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Influence of explant collection period, antibrowning strategy and growth regulators composition on *in vitro* propagation of Bhagwa pomegranate

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ABSTRACT

***In vitro* propagation is a rapid mean for production of disease-free and healthy plants. Using nodal segments for *in vitro* propagation of pomegranate cv. Bhagwa, the effect of time of explant collection and anti-browning strategy on culture establishment were investigated. A combination of growth regulators (BAP, NAA) and growth substances ($AgNO_3$, Adenine sulphate) were also tested for shoot bud initiation, shoot proliferation and *in vitro* rooting. In a culture cycle of April to July months, the maximum explant establishment (58.07%) was recorded in the month of April. The medium supplemented with activated charcoal (2.5 g l^{-1}) induced significantly higher culture establishment (63.9%) than control and the treatments involving pre-culture shaking of explants with polyvinylpyrrolidone (PVP) (1.0%) solution or antioxidants (100 mg l^{-1} citric acid + 150 mg l^{-1} ascorbic acid) solution. For shoot bud initiation from nodal segments, different levels of BAP (0.5, 1.0, 1.5 and 2.0 mg l^{-1}) alone or with NAA (0.25 mg l^{-1}) were tested. The significantly highest response (85.17%) was achieved on MS medium supplemented with BAP (1.0 mg l^{-1}) + NAA (0.25 mg l^{-1}). The $AgNO_3$ (1.5 mg l^{-1}) enabled maximum survival of cultured shoots during sub-culture stage. The MS medium containing BAP (0.5 mg l^{-1}) + NAA (0.5 mg l^{-1}) + $AgNO_3$ (1.5 mg l^{-1}) + Adenine sulphate (40 mg l^{-1}) was good for shoot multiplication. The half strength MS medium supplemented with NAA (1.0 mg l^{-1}), was suitable for rooting of shoots *in vitro*.**

Key words: *Punica granatum*, explant establishment, *In vitro*, plantlet regeneration.

INTRODUCTION

Pomegranate (*Punica granatum*) is well known for its delicious fruits which have high nutritional and medicinal properties. It is cultivated in different countries across the globe, but India is its largest producer with an annual production of 2.3 million tons from an area of 0.93 million ha (Anonymous, 1). In India, it is mostly cultivated in the tropical climate of the states Maharashtra, Karnataka, Gujarat and Andhra Pradesh. 'Bhagwa', 'Mridula', 'Arakta', 'Ruby' and 'Ganesh' are the different cultivated varieties of pomegranate in India. 'Bhagwa', due to medium size fruits, deep red colored arils and soft seeds is the major cultivated variety. Since its recommendation for commercial cultivation under Punjab in 2013 by Punjab Agricultural University, Ludhiana, its popularity is rapidly increasing among growers. For commercialization of a variety, the availability of adequate planting material is a pre-requisite. Tissue culture offers rapid production of healthy, uniform and disease free plants (Chauhan and Kanwar, 5). The mature explants like nodal segment have been reported to be responsive explant for obtaining true to type plantlets (Naik *et al*., 12 ; Singh *et al*., 15). The establishment of cultures from mature explants in pomegranate is seriously affected by the oozing phenolic exudates. The phenolics cause browning of the media and also induce explant mortality (Martini *et al*., 9). The use of various anti-browning measures like addition of activated charcoal, transfer of explants to fresh medium at regular interval (Murkute *et al*., 10), pre-treatment with antioxidant (El-Agamy *et al*., 6) or polyvinylpyrrolidone (Foughat *et al*., 7) solutions have been suggested to counter the problem of phenolics. But, all of these have not been compared together. The woody trees also exhibit episodic growth (Naaz *et al*., 11), the pattern of which further gets modified with change in the growing climate. For instance, pomegranate remains evergreen under the tropical climate of Maharashtra and Karnataka states while behaves as semi-deciduous in subtropics of Punjab. In Punjab, vegetative growth starts in March, ceases in August and becomes semi-deciduous in the winter months of December-January. The season of explant collection has been reported to affect the culture establishment through its influence on microbial contamination in woody trees (Martini *et al*., 9). Further, the *in vitro* regeneration of pomegranate has been reported to be a relatively recalcitrant (Benson, 3). The use of ethylene inhibitor like silver nitrate has been found to promote regeneration, but its effect may be genotype, species or explant dependent (Naik and Chand, 13).

