MICROPROPAGATION OF ENDANGER PLANT BRYOPHYLLUM PINNATUM WITH ITS QUANTITATIVE SCREENING AND THIN LAYER CHROMATOGRAPHY.

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Abstarct: To establish a standardized protocol for in vitro propagation of endangered plant *Bryophyllum pinnatum* using leaf explants, for long term conservation with its quantitative, quantitative screening and thin layer chromatography analysis to make the plant commercially available. The highest rate of shoot proliferation (85%) and number of shoots per explant 10 was obtained on media BAP 2.5mg/l + Kn0.5mg/l with average shoot length 2.0±0.66. The maximum shoot regeneration showed 80±1.45 in BAP 1.5mg/l + NAA 0.5mg/l + IBA 0.5mg/l .The largest effect on root formation occurred in BAP 0.5mg/l+ IBA 0.5mg/l +NAA 2.0 mg/l and 45±0.0 percentage of rooted explants with 9±11.5 number of roots produced. The plants were maintained at about 70% relative humidity in the greenhouse with 75% shading with 98% of survival rate was achieved after 6 weeks. The phytochemical screening of the Ethanolic and Aqueous leaf extracts of *Bryophyllum pinnatum* showed the presence of alkaloids, flavonoids, carbohydrates, saponins, terpinoids, oxalate, streroids, tannins, glycosides, protein and phenolic compounds with presence of rich amount of chlorophyll (0.7236µg/ml), Free amino acid (234.96µg/ml), protein (24.402µg/ml) and carbohydrate (513.90µg/ml). Thin layer chromatography showed the presence of amino acids (0.77cm) and cardiac glycosides (0.88cm) from leaf extracts.

Keywords:-Micropropagation, Growth regulators, Ethanolic and Aqueous leaf extracts, Phytochemical screening, Thin layer chromatography.

I. INTRODUCTION:

Plants are essential source of medicines and play a key role in world health (**Constebel, 2000**). Among them ***Bryophillum pinnatum*** is one. Genus *Bryophyllum* of the family Crassulaceae is a valuable medicinal and ornamental plant. These plants are cultivated as ornamental house plants and rock or succulent garden plant (**Kulka, 2006**). It has a definite ornamental value (**Souza-Brito etal, 1993**) because of its beautiful inflorescence. It is a popular house plant and become naturalized in temperate region of Asia, the Pasific,Caribbean, Austrelia, Newzeland, West Indies, Mascarenes, Galapagoes, Polynesia And

Hawai (**Zamore etal, 1998).** In South Eastern Nigeria, this herb is used to facilitate the dropping of the placenta of a newly born baby (**Daziel, 1995**). In India, many indigenous plants are used in herbal medicine to cure diseases and heal injuries. So this plant is used

in ethno medicine for treatment of earache, burns, abscesses, ulcer, insect bites, diarrhea and

Lithiasis (**Chopra et al, 1956**).

Tissue culture methods are recognized as excellent tools for medicinal plants propagation, allowing the production of pathogen-free plants, under controlled conditions and with independence of climatic factors. Among them, micropropagation protocol is a necessary step for conservation and development **(Sajc *et al.,* 2000)**. It has many advantages over conventional methods of propagation, which suffers from several limitations.

These plants are also used by the tribals of Kerela for treating Cancer symptoms (**Paranjpe, 2005), (Mathew, Unithan 1992).** This plant induce the typical symptoms of cardiac poising but repeted small doses also cause cotyledons, an intoxication affecting the nervous and muscular systems of small animals, particularly sheep in the karoo area of South Africa ***Bryophillum pinnatum*** is rich in alkaloids, terpines, glycosides, flavonoids, steroids and lipids (**Marriage and Wilson, 1971**). The leaves contains a group of chemicals called bufadianelids which are very active (**Franke etal, 1987**) and very similar in structure and activity as two other cardiac glycosides digoxin and digotoxin which possesses antibacterial, antitumorus cancer preventive and insecticidal actions **(Chang etal, 1989**).

Chromatography techniques are extensively used in bio analysis for the separation, isolation and purification of drugs and their metabolites. Due to its medicinal importance this plant is overexploited and there is a decline in the population of ***Bryophillum pinnatum***.

The aim of this study is invitro micropropagation through various growth hormones used and screening of phyto compounds through TLC method.

