Applied Biological Research 24(2): 210-218; (2022)
DOI: 10.5958/0974-4517.2022.00025.8

In vitro CLONAL PROPAGATION OF POMEGRANATE (*Punica granatum* L.) cv. BHAGWA

R. Kiruthika Devi, C. Kavitha*, K. Soorianathasundaram, and K. Hemaprabha

Department of Fruit Science, Horticultural College & Research Institute,
Tamil Nadu Agricultural University, Coimbatore - 641 003, Tamil Nadu (India)
*e-mail: *ck77@tnau.ac.in*

(Received 16 November, 2021; accepted 2 May, 2022)

ABSTRACT

An attempt was made to standardize *in vitro* clonal propagation of pomegranate cv. Bhagwa, using nodal segment and shoot tip explants at TNAU, Coimbatore, India. Direct organogenesis of pomegranate was tried in MS medium with growth regulator combinations *viz.*, benzyl amino purine (BAP), α-naphthalene acetic acid (NAA) and adenine sulphate for *in vitro* shoot regeneration and indole butyric acid (IBA) for *in vitro* rooting. Among the explants evaluated, nodal segments responded better for culture establishment. Pre-treatment of nodal segments with 0.5% carbendazim for 30 min followed by surface sterilization with 70% ethanol for 30 sec and 0.5% sodium hypochlorite for 10 min gave highest survival rate (81.7%) with minimum microbial contamination (13.3%). Phenolic exudation was reduced by treating explants in antioxidant solution consisting of citric acid (100 mg L^{-1}) and ascorbic acid (150 mg L^{-1}) for 20 min and then cultured on basal MS medium with polyvinyl pyrrolidone (100 mg L^{-1}) resulted in maximum survival (98.3%). The earliest shoot evocation response (4.4 days), higher shoot proliferation (91.7%) and maximum shoot number (4.6) were observed on basal MS medium with adenine sulphate (40 mg L^{1}). Half strength MS medium supplemented with IBA (1 mg L^{-1}), recorded minimum days to root initiation (16.9) and maximum rooting percentage (71.7%) with higher number of roots explant^{-1} (3.7) and lengthier roots (3.93 cm). Rooted plantlets transferred to hardening media with pot mixture and vermicompost (1:1) exhibited an *ex-vitro* survival of 81.7%.

Keywords: Micropropagation, nodal segment, phenolic exudation, pomegranate, rooting, shoot tip

INTRODUCTION

Pomegranate (*Punica granatum* L.) is an economically important fruit crop suitable for cultivation in tropical, sub-tropical and semi-arid climatic conditions. It is native to Iran, Afghanistan and North-Western Himalayas. It has been widely cultivated throughout drier parts of Southeast Asia, Malaysia, the East Indies and Tropical Africa (Johanningsmeier and Harris, 2011). Worldwide, India occupies 1st position with respect to the area (2.73 lakh ha) and production (30.63 lakh tonnes) of pomegranate (Hedge, 2021). In India, Maharashtra is the leading producer followed by Gujarat, Karnataka, Andhra Pradesh, Madhya Pradesh, Rajasthan, Telangana, Tamil Nadu and Chhattisgarh (Gaiker and Yadav, 2022). The highest productivity of pomegranate is registered in Tamil Nadu (23.32 metric tonnes ha^{-1}) followed by Gujarat, Andhra Pradesh and Telangana (Tewari and Avinashilingam, 2020). Pomegranate is commercially grown for its sweet acidic fruits, which serve as a rich source of minerals, vitamins, polyphenols and tannins. The juice is refreshing and contains both glucose and

fructose (Gil *et al*., 2000). The leaves, immature fruits and fruit rind are traditionally used for their medicinal properties and for tanning of leather (Deepika and Kanwar, 2010).

