In vitro REGENERATION OF *Punica granatum* L. PLANTS FROM DIFFERENT JUVENILE EXPLANTS

Raj Deepika* and Kamlesh Kanwar

Department of Biotechnology
Dr Y S Parmar University of Horticulture and Forestry, Solan 173 230, HP, INDIA

*Corresponding author's present address:
Forestry and Agricultural Biotechnology Institute (FABI), Department of Genetics
Faculty of Natural and Agricultural Sciences, University of Pretoria
Pretoria 0002, SOUTH AFRICA
e-mail: rajdeepika@gmail.com

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A B S T R A C T

Reliable and reproducible protocols to get healthy and well formed plants from juvenile explants of the pomegranate (*Punica granatum* L.) cv. 'Kandhari Kabuli' have been developed. Calli were initiated from cotyledon, hypocotyl, leaf and internode sections excised from 30 days old *in vitro* germinated seedlings. The best media for callus induction from cotyledon, hypocotyl, internode and leaf explants were MS medium supplemented with 13.0 µM NAA and 13.5 µM BA, 13.0 µM NAA and 18.0 µM BA, 5.0 µM IBA and 9.0 µM BA, 8.0 µM NAA and 9.0 µM kinetin, respectively. The highest percentage of callus was obtained from cotyledon explants (85.50) followed by hypocotyl (79.67), internode (79.47) and leaf (75.48) explants. The calli thus obtained showed differentiation on MS medium supplemented with 9.0 µM BA and 2.5 µM NAA. Cotyledon derived callus showed the highest regeneration rate (81.97%, with mean number of 16.47 shoots per explant) followed by hypocotyl, internode and leaf derived calli. *In vitro* rooting was best on half strength MS medium containing 500 mg l^{-1} of activated charcoal. The plantlets with well formed root systems were transferred to plastic cups containing cocopeat followed by transfer to earthen pots containing soil and sand (1:1).

Key words: callus cultures, juvenile explants, *Punica granatum* L.

INTRODUCTION

Pomegranate is an economically important species of the tropical and subtropical regions of the world due to its delicious edible fruits, and pharmaceutical and ornamental usage (Jayesh and Kumar, 2004). It is considered native to Iran, Afghanistan and Southern Pakistan's Baluchistan region to the Himalayas in Northern India. Pomegranate has been naturalized over the whole Mediterranean region and the Caucasus since ancient times. It has been widely cultivated throughout India, drier parts of Southeast Asia, Malaysia, the East Indies and tropical Africa. The total area under pomegranate cultivation in India is 100,000 ha yielding 0.45 million tons of fruit per year. It is largely used as a dessert. The seeds along with the fleshy pulp are dried and used as condiment. The fruit juice is a good source of sugars, vitamin C, vitamin B, pantothenic acid, potassium, antioxidant polyphenols and a fair source of iron. Some parts of the pomegranate tree (leaves, immature fruits, fruit rind, flower buds) have been used traditionally for their medicinal properties and also for tanning of leather.

Several studies have been conducted on micropropagation of pomegranate trees over the past several years. Protocols have been developed for regeneration of *Punica granatum* L. plantlets *in vitro* through either organogenesis from callus derived from leaf segments (Omura et al., 1987; Murkute et al., 2002), cotyledons (Murkute et al., 2002; Raj Deepika and Kanwar, 2008; Kanwar et al., 2010), anthers (Moriguchi et al., 1987) or through embryogenesis from various seedling explants (Jaidka and Mehra, 1986), petals (Nataraja and Neelambika, 1996) and immature zygotic embryos (Bhansali, 1990; Kanwar et al., 2010). *In vitro* propagation of pomegranate through axillary shoot proliferation from nodal segments (Zhang and Stolz, 1991; Naik et al., 1999; Naik et al., 2000; Murkute et al., 2004; Kanwar et al., 2004), shoot tips (Murkute et al., 2004) and cotyledonary nodes (Sharon and Sinha, 2000) has also been reported. However, to date there has been hardly any report done, comparing *in vitro* regeneration of the complete plantlet from seedling derived cotyledon, hypocotyl, internode and leaf explants of *Punica granatum* L. cv. 'Kandhari Kabuli' through callus induction.

MATERIAL AND METHODS

Source material

Fruits of *Punica granatum* L. cv. 'Kandhari Kabuli' were collected from the high yielding, 6 year old tree growing in the fields of the Department of Fruit Sciences, Dr Y S Parmar University of Horticulture and Forestry, Solan (HP) 173 230, India. The flowers were allowed to self pollinate by bagging them in March and April 2006 during the first flush of flowering. After self pollination the bags were removed and the fruit was allowed to ripen. The mature ripened fruits were harvested in September. The juicy

pulp was removed from the seeds and the seeds were allowed to dry in the sun. The seeds were stored in a dark place for further use.

Establishment of seedlings

Seeds of *Punica granatum* L. cv. 'Kandhari Kabuli' collected from ripe fruits were kept under running tap water for one hour followed by treatment with a 5% (v/v) aqueous solution of teepol and rinsed 5-6 times with distilled water. The seeds were then surface sterilized by treating them with 0.2% bavistin (Carbendazim, United Phosphorous Ltd., Gujarat, India) for 5 minutes followed by three washings with autoclaved distilled water. They were then treated with 15% sodium hypochlorite for 20 minutes followed by three washings with autoclaved distilled water. Seeds were finally treated with 70% ethanol for 30 seconds and rinsed thoroughly with autoclaved distilled water. Seeds were kept in autoclaved distilled water for 24 hours so as to soften the seed coat. The zygotic embryos were excised by making a horizontal cut on the broad part of the seed coat and placed on the MS (Murashige and Skoog, 1962) basal medium. The cultures were incubated at 25 °C (±2 °C) under a 16 hour photoperiod for germination.

