BIOTECHNOLOGICAL APPROACHES FOR PROPAGATION, CONSERVATION AND IMPROVEMENT OF IMPORTANT BAMBOOS

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Topics to be touched during the presentation:

i. Bamboo micro-propagation
ii. Conservation strategies
iii. Genetic fidelity of TC raised plants
iv. *In vitro* flower induction – for viable hybrid seeds?
v. Field performance of TCPs
vi. Possibilities of Genetic manipulations and overcoming the barriers.
vii. Genetic diversity evaluation
viii. Bamboo leaves as fodder
ix. Acknowledgements

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Issues involved

- Bamboos are considered non-timber forest produce and hence neglected.
- No efforts are made to raise bamboo nurseries until recently.
- Most of the bamboo plantations are in the private land and are grown through use of offsets.
- Low commercial utilization of bamboos.
- Gregarious flowering of *Dendroclamus strictus, Bambusa bambos* and *Melocanna baccifera* in many parts of the country creating social problems.
- Bamboos extraction not done systematically and this damages the bamboo strands.
- No survey reports about the actual assessment of bamboo stocks.
- Being cross pollinated, natural variability is of common occurrence.
- Variability in terms of low lignin and high cellulose is required by paper industry.
- Greater need for generating planting materials with desired characteristics possibly through micro-propagation.

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Areas of focused attention at CSIR-IHBT

- ✓ Collection,Conservation and Characterization of bamboo germplasm
- ✓ Selection and propagation of elites both by macro- and micro-propagation
- ✓ Training in bamboo propagation and agro-technologies
- ✓ Improvement through seedling selections and transgenics
- ✓ Induction of bamboo flowering *in vitro*
- ✓ Setting up demonstration plots of TCPs
- ✓ Ensuring availability of planting stocks for the farmers, foresters, NGOs, and DRDA(District Rural Development Agency)

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Status of Bamboo Species at CSIR-IHBT

- *In vitro* grown species :*Bambusa balcooa, B.bambos, B. multiplex, B. nutans, Dendrocalamus asper, D. giganteus, D.hamiltonii, D. membranaceus, Melocanna baccifera, Phyllostachys pubescens.*

- Species grown in the nurseries: *Arundinaria falconerii, Bambusa bambos, B. balcooa, B. distegia, B. multiplex, B. nutans,,|B.nana, B.pallida, B. tulda, B. ventricosa, B. vulgaris, Chimonobambusa quadrangularis, Dendrocalamus asper, D.barbatus, D. giganteus, D. hamiltonii, D. hookerii, D. membranaceus, D. sinicus, D. strictus, D. tibeticus, D. yunnanensis, Diongzhura tumidinoda, Guadua angustifolia, Ochlandra travancorica, Phyllostachys aurea, P. bambusoides, P. pubescens, P. nigra, Sasa auricoma, Melocanna baccifera,* and *Thyrsostachys siamensis.*

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Micropropagation

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The micropropagation protocols for all the aforementioned bamboo species have been developed using either:

i. Auxiliary bud proliferation using nodal explants of precocious branches and rooting or

ii. Formation of compact and nodular callus, differentiation of somatic embryos, their germination and production of rooted plants.

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- While efficient micropropagation systems (shoot cultures and somatic embryos) from lab to land were developed, protocols for slow growth of multiplying cultures were also standardized for efficient stock piling

- However, this requires strictly programmed schedules

- And therefore, methods for short and mid-term conservation were also standardized so as to ensure availability of mother stocks to meet the requirements.

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Selection of Elite Stocks

- Seed collection from various locations.
- Clump-wise germination in nursery beds.
- Seedlings observed for 3-4 years in the fields for the following characteristics :
  - i) No. of culms produced annually,
  - ii) The length of culms, and
  - iii) Thickness of culms at the third internode from the base.
- Selection of promising ones – (Year 2)
- Final selection of elites – (Year 3)
- Initiation of aseptic cultures using explants from thus selected field grown plus clumps.

Micropropagation of *D. hamiltonii*
a) Auxiliary bud proliferation and rooting

Nodal Explant

Sprouted Bud

Multiple Shoots

Rooting

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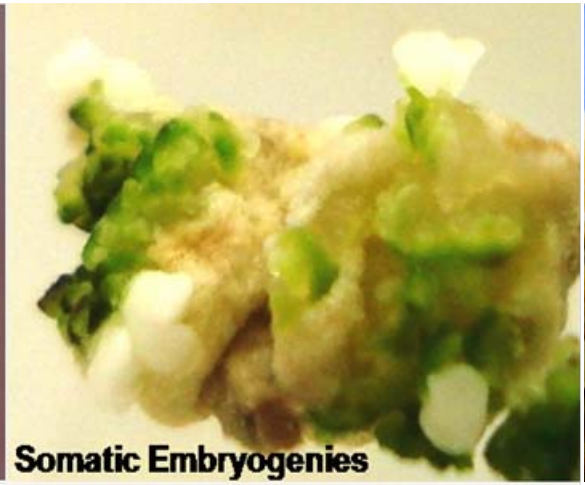

b) Indirect somatic embryogenesis in *Dendrocalamus hamiltonii*

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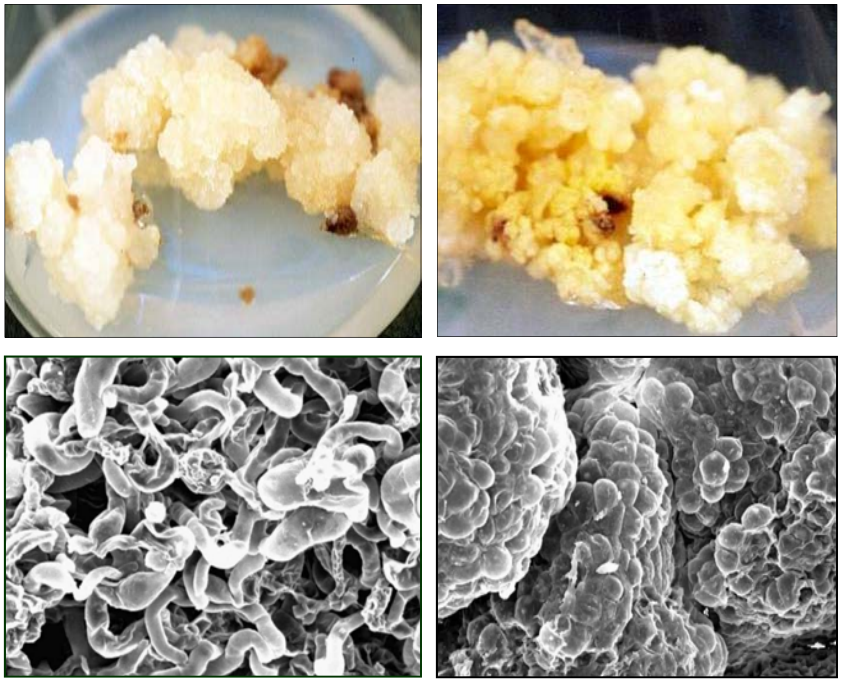
Somatic Embryogenesis

Callus induction:

- Incorporation of 2,4-D in the medium absolutely essential.
- Best response in MS + 2,4-D + BAP (2.0 mg/l each)
- Callus initiation takes about 2 weeks .

