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In vitro culture studies on *Hemigraphis alternata* (Burm.f.) T. Ander. (Acanthaceae): A Potential medicinal herb

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Abstract
The current situation of medicinal plants and increasing demand of plant derived drugs suggest an immediate need to conserve our medicinal plant resources. The present study attempted to develop *in vitro* micropropagation protocol for the important medicinal plant *Hemigraphis alternata* (Burm.f.) T. Ander. From this experiment it is clear that higher amount of cytokinins and lower auxin induce shoot regeneration and high amount of auxin and lower amount of cytokinins induce root regeneration. Similarly the most effective shoot regeneration was found in the M.S. medium supplemented with KIN 2 mg/l. It shows an effective rooting in the ¼ strength M.S. medium supplemented with NAA 0.5 mg/l. large numbers of multiple shoots were also formed in the M.S. medium is supplemented with KIN 2 mg/l.

Keywords: *In vitro* culture, *Hemigraphis alternata*, Acanthaceae, medicinal herb

Introduction
Plant tissue culture is a technique of culturing plant cells, tissues and organs on synthetic media under aseptic environment and controlled conditions of light, temperature and humidity. The development of plant tissue culture as a fundamental science, it was closely linked with the discovery and characterization of plant hormones and has facilitated our understanding of plant growth and development. Furthermore, the ability to grow plant cells and tissues in culture and to control their development forms the basis of many practical applications in agriculture, horticulture, phytochemistry and also a prerequisite for plant genetic engineering [1].

Plants have been the basis of many traditional medicine systems throughout the world for thousands of years and it continue to provide mankind with new remedies. Plant based medicines initially dispensed in the form of crude drugs such as herbal formulations, now it could serve as the basis of novel drug discovery. The use of plants as medicines has involved to the isolation of active compounds [2]. Isolation and characterization of pharmacologically active compounds from medicinal plants are being continuously increased in nowadays. *Hemigraphis alternata* (Burm.f.) T. Ander. belong to the family Acanthaceae. It is an exotic plant adapted to India is an excellent garden plant. In folk medicine, the leaves of *H. alternata* are ground in to a paste and it is applied on fresh cut wounds. Because it has very good wound healing activity. More over the wound healing property of this potential herb was also proved experimentally [3]. Plant tissue culture helps in providing a basic understanding of physical and chemical requirements of cell, tissues, organs and their growth and development. The establishment of cell, tissue and organ culture and regeneration of plantlets under *in vitro* conditions has opened up new avenues in the area of plant biotechnology. In addition to that, such *In vitro* studies also lead to reveal active principles behind various potential medicinal herbs [4].

Materials and methods
Classification and Description of the Plant
Kingdom: Plantae
Class: Dicotyledons
Sub class: Gamopetalae

Order: Lamiales
Family: Acanthaceae
Genus: *Hemigraphis*
Species: *Hemigraphis alternata* (Burm.f.) T. Ander.
Synonyms: *Ruellia alternata* Burm.f.
Hemigraphis colorata Hall.
Fl.&Fr.: Feb. – May
Habitat: Grown as garden plant
Distribution: Native of Central America
Local Name: *Murikootti*

Description of the Plant: It is a prostrate tropical perennial herb and it typically grows to 6 -9 in. tall and spreads indefinitely along the ground rooting at the stem nodes as it goes. It is primarily valued as a ground cover for its colourful foliage. Leaves are ovate-cordate leaves toothed, puckered (to 3 in long) are metallic silvery gray-green above and purple beneath. Inflorescence is raceme in which tiny, five-lobed, bell-shaped, white flowers. Flowers are insignificant (Fig.1).

Fig.1: Images of *Hemigraphis alternata* (Burm.f.) T. Ander.

Preparation of nutrient medium

For the *in vitro* regeneration, the nutritional media which contains macro and micro elements, sucrose, vitamins, amino acids and growth regulators. Agar is used as the gelling agent for the culture. In the present study M.S. medium was used (Table-1) [5]. For the preparation of the stock solution, the chemicals such as sucrose 3%, $CaCl_2$, growth regulators, agar 0.8%, etc were weighed accurately and mixed by adding required amount of double distilled water. Finally the pH is adjusted to 5.6-5.8. (1 normal NAOH and HCL were used for adjusting the pH). The solution is then subjected to water bath at 80^0C for about 20 minutes with continues stirring. Then the medium is transferred into the pre sterilized culturing tubes before solidify. It was then autoclaved (20 minutes, 15 psi pressures at 120^0C). The autoclaved tubes containing the medium are kept to solidify and cool before inoculation. The materials like blades, scalpel, conical flask, beakers, Petri dishes, etc used for this process were surface sterilized by treating with detergent and water. It was then rinsed with double distilled water about 3-4 times and autoclaved it for 30 minutes after covering all with aluminum foil.

