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Production of Resveratrol from Callus Cultures of *Artocarpus lacucha* Buch.–Ham.

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Abstract

Production of resveratrol from callus cultures of *Artocarpus lacucha* Buch.-Ham. was investigated. Callus of *A. lacucha* was established and cultured at 25 ± 2 °C in Murashige and Skoog (MS) medium supplemented with various combinations of plant growth regulators, auxin and cytokinin. The accumulation of resveratrol in the cell cultures was determined by TLC densitometric method. The results revealed that callus cultures of *A. lacucha* produced and accumulated relatively high level of resveratrol. The *A. lacucha* callus cultures produced resveratrol simultaneously during the rapid growth phase of the culture cycle, suggesting that the production of resveratrol in this plant was growth-associated. Callus generated from shoots of *A. lacucha* produced and accumulated the highest yield of resveratrol (0.79 mgg^{-1}DW) when cultured in MS medium supplemented with 4.5 μM of 2, 4-dichlorophenoxy acetic acid (2,4-D) and 4.4 μM of Benzylaminopurine (BAP), which was remarkably higher than that found in the heartwood of the intact plant.

Keywords: *Artocarpus lacucha* Buch.-Ham., resveratrol, callus culture, TLC densitometry

1. Introduction

Artocarpus lacucha Buch.-Ham., commonly known as "Mahat", belongs to the family Moraceae which is widely distributed in the South and Southeast Asia regions (5). It has been used in the Thai traditional medicine for centuries. In addition to oxyresveratrol, resveratrol and other flavonoids compounds in the heartwood of the *A. lacucha* have been reported to be used as therapeutic agents in human health (9). Resveratrol possesses the effective pharmacological properties such as anticancer (3), antioxidant, anti-inflammatory, antibacterial (6) as well as protection against coronary heart disease (1, 2) and dementia (4). Currently, *A. lacucha* becomes a popular among other medicinal plants. While resveratrol can be extracted from a selected number of plants, it is not suitable for many applications in the pharmaceutical sector. Moreover, the number of *A. lacucha* in the wild has been dramatically decreased.

Plant tissue culture (PTC) has been known as a potential tool for the production of highly defined secondary metabolites from various plant species. Production of oxyresveratrol by callus cultures of *A. lacucha* has been previously reported (7), but there has been no reported dealing with resveratrol production through PTC system. Here we reported that callus of *A. lacucha* could be easily established from various explants and these cultures produced and accumulated resveratrol simultaneously during the growth phase of the culture cycle when grown in MS medium supplemented with various combinations of plant growth regulators.

2. Materials and Methods

2.1 Plant materials

Seeds and heartwood of *A. lacucha* were collected from Sisaket Province, Thailand, in March 2012.

2.2 Establishment of callus cultures

Seeds of *A. lacucha* were surface sterilized and germinated on a hormone-free MS medium containing 3.0% sucrose and 0.8% agar. After 8 weeks of cultivation, the seedlings were cut into pieces of explants (roots, cotyledons, leaves, stems and shoots) and all explants were transferred onto the same MS medium supplemented with 4.5 μM 2,4-D: 4.4 μM BAP; 4.5 μM 2,4-D: 8.8 μM BAP; 4.5 μM 2,4-D: 0.44 μM kinetin; and 4.4 μM 1-Naphthaleneacetic acid (NAA): 4.4 μM BAP. The generated callus from 4 weeks cultures from each explant was then transferred onto the fresh MS medium followed by subculturing every 4 weeks. All cultures were grown and maintained at 25±2 °C under cool white light (16/8 photoperiod). Growth of the callus on MS agar medium was monitored by determination of diameter.

2.3 Extraction of resveratrol

During cultivation, callus cultures were withdrew and dried in oven at 60°C for 4 h. Resveratrol was extracted from the callus using the modified method described by Maneechai *et al*., (7). Briefly, dried callus and heartwood of *A. lacucha* (1 g) was extracted at 45°C with 250 ml ethanol in a Soxhlet apparatus for 8 h. The organic layer, after removal of the organic solvent and drying in desiccators, was collected. This resulting extract (1 mg) was then dissolved in 10 ml methanol to give a sample solution.

2.4 Preparation of standard solutions

A standard solution with an accurate concentration of 0.10 mg/ml was prepared by dissolving 10 mg of resveratrol in 100 ml of methanol. Six additional standard solutions (0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 mg/ml) were prepared from the standard solution by serial dilution.

2.5 Determination of resveratrol contents

Resveratrol content in each sample of dried callus and heartwood of *A. lacucha* extract was determined by a Camag TLC system, which included an automatic TLC sampler, a TLC scanner and the CATS software. Chromatography was performed on a TLC aluminum sheet (silica gel 60 F_{254} plate, 20x10 cm) using chloroform: ethyl acetate: formic acid (2.5: 1: 0.1) as the mobile phase (8). Each sample (15 μl) was applied as a band in triplicate. Plates were developed in a glass tank pre-equilibrated with the mobile phase. The solvent was allowed to run up the plate to a height of 8 cm. Chromatograms were evaluated by measuring the peak area with the TLC scanner in the absorbance mode at 254 nm. In this study, retardation factor of resveratrol was 0.40±0.03 (8).

