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Full Length Research Paper

In vitro propagation of *Phaleria macrocarpa,* God's Crown

Nazirah Abdullah*, Nor Hasnida Hassan, Siti Suhaila A. Rahman, Haliza Ismail, Rozidah Khalid and Muhammad Fuad Yahya

Forestry Biotechnology Division, Forest Research Institute Malaysia (FRIM) 52109, Kepong, Selangor, Malaysia.

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***Phaleria macrocarpa* (Scheff.) Boerl, also known as God's Crown, belongs to Thymelaeaceae family posses several medicinal properties such as anti-proliferative, anti-inflammatory and anti-angiogenic. In this paper, an attempt was made to develop a protocol for *in vitro* propagation of *Phaleria macrocarpa.* Seeds were used as explants for culture initiation and germinated *in vitro* after surface sterilization. Shoots multiplication in MS basal media supplemented with different concentrations of cytokinins were studied. BAP at 0.25 mg/l showed the most effective concentration for shoot multiplication with the highest number of shoots produced (4.27) and shoot elongation. For *in vitro* rooting, shoots were transferred to ½ MS basal media supplemented with auxins at different concentrations. The highest rooting frequency (83.33%) was observed in ½ MS basal medium. Regenerated plantlets were transferred to the nursery, 50% survivability was achieved and successfully acclimatized in mixed soil. The acclimatized plantlets were normal without any phenotypic aberration.**

Key words: *Phaleria macrocarpa,* shoot multiplication, root induction and acclimatization.

INTRODUCTION

Phaleria macrocarpa (Scheff.) Boerl, also known as God's Crown or Mahkota Dewa, is a medicinal plant belongs to the Thymelaeaceae family. It is a native plant from the island of Papua New Guinea (Irian Jaya), Indonesia. This plant grows to 5 - 18 m tall and can be found up to 1,200 m above sea level (Winarto, 2003; Burkill, 1966). Fruits from this plant are greenish and later turn to dark red colour when matured whereby some of the native find them attractive. Therefore, some of the Indonesians considered *P. macrocarpa* as a landscape plant. Traditionally, *P. macrocarpa* is used by the locals as a herbal drink either singly or mixed with other medicinal plants to cure illnesses such as cancer, hypertension and diabetes mellitus (Kurnia et al., 2008).

A wide variety of bioactive compounds can be found in *P. macrocarpa* such as flavanoids, alkaloids, polyphenols and saponins (Tambunan and Simanjuntak, 2006). According to recent *in vitro* studies, this plant extract exhibit anti-proliferative, anti-inflammatory and anti-angiogenic properties which make this plant ideal for the treatment or prevention of cancer. These bioactive compounds can be found in different parts of *P. macrocarpa* fruit (Tjandrawinata et al., 2010; Hendra et al., 2011). Since many findings support the medicinal values of *P. macrocarpa*, the demand for this plant is gradually increasing. Many products contain *P. macrocarpa* fruit extracts are currently sold in the market, for example in herbal tea and instant coffee. In order to cater for the increasing demand, there is a need for the continuous supply of raw materials. Cultivation of the species is one of the means to ensure sustainable supply of the plant materials.

The important aspects that need to be considered in commercialization are the quality and quantity of the raw materials. Therefore, mass propagation *in vitro* is considered as a relevant approach since this method can fulfil the requirements, given that good mother plant source is used. Besides that, production of this plant by *in vitro* will open up new research area where the targeted active compounds can be mass produced without the needs to produce the whole plant by using cell and organ cultures. Some of the examples were cell suspension culture (Dong et al., 2010), callus culture (Anna et al.,

*Corresponding author: nazirah@frim.gov.my. Tel: 603-6279 7646 Fax: 603-62804614.

2005) or organ culture (Cai et al., 2012; Nosov, 2012; Sakunphueak and Panichayupakaranant, 2010).

