Indian J.Sci.Res. 08 (2): 41-45, 2018 ISSN: 0976-2876 (Print)
ISSN: 2250-0138 (Online)

MICROPROPAGATION OF *Centella asiatica* THROUGH TISSUE CULTURE AN ATTEMPT TO DEVELOP AN EFFICIENT PROTOCOL FOR LARGE SCALE PRODUCTION

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ABSTRACT

Centella asiatica **of Apiaceae is an important medicinal herb, found in the moist shady places as a wild species. Because of its medicinal value people do harvest the species from its natural population. This plant has been listed for immediate conservation by the Medicinal Plant Board, Govt. of India. Keeping this idea in mind tissue culture study was done to select suitable combination and concentration of the growth regulators so that it may be considered as an efficient protocol that shall be exploited at commercial scale to provide large number of uniform planting material. MS basal medium was supplemented with 3% sucrose, seven different concentrations of BAP and KN with 0.5 mg/l NAA, separately. It was noted that MS + 3% sucrose, gelled with 0.8% agar + 4.0 mg/l BAP + 0.5 mg/l NAA gave the highest percentage of response that was 92.64% with respect to shoot bud initiation which was 4.68 in number after 12th day of inoculation. At the similar concentrations KN + NAA gave only 58.84% response, with 2.24 number of shoot buds after 16th day of inoculation. On subculture in the same culture conditions MS + 4.0 mg/l BAP + 0.5 mg/l NAA induced 8.72 numbers of axillary shoots per explants. 4 week old plantlets were used for rooting in MS + 0.3 to 1.5 mg/l IBA. Here again the maximum number of roots was obtained in MS + 0.5 mg/l IBA which was 4.5 / plantlet on an average.**

KEYWORDS: *Centella asiatica,* Apiaceae, Micropropagation, Tissue Culture, Growth Regulators, Axillary Shoots

Abbreviation: BAP – 6 Benzyl Amino Purine
KN = Kinetin
NAA = Naphthalene Acetic Acid
IBA = Indole Butyric Acid
MS = Murashige and Skoog

Medicinal plants were used by the ancient people, but along with the advent of Allopathic medicine the importance of medicinal plants was eclipsed for a particular time. However, it has been observed that there is a U turn regarding the use of herbal medicine all over the World and even the developed country like USA, there are utilization of herbal medicines or drug derived from the medicinal plants. Therefore, one in four prescriptions filled in USA, is either a synthesized form or derived from plant materials. (Srivastva *et al*; 1997). Due to increasing demand of the medicinal plants at global level, there is resurgence in the trade of herbal medicines as well as the medicinal plants to be used as a raw material for the preparation of different drugs. It is estimated that Europe, annually, imports about 400, 000 tonnes of medicinal plant material from Africa and Asia. The expected value is 1 billion US, Dollar.

Due to over exploitation of the medicinal plants in their natural population, The Government of India created 30- *in-situ* and 15 *ex-situ* Medicinal Plant Conservation Areas and Parks. Similarly, Central Institute of Medicinal and Aromatic Plants (CIMAP) at Lucknow is also dealing with conservation of medicinal plants.

Micropropagation through tissue culture is the best alternative for the conservation of medicinal plants. We get several literatures with respect to micropropagation of medicinal plants. Some of them may be cited here. Johnson *et al*; (1997) in *Saussurea lappa*, Ajit Kumar and Seeni (1998) in *Aegle marmelos*; Patra *et al*; (1998) in *Centella asiatica* through calus culture, Banerjee *et al*; (1999); in *Centella asiatica*, Kulkarni *et al*; (2000), in *Withania somnifera* from different explants; Manickam *et al*; (2000) in Indian Ginseng, Reddy *et al*; (2001), in *Coleus forskohlii* from leaf explants; Bhavish and Jasria (2003); in an endangered medicinal plant *Curculigo orchioides*; Faisal and Anish (2003); in *Tylophora indica*; Banniaamin *et al*; (2004) in *Cretavea magna* (Lour) DC; Gawada and Patratkar (2004); in *Eclipta alba*; Guo *et al*; (2007) in an endangered medicinal plants, *Saussurea involucrate*, Kar;

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Kyo, (2008) in different medicinal plants for transformation and production of useful secondary metabolites; Singh *et al*; (2009) in an endangered medicinal plant – Sarpgandha (*Rauvolfia serpentina*); Yaadwinder Sidhu (2010) reported micropropagation of medicinal plants through tissue culture, Tejovathi *et al*; (2011) an endangered medicinal plant – *Commiphora wightii* through tissue culture, Pandey *et al*; (2013) in an endangered plant *Psoralea corylifolia*, Lalabadi *et al*; (2014) in Tashnedari an endangered medicinal plant, Anju and Kumar (2017) gave a detail review for the micropropagation of different medicinal plants for their conservation and plantation. Keeping all these ideas in mind the present work was done.

