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In vitro Propagation of *Garcinia quaesita* Pierre

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***Abstract*—** *Garcinia quaesita Pierre has growing demand presently in the world for its remarkable medicinal value and has tremendous potential for introduction into home gardens as a cash crop. This experiment was designed to establish a protocol for in vitro propagation of Garcinia to fulfill the high demand of good quality planting material with the objectives of finding suitable sterilization and regeneration media.*

In experiment one, three treatments were tested to identify the best sterilization procedure using 1-3 cm long shoot tips cultured on Woody Plant Medium (WPM). As treatments, 0.1% Captan (1 hour) + 30% Clorox (20 minutes), 0.1% Captan (1 hour) + 30% Clorox (20 minutes) + 50% ethanol (5 minutes) and 0.1% Captan (1 hour) + 40% Clorox (20 minutes) + 50% ethanol (5 minutes) were used. Among those, treatment three showed the best performance (26.66% sterile culture, 2.22% bacterial contamination, 2.22% dead culture and 68.8% fungal contamination) compared to other treatments after 4 weeks. Therefore, treatment three was used for further experiments.

In experiment two, apical buds were cultured on WPM supplemented with three concentrations of BAP at 3.0, 5.0, 10.0 mgL^{-1} alone and 3.0 mgL^{-1} BAP with 0.5 mgL^{-1} NAA. The highest shoot length (0.68 cm), the highest numbers of leaves (1.55 ± 1.01) and the highest sterile culture percentage (44.55%) were observed in medium containing 3.0 mgL^{-1} BAP with 0.5mgL^{-1} NAA after 4 weeks (p=0.7089). There were not significant difference of shoot height among treatments. Leaf explant of Garcinia were cultured on WPM containing four levels of BAP (1, 2, 4, 8 mgL^{-1}) with 0.5 mgL^{-1} NAA. Callus development from the leaf explants couldn't observed during the time period and some were contaminated after 7 weeks.

WPM containing 3.0 mgL^{-1} BAP with 0.5 mgL^{-1} NAA was the best treatment for in vitro propagation of Garcinia.

Keywords—*Benzyl Amino Purine* (*BAP), Garcinia quaesita Pierre, Napthaleneacetic acid (NAA).*

I. Introduction

Garcinia quaesita Pierre has growing demand presently in the world for its remarkable medicinal value most important of which is anti-obesity effect. Garcinia has tremendous potential for introduction into home gardens as a cash crop and more likely to be booming in the future for its export products.Garcinia belongs to the family Clusiaceae. There are 10 species represented by the Sri Lankan genus including five endemic species. Among those *Garcinia quaesita* Pierre and *Garcinia zeylanica* Roxb can be seen commonly in Sri Lanka (Farzana *et al.*, 2010).

Garcinia quaesita Pierre *(Goraka)* plants are an important component of the natural ecosystem. It has enormous local and export market demand (Pushpakumara *et al.*, 2007). Garcinia fruit rinds are the edible part and important due to their chemical composition and health benefits. As well as Garcinia plants are one of the most economically and beneficially important plant and help to balance the natural ecosystem. Therefore it is extremely important to improve different propagation methods for cultivating Garcinia plants to develop high production for the local and export market.

Garcinia quaesita Pierre plant is slow-growing, evergreen tropical tree. And also a cross-pollinated plant. There are two types of plants with androecium or hermaphrodite flowers. In the early growth stage, it is difficult to identified male and bisexual plants by using morphological characteristics (Deodhar *et al.*, 2014). The plants bear flowers only 7-8 years after planting. Those may lead to the unavailability of good planting materials thus, limited production of *Garcinia* (Farzana *et al.*, 2010).

Garcinia species were propagated by seeds as a traditional propagation technique. The seeds are recalcitrant and high sensitivity to desiccation and freezing with a less shelf life. The method of seed propagation is more challenging due to its low percentage of germination, seasonal flowering behavior, and long juvenile seedling period. Therefore, it is difficult to raise seedlings throughout the year (Farzana *et al.*, 2010). In addition, 50% of plants are usually male in an orchard established with seedlings. Vegetative propagation methods can be a better solution to overcome these problems.

