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Effect of Auxin and Cytokinin on Phyllanthusol A Production by Callus Cultures of *Phyllanthus acidus* Skeels

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Abstract: Callus cultures of *Phyllanthus acidus* Skeels were established to verify whether they produce Phyllanthusol A as the intact plant does. Different growth regulator combinations were applied to MS medium to influence the level of production of Phyllanthusol A. The effects of various combinations of auxin and cytokinin on the growth and accumulation of Phyllanthusol A were investigated. MS medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) 1 mg l^{-1} and 6-furfurylaminopurine (kinetin) 1 mg l^{-1} was used to support the growth of callus cultures and the maximum amount of dry biomass (613 mg) was produced after 42 days of culture. High Performance Liquid Chromatographic analysis of methanol extracts from callus cultures of *P. acidus* revealed that the cultures produced Phyllanthusol A. The concentrations of the growth regulators á-naphthaleneacetic acid (NAA) and benzyladenine (BA) played a critical role in the production of Phyllanthusol A. The callus cultures accumulated 20 mg/g.dry weight of Phyllanthusol A in MS medium supplemented with NAA (2 mgl^{-1}) and BA (0.5 mgl^{-1}).

Key words: *Phyllanthus acidus* Skeels · Phyllanthusol A · Callus cultures · Growth regulators

INTRODUCTION

Plants belonging to genus *Phyllanthus* (Euphorbiaceae) produce useful secondary metabolites such as alkaloids, tannins, flavonoids, lignans, phenolics and terpenes [1]. Extractives from *Phyllanthus* have shown antinociceptive action in mice [2]. *P. acidus* Skeels belongs to genus *Phyllanthus* and has several vernacular names depending on the country in which it is found, e.g., Philippines: iba, Indonesia: cerme, Malaysia: chermai and Thailand: ma yom. It has been cultivated as a fruit tree in tropical Asia. The fruit of *P. acidus* is very acidic and is similar to lemon or grapefruit in that it contains 40 mg/100 g ascorbic acid. Several parts of *P. acidus* have been used in folk medicine. The latex from *P. acidus* is credited with emetic and purgative activity [3]. Furthermore, extracts from *P. acidus* have long been used for the treatment of diseases such as hypertension and respiratory ailments [4]. Rats fed with extracts from *P. acidus* showed a hepatoprotective effect against acute liver damage induced by carbon tetrachloride [5]. In addition, methanolic extracts of *P. acidus* possess strong antibacterial activity *in vitro* [6]. The root bark contains saponins, gallic acid and tannins and root extracts have long been used in the rehabilitation program for alcoholics in Thailand. Vongvanich *et al*. [7] isolated Phyllanthusol A from the root of *P. acidus*. Phyllanthusol A (Fig. 1) has been proposed as a possible antitumor

