MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society For Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

EFFECTS OF SUCROSE AND METHYL JASMONATE ON ALLIXIN PRODUCTION IN CALLUS OF GARLIC (*Allium sativum* L.)

Jeeraporn Chitphan[1, 2] Rachaporn Manmanasaree[1, 2] Pornchai Junta[2] Tanawat Ariya[2], Srisulak Dheeranupattana[3] and Lalida Shank[2*]

[1]*Interdisciplinary Program in Biotechnology, Graduate School, Chiang Mai University, Chiang Mai, Thailand, 50200*
[2]*Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand, 50200*
[3]*Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand, 50200*

*Corresponding Author: lalida.shank@cmu.ac.th

History
Received: 30 April 2022
Accepted: 15 November 2022

Keywords:

Allixin; Callus; Garlic; Methyl jasmonate; Sucrose

Abstract

Allixin is a nonsulfur-containing compound with red-brown color found to accumulate on the surface of garlic (*Allium sativum* L.) bulbs that have been stored long term, approximately 9 months to 2 years. This phytoalexin showed several unique biological properties such as antioxidative, antimicrobial, radical scavenging, and neurotrophic effects with the ability to inhibit the binding of aflatoxin B2 to DNA. Allixin is absent in fresh garlic and may possibly be produced via plant tissue culture technique. Effects of sucrose and methyl jasmonate (MeJA) on allixin production in callus of garlic were therefore investigated in this study. Germ-free garlic explants were prepared and cultured on Murashige and Skoog (MS) medium, which contained 3% (w/v) of sucrose in the presence of 5.0 mg/L of kinetin and 1.5 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) for 4 weeks. Garlic callus was subsequently subcultured onto the MS medium containing 0.5 mg/L of 2,4-D supplemented with different sucrose concentrations, varying from 3 to 6% (w/v) and in other sets by varying sucrose concentrations from 3 to 6% (w/v) in combination with either 25 or 50 µM of MeJA. After incubation for another 4 weeks, fresh calli were subjected to extraction with methanol and analysed by High-Performance Liquid Chromatography (HPLC) to observe allixin production in comparison with commercial standard. Sucrose at 4% (w/v) in MS medium provided the highest allixin content at 0.248 ± 0.01 mg/g among the concentrations tested. The combination of 25 or 50 µM MeJA with 4% (w/v) of sucrose further enhanced allixin content to 0.343 ± 0.02 mg/g and 0.949 ± 0.03 mg/g, while allixin was not detected in the control group of callus. Hence, induced garlic callus is a newfound source of allixin with a much more feasible production time. Moreover, low heat drying did not affect allixin content and offered convenience for storing garlic callus for future extraction.

INTRODUCTION

Garlic (*Allium sativum* L.) belongs to the family Alliaceae and is known as a prophylactic therapeutic medicinal plant [1]. Garlic is normally propagated from cloves and after harvest, the garlic bulbs are usually placed in storage houses. During the post-harvest stage, the bulbs are prone to fungal contamination that could lead to spoilage, discoloration, and disintegration of the tissues as well as mycotoxin contamination [2, 3]. After about 9 months to 2 years of storage, allixin accumulation can be found on the surface of garlic bulbs [4]. Production of allixin in garlic might occur in response to continuous microbial stress to help protect the garlic cloves against fragmentation by invasion of microorganisms. In addition, allixin was only

observed in stressed garlic bulbs around the surface of the necrotic area [5].

Allixin (3-hydroxy-5-methoxy-6-methyl-2-penthyl-4H-pyran-4-one) is a phenolic phytoalexin that firstly isolated and characterized in 1989. It is a nonsulfur-containing compound having a γ-pyrone skeleton structure. Allixin is a compound with red-brown color and can form crystalline [4]. This compound showed several unique biological properties such as antioxidative, antimicrobial and anti skin-tumor promoter activity *in vivo*, radical scavenging effect, inhibition of aflatoxin B2 binding to DNA, and inhibitory effect on aflatoxin B1 *in vitro* mutagenesis induction [6]. Neurotrophic effects have also been reported. Simple chemical analogs of allixin were found to have potent neurotrophic activity. Allixin may, therefore, be a useful starting substance for the development of pharmaceutical drugs for the treatment of neurodegenerative disorders and for neuronal regeneration in the brain [7]. However, the biosynthesis pathway of allixin is imprecise. Chemical synthesis of allixin has been reported by two research groups. The first method demonstrated by Hirokazu *et al.* completed the synthesis in 22 steps starting from D-mannose. Later, Yoshihiro *et al.* carried out the process in 5 steps from 5-methylfurfural [6, 8].

