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Application of Image J for the quantitative measurement of *in vitro* plants in *Hemigraphis alternata*(Burm. f.) T. Anderson

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ABSTRACT
An efficient system for the quantitative measurement of in vitro plants have been obtained by using plant image analysis software. Image J is an open source image processing program designed for scientific multidimensional images. In vitro regenerants were developed from nodal explants of Hemigraphis alternata on Murashige and Skoog (MS) basal medium supplemented with 3µM BAP. Individual elongated shoots were rooted on half strength MS liquid medium containing 2µM IBA. Assessment of the genetic stability of the plants using ISSR marker confirmed the uniformity of the micropropagated plants. The images ofin vitro regenerants were analyzed for measurements of quantitative characters and compared with manual data with no significant variation. Regenerated plantlets with well-developed shoots and roots were successfully transferred to soil with 98% survival rate

Keywords: *Image J, Smart root, In vitro regeneration, Hemigraphis alternata, Genetic stability*
Abbreviations: **BAP** – Benzyl Amino Purine; **IBA** – Indole -3-Butyric Acid; **MS**-Murashige and Skoog
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INTRODUCTION

Plant phenotyping problems can be addressed by the use of various computational tools [1] and such traits are important for all approaches of crop improvement programmes [2]. The manual phenotyping of *in vitro* regenerated plants are time consuming and often require sacrifice of plants due to contamination during the process.The image analysis tool opens a new platform for such analysis. Image J is an open source image processing program designed for scientific multidimensional images. It is highly extensible, with thousands of plugins and scripts for performing a wide variety of tasks, and a large user community. The software smart root is an operating system – independent freeware based on Image J. This software combines a vectoral representation of root objects with a powerful tracing algorithm which accommodates to a wide range of image source and quality. It supports a sampling based analysis of root system images in which detailed information is collected on a limited number of roots selected by the user according to specific research requirements [3]. Measured traits such as root length, root diameter, root insertion angle, lateral density, growth topology can be obtained from Smart root. *Hemigraphis alternata* (Burm. f.) T. Anderson commonly known, as red flame ivy plant, belongs to the family Acanthaceae. It is chiefly grown as an ornamental plant. This is a prostrate herb with rooting branches spread indefinitely on the ground with its creeping growth habit, leaves are opposite, broad, cordate and toothed. Flowers are white, small and in terminal head [4]. It is native to India and Indonesia. In folk medicine the plant is used for its wound healing activity [5, 6].

Traditionally, the leaves are consumed to cure gall stones, excessive menstruation and as a contraceptive. It has immense power to pacify vitiated pitta, fresh wound, cuts, ulcers, inflammations and skin complaints.

In the present investigation the *in vitro* regeneration from *Hemigraphis alternata*, an exotic medicinal plant and the measurements of its quantitative characters using Image J software were done for the first time.

MATERIALS AND METHODS

The plant was collected from Botanical garden, Department of Botany, University of Kerala. For the surface sterilization, the top cuttings consisted of 4-6 visible nodes were excised from newly formed shoots. The shoot cuttings were first washed in 20% (v/v) labolene detergent (Qualigens, India) for 10minutes and then in running tap water for 15 minutes followed by rinsing in distilled water. The washed shoot cuttings were surface sterilized in 0.1% (w/v) aqueous solution of mercuric chloride ($HgCl_2$) for 7 minutes, followed by rinse in sterile distilled water three times to remove any traces of mercuric chloride.

Sterilized shoot cuttings were chopped into 1-2 cm single node pieces and inoculated on Murashige and Skoog [7] basal medium supplemented with 3% (w/v) sucrose and 1, 2, 3, 4 or 5 µM BAP. The pH of the medium was adjusted to 5.7 using 0.1N NaOH or 0.1N HCl before adding 0.8% (w/v) agar (SRL, Mumbai)). After dissolving the agar by heating in a water bath, the medium was dispensed in 15ml aliquots into 25x150 mm culture tubes and autoclaved at 121^{o}C and 1.1Kg cm^2 pressure for 15minutes. All the cultures were incubated at 22±2^{o}C under a relative humidity of 50-60% and 12-hour photoperiod at a photon flux density of 50-60 µ ε m^{-2} s^{-1} light fluorescent tubes (Philips India Ltd., Mumbai). Observations were made 8weeks after inoculation.

