

# Effect of Growth Factor In Callus Induction and Bioactive Compounds In Seed Explant of Kaffir Lime (*Citrus hystrix* DC.)

Woro Anindito Sri Tunjung<sup>1\*</sup>, Vita Fatonah<sup>1</sup>, Ghea Putri Christy<sup>1</sup>, Sugeng Triono<sup>2</sup>, Lisna Hidayati<sup>1</sup>, Dwi Priyanto<sup>1</sup>, Yekti Asih Purwestri<sup>1</sup>, Aries Bagus Sasongko<sup>1</sup>, Hennisa<sup>1</sup>, Nur Faizah<sup>1</sup> and Ari Indrianto<sup>1</sup>.

1. Faculty of Biology Universitas Gadjah Mada, Jalan Teknika Selatan Sekip Utara Yogyakarta 55281 Indonesia.
2. Department of Chemistry, Faculty of Mathematic and Sciences, Universitas Gadjah Mada, Jalan Teknika Selatan Sekip Utara Yogyakarta 55281 Indonesia.

## Info Article

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\*Corresponding author  
Woro Anindito Sri Tunjung

Email:  
wanindito@ugm.ac.id

## ABSTRACT

Our previous study showed that kaffir lime leaf extracts may have anti-cancer properties. However, production of bioactive compounds is affected by environmental factors. Here, we present a method to control environmental conditions using *in vitro* culture techniques such as callus induction. Calluses were induced from seed embryo explants of kaffir lime on MS medium with combinations of 2,4-D and BAP at concentrations 1:0.5; 1:1; and 2:1, respectively. Forty and 60 days-old calluses were extracted using chloroform and ethyl acetate and analyzed by GC-MS. Results showed all combinations of 2,4-D and BAP were able to induce callogenesis from seed embryo explants of kaffir lime with no significant differences of callus initiation time, biomass, morphology and growth rates. However differences were detected in the bioactive compound profiles. In kaffir lime callus, both fatty acids and secondary metabolites were detected. Specifically, in 40 days-old calluses (exponential growth phase) we detected  $\alpha$ -pinene and 1,8-cineole in plants treated with 2,4-D: BAP at concentration 1:0.5 and 2:1. In 60 days-old calluses (stationary phase) we detected a number of compounds in plants treated with 2,4-D: BAP at concentrations of 1:0.5 and 2:1, including caryophyllene, linoleoyl chloride, thiogeraniol, stigmaterol, clianosterol, citronellal, neo-isopulegol, citronellol, geraniol, eugenol, cyclopropane, pristane, elemol and farnesol.

**Keywords:** 2,4-D (2,4-dichlorophenoxyacetic acid); Bioactive compounds; BAP (Benzyl amino purine); Callus; kaffir lime


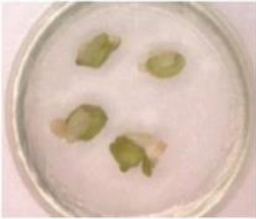
## INTRODUCTION

Kaffir lime (*Citrus hystrix* DC.) is a variety of citrus which is native to Indonesia, Malaysia and Thailand. Kaffir lime is used in food spices, dish flavorings and aroma therapy. According to (Aziman *et al.*, 2012), kaffir lime contains compounds that may be useful medicinally. Our previous study revealed that chloroform and ethyl acetate leaf extracts of kaffir lime have cytotoxic effect on cervical cancer and neuroblastoma cells (Tunjung, *et al.*, 2015). In addition, Hutadilok-Towatana *et al.* (2006) showed that extracts of kaffir lime leaves and fruit have antioxidant, antimicrobial, and anti-inflammatory activity.

Production of secondary metabolites are affected by external factors such as biotic and

abiotic environment. Unfortunately, the content of bioactive compounds in foods with medicinal properties are unstable throughout the year, which may reduce their efficacy. Thus we need biotechnological approach to maintain the quality and potentially increase the quantity of secondary metabolites. The tissue culture can be used to control environmental factors and stabilize secondary metabolite production (Constable, 1984). Callus induction *in vitro* allows a more consistent and uniform production of natural products. From an economical point of view, tissue culture is very profitable because it allows production of natural products in shorter time and does not need large areas, and in this way it is well-suited for use in urban environments.

