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Optimization of *in vitro* propagation of jamun variety Konkan Bahadoli

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ABSTRACT

Micro-propagation is a vital technique for round-the-year clonal multiplication of plants. Jamun is an indigenous fruit tree with great pharmacological importance. Two explants *viz*., shoot tips and nodal explants were cultured from January to May. The shoot regeneration from nodal explants was tested using 6-benzyl amino purine (BAP; 1.0-4.0 mg l^{-1}) and α-naphthalene acetic acid (NAA; 0-0.25 mg l^{-1}), while rooting was investigated with NAA (0.5-2.0 mg l^{-1}). Maximum explant establishment was recorded in May. The nodal explants showed better establishment response (50.0%) than shoot tips (40.7%). The maximum shoot regeneration (72.16%) and earliest evocation response from nodal segments (14.0 days) were obtained with combinations of BAP (2.0 mg l^{-1}) and NAA (0.25 mg l^{-1}). Silver nitrate ($AgNO_3$ @ 3.0 mg l^{-1}) enabled shoot proliferation. Maximum rooting occurred on the medium containing 0.5 mg l^{-1} NAA. The *in vitro* plants were hardened on the potting mixture of cocopeat (4): perlite (1): vermiculite (1).

Key words: *Syzygium cumini*, $AgNO_3$, Growth regulators, Micro-propagation, Nodal explant.

INTRODUCTION

Jamun (*Syzygium cumini* Skeels) is an important but underutilized indigenous fruit tree of Myrtaceae family. It originated in India, Burma, Ceylon and Andaman Islands (Zeven and de Wet, 23). The edible pulp of its fruits is rich in polyphenols, anthocyanins and minerals like potassium and calcium (Ghosh *et al*., 3). Apart from its nutritional importance, the tree also has a tremendous pharmacological significance. Every plant part of the tree has traditionally been utilized for curing one or the other ailments (Ayyanar and Subash-Babu, 2). However, medicinal importance of *jamun* is better known from the ability of its seeds to cure diabetes (Ayyanar and Subash-Babu, 2). Further, its ability to grow better in saline and shallow waterlogged conditions (Hebbara *et al*., 6) makes it a prospective tree for afforestation of salt affected areas. Despite multiple utilities, most of the *jamun* plantation is still of seedling origin and mainly found as avenue trees on the roadside or as windbreak rows in the orchards. This may be due to the unavailability of superior genotypes. In recent years, many improved varieties of *jamun* have been identified. Konkan Bahadoli is very promising as it has bold fruits and high TSS content (Singh *et al*., 20). For large scale cultivation of a fruit variety, a huge number of planting materials are required. Conventionally, *jamun* is propagated by seeds or vegetative means. The seeds of *jamun,* though are reported to be polyembryonic still, all the embryos of a seed do not germinate (Sivasubramaniam and Selvarani, 21), thus, liable to produce variant plant stand, which is undesired for a commercial grove. On the other hand, vegetative propagation can only be performed during a specific period of the year. Micro-propagation is an ideal means of year round, rapid multiplication of true-to-type plants in *Syzygium* (Raju and Divya, 16). For clonal propagation of a variety, the regeneration protocols should be optimized using meristematic explants like shoot tips and nodal explants (Jain and Babbar, 7; Naaz *et al.*, 15). Apart from explant type, explant collection period and genotype influence woody trees' in vitro propagation response (Martini *et al.*, 12; Gomes *et al.,* 4). Though micro-propagation studies using meristematic explants have been performed earlier in *jamun* (Jain and Babbar, 7; Naaz *et al.*, 15; Rathore *et al.*, 17), as such there is no report on micro-propagation of *jamun* variety Konkan Bahadoli. Hence, this study was conducted to identify the responsive explant, suitable period of explant collection and culture media for *in vitro* propagation of *jamun* variety Konkan Bahadoli.

