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Effect of plant growth regulators on *in vitro* regeneration of *Hemigraphis alternata* (burm. f.) t. Anderson

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Abstract
An efficient shoot regeneration protocol for direct plant regeneration from nodal explants of *Hemigraphis alternata*- an important medicinal plant has been established. The aim of the present work is to figure out the auxin-cytokinin synergism on *in vitro* regeneration in this plant. In vitro shoot regeneration and proliferation was done on Murashige and Skoog Medium(MS) medium supplemented with different concentrations of cytokinins viz 6-Benzyl amio purine(BA), Kinetin(Kn) and Thidiazuron(TDZ) singly or in combination with auxins viz, α Naphthalene acetic acid (NAA) or 2,4-Dichlorophenoxy acetic acid (2,4-D). Maximum number of shoots (10.39) with 4.72cm shoot length and highest regeneration frequency (87%) were obtained on MS medium supplemented with 3µM BA after 60 days of incubation. Among various combinations and concentrations of plant growth regulators tested optimum regeneration frequency (86%) of multiple shoot formation and highest number (11.67) of shoots per nodal explants was obtained in MS medium supplemented with a combination of BA (3µM) and NAA (0.75µM) after 60 days of incubation. The synergistic effects of α Naphthalene acetic acid (NAA) (0.75µM) with BA (3µM) increased the regeneration frequency compared to other treatments. The optimum frequency (98%) of rhizogenesis was obtained on half strength MS medium having 2µM IBA after 30 days of incubation. Regenerated plantlets with well developed root system were successfully acclimatized in vermiculate and transplanted to soil with 97% survival rate.

Keywords: plant growth, regeneration, *Hemigraphis alternata*

Introduction
Hemigraphis alternata belongs to family acanthaceae is a tropical low creeping perennial herb chiefly grown as an ornamental indoor and outdoor plant. The species is exotic and originated in south eastern Asia (i.e. on Java in Indonesia) and adapted to India. The plant is commonly known by several names such as Aluminium plant, Cemetary plant, Metal leaf, Red flame Ivy, Waffle plant, Java Ivy etc. It prostrates and spreads with rooting stems when grown on ground (Fig. 1), and on hanging baskets it cascades over beautifully [1]. The plant is well known for its wound healing potential [2, 3]. Traditionally, the leaves are consumed to mend gall stones, excessive menstruation and as a contraceptive [4]. The phytoconstituents present in *H. colorata* are phenols, saponins, flavonoids, terpenoids [5], coumarins, carbohydrates, carboxylic acid, xanthoproteins, tannins, proteins, alkaloids, steroids and sterol [6].
Plant tissue culture offers an effective and relevant technique for plant propagation [7]. The plantlets obtained from tissue culture are season independent, superior, genetically uniform and requires smaller space compared to the seed grown plants [8]. The response of plant growth regulators change during plant tissue culture can be varied from species to species and also this response depends on the ability of tissues to response and the types of plant growth regulators [9]. For instances auxins and cytokinins play a crucial role in plant propagation such as shoot development and multiplication and callus initiation and development [10].The require levels of plant growth regulators in these two steps may alter from species to species, so the auxins/cytokinins ratio play a considerable role in plant tissue culture [11, 12].

Fig 1: Habit of *Hemigraphis alternata*

2. Materials and Methods-
2.1 Plant material and surface sterilization
Young, healthy shoots of *H. alternata* were collected from the plants grown in Botanic Garden Department of Botany, University of Kerala, during the month of January to March. Healthy juvenile branches were trimmed off and the leaves were excised and the stem was cut into small segments each with at least one node. Explants were washed under running tap water for 30 minutes and soaked in a 10% (v/v) detergent, Labolene for 3 minutes. After thorough washing they were pretreated with 0.1% (w/v) Bavistin (BASF, Mumbai, India)

and surface sterilized with 0.1% $HgCl_2$(w/v) (Merck, India) solution for 1 to 2 minutes and finally rinsed four times with sterile distilled water. The explants were then rinsed with sterile distilled water and dipped in 70% alcohol. The surface sterilized nodal explants were inoculated vertically on to the sterile nutrient media under Laminar Air Flow Cabinet.