MATERIALS AND METHODS

Healthy branches of *Centella asiatica* were collected from the natural population growing in the University Campus in a beaker containing distilled water. They were brought in the laboratory, where the leaves were removed and branches were cut into smaller pieces of 1.5 cm. These nodal segments were placed in a conical flask and the mouth was wrapped with muslin cloth with the help of a rubber band. The flask was placed under the running tap water so that the water current after washing the surface of the explants was coming out. However, due to the muslin cloth the materials were simply rotating along with the water current. This was done for 50 minutes. The explants were washed with 2% (v/v) teepol, followed by the surface sterilization with 0.1% (w/v) mercuric chloride aqueous solution for 10 minutes. During this the flask was shaken manually, so that there was proper contact of the chemical with the surface of the plant material. After this the explants were rinsed thrice with sterilized distilled water to remove even the trace of the chemical. Because the stems of *Centella* are prostrate so both bacterial and fungal contamination creates problem in tissue culture. Therefore, the above treated explants were treated with 0.6% Bavistin a systemic fungicide and 0.05% Gentamycin an antibiotic for 10 minutes. These explants were again rinsed with sterile distilled water and stored at low temperature after wrapping it with pre-sterilized and pre-wetted muslin cloth.

Preparation of Culture Medium

Murashige and Skoog (1962) basal medium was prepared after dissolving all the ingredients and 30 g sucrose in 500 distilled water, different concentration and combination of growth regulators were added separately. The pH was adjusted at 5.8 with the help of N-HCl. 8 gm of agar was dissolved in 400 ml distilled water by heating them slowly.

Now these two were mixed and the volume was made 1000 ml. In the culture tubes 125 X 25 mm. 20 ml culture medium was dispensed carefully, while the tubes were present vertically in the culture tube stand. Finally the mouth of the tube was covered with suitable cotton plug wrapped with muslin cloth. The culture tubes were placed in the plastic basket and the plugs were covered with aluminum foil. These baskets were placed in the container of the autoclave and the lid was tightly closed to make it air tight. The autoclaving was done at 15 lb pressure so that the temperature became 121^0C. This was maintained for 15-20 minutes. Finally the pressure of the autoclave was released and the tubes were taken out with the help of heat proof pads. They were allowed to cool at room temperature. Then they were placed in the fridge at low temperature. After three to four days inoculation was done. Culture tubes showing contamination were discarded after autoclaving.

Inoculation was done in the aseptic chamber of Laminar Air Flow. First of all the base of the chamber was cleaned to remove the dust and then it was rubbed with ethyl alcohol, soaked in the cotton ball. The UV tube was put on so that the chamber was sterilized with it. This was done for 45 minutes. Then this light was put off and the white light was put on along with the fan, through which filtered air was coming inside. All the forceps, needles, scalpels, were also sterilized on the flame and stored in a beaker which was full of ethanol. Before inoculation hand gloves were also sterilized. Every time the tip of the forceps was heated to red in the flame and after cooling dipped in the alcohol. The culture tube was taken and its upper part was heated by rotating to evaporate the water vapour. Then the plug was removed while the mouth of the tube was above the flame. Now with the help of the sterile forceps, one explants was selected and placed vertically on the culture medium. The explants were pressed slightly to dip it in the medium for few mm. Due to this explants was placed vertically on the culture medium.

After this the cotton plug was placed properly. In this way all precautions were taken during inoculation. The culture tubes after inoculation were incubated in the culture room. All the tubes were placed in culture tube stand in the vertical position. The culture room was maintained at 26 ± 1^0C temperature, 68-72% relative humidity and 3000 lux light generated by white fluorescent tube light (Philips). The photoperiod was maintained at 16/8 light and dark

cycle. Observation was made on an alternate day to note if there was contamination, the day after shoot bud initiation took place, total number of explants inoculation and the number of explants showing response, number of shoot buds initiated etc. All the experiments were done in triplicate and the number of tubes inoculated was 20 in each culture. The mean of the data was taken for further analysis and discussion.

