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SELECTION AND *IN VITRO* MULTIPLICATION OF HIGH YIELDING CLONES OF *PLECTRANTHUS BARBATUS* [SYN.: *COLEUS FORSKOHLII* (WILLD.) BRIQ.]

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ABSTRACT
An improved regeneration Protocol has been developed with high frequency shoot organogenesis and plant establishment for *Plectranthus barbatus.* A systematic attempt was made to achieve a greater number of multiple shoots from elite high yielding genotypes. Healthy axillary and terminal buds were collected from healthy mother plants with high forskolin content (1.7% & 1.9%) for experiments. These nodal explants with axillary buds and shoot tips obtained from elite high yielding genotypes were used to regenerate shoots on MS media enriched with Cytokinins alone and together with auxins. Subculturing was done frequently, and after the third or fourth passage, the 150 numerous shoots increased. Regenerated shootlets were placed to a medium called 12 MS that was free of growth regulators and was ideal since it frequently had healthy roots (86.67%). These *in vitro* raised plants were acclimatized and established successfully in soil. The plants those hardened for 2 – 4 weeks in the mist chamber showed 93.3% survival. A detailed study was made to know the field performance of micro propagated plants and forskolin content.

Keywords: *Plectranthus barbatus, Cytokinins, forskolin, Micro Propagated*

Abbreviations: *BAP-6-benzyl amino purine; IAA - indole acetic acid; IBA – indole butyric acid; NAA - naphthalene acetic acid; AC - activated charcoal; $HgCl_2$ - mercuric chloride; MS -Murashige and Skoog medium*

INTRODUCTION

Plectranthus barbatus [syn.: *Coleus forskohlii* (Willd.) Briq., *C. barbatus.* (Andr.) Benth] belonging to the family Labiatae (Lamiaceae) is an aromatic herb, 30-40cm height with tuberous roots under the Sanskrit name "Makandi" and "Mayini". The drug Makandi is bitter, sharp and sweet to taste and produced in multiple fragrant tuberous roots, growing on hills.

Forskolin is a naturally occurring labdane diterpene that is produced from Coleus forskohlii roots and has antihypertensive, positive inotropic, and adenylcyclase-activating effects (De Souza *et al.,* 1983). It helps with nerve regeneration after trauma, reduces intraocular pressure in cases of glaucoma, and prevents platelet clotting (De Souza *et al.,* 1986; Valdes *et al*., 1987; Hussain *et al.,* 1992). It prevents hair from ageing (Keikichi *et al.,* 1988). Although diterpenoids are present in practically all plant parts, the roots are the primary source (Shah *et al.,* 1980), with forskolin serving as the principal diterpenoid (coleonol). However, being root as the economically important part this particular plant is over exploited and resulted in depletion of wild resources.

Finding a different plant source of forskolin became important due to its distinct pharmacological qualities and the growing demands of the pharmaceutical industry. The wild population provides an estimated 1000 tonnes of dried roots annually to the pharmaceutical industry. Limited additional attempts are being made to cultivate this species. Selecting and mass-producing high forskolin-yielding strains of *Plectranthus barbatus* would therefore be the most practical way to lower the cost of the raw material.

In an effort to find prospective forskolin genotypes, 38 genotypes obtained from diverse sites were screened by Vishwakarma *et al.* in 1988. Additionally, the plant grows at a rather slow pace, and environmental and/or geographic factors might affect the pattern of alkaloid accumulation. Stable forskolin manufacturing would be made possible via in vitro methods (Chandel and Sharma, 1997). However, in the present study a systematic attempt was made to achieve a greater number of multiple shoots from elite high yielding genotypes. Because of the wide variability in forskolin content (0.1 – 1.9%), in the wild and *in vitro* developed plants, the present study was

conceived with an idea of screening high yielding lines and developing a cost-effective protocol for mass propagation of true - to - type plants without any variations. The present investigation thus provides a means of desirable free healthy clones for extraction of the forskolin.

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIAL

Attur, Reddiarpalyam, Thiruvanamalai, Kallakurchi in Tamil Nadu, Hyderabad in Telangana, Bangalore, Sindgi, Belgaum in Karnataka, and Kholhapur in Maharashtra were among the areas from which planting material was gathered. In the medical plant garden cared for by M/s Rishi Herbal Technologies, Bangalore, Karnataka, India, germplasm was planted.

STAGE 'O': MOTHER PLANT SELECTION AND PREPARATION

Stage 'O' includes maintenance of high yielding germplasm, selection and collection of explants for tissue culture studies. Mother plants were sprayed with 100 ppm Bavistin three days before inoculation. Morphological studies were carried out for the selection of high yielding line. They were analyzed for their high forskolin content followed by Inamdar *et al*. (1984). Accession numberCF1 to CF10 were given to each line. Healthy axillary and terminal buds were collected from healthy mother plants with high forskolin content (1.7% &1.9%) for experiments.

STAGE: 1 PREPARATION AND STERILIZATION OF EXPLANTS

The axillary bud and shoot tip explants were initially treated for 5 minutes with detergent before being thoroughly washed for 30 minutes under a jet of tap water. Later, explants were given a 2-minute treatment with 70% alcohol (v/v) before being repeatedly washed with tap water. The final step of the surface sterilisation process was carried out aseptically in a Laminar Airflow Chamber. After subjecting to 0.1% $HgCl_2$ (7 min) followed by 0.05% $HgCl_2$ (4 min) disinfectants, explants were washed thoroughly 5 - 6 times with sterilized distilled water. Disinfected explants were aseptically placed on to the MS medium supplemented with 0.5 mg/l BAP.

From the established clean, mother cultures nodal explants were implanted on MS (Murashige and Skoog, 1962), B_5(Gamborg *et al.*, 1968) and SH (Schenk and Hildebrandt, 1972) media supplemented with 0.5 mg/l BAP in order to select most suitable medium for shoot multiplication and also to study the influence of salt formulations on growth and differentiation. In order to choose the best explant for shoot multiplication, the established clean, mother cultures were afterwards dissected into nodes with axillary buds and grown on MS media supplemented with 0.5 mg/l BAP. At the conclusion of 15 days, the quantity of regenerated shoots, the type, and the percentage frequency of responses were noted. The medium and explant on which high rate of multiple shoots proliferation obtained was selected for further experiments.

