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RAPID MICRO PROPAGATION TECHNIQUES FOR CONSERVING *Centella asiatica*- A VALUABLE MEDICINAL HERB.

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Abstract- The present investigation aims at developing rapid micro propagation protocol, which can be used for conservation of *Centella asiatica* and mass multiplication of a valuable medicinal plant to meet out the pharmaceutical demand. Attempts were made to evolve a low cost rapid micro propagation technology to conserve this valuable medicinal herb within a month of period. The combinations of BAP (1.0 mg L-1) with IAA (0.5 mg L-1) showed good callus proliferation. The combination of cytokinin with auxin (3:1) in modified MS media showed higher response in shoot elongation. Among the combinations of BAP with IAA (4:1) showed good response in shoots elongation. BAP (1.5 mg L-1) with IAA (0.5 mg L-1) showed higher shoot multiplication within a month and the difference were significant among the trials.
Keywords- Micropropagation, cytotoxin, auxin.

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Introduction

In India, approximately 1700 plant species are used in ayurveda, 500 for Siddha, 400 for Unani, 300 for Amchi systems of medicine with substantial overlaps of common plants among these systems. *Centella asiatica* (Bhrami 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 medicine for increasing memory power. In India, *Centella asiatica*, the plant frequently suffers due to growing modern agriculture, increasing 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. To cope up with this alarming situation, the micro propagation, a tissue culture technique is used for mass production and conservation of variability exists in the valuable medicinal herb.

Earlier in vitro propagation through callus cultures [8,15,18], axillary buds [6,23], shoot tips [21], leaf explants [2], stolons [20] and somatic embryogenesis [9,13] were reported in *Centella asiatica.* The present investigation aims at developing a viable cost effective protocol, which can be used for rapid mass propagation, in vitro biomass production and conservation of *Centella asiatica* to meet the pharmaceutical demand. Attempts were made to evolve a low cost rapid micro propagation technology to conserve this valuable medicinal herb.

Materials and methods

Explant Collection and Surface Sterilization

Centella asiatica were collected from the plants growing wild in the river banks of Cauvery. The plants were washed thoroughly under running tap water and the leaves and roots were trimmed off from the plant. Shoot pieces were excised from the stolons and kept under running tap water for 30 min. The nodal explants were washed in mild detergent, 1% (v/w) Teepol, for 5 min with constant agitation. The explants were surface sterilized with aqueous mercuric chloride (HgCl2, 0.1%) and Teepol (5 drops/100 mL) for 3 min. The solution was drained off and rinsed 4-5 times with sterile distilled water. The explants were then taken to the laminar airflow chamber. Under aseptic conditions the explants were again treated with mercuric chloride (0.1%) for 3 min followed by thorough washing in sterile distilled water. The explants were trimmed to the appropriate size, making them ready for inoculation.

Culture Media and Conditions

Throughout the study, different strengths of Murashige and Skoog [12] medium were used for experiments. Full strength MS basal medium and modified MS medium with different concentrations and combinations of BAP (1.0, 1.5, 2.0 mg L-1) and auxins (NAA; 0.5 mg L-1 and IAA; 0.5 mg L-1) for studying the in vitro multiplication responses (Table 1) 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 0.4% gelride instead of agar. The modified MS medium was enriched with the macronutrient CaCl2 from 332.02 to 440 mg/l and MgSO4 from 180.54 to 370 mg/l (Table 1).

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. Conical flask with cotton made cap containing 20 ml of medium were used. The cultures were maintained at 24 ± 2°C under 12 hour photoperiod with a light intensity of 35-40 µmol m-2 sec-1 irradiance provided by cool white fluorescent tubes.

One month after inoculation, the proliferated callus produced somatic embryo, from which the plants were emerged and they were kept in small plastic cups filled with soil and coir pith for ex vitro rooting and kept in humid chamber for providing proper humid conditions. The experiments were carried out with 12 different types of media composition. The percentage response, average number of shoots per explants was calculated (Table 2). Cultures were observed daily and the data were recorded at an interval of six days and finally after 4 weeks.

