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Growing Substrate and Light Intensity Influences Growth and Development of Tissue Cultured Pomegranate Plants During Secondary Hardening

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ABSTRACT

Pomegranate is a unique crop due to its drought tolerance capacity and pharmaceutical and nutraceutical values. It has special economic importance among fruit crop of arid and semi-arid regions. Area under pomegranate has been growing in India. In the Indian tropics pomegranate crop is severely affected by bacterial blight and several other fungal and bacterial diseases. Disease free new plantation can be established only if high quality planting material is available. Tissue culture offers the possibility of propagating disease-free pomegranate plantlets but success rate of transplanting in vitro plants to field is a challenge. Light, temperature, humidity and growing media play an important role in the acclimatization process. An effort was made to standardize a commercial secondary acclimatization process for in vitro propagated pomegranate plants. The secondary acclimatization experiments involved light conditions and growing substrates which help in controlling growing environment especially light, temperature and water relations. In this study, pomegranate plants subjected to three light cut off treatments (30%, 45% and 70%) in combination with four different growing substrates (M1, M2, M3 and M4). Treatment receiving average 282.72 micro mole /m2/sec natural light (21.81% total light) and growing substrate with a mixture of peat and cocopeat gave better plant growth and success in acclimatization.

***Key words*:** Secondary hardening, Pomegranate, Light intensity, Acclimatization, Growing substrates

Pomegranate *Punica granatum* L. belongs to the family *Punicaceae* and is one of the oldest table fruits of arid and semiarid sub-tropical regions of the world [1]. Pomegranate has great adaptability to salty soil as well as drought condition [2]. India is the second largest producer of pomegranate and it is mainly cultivated in the states of Maharashtra, Gujarat, Karnataka, Tamil Nadu, Uttar Pradesh, Haryana, and Andhra Pradesh. Pomegranate fresh fruits and juices are always in demands, apart from this; the processed products like pomegranate wine, pomegranate tea and candy are also gaining importance in the world. The fruit are rich in irons and calcium while it also has various medicinal properties. In addition, the tree is also valued for its pharmaceutical properties. It is used for treating dyspepsia and considered beneficial in treating leprosy [3]. The rind of the fruit and the bark of pomegranate tree are used as a traditional remedy against diarrhea, dysentery and intestinal parasites.

Pomegranate is commercially propagated by stem cuttings or by air layering just to obtain true to type planting material. However, it has lot of limitations like slow propagation as new plants require one year for establishment, low success, etc. This results in non-availability of clonal planting material through-out the year. Micro propagation technique is being exploited for efficient multiplication of several fruit crops.

Several efforts have been made for *in vitro* micropropagation of pomegranate plants through axillary bud, shoot tip, meristem culture, and direct organogenesis [4-5] but most of report does not give emphasis to the acclimatization of these plants to field conditions. However, for commercial propagation appropriate acclimatization protocol is of prime

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importance. Media optimization for primary hardening of pomegranate plants was also developed previously for successful plant growth and development [6]. 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 mortality sometimes up to 100 percent and very limited information is available for the acclimatization of *in vitro* produced pomegranate plants.

One of the important steps in micropropagation technique is the transfer of plantlets from *in vitro* culture to soil condition (hardening steps) as plantlets undergo several physiological, anatomical and morphological changes due to controlled environment during *in vitro* growth [7-8]. Now it is widely known that plants transfer to soil causes modification in its leaf anatomy and stomata [9-10]. Thus, development of primary and secondary 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 secondary hardening process in tissue culture grown pomegranate plants, continuation of our previous report [11] on primary hardening of pomegranate plantlets.

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 primary hardened pomegranate cv. Bhagwa plantlets were planted in 1000 cc polythene bags (with different growing substrate as per treatment). Plants were grown in poly cum shade house structures (4m gutter height) covered with 200-micron poly film on top and sides covered with 40 mesh insect net and additional shade net from inside on the top at gutter height to control light (depending on treatments). The plants were irrigated through overhead sprinkler irrigation and cared for by an experienced agronomist.

Growing substrate

Planting was done in the polybags filled with four different growing substrates depending on treatment, viz. M_1: 0-10mm blonde peat and added nutrient with pH 5.1-5.9 and EC 2-4µs/cm^2, M_2: 0-10mm blonde peat (like M_1) with cocopeat having Low EC (<1.2ms/cm^2), M_3: mixture of river bed soil + cocopeat (like M_2) + press mud cake (PMC) (33% each by volume) and M_4: having cocopeat (like M_2) + baggase in 1:1 ratio by volume. The volume of growing substrate in each treatment was kept constant. Each treatment was replicated thrice, with 150 plants in each replicate.

Light Intensity and duration

Three different light conditions were created 30, 45 and 70% light cutting by use of shading nets below the poly cover 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.). Photosynthetically Active Radiation (PAR) light intensities under different treatments, L30, L45 and L70 varied greatly under different treatments. In L30 treatment, average PAR light intensity was 282.71 µM/m^2/sec (21.81% to the outside light intensity). The treatment L45 has an average PAR light intensity of 155.71 µM/m^2/sec (12.01% to outside) and whereas, L70 has an average of 81.43 µM/m^2/sec PAR light intensity (only 6.28% to outside).

