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IN VITRO PLANT REGENERATION FROM ORGAN CULTURES OF *GMELINA ARBOREA* ROXB.

AMOD KUMAR, MD. SARFARAZ AHMAD[2] AND MD. NASEEM[1]

Plant Tissue Culture Lab., University Department of Botany,
B.R.A. Bihar University, Muzaffarpur-842001, India
1. Corresponding author
2. Deptt. of Botany, Gopeshwar College, Hathwa, Gopalganj

In vitro regeneration of plantlets was obtained using stem (node, internode) and shoot-tip of *Gmelina arborea* as explants. Explants were cultured on Murashige and Skoog's (1962) medium containing 0.8% agar and different combinations and concentrations of auxin and cytokinin. Techniques were developed for multiple shoot formation directly from nodal and shoot-tip explants as well as shoot regeneration from the callus. Kinetin (Kn) alone at 9.3 µM was most effective and induced the formation of direct multiple shoots in culture. 10.7 µM NAA (α−naphthalene acetic acid) and 9.3 µM Kn resulted in shoot differentiation from the callus. Callus was greenish - white, compact, hydrated and crystalline in appearance. Rooting of the excised shoots was obtained on medium (½ MS salts) fortified with IBA (indole-3 butyric acid) and NAA. 2,4-D (2,4-dichlorophenoxy acetic acid) was, however, suitable for callus induction, not for regeneration. Regenerants were genetically identical to the parent.

Key words: Callus induction, *Gmelina arborea*, mature tree, micropropagation, multiple shoots

Forest trees represent renewable natural resources and sustained forest yield whether for lumber and bioenergy is dependent on reforestation programme (Arya *et al.* 1994, Augustine and D'Souza 1997). In this background, micropropagation offers the potential for rapidly increasing tree clones for afforestation and conservation (Rathod *et al.* 2008, Sreelatha *et al.* 2008).

Gmelina arborea Roxb (Fam: Verbenaceae), commonly known as white teak is in high commercial demand for furniture, artificial limb, musical instruments, bridges etc. and has restricted cultivation in our locality. In addition to its high timber value, this plant is used for the treatment of various ailments like tuberculosis, gonorrhoea, cough etc. and is the source of many active compounds (Chopra *et al.* 1956, Satyanarayana and Rao 1986). The conventional methods of multiplication in *Gmelina* are time consuming and season-dependent and percentage of seed germination is also very low. There are relatively few reports available on the clonal propagation of this species (Yang *et al.* 1993, Kannan and Jasrai 1996, Kumar *et al.* 1997, Thirunavoukkarasu an Debata 1998). Young *et al.* (1993) and Kannan and Jasrai (1996) used explants collected from 1-month old *in vitro* seedlings and 3-5 years old *in vivo* grown plants respectively while Thirunavoukkarasu and Debata (1998) used axillary buds as explants collected from young shoots of 6 year old *Gmelina* tree.

It is highly desirable to develop an efficient *in vitro* protocol for mass propagation of this species to meet the growing demand for reforestation and timber industries. Our investigation is based on a separate line using explants collected from mature tree (12 years old) and the cultures were maintained under continuous cool white light (2000 lux) during the whole experiment. As the tissues of mature tree are recalcitrant, the tissue culture studies with explants taken from mature tree are of great significance. Hence, the present study was aimed at *in vitro* cloning of *G. arborea* through direct shoot regeneration using explants collected from *in vivo* grown mature tree (about 12 years old,

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Fig. 1).

MATERIALS AND METHODS

Node (8-10 mm), internode (10 mm) and shoot-tip (10-15 mm) collected from young shoots of mature tree (12 years old) during March to November were used as explants. Explants after washing with running tap water were treated for 2 min in 1% Cetavelon (Cetramide I.P. 20% w/v, isopropyl alcohol B.P. 10% v/v) solution followed by thorough washing in running tap water. They were then surface sterilized with 0.2% $HgCl_2$ solution for 5 min and finally rinsed 3-4 times with sterilized distilled water.

