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Reduction of polyphenolic exudation and enhancement of hardening efficiency in pomegranate tissue culture plants

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Punica granatum is one of the world's nutritious fruit, tolerating high drought. Bacterial blight is being an obstruct in the export of pomegranate from India. In the present study, a rigorous and efficient protocol for micropropagation of pomegranate plantlets is developed which can replace traditional plant breeding practices. Antioxidants controls polyphenolic exudation in micropropagation. For the first time, this report accomplishes to control polyphenols by modifying medium constituents. Woody plant (WP) medium containing benzyl-amino-purine (BAP) and kinetin (both at 1.5 mg l^{-1}) exhibited high bud break of about 99%. Further, it gave highest number of shoots of about 6.36 ± 0.22 with the length 6.12 ± 0.31 cm in multiplication stage. Shoots were successfully rooted in WP medium containing indole butyric acid (IBA) 1.5 mg l^{-1} with 5.62 ± 0.17 roots. This research emphasizes to preliminarily harden plant lets *in vitro* prior to hardening using innovative technique with increase in gelling strength and by creating nutrient competition. Nutrient stressed plants have a higher survival rate of 98% and water stressed plants have a survival rate of 96% when compared to conventional method. Therefore, mass propagation of pomegranate plants is possible to meet the demand of farmers in procuring disease-free plantlets.

Keywords: *Punica granatum*, polyphenolic exudation, woody plant medium, plant growth regulator

Introduction

Pomegranate botanically called *Punica granatum* (Bhagava) under the family Punicaceae is being cultivated in arid and semi-arid regions throughout the world. It contains a rich source of sodium, vitamin C, and phosphorus[1]. Pomegranate has many essential bioactive compounds like phenolics, flavonoids, ellagitannins, and proanthocyanidin compounds, also minerals mainly potassium, nitrogen, calcium, magnesium, and complex polysaccharides[2]. Few challenges in production of quality, disease free pomegranate are discussed further and possibilities to produce high yielding tissue culture plantlets are explored in this work. Capability to regenerate into individual plants from a single cell, tissue, and an organ under sterile condition is being the scope of tissue culture[3]. The phenolic compound is a complex malonic compounds exuding from the cut surfaces of explants which start to brown the medium by getting attached to the proteins through hydrogen bonding, finally forming quinones that are cyclic-polyphenol polymers in nature[4]. One of the major catastrophic threats for the pomegranate cultivation is blight disease caused by bacterium *Xanthomonas* species (Fig. 1) which effects huge economic losses in domestic and export produce by 80%. This bacterium

Fig. 1 — Bacterial blight necrosis symptoms observed in pomegranate (a) black necrosis (b) fruit baring oily rings (c) split fruit due to blight disease (d) blight infected leaf (e) black spots on pomegranate flower.

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was first found in the year of 1980 and started spreading widely from the year 2000 later it turned into plague from the last 10 to 12 years[5]. Plant tissue culture being a reliable weapon to produce disease free plantlets could majorly stop the spreading of blight disease from mother plant to air layered cuttings. High polyphenolic exudation in hardening is becoming critical parameters in mass production of pomegranate plantlets in tissue culture industries[6]. Preliminary hardening is the new technique carried out *in vitro*, we are adopting specially for pomegranate prior to primary hardening to increase the success rate in primary hardening. As primary hardening in green house employs the hardening chamber for the rooted plants from the lab, they get incubated for 20-30 days. This procedure enables the plants to adapt to natural climate[7]. This technique is slightly altered with respect to regular hardening parameters that enable humidity management based on moisture in the medium. Since pomegranate plants are sensitive to excess humidity, they will undergo vitrification at higher humidity in hardening chambers[8].

There are many attempts made to control this blight disease but complete control has not achieved so far in fields. So best option to control and avoid blight disease is to remove all infected mother plants and replant tissue culture disease free plants by farmers. In previous reports, attaining the speed to produce pomegranate plantlets was a challenge due to exudation[9].

