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Application of elicitors (jasmonic acid, salicylic acid and nanosheet) for *in vitro* growth and biochemical properties of Siam tulip (*Curcuma alismatifolia* cv. Maejo Impress)

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Abstract

Importance of the work: The Siam tulip (*Curcuma alismatifolia*) is an economic crop with the potential for producing bioactive compound through plant tissue culture.

Objectives: To investigate the effects of jasmonic acid, salicylic acid and two-dimensional nanomaterials (nanosheets) on the *in vitro* growth of *C. alismatifolia* cv. Maejo Impress and its phytochemical properties.

Materials & Methods: Two types of elicitors were tested at three concentrations and they were combined with a nanosheet. The control group utilized a basal Murashige and Skoog medium. The experiment units were assigned following a completely randomized design. After 6 wk of culture, growth, total phenolic content and antioxidant activity of the Siam tulip cv. Maejo Impress were studied.

Results: The addition of elicitors had no significant effect on the height of explants and new shoots, shoot multiplication and leaf colour. However, the Siam tulip cultured with 4 mg/L and 6 mg/L jasmonic acid (JA) had the highest ($p < 0.05$) total phenolic content and antioxidant activity compared to those cultured without elicitors.

Main finding: The optimal type and concentrations of elicitors were 4–6 mg/L JA for improving the accumulation of bioactive compounds in the micropropagation of *C. alismatifolia* cv. Maejo Impress without inducing shoot necrosis or death.

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Introduction

Siam tulip (*Curcuma alismatifolia*), a native species of North-eastern Thailand, has become an important tropical crop with high economic value for worldwide floriculture. This plant is a monocotyledonous perennial crop that grows from rhizomes. Besides being cut flowers and ornamental plants, *C. alismatifolia* is used as an herb or as a medicinal plant (Taheri et al., 2014; Theanphong and Mingvanish, 2017). Micropropagation of commercial crops is now widely used as it provides many benefits, such as large-scale, disease-free production and germplasm conservation, while in addition, it can be used for secondary metabolite production as demonstrated for several genera, including *Bosenbergia*, *Curcuma*, *Alpinia* and *Kaempferia* (Victório et al., 2011; Yusuf et al., 2013; Chaturvedi et al., 2014; Senarath et al., 2017; Jirakiattikul et al., 2021a,b). The genus *Curcuma* has been reported as a resource for polyphenols and essential oils. For example, *C. alismatifolia* contained essential oils, such as xanthorhizol and curcumene, with the former oil having several bioactive functions, such as anticancer, anti-inflammatory and antioxidant effects (Oon et al., 2015; Theanphong and Mingvanish, 2017).

Secondary metabolites are generated and accumulated in many parts of plants in tiny amounts with specific roles. One of these groups is phenolic compounds that have a wide range of biological activities, such as antioxidant, anti-inflammatory and antibacterial or fungal (Tanase et al., 2019). Consumers now prefer natural materials from plants rather than synthetic compounds, so products containing natural additives, such as antioxidants or phytochemicals, are in demand (Beristain-Bauza et al., 2019; Zhang et al., 2021). The quality of standard medicinal compounds varies with geographical, seasonal or climatic changes (Mishra, 2016). Plant tissue culture research has resulted in the production of diverse bioactive molecules that can be used in consumer products and provide more standardized products (Chandran et al., 2020; Wu et al., 2021). Plant growth regulators are one of the factors commonly used for regulating cell division, inducing axillary and adventitious shoot proliferation, or rooting (Gaba, 2005; Schaller et al., 2015). Besides plant growth regulators, other compounds or additives are used as elicitors, where the main goal of elicitor application is to improve the synthesis of secondary metabolites in medicinal plants (Narayani and Srivastava, 2017; Cardoso et al., 2019).