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The present study aimed to standardize *in vitro* plantlet regeneration protocol from nodal segments in pomegranate cv. Bhagwa by studying the effect of month of explant collection and different anti-browning strategies on explant establishment; by optimizing the combinations of growth regulators and growth substances for shoot regeneration, shoot proliferation and *in vitro* rooting.

MATERIALS AND METHODS

The study was carried out in Tissue Culture Lab of Punjab Agricultural University, Regional Research Station, Abohar (30°9'0" N, 74°11'0" E) during 2014-16. To find out the best month for culture establishment, the explants (nodal segments) were cultured from April to July. For this purpose, 30-40 cm long shoots were collected from 5 candidate 'Bhagwa' trees and were thoroughly washed under running tap water. The nodal segments of 2-3 cm length with one or two axillary bud(s) were prepared from middle of these shoots. The so prepared nodal segments were disinfected by dipping in antifungal and antibacterial solution mixture (0.1% Carbendazim + 100 ppm Streptocycline) for 30 min, followed by disinfection treatment with mercuric chloride ($HgCl_2$) @ 0.1% for 12 min, and washing with sterile distilled water thrice to remove the traces of mercuric chloride. In April month, the effect of various anti-browning strategies on explant establishment was also studied. The strategies included transfer of nodal segments to fresh medium after 3 days of first culture, addition of activated charcoal (2.5 g l^{-1}) in the medium and pre-culture shaking treatment of nodal segments in polyvinylpyrrolidone (PVP) (1.0%) or antioxidants (100 mg l^{-1} citric acid + 150 mg l^{-1} ascorbic acid) solution for 15 minutes. The direct culture of disinfected nodal segments on Murashige and Skoog (MS) medium containing BAP (1.0 mg l^{-1}) was taken as control.

The basal MS medium was supplemented with different growth regulators depending upon the desired *in vitro* response. For shoot initiation from nodal segments, the MS medium was supplemented with different concentrations of BAP (0.5, 1.0, 1.5 and 2.0 mg l^{-1}) alone or in combination with NAA (0.25 mg l^{-1}). The shoots excised from nodal segments did not grow further after their transfer to fresh medium. To enable their growth, $AgNO_3$ (1.0, 1.5, 2.0 and 3.0 mg l^{-1}), an anti-ethylene compound was added in the medium. To induce shoot proliferation, individual shoots of 2.0 to 2.5 cm length were cultured in the presence of BAP (0.5 mg l^{-1}) + NAA (0.5 mg l^{-1}) + $AgNO_3$ (1.5 mg l^{-1}) + variable levels of Adenine sulphate (0, 20, 40 and 60 mg l^{-1}). For root induction, the shoots of 2.5 to 3.0 cm length were cultured on ½ MS medium alone or with various levels of NAA (0.5, 1.0 and 2.0 mg l^{-1}) for two culture cycles. All the media were fortified with 100 mg l^{-1} myo-inositol and 3.0% sucrose and solidified with 0.35% clerigar (Himedia). The pH of the media was adjusted to 5.8 and sterilized at 121.5°C temperature, 15 lbs/inch^2 pressure for 20 min. The cultures were incubated under 16/8 hr light/dark cycle with white light of 2000-3000 lux intensity, at a constant temperature of 25±2°C.

The data on explant establishment was recorded after 20 days of culture. The data on percent shoot bud initiation and shoot proliferation were recorded after 30 days of culture. However, the data on percent rooting was recorded after 60 days of culture, as after 30 days, no roots were induced on either of the media. The experiments were conducted in completely randomized block design, with three replications per treatment. For the experiments on culture establishment and shoot bud initiation, 25 explants constituted a replication, while for studying the *in vitro* culture survival, shoot multiplication and *in vitro* rooting, 15 shoots were used per replication. For testing the statistical significance, the data was analyzed using one-way analysis of variance and the treatment means were compared by the Tukey's test at p = 0.05 using JMP Pro software, SAS Institute, Cary, NC.

