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Multiple Shoot Induction of Pomegranate (*Punica granatum* L.) Through Different Juvenile Explants

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ABSTRACT
The explants were subjected to surface sterilization by treatment involving $HgCl_2$ (0.5 %). Reliable and reproducible protocol to get healthy plants from different juvenile explants like stem segment, meristem and nodal segment for pomegranate cv 'Bhagava' has been developed. Out of these three explants, multiplications of shoots were observed in nodal segment on MS medium fortified with 3 mg/l BAP. MS medium containing 0.5 mg/L of GA_3 was found to best shoot elongation. Elongated shoots were transferred in MS media fortified 0.1 to 0.5 mg/L of NAA and 0.1 to 0.5 mg/L of IBA for rhizogenesis but root initiation was not started within 25 days incubation period as it may require some more time. Present study is carried out with an aim to develop a protocol for commercial production of pomegranate. Multiple of shoots showed potential micropropagation protocol to be used in large-scale disease free true to type clonal multiplication.
Key Word: *Micro-propagation, Regeneration, multiplication, punnica granatum.*

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INTRODUCTION

Pomegranate is a multipurpose species having both economic and ecological significance. The pomegranate tree, especially its fruit, possesses a vast ethnomedical history and represents a phytochemical reservoir of heuristic medicinal value. In 'Unani' system of medicine, pomegranate fruit extracts are served as a remedy for diabetes [8]. It retains its flavor and it can be preserved as such for over a year if it is properly filtered, bottled and preserved by using 0.1 percent sodium benzoate. The juice of pomegranate is believed to be beneficial for leprosy patients. The uses of juice, peel and oil have also been shown to possess anticancer activities, including interference with tumor cell proliferation, cell cycle, invasion and angiogenesis [3]. Powdered flower buds are used for the treatment of bronchitis; while fruit rind, decoction of bark, fresh leaves, and young fruits are used for the treatment of gastro-intestinal disorders [1]. Juice and peels possess potent antioxidant properties, while juice, peel and oil are all weakly estrogenic and heuristically of interest for the treatment of menopausal symptoms and sequellae.

Pomegranate seeds germinate readily even when merely thrown on to the surface of loose soil and the seedlings spring up with vigor. Grafting has never been successful but branches may be air-layered and suckers from a parent plant can be taken up and transplanted. Propagation of pomegranate by cutting is the most convenient as well as the cheapest method of obtaining a fully developed stronger tree in considerably less time. In order to reduce the high mortality of rooted cuttings under field conditions, it is highly desirable to build a healthy and well developed root system for enabling better field establishment of pomegranate trees through the usage of appropriate plant growth regulators [7]. The use of tissue culture techniques for fruit tree propagation has increased significantly. Micropropagation helps in overcoming difficulties that arise due to vegetative propagation, producing true-to-type plants and mass production of planting materials [9].

MATERIAL AND METHODS

Plant material was obtained from Department of Horticulture, JNKVV, Jabalpur and a private Horticulture Nursery of Nagpur. Small plants were transplanted in polyhouse at Biotechnology Centre, JNKVV and maintained as insect and disease free. Different juvenile explants that are Stem segment up to size 2.5 to 3 cm, meristem size 1.5 to 2 cm and nodal segment size 2 to 3 cm from pomegranate 'Bhagava' cultivars were collected from mature plants.

Explants sterilization

Initially the juvenile explants of pomegranate were excised from plants maintained insect and disease free in polyhouse with scissor and cleaned under running tap water for about 10 to 15 min after which they were again washed for 4 to 5 times with distilled water. Then these explants were treated with Tween-20 by adding 3-4 drops for 20 min with intermittent shaking. The explants were rinsed with distilled water until foam disappears. Further sterilization was carried out inside the laminar air flow chamber. Explants were treated with 0.5% $HgCl_2$ solution for 2 min. Sterilizes explants were washed thoroughly with autoclaved distilled water for 4 to 5 times to overcome the poisonous effect of $HgCl_2$. Finally the explants were inoculated in the MS medium fortified with combination of different growth regulators.

Inoculation of explants

MS medium fortified with different growth hormones were tested for micro-propagation of pomegranate cultivar 'Bhagava'. For initiation, MS media fortified with BAP, Kinetin and NAA was tested separately. The explants were Stem segment, meristem and nodal segment were excised with sterilized forceps and scalpels under laminar air flow and 8-10 explants were inoculated on a single plate. Plates were sealed with parafilm. Plates were incubated under 16 h photo periods at 25 ± 2°C. Explants were sub cultured on same medium at 14 days interval.

