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Standardization of *in vitro* propagation protocol for pomegranate cv. Super Bhagwa

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Abstract
The present paper demonstrates the effect of different media and combinations of growth regulators to standardize different stages of *in vitro* propagation protocol for mass production of pomegranate cv. Super Bhagwa. In this investigation, shoot tips were used as explant and explant necrosis, culture contamination and culture establishment were found to be 7.61%, 16.39% and 76.66%, respectively. The combination of α-Naphthaleneacetic acid (NAA) and 6-Benzylaminopurine (BAP) was more responsive for culture establishment. T6 medium (Modified MS + Adenine Sulphate (60mg/l) + L-Arginine (60mg/l) + 1mg/l Kinetin + 0.1mg/l TDZ + 0.2 mg/l NAA + Undisclosed media supplements) registered maximum proliferation rate (3.800), however, MS medium supplemented BAP + very low concentration of TDZ/ZR and adenine sulphate (60 mg/l) along with 0.1/0.2 mg per litre NAA produced tallest shoot and high average shoot length.
In vitro rooting of proliferated shoots on Woody Plant medium (Lloyd and McCown, 1980) (WPM medium), irrespective of growth regulator concentration and type, took lesser number of days for root initiation as compared to half strength MS medium (Murashige and Skoog, 1962). Major roots per shoot (5.00) and the rooting percent (76%) were found highest in WPM medium supplemented with 1.0 mg/l NAA as compared to other treatments. Use of half strength concentration of MS medium and full strength WPM was an attempt to reduce the salt concentration to enhance rooting *in vitro*.
Arbuscular Mycorrhizal Fungi (AMF; predominantly *Glomus intraradices*) inoculated plants depicted higher relative leaf water (85.27%) and more leaf chlorophyll content (2.17) as compared to non-inoculated micropropagated pomegranate plants of cv. Super Bhagwa.

Keywords: arbuscular mycorrhizal fungi, *In vitro* propagation, pomegranate, *Punica granatum*

Introduction
The pomegranate (*Punica granatum* L.), belongs to family Lythraceae, has grown leaps and bounds during the last decade. The pomegranate is one of the oldest known edible fruits. It is an economically important crop of the semi-arid tropics of the world due to its highly nutritious edible fruits, high returns, great export demand, versatile adaptability, low irrigation water requirement and pharmaceutical and ornamental usage (Bhandari, 2012; Singh *et al.*, 2012; Singh *et al.*, 2016) [1, 2, 3]. The fruit is a good source of sugars and Vitamin C, whereas seeds with arils are sun-dried and commercially marketed as condiment or spice, food flavoring agents, besides, having huge market potential as pomegranate seed oil owing to its immense medicinal properties (Parmar and Kaushal 1982; Pal and Singh, 2017.) [4, 5]. The plant is well adapted to drought with high distribution extending from Iran to Himalaya in Northern India and has been cultivated throughout the Mediterranean region (Soloklui *et al.*, 2012; Singh *et al.*, 2015) [6, 7]. Pomegranate is conventionally propagated through air layering, hard wood and soft wood cuttings (Chandra *et al.*, 2014; Pal and Singh, 2017) [8, 5]. But these traditional propagation methods do not ensure disease free and healthy plants.
Mass multiplication through tissue culture is necessary to meet the rapidly increasing demand for elite pomegranate planting material. Both direct and indirect organogenesis including somatic embryogenesis have been successfully attempted in pomegranate for plant propagation and other purposes. Micropropagation in pomegranate can be initiated through regeneration of existing meristems or regeneration from adventitious meristems or through regeneration by somatic embryogenesis (Naik *et al*., 1999, 2000; Singh *et al*., 2007; Kajla *et al*., 2013; Guruanna *et al*., 2018) [9, 10, 11, 12, 13]. Healthy and disease free planting material is a key to the productive pomegranate orchard. Since 2005, pomegranate cultivation in India is severely affected by a bacterial disease causing huge economic losses (Sharma *et al*., 2017) [14]. Urgent supply of disease free elite planting material is possible through tissue culture to non-