STAGE: 2 &3 SHOOT MULTIPLICATION AND ROOTING

The ability of shoot multiplication was studied by culturing suitable explant in selected medium supplemented with different plant growth regulators (either individually or in combinations). Various shoot regeneration media tried for optimization of protocol for rapid shoot multiplication for stage 2. Single shoots with 3 - 4 nodes were excised from multiple shoot cultures and transferred to rooting medium directly in stage 3. In order to induce roots, the removed shoots were added to MS 12 strength with or without AC and hormone supplements. Plantlets that had roots were gently taken out of the culture vessel without disturbing them. To get rid of the agar that had adhered to the roots, these were thereafter carefully rinsed. To prevent fungus contamination, washed plantlets were covered in 100 ppm of the systemic fungicide bavistin. In protrays with net pots, treated plantlets were implanted in soilrite that had been autoclaved. These specimens were put in a polyhouse with a misting system. To promote airflow, the polyhouse's side coverings were occasionally rolled up. The ideal temperature of 24°C was maintained with a high humidity of 95%. The temperature inside the polyhouse was lowered with the help of timely misting. Plantlets were slowly acclimatized by increasing the exposure period from 1 hour to several hours.

ANALYSIS OF FORSKOLIN

Plant roots were harvested, dried, and ground into a fine powder. At 70 °C for two hours, benzene was used to extract the powdered roots. The benzene extracts are purified and vacuum-concentrated. HPLC was subsequently used to determine the residue's forskolin content.

CHEMICALS

Hexane, methylene chloride, chloroform (Spectroscopy grade, parachem, India), benzene, ethyl acetate (AR grade, BDH, India).

MOBILE PHASE AND CHROMATOGRAPHIC PARAMETERS

A mixture of 70:20:10 (v/v) hexane, ethylacetate, and methylene chloride was prepared and degassed before use. For 30 minutes, the column was in an equilibrium state. With a back pressure of 200 psig, the mobile phase was pumped at a rate of 0.8 ml/min. With mobile phase, the injector and detector were flushed. The potentiometer chart speed was set to 0.5 cm/min, and the refractive index detector was adjusted to 4x. In the analysis work, the injection volume was 10 ml and 20 ml. For a semi-preparatory analysis of the plant extract, the parameters were altered. They were as follows: 0.5 ml/min flow rate, 100 psig pressure, 2 cm/min chart speed, 32x detector attention, and 100 ml injection volume.

METHODS

Forskolin samples (25 mg) were dissolved in 0.5 ml of chloroform before being diluted in the mobile phase to 10 ml. The internal standard solution was made by combining mobile phase (0.5 mg) with chloroform (10ml). Forskolin was created in triplicates to flow the stock solutions at concentrations of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2, 2.25, and 2.5 mg/ml, with the internal standard's concentration being consistently held at 3 mg/ml. The column was injected with 20 ml of each solution, and the outcomes were recorded.

RESULTS AND DISCUSSIONS

Notable progress has been achieved in the past years after the recognition of important four stages in micropropagation (stage 1 to stage 4) by Murashige during the year 1974. The scheme of ‘stage 0’ is introduced for micropropagation systems later by Debergh and Maene (1981). Thus for any species in clonal propagation for the establishment of plants, the following defined steps are adopted (George, 1993).

Stage 0: Mother plant selection and preparation
Stage 1: Initiation of an aseptic culture of the explant
Stage 2: Multiplication of shoots or other propagules
Stage 3: Rooting of shoots
Stage 4: Plant establishment/Acclimatization

STAGE ‘O’: MOTHER PLANT SELECTION AND PREPARATION

The choice of mother plants should be made with care before micropropagation begins. They must not exhibit any illness symptoms and must be representative of the variety or species. In order to successfully cultivate the chosen plant in vitro, it might be desirable to modify it or some of its components. By providing stock plants with the proper environmental conditions and chemical pre-treatments, it is possible to increase growth, morphogenesis, and rates of in vitro propagation (George, 1993).

To select elite mother plants a rigorous survey was conducted in various geographical locations and naturally selected disease-free mother plants, which are luxuriant in growth. Root biomass and forskolin content were analyzed for final selection of high yielding lines. Cuttings measuring 10-12cms with three to four nodes from the selected plants were planted individually in rows and a clonal population of these selected lines was marked and maintained (CF1 – CF10). After 6 months roots were harvested and analyzed for the average number of roots, fresh weight, dry weight and forskolin content. The results were summarized in the Table-1.

Table 1: Analysis of results for the selection of selected clones of CF1 to CF10 in *Plectranthusbarbatus* (syn.: *Coleus forskohlii*)

Code accession Number	Average no. Of roots	Average Fresh wt. (Kg)	Average Dry wt. (Kg.)	Forskolin Content (% dry weight)
CF1	7.5	0.463	0.041	0.66
CF2	7.8	0.482	0.043	0.68
CF3	5.8	0.384	0.034	0.58
CF4	7.4	0.464	0.041	0.64
CF5	8.4	0.543	0.049	0.73
CF6	6.4	0.416	0.037	0.6
CF7	13.6	0.854	0.076	1.5
CF8	11.6	0.843	0.075	1.4
CF9	17.2	0.923	0.083	1.9
CF10	6.6	0.426	0.038	0.6

These naturally selected lines were further subjected to stability studies for three subsequent generations. Interestingly the high yielding lines i.e. CF7 and CF9 are very stable and the content of forskolin was high with more root biomass in all the three generations. These two lines were considered as high yielding genotypes as they are exhibiting the ability to synthesize more forskolin. It is evidenced from the reports of Sen and Sharma (1991b) and Sen *et al.* (1992) that only 0.5 – 0.7% forskolin is present in natural population of *Coleus forskohlii*. In the present study it is very clearly indicated that the variations in forskolin content were ranging from 0.6 – 1.9% on dry weight basis (Table.1). Of all the lines tested CF9 is expressing its maximum potentiality with a yield of 1.9% followed by CF7 with 1.5% forskolin (Table.1). Hence, the selected lines were considered as elite mother plants and further experimentation was continued with these lines.

