

## Enhancement of plumbagin production from *Drosera peltata* Thunb. using different elicitors

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### ABSTRACT

*In vitro* shoot clumps of *Drosera peltata* were cultured on semi-solid half-strength MS medium with topping by liquid half-strength MS medium supplemented with various concentrations and types of biotic and abiotic elicitors (50-150 mg/L yeast extract, 50-150 mg/L chitosan, 50-200 µM methyl jasmonate, 1-4 mM cyclodextrin, 10-100 µM silver nitrate, 5-20 mg/L 2,4-D, 1-10 mM L-alanine and 50-150 µM salicylic acid for 3 days. Shoot clumps were investigated for plumbagin accumulation using HPLC. Highest plumbagin content ( $3.45 \pm 0.90$  mg/g dry wt) was detected when 5 mg/L 2,4-D was used as the elicitor, almost 3 times higher than the control treatment ( $1.22 \pm 0.08$  mg/g dry wt) while other elicitors also significantly stimulated higher plumbagin content than the control treatment. Results revealed that relatively high concentrations of 2,4-D, commonly used in tissue culture media, elicited high plumbagin contents from *in vitro* *D. peltata* shoot clumps.

*Keywords:* *Drosera peltata*, Elicitation, Plumbagin

### INTRODUCTION

*Drosera indica* L., *D. burmannii* Vahl., and *D. peltata* Thunb. are important Thai *Drosera* species that have long been used as traditional medicines. The main bioactive compound found in *D. peltata* is plumbagin, a naphthoquinone which displays various pharmacological activities to treat dysentery, headaches, rheumatic pain and traumatic injuries (Perry and Metzger, 1980; Duke and Ayensu, 1985), while also exhibiting antibacterial (Didry et al., 1998), antimicrobial (Ravikumar et al., 2003) antifungal (Tien et al., 2014), antioxidant and anticancer properties (Balaji and Asirvatham, 2015). Because of these distinguished pharmaceutical abilities, *D. peltata* is interesting as an alternative source of plumbagin production.

Different tissue culture techniques have been used to study secondary metabolite production in many plants (Jawahar et al., 2014) including *Drosera* species (Marczak et al., 2005). Investigations regarding secondary metabolite

production improvement have several possible approaches including selection of high producing cell lines, optimization of medium components, alterations in environmental factors, and elicitation organization of the biochemical capability of cultivated plant cells (Dornenburg and Knorr, 1995). Elicitation is an effective method to enhance metabolite biosynthesis in *in vitro* cultures. Elicitors are generally classified into abiotic and biotic depending on their nature and origin. They have been successfully used to treat cultured cells or tissues (Radman et al., 2003). Improved elicitation techniques for plumbagin production have been reported in several plants using both abiotic and biotic elicitors as *Drosophyllum lusitanicum* (Nahálka et al., 1996), *Plumbago rosea* (Komaraiah et al., 2003), *P. indica* (Karadi et al., 2007; Martin et al., 2011; Jaisi et al., 2013; Jaisi and Panichayupakaranant, 2016; Jaisi and Panichayupakaranant, 2017), and *Drosera* species such as *D. carpensis* (Krolicka et al., 2008), *D. burmannii* (Putalun et al., 2010), and *D. indica* (Juengwatanatrakul et al., 2011). Results from previous studies revealed that an *in vitro* culture of *Drosera* species produced relatively higher plumbagin content than *Plumbago* species. In addition, *Drosera* species have added advantages over *Plumbago* species because their life cycle is shorter. Furthermore, culture systems of *Drosera* species are more efficient and easier to handle and harvest for plumbagin production than *Plumbago* species. Elicitation mechanisms for plumbagin production in *D. indica* and *D. burmannii* have been studied and reported; however, *in vitro* elicitation methods to enhance plumbagin production in *D. peltata* have not been assessed. Hence, selected biotic and abiotic elicitors were tested to determine the optimum types and concentrations for improvement of plumbagin production in *in vitro* cultured *D. peltata*.