Another technique that can be used for clonal propagation of *G. quaesita*, as reported previously, is grafting (Deodhar *et al.*, 2014). Grafting was successful but air layering and stem cutting were not successful (George *et al.*, 1994). Successful clonal propagation is seasonally dependent, requiring space and showing more and more grafted plants change in the canopy with reduced fruit yield, thus, there are limited applications. Availability of rootstock is another limitation for large-scale successful grafting of Garcinia (Deodhar *et al.*, 2014). *In vitro* propagation through tissue culture techniques could be an alternative solution to solve the problems pertaining to Gracinia (Harahap *et al.*, 2014). *In vitro* propagation is expected to solve the problems of uniform, good performance, and continuous annual supply of plant material by mass propagation (Rostika *et al.*, 2008). Therefore, the research was conducted to develop a shoot multiplication protocol for *Garcinia quaesita* Pierre using *in vitro* techniques.

II. Materials and Methods

Apical buds were collected from fruit-bearing *Garcinia quaesita* Pierre mother plants which were grown in the net house of the Central Research Station, Matale, Sri Lanka. Captan fungicide was sprayed in four days interval and Bordeaux mixture was used as pretreatments before collecting explant to reduce fungal contamination. All the laboratory experiments were conducted in the Central research station,

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Matale, Sri Lanka.Woody Plant Medium (WPM) was used as the basal medium with different hormonal combinations for all the experiments and sterilization treatments.

2.1: Experiment 01: To find out the best sterilization method

About 2-3 cm long and 2-3 mm diameter apical buds were separated from branches and thoroughly cleaned with cotton wool, soaked with T-20 (Tween twenty). Then the buds were washed for 30 minutes with running tap water and Teepol (detergent) to remove debris. Subsequently, cleaned buds were rinsed 2-3 times with distilled water and put into 0.1% Captan fungicide solution with Teepol and shake for 1 hour. Then the buds were washed thoroughly 3-4 times with distilled water. The experiment was done in 3 replicates with 15 culture tubes for each replicate. Following treatments were used with time duration to find out the best sterilization method.

Treatments

Treatment 01(Z_1): 0.1% Captan (1 hour) + 30% Clorox (20 minutes)

Treatment 02 (Z_2): 0.1% Captan (1 hour) + 30% Clorox (20 minutes) + 50% Ethanol (5 minutes)

Treatment 03 (Z_3): 0.1% Captan (1 hour) + 40% Clorox (20 minutes) + 50% Ethanol (5 minutes)

In Z_1, 30% Clorox with 2 drops of Teepol was put into the beaker and kept on a shaker for 20 minutes. Then the apical buds were washed with distilled water and again washed with sterile distilled water under the Laminar Air Flow (LAF) cabinet.

In Z_2, 30% Clorox with 2 drops of Teepol was put into a beaker and kept on a shaker for 20 minutes.After that apical buds were washed with distilled water and put in 50% ethanol and kept on a shaker for 5 minutes. Then they were washed with distilled and sterile distilled water under the LAF cabinet.

In Z_3, 40% Clorox with 2 drops of teepol was put into a beaker and kept on a shaker for 20 minutes.After that apical buds were washed with distilled water and put in 50% ethanol and kept on shaker for 5 minutes. Subsequently, apical buds were washed with distilled and sterile distilled water under the LAF cabinet.

Single bud was introduced into each test tube containing 10 ml of WPM. The cultures were kept in air-conditioned culture room at 22 ± 1 ^{0}C under 24 hours light (3000 lux) with 75% relative humidity.

Sterile culture percentage, fungal and bacterial contamination percentages, and dead or phenolic browning percentage data were recorded at one-week interval for one month period.

2.2: Experiment 02: Direct shoot regeneration using apical buds

About 2-3 cm long and 2-3 mm diameter with light green color apical buds were thoroughly cleaned with cotton wool, soaked in T-20. Then they were washed 30 minutes under running tap water with Teepol to remove debris. The cleaned buds were washed 2-3 times in distilled water and put into 0.1% Captan fungicide solution with Teepol and it was kept on a shaker for 1 hour. After that, the apical buds were washed thoroughly 3-4 times with distilled water. Next, the buds were kept in 40% Clorox with 2 drops of Teepol and kept on a shaker for 20 minutes (the best sterilization method selected from experiment 01).Subsequently, the apical buds were washed with distilled water and put in 50% ethanol, and kept on shaker for 5 minutes. Then they were washed with distilled and sterile distilled water under the LAF cabinet.