Elicitation is defined as a process of enhanced synthesis of secondary metabolites or phytochemical compounds (Patel and Krishnamurth, 2013). Elicitors activate or stimulate many types of physiological processes that have been studied and investigated based on plant stress responses from both abiotic and biotic stresses (Patel and Krishnamurth, 2013; Shakya et al., 2019). Some elicitors can act as plant hormones, such as salicylic acid (SA) and jasmonic acid (JA). Both molecules are key signal compounds for defense gene expression. For example, SA generally regulates pathogen resistance (Patel and Krishnamurth, 2013). In plant tissue culture, SA is used as an elicitor for improving growth and inducing phytochemical compound production in many plants (Mahalakshmi et al., 2013; Wen et al., 2019), while JA mostly regulates responses to abiotic stresses, such as drought or salt stress (Wang et al., 2020; Raza et al., 2021). In some studies, researchers have applied both SA and JA to improve *in vitro* growth and secondary metabolite production in medicinal plants (da Silva, 2012; Narayani and Srivastava, 2017; Jirakiattikul et al., 2021a,b).

Nanomaterials are gradually emerging for use in the agricultural sector. They have been successfully applied in plant tissue cultures in the form of nanoparticles for surface decontamination, nutrient delivery, growth induction, genetic transformation and as plant inducers for secondary metabolite production (Kim et al., 2017). Most research has been conducted into the form of the nanoparticles (NPs); however, recent advances in nanoscience are moving toward two-dimensional nanomaterials, the so-called nanosheets (NSs). They are anisotropic materials with a thickness in the molecular range but with lateral dimensions on a micrometer or millimeter scale. Prominent examples include graphene, clays, transition metal dichalcogenides and transition metal oxides. For the latter, the current work specifically investigated titanate NSs, derived from soft chemical exfoliation of the layered potassium titanate precursor (Maluangnont et al., 2013).

One of the common NPs used is titanium dioxide (TiO_2) which has been used to sterilize plant parts in the initiation step (Mandeh et al., 2012). There are reports showing the positive effects of NPs to improve seed germination, increase *in vitro* growth and enable genetic modification, including enhancing the production of secondary metabolites (Ghorbanpour, 2015; Ali et al., 2019). However, the application of NSs in plant tissue culture is new and has rarely been reported. Therefore, the aim of this study was to investigate the effect of elicitors (JA, SA) and NSs on *in vitro* plant growth and total phenolic content production, as well as antioxidant activities.

Materials and Methods

Explant and plant material preparation

Young inflorescence flower samples were collected from plants located in a greenhouse at the University of Phayao, Thailand. Inflorescence segments about 10 cm were washed and immersed in 70% EtOH for 1 min and then soaked in 1.2% sodium hypochlorite (volume per volume) for 30 min. Finally, the explants were rinsed three times using sterile distilled water. These explants were cultured on Murashige and Skoog (MS) medium with 2 mg/L N6-benzylaminopurine (BAP) in glass bottles. All cultures were maintained in a culture room at 24±1°C with a 16 hr light and 8 hr darkness photoperiod with 40–50 μM/m^{2}s irradiance provided by cool white, light emitting diode (LED) fluorescent bulbs (Zeberg; LED T8; 22 W; Bangkok, Thailand). Subcultures aged 4 wk were required to produce adequate plant material.

Effect of elicitors (jasmonic acid, salicylic acid and nanosheet) on in vitro growth of C. alismatifolia cv. Maejo Impress

Shoots regenerated from bracteoles were used for shoot initiation. Initial adventitious shoots produced more plants after being transferred into MS supplemented with 2 mg/L BAP (5 passages). After clonal multiplication, the starting explant used for testing elicitors was proliferated shoots in aseptic glass bottles. These explants were trimmed to a length at the pseudostem of about 3–5 cm (Fig S1).

Two types of elicitors (JA and SA) were tested, each at three concentrations. The JA concentrations were 2 mg/L, 4 mg/L and 6 mg/L, while for SA, they were 0.5 mg/L, 1 mg/L and 2 mg/L. The other six treatments were JA and SA at each of the above relevant concentrations in combination with the nanosheet (NS) at 10 mg/L. The control consisted of the basal MS medium without elicitors or NS. This experiment used a completely randomized design. All 13 treatments were conducted with six replicates using glass bottle, having four plantlets as one replicate. All chemicals were AR grade. The titanate nanosheets were kindly provided by Dr. T. Maluangnont (King Mongkut's Institute of Technology Ladkrabang, Thailand). After culture for 6 wk in a controlled room (as described as above), the first three replicates were used for growth characteristics (length of new shoots and explants, shoot number, leaf color using a SPAD-502 meter (Konica Minolta; Japan), leaf number and length, root number and root length. The remaining three replicates were combined with the first three replicates to provide three samples for each treatment and these were used for the biochemical evaluation.