## MATERIALS AND METHODS

### Explant preparation

Seeds of *D. peltata* from natural plants (Loei Province, Thailand) were surface sterilized for 10 min with 15% (v/v) Clorox<sup>®</sup> solution added with 0.01% (v/v) Tween 20, and then washed in sterilized distilled water 3 times. Sterilized seeds were sown on semi-solid half-strength MS (Murashige and Skoog, 1962) medium supplemented with 0.2% (w/v) Gelrite and 3% (w/v) sucrose. The seeds were incubated under cool white fluorescent light ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 12 h photoperiod at  $25 \pm 2$  °C. Forty-five-day-old seedlings were subcultured to the fresh medium once every 4 weeks. At the third subculture, shoot clumps were used for the experiment.

### Elicitation experiment

*In vitro* 8 weeks-old shoot clumps of *D. peltata* (with approximately 0.8-1.0 g of fresh weight per clump) cultured on semi-solid half-strength MS medium were overlaid with liquid half-strength MS medium supplemented with various concentrations and types of elicitors as yeast extract (50-150 mg/L), chitosan (50-150 mg/L), methyl jasmonate (50-200  $\mu\text{M}$ ), cyclodextrin (1-4 mM), silver nitrate (10-100  $\mu\text{M}$ ), 2,4-D (5-20 mg/L), L-alanine (1-10 mM) salicylic acid (50-150  $\mu\text{M}$ ) and elicitor free medium as control group. Treated cultures were incubated for 3 days, then *in vitro* treated shoot clumps were removed and analyzed for plumbagin contents. Each treatment consisted of 3 replicates. All experiments were repeated twice. Data were

analyzed by mean comparisons using analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) at significance level of  $P \leq 0.05$ .

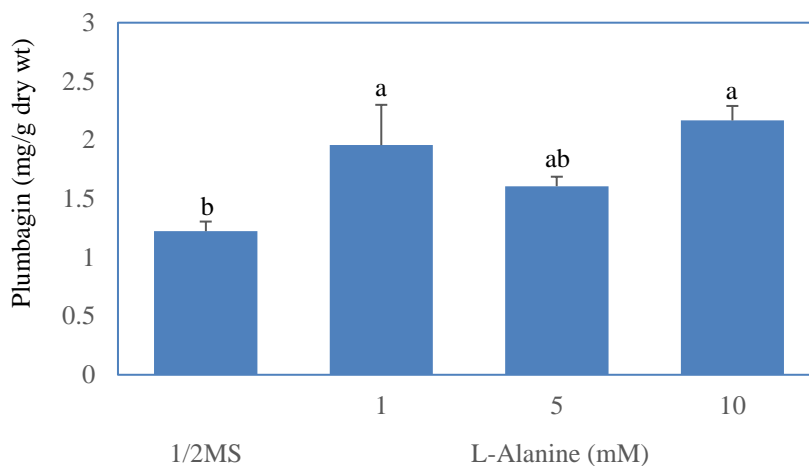
### **Plumbagin analysis by HPLC**

Plumbagin was extracted from the cultured explants and analyzed following the protocol of Marczak et al. (2005) with some modifications. In short, shoot clumps were dried at 50 °C for 2 days. The dried sample was then ground, dissolved with methanol and the mixture was sonicated for 15 min at 25 °C. The suspension was subsequently centrifuged at 10,000 rpm for 15 minutes and the supernatant was harvested. Later, the supernatant was filtered through 0.45 µm membrane filter (VertiClen™) and the content was analyzed for plumbagin using an HPLC (Perkin Elmer, series 200) system with a C18 column (Restek, 4.6 mm ID x 50 mm length, 3 µm particle size). The 10 µl injection volume of each sample was eluted by the mixture mobile phase of methanol and 0.4% acetic acid in water (70:30 v/v) at a flow rate of 1.0 ml min<sup>-1</sup>. The presence of plumbagin was detected by a UV detector at 270 nm and content was calculated by comparison with the standard curve of genuine plumbagin (Sigma-Aldrich, USA) using Total Chrom™ workstation (version 6.2.) software (Perkin-Elmer Instrument HPLC).

