In vitro clonal propagation of bael (*Aegle marmelos* Corr.) CV. CISH-B1 through enhanced axillary branching

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ABSTRACT

Rapid clonal micropropagation protocol of *Aegle marmelos* (L.) Corr. cv. CISH-B1 was achieved by nodal stem segment of mature bearing tree. Three centimeter long shoots having one axillary bud excised from 10-15th nodal region of shoots during September gave quick *in vitro* bud burst (5.33 days) when cultured on MS medium supplemented with BAP, 8.84 μM + IAA 5.7 μM . The maximum number of proliferated shoots (9.0/explant) were obtained on same medium supplemented with BAP 8.84 μM + IAA 5.7 μM . The micro shoots were rooted (100 %) on $\frac{1}{2}$ strength MS medium supplemented with IBA 49.0 + IAA 5.7 μM . *In vitro* rooted plants were acclimatized on autoclaved coconut husk containing $\frac{1}{2}$ strength MS plant salt mixture and under shade net house (50 % shade 70-80 % RH). The plants were established in the field after acclimatization. The micropropagated plants were tested for its genetic fidelity using 13 RAPD, 3 ISSR and 2 DAMD primers. Profile obtained by all the three Single Primer Amplification Reaction (SPAR) technique from mother tree and micropropagated plants revealed genetic integrity of micropropagated plants with that of mother tree. [Physiol. Mol. Biol. Plants 2008; 14(4) : 337-346] E-mail : chauhanu@rediffmail.com

Key words : Micropropagation, *Aegle marmelos*, PCR, ISSR, RAPD, DAMD, SPAR

Abbreviations : IAA - Indole-3-acetic acid, IBA - Indole-3-butyric acid, BAP - Benzyl amino purine, ADS - Adenine sulphate

Bael (*Aegle marmelos* Corr.), originated in India, is more prized for its medicinal virtues than its edible quality. It belongs to family Rutaceae. The tree grows wild in dry forests on hills and plains of central and southern India, Burma, Pakistan, Bangladesh, Sri Lanka, Northern Malaya, Java, and Philippine Islands. Various parts of the bael plant are being used in many Ayurveda and Unani patented drugs in India for treatment of a variety of diseases. The fruits and roots of *A. marmelos* Corr. posses antiamebic and hypoglycaemic activity (Chopra *et al.*, 1956; Ponnachan *et al.*, 1993). The alkaloid 'aegeline' present in the leaf is effective antiasthmatic agent (Harvey, 1968). The bark contains tannin and the coumarin, aegelinol; also the furocoumarin, marmesin; umbelliferone, a hydroxy coumarin; and the alkaloids, fagarine and skimmianine. Marmelosin (C₁₃H₁₂O₃) derived from the pulp is laxative and diuretic. The bael

fruit is commonly multiplied by seed in nurseries and the seedlings show great variation in morphological and biochemical characters due to heterozygous nature of plant. However, its commercial orcharding is not expanding at a faster rate due to severe shortage of planting material. Conventional methods of its propagation (budding and soft wood grafting) are season dependent, slow and labor intensive. Micropropagation techniques have been gainfully employed in mass multiplication of various fruit species (Mishra *et al.*, 2005). Clonal micropropagation of *Aegle marmelos* from mature nodal explant has been developed by Varghese *et al.* (1993) and Ajithkumar and Seenii (1998). But the real physiological state of explant (position of node from where explant is excised, size of explant, number of buds to be retained per explant and season of collection etc.) have not been studied which greatly influences the repeatability of any micropropagation protocol. Further, none of the micropropagation protocols have included testing of genetic fidelity of *in vitro* raised

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plants, which gives an incomplete picture of any micropropagation protocol. However, most of the protocols are genotype dependent particularly in perennial trees. It is therefore imperative to develop rapid multiplication technique using micropropagation tool in improved cultivar of bael cv. CISH-B1.

MATERIALS AND METHODS

Plant material

Twigs were collected from 14 year old trees of *Aegle marmelos* Corr. cv. CISH-B1, grown in the germplasm block at R.B. Road campus of Central Institute for Subtropical Horticulture, Rehmankhera, P.O. Kakori, Lucknow, India. The 30-40 cm long shoots were brought to the laboratory in an aqueous solution of ascorbic acid (566 μM) and citric acid (474.8 μM).

