SOMATIC EMBRYOGENESIS OF DREPANOSTACHYUM FALCATUM AN IMPORTANT HILL BAMBOO-A RAPID MEANS OF MICROPROPAGATION

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Why Bamboo's

- Due to steep increase in market demand for bamboo's. Naturally occurring stands are being harvested with minimal conservation or reforestation programmes.
- Also problems associated with bamboos like seeding and availability of limited number of propagules leads to a situation where there is going to be severe shortage of Bamboos.

DREPANOSTACHYUM FALCATUM

SYSTEMATIC POSITION

Division : Angiosperms
Sub – Division : Monocotyledons
Family : Poaceae (Gramineae)
Sub-family : Bambusoideae
Genus : Drepanostachyum
Species : *falcatum*

***Drepanostachyum falcatum* is one of the economically important bamboo commonly known as hill bamboo or Ringal, Gol Ringal. It is a shrubby species with the flowering and seedling cycle of 28-30 years.**

DISTRIBUTION

Hills of North – Western India
Himachal Pradesh, Garhwal, Kumaun,
Kashmir & Western Nepal at elevations between 1200-1500m

ECONOMIC IMPORTANCE

- Basic raw material for huts, tools, utensils and other item.
- Culms much valued for fishing rods, pipes, arrows, etc
- Leaves are used for thatching, roofing, etc.
- Edible young shoots, used as a animal fodder in winter.
- Used in compounding many medicines
- As a tool for soil and water conservation
- Important indicator of disturbances

OBJECTIVES

To develop a tissue culture protocol for rapid and large scale multiplication of *Drepanostachyum falcatum* which would involve :

- **Induction of Somatic Embryogenesis.**
- **Development of Somatic Embryos.**
- **Direct regeneration of S.E. into plants**
- **Development of in vitro shoot from S.E. and *in vitro* shoot multiplication.**
- ***In vitro* rooting.**
- **Hardening and acclimatization of tissue culture raised plants.**

PROBLEMS ASSOCIATED WITH CONVENTIONAL PROPAGATION OF Hill bamboo

Limited availability of seeds due to

- Flowering after long intervals (28-30 years).
- Poor seed set
- Short viability period
- Seed sterility.
- Large-scale consumption of seeds by rodents and wild animals

Vegetative propagation by rhizomes & culm-cuttings

- Laborious and cost intensive.
- Offsets & rhizomes : Non-availability of propagules in required numbers.

REGENERATION PATHWAY

Somatic Embryogenesis

Drepanostachyum falcatum
Explants- Nodal segments with axillary bud. Harvested from mature clump.

METHODOLOGY

COLLECTION OF EXPLANT

Axillary bud proliferation : Nodal segments measuring about 2-3 cm were collected from the shoots of mother plant.

SURFACE STERILIZATION

Nodal segments were cleaned with 70% ethanol soaked cotton followed by surface sterilization with 0.1% $HgCl_2$ for 12 minutes and finally washed 3-4 times with autoclaved distilled water.

RESULTS

AXILLARY BUD INDUCTION

90-95% bud break response was recorded when nodal segments were collected during the months of Feb-March and Sept.-Oct.

Maximum bud break response of about 95% was obtained on liquid MS medium supplemented with 5 mg/l BAP. 10-12 shoots proliferated during bud break.

On hormone free medium only 30% of explant exhibited bud break.