II. MATERIAL & METHODS:

2.1. Collection of Explants:

Actively growing, healthy and disease free young shoots with green leaves of ***Bryophillum pinattum*** collected from the green house of Tectona Biotech Resource Centre (TBRC), Bhubaneswar, Odisha, India.

2.2. Surface Sterilization:

The younger and clean leaves of Bryophillum pinnatum have been washed with 2% (v/v) detergent Teepol (Qualigen, India) and rinsed numerous times with jogging tap water. The explants were surface sterilized in 0.1% (w/v) aqueous mercury chloride for 3-4 minutes accompanied by means of 4 washing with sterile distilled water.

2.3. Inoculation of Explants, Shoot bud Initiation & Multiplication:

As it's an instantaneous micro propagation procedure all sterilized leaves explants had been belittled and dipped in the one of a kind concentration of PGRs, BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/l) with particular extent of Kin (0.5mg/l) at the MS supplemented medium. The cultures were maintained at 25±2ºC less than 16h photoperiod light at cool, white fluorescent lamps. After remark for 4-5 weeks the shoot buds were transferred without delay again to MS medium supplemented with various concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5mg/l) and Kn (0.5mg/l) respectively in mixture for shoot regeneration and multiplication. The cultures had been maintained with the aid of normal subcultures at 1-2 week intervals on fresh medium with the same compositions.

2.4. Root Induction & Acclimatization:

All shoots were transferred to rooting medium, which consisted of MS supplemented with exceptional concentrations of IBA (0.5-2.5 mg/l and NAA (0.5, 1.0mg/l) and mixture of IBA (0.5 - 2.5mg/l), NAA (0.5-2.5 mg/l) and BAP (0.5-2.5 mg/l). All the cultures had been incubated in culture room at 25 ± 2°C, light depth (3000lux) with a photoperiod of 16 hours with 60-70% relative humidity. The cultures were monitored and the statistics have been recorded at each week interval. After 15-20 days of way of life, the sufficient rooted plantlets had been dipped in bavistin solution for approx 2-3 mins and planted cautiously inside the poly luggage containing soil mixtures (organic soil blended with lawn soil 1:1). They were maintained at about 70% relative humidity in the greenhouse with 75% shading to supply newer leaves / roots. Plants are maintained underneath shade with controlled temperature and humidity to produce more modern leaves/roots. They are ready to be transferred in open nursery.

III. STATISTICAL ANALYSIS:

Data were collected on shoot and root regeneration. The experiments were laid out in completely randomized design (CRD). Each treatment was replicated thrice and 10 test tubes were used per replication. The data collected was analyzed by SPSS software (Version 13.00) and the means were compared by one way ANOVA.

IV. BIOCHEMICAL ANALYSIS :

4.1 Chlorophyll Estimation by Acetone Method:

Chlorophyll is soluble in acetone. When the sample is macerated in acetone, chlorophyll gets dissolved in it. The optical density of the extract is measured at 663 and 645 nm wavelengths using spectrophotometer because at these wavelengths, maximum absorption of chlorophyll "a" and "b" takes place respectively. Using the absorption coefficients, the amount of chlorophyll is calculated (**Arnon, 1949**).

4.1.1. Extraction of chlorophyll:

1 gram of finely cut fresh leaves were taken and ground with 20 – 40ml of 80% acetone and then centrifuged at 5000 –10000 rpm for 5mins. The supernatant was transferred to a flask and the procedure was repeated till the residue becomes colorless. The final volume in the flask was made up to 100ml by addition of 80% acetone. The absorbance of the solution was taken at 645nm and 663nm against the solvent (80% acetone) as blank.

The concentrations of chlorophyll a, chlorophyll b and total chlorophyll were calculated using the following equation:

$$(\text{Mg})\ \text{chlorophyll a per (gm) tissue} = \{12.7(A_{663}) - 2.69\ (A_{645})\} \times \{V \div (1000 \times W)\}$$

Chlorophyll b per (gm) tissue = $\{22.9(A_{645}) - 4.68 (A_{663})\} \times \{V \div (1000 \times W)\}$

Total chlorophyll per (gm) tissue= Chl a + Chl b

Where, A = absorbance at specific wavelengths, V = final volume of chlorophyll extract,

W = fresh weigh of tissue extracted

4.2. Protein estimation by Lowry's method:

The phenolic group of tyrosine and trytophan residues (amino acid) in a protein will produce a blue purple color complex , with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different proteins. Most proteins estimation techniques use Bovin Serum Albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability. The method is sensitive down to about10 µg/ml and is probably the most widely used protein assay despite its being only a relative method, subject to interference from Tris buffer, EDTA, nonionic and cationic detergents, carbohydrate, lipids and some salts. The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9 to 10.5 is essential.