One healthy apical buds per test tube were transferred to culture tubes containing WPM supplemented with three BAP levels (3.0, 5.0, 10.0 mgL^{-1}) and medium containing 3.0 mgL^{-1} BAP and 0.5 mgL^{-1} NAA. All the cultures were kept in a growth room under 22±1 ^{0}C temperature, 24 hours light (3000 lux), and 75% relative humidity. All treatments were replicated 3 times with 15 culture tubes for each replicate.

Treatments

T_1– woody plant medium with 3.0 mgL^{-1}BAP (Control)

T_2 – woody plant medium with 5.0 mgL^{-1}BAP

T_3– woody plant medium with 10.0 mgL^{-1}BAP

T_4– woody plant medium with 3.0 mgL^{-1}BAP + 0.5 mgL^{-1}NAA

Sterile culture percentage, dead and contamination percentages, numbers of leavesand length of shoots were recorded at one-week intervals for a period of one month.

2.3: Experiment 03: Indirect shoot regeneration using leaf explants

Healthy immature semi hard leaves were collected from pretreated *Garcinia quaesita* Pierre plants and followed the sterilization procedure. The leaves were thoroughly cleaned with cotton wool, soaked with T-20 (Tween twenty). Then the leaves were washed for 30 minutes under running tap water with Teepol to remove debris. Subsequently, cleaned leaves were washed 2-3 times and put into 0.1% Captan fungicide solution with Teepol and it was kept on a shaker for one hour. Then the leaves were washed thoroughly 3-4 times with distilled water. Next, the leaves were kept in 40% Clorox with 2 drops of Teepol and kept on the shaker for 20 minutes (the best sterilization method selected from experiment 01).After that the leaves were washed with distilled water and put 50% ethanol and kept on a shaker for 5 minutes. Then they were washed with distilled and sterile distilled water under the LAF cabinet.

Then sterilized leaves were cut into the 1 x 1 cm (1 cm^2) size pieces under aseptic condition. The prepared explants were inoculated into 250 ml jars (three pieces per jar) containing 15 ml full strength WPM supplemented with four BAP levels (1, 2, 4, 8 mgL^{-1}) and 0.5 mgL^{-1} NAA. All treatments were replicated 3 times with 15 culture tubes for each replicate. The cultures were kept in a growth room under 8/16 hour light/dark (3000 lux), 22 ± 1 ^{0}C temperature and 75% relative humidity.

Treatments

Y_1 - woody plant medium with 1 mgL^{-1} BAP + 0.5 mgL^{-1} NAA (Control)

Y_2 - woody plant medium with 2 mgL^{-1} BAP + 0.5 mgL^{-1} NAA

Y_3 - woody plant medium with 4 mgL^{-1} BAP + 0.5 mgL^{-1} NAA

Y_4 - woody plant medium with 8 mgL^{-1} BAP + 0.5 mgL^{-1} NAA

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Statistical Analysis

Complete Randomized Design (CRD) was used as experimental design. ANOVA was used to analyze the significant difference between treatments. Experiments were analyzed by using SAS programme software with a 95% confidence level. Mean separation was done by Duncan's multiple range test (DMART).

III. Results and Discussion

3.1: Experiment 01: To find out the best sterilization method

There was no significant difference between sterilization treatments. According to the Table 3.1, in Z_3, explants which were sterilized in three step process (1 hour 0.1% Captan followed by 40% Clorox in 20 minutes and 5 minutes in 50% ethanol) resulted 68.8% of fungal contamination, 2.22% bacterial contamination and 2.22% dead culture percentage. However, 26.66% success cultures resulted than other two treatments (Plate 1). Garcinia shoot tips showed high fungal contamination after 2 weeks of culture. Deodhar *et al.*, (2014) reported that microbial contamination was the major limiting factor of Garcinia culture establishment and 10% to 70% of inoculated cultures were contaminated after the fourth day.