traditional belts of pomegranate cultivation that can avoid the spread of this deadly disease through infected planting material. Besides these, *in vitro* raised plants are disease free, precocious and highly amicable to mechanized farming. The present work attempted to overcome limitations in protocols that were followed by previous researchers. Plants propagated through tissue culture generally suffers from high field mortality due to improper hardening, before acclimatization, theses plantlets have poorly formed and weak root system, unfavorable nutritional and environmental conditions and poorly developed cuticle and/or nonfunctional stomata. The success rate of tissue culture raised plantlets can effectively be improved by proper hardening using bioagents during secondary hardening stage like Arbuscular Mycorrhizal Fungi. Many fruit crops have a symbiotic Mycorrhizal relationship and exhibit a high degree of dependence on this symbiosis for normal development and improved field performance (Aseri *et al.*, 2008) [15]. Arbuscular Mycorrhizal Fungi (AMF) can mitigate the effects of extreme variation in temperature and water stress by improving water and nutrients uptake through increased rhizosphere exploring area (Singh *et al*., 2012; Singh *et al*., 2016) [2, 3].

Materials and Methods

The study was carried out at Tissue Culture and Biotechnology Laboratory as well as experimental polyhouse of the ICAR-National Research Centre on Pomegranate, Solapur, India, located at 17°68' N latitude, 75°91'E longitude and 483.5 m altitude from mean sea level, during the period of 2018-2019.

Sterilization of glassware

All the glassware *viz.*, beakers, flasks, measuring cylinders, test tubes, petridishes, jam bottles etc. were washed with laboratory detergent followed by running tap water to remove the detergent residue and then rinsed with double distilled-water then sterilized in an autoclave at 121°C for 40 minutes (15 lbs./inch^2) and oven dried (hot air oven) for 4 hours at about 150 ºC.

Explant selection

For *in vitro* propagation, apparently healthy plants were selected for excising the explants. Use of juvenile explant as a source of explants has been reported in many woody plants. One type of explant of pomegranate cv. Super Bhagwa i.e. shoot tips (3-4 cm long) were collected from the field-grown mature pomegranate plants.

Pre-treatment for explant sterilization

The explants were washed in running tap water. Pre-treatment of explant with the solution of Carbendazim (50%) [0.1%] + Ridomil Gold® (0.1%) [Mancozeb (64%) + Metalyxyl–M (8%)] + Bactronol-100® [2- bromo 2-nitropropane-1, 3-diol] (0.05%) for 30 minutes, followed by two washings with autoclaved double distilled water under Laminar Air Flow and surface sterilization with $HgCl_2$ (0.1% for 4 min) prepared in autoclaved double distilled water, followed by four washings with autoclaved double distilled water.

In vitro culture establishment

The experiments were laid out in complete randomized design with three replications having 10 tubes in each replication. Completely sterilized explants were inoculated on MS medium (Murashige and Skoog, 1962) and Quoirin and Lepoivre (1977) Q and L medium [16, 17] with various mineral salt formulations and different combinations of BAP and NAA. Observations on average culture necrosis, average culture contamination and culture establishment percent were recorded.

In vitro multiplication

The cultured explants were aseptically removed from establishment medium and sub cultured in multiplication medium after 25 days after inoculation in the establishment medium. Media compositions with different combinations of mineral salts were supplemented with 60 mg/l Adenine Sulphate and, 60mg/l L-Arginine and growth regulators such as BAP, NAA, Kinetin, Zeatin Riboside and Thidiazuron (TDZ) for shoots proliferation. Observations were recorded on parameters such as average number of side shoots per sprout, average shoot growth rate (cm) and tallest shoot length.

