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Investigations on the Effects of Auxin, Cytokinin, Season and Genotype on Micropropagation of *Centella asiatica* for Ayurvedic Medicines

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***Abstract* – Ayurvedic treatments required individual parts of medicinal plants for the preparation of medicines in different dosages for various diseases. Explants collected from the vallam village of Ramanathapurm district, inoculated in auxins and cytokinins ((2, 4-D, NAA, IAA, IBA, BAP and KN) either alone or in different concentration and combinations in intermediate season and maintained for 60days. Explants were curled and remained fresh after 60days of inoculation. Freshness and response of explants reveals that the micropropagation of *Centella asiatica* is mainly auxin, cytokinin, genotype and seasonal dependent.**

***Keywords* – *Centella asiatica,* Seasons, Growth Hormones, Genotypes, 2,4-D-2,4-Dichlorophenoxy Acetic Acid, NAA-Naphthalene Acetic Acid, IAA-Indole 3-Acetic Acid, IBA-Indole 3 Butyric Acid, BAP-Benzyl Amino Purine, KN-6 Furfuryl Adenine.**

I. Introduction

Centella asiatica L. is one of the important medicinal herbs in Asian subcontinents. It is a small herbaceous creeper belonging to the family Apiaceae and comprises of over 50 species [7]. This medicinal herb is native to Australia, Pacific Islands, New Guinea, Melanesia, Malaysia, Northern Iran and Asia [24]. Its common names are Gotu Kola, Asian Pennywort, Antanan, Pegaga, Kula kud and Brahmi. The name 'Brahmi' is also shared with *Bacopa monnieri* L as both the medicinal herbs were found to have similar medicinal properties [5]. *C. asiatica* is wildly grown at different elevations and grows extremely well in marshy places under different geographical conditions in India [24]. The whole plant of *C. asiatica* contains four important bioactive compounds, collectively known as centellosides, that includes major principle compounds such as asiaticoside, madecassoside, asiatic acid and madecassic acid [2] - [4], [23], [25], [26], [28].

C. asiatica is one of the key sources of herbal medicines in Indian system of traditional medicines, namely ayurvedic and siddha and also in traditional Chinese herbal medicines. Considerable amount of research on pharmacological aspects of this medicinal herb lead to clear understanding of its medicinal properties and presently, *C. asiatica* forms a major components in various herbal formulations. The estimated annual requirement of *C. asiatica* is 12,700 tonnes of dry mass, primarily met through natural population [1], [20]. Because of large scale and unrestricted exploitation, coupled with limited cultivation and insufficient attempts for replacement, wild stock of *C. asiatica* has been depleted in many parts of India [20]. However, information on appropriate technologies for commercial cultivation of *C. asiatica* is scanty.

Due to diverse use of *C. asiatica*, there is an ever increasing demand for raw materials for preparation of herbal products by pharmaceutical industries. In India *C. asiatica* is one of the chief components of the herbal medicines. The Indian pharmaceutical companies largely depend on natural populations of *C. asiatica,* leading to rapid decrease in availability of this precious medicinal herb. Thus, the role of cell culture technique to develop alternative production system for *Centella* metabolites is felt very important. Insufficient availability of raw materials is mainly due to restricted vegetative growth only during wet season in most of the agricultural lands. Besides, there is a serious concern regarding the purity of raw materials since the raw materials are sourced from agricultural field, often polluted with agrochemicals (fungicides and pesticides) during cycling of various seasonal crops such as cotton, vegetable crops, cereals and pulses. It was cautioned that paddy, wheat, mustard, potato, cotton, tea, tomato, sugarcane, grape and urban soils were found polluted with persistent organochlorine insecticide residues. DDT and HCH concentrations were found to be higher in paddy fields in many part of India [8]. Thus, utilization of *C. asiatica* by sourcing from agricultural lands is expected to contain residues of various toxic agrochemicals and high risk is involved [8]. Search for alternative methods for the production of phytochemicals of medicinal importance utilizing biotechnological approaches, especially plant tissue culture is reported [22]. Considering its wide range of medicinal properties in addition to over exploitation, wild plants of *C. asiatica* are heavily depleted [20]. Effort towards utilization of Indian wild germplasm of *C. asiatica* for production of useful bioactive compounds is reported using *in vitro* cell culture system [14]. Though many researches were done for high yielding of important metabolites by cell culture technique, ayurvedic treatments required individual parts of medicinal plants for finalzing the dosage based on the activity towards the diseases. Therefore, the present study revealed the effects of auxins, cytokinins and seasons on micropropagation is focused.