RESULTS AND DISCUSSION

Culture establishment is the first step for the *in vitro* propagation. The success of culture establishment from *ex vitro* source in woody trees is partly determined by the extent of microbial contamination (Martini *et al.*, 9). The month of culture had influence on percent microbial contamination in this study. The minimum microbial contamination (19.86%) was observed in May month while the maximum microbial contamination (71.30%) was observed in July month (Fig. 1A). However, the established explants (refers to the surviving aseptic explants here) were significantly highest (58.07%) in the month of April (Fig. 1B). The next best response in this regard was observed in June month. There was a surge of new vegetative flush in this month. The results indicate that beside the microbial contamination, the growth state of the trees also influence culture establishment. In grapes, the maximum culture establishment was observed in the month of April, which was linked to low phenolic content during this month (Singh *et al.*, 14). We did not estimate the level of polyphenols in this study. However, estimation of the same could reveal the actual relationships of polyphenols with the culture establishment in pomegranate.

The phenolics exuding from cut ends of the explants is one of the cause of explant mortality, which

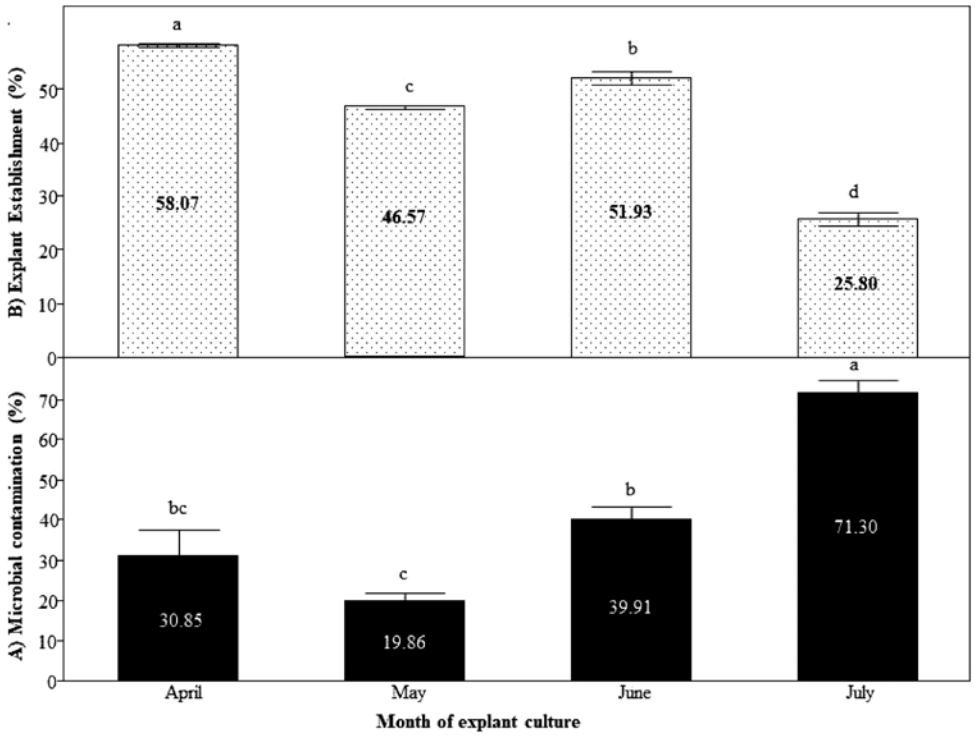

Fig. 1. Influence of month of explant culture on percent microbial contamination and explant establishment. Bars not sharing a common letter are significantly different as per Tukey's test at p value ≤ 0.05.