Explant and growth conditions

Three different sub-experiments were undertaken to optimize ideal explant. Shoots obtained from different nodal position (1-5, 6-10, 11-15, 16-20th nodes), length (1, 2, 3 and 4 cm) and number of buds/explant (1, 2, and 3). The nodal explants were washed thoroughly with 2-3 drops of Tween-20 (a liquid detergent) and were rinsed in running tap water for 30 min. To control the bacterial and fungal contamination, explants were treated with 5.24 μM carbendazime + 303.5 μM rifampicin in 100 ml of distilled water and 2-3 drop of Tween-20 for one hour. The segments were washed under running tap water for 30 minutes. To overcome the problem of *in vitro* oxidative browning, the explants were given a pre-treatment with an antioxidant solution comprising ascorbic acid (566 μM), Polyvinylpyrrolidone (250 $\times 10^{-2}$) and citric acid (474.8 μM) for 1 hours. The explants were then rinsed with sterile double distilled water and brought under laminar air flow hood for surface sterilization. The explants were treated with 3.68 μM HgCl_2 for 6 minutes and washed with sterile distilled water (3-4 times) and dried on pre sterilized filter paper sheets. There after, the explants were placed on MS medium (Murashige and Skoog, 1962) fortified with treatments were supplemented with BAP (0, 2.21, 4.42, 8.84, 13.26 μM), Kinetin (0, 2.32, 4.64, 9.28, 13.92 μM), IAA (2.85 and 5.7 μM). All the treatments were added 0.8 % (w/v) agar (Hi-media, India) and 3 % (w/v) sucrose (Qualigens, Glaxo Fine Chemicals, India). The pH of the medium was kept 5.8 prior to autoclaving. Observations were recorded on morphogenetic characters such as

days taken for bud break, number of axillary shoots per explant, number of leaf per explant and length of axillary shoots (cm) after 30 days of culture of the explants.

Microshoots obtained *in vitro* were subjected to auxin(s) such as IBA (49.0 and 73.5 μM), IAA (2.85, 5.7 and 8.55 μM) in MS basal medium (Full and $\frac{1}{2}$ strength) supplemented with 3 % sucrose, 0.8 % agar. Observations were recorded on per cent rooting, number of roots, length of roots, diameter of roots and weight of roots. All cultures were incubated at 25 ± 2 °C and were exposed to a photoperiod of 16/8 hours light and dark cycling under 50 $\text{mmol m}^{-2} \text{s}^{-1}$ quantum flux density provided by cool day light fluorescent tubes (40 W, Philips, India) with 70 % $\pm$ 5 % relative humidity maintained in the culture room.

Acclimatization studies

Eight week old rooted shoots, were transferred to glass bottles (450 ml volume) containing different carrier substrates viz. autoclaved soil, soil + sand + FYM (1:1:1) and coconut husk. The rooted shoots were placed in each carrier substrate and replicated thrice. All the treatments were supplemented with $\frac{1}{2}$ MS plant salt mixture (Hi-Media, India). Initially the bottles were incubated in the culture room under 50 $\text{mmol m}^{-2} \text{s}^{-1}$ quantum flux density and at 25 °C temperature for about 15 days, and then they were shifted to plant growth chamber where illumination was increased to 100 $\text{mmol m}^{-2} \text{s}^{-1}$ quantum flux density with high humidity (70-80 %). The plants were then shifted to shade net house (50 %) provided with misting. The caps of the bottles were loosened gradually and the plants were shifted to pots. Once the plants develop 4-6 leaves, they were shifted to field during the month of July and August. The data pertaining to growth and survival was recorded periodically.

Biochemical Studies

The regenerated plants of bael were analyzed for different biochemical parameters viz. chlorophyll, reducing sugar, nitrate reductase activity and protein at various regeneration stages (*in-vitro*, acclimatized, field established and control). The chlorophyll (a, b and total) was estimated as per the method described by Arnon (1949). The reducing sugar was estimated by the method suggested by Ranganna (1986). Nitrate reductase activity (NRA) was measured by the method of Srivastava (1975) The total soluble protein was estimated as per the method suggested by Lowry *et al.* (1951).