There are reports stating the usage of potassium iodide in the medium to increase the antioxidant potential and to induce tolerance against stress. However, some researchers published that potassium iodide increased the oxidation of specific compounds when it comes in contact with secondary metabolites (polyphenol) during injury[10]. Therefore, present study has explored the possibilities to omit or alter the concentrations of potassium iodide and some other constituents which can eliminate browning during subcultures. Further, the desire of this work is to develop a reliable protocol for production of blight free pomegranate plantlets through plant tissue culture by controlling polyphenolic exudation that in turn increases bud break from explants and transforms mass propagation more viable.

Materials and Methods

Healthy mother plant is obtained from Indian Institute of Horticulture Research (IIHR), Bangalore, India and maintained in green house conditions located in 12°35'N latitude, 77°51'E longitude and 772 m altitude, Tamil Nadu. *In vitro* experiments is carried out in Tissue Culture Laboratory, M/s BCX Bio Organics, Bangalore.

Preparation of the Culture Medium

Woody plant medium[11] was prepared for each stage with different combinations of plant growth regulators (PGRs) and dispensed 50 ml per bottle into 250 ml glass bottles. The medium sterilized by autoclaving at 121°C and 15 psi pressure for 15 minutes. pH is adjusted to 5.6 ± 0.2 prior to autoclaving. All the mediums were labeled with respective trial names and incubated for minimum 72 hours before explant preparation and initiation.

Explant Selection, Sterilization and Initiation

Axillary buds were collected from 2–3 years old plant. Initially, the explants were washed with running tap water for 30 minutes after which they were again washed for 3 to 4 times with sterile demineralized water (DM). Then these explants were treated with detergent (Twenty 20) solution for 10 minutes. After rinsing, they were immediately soaked in streptomycin (0.2%) with carbendazim (0.2%) solution for 20 minutes[12]. Further work is carried out inside the laminar air flow chamber. Explants were surface sterilized with 2 different surface sterilants to check the efficacy in explant sterilization. Mercuric chloride ($HgCl_2$) (0.1%) with a treatment time ranging between 1-5 minutes and 4% sodium hypochlorite ($NaOCl_2$) with treatment time ranging between 15-30 minutes. After treatment, they are washed thoroughly with DM water[13]. Finally, the explants were inoculated in the WP medium fortified with various concentrations of BAP (0.5, 1, 1.5, 2 mg l^{-1}) and kinetin (0.5, 1, 1.5, 2 mg l^{-1}) in combinations with labels (IM1 to IM9). The medium was supplemented with 3% sucrose and 0.8% agar. After inoculating the explant into medium, the cultures were incubated at 25°C under 12 hrs photoperiod with white fluorescent lamps. After 2-3 weeks of incubation, the contamination free sprouted buds were transferred to the multiplication medium[14]. Observations on the percentage of contamination, bud sprouting etc. were recorded.

Multiplication

After 28 days, the cultures are sub-cultured aseptically in multiplication medium comprising concentrations (0.5, 1, 1.5, 2 mg l^{-1}) with labels (MM1 - MM4) of two different PGR's BAP and

kinetin having WP medium as basal medium with 3% sucrose and 0.8% agar for inducing shoot proliferation and multiplication[15]. Observations on the number of shoots, number of leaves and length of the shoots were recorded.

Control of Polyphenolic Exudation

Wound during culture trimming in pomegranate secretes polyphenolic exudation as a result of secondary metabolism. These polyphenols cause browning of medium and in turns toxic to the *in vitro* cultures finally hindering the growth. To address this, four ingredients being the part of standard mediums were used in different concentrations and in some trials, it is completely eliminated to check the influence in arresting the exudation. Potassium iodide (PI), ascorbic acid (AA), activated charcoal (AC) and cupric sulphate (CS) were taken for studying the influence of these ingredients in polyphenol exudation. Browning due to oxidation which results in losses up to 50% of plant growth[16]. In present study, the trial was set with decreased concentration of PI than the standard usage which is 0.83. Trial is set between 0.8, 0.5, 0.3 to 0 mg l^{-1} of multiplication medium (Fig 2).