affect the ultimate culture establishment (Martini *et al*., 9). Out of different anti-browning treatments, the maximum culture establishment (63.9%) was recorded with culture of nodal segments on the medium containing activated charcoal. It was significantly higher than control (58.1%) and pre-culture treatments of nodal segments with either PVP (1%) solution or antioxidants (100 mg l^{-1}citric acid + 150 mg l^{-1}ascorbic acid) solution (Table 1). The treatment pairs of activated charcoal in the medium and subculture of nodal segments to fresh medium after 3 days of culture were statistically similar for percent culture establishment. Murkute *et al*. (10) evaluated many of these strategies for overcoming the phenolics associated browning and explant mortality in pomegranate and found sub-culturing of nodal segments at 1, 2 and 3 days after culture as the best strategy for culture establishment. However, sub-culture of the nodal segments to fresh medium is a little bit laborious exercise than their single culture on the medium containing activated charcoal. The probability of spread of fungal infection from infected to non-infected explants is also increased during frequent sub-culture, as the fungal contamination continues to appear even up to 15 days after culture.

The data on percent shoot bud initiation from nodal segments is presented in the Table 2. In the medium with BAP (0.5 to 2.0 mg l^{-1}) alone, the highest percent shoot initiation response (63.00%) was achieved with BAP (1.0 mg l^{-1}). The supplementation of NAA (0.25 mg l^{-1}) with either of the BAP (0.5 to 2.0 mg l^{-1}) level in the medium improved the shoot bud initiation response than their solo application (Table 2, Fig. 2a). The significantly highest response (85.57%) was obtained on MS medium containing BAP (1.0 mg l^{-1}) and NAA (0.25 mg l^{-1}). The significantly highest number of shoots (4.50) was induced on MS medium containing BAP (2.0 mg l^{-1}). This was followed by MS medium enriched with BAP (1.0 mg l^{-1}) and NAA (0.25 mg l^{-1}), while the minimum number of shoots were obtained on MS medium with BAP (0.5 mg l^{-1}) (Table 2). BAP is known to be superior cytokinin in terms of metabolization by plant tissues or for its ability to synthesize other natural hormones, such as zeatin within the tissue (Malik *et al*., 8). The improved shoot bud initiation after supplementation of NAA (0.25 mg l^{-1}) with the BAP (1.0 to 2.0 mg l^{-1}) in our study points to the synergistic cross talk of cytokinin and auxin for shoot meristem formation (Zhao *et al*., 16).

The shoots excised from nodal segments did not grow further upon their culture to fresh medium and showed tissue necrosis from top to downwards. It has been reported that ethylene released from the explants is responsible for recalcitrant response

Table 1. Effect of different anti-browning treatments on *in vitro* culture establishment from nodal segments.

Treatment[#]	Culture establishment (%)
Single culture of nodal segments (control)	58.1[b]
Subculture of nodal segments to fresh medium after 3 days of culture	59.4[ab]
Culture of nodal segments on medium containing 2.5 g l^{-1} activated charcoal	63.9[a]
Pre-treatment of nodal segments with antioxidant solution (100 mg l^{-1} citric acid + 150 mg l^{-1} ascorbic acid) for 15 minutes	28.2[d]
Pre-treatment of nodal segments with polyvinylpyrrolidone (1.0%) solution for 15 minutes	48.6[c]

[#]Medium: MS + BAP (1.0 mg l^{-1}). Within a column, values not sharing common superscript letters are different as per Tukey's test at $P \leq 0.05$.