DNA isolation

Total genomic DNA was isolated from 2 g each of micropropagated (acclimatized), mother and wild plant leaf tissue as described by Dellaporta and Wood, 1983 (CTAB method). The DNA pellet was dissolved in TE (200 µl) and stored at -20 °C.

PCR using RAPD, ISSR and DAMD primers

The RAPD reactions were carried out according to Williams *et al.* (1990) for bael. The ISSR-PCR conditions were carried out as proposed by Gupta *et al.* (1994) with minor modification. Total reaction were carried in total volume of 25 µl.

The gel electrophoresis was carried out using a submarine horizontal agarose slab gel as described by Sambrook *et al.* (1989). The PCR product obtained were separated on 1.5 agarose gel, stained with ethidium bromide and documented in a gel documentation system (Alpha Innotech Corporation, USA).

RESULTS

Effect of season on explant collection

It is evident from data that explants excised during September-October was found ideal because 85.7 % explant shows in vitro bud burst where as bud burst frequency reduced in other months. Least in born contamination was observed in September-October.

Effect of position of node, size and number of buds of explant

The apex portion of explants (1-5th node) did not respond in culture due to very soft and succumbs toxic effects on sterilents. Whereas, those from 11-15th nodal stem segment proved to be the best explants where most of the explants showed bud burst (87 %) in just 6.67 days. Bud burst frequency reduced and time taken for bud induction enhanced with hard and brown explants from 15-21 nodal segment.

Size and number of axillary buds present in the explant significantly affects the bud induction frequency. 3 cm long having one axillary bud gave quicker bud induction being 6.67 days in both observations.

Shoot multiplication

Quicker bud burst (5.33 days) were recorded (Table-1) when explants were inoculated in MS+BAP 8.84 µM +IAA 5.7 µM, while more number of axillary shoot

Fig. 1. In vitro clonal propagation of *A. Marmelos* Corr. A. Shoot bud initiation. B. Microshoot proliferation. C. Rooting D. Clonal plant established in the field 10 months after transplantation.

(5.33 shoots/explant) were recorded when explant was incubated on and MS+ Kinetin 13.92 µM +IAA 5.7 µM. However, MS+BAP 8.84 µM /I+IAA 5.57 µM containing proliferating medium (Fig-1) produced 9.67 microshoots/explant. This treatment also gave maximum leaves/explant (15.33) and highest shoot length (3.1 cm).

In vitro rooting of isolated shoots

From perusal of data (Table-2), among the different strength of media, IBA and IAA levels; ½ strength MS+IBA 49.0 µM and IAA 5.7 µM produced the 100 % rooting as compared to other treatments. While more number of roots (2.33), root length (5.0 cm) and root diameter (2.70 mm) were recorded with Full MS +IBA 49.0 µM +IAA 5.7 µM.

Hardening of in vitro plantlets

Among the various carrier substrates (Table-3) tested for acclimatization, coconut husk fortified with ½ strength MS plant salt mixture proved to be ideal

Table 1. Effect of phytohormones on *in vitro* shoot multiplication of *Aegle marmelos* Corr.