In vitro rooting

Proliferated shoots were transferred in half strength (Macro) MS medium and WPM (Woody plant medium) medium (Lloyd and McCown, 1980) [18], supplemented with auxins like Indole 3-butyric acid (IBA) and NAA which stimulates the formation of roots along with activated charcoal. Observations like average number of days to root initiation, average number of roots per micro-shoot and average rooting percentage were recorded.

Biohardening

In vitro raised plantlets of pomegranate cv. Super Bhagwa (60 days old rooted primary hardened plantlets) were taken for bio-hardening. Plantlets were maintained in a polyhouse with temperature range of 27± 2 ºC. Humidity was maintained at 85% using fogger (Netafim^{TM}). Plantlets were watered on alternate days. The commercial products of AMF available as Josh® (Cadila Pharmaceuticals Ltd. Ahmedabad, Gujarat) having *Rhizophagus intraradices* (Syn. *Glomus intraradices*) was used containing 60000 infectious propagules per kg of product. Thirty (30) days old rooted plantlets from sterile hardening medium (cocopeat: perlite: vermiculite, 1:1:1) were transferred to presterilized potting mixture (sand: soil: FYM, 1:1:1). At the time of transfer, 5 g of Josh® containing 300 infectious propagules per kg of potting mixture was placed near the root zone of the *in vitro* raised plants. Relative leaf water content (%) and total leaf chlorophyll (mg/100 g of FW) were recorded at 60 days after treatment. Control was maintained without microbial treatment.

Relative leaf water content (RLW)

The RLW in the recently measured leaf was determined using method suggested by Weather ley (1950) [19]. Leaves were collected and 8mm diameter discs were made. Fresh weight of this discs were measured and then floated over distilled water in petridish for 4-6 Hours. These discs were then surface dried by placing them in between 2 sheets of Whatman's filter paper and saturated weight of this discs were recorded. After that, the sample were dried in an oven at 70ºC for 24 h. or till they showed no change in their weight after two consecutive drying. The dry weight of the sample was then recorded
The RLW was then estimated by using following formula

$$\text{RLW (\%)} = \frac{\text{Fresh weight – oven dry weight}}{\text{Turgid weight-oven dry weight}} \text{ x100}$$

Chlorophylls

The chlorophyll content (chlorophyll a, b and total chlorophyll) of the leaves was analyzed at 60 days after inoculation following the method as suggested by Barnes *et al.* (1992) [20]. Fully mature open leaves were chosen as the experiment sample for chlorophyll estimation. Accurately weighed 0.5 g of clean leaf sample was immersed in 10 ml of Dimethyl Sulphoxide (DMSO) AR. The samples are incubated at 70 ºC for four hours in hot air oven. It was then out and 1 ml of the solution was diluted to 5 ml with DMSO and the sample was read on a spectrophotometer at 645 and 663 nm using pure DMSO as blank. Total chlorophyll was calculated according to the following formula.

$$\text{Total Chlorophyll (mg/g)} = \frac{(20.20 \times OD645) + (8.02 \times OD663) \times \text{Volume} \times \text{Dilution}}{1000 \times \text{Weight of sample}}$$

Statistical analysis

The data obtained from the experiment were subjected to analysis of variance (ANOVA) which was done by using freely available online software WASP 2.0 (Web-based Agri Stat Package) developed by ICAR-CCARI, Goa.

Results and Discussion

Effect of basal media on *in vitro* culture establishment

In the present investigation, different media and growth regulators combinations were used to standardize different stages on *in vitro* propagation protocol for mass production of pomegranate cv. Super Bhagwa. Though successful, micropropagation of pomegranate suffers many problems during culture establishment that include rate of contamination, secretion of phenolic compounds by explant or propagules. Overcoming these problems during culture establishment, effects of various growth regulators and media compositions for micropropagation of pomegranate have been observed and discussed. Figure 2 showed percentage of culture establishment for various treatments on shoot tip explants. Culture establishment by shoot tips inoculated in MS medium having half strength macro nutrients, 1mg/l BAP and 0.1mg/l NAA alongwith 30 mg/l each of Adenine Sulphate and Arginine was found significantly better as far as explant necrosis (7.61%), culture contamination (16.39%), and culture establishment (76.66%), are concerned (Figure 1).

