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Optimization of regeneration using different plant growth regulator in Pomegranate (*Punica granatum* L.) cv.'Bhagwa' cultivar

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Abstract
A micropropagation technique is used for the pomegranate variety 'Bhagwa' with nodal segments as explants in present research. In current study, disease free culture were developed using a mixture of different concentrations sterilizing agents like carbendazim-50 per cent, cefotaxime, kanamycin, ketokenazol, and mercuric chloride ($HgCl_2$). In addition to this, the phenolic exudates interfere with explant growth during *In vitro* condition. Consequently, the best approached proven to be transfer of explants at a regular interval of 24 h is best approach for phenolic exudates. Nodal segments were cultured on full strength Murashige and Skoog (MS) for shoot induction. The media was prepared supplemented with 6-benzylaminopurine (BAP), 0 to 2.5 mgl^{-1} and Kinetin (KIN), 0 to 1.0 mgl^{-1} produced maximum number of shoots (2.38±0.02) among all the treatments tested for shoot induction. Moreover, BAP with NAA proved to be best combination with adenine sulphate (30 mgl^{-1}) and silver nitrate (1 mgl^{-1}) for multiple shoot development gives produced more than 12 shoots (13.26±0.26) in 21 days intervals. Maximum number of root length and number of root recorded using ½ WPM with low concentration of IBA. The plantlets with well-formed root systems were progressively acclimatized in the greenhouse using soil, cocopeat (3:1) mixture and then transferred. Moreover, scanning electron microscopy (SEM) studies were carried out to observe and differentiate stomata and morphology of *in vitro* Pomegranate (*Punica granatum* L.) cv.'Bhagwa'

Keywords: Explant, micropropagation, multiplication, SEM, rooting

Introduction
Pomegranate (*Punica granatum* L.) is widely classified in a distinct family (Punicaceae) consis ting of only one genus and two species; *P. granatum* and *P.protopunica* (El-Agamy *et al.* 2010) [3]. It is an economically significant fruit crop of the world's tropical and subtropical regions (Jayesh and Kumar 2004) [6]. It has chromosomes 2n=2x=16, 18 (Simmonds and Smartt 1976) [20]. Pomegranate is native to Iran and is grown widely in Mediterranean countries such as Spain, Morocco, Egypt, California and Afghan and Baluchistan. The Burma, China, and Japan have also evolved to some degree. The estimated area under pomegranate was 1.03 lakh ha with total production of 17.06 lakh MT and 9.05 toone / ha productivity (2018-19). In India, though pomegranate is cultivated by a number of states, cultivation is highest in Maharashtra followed by Gujarat and Karnataka.
Pomegranate is used for the nutritious value of its fruit, for the medicinal properties of different parts of the tree and for ornamental purposes (Fadavi *et al.* 2005) [4]. Seeds and fleshy pulp are dried for use in Indian and Pakistani cuisines as condiments. The fruit is a rich source of minerals, vitamins, antioxidants, tannins, and polyphenols. Pomegranate is a rich source of carbohydrate (14.5%), protein (1.6%), fat (10 mg/100 g), calcium (10 mg/100 g), phosphorus (70 mg/100 g), iron (0.3 mg/100gm), and vitamin C (65 mg/100 g). This is also high in potassium, riboflavin, niacin, ascorbic acid (Korkmaz and Aşkın 2013) [8]. Pomegranates can be cultivated in a broad range of soils, from acidic sandy loams to alkaline calcareous soils, and can even be suited to the drought (Sepulveda *et al.* 2000) [18]. Considering the fact that India has all the favourable conditions for pomegranate cultivation, the annual production is much lower due to a selected variety's scarcity of good quality disease-free planting material. This demands the immediate creation of an effective method of propagation for the large-scale

cultivation of pomegranate plants of a specified variety. The modern method of seed propagation is not favoured because of the resultant variation of tree and fruit character; while the distribution of hardwood or softwood cuttings does not generate disease-free and stable plants (Deepika and Kanwar 2010) [2]. Besides this, the spread of bacterial disease induced by *Xanthomonas axonopodis* pv. *punicae* has emerged as a major constraint in Pomegranate development in India(Sharma *et al*. 2017) [19]. This disease spread through the soil, rendering it difficult to monitor. Although traditional vegetative propagation is easy and true to sampling form, it has had many disadvantages, such as poor safety, slow rate of spread, and so on.

Nevertheless, plant multiplication is conducted in commercial tissue culture, via shoot proliferation. This is favoured mostly because of its real existence and genetically healthy plantlets.

In vitro pomegranate propagation was recorded from nodal parts (Murkute *et al*. 2004; Naik *et al*. 1999; Naik *et al*. 2000) [10, 13, 14], shoot tips (Murkute *et al*. 2004) [10], and cotyledonary nodes (Madhuri and Shailja 2000) [9] by axillary shoot proliferation. Regeneration of *In vitro P.granatum* plantlets can occur by organogenesis from the leaf segment generated callus (Deepika and Kanwar 2010) [2].

In order to explore propagation by *In vitro* technique in pomegranate over traditional propagation, overcoming such work using various parts and major problems associated with tissue culture is very important. Besides this, micro propagation by proliferation of the axillary bud for the production of disease-free and elite planting material in leading commercial varieties is gaining significant importance. Despite this, pomegranate production in India is very low, as farmers face a dire threat of disease. Farmers are not willing to take a step forward to solve this issue as the disease will impact the whole farm in a short time. Alternative choice is by micropropagation to solve this issue and improve productivity. In view of this fact, the present investigation was carried out in the famous pomegranate variety 'Bhagwa'.

Material and Methods

The work is establishment of large scale micro propagation protocol of "Bhagva" cultivar, was done at Tissue culture laboratory, Department of Biotechnology, Junagadh Agricultural University, Junagadh during the period 2019-20.

Explant selection

The mother plant selection was done in pomegranate field of progressive farmer near Junagadh district. Nodal segment of about 1 to 2 cm long pomegranate "Bhagva" cultivar were collected from mother plant.

Preparation of culture medium

Two different media; MS (Murashi and skoog, 1962) was prepared and dispensed in50 ml glass bottle (Borosil, India). The different media supplemented with organic acids and vitamins. In addition to this, sucrose were autoclaved with media as energy source and sterilized at 121 ºC and 15 psi pressure for 15 min. The media prepared and adjusted pH 5.6 ± 0.01 prior to autoclaving.

