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A Rapid and Cost Effective Hardening Method for Tissue Culture derived Banana Plantlets

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Key words: *In vitro* culture, Musa sp., Robusta, Commercial propagation, Micropropagation, Acclimatization, Cost reduction

Abstract

The present study was aimed to develop a cost effective method for the hardening of tissue culture derived plants of banana var. Robusta. Two approaches were investigated to reduce cost. First, the shoots inoculated in rooting medium were incubated in (i) growth room under controlled temperature and light conditions for four weeks (RL) (ii) shadehouse exposed to natural light and ambient temperature (RP). The plants obtained from these treatments were given five hardening treatments (a) direct planting in polybags (filled with 1 : 1 Soil and leaf mould) (DP), (b) DP plants covered individually with transparent polybags (DPC), (c) DPC plants placed in tray of water (up to 3 cm height)-DPCW (d) DP plants kept in polytunnel (DPP) (e) DP plants placed in tray of water and kept in polytunnel DPWP, (f) control plants (CN)-primary hardened in potrays kept in polytunnel for four weeks followed by transplantation to polybags (filled with3:1 Soil and leaf mould). RP Plants were greener and sturdier with less hyperhydricity. Rooting outside the lab (RP) reduced the cost as the growth room space during rooting could be saved and so there is saving in electricity of lighting and climate control. 80% of plants in control and 50% plants in direct planting (DP) survived. In all other treatments 100% survival was observed. RP-DPCW and RP-DPWP plants showed the best performance in terms of visible appearance and accumulation of biomass. The method employed could reduce the hardening period from 12 weeks (CN) to 8 weeks. The cost reduction occurs in this process by a) omitting primary hardening process which requires costly potting media, manpower and climate controlled greenhouse, b) Lab space could be saved as incubation for rooting can be done in shade house thereby saving electricity c) As there is 4 weeks saving in hardening, shade house space could be utilized for hardening more plants. The method could be adopted for hardening in small

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nurseries to large industries and can result in cost reduction of plantlets. Saving of 4 weeks for hardening will be highly beneficial to the micropropagation industries for delivering plants to farmers in time during peak planting seasons.

Introduction

Bananas (*Musa* sp.) are well-known as energy-rich food with a good content of mineral salts and vitamins (Chandler 1995). It is probably the cheapest fruit available throughout the year. Main trouble in inconsistent planting of bananas is the difficulty in getting high-quality disease-free planting materials through field propagation. The farmer-produced suckers are good transmitters of insect pests and diseases. But this problem was overcome by micropropagation which is an efficient method for the mass production of morphologically, physiologically and genetically identical clones within a short time regardless of seasonal and other factors (Chandra et al. 2010, Singh 2002). *In-vitro* micropropagation is widely used for commercial propagation of bananas (Al-Amin et al. 2009, Lule et al. 2011). The advantages of TC grown banana over conventionally grown banana in yield and quality is well established (Bhojwani and Razdan 1983) and constant improvement in the productivity, profitability, stability and sustainability of the banana plantation system could be achieved (Rao 2005). As the plants are micropropagated under controlled conditions, they cannot be planted directly in the field. Susceptibility of the plants to environmental stress, the presence of poorly developed stomata, thin cuticles, hyperhydricity, and a partly heterotrophic nature are the common challenges of the banana hardening process (Hazarika et al. 2006, Chandra et al. 2010). Their direct plantation in field causes high mortality, resulting in loss to the farmers. Micropropagated plants are, therefore, slowly acclimatized for the field conditions in greenhouses and shade houses before being released to the farmers for plantation (Hazarika et al. 2003).

Micropropagation is a labour-intensive, skill-demanding, high cost technique and cost per plant becomes too high in comparison with the conventional planting material. The cost of TC plantlets becomes relatively high due to the requirement of laboratory process as well as long acclimatization period about 12weeks (Ikram-ul-Haq et al. 2007, Vasane and Kothari 2006, 2008). Main production costs of micropropagation technique are equipments, chemicals, glass-ware, greenhouse infrastructures and labour. Labour costs usually represent 40-50% of the total cost and electricity was second, at 20-30 % (Tomar et al. 2008).