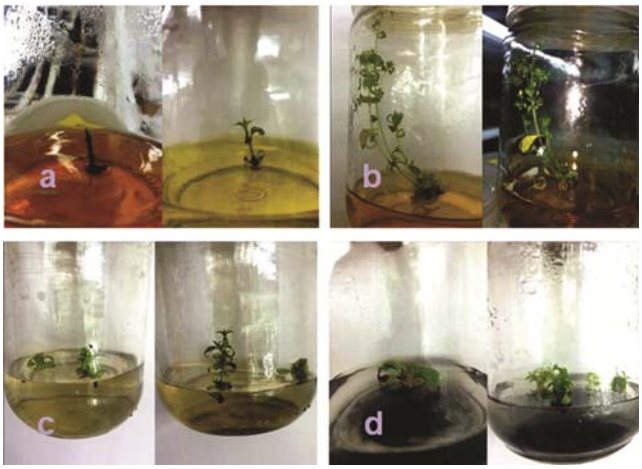

Fig. 2 — Concentrations of the ingredients on the medium (a) Left side - Release of polyphenols due to the polyphenol exudation, Right side - control of polyphenols by removing potassium iodide (b) Left side - 25 mg l^{-1} concentration of ascorbic acid plant growth was seen as good but medium was not solidified, Right side - by increasing the concentration of ascorbic acid plant grows well (c) Left side - Increased concentration of activated charcoal reduced the polyphenol but also absorbs the required nutrients for plants Right side - No polyphenol exudation was seen at the concentration of 200 mg l^{-1} of activated charcoal, (d) Left side - increased concentration of cupric sulphate toxicity effect on medium Right, side - 1 mg l^{-1} of cupric sulphate controlling the polyphenol exudation.

Ascorbic acid trial was set between 25-100 mg l^{-1} however increase of ascorbic acid concentration in the medium, gradually affected the gelling of medium. Addition of ascorbic acid at a rate of 5-30 mg l^{-1} concentration explant initiation medium has reduced the polyphenolic exudation. Activated charcoal being a strong phenol adsorbent that reduces phenolic browning when it is used in the concentration of 200 mg l^{-1} was found to be best in controlling polyphenolic exudation[17]. However, addition of 300 mg l^{-1} activated charcoal into medium adversely affected culture establishment and shoot growth[18]. In this work, concentration of activated charcoal was set between 100-200 mg l^{-1}. Cupric sulphate, being a strong metal ion, it is expected to have a direct effect on polyphenols and metal chelation in plants[19]. The concentration of CS ranging from 0.25-1 mg l^{-1} was set to find the influence of cupric sulphate on arresting polyphenols release in the medium.

Rooting

At least 3-4 cm long healthy culture shoots are selected from multiplied cultures and subjected to rooting induction *in vitro*. Rooting medium (RM) was supplemented with basal medium containing ½ strength of Murashige and Skoog (MS) medium with concentrations of NAA, IBA and IAA (0.5, 1.0 and 1.5 mg l^{-1}) with labels RM1-RM9 along with 0.2% activated charcoal, 3% sucrose and 0.8% agar. After 8-10 days, the rooted explants were transferred to an auxin-free half-strength medium for further elongation of the roots. Observations of percentage of rooting, number of roots and length of roots were recorded.