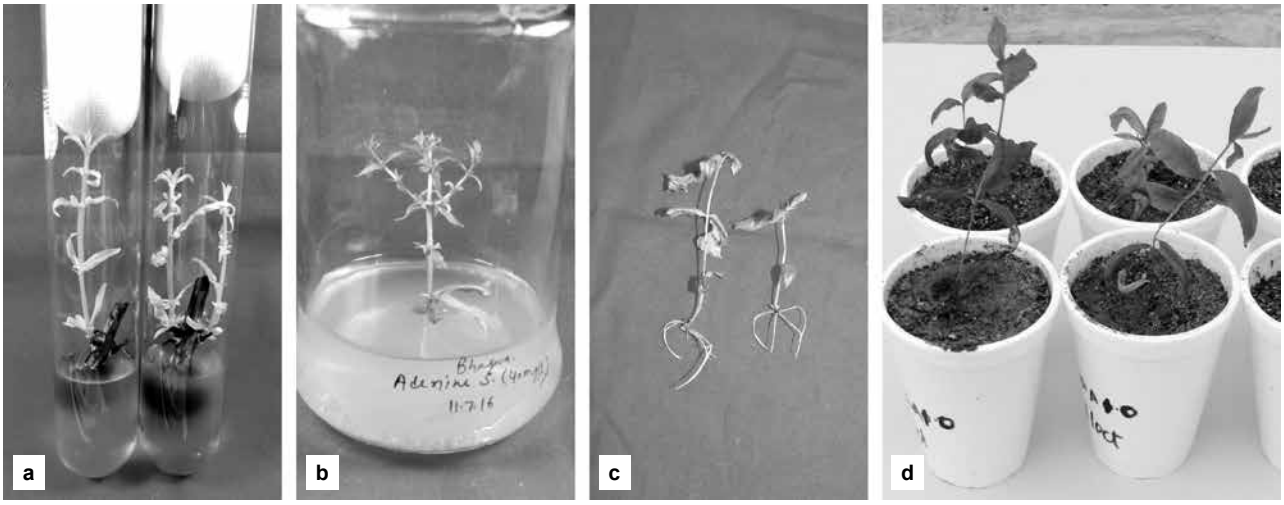

Fig. 2. *In vitro* plantlet regeneration from nodal segments in pomegranate cv. Bhagwa. *In vitro* shoot initiation on MS medium supplemented with BAP (1.0 mg l^{-1}) and NAA (0.25 mg l^{-1}) (a), shoot proliferation on MS medium supplemented with BAP (0.5 mg l^{-1}), NAA (0.5 mg l^{-1}), $AgNO_3$ (1.5 mg l^{-1}) and Adenine sulphate (40 mg l^{-1}) (b), rooting of the *in vitro* excised shoots on ½ MS medium supplemented with NAA (1.0 mg l^{-1}) (c) and plantlets growing on sterile potting mixture of vermiculite (5): garden soil (1): sand (1): vermicompost (1) (d).

Table 2. Effect of different levels of BAP without or with NAA on percent shoot bud initiation and number of shoots/ nodal explant.

Growth regulator (mg l^{-1})	Shoot initiation (%)	Number of shoots/ explant
BAP (0.5)	38.40[e]	2.50[ef]
BAP (1.0)	63.00[c]	3.27[c]
BAP (1.5)	46.90[d]	2.53[e]
BAP (2.0)	29.00[f]	4.50[a]
BAP (0.5) + NAA (0.25)	69.00[bc]	2.67[e]
BAP (1.0) + NAA (0.25)	85.57[a]	3.47[b]
BAP (1.5) + NAA (0.25)	74.49[b]	2.33[f]
BAP (2.0) + NAA (0.25)	75.19[b]	3.00[d]

Within a column, values not sharing common superscript letters are different as per Tukey's test at $P \leq 0.05$.

of plant tissues under *in vitro* conditions (Benson, 3). The addition of $AgNO_3$ in the medium helped in survival of the cultures. The maximum culture survival (91.33%) was observed at 1.5 mg l^{-1} $AgNO_3$, which was significantly higher over other concentrations (1.0, 2.0 and 3.0 mg l^{-1}) (Table 3). The results are in agreement with Naik and Chand (13), who illustrated the promotive effect of $AgNO_3$ on adventitious shoot bud initiation from cotyledonary explants in pomegranate. The $AgNO_3$ competes with ethylene for binding site at cellular membrane (Beyer, 4).

In a medium containing $AgNO_3$ (1.5 mg l^{-1}) + NAA (0.5 mg l^{-1}) + BAP (0.5 mg l^{-1}), the addition of Adenine sulphate at either of the concentration (20, 40 or 60 mg l^{-1}), significantly improved the rate of shoot multiplication over control. The highest shoot multiplication rate (2.35) was observed with 40 mg l^{-1} Adenine sulphate (Fig. 2b, Table 4). The significantly higher shoot length (3.32 cm) was observed with 60 mg l^{-1} Adenine sulphate. The new shoots in most of

Table 3. Influence of different levels of $AgNO_3$ on the survival of *in vitro* cultures during sub-culture stage.