Determination of effects of elicitors (jasmonic acid, salicylic acid and nanosheet) on total phenolic content and antioxidant activities

Whole *in vitro* plantlets from the 13 treatments were dried at 60°C for 72 hr. Samples were ground and the powder was macerated with 70% EtOH, filtered and evaporated. The total phenolic content and the antioxidant activities were determined based on three replicates of each treatment. The total phenolic content was analyzed using the Folin-Ciocalteu method, modified from Jirakiattikul et al. (2021a). The absorbance values of samples were read using an AccuReader$^+$ microplate reader (Metertech; Taiwan) at a wavelength of 765 nm. The values of the total phenolic content were expressed in milligrams of gallic acid equivalents (GAE) per gram dry weight (DW) of a sample. The antioxidant activities were evaluated using both the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the ferric reduction antioxidant power (FRAP) methods, as described by Xiao et al. (2020). In addition, the total phenolic content and antioxidant activities of *in vitro* and field-grown leaves were studied and compared. *In vitro* leaves were collected from plantlets grown on basal MS supplemented with 2 mg/L BAP, whereas the field-grown leaves were collected from plants aged 4 mth derived from rhizomes and cultivated in pots under greenhouse conditions.

Statistical analysis

Data were analyzed based on analysis of variance. Mean ± SD values were reported and compared using Duncan's multiple range test to determine significant ($p < 0.05$) differences using the SPSS version 28.0 software (IBM Corp.; Armonk, NY, USA).

Results

Effects of elicitors (jasmonic acid, salicylic acid and nanosheet) on in vitro growth

The leaf number, leaf length, root number and root length were considerably affected by elicitor addition and

there were significant effects on growth. However, there were no significant differences among treatments for the length of explants and new shoots, shoot number and leaf color (Table 1). Adding elicitors and NS did not significantly decrease the shoot length of explants, number of new shoots, leaf color and root numbers.

The number of leaves and shoots in the 1 mg/L SA treatment was the highest, being significantly more than for shoots grown on the control medium (Table 1). The eight treatments with elicitors and NS were not significantly different from the control (2.37±0.09) while the remaining five treatments had significantly fewer leaves (2 mg/L JA, 4–6 mg/L JA + NS and 0.5–1 mg/L SA + NS), as shown in Table 1. Shoots grown with 2–4 mg/L JA alone and 4 mg/L JA + NS had significantly shorter leaves compared to the control (Table 1).

For root growth, both root number and root length were measured and evaluated, as shown in Table 1. The highest root number was observed with 2 mg/L JA alone (4.07±0.29 roots) and was significantly higher than for the control (3.29±0.19), as shown in Table 1. The addition of SA and NS significantly reduced the root number. JA alone at any concentration and 0.5 mg/L SA significantly decreased root length compared to the control. Growth on JA or SA combined with NS considerably decreased root length from 2.83±0.24 cm for the control to 1.44–1.70 cm (Table 1). There were no significant differences in the growth appearance among the *in vitro C. alismatifolia* shoots cultured with the various concentrations of JA and SA in combination with 10 mg/L NS for 6 wk (Fig. 1). The *in vitro* shoots treated with elicitors did not show any obvious decreases in leaf growth and root number; however, the root length was adversely affected.