Table 2: Analysis of results for the forskolin samples as per the chromatograms for the selectedclones of CF1 to CF 10 in *Plectranthus barbatus* (syn. : *Coleus forskohlii*)

Forskolin Sample	**RT**	**Area**	**Height**	**Assay**
Standard	6.19	35019899	968025	95%
CF1	6.62	18931482	58040	0.66%
CF2	6.58	18897573	53304	0.68%
CF3	6.62	15754742	46090	0.58%
CF4	6.18	13273485	34624	0.64%
CF5	6.20	151323320	40058	0.73%
Forskolin Sample	RT	Area	Height	Assay
Standard	11.43	2881.7220	96.8025	95%
CF6	11.45	590.4268	17.4672	0.6%
CF7	11.48	659.9963	20.3830	1.5%
CF8	11.47	658.0758	20.5985	1.4%
CF9	11.46	698.1965	23.2393	1.9%
CF10	11.42	619.7429	17.5901	0.6%

In the present study, selected elite mother plants of *Plectranthus barbatus* [syn.: *Coleus forskohlii* (Willd.) Briq.]are maintained and proved to be the most suitable source of explants throughout the year. Healthy juvenile axillary shoots were used to establish mother cultures for micropropagation. Three days prior to inoculation the plants were sprayed with 100 ppm bavistin, a systemic fungicide, to ensure the explants fungal free. The breeding programs for the selected lines is quite complex and homogeneity of the population is not guaranteed, especially in *Coleus* because of high degree of variations which are happening spontaneously (Sen and Sharma,1991b). This is not only a complexity and time consuming but also a labour intensive character of afore mentioned approach. Hence, it is always preferable to select naturally available high yielding lines from the wild and developing a suitable micropropagation system to ensure true - to - type plants. In the present study a reliable micropropagation system was developed in *Plectranthus barbatus* (syn.: *Coleus forskohlii*) from selected adult mother plants as followed by Beruto *et al* (1989), Beruto and Debergh (2004) in *Ranunculus asiaticus.*

STAGE'1': INITIATION OF AN ASEPTIC CULTURE OF THE EXPLANT

Getting an aseptic culture of the chosen plant material is the traditional second stage in the micropropagation procedure. Explants must first be moved into a culture environment free of any visible microbial contamination if this stage is to be successful. Thus the initiation of clean cultures is the foremost and important stage for successful establishment of protocols for micropropagation. To achieve this objective, the right choice of explant is often always either shoot meristem or axillary buds. The predominant reason of this choice is due to their high degree of stability and plasticity (George,1993).

Various surface sterilization procedures were employed in order to obtain clean mother cultures. *Plectranthus barbatus* (syn.: *Coleus forskohlii*) being a tender herb with lots of pubescence is a real challenge to obtain healthy and clean cultures without browning. Hence, many researchers tried with seed material as an initial source of explants rather than that of mature plant (Sen and Sharma, 1991b). To optimize a surface sterilization various

treatments were followed to obtain clean and healthy cultures. Of all the procedures tried , 0.1% $HgCl_2$ (7 min) followed by 0.05% $HgCl_2$ (4 min)procedure is best which resulted in highest percentage (86.7%) of healthy cultures without contamination (Figure 1A). The present study is targeted to establish clean cultures from mature plants because of the following reasons.

* Seeds might be heterozygous in nature and result in not uniform populations.
* As the present investigation is aimed at selection of high yielding lines and their multiplication through tissue cultures, it is a must to develop protocols from mature plants rather than seedling raised plants.

STAGE '2': MULTIPLICATION OF SHOOTS OR OTHER PROPAGULES

Somatic embryos, axillary or adventitious shoots, tiny storage or propagative organs, or newly regenerated shoots can all contribute to growth. Prior induction of meristematic centres, from which adventitious organs may grow, will be part of stage '2' .Hence, the multiplication of the explants and the most rapid rates of multiplication are crucial stages and genotype independent. In order to achieve rapid proliferation of healthy multiple shoots various factors were optimized in the present study.

MEDIA EVALUATION

The degree of success in any technology employing plant cell, tissue or organ culture is dependent on choice of nutritional compounds (Gamborg and Phillips, 1998). Hence in the present study, nodal explants were cultured on three different media viz., MS, B_5 and SH in order to study the effect of different salt formulations and concentrations of these media on morphogenesis (Table:3).

Table 3: Effect of different media on multiple shoot proliferation from nodal explant of *Plectranthus barbatus* (syn.: *Coleus forskohlii*)

Media + 0.5mg/1 BAP	**Percent frequency Of cultures With browning**	**Percent frequency Of cultures with Multiple shoot buds**	**Average length of shoots (cm)**
B	51.65	48.35	3.33
MS	15.00	85.00	4.67
SH	23.35	76.65	2.67
F- Value	79.88**	79.18**	23.52**
SEm±03.35		03.05	00.32
CD at 5% level	07.92	07.21	00.76

*** Significant at 1% level*

Data represents average of 3 experiments with 10 replications.

MS medium promoted maximum percent of multiple shoots and this response (85%) was rather low on B_5(48.35%) and SH (76.65%) media. There was no remarkable change in length of shoots, though the percent frequency of multiple shoot buds varied depending on the media. The data subjected for statistical analysis indicate the significant difference in response with respect to different media with cultures showing browning (F= 79.88**), cultures with multiple shoot buds (F= 79.18**) and shoots with average elongation (F= 23.52**).

In vitro propagation of plants belonging to Lamiaceae showed optimum growth on MS medium (Jaideep *et al.*, 2000). The preference of MS medium was already found in *Plectranthus barbatus* for the development of multiple shoots and callus cultures (Krombholz*et al.*,1990; Sen and Sharma, 1991b; Sen *et al.*, 1992; Mukherjee *et al.*, 1996; Malathy and Pai,1999; Reddy *et al.*, 2001; Bhattacharya and Bhattacharya,2001; Balasubramanya*et al.*, 2012). The difference in morphogenic response on tested media could be attributed to the high content of nitrogen in MS medium. The nitrogen NO3 -/ NH4 + ratio is considered as an important factor on nitrogen uptake and pH regulation during plant tissue culture (George, 1993). Thus in Plectranthus barbatusMS medium is proved to be the best suitable medium among tested media indicating the high salt requirements for shoot sprouting and proliferation.

