Indian J. Applied & Pure Bio. Vol. 28(2), 181-192 (2013).

In vitro plant regeneration of *Bauhinia variegata* Linn. through direct and indirect shoot bud development from different explants on MS solid and liquid media

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ABSTRACT

The conventional methods of propagation of *Bauhinia variegata* Linn., sexual as well as vegetative, are impeded with many problems that restrict their multiplication on a large scale. A fairly long time gap between pod formation and maturation hampers adequate supply of seeds in natural conditions. Therefore, to meet the ever increasing demand of planting material, an *in vitro* regeneration protocol of this plant species is very important. Cotyledons, hypocotyls and leaves were used as explants for *in vitro* plant regeneration of *Bauhinia variegata* through direct and indirect shoot bud development on MS solid and liquid media supplemented with various concentrations of cytokinin and auxin in combination or singly with coconut water. A very high number of shoot buds were regenerated both in liquid and semi solid cultures in all explants. However, liquid media proved its supremacy over semi solid culture.

ABBREVIATIONS

2iP- N^6-(Δ^2-isopentenyl)adenine, ANOVA- analysis of variance, BAP- N^6- benzylaminopurine, CW- Coconut water, DMRT- Duncan's multiple range test, Kn- Kinetin, MS- Murashige and Skoog medium, NAA- α-Napthalene acetic acid, PGR- Plant growth regulator, SE- standard error

Key words : 2iP, BAP, Bauhinia variegata, Cotyledon, Hypocotyl, In vitro Regeneration.

Bauhinia variegata Linn., commonly called orchid tree, grows in humid tropical countries, especially in the acid and degraded soils to which it can restore fertility owing to its natural nitrogen fixing ability[1]. With the expanding biomass requirements due to population explosion, the demand for quality and quantity of firewood based products is continuously increasing leading to shrinking of forest cover with the species of *Bauhinia*. Besides, an infusion from the bark is used as an astringent, tonic, and for treating scrofula, skin disorders and ulcers. The decoction of the roots is used in dyspepsia and act as an antidote to snake poison[12]. Further, phytochemical studies revealed the presence of several flavonoids[16]. The anti-inflammatory and antibacterial activity of all the extracts of *Bauhinia variegata* has also been reported[32,31]

The conventional methods of propagation of *Bauhinia*, sexual as well as vegetative, are impeded with many problems that restrict their multiplication on a large scale. A fairly long time gap between pod formation and maturation hampers adequate supply of seeds in natural conditions[20]. Micropropagation of this tree species offers a rapid means of producing clonal plant stock for afforestation, woody biomass production and conservation of elite germplasm[25]. Several investigations regarding developing suitable methodology for rapid propagation of elite germplasm of *Bauhinia* species from mature explants have been reported[7]. In order to meet the ever increasing demand of planting material, an *in vitro* regeneration protocol using seedling[38] and mature nodal explants in *Bauhinia vahlii*[10] was developed.

The protocol for direct regeneration of plantlets would provide a suitable system for their genetic transformation[9]. Although regeneration of plantlets of *Bauhinia variegata* through somatic embryogenesis has been developed[5], regeneration via shoot bud production using cotyledon, hypocotyl and leaf explants has not been reported so far. Moreover, a comprehensive study of the effects of various plant growth regulators as well as other media supplements on the direct and indirect plant regeneration via organogenesis of *Bauhinia variegata* in different cultural systems has not been made yet.

Therefore, the present study dealt with the direct and indirect *in vitro* plant regeneration through shoot bud development from cotyledons, hypocotyls and leaf explants of *Bauhinia variegata* emphasizing the impact of supplemented growth regulators and physical state of the medium. The plant regeneration through direct differentiation of shoot buds would be an effective measure for conservation purpose of this plant species while the callus mediated organogenesis would possibly help in maintaining optimum genetic diversity causing minimum damage to the natural population. The protocol offers an efficient and practicable system for genetic transformation and clonal propagation for plantation forestry.