## **RESULTS AND DISCUSSION**

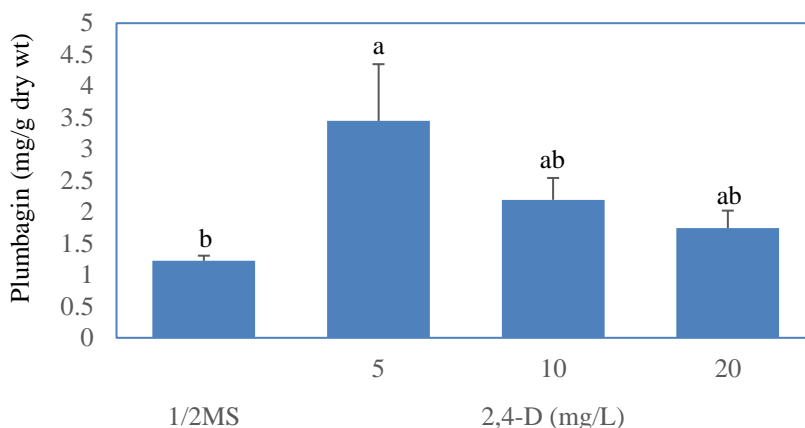
Modern methods for secondary metabolite production are currently being investigated and improved by biotechnological techniques which include organizing nutrient arrangement, modifying environmental conditions and applying specialized techniques as immobilization, hairy root culture, permeabilization, precursor feeding and especially elicitation to increase secondary metabolite yields (Bourgaud et al., 2001; Rao and Ravishankar, 2002). Elicitation is a phenomenon whereby plant cells respond to treatment by biotic or abiotic elicitors to produce the desired secondary metabolites. Biotic elicitors are generally prepared from microbial extracts while abiotic elicitors usually refer to chemical and physical factors that stimulate secondary metabolite production in plants. Abiotic elicitors have been widely reported to stimulate secondary metabolites production in several plants (Naik and Al-Khayri, 2016). Using the optimal type and concentration of elicitor are crucial to enhance secondary metabolite improvement. In our study, effects of elicitors on plumbagin production from *in vitro* shoot clumps of *D. peltata* were observed and reported. Cultures were pretreated with various types and concentrations of biotic and abiotic elicitors (yeast extract, chitosan, methyl jasmonate, cyclodextrin, silver nitrate, 2,4-D, L-alanine and salicylic acid) for 3 days and then harvested for plumbagin analysis. The shoot clump explants treated with all elicitors produced plumbagin content higher than the control (Figure 1-8), which indicated that plumbagin mainly accumulated in the intracellular levels. Low extracellular content of plumbagin (less than 0.001 mg/g dry wt) was also released and detected when shoot clumps were treated with L-alanine, detected by color change of the culture medium (extracellular levels). Highest plumbagin content was observed for the medium added with 10 µM of L-alanine ( $2.17 \pm 0.12$  mg/g dry wt), at higher than the control ( $1.22 \pm 0.08$  mg/g dry wt) (Figure 1). Similar results have been observed for *Plumbago*

*indica* root cultures. Higher extracellular content of plumbagin was mainly detected when L-alanine was used as the elicitor (Jaisi and Panichayupakaranant, 2016, 2017).