2.2 Culture media and culture conditions

Murashige and Skoog Medium(MS) [13] supplemented with 3%(w/v) sucrose was used as basal medium for the present study. MS Medium augmented with various concentrations of BAP and Kin ranging from 1.0 to 5.0 µM was used for culture initiation from the nodal segments. The ₚH of the medium adjusted to 5.8 using 0.1N NaOH or 0.1N HCl. The media were solidified with 0.8%(m/v) agar. Twelve ml of medium (15 replicates) was poured in each culture tube and were autoclaved at 121^0C for 18 minutes The cultures were incubated at 23 ± 2^0C under 12hrs photoperiod with a photosynthetic photon flux density of 50-60 µ ε m^{-2} s^{-1} provided by Wight fluorescent tubes (Philips India Ltd., Mumbai). All the experiments were repeated thrice.

2.3 Multiplication of Shoots

MS medium supplemented with different concentrations of BAP 3µM and Kinetin 4µM and NAA (0.5, 0.75, 1.0, 1.5 & 2.0 µM) were used for multiple shoot formation. The *in vitro* regenerated shoots from the meristem of nodal explants were chosen for further multiplication of shoots. The cultures were multiplied by subculturing the *in vitro* shoots. The *in vitro* grown shoots were cut into 2-3cm long segments (each with at least one node) and subcultured on fresh medium every 3 to 4 weeks. The percentage of response, number of shots from explants, shoot length was recorded after 8 weeks of culture.

2.4 Rooting and Acclimatization

For rooting, *in vitro* produced shoots about 4-5cm long were excised and transferred to ½ strength MS Basal Medium supplemented with different concentrations of IBA and NAA ranging from 1-5 µM to induce *in vitro* roots. After rooting, regenerated plantlets were washed carefully and transferred to pots containing sterile vermiculate. Potted plantlets were covered with a transparent poly bags to ensure high humidity and watered every 3 days. Polybags were opened after 3 weeks in order to acclimatize plants to field conditions. After 4 weeks, these plants were removed from the planting substrates and transferd to pots containing normal garden soil and maintained in a glasshouse under normal day light conditions.

Statistical Data Analysis

All the experiments were carried out with 15 replicates and repeated thrice. Datas were subjected to Analysis of Variance by ANOVA and the significance of difference was calculated by Duncan's Multiple Range Test using SPSS software(ver.22).

3. Result and Discussion

3.1 Establishment of cultures

Initiation of shoot buds from the nodal meristem observed after four to five days of inoculation (Fig. 2). Second and third nodes were found to be the most suitable for culture initiation. 0.1% $HgCl_2$ was effective for surface sterilization of all the nodes except first node. An optimum of 10.39 ± 1.49 shoots per explants with 4.72 ± 0.26 cm length were obtained in MS medium supplemented 3µM BAP. MS medium augmented with KIN produced less number of shoots than BAP. A maximum of 7.78 shoots were produced at a concentration of 4µM. Among the cytokinins tested, BAP was found to be the most suitable for shoot initiation and multiplication from nodal explants (Table. 1). The regeneration of meristem was achieved through the selection of explants and by the use of different cytokinins. Sebastian *et al*., 2017 [14] also used nodal explants of *Hemigraphis alternata* and produced multiple shoots on MS medium supplemented with BAP and KIN.

In vitro propagation by nodal cuttings promoted the development of a preexisting morphological structure and the nutritional and hormonal conditions of the medium break the dormancy of the auxiliary bud which promoted its rapid development [15]. Effectiveness of BAP for shoot regeneration has also been reported by many other authors in *Saraca asoca* [16], *Tridax procumbens* [17], *Cicer arietinum* [18] and *Lavandula officinalis* [19].