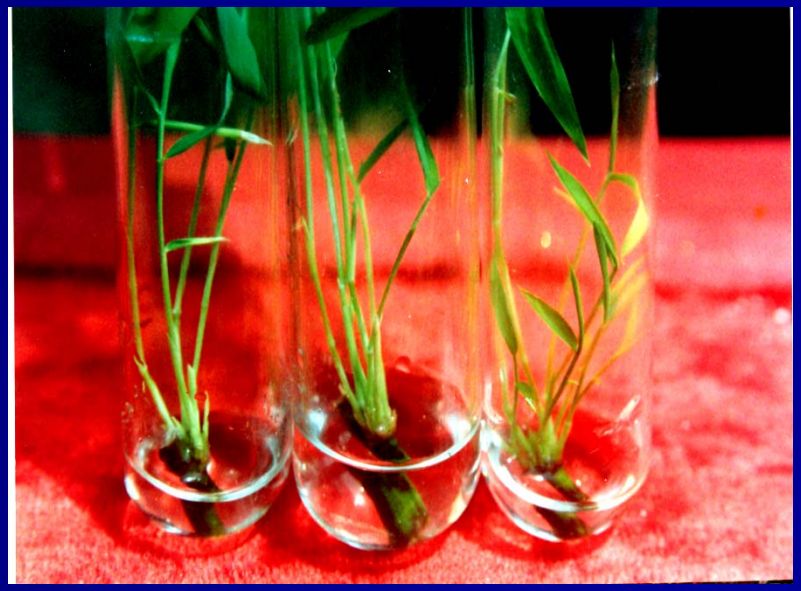


Table 1: Effect of Cytokinin (BAP) in MS medium on axillary bud induction using nodal segments. Data recorded after 4 weeks.

Hormonal Concentration BAP (mg/l)	Response %	Average shoot number	Average shoot length (cm)
0.0	33.33 ± 0.57	1.25 ± 0.13	1.61 ± 0.14
1.0	46.14 ± 0.34	1.83 ± 0.21	1.80 ± 0.13
3.0	70.83 ± 0.58	4.75 ± 0.27	2.48 ± 0.14
5.0	95.83 ± 0.58	12.08 ± 0.19	2.08 ± 0.19
7.0	87.50 ± 0.60	9.08 ± 0.23	2.05 ± 0.10
9.0	75.00 ± 0.60	7.00 ± 0.21	1.67 ± 0.10
11.0	58.50 ± 0.44	4.75 ± 0.28	0.93 ± 0.08
Significance	***	***	***
CD	1.61	0.64	0.38

***-Significance at 0.1%

± Values represent the Standard deviation

SOMATIC EMBRYOGENESIS

Explants - Nodal segments

- Leaf sheath base (harvested from aseptically grown shoot cultures)

Induction of embryogenic callus

Multiplication of somatic embryogenic callus

Maturation of somatic embryos

Germination of somatic embryos

Micropropagation of shoots

***In vitro* rooting of micro propagated shoots**

Hardening and acclimatization

Field transfer of plantlets

Five parameters were used to assess embryogenic response as reported by Lazzeri *et al.* (1987).

$$\text{Embryogenic frequency} = \frac{\text{Number of embryogenic cultures}}{\text{Total cultures inoculated}}$$

$$\text{Callus Multiplication rate} = \frac{\text{Final fresh weight of callus}}{\text{Initial weight of callus inoculated}}$$

Mean embryo number = Mean number of somatic embryos per embryogenic callus

Embryogenic efficiency = Embryogenesis frequency x Mean embryo number

$$\text{Germination frequency} = \frac{\text{Number of embryos that germinated}}{\text{Total number of embryos cultured}}$$

Somatic Embryogenesis

Induction of callus

20µM 2,4-D induced 70% callus in leaf sheath base and 85% callus in nodal segments and is the best callus induction hormone.

MS medium was found to be suitable for callus induction as compared to B5 medium

Morphologically three types of callus were produced, friable, compact nodular and mucilaginous. Only compact callus is embryogenic in response.

