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In Vitro Shoot Proliferation from different Explants in Pomegranate (*Punica granatum* L.)

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ABSTRACT: Explants and antioxidants have a significant impact on pomegranate *in vitro* culture. Especially in mature explant, explants and medium browning is a major problem in pomegranate due to the exudation of high amount of phenols. Browning and latent contamination are challenges of the study. As a result, the current study was carried out in order to offer a suitable technique for in vitro cultivation with the least amount of phenolic exudates in order to create virus-free plants that are true to type. Due to the existence of pre-existing meristems, cytokinins have been demonstrated to be beneficial for the induction of numerous shoots from apical/axillary explants in the current study. In fact, such a characteristic has been fully used in the species for micropropagation. Various plant growth regulators, such as cytokinins (BAP/KN) and auxins (IAA/2,4-D), were applied to explants at different concentrations. In shoot apex explants, maximum shoot proliferation was recorded on a medium containing 2.5 mg/l BAP, followed by 3.0 mg/l BAP and 2.0 mg/l 2,4-D with 100% frequency. With a 100% frequency, 2.0 mg/l BAP, 2.0 mg/l Kn, and 2.0 mg/l 2,4-D produced the most shoot proliferation in the Nodal section. At higher concentrations (4.0-5.0 mg/l), bud induction was entirely suppressed. At 1.0 mg/l and 5.0 mg/l Kn, the minimum number of shoot buds (1.3) was reported 100% frequency. The induction of shoot buds was gradually decreased as the concentration of BAP (> 2.0 mg/l) was increased.

Keywords: Pomegranate, shoot proliferation, Micropropagation, Tissue culture.

INTRODUCTION

The pomegranate (*Punica granatum* L.) is a member of the Punicaceae family. It is endemic to Iran and has spread over Asia, Africa, and Europe's Mediterranean region (Sepulveda *et al.,* 2000). It contains chromosomes with 2n=2x=16, 18 Smith 1976. Pomegranate was one of the first five fruit crops domesticated by mankind in 2000 BC (date palm, fig, olive, grape, and pomegranate). Pomegranates come in a variety of ecotypes, including farmed (*Punica granatum* L.), wild (*Punica protopunica*) and ornamental varieties (Japanese Dwarf pomegranate - *Punica granatum* var. Nana). Pomegranate is a hardy plant that grows in saline soil and drought**.**

Pomegranates are rated a super fruit because of its excellent medicinal capabilities and numerous health advantages (da Silva *et al.* 2013). Pomegranate fruit includes ellagitannins, notably punicalagins, anthocyanins, flavonols, and flavonoids, among other beneficial components (Salgado *et al.,* 2012; Yuan *et al.,* 2018). Pomegranate is valued highly for its delicious edible fruits are rich in sugars, vitamins, polysaccharides, polyphenols, and minerals (Ferrara *et al.,* 2014). Because of its widespread use in the pharmaceutical and food industries, the demand for high-quality pomegranate planting material is rising.

It is grown on 2.16 lakh ha in India, with a production of 27.95 lakh tonnes and a productivity of 12.94 tonnes per hectare Anonymous 2016-17a. It I is grown on 2857 ha in Rajasthan, with a production of 10379 tonnes and a productivity of 3.63 tonnes per hectare. Rajasthan's pomegranate-producing districts include Jalore, Chittorgarh, Barmer, Bhilwara, and Jodhpur. With 847 ha of land, 3134 tonnes of production, and 3.7 tonnes per hectare of productivity, Jalore is the most productive of these districts (Anonymous 2016-17b).

Pomegranates are propagated commercially through stem cuttings (Hardwood cutting) or air layering. These methods are time-consuming and labor-intensive, and they have other drawbacks such as a low success rate and the fact that new plants need a year to develop. As a result, plantlets are not available throughout the year. Furthermore, this conventional method of propagation does not offer disease-free and healthy plants (Kanware *et al*., 2010). Despite a large rise in pomegranate planting acreage, the fruit's production is severely hindered by diseases and insect pest attacks (Cocuzza *et al*., 2016; Pathania *et al*., 2019). Therefore, to increase production, quick propagation methods are required. Seeds are not a highly dependable means of propagation since they cause population heterozygosity. Furthermore, employing hardwood and softwood cuttings for plant propagation does not guarantee the development of disease-free clones (Desai *et al*., 2018).

