Plant Tissue Cult. & Biotech. **34**(1): 25-33, 2024 (June) **ISSN 1817-3721, E-ISSN 1818-8745**
DOI: https://doi.org/10.3329/ptcb.v34i1.74355

PTC&B

Development of a Suitable *in vitro* Regeneration Protocol for Rahangala Pear *(Pyrus pyrifolia* L.) in Sri Lanka

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Keywords: Pear, Rahangala, Plant growth regulators, Shoot multiplication, Root induction, Acclimatization

Abstract

The limitation of planting material is the major drawback to expanding pear cultivation in Sri Lanka. Hence, an *in vitro* protocol was developed to produce planting material for the pear variety of Rahangala. Pear shoots were used as explants and surface sterilization of explants was done using 0.5% and 1% (w/v) $AgNO_3$ for 15 min, 5% and 10% (v/v) NaOCl for 5, 10 and 15 min. *In vitro* shoot multiplication and root induction were found to be suitable in full and half strengths of MS medium respectively supplemented with various concentrations of BAP (1, 1.5, 2, 2.5, 3 mg/l), IBA (0, 0.5, 1, 2, 3, 4 mg/l) and NAA (0.1 mg/l). Acclimatization was conducted in pots containing different proportions of topsoil, compost, coir dust and sand. Completely Randomized Design (CRD) was used as an experimental design for this study. During sterilization of explants, the survival rate of 86.6% was recorded after 3 weeks where the treatment was done with 1% $AgNO_3$, and 10% NaOCl for 10 min. BAP (3.0 mg/l) produced the best shoot multiplication (1.46 ± 0.2). Significantly ($p<0.05$), the highest mean number of roots (1.93 ± 0.98), average length (60.6 mm), and rooting percentage (68%) were observed in ½ MS medium with 4.0 mg/l IBA. After five weeks of transplantation in pots, 100% of plantlets survived in topsoil, compost, coir dust, and sand in a ratio of 1:1:1:1.

Introduction

The pear is the third most important temperate fruit in world production and belongs to the family Rosaceae. In Sri Lanka, the *Rahangala* variety has been recommended for cultivation by the Department of Agriculture, Sri Lanka which is a high-quality and high-yielding cultivar in Sri Lankan conditions (Wijeratne 2005). In Sri Lanka, the expansion of pear cultivation was limited due to the lack of planting materials.

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However, the pear cultivars presently grown in Sri Lanka do not produce seeds in adequate quantities and also seeds cannot be used when producing true-to-type planting materials due to extreme genetic variations in seeds. The main advantage of tissue culture technology is that high-quality and uniform pear planting materials can be obtained on a year-round basis under disease-free conditions irrespective of season and weather (Loberant and Altman 2010). This method enables to production of more planting materials in a shorter period with less labour input and at a lower cost (Bommineni et al. 2001).

In Sri Lanka, the traditional propagation of pear by grafting with a wild variety *Gommelikanda* is not completely satisfactory due to the difficulties of graft compatibility resulting in low survival rates of 30-40%. The success rate of grafting was only about 30% of rootstocks from cuttings (personal communication). Most of the commercial pear plantations in Sri Lanka are now 50-65 years old and have ceased their commercial-bearing life. Therefore, it is recommended new cultivation of pears, but the cultivation of large numbers of trees through conventional methods results in a high initial cost. Tissue culture techniques, particularly micro-propagation play a vital role in the production of a large number of self-rooted clones of pear cultivars. This eliminates the challenges involved in budding and grafting (Haq and Kaloo 2010). Thus, the study was focused to develop a suitable protocol for production of planting materials locally grown Rahangala pear through plant tissue culture techniques.

Materials and Methods

Shoots were collected from field-grown Rahangala pear trees in Agriculture Research Station, Rahangala, Sri Lanka. Shoots were cut into 5 cm lengths containing 2-3 axillary buds. In the entire cultures, MS basal medium was used. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH /HCl. Media-containing jars were subjected to sterilization using an autoclave (SA-300VF- F-A500) at 121°C under 1.06 kg/cm^3 steam pressure for 20 min. Cultures were incubated at 23 ± 1°C under 16 hrs photoperiod using white fluorescent bulbs (1000 Lux) in a culture room.