Preliminary Hardening to Field Establishment

Hardening refers to the process of acclimatizing plants from indoor nature to the outdoors. Moisture control is performed by varying gelling strength of the medium. Two methods for *in vitro* preliminary hardening is performed, one by increasing agar strength and another, by creating nutritional competition among plants through stress. In this stage, plantlets were transferred into half strength WP medium with different concentrations of agar (5.7 g l^{-1}, 5.9 g l^{-1}, 6.1 g l^{-1} and 6.4 g l^{-1} of medium with labels HM1-HM4). After 4 weeks of incubation, plantlets under stress are ready for hardening. During this preliminary hardening the new regenerates gets hardened for its subsequent soil transfer. Plantlets

were incubated under normal room temperature with 60 days of photoperiod. Well rooted plantlets were removed from the culture medium, washed gently with tap water to remove the adhering gel and transplanted to polyethylene bags with potting mix sand: red soil: coir pith (1:1:1)[20]. Further, the plantlets were hardened in shade house for a period of 6 weeks and then transferred to the field. Plants health under field condition and field establishment is also monitored in farmers site.

Statistical Analysis

The data obtained from the experiments conducted for initiation, multiplication, control of polyphenolic exudation and rooting were statistically analysed using Xl Stat - Microsoft Office 2019, for determining mean and standard errors as all trials were performed in triplicates.

Results & Discussion

Explant Sterilization and Bud Sprouting

In this study, among the two different treatments, significantly less percent of contamination of about 12% with 58% bud sprouting was observed with 0.1% $HgCl_2$ for 4 minutes. The detailed results are presented in Table 1. WP medium supplemented with BAP and kinetin (1.5 mg l^{-1} and 1.5 mg l^{-1}) resulted in the highest number of shoots, good number of leaves and length. The highest bud break (99%) was obtained in the medium IM9 whereas, the lowest bud break rate (90%) obtained in the medium IM1. Among all trials, it was found that IM9 media which was a combination of BAP and kinetin (1.5 mg l^{-1} and 1.5 mg l^{-1}) was most effective in achieving efficient bud break rate. The detailed results are given in Table 2 and Figure 3a. Pathak *et al* reported the use of sodium hypochlorite and mercuric chloride for surface sterilization with success rates 75.75% and 95% in axenic cultures respectively. In this study, the sterilants treated with $HgCl_2$ (0.1%) for 4 minutes and $NaOCl_2$ (4%) for 30 minutes was found to be best and resulted in low level of contamination. Poudyal *et al* that WP medium supplemented with different combinations of BAP and kinetin for surface sterilization. In the present investigation, the WP medium fortified with different combinations of BAP and kinetin and resulted as about 99% of bud sprouting rate.

Table 1 — Effect of different surface sterilization treatments on pomegranate explants

S.No	Sterilization agents	Concentrations (%)	Time (minutes)	Total contamination (%)	Bud sprouting (%)	Non- responsive (%)
1	$HgCl_2$	0.1%	1	62	88	12
			2	30	74	23
			3	18	65	36
			4	12	58	42
			5	2	42	67
2	$NaOCl_2$	4%	15	58	89	7
			20	46	66	15
			25	38	44	23
			30	24	33	48

Table 2 — Effect of medium along with different combination of BAP and kinetin on initiation

Medium Trials	Plant growth regulators (mg l^{-1})		Growth parameters			
	BAP	Kinetin	Sprouting (%)	No. of shoots/ culture (± SE)*	No. of leaves/ culture (± SE)*	Average length of shoots (cm) (± SE)*
IM1	0.5	0.5	90	0.19 ± 0.09	1.58 ± 0.64	0.46 ± 0.71
IM2	0.5	1.0	94	0.33 ± 0.07	1.04 ± 0.59	0.58 ± 0.27
IM3	0.5	1.5	98	0.53 ± 0.26	0.11 ± 0.06	1.05 ± 0.13
IM4	0.1	0.5	92	0.25 ± 0.11	0.85 ± 0.40	0.98 ± 0.19
IM5	0.1	1.0	95	0.45 ± 0.22	0.36 ± 0.18	1.13 ± 0.74
IM6	0.1	1.5	97	0.59 ± 0.28	1.16 ± 0.79	1.11 ± 0.17
IM7	1.5	0.5	95	0.79 ± 0.45	0.59 ± 0.16	0.32 ± 0.51
IM8	1.5	1.0	97	0.88 ± 0.59	0.46 ± 0.12	1.18 ± 0.80
IM9	1.5	1.5	99	0.17 ± 0.02	0.43 ± 0.20	1.22 ± 0.90