Effects of elicitors (jasmonic acid, salicylic acid and nanosheet) on total phenolic content and antioxidant activities

There were significant differences in the levels of total phenolic content and antioxidant activity among the treatments (Figs. 2–4). The highest total phenolic content was in the

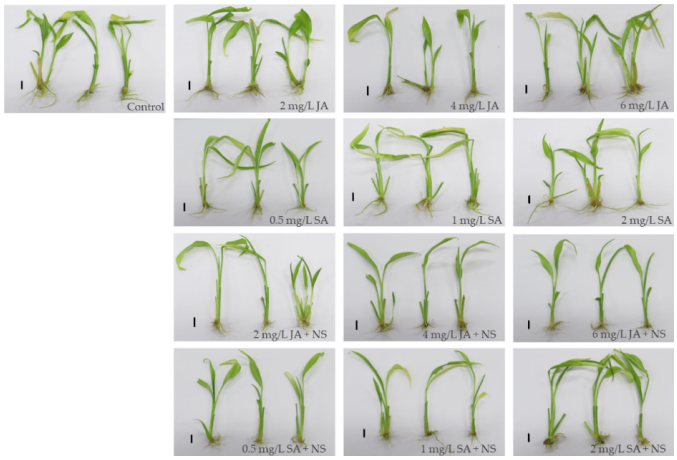

Fig. 1 Growth appearance of *in vitro* shoots cultured on Murashige and Skoog medium supplemented with various elicitors—jasmonic acid (JA), salicylic acid (SA) and nanosheets (NS)—for 6 wk, where vertical scale bar = 1.0 cm

Table 1 Influence of elicitor addition on *in vitro* growth and development of *Curcuma alismatifolia* cv. Maejo Impress cultured for 6 wk

Treatment	Shoot length of explants (cm)	Shoot length of new shoots (cm)	Number of shoots	Leaf color (SPAD reading score)	Number of leaves	Leaf length (cm)	Number of roots	Root length (cm)
Control	4.76±0.77	0.55±0.62	0.22±0.24	16.21±2.38	2.37±0.26^{ab}	7.26±1.02^{a}	3.29±0.5^{6b-d}	2.83±0.72^{a}
2 JA	4.87±0.63	0.84±1.38	0.15±0.34	15.43±3.47	1.89±0.44^{cd}	5.87±1.0^{8b}c	4.07±0.8^{6}a	1.70±0.24^{c}
4 JA	4.75±0.67	0.30±0.72	0.15±0.24	14.67±2.65	1.93±0.37^{b-d}	5.65±1.3^{9}c	3.33±0.76^{a-c}	1.48±0.26^{c}
6 JA	5.28±0.58	0.77±0.88	0.26±0.28	16.31±2.77	2.11±0.47^{a-d}	6.56±0.84^{a-c}	3.93±0.64^{ab}	1.66±0.25^{c}
0.5 SA	4.72±1.08	0.32±0.48	0.11±0.17	14.60±2.94	2.15±0.48^{a-d}	6.82±0.86^{ab}	3.26±0.74^{b-e}	2.17±0.42^{b}
1 SA	4.63±0.93	1.27±1.43	0.41±0.46	16.85±5.79	2.41±0.43^{a}	7.02±0.85^{a}	3.89±0.47^{ab}	2.66±0.37^{a}
2 SA	4.56±0.92	0.68±0.91	0.22±0.29	14.21±5.07	2.22±0.55^{a-d}	6.20±1.20^{a-c}	2.89±1.03^{c-f}	2.64±0.57^{a}
2 JA + NS	5.00±0.50	0.35±1.06	0.11±0.33	17.28±2.39	2.30±0.45^{a-c}	6.31±0.77^{a-c}	3.48±0.76^{a-c}	1.52±0.25^{c}
4 JA + NS	4.60±0.66	0.04±0.11	0.04±0.11	16.38±2.82	1.89±0.58^{cd}	5.63±1.1^{2}c	2.56±0.78^{d-g}	1.44±0.25^{c}
6 JA + NS	4.86±0.59	0.24±0.40	0.15±0.24	16.67±1.73	1.89±0.2^{9c}d	6.30±0.77^{a-c}	2.89±0.82^{c-f}	1.46±0.20^{c}
0.5 SA + NS	4.63±0.78	0.70±1.17	0.19±0.30	14.39±4.01	1.78±0.2^{4}d	6.29±0.97^{a-c}	2.33±0.7^{8f}g	1.70±0.47^{c}
1 SA + NS	4.66±0.35	0.19±0.38	0.07±0.15	16.42±3.35	1.78±0.2^{9}d	6.28±0.95^{a-c}	2.00±0.5^{0}g	1.70±0.42^{c}
2 SA + NS	4.80±0.64	0.23±0.48	0.07±0.22	17.37±4.06	2.00±0.33^{a-d}	6.76±1.31^{ab}	2.52±0.5^{0c-}g	1.49±0.36^{c}
F test	ns	ns	ns	ns	*	*	*	*

