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INDUCTION AND MAINTENANCE OF CALLUS FROM LEAF EXPLANTS OF *PHALERIA* *MACROCARPA*

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Abstract - *In vitro* and *in vivo* leaves explants of *Phaleria macrocarpa* was selected to study their response towards callus induction. The callus was induced on MS medium supplemented with various concentrations of NAA alone or with inclusion of BAP. Of the two explants, *in vitro* leaves demonstrated a better performance for callus induction as compared to *in vivo* leaves. The result of surface sterilization used in this study showed that seed explants successfully produced 80% clean culture whereas *in vivo* leaves produced 23.33% clean cultures. The *in vitro* leaves derived from the aseptic seeds responded well to all treatments. Explants showed evidence of response to the treatment by swelling as early as 3 days in culture and whitish callus can be seen after three weeks of inoculation. The highest frequencies of callus induction (100%) was obtained using the MS medium containing 3% (w/v) sucrose supplemented with 2.0 mg/L NAA + 0.5 mg/L BAP (Treatment 5) and 3.0 mg/L NAA + 0.5 mg/L BAP (Treatment 6) using *in vitro* leaves. Later, callus in Treatment 6 turned to white, became loose and spongy which was more suitable for continuous subculture and suspension culture.

Keywords: Callus; Murashige and Skoog (MS) media; 6-benzyloaminopurine (BAP); α -naphthalene acetic acid (NAA); *Phaleria macrocarpa*

I. INTRODUCTION

Phaleria macrocarpa (Scheff.) Boerl is a medicinal plant belongs to the Thymelaeaceae family. It is also known as God's Crown or Mahkota Dewa. The plant originates from Papua Island, Indonesia and grows in tropical areas. *P. macrocarpa* is a complete tree consist of the stem, leaves, flower and fruits. The colour of the fruits before ripe is green and it turns to red when fully ripe. The seeds are well known for its unpleasant taste and chemical property [1]. The plant has been traditionally used as an indispensable medicinal plant in Malaysia and Indonesia.

The use of this plant medically has been well documented. Parts of *P. macrocarpa* that are used for medicinal treatment are the stem, leaves and fruit. For centuries, the old folks have used the fruit and leaves to counter diabetes, liver diseases, vascular problems, cancer and high blood pressure [2]. Traditionally, *P. macrocarpa* is reported to be used by the locals in Malaysia and Indonesia as an herbal drink either single or in combination with others medicinal plants to cure illnesses such as cancer, hypertension and diabetes mellitus [1]. Studies also shown that *P. macrocarpa* contains plant secondary metabolites that can combat fevers and have anticancer properties [2, 3, 4]. *P. macrocarpa* fruit extracts is traditionally used to prevent arteriosclerosis and reduced cholesterol level [5, 6]. In addition, research also revealed that *P. macrocarpa* has anti-microbial, anti-inflammatory and its antioxidant activity [7, 8]. This plant is also capable of controlling impotency, dysentery, hemorrhoid, diabetes mellitus, allergies, liver and heart diseases, kidney disorders, blood diseases, arthritis, rheumatism, high blood pressure, stroke, migraine and various skin diseases [2, 7, 8].

Plant tissue culture is a technique that is widely used to produce clones of a plant under sterilized condition. The plant tissue can be grown in test tubes in which the medium condition can be manipulated. Suitable nutrient medium aided with plant growth regulators plays crucial roles in the development of the plant *in vitro* [9]. Callus is undifferentiated cell mass or tumours induced from any tissue removed from a plant *in vitro*. Callus is formed by adjusting the combination ratio of plant growth regulators, normally auxin and cytokinin in the growth medium [10]. Callus culture has been widely utilized to produce a transgenic plant which is resistant to pest and shows desire characteristic [11, 12, 13]. In this study, the effects of different combinations of α -naphthaleneacetic acid (NAA) and 6-Benzylaminopurine (BAP) on callus induction from *in vivo* and *in vitro* leaves explants of *P. macrocarpa* were studied.



II. MATERIALS AND METHODS

A. Plant materials

Fruits and wildings of *P. macrocarpa* sized about 2 feet were purchased from Forest Research Institute of Malaysia (FRIM) and wet markets in Kuala Pilah, Malaysia. The wildings were maintained at UiTM Negeri Sembilan, Malaysia. The fruits were cleaned from dirt with wet tissues and kept in refrigerator prior to used.