Establishment of callus cultures

Thirty day-old seedlings were used as an explant source. The cotyledons without proximal ends were transversely cut into two halves (0.5-0.8 cm); hypocotyls were cut into small segments (0.5-0.8 cm). The margins of the first leaf were removed before cutting into small segments (0.3-0.5 cm) and the internode region was also cut into small segments (0.5-0.8 cm). Different concentrations of NAA ranging from 7.0 µM to 16.0 µM were used in combination with 4.5 µM to 18.0 µM BA for the induction of callus from cotyledon and hypocotyl. The leaf and internode explants were cultured on MS medium supplemented with various combinations of NAA (0.0 to 10.0 µM), IBA (2.5 to 7.5 µM), BA (0.0 to 13.5 µM) and kinetin (0.0 to 9.0 µM) as presented in Tables 1 and 2.

All the experiments were conducted using 100 ml borosil flasks each containing 20 ml medium. Cultures were maintained at 25°C (±2°C) with 35 µ mol m^{-2} s^{-2} photon flux density provided by cool white fluorescent tubes under 16 hours of photoperiod and 60% relative humidity for six weeks. The percentage of callus-forming explants, morphological characters of callus such as growth, colour and type were recorded. Each treatment consisted of five replicates (culture flasks) and the experimental unit was five explants per flask. The experiment was replicated three times.

Callus differentiation, shoot bud induction and shoot regeneration

For shoot regeneration studies, the callus obtained from all the explants was cut into small pieces (0.6-0.8 cm^2) and subsequently subcultured on the same medium for callus proliferation as well as on MS

medium supplemented with various combinations of BA (0.0 to 11.0 µM) and NAA (0.0 to 5.0 µM), kinetin (0.0 to 11.0 µM) and NAA (0.0 to 5.0 µM) as well as TDZ (3.0-11.0 µM) alone for shoot bud induction (Tab. 3). Cultures were maintained at 25 °C (±2 °C) under 16 hours of photoperiod to promote callus proliferation and shoot bud induction. After shoot bud differentiation, the callus pieces along with the shoot buds were transferred to the same medium. The observations were taken for per cent callus pieces showing shoot bud induction and average number of shoots per callus piece. Each treatment consisted of eight replicates (culture flasks) and the experimental unit was three callus pieces per flask. The experiment was replicated thrice.

In vitro rooting

Regenerated microshoots (1.5-3.0 cm) were excised from the callus and transferred to half strength MS medium supplemented with different concentrations of NAA, IAA, IBA ranging from 0.25 µM to 2.0 µM and activated charcoal from 100 mg l^{-1} to 600 mg l^{-1}. The cultures were maintained under a 16 hour photoperiod at 25 °C (±2 °C). Observations were recorded after four weeks of incubation for per cent rooting based on number of shoots forming roots, number of roots per shoot and length of roots (cm). Each treatment consisted of 10 replicates (culture tubes). The experimental unit was one shoot per culture tube. The experiment was replicated thrice.

Acclimatization of *in vitro* raised plantlets

One month later, well-rooted plants were removed from the medium and their roots were gently washed with tap water to remove the sticking medium. Regenerated plantlets were dipped in 0.01% bavistin (Carbendazim, United Phosphorous Ltd., Gujarat, India) solution for 15-20 minutes and transferred into plastic cups containing sterilized cocopeat. The potted plants were covered with transparent plastic bags to maintain the humidity. Acclimatization to the external environment was done by removing the transparent plastic bags gradually to reduce the humidity. Plants were nourished with 1/10 strength MS basal medium (devoid of sucrose) every other day. Subsequently, the plantlets were transferred to earthen pots containing soil : sand (1:1). After four weeks, the observations were recorded for per cent survival of plants in the earthen pots.

Statistical analysis

The experiments were conducted in a completely randomized design (CRD). The data recorded on different parameters were subjected to analysis of variance using CRD (Gomez and Gomez, 1984). Arcsine transformation was applied for the data expressed in percentages.

RESULTS

Establishment of callus cultures

The experiment was carried out to study the effect of different treatments,

explants and their interaction on per cent callus induction. The callus initiation was observed at the cut ends and wounded regions of both the explants after two weeks of culture. The whole surface of the explant was covered with the callus within six weeks of incubation (Fig. 1). Data in Table 1 shows that the plant growth regulators had a significant effect on per cent of explants showing callus formation in cotyledon and hypocotyl explants while the control without growth regulators, did not respond at all. The MS medium supplemented with 13.0 µM NAA and 13.5 µM BA resulted in the highest callus induction (85.50%) from cotyledon explants followed by the treatment consisting of MS medium supplemented with 16.0 µM NAA and 13.5 µM BA which resulted in 73.67% callus induction. The best medium for callus induction from hypocotyl explant was MS medium supplemented with 13.0 µM NAA and 18.0 µM BA which caused 79.67% callus induction followed by treatment consisting of MS medium supplemented with 16.0 µM NAA and 18.0 µM BA inducing 70.15% callus. The cotyledon and hypocotyl explants showed an increased percentage of callus induction as the concentration of NAA increased from 7.0 µM to 16.0 µM in combination with all the concentration of BA ranging from 4.5 µM to 18.0 µM. In general, the cotyledon explants yielded a significantly higher callus induction percentage (51.53%) as compared to hypocotyl explants (44.47%), irrespective of different media combination.

