conservation of rare, threatened and endangered germplasm (Rao, 2004). Multiplication of *C. asiatica* through shoot tip, leaf and meristem culture has been reported (Tiwari et al., 2000; Nath and Buragohain, 2003; Martin, 2004; Mohapatra et al., 2008). This biotechnological approach helps to produce secondary metabolites and other bioactive compounds from the cultured tissues that avoid the use of natural plants for the extraction processes. The aim of the present study was to develop high frequency multiple shoots regeneration of *C. asiatica* from leaf explants utilizing the least number and various concentrations of plant growth regulators.

Materials and methods:-

Plant material: The young leaves of *C. asiatica* were collected from the campus of KAHM Unity Women's College, Manjeri, Malappuram, Kerala.

Surface sterilization of explant: The young leaves were first washed with running tap water for 15 minutes to remove the soil particles and other extraneous fine particles. Explants were soaked in 1% Tween 20 for 10 minutes and washed thoroughly with running tap water. The explants were immersed in 0.5% bavistin for 1 hour and gently washed twice with tap water and double distilled water.

Callogenesis: To reduce the contamination of the MS medium at the time of transfer of the explants, all inoculation processes were carried out inside the laminar air flow chamber (LAF). Before starting the work, the culture flasks with growth medium, sterilized forceps and knife, spirit lamp, 70% alcohol, sterile double distilled water and others needed for the inoculation process were surface sterilized using UV light for 30 minutes. The explants were sterilized using 0.1% HgCl_2 for 2-3 minutes, followed by washing with 70% alcohol for 3-5 minutes and sterile double distilled water for 5-6 times. The undesirable and dead portions of cut ends of the leaves were removed using the sterile knife. The leaf explants were placed horizontally in the culture flasks containing the media (MS+2,4-D1mg/l+KIN0.5mg/l+TRY4mg/l, MS+2,4-D0.5mg/l+KIN1mg/l+TRY2mg/l, 2,4-D1mg/l+KIN0.5mg/l and 2,4-D0.5mg/l+KIN1mg/l) with the help of sterile forceps and all the processes were done in front of the spirit lamp to keep a sterile ambience. The culture flasks were closed carefully and sealed with Klin film. Five replicas for each treatment were used for the present study. The cultures were maintained at $25 \pm 2^\circ\text{C}$, with a photoperiod of 8 h daylight and light intensity of $1500 \mu\text{Em}^{-2}\text{S}^{-1}$.

Shoot multiplication: After four weeks, the callus was transferred to the shoot multiplication media (MS+KIN0.1mg/l+BAP0.5mg/l, MS+KIN0.5mg/l+BAP0.3mg/l, MS+KIN1mg/l+BAP0.1mg/l, MS+BAP1mg/l+NAA0.5mg/l and MS+BAP0.5mg/l+NAA1mg/l). The cultures were maintained at $25 \pm 2^\circ\text{C}$, with a photoperiod of 8 h daylight and light intensity of $1500 \mu\text{Em}^{-2}\text{S}^{-1}$ for regeneration of multiple shoots.

Results and discussion:-

Plant tissue culture techniques for medicinal plants have been well established today which act as a powerful tool for germplasm conservation. Plant biotechnology comprises various cultural methods of plant organs which facilitate the experimental approaches for improvement of medicinal plants and crops.

Callogenesis: Callogenic shoot multiplication is a characteristic feature observed in micropropagation of *C. asiatica* (Bibi et al., 2011). Calli characteristics and induction response vary depending on explant source, explant size, media components and presence of plant growth regulator in media along with other culture conditions (Zia et al., 2007). In our study, leaf explants of *C. asiatica* developed callus at cut surfaces and subsequently covered the entire surface of explants within 21 days (Fig. 2A and 2B). Callogenesis was not observed in MS medium without growth hormones. Different concentrations of plant growth regulators including KIN and 2,4-D together with an amino acid - Tryptophan were used to induce callogenesis from *Centella* leaves.

Calli formed in the study were non-embryogenic, granular and friable. At the initial stage, the calli were cream coloured which gradually turned to green within 28 days (Fig. 2C). Previous studies reported that MS medium supplemented with BA/KIN along with 2,4-D induced best callogenic response in *Oryza* seeds (Al-Khayri and Anderson, 1995). However our study revealed that non-embryogenic callus induction was better on MS medium supplemented with 2,4-D1mg/l+KIN0.5mg/l+TRY4mg/l and 2,4-D0.5mg/l+KIN1mg/l+TRY2mg/l compared to

other growth regulator combinations. However, a fewer callogenic response was observed when explants grown on MS medium supplemented with 2,4-D1mg/l+KIN0.5mg/l and 2,4-D0.5mg/l+KIN1mg/l. The significant role of tryptophan on callogenesis is highlighted in our study which can be clarified by further biochemical studies.