Table I. Effect of different combination of 2,4-D and BAP on callus induction from kaffir lime seeds explants culture on MS medium

2,4-D (ppm)	BAP (ppm)	Type of response	Type of induction (%)	Callus initiation time (days)	Capacity for growth	Consistence Apperance	Figure
0	0	Germinate	0	-	-	-	
1	0.5	Callus	100	9.75 <sup>a</sup> ±3.24	Good Multiplication	Fariable yellowfish	
1	1	Callus	100	9.25 <sup>a</sup> ±3.11	Good Multiplication	Fariable yellowfish	
2	1	callus	100	11.38 <sup>a</sup> ±5.07	Good Multiplication	Fariable yellowfish	

\*same letter means there are no significantly different at the 95% confidence level according to a Duncan's Test

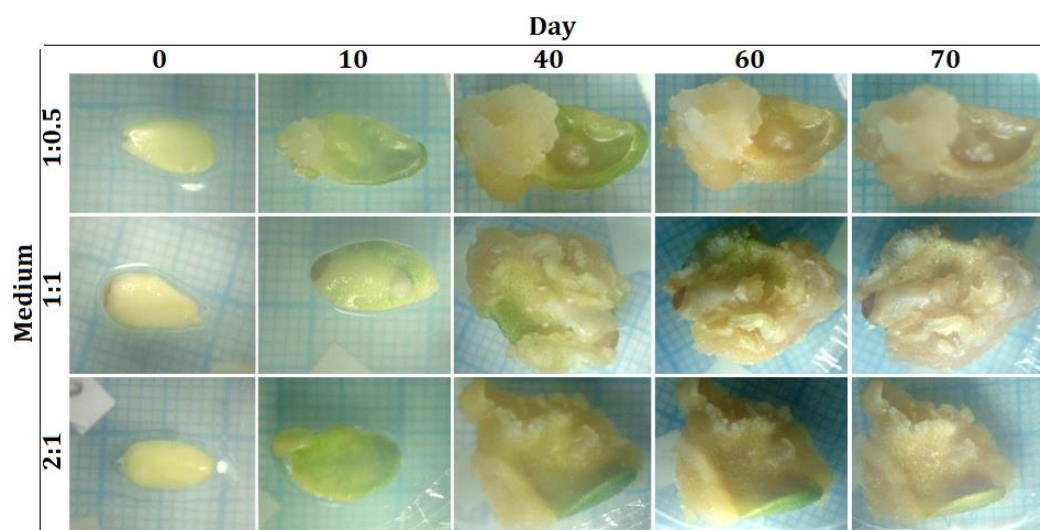


Figure 1. Callus growth and development

Calluses are induced using explants that are inoculated aseptically into a culture medium with addition of various combinations of plant growth regulators. Citrus embryo explants have been shown to be the most responsive to callus induction and proliferation (Jajoo, 2010) thus we used seed explant in this study. Furthermore, a previous study by Ramdan *et al.* (2014) showed that callus induction using embryos of *Citrus rootstocks* was succesful after addition of

synthetic Auxin 2,4-D (2,4-diclorophenoxy acetic acid) and synthetic Cytokinin BAP (Benzylaminopurine). 2,4-D accelerate cell's proliferation and cell's division whereas BAP plays a role in stimulating cell division. Therefore the objectives of this study were to induce callus formation from kaffir lime seeds in MS medium with combinations of 2,4-D and BAP, and to analyze the profiles of bioactive compounds in kaffir lime callus.

## MATERIAL AND METHODS

### Collection of kaffir lime fruits

Fruits were collected from Kaffir lime plantation at Kali Duren, Candirejo, Borobudur Magelang, Central Java, Indonesia.

### Sterilization and callus induction of kaffir lime seeds

Fresh kaffir lime fruits were cut and the seeds then be shelled to discard their outer layer. Seeds were sterilized with NaClO 5.25% (Sigma-Aldrich) for 5min by gently shaking in aseptic condition to protect the seeds from contaminant. Mature embryos were excised from seeds aseptically and induced on MS (Murashige and Skoog, 1962) standard medium which contained 8g/L agar (Bioagar, Swallow globe) and 30g/L sucrose. The pH of the medium was adjusted to 5.8-6.3. Combinations of 2,4D (Merck Millipore) and BAP (Merck Millipore) were applied at relative concentrations of 1:0.5 (ppm); 1:1 (ppm); and 2:1 (ppm) for callus induction. MS0 (without addition of plant growth regulators) was used as control. Culture was incubated at  $\pm 25^{\circ}\text{C}$  in dark condition.