Two types:



Type I
Fast growing, friable
(No response)

Type II
Comparatively slow growing,
nodular and compact

(Somatic embryos are initiated as ivory white well formed structures)

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Somatic Embryogenesis

Nodular callus

Somatic embryogenesis

SE germination

Regenerated plantlets

Rooted plantlets

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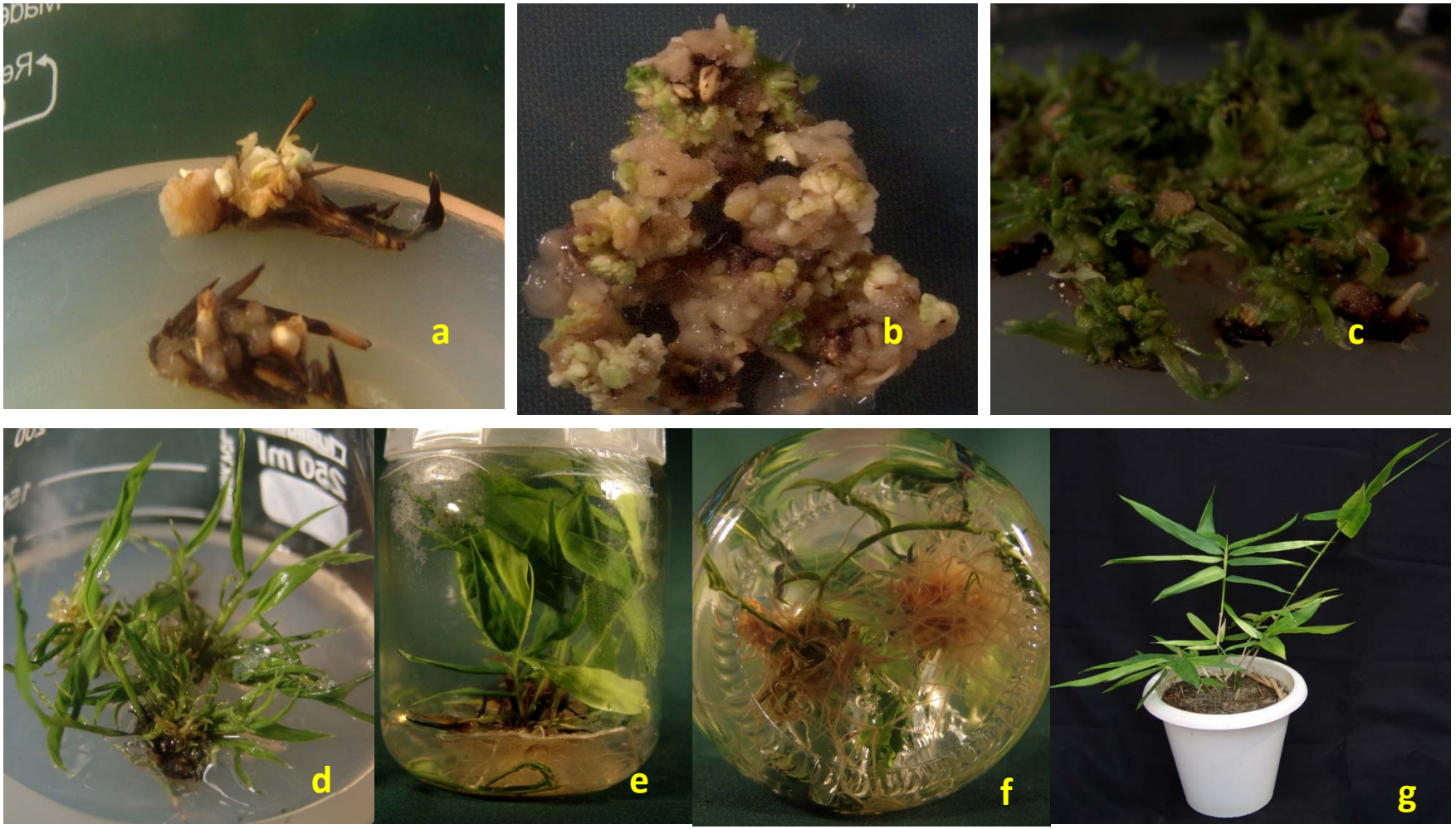
Somatic embryogenesis in *Bambusa nutans*



a) Induction of callus; **b)** Nodular, compact callus showing embryoid induction; **c)** Germination of somatic embryos; **d)** Germinated embryoids with shoots and roots; **e-f)** rooted plantlets; **g)** Hardened plant in a pot

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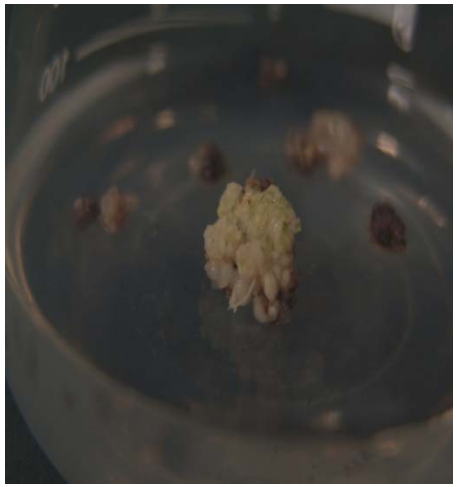
Dendrocalamus asper

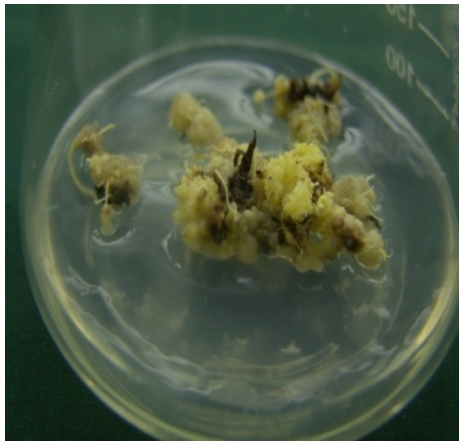
Multiple shoot induction

Proliferated shoots on BAP

Rooting on IBA



Induction of embryogenic callus



Formation of somatic embryos

Acclimatized plants

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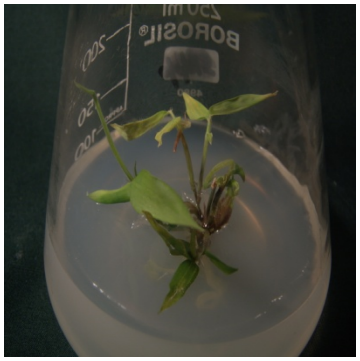
Micropropagation protocol for *Dendrocalamus membranaceus*