II. Materials And Methods

A. Plant collection and maintenance

Survey was carried out in paddy field of vallam village, Ramanathapuram District of Tamilnadu for collection of

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wildly grown plants of C. *asiatica*. More than 50 plants were collected for the survey. Some qualitative parameters such as leaf colour, leaf shape, colour of internodes, colour of stolon, colour of bracts, colour of ovary wall, colour of flowers, colour of epigynous disc, fruit shape, colour of style, colour of anther and colour of filament were observed based on the descriptors developed earlier by [11] and documented. In addition, quantitative parameters such as leaf length, leaf width, number of leaves, number of leaf aurides, number of petioles, petiole length, diameter of petiole, diameter of stolen, internodal length, length of roots, length of flower pedicel, etc. were observed. Some of the prominent features such as leaf size, leaf colour and leaf aurides were documented by photography. Plants were collected in adequate numbers and they were transplanted to earthen pots containing soil, sand and vermicompost (6:1:2) and maintained under the natural shade in Thassim Beevi Abdul Kader College for Women, Kilakarai, Ramanathapuram District.

B. Media and culture conditions

Full strength MS (Murashige and Skoog, 1962) medium containing sucrose (3%) was used as basic media for all the experiments. The media was augmented with different concentrations and combinations of growth regulators such as BAP, KN, 2, 4-D, NAA, IAA and IBA depending upon the experimental design. The pH of the media was adjusted to 5.8, gelled with 0.7% agar (Hi Media, India) and autoclaved at 121^{o}C for 20 minutes. All the cultures in various experiments were maintained either at room temperature or at 23^{o}C ± 25^{o}C depending upon the experimental conditions and grown under 16h photoperiod with irradiance provided by cool white fluorescent tubes (Phillips, India). The number of explants cultured in each treatment was ranged from 30. The duration of culture was ranging from 30 – 60 days.

C. Optimization of surface sterilization for leaf explants of C. asiatica

Commonly used disinfectant, $HgCl_2$ (0.1%) was used to standardize the disinfection protocol for leaf explants of *C. asiatica* in order to improve the establishment of primary culture without microbial contamination. Well expanded youn leaves of *C. asiatica* were surface sterilized with $HgCl_2$ (0.1%) individually at different durations ranging from 2 - 10 min and thoroughly rinsed with sterile distilled water to remove the traces of these two disinfectants. Explants of 1 cm^2 were cut from the whole leaves and placed on sterile blotting paper. Leaf explants were cultured on MS medium supplemented with 2, 4-D (0.1 mg/l) and BAP (1 mg/l) [13] and incubated at 23^{o}C ± 25^{o}C for 60 days and data on the recovery of explants in each treatment was documented.

D. Preparation of media for micropropagation of Centella asiatica

Appropriate volume of all the stock solutions for 1000ml media (stock - 50ml, Stock B1 - 5ml, Stock B2 - 25ml, Stock B5 - 5ml, Stock C - 5ml) were taken in measuring cylinder and solutions were fortified with varying concentrations of four commonly used auxins (2,4-D, NAA, IAA, IBA) either alone or in combination with two cytokinins (BAP and KN) at different concentrations (Table I).