EXPLANT EVALUATION

To trigger morphogenic potentiality type and nature of explant is a major determining factor. Plant regeneration often depends upon the presence of organized explant tissue. Basically micropropagation technique is defined as usage of axillary and terminal buds as initial source of explants (Morel, 1960). Hence, axillary bud and terminal bud that have preformed meristems are selected for regeneration of shoot buds in the present study and the results are as follows (Table: 4). The better morphogenic potentiality of nodal explant over shoot tip explant might be due to natural apical dominance of shoot tip. Hence, this affected both time period of response and also the number of multiple shoot buds.

Table 4: Influence of various explants on multiple shoot induction on MS media fortified with 0.5 mg/l BAP in*Plectranthus barbatus (syn.: Coleus forskohlii).*

Explant Type	Percent frequency Of shoot Sprouting	Shoot no. / explants	Shoot length (cm)
Nodal	96.67	35.33	5.33
Shoot tip	76.67	18.67	4.33
F- Value	18.00**	50.00**	4.50^{NS}
SEm±04.71		00.75	0.47
CD at 5% level	13.07	02.08	

** Significant at 1% level* *NS: Non-Significant*
Data represent with average of 3 experiments with 10 replications

The data subjected for statistical analysis indicate the significant difference with respect to different explants in percentage of frequency of cultures responding (F= 18.00**), multiple shoot buds per explant (F= 50.00**) and shoots with average elongation (F= 4.50NS). In accordance with the optimum response found with nodal explant in terms of induction of high percent frequency (96.67%) and number (36) of multiple shoots, it is selected further to assess the efficacy of other cultural factors on morphogenic potentiality. Bhattacharya and Bhattacharya (2001) reported a maximum of 12 multiple shoots from nodal explants of *Coleus forskohlii*on MS medium fortified with 0.46 mM KN and 0.57 mM IAA. However, in the present study there are more than 35 multiple shoots from nodal explants inoculated on MS medium fortified with 0.5 mg/l BAP. This is in advancement over the existing reports in *Plectranthus barbatus.*

The finest results come from explants made up of meristems that are going through regular mitoses. Only clones generated from meristem, shoot tip, and nodal bud cultures are thought to be genetically stable since they are frequently phenotypically uniform (Hu and Wang, 1983). As a result, the use of axillary buds in the current study has established itself as the most practical and trustworthy approach for true-to-type in vitro propagation.

EFFECT OF CYTOKININS

Various concentrations of growth regulators in the medium trigger differential morphogenetic responses by inducing meristematic action of explants. To identify best suitable type and concentration of hormone that influences the morphogenic potentiality in terms of percent frequency, MS medium with different concentrations of BAP, KN and TDZ were employed. BAP was more effective than KN and TDZ for proliferation of axillary buds. The results (Table: 5) showed that the optimal concentration of cytokinin required to regenerate maximum number of shoot buds is 0.5 mg/l BAP (Figure:1B). Increase in concentrations of BAP (>0.5 mg/l) decreased the regenerative potentiality and resulted in callus formation at the cut ends and exhibited various manifestations of BAP toxicity. Shoots were very stunted (less than 15mm.) and abnormal at higher concentrations of BAP (>2 mg/l). Shoot elongation was marked, when medium is fortified with KN (0.5 mg/l) and the result was unsatisfactory with regard to multiplication (55%) and induced lesser number of shoots (10.67) than BAP (0.5 mg/l) and TDZ (0.005 mg/l).The significance of varied concentration of hormones on the length of regenerated shoots was not found and data not shown. Thus the observations displayed that the supplementation of a cytokinin to the culture media positively influenced and of the three cytokinins tested, BAP resulted significantly more shoots per explant than the other two. This result is in advancement over earlier reports (Sen and Sharma, 1991b; Suryanarayan and Pai, 1998: Bhattacharya and Bhattacharya, 2001).

The data subjected for statistical analysis indicate the significant difference in responding with respect to different concentrations of cytokinins with average number of shoots (F= 2083.71**) percentage of frequency of cultures responding subject to >10 multiple shoots (F= 1150.74**) and percentage of explants showing callus (F= 485.82**).BAP proved to be the most suitable cytokinin and was significantly better than KN for inducing shoot bud induction may be due to the inhibition of callusing and at the same time the decrease in shoot production in high BAP may be due to the inhibition of shoot initiation (Faisal and Anis, 2003). It is the first report that TDZ is supplemented to the culture medium for studying morphogenic response in *Plectranthus barbatus* cultures. However, the effected leaf morphology could not serve TDZ as a system of choice for better and enhanced shoot multiplication in the present study. Repeated subcultures of nodal explants at 4 – week intervals on MS supplemented with 0.5 mg/l BAP enabled mass multiplication of shoots without any evidence of decline. Not all

buds elongated but gradual removal of the growing shoots at each sub-culturing enhanced the elongation of dormant buds.

Table 5: Effect of cytokinins on shoot multiplication in *Plectranthus barbatus* (syn.: *Coleus forskohlii*)

Hormone (mg/l)	**Average no. Multiple shoots Showing Per explants**	**Percentage of explants Showing (<10) Multiple shoots**	**Percentage of Explants Showing callus At cut ends**
BAP 0.5	45.33	95.00	0.00
1.0	20.67	88.35	21.65
1.5	10.33	55.00	35.00
2.0	5.67	0.00	48.35
2.5	5.67	0.00	76.65
3.0	1.33	0.00	98.35
KN 0.5	10.67	55.00	0.00
1.0	9.00	0.00	0.00
1.5	6.67	0.00	6.65
2.0	5.67	0.00	28.35
2.5	2.33	0.00	55.00
3.0	1.00	0.00	70.00
TDZ 0.001	35.33	26.65	0.00
0.005	40.67	95.00	8.35
0.010	30.33	85.00	50.00
0.050	10.33	76.65	96.65
F – Value	2083.71**	1150.74**	485.82**
SEm±	0.42	1.70	2.25
CD at 5% level	0.82	3.33	4.41

*** Significant at 1% level*
Data represents average of 3 experiments with 20 replications.

EFFECT OF CYTOKININS IN DIFFERENT COMBINATION

The rate of multiplication depends not only on choosing the best explant but also on using the right combination of growth regulators and medium for that particular organ or tissue (Mathews, 1987). By supplementing the medium with various concentrations of BAP and KN, these in vitro-grown shoots from nodal explants were then used to evaluate the ideal growth regulator requirements for optimum multiplication of shoot buds. This is the first paper that looked at cytokinins in various combinations, and the findings are shown in Table 6. With the administration of cytokinins in combination, significant proliferation of many shoots was observed on the medium. Average 41 multiple shoots were produced by the medium enriched with KN and BAP (0.25mg/l) and 0.25mg/l, respectively.