Data in Table 2 shows that the explants in control medium did not respond at all. It was observed that there was an increase in the percentage of explants showing callus formation with increasing concentrations of NAA from 6.0 µM to 10 µM or IBA (2.5 µM and 5.0 µM) in combination with BA or kinetin (4.5 µM and 9.0 µM). The highest callus induction from internode explants was achieved in treatment consisting of MS medium supplemented with 5.0 µM IBA and 9.0 µM BA (79.47%) followed by treatment consisting of MS medium supplemented with 8.0 µM NAA and 9.0 µM BA (74.50%). On the other hand, the highest per cent callus induction from leaf explants was obtained in treatment consisting of MS medium supplemented with 8.0 µM NAA and 9.0 µM kinetin (75.48%) followed by treatment consisting of MS medium supplemented with 8.0 µM NAA and 9.0 µM BA (74.08%). In general, the leaf explants yielded a significantly higher response (54.06%) than the internode explants (48.14%) where it took six weeks for optimum growth of callus on the explants in comparison with five weeks in internode explants.

Effect of different combinations and concentrations of these plant growth regulators supplemented in MS medium, on type, colour and growth of callus derived from internode, as well as leaf segments, was also studied. The results showed that the callus obtained from leaf explants on MS medium supplemented with 8.0 µM NAA and 9.0 µM kinetin was compact, yellowish green in

Table 1. Effect of different concentrations of NAA, alone and in combination with BA supplemented in MS medium, on per cent callus induction from cotyledon and hypocotyl explants after six weeks of culture

Plant growth regulators [µM]		Per cent of explants forming callus		Mean
NAA	BA	Cotyledon pieces	Hypocotyl segments	
0.0	0.0	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00 (0.00 ± 0.00)	00.00 (00.00)
7.0	0.0	26.14 ± 0.60 (30.75 ± 0.39)	32.24 ± 0.32 (34.86 ± 0.20)	29.19 (32.67)
10.0	0.0	32.10 ± 0.79 (34.51 ± 0.49)	39.59 ± 0.43 (38.99 ± 0.25)	35.84 (36.75)
13.0	0.0	48.17 ± 0.61 (43.95 ± 0.35)	42.63 ± 0.02 (40.77 ± 0.01)	45.40 (42.36)
16.0	0.0	42.47 ± 1.45 (40.67 ± 0.84)	40.63 ± 0.32 (39.60 ± 0.19)	41.55 (40.13)
7.0	4.5	32.58 ± 1.11 (34.80 ± 0.68)	26.82 ± 1.20 (31.18 ± 0.77)	29.70 (32.99)
10.0	4.5	45.29 ± 0.68 (42.30 ± 0.39)	31.90 ± 0.84 (34.38 ± 0.52)	38.60 (38.34)
13.0	4.5	52.01 ± 0.90 (46.16 ± 0.52)	48.14 ± 0.56 (43.94 ± 0.32)	50.08 (45.05)
16.0	4.5	49.26 ± 0.61 (44.58 ± 0.35)	44.15± 2.31 (41.64 ± 1.34)	46.71 (43.11)
7.0	9.0	46.32 ± 0.67 (42.89 ± 0.39)	35.79 ± 0.32 (36.75 ± 0.19)	41.06 (39.82)
10.0	9.0	53.00 ± 1.61 (46.72 ± 0.93)	41.25 ± 0.50 (39.96 ± 0.29)	47.13 (43.34)
13.0	9.0	68.32 ± 0.08 (55.75 ± 0.03)	50.95 ± 0.78 (45.54 ± 0.45)	59.64 (50.65)
16.0	9.0	61.74 ± 0.78 (51.79 ± 0.46)	46.48 ± 0.88 (42.98 ± 0.45)	54.94 (47.89)
7.0	13.5	67.43 ± 0.69 (55.21 ± 0.42)	41.08 ± 0.60 (39.86 ± 0.35)	54.25 (47.53)
10.0	13.5	68.00 ± 1.08 (55.56 ± 0.66)	51.85 ± 0.90 (46.06 ± 0.52)	59.92 (50.81)
13.0	13.5	**85.50 ± 1.25 (67.63 ± 0.56)**	64.85 ± 0.85 (53.64 ± 0.51)	75.18 (60.64)
16.0	13.5	73.67 ± 0.97 (59.14 ± 0.63)	41.08 ± 0.60 (39.86 ± 0.35)	57.37 (49.50)
7.0	18.0	48.70 ± 0.35 (44.26 ± 0.20)	42.84 ± 1.77 (40.88 ± 1.03)	45.77 (42.57)
10.0	18.0	50.19 ± 0.04 (45.11 ± 0.02)	61.86 ± 0.65 (51.86 ± 0.38)	56.02 (48.49)
13.0	18.0	68.48 ± 0.32 (55.85 ± 0.20)	**79.67 ± 0.17 (63.20 ± 0.12)**	74.07 (59.52)
16.0	18.0	61.14 ± 0.61 (51.44 ± 0.36)	70.15 ± 0.51(56.89 ± 0.32)	65.65 (54.16)
Mean		51.53 (45.24)	44.47 (41.08)	

Effect – $CD_{0.05}$ Treatment – 1.99 (1.21) Explant – 0.48 (0.29) Treatment X Explant – 2.81 (1.71)

Figures in parentheses are arcsine transformation values

Table 2. Effect of different concentrations of NAA in combination with BA and kinetin, and different concentrations of IBA together with BA and kinetin supplemented in MS medium, on per cent callus induction from internode and leaf explants after six weeks of culture