Pomegranate is gaining prominence as an important export-oriented fruit crop in India and the increasing demand for internal consumption and export has been a driving force for its wider cultivation in India. The conventional method of propagation in pomegranate which involves rooting of hard wood cuttings and layering is highly time-consuming and labour-intensive (Kanwar *et al.*, 2010; Singh and Patel, 2014). In addition, infestation by bacterial blight is a critical and major constraint in pomegranate production which causes drastic reduction in yield and its fruit quality; and infected cuttings/layers serve as the main source of inoculum for its spread. Hence, development of an efficient propagation method for large scale multiplication of quality planting material in pomegranate is warranted. Micropropagation aids in rapid and mass production of true to type, healthy and disease free planting materials (Susmita *et al*., 2016; Parth *et al*., 2018; Rachana *et al*., 2019) and *in vitro* propagation protocol for *P. granatum* L. from various explants *viz*., leaf segments, cotyledons (Murkute *et al.*, 2002; Kanwar *et al.*, 2010; Bharose *et al*., 2014), anthers (Naik *et al.*, 1999) petals and immature zygotic embryos (Kanwar *et al.*, 2010) and nodal segments (Deepika and Kanwar, 2010; Usharani *et al*., 2014) is reported earlier. However, the problem of excessive phenolic exudation, browning of explants and slow shoot growth are major bottlenecks in *in vitro* pomegranate propagation (Kantharajah *et al.*, 1998; Singh and Patel, 2014; Naik and Chand, 2011). In present study the explants were treated with antioxidants like citric acid and ascorbic acid, and growth regulators like benzyl amino purine (BAP), α-naphthalene acetic acid (NAA) and adenine sulphate (AdS) to address these bottlenecks in *in vitro* pomegranate propagation with the hypothesis that antioxidants may inhibit the oxidation, reduce browning of explants by scavenging free radicals, while growth regulator-combination with BAP, NAA and AdS may aid in growth enhancement by promoting shoot proliferation in the explant. In India, pomegranate cv. Bhagwa is a leading commercial cultivar predominantly due to its wide consumer appeal. Hence, the present study was aimed to identify the most suitable explant and assess the influence of different growth regulators on micropropagation of pomegranate cv. Bhagwa.

MATERIALS AND METHODS

Explant preparation and sterilization

Newly emerged 6 weeks old shoots containing dormant buds were collected from disease free 3 years old mother plants of pomegranate cv. Bhagwa maintained under polyhouse condition. The leaves and other unwanted parts in the collected shoots were removed and the explants *viz*., shoot tip (1-2 cm) and nodal segment (2-3 cm) were prepared by trimming with sterile blade. Explants were thoroughly washed with running tap water to remove the dust particles followed by Teepol® wash for 10 min. Explants were pre-treated with fungicide carbendazim solution (0.25 and 0.5%) for 30 min and rinsed with distilled water. The explants were then treated with streptomycin (100 mg 100 mL^{-1}) for 20 min followed by washing in distilled water. Later, in the laminar air flow chamber, explants were sterilized with 70% ethanol for 30 sec followed by three sterile water wash. Finally, the explants were treated with sodium hypochlorite (NaOCl, 0.25 and 0.5%) for 3 different durations (5, 10 and 15 min), followed by two times washing in sterile water and then inoculated in the basal Murashige and Skoog (MS) medium enriched with 30 g L^{-1} sucrose. The pH of the prepared medium was adjusted between 5.6 - 5.8 and agar-agar (8 g L^{-1}) was added for solidification. All the cultures were maintained at 25 ± 2°C with 70-80% relative humidity with a photoperiod of 16/8 h (light intensity of 2500-3000 lux).

Control of phenolic exudation

To control phenolic exudation, explants were pre-soaked in antioxidant solutions *viz*., citric acid (100 mg L^{-1}) and ascorbic acid (150 mg L^{-1}) alone or in combination for 20 min followed by inoculation

in three different media combinations *viz*., MS basal medium, MS + polyvinyl pyrrolidone (PVP, 100 mg L^{-1} and MS + activated charcoal (AC) 200 mg L^{-1} and the influence of antioxidants on explants with respect to survival percentage was evaluated.

In vitro shoot regeneration

MS medium fortified with different concentrations of BAP (1 and 2 mg L^{-1}), NAA (0.5 and 1.0 mg L^{-1}) and AdS (20, 30 and 40 mgL^{-1}) were used for shoot regeneration and multiplication. After 20-25 days of initial culturing, explants were sub-cultured in the same medium combinations for three subsequent times to facilitate induction of multiple shoots. Observations on days for shoot initiation, shoot proliferation percentage, number of shoots, number of leaves and shoot length were recorded.

In vitro rooting

Multiple shoots ≧ 7.0 cm in length were transferred to rooting media for root induction. Observations on the number of days taken for rooting, rooting percentage, number of roots and root length were recorded.

Hardening of in vitro rooted plantlets

Rooted plantlets were carefully taken out of the rooting medium without any damage and were thoroughly washed under running tap water to remove traces of medium. The plantlets were then planted in micro-pots filled with different hardening media consisting of pot mix, cocopeat and vermicompost and shifted to shadenet house with 50% shade by covering each pot with transparent polythene covers to maintain high humidity. After 15-20 days, during new leaf emergence polythene covers were removed to reduce excessive humidity.

Statistical analysis

The experiment was carried out in completely randomized block design with three replications and each replication consisted of 20 explants. The data generated from the various experiments were subjected to statistical analysis as per the standard procedures (Sukhatme and Panse, 1954).