Though regeneration from existing meristems (shoot tip and nodal bud), vegetative and reproductive plant components has been attempted with some notable results, pomegranate cell and tissue culture remains difficult. The most widely utilised organogenesis approaches have relied on seedling-derived plant material (Chauhan & Kanwar, 2012). Although this strategy is ineffective for elite cultivars. The goal of this study is to develop a dependable methodology for callus induction and organogenesis in vitro in order to create plants that are true to type.

Explants have a significant impact on pomegranate *in vitro* culture. As a result, the current study was carried out in order to offer a dependable in vitro growth strategy for producing virus-free plants that are true to type. According to Kabir *et al*. (2021) ninety percent of the cultured explants responded to form shoots from 30 days old *in vitro* raised seedlings after 90 days of culture initiation in MS containing 1.0 mg/l IBA + 0.1 mg/l NAA. The average number of shoots per explant was 10.0 ± 2.20, shoot length of 12.0 ± 2.40 cm, node per regenerated shoot was 9.0 ± 1.60 and the leaf number was14.0 ± 1.40.

The lethal browning of explants and culture medium makes it difficult to establish in vitro culture of various plant species, particularly woody plants. The many strategies used to prevent browning's negative consequences. Antioxidants such as activated charcoal (AC), polyvinylpyrrolidone (PVP), citric acid and others are used to reduce the negative effects (Weatherhead *et al.,* 1978).

Because of a browning problem during the initial establishing stage of *in vitro* culture, *in vitro* propagation of woody plants is difficult to grow (Pirttila *et al.* 2008; Krishna *et al.,* 2008). Because of the leaching of phenolic compounds and secondary metabolites from the cut surface, subsequent morphogenesis response and rooting of explants is hampered. 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.,* 2013). Due to the exudation of large amounts of phenols, especially in mature explants, explants and medium browning are a severe problem (Naik and Chand 2010). Phenolic compound leads to oxidation and turns the tissue brown or black and the oxidation product inhibit enzyme activity which results in killing of tissue browning of media and ultimately results in poor establishment of cultures. Phenols are a class of chemical compounds that include a wide range of plant components that share an aromatic ring with one or more hydroxyl elements (Onuoha *et al*., 2011). Many attempts to replicate pomegranate utilising tissue culture techniques employing mature plant shoot tip and nodal segment explants (Kantharajah *et al*., 1998 and Kanwar *et al.,* 2009). The activated charcoal supplementation of 200 mg/l in culture media was found best antioxidant for maximum shoot bud induction in nodal segment through controlling of accumulation of phenolic compounds.

MATERIALS AND METHODS

Punica granatum was the subject of the current study. Explants were collected from healthy trees maintained at the Department of Plant Breeding and Genetics, S.K.N. College of Agriculture, Jobner, and included shoot apexes and nodal segments. Different surface sterilisation agents were used to sterilise explants. Explants were extensively cleansed with running tap water for 20 minutes before being rinsed again with liquid detergent (RanKleen) for 10 minutes while vigorously shaking. Explants were cleaned with running tap water for 5 minutes after washing with detergent to remove any evidence of detergent. Finally, explants were surface sterilised with 0.1 percent $HgCl_2$ for 2-5 minutes in a laminar air flow cabinet, depending on the explant.

A. Induction of Shoot proliferation

For shoot proliferation, shoot apex and nodal segments were placed on MS medium supplemented with various concentrations of cytokinins (BAP/Kn1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 mg/l) and auxins (IAA/2,4–D 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 mg/l).

RESULTS AND DISCUSSION

When Shoot apex explants on medium supplemented various concentration of plant growth regulators were inoculated, it reacted by an approach of shoot bud break at different levels of BAP with 100 % frequency. At 2.5 mg/l BAP, the maximum shoot bud induction (2.0) was recorded, followed by 1.9 buds at 3.0 mg/l BAP (Fig. 1). Similarly to the nodal segment, raising the level of BAP in the shoot apex promoted shoot bud induction up to 2.5 mg/l. However, higher levels of BAP had an inhibitory influence on bud proliferation (Table 1).