Selection of Explant and pre treatment

Selected mother plant which is disease free healthy pomegranate 'Bhagava' cultivar were sprayed three days with 1000 mgl^{-1} bavistin (50% carbendazim) before collection of nodal segments of about 1 to 2 cm long. Single node from selected 4-5 year old mother plants were collected during November, 2019. Excised nodal segments were thoroughly washed under running tap water for about 5 to 10 min followed by washing with distilled water and 1 to 2 drops of surfactant, Tween-20.

Nodal segment surface sterilization

Isolated nodal segment were treated with different antimicrobial agents of varying combinations of duration and concentration were tested for establishing axenic explant cultures. Explants were treated with Bavistin 1gm/l (50% carbendazim) for 10min, ketakanazol (200mg/l for 10min), cefotaxime (1gm/l for 7 min) Kanamycin (1gm/l for 5 min). Two wash of sterilized water in each treatment were given. Besides this, explants were treated finally with mercuric chloride (1000 mg/l, 3min) after the above anti-microbial treatments (Table 1).

Control of browning

Generally, woody plants like pomegranate contain high amount of polyphenolic compound which may interfere the initiation of nodal segment *In vitro* condition. Therefore, to overcome this issue during initiation stage addition of activated charcoal at 2.5mg/l not much resolved as compare with transfer of explants every 24 hr (Table 2)

Inoculation of explant for initiation

For shoot initiation several media are available but commonly use media in pomegranate *viz*. MS along with different plant growth regulator like BAP(0,0.2,0.5,1,1.5,2,3 mgl^{-1}) and NAA (0,0.1,0.2,0.5, mgl^{-1}) with 3% sucrose and 0.8% Bacteriologial grade Agar were used in experiment. The explants were transferred to the same medium combinations for three subsequent subcultures for identification of the best shoot induction medium. Observations on number of shoots, number of leaves and length of the shoots were recorded (Table No 3).

Sub culturing

Sub culturing of explants was done regularly at three weeks interval on the fresh medium as per the treatments.

Multiple shoot development

Cytokinins group plant growth regulator were used for multiple shoot induction *viz* BAP and Kn with adenine sulphate ($30mgl^{-1}$) and silver nitrate ($1mgl^{-1}$) at different sub culturing stage. The media was prepared according to MS basal composition with 3% sucrose and 0.4% clarigel. Observation like number of shoots, length of shoots (cm), and number of leaves were recorded (Table No 4).

Root induction

Shoots of 2-3 cm length or with 4-5 leaves were transferred for root induction. The root organogenesis was observed on MS medium and medium fortified with different levels of auxins (Table No 5). Observations on rooting percentage, number of roots and length of roots were recorded.

Hardening of tissue culture plantlets

In vitro grown plantlets were removed from culture bottles and washed with distilled water to remove medium sticking to roots. The plantlets were dipped into 0.1% bavistin for 10 minutes and then planted in plastic try containing coco peat for primary hardening. These plantlets were grown under polyhouse condition at 65% humidity and 24±1 ºC temperature for 21 days. The plan lets were shifted to shade net house for secondary hardening. The planlets were planted in polythene bags containing different potting mixture like soil, FYM, and Coco peat. The harded plantlets were then

exposed to the direct sunlight to get acclimatized for the natural environmental conditions (Table No 6).

Scanning electron microscopy (SEM) studies

The leaves were sliced in square form with a diameter of roughly 3 mm x 3 mm and immersed in a mixed solution of 8 percent Glutaraldehyde and Sorencen Buffered Phosphate with a ratio of 1:1 per hour. The leaf specimens were washed with a solution of Sorencen Buffered Phosphate and purified water at a ratio of 1:1 for 5 minutes. Specimens were then soaked in a 4 percent mixed solution of Osmium and distilled water with a 1:1 ratio. After about 14 hours at low temperatures, the process of dehydration in the vapours of the vessel was carried out. Every 15 minutes the concentration of ethanol was increased from 10 percent to 100 percent. Specimens were immersed in a combination of 100% ethanol, respectively. Immediately after this, the leaf extracts were then immersed for 20 minutes in a 100 percent ethanol solution, repeating them four times. Next, use of Bal-Tec CPD 030 Critical Point Dryer was performed Specimens were placed on 12.5 mm diameter aluminium using Conducting Carbon Cement (LEIT-C), held in a drying jar and filled with a thin gold film (40-60 nm) using EVD 18 ZEISS sputter coater Coating Process. Under the scanning electron microscope, structures such as stomata and the abaxial surface texture of the leaves were observed and recorded.

Statistical analysis

The data were analysed in completely randomized design (CRD) according to the described method by Snedecor and Cochran (Snedecor & Cochran, 1989) to find out the significance of difference during initiation, mass multiplication, rooting and hardening stages. The data were subjected to one way analysis of variance (ANOVA) followed by Duncan multiple range test (DMRT).

Results and Discussion

The present experiment aim was to establish, optimize the protocol of pomegranate tissue culture for large scale production under low cost. We have successfully develop a protocol for the micropropagation of pomegranate 'Bhagwa' cultivar using two various media combination MS basal and, WPM for rooting.

Sterilization stage

Illustrated data in Figure. 1 and Table 1 shows the effect of $HgCl_2$, Carbendazim-50%, Cefotaxime, Kanamycin, and Ketokenazol at different concentrations on *In vitro* sterilization of nodal parts of Bhagwa cultivar. The effects of these concentrations on contamination and survival percentages were expressed. The maximum percentages of contamination were recorded in T5 and the minimum percentages of contamination were recorded in T3.