Generally, the production of secondary metabolites from vegetative population is minimal compared with *in vitro* cultures. In the case of allixin, the amount produced in raw garlic with necrotic tissue areas after storage for about 2 years was 1400 mg/g fresh garlic [5].

Therefore, plant tissue culture technologies have attracted our attention as the alternative means for studying and enhancing the production of allixin. Plant tissue culture conditions such as temperature, light, and humidity as well as the addition of supplements in media can be controlled and optimized [9, 10]. Sucrose is the main source of carbon energy for *in vitro* cultures. The addition of an external carbon source to the agar media often enhances the regeneration of cells and proliferation of callus [11]. Sucrose serves not only as an energy source, but also controls other factors such as promoting the production of secondary metabolites in plants [12]. In addition, the use of external hormones such as methyl jasmonate (MeJA) to the plant cell culture can stimulate the biosynthesis of phytoalexins and other secondary metabolites, for examples, alkaloids, shikonine and toxoids. MeJA has also been demonstrated to trigger physiological responses linked to pathogen assault, including the modulation of phytoalexin metabolism. It also can stimulate molecular signal transduction, induce production of antioxidant enzymes, promote expression of defense-related genes, which in turn results in the production and induction of phytoalexins *in vitro* culture [13-15].

This research aimed to study the effects of sucrose and MeJA on the production of allixin in callus of garlic. The utilization of a garlic callus culture stimulated with sucrose and MeJA is an alternate way for allixin manufacturing with a faster production time for future applications.

MATERIALS AND METHODS

Materials

Garlic (*Allium sativum* L.) bulbs were purchased from a farmers market hosted by Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand. Garlic cloves were used as starting materials for callus induction. Allixin standard was purchased from MuseChem in New Jersey, USA. All chemicals were HPLC grade.

Plant Tissue Culture Preparation

Prior to incubation, the explants were washed under running water for 30 min. Subsequently, the explants were surface sterilized with 0.1% (w/v) mercuric chloride with 3 drops of Tween 20 for 15 min, followed by 70% ethanol for 30 s and rinsed in sterile distilled water for 15 min three times. The explants were cut into approximately 6.0-8.0 mm in length before placing on MS medium.

Callus Induction

For garlic callus induction, MS medium [16] with 3% sucrose was supplemented with 1.5 mg/L of 2,4-dichloropheoxyacetic acid (2,4-D) and 5.0 mg/L of kinetin following the method applied by Khan *et al*. [17]. The medium pH was adjusted to 5.8 ± 0.02 using 1 N of NaOH or HCl and sterilized by autoclaving at 121 °C and 1 atm of pressure for 15 min. The sterile garlic cloves were transferred to medium for callus induction after the medium was perfectly solidified. The cultures were incubated in a growth chamber (Contherm phytotron climate simulator, New Zealand) at 25 ± 2 °C with the relative humidity of 70% under photoperiod of 16 h light and 8 h dark at 2000-2500 lux from General Electric white fluorescent tube. The distance between the fluorescent tube and the culture bottles was approximately 60 cm. Weight and morphology of callus were taken and recorded on days 7, 14, 21, and 28. The callus was transferred from the garlic explant at the age of 28 days onto the subculturing medium and incubated in the growth chamber for another 28 days prior to another subculturing onto the basal medium with different treatments.

Elicitation of Callus with Different Sucrose Concentrations

Garlic callus was subcultured onto MS medium with different sucrose concentrations, varying from 3 to 6% (w/v) supplemented with 0.5 mg/L of 2,4-D. The concentration of 2,4-D was reduced to 0.5 mg/L to prevent further shoot development and to promote multiplication of garlic callus. MS medium with 3% sucrose served as the control. The treatment and control cultures were prepared in triplicate, with three individual samples examined for each treatment. Fresh weights of calli were recorded on days 7, 14, 21, and

28. Callus from days 7, 14, 21, and 28 were harvested and extracted with methanol, respectively, and the amount of allixin was estimated according to commercial standard by HPLC analysis.

Elicitation of Callus with Sucrose in Combination with Different Concentrations of MeJA

Garlic callus was subcultured onto MS medium supplemented with 0.5 mg/L of 2,4-D with different sucrose concentrations, varying from 3 to 6% (w/v) in combination with different MeJA concentrations (25 and 50 µM). MS medium with 3% sucrose devoid of MeJA was used as control. The treatment and control cultures were prepared in triplicate, with three individual samples examined for each treatment. Calli from days 7, 14, 21, and 28 were harvested. Fresh and dry weights of the callus samples were recorded from days 7, 14, 21, and 28 prior to extraction with methanol. Allixin was quantified using HPLC analysis.