The shoot explants were prepared by removing leaves. Initially, explants were put into the beaker and subjected to running tap water for 30 min. to eliminate surface contamination. Then explants were washed three times using liquid Vim (a detergent) and tap water with adding 2-3 drops of Tween 20 for 15 min. followed by 3 washes in distilled water. Then excised shoots were disinfected with 0.06% Topsin fungicide for 1 hour and subsequently, subjected to 70% ethanol for 1 min. Next, immersion in 600 mg/l ascorbic acid for 3 min was done. Finally, washed with 10% H_2O_2 for 3 min and rinsed with sterilized distilled water after applying each disinfection step. After surface sterilization, twelve treatments were laid by treating shoots in two concentrations of w/v = 0.5% and 1% of Silver nitrate ($AgNO_3$) for 15 min. followed by washing with sterilized distilled water (Table 1). Subsequently, explants were sterilized by shaking with two

different concentrations of v/v = 5% and 10% of NaOCl for 5, 10, and 15 min. by adding 2 drops of Tween 20. Finally, shoots were washed three times with double autoclaved distilled water. The experiment was done in 3 replicates with 15 samples in each replicate.

Table 1. Surface sterilization of pear shoots using different concentrations of $AgNO_3$ and NaOCl treatment by 15 min.

Treatments (T)	Silver nitrate (%) (mg/l)	Sodium hypochlorite (%) (mg/l)	Exposure time for NaOCl (min.)
Control	0	0	0
T1	0.5	5	5
T2	0.5	5	10
T3	0.5	5	15
T4	0.5	10	5
T5	0.5	10	10
T6	0.5	10	15
T7	1.0	5	5
T8	1.0	5	10
T9	1.0	5	15
T10	1.0	10	5
T11	1.0	10	10
T12	1.0	10	15

Surface sterilized (from the best sterilized method) shoots obtained from the previous experiment were sub-cultured into MS medium containing different concentrations (1, 1.5, 2, 2.5 and 3 mg/l) of benzil amino purine (BAP). The average number of shoots and shoot length were recorded after four weeks of culturing. Selected multiplied shoots with 2.0 cm length were transferred to the MS medium with six different concentrations (0, 0.5, 1, 2, 3 and 4 mg/l) of indole-3-butyric acid (IBA) with 0.1 mg/l naphthalene acetic acid (NAA) in full and half strength of MS media. All treatments were replicated 3 times with 15 samples in each replicate. Rooted plants were removed from the medium and rinsed with sterilized distilled water. Plants were transferred to pots containing different combinations of sterilized topsoil, compost, coir dust and sand in 4 treatments with 5 replications mentioned in Table 2. 100 ml of 1 mg/l Captan fungicide solution and 100 ml of 1 mg/l Albert's liquid fertilizer solution were added to each pot. All the pots were covered with a single propagator and placed in a protected house. All experiments were arranged according to the Completely Randomized Design

(CRD) and collected data were analyzed statistically and evaluated by ANOVA procedure using R Studio with 95% confidence interval and mean separation was done by using the least Significant Difference (LSD) test. In Figs 3-4 bars denoted by the same letters are not significantly different from each according to the least significant difference at 0.05 level of probability.

Table 2. The treatment composition of acclimatization of pear plant.

Treatment	Media Composition
T1	Topsoil 1: Compost 1: Coir dust 1: Sand 1
T2	Compost 1: Sand 1
T3	Sand 1 : Coir dust 1
Control	Topsoil

Results and Discussion

Several disinfection treatments were evaluated involving $AgNO_3$ and NaOCl. Scare information is available about the use of $AgNO_3$ as a disinfectant in *in vitro* shoot cultures of pear. According to Wegayehu et al. (2015), NaOCl hampers the growth rate of fungi and bacteria which leads to reduced contaminations on growth media. Also, Nacheva and Ivanova (2017) indicated that $AgNO_3$ has a great ability to inhibit microorganisms and act as an antimicrobial agent. Therefore, the present study developed a new procedure of surface sterilization for pear shoots. In the same experiment, three weeks after the incubation of explants, 100% contamination was observed in the control treatment. The highest incidences of fungal and bacterial contamination were observed after 10-20 days from incubation. Fungal contaminations were reduced in treatments containing 1% $AgNO_3$ with 10% NaOCl.

The maximum number of surviving shoots after three weeks of culture establishment was recorded in treatment 11 (1% $AgNO_3$ + 10% NaOCl for 10 min. at $p < 0.05$ level (Fig. 1). As mentioned in Table 3, the maximum amount of shoots per explant (1.46 ± 0.2) and the highest shoot length (14.6 mm) were observed in T5 where MS medium supplemented with 3 mg/l BAP (Fig. 2). According to the Karimpour et al. (2013), similar result was founded by producing highest number of proliferated shoots (2.6-3.6) at 3 mg/l BAP concentration for the Sebri pear cultivar. According to Haq and Kaloo (2010), the best result for shoot multiplication was obtained on MS medium supplemented with a combination of BAP 2.2 mg/l, IBA 2.0 mg/l and Kn 1 mg/l. According to Samanmalie et al. (2019), the highest shoot multiplication was observed in 4 mg/l BAP for apple (*Malus pumila*) which also belongs to the family Rosaceae.