Since from many research techniques of *in vitro* regeneration has been practised in number of plant species. *In vitro* propagation of pomegranate plants through nodal explants of mature mother plant has been reported earlier (Kaji *et al*. 2013; Singh and Patel 2014; Singh *et al*. 2013) [7, 22, 24]. Therefore, the first objective of the study was to establish successful surface sterilization techniques using different surfactants for disease-free culture. Surface sterilization of any explant is a crucial step in plant tissue culture. The plant cell does not die and only the contaminants should be eliminated during sterilization The effectiveness of sterilization methods is dependent on the specific concentration and application of antimicrobial agents. (Oyebanji *et al*. 2009) [15]. Present investigation, use of treatment number 3 shows the ($HgCl_2$, Carbendazim-50%, Cefotaxime, Kanamycin, and Ketokenazol) effective compared to others. Similarly effective surface sterilization with lowest contamination (15%) was achieved with $HgCl_2$ (0.10%) for 3 minute recorded (Guranna and Hoolageri 2017) [5].

In addition to this, various treatments, surface sterilization of double nodal explants containing nodes with $HgCl_2$ at 0.1% for 3 minutes resulted in maximum establishment aseptic culture (55%), lowest contamination (15%) and lesser intensity of browning (+) when incubated onto MS medium containing BAP 1 mgl^{-1} + $AgNO_3$ 1 mgl^{-1} + activated charcoal 2000 mgl^{-1} were recorded (Prabhuling and Huchesh 2018) [17].

Fig 1: Phenolic exudation treatment after transfer of explants every 24 hours

Phenolic exudation in shoot tip explant

The browning of cultures is a major obstacle to developing explants in perennial fruit crops, which subsequently simplifies the *In vitro* techniques. Pomegranate have the same problem in establishment of *In vitro* cultures due to high phenolic contents. Several treatments were tried including use of adsorbents and antioxidants, transfer of explants every 24 hours is effective compare to all other treatments. Although several research (Murkute and Patil 2003; Prabhuling and Huchesh 2018; Singh *et al*. 2011; Singh and Patel 2016) [11, 17, 21, 23] found several treatments were tried including use of adsorbents and antioxidants with different explants *viz*., leaf segment, cotyledon, nodal segment and shoot tip. The activated charcoal (adsorbent) and ascorbic acid (antioxidant) could not alter the extent of browning at various concentrations. The sub culturing of explants thrice, at an interval of 24 hrs. could have control browning in all explants used, except cotyledon. Pomegranate nodal segment was establish on MS basal medium and reducing of browning was done using different treatment like ascorbic acid, activated charcoal, PVP, and regular interval transfer with various concentration. Among the seven different treatments used to minimize the browning issue, transfer of explants every 24 hours for two sub culturing is effective compared to all others treatments (Table 2, Fig 1). Using transfer of explants with regular interval gives 90% survival of explants during initiation stage.

Effect of different growth regulators on shoot initiation

In the present experiment, the nodal shoot proliferation raged from 42% to 91% in MS basal medium and it was observed

that bud break at 12 days after initiation. It is established fact about concentration of cytokinin have a positive formative effect on shoot initiation. Two PGRs used *viz*; 6-benzylaminopurine (BAP) and 1-napthalacetic acid(NAA) with various concentration. In context to nodal explant significantly maximum percent of shoot initiation was recorded with highest number of shoots (2.38±0.02^{b}), length of shoot (1.23±0.06$^{a)}$, number of nodes (2.31±0.33$^{a)}$, and no.of leaves (2.26±0.47^{a}) using BAP (0.2 mgl^{-1}) and NAA (0.1mgl^{-1})respectively (Table 3, Figure 2). In shoot initiation stage two different plant growth hormones were used for shoot proliferation. However, combination of BAP (0.2 mgl^{-1}) and NAA (0.1 mgl-1) considerably higher response for various parameters. Similarly, (Singh *et al*. 2013) [24] the maximum culture establishment was obtained with treatment 2.0 mg/l BAP + 0.1 mg/l NAA + 0.5 mg/l GA which also gave the earliest bud sprouting (5.67 days) as compared to control using cotyledonary nodal segmant. Besides this, (Usharani *et al*. 2014) [26] Shoot proliferation was induced in nodal segments on Murashige and Skoog media containing various combinations of 6 – Benzylaminopurine (BAP) (0 – 2 mg L^{-1}) and 1-naphthalacetic acid (NAA) (0 – 1 mg L^{-1}). Maximum number of shoots (6.2) was produced in N8 media with BAP 1.5 mg L−1 and 1-naphthalene acetic acid (NAA) 0.5 mg L−1 Amaximum shoot length of 7.7 cm with highest number of leaves (13) was recorded on the same medium.

Fig 2: A shoot development from nodal segments of pomegranate cultivar 'Bhagwa' after 12 day of culture on MS basal medium

Multiplication of initiated shoot

Effect of plant growth regulator on multiplication of explants such as number of shoots per explant, length of shoots (cm), and number of leaves is illustrated (Table 4, Figure 3). Two different cytokinins (BAP and Kn) using with various combination for multiple shoot induction. In MS basal medium supplemented with BAP @ 0.2 mgl-1, Kn @ 0.1 mgl-1, silver nitrate 1mgl-1, and Adenine sulphate 30mg-1 gives highest number of leaves (31.77±0.55a) and length of shoot (7.19±0.07a), and number of shoots (13.26±0.26a) in 21 days interval with three sequential sub culturing. This may be attributed due to synergistic effect of BAP as a cell division and $AgNO_3$ as an ethylene inhibitor at higher level. It is evident from the fact that, with decrease in level of BAP and $AgNO_3$ resulted in declined in percent response. The results is correlates with several experiment conducted for multiplication of pomegranate genotype. This was in accordance with findings of (Patil *et al*. 2011) [16] in pomegranate. Similarly, effect of BAP in accordance (Naik *et al*. 1999) [13] with axillary shoot proliferation using nodal segment were correlate with this study. Role of silver nitrate were (Naik and Chand 2003) [12] also characterized in pomegranate cultivars.