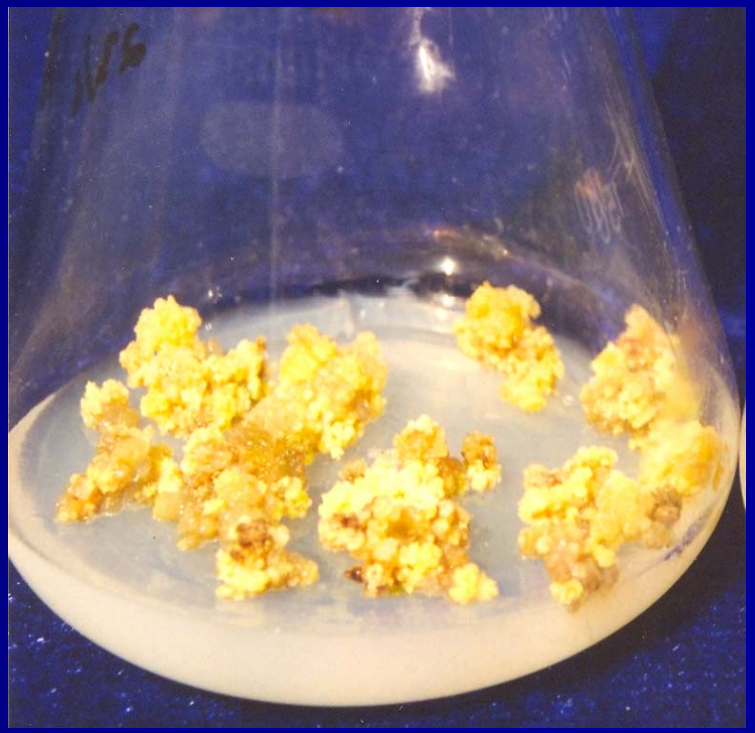


Table 16: Effect of hormone (2, 4-D) on induction of callus from leaf base and nodal segments on MS medium. Data recorded after 4 weeks.

Hormonal concentration 2,4-D (µM)	Response (%) (Leaf base)	Response (%) (Nodal segments)	Fresh Callus wt. (gm) (Nodal segments)	Fresh Callus wt. (gm) (Leaf base)
Control	-	-	-	-
10	60.00 ± 0.52	65.00 ± 0.23	0.0258 ± 0.0013	0.0156 ± 0.0014
20	70.00 ± 0.58	85.00 ± 0.29	0.0302 ± 0.0009	0.0196 ± 0.0007
30	65.00 ± 0.29	65.00 ± 0.29	0.0270 ± 0.0008	0.0186 ± 0.0010
40	49.90 ± 0.19	55.00 ± 0.17	0.0156 ± 0.0009	0.0178 ± 0.0009
50	40.00 ± 0.23	45.00 ± 0.17	0.0090 ± 0.0007	0.0120 ± 0.0008
Significance	***	***	***	***
CD	1.24	0.74	0.003	0.004

***-Significance at 0.1%

± Values represent the Standard deviation

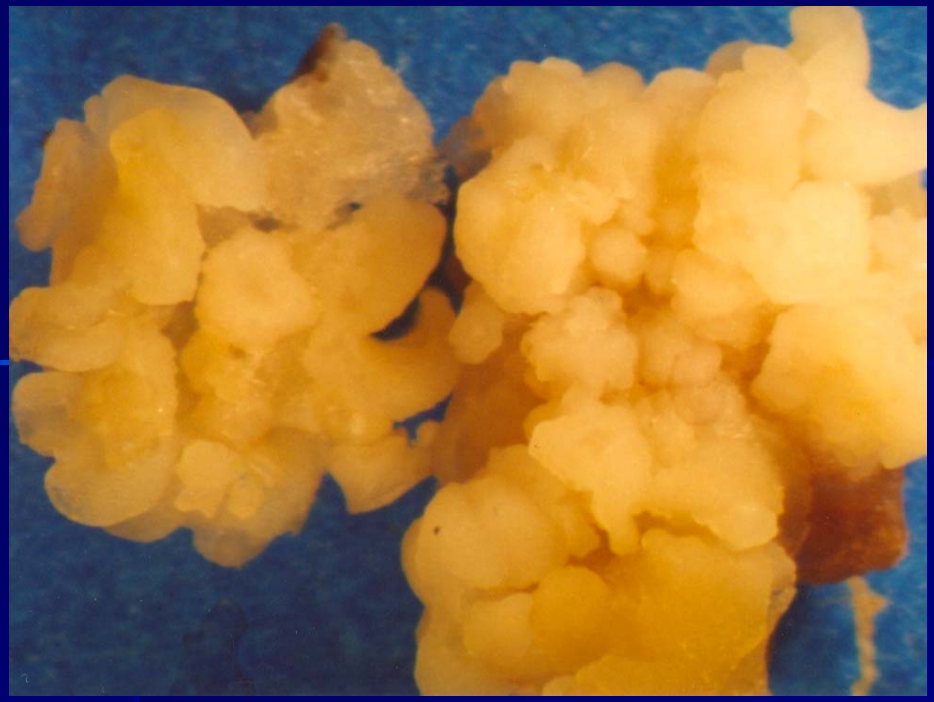
Multiplication of embryogenic callus

For multiplication of callus, MS medium supplemented with 10 µM 2,4-D and 0.88 µM BAP was found to be highly efficient with 18-20 embryos per culture. On the multiplication medium globular and scutellar somatic embryos were formed within 12 weeks

Subculture duration of 4-6weeks is found to be optimum as beyond this non embryogenic callus increases in quantity.

