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EFFECT OF DIFFERENT SURFACE STERILIZATION METHODS AND DIFFERENT PLANT GROWTH REGULATORS ON CULTURE ESTABLISHMENT FROM POMEGRANATE NODAL EXPLANTS OF cv. BHAGWA

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Abstract –The present investigation was conducted to know the effect of different surface sterilization methods and different plant growth regulators on culture establishment from Pomegranate nodal explants of cv. Bhagwa with two factors: Sterilizing agents(S): 0.1% Mercuric chloride ($HgCl_2$) and 1% Sodium hypochlorite (NaOCl) each at two different timing intervals i.e., 5 min and 10 min and growth regulators(G) G1: BAP 2 mg L^{-1}, G2: Kinetin 2 mg L^{-1}, G3: 2-ip 2 mg L^{-1}, G4: zeatin 2 mg L^{-1}). The results revealed that highest percentage of uncontamination was recorded in S2: 0.1% HgCl2 for 10min (93.33%) with lowest fungal contamination (5.83%) and bacterial contamination (0.83%) with no significant effect of growth regulators on contamination. Highest regeneration efficiency was recorded in BAP 2 mg L^{-1} (58.33%) with individual effect of sterilizing agent $HgCl_2$ at 0.1% for 5 min in less number of days (9.53).

INTRODUCTION

Pomegranate (*Punica granatum* L.) is a significant fruit crop that thrives in tropical and subtropical areas worldwide. It is categorized within the Punicaceae family, which contains only one genus (Punica) and two species, namely P. granatum and P. protopunica. While it is primarily grown for its delectable fruits, various plant parts, such as the leaves, stem, bark, root, and fruit rind, contain valuable tannins and alkaloids that enhance its commercial appeal. Pomegranate is cultivated across India, from Kanyakumari to Kashmir, although commercial farming is mainly concentrated in Maharashtra (Desai *et al.,* 2018).

Currently, India ranked seventh globally in pomegranate production, with approximately 2,75,500 hectares under cultivation. In Telangana, pomegranate cultivation spans 3,732 acres, yielding an annual production of 15,153 metric tons and a productivity rate of 4.1 metric tons per acre (Dept. of Horticulture, Government of Telangana, Telangana, 2023).

Traditional propagation methods, which include hard and softwood cuttings, are not only labor-intensive but also time-consuming, often requiring up to a year for the establishment of new plants. Additionally, there is no assurance that all plants produced through these methods will be completely disease-free clones. On the other hand, propagation through seeds results in a heterozygous population, leading to significant variations in tree and fruit characteristics (Desai *et al.,* 2018).

The current supply of planting material from existing plantations is inadequate to satisfy the growing demand. In developed nations,

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micropropagation is being utilized commercially for the mass production of disease-free planting material for a variety of fruit crops (Gorad *et al.*, 2018). While most research on the micropropagation of pomegranate has focused on foreign cultivars, there has been limited investigation into Iranian pomegranate cultivars (Abadi *et al.*, 2020).

MATERIAL AND METHODS

The work was done to establish contamination free initiation cultures of pomegranate cv. Bhagwa using nodal explants by using sterilizing agents(S) and growth regulators(G) each at 4 levels (S1: 0.1% $HgCl_2$ for 5min, S2: 0.1% $HgCl_2$ for 10 min, S3: 1% NaOCl for 5min, S4: 1% NaOCl for 10 min and G1: BAP 2 mg L^{-1}, G2: Kinetin 2 mg L^{-1}, G3: 2-ip 2 mg L^{-1}, G4: zeatin 2 mg L^{-1}) with total sixteen(16) treatments in Factorial Completely Randomized Design (FCRD).

Selection of planting material

Healthy mother plants which are devoid of diseases and pest infestation were selected. Two days prior to the collection of explants, the mother plant was sprayed with fungicide Bavistin (0.2mg L^{-1}) solution in the morning or evening hours. Explants were collected at the beginning of march and summer season in the early morning as season of collection also matters in micropropagation.