Though the average number of multiple shoots regenerated on this medium is on par with 0.5 mg/l BAP, the morphological nature is varied. Callus from the cut ends was observed as the concentrations of growth regulators increased. Combination of cytokinins proved to develop healthy shoots with short internodes. Proliferation drastically improved after 2nd and 3rd subcultures. Many cultures regenerated more than 150 shoot buds on this medium.

The statistical F-test results suggest a significant difference between cultures that received different treatments in terms of the average number of shoots (F= 1930.09**) and the proportion of explants that had callus (F= 916.40**). The findings show that particular and well-defined hormone requirements are needed for the differentiation of shoots, roots, or both, and that even a small change in the necessary quantity affects how the cultured explants grow. (Ali *et al.*, 1996). Malathy and Pai (1998) in *Hemidesmus indicus* and Komalavalli and Rao (2000) in Gymnemasylvestre both concur with the outcome. In addition to blocking the already formed shoots, callus production from the cut ends was evident at greater concentrations and may have covered shoot formation sites in Hemidesmus indicus, according to Sreekumar *et al.* (2000).

Table 6: Effect of combination of cytokinins for multiple shoot bud induction in *Plectranthus barbatus* (syn.: *Coleus forskohlii*)

Hormone treatment (mg/l)		**Average no. of Multiple shoots. Explants**	**Percentage of explants showing Callus**
BAP	**KN**		
0.25	0.25	41.00	26.65
0.50	0.50	10.33	10.00
0.25	0.50	30.33	33.35
0.50	0.25	19.67	75.00
F – Value		1930.09**	916.40**
SEm±		0.54	1.60
CD at 5% level		1.25	3.69

*** Significant at 1% level*
Data represents average of 3 experiments with 20 replications

EFFECT OF CYTOKININS AND AUXINS IN DIFFERENT COMBINATIONS

It is a well-known fact that morphogenesis, which results in the creation of full plantlets, requires the right ratio of cytokinin and auxin. This study also noticed the multiple shoot regeneration's growth-stimulating effect on MS medium enriched with various auxin and cytokinin combinations (Table: 7). Within 10 days of subculture, shoot emergence was seen (up to 14.67 shoots) in the medium BAP(1mg/l) and NAA(1mg/l). The number of shoots per explant was dramatically decreased in this instance, but shoot length was unaffected by the addition of either NAA or IAA to the MS medium supplemented with various concentrations of BAP. With the addition of IAA and KN, only 12 shoots per explant were produced in Coleus forskohlii (Bhattacharya and Bhattacharya, 2001). Whereas, Sen and Sharma (1991b) in contrast reported 20 number of shoots in the medium fortified with BAP and IAA (2.0 and 0.05mg/l).

Table 7: Effect of Cytokinins and Auxins on Multiplication of Shoots in nodal Explants of *Plectranthus barbatus* (syn.: *Coleus forskohlii*)

Growth Regulator (mg/l)		**Percentage of Explants Showing Multiple shoot Formation**	**Average no. Of shoots Regenerated / Explants**	**Percentage of explants Showing root formation**
BAP	**NAA**			
0.5	0.5	56.7	1.00	13.33
1.0	1.0	63.3	6.33	36.67
1.0	1.0	83.3	14.67	43.33
2.0	2.0	73.3	8.33	66.67
2.0	2.0	66.7	1.00	86.67
BAP	IAA			
0.5	0.5	53.3	1.00	23.33
1.0	0.5	56.7	4.33	30.00
1.0	1.0	76.7	12.33	30.00
2.0	1.0	73.3	6.33	56.67
2.0	2.0	66.7	1.00	83.33
F – Value		5.54**	185.27**	1921.90**
SEm±		4.71	0.52	5.58
CD at 5% level		9.83	1.08	11.64

*** Significant at 1% level*
Data represents average of 3 experiments with 10 replications.

The statistical analysis of the data reveals a significant difference between the cultures that were subjected to the different treatments in terms of the percentage of cultures that responded (F= 5.54**), the average number of shoots

(F= 185.27**), and the percentage of explants that showed root formation (F=1921.90**). Auxins and cytokinins work together to promote many plant species' in vitro multiplication and shoot growth (George,1993). The number of shoots per explant was greatly reduced in our case, nevertheless, as a result of adding NAA or IAA to the multiplication media, and the outcome is consistent with an earlier report revealed in Cunilagalioides (Fracaro and Echeverrigaray, 2001). In contrast the effective role of NAA/IAA in combination with BAP for shoot induction of multiple shoots has been reported in *Rauwolfia* (Ghosh *et al.*, 2001).

MULTIPLICATION FROM LONG - TERM CULTURES

The research that is now available on *Plectranthus barbatus* (syn.: *Coleus forskohlii*) shows unequivocally that long-term cultures have never been attempted. The strength of the initial explants' capacity for regeneration across various passages was examined in the current investigation. Maintaining long-term cultures is important for the preservation of genetic material. *Plectranthus barbatus* is prone to variations and abnormalities and after 3–4 sub-cultures on multiplication media tends to reveal anomalies in leaf morphology, making the current study—which is concerned with selection of high producing lines—extremely important.

Sharma et al. and Sen and Sharma (1991b) also reported on related observations (1992). Bhattacharya and Bhattacharya (2001) suggested single passage cultures to prevent these problems. But in the current work, a successful methodology for maintaining long-term cultures was found and optimised. BAP (0.25 mg/l), a very low dosage of cytokinin, was initially used to start cultures. These axillary and terminal buds were placed on multiplication media for a single sub-culture lasting only 10 days after receiving clean cultures. These expanded shoot buds were later transplanted into basal medium. Sub-cultures were routinely conducted every 25 days.

Axillary and terminal buds from these cultures were removed and placed to multiplication media whenever the requirement for multiplication arose again. Cultures were kept for three years without any abnormalities by using this procedure. The rate of recuperation and caulogenic potentiality retention were both satisfactory. Therefore, two to three passages of cytokinin elimination are necessary for long-term maintenance. Otherwise, anomalies such as callusing at the cut edges would develop. This demonstrates that high cytokinin levels are required to start shoot proliferation, but that after induction has begun, the concentration must be lowered. This result is consistent with Stimart's findings (1986).