In this experiment, the average number of roots per shoot, average length (mm) of the roots, and rooting percentage were recorded after 10 weeks of culture initiation. Root

emergence commenced in T10 (½ MS + 4 mg/l IBA) and T4 (½ MS + 3 mg/l) after 4th week. While other treatments had delayed root emergence. Explants on half-strength MS medium showed short and thick roots while shoots on full-strength medium produced long roots. Further, callus formation was observed at the base of the shoot within the experimental period when IBA concentration decreased. When comparing full and half-strength MS media with the control treatment (0 mg/l IBA), no root formation was observed in the full-strength MS medium. The highest average number of roots (1.93 ± 0.98) was produced in the half-strength medium supplemented with 4 mg/l IBA (Fig. 3). According to Haq and Kaloo (2010), the highest rooting was observed on 1.0 mg/l IBA with an average number of 4.25 ± 1.2 roots per shoot for *Pyrus pyrifolia* (Burm. f.) Nakai.

Table 3. Effects of different concentrations of BAP on shoot multiplication after 4 weeks of culture initiation.

Treatment (T)	BAP (mg/l)	No. of Shoots (Mean ± S.E)	Length of the Shoots (mm)
T1	1.0	1.06 ± 0.1^{b}	11.48^{ab}
T2	1.5	1.20 ± 0.2^{ab}	9.66^{b}
T3	2.0	1.13 ± 0.1^{b}	8.76^{b}
T4	2.5	1.40 ± 0.05^{b}	10.07^{b}
T5	3.0	1.46 ± 0.2^{a}	14.6^{a}

Fig. 1. Sprouted shoots after 4 weeks of culture initiation.

Fig. 2. Shoot multiplication of Rahangala pear.

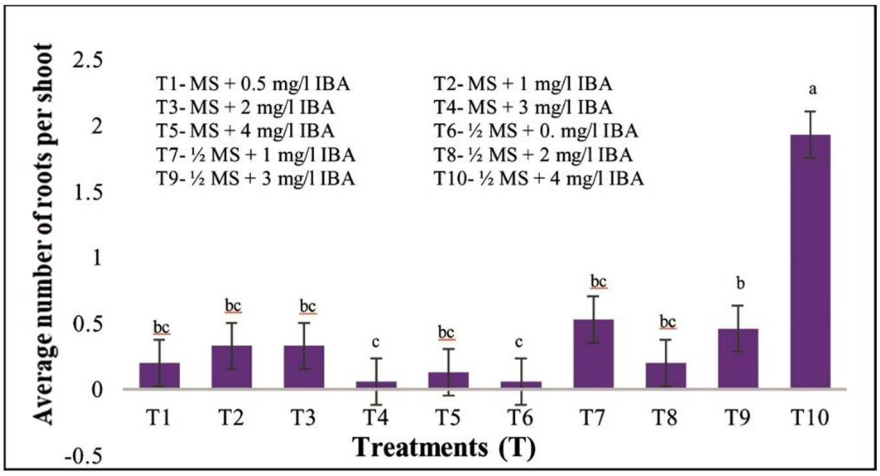

Fig. 3. Effect of different treatments on average number of roots per shoot.

The highest average root length (60.6 mm) was observed in the half-strength medium supplemented with 4 mg/l IBA. Full MS medium supplemented with 4 mg/l IBA resulted 34 mm average length by producing few numbers of roots per shoot (Fig. 4 and 6). Root induction experiment conducted on MS medium by Eshbekova et al. (2021) on *Pyrus communis* obtained maximum average root length per explant (6.5 cm) with a combination of IBA at 1 mg/l and NAA at 0.5 mg/l.

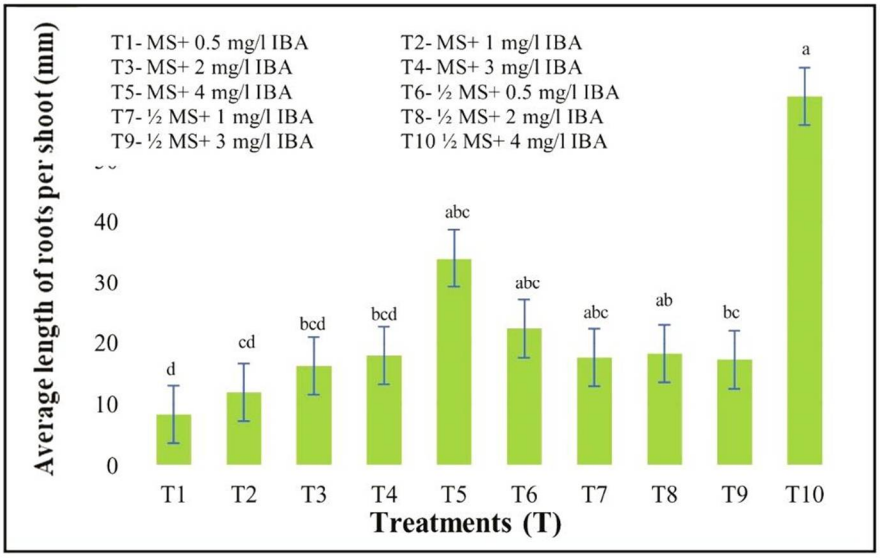

Fig. 4. Effect of different treatments on average length of roots after 10 weeks of culture initiation.