RESULTS AND DISCUSSION

In present study, pretreatment of carbendazim (0.25 and 0.5%) for 30 min + surface sterilization with 70% ethanol for 30 sec and sodium hypochlorite (0.25 and 0.5%) for 5, 10 and 15 min were used to assess the survival, contamination and mortality percentage of shoot tip and nodal segment explants. In case of shoot tips, the pre-treatment of 0.25% carbendazim for 30 min + surface sterilization with 70% ethanol for 30 sec followed by 0.25% NaOCl treatment for 5 min showed highest survival percentage of 68.33% (Fig. 1). In case of nodal segments, the pretreatment of 0.5% carbendazim for 30 min + surface sterilization with 70% ethanol for 30 sec followed by 0.5% NaOCl treatment for 10 min recorded the highest survival percentage of 81.66%. These finding were in line with the earlier findings in other pomegranate varieties (Damiano *et al.*,2008; Kalalbandi *et al.*, 2014; Satheesh and Sridharan, 2014).–Explants collected from the field grown plants harbour large populations of microbes which have to be disinfected before inoculation into the culture medium. Microbial contamination generally increases the mortality of cultures. The latent infections in explants might cause variability in growth, tissue necrosis with reduction in shoot proliferation and rooting. Hence, successful disinfection of explants is a pre-requisite for *in vitro* culture (Singh *et al.*, 2010). Selection of sterilizing agent and duration of exposure are critical because the explant should not lose its biological activity and only contaminants should be eliminated during sterilization (Tiwari *et al.*, 2012). In present study, the use of carbendazim (a broad-spectrum systemic fungicide); ethanol (a powerful sterilizing agent) and sodium hypochlorite (an effective bactericide at micro-molar level) are effective to control fungal and bacterial contaminants (Singh *et al.*, 2011).

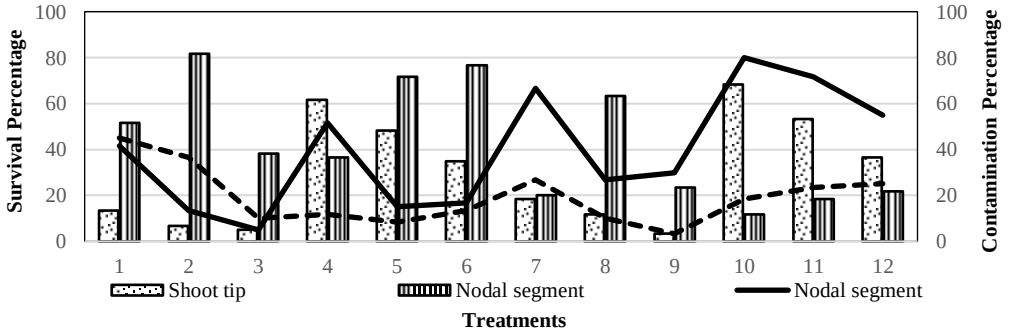

Fig. 1: Effect of sterilization treatments on survival and contamination percentage of explants in pomegranate cv. Bhagwa; S1-S6 have common treatment of 0.5% carbendazim for 30 min + 70% ethanol treatment for 30 sec; with varying 0.5% NaOCl treatment for 5, 10 and 15 min in S1, S2 and S3, respectively, and 0.25% NaOCl treatment for 5, 10 and 15 min in S4, S5 and S6, respectively. Similarly, S7-S12 have common treatment of 0.25% carbendazim for 30 min + 70% ethanol treatment for 30 sec with varying 0.25% NaOCl treatment for 5, 10 and 15 min in S7, S8 and S9, respectively, and 0.25% NaOCl treatment for 5, 10 and 15 min in S10, S11 and S12, respectively.

Anti-browning treatments on culture establishment

Generally, woody plants are recalcitrant for *in vitro* propagation as browning due to exudation of phenols is a major problem during initial culture establishment and further morphogenesis and *in vitro* rooting are hampered (Krishna *et al.*, 2008). Browning of explant and medium is most commonly encountered in pomegranate when mature plant parts are used for culture establishment (Naik and Chand, 2011). In present study, the explants were treated with antioxidants *viz.*, ascorbic acid and citric acid for 20 min and then cultured on MS medium alone or with AC and PVP and their influence on survival percentage evaluated. Among the treatments, pre-treatment with citric acid (100 mg L^{-1}) and ascorbic acid (150 mg L^{-1}) for 20 min followed by inoculation in basal MS + PVP 100 mg L^{-1} resulted in the highest survival percentage of nodal segments (98.3%) and shoot tips (93.3%) (Fig. 2). Antioxidants are reducing agents which inhibit the oxidation of labile substrates. In present study, the combination of citric acid and ascorbic acid delayed browning. Citric acid as a chelating agent binds to the ions responsible for activating polyphenol oxidative enzymes and ascorbate in ascorbic acid scavenges oxygen radicals produced during tissue damage and thereby protects the explants from oxidative injury (Panaia, 1998). Thus, antioxidants combination with citric and ascorbic acid might have reduced the browning of explant tissues by scavenging free radicals and addition of PVP in basal MS medium reduced browning by adsorbing the phenolics leached out into the medium from the cut ends of explants (Ahmad *et al.*, 2013). Hence, the results obtained are in agreement with those of Preece and Compton (1991), Patil *et al.* (2011), Satheesh and Sridharan (2014) and Singh and Patel (2014) in pomegranate.