Sterilized explants (node, internode and shoot-tip) were aseptically cultured on MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.8% agar (Difco-Bacto) and various concentrations (2.2 µM to 26.9 µM) and combinations of auxins (2,4-D, NAA) and cytokinins (Kn, BA). The pH of the medium was adjusted to 5.6 prior to autoclaving at 121^0C for 20 min. Cultures (15 ml medium dispensed in each culture tube, borosilicate glass culture tubes, 150 X 25 mm) were incubated at 25 ± 2^0C with constant cool white fluorescent light (2000 lux) during whole experiment. Ten replicates were maintained for each experiment and was repeated once.

Callus induction lasted for 5-6 weeks, sub-culture of callus 5 weeks, shoot regeneration and elongation 5 weeks and rooting 4 weeks, the time of each stage was fixed. Effect of seasonal variation (Mar-May, June-Aug, Sept-Nov, Dec-Jan) on regeneration capabilities of explants was also determined on suitable hormonal combinations (9.3 µM Kn + 10.7 µM NAA, 9.3 µM Kn, Table 3) of culture.

RESULTS AND DISCUSSION

Shoots were obtained directly from nodal and shoot-tip explants after 10 d of inoculation on MS medium supplemented with Kn / BA (6-benzyl adenine) while internodal and nodal segments produced callus on MS medium fortified with NAA + Kn / 2,4-D or 2,4-D+Kn (Table 1,2), different hormones were used within a range of 2.2 µM to 26.9 µM.

Shoot regeneration

Among cytokinins tested, the best result was achieved with 9.3 µM Kn which generated 6-7 shoots/culture and 4-5 shoots/culture from nodal (Fig. 3) and shoot-tip (Fig. 2) explants respectively after 10 d of implantation. Inoculation of shoot-tip on MS medium without any growth hormone resulted in single shoot whereas nodal and internodal segments failed to regenerate. BA at different concentrations (2.2-22.2 µM) could neither improve the number of proliferating shoots nor overall growth of the shoots. Kn in combination with NAA/2,4-D also failed to regenerate shoot from nodal and shoot-tip segments rather caused undesirable callusing (Table 1). Callus was also obtained on shoot regeneration medium fortified with 9.3 µM Kn at the basal end of nodal segment (Fig. 3) after 18 d of culture.

In the present investigation, the superior effect of Kn for high frequency shoot regeneration of *Gmelina* was reported. Perusal of literature, however, reveals the superior effect of BA on shoot regeneration as was observed in *Sterculia urens* (Purohit and Dev 1996) Pineapple (Devi *et al*. 1997) and *Cleome gynandra* (Naseem and Jha 1997). Further, a fine balance of exogenous auxins & cytokinin / cytokinin alone is necessary before successful generation can occur. This was also confirmed in many plants by Dwari and Chand (1996), Naseem and Jha (1997) and Shahzad *et al*. (1999).

Table 1: Effect of different hormones on shoot multiplication in nodal (N) and shoot-tip (ST) cultures of *Gmelina arborea**

Hormones (µM) (MS medium)	% of culture response		No. of shoots/ culture		Other response
	N	ST	N	ST	
BM	-	-	-	-	-
2, 4-D					
4.5	82	79.4	-	1.4 ± 0.3	Hypertrophy & callusing
9.05	90	88.4	-	1.8 ± 0.3	Callus (greenish white)
22.6	92.2	91.6	-	1.0 ± 0.3	Vigorous callus
Kn					
9.3	95	92.5	6.0 ± 0.7	4.5 ± 0.6	Multiple shoots, callus
14	90.4	-	5.2 ± 0.4	-	Shoots, callus
23.2	86.4	83	3.2 ± 0.4	1.6 ± 0.8	Shoots, callus
BA					
8.9	91.4	89.6	3.9 ± 0.3	2.5 ± 0.3	Shoots, poor callus
22.2	90.8	88	2.8 ± 0.5	2.5 ± 0.6	Shoots, callus
NAA + Kn					
5.4 + 9.3	90.3	85	-	-	Vigorous callus
10.7 + 9.3	93.5	91.3	-	-	Excellent callus
2, 4-D + Kn					
4.5 + 9.3	91.2	89	-	-	Good callus
9.1 + 4.7	92.5	88.6	-	-	Good callus
9.1 + 9.3	92.2	90.6	-	-	Good callus