TABLE 3.1. Success culture, fungal and bacterial contaminations and phenolic browning or dead culture percentages

	Treatment 01 (Z_1)	**Treatment 02 (Z_2)**	**Treatment 03 (Z_3)**
Success culturepercentage	4.44	8.89	26.66
Fungal contamination percentage	91.11	86.67	68.8
Bacterial contamination percentage	4.44	4.44	2.22
Phenolic browning or dead percentage	0	0	2.22

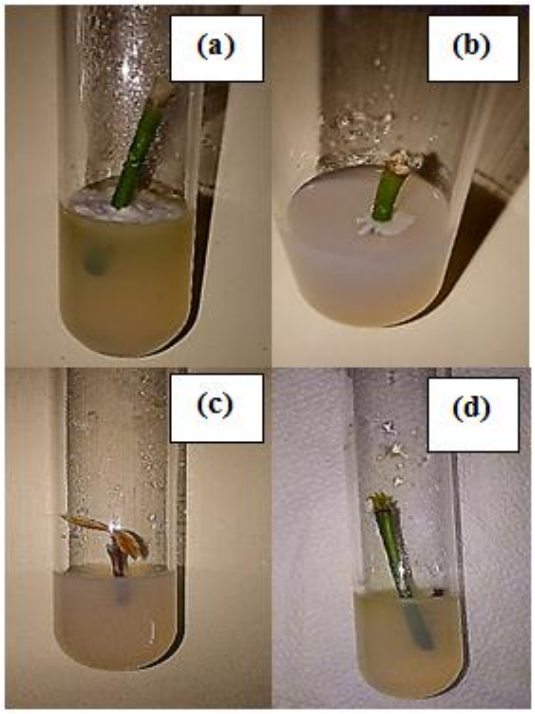

Plate 1. Contamination cultures. (a) fungal contamination, (b) bacterial contamination, (c) dead culture and (d) success culture

Many researchers reported that several sterilization methods for Garcinia explants. Deodhar *et al*., (2014) reported that they used 70% ethanol and $HgCl_2$ for *Garcinia indica* Choiss. They reported that they have observed 10% to 70% contaminated cultures. However, 50% ethanol and high concentration (40 %) of Clorox was used instead of 70% ethanol and $HgCl_2$ in this experiment and resulted 73% contaminated cultures. In addition, Manvi and Parasharami, (2019) reported that *Garcinia indica* Choiss explants were treated with sterile distilled water, T-20, 10% Savlon, 0.1% PVP, and 1% anti-fungal agent Bavistin, then aseptically treated with 0.75% $HgCl_2$ and dried for 60 minutes before inoculations, resulted 87% of sterile buds. However, only 26.66% of success cultures wereresulted by using 0.1% Captan (1 hour) + 40% Clorox (20 minutes) + 50% Ethanol (5 minutes).

Average number of success culture, fungal and bacterial contaminations, and phenolic browning or dead culture with their standard deviation is shown in Table 3.2.

TABLE 3.2. Average number of Success culture, fungal and bacterial contaminations and phenolic browning or dead culture

	Contamination		**Phenolic browning or dead culture**	**Success culture**
	Fungal	**Bacterial**		
Treatment 01 (Z_1)	1.33^a	0.12^a	0.00^a	0.17^a
Treatment 02 (Z_2)	1.19^a	0.17^a	0.00^a	0.29^{ab}
Treatment 03 (Z_3)	0.99^a	0.08^a	0.08^a	0.53^b

Note: Means with same letters in a column are not significantly different at $p < 0.05$

3.2: Experiment 02: Direct shoot regeneration using apical buds

3.2.1: Length of shoots

There was no significant difference between the four treatments for shoot length ($p = 0.7089$). However, the highest shoot length (0.68 cm) was observed in woody plant medium contained 3.0 mgL^{-1}BAP + 0.5 mgL^{-1}NAA (Table 3.3).

TABLE 3.3. Effects of different concentrations of BAP levels on shoot elongation

Treatments	**Concentration of BAP/ NAA (mgL^{-1})**	**Mean shoot length (cm)**
T_1	3.0 BAP	0.59^a
T_2	5.0 BAP	0.43^a
T_3	10.0 BAP	0.61^a
T_4	3.0 BAP + 0.5 NAA	0.68^a

Note: Means with same letters in a column are not significant at $p < 0.05$

In similar study Joshi *et al.*, (2015) reported that MS medium containing 3 mgL^{-1} BAP and 0.5 mgL^{-1} NAA resulted the best hormonal concentration for multiple shoot induction and 5 cm maximum shoot length resulted from *Garcinia indica* Choiss seed explants. Farzana *et al.*, (2010) reported that MS medium supplemented with 20 mgL^{-1} BAP showed a higher percentage of node growth and shoot elongation of Garcinia. Therefore, probably higher concentrations of BAP level are needed for significant growth of the shoots in *Garcinia quaesita* Pierre apical bud explants.