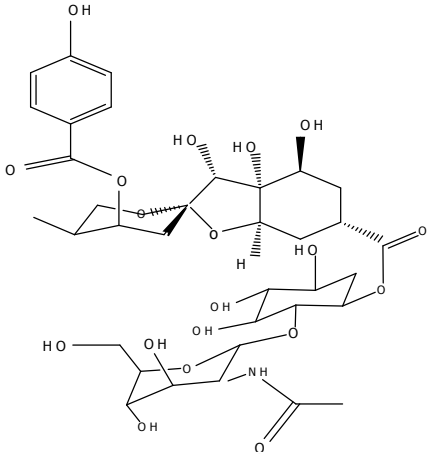

Fig. 1: Phyllanthusol A

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agent [8]. As the development of resistance by tumor cells to chemotherapeutic agents is a major problem in cancer treatment, there is great effort to counter this resistance by finding compounds with novel cytotoxic mechanisms [9]. Phyllanthusol A has attracted considerable attention, as it exhibits cytotoxicity against BC and KB cell lines *in vitro*. This is the first investigation that has focused on callus cultures as an alternative production source of the antitumor agent, Phyllanthusol A. Plant cell and tissue culture have been suggested as a feasible technology for the production of many plant secondary metabolites. For example, ginsenoside from *Panax gingseng*, rosmarinic acid from *Coleus blumei*, shikonin from *Lithospermum erythrohizon*, diosgenin from *Dioscorea*, ubiquinone-10 from *Nicotiana tabacum*, berberin from *Coptis japonica* and podophyllotoxin from *Juniperus chinensis* accumulated at much higher levels in cultured cells than in intact plants [10-12]. The aim of this study is to develop conditions for callus cultures of *P. acidus* by the manipulation of different combinations of plant growth regulators, with the aim of inducing Phyllanthusol A production.

MATERIALS AND METHODS

Establishment of Callus Cultures: Plant materials were collected in Phitsanulok province. Young leaves and stems were surface sterilized in commercial sodium hypochlorite solution (5% active chlorine) for 20 min and then rinsed 3 times with sterile distilled water. Explants of ca. 1 cm. were excised and individually transferred into 4-ounce glasses containing 20 ml of Murashige and Skoog (MS, 1962) culture medium, with 3 % (w/v) sucrose and differing combinations of NAA, 2, 4-D, BA and kinetin. To test the effect of growth regulators, 32 treatments with factorial combinations of four levels of NAA, kinetin, NAA and BA (0, 0.5, 1 and 2 mgl^{-1}) were designed. The pH value of the cultured medium was adjusted to 5.8 prior to autoclaving (121 °C, 15 min) and the medium was solidified with 8 g/l agar. The culture conditions were maintained at 25 ± 1 °C with a 16-hour photoperiod under a photon flux of 1,500-2,000 lux, provided by fluorescent lamps. The explants were cultured for 42 days and each treatment was repeated 3 times.

Growth Measurement: Growth measurement was measured as the dry weight (DW) of callus cultures grown for 42 days by harvesting the biomass, followed by lyophilization.

Extraction and Determination of Phyllanthusol A: The extraction and analysis of Phyllanthusol A was modified from Vongvanich et al. (2000). Each of the 32 lyophilized extracts from treated tissue samples (0.5 g) was ground to a powder, followed by extraction with methanol for 24 h. The extracts were then concentrated under vacuum and re-dissolved in a small amount of the same solvent before separation using HPLC. The Phyllanthusol A content of each sample was determined using HPLC performed on a reversed-phase column (C18 Supelco ODS Hypersil 5 µm, 4 x 250mm) in an Agilent 1100 liquid chromatograph with a diode array (UV detection at 254 nm) by isocratic elution with MeOH/ H_2O (50:50 v/v) as a mobile phase. The flow rate was set at 0.5 ml/min and the oven temperature was set to 40°C. The injection volume was 20µl. The methanolic extract of Phyllanthusol A was identified by comparing its retention time with that of a reference sample of Phyllanthusol A. Statistical analyses were performed with ANOVA. The Duncan multiple range test (DMRT) was used to compare means, with a significant level of 5%. Data presented in tables correspond to the mean values of three replicates.

RESULTS AND DISCUSSION

Establishment of Callus Cultures of *P. acidus*: Callus cultures were initiated from young stems and leaves of *P. acidus* obtained from a natural tree source in Phitsanulok province. Growth regulators 2, 4-D, NAA, BA and kinetin are frequently used to induce callus tissues in many plant species [13].They were selected for initiation of callus cultures of *P. acidus*. Furthermore, MS medium supplemented with NAA, BA and kinetin were utilized by previous researchers for *in vitro* cultures of several *Phyllanthus* species [14-16]. The overall response to plant regulators in stem segments was superior, while leaf explants resulted in poor callus induction. Friable greenish-yellow or beige callus was successfully induced from wound sites in the young stem explants at a culture time in the range of 5-10 days. Callus cultures were subcultured every 30 days. The callus cultures of the third sub-culture were used to investigate the effect of 32 different combinations of growth regulators on growth and Phyllanthusol A production. All combinations of growth regulators induced callus growth without an organogenesis response over 42 days of cultivation.