*Data are in the form of mean ± SE of three replicates. ± SE = standard error

Multiplication

WP medium was fortified with BAP and kinetin (both at 1.5 mg l^{-1}) resulted in the highest number of shoots (6.36 ± 0.22) and also number of leaves, length of the shoots was recorded as 24.39 ± 0.33 cm and 6.12 ± 0.31 cm respectively (Table 3 and Fig. 3b). Different medium trials were carried out for multiplication and the results are presented in Table 3. Yancheva and Kondakova (2016) reported that WP medium containing 1 mg l^{-1} BAP and 0.5^{-1} mg l^{-1} kinetin where they recorded number of shoots being 11.21 ± 0.14 cm and length of shoots about 6.54 ± 0.85 cm, respectively. In this work, in the shoot of multiplication, M3 medium trial exhibits the highest number of shoots which is about 6.36 ± 0.20 cm and length of shoots was recorded as 24.39 ± 0.33 cm.

Control of Polyphenol Exudation

The result of present study observed that polyphenolic exudation can be controlled by supplementing medium with various antioxidants such as potassium iodide, ascorbic acid, activated charcoal and cupric sulphate. Activated charcoal (200 mg l^{-1}) has reduced browning but it decreased the intensity of plant growth. Similar case was observed in cupric sulphate (1 mg l^{-1}) where although no browning was observed, intensity of plant growth was reduced to 20%. In this experiment, almost no exudation was found when WP medium was fortified with potassium iodide 0.3 mg l^{-1}, ascorbic acid 100 mg l^{-1} activated charcoal 200 mg l^{-1} and cupric sulphate 1 mg l^{-1} respectively (Table 4 and Fig. 2). It is reported that the browning of explants due to the

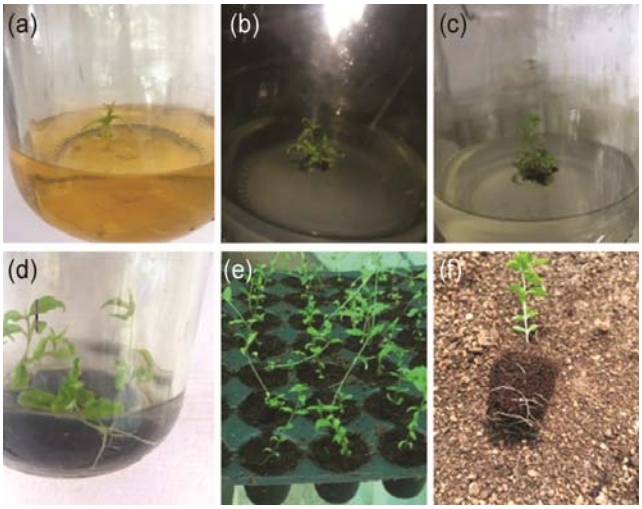

Fig. 3 — Stages of pomegranate in tissue culture (a) Initiation (b) Multiplication (c) Rooting (d) Hardening (e) Preliminary hardening (f) Secondary hardening.

Table 4 — Combination of potassium iodide and ascorbic acid in the control of polyphenols and in controlling browning effect