STAGE '3': ROOTING OF SHOOTS

The regenerated microshoots from nodal culture, measuring 7 to 8 cm in length, were placed in various rooting media (Table:8). Within 10 days of the inoculation, root regrowth was visible from the shoots' basal cut part (Figure:1C). It's interesting that shoots implanted on 12 MS basal media had the strongest root regeneration response, demonstrating that shoot rhizogenesis is independent of growth regulators. All growth regulators consistently work to induce root development at a range of percent frequencies. However, the callus growth at the shoots' cut margins is the most important finding.

With an increase in growth regulator concentration, the percent frequency and callus mass produced greatly improved. On basal medium-cultured shoots, the cut margins had little to no callus, if any at all. Therefore, basal medium is the most suited of all the rooting media tested, with a high frequency of healthy roots (86.67%). Only 8.35% of the shoots on these media display callus, and even the amount of callus present in these shoots is insignificant. Roots had callus on the surface and were stunted in the presence of NAA and IBA. In rhizogenic studies, IAA (0.5mg/l) had the second-best response (76.65%), followed by NAA (0.5mg/l) with 51.65% and IBA (0.5mg/l) with 61.65%. The benefit of employing basal media for roots was also validated by Reddy et al. (2001) in their investigation of *Plectranthus barbatus*. Bhattacharya and Bhattacharya (2001) and Sharma et al. (1991) both observed greater rooting response in media containing IAA for *Coleus forskohlii*. IAA caused callus in the current investigation, demonstrating its unsuitability for use in rhizogenic experiments and confirming the benefit of employing basal medium for rhizogenesis.

The data subjected for statistical analysis indicate the significant difference to different treatments of auxins with percent frequency of response for rooting (F= 33.60**) percentage of cultures with callus (F= 29.58**) average number of roots (F= 31.83**) and with average length of roots (F= 15.66). The maximum frequency of best root formation (93.35%) was achieved on ½ MS basal medium supplemented with 0.05% AC. Several workers reported the role of activated charcoal on rooting response (Misra 2002) and described it as an essential component for vigorous root formation. This incidence of root formation in auxin free medium may be due to the presence of higher quantity of endogenous auxin in *in vitro* raised shootlets(Faisal and Anis, 2003). The fortification of activated charcoal to the rooting medium proved to be most fruitful for high root frequency and elongation.

Table 8: Effect of different hormone treatments (with Activated charcoal) for *in vitro* rooting in *Plectranthus barbatus* (syn.: *Coleus forskohlii*)

Media + Hormone (mg/l)	Percentage Of explants Showing Rooting	Percentag of explants showing callus	Average No. of Roots/ Explants	Average Root Length (cm)
1/2MS + 0.50 NAA	51.65	48.35	13.33	10.00
1/2MS + 1.00 NAA	26.65	73.35	10.67	9.33
1/2MS + 1.50 NAA	8.35	91.65	9.33	8.67
1/2MS + 2.00 NAA	0.00	100.00	4.33	8.67
1/2MS + 0.50 IAA	76.65	23.35	17.67	11.67
1/2MS + 1.00 IAA	46.65	53.35	14.67	10.67
1/2MS + 1.50 IAA	26.65	73.35	10.67	8.67
1/2MS + 2.00 IAA	6.65	93.35	4.67	7.67
1/2MS + 0.50 IBA	61.65	38.35	11.67	9.67
1/2MS + 1.00 IBA	41.65	26.67	9.67	9.33
1/2MS + 1.50IBA	20.00	80.00	8.67	7.33
1/2MS + 2.00 IBA	6.65	93.35	3.67	6.67
1/2MS control	86.67	8.35	17.33	11.33
F – Value	11.20**	394.22**	133.38**	9.57**
SEm±	2.38	2.05	0.55	0.66
CD at 5% level	4.91	4.23	1.13	1.36

*** Significant at 1% level*
Data represents average of 3 experiments with 20 replications.

STAGE '4': PLANT ESTABLISHMENT / ACCLIMATIZATION

The ultimate success of in vitro reproduction is determined by how well plants take root in the soil (Saxena and Dhawan, 1999). For many plant species, acclimatisation of in vitro plantlets to greenhouse or field conditions is a crucial step that takes time and money and limits the commercial utility of the micropropagation procedure.

Well rooted plantlets from the culture vessels gently removed keeping the roots intact. The plantlets transferred to a container of warm water and gently rinsed the agar-media off the roots. The regenerants were planted in a plastic bag with 1: 1: 1 (soil: sand: manure) sterile mix. The plant is wrapped in plastic paper and placed in a tent made with plastic wrap. The tents are opened to allow air exchange briefly and for the passage of diffuse light. Gradual acclimatization of the plants exhibited very high degree of survival (Figure: 1D). Two different sets of plants were maintained. One set was given with ½ MS liquid medium without any vitamins and sucrose. Another set was provided with normal tap water. Interestingly plants supplied with tap water exhibited relatively more survival rate than plant fed with nutrient medium. The reason for this differential behaviour is plants supported with nutrients resulted in fungal growth of the plant eventually leading to the necrosis and finally death. A two – week period of gradual lowering of humidity from 98% to 70% had no effect upon the plantlets. After one week, some air let for 1 hour each day and increased gradually to several hours per day. After third week, the wrap is removed and plants are allowed to adjust to ambient conditions. The plantlets now performed all of their own photosynthesis and adapted to lower relative humidity by developing a waxy cuticle and regulating stomata function. Shoot growth resumed at week eight and by fourteen, there were at least two new leaves on each plant. Leaves produced *in vitro* were abscised by week fourteen.