Table 1: M.S. medium composition

Constituents	Chemical Formula	Amount (mg/l)
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650.00
Potassium nitrate	KNO_3	1900.00
Calcium chloride	$CaCl_2.7H_2O$	440.00
Magnesium sulphate	$MgSO_4.7H_2O$	370.00
Potassium dihydrogen phosphate	KH_2PO_4	170.00
Micronutrients		
Manganese sulphate	$MnSO_4.4H_2O$	22.30
Zinc sulphate	$ZnSO_4.7H_2O$	8.60
Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Sodium molybdate	$Na_2MO_4.2H_2O$	0.025
Copper sulphate	$CuSO_4.5H_2O$	0.25
Cobalt chloride	$CoCl_2.6H_2O$	0.25
Iron		
Disodium ethylene diamine Tetracetate	NaEDTA	37.30
Ferrous sulphate	$FeSO_4.7H_2O$	27.80
Sugar		
Sucrose		3000.00

Growth Regulators	Chemical formula	Molecular weight
Auxins IBA NAA	Indole-3 butyric acid($C_{12}H_{13}NO_2$) Naphthalene Acetic Acid($C_{12}H_{10}O_2$)	203.20 186.20
Cytokinins BA KIN	6-Benzyl-Adenine($C_{12}H_{11}N_{15}$) Kinetin(6-furfuryl amino purine) ($C_{10}H_9N_5O_5$)	225.00 215.20

Explant sterilization

Nodes of the plant *H. alternata* were used as explants for the present study. Healthy and good looking nodes were selected. This was then washed under running tap water to remove the dirt. The plant part then washed in neutral detergent Labolene for 5-10 minutes. The material was then washed 3-4 times in double distilled water to remove all the traces of the detergent. It was then surface sterilized by 1% aqueous mercuric chloride solution for 10 minutes and after decanting the sterilant, explants were washed with sterile double distilled water.

Inoculation

Inoculation was done in laminar air flow cabinet for creating the aseptic condition. Before the inoculation, the blades, scalpel, forceps, etc were flame sterilized by dipping in the alcohol and subjected to flame. The explant was then placed properly in the nutrient medium by pressing it gently. The rim of the culture tube was also flamed before and after the inoculation to avoid the microbial contamination and covered tightly by pre sterilized nonabsorbent cotton plugs. These were all then transferred to the incubation chamber. The culture tubes were maintained at temperature 25 ± 2^0C with relative humidity 60% and a photoperiod of 16/8 hrs with 1000 lux luminance. Followed by regular observation were made.

Results and Discussion

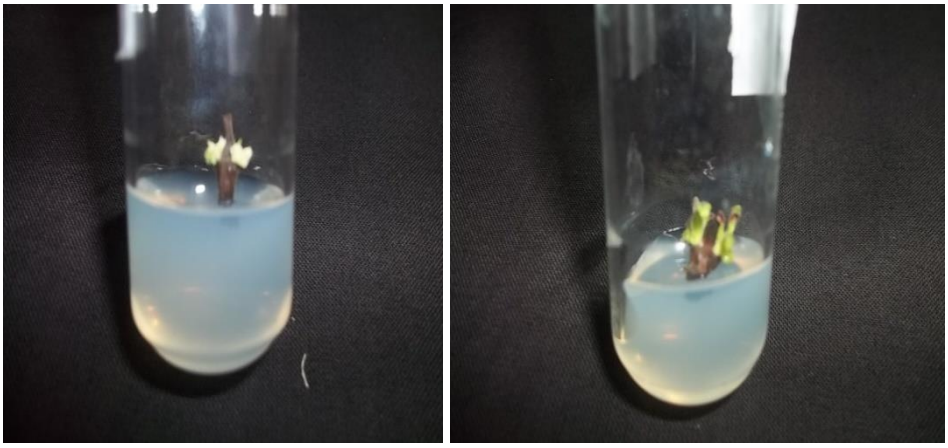
For the present study M.S. medium supplemented with varying concentration of growth regulators are used. The inoculated nodal explants of *H. alternata* is cultured under aseptic and controlled environmental condition in the tissue culture lab. The *in vitro* reponse of the plant is observed and noted at regular intervals of time. For the shoot initiation M.S. medium supplemented with different concentration of auxin with cytokinins and cytokinins alone is used and for root initiation M.S medium or ¼ M.S medium alone with auxin and auxin with cytokinins are used. The following 10 different concentration of growth regulators are used for shoot and root regeneration of the study (Table 2,3&4; Fig.2, 3 & 4).