Treatment (μM)	Shoot-bud induction			Proliferation		
	Shoot bud initiation (Days) length	No. of axillary shoots/explant	Length of axillary shoots (cm)	Average no. of micro shoots/explant	Average no. of leaves/explant	Average of micro shoots/explant (cm)
Control	12.33	1.0	0.47	1.33	2.33	0.57
BAP 2.21+IAA 2.85	8.33	1.67	3.63	3.67	5.67	1.23
BAP 2.21+IAA 5.7	7.67	1.33	3.63	4.67	6.0	1.43
BAP 4.42+IAA 2.85	7.33	1.33	3.80	4.67	9.33	2.33
BAP 4.42+IAA 5.7	7.33	2.0	3.70	5.33	10.67	2.43
BAP 8.84+IAA 2.85	7.0	2.33	4.13	7.67	12.67	2.73
BAP 8.84+IAA 5.7	5.33	5.0	5.43	9.67	15.33	3.1
BAP 13.26+IAA 2.85	6.33	3.33	4.83	7.33	13.67	2.3
BAP 13.26+IAA 5.7	6.33	2.67	4.57	8.0	14.33	2.33
Kinetin 2.32+IAA 2.85	7.67	1.33	3.37	3.33	4.67	0.83
Kinetin 2.32+IAA 5.7	7.33	1.67	3.57	3.67	6.0	1.07
Kinetin 4.64+IAA 2.85	7.0	1.33	3.80	4.0	9.0	1.5
Kinetin 4.64+IAA 5.7	6.67	2.0	3.60	4.33	9.67	1.63
Kinetin 9.28+IAA 2.85	6.33	2.0	3.60	5.33	13.0	2.22
Kinetin 9.28+IAA 5.7	6.33	2.33	3.77	6.0	15.0	2.33
Kinetin 13.92+IAA 2.85	6.0	2.67	4.20	6.0	14.0	2.4
Kinetin 13.92+ IAA 5.7	6.0	5.33	4.43	8.67	15.33	2.77
SE(m) $\pm$	NS	0.31	0.18	0.44	0.56	0.07
CD (0.05)	NS	0.87	0.50	1.25	1.59	0.20

substrate as maximum plant survival (83.33 %) in this substrate. Plant grew taller (5.53 cm), produced more leaves (7.66) and roots (2.33) in coconut husk followed by Sand+Soil+FYM and Soil during the study.

Biochemical analysis of regenerated plants

The regenerated plants showed significant effect on different growth conditions as regard to biochemical contents (Table-4). A gradual increase in chlorophyll synthesis was observed during acclimatization and field condition. Highly chlorophyll synthesis was recorded when plants were shifted from photo-heterotrophic mode

of nutrition to photo-autotrophic (acclimatization) mode of nutrition. Maximum chlorophyll synthesis was observed in *in vitro* regenerated field established plants (0.118 mg/g). Reducing sugar was recorded lower (3.22 mg g⁻¹) in *in vitro* grown plants then *in vivo* grown plants. The nitrate reductase activity (NRA) was recorded higher (1.35 n mol, NO₂, h⁻¹g⁻¹ FW) in *in vitro* grown plants and decreased gradually during acclimatization and least in *in vivo* (control) grown plants. It is evident from the data that protein levels remain low during acclimatization (0.048 $\mu\text{g g}^{-1}$) as compared to control plant.

Table 2. Effect of different concentrations of IBA and IAA using along with full strength and half strength MS medium on root induction of isolated shoots of *Aegle marmelos* Corr.

Treatment (μ M)			Rooted explant (%)	No. of roots	Root length (cm)	Root diameter (mm)	Root weight (gm)
MS	IBA 0.0	IAA 0.0	0.0	0.0	0.0	0.0	0.0
		IAA 2.85	15	1.33	1.3	1.23	0.002
		IAA 5.7	25	1.33	0.18	1.33	0.013
		IAA 8.55	20	1.67	2.06	2.13	0.003
	IBA 49.0	IAA 0.0	45	1.0	2.17	1.33	0.002
		IAA 2.85	60	1.0	3.9	2.23	0.006
		IAA 5.7	90	1.33	5.0	2.70	0.022
		IAA 8.55	61.67	2.33	2.9	1.70	0.007
	IBA 73.5	IAA 0.0	41.67	1.0	3.57	1.15	0.005
		IAA 2.85	45	2.0	4.8	2.11	0.014
		IAA 5.7	50	1.33	4.53	1.84	0.016
		IAA 8.55	20	1.33	2.83	1.91	0.015
$\frac{1}{2}$ MS	IBA 0.0	IAA 0.0	0.0	0.0	0.0	0.0	0.00
		IAA 2.85	20	1.33	2.47	1.80	0.003
		IAA 5.7	30	2.0	1.83	2.10	0.002
		IAA 8.55	40	1.0	2.97	2.43	0.007
	IBA 49.0	IAA 0.0	65	1.0	3.97	2.32	0.028
		IAA 2.85	80	1.0	3.07	1.88	0.016
		IAA 5.7	100	2.33	4.07	1.88	0.043
		IAA 8.55	81.67	1.67	2.97	2.01	0.034
	IBA 73.5	IAA 0.0	41.67	1.33	2.8	1.95	0.009
		IAA 2.85	51.67	1.67	2.33	1.65	0.016
		IAA 5.7	60	1.33	3.67	1.41	0.015
		IAA 8.55	41.67	1.67	2.90	1.16	0.026
CD	Media		2.0	NS	NS	NS	0.002
	IBA		2.4	0.33	0.36	0.22	0.002
	IAA		2.82	NS	0.41	0.26	0.003
	Media X IBA		3.46	0.46	0.50	0.32	0.003
	Media X IAA		3.99	NS	0.58	0.37	0.004
	IBA X IAA		4.89	0.66	0.71	0.45	0.005
	Media X IBA X IAA		6.91	NS	1.01	0.63	0.007