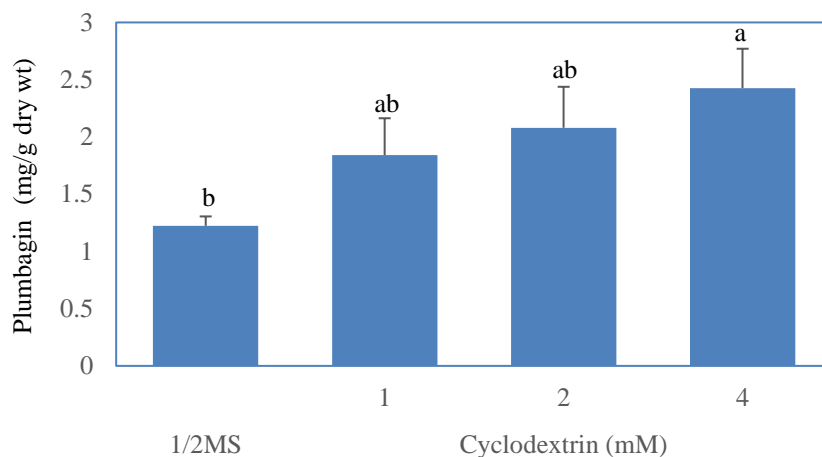
**Figure 1** Effect of L-alanine on plumbagin production from *in vitro* shoot clumps of *D. peltata* after 3 days of pretreatment with different concentrations (1, 5 and 10 mM) of L-alanine. Values are indicated as mean  $\pm$  SE. Different letters above the bar graphs represent significant difference at  $P \leq 0.05$  level.

Highest plumbagin content ( $3.45 \pm 0.90$  mg/g dry wt) resulted when 5 mg/L 2,4-D was used as the elicitor. This content was nearly 3 times higher than the control (Figure 2). However, increasing 2,4-D concentration decreased plumbagin production from *D. peltata* shoot clumps. 2,4-D is known to induce reactive oxygen species (ROS) which cause detrimental effects in plants. To combat this, plants produce several antioxidants to inhibit free radical activities, and plumbagin, one of the main bioactive compounds in *Drosera* species, may also be produced as an antioxidative response (Pazmiño et al., 2012) to plant stress (Shariatpanahi et al., 2006). Relative effect of 2,4-D on stimulation of secondary metabolite production has been found in carrot. Mousavizadeh et al. (2010) showed that applying 2,4-D into the medium caused an increase in anthocyanin accumulation in carrot suspended cells; however, increasing 2,4-D concentration reduced plumbagin content in *D. peltata*.



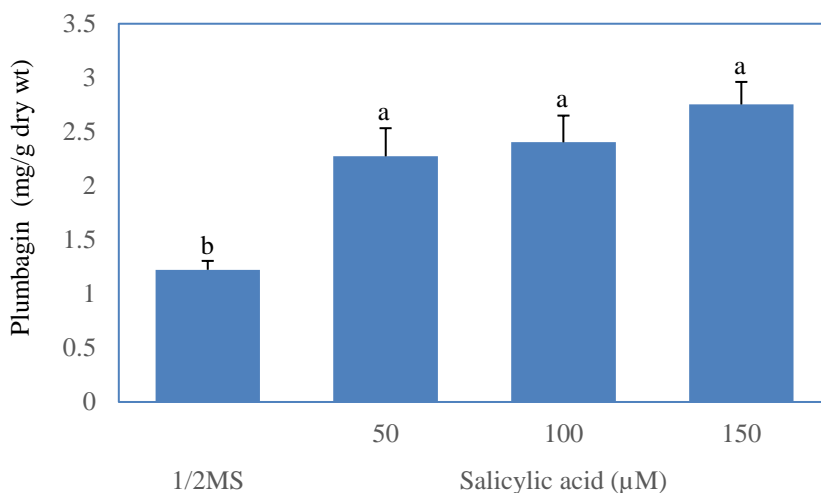
**Figure 2** Effect of 2,4-D on plumbagin production from *in vitro* shoot clumps of *D. peltata* after 3 days of pretreatment with different concentrations (5, 10 and 20 mg/L) of 2,4-D. Values are indicated as mean  $\pm$  SE. Different letters above the bar graphs represent significant difference at  $P \leq 0.05$  level.