Effect of Growth Regulators on Callus Cultures Growth: The production of secondary metabolites in callus cultures is controlled by environmental factors and by

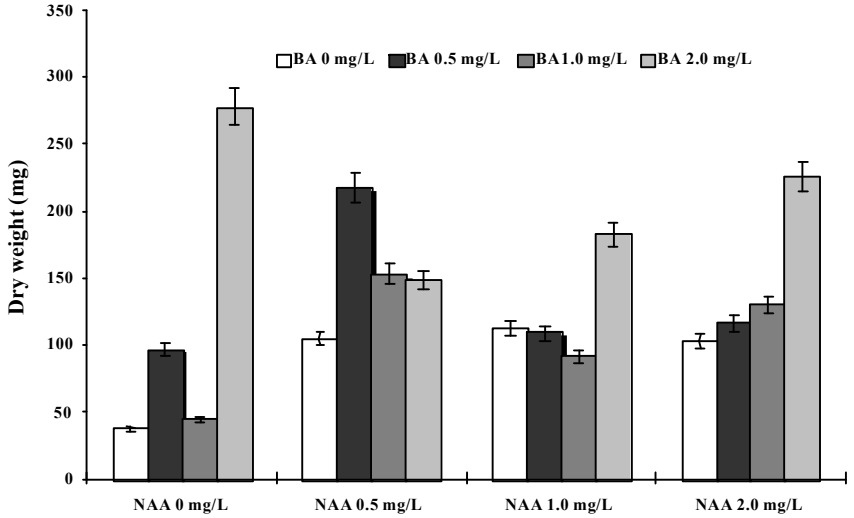

Fig. 2: The Effect of Different Combinations of NAA And BA on the Growth of Callus Cultures of *P. acidus* After 42 Days of Culture. (Values represent the mean n = 3, ± SE, P< 0.5)

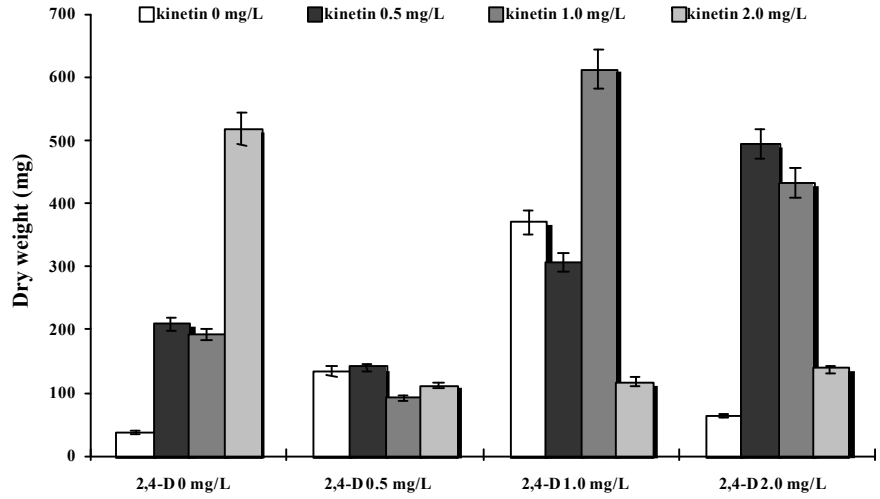

Fig. 3: The Effect of Different Combinations of 2,4-D and Kinetin on the Growth of Callus Cultures of *P. acidus* after 42 Days of Culture. (Values represent the mean n = 3, ± SE, P< 0.5)