4.2.1. Sample preparation:

The plant sample was prepared by crushing fresh leaves (1mg leaves) per 1ml distilled water. Different concentrations of BSA (0.2, 0.4, 0.6, 0.8, 1ml) and plant leaf sample (0.2, 0.5, 1 ml) were taken in different test tubes with replications and volumes were made up to 1ml using distilled water. The test tube with 1 ml distilled water was served as blank.

4.2.2. Estimation of protein:

5 ml of Reagent I was added to each test tube and incubated for 10 minutes. After incubation, 0.5 ml of reagent II was added to each test tube and mixed properly using vertex mixture and then left for incubation for 30 minutes. After 30 minutes of incubation the absorbance of each test tube was measured at 660 nm and the standard graph was plotted of BSA. The amount of protein present in the leave sample was estimated from the standard graph.

4.3. Estimation of Carbohydrate by Anthrone Method:

Carbohydrate is first hydrolyzed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloured product with absorption maximum at 630 nm. Carbohydrate content was calculated by using method by **Ashwell (1957**).

4.3.1. Sample Preparation:

100 mg of the sample was weighed and hydrolyzed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cooled to room temperature. Then it was neutralized with solid sodium carbonate until the effervescence ceases. The volume was made up to 100 ml and centrifuge at 10,000rpm for 20 mins. The supernatant was collected and 0.5 and 1 ml aliquots were taken for analysis.

4.3.2. Estimation process:

The standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard.'0' serves as blank. The volume was made up to 1 ml in all the tubes including the sample tubes by adding distilled water. Then 4 ml of anthrone reagent was added and was heated for eight minutes in a boiling water bath. Cooled rapidly and read the green to dark green colour at 630 nm. A standard graph was drawn by plotting

concentration of the standard on the X axis versus absorbance on the Y axis. From the graph the amount of carbohydrate present in the sample tube was calculated.

4.4. Thin Layer Chromatography:

The plant extract was subjected to TLC to confirm the presence of amino acid and Cardiac glycosides. These plates were kept in oven at 50º - 60º C for 15 – 20 minutes of activation. The Previously prepared solvent extract of *Bryophyllum pinnatum in vivo* leaves, roots, callus and regenerated leaves were used for TLC. 15 μ l of each extracts were loaded on the analytical plate (2.5 cm above from the bottom) and dried in air for thirty minutes. The spotted plates were kept in a previously saturated developing chambers containing mobile phase, ethyl acetate: methanol: water in the ratio 10:2:1 and allowed to run 3/4th of the height of the prepared plates. In which solvent was the mobile phase. The different bands of chromatograms were observed under UV light and Iodine stain. Chloroform was used as a spraying reagent for detection of Cardiac glycosides and iodine stain is used for amino acid detection. The Rf values were calculated by using the formula:

$$Rf = \frac{\text{Distance travelled by extract}}{\text{Distance travelled by solvent}}$$

V. RESULT AND DISCUSSION:

5.1. Clonal Propagation:

5.1.1. Shoot Initiation

An effective protocol was developed for the *in vitro* clonal propagation of *Bryophyllum pinnatum*. Although it was a direct regeneration technique, leaf explants were cultured on MS medium supplemented with different concentration of BAP and Kn respectively. But the best result was observed in BAP 1.0mg/l + Kn 0.5mg/l with 70% of shoot initiation within 15-20 days and average shoot length were 1.5±0.15 (Table -1). A cytokinin supplement to MS was essential to induce shoot proliferation. A combination of cytokinin and auxin improves the percentage of shoot regeneration as well as the shoot number and shoot length. *Bryophyllum pinnatum* produced 2.8 times more shoots in 10-6M BAP as comparedto water (**Duen and Riserberg**).