By contrast, increasing concentration in both cyclodextrin and salicylic acid also resulted in increasing plumbagin accumulation in *D. peltata* shoot clumps (Figures 3 and 4). Cyclodextrin at 4 mM gave the highest content of plumbagin ( $2.43 \pm 0.34$  mg/g dry wt), almost twice as high as the control. Effect of cyclodextrin on secondary metabolite has been reported to stimulate secondary metabolite production in a number of plants e.g. decursinol angelate in *Angelica gigas* (Cho et al., 2003), resveratrol in *Vitis vinifera* (Zamboni et al., 2006; Lijavetzky et al., 2008), ajmalicine in *Catharanthus roseus* (Almagro et al., 2011), taxane in *Taxus x media* (Sabater-Jara et al., 2014), and anthraquinone in *Morinda citrifolia* and *Rubia tinctorum* (Perassolo et al., 2016). However, no publications on the use of cyclodextrin for plumbagin production in *Drosera* species have been studied and reported. Here, we improved production of plumbagin content in *D. peltata* using cyclodextrin.



**Figure 3** Effect of cyclodextrin on plumbagin production from *in vitro* shoot clumps of *D. peltata* after 3 days of pretreatment with different concentrations (1, 2 and 4 mM) of cyclodextrin. Values are indicated as mean  $\pm$  SE. Different letters above the bar graphs represent significant difference at  $P \leq 0.05$  level.

Figure 4 indicated that all salicylic acid concentrations significantly induced higher plumbagin production over the control. Highest plumbagin content was found on the medium elicited with 150  $\mu$ M salicylic acid ( $2.75 \pm 0.21$  mg/g dry wt) at almost 2.5 times higher than the control. Similar results were previously reported for enhancement of 7-methyljuglone in *D. capensis* (Ziaratnia et al., 2009) and stimulation of plumbagin by salicylic acid in *D. burmannii* (Potalun et al., 2010).

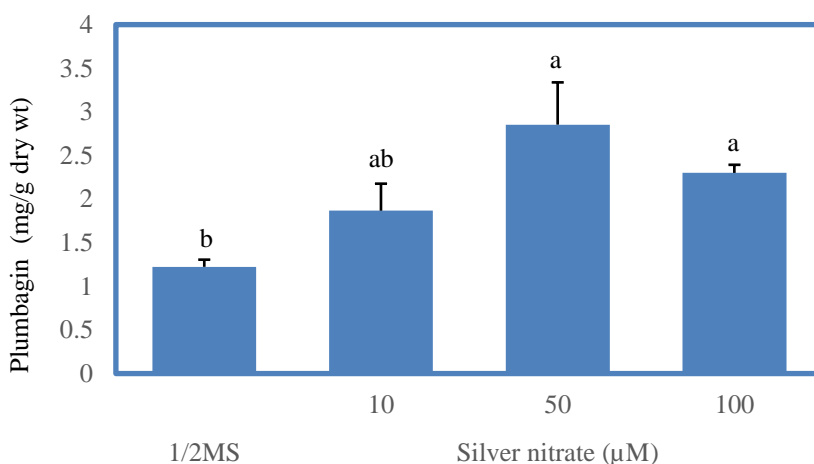


**Figure 4** Effect of salicylic acid on plumbagin production from *in vitro* shoot clumps of *D. peltata* after 3 days of pretreatment with different concentrations (50,

100 and 150  $\mu\text{M}$ ) of salicylic acid. Values are indicated as mean  $\pm$  SE. Different letters above the bar graphs represent significant difference at  $P \leq 0.05$  level.

Plumbagin production in shoot clumps of *D. peltata* treated by silver nitrate, yeast extract and methyl jasmonate gave similar results. However, plumbagin production using higher concentrations of these elicitors decreased. Results showed that addition of 50  $\mu\text{M}$  silver nitrate, 100 mg/L yeast extract, and 100  $\mu\text{M}$  methyl jasmonate induced the highest contents of plumbagin at  $2.85 \pm 0.48$ ,  $2.51 \pm 0.10$  and  $2.03 \pm 0.36$  mg/g dry wt, respectively.