Seeds of *Bauhinia variegata* were collected from the forests of Santiniketan, Birbhum, West Bengal, India. They were washed in 2% (V/V) detergent solution "Teepol" (Qualigens, India) and subsequently surface sterilized in 0.1% (w/v) aqueous mercuric chloride solution for 12 minutes. After rinsing 4 times with sterile distilled water seeds were transferred to moist cotton beds in 250 ml Erlenmeyer flask and incubated in the culture room for 7 days under 10 hours of photoperiod of 37.5 μ mol. $m^{-2}s^{-1}$ light intensity. Cotyledons, hypocotyls and the leaves were excised from the 7-day old axenic seedlings and were inoculated onto MS basal medium[28] supplemented with 2iP (0.5 to 4 $mg.dm^{-3}$) combined with 15% v/v CW for *de novo* formation of shoot buds. Shoot buds produced at higher concentrations of 2ip were sub-cultured on MS media supplemented with lower levels of 2iP (0.1 $mg.dm^{-3}$) or with complete omission of 2iP for subsequent leafy shoot emergence. Similar experiments were carried out in liquid MS media through filter paper bridge technique.

Cotyledon explants were inoculated onto MS basal media supplemented with various concentrations and combinations of NAA (0.2-4.0 $mg.dm^{-3}$) and BAP (0.2- 4.0 $mg.dm^{-3}$) for initiation of callus and subsequent shoot bud regeneration through organogenesis. Friable callus after 60 days, were sub-cultured on MS media with various concentrations of BAP (0.2 to 4.0 mg dm^{-3}) in combination with a constant level of NAA (0.2 $mg.dm^{-3}$) for regeneration of shoot buds. Shoot buds were transferred to both on the solid and liquid media with lower levels of BAP (0.1-1.0 $mg.dm^{-3}$) and NAA (0.2 $mg.dm^{-3}$) for emergence of leafy shoots.

Ten replicates were used per treatment. The pH of the media was adjusted to 5.8 prior to autoclaving. The media were solidified with 0.8% w/v agar. Routinely, 25 ml molten medium was dispensed into culture tube (25x 150mm), plugged with non-absorbent cotton and subsequently sterilized at 121ºC and 102 $x10^{-6}$ $kg.m^{-2}$ pressure for 15 min. Cultures were incubated at 25 ± 2ºC under a light intensity 37.5 μ $mol.m^{-2}s^{-1}$ under a photoperiod of 10 hours.

The regenerated shoots were excised from the parent culture and transferred to half strength MS semi-solid medium with different concentrations of NAA (0.5to 4 $mg.dm^{-3}$) for induction of roots. *In vitro* grown plants with well developed root systems were first washed under running tap water and transferred to plastic cups containing sterile sand-soil mixture (1:1) with adequate water. Surviving plants were finally transplanted to the soil on earthen pots. The process of transfer and gradual acclimatization to the natural conditions were stringently monitored.

The experimental units were assigned to "randomized complete block design" with single replicate per block. The mean values of different morphogenetic responses were shown along with their respective standard errors (SE) and were analyzed by analysis of variance (ANOVA). After obtaining a significant F value ($\alpha = 0.05$) the treatment means were separated by Duncan's Multiple Range Test (DMRT) in case of single Factor ANOVA only. Statistical analyses were performed according to Little and Hills[22].

Direct shoot bud regeneration in Bauhinia variegata:

The effects of 2iP on shoot bud regeneration from de-embryonated cotyledon, hypocotyl and excised leaf are shown in Table-1. Direct differentiation of shoot buds was monitored in various explants both in the PGR supplemented and PGR-free media. PGR-free control did not exhibit shoot bud formation in any of the explants. However, considerable enhancement in response was achieved with the application of 2iP combined with 15% v/v CW. In all the instances the explants that failed to initiate shoot bud differentiation remained in a swollen green condition throughout the culture period, the excised cotyledon showed best response, in presence of 2mg dm^{-3} 2iP combined with 15% v/v CW (Figure 1: E). Compared to control, the use of 2iP exhibited marked morphogenetic changes, although very high concentration suppressed the shoot bud regeneration potential of the same explants. In case of excised leaf and hypocotyl explants (Figure 1: F), best response was also achieved at 2mg dm^{-3} 2iP and the maximum number of shoot buds were 90 ± 1.41 and 38 ± 0.16 respectively.

Cultures grown in liquid media through filter paper bridge technique showed higher frequencies of shoot bud regeneration compared to semisolid media having the same PGR levels (Table-1 and Figure 1: G, H and I). In general, a very high number of shoot buds were regenerated in liquid cultures in all explants and the best responses were observed at 2iP 2 mg.dm^{-3}.