### Callus growth

Callus growth was determined by measuring callus initiation time, percentage of callogenesis, callus biomass, and callus morphology. The callus growth curve was obtained from callus biomass and growth was measured every 5 days until 70 days of culture.

### Analysis of callus bioactive compounds

Based on the curve of callus growth, 40 and 60 days-old calluses were chosen to be macerated using chloroform and ethyl acetate. The extracts were then analyzed by GCMS Shimadzu GCMS-QP 2010S, with a non polar column AGILENT HP-5 MS with 95% dimethyl polysiloxane and 5% diphenyl polysiloxane to determine the profile of callus bioactive compounds. Gas chromatograph linked to a mass spectrometer available at Organic Chemistry Laboratory, Universitas Gadjah Mada, Yogyakarta, Indonesia.

## RESULTS AND DISCUSSION

Callus was initiated from the apical pole of the embryo. All regions of explants displayed swelling. Medium with all combinations of 2,4-D and BAP (1:0.5; 1:1; 2:1) can induce calluses from seed explants after 7 days of inoculation. On the contrary, control (MS medium only) was not able to induce callus formation. Furthermore, in the

control group seeds germinated after a few days of inoculation. It showed the important role of growth regulators in callus induction. Effects of different combination of 2,4-D and BAP on callus induction from kaffir lime seed explants cultured on MS medium (Table I). All combination's mediums of 2,4-D and BAP were able to induce callus formation. Each treatment required different times to reach 100% callus formation; however there was no significant difference in callus initiation time (Table I). All calluses have friable texture and typical odor. Callus's texture is one of the markers used to determine the quality of a callus. A friable callus can be used to generate cell suspension cultures. Calluses were yellow, transparent and shiny, and has a typical flavor that is similar to the kaffir lime's leaf. During its development, calluses change their colour to brownish indicating that the callus has entered its death phase. This process indicated decreasing nurients and accumulation of secondary metabolites. Browning was detected on the 50<sup>th</sup> day. Callus growth and development during 70 days of *in vitro* callus culture (Figure 1).

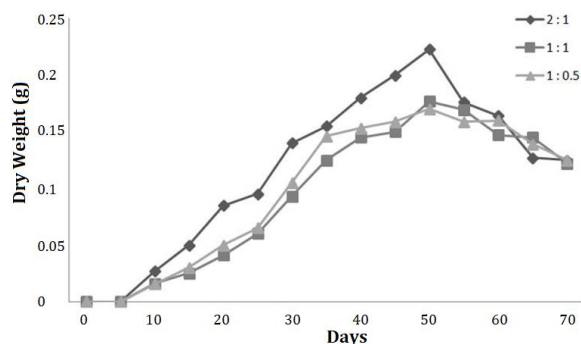


Figure 2. Growth curve of callus of *Citrus hystrix* DC. During 70 days of culture

The callus dry weight was used to determine the growth curve (Figure 2). The rate of callus growth forms a sigmoid curve. Growth curves from 3 combinations of medium with 2,4-D:BAP showed a similar pattern that presented sigmoidal shape, with 3 phases: lag phase, exponential and stationary phase. The lag phase occurred from the beginning of the culture until the 10<sup>th</sup> day after inoculation (DAI), during preparation for cell division. The exponential phase is the period of maximum cell division, and formation of a mass of unorganized cells occurred from the 10<sup>th</sup> DAI to the 50<sup>th</sup> DAI. The last phase was the stationary phase that started at the 50<sup>th</sup> DAI. Callus growth was stagnant, where no cell division or weight change occurs and the callus changed colour to brownish.

At the end of the 60<sup>th</sup> DAI callus growth began to decline due to nutrient reduction. The *in vitro* culture was stopped at 70 DAI because the callus had turned brown. Further studies regarding methods to subculture kaffir lime callus are needed.

This study revealed that all combination of 2,4D and BAP were able to induce callogenesis from seed embryo explants of kaffir lime with no significant differences in callus initiation time, biomass and morphology. Callus texture was friable. Callus growth curves were sigmoidal in shape, with three phases, a lag phases (0-10 DAI), exponential phase (10-50 DAI) and stationary phase (50-60 DAI).