Fig 1: Effect of basal media on *in vitro* established cultures on Modified MS media (half macro nutrients) + 30 mg/l Adenine Sulphate + 30 mg/l L-Arginine + 1mg/l BAP + 0.1mg/l NAA culture establishment

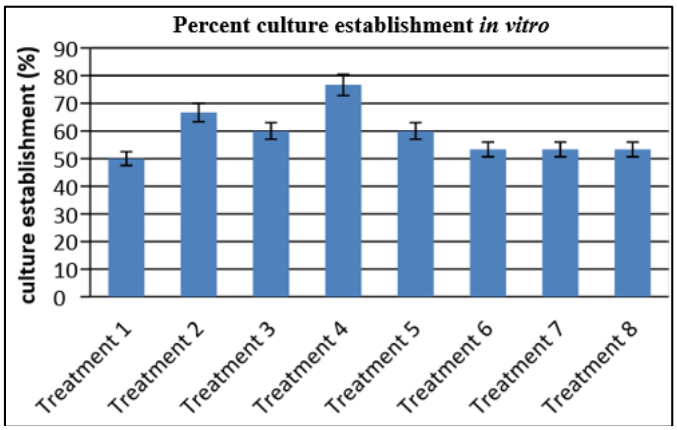

Fig 2: Effect of basal media on *in vitro* culture establishment

Treatment 1: Modified MS media (400mg/l NH_4NO_3) + 30mg/l Adenine Sulphate + 30mg/l L-Arginine + 1mg/l BAP + 0.25mg/l NAA.

Treatment 2: Modified MS media (400mg/l NH_4NO_3) + 30mg/l Adenine Sulphate + 30mg/l L-Arginine + 1mg/l BAP + 0.1mg/l NAA.

Treatment 3: Modified MS media (half macro nutrients) + 30mg/l Adenine Sulphate 30mg/l L-Arginine + 1mg/l BAP + 0.25mg/l NAA.

Treatment 4: Modified MS media (half macro nutrients) + 30mg/l Adenine Sulphate + 30mg/l L-Arginine + 1mg/l BAP + 0.1mg/l NAA.

Treatment 5: Modified MS media (3/4th macro nutrients) + 30mg/l Adenine Sulphate + 30mg/l L-Arginine + 1mg/l BAP + 0.25mg/l NAA.

Treatment 6: Modified MS media (3/4th macro nutrients) + 30mg/l Adenine Sulphate + 30mg/l L-Arginine 1mg/l BAP + 0.1mg/l NAA.

Treatment 7: Q and L media + 30mg/l Adenine Sulphate + 30mg/l L-Arginine + 1mg/l BAP + 0.25mg/l NAA.

Treatment 8: Q and L media + 30mg/l Adenine Sulphate + 30mg/l L-Arginine + 1mg/l BAP + 0.1mg/l NAA.