Emergence of leafy shoots (Figure 1: D) from shoot buds primarily depended upon the applied PGRs. Complete omission of 2iP exhibited lower frequencies of leafy shoot development compared to low levels of 2iP (0.1mg.dm^{-3}). Similar trend of responses were obtained both in semi-solid and liquid systems with minor variations. The application of lower concentration of 2iP proved to be effective, while kinetin and BAP at low levels failed to initiate the leafy shoot emergence (Table-2). NAA at 0.1 mg.dm^{-3} induced sporadic callus formation.

Callus mediated organogenesis:

Formation of callus was not observed in PGR free medium as well as in the media supplemented with only NAA or BAP (Table 3). Combined application of NAA and BAP showed considerable amount of callus formation (Figure 1: A and B). Growth of the callus was faster when the BAP level was kept lower than NAA. High level of NAA differentiated roots from the callus. NAA at lower level (0.2 mg.dm^{-3}) combined with various levels of BAP (0.2-4 mg.dm^{-3}) were applied for differentiation of shoot buds from callus. The shoot bud differentiation (Figure 1: C) from callus was found optimum at BAP 2 mg.dm^{-3} combined with NAA (0.2 mg.dm^{-3}) while very high concentration of BAP suppressed this augmentative effect (Table-4). The emergence of leafy shoots from organogenic callus mass (Table 5) was always higher in liquid cultures (57.6 ± 2.5) compared to semi-solid medium (43.2 ± 1.5).

Induction of roots on the regenerated shoots, excised from the parent culture, was observed at all the concentrations of NAA tested (0.5 to 4 mg.dm^{-3}). Best response was obtained at 1 mg.dm^{-3}. *In vitro* grown plants with well developed root systems were successfully transplanted to the sand-soil mixture (1:1) with adequate water on plastic cup. Survivals of the transplanted plants were more than 90%. Transplanted plants showed normal seedling morphology and growth (Figure 1: J).

Direct organogenesis:

Development of shoots in *Bauhinia*

variegata took place through *de novo* differentiation of shoot buds from cotyledon, hypocotyl and leaf explants. In general, such type of morphogenesis is common in leguminosae[18]. However, further development of shoot buds into complete plantlet is a rare phenomenon. Similar pattern of growth and morphogenesis was followed in case of callus mediated organogenesis. Khattar and Mohan Ram[21] reported the production of shoot buds in *Sesbania grandiflora* and *Sesbania sesban* using various plant organs as explants. Direct regeneration of shoot buds has also been reported in a number of tree legumes viz., *Albizia lebbeck, Dalbergia paniculata* using different cytokinins at various levels[33,15].

Although swelling occurred in various explants under different cultural conditions, *de novo* differentiation of shoot buds and further development of plantlets did not occur easily and prudent application of PGRs was necessary to achieve that. Thus it was observed that exogenous PGRs were crucial for improved development of shoot buds as well as for plantlet formation which is in conformity with the observation of Banerjee *et al*.[6, 4] in another leguminous species.

In the experimental time frame, less than half of the explants developed swelling in the control, indicating a dormant stage of the explants. However, formation of shoot buds was triggered by the application of 2iP. Therefore, it appears that the inactiveness of explants in the control could be due to lack of adequate endogenous growth regulators[35]. Although shoot bud development was common for each of the treatments, the differential response of the explants to the exogenous 2iP was apparent from the differences in the number of shoot buds developed from the respective explants. Previous reports on the culture of trees exhibited the effectiveness of BAP but the present investigation clearly demonstrated the supremacy of 2iP. The development of adventitious shoot buds has also been reported in presence of 2iP in leaf explants of *Garcinia mangostana* and *Pinus strobes*[11,15].

Development of adventitious shoot buds directly from cotyledon explants was more frequent than those of the hypocotyls as well as the leaf, possibly because greater amount of nutrients are generally stored in the first formed leaves of the plant[8]. When the embryonic axis is decapitated at the junction of the cotyledon, mobilization of the food from the cotyledon to the embryonic axis is interrupted and thus the ample amount of food is retained in the cotyledons. The high frequency of shoot bud development, in general, suggests the presence of required amount of necessary growth regulators in the excised cotyledons. This is in accordance with the report in *Vigna radiata*[3].