Fig 2: Initiation of shoots from nodal explant in 2 µM BAP

Table 1: Effect of cytokinins (BAP and Kinetin) on shoot induction from nodal explants of *Hemigraphis alternata* after 60 days

Concentration of BAP (µM)	Concentration of Kinetin (µM)	Percentage of Response (%)	Number of Shoots (Mean±SD)	Shoot Length (Mean±SD)
1	-	81	5.03 ± 0.25^{d}	6.67 ± 0.10^{a}
2	-	85	6.75 ± 0.69^{bc}	5.23 ± 0.30^{b}
3	-	87	10.39 ± 1.49^{a}	4.72 ± 0.26^{bc}
4	-	83	8.04 ± 0.77^{b}	5.41 ± 0.47^{b}
5	-	83	5.87 ± 0.44^{cd}	4.20 ± 0.30^{c}
-	1	79	3.67 ± 0.42^{c}	1.20 ± 0.25^{b}

-	2	81	5.90 ± 0.56[b]	1.08 ± 0.14[b]
-	3	83	6.92 ± 1.52[ab]	1.83 ± 0.28[a]
-	4	84	7.78 ± 0.64[a]	1.39 ± 0.45[ab]
-	5	80	6.63 ± 0.40[ab]	1.36 ± 0.36[ab]

The experiments were carried out with 15 replicates and repeated thrice. Mean separation was analyzed by ANOVA using SPSS Software (Ver.22). Means within a column followed by same letters are not significantly (P <0.05) different as determined by Duncan's Multiple range test.

3.2 Multiplication of shoots *in vitro*

The *in vitro* developed shoots were multiplied by repeated subculture on MS medium supplemented with 3µM BAP and 4µM Kinetin in combination with 0.5-2.0 µM NAA. This process of shoot multiplication by repeated subculture has reported in species like *Stevia rebaudiana* [20]. An average of 11.61 ± 1.15 shoots was produced from each explants after 60 days in 3 µM BAP in combination with 0.75 µM NAA (Fig. 3). Rooting of *in vitro* shoots were prominent in all the combinations of BAP and NAA tried. Rhizogenesis simultaneous with caulogenesis were reported in presence of cytokinins in *Tylophora indica* [21]. This medium composition for shoot multiplication was found good for shoot elongation also. The combination of NAA with Kinetin resulted in the decreased number of shoots than kinetin alone, hence kinetin NAA combination was found to be in effective for multiple shoot induction from nodal explants (Table. 2).

The protocol reported here improved the number of *in vitro* shoots multiplied per explant and showed higher efficiency than the other preexisting employed methods. The effectiveness of cytokinin auxin combination for shoot multiplication was also reported earlier in *Musa paradisiaca* [22], *Pinguicula gigantean* [23] and *Vitex negundo* [24].

The shoots and leaves developed from the nodal meristem were small in the early stages of incubation but the size of the leaves and the thickness of stem increased and the shoots were elongated in the last two weeks. Well-developed leaf system supports the chances of survival of *in vitro* raised plantlets during hardening and field transfer [25]. Plantlets with a high number of well-developed leaves adapt quickly to natural environment as they are photosynthetically more efficient compared to those with smaller and fewer leaves [26].

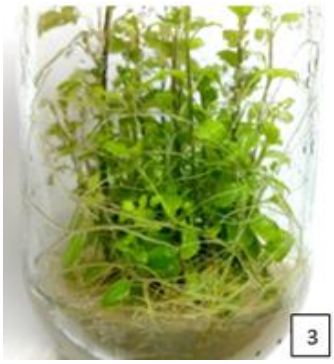

Fig 3: Multiple shoot induction in 3 µM BAP and 0.75 µM NAA

Table 2: Effect of BAP and Kinetin in combination with NAA on multiplication of shoots after 60 days

Concentration of BAP (µM)	Concentration of inetin (µM)	Concentration of NAA (µM)	Percentage of Response (%)	Number of shoots (Mean ± SD)	Shoot Length (Mean ± SD)
3	-	0.5	82	9.19 ± 0.66[b]	2.26 ± 0.38[a]
3	-	0.75	86	11.61 ± 1.15[a]	2.16 ± 0.60[ab]
3	-	1.0	83	8.1 ± 0.84[bc]	1.76 ± 0.36[b]
3	-	1.5	84	6.49 ± 1.5[cd]	1.89 ± 0.35[ab]
3	-	2.0	80	5.23 ± 1.14[d]	1.62 ± 0.48[b]
-	4	0.5	81	3.0 ± 0.81[c]	1.42 ± 0.07[a]
-	4	0.75	84	5.16 ± 0.98[b]	1.46 ± 0.10[a]
-	4	1.0	84	4.85 ± 0.89[b]	1.35 ± 0.18[a]
-	4	1.5	83	6.42 ± 0.53[a]	1.49 ± 0.13[a]
-	4	2.0	80	3.85 ± 0.88[c]	1.44 ± 0.44[a]

