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Optimization of Air Temperature and Medium pH Enhanced Growths and 1′-Acetoxychavicol Acetate (ACA) Content of Galangal (*Alpinia galanga*) Plantlets *in vitro*

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Abstract

Galangal (*Alpinia galanga*) is a tropical rhizome-producing plant that produced 1′-acetoxychavicol acetate (ACA) which has been used as a traditional herb in South-Eastern Asian. However, the environmental factors in the fields such as microorganisms, heavy metal, dust, etc. affected the growth and ACA content in galangal. Therefore, the aim of this research was to investigate the responses of growths and ACA content of galangal plantlets on different air temperatures and medium pH. The galangal *in vitro* plantlets were cultured on modified Murashige and Skoog (1962) under different air temperatures (15, 20, 25, 30 and 35°C) and medium pH (4.0, 5.5, 7.0 and 8.5) for 5 weeks. Fresh weight and dry weight of shoots, roots and rhizomes, and ACA content were assessed. The fresh weight and dry weight of shoots, roots and rhizomes were enhanced at 20-35°C air temperature. The ACA content in galangal rhizomes was greatest at 30°C air temperature (28.0 μg g^{-1} rhizome dry weight). In addition, the medium pH at 5.5-7.0 promoted the fresh weight and dry weight of shoots, roots and rhizomes of galangal plantlets. The ACA content in galangal rhizomes was greatest when cultured on medium pH at 7.0 (28.2 μg g^{-1} rhizome dry weight). Moreover, the air temperature and medium pH were positively related to ACA production (r^2= 0.97 and r^2= 0.90, respectively). It is deliberated that the growth and ACA content in galangal *in vitro* plantlets can be promoted under condition of 30°C air temperature and medium pH at 7.0.

Keywords: Zingiberaceae, secondary metabolite, medicinal plant, plant tissue culture

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1. Introduction

Galangal (*Alpinia galanga*) is a tropical rhizome-producing plant belonging to Zingiberaceae family. It has been used as a traditional herb in South-Eastern Asian for centuries [1]. Recently, the 1′- acetoxychavicol acetate (ACA) (Figure 1) found in the galangal rhizome has been reported to have properties of anti-cancer [2], anti-tuberculosis [3], anti-allergy [4] and anti-HIV (Human immunodeficiency virus) [5]. However, plant growth and medicinal production in the fields are limited by environmental factors [6-9]. Moreover, the contaminations from the fields such as microorganisms, heavy metal, dust, etc. may be serious causes of being harmful to medicinal plant consumers [10, 11]. Therefore, the advancement of alternative methods is producing plants in *in vitro* culture systems where enhanced growth and medicinal content and no risk of the contamination are presumably obtained.

In vitro culture has been proved to be useful for plant micropropagation and being important tools for plant breeding as well as plant molecular studies [12]. Recently, there has been the immense interest on using of *in vitro* culture systems for production of medicinal plants such as *Adhatoda zeylanica* (vasicine), *Pilocarpus microphyllus* (Pilocarpine) and *Valeriana glechomifolia* (valepotriates), due to its potentiality to control quality and quantity in growth and secondary metabolite production by controlling the *in vitro* environments [13-15].

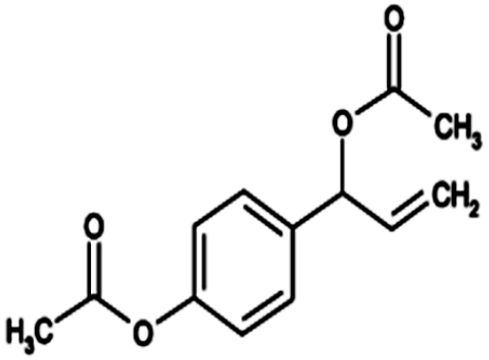

Figure 1 Molecular structure of 1′-Acetoxychavicol acetate (ACA) [3]

Air temperature plays an important role in every phase of plant growth and development since it regulates the differential of physiological processes under *in vitro* culture [16]. It has been shown that the optimal temperature induced growth and secondary metabolites in *Perilla frutescens* [17], wheat [18], *Catharantus roseus* [19], *Hypericum perforatum* [20] and *Eleutherococcus sessiliflorus* [21, 22]. In addition, medium pH plays a role on the available of nutrient and nutrient uptake which is optimal medium pH promote plant growth [23, 24], resulting in induction the plant metabolisms which raised the secondary metabolites in *Beta vulgaris* [25, 26], *Vigna angularis* [27], *Datura stramonium*, *Catheranthus roseas* and *Tagetes patula* [28] and *Panax ginseng* [29].