Banana plant production via low cost technology by improving process efficiency and better utilization of resources is reported by Savangikar et al. (2002) and Savangikar (2004). Low cost option should reduce the rate of tissue cultured plantlets without compromising the quality of plants (Prakash et al. 2004). Ganapathi et al. (1995) achieved cost reduction of 31.2% by the use of tap water, Sugar and reduced salt components in tissue culture medium for banana micropropagation. The utilization of culture media,

vessels and low cost substitutes for tissue culture plant production was successfully investigated in several other species (Sujatha and Chandran 1997, Varshney et al. 2000, Kadota and Niimi 2004, Piatezak et al. 2005, Anonymous 2004, 2010, Hung et al. 2006, Huang and Lee 2010). Batool et al. (2020) reported the effects of different potting media during hardening of tissue-culture raised banana plants. Suada et al. (2015) reported the phytostimulatory and hardening period reducing effects of plant associated bacteria on micropropagated *Musa acuminata* var. Grand Naine. It is necessary to develop low cost technologies by improving the process efficiency and better utilization of resources. Keeping the above facts, the current study was aimed to evaluate the cost-effectiveness of the hardening without use of the costly greenhouse infrastructures and standardize a direct hardening method for the production of banana plants tissue culture (TC) derived.

Materials and Methods

Banana (*Musa acuminata* var. Robusta) shoot tip cultures were initiated from suckers that were collected from a farmer's field. In the laboratory, suckers were trimmed to 3-4 cm^3 pieces containing the intact shoot meristem. They were surface sterilized with 0.1% (w/v) mercuric chloride for 10 min followed by several washes with sterile distilled water. Leaf-sheaths were removed, and the suckers were trimmed further into 0.5-1.0 cm^3 pieces containing intact meristems. Shoot tips were then inoculated onto MS medium supplemented with 13.31 μM 6- benzyl amino purine 30 g/l sucrose, and 7 g/l agar MS. Cultures were incubated at 27°C under a light intensity of 50-60 $\mu mol\ m^{-2}s^{-1}$ with a 16- h light (fluorescent tube lights - Philips, India)/8-h dark photoperiod. Shoot multiplication was done by splitting shoot clumps into 2-3 pieces and inoculating the pieces onto fresh MS medium. Multiple meristem cultures were regularly subcultured at 4-wk intervals. After getting the desired number of plantlets from multiple shoot cultures, in vitro rooting of shoots was carried out. For this, uniform-sized shoots with a base diameter of 0.6-0.7 cm and at least three leaves were separated and removed from the shoot cultures. Five shoots were transplanted in each bottle containing rooting media (MS + 1 mg/l Indole 3-butyric acid-IBA).

In order to develop a faster and cost effective hardening process, the following treatments were given to TC plants. First, the plants under rooting treatments were kept in (i) growth room under controlled temperature and light conditions for four weeks-(RL) (ii) Shade house exposed to natural light and ambient temperature (RP). The plants obtained from these treatments were given five hardening treatments (a) direct planting in polybags (DP), (b) DP plants covered individually with transparent polybags (DPC), (c) DPC plants placed in tray of water (water upto 3 cm height)-DPCW (d) DP plants kept in polytunnel (DPP) (e) DP plants placed in tray of water and kept in polytunnel-DPWP, (f) control plants-primary hardened in potrays filled with cocopeat kept in polytunnel for four weeks followed by transplantation to polybags filled with 3:1 soil and leaf-mould (CN). All hardening trials were conducted at the Botanical garden of University College,

Thiruvananthapuram. Rooted plants (both RL and RP) with a minimum of three roots and a height of 4-5 cm were removed from culture bottles. The agar sticking to roots was removed by washing carefully with tap water. For direct planting trials the washed plantlets were directly planted in polybags (20 cm height, 11 cm diameter) filled with soil and leaf mould (3:1). Every treatment includes ten replicates, were watered daily. The trays were prepared from tarpaulin sheets and it was filled with water at 3 cm height. Transparent polythene sheets (100 microns) were used for making polytunnels. 30 ml of chemical fertilizer solution (4 g/l of Factamfos (N-20: P-20: S-15) and 1 g/l of Potash and 1 g/l micronutrients) were applied to the polybags in 4 days interval. The transparent polytunnel and water trays were removed after 14 days. All the plants were kept in a shade house and watered daily. Control plants were planted in polybags after 4 weeks of growth in potrays and maintained in polyhouse with regular watering and fertilizer application.