Shoots developed through nodal cultures were isolated and cultured on medium containing ½ MS salts, 1.5% sucrose and 1, 2, 3, 4 or 5µM IBA (1-5µM) for rooting. Shoots were kept in same medium for four weeks. Observations were made after 8 weeks.

The rooted plantlets having 2 to 3 roots were washed well in tap water to remove agar from roots and transferred in plastic cups filled with sterile vermiculate watered daily and covered with punctured polythene bags to maintain humidity. 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.

The genetic uniformity of the *in vitro* raised plantlets was tested using Inter Simple Sequence Repeats (ISSR) markers. Extraction of genomic DNA from frozen leaves was carried out according to Dellaporta method [8], with minor modification. Three ISSR primers (UBC818, UBC826, and UBC841) synthesized from Integrated Data Technologies (IDT), USA were used to amplify the genomic DNA from*in vitro*regenerated plantletsand mother plant as control. The reaction mixture contained 4 pmol of each primer, 0.2mM of deoxynucleotides, 2.5 µl of buffer with $MgCl_2$, 1 unit Taq polymerase and 50 ng of DNA template. After 2 min at 95°C, 40 cycles were performed with 30 secs at 94°C, 1 min annealing at the optimized temperature, and 2 min extension at 72°C, and a final extension step of 10 min at 72°C. The PCR was carried out using Eppendorf 96 well thermo cycler. After the PCR, the amplified DNA products were electrophoresed on 1.8% agarose gels with ethidium bromide (10ug/ul) and products visualized under ultraviolet light using gel documentation (BioRad) system.

Data analysis

Images of regenerated shoots before rooting and rooted plants after 4 weeks were taken using Canon DSLR Camera and the images were analyzed using Image J Plant image analysis software and measurements were obtained. Shoot length, leaf length, petiole length and leaf area were determined using image J and root characters such as primary, lateral root number and length were analyzed using image J Plugin Smart root software. The data were then compared with manual measurements using graphical method.

All the experiments were conducted with a minimum of 15 replicates per treatment and repeated thrice. The data analyzed statistically using SPSS ver.22. The significance of differences among the means was carried out using Duncan's multiple range test (DMRT) at $P \leq 0.05$. The results are expressed as the means ± SD.

RESULTS AND DISCUSSION

The nodal segments inoculated on basal MS medium showed bud initiation and multiple shoot formation after 3 weeks of culture. Culture initiation from the nodal explants were noticed within 7 days of inoculation (Fig.1) followed by the development of multiple shoots within 30 days of incubation (Fig.2).MS medium supplemented with 3µM BAP produced an average of ten shoots per node having an average length of 4.66cm shoots after 60 days (Fig.3). Reduction in shoot number noticed in other concentartions of BAP (Table 1). Our results are in agreement with earlier findings as observed in *Sesamum indicum* [9], *Hybanthus enneaspermus* [10], *Enicostemma littorale* [11] and *Enicostemma axillare*[12].Root initiation from *in vitro* shootswas noticed within 7 days. A maximum of 19.6 roots per shoots having 4.56 cm length were obtained in presence of 2 µM IBA within 30 days of culture (Table 2). The roots were thin and slender (Fig.4). The rooted plantlets of 4-5cm height with fully expanded leaves and well developed roots were then deflasked and were subsequently transferred to sterile vermiculate taken in plastic cup for hardening(Fig.5). Hardened plants were transferred to soil(Fig.6) and 96% of the plantlets were survived in the greenhouse.

Table. 1 Effect of cytokinins BAP on shoot induction from nodal explants of *Hemigraphis alternata*