The experiments were carried out with 15 replicates and repeated thrice. Mean separation was analysed by ANOVA using SPSS Software (Ver.22). Means within a column followed by same letters are not significantly (P <0.05) different as determined by Duncan's Multiple range test.

3.3 *In vitro* rooting of Microshoots

Roots have an essential role in plant growth and development in supplying water and nutrients to the plant from the environment [27]. In the present study, IBA was found to be most effective for induction of roots from the cut ends of shoots. About 98% of shoots were rooted *in vitro* on half strength of MS medium augmented with IBA. Maximum number of roots were repeated on half strength MS medium augmented with 2 µM IBA (Fig. 4). An average of 19.63 ± 0.55 roots was produced from *in vitro* shoots within 4 weeks on this medium combination (Table. 3). The percentage of rooted shoots was found to be less in NAA. Our result signifies that half strength of MS Medium is appropriate for *in vitro* rooting and is in line with the research work reported by many authors in *Rotula aquatic* [28], *Passiflora foetida* [29], *Pyrus elaeagrifolia* [30]. Sebastian *et al*., 2017 [14] also used IBA and NAA for *in vitro* rooting of shoots.

Fig 4: Rooting of *in vitro* developed shoots in 2 µM IBA

Table 3: Effect of auxins (IBA and NAA) on *in vitro* root induction from *in vitro* raised shoots after 4 weeks

Concentration of IBA (µM)	Concentration of NAA (µM)	Percentage of Response (%)	Number of Roots (Mean ± SD)	Root Length (Mean ± SD)
1	-	94	14.91 ± 0.20^{c}	3.91 ± 0.11^{a}
2	-	98	19.63 ± 0.55^{a}	2.86 ± 0.70^{c}
3	-	94	16.37 ± 0.44^{b}	3.14 ± 0.22^{bc}
4	-	93	12.25 ± 0.44^{d}	3.18 ± 0.09^{b}
5	-	93	9.67 ± 0.39^{e}	2.22 ± 0.24^{d}
-	1	91	8.23 ± 0.69^{c}	2.86 ± 0.18^{a}
-	2	93	10.24 ± 1.76^{bc}	2.49 ± 0.35^{a}
-	3	86	14.24 ± 1.43^{a}	2.31 ± 0.11^{a}
-	4	88	10.85 ± 0.30^{b}	2.62 ± 0.75^{a}
-	5	89	9.26 ± 0.75^{bc}	2.50 ± 0.87^{a}

The experiments were carried out with 15 replicates and repeated thrice. Mean separation was analyzed by ANOVA using SPSS Software (Ver.22). Means within a column followed by same letters are not significantly ($P < 0.05$) different as determined by Duncan's Multiple range test.

3.4 Hardening and Acclimatization of Plantlets

After 4 weeks in rooting medium, the rooted plantlets having roots were washed well in tap water to remove agar from roots and transferred in plastic cups filled with sterile vermiculate and covered with punctured polythene bags to maintain humidity and plants were irrigated daily (Fig. 5). After 30 days, the polythene bags were removed and the plants were transferred to clay pots (7.5 inch) containing garden soil and sand (1:3) and were kept under greenhouse conditions About 97% of the plants were hardened successfully. All the acclimatized plants exhibited normal growth and true to type morphology under natural conditions (Fig. 6).

Fig 5: Hardening of *in vitro* plants

Fig 6: Established plant

4. Conclusion

The present investigation reported very high rate of multiplication in *Hemigraphis alternata*. *Ex vitro* rooting of *in vitro* shoots were successfully obtained which saved time, energy and cost of production of micropropagated plantlets. This method offers an alternate method for the mass propagation of *Hemigraphis alternata*, an exotic medicinal plant.

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