Plant growth regulators [µM]				Per cent of explants forming callus		Mean
NAA	IBA	BA	Kinetin	Internode	Leaf	
0.0	0.0	0.0	0.0	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00 (0.00 ± 0.00)	0.00 (0.00)
6.0	-	4.5	-	41.04 ± 0.59 (39.84 ± 0.34)	30.18 ± 0.04 (33.33 ±0.03)	35.61(36.58)
8.0	-	4.5	-	51.47 ± 0.71 (45.84 ± 0.41)	51.72 ± 0.79 (45.99 ±0.45)	51.59 (45.92)
10.0	-	4.5	-	46.82 ± 1.20 (43.18 ± 0.69)	43.62 ± 0.67 (41.34 ±0.39)	45.22 (42.26)
6.0	-	9.0	-	71.81 ± 1.68 (57.95 ± 1.08)	70.19 ± 0.57 (56.91± 0.36)	71.00 (57.43)
8.0	-	9.0	-	74.50 ± 1.21 (59.68 ± 0.79)	74.08 ± 0.52 (59.40± 0.34)	74.29 (59.54)
10.0	-	9.0	-	51.15 ± 0.56 (45.66 ± 0.32)	72.17 ± 0.01(58.16 ± 0.01)	61.66 (51.91)
6.0	-	-	4.5	30.21 ± 0.01(33.35 ± 0.01)	48.17 ± 0.56(43.95 ± 0.32)	39.19 (38.65)
8.0	-	-	4.5	57.84 ± 0.36 (49.51 ± 0.21)	60.81 ± 0.57(51.25 ± 0.33)	59.32 (50.38)
10.0	-	-	4.5	43.17 ± 1.53 (41.07 ± 0.89)	58.18 ± 0.57(49.71 ± 0.33)	50.69 (45.39)
6.0	-	-	9.0	25.15 ± 0.57 (30.10 ± 0.38)	69.10 ±0.07 (56.23 ± 0.04)	47.13 (43.17)
8.0	-	-	**9.0**	73.23 ± 1.13 (58.85 ± 0.73)	**75.48 ± 0.33(60.32 ± 0.22)**	74.36 (59.59)
10.0	-	-	9.0	59.22 ± 0.57 (50.31 ± 0.33)	64.85 ± 0.33(53.64 ± 0.20)	62.03 (51.98)
-	2.5	4.5	-	23.22 ± 1.94 (28.77 ± 1.31)	58.85 ± 0.30(50.10 ± 0.17)	41.04 (39.44)
-	**5.0**	**9.0**	**-**	**79.47 ± 0.32 (63.06 ± 0.23)**	71.39 ± 0.64(57.67 ± 0.41)	75.43 (60.36)
-	7.5	13.5	-	32.80 ± 0.35 (34.94 ± 0.21)	28.25 ± 0.56(32.10 ± 0.36)	30.52 (33.52)
-	2.5	4.5	4.5	40.44 ± 0.26(39.49 ± 0.15)	32.18 ± 0.55(34.56 ± 0.34)	36.31 (37.03)
-	5.0	9.0	9.0	64.94 ± 1.41(53.70 ± 0.84)	63.85 ± 1.22(53.05 ± 0.73)	64.39 (53.37)
Mean				48.14 (43.07)	54.06 (46.54)	

Effect	**$CD_{0.05}$**
Treatment	1.58 (0.98)
Explant	0.53 (0.32)
Treatment X Explant	1.50 (1.31)

Figures in parentheses are arcsine transformation values

a b c d

Figure 1. Callus induction from juvenile explants of *Punica granatum* L. cv. 'Kandhari Kabuli' after six weeks of culture

a) Callus obtained from cotyledon explants on MS medium supplemented with 13.0 µM NAA and 13.5 µM BA
b) Callus obtained from hypocotyl explants on MS medium supplemented with 13.0 µM NAA and 18.0 µM BA
c) Callus obtained from internode on MS medium supplemented with 5.0 µM IBA and 9.0 µM BA
d) Callus obtained from leaf explant on MS medium supplemented with 8.0 µM NAA and 9.0 µM BA

colour and showed very good callus growth (1.0-1.5 cm). On the other hand, the callus obtained from internode explants on MS medium supplemented with 5.0 µM IBA and 9.0 µM BA was compact nodular, creamy yellow in colour and showed excellent callus growth (1.5-2.0 cm).

The calli obtained from all the explants were proliferated on the same respective medium by subculturing two times at an interval of four weeks.

Callus differentiation, shoot bud induction and shoot regeneration

Small callus pieces (0.6-0.8 cm^2) derived from all the explants were cultured on callus differentiation and shoot regeneration media containing various concentrations and combinations of plant growth regulators (BA, NAA, kinetin and TDZ), to observe the regeneration potential of calli. Table 3 reveals that the best medium for the induction of shoot buds was MS basal medium supplemented with 9.0 µM BA and 2.5 µM NAA followed by MS medium supplemented with 11.0 µM BA and 5.0 µM NAA. The callus differentiation was observed in 5 to 71 per cent of calli derived from all the explants.

The highest rate of shoot regeneration was observed in cotyledon (81.97%) followed by hypocotyl (70.57%), internode (68.43%) and leaf derived callus (65.32%). The data shows that 24.76%, 18.86%, 20.41%, 16.53% calli differentiated into shoots from cotyledon, hypocotyl, internode and leaf derived callus, respectively. Cotyledon derived callus yielded the best response compared with all the other explants.