Table 3. Effect of substrate on acclimatization of micropropagated plants of *Aegle marmelos* Corr.

Substrate	Plant Height (cm)	No. of leaves/plant	No. of roots/plant	Length of roots (cm)	No. of shoots/plant	Survival of plants (%)
Soil	4.33	3.00	1.50	0.899	1.60	31.66
Soil+Sand+FYM	4.63	7.33	1.66	1.40	2.33	63.33
Coconut husk	5.53	7.66	2.33	1.60	3.00	83.33
SEm $\pm$	0.156	0.430	0.319	0.745	0.396	2.886
CD (0.05)	0.540	1.487	1.102	0.257	1.371	9.976

Table 4. Biochemical analysis of nodal stem segment-regenerated plants of *Aegle marmelos* Corr.

Treatment Condition	Biochemical Attributes					
	Chlororophyll			Reducing sugar (mg g ⁻¹)	Nitrate reductase assay (n mol, NO ₂ , h ⁻¹ g ⁻¹ FW)	Protein (µg g ⁻¹)
	'a'	'b'	'a+b' (mg/g)			
<i>In vitro</i>	0.022	0.020	0.042	3.227	1.353	0.048
Acclimatized	0.048	0.045	0.093	4.890	1.117	0.028
Field grown	0.061	0.057	0.118	7.427	0.683	0.063
Mother/control	0.127	0.099	0.226	9.55	0.330	0.071
CD (0.05)	0.014	0.014	0.013	1.135	0.203	0.008
SEm ±	0.004	0.004	0.004	0.343	0.061	0.002

Fig. 2. DNA fingerprinting pattern of mother plant (lane 1). Micropropagated plants (lane 2-10), Lane M is lambda DNA-Eco R I+Hind and Lane M1-100 bp ladder and Line M1 -1 and Lane 100 bp Ladder and C- Negative control obtained by : A. RAPD (OPA 2), B. ISSR (Mp2) and C. DAMD (33.6)

Molecular analysis of regenerated plants

A total of 13 RAPD (Fig-2, a), 3 DAMD (Fig-2, b) and 2 ISSR (Fig-2, c) primers selected to test genetic fidelity of micropropagated plants of bael c.v. CISH-B1 recorded that they exist no genetic variation in mother tree and micropropagated plants.

DISCUSSION

Micropropagation of mature trees employing vegetative explants has been a difficult task and lagging behind that of herbaceous plants due to various factors, like juvenility vs. maturity, inherent slow growing habit, exogenous and endogenous infection, presence of phenolic compounds, long complex life cycles, great genetic variations, etc. (Bonga and Durzan, 1986; Durzan, 1985; Zimmerman, 1985; Bajaj, 1991 and 1997). It is well established that *in vitro* propagation of plant species is influenced by several factors, like genotype, age and source of initial tissue/organ which in turn are related to their endogenous hormonal status (George, 1993).