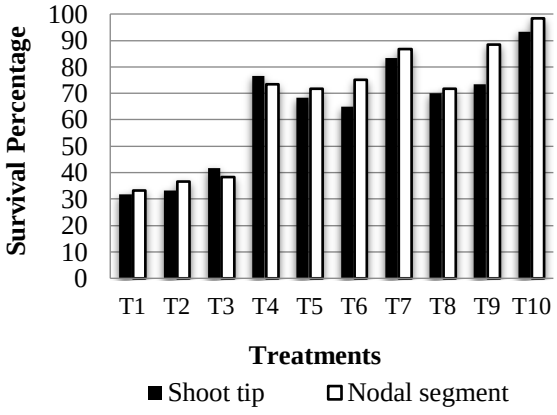

Fig. 2: Effect of antioxidant treatments on survival percentage of explants in pomegranate cv. Bhagwa; T_1 - Control (No antioxidant pre-treatment), T_2 - Citric acid (100 mg L^{-1}) + MS; T_3 - Ascorbic acid (150 mg L^{-1}) + MS; T_4 - Citric acid (100 mg L^{-1}) + ascorbic acid (150 mg L^{-1}) + MS; T_5 - Citric acid (100 mg L^{-1}) + MS with activated charcoal [AC] (200 mg L^{-1}); T_6 - Ascorbic acid (150 mg L^{-1}) + MS with AC (200 mg L^{-1}); T_7 - Citric acid (100 mg L^{-1}) and ascorbic acid (150 mg L^{-1}) + MS with AC (200 mg L^{-1}); T_8 - Citric acid (100 mg L^{-1}) + MS with polyvinyl pyrrolidone [PVP] (100 mg L^{-1}); T_9 - Ascorbic acid (150 mg L^{-1}) + MS with PVP (100 mg L^{-1}); and T_{10} - Citric acid (100 mg L^{-1}) and ascorbic acid (150 mg L^{-1}) + MS with PVP (100 mg L^{-1})

***In vitro* shoot regeneration**

In plant tissue culture, specific concentration of plant growth regulators is needed to induce multiple shoots but may vary from species to species and also depends on the source of explant. Eight different combinations of growth regulators *viz.,* basal MS medium, basal MS medium supplemented with BAP (1.0 and 2.0 mg L^{-1}), basal MS medium supplemented with NAA (0.5 and 1.0 mg L^{-1}) and basal MS medium with adenine sulphate (20, 30 and 40 mg L^{-1}) were used to assess the significant influence of growth regulators on shoot proliferation. Among treatment combinations evaluated for shoot proliferation, for shoot tips, basal MS medium supplemented with 20 mg L^{-1} adenine sulphate recorded early shoot initiation (9.77 days), highest shoot proliferation (58.33%), maximum number of shoots explant^{-1} (3.92), lengthier shoot (4.23 cm) and maximum number of leaves shoot^{-1} (10.22); and for nodal segments, the basal MS medium supplemented with 40 mg L^{-1} adenine sulphate showed early shoot initiation (4.4 days), highest shoot proliferation (91.7%), maximum number of shoots explant^{-1} (4.57), lengthier shoots (8.67 cm) and maximum number of leaves shoot^{-1} (13.17) (Table 1). Among the explants, nodal segments gave better response as compared to shoot tips (Plate 1). In pomegranate, a combination of cytokinin (BAP, kinetin and adenine sulphate) and auxin (IBA and NAA) have been most commonly employed for culture initiation and shoot proliferation. Adenine sulphate was used as growth regulator for shoot proliferation and

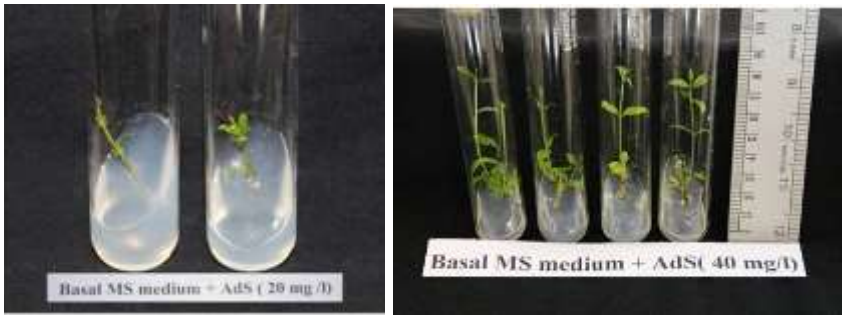

Plate 1: *In vitro* shoot proliferation of pomegranate cv. Bhagwa explants

Table 1: Effect of growth regulators on *in vitro* shoot regeneration of pomegranate cv. Bhagwa