Fig 3: Development of multiple shoots after 21 day of culture on the same medium

Effect of growth regulator (IBA and IAA) on root growth

Effect of different growth regulators on root regeneration in pomegranate cv. 'Bhagwa' is depicted in Table No.5 (Figure 4). Root initiation was found 100% in all treatments using media supplemented at ½ WPM with different concentration of IBA and IAA. In rooting medium after 12 -15 days root started initiation. Best response was seen in sole IBA with 0.3mgl^{-1}. The result found significantly higher in response to root length (2.16±0.31^{a} cm) and number of roots (2.51±0.15^{a}) in Bhagwa cultivar. Superior percent response to growth regulator was recovered with treatment of MS+0.2 mgl^{-1} BAP+ 0.1mgl^{-1} KIN + $AgNO_3$ 1.0 mgl^{-1} + Adenine sulphate 30 mgl^{-1} + 250 mgl^{-1} clarigel gives highest number of leaves (31.77±0.55^{a}) and length of shoot (7.19±0.07^{a}), and number of shoots (13.26±0.26^{a}) in 21 days interval. Half strength WPM proved superior in promoting *In vitro* root growth with lower sole concentration of 0.3 mgl^{-1} IBA showed significantly early root initiation with better root growth. This may be due to effectiveness of IBA in promoting root growth as it was efficiently absorbed and utilized. This may be also attribute to the fact that root growth requires reduced levels of nutrients so as to enhance more absorption of rooting hormones in medium. Poor growth was observed using NAA and IBA with higher concentration. Similar result were reported by (Chaugule *et al*. 2005) [1] (Soukhak *et al*. 2012) [25] various researchers in their study.

Fig 4: Root development in shoot cultured on half strength WPM medium at 21 days

Harding of tissue culture plantlets

The data pertaining to 100% survival of *in vitro* raised plantlets of pomegranate cv Baghwa in different potting mixture has be utilized in presented study (Table No 6, Figure 5). Harding of tissue culture, five different composition used for adaptation of plantlets produced *In vitro* condition, soil and cocopeat (3:1) proved to be the effective medium compared to others combination. The plants grown in soil with cocopeat after 12 days interval produced maximum number of shoots (2.25 ± 0.12^{a}), length of shoots (3.77 ± 1.85^{a}), number of leaves (5.94 ± 0.21^{a}), number of roots (3.59 ± 0.71^{a}), and length of roots (2.80 ± 0.24^{a}) respectively compare to all other combination. The second best response was seen in case of soil with vermiculture (3:1) mixture gives number of shoots (1.89 ± 0.26^{ab}), length of shoots (3.32 ± 0.44^{b}) comparatively lesser than soil with cocopeat combination. Efficaciously hardened pomegranate plants were moved to polyhouse and promoted to acclimatize in the soil. Five different treatment were used for primary hardening of rooted plantlets, soil with cocopeat (3:1) had resulted as an excellent composition for hardening of the *in vtiro* plants. The results obtained showed that BAP induced shoots while IBA induced roots.

Fig 5: Well-rooted plantlet acclimatization for hardening

Scanning Electron Microscopy (SEM) Analysis

Figs. 6(a-f) shows that magnification at 250 X, abaxial surface and stomata during initiation stage length 13.80 µm; width 17.97 µm whereas, during multiplication stage length 10.61 µm/17.79 µm width and during rooting stage length 11.34 µm/ 15.44 µm width. Distinct changes observed in stomata width in different stages were observed in Bhagwa cultivar. Scanning Electron Microscopy (SEM) studies were carried out to observe and differentiate the morphology of *In vitro*. SEM techniques were done to observe the stomata and microcharacters of the abaxial surfaces of *In vitro* Pomegranate genotype. Similar result also were observed scanning electron microscopy studies and *In vitro* regeneration of *Passiflora edulis Sims* var. edulis for conservation (Veeramohan *et al*. 2013) [27]. Thus, development of efficient protocol by using various plant growth regulators combination with silver nitrate and adenine sulphate for mass multiplication of pomegranate "Bhagwa" cultivar gives high frequencies plantlets through nodal explant.

