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Enhanced Growth Performance and Acclimatization of *In Vitro* Cultured Pomegranate (*Punica granatum* L.) Plants Due to Synergistic Effect of Media and Light Intensity

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ABSTRACT

Pomegranate (*Punica granatum* L.) is a fruit crop of arid and semi-arid tropics and sub-tropics for its capacity to grow in saline soil and adaptability to drought conditions but more prone to bacterial blight and several other diseases. To establish disease free plantation in new areas, availability of quality planting material in large quantities appeared as major challenge through conventional propagation. Tissue culture laboratory at Jain Irrigation Systems Ltd. Jalgaon has developed an efficient *in vitro* propagation method for pomegranate. Being a delicate plantlet with unique physiology, acclimatization of pomegranate plantlets is difficult and has a low success rate. Light, temperature, humidity and growing media play an important role in the acclimatization process. Towards this end, an effort was undertaken to standardize a commercial acclimatization process for *in vitro* propagated pomegranate plants. The tissue culture grown plants were subjected to three different light intensities (L30, 30% light cut off; L45, 45% light cut off and L70, 70% light cut off) and four different media types *viz.* peat with perlite (M1), peat with 30% added wood fiber (M2), cocopeat (M3) and Soilrite (M4). Average light quantity received in L30, L45 and L70 were 282.71, 155.71 and 81.43 µM/m^2/sec respectively. For primary hardening (acclimatization), the plants grown in combination of L45+M2 responded synergistically to high survival rate (79.57%), better plant height, stem girth, leaf area, number of leaves as well as number of nodes. Thus, the present study has provided a path on selection of media, and light intensity for acclimatization of tissue culture grown pomegranate plant for primary hardening process.

***Key words*:** Primary hardening, Acclimatisation, Pomegranate, Tissue culture, Light intensity, Media

Pomegranate (*Punica granatum* L.) is an economically important fruit crop cultivated in arid, semiarid and sub-tropical regions of the world because of its unique capacity to grow in variety of soils, ranging from acidic sandy loam to alkaline calcareous soils and adaptability to drought conditions [1-2]. The pomegranate has several antimicrobial, antihyperglycemic, antiproliferative, anti-inflammatory, antiatherogenic, antitumoriogenic, radioprotective, nutraceutical effects due to antioxidant and free radical scavenging properties [3]. Fruits as well as flowers, bark, leave and seed contains bioactive metabolites which help to reduce blood pressure and also combat diabetes and cancer [4]. Due to high medicinal and nutraceutical effects, there is growing health awareness about pomegranate fruit and eventually increasing its demand. This warrants more cultivation of pomegranate plantation which is a challenging issue due to its susceptibility to various bacterial blight, fungal wilt diseases and insect pest infestation. This eventually declined the large-scale plantation of pomegranate [5]. Due to this, quality planting material on large scale is in high demand. During 2007 to 2010 bacterial blight caused by *Xanthomonas compastris cv*. *punicae* imposed serious threat on pomegranate cultivation in India. The disease spread to new cultivation areas with the planting material.

Micropropagation through plant tissue culture is an efficient technique for rapid clonal multiplication high quality true to the type plants. Besides true to the type and efficient

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multiplication tissue culture also enables to produce disease free planting material. Conventional vegetative propagation of pomegranate carried out by cuttings [6-8] and air layering. Several efforts have been made for *in vitro* micropropagation of pomegranate plants through axillary bud, shoot tip, meristem culture, and direct organogenesis [9]. Tissue culture laboratory of Jain Irrigation Systems Ltd., Jalgaon also developed a protocol for commercial micropropagation of pomegranate. Establishment of *in vitro* plantlets in the field needs gradual acclimatization. There is high percentage of mortality sometimes up to 100% and very limited information is available for the acclimatization of *in vitro* produced pomegranate plants.

In micropropagation, one of the important steps is transfer of plantlets from *in vitro* culture to soil condition. Tissue culture plantlets undergo several physiological, anatomical and morphological changes due to controlled environment during *in vitro* growth [10-11]. Now it is widely known that plants transferred to soil causes modification in its leaf anatomy and stomata [12-13]. Thus, development of hardening techniques of micro-propagated pomegranate for commercial production was considered as an essential step for successful survival of planting material in field. Therefore, the main objective of the study is to standardize the method of primary hardening process in tissue culture grown pomegranate plants.