Result and Discussion

Establishment of Cultures

The shoot explants of *Centella asiatica* cultures were successfully established in modified MS medium. Only 80% of the cultures could be established and major loss was due to fungal contamination. Emergence of two to three leaves was noticed per culture within 25 days in modified MS medium with BAP: IAA hormone combinations of 2:1, 3:1 and 4:1 (Fig. 1c). But the cultures did not show any shoot induction response in full strength MS medium with auxin: cytokinin hormone combinations. Similar results were reported in the same species [4] and also in other medicinal plants [14,19,24]. Modified MS + 1.5 mg BAP + 0.5 mg IAA medium responded very well and callus was developed 10 days after inoculation. The somatic embryo was developed in 15 days after inoculation. The full shoot growth was established 27 days after inoculation. The similar response was noticed in modified MS + 2.0 mg BAP + 0.5 mg IAA medium.

Growth Regulators

The explants were cultured in full strength MS medium fortified with different concentrations of BAP (1.0, 1.5 and 2.0 mg L-1) and in combination with IAA (0.5 mg L-1) for studying the various in vitro responses (Table 2). The combinations of BAP (1.0 mg L-1) with auxins, IAA (0.5 mg L-1) showed good callus proliferation (Fig.1a). The combination of cytokinin with auxin (3:1) in modified MS media showed higher response in shoot elongation (Fig. 1c). Among the combinations of BAP with auxins IAA (4:1) showed good response shoots elongation (Fig. 1b). BAP (1.5 mg L-1) with IAA (0.5 mg L-1) showed higher shoot multiplication and the difference were significant among the trials. Maximum number of leaves was observed in combinations of BAP (1.5 mg L-1) and IAA (0.5 mg L-1). Rooting was noticed in all the trials but the percentage of response was very low (20%).

Similar to our observations, George also reported that BAP alone showed good shoot induction in *C. asiatica*5. The use of BAP as an efficient cytokinin for the axillary bud multiplication has been reported in several plants such as *Artemisia annua* [24], *Heracleum canndicans* [25], *Tinospora cordifolia* [16]. BAP with IAA combinations showed superiority to NAA combinations, which was in agreement with the same plant [22]. The potential of BAP in combination with IAA was demonstrated in *Plumbago rosea*[6] and *Alpinia calcarata* [1]. Full strength MS media with growth regulators such as, 0.5 mg/l of BAP in combination with 0.01 mg/l NAA has been reported to give optimum results in *Utleria salicifolia* [4]

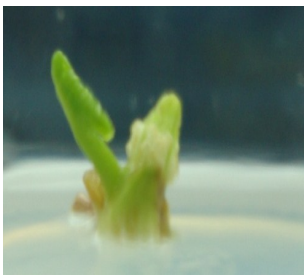

Fig.1a- Good callus proliferation **Fig.1b-** MS + 2.0 mg BAP + 0.5 mg IAA

Fig.1c-The established shoot in full strength MS with 3 BAP: 1 IAA medium in 25 days after inoculation.

Effect of Enzyme activity in modified MS medium

Trials for developing commercially feasible micro propagation method was initiated by culturing the shoot explants established in the modified MS medium in the combination of BAP (1.0, 1.5 and 2.0 mg L-1) with IAA (0.5 mg L-1). Percentage of culture response (80%) was same in the BAP (1.5 and 2.0 mg L-1) with IAA (0.5 mg L-1) the treatments. Difference in the strength of basal medium and cytokinin concentration showed considerable variation in morphogenic responses. BAP at higher concentration (1.5 and 2.0 mg L-1) showed effective shoot proliferation. Rooting was negligible in both treatments.

The modified MS medium containing higher dose of Ca2+ ions (440.00 mg/l) and two folds of Mg2+ ions (370.00 mg/l). The stability of the cell membrane is highly influenced by Ca2+. A shortage of Ca2+ results in an increased leakage of low-molecular compounds out of the membrane. A severe Ca2+ deficiency causes total degradation of the membrane. Ca2+ Stabilizes the membrane by interactions with phosphates, carboxylate groups of