The experiment was monitored daily and data were recorded weekly on plant growth. The growth parameters like percentage of survival, the height of the plants, and number of the leaves, length and width of the longest leaf, fresh weight, root number, and root length were recorded after 8 weeks (direct planting) and 12 weeks in control plants. All the data were statistically analyzed by one-way ANOVA, and the significant variations among the means were analyzed by Duncan's multiple range test. The statistical analyses were carried out by using the software SPSS 13.0 for Windows.

Results and Discussion

In the present study we have tried to substitute the TC laboratory condition to shade house environment for rooting, where the shoots were transferred to rooting medium and sterile bottles were transferred to shade house and incubated in shade house for 4 weeks (RP). This was compared to standard conditions of 4 weeks incubation of cultures in rooting medium in growth room (RL). The RP plants looked greener and shorter in cultures after 4 weeks, in comparison to RL plants (Fig. 1A-D). The humidity in shade house was 80-95%. The temperature was monitored in shade house and was about 22-32^{o}C. The acclimatization of RP and RL plants in different treatments were comparable. But as the growth room space, electricity for cooling and lighting was not required in the case of RP plants and so this method is cost reducing. Chen (2016) demonstrated the Cost analysis of micropropagation of Phalaenopsis, and found that most of the electrical energy is used for cooling (74% electricity consumption).

CN plants of RP group also showed 100% survival. On the other hand, the CN plants of RL group showed 80% survival. Probably the RP group plants got adapted to the fluctuating temperature and humidity conditions during rooting in the shade house. DP plants of RL group showed less survival rate (50%) compared to the DP plants of RP group (70%). This shows that RP group plants are hardier compared to that of the RL group plants. The incubation of culture bottles in shade house conditions is better than

that in growth room in terms survival of plants during hardening. The direct hardening with individual plants top covered with transparent plastics sheet and with or without water trays for the first 3-4 weeks was the most effective way to maintain high humidity around the plants. All plants of direct planting treatments survived except plants of DP and CN treatment. In treatments other than DP and CN treatments, the plants were protected from variations of humidity by covering with individual polythene covers or kept inside a polytunnel. Here, we have also observed the effect of humidity on plant growth, as treatments covered with plastic bags had the highest degree of growth and no leaf tip burning. This is because closed plastic bags trap moisture in the vicinity of plants while leaving them open will lose moisture through transpiration. From this it is clear that for initial acclimatization, proper humidity must be available constantly around the plant.