S.No	Ingredients	Concentrations (± SE)*	Extent of polyphenol exudation (± SE)*	Plant growth (%) (± SE)*
1	Potassium iodide (mg l^{-1})	0.8 ± 0.12	****	30 ± 0.27
		0.5 ± 0.84	***	50 ± 0.92
		0.3 ± 0.67	**	70 ± 0.31
		0	*	90 ± 0.56
2	Ascorbic acid (mg l^{-1})	25 ± 0.80	****	20 ± 0.58
		50 ± 0.13	***	40 ± 0.83
		75 ± 0.45	**	60 ± 0.17
		100 ± 0.23	*	90 ± 0.23
3	Activated charcoal (mg l^{-1})	100 ± 0.28	****	80 ± 0.29
		125 ± 0.18	***	50 ± 0.19
		150 ± 0.75	**	30 ± 0.80
		200 ± 0.62	*	10 ± 0.29
4	Cupric sulphate (mg l^{-1})	0.25 ± 0.34	***	70 ± 0.58
		0.50 ± 0.89	**	50 ± 0.63
		0.75 ± 0.14	*	40 ± 0.49
		1.00 ± 0.79	*	20 ± 0.11

*Rating of Polyphenols

* Explant remained green, no exudate observed in the medium

** Explant turned slightly brown, light brown exudate observed in the medium

***Explant turned into intermediate browning which observed in the medium

**** Explant turned completely brown, exudate observed in the medium

*Data are in the form of mean ± SE of three replicates. ± SE= standard error

Table 3 — Effect of different concentrations of BAP and kinetin on multiplication

Medium trials	BAP (mg l^{-1})	Kinetin (mg l^{1})	No. of shoots/ explant (± SE)*	No. of leaves/ explant (± SE)*	Average length of shoots (cm) (± SE)*
MM1	0.5	0.5	2.15 ± 0.17	11.08 ± 1.36	4.27 ± 1.19
MM2	1.0	1.0	4.04 ± 0.48	12.19 ± 0.38	3.80 ± 1.16
MM3	1.5	1.5	6.36 ± 0.42	24.39 ± 0.73	6.12 ± 0.93
MM4	2.0	2.0	4.83 ± 1.14	21.05 ± 1.19	5.09 ± 0.76

*Data are in the form of mean ± SE of three replicates. ± SE = standard error

Table 5 — Effect of different concentration and combination of NAA, IBA and IAA on root induction*

Medium trials	Plant growth regulators NAA	IBA	IAA	Rooting (%) (± SE)*	No. of roots (± SE)*	Length of roots (cm) (± SE)*
RM1	0.5	-	-	95 ± 0.48	4.55 ± 0.30	-
RM2	-	0.5	-	99 ± 0.21	4.79 ± 0.10	4.64 ± 0.30
RM3	-	-	0.5	0	0	0
RM4	1	-	-	96 ± 0.50	2.76 ± 0.12	-
RM5	-	1	-	100 ± 0.89	3.82 ± 0.23	2.84 ± 0.30
RM6	-	-	1	0	0	0
RM7	1.5	-	-	95 ± 0.91	4.59 ± 0.25	-
RM8	-	1.5	-	100 ± 0.34	5.62 ± 0.17	6.52 ± 0.14
RM9	-	-	1.5	0	0	0

*Data are in the form of mean ± SE of three replicates. ± SE= standard error

exudation of polyphenols has a critical problem in establishing cultures of plant species. In the present investigation, the polyphenolic content was completely controlled by removing potassium iodide and also less degree of browning was observed. According to Kever *et al*[21] control of defoliation was done by cobalt chloride. Present study investigated the controlling of potassium iodide resulting in the reduction of the polyphenolic exudation.

Rooting

WP medium fortified with different concentrations of auxins (NAA, IBA and IAA) was used for rooting experiment. Nine different media (RM1-RM9) trials were carried out for the root induction. Results presented in Table 5 clearly showed the percentage of rooting, number of roots and length of roots. Thus, the data signifies that WP medium (RM8) containing higher concentration of IBA (1.5 mg l^{-1}) was found to be best for root induction. For the root proliferation, various combinations of WP medium were used. The WP medium trials showed the highest number of roots about 5.62 ± 0.17. Lebedev *et al*[22] evidenced the increase in the concentrations of the agar, reduces the rooting efficacy.