At all levels of BAP (1.0-5.0 mg/l), the nodal segments began to proliferate shoot bud within 16–19 days of incubation. At 2.0 mg/l BAP, the maximum number of shoot buds (2.1) induction was observed (Fig. 2). At 1.0 mg/l BAP, the smallest number of shoot buds (1.4) was found. At all levels of BAP (1.0-5.0 mg/l), the frequency of shoot bud proliferation was 100%. Induction of shoot buds was aided by increasing the concentration of BAP to 2.0 mg/l. However, when the concentration of BAP (> 2.0 mg/l) increased, the induction of shoot buds decreased (Table 2).

Within 16 - 20 days of inoculation, nodal explants cultured on MS medium supplemented with varied amounts of Kn (1.0-5.0 mg/l) started sprouting. Multiple shoot bud induction was detected at all levels of Kn with 100% frequency, just like BAP. At 2.0 mg/l, maximum shoot bud induction (1.8) was observed with 100% frequency (Fig. 3). At 1.0 mg/l and 5.0 mg/l Kn, the minimum number of shoot buds (1.3) were reported 100% of the time. Shoot bud induction was enhanced up to 2.0 mg/l by increasing the concentration of Kn in the medium. After 2.5 mg/l, the number of shoot bud inductions decreased as the level of Kn increased (Table 2).

When shoot apex explants were inoculated in MS Medium supplemented with various concentrations of 2,4-D (1.0-5.0 mg/l), the following results were obtained. Bud proliferation in shoot apex explants began after 12-14 days of incubation. The largest number of shoot buds (1.9) was observed at 2.0 mg/l, while the lowest (1.3) was observed at 1.0 mg/l of 2,4-D. (Fig. 4). At higher concentrations (4.0-5.0 mg/l), bud induction was entirely suppressed (Table 1).

Auxins elicited a substantial difference in response in pomegranate explants as cytokinins in the current study. Auxins (IAA/2,4-D) generated shoot bud and callus in the nodal segment and shoot apex, but only callus proliferation was observed in leaf explants. Fougat *et al.* (1997) reported callus induction in pomegranate cotyledon and leaf explants.

One of the most essential aspects in optimising the tissue culture technique is the type of explant. Explants such as leaf, petiole, cotyledonary leaf, hypocotyle, epicotyle, embryo, internode, and root have a substantial impact on plant tissue culture. Several factors influence an explant's in vitro response, including the organ from which it was produced, the explant's physiological state and its size. Shoot bud differentiation, callus induction, root induction, and organogenesis may all be affected by the orientation of the explant on the medium and the inoculation density (Khan e*t al.*, 1988; Sujatha and Mukta 1996; Gubis *et al*. 2003; Alagumanian *et al.,* 2004; Ali and Mirza 2006; Kumar *et al.,* 2011). This may be due to the different level of endogenous plant hormones present in the plants parts.

In this study, cytokinins (BAP/Kn) and auxins (IAA/2,4-D) were applied to nodal segment, shoot apex, and leaf explants. Under MS media supplemented with 2.0 mg/l BAP, nodal segment explants had the most shoot proliferation of all the explants. These findings are consistent with those published in pomegranate by Kalalbandi *et al.,* (2014).

Fig. 1. Shoot bud proliferation in shoot apex explant on MS medium supplemented with 2.5 mg/l BAP.

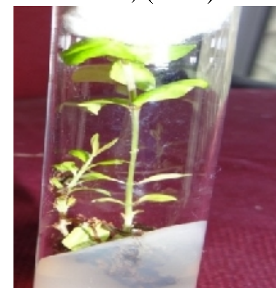

Fig. 2. Shoot bud proliferation in nodal segment explant on MS medium supplemented with 2.0 mg/l BAP.

Fig. 3. Shoot bud proliferation in nodal segment explant on MS medium supplemented with 2.0 mg/l Kn.

Fig. 4. Shoot bud proliferation in shoot apex explant on MS medium supplemented with 2.0 mg/l 2,4-D.