Table II. Profil of bioactive compounds of kaffir lime seeds extracted by ethyl acetate and chloroform

Peak	Ethyl acetate extract	Chloroform extract
1	Methyl ester myristic acid	2-heptenal (Z and E)
2	Methyl ester Oleic	E-2-decenal
3	Methyl ester Palmitic	2,4-decadienal, (E,E)
4	Methyl ester Linolelaidic	E-2-undecenal
5	Methyl ester Elaidic	cyclododecene
6	Methyl ester 10-octodecanoid	
7	Methyl ester Stearic	

We analyzed bioactive compounds in ethyl acetate and chloroform extracts from seeds and calluses. Thus we can compare bioactive compounds from seeds and calluses derived from seed explants. Bioactive compounds of kaffir lime seeds of each peak (Table II). This result showed that bioactive compounds detected in kaffir lime seeds are mostly derived from fatty acid. The seed is an embryonic plant enclosed with a protective outer covering. Thus, compared to the other part, seeds usually have many primary metabolic compounds necessary for embryo nutrition.

Profiles of callus bioactive compounds were analyzed by GC-MS using 40 and 60 days-old calluses to compare bioactive compounds produced in the exponential and stationary phases (Table III and Table IV). In total from both solvents, chromatograms of 40 days-old calluses showed fewer peaks than 60 days-old callus. In addition, ethyl acetate and chloroform were capable to dissolving secondary metabolites compounds detected on both medium 1:0.5 and 2:1 (Table IV).

Table III. Number of bioactive peak on GC-MS chromatogram

Callus days-old	Solvent	Peaks detected in medium*		
		1:0.5	1:1	2:1
40	Ethyl acetate	10	8	7
	Chloroform	5	10	5
60	Ethyl acetate	6	7	5
	Chloroform	15	25	17

Bioactive compounds detected in ethyl acetate and chloroform extract in both 40 and 60 days-old callus in all combinations of 2,4-D and BAP were mostly derived from fatty acids (Table IV). This phenomenon possibly resulted because calluses are still in the growth period and therefore most of the cells synthesize more primary metabolites.

Common and uncommon bioactive compounds in each group. Bioactive compounds that were common to all groups regardless of solvent were fatty acids such as palmitic and oleic acid (Table IV). There was a decrease of these common compounds found in combinations of 2,4-D: BAP of 2:1 ppm. This might be due to an increase in the concentration of the 2,4D in the medium. The different hormone concentrations in medium lead to the alteration of callus bioactive compounds synthesis. On the other hand, both fatty acid and secondary metabolites were detected in callus extracts. Callus induction was able to initiate the formation of secondary metabolites that were detected in a phase-specific manner. In contrast to the seed, the callus is an aggregate of meristematic cells that can differentiate into different types of tissue. Thus, secondary metabolic compounds were synthesized in callus and were more varied than in seeds. The various types of bioactive compounds in calluses were also a result of induction from 2,4-D and BAP in medium.

This study showed that different solvents dissolve different secondary metabolites. Ethyl acetate was capable of dissolving secondary metabolite compounds that were predicted such as  $\alpha$ -pinene and 1.8-cineole. They were detected only in 40 days-old calluses cultured in medium with combinations of 2,4-D and BAP 1:0.5 and 2:1. On the other hand, chloroform was capable of dissolving different types of secondary metabolites in all treatments of 60 days-old calluses which were not detected in ethyl acetate extracts.

Table IV. Profile bioactive compounds of 40 and 60 day old calluses extracted by ethyl acetate and chloroform.