B. Explants Preparation

3 young *in vivo* leaves of *P. macrocarpa* wildings were collected. Initially, the surface of leaves was wiper with a wet tissue. Next, the leaves were washed thoroughly under running tap water for 5 minutes. Leaves were then soaked for 10 minutes in sterile distilled water with a 1 drop of Tween 20 and finally washed thoroughly with sterile distilled water.

C. Surface sterilization of explants

At laminar airflow, the *in vivo* leaves were washed again using sterile distilled water and a drop of Tween 20 with gentle agitation for another 20 minutes. The explant was then soaked in 20% Clorox for 10 min and then washed few times with sterile distilled water and finally air dried for 10 min.

For *in vitro* germination of *P. macrocarpa*, mature and healthy fruits were first washed with sterile distilled water with few drops of Tween 20 followed by rinsed with sterile distilled water for few times. The fruits were then agitated in 20% of Clorox for 10 min before rinsing several times with sterile distilled water and were air dried for 10 min. Seed was then dissected from the fruits under aseptic conditions and transferred onto MS0 regeneration medium. The explants grown into full plantlets within 3 weeks. The young aseptically *in vitro* leaves were then cut and used as an explant

D. Establishment of callus culture

For the establishment of callus culture, the *in vivo* and *in vitro* leaf sheath explants were cut into 1x1 cm² pieces and cultured on MS medium [14] supplemented with 3% sucrose, 0.7% agar and various concentrations of NAA with or without combination of BAP (Table 1). Explants were also cultured on MS medium without growth regulator as a control.

All cultures were maintained in continuous darkness. Cultures were transferred onto fresh media every 4 weeks. Observation was made every day for callus formation and contamination. Cultures that were contaminated with bacteria or fungi is

recorded and discarded by autoclave. The experiment was performed with five replicates per treatment.

Table 1: Different media treatments on MS medium with plant growth regulators for callus induction

Treatment	Plant growth regulators (mg/L)	
	NAA	BAP
T1	1.0	-
T2	2.0	-
T3	3.0	-
T4	1.0	0.5
T5	2.0	0.5
T6	3.0	0.5

III. RESULT

A. *In vitro* germination of *P. macrocarpa* seedling

Fruit of *P. macrocarpa* were relatively hard coated as observed in the experiment. The *in vitro* growth of the dissected seed responded positively by the end of the 2nd week. The first sign of germination was the emergence of hypocotyls, followed by development of leaves on the subsequent week. 99% of the aseptic seed successfully grown into plantlets in the MS0 germination medium (Figure 1A).

B. Percentage of clean culture after surface sterilization

Surface sterilization is the most critical part in plant tissue culture. Establishment of this step is necessary for the continuity of *in vitro* study. In this study, the percentage of clean cultures and contamination by bacteria and fungi were observed every day and the results was recorded after 4 weeks in culture. Results obtained were tabulated in Table 2. For *in vivo* leaf explant, the surface sterilization technique produced 23.33% clean cultures, while 23.33% of bacterial contamination and 53.55% of fungal contamination were recorded after 4 weeks in culture. The *in vitro* seed successfully produced 80% clean culture. Surface sterilization of the seed explant resulted in 15% and 5% of bacterial and fungal contamination respectively.

Table 2 Percentage of clean culture, bacteria and fungi contamination after 4th weeks in culture

	Percentage (%)	
	<i>In vivo</i> leaves	Seed
Clean culture	23.33	80
Bacteria	23.33	15
Fungus	53.33	5

The bacteria contamination appeared as small spot of creamish white growth [15] and can be seen around the base of shoots in the agar-gelled medium (Figure 1B). Fungal contamination exhibits woolly or cottony in texture that covers the explant (Figure 1C) [16]. Bacterial contamination was detected after 3 days of culture whereas fungal contamination was first observed after 5 days of culture.