MATERIALS AND METHODS

Establishment of P. macrocarpa in tissue culture

Phaleria macrocarpa fruits were collected from Bangi, Malaysia. Seeds were used as explants for *P.macrocarpa* culture initiation. The seeds were soaked in sterile distilled water and Tween 20 as surfactant for 5 min followed by washing with distilled water and soaking in 95% Ethanol with 1 drop of Tween 20 for 30 min. The ethanol-soaked seeds were then flamed to remove any potential contaminant. The seed-coat was removed to induce faster germination. Seeds were then cultured in Murashige and Skoog (MS) basal medium supplemented with 0.5 mg/l 6-Benzylaminopurine (BAP). All cultures were incubated at 22 ±2 ^{o}C under a 16 h photoperiod.

Shoot multiplication

Apical shoots of *P. macrocarpa*, 1.5 to 2 cm long, were used as explants for shoot multiplication study. Murashige and Skoog (1962) (MS) basal media supplemented with various concentrations of cytokinins with a range of 0.0 – 2.5 mg/l BAP and 0.0 – 2.5 mg/l kinetin (KIN) was used for shoot multiplication. MS basal medium without the addition of plant growth regulator was used as control. The media were prepared with the addition of 3% sucrose, and solidified with the combination of 0.4% bacto-agar and 0.15% gelrite; pH of the media was adjusted to pH 5.8 prior to autoclaving at 121°C for 15 min. About 12 explants were used in each treatment (4 explants with three replicates). All cultures were incubated in the culture room at 22 ±2 ^{o}C under a 16-h photoperiod for eight weeks. Data for shoot multiplication and shoot height were collected after eight weeks in culture.

Root induction

Shoots, 3-4 cm long, were used as explants for *in vitro* rooting. Half strength MS basal media were used in rooting experiment with the addition of root inducing plant growth regulator, IBA and NAA at various concentrations (Indolebutyric acid, IBA: 0.0 - 2.0 mg/l; Naphthalene acetic acid NAA: 0.0 - 2.0 mg/l). ½ MS basal media without the addition of plant growth regulator was used as control. The media were prepared with the addition of 3% sucrose and solidified with 0.3% gelrite; pH was adjusted to pH 5.8 before adding agar prior to autoclaving at 121°C for 15 min. About 12 explants were used in each treatment (4 explants with three replicates). Cultures were incubated in the culture room at 22 ±2 ^{o}C under a 16-h photoperiod for four weeks. The percentage of shoots rooted was calculated.

Acclimatization

After four weeks in rooting media, the rooted plantlets were transferred to a weaning chamber (80% humidity with 50% light) for acclimatization process. Three types of potting media were tested; mixed soil (soil: sand: coconut husk = 1:1:1), jiffy and vermiculite. The percentage of plantlets survival was calculated after one month acclimatized in the weaning chamber

RESULTS AND DISCUSSION

In vitro germination

P. macrocarpa seeds cultured *in vitro* started to germinate after only one week in culture with the emergence of epicotyls and hypocotyls. The number of new shoots produced was higher, range from three to five shoots per seed as compared to the seed germinated *ex vitro* which produced only one shoot per seed. The *in-vitro* germinated seed produced more than one shoot was due to the exogenous cytokinin supplied in the media (Personal observation). Seeds were used as a source for culture initiation due to their having less contact between embryos and sterilizing agent during sterilizing. Therefore, the sterilization method for seed sterilization can be more stringent than vegetative tissue (Akin-Idowu et al., 2009).

Shoot multiplication

After two weeks in culture, new axillary shoots started to emerge at the bases and nodes of the explants. Most of the shoots cultured in media with BAP showed swollen and callus formation at the basal part of the shoots. However, no swollen or callus formation observed in media without BAP (control). Based on the recorded data, the explants cultured in the MS basal medium supplemented with 0.25 mg/l BAP showed the highest mean number of shoots, 4.27 with average shoot height of 0.94 cm but there is no significant difference with 0.1 mg/l and 0.5 mg/l BAP. However, there are significant difference in shoot production with control and 2.5 mg/l BAP (Table 1; Figure 1a). MS supplemented with 0.1 and 0.5 mg/l BAP showed similar result in terms of number of new shoots produced and average shoot height. From the overall observations, explants in MS basal media treated with 0.1 and 0.25 mg/l BAP produced healthy shoots according to their leaflet size and colour. In both BAP and KIN treatment, it was observed that axillary shoots were

Table 1. Effect of different concentrations of BAP and KIN in MS basal media for shoot multiplication of *P. macrocarpa,* after eight weeks in culture.