In this study, galangal, as a tropical and rhizome-producing plant species, was chosen as a model plant for rhizome-producing species. The objective of this study was to investigate the growth and ACA content in galangal plantlets *in vitro* under different air temperatures and medium pH. The relationships between air temperature, medium pH and ACA content were also reported.

2. Materials and Methods

2.1 Preparation of plant materials

Buds of galangal (*Alpinia galanga*) rhizomes from Nakhon Pathom province, Thailand were surface-sterilized twice by soaking for 15 min each with 20% Clorox® (5.25% (w/v) sodium hypochlorite solution (Clorox Co. Ltd., USA). The sterilized buds were cultured for 3 weeks on Murashige and Skoog medium [30] with 0.013 μM N_6–Benzyladenine (BA) and containing 3% (w/v) sucrose and 0.25% (w/v) Phytagel® (Sigma, USA). The medium pH was adjusted to 5.7 before autoclaving. All cultures were grown under condition of 25±2°C air temperature, 60±5% relative humidity (RH), 60±5 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF) provided by fluorescent lamps (TLD 36 W/84 Cool White 3350 Im, Philips, Thailand) and 16 h d^{-1} photoperiod. To increase number of shoot materials, newly developed shoots in clumps were excised and cultured on the same medium and under the same conditions as previously mentioned.

2.2 Effect of air temperature on growth and ACA content

The galangal shoots (2.0±0.25 cm height) were cultured *in vitro* on a modified MS medium with 0.036 μM N_6–Benzyladenine (BA), 3% (w/v) sucrose and 0.25% (w/v) Phytagel®. The medium pH was adjusted to 5.7 before autoclaving. To investigate the effect of air temperature and ACA content, the galangal shoots were cultured at 15±2, 20±2, 25±2 (control), 30±2 or 35±2°C in plant growth chambers (SANYO growth cabinet; Model MLT 350 HT, SANYO Co. Ltd., Japan). Other culture conditions were controlled similarly as mentioned in plant material preparation.

2.3 Effect of medium pH on growth and ACA content

To investigate effect of medium pH on growth and ACA content, the 2.0±0.25 cm long galangal shoots were cultured *in vitro* on the modified MS medium with 0.036 μM N_6–Benzyladenine (BA), 3% (w/v) sucrose and 0.25% (w/v) Phytagel®, and the medium pH was adjusted to 4.0±0.2, 5.5±0.2 (control), 7.0±0.2 or 8.5±0.2 before autoclaving. All shoots were cultured in the plant growth chambers under condition of 30±2°C air temperature, 60±5% relative humidity (RH), 60±5 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF) provided by fluorescent lamps (TLD 36 W/84 Cool White 3350 Im, Philips, Thailand) and 16 h d^{-1} photoperiod.

2.4 Growth analysis

On day 35 after culture, fresh weight and dry weight of shoots, roots and rhizomes were determined according to the method described by Lutt *et al.* [31]. Dry weight of shoots, roots and rhizomes were determined after drying the tissue at 110°C in a hot-air oven (Memmert, Model 500, Germany) for 48 hours.

2.5 Extraction and determination of 1′-Acetoxychavicol acetate (ACA) content

The ACA in rhizomes was extracted by Hexane (99% (v/v), LAB-SCAN Co. Ltd., Thailand) for 72 hours followed the method of Palittapongarnpim *et al.* [3]. A 1-mL extract solution (unknown concentration) in a 1.5-mL Eppendoff tube was solvent-evaporated in a fume hood until the weight of extract was stable. The crude extract was then dissolved in dimethylsulphoxide (DMSO) (99.5% (v/v), CARLO ERBA REAGENTI, Thailand) to prepare a ACA stock solution having concentration of 10 mg mL^{-1}.

