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Rekha Choudhary
Research Scholar, Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

Mohan Lal Jakhar
Professor, Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

Rakesh Kumar Prajapat
Assistant Professor, School of Agriculture, Suresh Gyan Vihar University Jaipur, Rajasthan, India

Ravi Kumar
Research Scholar, Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

Komal Sekhawat
Research Scholar, Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

Swarnlata Kumawat
Research Scholar, Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

Corresponding Author:
Rekha Choudhary
Research Scholar-Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

In vitro effect of different antioxidants *viz*., activated charcoal, ascorbic acid, citric acid and polyvinylpyrrolidone on callus and shoot induction in Pomegranate (*Punica granatum* L.)

Rekha Choudhary, Mohan Lal Jakhar, Rakesh Kumar Prajapat, Ravi Kumar, Komal Sekhawat and Swarnlata Kumawat

Abstract
Leaf, Shoot apex and Nodal segment explant of pomegranate were placed on Murashige and Skoog Medium (MS medium, 1962) supplemented with different concentration of cytokinins and auxins for callus induction. Antioxidants *viz:* activated charcoal, ascorbic acid, citric acid and polyvinylpyrollidone were used to control the accumulation of phenolic compounds in the culture medium and enhance the rate of micropropagation. In the present investigation efforts were made to assess the role of antioxidants to minimize the release of phenolic substances at most responsive levels worked out in the present investigation that is maximum shoot bud induction in nodal segment at 2.0 mg/l BAP at activated charcoal (200 mg/l) and callus proliferation in leaf explant at 1.5 mg/l BAP at 200 mg/l of activated charcoal.

Keywords: Pomegranate, callus induction, tissue culture, micropropagation

1. Introduction
Pomegranate (*Punica granatum* L.) belongs to the family "Punicaceae". It is native from Iran and spread throughout the Mediterranean region of Asia, Africa and Europe [1]. It has 2n=2x=16, 18 chromosomes [2]. Pomegranate was domesticated in 2000 BC and was one of the first five fruit crops (date palm, fig, olive, grape and pomegranate) domesticated by mankind. Pomegranate has different ecotypes *viz.*, cultivated (*Punica granatum* L.), wild types (*Punica protopunica*) and ornamental forms (Japanese Dwarf pomegranate - *Punica granatum* var. Nana). Pomegranate has great adaptability to saline soil and drought conditions. In India, it is cultivated over 2.16 lakh ha with a production of 27.95 lakh tones and productivity of 12.94 tones/ha [3]. In Rajasthan, it is cultivated over 2857 ha area with production of 10379 tones and productivity of 3.63 tones/ha. Jalore, Chittorgarh, Barmer, Bhilwara and Jodhpur are major pomegranate producing district of Rajasthan. Out of these districts Jalore is leading district with 847 ha area, 3134 tones production and 3.7 tones/ha productivity [4]. Pomegranate is commercially propagated by stem cuttings (Hardwood cutting) or by air layering. These methods are time consuming, labor intensive process and it has other limitations like low success rate and new plants require one year for establishment. This results in non-availability of plantlets throughout the year. Further, this traditional propagation method does not ensure disease free and healthy plants [5].
Pomegranate cell and tissue culture is not easy though regeneration from existing meristems (shoot tip and nodal bud), vegetative and reproductive plant parts have been attempted with some noteworthy success. The present investigation has been undertaken to establish reliable protocol for callus induction and organogenesis under *in vitro* conditions to produce true to type plants. *In vitro* culture of pomegranate are greatly affected by explants. Thus, the present investigation has been undertaken to suggest a reliable protocol for *in vitro* culture to produce true to type and virus free plants

2. Material and Methods
The present research work was conducted on *Punica granatum*. Leaves, shoot apexes and nodal segments were used as explants and obtained from healthy trees grown at Department of plant Breeding and Genetics, S.K.N. College of Agriculture, Jobner. Explants were sterilized

by using different surface sterilization agents. Explants were washed thoroughly in running tap water for 20 minutes, these were again washed with liquid detergent (RanKleen) for ten minutes with vigorous shaking. After washing with detergent, explants were again washed with running tap water to remove any trace of detergent for 5 minutes. Finally explants were surface sterilized with 0.1 per cent $HgCl_2$ in a laminar air flow cabinet for 2-5 minutes according to explant.