Analysis	Conc.of BAP (1µM)	Shoot Number (Mean ±SD) (cm)	Shoot Length (Mean±SD) (cm)	Leaf Length (Mean± SD) (cm)	Petiole Length (Mean± SD) (cm)	Leaf Area (Mean± SD) (cm)
Image J	1	4.90 ± 0.70^{d}	6.26 ± 0.30^{a}	2.26 ± 0.25^{a}	2.21 ± 0.20^{a}	5.76 ± 0.32^{a}
	2	6.43 ± 0.56^{c}	5.30 ± 0.52^{b}	1.66 ± 0.15^{b}	2.73 ± 0.20^{b}	4.33 ± 0.25^{b}
	3	11.06 ± 0.30^{a}	4.46 ± 0.44^{c}	1.40 ± 0.10^{b}	2.96 ± 0.15^{b}	4.56 ± 0.25^{b}
	4	7.93 ± 0.05^{b}	5.50 ± 0.26^{b}	1.53 ± 0.30^{b}	1.90 ± 0.10^{b}	5.73 ± 0.30^{a}
	5	5.73 ± 0.15^{c}	4.10 ± 0.10^{c}	1.36 ± 0.05^{b}	2.16 ± 0.20^{b}	5.43 ± 0.45^{a}
Manual	1	5.00 ± 0.10^{e}	6.56 ± 0.05^{a}	2.06 ± 0.11^{a}	2.06 ± 0.05^{d}	5.86 ± 0.05^{a}
	2	6.63 ± 0.20^{c}	5.20 ± 0.10^{c}	1.63 ± 0.05^{b}	2.26 ± 0.05^{b}	4.86 ± 0.05^{a}
	3	10.3 ± 0.10^{a}	4.66 ± 0.05^{d}	1.33 ± 0.05^{d}	2.56 ± 0.05^{a}	5.20 ± 0.60^{bc}
	4	8.03 ± 0.05^{b}	5.36 ± 0.05^{b}	1.50 ± 0.00^{c}	2.23 ± 0.05^{ba}	5.66 ± 0.32^{ab}
	5	5.76 ± 0.05^{d}	4.13 ± 0.05^{e}	1.36 ± 0.05^{d}	2.13 ± 0.05^{cd}	5.36 ± 0.05^{abc}
Type Df(n-1)=1		0.332^{NS}	0.420^{NS}	1.538^{*}	8.321^{NS}	3.81^{*}
Concentration Df(n-1)=4		304.30^{***}	69.39^{***}	30.36^{***}	30.54^{***}	16.31^{***}
Type* ConcentrationDf(n-1)=		2.34^{*}	0.79^{NS}	0.42^{*}	8.69^{NS}	1.66^{NS}

Means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Duncan's Multiple Range test. NS- non significant, ***F value is highly significant at $p<0.001$ level, **significant at $p<0.01$ level, *significant at $p<0.05$ level.

Plant image analysis tools such as Image J and Smart root were used for the quantitative analysis of *in vitro* shoots and the values were compared with the manual measurements (Fig.7, 8, 9, 10). The characters such as shoot length, leaf length, petiole length and leaf area were calculated using image J. The data obtained showed close similarity with the data obtained from manual experiments. The measurements obtained from image J and manual calculation were shown in Table1, 2. This validates the use of image analysis software for the analysis of *in vitro* developed shoots without sacrificing them for data collection. Similarly, for root measurements smart root software were used and showed almost similarity with the manual calculations. The use of Smart root for root measurements found to be effective in plants like Maize and Arabidopsis.

The ISSR primers used in this study generated 23 scorable monomorphic bands ranging in size from 150 bp to 1300 bp. The number of bands for each primer was from 5 (UBC841),8(UBC826) to 10 (UBC818) respectively with an average of 7.6 bands per ISSR primer (Fig. 11, 12, 13).

Genetic uniformity testing of regenerated plants was in consonance with earlier reports [13, 14] where morphological traits and molecular studies were considered as a powerful tool for studying and monitoring the variations arising in the cultured plant tissues. The ISSR technique has been used successfully for analyzing the genetic stability of species

propagated through in vitro techniques [15, 16]. The present study concludes that a reproducible protocol for axillary shoot multiplication of *H. alternata* has been successfully described. The tissue derived plantlets exhibited no morphological variation composed to mother plant which was further confirmed by ISSR markers.

Genetic uniformity or otherwise known as clonal fidelity is one of the most important prerequisites for successful micropropagation of any plant species. The uniformity of the plants was further confirmed using ISSR markers.