Combination 2,4D:BAP (ppm)	Common Bioactive Compounds			Different Bioactive Compounds			
	40 & 60 DAI	40 DAI	60 DAI	40 DAI		60 DAI	
				Ethyl Acetate	Chloroform	Ethyl Acetate	Chloroform
1 : 0.5	Palmitic acid; Oleic acid	Palmitic acid; Dioctyl adipate	Palmitic acid; Oleic acid; Stearic acid; Dioctyl adipate; Stigmasterol; Clionasterol	Alpha-pinene; 1,8- Cineole; 1,6- Dioxacyclododecane -7, 12-dione; Ethyl oleate; Methyl palmitate; ethyl palmitate	Methyl elaidate; Methyl palmitate; Ethyl palmitate	Cyclodecena	(Z)-2Decenal; cis-13- octadecenal; Cyclohexane eicosyl; Caryophyllene; Androstan-17-one, 3- ethyl-3-hydroxy- (5.alpha); Palmitin,2- mono; Linoleolyl chloride; Cholesta-4,6- dien-3 ol, benzoate, (3.beta)-;
1 : 1	Palmitic acid; Oleic acid			1,6- Dioxacyclododecane -7, 12-dione;1- Phenanthrenecarbox ylic acid; 1,2,3,4,4a,9,10,10a- octahydro-1,4a- dimethyl-7-(1- dimethylethyl)-,R- (1.alpha, 4a, beta, 10a.alpha); Stearic acid	Hexanal; Isooctane; 2- Pentatone; 4- hydroxy-4- methyl-; (Z)- 2Decenal; Evodinol; Stearic acid	Seselin; Stearic acid; Stearic acid; Margaric acid	Isododecane; Hexadecane; Pristane; Methyl palmitate; Methyl linoleluidate; Methyl elaidate; Methyl stearat; 1,3- Dipalmitin; Oleol; Cyclohexenale, eicosyl; dihydroergosterol; Margaric acid
2 : 1	Palmitic acid			2-Pentanone; 4- hydroxy-4-methyl-; Alpha-pinene; 1,8- Cineole; Methyl palmitate	Methyl elaidate; Oleic acid; Methyl palmitate		Citronellal; neo- isopulegol; citronellol; Geraniol; (Z)-2- Decenal; Eugenol; Cyclopropane, -1(-2- methylene-3-butenyl)- 1-(1- methylenepropyl); Elemol; Nerolidol Z dan E; Farnesol; Palmitin, 1,3-di-; Glyceryl palmitate; Thiogeraniol

However calluses with combinations of 2,4-D and BAP 1:0,5 and 2:1 produced more secondary metabolites than combinations of 1:1. Both combinations have the same ratio, in which the amount of BAP is half of 2,4-D. This data reveals that the ratio of 2,4-D and BAP have a very important impact to production of bioactive compounds.

This study indicates that production of secondary metabolites in kaffir lime calluses occurs in both exponential and stationary phase. The number and type of secondary metabolites detected in 40 and 60 days-old calluses was different according to the solvent used in

extraction. Production of secondary metabolites produced during both exponential and stationary phase is supported by previous studies. According to study in tea cell suspension culture (Muthaiya *et al.*, 2013), production of catechin is higher during the stationary phase rather than in both the lag phase and exponential phase. However based on a review (Shahzad *et al.*, 2013), it has been shown that some plants such as *Haplopappus*, *Rosa*, *Datura*, *Scopolia*, *Andrographis*, *Morinda* and *Poppulus* were able to produce secondary metabolites in the early exponential phase. The secondary metabolites are anthocyanin formation by *Haplopappus* and *Poppulus* cells, phenolic

production by *Rosa* cells, tropane alkaloid by *Datura* and *Scopolia*, sesquiterpene lactone by *Andrographis*, anthraquinone by *Morinda*. These data reveal that different species have different production patterns of bioactive compound.

Many bioactive compounds detected in this study are known to have a medicinal role. Our results warrant the further investigation of kaffir lime callus extracts for a more complete understanding of their potential for therapeutic use. Secondary metabolites detected in callus extract (Table V).

From this study it can be concluded that the combinations of 2,4-D and BAP are able to induce callogenesis which yields friable callus. Proportion difference of 2,4-D to BAP has no significant effect on callus initiation time, biomass and morphology. However differences were detected in the bioactive compound profiles. In kaffir lime callus, both fatty acids and secondary metabolites were detected. Specifically, in 40 days-old calluses (exponential growth phase) we detected  $\alpha$ -pinene and 1,8-cineole in plants treated with 2,4-D: BAP at concentration 1:0.5 and 2:1. In 60 days-old calluses (stationary phase) we detected a number of compounds in plants treated with 2,4-D: BAP at concentrations of 1:0.5 and 2:1, including caryophyllene, linoleoyl chloride, thiogeraniol, stigmasterol, clionasterol, citronellal, neo-isopulegol, citronellol, geraniol, eugenol, cyclopropane, pristane, elemol and farnesol.

## CONCLUSION

All combinations of 2,4-D and BAP were able to induce callogenesis from seed embryo explants of kaffir lime with no significant differences of callus initiation time, biomass, morphology and growth rates. However differences were detected in the bioactive compound profiles. Those secondary metabolites detected in callus are known for their anti-cancer, anti-oxidant, anti-inflammatory, anti-fungal, antibacterial properties etc. This finding suggests that kaffir lime callus might be potential resource for large-scale industrial production of secondary metabolites, which is valuable in pharmaceutical industry.

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