Table 1: Effect of PGRs on shoot induction of *Bryophyllum pinnatum:*

Sl No	Concentration of PGR	No of shoots per plant	Percentage of Shoot Initiation	Average shoot length
1	BAP0.5+Kn0.5	1-2	64%	1.2±0.20
2	BAP1.0+ Kn0.5	1	70%	1.5±0.15
3	BAP1.5+Kn0.5	2	68%	2.2±0.52
4	BAP2.0+Kn0.5	1-2	60%	1.5±0.32
5	BAP2.5+Kn0.5	1	48%	2.0±0.22
6	BAP3.0+Kn0.5	1	40%	1.0±0.10

5.1.2. Multiplication:

Different configuration of BAP supplemented with Kn (0.5mg/l) added with MS medium. In which the highest rate of shoot proliferation was 85% followed by 10 numbers of shoots per explants on the media supplemented with BAP 2.5mg/l + Kn 0.5mg/l.(**Table-2**). On the other hand, least shoots were recorded in MS medium that contained BAP 0.5mg/l+ Kn 0.5mg/l and number of shoot per explants were 6.

S. Baghel studied High frequency of shoot regeneration was achieved on MS medium with BAP (1 mgl).

Table 2: Effect of PGR on shoot multiplication in *Bryophyllum pinnatum*

Sl No	Concentration of PGR	No of shoots per plant	Percentage of Shoot Initiation	Average shoot length
1	BAP0.5+Kn0.5	6	50%	1.7±0.80
2	BAP1.0+ Kn0.5	9	72%	2.8±0.23
3	BAP1.5+Kn0.5	6	69%	2.2±0.18
4	BAP2.0+Kn0.5	8	70%	1.5±0.35
5	BAP2.5+Kn0.5	10	85%	2.0±0.66
6	BAP3.0+Kn0.5	8	80%	2.0±0.58

5.1.3. Rooting :

Elongated shoots were excised from parent culture and transfer onto full strength basal MS medium with growth regulators. Inclusion of BAP (0.5–2.5 mg/l) , NAA and IBA (0.5 mg/l) with 2 % sucrose induced the rooting within 3 weeks of culture. The highest percentage of rooting was observed 32% with MS medium supplemented with BAP (1.5 mg/L) in combination with NAA (0.5mg/l) and IBA (0.5mg/l) with 5 no. of roots and with maximum 6.3 cm length in 22 days. Hakan study show the auxin group hormones (IAA, IBA, and NAA) do not have an apparent effect on rooting percentage, these hormones were detected to affect the morphological characteristics of the newly generated plants, especially root generation. Different concentrations of BAP, IBA and NAA had highly significant effect on the shoot and root regeneration.

Table 3: Effects of PGRs on Rooting

<table>
<tr><th rowspan="2">Sl No</th><th colspan="3">Concentration of PGRs (Mg/l)</th><th rowspan="2">Number of roots</th><th rowspan="2">Root length (cm)</th><th rowspan="2">Percent of rooting (Mean ±SE)*</th></tr>
<tr><th>BAP</th><th>NAA</th><th>IBA</th></tr>
<tr><td>1</td><td>0.5</td><td rowspan="3">0.5</td><td rowspan="3">0.5</td><td>3</td><td>2.02 ±0.46</td><td>10±0.66</td></tr>
<tr><td>2</td><td>1.0</td><td>4</td><td>3.12±0.93</td><td>20±0.33</td></tr>
<tr><td>3</td><td>1.5</td><td>5</td><td>4.86±0.13</td><td>32±1.52</td></tr>
</table>

4	2.0			3	3.25±0.02	25±2.33
5	2.5			2	2.15±0.05	10±0.57

5.1.4. Acclimatization & Hardening:

The acclimatization of rooted plants in ex vitro conditions was carried out with the plants bearing well-developed roots transferred to small pots containing soil mixtures (organic soil mixed with garden soil 1:1). They were maintained at about 70% relative humidity in the greenhouse with 75% shading. A survival rate 98% was achieved after 6 weeks. About 80 % of the plantlets established within 15 days of transfer. The plants grew well and attained 4–5 cm height within 1 months of transfer

Fig 1: A- Initial culture, B- Shoot induction, Multiplication, C- Rooting, D- Hardening

5.2. Biochemical analysis:

5.2.1. Estimation of Protein:

Protein was estimated by Lowry's method. Biochemical studies on leaf extracts of *B.pinnatum* revealed from the standard curve (fig-2) of BSA that approx 24.402µ g per ml of protein present in 1gram of leaves sample(Table-4) .