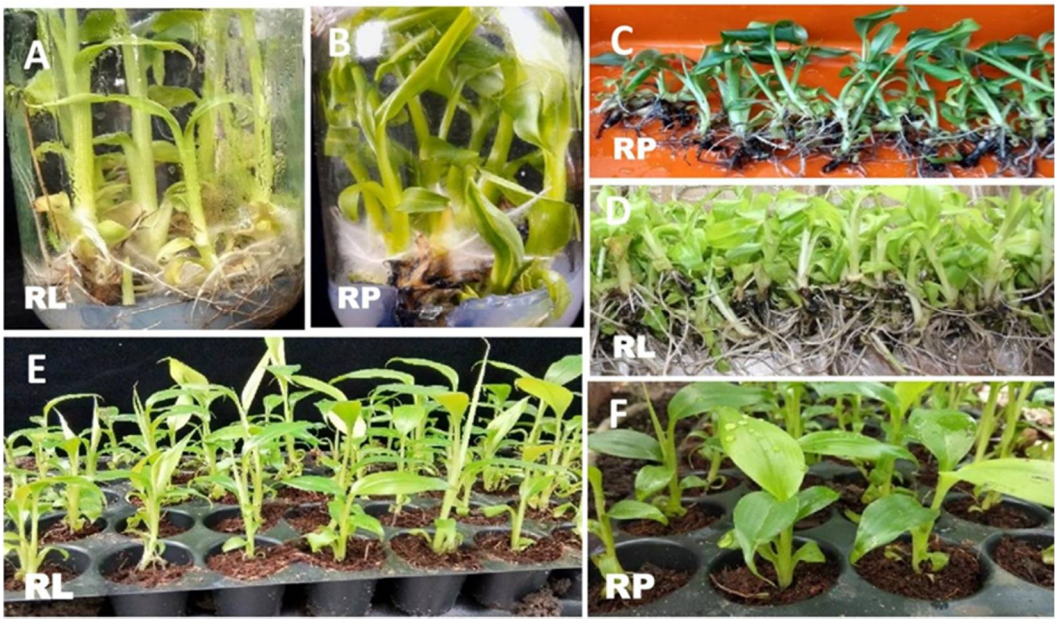

Fig. 1. (A & B) Banana plantlets of RL (in culture room) and RP (in polyhouse) in rooting after 4 week's incubation; (C & D) Uprooted banana plantlets from RP and RL treatments; (E) Primary hardened banana plants of RL-control group; (F) Primary hardened banana plants of RP-control group .

Plants reaching a height of about 1ft are considered as a marker for completion of hardening period and readiness of the plants for planting out in the field. All the plants of the direct planting treatments reached 1ft height within 8 weeks. On the other hand, the control plants of both RL and RP group took 12 weeks to achieve the hardening. Probably in CN plants, the plants produced roots in the primary hardening process are thinner due to limited availability of the space and medium within the potrays. The roots are found to completely fill up the cavity of the potrays and reached stagnation in

shoot growth also. The fertilizer applied also will not be retained in the limited amount of medium. Most of these roots are replaced in the secondary hardening step and the plant has to restart it shoot growth phase and take the full period 8 weeks for the hardening to achieve the required height. In the case of direct planting method, the plants are directly exposed to the soil and the sufficient space available for the plant to sustain the growth. Initially the roots produced are thin. But roots produced after 2-3 weeks are sturdier and longer. The shoot growth does not reached stagnation as sufficient water and nutrients are available in the potting medium or are supplied as fertilizer mix which will be more retained in the soil matrix.

After 8 weeks of direct hardening the banana plants reached a size for field plantation. The observed response was highly significant as the normal hardening period of banana is about 12 weeks. Shoot growth parameters, showed comparable trend within the RL and RP groups except control. Plant height was found significantly higher in DPCW in both RL and RP when compared to control at eighth week (Figs 2-3). All the growth characteristics were observed to be highest in DPCW of RL and RP treatments proved positive effect of direct hardening with top covering and keeping in water tray.

Fig. 2. Growth of tissue cultured banana plants of RL group after 8 weeks of planting (A) CN-RL*; (B)- DPC-RL; (C) DPCW-RL; (D) DP-RL; (E) DPP-RL; (F) DPWP-RL. (The bar represents 5 cm). *CN-RL photos were taken after 12 weeks of planting

