IN VITRO PROPAGATION OF *DENDROCALAMUS HAMILTONII* THROUGH SOMATIC EMBRYOGENESIS

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INTRODUCTION OF *D. hamiltonii*

FAMILY- Graminae/Poaceae

SUB-FAMILY- Bambusoideae

DISTRIBUTION- India, Bhutan, Nepal, Myanmar , Thailand. In India it is found in the North-West Himalaya, Sikkim, Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland and Tripura.

USES- Forms the basic raw material for walls of native huts, construction purposes, basket-making, mats, water and milk vessels, fuel, and floats for timber-rafts.

EDIBLE BAMBOO- Tender shoot for preparation of ‘'hiyup' a sour pickle

FLOWERING CYCLE- 30- 40 YEARS

PROBLEMS ASSOCIATED WITH CONVENTIONAL PROPAGATION

- Conventional methods include rhizomes, culm cuttings and seeds.

- Conventional methods, sexual as well as vegetative, are beset with many problems such as availability of the propagules, bulkiness and transport of propagules and low survival.

- Propagation with seeds is cheaper and easier. However, it is unreliable due to their monocarpic habit, limited availability, poor seeds set during off season flowering, short viability period, seed sterility and large scale consumption of seeds by rodents and wild animals.

- Due to such problems mass multiplication of bamboo cannot be achieved through conventional methods. In such condition, *in vitro* methods remain an alternative solution.

The regeneration pathways used for micropropagation of plants is through somatic embryogenesis.

Somatic embryogenesis may also resets the zero stage in bamboos thus plants produced can be relied on to live their full life span. Keeping all this in view, protocol for somatic embryogenesis in *Dendrocalamus hamiltonii* using vegetative explants was attempted.

METHODOLOGY

- Small segments cut and isolated from *in vitro* proliferated axillary shoots were used as explant material for somatic embryogenesis.

- They were inoculated on MS medium supplemented with 2,4-D.

To study the effect of auxin alone and in combination with cytokinin on proliferation of embryogenic callus, callus was cultured on medium supplemented with various combinations of phytohormones.

- For improvement in proliferation rate of embryogenic callus, it was transferred to other hormonal combinations. 2, 4-D was used (0-50 µM) alone and in combination with BAP. Sucrose was supplemented in MS medium at concentration from 2-10% to assess its effect on maturation of somatic embryos.

- For germination, coleoptillar embryos were cultured on MS medium. For this hormones like BAP, GA_3 (5-15µM) were tried in MS medium and were incubated in light for 6-8 weeks. Two stage embryo germination was recorded. In stage 1, few embryos germinated into both shoot and root system. These embryos were scored and germination percentage was calculated. In stage 2, majority of the embryos germinated into shoots only. These embryos were scored and the shoots regenerated were harvested for further *in vitro* shoot multiplication.

- Shoots produced by the somatic embryos in germination medium were harvested from embryogenic cultures and were multiplied on MS medium supplemented with cytokinin BAP.

- *In vitro* rooting was achieved when *in vitro* raised shoots were given two step treatment of high IBA for 7 days followed by transfer to hormone free MS medium.

- The plantlets developed via rooting of *in vitro* shoots obtained from somatic embryos were hardened and acclimatized by the standardized procedure.

INDUCTION OF CALLUS

Small segments cut and isolated from *in vitro* proliferated axillary shoots were used as explant material for somatic embryogenesis. Callus formation initiated in one week of culture. A reasonable amount of callus developed after 4 weeks of incubation on MS medium supplemented with 5-30µM 2, 4-D. Callus formation initiated from cut ends of the segments. 15µM 2, 4-D was found to be the best for induction that had resulted in 72.22% callus development from the explants. The callus size varied from 2-4 mm in diameter weighing from 10-30 mg in fresh weight in 4-5 weeks. Decreased frequency of induction and browning of callus was noticed at increased 2, 4-D concentration (20-30µM). On hormone free medium, no callus formation occurred and the cultured segments dried up in due course of time.

➢ Effect of hormone (2, 4-D) on induction of callus. Data recorded after 4 weeks.