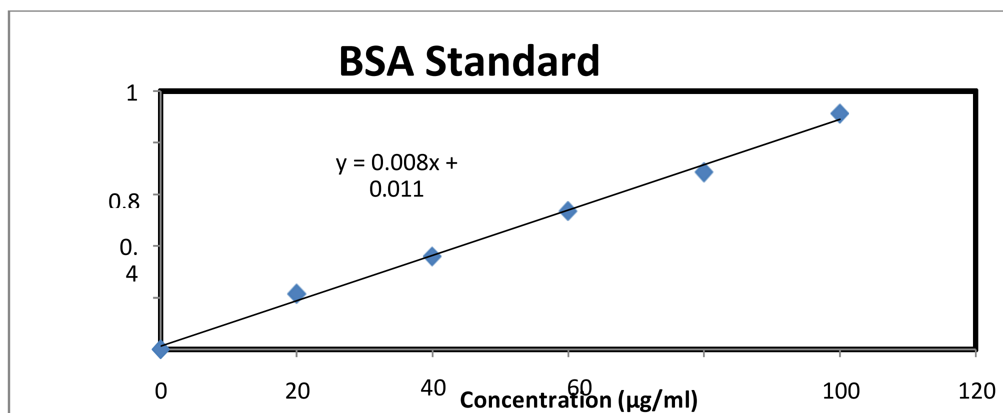

Fig 2 : Standard Graph For protein estimation

Sl no		Volume of protein(ml)	Conc. Of protrin (µg/ml)	Volume of distilled water (ml)	Volume of reagent 1(ml)		Volume of reagent 2 (ml)		Od at 660nm
1	Blank	0	0	1	5	Incubation For 10 m i n	0.5	Incubation For 30 m i n	0
2	Bsa	0.2	20	0.80	5		0.5		0.215
3		0.4	40	0.60	5		0.5		0.359
4		0.6	60	0.40	5		0.5		0.536
5		0.8	80	0.20	5		0.5		0.686
6		1	100	0	5		0.5		0.913
7	B. Pinnatum leaf	0.5	Unknown	0.80	5		0.5		0.117
8		1.0	Unknown	0.50	5		0.5		0.218

Table 4 : Total protein contents by Lowry Method:

5.3. Estimation of carbohydrate:

From the standard graph of glucose (Fig-3) the amount of carbohydrate present in the *B. pinnatum* fresh leaf extract was estimated to be approximately 513.90 µg/ml (Table-5).