Effect of basal medium and growth regulators on *in vitro* shoot proliferation

Table 1 confirmed the higher proliferation rate (3.8 shoots per micro-shoots) on Modified MS medium supplemented with Adenine Sulphate (60mg/l), L-Arginine (60mg/l), 1.0 mg/l Kinetin, 0.1mg/l TDZ and 0.2mg/l NAA [T(M)6], however, this treatment was at par with the performance with T(M)5, T(M)7, T(M)8, and T(M)9. This combination was significantly superior over remaining treatments. However, micro shoots on MS medium supplemented with 60mg/l Adenine Sulphate, 60mg/l L-Arginine, 1mg/l BAP, and 0.1mg/l NAA registered tallest shoot length (8.18cm) 30 days after inoculation. Modified MS medium with 60mg/l Adenine Sulphate, 60mg/l L-Arginine, 1mg/l BAP, 0.1 mg/l TDZ and 0.2mg/l NAA (8.18 cm) and Modified MS + Adenine Sulphate (60mg/l) + L-Arginine (60mg/l) + 1mg/l BAP + 0.1mg/l Zeatin Riboside + 0.1mg/l NAA (7.66cm) registered at par results for the tallest shoot length and these two treatments were significantly superior to other treatments. T(M)7 (Modified MS + Adenine Sulphate (60mg/l) + L-Arginine (60mg/l) + 1mg/l BAP + 0.1mg/l TDZ + 0.1mg/l NAA + Undisclosed media supplements) resulted into highest average shoot length (3.78 cm). The combination of BAP and NAA was responsive in many fruit tree species (Zimmerman and Swartz, 1994) [21]. For the shoot regeneration in pomegranate, cytokinin is effective when used in combination

with an auxin (Naik *et al*., 1999; Singh *et al*., 2010, Naik and Chand, 2011, Patil *et al*., 2011; da Silva *et al*., 2013) [9, 22, 23, 24, 25]. Murkute *et al*. (2004) [26] obtained the highest shoot proliferation in *in vitro* regeneration of pomegranate cv. 'Ganesh' on MS basal medium supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA. Singh and Khawale (2003) [27] found half-strength MS medium supplemented with 1.0 mg/l BA+ 1.0 mg/l Kin along with 200 mg/l activated charcoal (AC) as the best medium for *in vitro* establishment of nodal segments of pomegranate cv. 'Jyoti'. Singh *et al*. (2007) [10] carried out *in vitro* clonal propagation of pomegranate cv. 'G137' using nodal segments and shoot tips of mature trees and reported MS basal medium supplemented with 1.0 mg/l BAP, 1.0 mg/l kinetin and 0.1 mg/l NAA produced the highest number of shoots per explant along with the longest shoot. Patil *et al*., (2011) [24] found that the nodal explants grown on MS medium containing 1.8 mg/l BAP, 0.9 mg/l NAA, 1 mg/l silver nitrate and 30 mg/l Adenine Sulphate had the highest proliferation rate (10 to 15 shoots/explants). These findings are in close conformity with the results found in present investigation.

Fig 3: *In vitro* shoot proliferation on T8 medium

Table 1: Effect of basal medium and growth regulators on *in vitro* shoot proliferation

Treatment	Proliferation rate per micro shoot *	Tallest shoot length (cm) at 30 DAI	Avg. shoot length (cm) at 30 DAI
T(M)1	2.000	4.260	2.460
T(M)2	2.400	4.820	3.080
T(M)3	3.400	5.960	3.560
T(M)4	2.400	5.260	3.180
T(M)5	3.600	4.900	3.580
T(M)6	3.800	5.400	3.560
T(M)7	3.600	5.580	3.780
T(M)8	3.600	8.180	3.640
T(M)9	3.400	7.660	3.680
CD (p=0.05)	0.715	0.879	0.409

*Side shoot and vertical splitting

T(M)1: Modified MS + 1mg/l BAP + 0.1 mg /l NAA Undisclosed media supplements.
T(M)2: Modified MS + 1mg/l BAP + 0.2 mg/l NAA + Undisclosed media supplements.
T(M)3: Modified MS + Adenine Sulphate (60mg/l) + L-Arginine (60mg/l) + 1 mg/l BAP + 0.1 mg/l NAA + Undisclosed media supplements.
T(M)4: Modified MS + Adenine Sulphate (60mg/l) + L-Arginine (60mg/l) + 1mg/l BAP + 0.2mg/l NAA + Undisclosed media supplements.
T(M)5: Modified MS + Adenine Sulphate (60mg/l) + L-Arginine (60mg/l) + 1mg/l Kinetin + 0.1mg/l TDZ + 0.1mg/l NAA + Undisclosed media supplements.
T(M)6: Modified MS + Adenine Sulphate (60mg/l) + L-Arginine (60mg/l) + 1mg/l Kinetin + 0.1mg/l TDZ + 0.2mg/l NAA + Undisclosed media supplements.
T(M)7: Modified MS + Adenine Sulphate (60mg/l) + L-Arginine (60mg/l) + 1mg/l BAP + 0.1mg/l TDZ + 0.1mg/l NAA + Undisclosed media supplements.
T(M)8: Modified MS + Adenine Sulphate (60mg/l) + L-Arginine (60mg/l) + 1mg/l BAP + 0.1mg/l TDZ+ 0.2mg/l NAA + Undisclosed media supplements.
T(M)9: Modified MS + Adenine Sulphate (60mg/l) + L-Arginine (60mg/l) + 1mg/l BAP + 0.1mg/l Zeatin Riboside + 0.1mg/l NAA + Undisclosed media supplements.