Parameters monitored

All the parameters were measured on 30th day then on 45th day and finally on 60th day after secondary hardening. Height of the plants (cm) was measured from the base of the stem in the polybag to the angle made between the youngest and 1st open leaf. Girth of stem (mm) was measured at 2 cm from the base of the plant in the polybag. Numbers of leaves were counted. Number of nodes (cm), root volume (ml), fresh and dry weight of root and shoot (gm per plant) and percent mortality were also recorded.

Experimental design and statistical analysis

The experiment was designed in a factorial completely randomized design with three replicates. Data analysis was performed using a R based program and means were separated by Duncan's Multiple Range Tests (DMRT) at P value 0.05.

RESULTS AND DISCUSSION

The experimental strategy was to optimize secondary hardening process for *in vitro* produced pomegranate plants by using different light intensities and growing substrates. By studying the survival and changes in growth parameters such as plant height, stem girth, number of nodes, number of leaves and biochemical character such as fresh and dry weight of shoot and root of plants as well as root volume, an integrated protocol was established to optimize the secondary hardening process.

Effect of light and growing substrate plant growth

Growth after 30 days

Under L30 (30% light cut-off) condition after 30 days, the morphological parameter like plant height, stem girth were significantly high in M_1 growing substrate 6.93 cm and 2.17 mm as compared to M_2, M_3 and M_4 growing substrate whereas number of nodes were recorded maximum with M_2 and M_4 (3.33) and number of leaves were highest with M_4 growing substrate. In biometric parameters, average fresh shoot weight and dry shoot weight was observed 11.17 gm and 2.59 gm in growing substrate M_2. The ratio of fresh shoot weight to dry shoot weight is 1:0.23 whereas ratio of fresh roots to dry roots is 1:0.13 and the root volume is 6.67 ml in M_1 growing substrates (Table 1).

Under L45 (45% light cutoff) condition after 30 days, plant height and stem girth were highest with M_1 (6.93 cm and 1.83 mm) respectively but there was no significant change in number of nodes in other growing substrates whereas highest number of leaves (18.33) were observed in M_2 and M_3. Average fresh shoot weight was highest in M_1 substrate i.e., 10.64 gm with dry shoot weight 2.50 gm and the root volume 8 ml (Table 1). Under L70 (70% light cutoff) condition after 30 days, plant height, stem girth, number of nodes and total number of leaves in M_1 growing substrate were 6.67 cm, 2.10 mm, 7 and 20 respectively. Highest fresh shoot weight (13.60 gm) and dry shoot weight (3.15gm) was also recorded in M_1 growing substrate. Similarly fresh and dry root weight was also highest in M_1 growing substrate as 4.96 gm and 0.59 gm respectively with the root volume of 8 ml (Table 1).

Table 1 Profile of morphological features and biometric parameters of secondary hardened pomegranate plants after 30 days of duration