plant material. These factors are medium component, pH, temperature, etc. Plant growth regulator such as auxins and cytokinin has shown the remarkable affects on growth and differentiation and thus metabolism of cultured cells [17, 18,]. In order to obtain callus biomass and phyllanthusol A in high concentration, therefore, experiments were carried out varying of growth regulators of the medium. The effects of various concentrations of auxins (2, 4-D, NAA) and cytokinins (kinetin, BA) on the growth of callus cultures derived from stem segment explants are presented in Figs. 2 and 3. Four concentrations of NAA, 2, 4-D, BA and kinetin (0, 0.5, 1, 2 mg l^{-1}) were varied in 32 combinations. All treatments were established with a fresh weight of 0.5 g of callus cultures. The stem-derived calluses showed a steady growth with a maximum up to 42 days and decreased by the 60th day. Calluses contained water content approximately 90% of fresh weight. Fig. 2 shows that increasing level of BA resulted in a high cell dry biomass (278 mg). In contrast, NAA in all concentrations had less effect in enhancing cell biomass. Combination of BA and NAA at equal concentrations ratio seemed to increase cell dry weight, but still lower than that of 2 mg l^{-1}BA alone. Fig.3 illustrates the dry mass of callus cultures grown in MS medium with different concentrations of 2, 4-D and kinetin. Calluses cultured in medium without growth

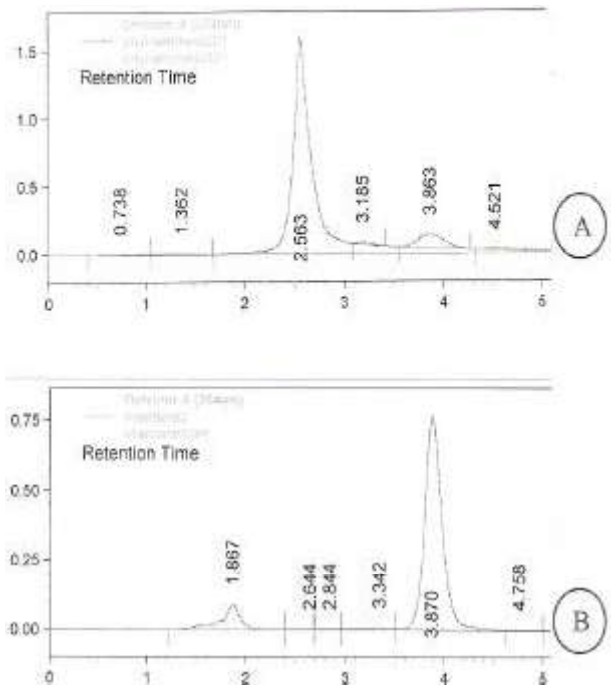

Fig. 4: HPLC Chromatogram of Phyllanthusol A in Callus Cultures Derived from the Stem of *P. acidus* (A) and Authentic Phyllanthusol A (B)

Table 1: The Effect of Different Concentrations of NAA and BA on Phyllanthusol A Accumulation of *P. acidus* Callus derived from stem cultured on MS Medium for 42 Days

NAA (mg/l)	BA (mg/l)	Phyllanthusol A (mg/g DW)	NAA (mg/l)	BA (mg/l)	Phyllanthusol A (mg/g DW)
0	0	0.071^{d}	0	1	0.27^{d}
0.5	0	0.98^{d}	0.5	1	1.74^{d}
1	0	10.29^{c}	1	1	0.27^{d}
2	0	12.73^{b}	2	1	0.37
0	0.5	0.71^{d}	0	2	0.250^{d}
0.5	0.5	0.23^{d}	0.5	2	0.991^{d}
1	0.5	0.283^{d}	1	2	0.16^{d}
2	0.5	20.23^{a}	2	2	1.36^{d}

Means in the same column with different letter(s) differ significantly according to Duncan's multiple range test (P <0.05)

Table 2: The Effect of Different Concentrations of 2,4-D and Kinetin on Phyllanthusol A Accumulation in *P. acidus* Callus derived from stem cultured on MS Medium for 42 Days