Experiment details

T_1: $S_1 \times G_1$	0.1% $HgCl_2$ (5 min) + BAP 2 mg/l
T_2: $S_1 \times G_2$	0.1% $HgCl_2$ (5 min) + Kn 2 mg/l
T_3: $S_1 \times G_3$	0.1% $HgCl_2$ (5 min) + 2-ip 2 mg/l
T_4: $S_1 \times G_4$	0.1% $HgCl_2$ (5 min) + Zeatin 2 mg/l
T_5: $S_2 \times G_1$	0.1% $HgCl_2$ (10 min) + BAP 2 mg/l
T_6: $S_2 \times G_2$	0.1% $HgCl_2$ (10 min) + Kn 2 mg/l
T_7: $S_2 \times G_3$	0.1% $HgCl_2$ (10 min) + 2-ip 2 mg/l
T_8: $S_2 \times G_4$	0.1% $HgCl_2$ (10 min) + Zeatin 2 mg/l
T_9: $S_3 \times G_1$	1% NaOCl (5 min) + BAP 2mg/l
T_{10}: $S_3 \times G_2$	1% NaOCl (5 min) + Kn 2mg/l
T_{11}: $S_3 \times G_3$	1% NaOCl (5min) + 2-ip 2mg/l
T_{12}: $S_3 \times G_4$	1% NaOCl (5min) + Zeatin 2mg/l
T_{13}: $S_4 \times G_1$	1% NaOCl (10min) + BAP 2mg/l
T_{14}: $S_4 \times G_2$	1% NaOCl (10min) + Kn 2mg/l
T_{15}: $S_4 \times G_3$	1% NaOCl (10min) + 2-ip 2mg/l
T_{16}: $S_4 \times G_4$	1% NaOCl (10min) + Zeatin 2mg/l

Collection of explants and sterilization

Node of pomegranate was selected as explant. The explants collected were trimmed into 1.5 to 2cm by removing leaves. Explants were washed under running tap water for 5 min and treated with 0.1% Tween-20 solution for 5 min followed by washing with distilled water for 3-4 times. The explants were then treated with 0.2% fungicide (bavistin) + 0.02% bactericide solution for 5 min followed by washing with distilled water for 3-4 times. After this, entire experimental material was shifted to laminar air flow chamber which was sterilized prior to use by UV light and IPA solution. All the explants were transferred into sterile glass beaker and treated with 70% ethanol for 1min. Finally, the explants were surface sterilized with 0.1% (w/v) $HgCl_2$ solution and 1% NaOCl solution for 5-10min followed by washing explants for 3-4 times with sterile distilled water to remove the traces of $HgCl_2$.

Inoculation of explants

The surface sterilized explants were dried on sterile filter paper, exposed cut ends were trimmed and transferred on to the initiation media with composition of different hormones in the laminar air flow chamber with continuous air flow inside.

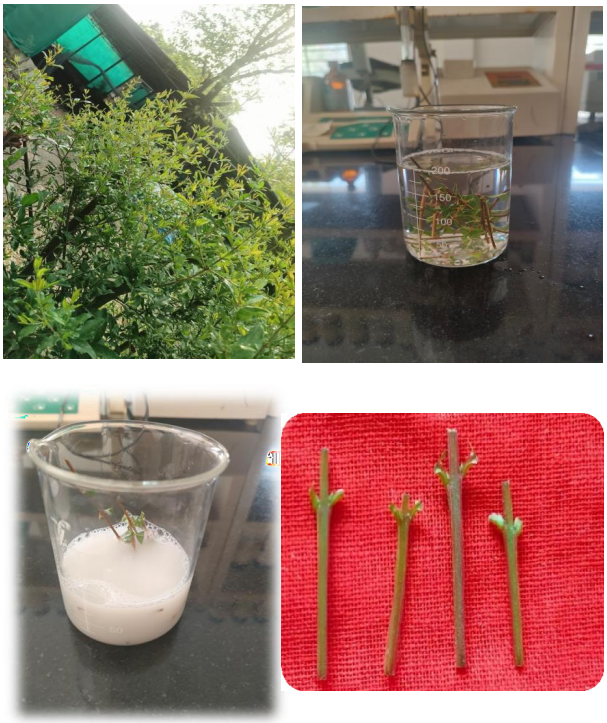

Fig. 1, 2, 3, 4. Mother plant from which explants are collected followed by washing and surface sterilization of nodal explants of Pomegranate cv. Bhagwa

Establishment of cultures

The explants were cultured for 15 days under 12/12 hr light/dark photoperiod. After 15 days, the

explants with developing shoot buds were subcultured on to the same fresh media and cultured for another 15 days under 12/12 hr photo period. Regenerated explants with shoots were used for further stages of micropropagation. Culture bottles with explants were kept in the growth room where temperature of 27 °C, light intensity of 1800-2000 lux was maintained.