JA = jasmonic acid; SA = salicylic acid; NS = nanosheet;

Values (mean ± SD) in each column superscripted with different lowercase letters are significantly ($p < 0.05$) different; ns = non-significantly ($p > 0.05$) different; * = significantly ($p < 0.05$) different.

shoots grown with 4 mg/L JA + NS (9.46±1.49 mg GAE/g DW) or 6 mg/L JA + NS (9.19±1.27 mg GAE/g DW), as shown in Fig. 2 that were significantly different from the total phenolic content in the shoots grown in the control (7.06±0.40 mg GAE/g DW).

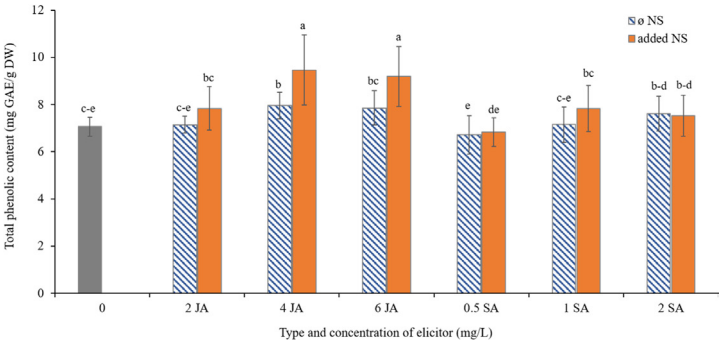

Fig. 2 Average total phenolic content of whole *in vitro* plantlets (EtOH extract) cultured in Murashige and Skoog medium supplemented with different concentrations of jasmonic acid (JA) and salicylic acid (SA) with and without nanosheets (NS), where values are mean ± SD (n = 12) and different lowercase letters above bars indicate significant (p > 0.05) differences.

The levels of antioxidant activity analyzed using DPPH and FRAP revealed the significant differences among treatments (Figs. 3–4). The shoots grown on 4–6 mg/L JA alone or 4–6 mg/L JA + NS had the greatest level of antioxidant activity that were significantly greater than for the control (Figs. 3A and 3B). The greatest FRAP antioxidant activity was detected in shoots grown on 4–6 mg/L JA alone and on 4–6 mg/L JA + NS (Fig. 4A). However, the levels of antioxidant activity of shoots grown in any treatment with elicitors were not significantly different from the control. The addition of SA tended to decrease the antioxidant activity compared to the addition of JA (Fig. 4B). The total phenolic content detected in this study had positive correlations with antioxidation activity, suggesting that the total phenolic compounds might have been generated as a response to the stress induced by the elicitors (Fig. S2). Furthermore, there were strongly positive correlations between the levels of antioxidant activity detected using DPPH (ascorbic acid versus Trolox), FRAP (Fe (II) versus Trolox) and DPPH versus FRAP (Fig. S3). The comparison of biochemical testing of plant materials from *in vitro* and field-grown plants is shown in Fig. S4.