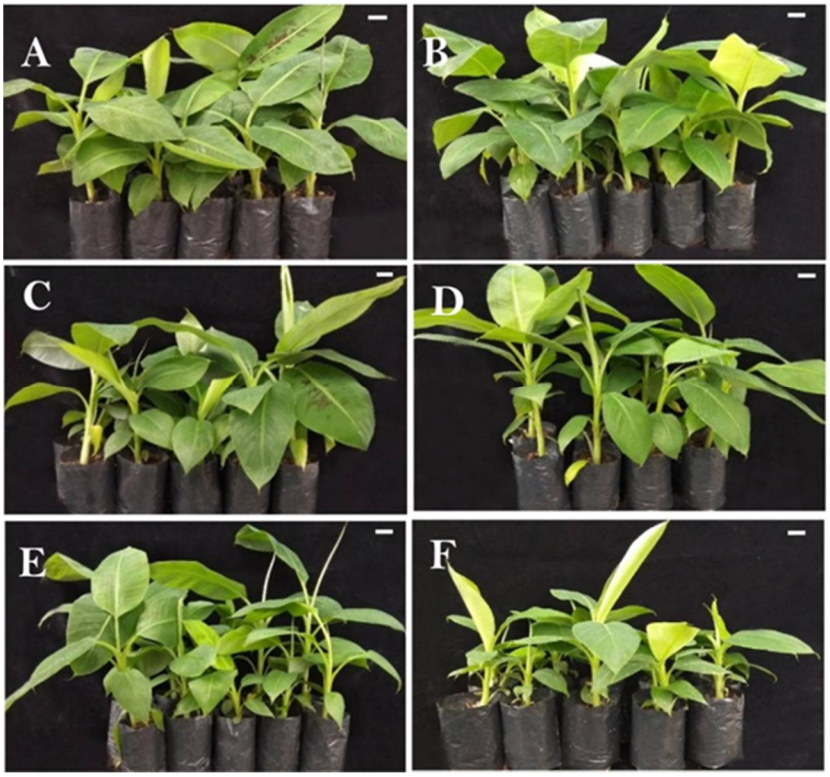

Fig. 3. Growth of tissue cultured banana plants of RL group, after 8 weeks of planting. (A) CN-RP*; (B) DPC-RP; (C) DPCW-RP; (D) DP-RP; (E) DPP-RP; (F) DPWP-RP. (The bar represents 5 cm).* CN-RP photos were taken after 12 weeks of planting

The plants of RL-DPCW group attain more leaf length (24.50 ± 1.08) and width (12.10 ± 0.53) which was almost on par with RP-DPCW (24.12 ± 0.32 and 11.61 ± 0.55). Number of leaves in RL-DPCW (8.10 ± 0.27) was comparable to that of RP-DPCW (Table 1). The leaf length and plant height of RP-DP were comparable with that of RL-DP. Plants of all the treatments of RL and RP were significantly better than that of CN (at the 8th week). The study indicates that simple changes in culture environmental conditions during hardening significantly improved the overall survival rate and cost reduction in *in-vitro* propagated banana plants during lab to land transfer.

The banana plants showed good root growth in all treatments of both RL and RP groups. The mean root length in DPC, DPP, DPCW of RL and RP were comparable and were significantly different from that RL-DP and RP-DP treatments (Table 2). The total root length of DPC, DPCW, DPP, DPWP of RP group showed comparable performance with respect to total root length of DPCW, DPP, DPWP of RL group, which were significantly different from that of CN (Table 2, Fig. 4). The total root length in plants of RL-DP and RP-DP treatments were comparable. Total root length was found significantly higher in DPCW of both RL and RP and was not significantly different from RL-DP, RL-

DPWP, RP-DPC, RP-DPP and RP-DPWP plants. While analyzing the fresh weight, both RL and RP plants attained significantly higher fresh weight than CN and DP plants. The fresh weight of RL-DPCW (60.88 ± 4.57) which was almost at par with RL-DPCW (59.72 ± 4.96) and was significantly different from that of DP plants (Table 2). The fresh weight of RL-DP treatment was comparable to CN and RP-DP group. Increase in root length has potential to collect more nutrients from soil and an increase in biomass indicated plant vigour, which could have bearing on banana yield.

Table 1. Growth parameters of tissue cultured banana plants under hardening. Data were recorded on 8th week of planting (except CN treatment where it was recorded after 12 weeks).