2,4-D (mg/l)	kinetin (mg/l)	Phyllanthusol A (mg/g DW)	2,4-D (mg/l)	Kinetin (mg/l)	Phyllanthusol A (mg/g DW)
0	0	0.016^{e}	0	1	ND
0.5	0	0.85b^{c}	0.5	1	0.15^{de}
1	0	0.720^{bcd}	1	1	ND
2	0	0.26^{cde}	2	1	1.14^{b}
0	0.5	0.80^{bc}	0	2	0.121^{de}
0.5	0.5	0.26^{cde}	0.5	2	1.12^{b}
1	0.5	0.29^{cde}	1	2	10.22^{a}
2	0.5	ND	2	2	0.12^{de}

Means in the same column with different letters differ significantly according to Duncan's multiple range test (P <0.05)

regulator showed the least dry biomass (37 mg). The maximum dry weight (613 mg) was observed for cultures containing 2, 4-D (1 mg l^{-1}) and kinetin (1 mg l^{-1}) and it was 16.5 times the weight of the control. The interaction of 2, 4-D and kinetin had a significant effect on cell dry weight. Among single growth regulators, kinetin alone increased cell biomass at the higher concentration (2 mg l^{-1}). These results demonstrated that, of the two cytokinins, BA and kinetin, 2 mg l^{-1}kinetin supported growth of callus and provided higher biomass than that of the BA. However, of the two auxins, 2, 4-D had more effect on growth of callus when compared to the NAA.

The Effect of Growth Regulators on Phyllanthusol A Production: HPLC analysis was used to detect the presence of Phyllanthusol A. The peak corresponding to Phyllanthusol A was identified by comparison with the elution time of the corresponding standard material and by an increase in the corresponding peak area when the reference standard was added to the sample (Fig. 4). Data presented in Tables 1 and 2 demonstrate the effects of growth regulators on the content of Phyllanthusol A. It was found that 2,4-D, kinetin, NAA and BA have a significant effect on Phyllanthusol A production in callus cultures of *P. acidus*. The accumulation of Phyllanthusol

A was observed for each of the combinations of NAA and BA as well as 2, 4 D and kinetin. Phyllanthusol A contents were varied from 0 to 20.23 mg g^{-1}DW. Callus grown in MS medium without growth regulator produced Phyllanthusol A in amount of 0.016-0.071 mg g^{-1}DW. Among single growth regulators, increasing in level of NAA stimulated accumulation of Phyllanthusol A while 2, 4-D performed contrast results. The callus gave very low response in product accumulation to BA and kinetin. Though the combinations NAA and BA were less effective than combinations of 2, 4 D and kinetin in promoting callus growth, they caused a marked increase in Phyllanthusol A production. The greatest Phyllanthusol A production (20.23 mg g^{-1}DW) resulted from the presence of 2 mg $l^{-1}$of NAA and 0.5 mg $l^{-1}$of BA, as compared to other combinations. We did not detect Phyllanthusol A in callus cultures grown on medium with the combination of growth regulators that produced the maximum dry weight. Taniguchi *et al*. [19] reported that addition of NAA (10 μM), BA (10 μM) to LS medium could enhance the production of triterpenes in callus cultures of *Eriobotrya japonica*. In addition, the shoot culture of *Mentha arvensis* produced terpenoid when the cultures were grown on MS medium supplemented BA (5 mgl^{-1}), NAA (0.5 mgl^{-1}) [20]. Similar observations have been reported for callus cultures of *Eucommia ulmoides*. Callus cultures of *E. ulmoides* showed high levels of accumulation of pinoresinol di-o-β glucoside when 3 mg l^{-1} of NAA and 4 mg l^{-1} of BA were added to the growth medium [21].

In conclusion, this is the first report on the establishment of callus cultures of *P. acidus* in order to produce Phyllanthusol A by varying the level of growth regulators. The amount of Phyllanthusol A in callus derived from stem (20.23 mg g^{-1}DW) is higher than that in root (10 mg g^{-1} DW).

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