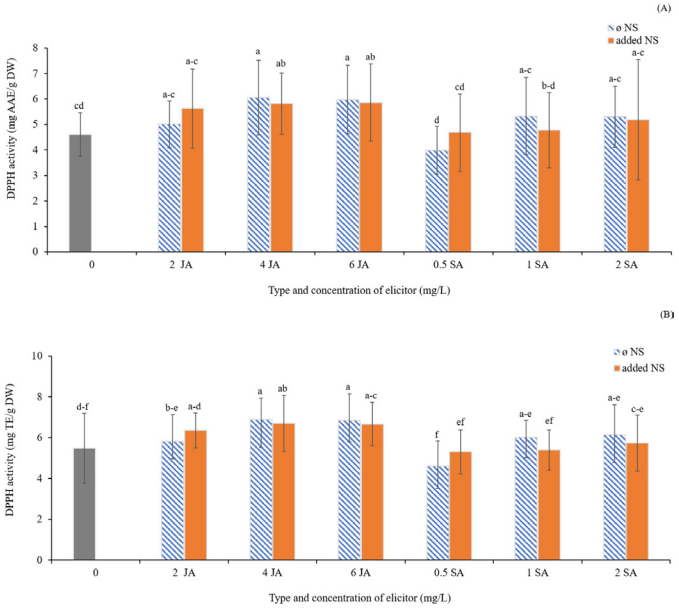

Fig. 3 Average antioxidant activity based on 1,1-diphenyl-2-picrylhydrazyl (DPPH) of whole *in vitro* plantlets (EtOH extract) cultured in Murashige and Skoog medium supplemented with different concentrations of jasmonic acid (JA) and salicylic acid (SA) with or without nanosheets (NS) expressed in: (A) ascorbic acid equivalents (AAE); (B) Trolox equivalents (TE), where values are mean ± SD (n = 12) and different lowercase letters above bars indicate significant (p > 0.05) differences.

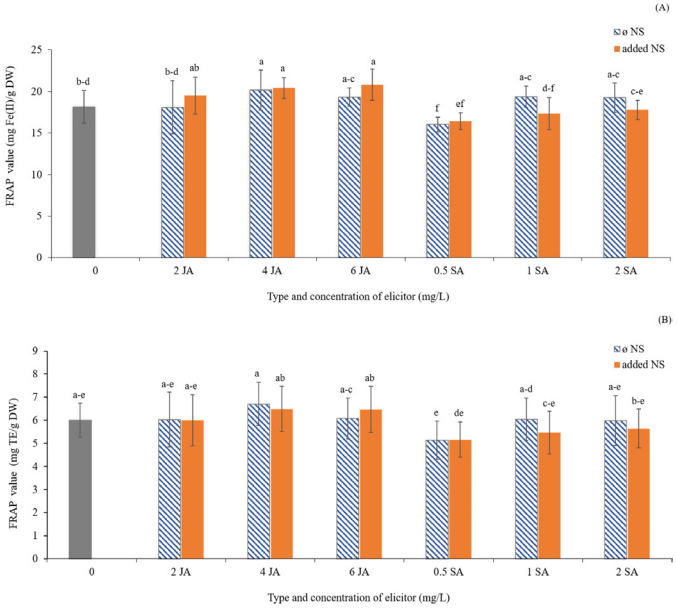

Fig. 4 Average antioxidant activities using ferric reduction antioxidant power (FRAP) of whole *in vitro* plantlets (EtOH extract) cultured in Murashige and Skoog medium supplemented with different concentrations of jasmonic acid (JA) and salicylic acid (SA) with or without nanosheets (NS) expressed in: (A) milligrams Fe(II)/g dry weight (DW); (B) milligrams Trolox equivalents (TE)/g DW, where values are mean ± SD (n = 12) and different lowercase letters above bars indicate significant (p > 0.05) differences.

Discussion

Many natural plant effectors are known to function as a part of defensive mechanisms against internal and external stimulators/stresses. These can be signaling components, regulatory proteins and reactive oxygen species (ROS), among which, the levels of phytohormones, such as SA and JA, are increased in plant tissues infected by pathogens, as part of the initiate numerous processes for survival, including the production of secondary metabolites, mainly phenolic compounds (Thakur and Sohal, 2013). The production and accumulation of phenolic compounds, so-called bioactive compounds, is the very first stage of defense at the infectious site of pathogens, as well as providing protection against other stresses (Pratyusha, 2022). Additional advantages of phenolic compounds are their antioxidant, anti-inflammation, anti-aging, anticancer and antimicrobial characteristics (Tanase et al., 2019).

Plant *in vitro* cultures have great potential for mass, pathogen-free explant production, conservation for diverse species, and useful metabolites, including off-season production (Neelakandan and Wang, 2012). Thus, *in vitro* cultures are an alternative source for the production of secondary metabolites. Organic and inorganic factors added to the growth medium may induce explant growth and secondary metabolite production. Under stress, plants often produce compounds with significant biological activity, while the scavenging activity of plant metabolites may help to counterbalance the increase in oxidative stress. Therefore, elicitation of plant metabolites could be a very promising method to increase the production of interesting biologically active substances (Jirakiattikul et al., 2021a,b). Notably, in the current study, the addition of JA and SA in the presence and absence of nanosheet did not significantly retard overall *in vitro* plant growth. Furthermore, the production of phenolic compounds and their related antioxidant activity in the Siam tulip (cv. Maejoe Impress) was enhanced.