Rooting method	Treatment code	Method	Total hardening period	Survival %	No. of leaves*	Height of plants*	Length of leaves*	Width of leaves*
RL	T20	CN#	12	80	7.60 ± 0.33	37.41 ± 1.72	21.68 ± 0.85	10.14 ± 0.39
	T23	DP	8	50	7.00 ± 0.36	37.59 ± 2.43	20.93 ± 2.01	10.00 ± 0.88
	T21	DPC	8	100	7.50 ± 0.40	37.09 ± 2.59	24.32 ± 1.10	11.73 ± 0.28
	T22	DPCW	8	100	8.10 ± 0.27	42.93 ± 2.34	24.50 ± 1.08	12.10 ± 0.53
	T24	DPP	8	100	6.75 ± 0.55	39.46 ± 3.70	21.56 ± 1.54	10.50 ± 0.78
	T25	DPWP	8	100	7.09 ± 0.25	38.97 ± 3.20	21.06 ± 1.45	10.76 ± 0.70
RP	T31	CN#	12	100	6.60 ± 0.37	36.10 ± 2.78	24.35 ± 1.21	11.10 ± 0.37
	T28	DP	8	70	7.50 ± 0.26	39.60 ± 2.74	22.30 ± 1.75	10.65 ± 0.52
	T26	DPC	8	100	7.50 ±0.25	43.70 ± 1.65	20.90 ± 1.40	10.95 ± 0.17
	T27	DPCW	8	100	7.75 ± 0.26	40.70 ± 3.02	24.12± 0.32	11.61 ± 0.55
	T29	DPP	8	100	7.40 ± 0.22	41.90 ± 1.98	21.90 ± 0.79	10.25 ± 0.38
	T30	DPWP	8	100	6.90 ± 0.23	40.30 ± 3.54	21.95 ± 1.63	9.65 ± 0.67

RL – Incubation of plants in rooting under lab conditions, RP- Incubation of plants in rooting under shade house. CN- Control plants – primary hardened in potrays kept in polytunnel for 4 weeks followed by 8 weeks secondary hardening in shade house; DP- direct planting in polybags; DPC- DP plants covered individually with transparent polybags; DPCW - DPC plants placed in tray of water (water upto 3 cm height); DPP- DP plants kept in polytunnel; DPWP - DP plants placed in tray of water and kept in polytunnel.

* The values are Mean ± Standard error of 10 replications. The values are not significantly different from each other by ANOVA ($P \leq 0.05$). #CN plants –data recorded after 12 weeks.

Among all the hardening treatments investigated, direct hardening of plantlets with top covering and, keeping in water tray exhibit the best result with regards to the growth parameters. After 8 weeks of planting all the growth parameter of control plants were significantly less than the other hardening methods. It is obvious from the result that direct planting group without covering to maintain moisture resulted in loss of plants and poor initial growth, compared to covered plant groups. The hardening treatments of RL and RP found to provide comparable results in growth parameters via direct hardening.