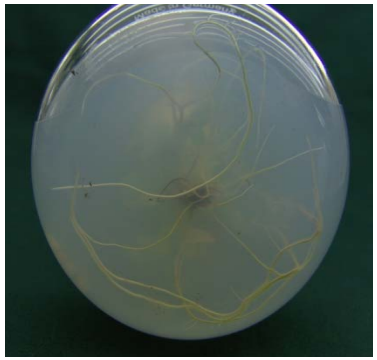
i) Through nodal explants

Sprouting of node on BMS in 10-days

Multiple shoot formation on 8.8µM BAP and 2.3µM Kn



Rooting of microshoot on 4.4µM BAP+5.4µM NAA



Rooting on 1 mg/l BAP+1 mg/l NAA

Ex vitro hardened plantlet on soil:sand (1:1) potting mix

Sprouting of seed on BMS

Plantlet formation on 8.8µM BAP+4.6µM Kn

Hardened plantlet on soil:sand (1:1) potting mix

ii) Through *in vitro* seed germination

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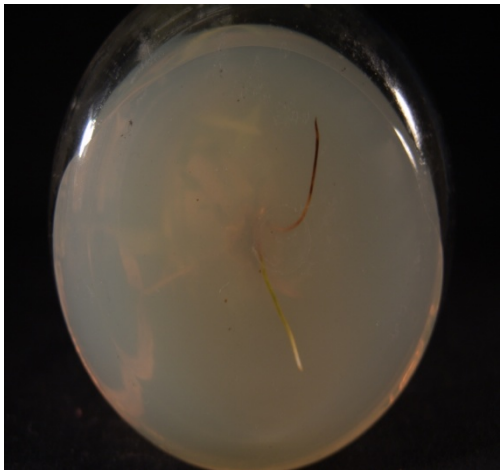
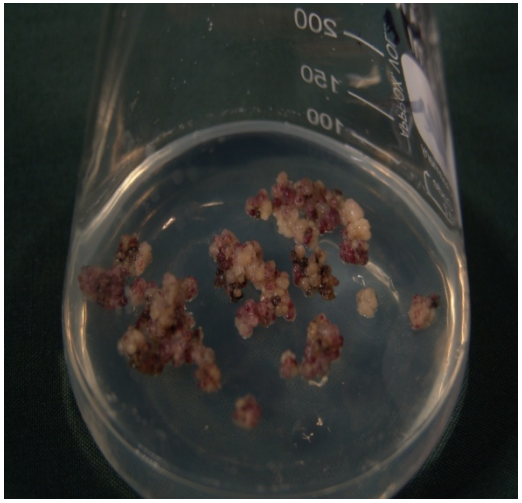
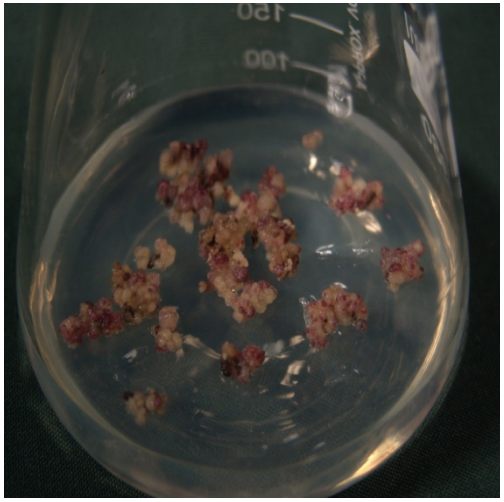
Phyllostachys pubescens

Multiple shoot proliferation



Rooting





Formation of somatic embryos

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Guadua angustifolia

Multiple shoot induction and rooting on liquid medium

Proliferated shoots

Hardened plant

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Hardening of TCPs

Initial hardening in pure sand for 2 weeks.

Subsequent transfer to a mixture containing river bed sand: garden soil: farm yard manure in the ratio of 1:1:1 in poly-sleeves.

Plantlets with six leaves showed 82.3% survival.

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Conservation Strategies

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CONSERVATION STRATEGIES:

Slow growth could be induced in shoot cultures and somatic embryos by using methods such as

- Reduced nutrients (minimal medium) of medium
- Adding growth retardants to medium
- Keeping cultures at low temperature
- Addition of osmoticum (sucrose, mannitol, PEG etc.)
- Change in gaseous environment
- Liquid paraffin overlay
- Alteration in photoperiod

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- Slow growth was induced in *D. hamiltonii* somatic embryos by maintaining them under mineral oil overlay for 1 year under culture lab conditions (16 h photoperiod, 25 ± 2˚C).

- When retrieved at different intervals, 80% viability and 60% germination was recorded after 1 year

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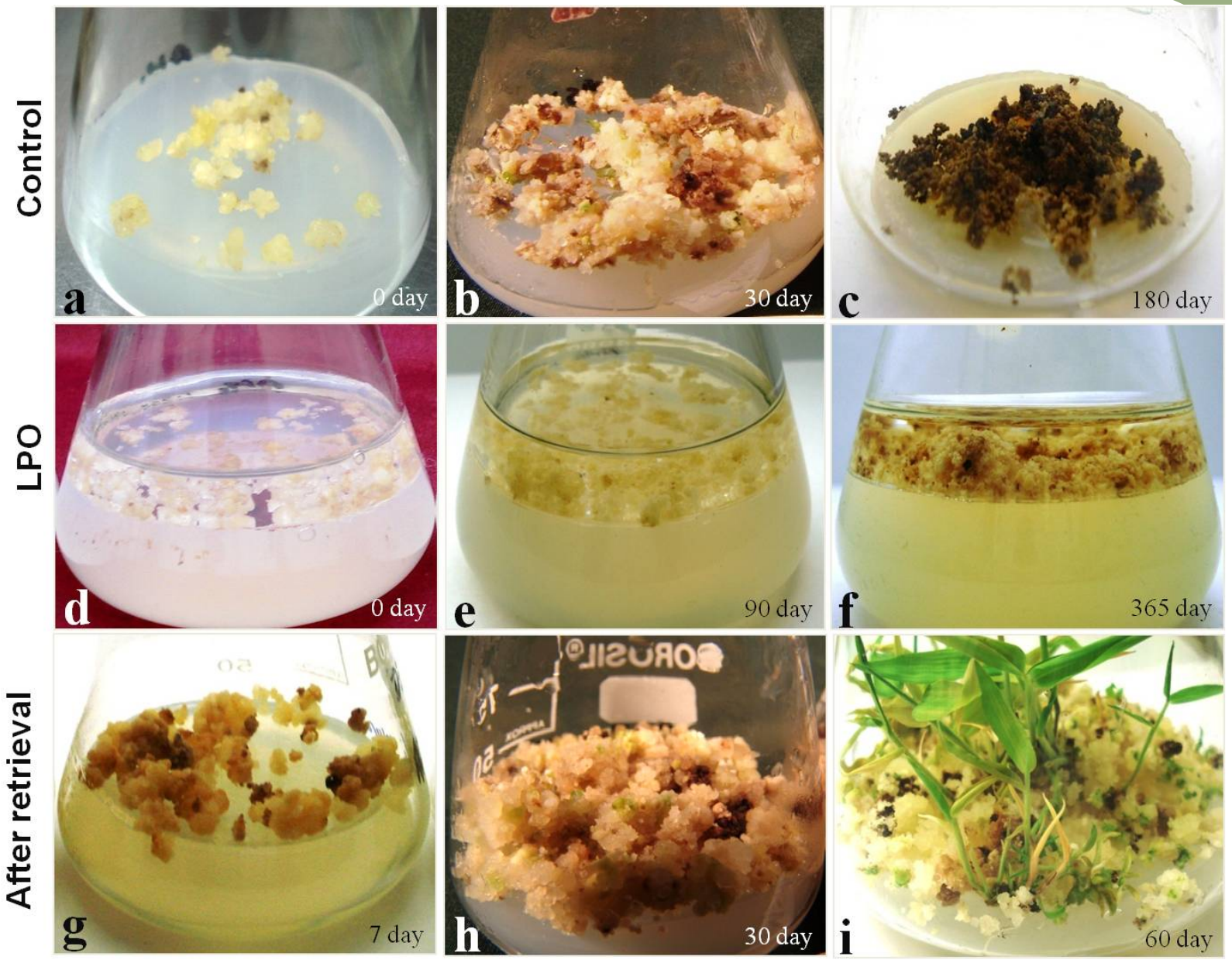
- **Different growth retardants like paclobutrazol, abscisic acid, ancymidol and picloram at different concentrations (0, 0.125, 0.25, 0.50 and 1 mg/l) were tested. A regular retardation of growth of *Dendrocalamus hamiltonii* cultures was observed with increasing concentrations**