RESULTS AND DISCUSSION

From the perusal of the data as represented by the graph no. 1, it is evident that highest percentage of response with respect to shoot bud initiation was obtained in MS + 4.0 mg/l BAP + 0.5 mg/l KN that was 92.64, followed by 78.18% in the medium supplemented with MS + 3.0 mg/l BAP + 0.5 mg/l IBA. It may be further noted by graph no. 2, that at the similar concentrations of KN + NAA the highest percentage of response was 58.84 and 46.72 respectively. The shoot bud initiation was noted after 12th day of incubation in MS + 4.0 mg/l BAP + 0.5 mg/l NAA, and after 14th day in MS + 3.0 mg/l BAP + 0.5 mg/l NAA. At the similar concentrations of KN + NAA the time taken for bud initiation was 16th and 19th day respectively. The number shoot buds were also observed. Here it may be noted that maximum shoot buds were initiated on the nodal explants inoculated in MS + 4.0 mg/l BAP + 0.5 mg/l NAA, which was 4.68, followed by MS + 3.0. mg/l BAP + 0.5 mg/l NAA which was 3.82 respectively. In the explants inoculated in similar concentration of KN + NAA the number of shoot buds was 2.24 and 1.84 respectively.

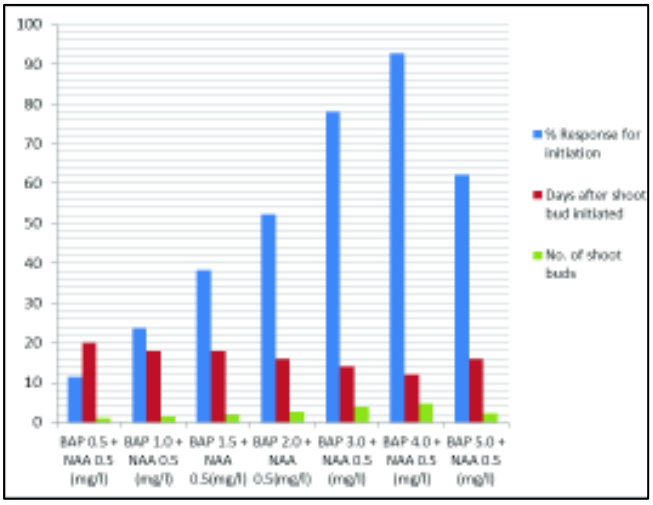
Similarly, from the graph no.3, the shoot buds when sub cultured gave maximum multiple shoots in MS + 4.0 mg/l BAP + 0.5 mg/l NAA which was 8.72 followed by MS + 3.0 mg/l BAP + 0.5 mg/l NAA that was 6.54. Similarly, from the graph no. 4, it is clear that maximum number of roots per plantlet was 4.62 in MS + 0.5 mg/l IBA followed by 3.24 in MS + 0.4 mg/l IBA. The minimum period taken for root initiation was 12 days where the maximum number of roots was initiated.

Internodal explants of *Centella asiatica* were inoculated in seven different concentrations (0.5-5.0 mg/l) of BAP and KN along with 0.5 mg/l NAA, supplemented in MS basal; medium. It was noted that the nodal explants initiated shoot buds in different growth regulators with different percentage of response 11.42 to 92.64, the days taken for initiation varied from 20 to 12 days and number of shoot buds 1.0 to 4.68. It is clear from the data that there was gradual increase in all the parameters considered here from 0.5 mg/l to 4.0 mg/l which again declined at 5.0 mg/l concentrations of the growth regulators. It was further noted that BAP at its different concentrations was found more efficacious than that of the KN at the same concentrations.