Sample Preparation and Extraction

Fresh callus was collected for extraction or dried in an oven at 40 °C for 24 h before extraction. Samples were extracted by mashing in a mortar with pestle and homogenized with methanol in 5-fold volume to fresh weight of the sample. The extracts were soaked in methanol at 4 °C for 24 h before centrifugation at 9000 xg at 4 °C for 20 min. The supernatants were filtered through a 0.45 µm polytetrafluoroethylene membrane filter and kept in glass vials at 4 °C until use.

Analysis of Allixin Content in Garlic Callus Using HPLC Method

Quantitative analysis was carried out on a VertiSep UPS C-18 column (150 mm x 4.6 mm ID) at 30 °C, equipped with UV detector (280 nm) operated by HPLC system from Agilent, USA. Chromatographic separation was carried out using a gradient elution. The mobile phase was a mixture of methanol and deionized water (40:60, v/v) and a mixture of methanol and deionized water (90:10, v/v). The mixture was filtered using membrane filter (0.45 µm) and degassed in an ultrasonic bath for 15 min before being analyzed. The flow rate was kept at 0.6 mL/min with sample injection of 10 µL. Allixin was identified according to the retention time of its corresponding standard. Quantification was carried out using the calibration curves.

Statistical Analysis

Statistical analysis of the quantitative data was performed using One Way Analysis of Variance (ANOVA) with a significance level of 0.05 and multiple (pairwise) comparisons were performed by Least Significant Difference Test (LSD Test) from the Statistix software version 8 for windows and Microsoft Excel 2021 version for standard deviation (SD). All experiments were repeated at least three times and the results are given as the mean of three independent experiments ± standard deviation (SD).

RESULTS AND DISCUSSION

Callus Induction with 2,4-D and Kinetin

The appearance of calli on explants was an indicator of growth during *in vitro* culture. Callus formation on explants was stimulated by nutrients present in the media and growth regulators. In this study, clove explants of garlic were placed on MS medium with 3% sucrose supplemented with 1.5 mg/L of 2,4-D and 5.0 mg/L of kinetin. Callus induction from garlic cloves was successfully achieved. Callus started growing within 14 days (Figure 1C) and appeared clearly on a new shoot and around cut wound within 28 days of incubation (Figure 1E). Callus was friable with yellowish color (Figure 1F).

Figure 1. Representative morphology of garlic callus. (A) Clove Explants, (B) 7 days old culture, (C) 14 days old culture, (D) 21 days old culture, (E) 28 days old culture, (F) friable callus with yellowish color

In general, the application of combined auxin and cytokinin in MS medium resulted in the fastest response and the highest frequency of callus induction. Auxins induce callus formation, proliferation and somatic embryogenesis while cytokinins induce mostly shoot and root differentiation and elongation [18, 19]. The combination of 0.5 mg/L of 2,4-D and 0.5 mg/L of kinetin resulted in better callus production compared with the application of auxin alone for callus induction from root explants of Allium sativum L. [20]. In addition, Robledo-paz *et al*. [21] reported that application of 4.5 µM of 2,4-D and 4.6 µM of kinetin optimized callus production from root-tip culture of Allium sativum L. The addition of 2,4-D and kinetin in MS medium was demonstrated as the best composition to induce calli from root tips of garlic by Khan *et al*. [22], which resulted in large amount of friable callus. When garlic clove explants were cultured in MS medium in the presence of both 2,4-D and kinetin, growth of friable callus was also observed in this study. Our data agreed with those concluded by Khan *et al*. [22].

Effects of Sucrose Concentrations on Callus Growth

As plant cells and tissue in a culture medium lack the autotrophic ability, added sucrose serves as the main source of carbon energy for in vitro cultures. The addition of an external carbon source to the medium enhances the regeneration of cells and proliferation of callus [12]. MS medium supplemented with 0.5 mg/L of 2,4-D with different sucrose concentrations, varying from 3 to 6% (w/v) were used in this experiment. Callus culture of garlic treated with sucrose was monitored for growth via fresh weight. The result of garlic callus elicitation with sucrose after 28 days of culturing showed that the MS medium elicited with 3% sucrose gave the highest fresh weight, followed by that of 4% sucrose, 5% sucrose or 6% sucrose, respectively (Figure 2.).