In the present study, the result on the pot establishment of the rooted plants show that hardening of the plants weaned away from the culture vessel at least for a minimum period of 2 weeks was a prerequisite for successful establishment. Plants transferred directly into the nursery showed 43.33% survival and those hardened for 2 – 4 weeks in the mist chamber showed 73.3% survival. A detailed study was made to know the field performance of micropropagated plants developed by both methods. Percentage of survival was calculated for different batches and results were given in Table: 9

Table 9: Effect of rooting method on percentage of survival in *Plectranthus barbatus* (syn.: *Coleus forskohlii*)

Nature of Plant	Percent frequency of plantlets survived at net pot level	Percent frequency of plants survived at field level	Percentage of mortality
Plantlets through *in vitro* rooting	73.33	65.00	8.33
Plantlets directly transferred tothe field level	–	43.33	33.33
F – Value SEm ± CD at 5% level	28.42** 3.75 10.43	36.76** 5.44 13.33	26.40** 6.45 15.80

*** Significant at 1% level*
Data represents average of 3 experiments with 20 replications

The data subjected for statistical analysis indicate the significant difference to different procedure of acclimatization with percent frequency of plantlets survived at net pot level (F= 28.42**) percent frequency of plantlets survived at field level (F= 36.76**) and percent of mortality (F= 26.40**). Thus out of total 1500 micropropagated plants obtained through *ex vitro* rooting transplanted into the polythene bags during the period of investigation 1446 plants (96.4%) successfully established. Thus a potting mixture misting at 4 hours interval was the most suitable factor to achieve higher percentage of establishment of the plants. The high survival rate of *in vitro* grown plants of *Plectranthus barbatus* indicates that this procedure could be easily adopted for large-scale cultivation.

The micropropagated plants cultivated in the field for 6 months showed uniform morphology and growth indicated the stability of the plants free of visible abnormalities and the same opinion was expressed by Bhattacharya and Bhattacharya (2001) and Reddy *et al*. (2001) in this species. After 6 months of growth under uniform conditions in the field, marked difference was observed between plants raised through conventional stem cuttings and nodal explant cultures. The enhanced performance of these plants could only be related to their health and conditions under which they were nourished (Wysokinska, 1993).

FORSKOLIN IN *IN VITRO* RAISED PLANTS

Raising high yielding material for an adequate supply of raw materials for commercial production to fulfil demand is one of the key goals in creating micropropagation protocols. Therefore, it was crucial to estimate the forskolin content of in vitro produced material. After six months of growth, in vitro and field-grown plants were harvested, and the whole output of tuberous roots was shade-dried and pulverised. These samples' forskolin content was determined using the technique recommended by Inamdar et al. (1984), and it was compared to samples taken from CF7 and CF9. The forskolin content estimated was about 1.5% and 1.9% on a dry weight basis respectively (Figures: 2A & B).

The potential of shoot cultures and plantlets *in vitro* as a source of forskolin has been demonstrated (Sen and Sharma, 1991b; Sen *et al.,* 1992). More or less differentiated axenic cultures, namely transformed and untransformed root cultures (Krombholz *et al.,* 1992; Mukherjee *et al.,* 1996; Mersinger *et al.,* 1988), shoot cultures (Sen and Sharma, 1991b; Suryanarayan and Pai,1998; Bhattacharya and Bhattacharya, 2001) and shoot – forming callus cultures (Reddy *et al.,* 2001; Balasubramanya *et al*.,2012) were reported to produce forskolin. On the other hand, there is no literature currently accessible on in vitro multiplication and the screening of high producing lines. A schematic and methodical approach was used in the current experiment to produce a large number of planting stocks with a high forskolin yield (1.9%). Plantlets are true – to – type for forskolin content with regard to growth and biomass. The result is very worth promising. The root biomass of *in vitro* raised plants was compared with vegetatively propagated plants, which were raised from the same selected mother plants i.e., CF7 and CF9. The forskolin content of these vegetatively propagated CF7 and CF9 are 1.0% and 1.2%. The regenerated plantlets did not show detectable phenotypic variation.

In vitro raised plants were much healthier with broader leaves. The root number and biomass is relatively higher (Table: 10) and analysis of results of the forskolin samples were given in (Table: 11A&B). The availability of growth regulators and ongoing provision of a well-balanced diet may be responsible for this. This demonstrates unequivocally the benefit of micropropagation over traditional propagation.

TABLE 10: Comparative analysis of *in vitro* grown plants obtained in a period of 6months raised from each selected mother plant of *Plectranthus barbatus* (syn.: *Coleus forskohlii*)

Clone	Content Offorskolin	Vegetative Progagation			Propagation throughtissue culture		
		No. of Plants obtained	Root Biomass (on dry weight basis/Kg)	Forskolin content(on dry weight)	No. of Plants obtained	Root Biomass (on dry weight basis/Kg)	Forskolin content(on dry weight)
CF 7	1.5	462	0.076	1.0	1508	0.089	1.5
CF 9	1.9	493	0.082	1.2	1592	0.098	1.9

Table 11A: Analysis of results for the forskolin samples as per the chromatograms for the *in vitro* grown *Plectranthus barbatus* (syn.: *Coleus forskohlii*) plants

Forskolin sample	**RT**	**Area**	**Height**	**Assay**
Standard	11.43	2881.7220	96.8025	95%
In vitro grownCF7	11.44	858.9346	23.2003	1.5%
In vitro grownCF9	11.44	809.5634	21.5901	1.9%

Table 11B: Analysis of results for the forskolin samples as per the chromatograms for the vegetatively propagated *Plectranthus barbatus* (syn.: *Coleus forskohlii*) plants

Forskolin sample	**RT**	**Area**	**Height**	**Assay**
Standard	11.43	2881.7220	96.8025	95%
vegetatively propagated CF7	11.45	858.0432	20.2003	1.0%
vegetatively propagated CF9	11.44	836.6439	21.4864	1.2%