Preliminary Hardening and Field Establishment

WP medium was gelled with agar (5.7 gm l^{-1}, 5.9 gm l^{-1}, 6.1 gm l^{-1} and 6.4 gm l^{-1} of medium). Four different media trials were carried out for preliminary hardening. Of these, HM3 medium was found to be more effective in rooting efficacy and mortality rate. Results presented in Table 6 clearly proved that higher concentration of agar showed 93% rooting efficacy and 4% mortality rate. In this

Table 6 — Efficacy of preliminary hardening

Medium Trials	Agar concentrations (gms)	Rooting efficacy (%) (± SE)*	Mortality rate (%) (± SE)*
HM1	5.7	87 ± 0.51	90 ± 0.71
HM2	5.9	89 ± 0.27	46 ± 0.43
HM3	6.1	93 ± 0.48	4 ± 0.81
HM4	6.4	-	Not applicable

*Data are in the form of mean ± SE of three replicates. ± SE= standard error

Table 7 — Effect of nutrient stress, water stress and both nutrient & water stress in survival frequency in field conditions

S.No	½ MS medium (ml)	Control (%)	Survival frequency with nutrient stress (%) (±SE)*	Survival frequency with water stress (%)(±SE)*	Survival frequency in combination of nutrient and water stress (%)(±SE)*
1	50	50 ± 0.23	70 ± 0.29	53 ± 0.23	96 ± 0.23
2	25	75 ± 0.32	74 ± 0.13	56 ± 0.23	98 ± 0.23

*±SE= standard error

experiment, effect and response of plant on nutrient, water stress individually and also both in combination were recorded. The results given in Table 7 shows that high survival rate percentage (98%) was observed in medium with combination of nutrient and water stress. Field establishment and survival frequency was remarkably significant in our *in vitro* hardening method when compared to conventional method in farmers site (Fig. 4). Similarly, studies[23] has reported that *in vitro* hardening has remarkably improved the hardening efficacy of *Phoenix dactylifera* (L.).

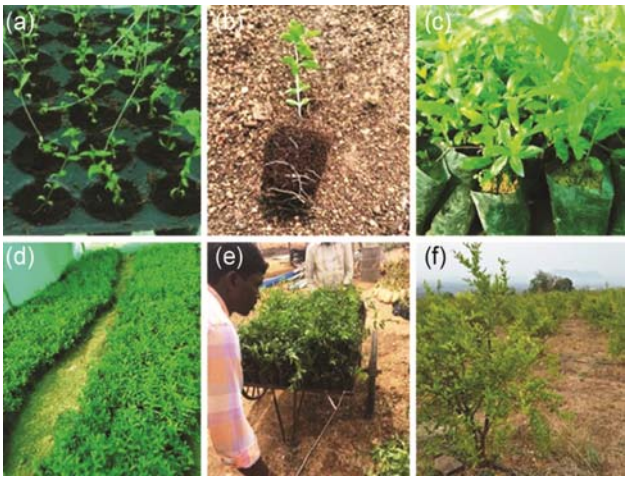

Fig. 4 — Preliminary hardening to field establishment (a) Preliminary hardening stage (b) Hardened pomegranate, plantlet, (c) Secondary hardening, (d) Plantlets kept under polyhouse, (e) After 45 days plantlets transferred to the farmers, (f) Field establishment of 5-month pomegranate plants.

Conclusion

The mass production of pomegranate plantlets was difficult by *in vitro* propagation method. Therefore, the objective of this study is to produce disease free pomegranate plantlets through removing the polyphenolic exudation and minimizing the mortality rate during hardening. Compared to the conventional technique, our new technique registered almost 96% survival in hardening and 98% survival in farmer's field whereas conventional method has exhibited high mortality rate in hardening as well as field establishment. So, this method of *in vitro* hardening will an efficient alternative for acclimatizing pomegranate tissue culture plants that will significantly reduce production cost in the culture conditions. Further, this protocol can be applied to many other plants in tissue culture that are economically important with slight modifications based on family and habitat to achieve best hardening efficiencies and better field establishment.

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