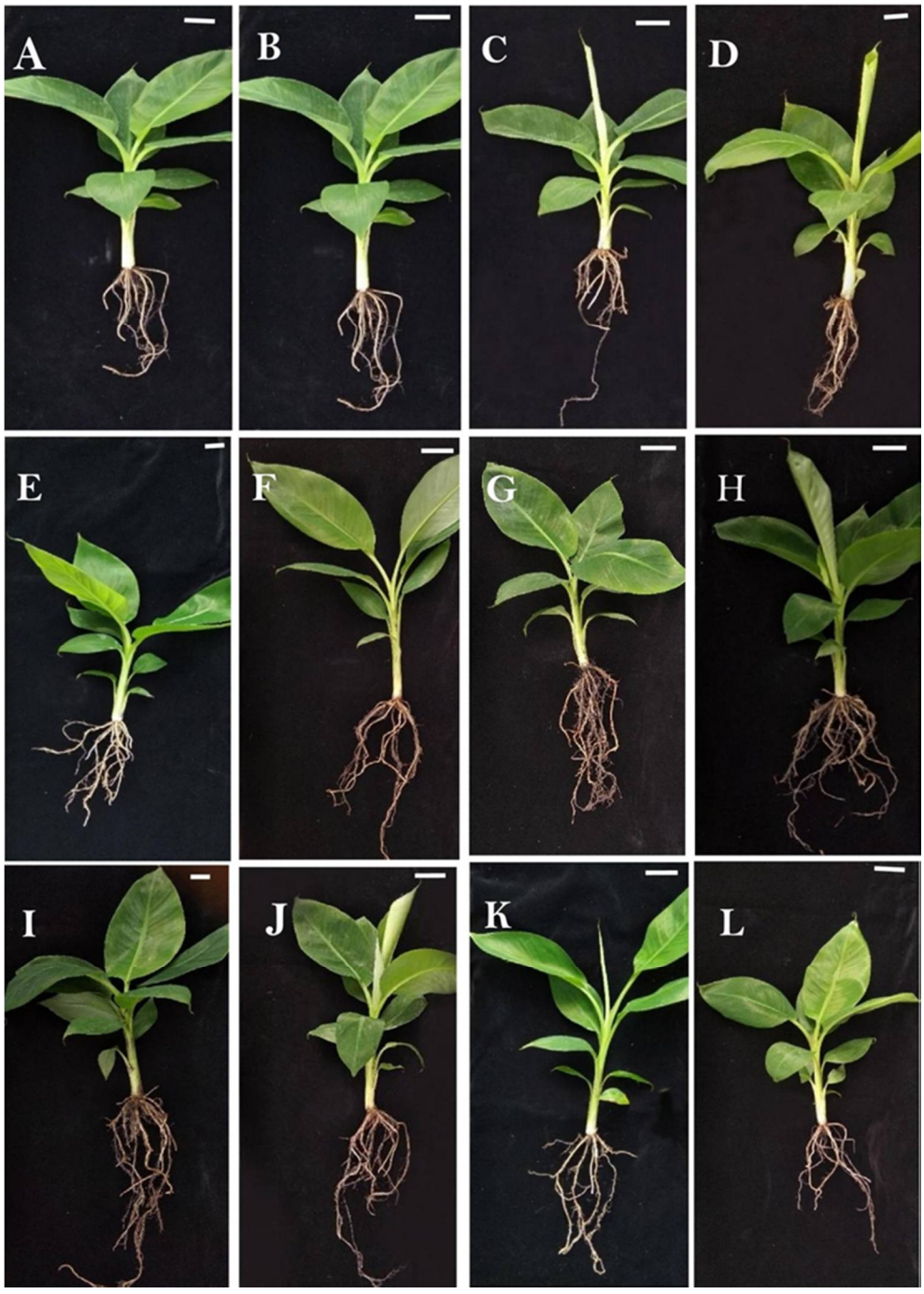

Fig. 4. Root system of tissue cultured banana plants of RP and RL, after 8 weeks of planting, Control after 12 weeks. (A) DPWP-RP; (B) DP-RP; (C) DPC-RP; (D) DPCW-RP; (E) DPP-RP; (F) CN-RP; (G) DPWP-RL; (H) DP-RL; (I) DPC-RL; (J) DPCW-RL; (K) DPP-RL; (L) CN-RL. (The bar represents 5 cm).

Table 2. Root parameters and fresh weight of hardened plants (after 8 weeks of planting)

Rooting Method	Treatment	Treatment code	Mean root length (cm)*	Total rootlength/ plant (cm)*	Fresh weight (gm)*
RL	CN#	20	9.80 ±1.39 c	125.40 ± 5.68 d	44.38 ± 1.61 bc
	DP	23	10.60 ± 0.67 c	138.40 ± 6.40 cd	41.96 ± 5.64 c
	DPC	21	11.20 ± 0.58 bc	154.20 ± 4.18 bcd	53.20 ± 4.93 abc
	DPCW	22	13.00 ± 0.44 ab	185.60 ± 6.80 a	59.72 ± 4.96 a
	DPP	24	11.40 ± 0.67 abc	158.60 ± 12.67 abc	49.10 ± 2.68 abc
	DPWP	25	12.80 ± 0.58 ab	182.40 ± 12.19 ab	55.36 ± 3.77 ab
RP	CN#	31	10.20 ± 0.37 c	127.20 ± 6.71 d	44.24 ± 3.62 bc
	DP	28	10.20 ± 0.80 c	139.00 ± 5.84 cd	44.12 ± 5.74 bc
	DPC	26	11.40 ± 0.40 abc	170.4 ± 7.75 ab	49.68 ± 5.26 abc
	DPCW	27	13.40 ± 0.40 a	188.00 ± 17.70 a	60.88 ± 4.57 a
	DPP	29	11.60 ± 0.24 abc	160.80 ± 7.95 abc	48.74 ± 2.97 abc
	DPWP	30	13.20 ± 0.58 ab	180.20 ± 11.27 ab	57.18 ± 4.69 ab