Table I: Different combinations and concentration of hormones

Name of the hormones and combinations	Concentrations
BAP	1mg/l
BAP	2mg/l
BAP + KN	2mg/l + 0.5mg/l
BAP + 2,4D	1mg/l + 1mg/l
BAP + 2,4D	2mg/l + 2mg/l
BAP + KN	1mg/l + 1mg/l
BAP + KN	2mg/l + 1mg/l
IBA	1.5mg/l
IBA + KN	1mg/l + 3mg/l
IBA + KN	1mg/l + 2mg/l
NAA + BAP	1mg/l + 2mg/l

30grams of sucrose was dissolved with the solutions completely and pH was adjusted to 5.8-5.9. After adjusting the pH, final volume of media was made to 1000ml. 7.4 grams of agar was added and boiled to get the transparent solution and transferred to tissue culture bottle for autoclaving at 121^{o}C for 20minutes and cooled to room temperature.

E. Preparations of Explants for micropropagation

Surface sterilization and preparation of explants

Healthy young leaves were collected from the herbal garden of Thassim Beevi Abdul Kader college for Women, Kilakarai, Ramanathapuram District. Collected leaves were washed under running tap water to remove the soil and dust particles and surface sterilized with 0.1 % mercuric chloride, at the finalized duration (4minutes) from the previous experiment. After 4minutes, mercuric chloride was discarded and the leaves were washed with sterile distilled water thrice to remove the trace of mercuric chloride. Water was fully dropped out from the leaves. Photographic presentation of surface sterilization of leaves was given in Fig.1.

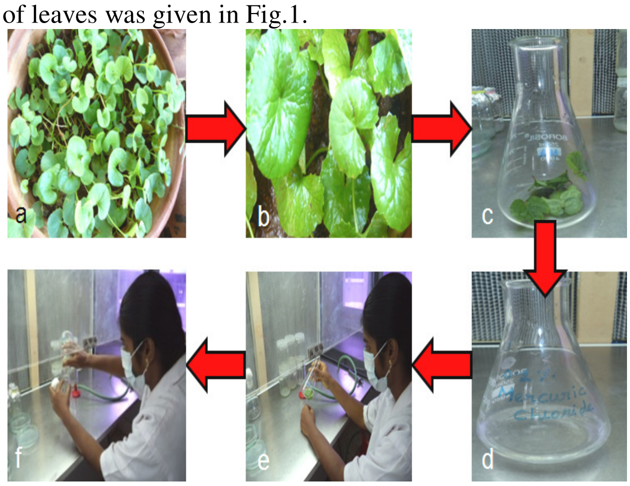

Fig.1. Steps involved in surface sterilization of leaf explants

a) Pots containing the wild type of *Centella asiatica,* b) Selection of leaf as an explants, c) collected leaves in conical flask for surface sterilization, d) 0.1% of mercuric chloride for surface sterilization of leaves, e,f) final step of surface sterilization

Surface sterilized leaves of 1 cm^2 were cut from the whole leaves and placed on sterile blotting paper to remove the water in order to avoid the microbial contamination. Leaf explants were cultured on MS medium supplemented with different concentration and combinations of auxins and cytokinins and incubated at 23°C ± 25°C for 60 days and data on the recovery of explants in each treatment was documented. Photographic presentation of explants preparation for inoculation was given in Fig 2.

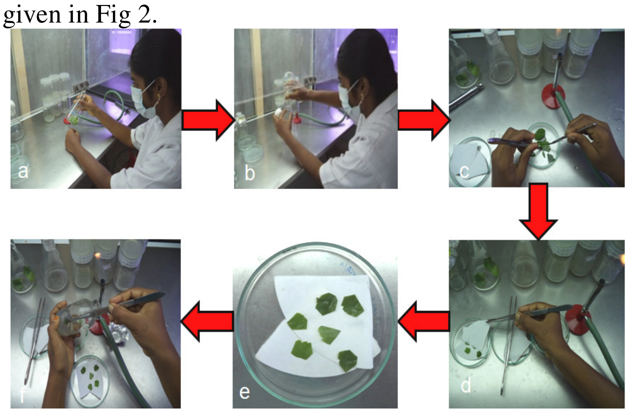

Fig.2. Preparation of surface sterilized leaves for inoculation
a, b) final step of surface sterilization protocol,
c) preparation of small fragments from whole leaves,
d) drying of explants in blotting paper, e) dried explants,
f) inoculation of explants on the surface of medium