2,4-D (µM)	Response %	Fresh Callus wt. (gm)
Control	00.00 ± 0.00^{e}	00.00 ± 0.00^{f}
5	38.66 ± 2.67^{c}	0.020 ± 0.00^{c}
10	52.78 ± 2.78^{b}	0.030 ± 0.00^{a}
15	72.22 ± 2.78^{a}	0.030 ± 0.00^{b}
20	44.22 ± 2.89^{c}	0.030 ± 0.00^{b}
25	41.33 ± 0.00^{c}	0.020 ± 0.00^{d}
30	30.55 ± 2.78^{d}	0.020 ± 0.00^{e}
	F= 87.99**	**F= 408.39****

** = P<0.01, *= P<0.05

Values are given as the mean ± SE. Values followed by the same letters in superscript within the column are not significantly different at the 5% level (Duncan's multiple range test)

➢CALLUS INDUCTION

Effect of culture condition

Growth and develpoment of callus needed dark condition. The callus multiplied 2-3 times (fresh weight) in dark condition in 4 weeks. Callus when subcultured and kept in light conditions in culture room, turned into compact green callus with little growth.

MULTIPLICATION OF EMBRYOGENIC CALLUS

Effect of phytohormones

Prolonged subculturing on MS medium supplemented with 2, 4-D alone (5µM-30 µM) did not produce somatic embryos and only non embryogenic callus multiplied. The development of somatic embryos occurred only when BAP is added with addition of BAP in the MS medium supplemented with 2, 4-D. It was observed that the cultures, which were established on 15µM 2, 4-D became embryogenic when transferred on MS medium supplemented with reduced level of 2, 4–D and BAP. On this medium number of globular embryos developed with 2.45 folds callus multiplication rate. Therefore, after 12 weeks of culture on induction medium the calli developed were transferred to multiplication medium (MS + 10µM 2, 4-D + 5µM BAP). On this medium proembryo initials developed further into globular stage of embryos. The embryogenic cultures were maintained on medium 10µM 2, 4–D and 5µM BAP referred to as multiplication medium. The callus cultures were subcultured in every 4 weeks to multiply the embryos and callus and to maintain the embryogenic potential of callus.

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Microscopic studies revealed the presence of proembryos in the callus that developed to globular stage somatic embryos after 2-3 subcultures on multiplication medium in dark conditions. Different developmental stages of somatic embryos were present in established embryogenic callus (after 5-6 subculture) as observed under stereozoom microscope. Three types of embryos were observed i.e. white round glossy smooth bodies (globular), white cup shaped (scutellar) and yellow tubular (coleoptillar) somatic embryos during various phases. Globular stage was prominent on multiplication (embryogenic) medium whereas scutellar and coleoptillar stages were prominent on maturation medium.

➢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.