Adventitious buds were also induced on hypocotyls segments in presence of BAP in *Picea* and their number increased with relative closeness of the hypocotyl segments to the shoot apex[19]. This suggests that a factor was possibly transported from the seedling apex to the hypocotyls, which in turn promoted the development of adventitious shoot buds. It is also evident in their report that CW stimulated the development of shoot buds. In general, CW is known to promote callus formation and also plays a suppressive role in the fo rmation of shoot buds. However, in our investigation the results were contrary to the general trend.

CW is an undefined complex mixture of organic substances that has been successfully employed for culturing different plant species[2,39]. The variable response of the different explants might be due to specific action of coconut as reported in orchids[35]. Although exogenous 2iP is regarded as the most crucial factor for the induction of shoot buds, very high concentration of 2iP proved ineffective as well. However, it could be pointed out that 2iP is an absolute prerequisite for the direct induction of shoot buds of this plant species. The response is enhanced with the addition of CW.

Leafy shoot emergence from the regenerated shoot buds could be achieved in MS basal medium supplemented with low level of 2iP which is in agreement of Maity *et al.* (2005)[23]. The optimum percentage of leafy shoot emergence was recorded at 0.1mg.dm^{-3} 2iP combined with 15% (v/v) CW. However, the incorporation of NAA plays no beneficial effect; instead it exhibited an inhibitory effect. Similar responses have been recorded in *Arachis hypogaea*, *Vanilla sp.*[26,24,4] and thus considered as a common phenomenon among the herbs and trees as well. In nature, this type of increase in number of seedlings per explants would definitely allow the plants to compensate to some extent the recalcitrant nature of the genus.

The same experiment when performed in liquid medium through filter paper bridge technique, better response was achieved. The optimum number of shoot buds generated in liquid media was higher in all explants compared to those formed in semi- solid media (Tables 3and 4). Comparative assessment of liquid and semi-solid cultures revealed that the former system was more economic in terms of saving of time and media requirements. Besides it was easy to retrieve uniform plantlets from liquid media than from the solid ones.

Callus mediated organogenesis:

Plantlet regeneration from induced callus was achieved only in excised cotyledons while the callus originated from other explants failed to differentiate shoot buds. Cytokinin as BAP could induce shoot bud in these calli, which is in agreement with Venkatachalam *et al.*[40]. BAP proved better than other cytokinin tested in terms of induction of shoot bud and subsequent multiplication, which corroborated the results of Vishwanath and Jayanthi[41]; Gupta *et al.*,[17] and Banerjee *et al.*,[4].

The variability in regeneration frequency shown by the different calli reflects a variable interaction of physiologically heterogenous explants with the tissue culture medium[29]. Since experimental observations were made at discrete intervals, it was difficult to determine the exact time of initiation of shoot buds from callus. Therefore, further study is required to verify that by critically monitoring the responses of the explants throughout the culture regime. Therefore, the organogenic response of calli of *Bauhinia variegata* varied markedly depending on the source of callus line *i.e.*, the type of explants used for the induction of callus and the supplements of basal medium, particularly the type and concentrations of cytokinin; which is supported by Sen[36] and Banerjee *et al.*,[4] in other plant species.