III. Results

A. *Morphological identification of Centella asiatica*

Vegetative and reproductive features of *C. asiatica* collected from vallam village, were documented according to the parameters developed by [11] in Table II.

Table II: Qualitative parameters of *Centella asiatica.* Qualitative parameters were collected from 50 plants of *Centella asatica*

Parameters	**Characters**
Plant Description	Slender prostrate herb, creeping perennial, rooted at nodes, long internodes
Leaf description	Orbicular-reniform, margin crenate or sub entire
Leaf Colour	Green
Leaf Base	Broadly widened
Colour of stolon	Light red
Colour of petals	Purple
Ovary wall colour	Light Green
Epigynous disc colour	Purple
Fruit shape	Ovoid
Bracts colour	Light Green
Style colour	Light pinkish Green
Anther colour	Purple with white patches
Filament colour	Light green

Few qualitative parameters such as leaf colour, leaf margin, colour of bracts, ovary, epigynous disc and anther were observed and photographed (Fig III). The striking features were documented with the leaf colour ranging from light green – dark green, colour of petals (pink), colour of epigynous disc was purple, colour of anther (purple). In addition, the overall morphology and plant vigor was completely documented. It was observed that root length of the plant was longer (65.4 mm) and exhibits with deeper root system.

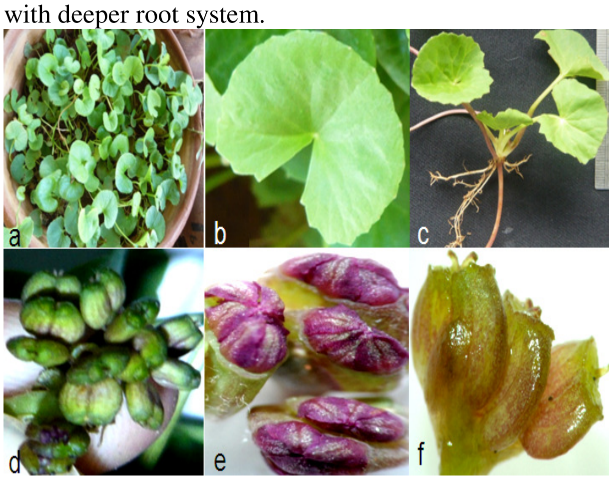

Fig III: Morphological characteristics of *Centella asiatica.* a) Establishment of *C. asiatica* in earthen pots for sourcing leaf tissue to establish aseptic cultures. b) enlarged figure showing the leaf morphology texture and aurides, c) whole plant with roots, d,e,f) colour intensity of floral parts at different stages and fully ripened fruit

B. *Optimization of surface sterilization for leaf segments of C. asiatica*

To improve the establishment of primary culture, commonly used surface disinfectant $HgCl_2$ (0.1%) was tested to optimize the disinfection protocol for leaf explants of *C. asiatica.* In control, leaf explants developed both bacterial and fungal contamination within a week of culture without any recovery of explants. However, explants disinfected with $HgCl_2$ (0.1%) for different durations ranging from 1 - 5 minutes recorded varying percentage of recovery depending upon the duration of treatments. Leaf explant disinfected with $HgCl_2$ (0.1%) for different duration of treatments lead to optimization of disinfection protocol for efficient establishment of culture (Table III).