Table 4 also reveals that the highest number of shoots per callus pieces were formed on MS medium supplemented with 9.0 µM BA and 2.5 µM NAA (Fig. 2). The maximum

Table 3. Effect of different concentrations of BA or kinetin in combination with NAA and TDZ singly supplemented in MS medium, on shoot bud induction from callus after four weeks of culture

Plant growth regulators (µM)				Per cent of explants forming shoots				**Mean**
BA	Kinetin	NAA	TDZ	Cotyledon	Hypocotyl	Internode	Leaf	
0.0	0.0	0.0	-	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00 (0.00 ± 0.00)	0.00 (0.00)
3.0	-	2.5	-	9.60 ± 0.38 (18.04 ± 0.37)	4.27 ± 0.13 (11.92 ± 0.18)	8.13 ± 0.07 (16.57 ± 0.07)	3.07 ± 0.06 (10.08 ± 0.10)	6.27 (14.15)
5.0	-	2.5	-	23.15 ± 1.76(28.73 ± 1.19)	16.23 ± 0.15 (23.76 ± 0.11)	18.45 ± 0.22 (25.44 ± 0.16)	15.15 ± 0.03 (22.91 ± 0.02)	18.25 (25.21)
7.0	-	2.5	-	48.75 ± 4.62 (44.27 ± 2.66)	35.42 ± 0.22 (36.52 ± 0.13)	39.27 ± 0.12 (38.80 ± 0.07)	30.05 ± 0.08 (33.24 ± 0.05)	38.37 (38.21)
9.0	**-**	**2.5**	-	**81.97 ± 0.96 (64.89 ± 0.71)**	**70.57 ± 0.23 (70.57 ± 0.15)**	**68.43 ± 0.12 (55.82 ± 0.07)**	**65.32 ± 0.09 (53.92 ± 0.06)**	**71.57 (57.94)**
11.0	-	5.0	-	53.61 ± 2.38 (47.08 ± 1.37)	48.28 ± 0.12 (44.02 ± 0.07)	50.20 ± 0.06 (45.12 ± 0.03)	45.27 ± 0.12 (42.29 ± 0.07)	49.34 (44.62)
-	3.0	2.5	-	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00 (0.00 ± 0.00)	0.00 (0.00)
-	5.0	2.5	-	15.63 ± 2.95 (20.71 ± 2.27)	9.27 ± 0.12 (17.72 ± 0.12)	10.15 ± 0.03 (18.58 ± 0.03)	7.28 ± 0.11 (15.66 ± 0.12)	10.58 (18.77)
-	7.0	2.5	-	25.39 ± 2.39 (28.32 ± 1.55)	14.32 ± 1.91 (24.14 ± 1.62)	20.21 ± 0.03 (26.71 ± 0.02)	10.23 ± 0.04 (18.65 ± 0.04)	17.54 (24.43)
-	9.0	2.5	-	33.51 ± 2.42 (32.69 ± 1.46)	31.47 ± 0.08 (34.13 ± 0.05)	30.50 ± 0.22 (33.52 ± 0.14)	28.28 ± 0.13 (32.13 ± 0.08)	30.94 (33.78)
-	11.0	5.0	-	32.17 ± 3.50 (37.16 ± 2.13)	26.54 ± 0.20 (31.01 ± 0.13)	25.20 ± 0.06 (30.13 ± 0.04)	15.30 ± 0.10 (23.03 ± 0.08)	24.80 (29.67)
-	-	-	3.0	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00 (0.00 ± 0.00)	0.00 (0.00)
-	-	-	5.0	10.01 ± 0.55 (18.43 ± 0.53)	2.32 ± 0.09 (8.76 ± 0.17)	3.50 ± 0.14 (10.76 ± 0.23)	4.65 ± 0.13 (12.45 ± 0.17)	5.12 (12.60)
-	-	-	7.0	12.66 ± 0.28 (20.84 ± 0.24)	4.26 ± 0.03 (11.91 ± 0.04)	9.28 ± 0.01 (17.73 ± 0.01)	6.18 ± 0.07 (14.40 ± 0.09)	8.10 (16.22)
	-	-	9.0	25.88 ± 2.80 (30.51 ± 1.87)	20.53 ± 0.19 (26.94 ± 0.13)	22.92 ± 0.05 (28.60 ± 0.04)	18.37 ± 0.11 (25.38 ± 0.08)	21.92 (27.86)
	-	-	11.0	23.89 ± 2.15 (29.22 ± 1.43)	18.25 ± 0.06 (25.29 ± 0.04)	20.27 ± 0.03 (26.76 ± 0.02)	15.38 ± 0.11 (23.09 ± 0.08)	19.45 (26.09)
Mean				24.76 (26.58)	18.86 (21.95)	20.41 (23.41)	16.53 (20.45)	

Effect – $CD_{0.05}$ Treatment – 1.57 (1.02) Explant – 0.79 (0.51) Treatment X Explant – 3.15 (2.04)

Figures in parentheses are arc sine transformation values

Table 4. Effect of different concentrations of BA or kinetin in combination with NAA and TDZ singly supplemented in MS medium, on number of shoots regenerated from callus after four weeks of culture