Effect of basal medium and growth regulators on *in vitro* rooting

In vitro rooting of micro shoots on WPM medium, irrespective of growth regulation concentration and type, took lesser number of days (15.200) for root initiation as compared to half strength MS medium. As depicted in Table 2, number of major roots per shoot (5.000) and the rooting percent (76.00%) was found significantly more in WPM medium supplemented with 1.0 mg/l (NAA). Usually role of low salt medium and auxins are very pertinent in rooting of micro shoots *in vitro*. Use of half strength concentration of MS and full strength WPM was an attempt to reduce the salt concentration to enhance rooting *in vitro*. Kantharajah *et al.* (1998) [28] also reported rooting of pomegranate (cv. Wonderful) on WPM medium supplemented with 2mg/l NAA. Naik and Chand (2011) [23] reported the half strength WPM and MS medium for *in vitro* rooting of pomegranate with varying success. Singh *et al.* (2013) [29] also reported NAA as effective growth regulator to induce rooting in pomegranate at concentration of 0.5 mg/l along with 200 mg/l activated charcoal. ValizadehKaji *et al.* (2013) [30] used 0.54 µM NAA and 4.9 µM IBA on half strength WPM medium for *in vitro* rooting of pomegranate micro shoots of two Iranian pomegranate varieties.

Fig 4: *In vitro* rooting on T6 medium

Table 2: Effect of basal medium and growth regulators on *in vitro* rooting

Treatment	Days to root initiation	No. of major roots	Rooting (%)
T(R)1	18.400	2.000	52.000
T(R)2	17.600	2.000	64.000
T(R)3	19.400	3.200	48.000
T(R)4	18.200	3.200	56.000
T(R)5	15.600	2.400	72.000
T(R)6	15.200	2.600	72.000
T(R)7	17.200	4.400	64.000
T(R)8	17.400	5.000	76.000
CD (p=0.05)	2.067	0.827	12.883

T(R)1: MS (macro half) + Activated charcoal (800mg/l) + 0.5mg/l IBA
T(R)2: MS (macro half) + Activated charcoal (800mg/l) + 1.0 mg/l IBA
T(R)3: MS (macro half) + Activated charcoal (800mg/l) + 0.5mg/l NAA
T(R)4: MS (macro half) + Activated charcoal (800mg/l) + 1.0mg/l NAA
T(R)5: WPM + Activated charcoal (800mg/l) + 0.5mg/l IBA
T(R)6: WPM + Activated charcoal (800mg/l) + 1.0mg/l IBA
T(R)7: WPM + Activated charcoal (800mg/l) + 0.5mg/l NAA
T(R)8: WPM + Activated charcoal (800mg/l) + 1.0mg/l NAA