MATERIALS AND METHODS

The present study was conducted at the Tissue Culture Laboratory of PAU- Dr. JC Bakhshi Regional Research Station, Abohar during 2020-21. The 20-30 cm long current season shoots were collected from 5-year-old Konkan Bahadoli trees and thoroughly cleaned with plain water. The nodal segments (2-3

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cm size) with an axillary bud (s) or shoot tips of 1-2 cm length were prepared from these shoots. The so prepared shoot apices and nodal explants were soaked in an antifungal and antibacterial mixture of 1.0% Carbendazim 50% WP + 0.01% Streptocycline for half an hour followed by 0.1% mercuric chloride ($HgCl_2$) treatment for 2.0 and 5.0 minutes, respectively. The disinfected explants were rinsed in sterile water thrice to remove the $HgCl_2$ residues.

The full strength MS medium (Murashige and Skoog, 14) was used as the basal medium. The media were fortified with different growth regulators for a desired *in vitro* response. The pH of the media was set to 5.8 before adding the solidifying agent clerigar (0.5%) (Himedia, Mumbai, India). The media was sterilized at 121°C temperature, 15 lbs/inch^2 pressure for 20 min. To find the ideal period for culture initiation, the explants were cultured during January-May on MS medium enriched with 6-Benzyl amino purine (BAP, Sigma-Aldrich, USA) @ 2.0 mg l^{-1}. For shoot regeneration, the nodal segments were inoculated on MS medium fortified with BAP (1.0, 2.0, 3.0 and 4.0 mgl^{-1}) and α-naphthalene acetic acid (NAA, Sigma-Aldrich, USA) (0, 0.25 mg l^{-1}). For shoot multiplication, 2.0-2.5 cm long regenerated shoots were excised and subcultured on the medium containing an anti-ethylene compound, silver nitrate ($AgNO_3$) (1.0 to 4.0 mg l^{-1}). Five different doses of NAA (0, 0.5, 1.0, 1.5 and 2.0 mg l-1) were tested for rooting of micro-shoots. The cultures in all the experiments were incubated at 25±2°C under 16-hour photoperiodic cycle managed by white light of 2,000-3,000 lux intensity. The data on per cent establishment and per cent explant browning was taken after 25 days of culture. The shoot regeneration and multiplication data were noted after 35 days of culture. The data on rooting (%), root number and root length were taken after 70 days of culturing.

The experiments in the study were performed in a completely randomized block design (CRD), with three replications per treatment. For the experiments on culture establishment and shoot initiation, 18 explants formed a replication, while for shoot multiplication and rooting, 10 shoots and 7 shoots were used per replication. Statistical analysis of data was performed using ANOVA and the treatment means were separated by Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ in RStudio (4.0.2) software (R Studio Team, 18).

RESULTS AND DISCUSSION

Prior knowledge of the appropriate time for culture initiation is important for pursuing micro-propagation from field trees. In the culture cycle of January-May, the maximum explant establishment (for both nodal segments and shoot tips) was obtained in May (Table 1). This period has also been reported as suitable for culture establishment of guava, another fruit tree of Myrtaceae family (Amin and Jaiswal, 1). This month, the higher establishment in May was related to the lowest microbial contamination for the two explants (shoot tips: 53.66%; nodal segments: 46.26%) (Fig. 1).

The lower microbial contamination in this month could be due to its higher temperature that retards pathogen proliferation. For pomegranate micro-propagation, the lowest contamination was reported

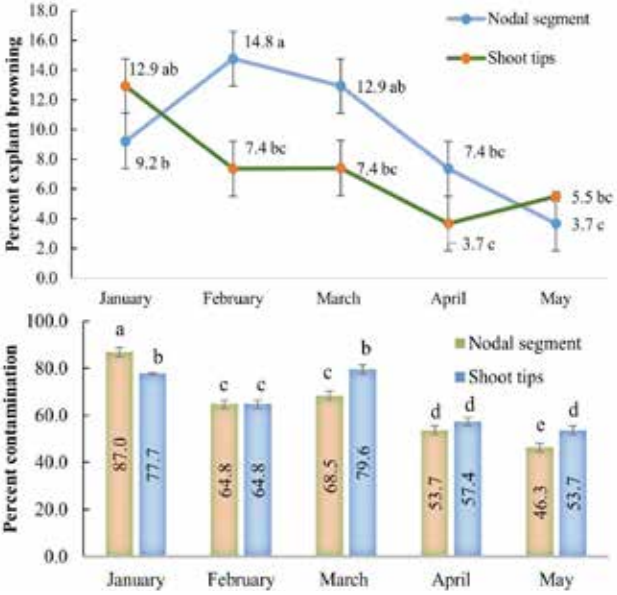

Fig. 1. Influence of culturing months on per cent contamination and per cent browning of two explants. The results are compared based on the interactive critical differences of two factors (Explant × month of culture). The bars with different alphabets differed statistically at $P \leq 0.05$.