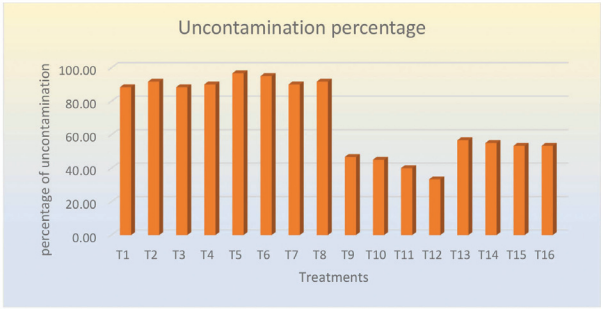

Fig. 1. Effect of different sterilizing agents and media with growth regulators on percentage of uncontaminated explants.

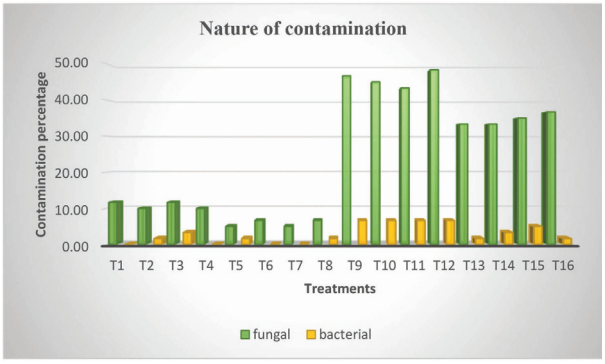

Fig. 2. Effect of different sterilizing agents and media with growth regulators on nature of microbial contamination (Fungal and Bacterial)

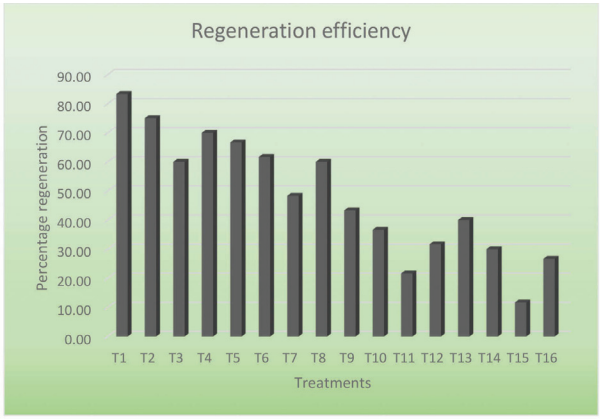

Fig. 3. Effect of different sterilizing agents and media with growth regulators on percentage regeneration efficiency

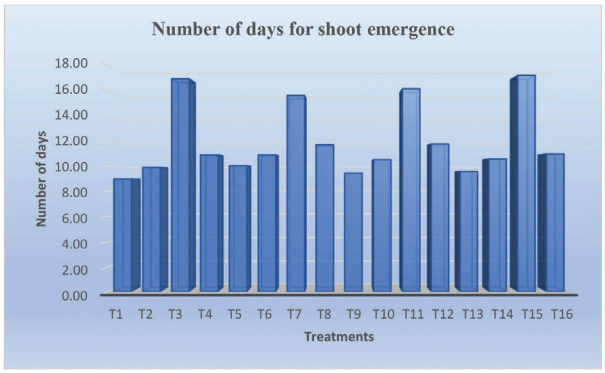

Fig. 4. Effect of sterilizing agents and growth regulators on number of days for shoot emergence

RESULTS AND DISCUSSION

Highest percentage of uncontamination was recorded in 0.1% $HgCl_2$ for 10min (93.33%) irrespective of different cytokinins used from the Table 1 data. Similar results were reported by Kalalbandi *et al.* (2014) in pomegranate and Gorad *et al.* (2018) also in pomegranate.