BAP (mg/l)	KIN (mg/l)	Regeneration percentage (%)	Number of shoots (Mean ± SE)	Shoot length (cm) (Mean ± SE)
-	-	8.3	1.08 ± 0.08 ^{b}	1.39 ± 0.2^{ab}
0.1	-	66.7	3.58 ± 1.29 ^{a}	1.11 ± 0.2 ^{b}
0.25	-	83.3	4.27 ± 0.37 ^{a}	0.94 ± 0.05 ^{b}
0.5	-	83.3	3.58 ± 0.17 ^{a}	1.07 ± 0.09 ^{b}
1.0	-	50	2.58 ± 0.58^{ab}	1.07 ± 0.04^{b}
2.5	-	8.3	1.33 ± 0.33 ^{b}	1.71 ± 0.25^{a}
-	0.1	66.67	3.17±0.58^{c}	0.85±0.18^{ab}
-	0.25	83.33	3.67±0.71^{bc}	0.69±0.11^{ab}
-	0.5	91.67	6.00±0.75^{a}	0.56±0.02^{b}
-	1.0	100	5.58±0.65^{ab}	0.61±0.03^{ab}
-	2.5	75	3.48±0.68^{bc}	0.81±0.18^{ab}

Means in a column with different small letters are significantly different at $p \leq 0.05$ by Duncan New Multiple Range Test.

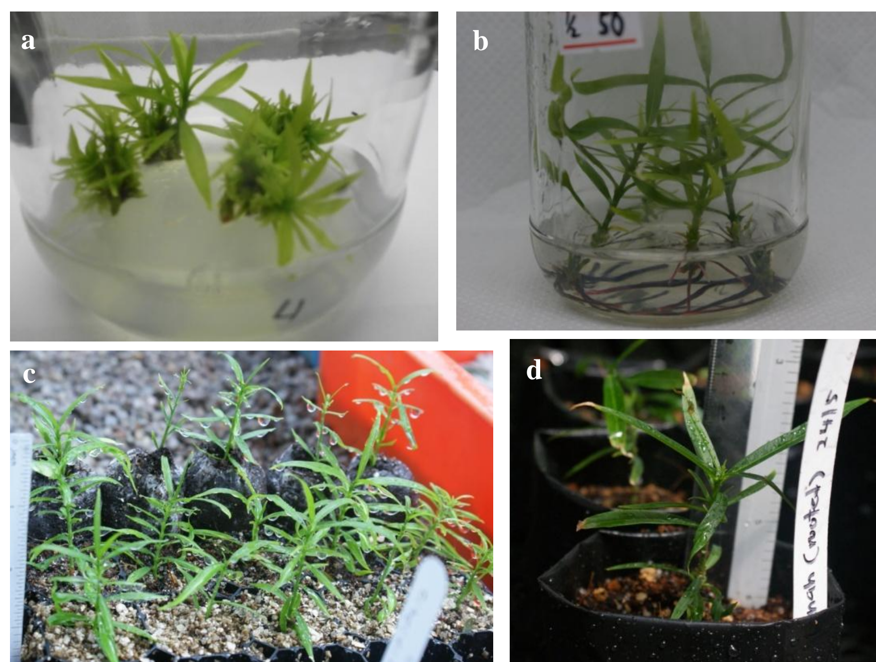

Figure 1. Micropropagation of *P. macrocarpa* a) *P. macrocarpa* shoots in MS basal media supplemented with 0.25 mg/l BAP; b) Rooting of *in vitro* shoots in ½ MS basal media; c) Acclimatized plantlets in weaning chamber; d) *P.macrocarpa* tissue culture plantlets in the polybag.

emerged from basal region and nodal segment along the explants. However, in BAP media, most of the shoots emerged from the basal of the explants. The axillary shoots that emerged from the basal region showed positive growth, elongate and can be subculture to a new media after three month. However, the axillary shoots that emerged from the nodes along the explants showed slower growth and some of them were stunted.