In this experiment, exposure of leaf explants to $HgCl_2$ (0.1%) for shorter duration (0 & 2 minutes) recorded poor recovery of explants. Recovery of aseptic leaf explants could be further enhanced to 20% when leaf tissues were subjected to disinfection for 4 min. Exposure of leaf explants to $HgCl_2$ (0.1%) for 4 min did not cause any browning and 80% of the recovered explants and remains fresh. However, rapid browning followed by death of leaf explants was observed when leaf tissues were exposed to $HgCl_2$ (0.1%) for longer duration (5minutes). $HgCl_2$ was found more effective in the duration of 4 minutes for the establishment of culture.

Table III: Effect of $HgCl_2$ on surface sterilization of leaf tissues of *C. asiatica*. Leaf tissues were surface sterilized with 0.1% HgCl2 for different durations and data collected after 60 days of primary culture on MS medium supplemented with 2, 4-D (0.1 mg/l) and BAP (1 mg/l).

$HgCl_2$ treatment duration	No of explants with microbial contamination			Browning	RE	% of explants recovery
	F	B	F&B			
0	7	2	1	0	0	0
1	2	5	3	0	0	0
2	3	2	5	0	0	0
3	4	2	2	0	0	20
4	1	0	1	0	8	80
5	0	0	0	8	3	30

***F-Fungal Contamination, B-Bacterial Contamination, F&B-Fungal and Bacterial Contamination, RE-Recovered explants

C. Effect of auxins and cytokinins on shoot induction

Explants were processed by following the protocol developed from the previous experiment and cultured on full strength MS medium fortified with varying concentrations of four commonly used auxins (2,4-D, NAA, IAA) either alone or in combination with two cytokinins (BAP and KN) at different concentrations. Leaf explants cultured on 11 different combinations of media were incubated at room temperature under natural diffuse light for 60 days was documented. Explants inoculated in Basal medium was found fresh and curled. BAP (1mg/l) started to produce callus from the edges of the explants. Edges of the explants on the following medium were turned to brown after 60 days of inoculation (BAP (2mg/l), BAP (2mg/l) + KN (0.5mg/l), BAP + 2,4-D (2mg/l), BAP (2mg/l) + KN (1mg/l), IBA (1mg/l) + KN (3mg/l), IBA (1mg/l) + KN (2mg/l), IBA (1.5mg/l)). NAA + BAP (1mg/l), BAP (1mg/l) + 2,4,D, NAA (2mg/l) + BAP (1mg/l) medium enhanced the callus production from explants than other combinations and concentrations of hormones (Fig 4).

D. Effect of seasons on micropropagation

To study the effect of different seasons on callus and shoot induction, leaf tissues were collected from the month of December to February (Intermediate season) and they were inoculated on MS medium supplemented with varying concentrations of four commonly used auxins (2,4-D, NAA, IAA) either alone or in combination with two cytokinins (BAP and KN). Leaf explants cultured on 11 different combinations of media and basal medium were incubated at room temperature under natural diffuse light for 60 days. Seasons are playing an important role n the invitro propagation. NAA + BAP (1mg/l), BAP (1mg/l) + 2,4-D , NAA (2mg/l) + BAP (1mg/l) medium stimulated the callus production from explants than other combinations and concentrations of hormones. Though the response is based upon the concentration and combinations of hormones, season was played main role in micropropagation.