*Data scored at effective hormonal combinations, mean of 20 replicates, growth period - 21 d

Table 2: Response of phytohormones on callus biomass in nodal (N) and internodal (IN) cultures of *Gmelina arborea**

Hormones (µM) (MS medium)	Callus biomass Fresh weight in mg		Other response
	N	IN	
2, 4-D			
9.05	1850 ± 45.7	2030 ± 47.5	-
22.6	2400 ± 55.80	3850 ± 82.2	-
Kn			
9.3	530 ± 10.25	-	Multiple shoots (N)
23.2	370 ± 9.60	-	Shoots (N)
2, 4-D + Kn			
4.5 + 9.3	2800 ± 62.4	3040 ± 55.80	Callus green-white
9.1 + 9.3	2955 ± 65.45	3260 ± 55.84	-
22.6 + 23.2	1710 ± 15.64	1830 ± 30.70	Browning
NAA + Kn			
5.4 + 9.3	3250 ± 70.6	3530 ± 52.20	Callus green
10.7 + 9.3	3300 ± 65.45	3627 ± 72.60	White

*Data scored at effective hormonal combinations, mean of 20 replicates, growth period - 25 d

Table 3: Effect of seasonal variation on regeneration of stem (S) and shoot-tip (ST) segments of *Gmelina arborea***

Age of explant (Duration in month)*	Explant	Total no. of explants treated	Number regene-rating	% regeneration	
Mar-May	S	40	38	94-95	fast growth
	ST	40	37	92.5	fast growth
June-Aug	S	25	22	88	fast growth
	ST	25	21	84	fast growth
Sept-Nov	S	30	23	76.6	slow growth
	ST	30	21	70	slow growth
Dec-Jan*	S	30	14	46.6	retarded growth
	ST	30	13	43.3	retarded growth

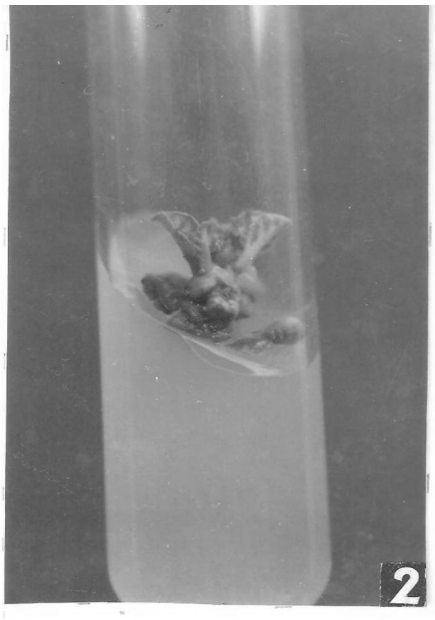
* February not counted

** Explants were taken from 12 years old *in vivo* grown plant, period was counted from the emergence of young shoots after leaf fall (from March); growth period -21 d; media – MS + Kn (9.3 µM), MS + NAA (10.7 µM) + Kn (9.3 µM)

2,4-D (4.5-22.6 µM) was not suitable for shoot regeneration, however, callusing was more frequent with an optimum response at 22.6 µM 2,4-D. The similar effect of 2,4-D was also reported by Harikrishnan and Hariharan (1996) and Shahzad *et al.* (1999) (Table 1, 2). Kn above 23.2 µM had adverse effect on shoot regeneration. *In vitro* raised shoots through direct multiplication were genetically identical to mother plant with deep green leaves.