L	M	Plant height (cm)	Stem girth (mm)	No. of nodes	No. of leaves	Fresh shoot weight (gm)	Dry shoot weight (gm)	Fresh root weight (gm)	Dry root weight (gm)	Root volume (ml)
L30	M_1	6.93^{a}	2.17^{a}	2.67^{b}	17.33^{c}	10.39^{b}	2.49^{b}	4.61^{bc}	0.57^{b}	6.67^{b}
	M_2	6.07^{bc}	1.90^{bc}	3.33^{b}	17.33^{c}	11.17^{b}	2.59^{b}	4.48^{bcd}	0.58^{b}	6.67^{b}
	M_3	4.63^{d}	1.80^{c}	3.00^{b}	20.00^{ab}	8.00^{cd}	2.04^{c}	4.03^{bcde}	0.53^{bcd}	7.33^{b}
	M_4	6.47^{abc}	1.83^{c}	3.33^{b}	20.67^{a}	10.31^{b}	2.56^{b}	6.09^{a}	0.76^{a}	12.00^{a}
L45	M_1	6.93^{a}	1.83^{c}	3.00^{b}	18.00^{bc}	10.64^{b}	2.50^{b}	4.35^{bcd}	0.54^{bc}	8.00^{b}
	M_2	5.87^{c}	1.50^{d}	3.00^{b}	18.33^{abc}	8.21^{c}	1.87^{cd}	3.03^{ef}	0.43^{cd}	5.33^{b}
	M_3	6.40^{abc}	1.43^{d}	3.00^{b}	18.33^{abc}	6.28^{e}	1.60^{d}	2.7^{9f}	0.40^{d}	5.33^{b}
	M_4	6.73^{ab}	1.47^{d}	3.00^{b}	18.00^{bc}	6.75^{de}	1.72^{cd}	4.36^{bcd}	0.47^{bcd}	7.33^{b}
L70	M_1	6.67^{abc}	2.10^{ab}	7.00^{a}	20.00^{ab}	13.60^{bc}	3.15^{a}	4.96^{ab}	0.59^{b}	8.00^{b}
	M_2	6.37^{abc}	1.50^{d}	7.00^{a}	18.33^{abc}	8.27^{b}	1.98^{c}	3.40^{def}	0.41^{cd}	6.67^{b}
	M_3	5.90^{bc}	1.50^{d}	6.67^{a}	17.00^{c}	7.43^{cde}	1.82^{cd}	3.59^{cdef}	0.48^{bcd}	7.33^{b}
	M_4	6.33^{abc}	1.53^{d}	6.33^{a}	19.00^{abc}	7.14^{cde}	1.72^{cd}	4.35^{bcd}	0.50^{bcd}	8.00^{b}
SEM		0.2546	0.0183	0.2020	2.2702	0.6776	0.0426	0.4634	0.0058	2.5959
L30		6.03^{a}	1.93^{a}	3.08^{b}	18.83^{a}	9.97^{a}	2.42^{a}	4.80^{a}	0.61^{a}	8.17a
L45		6.48^{a}	1.56^{b}	3.00^{b}	18.17^{a}	7.97^{c}	1.93^{c}	3.63^{b}	0.46^{b}	6.50b
L70		6.32^{ab}	1.66^{b}	6.75^{a}	18.58^{a}	9.11^{b}	2.17^{b}	4.08^{b}	0.50^{b}	7.50ab
SEM		0.2546	0.0183	0.2020	2.2702	0.6776	0.0426	0.4634	0.0058	2.5959
M_1		6.84^{a}	2.03^{a}	4.22^{a}	18.44^{a}	11.54^{a}	2.72^{a}	4.64^{a}	0.57^{a}	7.56^{ab}
M_2		6.10^{bc}	1.63^{b}	4.44^{a}	18.00^{a}	9.22^{b}	2.15^{b}	3.63^{b}	0.47^{b}	6.22^{b}
M_3		5.64^{c}	1.58^{b}	4.22^{a}	18.44^{a}	7.24^{d}	1.82^{c}	3.47^{b}	0.47^{b}	6.67^{b}
M_4		6.51^{ab}	1.61^{b}	4.22^{a}	19.22^{a}	8.07^{c}	2.00^{bc}	4.93^{a}	0.58^{a}	9.11^{a}
SEM		0.2546	0.0183	0.2020	2.2702	0.6776	0.0426	0.4634	0.0058	2.5959
L		6.28	1.71	4.28	18.53	9.01	2.17	4.17	0.52	7.39
M		6.28	1.71	4.28	18.53	9.01	2.17	4.17	0.52	7.39

Growing substrate M_1: Peat, M_2: Peat with cocopeat, M_3: Soil+cocopeat + PMC and M_4: Cocopeat + baggase and light intensities L_1: 30% light cutoff, L_2: 45% light cutoff and L_3: 70% light cutoff. 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

Table 2 Profile of morphological features and biometric parameters of secondary hardened pomegranate plants after 45 days of duration