Antioxidants *viz:* activated charcoal, ascorbic acid, citric acid and polyvinylpyrollidone were used to control the accumulation of phenolic compounds in the culture medium and enhance the rate of micropropagation was worked out at most responsive level of plant growth regulators.

2.1 Induction of callus and Shoot

Leaf, shoot apex and nodal segments were placed on MS medium supplemented with different concentration of cytokinins (BAP/Kn 1.0, 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) and antioxidants activated charcoal (100, 200, 300 and 400 mg/l), ascorbic acid (50,100,150 and 200 mg/l), citric acid (25, 50, 75 and 100 mg/l) and polyvinylpyrrolidone (10, 20, 30 and 40 mg/l) for callus and shoot induction.

3. Results

3.1 Effect of Antioxidants

Under *in vitro* condition accumulation of inhibitory substances and/or secondary metabolites in the growth medium is a major problem more frequently associated with micropropagation and root formation in woody perennials. These substance leaches from the cut surface of the explant and oxidizes later results in turning of media brown. Browning of tissue process is caused by the oxidation of tannin and polyphenols and the formation of guinones which are highly reactive and toxic to the tissue. Phenolic compounds contain at least one hydroxyl group on the benzene ring. Several oxidases such as monophenolase (Tryosinase), polyphenol (Cate-choloxidase) oxidized the hydroxyl group resulting in the formation of guinone and water. Plant. Medium browning is a major problem in pomegranate due to the exudation of high amount of phenols, essentially in nodal segment and shoot apex explants. In the present investigation efforts were made to assess the role of antioxidants to minimize the release of phenolic substances at most responsive levels worked out in the present investigation that is maximum shoot bud induction at 2.0 mg/l BAP in nodal segment and maximum callus proliferation in leaf explant at 1.5 mg/l BAP.

When activated charcoal (100-400 mg/l) was added in the basal medium with micropropagation protocol for nodal segment (2.0 mg/l BAP), it induced shoots at all the level of activated charcoal ranging from 2.1 – 2.5 within 17-19 days of incubation. Maximum number of shoot bud (2.5) was observed at 200 mg/l activated charcoal with low browning intensity in the culture medium (Table 1 and Fig. 1).

When activated charcoal was added in the basal medium for leaf (1.5 mg/l), it induce callus proliferation at all the levels of activated charcoal (100 – 400 mg/l). Maximum callus weight (0.85 g) induced at 200 mg/l of activated charcoal followed by 300 mg/l (Fig. 2). Minimum callusing (0.82 g) reported at 100 mg/l activated charcoal (Table 2). When ascorbic acid was added in the MS medium with 2.0 mg/l BAP for nodal segment explants along with different level of ascorbic acid. Maximum shoot bud (2.3) induction was observed at 100 mg/l ascorbic acid in nodal segment. Ascorbic acid showed medium browning in different levels except at 100 mg/l in nodal segment explants.

Supplementation of ascorbic acid (50 – 200 mg/l) in the MS Medium with 1.5 mg/l of BAP for leaf explants for maximum callus proliferation. Maximum callus proliferation was observed (0.83 g) at 100 mg/l of ascorbic acid and minimum callus weight (0.80 g) at 200 mg/l of ascorbic acid.

Supplementation of citric acid in the basal medium containing 2.0 mg/l BAP induced shoots at all the levels (25 – 100 mg/l) in nodal segment explants. Maximum bud shoot induction was observed at 50 mg/l level of citric acid followed by 25 mg/l and 75 mg/l levels. Lower level of citric acid were insufficient to prevent the phenolic accumulation in culture media. The effectiveness of citric acid was less in comparison to activated charcoal.

When different levels of citric acid (25 – 100 mg/l) were added in the basal medium of leaf explants with 1.5 mg/l of BAP. Maximum callus weight (0.80 g) was observed at 25 mg/l of citric acid. Minimum callus weight (0.77 g) was observed at 75 mg/l of citric acid.

Addition of polyvinylpyrrolidone in different levels (10 – 40 mg/l) with responsive level of plant growth regulator induced shoot bud from nodal segment explants at all the levels. Maximum shoot bud (2.1) induction was observed at 20 mg/l as well as 30 mg/l of polyvinylpyrrolidone followed by 10 mg/l and 40 mg/l within 17 -19 days of shoot induction.