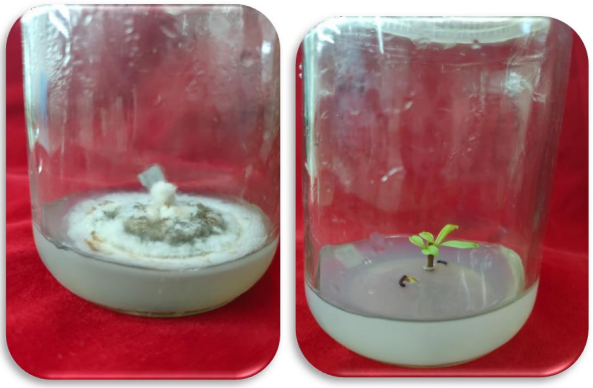

Fig. 1,2. Highest fungal contamination and bacterial contamination was recorded in S3: NaOCl 1% for 5min

Fig. 3. Response of explants to different growth regulators: 2-ip, Zeatin, Kinetin, BAP respectively

Table 1. Effect of different sterilizing agents and media with growth regulators on percentage of uncontaminated explants in Pomegranate cv. Bhagwa

Factors	G1	G2	G3	G4	Mean S
S1	88.33	86.66	88.33	88.33	87.91
S2	96.66	95.00	90.00	91.66	93.33
S3	46.66	45.00	40.00	33.33	41.25
S4	56.66	55.00	53.33	53.33	54.58
Mean G	72.08	70.41	67.91	66.66	
Factor	SE(m) ±			CD at 1%	
Factor A	1.55			4.51	
Factor B	1.55			NS	
A x B	3.11			NS	

The fungal and bacterial contamination was recorded maximum when 1% NaOCl for 5min (45.83%, 6.66%) was used and minimum contamination was observed in case of 0.1% $HgCl_2$ for 10 min (5.83%, 0.83%) from the data represented in Table 2 and 3. Similar results were reported by Debbarma *et al.* (2016) in grape. From the observations, as the time period of both sterilizing agents ($HgCl_2$, NaOCl) increased from 5min to 10 min keeping the concentration constant i.e., $HgCl_2$ at 0.1%, NaOCl at 1%, contamination was decreased.

Pomegranate is a semi woody shrub which exudates phenols as the part of defense mechanism in response to biotic and abiotic stress. The excision of Pomegranate explant from mother plant stimulates the phenol exudation by activating oxidative enzymes which results in browning and mortality of explants. The study revealed that there

Fig. 4, 5. Percentage of phenol exudation

Table 2. Effect of different sterilizing agents and media with growth regulators on nature of microbial contamination- Fungal contamination in Pomegranate cv. Bhagwa

Factors	G1	G2	G3	G4	Mean S
S1	11.66(19.87)	10.00(18.42)	11.66(19.87)	10.00(18.42)	10.83(19.15)
S2	5.00(12.91)	6.66(14.75)	5.00(12.91)	6.66(14.75)	5.833(13.83)
S3	46.66(43.07)	45.00(42.11)	43.33(41.14)	48.33(43.07)	45.833(42.35)
S4	33.33(35.23)	33.33(35.23)	35.00(36.25)	36.66(37.24)	34.58(35.99)
Mean G	24.16(27.77)	23.75(27.63)	23.75(27.54)	25.41(28.37)	
Factors	SE(m) ±	CD at 1%			
Sterilizing agents (S)	0.51	1.48			
Growth regulators (G)	0.51	NS			
S x G	1.02	NS			

*Three replications were performed
*Figures in parenthesis are Arc Sin values

Table 3. Effect of different sterilizing agents and media with growth regulators on nature of microbial contamination- Bacterial contamination in Pomegranate cv. Bhagwa

Factors	G1	G2	G3	G4	Mean S
S1	0.00(1.00)	1.66(1.48)	3.33(1.96)	0.00(1.00)	1.25(1.36)
S2	1.66(1.48)	0.00(1.00)	0.00(1.00)	1.66(1.48)	0.833(1.24)
S3	6.66(2.73)	6.66(2.73)	6.66(2.73)	6.66(2.73)	6.66(2.73)
S4	1.66(1.48)	3.33(1.96)	5.00(2.44)	1.66(1.48)	2.91(1.84)
Mean G	2.50(1.67)	2.91(1.79)	3.75(2.03)	2.50(1.67)	
Factors		SE(m) ±		CD at 1%	
Sterilizing agents (S)		0.17		0.50	
Growth regulators (G)		0.17		NS	
S x G		0.35		NS	