To develop colorimetric assay for ACA content by UV, the ACA stock solution diluted to prepare standard solution (0, 4, 8, 16, 32, 64, and 128 μg mL^{-1}) was determined using a spectrophotometer (HACH DR/4000, USA). The absorbance of standard solution was measured at 234 nm. A standard curve plotted by the absorbance of standard ACA showed high linearity of standard curve ($r^2 \geq 0.99$). Hereafter, the ACA content of samples were then quantified by comparing its absorbance to the standard curve.

2.6 Statistical analysis of data

The experimental designs were Completely Randomized Design (CRD) with 5 replications per treatment. Statistical significance was determined by one-way analysis of variance (ANOVA) using the SPSS software (SPSS for Windows, SPSS Inc., USA). Differences between means were assessed with the Duncan's New Multiple Range Test (DMRT).

3. Results and Discussions

3.1 Effect of air temperature on growth and ACA content

Fresh weight of shoots, roots and rhizomes of galangal plantlets were greatest at 35, 25 and 25°C, respectively, and greater 2.4, 2.4 and 1.5 folds than those of the plantlets at 15°C, respectively (Table 1). Shoot dry weight, root dry weight and rhizome dry weight were greatest at 30°C and greater 1.8, 2.5 and 1.6 folds than those of the plantlets at 15°C, respectively (Table 1). In addition, ACA content in the galangal plantlets increased with increasing air temperature from 15 °C, reached the maximum at 30°C (28.0 μg g^{-1} rhizome dry weight), and decreased when the air temperature was over 30° C. The ACA content at 30°C was greater 2.2 folds than those of the galangal rhizomes at 15°C. Moreover, the ACA content in galangal rhizomes was positively related to air temperature $r^2 = 0.97$ (Figure 2).

It is well known that the galangal naturally grows in the temperate and tropical regions, where the air temperature varies from 20-35°C. The present study showed that the fresh weight and dry weight of shoots, roots and rhizomes of galangal plantlets were greatest when cultured at 20-35°C. It has been similarly observed in *Perilla frutescens* [17] and *Catharantus roseus* [19]. However, the fresh weight and dry weight of galangal plantlets significantly reduced when cultured at 15 °C. In general, the growth reduction by decreasing air temperature (20 to 15°C) may be involved with the reduction of enzyme activities, resulting in the reduction of plant growth and development, and secondary metabolites production [24, 32]. It has been similarly observed in *Hypericum perforatum* [20]. In the present study, the ACA of galangal rhizomes cultured under air temperature was lower than 20°C or higher than 30°C to reduce the ACA content. Since air temperature leads to the commute the plant metabolisms such as enzyme activity, resulting in decline secondary metabolite contents [4, 20, 33-35]. The results showed that the air temperature was closely related to ACA content in galangal plantlets (Figure 2). Similarly, other plant species showed the relationship between air temperature and secondary metabolite contents, such as, wheat [18], *Hypericum perforatum* [20] and *Eleutherococcus sessiliflorus* [22]. It could be concluded that the fresh weight and dry weight of shoots, roots and rhizome, and ACA content of galangal rhizomes cultured at 20-35°C was enhanced under *in vitro*.

Table 1 Effect of air temperature on fresh weight (FW) and dry weight (DW) (mg plantlet^{-1}) of shoots, roots and rhizomes of galangal plantlets cultured *in vitro* for 5 weeks onmodified MS medium under a condition of 60±5% RH, 60±5 μmol m^{-2} s^{-1} PPF and 16 h d^{-1} photoperiod (n = 5).

Air temperature (°C)	Shoot		Root		Rhizome	
	FW	DW	FW	DW	FW	DW
15 ± 2	189 c	73 b	7 b	2 c	941 b	282 b
20 ± 2	348 b	122 a	14 a	3 bc	1321 a	396 a
25 ± 2	363 b	127 a	17 a	5 a	1422 a	431 a
30 ± 2	436 a	131 a	15 a	5 a	1344 a	447 a
35 ± 2	445 a	121 a	14 a	4 ab	1274 a	382 a
ANOVA	**	**	**	**	*	**

Means with the different letters are significantly different at $P \leq 0.05$ (*) and $P \leq 0.01$ (**) by Duncan's New Multiple Range Test.