Callus Induction

Table 1 & 2 show that callus was obtained from internodal and nodal explants (Fig. 3,4) after 10 and 15 d of inoculation respectively on different hormonal regimes. Of the combination tested, the best callus biomass (fresh wt.) and % response were achieved from internodal segment on 10.7 µM NAA + 9.3 µM Kn and 9.1 µM 2,4-D + 9.3 µM Kn after 21 d of culture (Fig. 5). The callus was greenish-white, compact, hydrated and crystalline in appearance. Calli derived from these segments were transferred on to the media with different combination of NAA (5.4-26.9 µM) & Kn (4.7-23.2 µM) upto two passages to determine regeneration potential. No callus mediated regeneration was obtained on any combination except 10.7 µM NAA and 9.3 µM Kn which regenerated few shoot buds (Fig. 6), calli on other combinations



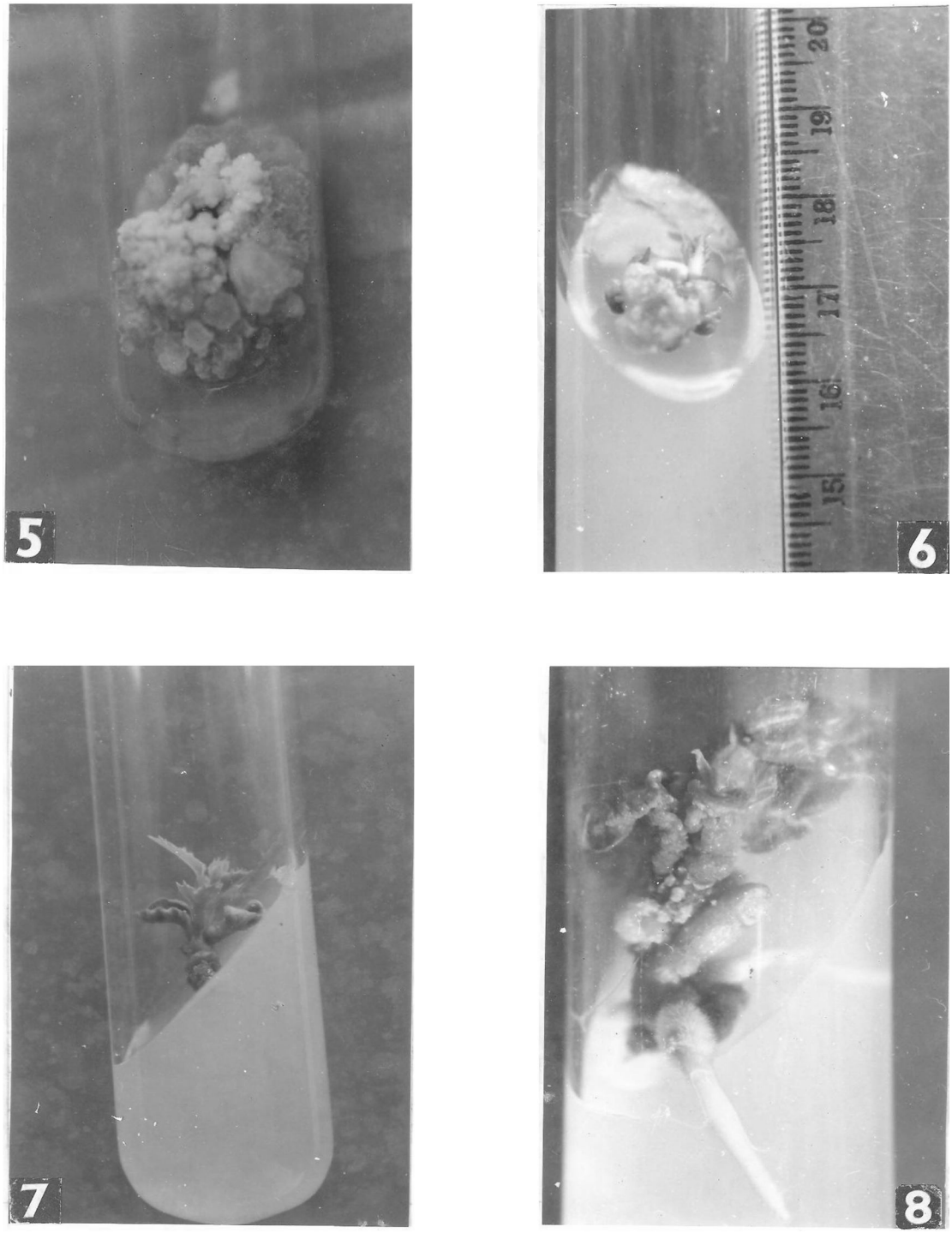
Figures 1-4: 1. *In vivo* grown *Gmelina arborea* (12 years old), 2. 25 d old culture showing shoots formation from shoot-tip culture on MS medium with 9.3 µM Kn, 3. 25 d old culture showing multiple shoots formation from nodal segment on above medium (Fig. 2), mark callus at basal end, 4. Callus induction from both the ends of internodal segment on MS medium containing 10.7 µM NAA and 9.3 µM Kn, 15 d old culture