Most of the hardwood species produce phenolic compounds after wounding (George and Sherrington, 1984), the same problem of leaching of phenols and browning of explants was also observed in *A. marmelos*. The phenolic substances on oxidation gets converted into quinines which actually affect tissue blackening and inhibit new *in vitro* morphogenetic responses in plants, particularly in tree species. To check the phenolic exudation, explants right from the time of collection to inoculation were kept submerged either in water or antioxidant solution comprising ascorbic acid, citric acid and PVP as it is well known that reduction in the direct contact of explants with oxygen reduces the rate of oxidation of phenols at the wounded sites (Elmore *et al.*, 1990). The explants were also given a pretreatment with antioxidant solution for one hour. Since, PVP, a polyamide, is known to be the most potent antioxidant, as it absorbs phenols through hydrogen bonding and thus prevents their oxidation. Such antioxidant has been

used by many workers to control browning of explant of neem (Gautam *et al.*, 1993). Ajithkumar and Seeni, 1998 found citrate and ascorbate as potent agent to control oxidative browning. However, our results clearly indicate that PVP was found effective in controlling oxidative browning in *A. marmelos*.

Nodal stem segment were found to be the best explant. The reason for suitability of nodal stem segment was the presence of protected axillary buds, which during surface sterilization do not get damaged. Nodal stem segment proved to be a better explant (*A. marmelos*) as suggested by Ajithkumar and Seeni (1998).

Besides, explants of mature bael tree exhibit the problem of exogenous and endogenous contamination. In our study mercuric chloride (3.68 μM) for 6 min was found to be most effective treatment for surface sterilization of nodal stem segments in bael. Similar results were also reported in *Crataeva nurvala* (Sharma and Pandey, 1996) and *Myrica esculenta* (Bhatt and Dhar, 2004). Establishment of aseptic cultures of bael was not possible round the year since significant seasonal fluctuation influence the explant viability in culture. Explants collection season is one of the important factor in the establishment and growth of *in vitro* cultures (Tisserat, 1985; Siril and Dhar, 1997; Dhar and Upreti, 1999 and Bhatt and Dhar, 2004). Explant collected during September-October resulted in best response, this period showed not only maximum bud break but low infection. One to three centimeter long shoots are generally used in tissue culture of litchi (Chandra and Padaria, 1999) and guava (Mishra *et al.*, 2007). Moderate hard explant (11-15th node) has been found best in aonla micropropagation (Mishra *et al.*, 1998) having single bud proved to be the best explants showing a significant earliness in bud burst and increase in percentage of bud burst with maximum number of axillary shoots. The effect of explant size on growth and differentiation of cultures has been reported in *A. marmelos* (Surana, 1982) and *Tacomela undulate* (Vaishnawa, 1983).

BAP 8.84 μM + IAA 5.7 μM was found to be most prolific combination of the treatments with regard to number of shoots, leaves and length of axillary shoots of *in vitro* established culture. Among the two cytokinins (BAP, Kinetin), BAP was found to be most effective cytokinin in inducing multiple shoot formation (Dewan *et al.*, 1992). However, a combination of an auxin IAA with BAP augmented multiplication of shoots. The synergetic effect of auxin along with cytokinins on shoot multiplication and shoot bud induction has been reported

by several workers (Ajithkumar and Seeni, 1998; Kaur *et al.*, 1998; Nodye *et al.*, 2003; Bhatt and Dhar, 2004; Chand and Singh, 2004).

Microshoots incubated on $\frac{1}{2}$ strength MS medium fortified with IBA 49.0 μM + IAA 5.7 μM significantly enhanced the root induction. The number, length, diameter and weight was found to be significantly higher under this treatment. Successful rooting in regenerated shoots is a prerequisite for micropropagation/ cloning. In some reports, half-strength MS medium is used as basal medium for rooting (Ajithkumar and Senni, 1998; Joshi and Tengane, 1996). Also, there are reports regarding to use of full-strength MS medium supplemented with auxin for rooting in neem (Eeswara *et al.*, 1998).

The *in vitro* grown plants usually show rapid wilting when transferred to *ex vitro*, if care is not taken to maintain high humidity in their new environment. Susceptibility to wilting has been attributed to the several impairment of water maintenance mechanisms (Fila *et al.*, 1998) and poor regulation of leaf transplantation due to thin cuticle, lack of leaf epicuticular waxes (Al-Ahmad *et al.*, 1998) and poor stomatal regulation.