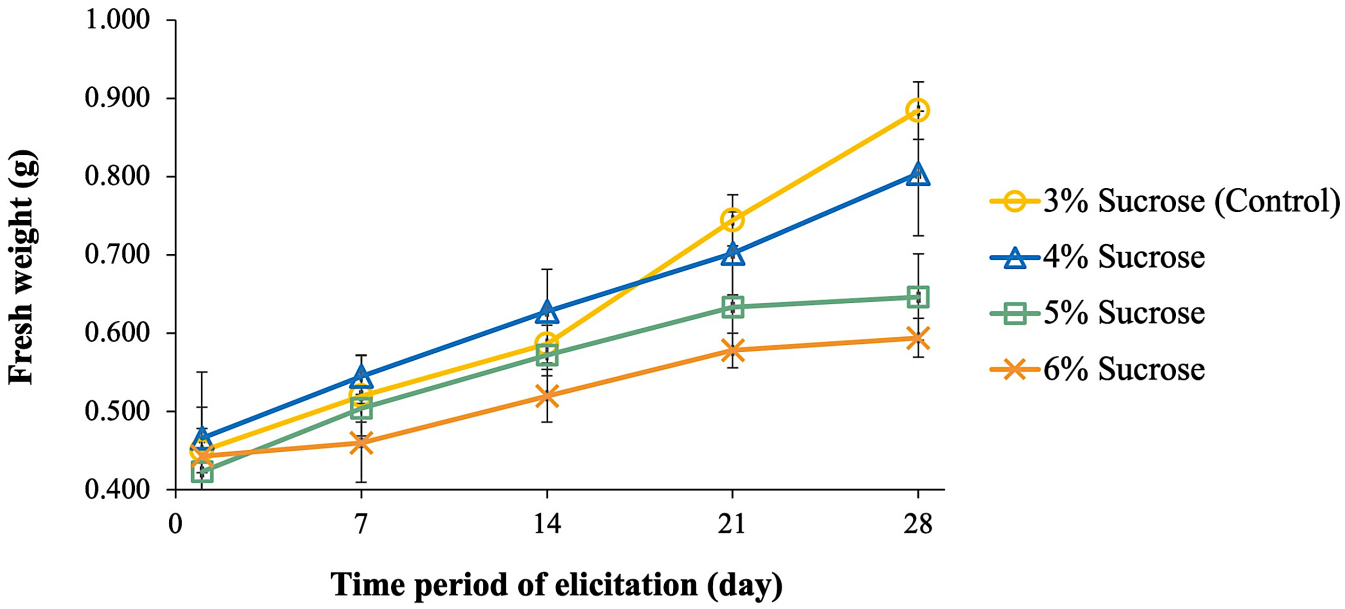

Figure 2. Garlic callus weight with sucrose elicitation

The scarcity of carbon sources will cause the explants to grow slower or even not at all, while too much carbon source will become toxic to the explants [11]. Normal concentration of sucrose in MS medium used at 3% (w/v) for this experiment was optimal to support plant cell growth during the in vitro incubation period. Extra sucrose (4, 5 and 6% w/v) did not promote growth of the garlic callus as much as those cultures grown in 3% w/v of sucrose.

HPLC Quantitative Analysis of Allixin

Effect of Sucrose Elicitation on Allixin Content of Garlic Callus Culture

Allixin content was analyzed for each treatment of callus and presented in Figure 3. Normal sucrose concentration (3% w/v) in MS medium did not result in accumulation of allixin in garlic callus. On the other hand, the amount of allixin increased when the callus of garlic was elicited with extra sucrose concentrations (4, 5 and 6% w/v). The best sucrose concentration for production of allixin was 4% (w/v), which yielded 0.248 ± 0.01 mg/g. Sucrose at 5% (w/v) yielded allixin at 0.245 ± 0.01 mg/g, followed by 6% (w/v) which yielded 0.122 ± 0.01 mg/g after 28 days of culturing.