- **The propagules remained healthy under slow growth conditions induced by growth retardants like ABA, paclobutrazol or ancymidol (0.25 mg/l) in combination with BA (1 mg/l) as compared to only growth retardants. Growth in tissues resumed normally 1 week, after retrieval .**



Slow growing *D. hamiltonii* somatic embryos stored under mineral oil overlay

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The slow growth protocol helped in:

- Ensuring steady availability of planting propagules as and when required
- Circumventing germination at an undesirable time and wastage of costly propagules

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Genetic Manipulations and Overcoming the Barriers

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Need for genetic improvement of bamboos

- It is impossible at present to set up the breeding programmes because of blocks in flowering, reproduction and seed set

- Traits required for improvements through genetic modification are:

✓ Reduced High lignin content
✓ Enhancement of fibre and paper making quality
✓ Resistance to Injuries caused due to sensitivity to frost
✓ Protection from diseases/gregarious flowering
✓ Lowered resistance to colouring
✓ Reduced Brittleness
✓ Lowered Fading and damage by moisture
✓ Resistance to damage by bleaching and attack by wood-boring insects

Development of transgenics

- Looking at the possibilities

- Recalcitrance of *Dendrocalamus hamiltonii* somatic embryos to *Agrobacterium* mediated transformation has been understood and overcome

- Transgenic plants were developed using *gus* reporter gene by the *Agrobacterium* mediated method of transformation

- Transgenic plants expressing thaumatin-like-protein(tlp) gene developed using the biolistic method of transformation

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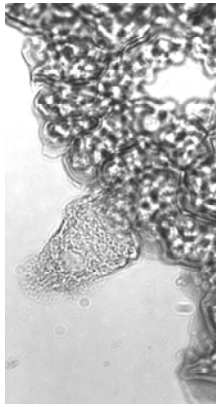
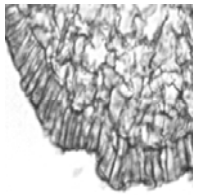
Blocks Encountered

Surface waxes

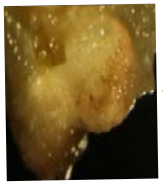
Necrosis due to polyphenol oxidation



Resists attachment of *Agrobacterium* infection



Thickened cell walls



Bamboo somatic embryos

Tween20 added in infecting culture to remove waxes

Co-cultivation on PVP containing medium to prevent polyphenol oxidation & necrosis

Culture on medium containing BA to prevent cell wall thickening

Successful infection & transformation by *Agrobacterium Strain GV 3101 harboring pB1121*

Plant defense

Breaking of plant defense

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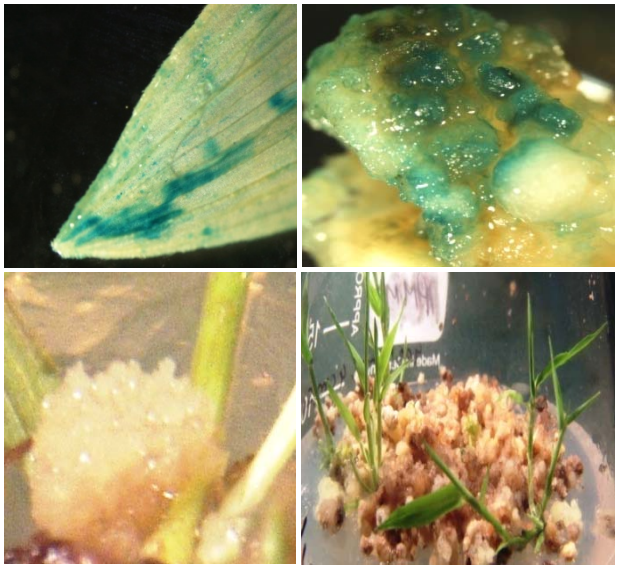
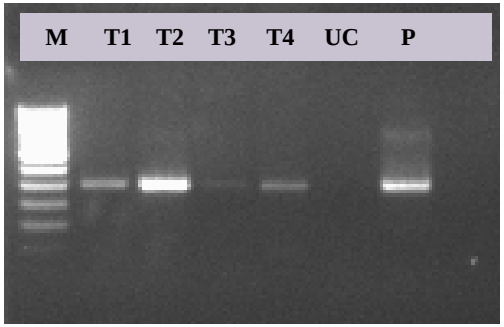
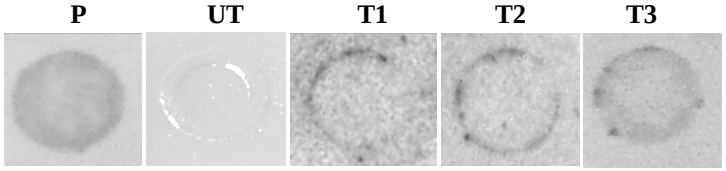
GUS expression in *Dendrocalamus hamiltonii* transformed by *Agrobacterium* strain GV3101 harboring pBI121



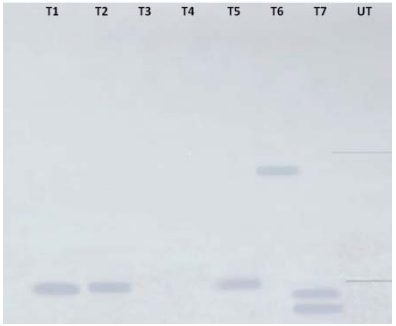
GUS expression in *Dendrocalamus hamiltonii* transformed by *Agrobacterium* strain GV3101 harboring pBI121



PCR amplification product of *npt*II gene in agro-transformed GUS positive somatic embryos



Dot blot showing a signal intensity equivalent to 45 ng of plasmid DNA



Southern blot analysis of transgenic lines probed with biotin labelled PCR amplified *gus* fragment

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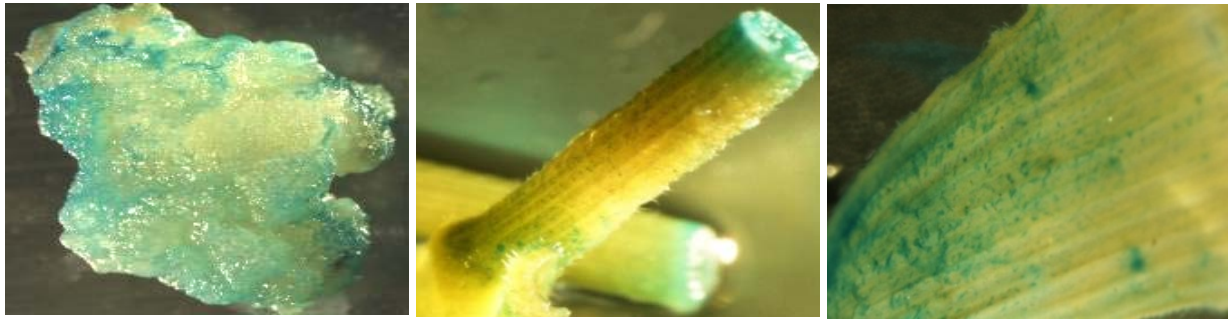
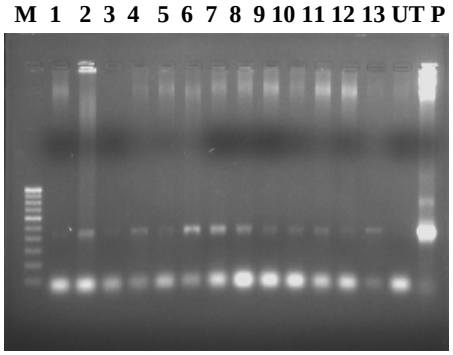
Biolistic gun BIORAD, PDS 1000 He mediated transformation