Shoot tip necrosis (STN) occurred in the explants treated with higher concentrations of BAP where the shoot tips and leaves were yellowed and wilted. The STN was also occurred in KIN however the number of explants effected were minimal. This finding is similar with *in vitro* micropropagation of another species belongs

Table 2. Effect of IBA and NAA on *in vitro* rooting of *P. macrocarpa* after four weeks in culture.

IBA (mg/l)	NAA (mg/l)	Percentage of shoots rooted (%)	Mean number of roots per shoot (Mean ± SE)
-	-	83.33	7.43 ± 1.09 [a]
0.1	-	66.67	2.93 ± 0.54 [b]
0.5	-	50	3.03 ± 1.75 [b]
1.0	-	66.67	2.83 ± 0.44 [b]
2.0	-	25	0.37 ± 0.23 [b]
-	0.1	**NR**	**NA**
-	0.5	**NR**	**NA**
-	1.0	**NR**	**NA**
-	2.0	**NR**	**NA**

Means in a column with different small letters are significantly different at p≤0.05 by Duncan New Multiple Range Test, **NR** = no response, **NA** = Not available.

to Thymelaeaceae family, *Pimelea spicata*. *Pimelea spicata* shoots cultured in MS media showed high percentage of shoot tip necrosis compared to half strength MS media though the shoot multiplication in MS media was higher (Offord and Tyler, 2009). According to Bairu et al. (2009), STN occurs due to a wide range of factors such as the action of the plant growth regulator, medium type, medium additives, calcium, boron and the culture condition to the plant's physiological effect, response and interaction. In this study, the number of new shoots produced was increased with the increasing of BAP concentration up to 0.25 mg/l, and thereafter decreased (Table 1). Similar effect was also noticed in other plant system (Nor Hasnida et al., 2011; Jain and Mudasir, 2010).

In studying the effect of KIN on the *P.macrocarpa* shoot multiplication, the highest number of shoots was observed in media supplemented with 0.5 mg/l KIN and have significant difference with control, 0.1 mg/l, 0.25 mg/l and 2.5 mg/l KIN (Table 1). 100% shoot induction frequency was achieved in 1.0 mg/l KIN suggesting that KIN is suitable for *P.macrocarpa* shoot multiplication. However, the growth of new shoots in KIN was slower compared to the shoots in BAP according to the average shoot height after several weeks. Most of the new shoots in media with KIN emerged at the nodes along the explants. Therefore, KIN was more probably suitable than BAP for vigorous shoot production, but there is no significant difference between shoot production in 0.5 mg/l KIN and 0.25 mg/l BAP. In KIN, the elongation of the new shoot has to be induced. Sub-culturing new shoots into a lower concentration of KIN may induced shoot elongation.

In vitro rooting

The effect of IBA and NAA on root induction is summarized in Table 2. In the present study, IBA was more active in promoting root induction. Root initiation was observed after one week in IBA while no root was observed in NAA even after four weeks in culture (Table 2). From the observation, the newly emerged roots were reddish in color and whitish at the root tips. After a month, the roots turned to dark red, nearly black color. No callus was observed from the shoots cultured in IBA. Roots were emerged at the basal part of the shoot through direct organogenesis. IBA is more preferable since IBA is more stable than NAA in tissue culture media during autoclaving and widely used in rooting experiment with positive effect as proven in many reports (Cuenca et al., 1999; Hussein et al., 2005; Kumar and Rao, 2006).

Interestingly, shoots cultured in ½ MS basal medium without any growth regulator were able to produce root after 1 week in culture and the frequency of rooting was higher than those supplemented with auxin (Figure 1b). The ability of *P. macrocarpa* to induce root in ½ MS basal medium suggests that this plant contain high endogenous auxin whereby the addition of exogenous auxin is not required (Table 2). The addition of auxin to plantlets that contain high endogenous auxin will affect their root development and also induce callus production. Further addition of exogenous auxin will inhibit the root development in *P. macrocarpa* shoots (Juliani et al., 1999). High endogenous auxin most probably the potential cause for callus induced from *P. macrocarpa* shoots cultured in media supplemented with cytokinin. The rooting ability of shoot without the addition of exogenous rooting hormone was also occurred in other plant species (George and Sherrington, 1984; Chan et al., 2009). Suitable carbon/nitrogen ratio in half strength MS medium may be more effective for root induction in *P. macrocarpa* compared to full strength MS basal medium (Data not shown). Similar effect of ½ MS media on root induction were observed in other plant species such as *Acacia auriculiformis*, *Pimelea spicata* and *Aquilaria agallocha* (Girijashankar, 2011, Offord and Tyler, 2009; He et al., 2005).