Table 1. Influence of explant type and month of culture on per cent culture establishment.

Month	Culture establishment (%)		
	Nodal segment	Shoot tip	Mean
January	1.83±1.83[f#]	9.23±1.87[e]	5.53±3.70[e]
February	20.33±1.87[d]	27.70±0.00[c]	24.01±3.68[c]
March	18.46±1.87[d]	13.00±1.83[e]	15.73±2.73[d]
April	38.80±0.00[b]	38.80±0.00[b]	38.80±0.00[b]
May	50.00±0.00[a]	40.70±1.87[b]	45.35±4.65[a]
Mean	25.90±8.40[a]	25.90±6.45[a]	

LSD ($P \leq 0.05$) Explants (A)=NS, Culturing months (B)= 2.38, Interaction of two factors (A×B) = 4.13

[#]The result values (means ± standard error) tagged with different alphabets differed statistically at $P \leq 0.05$. The explants were cultured on MS medium containing 2.0 mg l-1 BAP for culture establishment. The experiment was set up as factorial experiment with explant and month of culture as two factors.

in May under Abohar (South-West Punjab) conditions (Kumar *et al.,* 8). This month, the culture establishment response of nodal segments (50.0%) was significantly higher than shoot tips (40.7%). Similar to our results, the superiority of nodal segments over shoot tips have also been demonstrated in *jamun* (Naaz *et al.*, 15) and other crops like grapes (Singh *et al.*, 19), guava (Amin and Jaiswal, 1) and natural *Malus* hybrid (Martini *et al.*, 12). The per cent browning of two explants was also low in April and May (Fig. 1).

The evocation of shoots is the first *in vitro* response in explants containing pre-formed meristems. Combinations of BAP and NAA were tested to find out the best media for shoot regeneration in nodal segments. No shoot induction occurred on basal MS medium, while in the sole BAP treatments, shoot regeneration response varied according to its concentration in the medium. Maximum shoot regeneration (61.06%) occurred in the presence of 2.0 mg l-1 BAP; beyond this, the regeneration response was reduced. The supplementation of NAA (0.25 mg l^{-1}) with BAP further improved the regeneration and decreased the time of shoot evocation. Maximum shoot regeneration response (72.16%) and the earliest shoot initiation (14.33 days) were noted with BAP (2.0 mg l^{-1}) and NAA (0.25 mg l^{-1}) (Table 2, Fig. 2a).

The induction of shoots in the presence of BAP verified that cytokinins are essential for releasing buds dormancy in the meristematic explants (Naaz *et al.*, 15). We used BAP as the sole cytokinin for shoot initiation based on the published reports of its superiority over other cytokinins like kinetin and adenine sulphate (Jain and Babbar, 7; Naaz *et al.*, 15; Raju and Divya, 16). BAP is an excellent cytokinin due to its quick metabolization by plant tissues or its ability to synthesize other natural hormones in the reference plant tissue (Malik *et al.,* 11). The complementation of BAP with NAA (0.25 mg l^{-1}) proved synergistic in our study as it improved per cent shoot regeneration and elicited early shoot evocation response. Similarly, Raju and Divya (16) also found an invigorating effect of auxin (IBA) and BAP for *in vitro* propagation of *Syzygium densiflorum*. The better shoot regeneration response may be attributed to the cross-talk of the two hormones in the meristem.

On the one hand, cytokinin maintains meristematic activity, while the auxins at lower concentrations activate the buds (Muller and Leyser, 13). The shoot number increased significantly with the rise of BAP concentration up to 3.0 mg l^{-1} and declined at 4.0 mg l^{-1}. The maximum number of shoots (3.33/ explant) was obtained with 3.0 mg l^{-1} BAP, which was statistically on par with BAP (2.0 mg l^{-1}) + NAA (0.25 mg l^{-1}). Our results also validate the finding of Naaz *et al.* (15), who also noted a decreased regeneration response at higher doses of BAP. The minimum number of shoots (1.33/ explant) was obtained with 1.0 mg l^{-1} BAP.