Supplementation with polyvinylpyrrolidone in different levels (10 – 40 mg/l) for leaf explants for callus proliferation with 1.5 mg/l of BAP. Maximum callus weight (0.78 g) at 20 mg/l and minimum callus (0.75 g) induction at 30 mg/l of polyvinylpyrrolidone.

4. Discussion

Under *in vitro* condition accumulation of inhibitory substances and/or secondary metabolites in the growth medium is a major problem more frequently associated with micropropagation and root formation in woody perennial [6]. These substance leaches from the cut surface of the explant and oxidizes later results in turning of media brown. Browning of tissue process is caused by the oxidation of tannin and polyphenols and the formation of guinones which are highly reactive and toxic to the tissue. Phenolic compounds contain at least one hydroxyl group on the benzene ring. Several oxidases such as monophenolase (Tryosinase), polyphenol (Cate-choloxidase) oxidized the hydroxyl group resulting in the formation of guinone and water [7, 8]. Plant tissue contains these substances in separate pools or compartments. During tissue wounding or senescence these pools are integrated and oxidation process is initiated [9, 28]. After oxidation these component become toxic to the explants and results in retardation of growth and eventually lead to complete failure to survivability of the explants. Medium browning is a major problem in pomegranate due to the exudation of high amount of phenols, essentially in nodal segment and shoot apex explants [10]. Various attempts has been made to multiply pomegranate by using tissue culture techniques through shoot tip and nodal segment explants of mature plant [11-14]. However, the problem of browning and death of culture during *in vitro* propagation of pomegranate has been reported earlier by Sharon and Sinha [15] and also Murkute *et al.* [16]. Different attempts has been

made to eliminate browning problem in woody plant species like pre-socking of explants in antioxidants (activated charcoal, PVP, ascorbic acid, citric acid *etc*.) solution, incorporation of oxidants into medium, incubation of culture in to dark period and frequent subculturing of explants.

In the present investigation antioxidants (activated charcoal, ascorbic acid, citric acid and polyvinylpyrollidone) when incorporated singly in the basal medium along with micropropagation protocol (nodal segment, 2.0 mg/l BAP) induced multiple shoots at all the levels. Among the different antioxidants, activated charcoal 200 mg/l was found better in reducing of medium and explant browning. However, addition of 300-400 mg/l activated charcoal into the medium adversely affected shoot bud proliferation. Citric acid and ascorbic acid did not show much effect in browning control. PVP, 30 – 40 mg/l reduced browning in medium and explant to some extent, however, the number of shoot bud proliferation both in the nodal segment explant was lower in comparison to 200 mg/l activated charcoal. The appearance of explant was green in all the subculturing treatments.

Several studies have reported the use of antioxidants in browning control in perennial fruit plants [17-21]. Whereas, in the present study, ascorbic acid and citric acid was less effective in control of browning. In contrast with our results, Patil *et al.* [21] found best results in browning control with 150 mg/l ascorbic acid and 100 mg/l citric acid in pomegranate. Similarly, PVP was also found almost effective in browning control. Tyagi *et al.* [22] and Prajapati *et al.* [23] effectively controlled explant browning with PVP when added into medium. The effectiveness of different antioxidants in control of browning is varying among plants and species. This could be due to the specificity of these chemicals to certain plant and species. The specificity of PVP in browning control was also reported by Vaugh and Duke [24]. In current investigation addition of activated charcoal 300 - 400 mg/l reduced the growth of explants. It might be due to the absorption of nutrients from medium. Activated charcoal is a strong phenol adsorbent [25] that reduces phenolic browning in explants by way of absorption of toxic substances and phenols [26, 27] in culture media.