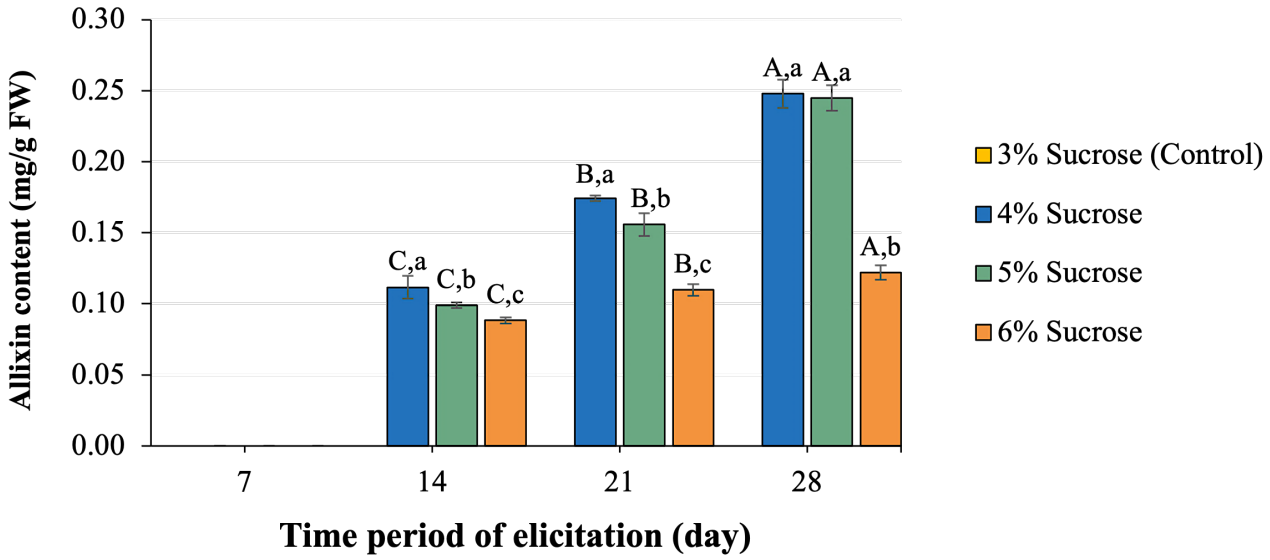

Figure 3. Effect of sucrose elicitation on allixin content of garlic callus culture. Values represent means of measurement made on three samples per treatment with statistically significance at $p < 0.05$ according to ANOVA. Uppercase letters represent statistic differences compared among different time periods. Lowercase letters represent statistic differences compared among different glucose concentrations. Values are expressed as means ± standard deviation (SD)

Additional sucrose did not only increase carbon energy in vitro culture, but was also able to regulate the production of secondary metabolites in the callus. An increase in secondary metabolite production with the application of 4% (w/v) sucrose in growth medium of Wedelia biflora was also described by Idris *et al*. [11]. They reported optimum production of stigmasterol in stem explants culture of Wedelia biflora when 4% sucrose was utilized among all concentrations tested (1-5% w/v). Furthermore, Modarres *et al*. [23] addressed that increase of sucrose concentrations in MS medium to 4% (w/v) enhanced the contents of caffeic acid and salvianolic acid in cell suspension culture of Salvia leriifolia Benth. Moreover, the highest amount of rosmarinic acid was observed in MS medium with 5% (w/v) of sucrose compared with that of the control.

Therefore, the production of allixin in callus of garlic could be improved by regulation of sucrose concentrations in MS medium. This study verified that the application of 4% and 5% (w/v) sucrose concentration in MS medium supplemented with 0.5 mg/L of 2,4-D helped increase the production of allixin from callus of garlic.

Effect of Sucrose in Combination with MeJA Elicitation on Allixin Content of Garlic Callus Culture

Methyl jasmonate is one of signaling hormones that is induced under stress and respond quickly to stimulate the creation of secondary metabolites when under stress. In plant tissue culture, MeJA has been widely used to produce secondary metabolites in various plant species. *[*9, 24, 25*]*. In this study, the effect of different sucrose concentrations, varying from 3 to 6*% (*w/v*)* in combination with either 25 or 50 µM of MeJA on allixin production by callus culture of garlic was investigated. Allixin contents in garlic callus treated with different concentrations of sucrose *(*3 to 6*% (*w/v*))* in combination with 25 µM of MeJA in both fresh and dry forms are presented in Figure 4A and Figure 4B, respectively. Those treated with different concentrations of sucrose *(*3 to 6*% (*w/v*))* in combination with 50 µM of MeJA in both fresh and dry forms are presented in Figure 5A and Figure 5B, respectively. Dried calli were tested to assure the stability of allixin in callus if kept in the dry form for future extraction.