Shoot multiplication: In the present study, callus induced from leaves were used to raise multiple clones of *C. asiatica* by supplying different concentrations of cytokinins including KIN and BAP. Within 14 days, callogenic response was enhanced in all KIN and BAP combinations used. Optimum callogenic response was observed in MS medium supplemented with 1mg/l KIN and 0.1mg/l BAP compared to other hormonal combinations (Fig. 2E), while minimum callus induction was on KIN0.1mg/l + BAP0.5mg/l. In the study, multiple shoots were initiated from the callus within 21 days. Among the hormonal combinations used, maximum number of multiple shoots (8 ± 2.6) was proliferated from the callus which was grown on MS medium supplemented with 1mg/l KIN and 0.1mg/l BAP (Fig. 1, Fig. 2E). In contrast, the number of multiple shoots was minima on KIN0.1mg/l + BAP0.5mg/l. Our study is in accordance with earlier studies on *C. asiatica* by Karthikeyan et al. (2009), that explains the positive role of KIN and BAP in shoot multiplication and callogenesis.

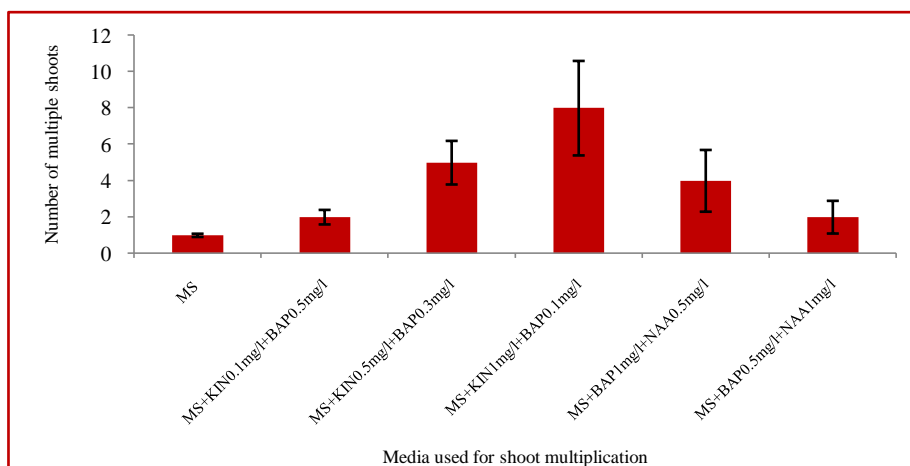


Fig.1:- Multiple shoot proliferation in *in vitro* *C. asiatica* on MS medium supplemented with different plant growth regulators within 28 days. The values are mean \pm SE and n=5.

The combinations of BAP and NAA were also used to analyze their role on multiple shoot induction and callogenesis in *C. asiatica*. The study revealed that both callus and shoot induction was lesser on MS+BAP/NAA compared to the combinations of KIN and BAP used. Within 14 days undifferentiated cream coloured calli induction was enhanced followed by multiple shoot induction within 21 days. After 14 days the callus turned to green which was showed the indication of shoot regeneration. Here, best callogenesis and shoot regeneration were observed on MS medium supplemented with 1mg/l BAP and 0.5mg/l NAA (Fig. 2D) compared to MS medium with 0.5mg/l BAP and 1mg/l NAA. The number of shoots regenerated from callus grown on 1mg/l BAP and 0.5mg/l NAA was 4 ± 1.7 within 28 days while that on MS medium supplemented with 0.5mg/l BAP and 1mg/l NAA was 2 ± 0.9 (Fig. 1). The result is supported by the absence of shoot multiplication on hormone free MS medium.

Conclusion:-

Our study provides a new protocol for the callogenic shoot proliferation from leaves within 8 weeks. MS medium supplemented with 1mg/l KIN and 0.1mg/l BAP induced optimum callogenic shoot proliferation that indicates the need of specific concentration of plant growth regulators in organogenesis. Enhancement of callus induction from the leaves by the supplementation of tryptophan together with 2,4-D and KIN provides a new experimental approach which reveals the positive role of tryptophan on non-embryogenic callus induction. Transfer of this callus to shoot multiplication medium will produce *C. asiatica* clones that in turn will help to extract medicinally important biocompounds without the harvesting of natural plants (Kim et al., 2004). Through tissue culture techniques, we can conserve this medicinally important threatened plant.