MATERIALS AND METHODS

All experiments were conducted at the tissue culture hardening facility at Tissue Culture Park of Jain Irrigation Systems Ltd., Jalgaon (Maharashtra, India). *In vitro* grown pomegranate plantlets were removed from glass bottles, cleaned with running tap water to remove agar medium sticking to roots and then planted in plastic trays containing media. Trays with plants were kept in green house for further growth and development with experimental conditions.

Growing conditions in green house

The Gothic arch type greenhouse with cooling pad and fan having eight meters bay and 32 meter length was used for the experimental treatments. The green house was equipped with thermal shade net, four-way misting system, thermometer, hygrometer and PAR meter to maintain and monitor temperature and humidity as well as light intensity. Temperature was maintained 25°±2°C and relative humidity was maintained 70-90%. Plants were under poly cover tunnel for initial one week after transplanting and humidity inside the tunnel was maintained at 90%. Plants were irrigated on daily basis.

Experimental treatments

Four types of media and three light cut off were used as treatment for the acclimatization of pomegranate plants. Potting media M1 was with 0-10mm blond peat with perlite and added nutrient and pH 5.1-5.9 and EC 2-4µs/cm^2. M2 media was with 0-10mm blonde peat with 30% added wood fiber and pH, EC like M1. Low EC (<1.2ms/cm^2) cocopeat was used as media 3 (M3) and Soilrite (having 75% Irish sphagnum moss plus 15% perlite with pH 5-6.5) was used as media 4 (M4). Three light conditions were created 30, 45 and 70% light cutting by use of shading nets and treatments were designated as L30, L45 and L70, respectively. Light intensity under different treatment was measured by a Quantum PAR light meter (Spectrum Technologies Inc.). Survival percentage and growth parameters namely, plant height (cm), plant girth (mm), leaf area (cm^2), number of leaves and number of nodes were determined after 30 days of growth. Leaf area (LA) was calculated using leaf length (L) and width (W) data by formula:

$$LA = -0.0477 + 0.0282*L + 0.0842*W + 0.965*L*W;\ R^2 = 0.999$$

as per Meshram *et al*. [14].

Experimental design and statistical analysis

Four media and three light cutoff treatments with three replicates were designed in a factorial completely randomized design (CRD). Three trays of plantlets per treatment with each tray have 96 plants in 20cc square cells were used in the experiment. Data analysis was performed using an R based program and means were separated by Duncan's Multiple Range Tests (DMRT) at P value 0.05.

RESULTS AND DISCUSSION

Acclimatization means the climatic adaptation of plantlets which has been moved to a new environment [15]. For plants acclimatization of *in vitro* plants, humidity and moderate light intensity are the main factors to be controlled beside temperature. Inside the greenhouse, plastic covering with frequent misting was used to maintain the relative humidity. Management of solar radiation is necessary as the high solar radiation itself may directly damage the plantlets and also influences temperature and relative humidity of growing environment. The experimental strategy was to optimize primary hardening process for *in vitro* produced pomegranate plants by using different light intensities and media compositions. By studying the survival and changes in growth parameters such as plant height, stem girth, leaf size, leaf area, number of leaves and biochemical character such as chlorophyll content, an integrated protocol was established to optimize the primary hardening process.

Table 1 Light (PAR) intensity measured in terms of µM /m^2/sec for three different treatments (L30, L45 and L70) at 1 hour of interval

Time	L30		L45		L70		Average light intensity outside
	Intensity	% to outside	Intensity	% to outside	Intensity	% to outside	
10:00	130.00	24.53	80.00	15.09	43.00	8.11	530.00
11:00	195.00	24.84	110.00	14.01	59.00	7.52	785.00
12:00	421.00	24.93	238.00	14.09	113.00	6.69	1689.00
13:00	432.00	22.33	247.00	12.76	130.00	6.72	1935.00
14:00	406.00	25.53	225.00	14.15	126.00	7.92	1590.00
15:00	235.00	17.28	100.00	7.35	54.00	3.97	1360.00
16:00	160.00	13.50	90.00	7.59	45.00	3.80	1185.00
Average	282.71	21.81	155.71	12.01	81.43	6.28	1296.29

Light intensity under different light treatments

Photosynthetically Active Radiation (PAR) light intensities under different treatments, L30, L45 and L70 varied greatly under different treatments (Table 1). In L30 treatment, average PAR light intensity was 282.71 µM/m^2/sec (21.81% to the outside light intensity). In the second treatment (L45) has an

average PAR light intensity of 155.71 µM/m2/sec (12.01% to outside) and whereas, for L70 has an average of 81.43 µM/m2/sec PAR light intensity (only 6.28% to outside).