Table. 2 Effect of IBA on root induction from nodal explants of *Hemigraphis alternata*

Analysis	Concentration of IBA (µM)	Primary Root Number (Mean± SD)	Lateral Root Number (Mean SD)	Primary Root Length (Mean ±SD) (cm)	Lateral Root Length (Mean ±SD) (cm)
Image J	1	14.90±0.14^{c}	3.70±0.28^{b}	3.65±0.21^{a}	0.50±0.14^{b}
	2	19.83±0.25^{a}	4.43±0.20^{a}	2.56±0.20^{c}	0.61±0.10^{b}
	3	16.03±0.15^{b}	3.10±0.10^{c}	3.16±0.15^{b}	0.90±0.10^{a}
	4	12.43±0.35^{d}	2.83±0.05^{c}	3.00±0.10^{b}	0.90±0.10^{a}
	5	9.63±0.15^{e}	2.50±0.10^{d}	2.10±0.10^{d}	0.76±0.15^{bc}
Manual	1	14.60±0.36^{a}	3.51±0.36^{b}	3.8±0.10^{a}	0.56±0.11^{b}
	2	16.16±0.51^{a}	4.30±0.30^{a}	2.56± 0.32^{c}	0.56±0.15^{b}
	3	16.50±0.26^{a}	3.46±0.45^{b}	3.30±0.20^{b}	0.70±0.17^{b}
	4	12.46±0.30^{ab}	2.63±0.25^{c}	3.16±0.37^{b}	1.13±0.15^{a}
	5	9.26±0.15^{b}	2.23±0.15^{c}	2.13±0.15^{c}	0.50±0.10^{b}
Type Df(n-1)=1		0.646^{NS}	0.414^{NS}	0.984^{NS}	0.749^{NS}
Concentration Df(n-1)=4		20.473***	55.3***	46.924***	12.953***
Type*ConcentrationDf(n-1)=		1.252^{NS}	1.52^{NS}	0.181^{NS}	3.5*

Means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Duncan's Multiple Range test. NS- non significant, ***F value is highly significant at $p<0.001$ level, **significant at $p<0.01$ level, *significant at $p<0.05$ level.

Figure **1**. Initiation of shoots from nodal explant **2**. Multiple shoot formation after 30 days **3**. Maximum shoot induction in 3mg/l BAP **4**. Root induction from *in vitro* shoots in IBA **5**. Hardening of *in vitro* shoots **6**. Established plants

Figure **7,8**. Measurement using Manual method **9**. Image J **10**. SmartRoot

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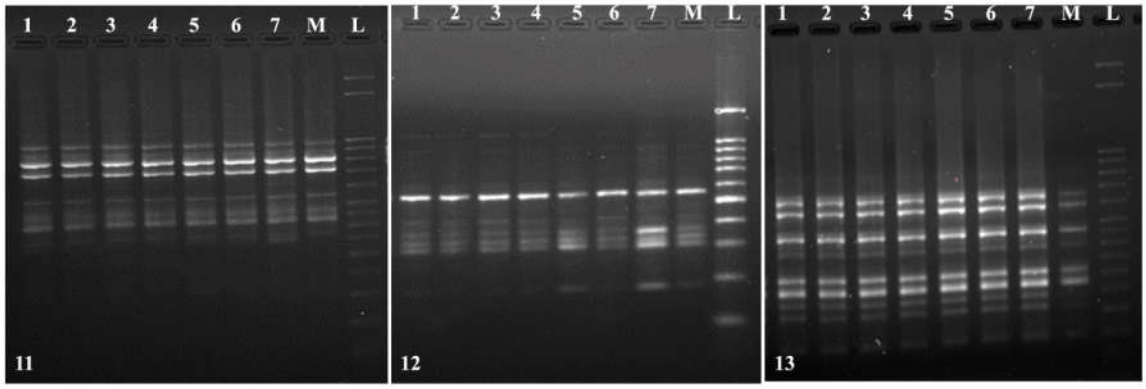

Fig 11, 12, 13: Genetic Fidelity using ISSR markers **11**. (UBC818), **12**. (UBC826) and **13**. (UBC841)

CONCLUSION

To conclude, that Plant image analysis tools provide an excellent system for studying quantitative analysis in *in vitro* systems and the protocol for multiple shoot formation described offers a potential system for improvement, conservation and mass propagation of *Hemigraphis alternata* from nodal explants.

DISCLOSURE

The present work has not involved any human participants and or animals

CONFLICTS OF INTEREST

The authors report that there is no conflict of interest regarding the publication of this paper.

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