Plant growth regulators (µM)				Number of shoots per piece of callus				Mean
BA	Kinetin	NAA	TDZ	Cotyledon	Hypocotyl	Internode	Leaf	
0.0	0.0	0.0	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00
3.0	-	2.5	-	1.18 ± 0.11	1.03 ± 0.03	1.12 ± 0.01	1.07 ± 0.07	1.10
5.0	-	2.5	-	6.54 ± 0.77	5.41 ± 0.12	4.12 ± 0.02	2.16 ± 0.03	4.56
7.0	-	2.5	-	7.94 ± 0.94	6.17 ± 0.04	5.15 ± 0.01	4.19 ± 0.02	5.86
9.0	**-**	**2.5**	**-**	**16.47 ± 2.20**	**12.75 ± 0.33**	**7.12 ± 0.06**	**5.15 ± 0.03**	10.37
11.0	-	5.0	-	7.62 ± 0.44	7.15 ± 0.03	4.19 ± 0.04	3.17 ± 0.04	5.53
-	3.0	2.5	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00
-	5.0	2.5	-	1.43 ± 0.30	1.07 ± 0.04	1.38 ± 0.15	1.06 ± 0.03	1.24
-	7.0	2.5	-	3.23 ± 0.57	2.15 ± 0.03	2.47 ± 0.16	1.25 ± 0.07	2.27
-	9.0	2.5	-	3.43 ± 0.28	3.33 ± 0.15	3.19 ± 0.03	2.55 ± 0.22	3.12
-	11.0	5.0	-	2.39 ± 0.26	2.03 ± 0.03	2.12 ± 0.01	1.67 ± 0.12	2.05
-	-	-	3.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00
-	-	-	5.0	2.27 ± 0.27	2.02 ± 0.04	1.82 ± 0.11	1.43 ± 0.04	1.88
-	-	-	7.0	2.15 ± 0.40	1.95 ± 0.03	1.66 ± 0.03	1.12 ± 0.01	1.72
	-	-	9.0	3.02 ± 0.51	2.38 ± 0.15	2.47 ± 0.09	2.12 ± 0.01	2.50
	-	-	11.0	2.02 ± 0.07	1.94 ± 0.02	1.67 ± 0.04	1.33 ± 0.21	1.73
Mean				3.73	3.09	2.40	1.77	

Effect	**$CD_{0.05}$**
Treatment	0.49
Explant	0.25
Treatment X Explant	0.99

a b c d

Figure 2. Shoot regeneration on MS medium supplemented with 9.0 µM BA and 2.5 µM NAA from cotyledon derived callus (a), hypocotyl derived callus (b), internode derived callus (c) and leaf derived callus (d)

average number of shoots per callus piece were observed from cotyledon derived callus (16.47 shoots) followed by hypocotyl (12.75 shoots), internode (7.12 shoots) and leaf (5.15 shoots) explants. The shoot buds grew in size and within six weeks shoots with an average shoot length of up to 2.07 cm were observed.

In vitro rooting

Data presented in Table 5 shows that the control (half strength MS basal medium), and half strength of MS basal medium supplemented with 0.25-2.0 µM IBA or 2.0 µM NAA did not show rooting. The maximum rooting (89.00%), average number of 6.57 roots per shoot and maximum average root length of 2.93 cm was achieved on half strength MS basal medium containing 500 mg l^{-1} activated charcoal followed by half strength MS medium supplemented with 0.50 µM NAA showing 83.00% rooting, 4.50 average number of roots per shoot and 2.12 cm of root length. The plantlets with well developed roots were obtained within four weeks of incubation on rooting medium (Fig. 3a&b). The plantlets were hardened in plastic cups containing cocopeat (Fig. 3c&d) and subsequently transferred to earthen pots containing soil and sand (1:1). The per cent survival of plantlets was 80% after 60 days of transfer and remained constant thereafter. The plant height increased over time from 3.26 cm at 0 day to 12.48 cm at 360 days of transfer. The average number of leaves shoot diameter and number of nodes also increased over time, indicating successful establishment of tissue culture raised plantlets (Fig. 3e).

DISCUSSION

Conventional breeding techniques of woody fruit trees is often difficult and slow because of high levels of heterozygosity and the long generation time between successive crosses as reviewed from time to time (Sriskandarajah et al., 1994; Naik et al., 1999; Naik and Chand, 2003; Singh et al., 2007). These difficulties necessitate the development of rapid and efficient regeneration protocol for *in vitro* propagation of elite genotypes.

Table 5. Effect of different rooting media on induction of roots from in vitro raised microshoots after six weeks of culture

Medium	Response	Per cent of shoots forming roots	Average number of roots per shoot	Average root length [cm]
½ MS basal medium	no response	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00	0.00 ± 0.00
½ MS basal medium + 0.25 µM NAA	rooting	71.00 ± 0.58 (57.42 ± 0.36)	2.10 ± 0.06	1.00 ± 0.29
½ MS basal medium + 0.50 µM NAA	rooting	83.00 ± 0.58 (65.6 6 ± 0.44)	4.50 ± 0.29	2.12 ± 0.22
½ MS basal medium + 1.00 µM NAA	rooting	61.00 ± 0.58 (51.36 ± 0.34)	3.10 ± 0.06	1.80 ± 0.15
½ MS basal medium + 2.00 µM NAA	callusing	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00	0.00 ± 0.00
½ MS basal medium + 0.25 µM IAA	rooting	32.00 ± 0.58 (34.45 ± 0.35)	2.20 ± 0.06	1.12 ± 0.07
½ MS basal medium + 0.50 µM IAA	rooting	77.67 ± 0.88 (61.81 ± 0.60)	3.77 ± 0.09	2.34 ± 0.14
½ MS basal medium + 1.00 µM IAA	rooting	41.33 ± 0.88 (40.01 ± 0.51)	2.50 ± 0.21	1.79 ± 0.09
½ MS basal medium + 2.00 µM IAA	rooting	30.00 ± 1.15 (33.20 ± 0.72)	2.27 ± 0.12	1.34 ± 0.09
½ MS basal medium + 0.25 µM IBA	callusing	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00	0.00 ± 0.00
½ MS basal medium + 0.50 µM IBA	callusing	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00	0.00 ± 0.00
½ MS basal medium + 1.00 µM IBA	callusing	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00	0.00 ± 0.00
½ MS basal medium + 2.00 µM IBA	callusing	0.00 ± 0.00 (0.00 ± 0.00)	0.00 ± 0.00	0.00 ± 0.00
½ MS basal medium + 100 mg l^{-1} activated charcoal	rooting	41.00 ± 0.58 (39.82 ± 0.34)	3.37 ± 0.12	1.43 ± 0.23
½ MS basal medium + 200 mg l^{-1} activated charcoal	rooting	52.67 ± 1.45 (46.53 ± 0.83)	2.90 ± 0.17	1.58 ± 0.19
½ MS basal medium + 300 mg l^{-1} activated charcoal	rooting	63.00 ± 2.52 (52.56 ± 1.51)	4.63 ± 0.09	1.41 ± 0.10
½ MS basal medium + 400 mg l^{-1} activated charcoal	rooting	63.67 ± 1.20 (52.94 ± 0.72)	5.17 ± 0.32	2.13 ± 0.10
½ MS basal medium+ 500 mg l^{-1} activated charcoal	rooting	**89.00 ± 0.58 (70.64 ± 0.53)**	**6.57 ± 0.20**	**2.93 ± 0.09**
½ MS basal medium+ 600 mg l^{-1} activated charcoal	rooting	60.00 ± 0.58 (50.77 ± 0.34)	3.37 ± 0.22	1.28 ± 0.14
$CD_{0.05}$		**2.55 (1.57)**	**0.42**	**0.38**