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3.2.2: Number of leaves per shoot

There was no significant difference shown among treatments in this study. After 4 weeks of culture establishment, 2 leaves were observed in sterile cultures. An average number of leaves with their standard deviation is shown in Table 3.4.

TABLE 3.4. Effects of different concentrations of BAP levels on production of leaves

Treatments	Concentration of BAP/ NAA (mgL^{-1})	Mean number of leaves with standard deviation
T_1	3.0 BAP	1.64 ± 0.64
T_2	5.0 BAP	1.24 ± 0.88
T_3	10.0 BAP	1.37 ± 0.68
T_4	3.0 BAP + 0.5 NAA	1.55 ± 1.01

Harahap *et al.*, (2014) reported that Mangosteen explants which is belongs to the same family treated with 5 mgL^{-1} BAP were produced more leaves and internodes than other concentration (0, 2.5, 7.5, 10 mgL^{-1}) of BAP. Rostika *et al.*, (2008) also reported that MS medium supplemented with 5 mgL^{-1} BA induced shoot growth up to 100% with largest numbers of leaves and shoots of Mangosteen.

3.2.3: Sterile culture percentage

Higher percentage (44.45%) of sterile culture was obtained in WPM contained with 3.0 mgL^{-1} BAP and 0.5 mgL^{-1} NAA (Plate 2 and Table 3.5). However, high fungal contaminations were observed after 3 weeks of the experiment. Deodhar *et al.*, (2014) reported that browning was also the major limitation of their study due to the high phenolic compound contained in *Garcinia indica* Choiss. Garcinia plants contain more latex and phenolic compounds therefore phenolic browning is happening. However, in this study there was not any phenolic browning culture resulted due to explants were collected early in the morning and immediately they were put into the water. As reported by Singh, (2018) the most successful removal of secondary products from explants can be done in two ways. Explants should be collected early in the morning, before sunrise (the plant does not like to be disturbed and not begin photosynthesis before sunrise), and immerse the plant in water to overcome phenolic browning.

TABLE 3.5.Effects of different concentrations of BAP levels on production of sterile culture

Treatments	Concentration of BAP/ NAA (mgL^{-1})	Sterile culture percentage (%)
T_1	3.0 BAP	33.34
T_2	5.0 BAP	35.56
T_3	10.0 BAP	28.89
T_4	3.0 BAP + 0.5 NAA	44.45

3.3: Experiment 03: Indirect shoot regeneration using leaf explants

In experiment 03 immature semi-hard *Garcinia quaesita* Pierre leaves were cultured on WPM supplemented with four BAP levels (1, 2, 4, 8 mgL^{-1}) with 0.5 mgL^{-1} NAA. However, in this study callus development from the immature leaf explants could not be observed and some explants were contaminated after 7 weeks.

Farzana *et al.*, (2010) reported that high average shoots were observed after 24 weeks of culture from immature leaves of Garcinia. However, they reported that immature and mature leaves performed differently with regard to induction of callus and shoot regeneration and showed that the immature leaves were better in shoot bud induction and callus initiation.

Plate 2. Sterile cultures

IV. CONCLUSION

Garcinia quaesita Pierre is a medicinally rich, economically valuable tree species in Sri Lanka. The study focused to investigate the best propagation method for producing good quality planting materials. Therefore, *in vitro* propagation method was selected to produce good planting material with experimenting the best sterilization method. The best sterilization treatment for *Garcinia quaesita* Pierre apical buds was observed in 0.1% Captan (1 hour) + 40% Clorox (20 minutes) + 50% ethanol (5 minutes) during this study. And also WPM containing 3.0 mgL^{-1} BAP with 0.5 mgL^{-1} NAA is the best hormonal combination for direct shoot multiplication of *Garcinia quaesita* Pierre apical buds. According to the study, callus was not formed during the experimental time period. However, it can be assumed more than 2 months is required for callus formation by using *Garcinia quaesita* Pierre leaf explants.

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