$AgNO_3$ (mg l^{-1})#	Survival of cultures (%)
1.0	66.93[c]
1.5	91.23[a]
2.0	86.23[b]
3.0	85.56[b]

#$AgNO_3$ was added to the MS medium containing BAP (0.5 mg l^{-1}) + NAA (0.5 mg l^{-1}). Within a column, values not sharing common superscript letters are different as per Tukey's test at $P \leq 0.05$.

Table 4. *In vitro* shoot multiplication and their growth with the use of Adenine sulphate.

Adenine sulphate (mg l^{-1})#	Shoot multiplication rate	Shoot length (cm)
0 (Control)	1.06[d]	3.06[b]
20	2.22[b]	3.08[b]
40	2.35[a]	2.99[b]
60	2.15[c]	3.32[a]

#Adenine sulphate was added to the MS medium containing BAP (0.5 mg l^{-1}) + NAA (0.5 mg l^{-1}) + $AgNO_3$ (1.5 mg l^{-1}). Within a column, values not sharing common superscript letters are different as per Tukey's test at $P \leq 0.05$.

the cases arose from the axillary buds of cultured shoots but sometimes also originated *de novo* from the base of the cultured shoots. Adenine sulphate has been known to increase shoot regeneration in common bean (Arias *et al*., 2) and *Syzygium cumini* (Naaz *et al*., 11). However, the magnitude of shoot multiplication in our study was much lower than *Syzygium cumuni*. This could be due to the genetic differences and variation in dose and stage of its use.

Tissue cultured plants require roots for becoming self-reliant upon *ex vitro* transfer. In our study, no rooting was induced on either of the tested media during the first culture cycle. However, sub-culture of the shoots on the same medium induced roots. The rooting was found to be highest (67.53%) in the shoots cultured on half strength MS medium supplemented with NAA (2.0 mg l^{-1}). It was statistically at par with half strength MS medium containing 1.0 mg l^{-1} NAA (Table 5, Fig. 2c).

The highest proportion of callus free roots (48.13%) was recorded on half strength MS medium containing 0.5 mg l^{-1} NAA. In pomegranate, rooting of the *in vitro* shoots has been achieved on hormone free half-strength MS medium alone or in the presence of growth regulators like NAA, IBA and additives like activated charcoal (Chauhan and Kanwar, 5). However, no rooting in the first culture and low rate

Table 5. *In vitro* rooting of the cultured shoots on ½ MS medium without or with growth regulator NAA.

Medium	Percent *in vitro* rooting	Percent callus free roots
½ MS	0.00[c]	0.00[d]
½ MS + NAA (0.5 mg l^{-1})	55.20[b]	48.13[b]
½ MS + NAA (1.0 mg l^{-1})	66.23[a]	44.13[a]
½ MS + NAA (2.0 mg l^{-1})	67.53[a]	38.33[c]

Within a column, values not sharing common superscript letters are different as per Tukey's test at $P \leq 0.05$.

of rooting in second subculture in our study, indicate that pomegranate cv. Bhagwa is either hard to root *in vitro* or the optimal medial requirement and incubation conditions for this response need to be standardized.

The rooted plantlets were transferred to sterile potting mixture of vermiculite (5): garden soil (1): sand (1): vermicompost (1) for further growth (Fig. 2d).

Under the subtropical conditions of Punjab, April was found to be the most suitable month for culture establishment from nodal segments of pomegranate cv. Bhagwa. The MS medium containing BAP (1.0 mg l^{-1}) and NAA (0.25 mg l^{-1}) was best for shoot bud initiation from nodal segments. The growth of the *in vitro* shoots could be restored with the use of anti-ethylene compound $AgNO_3$ (1.5 mg l^{-1}) in the medium. However, the media for shoot multiplication and rooting needs further refinement for use as a protocol for mass multiplication.

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