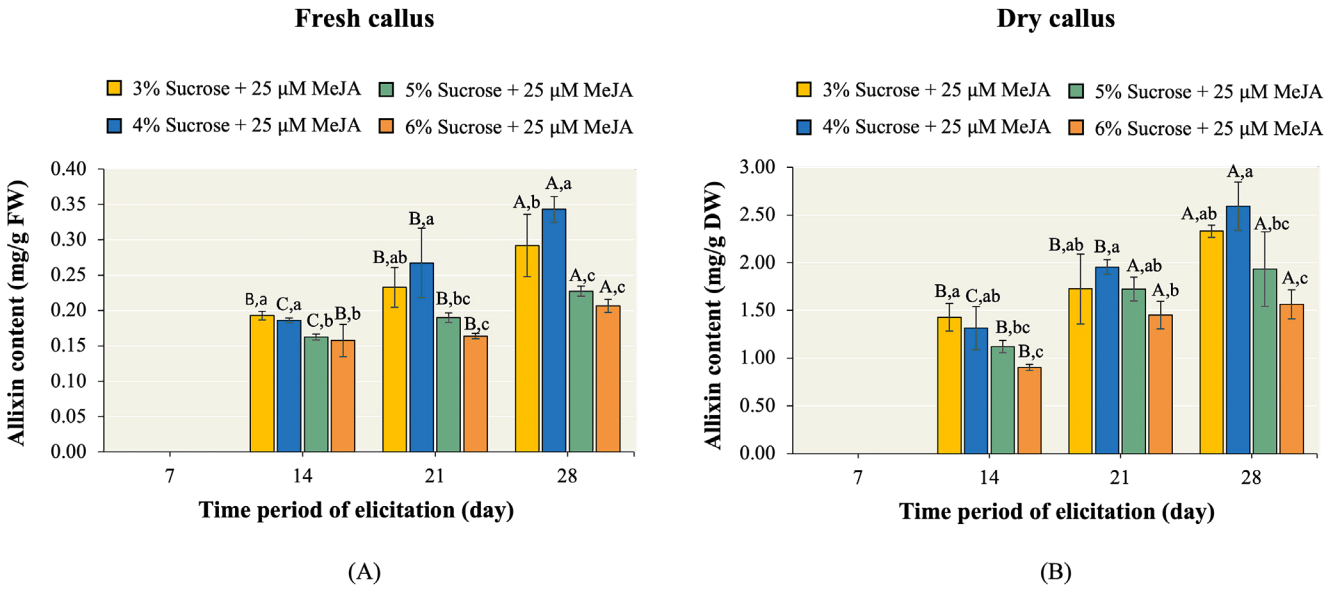

Figure 4. Effect of different sucrose concentrations, varying from 3 to 6% (w/v) in combination with 25 µM of MeJA on allixin production of fresh callus (A) and dry callus (B). Values represent means of measurement made on three samples per treatment with statistically significance at $p < 0.05$ according to ANOVA. Uppercase letters represent statistic differences compared among different time periods. Lowercase letters represent statistic differences compared among different glucose concentrations. Values are expressed as means ± standard deviation (SD)