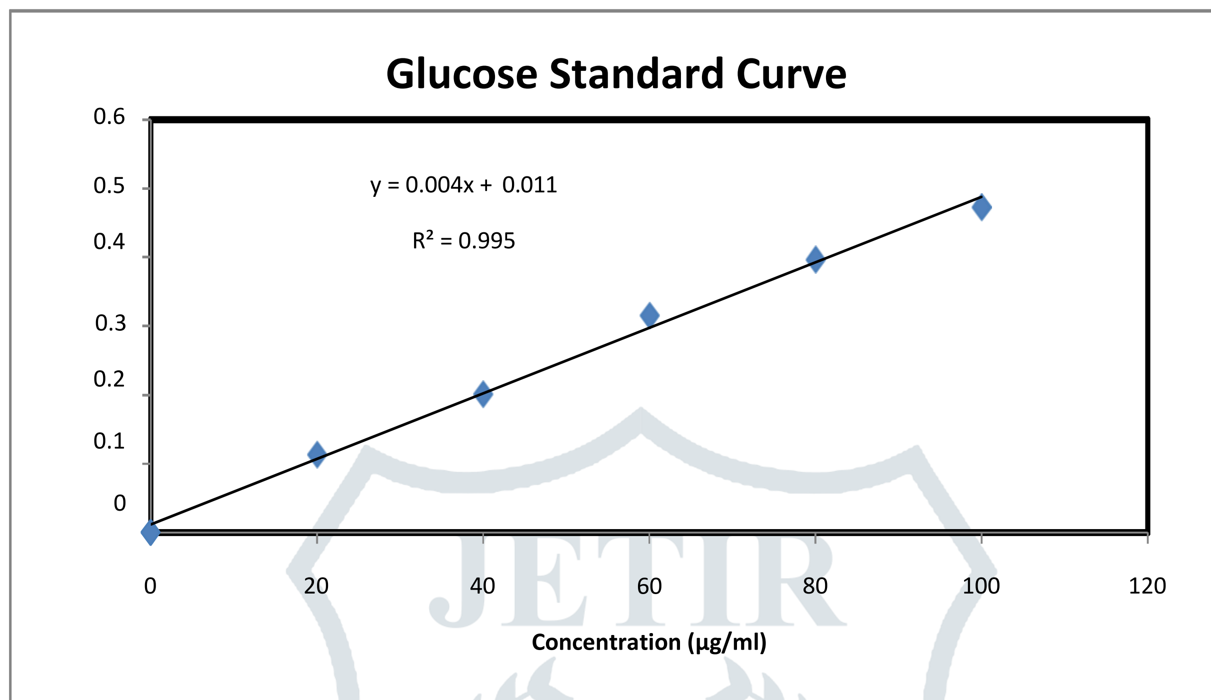

Fig- 3 Standard Graph of Glucose

Sl no		volume carbohydrate extracted (ml)	volume of distilled water (ml)	conc. of carbohydrate µg/ml	volume of anthron reagent (ml)		abs. at 630nm
1	Blank	0	1	0	4	incubation for 8 min	0
2	Glucose	0.2	0.8	20	4		0.113
3		0.4	0.6	40	4		0.201
4		0.6	0.4	60	4		0.315
5		0.8	0.2	80	4		0.397
6		1	0	100	4		0.473
7	Sample	0.5	0.5	Unknown	4		1.354
8		1	0	Unknown	4		2.698

Table 5: **Estimation of carbohydrate content**

5.4. Estimation of chlorophyll:

The total chlorophyll content was estimated using Arnon method. In the Spectrophotometric analysis, *B.pinnatum* leaf sample showed different variance in different absorbance and the result is shown in (**Table 6**). The absorbance Chlorophyll is measured as 0.319 at 645 and as 0.203 at 663. Based on these data the total chlorophyll content of the leaf extract comes to be 0.206 mg per gram sample.

Table: 6 Estimation of chlorophyll content in *bryphyllum pinnatum*

Sl. No.	Biochemical	Bryophyllum pinnatum	Wavelength in nm	Reference	Optical density
1	Chlorophyll	1	645	80% acetone	0.391
			663		0.203

5.5.Thin Layer Chromatography:

The TLC profiling of *in vitro callus, Regenerate plant,* and *in-vivo* leaf and root extracts in the methanol solvent system confirms the presence of amino acid and Cardiac glycoside. Ethyl acetate: methanol: water (10:2:1) was used as mobile phase. Among them in UV light and iodine stains two bands were clearly shown in the in vitro leaf extract. The Rf values were 0.88cm and 0.77cm respectively for amino acid and cardiac glycosides (Table 7). In regeneration plant extract, callus extract, root extract negligible bands were observed under UV light and Iodine stain.

Table: 7 TLC of *Bryophyllum pinnatum* methanol extract in mobile phase petroleum Ethyl acetate: methanol: water (10:2:1)

Pigment band	Bryophyllum pinnatum Explnat	Distance of pigment migrate For amino acid	Distance of pigment migrate For cardiac glycoside	Distance of solvent migrate	Rf value For amino acid	Rf value for cardiac glycoside
1	In vitro leaf	5.3cm	6.0cm	6.8cm	0.77cm	0.88cm
2	In vitro roots	2.2	2.3	6.8cm	0.32	0.33
3	Regenerate plant	1.0	0.2	6.8cm	0.14	0.02
4	In vivo callus	1.6	1.5	6.8cm	0.23	0.22