Effect of media compositions and light intensities on survival rate of plantlets

The compatibility of plantlets to different media as well as light conditions can be judged by the survival capacity or survival rate (%), thus the primary hardened plantlets subjected to four media compositions M1, M2, M3 and M4 with three levels of light intensities was studied for survival rate. The plantlets exposed to L70 showed 72.89, 71.67, 57.16 and 57.55 % survival rate when grown in M1, M2, M3 and M4 medium, respectively. Similarly, plants exposed to L45 showed 79.83, 79.57, 64.61 and 59.55% survival rate while, plantlets subjected to L70 showed 77.89, 80.58, 57.51 and 56.61 % survival rate in M1, M2, M3 and M4 medium, respectively (Table 2). About 70% light cutoff with peat moss-based media M2 recorded significant survival rate which was at par with M1 media. Among the light cutoff treatments 45% cutoff had shown 70.86% survival rate, but it was statistically similar to 30 and 70% cutoff which resulted 64.82 and 68.15% survival, respectively (Table 2). Effect of media composition on survival rate has been calculated in many primary hardened plant species. The media composition consisting of vermicompost and soil had resulted in high survival rate of tissue culture grown pomegranate plants [16-17]. Murkute *et al.* [18] achieved 50% survival of plants when transferred to vermicompost + soil mixture (1: 1).

Table 2 Effect of media compositions, M1 (peat with perlite), M2 (peat with wood fibre), M3 (Soilrite) and M4 (cocopeat) and light intensities L30 (30% light cutoff), L45 (45% light cutoff) and L70 (70% light cutoff) on survival rate during primary hardening tissue culture pomegranate plantlets. Each value represents the mean of three biological replicates. Different letters superscript on the values indicates significant difference whereas same letters indicate non-significant at P value 0.05. Means were separated by DMRT

Light	Survival rate (%)				Average
	Media				
	M1	M2	M3	M4	
L30	72.89abc	71.67bc	57.16d	57.55d	64.82a
L45	79.83ab	79.57ab	64.61cd	59.42d	70.86ab
L70	77.89ab	80.58a	57.51d	56.61d	68.15b
Average	76.87a	77.27a	59.76b	57.86b	

Effect of media compositions and light intensities on plant height and stem girth

To optimize the growing medium for primary acclimatization of tissue cultured pomegranate plants, four media compositions viz. M1 (peat with perlite), M2 (peat with wood fiber), M3 (Soilrite) and M4 (cocopeat) were used and further subjected to three different levels of light intensities viz. L30 (30% light cutoff), L45 (45% light cutoff) and L70 (70% light cutoff). Plant height and stem girth give appropriate understanding of growth and development of plants [19]. Among the lights, about 70% light cutoff result in significantly taller plants than 45% light cutoff however, the girth was not affected by these light treatments. All media treatments produced statistically similar plant height whereas, girth in M1 and M2 was significantly improved than M3 and M4.

Plantlets exposed to L30 (30% light cutoff) showed 10.6, 10.8, 11.3 and 10.5cm of average plant height grown in M1, M2, M3 and M4 medium, respectively. Similarly, plants exposed to L45 (45% light intensity) showed 10.8, 12.1, 11.3 and 9.9cm of average plant height and plants subjected to L70 (70% light intensity) showed 12, 10.4, 11.5 and 12.1cm of average plant height grown in M1, M2, M3 and M4 medium, respectively (Table 3). Changes in the stem girth was also noted, it was found that the plantlets treated with L30 light intensities showed 0.53, 0.63, 0.52 and 0.48 mm stem girth, plantlets given L45 light intensities showed 0.58, 0.54, 0.46 and 0.44 mm stem girth, while plantlets subjected to L70 light intensities showed 0.51, 0.52, 0.44 and 0.48 mm average stem girth when grown in grown in M1, M2, M3 and M4 medium, respectively (Table 3). Out of these two agronomical traits, there were not much noticeable changes in stem girth exposed to different media compositions and light intensities which may be due to poor secondary growth in the early stage of development.

Table 3 Effect of media compositions, M1 (peat with perlite), M2 (peat with wood fiber), M3 (Soilrite) and M4 (cocopeat) and light intensities L30 (30% light cutoff), L45 (45% light cutoff) and L70 (70% light cutoff) on plant height and stem girth of primary hardened tissue culture pomegranate plantlets. Different letters superscript on the values indicates significant difference whereas same letters indicate non-significant at P value 0.05. Means were separated by DMRT.