As shown in Fig. 5 the best rooting percentage (68%) resulted in T10 (½ MS + 4 mg/l IBA). In a similar study, Hlaing et al. (2019) reported a 67% rooting percentage in half-strength MS with 0.3 mg/l IBA dipping IBA in 10 seconds for *in vitro* shoots of *Pyrus betulifolia.*

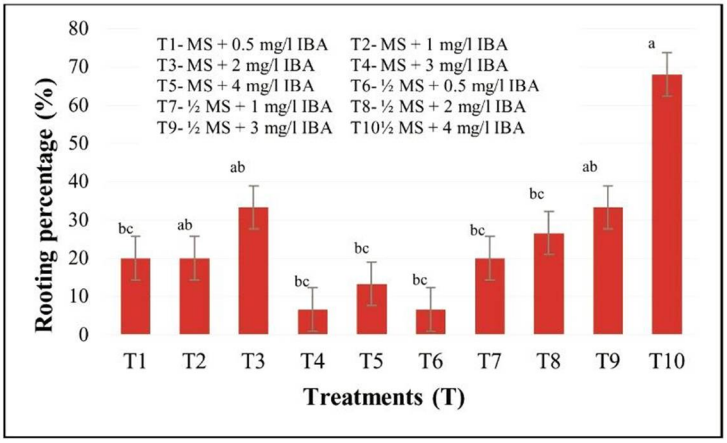

Fig. 5. Effect of different treatments for rooting percentage after 10 weeks of culture initiation.

The best rooting was found in T1 (topsoil, compost, coir dust and sand 1:1:1:1) resulted 100% rate of survival after two weeks (Fig. 7). However, there were no significant differences between T2 and T3. *In vitro,* raised roots could be adapted to the soil containing media after hardening conditions. In the acclimatization period, new leaves and roots were observed and roots became phenotypically strong. According to Hlaing et al. (2019), 39.74 % *in vitro* raised *P. betulifolia* plants survived in a mixture of sterile soil and perlite after two weeks of acclimatization.

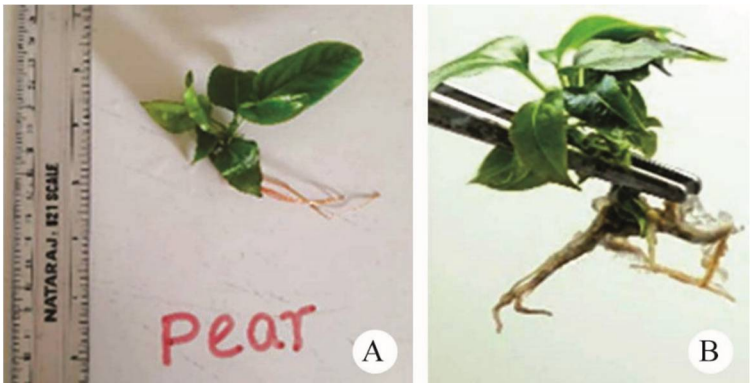

Fig. 6. (A-B). *In vitro* root induction of pear shoots in ½ MS medium with 4 mg/l IBA. A - Root induction after 3 weeks, B - Root induction after 10 weeks.

Fig. 7. Survived pear plants after 02 weeks of acclimatization in T1.

It can be concluded that 1% $AgNO_3$ + 10% NaOCl in 10 min. was the best treatment for surface sterilization of Rahangala shoots with a survival rate of (86.6%). The optimum amount of shoots per explant (1.46 ± 0.2) and highest shoot length (14.6 mm) were produced in MS medium supplemented with 3.0 mg/l BAP. Half-strength MS medium supplemented with 4.0 mg/l IBA was the best treatment for induction of roots of *in vitro* raised pear shoots. Moreover, a 100% survival rate was achieved after the transplantation to the growth medium composed of topsoil, compost, coir dust and sand in a 1:1:1:1 ratio. The results indicate the possibility of mass production of pears using locally available planting material through tissue culture technology.

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(Manuscript received on 22 February, 2024; revised on 02 April, 2024)