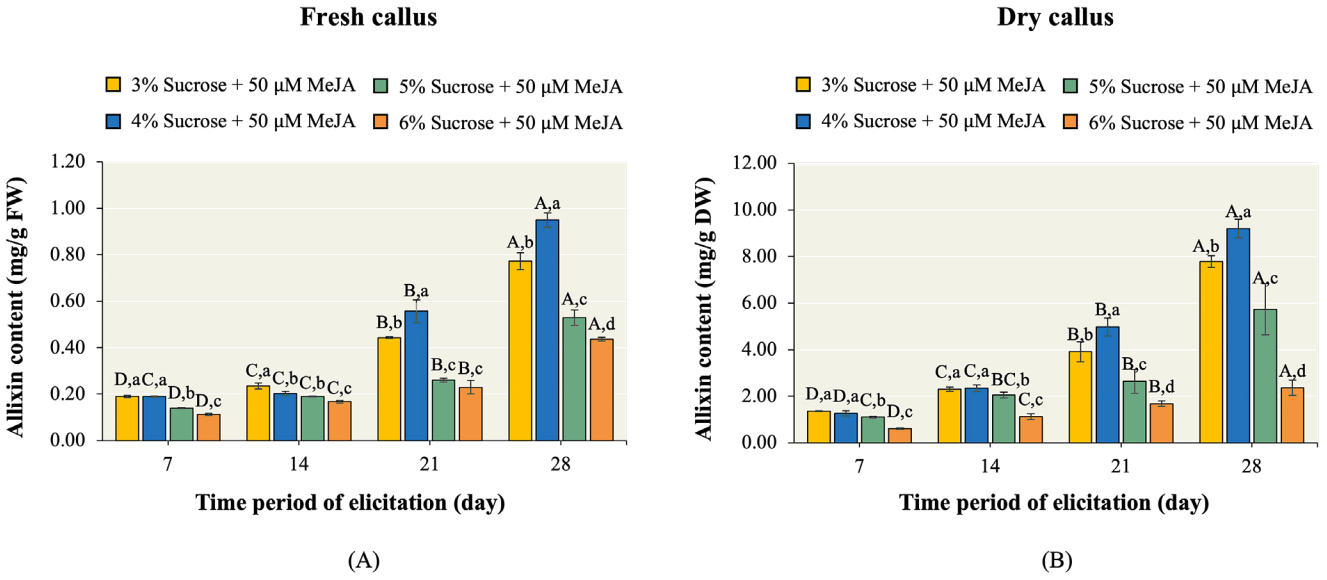

Figure 5. Effect of different sucrose concentrations, varying from 3 to 6% (w/v) in combination with 50 µM of MeJA on allixin production of fresh callus (A) and dry callus (B). Values represent means of measurement made on three samples per treatment with statistically significance at $p < 0.05$ according to ANOVA. Uppercase letters represent statistic differences compared among different time periods. Lowercase letters represent statistic differences compared among different glucose concentrations. Values are expressed as means ± standard deviation (SD)

The application of different sucrose concentrations, varying from 3 to 6% (w/v) in combination with 25 µM of MeJA did not promote accumulation of allixin in callus during 7 days of culturing. However, the amount of allixin increased when the callus of garlic was elicited with this combination for 14 days. After 28 days, the best combination was 4% (w/v) of sucrose with 25 µM of MeJA, resulting in 0.343 ± 0.02 mg/g FW and 2.590 ± 0.25 mg/g DW respectively, followed by 3% (w/v) of sucrose with 25 µM of MeJA (0.292 ± 0.04 mg/g FW and 2.330 ± 0.06 mg/g DW), 5% (w/v) of sucrose with 25 µM of MeJA (0.227 ± 0.01 mg/g FW and 1.933 ± 0.39 mg/g DW), and 6% (w/v) of

sucrose with 25 μM of MeJA (0.207 ± 0.01 mg/g FW and 1.565 ± 0.15 mg/g DW), respectively.

On the other hand, the application of different sucrose concentrations, varying from 3 to 6 % (w/v) in combination with 50 μM of MeJA showed the presence of allixin within the first week of culturing and the amounts of allixin were greater than those with 25 μM of MeJA for all sucrose concentrations, varying from 3 to 6% (w/v). The best combination was 4% (w/v) of sucrose with 50 μM of MeJA providing 0.949 ± 0.03 mg/g FW and 9.200 ± 0.40 mg/g DW, after 28 days of culturing, followed by 3% (w/v) of sucrose with 50 μM of MeJA (0.773 ± 0.04 mg/g FW and 7.784 ± 0.25 mg/g DW), 5% (w/v) of sucrose with 50 μM of MeJA (0.529 ± 0.03 mg/g FW and 5.433 ± 1.10 mg/g DW), and 6% (w/v) of sucrose with 50 μM of MeJA (0.436 ± 0.01 mg/g FW and 2.359 ± 0.33 mg/g DW), respectively.

Hampel *et al*. reported that MeJA was used as an elicitor of artemisinin accumulation in suspension cell culture of Artemisia annua. It was also shown that MeJA treatment offered higher production of artemisinin in intact plants. This finding was significant from a practical point of view since this plant is the only commercial source of artemisinin [13]. In addition, MeJA treatment extended the quality of peeled garlic cloves. MeJA treated garlic cloves could be stored at 0 °C for up to 4 months or at 5 °C for up to 3 months due to secondary metabolites produced to inhibit bacterial activity [26].

Therefore, this study proposes that the combination of 50 μM MeJA with 4% (w/v) of sucrose in MS medium supplemented with 0.5 mg/L of 2,4-D is the best to enhance the production of allixin from callus of garlic.

Effect of Drying on Stability of Allixin

In terms of oven-drying, water is usually removed by evaporation, and secondary metabolites may undergo degradation or changes within their chemical structure as well. Yukihiro *et al*. described allixin as volatile compound [28]. Accordingly, loss of allixin by drying process was investigated for all treatments in this study.

Garlic callus samples were dried in an oven at 40 °C for 24 h until their weights were stable. Fresh and dry weight of garlic callus elicited with 3% w/v of sucrose (control) are presented in Table 1. It was notable that allixin contents in the control samples remained undetectable throughout the 28 days of culturing. Fresh and dry weight of garlic callus elicited with 4% (w/v) of sucrose in combination with 50 μM of MeJA are presented in Table 1. Dry weight of garlic callus elicited with 3% w/v of sucrose (control) and that of garlic callus elicited with 4% (w/v) of sucrose in combination with 50 μM of MeJA decreased approximately 10-fold from fresh weight.