Transgenic with *gus*-reporter gene

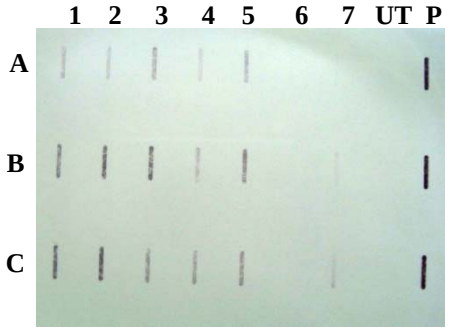


GUS expression in *Dendrocalamus hamiltonii* transformed by pBI121

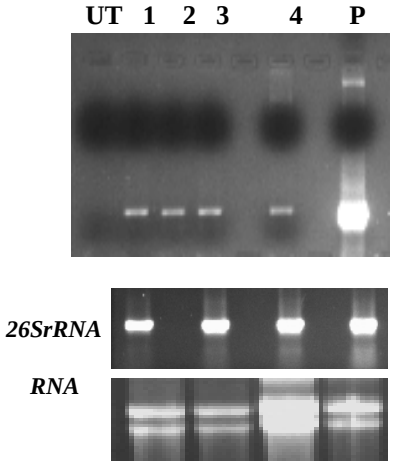
Development of *tlp* transgenics



PCR amplification product of *tlp* gene in transgenic plants



Slot blot of *tlp* transgenics



Transcript expression of *tlp* in transgenic shoots

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Genetic Diversity Evaluation

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AFLP Fingerprinting status of bamboo germplasm

Name of Species/ accessions	Samples procured / DNA isolation and pre-amplifications	AFLP Fingerprinting Status
D. hamiltonii,	111	111
D. strictus	40	40
D. giganteus	09	09
B. nutans	44	44
B. pallida	04	04
P. pubescens	41	41
Accessions representing other bamboo species	105	105
Total	354	354 accessions with Eight primer combinations

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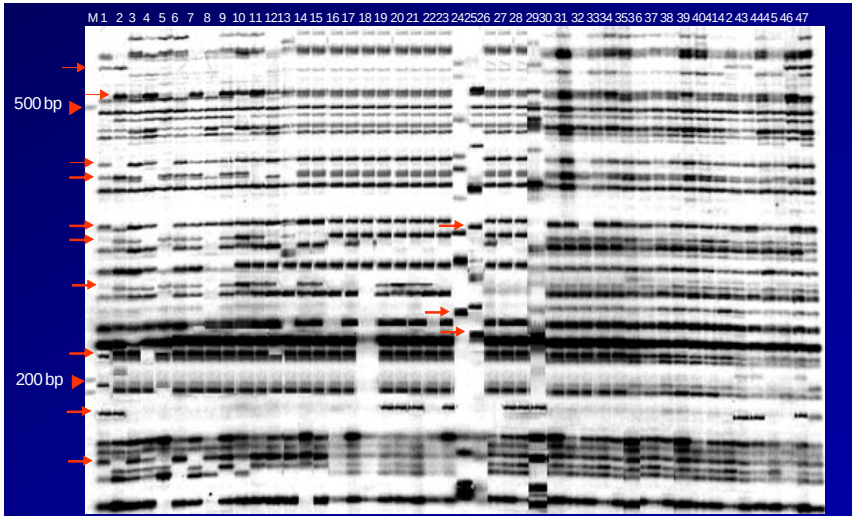
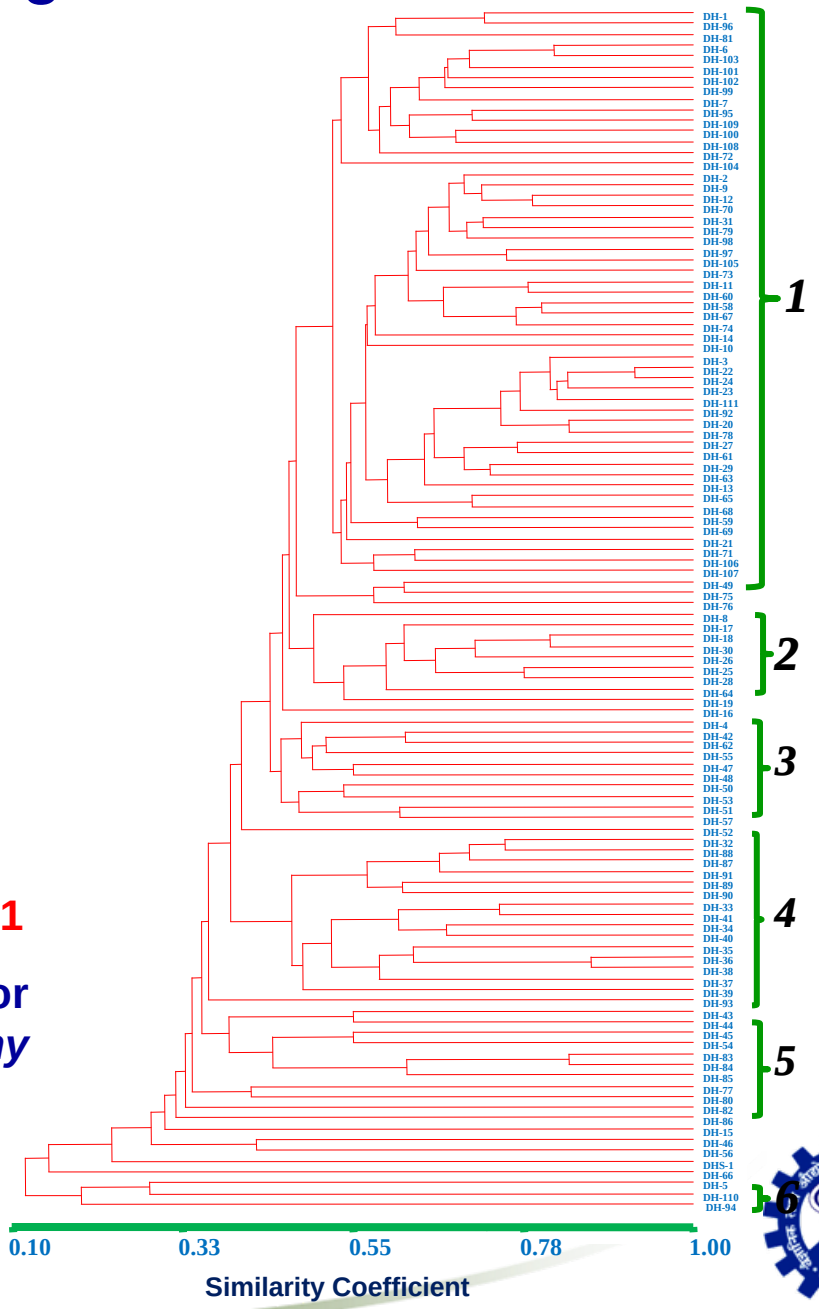
AFLP Fingerprinting of *D. hamiltonii*