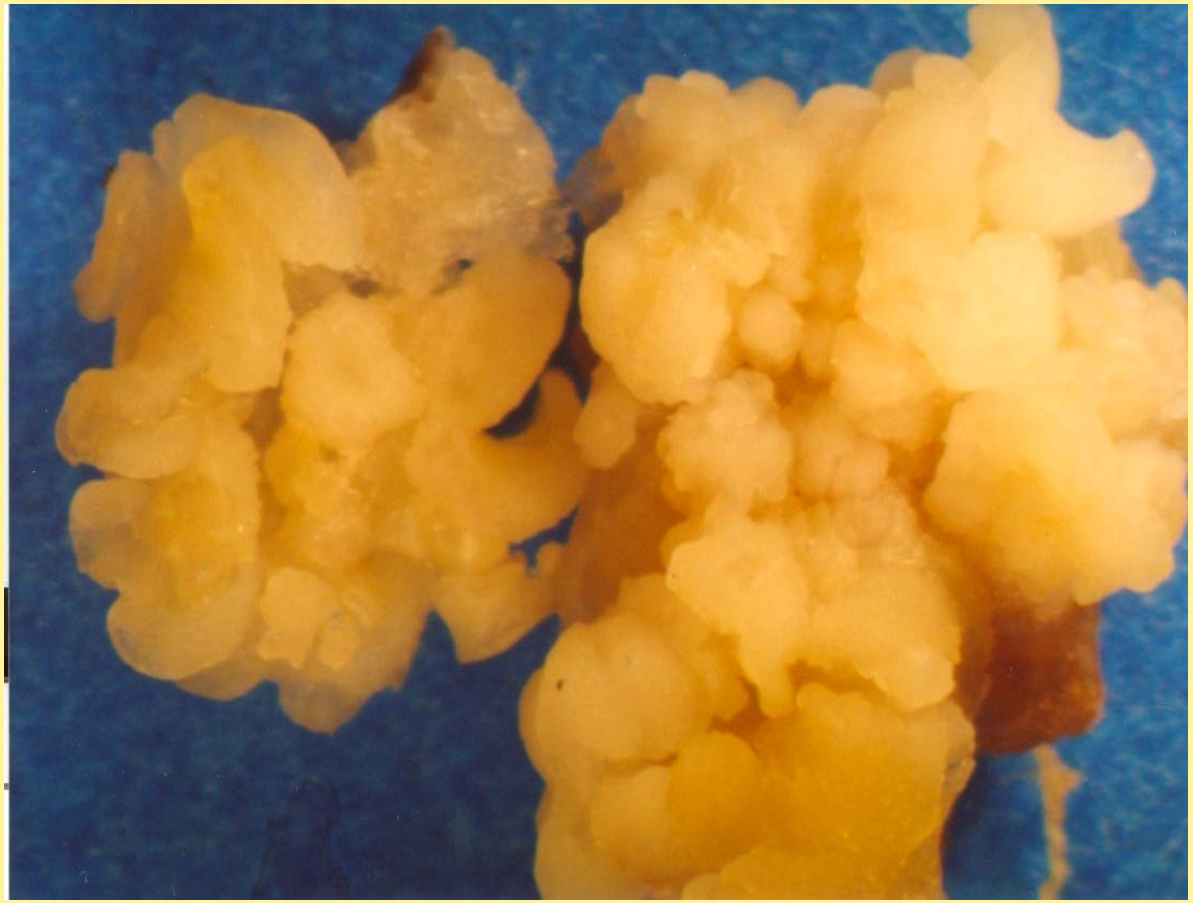
2,4-D + BAP (μM)	Fresh callus wt. (gm)	Callus multiplication rate	Mean no. of embryos
05 + 2.5	0.08 ± 0.00^{de}	1.65 ± 0.05^{e}	5.77 ± 0.15^{de}
05 + 5.0	0.10 ± 0.00^{c}	2.05 ± 0.08^{b}	6.33 ± 0.24^{cd}
05 + 10.0	0.07 ± 0.00^{e}	1.39 ± 0.06^{f}	5.23 ± 0.15^{e}
10 + 2.5	1.06 ± 0.01^{a}	1.99 ± 0.06^{bc}	8.60 ± 0.21^{b}
10 + 5.0	**0.12 ± 0.00^{b}**	**2.45 ± 0.03^{a}**	**9.67 ± 0.33^{a}**
10 + 10.0	0.09 ± 0.00^{cd}	1.83 ± 0.04^{cd}	6.97 ± 0.32^{c}
15 + 2.5	1.28 ± 0.08^{e}	1.28 ± 0.06^{f}	3.07 ± 0.23^{g}
15 + 5.0	2.08 ± 0.04^{c}	2.08 ± 0.07^{b}	4.10 ± 0.21^{f}
15 +10.0	1.68 ± 0.06^{de}	1.68 ± 0.07^{de}	1.60 ± 0.21^{h}
	F= 3070.45**	**F= 40.09****	**F= 108.40****

** = P<0.01, *= P<0.05

Values are given as the mean ± SE. Values followed by the same letters in superscript within the column are not significantly different at the 5% level (Duncan's multiple range test)

➢Multiplication of embryogenic callus

Somatic Embryos



Effect of sucrose concentration

Different concentration of sucrose 1-6% in MS medium was tried for multiplication of embryogenic callus. Sucrose at 3% in the MS medium showed best response in terms of multiplication rate of callus (2.85 folds) along with 9.66 embryos developed on an average per callus. At this concentration numbers of globular stage embryos developed. At 1% sucrose in MS medium reduced number of embryos was obtained. At higher concentration of sucrose (4-6%) embryo formation decreased. Thus, concentration of sucrose above 3% level in MS medium was not favorable for multiplication and maintenance of embryogenic callus.