Coconut husk as carrier substrates have marked effect on the plant height, number of leaves, number of roots, length of roots, number of shoots/explant and their survival per cent. This indicated that coconut husk was effective carrier substrate for acclimatization. The interphase of inorganic salt solution allows the restoration of photosynthetic process as well as the mechanism of stomatal opening and closing, deposition of epicuticular wax over leaves while the plants have free availability of essential availability of essential mineral salts because of liquid state of nutrient medium. The *in vitro* raised plants showed a uniform luxuriant growth with sturdy stem and good foliage when transplanted in soil

Clonal propagation and preservation of elite genotype, selected by their superior characteristics, require high degree of genetic uniformity amongst the regenerated plants. The occurrence of somaclonal variation is a drawback for both *in vitro* cloning as well as germplasm preservation method. Therefore, it is of immense significance to assure the genetic uniformity of *in vitro* raised plants at an early stage.

In case of nodal stem segment regenerated plants, lack of variation, gets a support from the fact that organized meristems are considered to be resistant to genetic changes (Shenoy and Vasil, 1992). Rout *et al.*

(1998) in ginger obtained monomorphic RAPD profiles for micropropagated plants regenerated from axillary bud proliferation. RAPD markers has recently been included in the *in vitro* studies to ascertain the genetic uniformity of tissue cultured plants, like *Pandanus amaryllifolius* (Gangopadhyay *et al.*, 2004), *Acacia mangium* (Nanda *et al.*, 2004) and *Foeniculum vulgare* (Benniei, *et al.*, 2004). ISSR techniques in case of pineapple to ascertain the genetic fidelity of plants produced through microshoots after storage in encapsulated form.

Acclimatization procedures have been attempted to increase *ex vitro* survival of plantlets upon transplanting (Dani & Hughes, 1995, 1997). Maximum mortality of micropropagated plants occurs during acclimatization phase because plantlets undergo a rapid and extreme change in physiological functioning when they are removed from *in vitro* condition to *in vivo* condition. In order to devise an efficient acclimatization procedure, it was imperative to investigate some of the crucial biochemical parameters during various cultural stages, which contributes towards growth and development of plants.

In the present experiment levels of protein remain low during *in vitro* phase of regeneration. The *in vitro* plants are generally photosynthetically incompetent (Preece and Sutter, 1993) and the cost of protein biosynthesis in terms of energy requirement remains higher if plants are photosynthetically inactive. Lower chlorophyll levels of *in vitro* plants clearly suggest lower or negligible rate of photosynthesis due to heterotrophic mode of nutrition that ultimately limits the biosynthesis of protein. The lower rate of metabolism of reduced nitrogen to the site of protein synthesis could be another reason for low protein synthesis. However, this needs to be further investigated. In general, total protein content decreased under sudden heat stress during acclimatization (Yali *et al.*, 2005). Synthesis of normal proteins in either soluble or membrane from usually decreased under heat stress conditions (Key *et al.*, 1981). A decrease in soluble protein content with increasing temperature has been detected in other species (Zhou *et al.*, 1995). Therefore it can be concluded that supplementation of essential nutrients during acclimatization and photoautotrophic development of micropropagated plant can increase survival rate. *In vitro* plants showed higher levels of reducing sugars. In micropropagation process, carbohydrate reserves are built up in leaves by manipulating sucrose in shoot multiplication stage prior to acclimatization. Nitrate reductase (NR) is one of the most important metalloflavoprotein regulatory enzyme

associated with the process of nitrate assimilation and plant growth. It was observed that the highest NR activity was observed during *in vitro* phase, which decreased significantly during acclimatization. Since, NR is substrate inducible enzyme (Haynes, 1964); the presence of high amount nitrate substrate in tissue culture medium probably augments the NR activity during *in vitro* phase. Reduced NR activity during acclimatization phase clearly suggests supplementation of nitrogenous compound during *in vitro*.

The genetic uniformity was also confirmed using molecular markers. There were no changes in the banding pattern observed in tissue culture plants as compared with that of mother plant, we conclude that our micropropagation protocol for bael can be carried out for a considerable length of time with no risk of genetic instability. The plants are being evaluated at field level.

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