2% sucrose was found to be the best for embryogenesis in which large number of globular stage embryos were developed.

MS medium was better than B5 medium at all salt strengths. Full strength MS medium showed high efficiency value of 18.4 embryos per culture.



Microscopic view of embryogenic callus

Fig1: Production of embryogenic callus.
Fig2: Development of globular embryoids in embryogenic callus after 8-10 weeks of culture.
Fig3: Proliferating embryogenic callus showing organization of globular, scutellar embryoids after 12 weeks of culture.

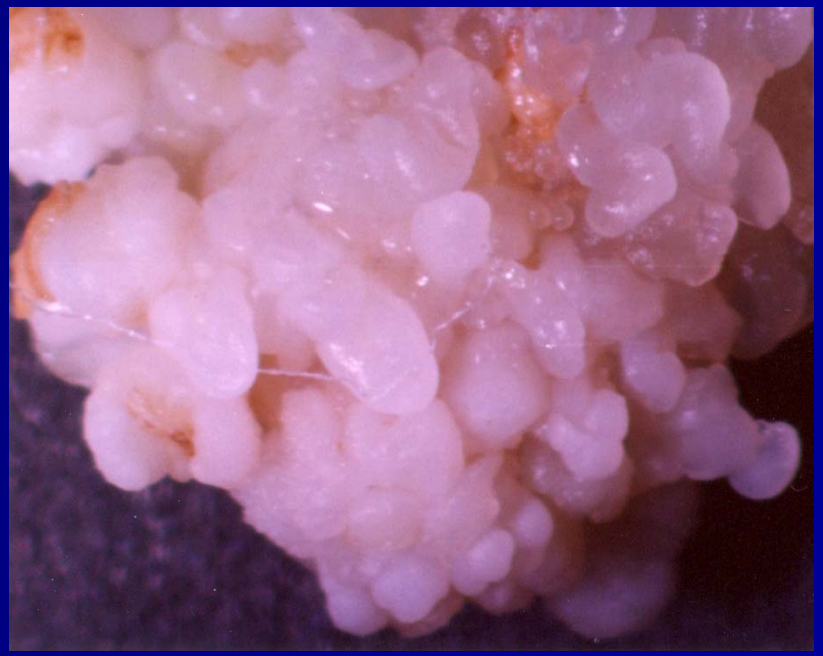


Table 19: Effect of phytohormonal combination (2, 4-D + BAP) on multiplication of embryogenic callus on MS medium. 50 mg of fresh wt. of callus was inoculated initially. Data recorded after 4 weeks.

Medium with phytohormone 2,4-D (µM)	BAP (µM)	Embryogenic response (%)	Fresh callus wt. (gm)	Callus multiplication rate (folds)	Mean no. of embryos	Embryogenic efficiency
10	0.66	77.00 ± 0.58	0.037 ± 0.003	2.616 ± 0.036	10.8 ± 0.583	8.316 ± 0.449
10	0.88	90.00 ± 0.58	0.142 ± 0.002	2.832 ± 0.033	18.6 ± 0.678	16.740 ± 0.610
10	1.10	75.03 ± 0.32	0.098 ± 0.002	1.964 ± 0.033	12.0 ± 0.548	9.000 ± 0.411
20	0.66	73.00 ± 0.29	0.121 ± 0.001	2.412 ± 0.028	11.0 ± 0.707	8.030 ± 0.516
20	0.88	71.00 ± 0.29	0.103 ± 0.002	2.056 ± 0.047	14.8 ± 1.625	10.508 ± 1.154
20	1.10	68.00 ± 0.58	0.093 ± 0.003	1.852 ± 0.064	10.2 ± 0.663	6.936 ± 0.450
30	0.66	70.00 ± 0.29	0.103 ± 0.003	2.068 ± 0.063	9.0 ± 0.707	6.300 ± 0.495
30	0.88	67.00 ± 0.29	0.103 ± 0.002	2.056 ± 0.047	8.2 ± 0.663	5.494 ± 0.444
30	1.10	56.00 ± 0.58	0.077 ± 0.002	1.540 ± 0.040	4.6 ± 1.030	2.576 ± 0.577
Significance		***	***	***	***	***
CD		1.33	0.023	0.096	3.18	2.24