The shoots were sub-cultured on the medium containing 2.0 mg l-1 BAP for shoot multiplication.

Table 2. Influence of BAP and NAA additions on *in vitro* shoot regeneration.

Treatments[#]		Days to regeneration	Shoot regeneration (%)	Shoot number
BAP (mg l^{-1})	NAA (mg l^{-1})			
0	0	0.00±0.00^{e}[##]	0.00±0.00^{f}	0.00±0.00^{d}
1.0	0	20.67±0.89^{b}	29.56±1.87^{de}	1.33±0.33^{c}
2.0	0	18.33±0.33^{c}	61.06±3.20^{b}	2.00±0.58^{bc}
3.0	0	21.00±0.58^{b}	34.96±1.67^{cd}	3.33±0.33^{a}
4.0	0	23.33±0.89^{a}	22.16±3.20^{e}	1.33±0.33^{c}
1.0	0.25	20.33±0.33^{b}	35.10±3.70^{cd}	1.67±0.33^{c}
2.0	0.25	14.33±0.67^{d}	72.16±3.20^{a}	3.00±0.58^{ab}
3.0	0.25	18.33±0.33^{c}	38.84±3.20^{c}	2.33±0.33^{abc}
4.0	0.25	23.00±0.58^{a}	27.74±3.20^{de}	2.00±0.58^{bc}

[#]MS was used as the basal medium. [##] The result values (means ± standard error) tagged with different alphabets differed statistically at $P \leq 0.05$.

Fig. 2. *In vitro* propagation of *jamun* variety Konkan Bahadoli. a. Shoot regeneration from the nodal segment on MS medium fortified with BAP (2.0 mg l^{-1}) + NAA (0.25 mg l^{-1}), b. Proliferation of micro-shoots on MS + BAP (2.0 mg l^{-1}) + $AgNO_3$ (3.0 mg l^{-1}), c. rooted micro-shoots on MS + NAA (0.5 mg l^{-1}), d. acclimatized Konkan Bahadoli plantlets on a potting mixture of cocopeat (4): vermiculite (1): perlite (1), e. grown up Konkan Bahadoli plant in polybag.

However, no new shoots or shoot growth was observed in this medium (data not presented). This medium was then supplemented with an anti-ethylene compound, $AgNO_3$. The rate of shoot multiplication continuously improved with elevation in the level of $AgNO_3$ from 1.0 to 3.0 mg l^{-1} but decreased significantly after that at 4.0 mg l^1 (Fig. 3). The highest rate of shoot multiplication (2.87) was noted with 3.0 mg l^1 $AgNO_3$ (Fig. 2b). The shoot proliferation capability of $AgNO_3$ may be due to is ethylene suppressing effects under *in vitro* systems (Mahmoud *et al.,* 10). The average shoot length was highest in the medium containing 2.0 mg l^1 $AgNO_3$, which decreased at higher doses.

The cultured shoots did not induce roots on basal MS medium, while in NAA supplemented media, the rooting took place only in the second cycle in 61-70 days (Table 3). The maximum rooting response (57.36%) and maximum number of roots (3.0) were recorded with 0.5 mg l^1 NAA (Fig. 2c). The maximum length of roots (0.63 cm) was noted with 1.0 mg l^{-1} NAA, which was statistically similar to 0.5 mg l^1 NAA but better than all other treatments. But, in all the NAA-containing media, intervening callus was observed at the shoot base. The leaf senescence was also observed in the medium containing NAA (1.5-2.0 mg l1). In contrast, at 2.0 mg l^1 NAA, the whole shoot turned into callus, followed by rooting, indicating that NAA above 1.0 mg l^1 is the higher dose for *in vitro* rooting of this variety.

NAA is reported as a strong auxin for *in vitro* rooting (Lal *et al.*, 9). Naaz et al. (15) found the best rooting response in *S. cumini* with 5.0 µM NAA. In our study, though NAA-induced rooting, the rooting frequency was sub-optimal (57.36%), and the duration of root initiation was also quite long. Further, at concentrations higher than 1.0 mg l^{-1}, NAA induced leaf fall. Higher auxin is known to elicit

Table 3. Effect of NAA supplementation of MS medium on *in vitro* rooting.