Representative AFLP profile of *D. hamiltonii*



- Total no of accessions analyzed = 111
- Clustered in 6 groups; one major group with Av SI :*0.75 with many internal clusters*
- Overall Av GSI: 0.42

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In vitro Flower Induction

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In vitro Flowering in *D. hamiltonii*

- unraveling the enigma

- *In vitro* flowering can serve as an ideal system for understanding the complexity of how vegetative shoots convert into flowering and finally how it leads to seed set

- This understanding can also lead to predict flowering in nature that will not only help us in conserving the right material but also preparing for strategizing the conservation methods

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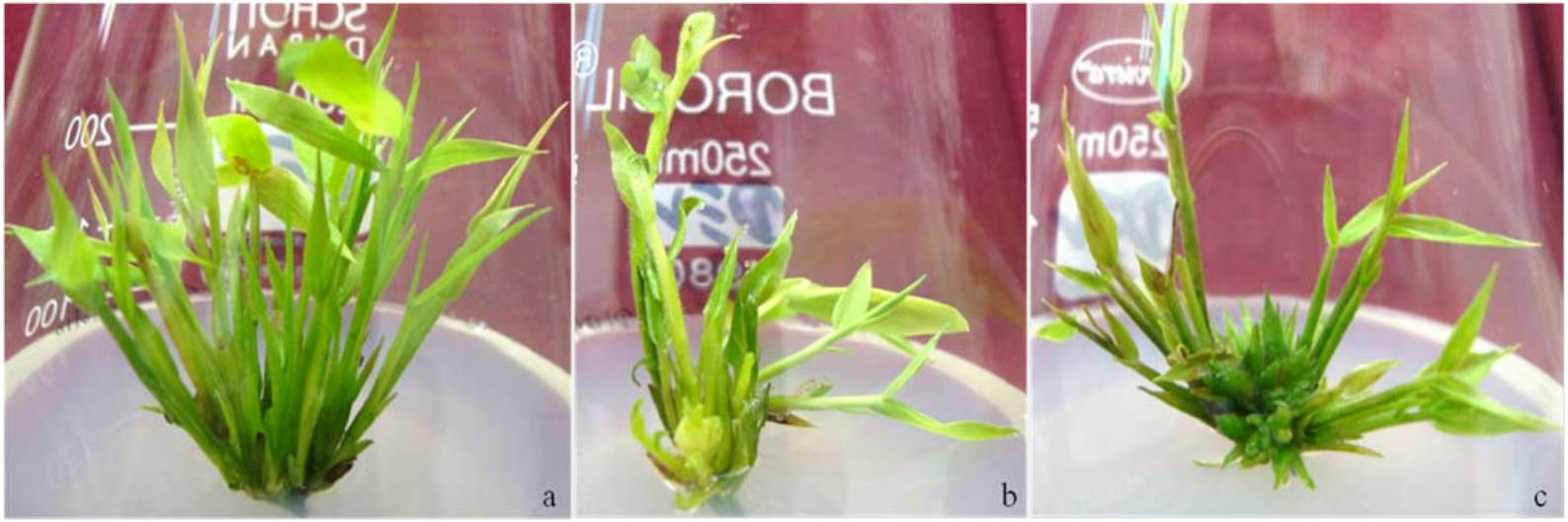

Vegetative shoot | About to flower shoot | Flowered shoot

In vitro flowers of *D. hamilltonii*

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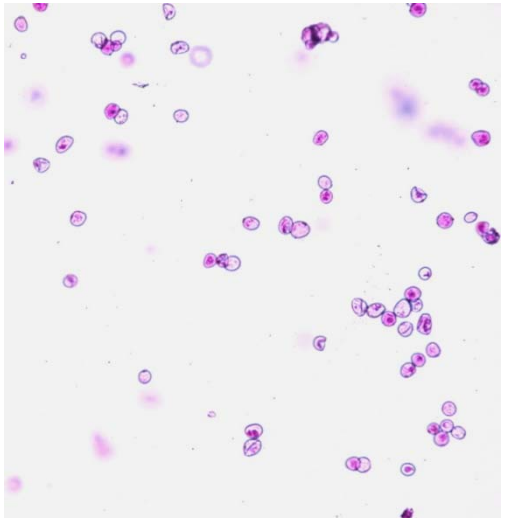
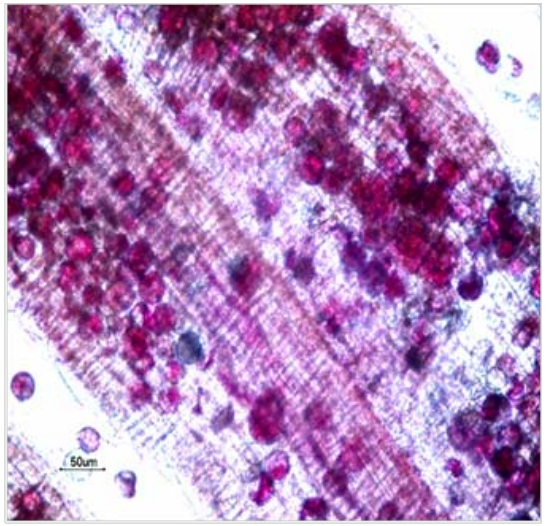
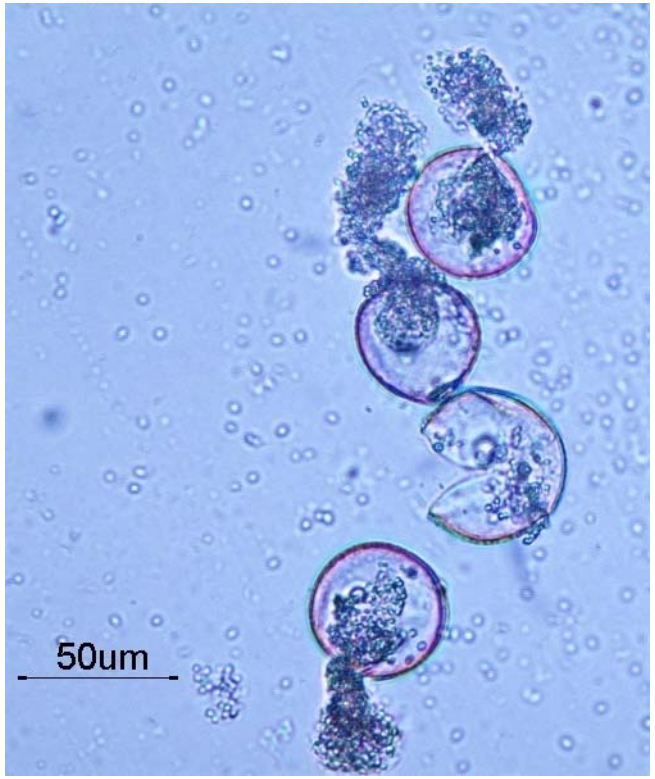