Figures in parentheses are arcsine transformation value

a b c d e

Figure 3. *In vitro* rooting and hardening of tissue culture raised plantlets of *Punica granatum* L.

a & b) *In vitro* rooting of microshoots on ½ strength MS medium containing 500 mg l^{-1} activated charcoal

c & d) Hardening of *in vitro* raised plantlets in plastic cups containing cocopeat

e) Hardening of in vitro raised plantlets in earthen pots containing soil : sand mixture

Juvenile explants were used to carry out the experiments as they have higher organogenic competence as compared to mature explants (Chakravarty and Goswami, 1999; Kanwar et al., 2008; Kanwar et al., 2010). The various *in vitro* responses were studied with respect to type of explants, plant growth regulators, their concentrations, their combinations and interaction with the explant. The results obtained from the present studies show that none of the explants responded to a growth regulator free MS medium. The cotyledon and hypocotyl explants cultured on MS medium supplemented with various combination and concentrations of NAA and BA showed formation of callus. Addition of NAA either singularly or in combination with BA to the medium was essential to induce callus from the explants. However, the percentage of callus formation observed was much higher compared to previous reports by Hammerschlag et al. (1985), Omura et al. (1987), Foughat et al. (1997), Murkute et al. (2002) and Chaugule et al. (2005). The cotyledon explants showed maximum callus induction (85.50%) on MS medium supplemented with 13.0 µM NAA in combination with 13.5 µM BA. Similarly, the maximum per cent callus induction from hypocotyl explant (79.67%) was observed on MS medium supplemented with 13.0 µM NAA along with 18.0 µM BA. The best medium for callus induction from internode and leaf explant was found to be MS medium supplemented with 5.0 µM IBA along with 9.0 µM BA and MS medium supplemented with 8.0 µM NAA and 9.0 µM kinetin showing 79.47% and 75.48% callus formation, respectively. Thus, the cotyledon explants were found to be

the best explant as compared to all other explants, for callus induction. A possible explanation may be that the young cotyledons are very active physiologically and are easily affected by exogenous plant growth regulators (Amin et al., 1999; Murkute et al., 2002; Naik and Chand, 2003). Similarly, Chaugule et al. (2005) reported that 78.94% of cotyledon explants of pomegranate formed callus on MS medium containing 0.4 mg l^{-1} NAA and 0.1 mg l^{-1} BAP. Murkute et al. (2002) also reported that cotyledon explants to be more responsive than the leaf explants.

Organogenesis is an outcome of the process of dedifferentiation followed by redifferentiation of cells. Dedifferentiation favours unorganized cell growth and the resultant callus has randomly divided meristems. Most of these meristems redifferentiate shoot buds and roots under favourable *in vitro* conditions (Bhojwani and Razdan, 1983). The callus obtained from cotyledon showed the maximum percentage (81.97%) of shoot regeneration followed by hypocotyl (70.57%), internode (68.43%) and leaf (65.32%) derived callus on MS medium supplemented with 9.0 µM BA and 2.5 µM NAA. About 18.41 shoots were obtained from cotyledonary callus after six weeks of culture. However, earlier reports on callus mediated organogenesis indicated that pomegranate is indeed a difficult species, if not a recalcitrant one, at least in respect of *in vitro* regeneration through organogenesis. The callus obtained from cotyledon and leaf explants of pomegranate showed a recalcitrant nature when cultured on different shoot regeneration medium (Chaugule et al., 2005). Similarly, shoot forming potential of the anther wall derived callus *Punica granatum* L. has been found to be very low, as only 10 out of 391 cultures showed shoot regeneration each producing 1-2 shoots per explant (Moriguchi et al., 1987). Callus derived from leaf segments of *Punica granatum* L. have been reported to exhibit very low shoot regeneration, from 10% to 15% with an average of only one shoot per explant (Omura et al., 1987).