N A A (mg l^1)	Days to root initiation	Rooting (%)	Number of roots per shoot	Average root length (cm)
Control (0.0)#	0.00±0.00$^{d##}$	0.00±0.00^{e}	0.00±0.00^{c}	0.00±0.00^{b}
0.5	61.00±0.58^{c}	57.36±1.87^{a}	3.00±0.58^{a}	0.53±0.08^{a}
1.0	65.67±0.67^{b}	48.13±1.87^{b}	1.33±0.33^{b}	0.63±0.08^{a}
1.5	69.00±0.58^{a}	42.53±1.87^{c}	0.33±0.33^{bc}	0.13±0.06^{b}
2.0	70.33±0.33^{a}	36.97±1.83^{d}	0.00±0.00^{c}	0.00±0.00^{b}

#The full strength MS medium was used as control. ##The result values (means ± standard error) tagged with different superscript alphabets differed statistically at $P \leq 0.05$.

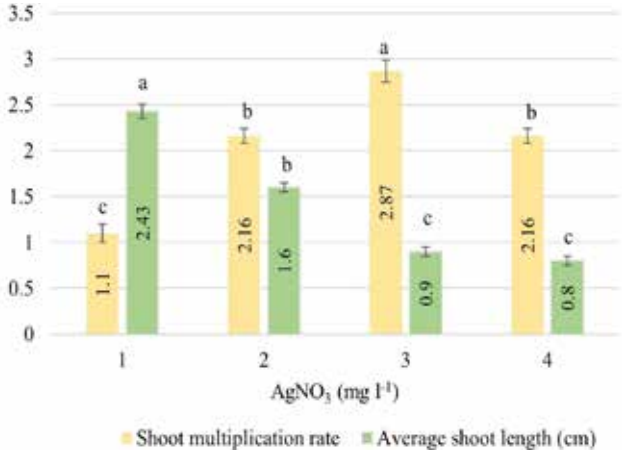

Fig. 3. Effect of silver nitrate ($AgNO_3$) on shoot multiplication. The bars with different alphabets differed statistically under Duncan Multiple Range Test ($P \leq 0.05$).

ethylene biosynthesis, which leads to an upsurge in endogenous ABA that causes leaf fall (Hansen and Grossmann, 5). The callus was also noted at the shoot base in all the tested NAA concentrations. This callus might be the cluster of root primordial cells that appear as callus cells under excess exogenous auxin supply in tissue culture. Such cells are in the differentiation process into root apical meristem (Yu *et al.,* 22). Raju and Divya (16) tested two auxins, indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) for rooting in *S. densiflorum* and reported 100% rooting with use of IBA (0.5 mg l^{-1}). Thus, it warrants further interventions to fine-tune the rooting medium of Konkan Bahadoli. However, the rooted plantlets were shifted to Styrofoam pots prefilled with sterile potting media [cocopeat (4): perlite (1): vermiculite (1)]. After 4-5 weeks of acclimatization, about 75% of the plants showed successful establishment (Fig. 2d and 2e).

In conclusion, prior knowledge of the appropriate time for culture initiation is essential for pursuing micro-propagation from field trees. May was found to be the suitable month for culture initiation from filed trees in Konkan Bahadoli on account of the lowest microbial contamination and highest establishment response. This month, the nodal segments showed better culture establishment response than shoot tips. The growth regulators combination of BAP (2.0 mg l^{-1}) and NAA (0.25 mg l^{-1}) was optimum for high-frequency shoot regeneration and the addition of $AgNO_3$ (3.0 mg l^{-1}) enabled shoot multiplication. The micro-shoots initiated roots on the MS medium with NAA (0.5 mg l^{-1}). The explant, time of culture and optimized media will help the micro-propagation of this promising *jamun* variety.

AUTHORS' CONTRIBUTION

Conceptualization of research (KK, JSB); Designing of the experiments (KK, HK); Contribution of experimental material (AK); Execution of field/lab experiments and data collection (HK, KK); Analysis of data and interpretation (HK, KK, PKA); Preparation of the manuscript (HK, KK, JSB, PKA).

DECLARATION

The authors declare no conflict of interest in the publication of this manuscript.

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