Effect of AMF inoculation on RLW and total leaf chlorophyll
High mortality and average field performance of improperly hardened *in* vitro raised plants are major concerns as far as commercial exploitation of these plants is concerned. Results in Figure 5 depicted better relative leaf water content (85.27%) and higher total leaf chlorophyll (2.17 mg/100 g of FW) in AMF inoculated plants as compared to non-inoculated tissue culture raised pomegranate plants of cv. Super Bhagwa (83.13% and 2.07 mg/ g of FW respectively).
Singh *et al.*, (2007) used AMF for hardening of *in vitro* raised pomegranate plants of cv. G-137 to avert transplantation shock of unfavorable *ex vitro* environmental conditions of pomegranate plantlets and found that mycorrhization resulted into faster growth as compared to non-mycorrhized control plantlets. Puthur *et al.* (1998) [31]; Rupnawar and Navale (2000) and Krishna *et al.* (2006) [33] and Singh *et al.* (2012 and 2016) [2, 3] also observed under pot culture experiments improved performance of AMF inoculated pomegranate plants in terms of more root and shoot biomass production, enhanced photosynthesis and better nutrient uptake due to improved soil exploration capacity of the root system and improved nutrient uptake with the help of hyphae and mycelia of AMF. They elaborated that the bio-hardening agents infect and establish themselves in the roots or rhizosphere of *in vitro* raised plants and help in mobilizing nutrients and increasing soil exploration capacity through their mycelia for better uptake of various nutrients.

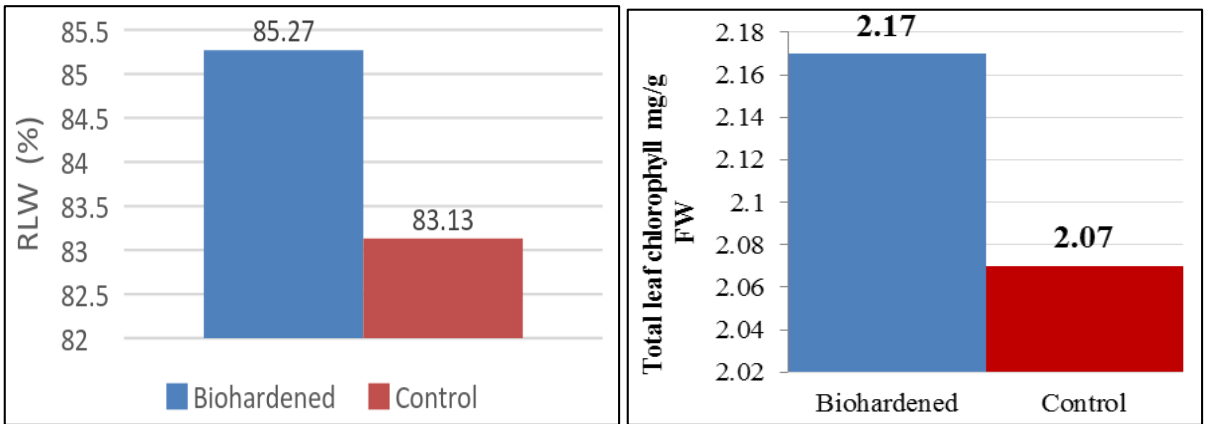

Fig 5: Relative leaf water content and total leaf chlorophyll content of bio-hardened and control saplings

Conclusion
The present investigation came out with a reproducible and efficient *in vitro* propagation protocol with emphasis on bio-hardening. Modified MS medium with combination of BAP/Kinetin either alone or with TDZ or ZR at low concentration alongwith adenine sulphate and 0.1 or 0.2 mg/l NAA proved effective for shoot proliferation. Pomegranate micro shoots on WPM medium supplemented with NAA and activated charcoal (800 mg/l) responded well for *in vitro* rooting. The present study advocated the use of plant beneficial microbes particularly AMF as bio-hardening to improve performance of *in vitro* propagated plantlets. Improved performance of AMF inoculated pomegranate plants might due to improved potting media exploration capacity root system with the help of hypahe and mycelia of AMF, high nutrient and water uptake and alleviation of stress response of the newly shifted saplings.

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Conflicts of interest
The authors declare that there are no conflicts of interest

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