C. Effects of NAA alone in formation of callus

Callus response of *P. macrocarpa* towards different NAA concentrations alone in MS medium was studied. It was observed that the *in vivo* leaves responded to the treatments after 7 days in culture with swelling at the edge of the leaves explant. For *in vivo* leaves, callus formation was first observed at fourth weeks in Treatment 1 followed by Treatment 3 and Treatment 2; at fifth week respectively. However, only 20% of *in vivo* leaves explants responded to callus formation in Treatment 1, while 30% and 20% of callus formation was observed in Treatment 2 and Treatment 3 respectively.

In *in vitro* leaves, the first respond of explant towards treatments was observed after 3 days in culture in which the explants pieces were started to enlarged and swelled. Later, whitish callus was seen after three weeks in Treatment 1 (Figure 1E) followed by Treatment 2 and Treatment 3; at fourth week respectively. In *in vitro* leaves explants, only 30% of explants responded to callus formation in Treatment 1, while 10% callus formation was observed both in Treatment 2 and Treatment 3.

D. Effects of NAA in combination with BAP on callus induction

The effect of auxin with combination of cytokinin for callus induction of *P. macrocarpa* was also studied. In this study three combinations of NAA (auxin) and BAP (cytokinin) was used. For *in vivo* leaves, callus formation was observed in Treatment 6 during the third week of culture. Unfortunately, no callus observation can be made in Treatment 4 and Treatment 5 due to total contamination. Percentages of explants that formed callus in treatment 6 was 20%. The callus exhibited white in colour and friable in texture.

In the *in vitro* leaves, all cultures were free from contamination in all treatments (4, 5 and 6). Percentage of callus formation in Treatment 4 was 66.67%, whereas 100% callus were observed in Treatment 5 and Treatment 6. Callus in Treatment 4, 5 and 6 exhibited white in colour and friable in texture.

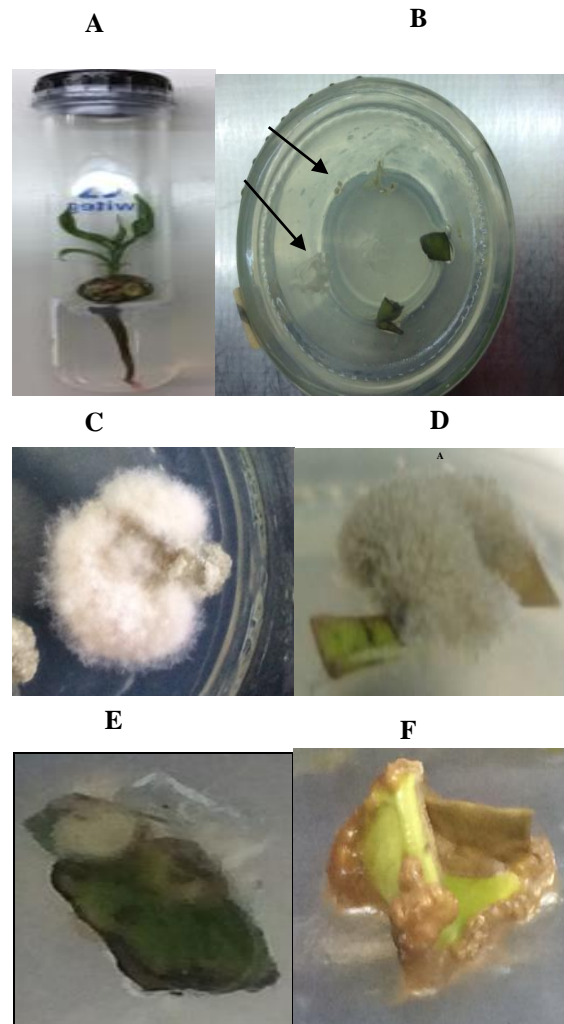


Figure 1: (A) *In vitro* regeneration of *P. macrocarpa* at 3rd weeks. (B) Arrows showed bacteria contamination that visible after 3 days in culture. (C) Fungal contamination exhibit woolly or cottony in texture that covered the explant after 10 days in culture. (D) Friable and whitish callus formed in *in vitro* leaf cultured on MS supplemented with 3.0 mg/L NAA + 0.5 mg/L BAP. (E) Small whitish callus formed in *in vitro* leaf cultured on MS supplemented with 1.0 mg/L NAA. (F) Brownish callus in *in vitro* leaf with no further respond after few weeks in culture.