Effect of subculture duration

Subculture interval of 4 weeks was found to be optimal duration for embryogenic callus cultures, where mean number of 9.67 embryos per callus developed. Subculture interval of more than 4 weeks resulted in the formation of non-embryogenic callus with browning in the callus.

Effect of culture conditions

Embryogenic callus multiplied in dark conditions. In dark conditions callus multiplication rate of 2-3 folds was obtained. Callus kept under light conditions resulted in development of chlorophyll with retarded growth in callus formation.

➢**Effect of subculture duration on multiplication of embryogenic callus on MS + 2, 4-D (10µM) + BAP (5µM). 50 mg of fresh wt. of callus was inoculated initially.**

Subculture interval	Fresh callus wt. (gm)	Callus multiplication rate (folds)	Mean no. of embryos
2 weeks	0.10 ± 0.00^{d}	1.92 ± 0.07^{c}	2.33 ± 0.60^{d}
4 weeks	$\mathbf{0.13 \pm 0.00^{c}}$	$\mathbf{2.65 \pm 0.06^{b}}$	$\mathbf{9.67 \pm 0.33^{a}}$
6 weeks	0.18 ± 0.00^{b}	3.60 ± 0.09^{a}	7.67 ± 0.24^{b}
8 weeks	0.20 ± 0.00^{a}	4.04 ± 0.07^{a}	5.30 ± 0.29^{c}
	F= 256.30**	**F= 23.44****	**F= 51.39****

** = P<0.01, *= P<0.05

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MATURATION OF EMBRYOS

The growth of embryos beyond globular and scutellar stage was only sporadic and embryos turned green with development of leafy structures when transferred to light conditions. For maturation and further development of embryos exposure of embryos on sucrose supplemented medium was found to be essential with the elimination of auxin 2, 4–D from the medium.

Embryogenic calli (with globular somatic embryos) when subcultured on MS medium with increased sucrose concentration (6%) without auxins in MS medium developed scutellar embryos which further developed into coleoptillar embryos.The percentage of such maturation was very low (2-3 coleoptillar embryos/callus). Concentrations higher than 6% of sucrose in MS medium resulted in browning of callus.

➢**Effect of sucrose in MS medium on maturation of embryos. 50 mg of fresh wt. of callus was inoculated initially. Data recorded after 4 weeks.**

Sucrose (%)	Formation of scutellar/ coleoptillar embryos
2%	1.40 ± 0.20bc
4%	1.43 ± 0.23bc
6%	3.33 ± 0.24a
8%	2.00 ± 0.00b
10%	1.16 ± 0.16d
	F= 21.28**

** = P<0.01, *= P<0.05

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GERMINATION OF EMBRYOS

Callus with mature coleoptillar embryos were isolated from the maturation medium. They were transferred to MS medium supplemented with BAP alone (5–15µM). On this medium it was found that around 5% somatic embryos developed direct shoot and root.

Rest of the embryos produced shoots and leafy structures. Addition of GA_3 to BAP supplemented MS medium did not improve the germination frequency of the embryos. The direct plantlets that developed from somatic embryos were very thin. The somatic embryos which germinated into shoot and root were separated and subcultured on hormone free medium for plant development. In the hormone free medium although shoot elongated but the root system remained very weak. These plants were later transferred to autoclaved soilrite for hardening and were kept in culture room for 2–3 weeks. However, these plantlets did not survive and died after leaf necrosis.

Large number of somatic embryos did not germinated into direct shoot and root system and only produced shoots on germination medium. These shoots were harvested for *in vitro* shoot multiplication.

➢Effect of phytohormones in MS medium on germination of embryos. Data recorded after 4 weeks.