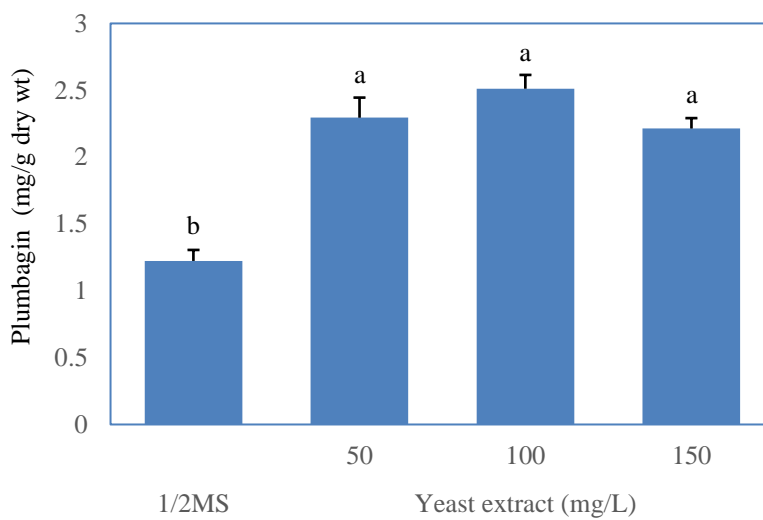
Silver nitrate heavy metal ions greatly affected plumbagin production in *D. peltata*. The results indicated that using 50  $\mu\text{M}$  silver nitrate as an elicitor induced plumbagin content 2.5 times higher than the control treatment. Metal ions such as Ag have been used to stimulate secondary metabolite production in several plants (Verpoorte et al., 2002). Successful published research showed secondary metabolite elicitation in *Brugmansia candida* (Angelova et al., 2006) using silver nitrate and tanshinone production in *Perovskia abrotanoides* (Arehzoo et al., 2015). Our results showing enhancement of plumbagin production in *D. peltata* using silver nitrate have not been reported elsewhere (Figure 5).



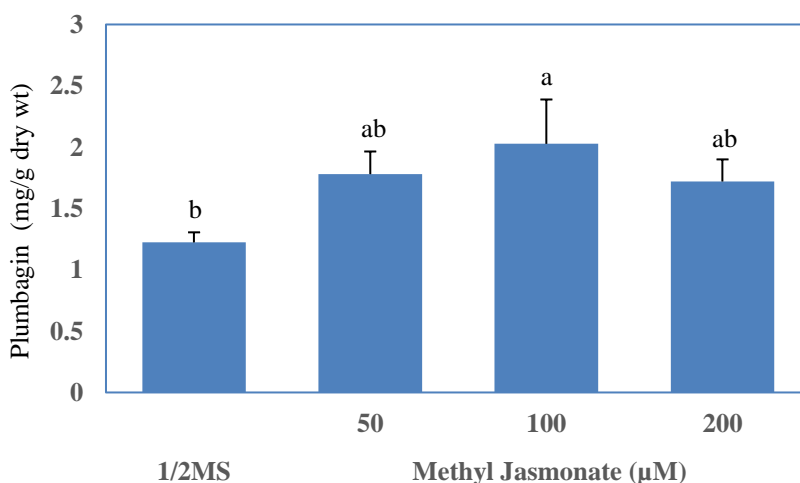
**Figure 5** Effect of silver nitrate on plumbagin production from *in vitro* shoot clumps of *D. peltata* after 3 days of pretreatment with different concentrations (10, 50 and 100  $\mu\text{M}$ ) of silver nitrate. Values are indicated as mean  $\pm$  SE. Different letters above the bar graphs represent significant difference at  $P \leq 0.05$  level.

Yeast extract and methyl jasmonate were formerly reported in different species to stimulate plumbagin production in *Plumbago indica* (Jaisi and Panichayupakaranant, 2016), rosmarinic acid production in *Solenostemon*

*scutellarioides* (Sahu et al., 2013), solasodine production by cultured cells of *Solanum hainanense* (Loc et al., 2014) and stevioside production from *in vitro* shoots of *Stevia rebaudiana* (Bayraktar et al., 2016). Among *Drosera* species, results revealed that yeast extract and methyl jasmonate could elicit higher plumbagin content in shoot clumps of *D. peltata* than values formerly reported in *D. burmannii* (Putalun et al., 2010) and *D. indica* (Juengwatanatrakul et al., 2011) using the same explant and elicitors as well as elicitation treatment period (Figures 6-7).