***-Significance at 0.1%

± Values represent the Standard deviation

Table 23: Effect of sucrose in MS medium on maturation of embryos. 50 mg of fresh weight of callus was inoculated initially. Data recorded after 4 weeks.

Sucrose (%)	Responding calli (%)	Efficiency of total no. of embryos	Efficiency of formation of scutellar/ coleoptillar embryos	Stages of embryos in callus
2%	75 ± 0.577	20.2 ± 0.753	3.2 ± 0.483	Globular/Scutellar
4%	70 ± 0.289	10.4 ± 0.966	4.0 ± 0.913	Globular/Scutellar
6%	70 ± 0.577	16.4 ± 0.876	11.4 ± 1.329	Scutellar/Coleoptillar
8%	45 ± 0.289	05.4 ± 0.658	3.0 ± 0.577	G/S/C Fused
10%	30 ± 0.577	05.2 ± 0.753	1.6 ± 0.516	G/S/C Fused
Significance	***	***	***	
CD	1.52	1.77	1.82	

***-Significance at 0.1%

± Values represent the Standard deviation

Maturation

3.5 µ M ABA showed highest efficiency of 18-20 embryos with maximum globular, scutellar and coleoptillar embryos.

4-6%sucrose also produced maximum efficiency of scutellar and coleoptillar embryos.

Fig: Coleoptillar stage somatic embryo.

Table 24: Effect of ABA in MS medium on maturation of embryos. 50 mg of fresh wt. of callus was inoculated initially. Data recorded after 4 weeks.

ABA (µM)	Responding calli (%)	Efficiency of total no. of embryos	Efficiency of formation of scutellar/ coleoptillar embryos	Stages of embryos in callus
0.5	65.0 ± 0.58	31.2 ± 0.48	5.20 ± 0.63	Globular mostly
1.5	90.0 ± 0.58	24.4 ± 1.56	8.20 ± 0.48	Globular/Scutellar
2.5	90.0 ± 0.29	22.2 ± 0.86	11.00 ± 0.91	Globular/Scutellar
3.5	99.7 ± 0.33	30.0 ± 0.71	20.20 ± 1.11	Scutellar & Coleoptillar
4.5	99.8 ± 0.15	31.6 ± 1.13	19.40 ± 1.13	Scutellar & Coleoptillar
5.5	95.0 ± 0.58	28.2 ± 1.38	15.20 ± 1.32	G,S,C
6.5	75.0 ± 0.58	06.8 ± 1.11	10.20 ± 0.86	Fused G/S/C
7.5	70.0 ± 0.58	10.0 ± 0.91	6.40 ± 0.88	Fused G/S/C
Significance	***	***	***	
CD	1.46	2.39	2.12	

***-Significance at 0.1%

± Values represent the Standard deviation

Germination

Maximum germination frequency of 65% was observed on MS medium supplemented with 5 µM BAP, other 35% embryos formed only shoots. Callus with mature coleoptillar embryos were selected for germination.

Fig1&2: Coleoptillar embryos in embryogenic callus forming shoots and roots on germination medium in light conditions.

Table 25: Effect of phytohormones in MS medium on germination of coleoptillar embryos. Data recorded after 4 weeks.