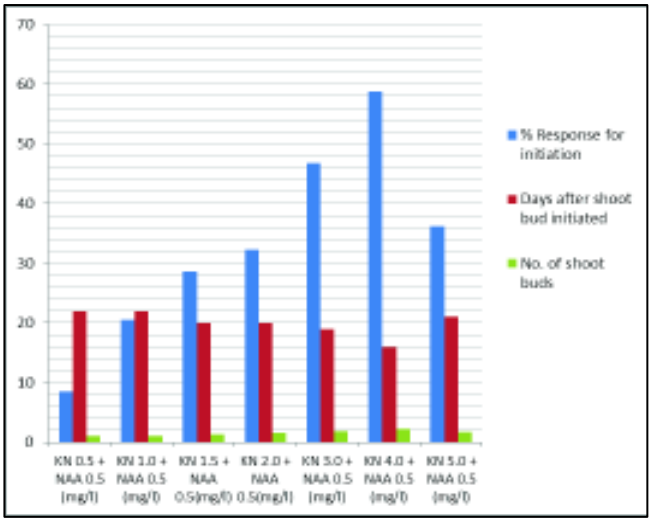
Micropropagation of different medicinal plants has been reported by different workers. Even in *Centella asiatica* we get report but in most cases the micro shoots have been initiated via callus induction. Present work was done with nodal explants, where we get pre-existing meristematic buds in dormant condition. Findings of present work corroborate with the findings of Arumugan and Gopinath (2012) who also reported that shoot multiplication in *Gloriosa superva* L. was the highest in the medium supplemented with 3.0 mg/l BAP.

Further the findings of the present work are in agreement with the findings of Ajit Kumar and Seeni (1998) in *Aegle marmelos*, Kulkarni *et al*; (2000) in *Withania somnifera*, Gawda and Pratkar (2004); Yaadwinder (2010) in different medicinal plants, Pandey *et al*; (2013) in *Psorelea corylifolia*,

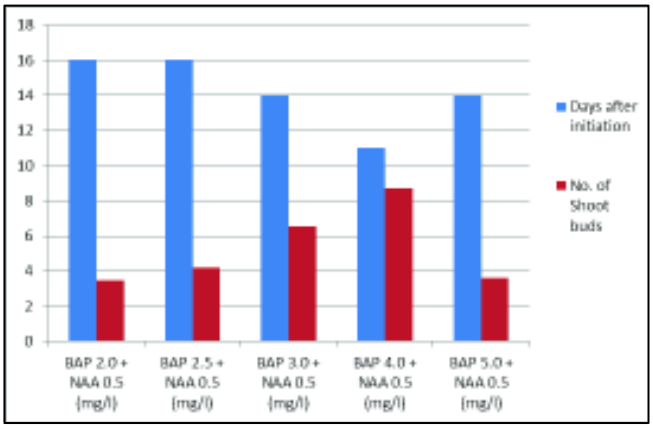
Rahman *et al*; (2015) in *Plectranthus amboimicus*, have developed multiple shoots from nodal or shoot apex of the medicinal plants mentioned above. Therefore, the nodal explants are more suitable for multiple shoot initiation for micropropagation because there are maximum plantlets which are true to their parents with respect to genotype, where as plantlets developed through callogenesis may differ among themselves, due to heterogenecity of cells in a particular callus.



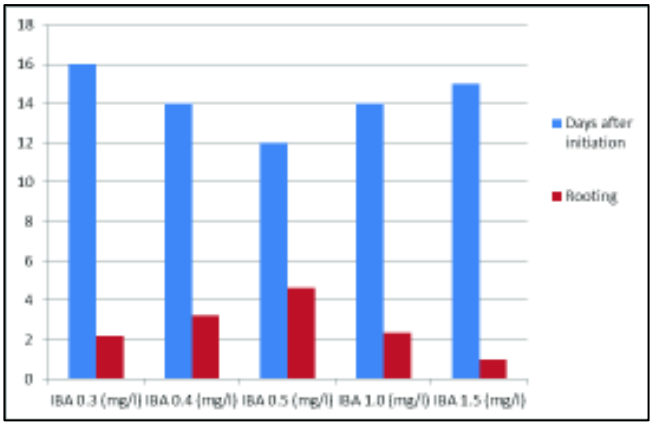
Graph 1: Showing impact of different concentration of BAP (0.5-5.0 mg/l) and NAA (0.5 mg/l) on percentage response, days of initiation of shoot buds and mean number of shoot buds per explants



Graph 2: Showing impact of different concentration of KN (0.5-5.0 mg/l) and NAA (0.5 mg/l) on percentage response, days of initiation of shoot buds and mean number of shoot buds per explants



Graph 3: Showing sub culture of plantlets raised above in different growth concentration for multiple shoot induction



Graph 4: Showing root induction in the four week old plantlets

ACKNOWLEDGEMENT

The work was done in Plant Biotechnology Laboratory, with the kind permission of Head, University Department of Botany, B.R. Ambedkar Bihar University, Muzaffarpur. All the authors are grateful to the Head for the kind permission granting.

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