2.6 Linearity

Linearity was determined over the range of 0.2-6.4 mg/spot. Six standard resveratrol solutions with different concentrations were prepared and loaded (15 ml each) onto the TLC plate to spot containing 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 mg/spot resveratrol. A plot of average area under curve (AUC) versus concentration (μg/spot) was constructed and linearity was expressed as the correlation coefficient (r^2).

2.4 Statistical analysis

Student's One-Way ANOVA ($p<0.05$) was performed using SPSS software. Data were presented as mean ± SD from three replications.

3. Results and Discussion

Callus of *A. lacucha* was successfully induced from root, stem, cotyledon, shoot and leaf explants of 4-week-old seedlings by using MS medium supplemented with 4.5 μM 2,4-D and 4.4 μM BAP (Figure 1). All calli were in the friable white-yellowish form (Figure 2). The response of stem, cotyledon, shoot and leaf segments to plant regulators were superior as compared to root segment. These results are in good agreement with that of Duangporn and Siripong (11) who reported that stem segment of *Phyllanthus acidus* higher responded to the plant regulators such as 2,4-D and kinetin, than the root segment.

Generally, growth of callus cultures is depended on environmental factors and nutrient composition in the culture medium particularly the combination of plant growth regulators (11). In this study, various combinations of 2, 4-D and BAP on growth of callus cultures of *A. lacucha* were investigated and the results were summarized in Table 1.

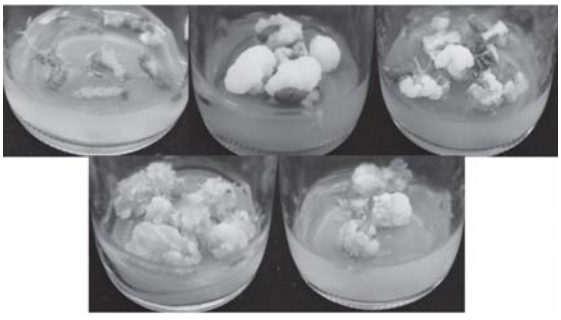

Figure 1. Callus established from the explants of *A. lacucha* on MS medium supplemented with 4.5 μM 2, 4-D and 4.4 μM BAP. a, root explants; b, cotyledon explants; c, leaf explants; d, stem explants; and e, shoot explants

Figure 2. Callus derived from shoot segment of *A. lacucha* growing on MS medium supplemented with 4.5 μM 2,4-D and 4.4 μM BAP

Table 1. The effect of plant growth regulators at various combinations on growth of *A. lacucha* callus cultures grown on MS agar medium

Callus from different explants	Growth of callus (as diameter, cm)			
	2,4-D: BAP (1: 1)	2,4-D: BAP (1: 2)	2,4-D: kinetin (1: 1)	NAA: BAP (1: 1)
Roots	$0.66\pm0.13^{c\underline{A}}$	$0.40\pm0.16^{a\underline{A}}$	$0.37\pm0.23^{a\underline{A}}$	$0.54\pm0.22^{bc\underline{A}}$
Cotyledons	$0.76\pm0.15^{d\underline{BC}}$	$0.69\pm0.12^{cd\underline{CD}}$	$0.66\pm0.12^{bc\underline{B}}$	$0.58\pm0.14^{ab\underline{AB}}$
Leaves	$0.68\pm0.09^{b\underline{AB}}$	$0.54\pm0.15^{a\underline{B}}$	$0.54\pm0.10^{a\underline{B}}$	$0.55\pm0.08^{a\underline{AB}}$
Stems	$0.76\pm0.12^{b\underline{BC}}$	$0.60\pm0.22^{a\underline{BC}}$	$0.65\pm0.18^{ab\underline{B}}$	$0.65\pm0.19^{ab\underline{B}}$
Shoots	$0.80\pm0.15^{a\underline{C}}$	$0.74\pm0.29^{a\underline{D}}$	$0.67\pm0.22^{a\underline{B}}$	$0.67\pm0.18^{a\underline{B}}$

Means in the same column with different letters are differed significantly according to Duncan's multiple range test ($p<0.05$)

Growth measuring by diameter of callus derived from shoot segment of *A. lacucha* was significantly higher than those derived from other explants ($p<0.05$). Among the various combinations of plant growth regulators tested, a combination of 2, 4-D and BAP at a ratio of 1.0:1.0 tend to give higher growth of callus than those of the other combinations. In addition, growth of callus of *A. lacucha* in MS medium containing 2,4-D and BAP was also higher than those of the callus grown in the medium containing kinetin and NAA, suggesting that *A. lacucha* callus cultures preferred 2,4-D and BAP for cell growth than kinetin and NAA. Based on this finding, 2, 4-D and BAP at a ratio of 1:1 was chosen for further experiments.