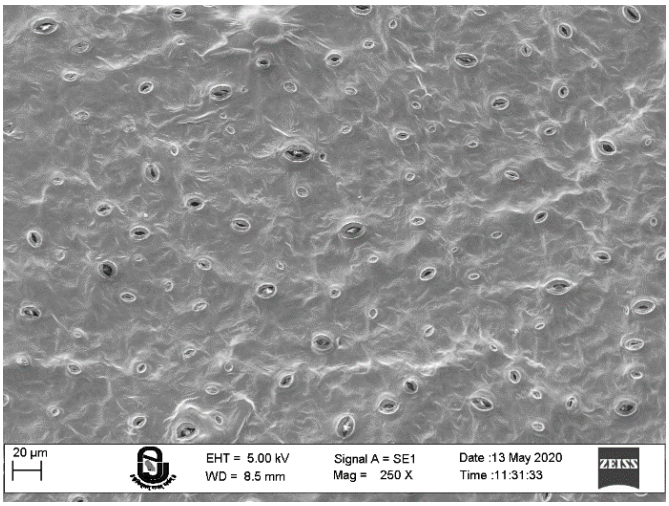

Fig 6: (a) Abaxial surface of leaf explant at initiation stage

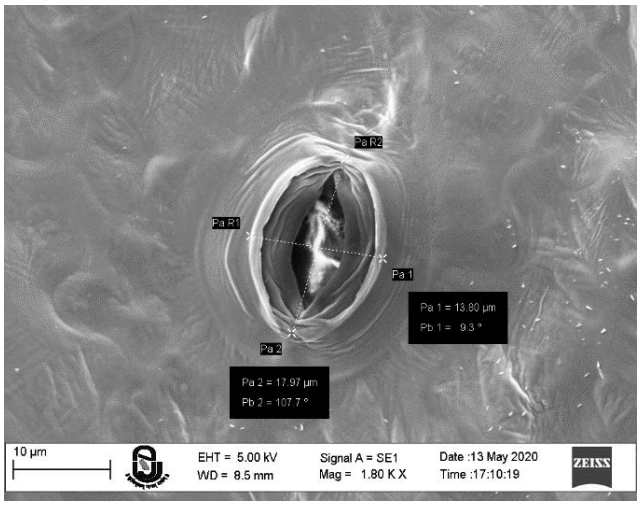

Fig 6: (b) stomata on the Abaxial surface of leaf explant at initiation stage

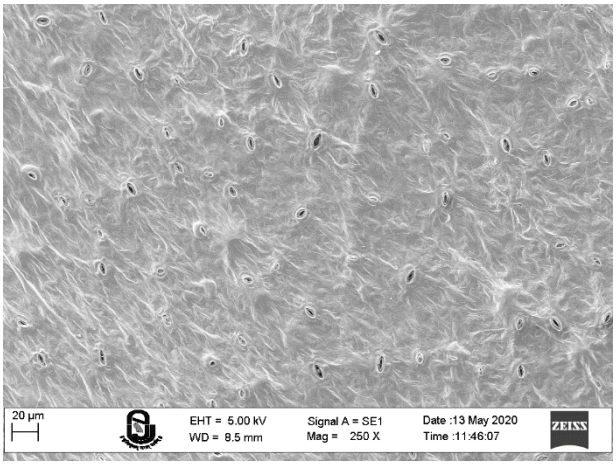

Fig 6: (c) Abaxial surface of leaf explant at multiplication stage

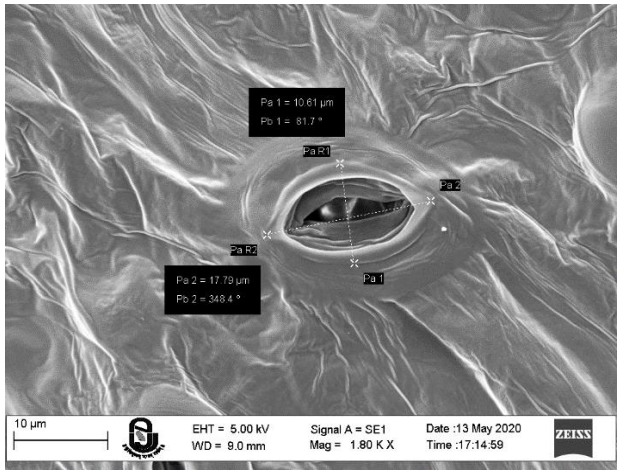

Fig 6: (d) Stomata on the Abaxial surface of leaf explant at multiplication stage

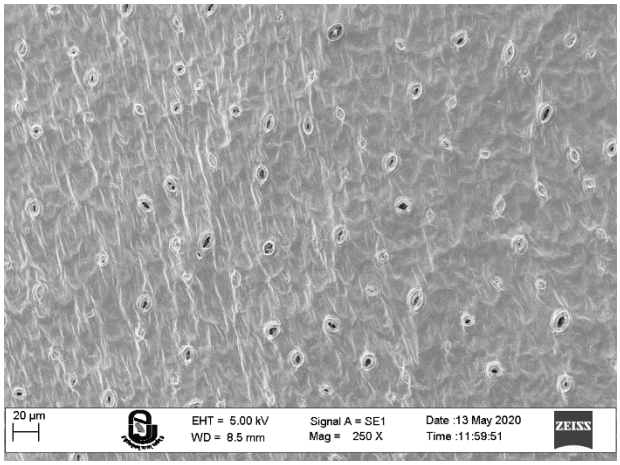

Fig 6: (e) Abaxial surface multiplication stage of leaf explant at rooting stage

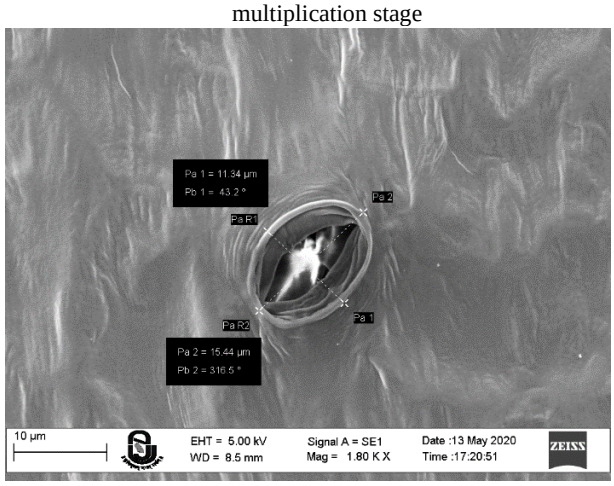

Fig 6: (f) stomata on the Abaxial surface of leaf explant at rooting stage

Table 1: Impact of different treatments for surface sterilization on grenade explants.

Treatment	Sterilizing agent	Concentration (mgl^{-1})	Time duration (Minutes)	Survival %
T1	Carbendazim-50%	500	12	76.5
	Cefotaxime	1000	12	
	Kanamycin	1000	8	
	Ketokenazol	1000	10	
	HgCl2	100	2	
T2	Carbendazim-50%	2000	10	55.63
	Cefotaxime	1000	10	
	Kanamycin	1000	8	
	Ketokenazol	500	15	
	HgCl2	100	3	
T3	Carbendazim-50%	1000	10	90.34
	Cefotaxime	1000	7	
	Kanamycin	1000	5	
	Ketokenazol	200	10	
	HgCl2	1000	3	
T4	Carbendazim-50%	3000	10	65.43
	Cefotaxime	1000	10	
	Kanamycin	1000	10	
	Ketokenazol	500	12	
	HgCl2	100	5	
T5	Carbendazim-50%	500	12	39.42
	Cefotaxime	500	12	
	Kanamycin	700	15	
	Ketokenazol	150	15	
	HgCl2	100	5	

Table 2: Effect of polyphenol control treatments on explants.

	Treatment details	Concentration (mgl-1)	Browning of explants	% Survival of explants
T1	Addition of ascorbic acid to media[a]	50	+++	18%
T2	Addition of ascorbic acid to media[a]	100	++	15%
T3	Transfer of explants every 24 hours[b]	0	+	90%
T4	Addition of PVP to media[c]	50	++	25%
T5	Addition of PVP to media[c]	100	+++	0%
T6	Addition of activated charcoal to media[d]	1	++	60%
T7	Addition of activated charcoal to media[d]	2	+++	22%

'+' indicates intensity of exudation and browning of the explants (+ = 25%, ++ = 50%, +++ = 75%, ++++ = 100%)[1].
[acd]MS media supplemented with 0.8% agar and 3% sucrose.
[b]Transfer of explants at every 24 hours for two subsequent days

Table 3: Effect *on In vitro* shoot induction with MS culture media using different combinations of cytokinines and auxins.