L	M	Plant height (cm)	Stem girth (mm)	No. of nodes	No of leaves	Fresh shoot weight (gm)	Dry shoot weight (gm)	Fresh root weight (gm)	Dry root weight (gm)	Root volume (ml)
L30	M_1	7.00^{ab}	2.55^{b}	11.80^{bc}	29.00^{bc}	22.81^{cd}	4.91^{de}	5.15^{def}	0.67d^{e}	10.67^{bc}
	M_2	5.48^{cd}	2.40^{bcde}	11.87^{bc}	28.60^{bc}	23.28^{bcd}	5.20^{cd}	6.42^{cd}	0.82^{bcd}	10.67^{bc}
	M_3	6.09^{abc}	2.52^{b}	6.67^{e}	16.67^{d}	18.31^{ef}	4.43^{def}	6.19^{cde}	0.79^{bcde}	11.33^{b}
	M_4	6.89^{ab}	2.46^{bc}	10.93^{c}	31.07^{b}	25.27^{bc}	6.20^{b}	10.96^{a}	1.21^{a}	13.33^{a}
L45	M_1	6.16^{abc}	2.43^{bcd}	12.53^{abc}	31.13^{b}	26.55^{b}	5.84^{bc}	6.13^{cde}	0.73^{cde}	8.67^{d}
	M_2	5.54^{cd}	2.13^{f}	10.93^{c}	30.93^{b}	20.88^{de}	4.88^{def}	3.95^{f}	0.65^{e}	8.00^{cd}
	M_3	5.79^{bc}	2.16^{f}	8.93^{d}	19.20^{d}	15.63^{f}	4.19^{ef}	4.96^{ef}	0.80^{bcde}	9.33^{d}
	M_4	4.93^{cd}	2.22^{def}	12.80^{ab}	30.67^{b}	17.86^{ef}	4.52^{def}	7.31^{bc}	0.88^{bc}	9.33^{cd}
L70	M_1	7.15^{a}	2.82^{a}	13.60^{a}	35.73^{a}	32.38^{a}	7.56^{a}	5.99^{cde}	0.86^{bc}	8.67^{d}
	M_2	4.35^{d}	2.29^{cdef}	12.27^{abc}	30.20^{b}	20.22^{de}	4.68^{def}	4.91^{ef}	0.72^{cde}	8.67^{d}
	M_3	5.84^{abc}	2.33^{cdef}	10.67^{d}	21.20^{d}	17.58^{f}	4.35^{f}	6.96^{bcd}	0.93^{b}	11.33^{b}
	M_4	5.62^{cd}	2.16^{ef}	10.40^{abc}	22.53^{c}	16.33^{ef}	4.13^{def}	7.39^{b}	0.91^{b}	11.33^{b}
SEM		0.5555	0.0156	0.9093	3.5489	4.6633	0.2510	0.6621	0.0090	1.3232
L_1		6.37^{a}	2.48^{a}	10.32^{b}	26.33^{b}	22.42^{a}	5.18^{a}	7.18^{a}	0.87^{a}	11.50^{a}
L_2		5.61^{b}	2.24^{b}	11.30^{a}	27.98^{a}	20.23^{b}	4.86^{a}	5.59^{c}	0.77^{b}	8.83^{c}
L_3		5.74^{ab}	2.40^{a}	11.73^{a}	27.42^{ab}	21.63^{ab}	5.18^{a}	6.31^{b}	0.86^{a}	10.00^{b}
SEM		0.5555	0.0156	0.9093	3.5489	4.6633	0.2510	0.6621	0.0090	1.3232
M_1		6.77^{a}	2.60^{a}	12.64^{a}	31.96^{a}	27.24^{a}	6.10^{a}	5.76^{b}	0.75^{bc}	9.33^{b}
M_2		5.12^{c}	2.27^{b}	11.69^{b}	29.91^{b}	21.46^{b}	4.92^{b}	5.10^{b}	0.73^{c}	9.11^{b}
M_3		5.91^{b}	2.34^{b}	8.76^{c}	19.02^{c}	17.17^{c}	4.32^{c}	6.04^{b}	0.84^{b}	10.67^{a}
M_4		5.81^{bc}	2.28^{b}	11.38^{ab}	28.09^{b}	19.82^{b}	4.95^{b}	8.55^{a}	1.00^{a}	11.33^{a}
SEM		0.5555	0.0156	0.9093	3.5489	4.6633	0.2510	0.6621	0.0090	1.3232
L		5.90	2.37	11.12	27.24	21.42	5.07	6.36	0.83	10.11
M		5.90	2.37	11.12	27.24	21.42	5.07	6.36	0.83	10.11

Growing substrate M_1: Peat, M_2: Peat with cocopeat, M_3: Soil+cocopeat + PMC and M_4: Cocopeat + baggase and light intensities L_1: 30% light cutoff, L_2: 45% light cutoff and L_3: 70% light cutoff. 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

Growth after 45 days

The results under L30 condition, with M_1 growing substrate showed 7 cm of plant height, 2.55 mm of stem girth, 11.80 number of nodes and 29 number of leaves and under L45 condition, M_1 media composition showed 6.16 cm plant height, 2.43 mm stem girth, 12.53 average numbers of nodes and 31.13 average number of leaves per plant whereas under L70 condition, M_1 growing substrate showed 7.15 cm plant height, 2.82 mm stem girth, 13.60 average numbers of nodes and 35.73 average number of leaves per plant. Most of these values were statistically higher in M_1 under all light cut off conditions. Average fresh shoot and dry shoot weight were also recorded in M_1 growing substrate under all three conditions. The result showed that M_1 under L70 condition gained the highest fresh and dry shoot weight per plant 32.38 gm and 7.56 gm respectively as compared to other growing substrates. The root fresh weight (5.99 gm) and dry weight (0.86 gm) were also observed highest in M_1 growing substrate as compared to others growing substrates. The root volume recorded in M_1 growing substrate under all three conditions was 10.67 ml (L30), 8.67 ml (L45) and 8.67 ml (L70) (Table 2).

The M_2 growing substrate also exhibited encouraging growth under different light intensity conditions. Under L30 condition, average plant height (5.48 cm), average stem girth (2.40), average numbers of nodes (11.87) and average number of leaves (28.60) respectively, these values were statistically at par with M_1 except plant height. The fresh shoot weight, dry shoot weight, fresh root weight, dry root weight and root volume were noted as 23.28 gm, 5.20 gm, 6.42 gm, 0.82 gm and 10.67 ml respectively in M_2. Whereas under L45 condition; average plant height, stem girth, numbers of nodes and average number of leaves were observed as 5.54 cm, 2.13 mm, 10.93 and 30.93 respectively. Average fresh shoot weight, dry shoot weight, fresh root weight, dry root weight and root volume were noted as 20.88 gm, 4.88 gm, 3.95 gm, 0.65 gm and 8.00 ml respectively while it was also observed under L70 condition as 4.35 cm, 2.29 mm, 12.27and 30.20 respectively. Fresh shoot weight, dry shoot weight, fresh root weight, dry root weight and root volume were noted as 20.22 gm, 4.68 gm, 4.91 gm, 0.72 gm and 8.67 ml respectively and root volume was recorded in M_2 growing substrate under all three conditions; under L30 condition (10.67 ml), under L45 condition (8.00) and under L70 condition (8.67) (Table 2).