Figure 3 showed growth pattern of *A. lacucha* callus cultures (as dry weight, DW) derived from different explants on MS medium supplemented with 4.5 μM 2, 4-D and 4.4 μM BAP at 25±2°C for 49 days. Growth of the callus was gradually increased and reached its maximum. After 35 days of cultivation, growth of the callus was gradually decreased. This might be due to the depletion of nutrients in the medium. Callus generated from shoot segment exhibited higher cell growth than those of the other callus cultures. The maximum cell growth of the shoot-derived callus was approximately 0.38 g DW.

The production of resveratrol from callus cultures of *A. lacucha* was investigated by culturing 0.2 g fresh cell callus in MS liquid medium supplemented with 4.5 μM 2,4-D and 4.4 mM BAP at 25±2°C for 49 days. The results were shown in Figure 4. During 7-21 days of cultivaton, resveratrol content in the callus cultures was hardly detected. The accumulation of resveratrol was gradually increased after 21 days of cultivation and reached its maximum within 35 days, which was correlated with the growth profile of the callus cultures. The maximum resveratrol content (0.79 mg/g DW) was detected in the callus derived from shoot segment, followed by the callus derived from stem segment. Based on the production profile of resveratrol and growth pattern of the callus, we concluded that the production and accumulation of resveratrol in the callus of *A. lacucha* was growth-associated (7).

The resveratrol content extracted from different calli and from heartwood of the intact plant was compared and the results were summarized in Table 2. It could be seen from this data that the resveratrol content in callus cultures was approximately 0.66±0.001-0.79±0.001 mg/g DW, which was about 4.7-5.6 times higher than that of the heartwood of the intact plant. This result revealed that *A. lacucha* callus is a good source for the production of resveratrol.

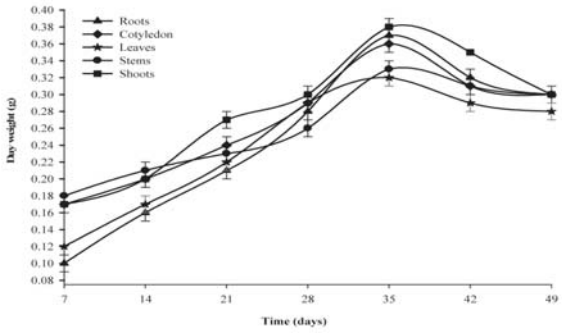

Figure 3. Growth of *A. lacucha* callus cultures on MS medium supplemented with 4.5 μM 2,4-D and 4.4 μM BAP at 25±2°C

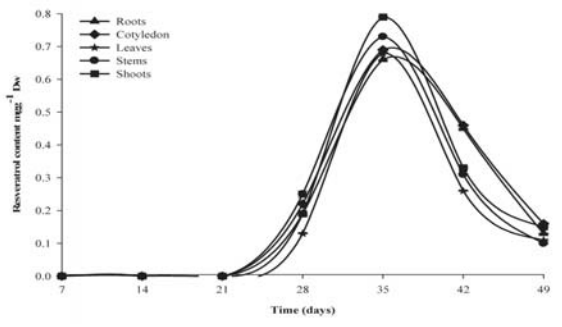

Figure 4. Production of resveratrol by *A. lacucha* callus cultures growing in MS medium supplemented with 4.5 μM 2,4-D and 4.4 μM BAP at 25±2°C

Table 2. Resveratrol content in the callus cultures and heartwood of the intact plant of *A. lacucha*

Sources	Resveratrol content (mg/g DW)
Root-derived callus*	0.66±0.001
Cotyledon-derived callus	0.69±0.003
Leaf-derived callus	0.68±0.002
Stem-derived callus	0.73±0.003
Shoot-derived callus	0.79±0.001
Heartwood of intact plant	0.14±0.020

* Callus from 35-day-old cultures; Data are mean of three replicates ± SD

Resveratrol can be found in various kinds of plant species, e.g., grape fruit and skin, root of Japanese knotweed, etc. The content of resveratrol produced in each plant is inversely related to anthocyanin biosynthesis (10). In grape fruit, the content of resveratrol is varied from 20 to 60 μg/g fresh skin (12). As compared to our results, the content of resveratrol in callus cultures of *A. lacucha* was comparable to that found in grape berry. This finding suggested that *A. lacucha* is one of the potential sources for the production of reveratrol for the industrial uses.

4. Conclusion

The callus of *A. lacucha* was successfully induced from the aerial part of the plant. All calli exhibited their abilities to synthesize and accumulate an active compound, resveratrol when cultivated on MS medium. Supplementation of 2, 4-D and BAP at a ratio of 1:1 gave the highest growth and resveratrol production by callus cultures of *A. lacucha*. This finding suggested that callus cultures of *A. lacucha* have a high potential to be used as a source of the resveratrol production. Further studies on cultural optimizations such as nutrient composition in the cultural medium and elicitation are needed in order to improve the resveratrol production efficiency of the callus cultures of this plant.

5. Acknowledgement

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6. References

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