68% pollen found viable when stained with Alexander's stain



Pollens showed plasmolysis when placed on germination medium, indicating poor pollen wall development

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- *In vitro* flowering, fertilization and seed set can pave the way for sustainable availability of propagules such as hybrid seeds for future use and bamboo improvement programmes

- Early prediction of flowering will help in timely collection and conservation of the right material

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Genetic fidelity of TC raised plants

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Genetic fidelity testing of tissue culture raise plants

Species wise genetic fidelity status of tissue culture raised plants

Name Species	Total No of tissue culture raised plant samples procured processed for DNA isolation	Genetic fidelity testing status using AFLP fingerprinting
D. hamiltonii	40	40
D. asper	40	*40*
B. nutans	20	20
Total	100	100

***100** tissue culture raised plant of 3 different species established in the field were tested with **10** selected AFLP primer combinations.*

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Genetic fidelity testing of tissue culture raise plants

AFLP primer combinations and amplifications pattern details across the tissue culture raised 40 plants of *D. hamiltonii.*

S.No.	Primer Combination	Total band	Monomorphic	Polymorphic
1.	E-AAC + M-CTG	69	69	0
2.	E-AAC + M-CAA	64	64	0
3.	**E-AAC + M-CAC**	**59**	**58**	**1**
4.	E-AAG + M-CAA	64	64	0
5.	E-AAG + M-CAG	67	67	0
6.	E-AGC + M-CAT	69	69	0
7.	E-AGG + M-CTG	64	64	0
8.	E-AGG + M-CAG	66	66	0
9.	**E-AGG + M-CAC**	**78**	**78**	**0**
10.	E-AGC + M-CAA	73	73	0
Total		**673**	**672**	**1**

AFLP data revealed that the tissue culture raised plants of D. hamiltonii maintained in IHBT fields are genetically stable

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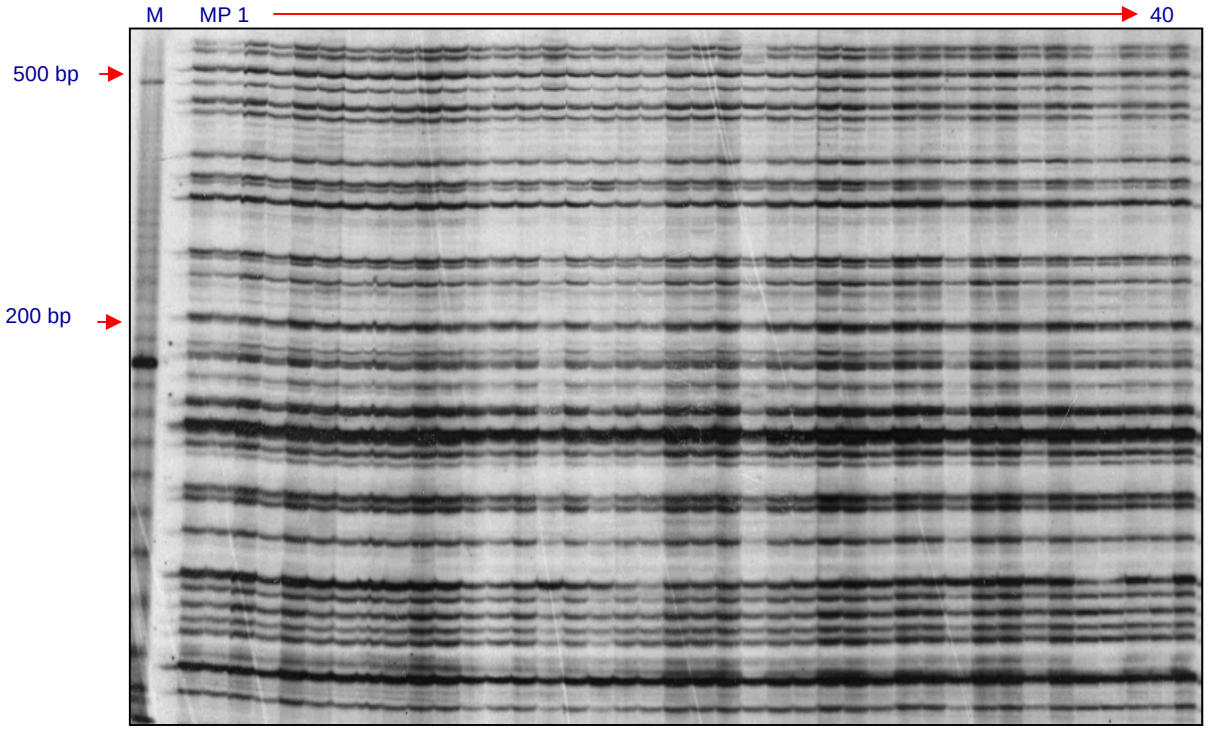

AFLP profile of 40 tissue culture raised plants of *D. hamiltonii* generated with primer combination E-AAC / M-CTG; M = 20 bp ladder plus (Cambrex), MP= Mother plant , 1-40 = Tissue culture raised plant

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Field Performance of TC raised bamboos

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Growth Performance of Somatic Embryo Raised Plantlets in the Fields.

A comparison of 6 month-old rooted plants raised through:

i) Nodal cuttings, *in vivo*.

ii) TCPs produced through somatic embrogenesis.

Transferred these to field in pits – 2'x2'x2' (Row to row distance: 6m)

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Growth Data of 6 Years (July, ’93 to Oct, ’98)

Year of observation	Total no. of culms produced*		Height of the longest culm (cm)		Diameter of the thickest culm** (cm)	
	In vitro Raised	Cuttings Raised	*In vitro* Raised	Cuttings Raised	*In vitro* Raised	Cuttings Raised
1993	18±0.81	3±0.39	270±3.61	32±0.56	1.73±0.09	8.0±0.47
1994	19±0.47	9±0.47	96±0.81	54±0.81	14.0±0.40	12.0±0.30
1995	10±0.47	5±0.47	380±2.14	690±3.03	15.0±0.35	15.3±0.22
1996	12±0.47	11±0.57	380±2.07	**510±0.47**	24.3±0.05	20.0±0.60
1997	12±0.57	10±0.47	**1105±5.05**	830±1.32	16.0±0.49	18.1±0.09
1998	**24±0.82**	**16±0.63**	1260±3.03	860±1.69	**27.5±0.23**	**23.4±0.10**

***Significance at p<0.01 evaluated with regards to control as field raised plants. Values are mean of four replicates with six plants each. **Data on height and thickness of culms was recorded every 12 months in the field after 1993.**

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Bamboos Leaves as Fodder

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Evaluation of Bamboo leaves for nutritive value as cattle feed using rumen contents from 3 adult male cattle

Bambusa bambos	*D. hamiltonii*
B. nutans	*D. hookerii*
B. tulda	*D. strictus*
B. ventricosa	*Melocanna baccifera*
B. vulgaris	*Phyllostachys aurea*
Dendrocalamus asper	*Sasa auricoma*

The results revealed that of all these species, *M. baccifera* proved to be best fodder in terms of maximum rate of gas production, higher *in vitro* dry matter (IVDMTD, 482 gkg -1 DM), organic matter degradability (IVOMTD; 548 g kg -1 OM) and microbial mass (19.6% of DM).