Table 3. Survival percentage of *P. macrocarpa* plantlets acclimatized in the weaning chamber.

Medium	Percentage of survival (%)
Jiffy	40
Mixed soil	50
Vermiculite	45

Plantlets with healthy roots were transferred to greenhouse for acclimatization purpose. Mixed soil was found suitable for *P. macrocarpa* plantlets with 50.0% survival rate and normal morphological appearance after one month transplanted (Table 3; Figure 1c and 1d). Acclimatization of *Aquilaria crassna*, a member from same family, Thymelaeaceae showed high survivability in mixture of soil: sand: coconut husk (1:1:1) (Mongkolsook et al., 2007). Coconut husk is porous which prevent overwatering, and it is a good absorbent which keep water in the medium at sufficient level. The combination of coconut husk, sand and soil is favoured for *P. macrocarpa* plantlets probably due to the trace amount of organic substance in the soil as well as good aeration and low water retention (Mengesha et al., 2013). Acclimatization is a crucial step in micropropagation due to several factors; leaves produced *in vitro* lack epicuticular waxes and have an under-developed functional stomata system which causes loss of water due to high transpiration rates (Pospicilova et al., 1999), their photosynthetic capability does not develop well *in vitro* (Borkowska, 2001), the roots produced *in vitro* also do not perform fully and they have less vascular tissue and hairy roots (George, 1993).

The present study has established a successful *in vitro* propagation protocol which can later be used for the large-scale micropropagation of *P. macrocarpa* for commercial plantation.

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REFERENCES

Akin-Idowu PE, Ibitoye DO, Ademoyegun OT (2009). Tissue culture as plant production technique for plant horticultural crop. Afr. J. Biotechnol. 8(16): 3782-3788.

Anna LPK, Maziah M, Nor'aini MF, Siti Khalijah D (2005). Effect of precursor supplementation on the production of triterpenes by *Centella asiatica* callus culture. Pak. J. Biol. Sci. 8(8): 1160-1169.

Bairu MW, Stirk WA, Staden JV (2009). Factors contributing to *in vitro* shoot-tip necrosis and their physiological interactions. Plant Cell Tiss. Organ. Cult. 98:239-248.

Borkowska B (2001). Morphological and physiological characteristics of micropropagated strawberry plants rooted *in vitro* or *ex-vitro*. Sci. Hortic. 89: 195-206.

Burkill IH (1966). A Dictionary of the Economic Products of the Malay Peninsular, Vol II. Kuala Lumpur: Ministry of Agriculture and Co-opeatives.

Cai Z, Kastell A, Knorr D, Smetanska I (2012). Exudation: An expanding technique for continuous production and release of secondary metabolites from plant cell suspension and hairy root cultures. Plant Cell Rep. 31(3): 461-477.

Chan LK, Lim SY, Pan LP (2009). Micropropagation of *Gynura procumbens (Lours.) Merr.* An important medicinal plants. J. Med. Plants Res. 3(3):105-111.

Cuenca S, Amo-Marco JB, Parra R (1999). Micropropagation from inflorescence stems of the Spanish endemic plant *Centaurea paui* Loscos ex Willk. (Compositae). Plant Cell Rep. 18: 674–679.

Dong J, Wan G, Liang Z (2010). Accumulation of salicylic acid-induced phenolic compounds and raised activities of secondary metabolic and antioxidative enzymes in *Salvia miltiorrhiza* cell culture. J. Biotechnol. 148: 99-104.

George EF (1993). Plant propagation by tissue culture (part 1). The technology, 2nd edn. Cambridge University Press, Cambridge.

George EF, Sherrington PD (1984). Plant propagation by tissue culture: handbook and directory of commercial laboratories. Exegetics, Bassingstokes. pp. 71- 83.

Girijashankar V (2011). Micropropagation of multipurpose medicinal tree *Acacia auriculiformis.* J. Med. Plants Res. 5(3):462-466.