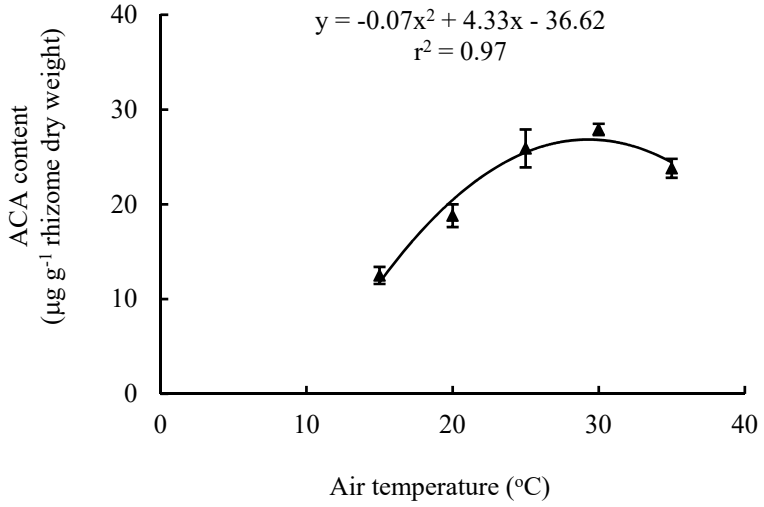

Figure 2 An increase in air temperature induced ACA content of galangal plantlets cultured *in vitro* for 5 weeks on modified MS medium under a condition of 60±5% RH, 60±5 μmol m^{-2} s^{-1} PPF and 16 h d^{-1} photoperiod.

3.2 Effect of medium pH on growth and ACA content

The galangal plantlets cultured on modified MS medium adjusted pH to be 5.5-7.0 showed the enhancement of growth, while those cultured on modified MS medium adjusted pH to be 4.0 or 8.5 showed the growth inhibition (Table 2). The fresh weight of shoots and roots of galangal plantlets were greatest when cultured on MS medium adjusted pH to be 7.0 and 5.5, respectively, and greater 1.4 and 5.8 folds than those of the plantlets cultured on MS medium adjusted pH to be 8.5, respectively (Table 2). The rhizome fresh weight of galangal plantlets was greatest when cultured on MS medium adjusted pH to be 5.5 and greater 1.2 folds than those of the plantlets cultured on MS medium adjusted pH to be 4.0 (Table 2). The dry weight of shoots and roots of galangal plantlets were greatest when cultured on MS medium adjusted pH to be 7.0 and 5.5, respectively, and greater 1.6 and 5.5 folds than those of the plantlets cultured on MS medium adjusted pH to be 8.5, respectively (Table 2). The rhizome dry weight of galangal plantlets was

greatest when cultured on MS medium adjusted pH to be 7.0 and greater 1.3 folds than those of the plantlets cultured on MS medium adjusted pH to be 4.0 (Table 2). Consistently, the results of this experiment showed that the growth of *in vitro* galangal plantlets was promoted on MS medium adjusted pH to be 5.5-7.0, but reduced on MS medium adjusted pH to be 4.0 or 8.5. In addition, the ACA content (μg g^{-1} rhizome dry weight) of *in vitro* galangal plantlets cultured on modified MS medium adjusted pH to be 7.0-8.5 showed the higher ACA content than those of plantlets cultured on the medium adjusted pH to be 5.5 and 4.0, respectively. The ACA content of the galangal rhizome plantlets cultured on MS medium adjusted pH to be 7.0 was greatest (28.2 μg g^{-1} rhizome dry weight), and greater 2.5 folds than those of the plantlets cultured on MS medium adjusted pH to be 4.0. Moreover, the ACA content in galangal rhizomes was related with medium pH and the relation could be expressed with a second order function with an r^2 =0.90 (Figure 3).