Table-2: *In vitro* response of shooting in *H. alternata* on M.S medium with varying growth regulators

Medium	***In vitro* response noticed at various time intervals**					
	5 Days	**10 Days**	**15 Days**	**20 Days**	**25 Days**	**3O Days**
BA 1.0 Mg/l	-	Shoot initiation	Shoot initiation	Shoot growth	Multiple shoot growth	Shoot elongation
BA 2.0 Mg/l	-	Green coloration	Bulging of explant	Shoot initiation	Shoot growth	Multiple shoot growth
KIN 1.0 Mg/l	-	Bulging of explant	Shoot initiation	Multiple shoot growth	Shoot elongation	Shoot enlargement
KIN 2.0 Mg/l	Swelling	Shoot initiation	multiple shoot growth	Shoot growth	Shoot enlargement	Leaf formation
KIN 1.0 Mg/l + BA 1.0 Mg/l	-	Shoot initiation	Shoot growth	Multiple shoot formation	Shoot elongation	Leaf formation
KIN 2.0 Mg/l + BA 2.0 Mg/l	-	Shoot initiation	Shoot elongation	Multiple shoot growth	Branching of shoot	Growth continues

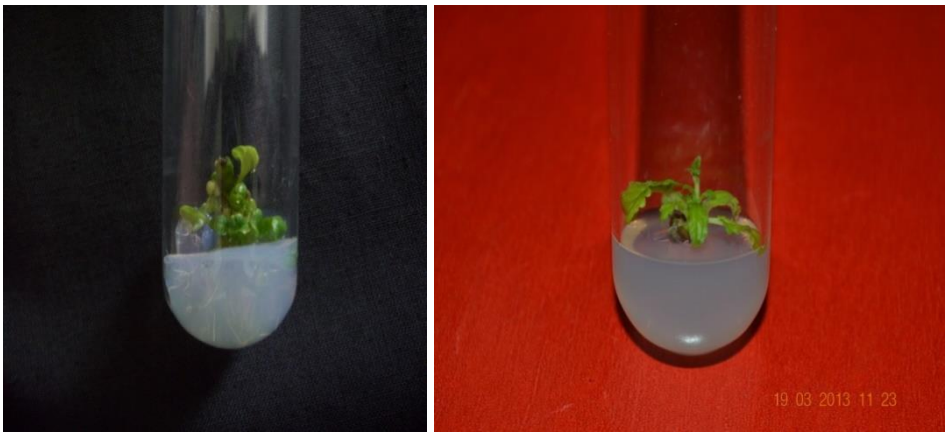
Table-3: *In vitro* response of rooting in *H. alternata* on M.S medium with varying growth regulators

Medium	*In vitro* response noticed at various time intervals					
	5 Days	**10 Days**	**15 Days**	**20 Days**	**25 Days**	**3O Days**
NAA 1.0 Mg/l +BA 6.0 Mg/l	-	No growth	Brown colouration of the medium	No growth	No growth	No growth
NAA 1.0 Mg/l + BA 4.0 Mg/l	Callus formation	Shoot initiation	Shoot initiation	Slow growth	Root initiation	Growth continues
NAA 1.0 Mg/l + KIN 4.0 Mg/l	-	Shoot initiation	Shoot growth	Multiple shoot growth	Root initiation	Growth continues
IBA 1.0 Mg/l + KIN 2.0 Mg/l	-	Shoot initiation	Shoot growth	Root development	Mutiple shoot growth	Growth continues
¼ MS+NAA 0.5 Mg/l	Callus formation	Root initiation	Shoot and root growth	root branching	Root elongation	Growth continues