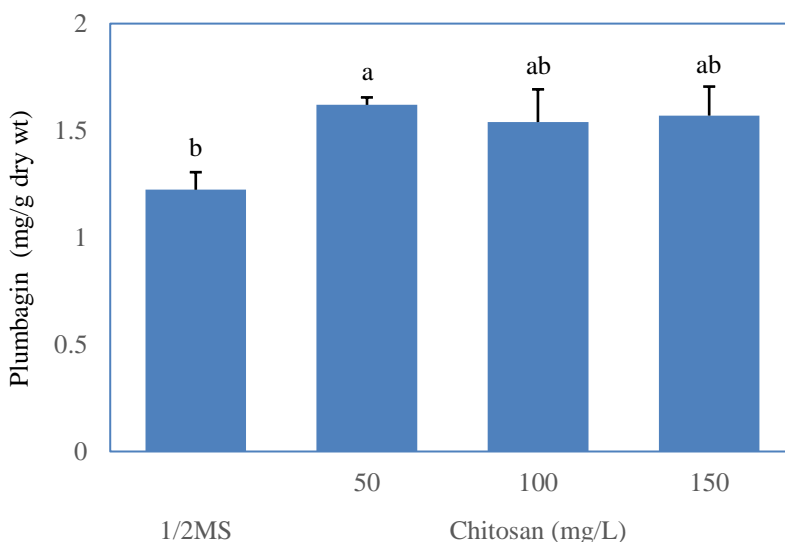
**Figure 6** Effect of yeast extract on plumbagin production from *in vitro* shoot clumps of *D. peltata* after 3 days of pretreatment with different concentrations (50, 100 and 150 mg/L) of yeast extract. Values are indicated as mean  $\pm$  SE. Different letters above the bar graphs represent significant difference at  $P \leq 0.05$  level.





**Figure 7** Effect of methyl jasmonate on plumbagin production from *in vitro* shoot clumps of *D. peltata* after 3 days of pretreatment with different concentrations (50, 100 and 200  $\mu$ M) of methyl jasmonate. Values are indicated as mean  $\pm$  SE. Different letters above the bar graphs represent significant difference at  $P \leq 0.05$  level.

Using different concentrations of chitosan (50-150 mg/l) to elicit plumbagin production in *D. peltata* shoot clumps was also studied. Results showed that 100 mg/L of chitosan induced the highest content of plumbagin ( $1.62 \pm 0.04$  mg/g dry wt). However, plumbagin content in all concentrations of chitosan showed no significant difference. This finding concurred with a previous report on plumbagin production in plantlets of *D. burmannii* using chitosan treatment (Putalun et al., 2010) (Figure 8).



**Figure 8** Effect of chitosan on plumbagin production from *in vitro* shoot clumps of *D. peltata* after 3 days of pretreatment with different concentrations (50, 100 and 150 mg/L) of chitosan. Values are indicated as mean  $\pm$  SE. Different letters above the bar graphs represent significant difference at  $P \leq 0.05$  level.

## CONCLUSIONS

Our results showed that *in vitro* shoot clumps of *D. peltata* respond to different elicitors at diverse plumbagin product rates. Here, 2,4-D (5 mg/L), silver nitrate (50  $\mu$ M) and salicylic acid (150  $\mu$ M) were the best elicitors to enhance the highest intracellular content of plumbagin at  $3.45 \pm 0.90$ ,  $2.85 \pm 0.48$  and  $2.75 \pm 0.20$  mg/g dry wt, respectively at 2.83, 2.33 and 2.25 times higher than the control treatment. In addition, elicitation treatment in *D. peltata* required only 3 days to generate higher plumbagin content. Our results revealed that improvement of

plumbagin production in *D. peltata* is possible using diverse elicitors. For *in vitro* production of plumbagin in *D. peltata*, it is, therefore, important to select the optimal type and concentration of elicitor.

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