Exogenous application of JA produces different effects on plant growth depending on the applied concentration, the *in vitro* plant stage, species and *in vitro* culture system (Kamińska, 2021). An elicitation with a low JA concentration (0.2–2 mg/L) in the MS basal medium increased the shoot fresh weight, root length and root number of potato, while the addition of JA at high concentrations (20–50 mg/L) suppressed the growth of potato explants (Zhang et al., 2006). When the concentration of JA was too high, it created a stressful environment, resulting in decreased growth and was possibly correlated with increased secondary metabolites as a response to stress encounters (Jirakiattikul et al., 2021b; Kamińska, 2021). Thus, JA itself can be used as an elicitor for phytochemical production in *in vitro* plant cultures. Plant interactions with SA were varied in the current study, with the addition of SA producing neither a significant negative effect on the growth appearance of the shoot, leaf and root of *in vitro C. alismatifolia* cultures nor on phytochemical production. For *in vitro Ajuga integrifolia* shoots, the addition of SA decreased the accumulation of biomass; notably, a high concentration of SA at 150 µM was seemingly optimal for inducing most of the secondary metabolite production (Abbasi et al., 2020). The study of Jirakiattikul et al. (2021a) in *in vitro Musa acuminata* L. cv. Gros Michel reported that the highest total phenolic and total flavonoid contents were in the 100 µM (13.8 mg/L) SA-treated shoots. A similar trend was observed in the micropropagation of *Boesenbergia rotunda* L, where the shoots treated with SA substantially decreased the growth index but slightly enhanced the accumulation of bioactive compounds and their antioxidant activities (Jirakiattikul et al., 2021b).

The treatment of *in vitro* cultures with selected plant elicitors is a promising strategy to enhance secondary metabolite biosynthesis. The addition of plant elicitors needs to be optimized to fine-tune the balance between plant growth and the required metabolites. The current study further applied nanosheets in combination with JA or SA. Then, the effects were observed on the growth of *in vitro C. alismatifolia* culture followed by the investigation of their potential for inducing polyphenol production.

In the current study, the addition of elicitors and NSs to the growth media of *in vitro C. alismatifolia* cultures did not affect shoot appearance (shoot length of explants, new shoot length, shoot number) and leaf color. Other leaf parameters, including leaf number and leaf length, were significantly decreased in the presence of the NS. A similar significant decrease was observed for root number and root length. However, considering the total phenolic content, it was found that the shoots grown in MS medium containing NS in combination with JA at 4–6 mg/L produced significantly more total phenolics than the control (elicitor-free) and JA alone. This finding demonstrated the beneficial effect of elicitors on the production of secondary metabolites in *C. alismatifolia* plants. Diverse effects of nanoparticles on certain plant species have been reported in the study by Gonçalves et al. (2021), with metal oxides having both positive and negative effects on shoot growth,

depending on the type of nanoparticles and the plant species. Media supplemented with naphthalene acetic acid and different ratios of gold and silver nanoparticles promoted biomass and secondary metabolite production in *Prunella vulgaris* L. (Fazal et al., 2019), with the radical scavenging activity increasing in accordance with the total phenolic and total flavonoid contents. This may explain how nanoparticles affect *in vitro* culture by activating plant defense mechanisms. There have not been many studies on the use of NSs to stimulate growth or phytochemical production, though they have been applied for crop protection against pathogens (El-Abeid et al., 2020).

The current study identified the optimal type and concentration of elicitor was 4–6 mg/L JA for improving the accumulation of bioactive compounds in the micropropagation of *C. alismatifolia* cv. Maejo Impress, without inducing shoot necrosis or death. Nevertheless, *in vitro* shoots of *C. alismatifolia* produced lower yields of bioactive compounds and lower levels of antioxidant activity compared to field-grown plants.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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