Treatments	Nodal segment					Shoot tip				
	Days to shoot initiation	Shoot proliferation (%)	No. of shoots	Shoot length (cm)	No. of leaves	Days to shoot initiation	Shoot proliferation (%)	No. of shoots	Shoot length (cm)	No. of leaves
MS (control)	8.57	61.67 (51.81)	2.90	2.77	6.92	16.73	33.33 (35.25)	1.97	0.80	5.04
MS + BAP 1.0 mg L^{-1}	6.43	66.67 (54.75)	3.23	6.03	9.70	10.60	53.33 (46.91)	3.63	3.63	9.55
MS + BAP 2.0 mg L^{-1}	5.33	81.67 (64.70)	4.20	7.80	12.06	13.90	40.00 (39.21)	2.83	2.13	7.55
MS + NAA 0.5 mg L^{-1}	11.47	46.67 (43.08)	1.77	1.53	4.75	18.27	25.00 (29.93)	1.63	0.63	3.84
MS + NAA 0.5 mg L^{-1}	15.43	38.33 (38.25)	1.13	1.37	3.05	20.10	16.67 (23.74)	1.10	0.40	2.93
MS + AdS 20 mg L^{-1}	7.56	65.00 (53.76)	3.17	5.20	8.86	9.77	58.33 (49.80)	3.92	4.23	10.22
MS + AdS 30 mg L^{-1}	6.00	76.67 (61.14)	3.80	7.13	10.53	12.40	45.00 (42.12)	3.47	2.97	8.30
MS + AdS 40 mg L^{-1}	4.40	91.67 (73.40)	4.57	8.67	13.17	15.40	36.67 (37.26)	2.20	1.40	6.81
Mean	8.15	66.04 (55.11)	3.10	5.06	8.63	14.65	38.54 (38.03)	2.59	2.03	6.78

Sources of variation/ observations	$CD_{0.05}$				
	Days for shoot initiation	Shoot proliferation (%)	No. of shoots	Shoot length (cm)	No. of leaves
Explants	0.33*	1.68*	0.12*	0.18*	0.18*
Treatments	0.66*	3.37*	0.24*	0.36*	0.37*
Explants x Treatments	0.93*	4.76*	0.35*	0.52*	0.53*

The values in parentheses are arcsine transformed values; * Significant at 5 % level

Table 2: Effect of growth regulators on *in vitro* rooting of pomegranate explants

Treatments	Days taken for root initiation	Rooting (%)	Number of roots	Root length (cm)
½ MS (control)	24.92	31.67 (34.23)	1.20	1.90
½ MS + IBA 0.5 mg L^{-1}	20.61	60.00 (50.79)	3.03	3.07
½ MS + IBA 1.0 mg L^{-1}	16.92	71.67 (57.86)	3.70	3.93
½ MS + IBA 1.5 mg L^{-1}	22.60	50.00 (45.00)	1.83	2.33
½ MS + IBA 0.5 mg L^{-1} + NAA 0.5 mg L^{-1}	25.00	28.33 (32.14)	1.10	1.73
½ MS + IBA 1 mg L^{-1} + NAA 0.5 mg L^{-1}	21.63	55.00 (47.88)	2.13	2.70
½ MS + IBA 1.5 mg L^{-1} + NAA 0.5 mg L^{-1}	27.37	20.00 (26.57)	0.40	1.00
½ MS + IBA 0.5 mg L^{-1} + AC 100 m gL^{-1}	17.32	63.33 (59.41)	3.33	3.57
½ MS + IBA 1 mg L^{-1} + AC 100 m gL^{-1}	23.93	45.00 (42.12)	1.53	2.13
½ MS + IBA 1.5 mg L^{-1} + AC 100 mg L^{-1}	27.23	23.33 (28.85)	0.53	1.20
½ MS + ½ MS + IBA 0.5 mg L^{-1} + NAA 0.5 mgL^{-1} + AC 100 mg L^{-1}	25.63	26.67 (31.07)	0.83	1.47
½ MS + ½ MS + IBA 1 mg L^{-1} + NAA 0.5 mg L^{-1} + AC 100 mg L^{-1}	24.47	40.00 (39.21)	1.40	2.07
½ MS + ½ MS + IBA 1.5 mg L^{-1} + NAA 0.5 mg L^{-1} + AC 100 mg L^{-1}	27.67	18.33 (25.30)	0.30	0.83
Mean	23.48	41.02 (39.52)	1.64	2.15
SE	0.36	1.85	0.14	0.12
$CD_{0.05}$	0.74*	3.81*	0.28*	0.25*