VI. CONCLUSION:

In conclusion it was clearly observed that rapid in vitro propagation of *Bryophillum pinatum* can be obtained by proper manipulation of leaf explants and concentration of plant growth regulators. Although it was a direct micropropagation technique so leaf explants were cultured on MS medium supplemented with different concentration of BAP and Kn respectively. But the best result was observed in BAP 1.0mg/l + Kn 0.5mg/l with 70% of shoot initiation within 15-20 days with average shoot length of 1.5±0.15 c.m. The highest rate of shoot proliferation was 85% with 10 numbers of shoots per explants on the media supplemented with BAP 2.5mg/l + Kn 0.5mg/l. Elongated shoots were excised from parent culture and transfer onto full strength basal MS medium with inclusion of BAP (1.5 mg/l) , NAA (0.5 mg/l) and IBA (0.5 mg/l) with 2 % sucrose induced the rooting within 3 weeks of culture in which 32% of rooting observed with 5 numbers of roots and 6.3 cm of maximum root length. Micro propagated plantlets were hardened, acclimatized and transferred to the field. These results can be further used for clonal propagation. Quantitative estimation of phytocompounds was done for observed the amount of phytocompounds present. Quantitative phytochemical analysis revealed that the plant has rich amount of chlorophyll (0.206 mg/gm), amino acid (24.402µg/gm) , and carbohydrate (513.90µ g/ml) In TLC profiling, determination of the presence of amino acid and cardiac glycosides from leaf extract in methanol solvent system , ethyl acetate: methanol: water (10:2:1) was used as mobile phase. In UV light and iodine stains two bands were clearly shown in the in vitro leaf extract. The Rf values were 0.88cm and

0.77cm respectively for amino acid and cardiac glycosides. In regeneration plant extract, callus extract, root extract negligible bands were observed under UV light and Iodine stain.

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VIII. REFFERENCES:

1. P.B. Marriage, and D.G. Wilson. Analysis of Organic acids of Bryophyllum pinnatum. *Can. J. Biochem.* 49: 282-295(1971).

2. Kulka RG, Kamgang R, Mboumi RY, Fondjo AF, Tagne MA, Ndille GP and Yonkeu JN: Hormonal control of root development on epiphyllous plantlets of *Bryophyllum* (*Kalanchoe*) *marnierianum*: role of auxin and ethylene. J Exp Bot. 2008; 59(9):2361-70.

3. Zamora AB, Gruezo SS and Siar SV 1998: Somaclonal variation: Breeder's tool for recovering potencial cultivars in ornamentals (*Kalanchoe*). Annual Scientific Conference of the federation of Crop Science Societies of the Philippines, Cebu City (Philippines) (Received for publication 3, 2009.

4. Chopra RN, Nayar SL and Chopra IC: Glossary of Indian Medicinal Plants vol. 1. Council of Scientific and Industrial Res, New Delhi 1956; pp 330.

5. Duane FH and Riserberg LH: Hormonal regulation of epiphyllous buds release in development in *Bryophyllum calycinum*. American Journal of Botany 1983; 70(6):912-915.

6. Dalziel, J. M., and J. I. Hutchinson (1955). Flora of tropical West Africa. Crown agent for overseas Government and Administration.

7. Souza-Brito, A.R.M. and A.A. Souza-Brito. 1993. Forty years of Brazilian medicinal plant research. *Journal of Ethnophormocology*, 39(1): 53-67.

8. P. Paranjpe. Indian Medicinal Plants forgotten Healers. Chaukhamba Sanskrit Pratisthan, 2Delhi,2005,194-195.

9. P.J. Mathew, and M.C. Unithan. Search for plants having anticancer properties used by the tribals of Wyandu, Malappuram and Palghat districts of Kerala. *Indian Aryavaidan.* 6:54-60(1992).

10. T. Yamagishi, M. Haruna, X.Z. Yan, J.J. Chang, and K.H. Lee. Antitumor agents, 110, Bryophyllin B, A Novel Potent cytotoxic Bufadienolide from *Bryophyllum Pinnatu.m J. Nat. Prod.* 52(5): 1071 -1079(1989).

11. R.A. McKenzie, F.P. Franke, and P.J. Dunster. The toxicity to Cattle and Bufadienolide content of six Bryophyllum species. *Aust Vet J*. 64 (10): 298-301(1987).

12. Constabel CP, Yip L, Patton JJ and Christopher ME: Polyphenol oxidase from hybrid poplar cloning and expression in response to wounding and herbivory. Plant Physiol 2000; 124: 285–296 .

13. Sajc L, Grubisic D and Vunjan-Novakovic :. Bioreactors for plant engineering: an outlook for further research. Biochem.Eng. J. 2000; 4: 89-99.

14. Arnon DI (Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiol , 1949 :24: 1–15.

15. Ashwell, G. Colorimetric analysis of sugars. Methods Enzymol. 1957: 3:73–105