RESULTS AND DISCUSION

During present investigation, observations were recorded for callus induction, multiple shoots formation and plant regeneration abilities. Ninety explants were inoculated in nodal segment, meristem and fifty of stem segment the MS medium fortified with each different concentration of BAP, KN and combination of BAP and NAA respectively and were kept under observation. The subsequent sub-culturing was done in another 15-20 days that was carried out in 3 replicate with having 90 explants. Analysis of variance showed that the mean sum of square due to different growth regulator combinations was highly significant at 5% probability level.

Micro-propagation in cultured explants

Under aseptic conditions, leaves were removed from the explants without damaging the axillary bud, lateral buds. The explants were inoculated on MS Media containing different concentration of growth hormone. The cultures were then incubated at 25±2°C with a photoperiod regime of 12 h at 1200 lux light intensity and at 50 to 60% humidity. Three explants namely stem segment, meristem and nodal segment were cultured on three different media having different concentrations of BAP, kinetin and combination of BAP and NAA respectively. The culture medium turned brown within 3 days of inoculation of explants. The secondary metabolite secretion was first noticed at cut ends of explants which gradually spread in to the surrounding medium. The first response of cultured explants was observed after 10-12 days and mostly independent of culture media combinations.

Initiation and Multiplication of explants

Results presented in table 1 and 2 clearly indicate the significant effect of growth regulator concentration on establishment of explants for direct multiple shoot induction from nodal segment and meristem (Fig 1&2). MS medium containing higher concentration of cytokinin were better suited for multiple shoot formation as compared to combination of BAP and NAA for 10-12 multiple shoots were observed from each explants.

For shoot initiation with nodal segment explants (Table 1) showed best initiation (76.3%) obtained with the media fortified with 3 mg/l BAP whereas, lowest initiation frequencies (47.41%) were obtained on combination of 1 mg/l BAP and 0.1 mg NAA which is nearly similar to the initiation frequencies to the control (39.26%) i.e. without growth regulators. BAP was found to be the best growth regulator when used alone as compared to in combination with NAA. Kinetin had moderate effect on regeneration being highest (64.07%) on 4 mg/l BAP and lowest (52.22%) on 1 mg/l BAP. Among nodal explants increasing trend in regeneration was observed up to 3 mg/l BAP. An increasing trend was also observed in shoot proliferation up to 4 mg/l kinetin. Multiple shoots obtained after one month of incubation were in bunches with an indefinite number of shoots from starting culture (Fig 2). Shoot initiation with stem segments also exhibited maximum results with BAP for establishment of explants but results were moderate as compared to meristem. The pattern of response of meristem explants to growth regulator

was similar as with the nodal explants. Maximum regeneration was observed with 3 mg/l BAP (58.89 %) and lowest with 1 mg/l (38.52%). 35.93 % regeneration was found with control. Kinetin was not found as successful as BAP with maximum regeneration on 4 mg/l (53.70%).

Table 1. Effect of plant growth regulators fortified with MS culture media on *in vitro* morphogenesis of nodal segment.

Sl.	Culture Media	Growth regulator (mg/l)			Morphogenic explants (%)	Number of shoots per explants
		BAP	KN	NAA		
1	MS1B	1.0	-	-	58.52	4.3
2	MS2B	2.0	-	-	69.26	6.6
3	**MS3B**	**3.0**	**-**	**-**	**76.30**	**9.3**
4	MS4B	4.0	-	-	72.96	7.3
5	MS5B	5.0	-	-	68.15	5.6
6	MS1KN	-	1.0	-	52.22	3.3
7	MS2KN	-	2.0	-	57.04	5.6
8	MS3KN	-	3.0	-	60.00	6.3
9	MS4KN	-	4.0	-	64.07	7.6
10	MS5KN	-	5.0	-	62.96	5.3
11	MS1B0.1N	1.0	-	0.1	47.41	2.6
12	MS1B0.2N	1.0	-	0.2	50.37	4.3
13	MS1B0.3N	1.0	-	0.3	56.67	6.3
14	MS1B0.4N	1.0	-	0.4	51.11	5.6
15	MS1B0.5N	1.0		0.5	52.96	3.3
16	Control	0.0	0.0	0.0	39.26	1.3

Data are given as mean ± SD of three replicates; means followed by same letters are not significant at 5% level.