Light	Media				Average
	M1	M2	M3	M4	
	Plant height (cm)				
L30	10.60abc	10.81abc	11.34abc	10.05bc	10.70b
L45	10.61abc	12.07a	11.50ab	9.95c	11.03ab
L70	11.96a	10.39bc	11.51ab	12.09a	11.49a
Average	11.06a	11.09a	11.45a	10.70a	
	Stem girth (mm)				
L30	0.54abc	0.63a	0.53abc	0.48bc	0.54a
L45	0.59ab	0.55abc	0.47bc	0.45c	0.52a
L70	0.58abc	0.52abc	0.44c	0.48bc	0.51a
Average	0.57a	0.57a	0.48b	0.47b	

Every woody plant at its early stage of development (starting from germination to small plantlet) does not form more secondary meristematic tissues [20]. Thus, considering plant height as a chief parameter we can conclude that a combination

of L30+M3, L45+M2 and L70+M4 was found to be the best for primary hardening. Use of peat for tomato plant cultivation was also found to be effective in comparison with other media types [21].

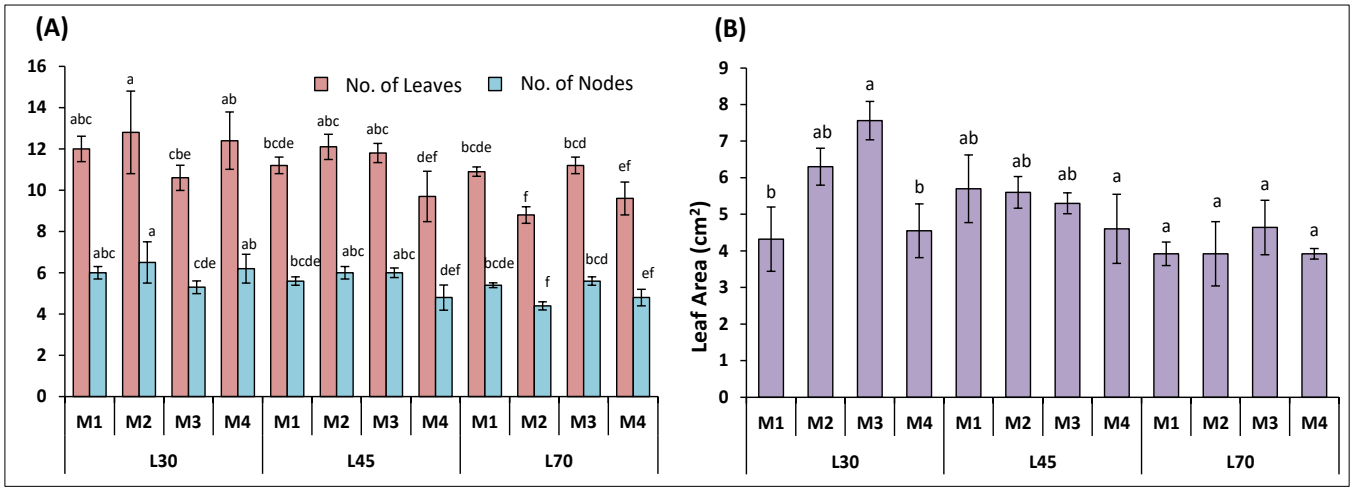

Fig 1 Effect of media compositions, M1 (peat with perlite), M2 (peat with wood fiber), M3 (Soilrite) and M4 (cocopeat) and light intensities L30 (30% light cutoff), L45 (45% light cut off) and L70 (70% light cut off) on number of leaves and number of nodes (A) and leaf area (B) of primary hardened tissue culture pomegranate plantlets. Each value represents the mean ±SE of three biological replicates. Different letters above bars indicate significant difference at P value 0.05. Means were separated by DMRT

Effect of media compositions and light intensities on leaf area, no. of leaves and no. of nodes