Table 1. Fresh and dry weight of garlic callus elicited with 3% w/v of sucrose (control) and 4% (w/v) of sucrose in combination with 50 μM of MeJA

Callus form	Elicitor	Weight (g)				
		Time period of elicitation (day)				
		1	7	14	21	28
Fresh	3% *(w/v)* sucrose	$0.450 \pm 0.03^{E, a}$	$0.520 \pm 0.06^{D, a}$	$0.586 \pm 0.03^{C, a}$	$0.744 \pm 0.04^{B, a}$	$0.884 \pm 0.04^{A, a}$
	4% *(w/v)* sucrose + 50 μM MeJA	$0.513 \pm 0.10^{AB, a}$	$0.449 \pm 0.05^{AB, a}$	$0.527 \pm 0.09^{AB, a}$	$0.551 \pm 0.16^{A, b}$	$0.402 \pm 0.09^{B, b}$
Dry	3% *(w/v)* sucrose	$0.044 \pm 0.00^{D, a}$	$0.052 \pm 0.01^{D, a}$	$0.065 \pm 0.00^{C, a}$	$0.077 \pm 0.01^{B, a}$	$0.095 \pm 0.01^{A, a}$
	4% *(w/v)* sucrose + 50 μM MeJA	$0.051 \pm 0.01^{A, a}$	$0.052 \pm 0.01^{A, a}$	$0.050 \pm 0.01^{A, b}$	$0.057 \pm 0.01^{A, b}$	$0.046 \pm 0.02^{A, b}$

Values represent means of measurement made on three samples per treatment with statistically significance at $p < 0.05$ according to ANOVA. Uppercase letters represent statistic differences compared in the same row. Lowercase letters represent statistic differences compared in the same column. Values are expressed as means ± standard deviation (SD)

Allixin contents in garlic callus treated with 4% (w/v) of sucrose in combination with 50 μM of MeJA in both fresh and dry forms which was the best combination are presented in Table 2. Each gram of dry weight of callus treated with 4% (w/v) of sucrose in combination with 50 μM of MeJA were found to contain allixin approximately 10-fold greater than those in the fresh ones at 28 days. The weight of garlic callus was reduced about 10 times due to evaporation from low heat drying, but all allixin content remained in the samples. Thus, with the drying, there were no changes in the amount of allixin. This gives us the convenience of dry treatment of garlic for storage for future extraction.

Table 2. Allixin contents in garlic callus treated with 4% (w/v) of sucrose in combination with 50 µM of MeJA

4 % (w/v) Sucrose + 50 µM MeJA	Allixin contents (mg/g)				
	Time period of elicitation (day)				
	1	7	14	21	28
Fresh callus	ND	0.189 ± 0.00	0.203 ± 0.01	0.557 ± 0.05	0.949 ± 0.03
Dry callus	ND	1.266 ± 0.11	2.345 ± 0.14	4.977 ± 0.38	9.200 ± 0.40

Values are expressed as average of three replicates ± SD.
ND: not determined

These results suggest that oven dry process at 40 °C insignificantly affected allixin content. However, this step helps provide convenience for storage. Another alternative method would be freeze-drying of the elicited callus which remains to be investigated for its effect on allixin recovery. However, freeze-drying may be an efficient alternative with shorter processing time and less impact on the degradation of the allixin due to heat. Gao *et al*. reported that freeze-drying of *Ziziphus jujuba* Mill. retained higher contents of secondary metabolites and preserved the antioxidant activity when compared to the oven dry process [27].

CONCLUSION

Callus formation from clove explants of garlic has been established through the combination of 1.5 mg/L of 2,4-D and 5.0 mg/L of kinetin in MS medium. Callus generated from this hormone combination was yellowish and friable in nature. Elicitation of garlic callus at all concentrations of sucrose combined with MeJA in this work enhance allixin content when compared to that the control. The addition of 4 % sucrose (w/v) combine with 50 µM MeJA in MS medium for a period of 28 days was of the best condition for allixin production. Moreover, garlic callus can be harvested, dried and stored without affecting the stability of allixin for future use.

ACKNOWLEDGEMENTS

Authors would like to extend their gratitude to the Interdisciplinary Program in Biotechnology, Graduate School, Chiang Mai University and Center of Excellence for Innovation in Chemistry (PERCH-CIC) for the financial support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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