Other promising bamboo cultivars considered as promising fodder for the ruminant live stock were : *D. hookerii, D. hamiltonii and D. asper.*

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Table 1. Average chemical composition (g kg^{-1} DM) of Bamboo leaves (n = 3)

Species	OM	CP	EE	CHO	NDF	ADF	ADL	HC	C	Ash	AIA
B1 (*Sasa auricoma*)	852.0	185.4	32.3	634.3	723.8	434.2	75.0	289.6	359.2	148.0	105.5
B2 (*B. nutans*)	882.5	182.2	24.9	675.4	785.3	438.6	73.1	346.7	365.5	117.5	81.9
B3 (*B. bambos*)	856.2	184.0	26.6	645.6	784.4	443.3	68.8	341.1	374.5	143.8	101.6
B4 (*Phyllostachys aurea*)	841.4	179.8	34.0	627.6	741.7	456.6	79.1	285.1	377.5	158.6	107.6
B5 (*B. tulda*)	867.9	146.1	20.1	701.7	782.8	465.7	84.2	317.1	381.5	132.1	94.3
B6 (*D. asper*)	846.7	176.2	35.6	634.9	762.1	468.8	58.9	293.3	409.9	153.3	98.1
B7 (*B. ventricosa*)	843.5	184.2	16.7	642.6	779.3	521.6	94.3	257.7	427.3	156.5	107.0
B8 (*D. strictus*)	853.3	161.2	13.9	678.2	774.7	532.3	85.9	242.4	446.4	146.7	106.9
B9 (*Melocanna baccifera*)	882.7	191.0	17.3	674.4	653.8	415.8	48.7	238.0	367.1	117.3	54.3
B10 (*D. hookerii*)	914.1	179.2	41.8	693.1	741.4	456.6	71.7	284.8	384.9	85.9	43.8
B11 (*B. vulgaris*)	846.8	193.0	32.2	621.6	765.5	453.2	92.5	312.3	360.7	153.2	110.1
B12 (*D. hamiltonii*)	862.2	203.9	47.3	611.0	731.5	416.6	56.7	314.9	359.9	137.8	94.4
Significance	*	**	**	NS	*	*	**	**	NS	**	**

ADF, acid detergent fiber; ADL, acid detergent lignin; AIA, acid insoluble ash; C, cellulose; CHO, total carbohydrate; CP, crude protein; EE, ether extract; HC, hemicellulose; NDF, neutral detergent fiber; OM, organic matter

The mean values in a column are statistically analysed for differences if any as NS, non-significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$

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Table 2. *In vitro* ruminal digestibility, ammonia-N and partitioning factor (PF) of different bamboo leaves

Bamboo type	24 h Gas (ml g^{-1} DM)	IVDMAD (g kg^{-1} DM)	IVDMTD (g kg^{-1} DM)	IVOMTD (g kg^{-1} OM)	Microbial mass (% of DM)	PF (mg ml^{-1})	ME* (MJ kg^{-1} DM)
B1	99.1	231.3	389.5	409.6	15.82	3.673	6.12
B2	93.1	211.8	276.1	325.6	6.43	3.108	5.98
B3	103.9	187.0	398.5	427.4	21.15	4.055	6.22
B4	91.8	163.0	289.0	297.6	12.6	3.120	5.91
B5	104.4	225.0	293.4	328.4	6.84	2.909	6.04
B6	110.0	273.0	455.5	500.1	18.25	4.294	6.31
B7	85.9	165.2	261.7	270.0	9.65	3.163	5.69
B8	100.2	188.2	299.9	298.1	11.17	3.104	6.04
B9	161.7	286.3	482.4	548.1	19.61	3.217	7.92
B10	129.4	201.0	344.9	439.2	14.39	3.285	6.88
B11	88.7	145.0	261.6	288.9	11.66	3.035	5.83
B12	146.4	297.5	456.1	504.0	15.86	3.271	7.54
Significance	**	**	**	**	**	**	*

1. B1 to B12 represents bamboo types as indicated in column 1 of table 1
2. IVDMAD, in vitro dry matter (DM) apparent digestibility; IVDMTD, in vitro DM true digestibility
3. PF, partitioning factor (ratio of DM truly degraded (mg) to gas volume (ml) at 24 h incubation)
4. *ME (MJ/Kg DM) = 2.20 + 0.136 Gp + 0.057 CP; (ME is the metabolizable energy; CP, crude protein in percent; Gp, the net gas production in ml from 200 mg dry sample after 24 h of incubation) (Menke and Steingass, 1988).
5. The mean values in a column are statistically analysed for differences if any as *P < 0.05; **P < 0.01

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Table 3. *In vitro* ruminal methane (ml/24 h) and fatty acid production (m*M*/30 ml) profile from different bamboo leaves

Bamboo type	CH_4	CH_4%	c2	c3	c4	c4i	c5	c5i	SCFA	c2:c3 ratio	lipo:gluco ratio	NH_3-N (mg $30ml^{-1}$)	Contribution to increased NH_3-N (%)#
B1	8.64	41.04	1.479	0.280	0.092	0.011	0.009	0.019	1.890	5.29	5.14	5.30	16.92
B2	7.96	39.45	1.306	0.244	0.084	0.010	0.004	0.012	1.661	5.35	5.38	4.80	8.50
B3	8.84	40.52	1.399	0.280	0.084	0.013	0.009	0.018	1.802	5.00	4.87	5.43	19.12
B4	8.06	40.81	1.355	0.246	0.085	0.007	0.009	0.019	1.721	5.51	5.28	5.06	13.21
B5	8.18	37.04	1.440	0.271	0.090	0.010	0.011	0.020	1.842	5.31	5.10	4.65	7.37
B6	9.10	39.81	1.398	0.295	0.091	0.011	0.010	0.020	1.826	4.73	4.62	5.20	15.87
B7	7.27	40.49	1.271	0.241	0.080	0.010	0.012	0.018	1.632	5.28	5.02	5.10	13.61
B8	8.58	40.00	1.288	0.254	0.085	0.008	0.013	0.015	1.664	5.06	4.90	4.50	3.91
B9	13.55	39.79	1.429	0.324	0.092	0.011	0.014	0.020	1.890	4.41	4.28	4.33	0.41
B10	10.35	38.45	1.331	0.283	0.090	0.010	0.012	0.018	1.743	4.71	4.57	4.83	9.23
B11	6.96	37.46	1.291	0.237	0.085	0.010	0.009	0.016	1.648	5.44	5.29	5.14	13.53
B12	12.35	40.24	1.368	0.292	0.090	0.011	0.014	0.018	1.793	4.68	4.53	5.33	14.30
Signi-ficance	**	*	-	-	-	-	-	-	*	*	*	*	-

1. B1 to B12 represents bamboo types as indicated in column 1 of table 1
2. c2, c3, c4, c4i, c5 and c5i represents acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acids
3. SCFA-Short chain fatty acids; c2:c3- acetate: propionate ratio; lipo:gluco ratio- ratio between lipogenic and glucogenic SCFA
4. NH3-N, ammonia nitrogen
5. # Substrate contribution to increased NH3-N = (NH3-N at 24 h – NH3-N of blank)/N content in the substrate
6. The mean values in a column are statistically analysed for differences if any as *P < 0.05; **P < 0.01

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Picture of all the authors

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Construction of a Bamboo Museum

covering an area of 3,600 sq. ft at CSIR-IHBT
Palampur -176 061 India

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! Thank You !

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