In the present investigation, various auxins and cytokinins were used for induction of shoot buds. The need to adjust auxins and cytokinin levels in the medium has been well documented in several fruit crops such as apple (Fan and Jiang, 1993), *Carica papaya* (Mondal et al., 1994), pear (Caboni et al., 1999), *Citrus acida* (Chakravarty and Goswami, 1999), pineapple (Akbar et al., 2003), and black cherry (Liu and Pijut, 2008; Canli and Tian, 2008). It was observed that BA stimulated higher shoot bud induction in comparison to other cytokinins. It has been reported that BA, its riboside and nucleotides are naturally occurring cytokinins in plant tissues (Te-chato et al., 2008) and are relatively stable in comparison with other cytokinins (Verdeil et al., 1994; Naik and Chand, 2003; Feeney et al., 2007). This may explain the improved response with BA. Adventitious shoots have been regenerated from the anther derived callus of *Punica granatum* L. on half strength MS medium sup-

plemented with 0.5 µM NAA and 2.0 µM BA (Moriguchi et al., 1987) and from leaf derived callus of *Punica granatum* L. var. Nana on half strength MS medium supplemented with 2.0 µM BA (Omura et al., 1987). The shoot regeneration from cotyledon and leaf derived callus of *Punica granatum* L. cv. 'Ganesh' was good on MS medium supplemented with 2.0 mg l^{-1} NAA and 2.0 mg l^{-1} BA (Foughat et al., 1997). A high frequency of shoot organogenesis from leaf derived callus of pomegranate was achieved on MS medium supplemented with 1.0 mg l^{-1} BAP (Kantharajah et al., 1998). Shoot differentiation was obtained in the cotyledon derived callus of *Punica granatum* L. cv. "Ganesh" on MS medium supplemented with 1.0 mg l^{-1} BAP and 0.5 mg l^{-1} NAA (Murkute et al., 2002). The percentage regeneration and average number of shoots per callus piece were higher in the present investigation compared to those in previous reports. A synergistic influence of BA in combination with NAA was evident from the results of the present investigation. It seems likely that this protocol offers high potential for mass propagation of this species.

A high frequency of root induction was obtained on MS medium containing 500 mg l^{-1} of activated charcoal within 10 days of culture. The maximum rooting percentage (89.00%) was noted in shoots derived from cotyledonary callus followed by shoots derived from hypocotyl (84.33%), internode (73.33%) and leaf (71.33%) callus. However, callusing was observed at the basal end of the shoot on half strength MS medium supplemented with IBA. About 83% of shoots showed rooting on half strength MS medium supplemented with 0.50 µM NAA. The promotive effect of growth regulator-free medium on root induction has been recorded on several fruit trees such as *Syzygium cuminii* (Yadav et al., 1990). Similar to our results, rooting in 85-90% of microshoots of *Robinia pseudoacacia* was observed on half strength MS medium containing 0.05% activated charcoal within 15 days of culture (Kanwar et al., 2008). MS medium containing 0.05% activated charcoal has also been used for the induction of roots in *Morus alba* L. (Agarwal and Kanwar, 2007). NAA induced rooting has already been reported in *Punica granatum* L. by several workers (Omura et al., 1987; Mahishni et al., 1991; Yang and Ludders, 1993; Amin et al., 1999; Naik et al., 2000; Naik and Chand, 2003; Zhu et al., 2003). On the contrary, rooting in 83.6% of regenerated shoots from cotyledon derived callus cultures of *Punica granatum* L. cv. 'Ganesh' was observed in half strength MS medium supplemented with 1.0 mg l^{-1} IBA by Murkute et al. (2004). Thus, the present study emphasizes the suitability of growth regulator-free MS medium with half strength and activated charcoal, for maximum rooting of *in vitro* raised microshoots.

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REGENERACJA *in vitro* ROŚLIN *Punica granatum L.* Z RÓŻNYCH EKSPLANTATÓW UZYSKANYCH Z MŁODYCH SIEWEK

Raj Deepika i Kamlesh Kanwar

STRESZCZENIE

Przedstawiono wiarygodny i odtwarzalny przebieg doświadczenia mającego na celu otrzymanie zdrowych i kształtnych roślin z eksplantatów uzyskanych z młodych siewek granatowca *Punica granatum* L. 'Kandhari Kabuli'. Kalusy otrzymano z wycinków liścienia, hypokotyla, liścia i łodygi z 30-dniowych sadzonek doprowadzonych do kiełkowania metodą *in vitro*. Najlepszą pożywką dla indukcji embriologicznej kalusa z eksplantatów pochodzących z liścienia, hypokotyla, łodygi i liścia były pożywki MS uzupełnione odpowiednio 13,0 µM NAA i 13,5 µM BA, 13,0 µM NAA i 18,0 µM BA, 5,0 µM IBA i 9,0 µM BA, 8,0 µM NAA i 9,0 µM kinetyny. Największy procent kalusów otrzymano z eksplantatów uzyskanych z liścienia (85,50), następnie z hypokotyla (79,67), łodygi (79,47) i liścia (75,48). Otrzymane w ten sposób kalusy wykazały zróżnicowanie na pożywce MS uzupełnionej 9,0 µM BA i 2,5 µM NAA. Kalus uzyskany z liścienia wykazał najwyższy wskaźnik regeneracji (81,97% ze średnią liczbą 61,47 pędów na eksplantant), następnie uzyskany z hypocotyla, łodygi i liścia. Ukorzenianie metodą *in vitro* dało najlepsze wyniki na zmniejszonej o połowę pożywce MS i zawierającej 500 mg l^{-1} węgla aktywnego. Roślinki z dobrze ukształtowanym systemem korzeniowym były przeniesione z plastikowych pojemników zawierających włókna kokosowe do glinianych doniczek zawierających ziemię i piasek (1:1).

Słowa kluczowe: hodowla kalusa, eksplantaty z młodych siewek, *Punica granatum* L.