IV. DISCUSSIONS

The potential used of different plant growth regulators for callus induction has been widely described. The application of auxin solely or with combination of cytokinin is reported to give remarkable effect on callus induction in many plant species. Similarly, in this study, the used of NAA as an auxin with or without combination of BAP (cytokinin) successfully initiate callus induction in leaf explants of *P. macrocarpa*.



Unfortunately, leaf explants cultured on MS medium free from any plant growth regulators did not respond to callus initiation nor any plant development can be observed. The explants eventually turned into brown in color and become necrosis after 3 weeks in culture (Figure 1F). Similarly, no sign of callus formation was recorded for *Oncidium* and *M. jalapa* cultured on PGR-free medium, and subsequently the explant turned into dark brown and died after few weeks in culture [17, 18].

The response of explants towards different treatments was observed visually on daily basis during the culture period. In the first and the second weeks, the explants turned to curved or curled (explants wavy edges). By the third weeks, the edge of the explant thickened and swell followed by the growth of callus on the following weeks. Leaf explants exhibit callus formation at the cut ends and gradually extended over the entire surface of the explants. Then the explants were gradually covered by thin layer of white callus starting from the wounded edge. Faster and greater callus formation was observed in MS medium supplemented with NAA and BAP as compared to MS with NAA alone. The fastest callus formation was recorded in explants cultured on Treatment 5 (MS supplemented with 2.0 mg/L NAA + 0.5 mg/L BAP) and Treatment 6 (MS supplemented with 3.0 mg/L NAA+ 0.5 mg/L BAP). Few weeks later after the callus were subculture on the same medium, the callus on Treatment 6 turned to white and became loose, and spongy which was more suitable for continuous subculture (Figure 1D). It was also observed that in comparison of two types of leaf explants, the *in vitro* leaf explant responded well to the treatments and gave higher percentage of clean culture and callus formation as compared to *in vivo* leaf. Position of leaf cutting may also contributes to callus formation. Leaf part that is closer to the stem showed to induce better callus compare to other part of the leaf.

Differences in callus growth is also influenced by the ability of explant tissues to absorb nutrients provided in the media. It is known that administration of auxin and cytokinin in culture media will stimulate callus formation through cell interaction and cell division. The callus texture also depends on the ratio of auxin and cytokinin [19]. Auxin plays an important role in regulating cell growth and elongation, while cytokinin is important in cell division [20]. Higher concentration of cytokinin than auxin in MS medium may induce callus faster as the number of cells undergoing division will increase [21]. In this study, great response of callus induction in *P. macrocarpa* using MS medium containing NAA with BAP were also in agreement with other studies [22, 23, 24, 25, 26]. The inclusion of BAP alone may cause explant to swell [27]. Nevertheless, the successful of NAA alone for callus induction has been reported for different plants such as

Cucumis anguria [28]; *Solanum dubium* [29], *Jatropha curcas* [30] and *Plumbago zeylanica* [31].

For maintenance of callus, it is necessary to subculture onto fresh same medium after every 30 days, otherwise, the callus became brown and dark and the growth was ceased. The production and oxidation of phenolic compound released by explants may cause browning callus [32]. Unfortunately, no callus observation can be made in treatment 4 and treatment 5 in *in vivo* leaves due to contamination occurred. It was observed that the growth of callus did not begin immediately after the explant were introduced onto the medium. It is due to the fact that the explant required time for absorption to the nutrient provided in the medium and time to adapt to the new environment such as media pH, temperature and culture medium.

V. CONCLUSION

Based on the current study, leaf explant of *P. macrocarpa* maintained in MS medium supplementation of NAA with combination of BAP under dark condition, was proved to be the best condition for optimum callus induction. Among the treatments, explant cultured on MS supplemented with 3.0 mg/L NAA + 0.5 mg/L BAP was shown to be the most effective for induction and production of friable callus. Loose, spongy and friable callus are suitable for a suspension culture. This type of callus has great potential for secondary metabolites and for particle bombardment to insert the gene of interest in the plant. Further investigation on secondary metabolites production using *P. macrocarpa* explants in the form of cell suspension culture is urgently needed. Therefore, the results of this study suggest a noble start for the future experiment.

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