Table 2. Effect of plant growth regulators fortified with MS culture media on *in vitro* morphogenesis of Meristem.

Sl.	Culture Media	Growth regulator (mg/l)			Morphogenic explants (%)	Number of shoots per explants
		BAP	KN	NAA		
1	MS1B	1.0	-	-	48.15	3.3
2	MS2B	2.0	-	-	52.59	4.3
3	MS3B	3.0	-	-	58.89	6.6
4	MS4B	4.0	-	-	54.07	5.3
5	MS5B	5.0	-	-	51.85	2.6
6	MS1KN	-	1.0	-	38.52	2.3
7	MS2KN	-	2.0	-	41.11	3.6
8	MS3KN	-	3.0	-	45.93	4.3
9	MS4KN	-	4.0	-	53.70	5.3
10	MS5KN	-	5.0	-	51.48	3.6
11	MS1B0.1N	1.0	-	0.1	46.67	2.6
12	MS1B0.2N	1.0	-	0.2	50.00	4.3
13	MS1B0.3N	1.0	-	0.3	54.07	5.3
14	MS1B0.4N	1.0	-	0.4	50.00	3.6
15	MS1B0.5N	1.0		0.5	48.52	2.3
16	Control	0.0	0.0	0.0	35.93	1.3

Data are given as mean ± SD of three replicates; means followed by same letters are not significant at 5% level.

Initial morphogenic study conducted with stem segment showed callus induction without direct shoot proliferation. MS medium fortified with 1 mg/l BAP and 0.4 mg/l NAA showed best result for callus induction (53.85%) where as lowest callus initiation was observed in medium with 1mg/l kinetin. Cytokinin alone at moderate quantities responded well for callus induction as more than 50 per cent explants initiated callus formation at 5 mg/l BAP concentration while 48 per cent responded in a similar manner with 5mg/l kinetin. During the second week of culture, explants became swollen and no callus proliferation was evident. The callus initiation started from the upper portion of explant usually not in contact with the culture medium. After 25-30 days of culture, callus initiating stem segment were counted (Fig 1).

Shoot elongation

Established explants regenerated in initiation culture were transferred in shoot elongation medium fortified with growth regulator GA_3 at the concentration of 0.1-0.5 mg/l (Table 3). These explants were kept under observation for 20-25 days (Fig 2). Best results were obtained in the media fortified with GA_3 at a concentration of 0.5 mg/l with shoot elongation ranging from 4-5 cm. Minimum shoot elongation (1

cm) was found in medium having GA_3 (0.1 mg/l). Table 3 shows effect of GA_3 on shoot elongation of pomegranate [2].

Table 3. Effect of plant growth regulators fortified with MS culture media for shoots elongation of different explants.

SI.	Culture Media	Growth regulator GA_3 (mg/l)	Shoot elongation response
1	MS0.1G	0.1	++
2	MS0.2G	0.2	++
3	MS0.3G	0.3	+++
4	MS0.4G	0.4	++++
5	MS0.5G	0.5	++++
6	Control	0.0	+

Where, + Less than 1 cm of shoots elongation; ++ 1-2 cm of shoots elongation; +++ 2-3 cm of shoots elongation; ++++ 4-5 cm of shoots elongation.