Hormonal concentration (µM) BAP	Hormonal concentration (µM) GA_3	Germination response (%)
0.0	0.0	50.0 ± 0.289
5.0	0.0	65.0 ± 0.289
10.0	0.0	50.0 ± 0.289
15.0	0.0	45.0 ± 0.289
5.0	2.5	59.0 ± 0.289
10.0	2.5	40.0 ± 0.577
15.0	2.5	45.0 ± 0.577
5.0	5.0	53.0 ± 0.577
10.0	5.0	45.0 ± 0.289
15.0	5.0	30.0 ± 0.289
Significance		***
C.D.		1.18

***-Significance at 0.1%

± Values represent the Standard deviation

Multiple shoots formation during germination of somatic embryos

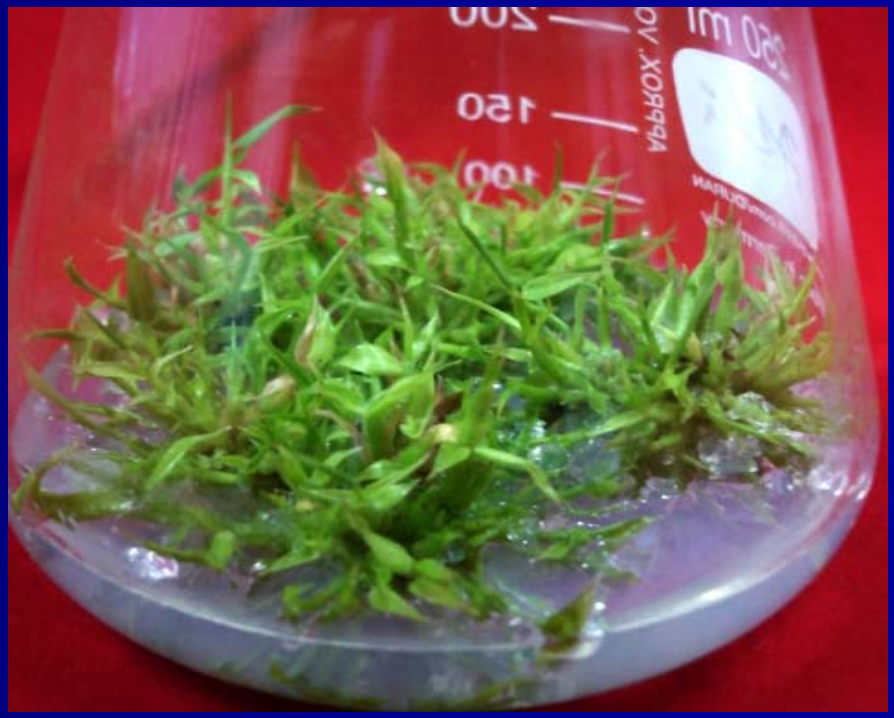
In vitro **multiplication of shoots derived from somatic embryos on MS+2.5mg/l BAP**