RL – Incubation of plants in rooting under lab conditions, RP- Incubation of plants in rooting under shade house. CN- Control plants-primary hardened in potrays kept in polytunnel for 4 weeks followed by 8 weeks secondary hardening in shade house; DP- direct planting in polybags; DPC- DP plants covered individually with transparent polybags; DPCW-DPC plants placed in tray of water (water upto 3 cm height); DPP- DP plants kept in polytunnel; DPWP - DP plants placed in tray of water and kept in polytunnel.

* The values are Mean ± Standard error of 10 replications. The values followed by the same alphabet are not significantly different from each other by Duncan's Multiple Range Test ($P \leq 0.05$).

#CN plants –data recorded after 12 weeks.

The cost reduction option should lower production costs without compromising on plant quality (Stephen and Dhanalakshmi 2014). *In vitro* growing plants of banana and potato, kept at 16-41°C at 750 µmol $m^{-2}S^{-1}$ under natural light, showed as good or better growth than in the controlled growth room (Kodym and Zapata-Arias 1999). Reduction in the cost of energy and labour in one step hardening could lower the production cost of micropropagated plants (Kodym and Zapata-Arias 2001). In the present study shade house incubation of cultures in rooting eliminates the requirement of electricity for lights, cooling for 4 weeks and the growth room space (which will be very scarce during peak production period). The growth room space could be utilized for production of more plantlets. The direct planting in polybags can save time and the growth of the banana plants was better. The elimination of primary hardening phase saved 4 week's labor cost, saving on costly potting media (vermiculite, perlite, coco peat, etc.), fertilizers and agrochemicals. The present study demonstrates that for conventional hardening (two step hardening - primary hardening and secondary hardening) is not essential for micropropagated banana. The control plants need extra 4 weeks to attain field plantable size in comparison with other treatments. The effective settlement of micro propagated

seedlings is strongly influenced by the physical, chemical, and biological properties of the potting mixture (Kansara et al. 2013). The choice of medium for hardening in tissue cultured plants is optional for laboratories (Cronauer and Krikorian 1984, Zimmerman 1991, Zimmerman and Fordham (1985), Zimmerman and Bhardwaj 1995). A previous study by Agrawal et al. (2010) showed that cost-effective *in vitro* production of banana could be achieved using substitutes of gelling agent (Isabgol) and carbon source (market sugar). In the present study we have directly transplanted the plantlets into soil - leaf mold mixture and it shortened the hardening process. This has also saved media cost in primary hardening.

Our result suggested that direct hardening is the best method which resulted in hardening within 8 weeks and plants appeared healthy by using cheap potting media (soil-leaf mould). The rate of survival was 100%. The low cost option should lower the cost of production without compromising the quality of the micropropagules and plants (Prakash et al. 2004). Tissue culture raised plants have an average hardening period of 12 week (Vasane and Kothari 2006, 2008, Choudhary et al. 2014). In the present study, hardening of tissue cultured banana Var. Robusta could be achieved within 8 weeks through direct hardening method. Keeping plants in the nursery for a long time means more work, more fertilizer, and more water. In addition, the costly greenhouse space could be utilized for hardening more plants in the same space. Also, the two-stage hardening requires more effort and a special medium in a temperature-controlled greenhouse/polyhouse for the primary hardening. This step can be omitted and significant cost savings can be achieved. It took only 8 weeks to prepare the plants with cent percent survival for transplantation into the field, reducing the hardening time by 4 weeks.

The present study has demonstrated a suitable alternative for hardening of TC derived banana plants, which resulted in saving time, growth room space, resources, electricity, shade house space and labour costs. When compared to the two-step hardening method (primary hardening followed by secondary hardening) used by commercial producers, the process of straight polybag planting saved four weeks. The methodology could be utilized for cost effective hardening of tissue cultured banana plants in commercial tissue culture facilities. The method could be adapted for hardening of tissue culture derived plants of other plant species also.

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