phospholipids and protein present in the membrane. Contrary to Magnesium, which is involved in the activation of many enzymes, calcium activates only a few enzymes like -amylase and ATPases. Calcium mainly stimulates membrane bound enzymes of which the activity is regulated by the structure of the membrane. IAA is involved in the transport of calcium10. Due to low calcium level in the cell, competitions with Mg2+ for cation binding sites are prevented, and are inactivation of enzymes avoided. In protein synthesis Mg2+ is involved at different levels. Magnesium forms a bridge between both ribosome subunits. In magnesium deficiency, the subunits will dissociate and protein synthesis stagnates. Magnesium is required for the activity of RNA polymerases, enzymes involved in the synthesis of RNA. A shortage of Mg2+ will block RNA synthesis10. Magnesium is also important for Ribulose Biphosphate Carboxylase activity. This CO2 binding enzyme is highly pH and Mg2+ dependent. The similar finding was observed that cis-diol borate complexes might influence the activity of membrane-bound enzymes9 in culture media. The higher dose of Ca2+ ions and Mg2+ ions in the modified MS medium may enhance the enzyme activity and protein synthesis could help the somatic embryo development and shoot elongation in *Centella asiatica* a valuable medicinal plant. Higher enzyme activity in the modified MS medium might be due to the higher dose of Ca2+ ions (440.00 mg/l) and two folds of Mg2+ ions (370.00 mg/l) and it reduces the time of culturing for micro propagation of C. asiatica.

In the present study, the sub culturing is not required for micro propagation; it could reduce the cost of production. In this study, the duration for shoot proliferation is a month after inoculation; it could also reduce the time of the micro propagation15. The solid MS medium with different combination of cytokinin and auxin could be used as alternative for liquid medium for rapid micro propagation of medicinal herb, *Centella asiatica*. The cost effectiveness by the reduction of time for shoot proliferation established during this study using C. asiatica shoot culture as a rapid micro propagation technique can be utilized in any other species.

Table 1- Nutritional composition of MS basal and modified medium

ELEMENTS	Basal MS medium (mg/l)	Modified MS medium (mg/l)
MACRO ELEMENTS		
$CaCl_2$	332.02	440
KH_2PO_4	170	170
KNO_3	1900	1900
$MgSO_4$	180.54	370
NH_4NO_3	1650	1650
MICRO ELEMENTS		
$CoCl_26H_2O$	0.025	0.025
$CuSO_45H_2O$	0.025	0.025
FeNaEDTA	36.7	36.7
H_3BO_3	6.2	6.2
KI	0.83	0.83
$MnSO_4H_2O$	16.9	16.9
$Na_2MoO_42H_2O$	0.25	0.25
$ZnSO_47H_2O$	8.6	8.6
VITAMINS		
Glycine	2	2
Myo-Inositol	100	100
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Thiamine HCl	0.1	0.1

Table 2- Response of shoot-bits explants of Centella asiatica to selected media composition

S. No	Media composition	Remarks
1.	Full strength MS + 1.0 mg BAP + 0.5 mg IAA	Callus developed 45 days after inoculation
2.	Full strength MS + 1.5 mg BAP + 0.5 mg IAA	Good callus proliferation was observed
3.	Full strength MS + 2.0 mg BAP + 0.5 mg IAA	Callus developed 35 days after inoculation
4.	Modified MS + 1.0 mg BAP + 0.5 mg IAA	Callus developed 18 days after inoculation.
5.	Modified MS + 1.5 mg BAP + 0.5 mg IAA	Callus developed 10 days after inoculation and Somatic embryo was developed. The full shoot growth was established 27 days after inoculation
6.	Modified MS + 2.0 mg BAP + 0.5 mg IAA	Callus developed 11 days after inoculation, Somatic embryo was developed. The shoot elongation was noticed in 30 days after inoculation
7.	Full strength MS + 1.0 mg IAA + 0.5 mg BAP	Browning of tissues leading to callus formation
8.	Full strength MS + 1.5 mg IAA + 0.5 mg BAP	Browning of tissues leading to proliferate callus formation
9.	Full strength MS + 2.0 mg IAA + 0.5 mg BAP	Callus developed 35 days after inoculation
10.	Modified MS + 1.0 mg IAA + 0.5 mg BAP	Callus developed 25 days after inoculation
11.	Modified MS + 1.5 mg IAA + 0.5 mg BAP	Callus developed 20 days after inoculation and approximate weight of the callus was 50 ± 5 mg/5 mg of explant
12.	Modified MS + 2.0 mg IAA + 0.5 mg BAP	Callus developed 20 days after inoculation and approximate weight of the callus was 50 ± 5 mg/5 mg of explant

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