MS = Murashige and Skoog medium; IBA = Indole-3-butyric acid; NAA = α-Naphthalene acetic acid; AC = Activated Charcoal; SE = Standard error deviation

elongation by earlier workers in pomegranate (Patil *et al.*, 2011; Satheesh and Sridharan, 2014; Singh and Patel, 2014; Bachake *et al*. 2019), because of its growth enhancement in meristem tips, promoting proliferation in axillary shoots and adventitious shoot formation from calli or from the explant and was found more effective than cytokinin (Van Staden and Drewes, 1992). The present findings are in line with those of Patil *et al.* (2011) and Satheesh and Sridharan (2014)

In vitro rooting

Rooting of *in vitro* generated shoots is a pre-requisite for generation of independent plants. In the present study, half strength MS medium supplemented with IBA alone, IBA with NAA, IBA with activated charcoal and IBA with NAA and activated charcoal were used for root induction and among which, IBA was found to be more effective than combination with NAA and activated charcoal. Among the treatment combinations, half MS medium supplemented with 1 mgL^{-1} IBA showed early root initiation (16.92 days), maximum rooting percentage (71.67%), maximum number of roots (3.70) $plantlet^{-1}$ and longest root (3.93 cm) (Table 2; Plate 2). Concentration of rooting media and auxin are the important factors that affect *in vitro* rooting (Kantharajah *et al.*, 1998). Half strength MS medium supplemented with NAA or IBA proved superior in promoting *in vitro* root growth (Heloir *et al.*, 1997; Omar *et al*., 2004). Similar results were reported by Soukhak *et al.* (2011), Bonyanpour and Khosh-Khui (2013) and Usharani *et al.* (2014) in different pomegranate cultivars.

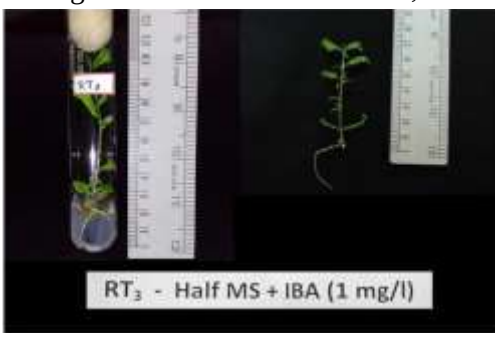

Plate 2: *In vitro* root regeneration in pomegranate microshoots

Hardening of plantlets

Acclimatization of *in vitro* derived plantlets to convert them to autotrophic is imperative prior to field planting for better survival. Fully developed plantlets with 3-4 pairs of leaves are to be removed from

the culture medium, washed carefully in order to remove remnant of culture medium, transferred to plastic cups containing various substrates. During the present investigation, *in vitro* derived plantlets of pomegranate cv. Bhagwa were hardened in different potting mixtures such as pot mix (1:1:1: of sand: red earth: FYM), cocopeat alone, pot mix + cocopeat (1:1), pot mix + vermicompost (1:1) and pot mix + cocopeat + vermicompost (1:1:1). Acclimatization of *in vitro* regenerated plantlets of pomegranate has been achieved by using different hardening media *viz.*, mixtures consisting of peat, perlite and sand, vermicompost, vermiculite, cocopeat and FYM with varying degree of success (Naik and Chand, 2003, Sharon and Sinha, 2000, Bonyanpour and Khosh-Khui, 2013, Bharose *et al.*, 2014). Plantlets can also be acclimatized in pots filled with sterile garden soil covered with polyethylene cups to maintain humidity (Shao *et al.*, 2003). In the present study, among the potting mixtures, combination of pot mix and vermicompost (1:1) showed the highest survival percentage (81.67%), early emergence of new leaf (12.97 days) and maximum number of leaves (4.80) (Fig. 3; Plate 3) and it may be attributed to the optimum growth conditions favoured by rich supply of nutrients, good water retention, good aeration and good drainage provided by the medium. The results obtained were parallel with the findings of Naik *et al.* (1999), Naik *et al.* (2000), Naik and Chand (2003) and Singh *et al.* (2013) in different pomegranate cultivars.

Plate 3: *Ex vitro* hardening of *in vitro* derived pomegranate palntlets

Conclusion: In the present study, nodal segments performed better than shoot tips with respect to overall response to *in vitro* regeneration and shoot proliferation. Hence, i*n vitro* propagation by nodal segment is effective for mass multiplication of quality planting materials in pomegranate cv. Bhagwa. The protocol resulted in maximum survival during hardening and hence the protocol can be deployed for mass multiplication of pomegranate cv. Bhagwa.

REFERENCES

Ahmad, L.N., Faisal, M., Anis, M. and Aref, I.M. 2013. *In vitro* callus induction and plant regeneration from leaf explants of *Ruta graveolens*. *South African Journal Botany*, **76**: 597-600.

Bachake, S.S., Jadhav, V.B., Deshpande, P.P., Tele, A.A., Banda, M.A., Adki, V.S., Gopika, M.K., Karanjule, P.G., Birajdar, S.B., Karwa, N.N., Mundhewadikar, D.M. and Singh, N.V. 2019. Standardization of *in vitro* propagation protocol for pomegranate cv. Super Bhagwa. *Journal of Pharmacognosy and Phytochemistry*, **8**(3): 2548-2553.