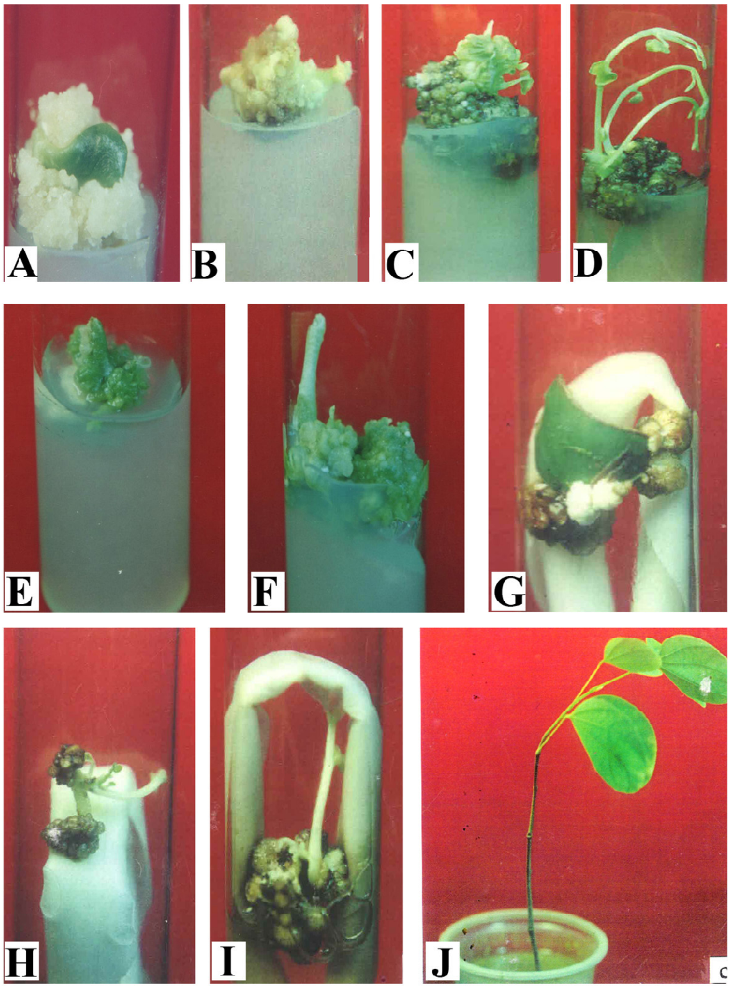

Figure captions

Figure: 1 A- J Direct and indirect regeneration of shoot buds of *Bauhinia variegata*

A. Friable callus from cotyledon explant
B. Friable organogenic callus
C. Numerous shoot buds with micro shoots developed on the surface of organogenic callus
D. Emergence of leafy shoots from tiny shoot buds
E. Direct shoot bud regeneration from cotyledon explant
F. Direct shoot bud regeneration from hypocotyl explant
G. Direct shoot bud regeneration from cotyledon explant on filter paper bridge
H. Direct shoot bud regeneration from hypocotyl explant on filter paper bridge
I. Direct shoot bud regeneration from leaf explant on filter paper bridge
J. Plantlet transferred to plastic cup

Table-1. Direct organogenesis in *Bauhinia variegata* cultured on MS solid and liquid media supplemented with various concentrations of 2iP and 15% CW after 60 days of incubation

2iP (mg.dm^{-3})	Mean number of shoot buds ± SE					
	Cotyledon		Hypocotyl		Leaf	
	Solid	Liquid	Solid	Liquid	Solid	Liquid
0.0	0	0	0	0	0	0
0.5	52.00±0.85^{d}	67.00±0.66^{d}	27±0.06^{b}	39.2±4.35^{b}	18±1.03^{d}	25.50±2.43^{d}
1.0	74.00±1.07^{c}	114.7±10.6^{c}	30±1.98^{b}	52.3±7.13^{a}	54±1.42^{c}	69.50±17.7^{b}
2.0	180.0±4.34^{a}	212.2±26.6^{a}	38±0.16^{a}	57.0±5.05^{a}	90±1.41^{a}	119.2±26.6^{a}
3.0	122.0±0.93^{b}	155.0±1.22^{b}	17±1.13^{c}	34.8±0.78^{b}	67±0.66^{b}	82.00±0.73^{b}
4.0	74.00±1.52^{c}	125.0±1.22^{bc}	11±0.86^{d}	17.0±1.36^{c}	17±0.66^{d}	52.30±7.13^{bc}

Mean values followed by same letter are not significantly different at 0.05 levels (DMRT)

Table-2. Leafy shoot emergence from shoot buds of *Bauhinia variegata* on MS solid and liquid media supplemented with different PGRs and 15% CW after 30 days of incubation

PGR(mg.dm^{-3})	Percentage of leafy shoot emergence ±SE	
	Solid media	Liquid media
0	25.35±1.18^{ab}	35.60±1.8^{b}
2iP-0.1	33.08±1.95^{a}	42.78±3.63^{a}
2iP-0.2	25.09±4.42^{ab}	33. 90±2.20^{a}
2iP-0.5Kn-0.1	16.9±2.11^{b}	27.33±1.67^{c}
No response	No response	
BAP-0.1	No response	No response
2iP-0.1+ NAA-0.1	Callus formation	Callus formation

Mean values followed by same letter are not significantly different at 0.05 levels (DMRT)

Table-3. Induction of callus from cotyledon explant of *Bauhinia variegata* (response recorded after 30 days of incubation)