NAA (mgl^{-1})	BAP (mgl^{-1})	Shoot proliferation (%)	Number of shoots/explants	Length of shoot (cm)	No. of nodes	No. of leaves
-	-	42.29	0.65 ± 0.01^{n}	0.82 ± 0.09^{hij}	1.14 ± 0.33^{d}	1.66 ± 0.38^{cde}
0.1	-	51.54	0.93 ± 0.02^{kl}	0.84 ± 0.17^{ghij}	1.14 ± 0.33^{d}	1.11 ± 0.16^{h}
0.2	-	56.46	1.17 ± 0.03^{j}	0.91 ± 0.07^{defghi}	1.38 ± 0.58^{cd}	1.72 ± 0.36^{bcd}
0.5	-	76.42	1.30 ± 0.08^{h}	0.83 ± 0.10^{ghij}	1.58 ± 0.88^{cd}	1.78 ± 0.62^{bcd}
-	0.2	50.64	0.96 ± 0.02^{k}	0.89 ± 0.03^{efghij}	1.14 ± 0.33^{d}	1.86 ± 0.39^{bcd}
0.1	0.2	90.73	2.38 ± 0.02^{b}	1.23 ± 0.06^{a}	2.31 ± 0.33^{a}	2.26 ± 0.47^{a}
0.2	0.2	75.71	1.78 ± 0.02^{de}	1.05 ± 0.08^{bcde}	1.52 ± 0.33^{cd}	1.68 ± 0.27^{bcde}
0.5	0.2	63.27	1.18 ± 0.02^{ij}	1.14 ± 0.05^{abc}	1.52 ± 0.33^{cd}	1.75 ± 0.25^{bcd}
-	0.5	71.91	1.54 ± 0.03^{g}	1.14 ± 0.10^{abc}	1.14 ± 0.33^{d}	1.22 ± 0.47^{gh}
0.1	0.5	82.17	1.85 ± 0.04^{cd}	1.03 ± 0.08^{bcdef}	1.82 ± 0.33^{bc}	1.41 ± 0.06^{efg}
0.2	0.5	63.58	2.56 ± 0.05^{a}	0.92 ± 0.07^{defghi}	1.14 ± 0.33^{d}	1.78 ± 0.05^{bcd}
0.5	0.5	71.36	0.91 ± 0.02^{kl}	0.65 ± 0.06^{k}	1.52 ± 0.33^{cd}	1.80 ± 0.10^{bcd}
-	1	57.11	0.59 ± 0.03^{n}	1.05 ± 0.10^{bcde}	2.08 ± 0.33^{ab}	1.57 ± 0.32^{def}
0.1	1	73.42	1.32 ± 0.02^{h}	0.91 ± 0.05^{defghi}	1.14 ± 0.33^{d}	1.06 ± 0.03^{h}
0.2	1	79.08	1.61 ± 0.04^{fg}	1.10 ± 0.07^{abc}	1.14 ± 0.33^{d}	1.29 ± 0.07^{fgh}
0.5	1	62.07	1.22 ± 0.04^{hij}	0.91 ± 0.01^{defghi}	1.52 ± 0.33^{cd}	1.87 ± 0.19^{bc}
-	1.5	44.02	0.84 ± 0.02^{lm}	0.99 ± 0.06^{bcdefg}	1.14 ± 0.33^{d}	1.96 ± 0.20^{b}
0.1	1.5	52.98	0.41 ± 0.04^{o}	0.73 ± 0.08^{jk}	1.14 ± 0.33^{d}	1.94 ± 0.38^{bc}
0.2	1.5	58.75	1.32 ± 0.03^{h}	1.15 ± 0.10^{ab}	1.82 ± 0.33^{bc}	1.85 ± 0.14^{bcd}
0.5	1.5	76.24	0.38 ± 0.03^{o}	1.04 ± 0.09^{bcde}	1.14 ± 0.33^{d}	1.66 ± 0.29^{bcd}
-	2	75.18	1.72 ± 0.05^{ef}	1.01 ± 0.23^{bcdef}	1.52 ± 0.33^{cd}	1.19 ± 0.14^{gh}
0.1	2	53.67	1.29 ± 0.03^{hi}	0.97 ± 0.06^{cdefgh}	1.14 ± 0.33^{d}	1.80 ± 0.29^{bcd}
0.2	2	81.24	1.95 ± 0.02^{c}	1.10 ± 0.05^{abc}	1.14 ± 0.33^{d}	1.93 ± 0.39^{bc}
0.5	2	72.54	0.77 ± 0.02^{m}	1.06 ± 0.11^{abcd}	1.82 ± 0.33^{bc}	1.75 ± 0.05^{bcd}
-	3	55.78	1.32 ± 0.03^{h}	0.79 ± 0.12^{ijk}	1.82 ± 0.33^{bc}	1.90 ± 0.25^{bc}
0.1	3	49.40	1.53 ± 0.03^{g}	1.05 ± 0.10^{bcde}	1.52 ± 0.33^{cd}	1.77 ± 0.34^{bcd}
0.2	3	75.48	1.91 ± 0.04^{c}	0.87 ± 0.05^{fghij}	1.14 ± 0.33^{d}	1.84 ± 0.36^{bcd}
0.5	3	61.41	1.16 ± 0.02^{j}	1.07 ± 0.09^{abcd}	1.52 ± 0.33^{cd}	1.87 ± 0.15^{bcd}

The maximum percentages of contamination were recorded in T5 and the minimum percentages of contamination were recorded in T3.

Table 4: Effect *on In vitro* shoot multiplication with MS culture media using different combinations of cytokinines and auxins.