Bharose, A.A., Khandagale D.J. and Damse, D.N. 2014. Regeneration media standardization of pomegranate (*Punica granatum* L.) cv. Bhagwa from shoot tip, cotyledonary node, hypocotyls and leaves. *Vegetos*, **27**(2): 338-342.

Bonyanpour, A. and Khosh-Khui, M. 2013. Callus induction and plant regeneration in *Punica granatum* L. 'Nana' from leaf explants. *Journal of Central European Agriculture*, **14**: 928-936.

Damino, C., Padro, M.D.A. and Frattarelli, A. 2008. Propagation and establishment *in vitro* of myrtle (*Myrtus communis* L.), pomegranate (*Punica granatum* L.) and mulberry (*Morus alba* L.). *Propagation of Ornamental Plants*, **8**: 3-8.

Deepika, R. and Kanwar, K. 2010. *In vitro* regeneration of *Punica granatum* L. plants from different juvenile explants. *Journal of Fruit and Ornamental Plant Research*, **18:** 5-22.

Gaiker, P.S. and Yadav, J. 2022. Existing position of pomegranate production among fruits in India. *Journal of Emerging Technologies and Innovative Research*, **9**(1): 314-317.

Gil, M.I., Tomas-Barberan, F.A., Hess-Pierce, B., Holcroft, D.M. and Kader, A.A. 2000. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agriculture and Food Chemistry,* **48**: 4581-4589.

Hedge, G.N. 2021. Role of non-government organization in horticultural development in India. *Indian Horticulture*, **66**(5): 59-62.

Heloir, M.C., Fournioux, J.C. Oziol, L. and Bessis, R. 1997. An improved procedure for the propagation *in vitro* of grapevine (*Vitis vinifera* cv. Pinot noir) using axillary-bud microcuttings. *Plant Cell Tissue and Organ Culture,* **49**: 223-225.

Johanningsmeier, S.D. and Harris, G.K. 2011. Pomegranate as a functional food and nutraceutical source. *Annual Reviews of Food Science Technology,* **2**: 181-201.

Kalabandi, B.M., Waskar, D.P., Khandare, V.S. and Gorad, D.S. 2014. Micropropagation studies on pomegranate var. Bhagwa. *Indian Journal of Horticulture*, **71**(4): 564-566.

Kantharajah, A., Dewitz, I. and Jabbari, S. 1998. The effect of media, plant growth regulators and source of explants on *in vitro* culture of pomegranate (*Punica granatum* L.). *Erwerbs-obtbau,* **40**: 54-58.

Kanwar, K., Joseph, J. and Deepika, R. 2010. Comparison of *in vitro* regeneration pathways in *Punica granatum* L. *Plant Cell Tissue and Organ Culture,* **100**: 199-207.

Krishna, H., Sairam, R.K., Singh, S.K., Patel, V.B., Sharma, R.R., Grover, M. and Sachdev, A. 2008. Mango explant browning: Effect of ontogenic age, mycorrhization and pre-treatments. *Scientia Horticulturae,* **118**(2): 132-138.

Murkute, A.A., Patil, S., Patil, B.N. and Kumari, M. 2002. Micropropagation in pomegranate, callus induction and differentiation. *South Indian Horticulture,* **50**(1-3): 49-55.

Naik S.K., Pattnaik, S. and Chand, P.K. 1999. *In vitro* propagation of pomegranate (*Punica granatum* L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree. *Scientia Horticulturae,* **79**: 175-183.

Naik, S.K. and Chand, P.K. 2011. Tissue culture-mediated biotechnological intervention in pomegranate: A review. *Plant Cell Reports,* **30**: 707-721.

Naik, S.K., Pattnaik, S. and Chand, P.K. 2000. High frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (*Punica granatum* L.). *Scientia Horticulturae,* **85**: 261-270.

Naik, S.K. and Chand, P.K. 2003. Silver nitrate and aminoethoxy vinyl glycine promote *in vitro* adventitious shoot regeneration of pomegranate (*Punica granatum* L.). *Journal of Plant Physiology*, **160**(4): 423-430.

Omar, R., Abdullah, M.A., Hasan, M.A. and Marziah, M. 2004. Development of growth medium for *Centella asiatica* cell culture *via.* response surface methodology. *American Journal Applied Science,* **1**(3): 215-219.

Panaia, M. 1998. *Rescuing Symonanthes bancroftii (Solanaceae), A Western Australian Native Species from Extinction Through In Vitro Micropropagation.* Honours Thesis, University of Western Australia, Perth, Australia.