In M_3 growing substrate, under L30, L45 and L70 conditions plant height (6.09, 5.79 and 5.84 cm), stem girth (2.52, 2.16 and 2.33 mm), numbers of nodes (6.67, 8.93 and 10.67) and number of leaves (16.67, 19.20 and 21.20) were observed. In case of biometric parameters, under L30, L45 and L70 conditions fresh shoot weight (18.31, 15.63 and 17.58 gm), dry shoot weight (4.43, 4.19 and 4.35 gm), fresh root weight (6.19, 4.96 and 6.96 gm), dry root weight (0.79, 0.80 and 0.93 gm) and root volume (11.33, 9.33 and 11.33 ml) were recorded (Table 2). In case of M_4 growing substrate, under L30, L45 and L70 conditions average plant height (6.89, 4.93 and 5.62 cm), average stem girth (2.46, 2.22 and 2.16 mm), average numbers of nodes (10.93, 12.80 and 10.40) and average number of leaves (31.07, 30.67 and 22.53) were observed. In case of biometric parameters, under L30, L45 and L70 conditions average fresh shoot weight (25.27, 17.86 and 16.33 gm), dry shoot weight (6.20, 4.52 and 4.13 gm), fresh root weight (10.96, 7.31 and 7.39 gm), dry root weight (1.21, 0.88 and 0.91 gm) and root volume (13.33, 9.33 and 11.33 ml) were noted (Table 2). Among all these condition and media compositions, our results showed that L70 with M_1 growing substrate showed better results than other conditions.

Table 3 Profile of morphological features and biometric parameters of secondary hardened pomegranate plants after 60 days of duration

L	M	Plant height (cm)	Stem girth (mm)	No. of nodes	No. of leaves	Fresh shoot weight (gm)	Dry shoot weight (gm)	Fresh root weight (gm)	Dry root weight (gm)	Root volume (ml)
L30	M_1	5.96^{abc}	3.50^{a}	26.13^{ab}	48.00^{ab}	44.97^{ab}	10.73^{abc}	7.99^{cd}	1.35^{def}	13.33^{b}
	M_2	5.16^{c}	2.99^{cd}	24.53^{bc}	44.87^{bc}	41.23^{bc}	10.14^{abc}	10.13^{abc}	1.67^{abcde}	12.67^{bc}
	M_3	6.06^{abc}	2.99^{cd}	16.53^{f}	28.07^{e}	27.90^{fg}	7.60^{de}	7.46^{d}	1.30^{ef}	11.33^{bcd}
	M_4	6.54^{ab}	3.05^{cd}	26.67^{ab}	49.80^{ab}	40.51^{bcd}	11.15^{ab}	11.43^{a}	2.01^{a}	16.00^{a}
L45	M_1	6.43^{ab}	3.24^{abc}	28.80^{a}	53.07^{a}	48.47^{a}	11.93^{a}	6.71^{d}	1.22^{f}	11.33^{bcd}
	M_2	5.73^{bc}	3.04^{cd}	25.60^{ab}	44.87^{bc}	36.96^{cde}	9.65^{bc}	7.60^{d}	1.47^{cdef}	10.67^{cd}
	M_3	5.71^{bc}	2.61^{e}	17.60^{ef}	29.00^{e}	24.66^{g}	7.22^{e}	8.52^{bcd}	1.62^{bcde}	12.00^{bcd}
	M_4	5.83^{abc}	2.75^{de}	27.73^{ab}	44.53^{bc}	33.88^{def}	9.28^{bcd}	10.56^{ab}	1.76^{abc}	12.67^{bc}
L70	M_1	6.76^{a}	3.42^{ab}	25.07^{b}	38.47^{cd}	41.54^{bc}	11.84^{a}	8.32^{bcd}	1.41^{cdef}	11.33^{bcd}
	M_2	6.41^{ab}	3.17^{abc}	20.27^{de}	28.93^{e}	32.36^{g}	9.04^{cde}	8.18^{cd}	1.59^{bcdef}	10.00^{d}
	M_3	6.26^{ab}	3.09^{bc}	18.67^{def}	26.80^{e}	28.99^{fg}	9.20^{bcde}	7.27^{d}	1.86^{ab}	10.67^{cd}
	M_4	6.23^{ab}	2.92^{cde}	21.73^{cd}	32.00^{de}	$31.44e^{fg}$	9.10^{cde}	8.36^{bcd}	1.73^{abcd}	11.33^{bcd}
SEM		0.0321	0.0394	3.5878	16.084	16.3633	1.4585	1.7883	0.0507	1.989
L30		5.93^{b}	3.13^{a}	23.47^{a}	42.68^{a}	38.65^{a}	9.91^{a}	9.25^{a}	1.58^{a}	13.33^{a}
L45		5.92^{b}	2.91^{b}	24.93^{a}	42.87^{a}	35.99^{ab}	9.52^{a}	8.35^{ab}	1.52^{a}	11.67^{b}
L70		6.42^{a}	3.15^{a}	21.43^{b}	31.55^{b}	33.58^{b}	9.79^{a}	8.03^{b}	1.65^{a}	10.83^{b}
SEM		0.0321	0.0394	3.5878	16.084	16.3633	1.4585	1.7883	0.0507	1.989
M_1		6.38^{a}	3.39^{a}	26.67^{a}	46.51^{a}	44.99^{a}	11.50^{a}	7.68^{b}	1.33^{c}	12.00^{ab}
M_2		5.77^{b}	3.07^{b}	23.47^{b}	39.56^{b}	36.85^{b}	9.61^{b}	8.64^{b}	1.58^{b}	11.11^{b}
M_3		6.01^{ab}	2.90^{b}	17.60^{c}	27.96^{c}	27.18^{c}	8.00^{c}	7.75^{b}	1.59^{b}	11.33^{b}
M_4		6.20^{ab}	2.91^{b}	25.38^{a}	42.11^{b}	35.27^{b}	9.84^{d}	10.11^{a}	1.83^{a}	13.33^{a}
SEM		0.0321	0.0394	3.5878	16.084	16.3633	1.4585	1.7883	0.0507	1.989
L		6.09	3.06	23.28	39.03	36.08	9.74	8.54	1.58	11.94
M		6.09	3.06	23.28	39.03	36.08	9.74	8.54	1.58	11.94