KIN (mgl^{-1})	BAP (mgl^{-1})	Number of shoots	Length of shoot (cm)	No. of leaves
-	-	9.57 ± 0.15^{g}	6.33 ± 0.16^{bcde}	9.51 ± 0.38^{q}
0.1	-	9.14 ± 0.09^{ghij}	6.26 ± 0.08^{bcdef}	8.57 ± 0.23^{q}
0.4	-	7.52 ± 0.23^{l}	5.42 ± 0.04^{fghij}	11.65 ± 0.24^{p}
0.6	-	8.76 ± 0.07^{ijk}	5.67 ± 0.14^{efghi}	15.61 ± 0.35^{mno}
-	0.2	8.54 ± 0.07^{k}	5.44 ± 0.13^{fghij}	18.32 ± 0.15^{kl}
0.1	0.2	13.26 ± 0.26^{a}	7.19 ± 0.07^{a}	31.77 ± 0.55^{a}
0.4	0.2	10.45 ± 0.03^{e}	5.37 ± 0.32^{ghij}	22.62 ± 0.20^{def}
0.6	0.2	9.51 ± 0.16^{gh}	5.87 ± 0.13^{defgh}	19.02 ± 0.22^{jk}
-	0.5	9.58 ± 0.07^{fg}	5.60 ± 0.24^{efghij}	23.61 ± 0.51^{cd}
0.1	0.5	8.53 ± 0.22^{k}	6.77 ± 0.21^{ab}	25.58 ± 0.24^{b}
0.4	0.5	7.77 ± 0.11^{l}	5.42 ± 0.30^{fghij}	16.47 ± 0.35^{mn}
0.6	0.5	6.84 ± 0.05^{m}	4.26 ± 0.20^{k}	20.94 ± 1.00^{fghi}
-	1.0	7.46 ± 0.03^{l}	5.29 ± 0.10^{ghij}	22.52 ± 0.76^{def}
0.1	1.0	9.30 ± 0.03^{ghi}	5.89 ± 0.10^{cdefgh}	25.16 ± 0.24^{bc}
0.4	1.0	11.44 ± 0.05^{bc}	6.74 ± 0.21^{abc}	22.87 ± 0.20^{de}
0.6	1.0	10.17 ± 0.04^{ef}	6.65 ± 0.34^{abcd}	11.86 ± 0.15^{p}
-	1.5	9.21 ± 0.62^{ghij}	5.23 ± 0.24^{hij}	14.63 ± 0.94^{o}
0.1	1.5	8.72 ± 0.19^{ijk}	5.33 ± 0.20^{ghij}	19.68 ± 0.27^{hijk}
0.4	1.5	7.74 ± 0.03^{e}	5.64 ± 0.29^{efghi}	21.76 ± 0.46^{defg}
0.6	1.5	10.71 ± 0.17^{de}	5.65 ± 0.39^{efghi}	19.61 ± 0.20^{ijk}
-	2	11.83 ± 0.13^{b}	4.75 ± 0.25^{jk}	15.01 ± 0.32^{no}

0.1	2	11.16±0.14^{cd}	5.20±0.16^{hij}	19.65±0.31^{hijk}
0.4	2	10.31±0.15^{e}	6.14±0.22^{bcdefg}	22.02±0.10^{defg}
0.6	2	8.71±0.15^{jk}	4.88±0.42^{ijk}	23.42±1.07^{cd}
0,3	3	9.43±0.11^{gh}	6.37±0.45^{abcde}	16.97±1.22^{lm}
0.1	3	9.72±0.05^{fg}	5.22±0.28^{hij}	20.23±0.50^{ghij}
0.4	3	8.93±0.02^{hijk}	5.68±0.19^{efghi}	22.27±0.93^{def}
0.6	3	10.71±0.02^{de}	5.15±0.39^{hij}	21.51±0.34^{efgh}

Data are in the form of mean ± SE. Means with the same letter along the column are not significantly different at p=0.05.

Table 5: Effect of various concentrations and combinations of NAA and IBA on *In vitro* root induction

IAA (mgl^{-1})	IBA (mgl^{-1})	Number of Roots	Root length (cm)
0	0	1.65±0.22^{f}	1.67±0.42^{def}
0.2	0	1.72±0.07^{ef}	1.84±0.24^{bcd}
0.5	0	1.82±0.07^{def}	1.80±0.39^{cde}
1	0	1.87±0.37^{cdef}	1.84±0.21^{cd}
0	0.3	2.51±0.15^{a}	2.16±0.31^{a}
0.2	0.3	2.05±0.26^{bcde}	2.08±0.29^{a}
0.5	0.3	2.15±0.18^{abcd}	1.66±0.06^{def}
1	0.3	1.91±0.15^{cdef}	2.10±0.13^{a}
0	0.5	2.07±0.78^{bcd}	2.09±0.25^{a}
0.2	0.5	2.13±0.20^{bcd}	1.62±0.09^{ef}
0.5	0.5	2.04±1.01^{bcde}	1.57±0.07^{f}
1	0.5	2.17±0.53^{abc}	1.78±0.01^{cde}
0	1	2.17±0.22^{abc}	1.78±0.04^{cde}
0.2	1	2.00±0.41^{bcde}	2.04±0.06^{ab}
0.5	1.0	2.12±0.03^{bcd}	1.97±0.17^{abc}
1	1	2.28±0.23^{ab}	2.07±0.21^{a}

Data are in the form of mean ± SE. Means with the same letter along the column are not significantly different at p=0.05

Table 6: Effect of the various hardening mixtures on primary hardening of the *In vitro* developed plantlets.

Composition	Number of shoots-L	Length of shoots(cm)	Number of leaves	Number of roots	Length of root (cm)
soil	1.67±0.35^{b}	2.69±0.11^{c}	3.31±0.70^{d}	2.68±0.12^{bc}	1.87±0.60^{c}
cocopeat	1.61±0.81^{b}	2.80±0.29^{c}	4.20±2.96^{c}	2.53±1.35^{c}	2.30±1.17^{b}
vermiculture	1.79±0.13^{b}	3.03±0.18^{bc}	5.30±1.65^{ab}	2.70±1.77^{bc}	2.08±0.18^{bc}
soil+cocopeat (3:1)	2.25±0.12^{a}	3.77±1.85^{a}	5.94±0.21^{a}	3.59±0.71^{a}	2.80±0.24^{a}
soil+vermictulre (3:1)	1.89±0.26^{ab}	3.32±0.44^{b}	5.20±2.97^{b}	3.27±0.21^{ab}	2.03±0.94^{bc}
cocopeat+vermiculture (2:1)	1.86±0.40^{ab}	3.26±0.91^{b}	5.13±1.02^{b}	3.06±1.31^{abc}	2.11±0.18^{bc}

The plants grown in soil with cocopeat after 12 days interval produced maximum number of shoots (2.25±0.12a), length of shoots (3.77±1.85a), number of leaves (5.94±0.21a), number of roots (3.59±0.71a), and length of roots (2.80±0.24a) respectively.