Photosynthesis of plant is dependent on nature of light, CO_2 intake, availability of water and suitable environmental condition. The more leaf surface area and total leaf number in a plant also support higher rate of photosynthesis [22]. Thus, during standardization of the medium of primary hardened pomegranate plants, the parameters like, total leaf area, no. of leaves as well as no. of nodes were measured. Changes in the number of leaves and number of nodes per plant was also noted, it was found that the plantlets subjected to L30 showed 12, 12.8, 10.6, 12.4 average number of leaves and 6, 6.5, 5.3, 6.2 average number of nodes when grown M1, M2, M3 and M4 medium, respectively. Likewise, plantlets exposed to L45 displayed 11.2, 12.1, 11.8, 9.7 average number of leaves and 5.6, 6.0, 6.0, 4.8 average number of nodes when grown M1, M2, M3 and M4 medium, respectively. The plantlets at L70 demonstrated 10.9, 8.8, 11.2, 9.6 average number of leaves and 5.4, 4.4, 5.6, 4.8 average number of nodes in M1, M2, M3 and M4 medium, respectively (Fig 1A). We found that the changes in the leaf area of plants were 4.32, 6.3, 7.56 and 4.55 cm^2 when grown in M1, M2, M3 and M4 medium, respectively during treatment with L30 (30% light cutoff). Similarly, plants exposed to L45 (45% light cut off) showed 5.7, 5.6, 5.3 and 4.6 cm^2 of average leaf area and plants treated at L70 (70% light cut off) showed 3.92, 3.92, 4.64 and 3.92 cm^2 of average leaf area when grown in M1, M2, M3 and M4 medium, respectively (Fig 1B). Here, the leaf area was significantly increased when plantlets grown in combination of L30+M3. However, considering all three parameters L30+M2 (30% light cutoff and peat with wood fiber) combination showed improved performance vis-a-vis other combinations. Vasane *et al*. [23] showed that, the use of PMC in media enhances the overall agronomical traits of primary hardened *in vitro* banana plant which include leaf area, size as well as number of leaves. In case of pomegranate primary hardening, use of peat found most suitable, probably due to the difference between the root architecture (adventitious roots and tap roots) of banana and pomegranate plants.

Light levels influenced leaf length, leaf area/leaf, number of leaves and number of nodes per plant significantly. Low light cut off (30%) produced higher number of nodes, more number of leaves, longer leaves and higher number of leaves. All these parameters were not affected by media treatment except leaf length. Peat with perlite or wood fiber produced significantly longer leaves.

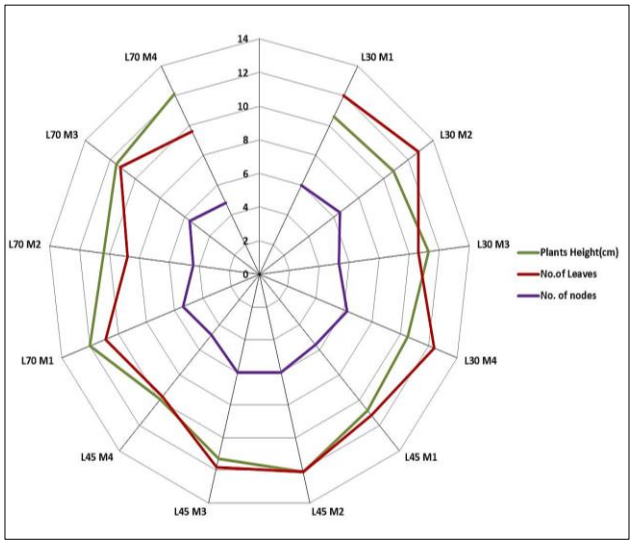

Fig 2 Effect of media compositions, M1 (peat with perlite), M2 (peat with wood fiber), M3 (Soilrite) and M4 (cocopeat) and light intensities L30 (30% light cutoff), L45 (45% light cut off) and L70 (70% light cut off) on plant height, leaf area, number of leaves, chlorophyll content and number of nodes shown by radar chart

Different types of media compositions were used to standardize hardening processes in pomegranate plants. Mahishni *et al*. [24] obtained over 80% successes in hardening of pomegranate plants using a potting mixture of 1: 1: 1 (v/v) peat: perlite: sand. Yang *et al*. [25] also obtained >90% success in hardening of rare pomegranate cv. 'Ruanzi' transfer to glasshouse conditions. The use of glass jar with polypropylene cap filled with peat: Soilrite (1:1) was found most effective for hardening of pomegranate plants [26]. Here, we can conclude that a combination of L30+M2, L45+M2 and L70+M2 enhance the chlorophyll content in the leaves of plant. The radar chart made by considering important parameters (Fig 2) shows L45+M2 combination favors the growth of pomegranate plants for primary hardening process.

CONCLUSION

In the light of these studies, an optimized protocol of primary hardening of tissue culture derived Pomegranate plantlet was established. The comparison of different media and light intensities and their effect on plant growth and development particularly, on tissue culture grown pomegranate plant is a crucial parameter in commercial scale tissue culture laboratory. Exposure to 45% light cutoff and combination of peat with wood fiber potting media appeared as the most suitable for overall growth of pomegranate plantlets under primary hardening process.

uthor contribution statement

BK, ABC and KBP conceived and designed the Experiment. KBP and KKM conducted the experiments and performed all analysis. KBP, BLJ and BK analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

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