BAP (µM)	Germination response (%)
Control	0.00 ± 0.00^{d}
5.0	**4.50 ± 0.28^{a}**
10.0	3.10 ± 0.16^{b}
15.0	2.50 ± 0.28^{b}
	F= 73.28**

** = P<0.01, *= P<0.05

Values are given as the mean ± SE. Values followed by the same letters in superscript within the column are not significantly different at the 5% level (Duncan's multiple range test)

➢Germination of somatic embryos

IN VITRO SHOOT MULTIPLICATION (derived from somatic embryos)

Shoots produced by the somatic embryos in germination medium were harvested from embryogenic cultures and were multiplied on MS medium supplemented with cytokinin BAP (5-30µM) under light conditions. A routine subculturing of these *in vitro* shoots every 3 weeks increased its number. Multiplication rate of nearly 13 folds was obtained on MS medium supplemented with 10µM BAP.

➢Effect of cytokinin (BAP) in MS medium on multiplication of shoots derived from somatic embryos. Data recorded after 4 weeks.

BAP (µM)	Mean shoot number	Mean shoot length (cm)	Multiplication rate
Control	13.70 ± 0.23^{f}	2.74 ± 0.13^{c}	03.42 ± 0.06^{f}
5.0	34.40 ± 0.59^{c}	4.09 ± 0.07^{a}	08.60 ± 0.15^{c}
10.0	**52.00 ± 0.58^{a}**	**3.90 ± 0.08^{a}**	**13.00 ± 0.14^{a}**
15.0	41.25 ± 0.69^{b}	3.26 ± 0.14^{b}	10.31 ± 0.17^{b}
20.0	31.07 ± 0.27^{d}	2.33 ± 0.12^{d}	07.77 ± 0.07^{d}
25.0	22.55 ± 0.64^{e}	1.68 ± 0.10^{e}	05.63 ± 0.16^{e}
	F= 649.30**	**F= 72.61****	**F= 652.97****

** = P<0.01, *= P<0.05

Values are given as the mean ± SE. Values followed by the same letters in superscript within the column are not significantly different at the 5% level (Duncan's multiple range test)

➢Multiplication of shoots derived from somatic embryos

IN VITRO ROOTING

90-95% success was obtained in *in vitro* rooting conditions. *In vitro* rooting was achieved when *in vitro* raised shoots were given two step treatment of high IBA for 7 days followed by transfer to hormone free MS medium.

➢Effect of IBA (two–step procedure) on *in vitro* rooting of *D. hamiltonii.*

IBA (µM)	Response %	Mean root number	Mean root length (cm)
10	09.09 ± 0.00^{fg}	2.11 ± 0.04^{f}	05.03 ± 0.09^{e}
20	12.12 ± 1.51^{f}	2.55 ± 0.08^{e}	05.39 ± 0.06^{d}
40	22.74 ± 2.62^{e}	2.74 ± 0.13^{e}	05.63 ± 0.08^{cd}
60	30.30 ± 1.51^{d}	3.27 ± 0.16^{d}	06.10 ± 0.07^{b}
80	54.54 ± 2.62^{b}	4.65 ± 0.21^{b}	05.89 ± 0.08^{bc}
100	**93.93 ± 1.52^{c}**	**9.77 ± 0.08^{a}**	**07.15 ± 0.10^{a}**
120	37.87 ± 1.51^{g}	4.14 ± 0.05^{c}	05.64 ± 0.07^{cd}
140	06.06 ± 1.52	1.55 ± 0.12^{g}	04.11 ± 0.12^{f}
	F= 273.74**	**F= 460.92****	**F=104.25****

Values are given as the mean ± SE. Values followed by the same letters in superscript within the column are not significantly different at the 5% level (Duncan's multiple range test)

In Vitro Rooting followed by pulse treatment in MS medium supplemented with 100µM IBA

HARDENING AND ACCLIMATIZATION

The tissue cultured raised plants were transferred in autoclave bottels containing soilrite. Plants produced were successfully hardened and acclimatized with 85- 90% survival rate. During hardening the shoots elongated, leaves turned greener and expanded. The plants became much healthier after hardening. After one month in the shade house the plants were transferred to polybags containing soil: sand: FYM in 1:1:1 proportion by volume and were irrigated with water. In next 2-3 months these plants developed rhizomes and were ready for field plantation.

Hardening

Acclimatization

Thanks