Table 1: Morphogenetic effect of various concentrations of cytokinins (BAP/Kn)and auxins (IAA/2,4-D) added singly in the MS medium Shoot apex explant.

Concentration (mg/l)	Shoot multiplication			Shoot multiplication			
	Response (%)	Days taken for sprouting	Number of shoot buds explants^{-1}	Concentration (mg/l)	Response (%)	Days taken for sprouting	Number of shoot buds explants^{-1}
BAP				**Kn**			
1.0	100	12.60	1.4±0.16	1.0	100	12.70	1.3±0.15
1.5	100	13.70	1.5±0.17	1.5	100	13.80	1.5±0.22
2.0	100	14.80	1.7±0.21	2.0	100	14.60	1.6±0.16
2.5	100	13.50	2.0±0.26	2.5	100	13.90	1.5±0.17
3.0	100	13.30	1.9±0.23	3.0	100	13.40	1.4±0.16
4.0	100	12.70	1.8±0.20	4.0	100	12.90	1.3±0.15
5.0	100	13.10	1.7±0.21	5.0	100	12.70	1.2±0.13
IAA				**2,4-D**			
1.0	100	12.20	1.2±0.13	1.0	100	12.10	1.3±0.15
1.5	100	13.10	1.4±0.16	1.5	100	13.30	1.6±0.16
2.0	100	14.20	1.6±0.16	2.0	100	14.50	1.9±0.18
2.5	100	13.10	1.7±0.15	2.5	100	13.40	1.5±0.17
3.0	100	13.00	1.6±0.16	3.0	80	13.10	1.4±0.16
4.0	100	12.90	1.5±0.17	4.0	-	-	-
5.0	90	12.60	1.4±0.16	5.0	-	-	-

(-) = No response

Table 2: Morphogenetic effect of various concentrations of cytokinins (BAP/Kn) and auxins (IAA/2,4-D) added singly in the MS medium Nodal segment explant.

Concentration (mg/l)	Shoot multiplication			Shoot multiplication			
	Response (%)	Days taken for sprouting	Number of shoot buds explants^{-1}	Concentration (mg/l)	Response (%)	Days taken for sprouting	Number of shoot buds explants^{-1}
BAP				**Kn**			
1.0	100	17.0	1.4 ±0.22	1.0	100	17.40	1.3 ±0.15
1.5	100	18.10	1.6±0.22	1.5	100	18.10	1.6±0.22
2.0	100	18.70	2.1±0.18	2.0	100	18.40	1.8±0.20
2.5	100	17.60	1.8±0.20	2.5	100	17.60	1.6±0.16
3.0	100	17.40	1.7±0.21	3.0	100	17.40	1.5±0.22
4.0	100	16.90	1.6±0.16	4.0	100	17.00	1.4±0.16
5.0	100	16.70	1.5±0.17	5.0	100	16.80	1.3±0.15
IAA				**2,4-D**			
1.0	100	16.00	1.4 ±0.16	1.0	100	16.10	1.3 ±0.15
1.5	100	17.10	1.5±0.17	1.5	100	17.10	1.5±0.17
2.0	100	18.40	1.7±0.15	2.0	100	18.50	1.8±0.13
2.5	100	17.40	1.5±0.17	2.5	100	17.50	1.6±0.16
3.0	100	17.20	1.4±0.16	3.0	80	17.30	1.5±0.17
4.0	100	17.10	1.3±0.15	4.0	70	17.00	1.4±0.16
5.0	100	16.70	1.2±0.13	5.0	-	-	-

(-) = No response

CONCLUSION

In this study, cytokinins (BAP/Kn) and auxins (IAA/2,4-D) were applied to nodal segment, shoot apex, and leaf explants. In shoot apex explants, maximum shoot proliferation was recorded on a medium containing 2.5 mg/l BAP followed by 3.0 mg/l BAP and 2.0 mg/l 2,4-D with 100% frequency. With a 100% frequency, 2.0 mg/l BAP, 2.0 mg/l Kn, and 2.0 mg/l 2,4-D produced the maximum shoot proliferation in the Nodal segment.

FUTURE SCOPE

Author may suggest to root induction programme for further research.

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