Generally, the weak acidic or weak basidic (pH 5.5-6.5) induce nutrient available for plant uptake, resulting in promoting the plant growth and development [24]. Similarly, the present study showed that the fresh weight and dry weight of shoots, roots and rhizomes of galangal plantlets were greatest when cultured at medium pH 5.5-7.0, and reduction when cultured at medium pH 4.0 or 8.5. Reduction the growth of galangal plantlets at 4.0 or 8.5 may be related to the nutrient available in the culture medium [36]. Since alteration of medium pH may reduce beneficial of nutrient and nutrient uptake, leads to decline plant metabolisms, resulting in reduction of plant growth [23, 24]. In addition, the ACA content was affected by alteration of medium pH. In the present study, the ACA content in galangal rhizomes cultured at medium pH 4.0 or 8.5 was reduced. Since medium pH induces the gradient of proton which involves driving the plant metabolisms, especially enzyme activity [37-39] resulting in reduction of secondary metabolite contents. The results showed that the medium pH was closely related to ACA content in galangal plantlets (Figure 3). Similarly, other plant species showed the relationship between medium pH and secondary metabolite contents, such as, *Vigna angularis* [27], *Datura stramonium*, *Catheranthus roseas* and *Tagetes patula* [28] and *Panax ginseng* [29].

Table 2 Effect of medium pH on fresh weight (FW) and dry weight (DW) (mg $plantlet^{-1}$) of shoots, roots and rhizomes of galangal plantlets cultured *in vitro* for 5 weeks on modified MS medium under a condition of 30±2°C air temperature, 60±5% RH, 60±5 μmol m^{-2} s^{-1} PPF and 16 h d^{-1} photoperiod (n=5).

Medium pH	Shoot		Root		Rhizome	
	FW	DW	FW	DW	FW	DW
4.0 ± 0.2	370 a	124 a	15 b	4 b	1109 b	333 b
5.5 ± 0.2	431 ab	133 a	35 a	10 a	1375 a	373 ab
7.0 ± 0.2	486 a	146 a	17 b	5 b	1320 ab	424 a
8.5 ± 0.2	343 b	93 b	6 c	2 c	1304 ab	391 ab
ANOVA	*	*	**	**	ns	*

Means with the different letters are significantly different at $P \leq 0.05$ (*), $P \leq 0.01$ (**) and non-significant (ns) by Duncan's New Multiple Range Test.

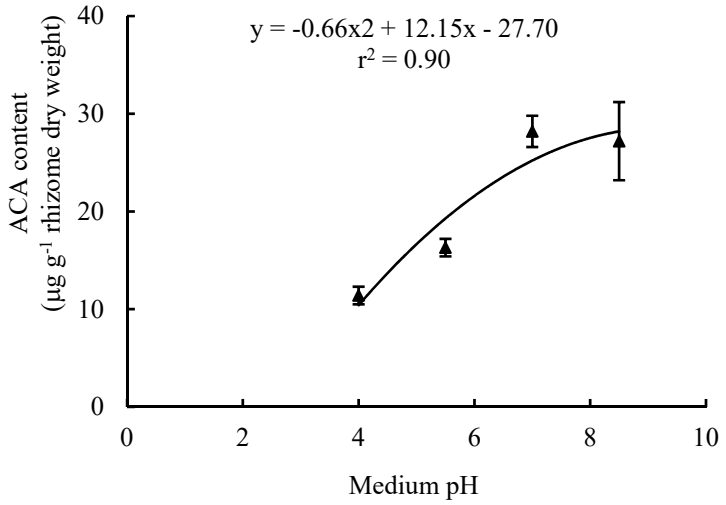

Figure 3 Increasing of medium pH enhanced ACA content of galangal plantlets cultured *in vitro* for 5 weeks on modified MS medium under a condition of 30±2°C air temperature, 60±5% RH, 60±5 µmol m^{-2} s^{-1} PPF and 16 h d^{-1} photoperiod.

4. Conclusions

Changes of air temperature and medium pH influenced on the growth and ACA content of galangal plantlets *in vitro*. Growth and ACA content in galangal rhizomes were induced at 20-30°C air temperature. In addition, the medium pH at 5.5-8.5 could enhance growth and ACA content in galangal rhizomes. However, to improve the efficiency of ACA production *in vitro*, an alternative method to increase of carbon source for biosynthesis by increasing the net photosynthetic rate of the galangal plantlets should be further studied.

5. Acknowledgements

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