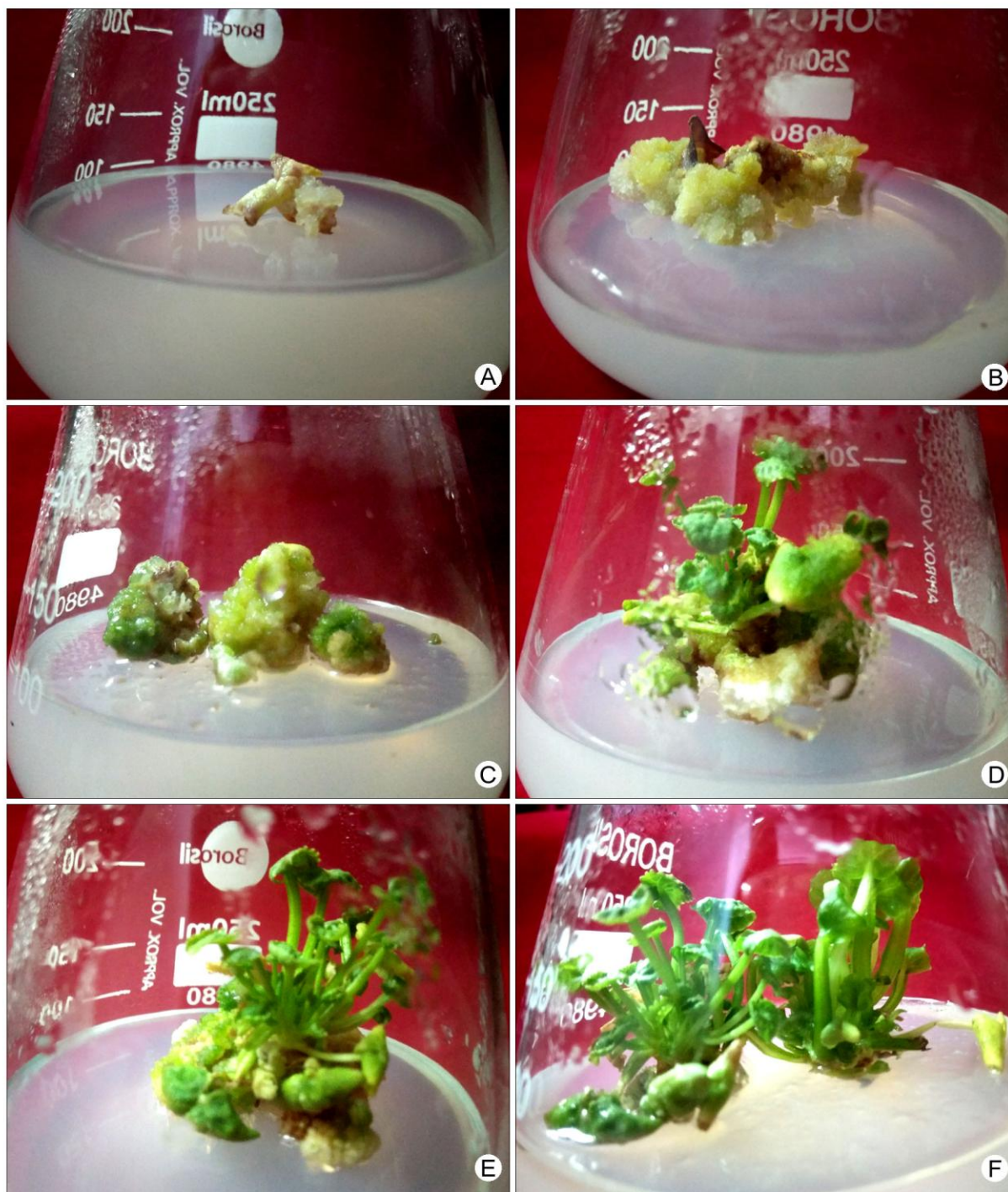


Fig. 2 A:- Callus induction from leaf of *C. asiatica* on MS medium supplemented with 2,4-D1mg/l+KIN0.5mg/l+TRY4mg/l within 14 days, **B.** Callus induction from leaf of *C. asiatica* within 21 days, **C.** Callus induction from leaf of *C. asiatica* within 28 days, **D.** Shoot proliferation from foliar callus on MS medium supplemented with 1mg/l BAP + 0.5mg/l NAA within 21 days, **E.** Shoot proliferation from foliar callus on MS medium supplemented with 1mg/l KIN + 0.1mg/l BAP within 21 days and **F.** Multiple shoots on MS medium supplemented with 1mg/l KIN + 0.1mg/l BAP within 28 days.

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