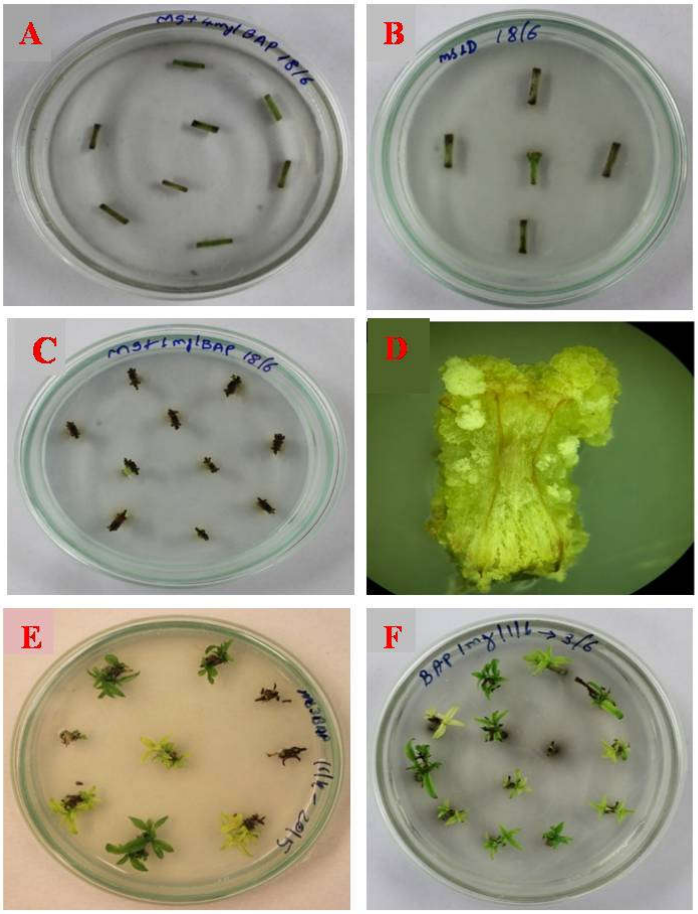

Fig. 1 Different explants used for micropropagation of Bhagava variety of pomegranate. A. Stem segments, B. Nodal segments, C. Meristems, D. Callus initiation of stem segments, E. Multiple shoot of nodal segments, F. Meristem explants

The 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 [11, 5, 6, 10]. *In vitro* clonal propagation of pomegranate is an efficient method to develop large number of plantlets from tiny explants as well as to overcome the problems related with traditional methods of vegetative propagation.[7]. Micropropagation from field grown plants is a very critical process with different problems such as microbial contamination, phenol exudation, etc. Phenol secretion from the cut ends of explants leads to browning of the medium and marked reduction in development of explants. [4, 5]. Establishment of *in vitro* cultures of woody plants is greatly affected by the browning of the medium and necrosis of explants. Browning is a result of the oxidation of phenolic substances

released from the cut ends of the explants by peroxides. In the present study problem of browning was overcome by subsequent transfer of explants to fresh medium. Browning of the culture medium followed by necrosis of the explants is a common problem which influenced the establishment of shoot cultures [5].

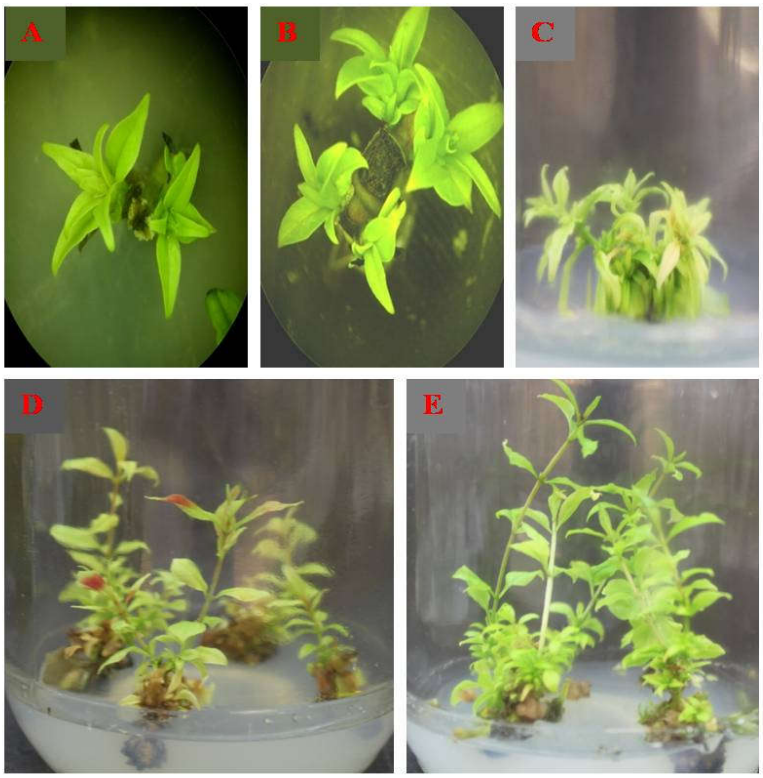

Fig 2 A, B & C Multiple shooting of nodal segments, D & E highest average growth response as recorded on MS medium containing 0.5 mg/L GA_3.

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