Conclusion

From the present study, it is evident that nodal segment found to be best explants, which was positively responded under different treatments of surface sterilization. Besides this, MS basal media along with shoot initiation @ BAP (0.2 mg l^{-1}), NAA (0.1 mgl^{-1}) gives best response. Similarly, shoot proliferation @BAP (0.2 mg l^{-1}), KIN (0.1 mgl^{-1}) with adenine sulphate (30 mgl^{-1}) and silver nitrate (1mgl^{-1}) gives more than ten multiple shoot in *in vitro* condition. In addition to this, root length and number roots were increase within 21 days period of time with low concentration of IBA (0.3 mgl^{-1}). SEM study also added new taxonomic details, particularly regarding the plant micromorphological and stomata aspects. Hence, it is expected that this protocol can be used for commercial plant tissue culture. Furthermore, efforts are needed to overcome the problem of toxicity, browning and contamination with reduce the mortality of culture.

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References

1. Chaugule R, More T, Patil R, Kamble A. Callus culture for rapid regeneration of pomegranate Journal-Maharashtra Agricultural Universities. 2005; 30:85
2. Deepika R, Kanwar K. *In vitro* regeneration of Punica granatum L. plants from different juvenile explants J Fruit Ornamental Plant Res. 2010; 18:5-22.
3. El-Agamy SZ, Mostafa RA, Shaaban MM, El-Mahdy MT. *In vitro* salt and drought tolerance of Manfalouty and Nab El-Gamal pomegranate cultivars Aus J Basic Appl Sci. 2010; 4:1076-1082.
4. Fadavi A, Barzegar M, Azizi M, Bayat M. Note. Physicochemical composition of ten pomegranate cultivars (*Punica granatum* L.) grown in Iran Food Science and Technology International. 2005; 11:113-119.
5. Guranna P, Hoolageri HC. Studies on Establishment of Aseptic Culture in Pomegranate cv. Bhagwa Annual Research & Review in Biology, 2017, 1-7.
6. Jayesh K, Kumar R. Crossability in pomegranate (*Punica granatum* L.) Indian Journal of Horticulture. 2004; 61:209-210.
7. Kaji BV, Ershadi A, Tohidfar M. *In vitro* propagation of pomegranate (*Punica granatum* L.) Cv.'Males Yazdi' Albanian Journal of Agricultural Sciences. 2013; 12:43.

8. Korkmaz N, Aşkın M. Effects of calcium and boron foliar application on pomegranate (*Punica granatum* L.) fruit quality, yield, and seasonal changes of leaf mineral nutrition. In: III International Symposium on Pomegranate and Minor Mediterranean Fruits, 1089, 2013, 413-422.
9. Madhuri S, Shailja S. Plant regeneration from cotyledonary node of *Punica granatum* L Indian Journal of Plant Physiology. 2000; 5:344-348
10. Murkute A, Patil S, Singh S. *In vitro* regeneration in pomegranate cv. Ganesh from mature trees Indian Journal of Horticulture. 2004; 61:206-208
11. Murkute AA, Patil S. Exudation and browning in tissue culture of pomegranate Agricultural Science Digest. 2003; 23:29-31.
12. Naik SK, Chand PK. Silver nitrate and aminoethoxyvinylglycine promote *In vitro* adventitious shoot regeneration of pomegranate (*Punica granatum* L.) Journal of Plant Physiology. 2003; 160:423-430.
13. Naik SK, Pattnaik S, Chand PK. *In vitro* propagation of pomegranate (Punica granatum L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree Scientia Horticulturae. 1999; 79:175-183.
14. Naik SK, Pattnaik S, Chand PK. High frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (*Punica granatum* L.) Scientia Horticulturae. 2000; 85:261-270.
15. Oyebanji O *et al*. Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds African Journal of Biotechnology, 2009, 8.
16. Patil VM, Dhande G, Thigale DM, Rajput J. Micropropagation of pomegranate (*Punica granatum* L.) Bhagava cultivar from nodal explant African journal of Biotechnology. 2011; 10:18130-18136.
17. Prabhuling G, Huchesh H. *In vitro* Regeneration in Pomegranate (Punica granatum L.) cv. Bhagwa using Double Nodal Segments Research Journal of Biotechnology. 2018; 13:8.
18. Sepulveda E, Galletti L, Sáenz C, Tapia M. Minimal processing of pomegranate var. Wonderful CIHEAM-Opitions Mediterraneennes. 2000; 42:237-242.
19. Sharma J *et al*. Pomegranate bacterial blight: symptomatology and rapid inoculation technique for Xanthomonas axonopodis pv. punicae Journal of Plant Pathology. 2017; 99:109-119.
20. Simmonds NW, Smartt J. Evolution of crop plants. Longman London, 1976.
21. Singh NV, Singh S, Patel V. *In vitro* culture establishment studies on pomegranate Indian Journal of Horticulture. 2011; 68:307-311.
22. Singh P, Patel R. Factors influencing *In vitro* growth and shoot multiplication of pomegranate The Bioscan. 2014; 9:1031-1035.
23. Singh P, Patel R. Factors affecting *In vitro* degree of browning and culture establishment of pomegranate Afr J Plant Sci. 2016; 10:43-49.
24. Singh P, Patel R, Kadam S. *In vitro* mass multiplication of pomegranate from cotyledonary nodal explants cv. Ganesh African journal of Biotechnology, 2013, 12.
25. Soukhak F, Khalighi A, Ghaemmaghami S. Study of Direct Adventitious Shoot Regeneration in Pomegranate (Punicagranatum cv. MalasSaveh) through Cotyledonary Explants International Journal of Agricultural Science and Research. 2012; 2:19-26.
26. Usharani T, Meenakshi S, Sunisha C, Mohandas S. *In vitro* shoot regeneration from nodal explants of pomegranate (*Punica granatum* L.) CV Bhagwa Advances in Applied Research. 2014; 6:66-71.
27. Veeramohan R, Haron NW, Taha RM. Scanning Electron Microscopy Studies and *In vitro* Regeneration of Passiflora edulis Sims var. edulis for Conservation International Journal of Environmental Science and Development. 2013; 4:586.