Growing substrate M_1: Peat, M_2: Peat with cocopeat, M_3: Soil+cocopeat + PMC and M_4: Cocopeat + baggase and light intensities L_1: 30% light cutoff, L_2: 45% light cutoff and L_3: 70% light cutoff. 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

Growth after 60 days

The morphological and biometric parameters were studied after 60 days of planting in secondary hardening (Table 3). Under L30 light condition, highest average plant height, stem girth, numbers of nodes, total number of leaves, average fresh shoot weight, dry shoot weight, fresh root weight, dry root weight and root volume were observed in M_4 media composition 6.54 cm, 3.05 mm, 26.67, 49.80, 40.51 gm, 11.15 gm, 11.43 gm, 2.01 gm and 16.00 ml respectively. Our results showed that under the L30 condition, M_4 growing substrate was suitable for better growth of secondary hardened pomegranate plants as compared to other growing substrates like M_1, M_2 and M_3. Under L45 conditions, highest average plant height, stem girth, numbers of nodes, total number of leaves, average fresh shoot weight, dry shoot weight, fresh root weight, dry root weight and root volume were observed with M_1 growing substrate mix composition and the values were 6.43 cm, 3.24 mm, 28.80, 53.07, 48.47 gm, 11.93 gm, 6.71 gm, 1.22 gm and 11.33 ml respectively. Under L70, highest average plant height, stem girth, numbers of nodes, total number of leaves, average fresh shoot weight, dry shoot weight, fresh root weight, dry root weight and root volume observed with M_1 media composition were 6.76 cm, 3.42 mm, 25.07, 38.47, 41.54 gm, 11.84 gm, 8.32 gm, 1.41 gm and 11.33 ml respectively. Overall, the influence of light on plant height and girth was non-significant however root fresh weight and root volume were significantly high under L30. Among the growing substrates mixture M_1 and M_4 gave better results.

Effect of growing substrate and light on survival and saleable plants

There was good survival of plants (>99%) in all treatments with most of the treatments exhibiting statistically at par values. The percent mortality after 30 days of duration in secondary hardened pomegranate plants are shown in (Fig 1). The result showed that, the lowest mortality (0.45%) was observed in M_4 whereas as highest mortality was observed in M_2, under 30% light cut off condition. The mortality percentage under 45% light cut off showed lowest mortality (0.20%) in M_4 growing substrate and highest in M_2 growing substrate. While under 70% light cut off, minimum mortality was observed in M_4 (0.65%) and maximum mortality was observed in M_2 and M_3 growing substrate (Fig 1). No mortality was observed after 30 days.