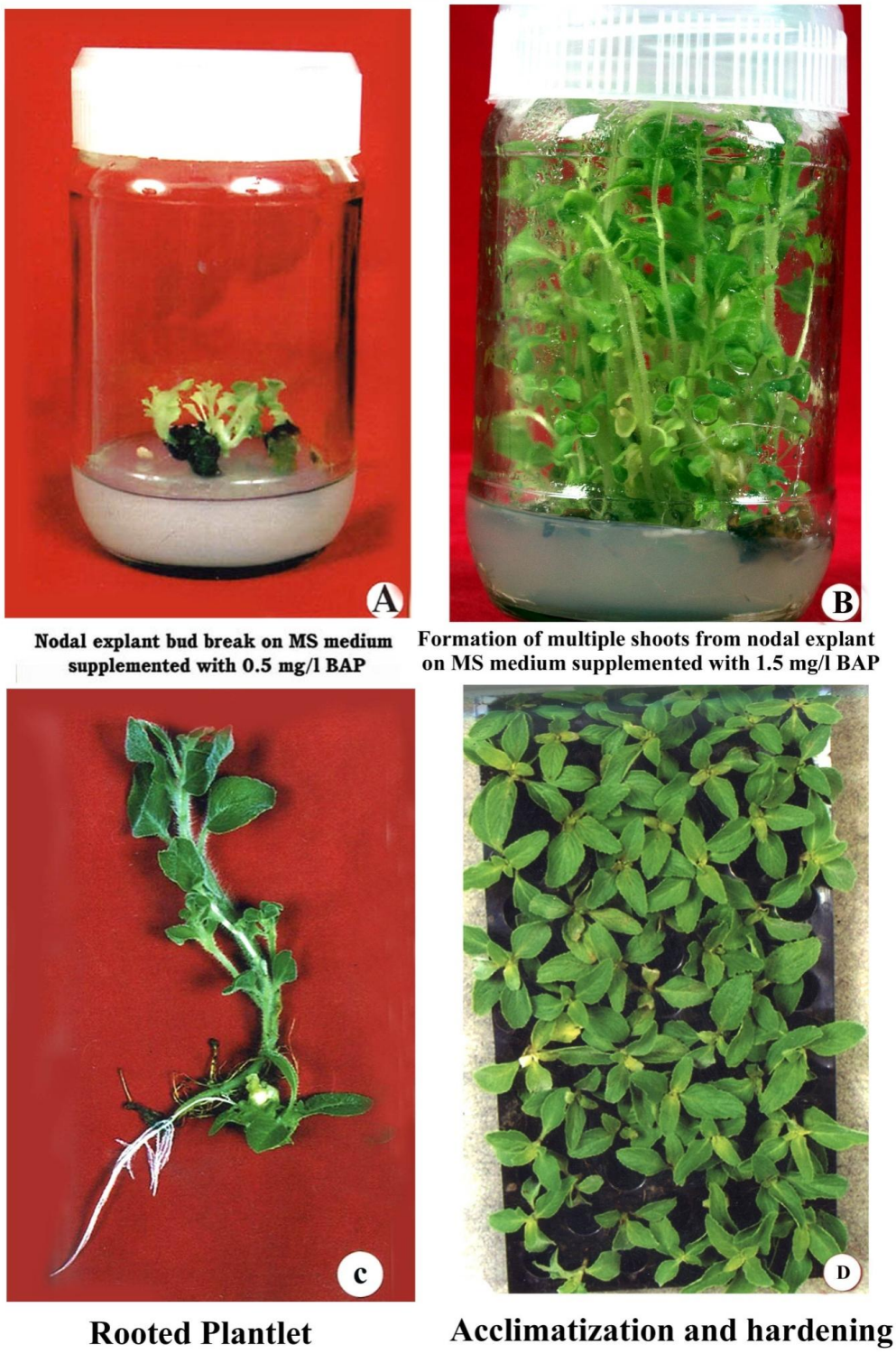
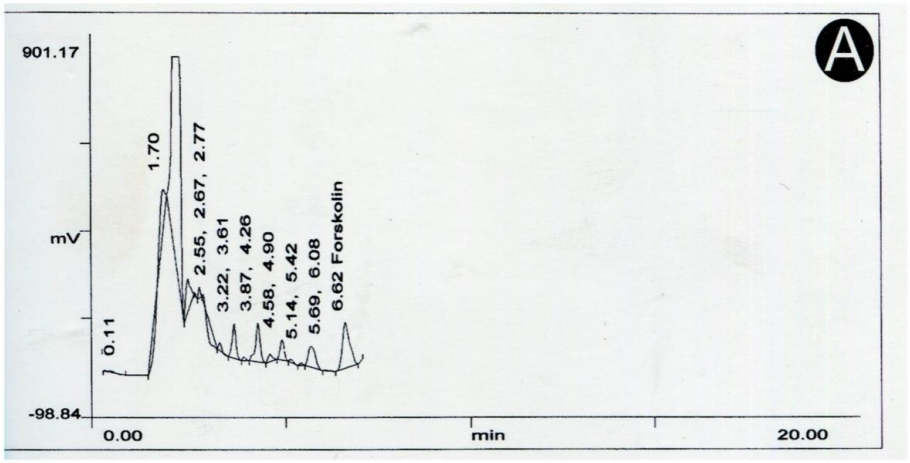
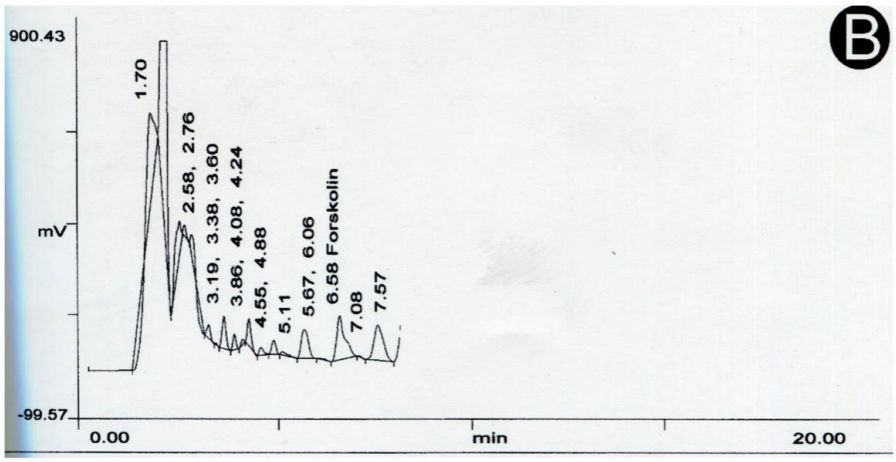

A
B
Nodal explant bud break on MS medium supplemented with 0.5 mg/l BAP
Formation of multiple shoots from nodal explant on MS medium supplemented with 1.5 mg/l BAP
c
D
Rooted Plantlet
Acclimatization and hardening

Figure 1



HPLC chromatogram for the naturally growing plants with 1.9%



HPLC chromatogram for the invitro grown plants with 1.7%

Figure 2

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