PGR (mg.dm^{-3}) NAA	BAP	Frequency of callus formation (%)	Nature of callus	Amount of callus*
0.0	0.0	No response	-	-
0.0	0.2	No response	-	-
0.0	0.5	No response	-	-
0.0	1	No response	-	-
0.0	2	No response	-	-
0.0	4	No response	-	-
0.0	8	No response	-	-
0.2	0	No response	-	-
0.5	0	No response	-	-
1	0	No response	-	-
2	0	No response	-	-
4	0	No response	-	-
8	0	No response	-	-
1.0	0.2	100	Friable white callus	++
1.0	0.5	100	Friable white callus	++
1.0	1.0	100	Friable white callus	+
2.0	0.2	100	Friable white callus	+++
2.0	0.5	100	Friable white callus	+++
2.0	1.0	100	Friable white callus	++
4.0	0.2	100	Friable white callus	++++++
4.0	0.5	100	Friable white callus	++++++
4.0	1.0	100	Callus with profuse rooting	++++++
8.0	0.2	100	Callus with profuse rooting	++++
8.0	0.5	100	Callus with profuse rooting	++++
8.0	1.0	100	Callus with profuse rooting	++++
8.0	2.0	100	Callus with profuse rooting	++++

* '+' sign denotes relative amount of callus (eye-estimated); '-' sign denotes no callus formation

Table-4. Regeneration of shoot buds from callus through organogenesis in *Bauhinia variegata* after 60 days of incubation

PGR (mg.dm^{-3}) BAP	NAA	Mean no. of shoot buds ± SE
0.0	0.0	No response
0.0	0.2	Soft, brownish and friable callus
0.2	0.0	Soft, brownish and friable callus
0.5	0.0	Soft, brownish and friable callus
1.0	0.0	Soft, brownish and friable callus
2.0	0.0	Soft, brownish and friable callus
4.0	0.0	Green nodular callus
0.2	0.2	46.30 ± 9.47
0.5	0.2	72.30 ± 9.17
1.0	0.2	121.5 ± 7.42
2.0	0.2	123.1 ± 7.22
4.0	0.2	65.60 ± 5.97

Table-5. Regeneration of plantlets through intermediary callus phase in *Bauhinia variegata* after 30 days of incubation

PGR(mg.dm^{-3})		Percentage of leafy shoot emergence ±SE	
BAP	NAA	Solid media	Liquid media
0.0	0.0	28.5±1.58	44.35±4.97
0.1	0.2	43.2±1.4	57.6±2.5
0.2	0.2	34.4±0.64	48.6±1.5
0.5	0.2	16.5±0.34	21.4±0.74
1.0	0.2	0	0

Mean values followed by same letter are not significantly different at 0.05 levels (DMRT)

The growth pattern has clearly demonstrated that a combination of auxin and cytokinin was absolute prerequisites for the induction and development of callus in *Bauhinia variagata*. Growth of the callus was enhanced when the amount of auxin was kept higher in the medium compared to cytokinin (Figure d). The present investigation has clearly demonstrated that higher ratio of exogenous NAA and BAP induced rooting which marked the end of morphogenetic development[30]. It is reasonable to conclude that improved efficiency of auxin in association with cytokinin is attributed to their role in DNA synthesis and mitosis[37].

Considering the results, it was revealed that upon sub-culturing the callus in a BAP (0.2mg.dm^{-3}) supplemented medium, shoot buds were regenerated (Figure e). Such response corroborated with earlier observations[14,13,4] in other plant species. BAP alone at higher concentration induced the development of greenish nodular callus while stimulation of shoot bud differentiation was achieved by supplementing the medium with NAA (0.2 mg dm^{-3}). Auxins and cytokinins might have acted synergistically to promote either cell division or cell expansion depending upon other factors within the cell, which react with other hormones. Thus the presence of NAA was crucial for the regeneration of shoot buds from callus[27].

Conversion of small shoot buds into leafy shoots was achieved in PGR free medium (Figure f). Supplementing the medium with low concentration of NAA and BAP stimulated the frequency of leafy shoot emergence. This experiment was further extended to compare the response in solid and liquid medium and proved the supremacy of liquid medium. The possible reason for this could be that the agar has high adsorptive capacity and hinders the uptake of cytokinin and other chemicals[8]. The inhibitory response at higher concentration of agar was probably due to the accumulation of invertase immediately below the explants[34].

The shoot buds and shoots obtained from the present *in vitro* culture method could therefore be used for the improvement of individual clones of this species through direct and indirect organogenesis. The techniques described here for the propagation of this commercially important species could be effectively applied both for rapid propagation as well as for conservation purposes.

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