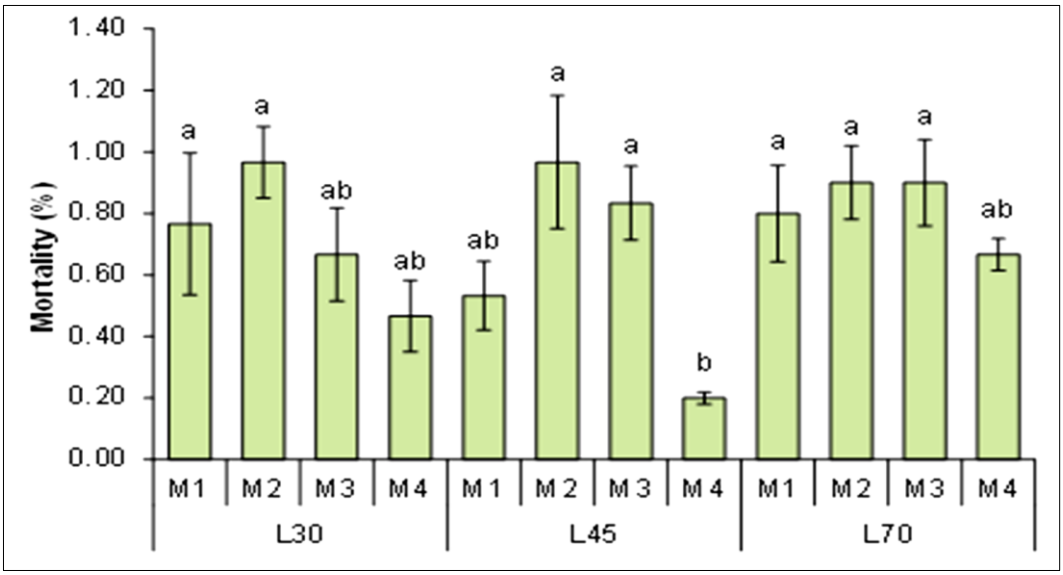

Fig 1 Mortality observations after 30 days of duration in secondary hardened Pomegranate plants.
Different letters superscript on the values indicates significant difference whereas the same letters indicate non-significant at P value 0.05.
Means were separated by DMRT

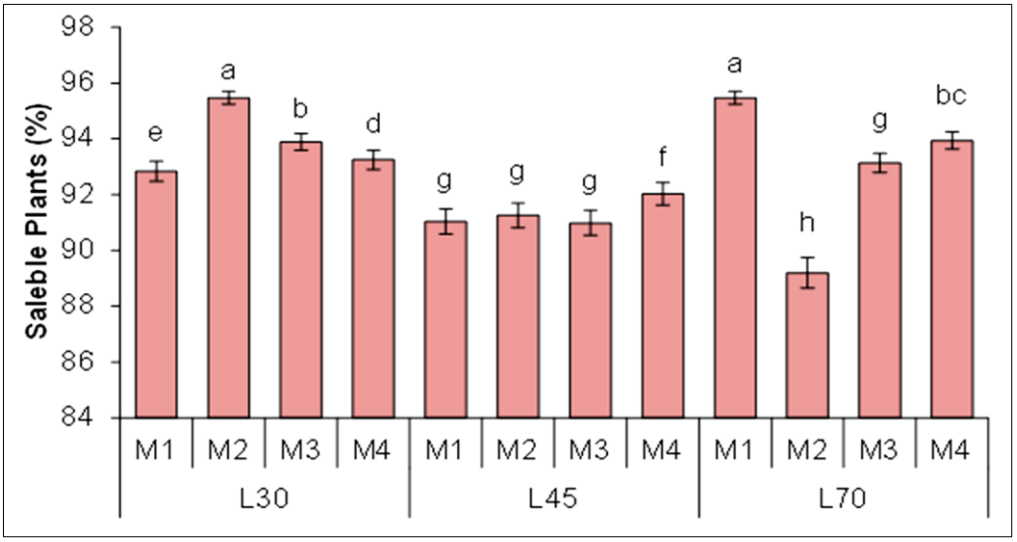

Fig 2 Saleable plants (%) after 45 days of duration in secondary hardened Pomegranate plants.
Different letters superscript on the values indicates significant difference whereas same letters indicate non-significant at P value 0.05.
Means were separated by DMRT

Days required to produce highest percent of saleable plant is the most important criteria of hardening process. Under L30 condition substrate M_2 resulted highest percentage (~ 96%) saleable plants whereas comparatively low in M_3 (~ 94%), M_4 (~ 93%) and M_1 (~ 92%) growing substrates. Similarly, under L45 condition M_4 media showed highest percentage (92%) saleable plants and under L70 M_1 growing substrate showed highest percentage (96%) of saleable plants (Fig 2). Our results suggests that, saleable plants after 45 days of duration were maximum (96%) in both L30 with M_2 and L70 with M_1 growing substrate whereas minimum (91%) in L70 with M_2 growing substrate.