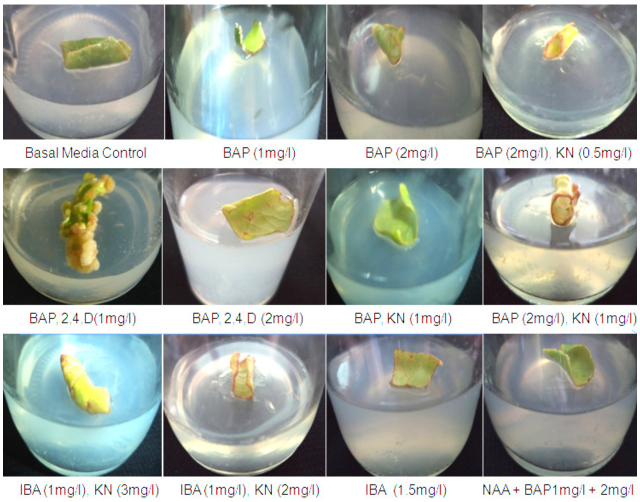

Fig.4. Response of explants for shoot induction on auxins and cytokinins

IV. Discussion

The present study has lead to the identification and characterization of *C. asiatica* collected from vallam village of Ramanathapuram district. Striking qualitative and quantitative features of *C. asiatica* was recorded. The high degree of genetic variability among various accessions of *C. asiatica* could be attributed to the existence of sexual reproduction and intermixing of different ecotypes through genetic recombination. The present and earlier studies therefore corroborate existence of wide range of variation among different ecotypes of *C.asiatica* morphologically, chemically and cytologically.

Development of *in vitro* culture system for wide range of application is primarily depends on efficient establishment of aseptic culture. Experiments carried out for improving the disinfection protocol for establishment of leaf tissues yielded promising results. Commonly used surface sterilants, $HgCl_2$ (0.1%) was found suitable for effective establishment of cultures. fungal contamination, especially when leaf samples were sourced during rainy season. In support of our findings, $HgCl_2$ (0.1%) was used for disinfection of leaf tissues of *C. asiatica* by various workers and proven to be one of the effective sterilants for establishment of aseptic leaf cultures [17], [16]. Our present study revealed that shoot induction was mostly influenced by three important factors in the order of season, genotypes and type of growth regulators used in the medium. Explants cultured in Intermediate season (December - February), indicated that callus induction was influenced by season. Intermediate season was not favour for callus induction and shoot induction as revealed by poor response of leaf tissues. In support of these findings, it was reported that efficient callus induction was often influenced by seasons in leaf tissues of *Ficus religiosa* [21] anther culture in *Oryza* species [10] and *Miscanthus sinensis* [6].

Considerable amount of work has been reported in *C. asiatica* on various aspects such as direct regeneration though meristem culture [9], [27], [16], somatic embryogenesis using leaf tissues [12], [20] and suspension cultures employing leaf derived callus [18], [17]. These reports revealed that *in vitro* morphogenesis in *C. asiatica* was often influenced by the types of auxins and cytokinins used in the media. Reference [12] reported high frequency somatic embryogenesis by culturing leaf tissues of *C. asiatica* on MS medium fortified with low concentration of 2,4-D (4.52 μM) along with varying levels of BAP (0.44 μM - 4.44 μM). When a similar combination was tested in our study with all the genotypes of *C. asiatica*, somatic embryogenesis could not be induced. This could be probably due to the influence of genotypes of *C. asiatica* among various genotypes as previously reported by many workers [12], [17].

Ever increasing demand for high quality of raw materials coupled with large-scale harvesting of *C. asiatica* lead to depletion of natural population in many countries, including India. It was reported that wildly growing *C. asiatica* became rare due to indiscriminate removal and over exploitation of *C. asiatica* by traders in India [19]. In conclusion, our present study revealed that induction of callus and shoot induction are primarily dependent on season, genotypes in addition to growth regulators. These findings are comparable with previous report in *C. asiatica* [13]. In India, whole plants of *C. asiatica* are harvested in larger quantity, mostly from agricultural field, where considerable amount of agrochemicals (fungicides and pesticides) are used for controlling pest and diseases of agricultural crops. Thus, wild plants of *C. asiatica* sourced from agricultural field are often exposed to harmful agrochemicals, leading to an accumulation of hazardous elements in the whole plants. So far, there is no separate cultivation practice for producing raw materials of *C. asiatica* to meet the ever increasing demand by the pharmaceutical industries in India. The preliminary study was done for multiple shoot production for the preparation of ayurvedic medicines.

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CONCLUSION

Micropropagation is influenced by some special features such as growth hormones, season genotype. A preliminary study must be one to identify the suitable methods for the invitro propagation of *Centella asiatica*

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