He ML, Qi SY, Hu LJ (2005). Rapid *in vitro* propagation of medicinally important *Aquilaria agallocha.* J Zhejiang Univ SCI. 6(8):849-852.

Hendra R, Shahida A, Ehsan O, Aspollah S, M Yunus S (2011). Antioxidant, anti-inflammatory and cytotoxicity of *Phaleria macrocarpa (Boerl.) Scheff* fruit. BMC Complementary Altern. Med. 11:110.

Hussein S, Rusli I, Anna LPK, Nor'aini MF, Siti Khalijah D (2005). Multiple shoots formation of an important tropical medicinal plant, *Eurycoma longifolia* Jack. Plant Biotechnol. 22: 349-351.

Jain AK and Mudasir B (2010). *In-vitro* propagation of Medicinal Plant *Portulaca grandiflora* Hook. World J. Agric. Sci. 6(3):327-330.

Juliani Jr HR, Koroch AR, Juliani HR, Trippi VS (1999). Micropropagation of *Lippia junelliana* (Mold.) Trone. Plant Cell Tiss. Organ Cult., 59: 175-179.

Kumar MS, Rao MV (2006). *In vitro* micropropagation of *Heliotropium indicum* Linn. - An Ayurvedic herb. Indian J. Biotechnol. 6:245-249.

Kurnia D, Akiyama K, Hayashi H (2008). 29- Norcucurbitacin derivative isolated from Indonesian medicinal plant, *Phaleria macrocarpa* (Scheff.) Boerl. Biosci., Biotechnol. Biochem. 72(2):618-620.

Mengesha A, Ayenew B, Tadesse T (2013). Acclimatization of *in vitro* propagated pineapple (*Ananas comosuss* (L.), var. Smooth cayenne) plantlets to *ex vitro* condition in Ethiopia. Am. J. Plant Sci. 4: 317-323.

Mongkolsook Y, Sumkaew R, Lichittammanit P, Wongwean P, Kaweekijthummakul W (2007). *In vitro* micropropagation of agarwood (*Aquilaria crassna*). Proceedings of the 45th

Kasetsart University Annual Conference, Bangkok, Thailand. 532-538 Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiol. 15(3): 473-497.

Nor Hasnida H, Nor Azah MA, Fadhilah Z, Haliza Ismail (2011). Effect of 6-benzylaminopurine (BAP) in different basal media on shoot multiplication of *Aquilaria hirta* and detection of essential oils in the *in vitro* shoots. Afr. J. Biotechnol. 10(51): 10500-10503.

Nosov AM (2012). Application of cell technologies for production of plant-derived bioactive substances of plant origin. Appl Biochem Microbiol 48(7): 609 – 624.

Offord CA, Tyler JL (2009). *In vitro* propagation of *Pimelea spicata* R.Br (Thymelaeaceae), an endangered species of the Sydney region, Australia. Plant Cell Tiss. Organ Cult., 98(1): 19-23.

Pospicilova J, Tischa I, Kadlecek P, Haisel D, Plzakova S (1999). Acclimatization of micropropagated plants to *ex-vitro* condition. Biol. Plant. 42(4):491-497.

Sakunphueak A, Panichayupakaranant P (2010). Increased production of naphthoquinones in *Impatiens balsamina* root cultures by elicitation with methyl jasmonate. Bioresour. Technol. 101: 8777-8783.

Tambunan RM, Simanjuntak P (2006). Determination of chemical structure of antioxidant compound benzophenon glycoside from *n*-butanol extract of the fruits of mahkota dewa (*Phaleria macrocarpa* (Scheff.) Boerl.) Majalah Farmasi Indonesia. 17(4): 184-189.

Tjandrawinata RR, Poppy FA,Tandrasasmita OM, Dina R, Asep A (2010). DLBS1425, a *Phaleria macrocarpa (Scheff.) Boerl*. Extract confers antiproliferative and proapoptosis effects via eicosanoid pathway. J. Exp. Ther. Oncol. 8(3):187-201.

Winarto WP (2003). Mahkota Dewa: Budidaya dan pemanfaatan Untuk Obat, Penebar Swadaya, Indonesia.