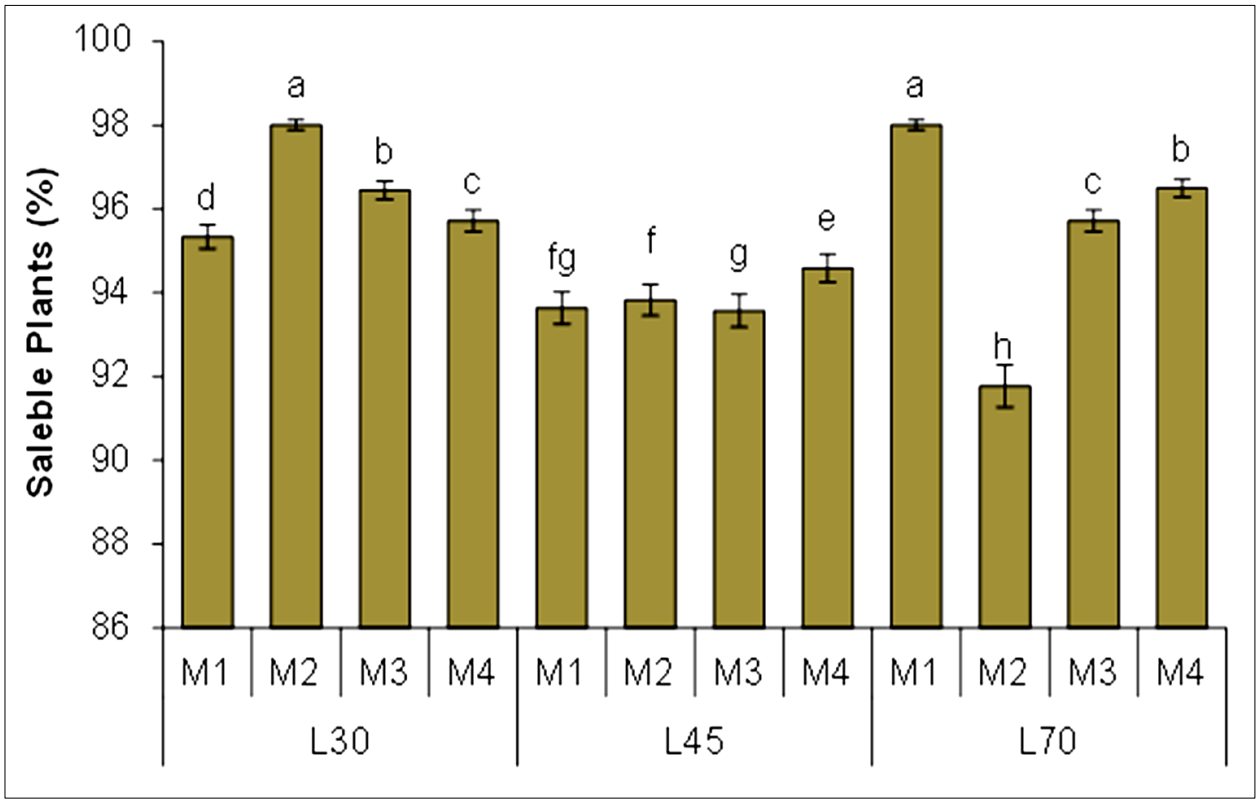

Fig 3 Saleable plants (%) after 60 days of duration in secondary hardened Pomegranate plants.
Different letters superscript on the values indicates significant difference whereas same letters indicate non-significant at P value 0.05.
Means were separated by DMRT

Saleable plants after 60 days of duration in secondary hardened pomegranate plants were found to increase (Fig 3). The results showed a maximum of 98% saleable plants in both L30 with M_2 and L70 with M_1 growing substrate while a minimum of 92% in L70 with M_2 growing substrate (Fig 3). From above observations we concluded that percentage of saleable plants was found to be higher significantly under L30 with M_2 and L70 with M_1 growing substrate as compared to other media compositions.

Acclimatization is the climatic adaptation of plantlets which has been moved to a newer environment [12]. For proper adaptation of plants (acclimatization) humidity and light intensity are the main factors to be controlled besides 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.

Use of different growing substrate for enhancement in growth and development of tissue culture plants was done in many plant species. The use of vermicompost and soil had resulted in high survival rate of tissue culture grown pomegranate plants [13-14]. Murkute *et al*. [15] achieved 50% survival of plants when transferred to vermicompost + soil mixture (1: 1). Mahishni *et al*. [16] 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*. [17] also obtained >90% success in hardening of rare pomegranate cv. 'Ruanzi' transfer to glasshouse conditions. The use of a glass jar with a polypropylene cap filled with peat: soil rite (1:1) was found most effective for hardening of pomegranate plants [18]. The results of present experiments are better than those of reported earlier. Use of peat for tomato plant cultivation was also found to be effective in comparison with other media types [19]. In growing substrate treatments produced statistically similar plant height whereas, plant girth in M_1 and M_4 was significantly better than M_2 and M_3. In comparison of plant height with stem girth, the plant height parameter showed much more noticeable changes than stem girth. The change in stem girth was not significant as every woody plant at its early stage of development does not form more secondary meristematic tissues [20].

The use of PMC in media enhances the overall agronomical traits of primary and secondary hardened *in vitro* banana plant which include leaf area, size as well as number of leaves [21]. In case of pomegranate primary hardening Patil *et al*. [22] found peat-based media most suitable, probably due to better air water ratio and nutrient availability.

CONCLUSION

The comparison of different media and light intensities and their effect on plant growth and development indicates that 30% light cutoff and combination of peat with cocopeat growing substrate is the most suitable for overall growth of pomegranate plantlets for secondary hardening process as it produces high number of saleable plants of good quality.

Author contribution statement

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

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