Parth, D., Ghanshyam, P., Bhumika, D., Shikha, D., Fenil, P. and Subhash, N. 2018. Development of an efficient micropropagation protocol through axillary shoot proliferation for pomegranate variety Bhagwa. *Annals of Agrarian Science,* **16**: 444-445.

Patil, V.M., Dhande, G.A., Thigale, D.M. and Rajput, J.C. 2011. Micropropagation of pomegranate (*Punica granatum* L.) 'Bhagava' cultivar from nodal explant. *African Journal of Biotechnology,* **10**(79): 18130-18136.

Preece, J.E. and Compton, M.E. 1991. Problems with explants exudation in micropropagation. *Biotechnology in Agriculture and Forestry,* **17**: 168-189.

Rachana, M.D., Ashok, K.P., Vibha, J. B., Smita, S. and Shekhawat, N.S. 2019. Cloning of mature pomegranate (*Punica granatum*) cv. Jalore seedless *via in vitro* shoot production and *ex vitro* rooting. *International Journal of Plant Research,* **2**(32): 181-189.

Satheesh, M. and Sridharan, T.B. 2014. Standardization of *Punica granatum* explants and callus induction through micropropagation - Indirect organogenesis. *International Journal of Research in Engineering Technology,* **3**(1): 19-27.

Shao, J.Z., Chen, C.L. and Deng, X.X. 2003. *In vitro* induction of tetraploid in pomegranate (*Punica granatum*). *Plant Cell Tissue and Organ Culture,* **75**: 241-246.

Sharon, M. and Sinha, S. 2000. Plant regeneration from cotyledonary node of *Punica granatum* L. *Indian Journal of Plant Physiology,* **5**: 344-348.

Singh, V.P.K., Chauhan, P., Kumari, P. and Seema K. 2011. Identification and prevention of bacterial contamination on explant used in plant tissue culture labs. *Alcohol,* **3**(4): 160-163.

Singh, J., Gupta, S. and Khoshe, P. 2014. *In vitro* regeneration of pomegranate (*Punica granatum* L.) from nodal explant. *International Journal of Advance Pharmacy Biology Chemical,* **3**: 734-736.

Singh, P. and Patel, R.M. 2014. Factors influencing *in vitro* growth and shoot multiplication of pomegranate. *Journal of Life Science.,* **9**(3): 1031-1035.

Singh, P., Patel, R.M. and Kadam, S. 2013. *In vitro* mass multiplication of pomegranate from cotyledonary nodal explants cv. Ganesh. *African Journal of Biotechnology,* **12**(20): 2863-2868.

Singh, S.K., Khawale, R.N. and Singh, S.P. 2002. Effect of season, type of explants and pre-treatments to minimize polyphenolics exudation on *in vitro* culture establishment in grape. *Indian Journal Horticulture,* **59**: 233-238.

Singh, S.K., Singh, A., Singh, N.V. and Ramajayam, D. 2010. Pomegranate tissue culture and biotechnology. *Fruits, Vegetables, Cereal Science and Biotechnology,* **4**: 35-44.

Soukhak, F., Khaligh, A. and Ghaemmaghami, S.A. 2011. Study of direct adventious shoot regeneration in pomegranate (*Punica granatum* cv. Malas Saveh) through cotyledonary explants. *International Journal of Agricultural Science and Research,* **2**: 19-26.

Suhasini, S.C., Patil, S.N., Prabhuling, G., Venkateshalu and Jagadeesh, S.L. 2017. A suitable explant for culture establishment in pomegranate (*Punica granatum* L.) *Advances in Bioresearch,* **8**(5): 33-36.

Sukhamate, P. and Panse, V. 1954. *Statistical Methods for Agricultural Workers.* Indian Council of Agricultural Research, New Delhi, India.

Susmita, S., Taramla, R. and Shiv, K. 2016. A review on the scope for increasing *in vitro* production of pomegranate (*Punica granatum* L.) cultivars and its application in the human health sector with emphasis on the Indian industry. *Plant Cell Biotechnology and Molecular Biology,* **16**(1&2): 60-73.

Tewari, P. and Avinashilingam, V. 2020. Supply chain of pomegranate and its value addition: Impact of improved variety Bhagwa. *Advances in Agriculture Horticulture and Entomology,* **111**: 1-6.

Tiwari, S.K., Tiwari, K.P. and Siril, E.A. 2012. An improved micropropagation protocol for teak. *Plant Cell Tissue Organ Culture,* **71**: 1-6.

Usharani, T.R., Meenakshi, S., Vijayalaksmi, Sunisha, C. and Mohandas, S. 2014. *In vitro* shoot regeneration from nodal explants of pomegranate (*Punica granatum* L.) cv. Bhagwa. *Advance in Applied Research,* **6**(1): 66-71.

Van Staden, J. and Drewes, F. 1992. The stability and metabolism of benzyl adenine glucosides in soyabean callus. *Journal of Plant Physiology,* **140**: 92-95.