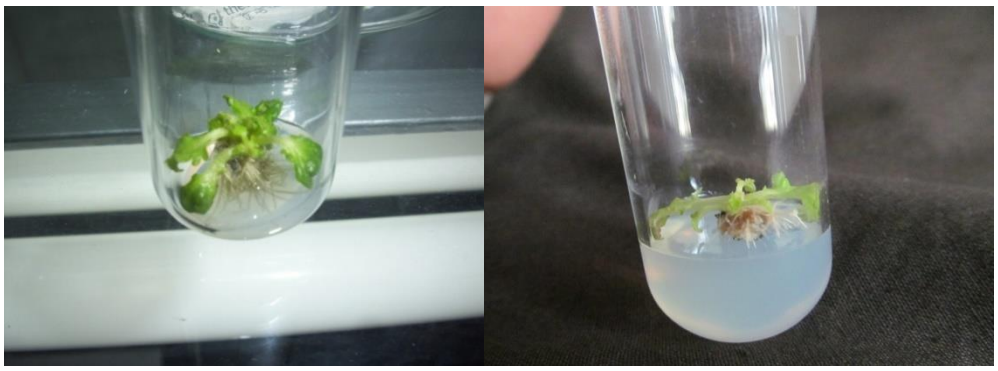
NAA 1.0 Mg/l + KIN 4.0 Mg/l KIN 1.0 Mg/l + BA 1.0 Mg/l

Fig. 2: Direct shoot regeneration in varying concentrations of growth regulators



KIN 2.0 Mg/l KIN 1.0 Mg/l

Fig. 3: Multiple shoot regeneration in varying concentrations of growth regulators



IBA 1.0 Mg/l + KIN 2.0 Mg/l ¼ M.S.Medium NAA 0.5mg/

Fig. 4: Root regeneration in varying concentrations of growth regulators

Table 4: Effect of auxin with respect to the Length of roots in *H. alternate*

Growth regulator	% of response	Length of roots
NAA 1.0 mg/l + **BA** 1.0 mg/l	-	-
NAA 1.0 mg/l + **BA** 4.0 mg/l	70	0.68±0.10
NAA 1.0 mg/l + **KN** 4.0 mg/l	70	0.70±0.23
IBA 1.0 mg/l + **KN** 2.0 mg/l	80	0.94±21
(1/4 M.S) NAA 0.2 mg/l	90	1.24±0.25

The present study was conducted to standardize micro-propagation of potential medicinal plant such as *H. alternata* Similar reports such as *in vitro* regeneration of plant species belongs to the family Acanthaceae are *Rhazya stricta Decne* [6], and *Adathoda vasica* Nees. [7] and *Justicia gendarussa* [8]. In this study, the medium is supplemented with kinetin gives more number of multiple shoots than other cytokinins. Some similar studies observed that, the micropropagation of an elite Medicinal Plant: Stevia rebaudiana Bert [9], they reported that M.S basal medium supplemented with sucrose (30 g L^{-1}), agar (7 g L^{-1}) and kinetin (2 mg L^{-1}) performed best in multiple

shoot proliferation, resulting more than 11 multiple shoots from a single shoot tip explant within 35 days of culture.

In the present observation on *in vitro* regeneration of *Hemigraphis alternata* in M.S. medium supplemented with the growth regulator Kinetin at a concentration of 2.0 mg/l is found to be more effective than the other cytokinins. Similar studies have been reported in the case of other plants species like *Stevia rebaudiana* Bertoni. [10], *Sinocalamus latiflora Munro* [11] and *Cucumis melo* L. [12]. It was also found that ¼ M.S. medium is more effective for the root initiation than all other auxins. Similar observations were made in *in vitro* Propagation of *Lychnophora pinaster* Mar.(Asteraceae) by Desouza *et al.*, 2007.

Conclusion

From the above studies the *in vitro* regeneration of *Hemigraphis alternata* on M.S. medium is supplemented with varying concentration of growth regulators helps to find out the most suitable concentration for the root and shoot regeneration from the nodal explants. From this experiment it is clear that higher amount of cytokinins and lower auxin induce shoot regeneration and high amount of auxin and lower amount of cytokinins induce root regeneration.

From this it is concluded that in *H. alternata*, the most effective shoot regeneration was found in the M.S. medium supplemented with KIN 2 mg/l. It shows an effective rooting in the ¼ strength M.S. medium supplemented with NAA 0.5 mg/l. large numbers of multiple shoots were also found in the M.S. medium is supplemented with KIN 2 mg/l. The present study is an attempt to standardize various factors which are responsible for micro-propagation of potential medicinal herb *Hemigraphis alternata*. Further studies are also required to reveal and compare the medicinal efficacy of micro-propagated plants with others.

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