Fig 1: Effect of activated charcoal (200 mg/l) on shoot bud induction in nodal segment explant of pomegranate supplemented with of 2.0 mg/l BAP

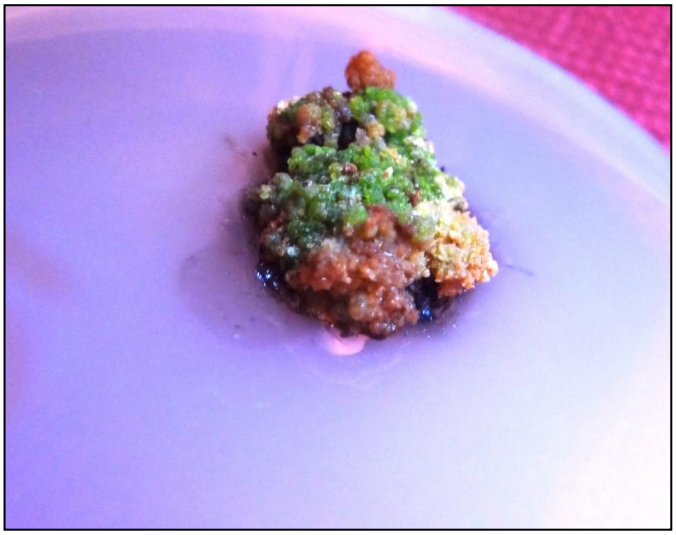

Fig 2: Effect of activated charcoal (200 mg/l) on callus induction in leaf explant of pomegranate supplemented with of 1.5 mg/l BAP.

Table 1: Effect of antioxidants on *in vitro* degree of browning and culture establishment of nodal segment explant in MS medium supplemented with 2.0 mg/l BAP in pomegranate

Antioxidant	Concentration (mg)	Days taken in shoot induction	Number of *de novo* shoot bud induction	Browning intensity
Activated Charcoal	100	18.2	2.2±0.13	++
	200	19.1	2.5±0.22	+
	300	18.6	2.3±0.21	+
	400	17.9	2.1±0.18	++
Ascorbic acid	50	17.8	2.0±0.21	+++
	100	18.9	2.3±0.26	++
	150	19.0	2.1±0.18	+
	200	18.3	2.1±0.18	++
Citric acid	25	17.9	2.0±0.26	+
	50	18.5	2.1±0.23	++
	75	19.1	2.0±0.15	++
	100	18.4	1.9±0.18	+++
Polyvinylpyrrolidone	10	17.8	2.0±0.26	+
	20	18.4	2.1±0.23	+
	30	19.0	2.1±0.10	++
	40	18.0	1.9±0.18	+++

(+++) = Intense browning, (++) = Medium browning, (+) = Low browning

Table 2: Effect of antioxidants on *in vitro* degree of browning and callus establishment of leaf explant in MS medium supplemented with 1.5 mg/l BAP in pomegranate.

Antioxidant	Concentration (mg)	Days taken in callus induction	Callus weight	Browning intensity
Activated Charcoal	100	25.8	0.82±0.02	++
	200	16.1	0.85±0.01	+
	300	27.1	0.84±0.01	+
	400	26.8	0.83±0.01	++
Ascorbic acid	50	27.2	0.81±0.01	+++
	100	26.1	0.83±0.01	++
	150	27.1	0.81±0.01	+
	200	26.3	0.80±0.02	++
Citric acid	25	27.9	0.80±0.01	+
	50	26.5	0.79±0.01	++
	75	28.2	0.77±0.02	++
	100	26.4	0.78±0.02	+++
Polyvinylpyrrolidone	10	27.8	0.76±0.01	+
	20	26.4	0.78±0.01	+
	30	28.0	0.75±0.01	++
	40	28.7	0.77±0.02	+++

(+++) = Intense browning, (++) = Medium browning, (+) = Low browning

5. Conclusion

Pomegranate (*Punica granatum* L.) belongs to the family "Punicaceae". It is native from Iran and spread throughout the Mediterranean region of Asia, Africa and Europe. Pomegranate has great adaptability to saline soil and drought conditions. Pomegranate is conventionally propagated by hard wood and soft wood cuttings. But, this traditional propagation method does not ensure disease-free and healthy plants. In addition, this method is a very time-consuming and labor-intensive process. Thus the present investigation has been undertaken to establish reliable protocol for micropropagation under *in vitro* condition with the help of the effect of different antioxidants *viz*., activated charcoal, polyvinylpyrrolidone, ascorbic acid and citric acid on callus induction and shoot proliferation. 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. Activated charcoal supplementation of 200 mg/l in culture media was found best antioxidant for maximum callus induction in leaf explants.

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