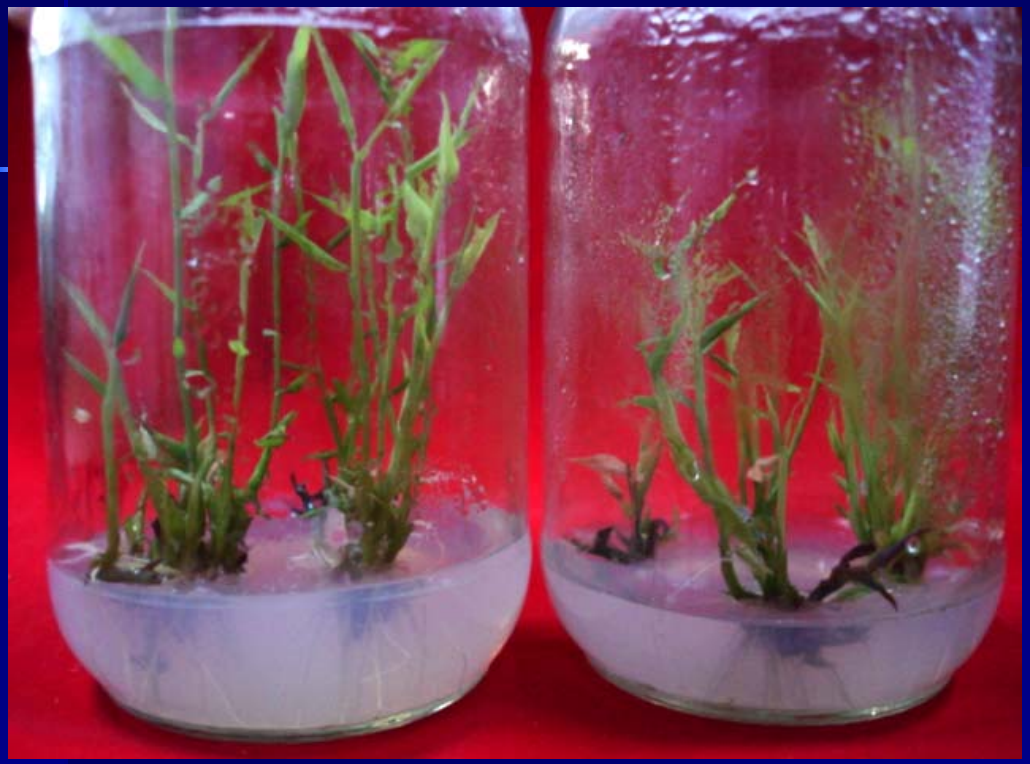
IN VITRO SHOOT MULTIPLICATION

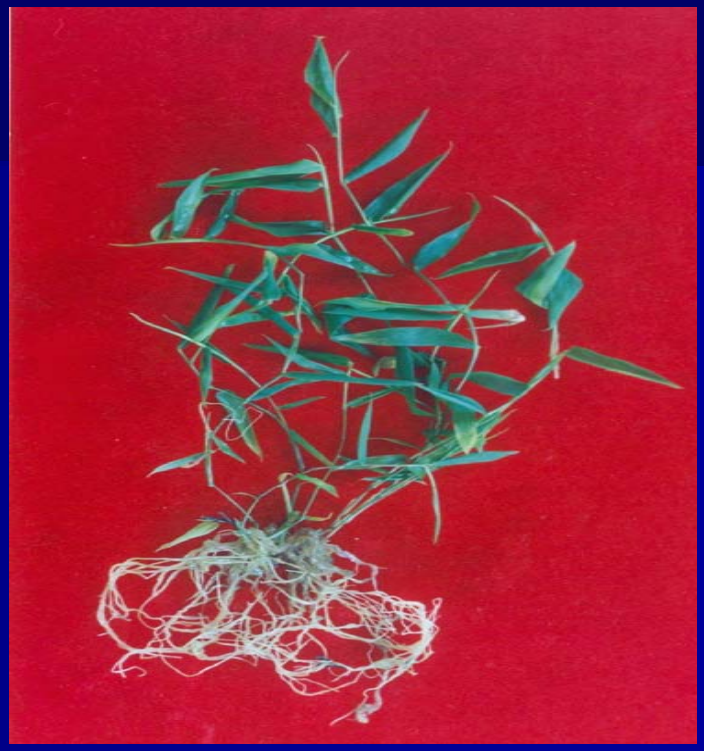
9-11 fold shoot multiplication was recorded on semi-solid MS medium supplemented with 3 mg/l BAP every 4 weeks of subculture.

4-5 shoots as a propagule gave maximum shoot multiplication rate i.e. 9-11 folds.

3% sucrose in the medium is optimum for shoot multiplication.







***In vitro* rooting of shoots derived from somatic embryos on MS+7mg/l IBA**

Hardening and acclimatization of plantlets raised through somatic embryogenesis

HARDENING & ACCLIMATIZATION

In vitro rooted plantlets were transferred to autoclaved cultured bottles containing vermiculite, supplied with ½ strength MS medium twice a week.

After 15-20 days hardened plantlets transferred to mist chamber and then transferred into polybags containing soil, sand and FYM in 1:1:1 ratio.

90% hardening and acclimatization along with field survival of plantlets was observed.

One year old tissue culture raised plant of *D. falcatum* in the field

CONCLUSION

This paper describes for the first time an effective regeneration and multiplication protocol for *in vitro* propagation of *Drepanostachyum falcatum: Through Somatic embryogenesis leading to direct regeneration of S.E's into plants and indirect plant regeneration through in vitro shoot multiplication.*

- **Two pathway plant regeneration from S.E.'s**
- **High rate of shoot multiplication (9-11 fold).**
- **Good rooting (95%).**
- **High transplantation rate of micropropagated plants are the major achievements of this study.**


Thanking you