finally turned brown.

The production of plantlets via callus phase, though has the potential for rapid multiplication, is not desirable because of getting high rates of genetically altered plants (Naseem and Jha 1997, Kumar and Kumar 1998).

Rooting and plantlet formation

Microshoots (2-3 cm) obtained from shoot-tip, nodal segment and regenerative callus were cultured on MS media of full, ½ and ¼ salt strengths supplemented with or without IBA



Figures 5-8. 5. Vigorous callus formation from internodal segment on MS medium containing 10.7 µM NAA and 9.3 µM Kn, 25 d old culture, 6. Regeneration of callus mediated shoot buds on MS medium (sub culture) containing 10.7 µM NAA and 9.3 µM Kn, 7. 21 d old isolated single plant on MS basal medium, 8. Complete rooted plant on medium (1/2 MS salts) with 5.4 µM NAA and 4.9 µM IBA, 25 d old culture; mark hypertrophy of shoot and root hairs.

(4.9-24.6 µM) and NAA (5.4-26.9 µM) singly and in combination. Regeneration of roots was difficult in all the combinations tested, about 50% rooting (stout roots with prominent hairs) were obtained from shoots on MS medium (½ salt strength) fortified with 4.9 µM IBA & 5.4 µM NAA after 15 d of culture but shoots subsequently deformed with hypertrophy (Fig. 8), regenerated roots were in direct contact with vascular tissues of shoots. However, Yang *et al.* (1993) and Thirunavoukkarasu and Debata (1998) reported root formation from *in vitro* shoots in *Gmelina* on MS and McCown's medium respectively simply in presence of IBA. Rooting was also not achieved on hormone free medium, this medium favoured only shoot elongation (Fig. 7). Induction of rooting in culture is, however difficult with woody species as was reported by Debergh and Maene (1981) and Pareek and Mathur (1997). Efforts are in progress to develop an efficient protocol for rooting and acclimatization.

Effect of seasonal variation on regeneration

Age of explants and seasonal variation (counted from the emergence of young shoots after leaf fall) greatly influenced shoot regeneration in culture (Table 3). The frequency of shoot regeneration was promising from explants collected during March to November, however, juvenile explants collected during March to August were most regenerative. This regeneration potential in explants might be due to high endogenous level of hormones and meristematic activity of explants. Influence of explant age and season of explant collection from field grown plants on better culture response was also confirmed by Conger (1987) Naseem and Jha (1994) and Haque *et al.* (1997).

The present investigation indicates that growth and proliferation of shoots from shoot-tip and nodal segments in *Gmelina* can be stimulated easily by using *in vitro* techniques. The protocol for multiple shoot regeneration as described in this paper may be used for rapid clonal multiplication of this species which is otherwise difficult and season dependent to propagate by conventional methods.

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