#Values in the parenthesis are square root transformed (x + 1) values

Table 4. Effect of different sterilizing agents and media with growth regulators on percent phenol exudation in Pomegranate cv. Bhagwa

Factors	G1	G2	G3	G4	Mean S
S1	0.00(1.00)	5.00(2.25)	3.33(1.96)	3.33(1.96)	2.91(1.79)
S2	5.00(2.44)	3.33(1.96)	5.00(2.44)	1.66(1.48)	3.75(2.08)
S3	3.33(1.96)	1.66(1.48)	0.00(1.00)	1.66(1.48)	1.66(1.48)
S4	1.66(1.48)	1.66(1.48)	1.66(1.48)	1.66(1.48)	2.08(1.60)
Mean G	2.50(1.72)	2.91(1.79)	2.50(1.72)	2.50(1.72)	
Factors		SE(m) ±		CD at 1%	
Sterilizing agents (S)		0.21		NS	
Growth regulators (G)		0.21		NS	
S x G		0.43		NS	

Values in the parenthesis are square root transformed (x + 1) values

Table 5. Effect of different sterilizing agents and media with growth regulators on percentage regeneration efficiency in Pomegranate cv. Bhagwa

Factors	G1	G2	G3	G4	Mean S
S1	83.33(65.92)	70.00(56.76)	60.00(50.76)	75.00(60.05)	72.08(58.37)
S2	66.66(54.72)	60.00(50.74)	48.33(44.02)	61.66(51.73)	59.16(50.30)
S3	43.33(41.13)	31.66(34.21)	21.66(27.69)	36.66(37.24)	33.33(35.07)
S4	40.00(39.21)	26.66(31.05)	11.66(19.87)	30.00(33.14)	27.08(30.82)
Mean G	58.33(50.25)	47.08(43.19)	35.41(35.59)	50.83(45.54)	
Factors		SE(m) ±		CD at 1%	
Sterilizing agents (S)		0.65		1.89	
Growth regulators (G)		0.65		1.89	
S x G		1.30		NS	

*Three replications were performed
*Figures in parenthesis are Arc Sin values

Table 4.1.1.5. Effect of sterilizing agents and growth regulators on number of days for shoot emergence in Pomegranate cv. Bhagwa

Factors	G1	G2	G3	G4	Mean S
S1	9.00	9.93	17.00	10.93	11.71
S2	10.06	10.93	15.66	11.73	12.10
S3	9.46	10.53	16.20	11.80	12.00
S4	9.60	10.60	17.26	11.00	
Mean G	9.53	10.50	16.53	11.36	
Factors		SE(m) ±		CD at 1%	
Sterilizing agents (S)		0.20		NS	
Growth regulators (G)		0.20		0.57	
S x G		0.40		NS	

is no significant difference among the sterilizing agents, WPM media with growth regulators and interaction between sterilizing agents and growth regulators.

Individual effect of both factors sterilizing agents and growth regulators was recorded with non-significant interaction effect with 72.08% bud proliferation of pomegranate nodal explants treated with 0.1% $HgCl_2$ for 5 min. As the time of exposure of explant to 0.1% $HgCl_2$ increased from 5min to 10min though contamination decreased, explant became necrotic and dead due to increased time exposure of explant (10 min) from the Table 5 data. Similar results were reported by Das *et al.* (2018) in jackfruit.

BAP 2 mg l^{-1} recorded the highest regeneration efficiency at the earliest (9.53 days) when compared to other treatments. Cytokinin BAP at 2 mg l^{-1} plays a crucial role in stimulating cell division and growth, induces the bud break of pomegranate

nodal explants cv. Bhagwa more effectively than other cytokinins used in the investigation. Similar results were reported by Theivanai *et al.* (2020) in grape.

CONCLUSION

From the observations, it can be concluded that, 0.1% $HgCl_2$ for 10 min worked effectively in reducing the contamination over 1% NaOCl. Highest regeneration efficiency was observed with BAP 2 mg/l within short period of time.

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