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Full Length Research Paper

Elicitation effect on production of plumbagin in *in vitro* culture of *Drosera indica* L.

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Influence of elicitors on plumbagin accumulation in *Drosera indica* whole plant culture was investigated. **Treatment of plantlets with 150 mg/l chitosan and 100 µM salicylic acid mostly stimulated plumbagin production after 3 days of elicitation, while 0.5 mg/ml yeast extract and 50 µM methyl jasmonate elicited at 6 days. Yeast extract (0.5 mg/ml) was the most efficient elicitor to enhance plumbagin production (2.69±0.03 mg/g dry wt.) by 5.4-fold over the control plant (0.50±0.01 mg/g dry wt.). Present results indicated the effectiveness of elicitation on plumbagin accumulation in *in vitro* culture of *D. indica*.**

Key words: *Drosera indica*, plumbagin, elicitors.

INTRODUCTION

The genus *Drosera* (Droseraceae), commonly called sundew, consists of approximately 170 species. *Drosera indica* has been commonly used in Thai traditional herbal remedy for treatment of stomached, eczema, and hepatitis (Chuakul, 2000). The major naphthoquinone found in *D. indica* is plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) (Zenk et al., 1969). Plumbagin exhibits a variety of therapeutic functions such as anti-microbial, anti-tuberculosis, bronchial infection, whooping cough, anti-asthma, anti-spasmodic, anti-cancer, anti-malarial, enhances *in vitro* phagocytosis of human granulocytes, anti-leprosy, anti-fertility, abortifacient, chitin synthetase inhibitor, immunomodulator, aphrodisiac, arteriosclerosis, insecticidal, anti-feedant, anti-malarial and hyper-glycaemic (Krolicka et al., 2008; Curreli et al., 2001; Lin et al., 2003; Likhitwitayawuid et al., 1998; Medentsey et al., 1998; Jayaram and Prasad, 2007; Tokunaga et al., 2004; Finnie and Van Staden, 1993). Because of its pharmacological benefits, high-plumbagin producing plants are of interest. Plant tissue culture plays an important role for micropropagation of *Drosera* species. (Jayaram and Prasad, 2005, 2007; Kawiak et al., 2003; Kim et al., 2004; Putalun et al., 2010). Production of plumbagin from plant tissue cultures is of importance because intact plants produce this compound only in small amounts. Elicitation is a tool extensively used for enhancing secondary-metabolite yields, and elicitors are compounds that induce plants to synthesize elevated levels of phytoalexins. There are reports, however, that the synthesis of secondary metabolites other than phytoalexins can also be stimulated by elicitors (Kamonwannasit et al., 2008; Putalun et al., 2007; Zhao et al., 2005). There are many useful elicitors that can influence the production of secondary metabolites of medicinal plants.

The studies of *in vitro* plumbagin production were reported on *Plumbago* species (Chuntaratin, 2006; Komaraiah et al., 2003; Lin et al., 2003) and *Drosera* species (Krolicka et al., 2008; Putalun et al., 2010). Unlike *Drosera*, *in vitro* culture of Plumbago must be used with special techniques such as immobilized cell culture and *in situ* adsorption (Komaraiah et al., 2003). Furthermore, *Drosera* shows the rapid growth in *in vitro* (Jayaram and Prasad, 2005). Thus, Drosera is considerably significant commercially for bioprospection of plumbagin. The impact of various elicitors on *in vitro* cultured *Drosera capensis* and *Drosera burmanii* have been reported in previous studies (Krolicka et al., 2008; Ziaratnia et al., 2009; Putalun et al., 2010), however,

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D. indica has not been investigated to date. In fact, no specific elicitor has a general effect on different plant species. Consequently, optimum concentration and type of elicitor is likely to vary from species to species. It would be interesting to find elicitors which can stimulate the maximum level of plumbagin production in plants culture of *D. indica*. Therefore, we have pursued the possibility using elicitor treatment of *D. indica* in order to improve plumbagin production in whole plants culture. In the present study, we were aimed to assess the enhancement of plumbagin production in *in vitro* cultured *D. indica* treated with various elicitors and exposure time.

MATERIALS AND METHODS

Chemical reagents

6-benzyladenine (BA) was purchased from Fluka Chemical (Buchs, Switzerland). Plumbagin and 2, 2'- Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Wako Pure Chemical (Osaka, Japan). Anti-plumbagin monoclonal antibody was obtained from Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan. Peroxidase-labeled anti-mouse IgG was purchased from Organon Teknika Cappel Products (West Chester, PA, USA). All other chemicals were standard commercial products of analytical grade.

Plant materials

D. indica seeds were obtained from Ubon Ratchathani, Thailand. The seeds were washed with sterile distilled water and were surface-sterilized in 2% (w/v) sodium hypochlorite for 20 min, washed with sterilized water and finally immersed in 70% (v/v) ethanol for 1 min before germinating on hormone-free half-strength (½) Murashige and Skoog (MS) medium containing 1 mg/l giberellic acid at 25±1°C under 16 h light/day. Plantlets were used as material for shoot induction.

Shoot induction

Stem segments from 4 weeks *in vitro* plantlets were subcultured on ¼ MS solid medium with 0.5 mg/l BA for shoot multiplication modified from Jayaram and Prasad (2005). After 4 weeks, multiple shoots were observed. The regenerated shoots were subcultured into 250 ml flasks containing 30 ml ¼ MS liquid medium, without hormones, for elongation. The medium was agitated on a rotary shaker (100 rpm at 25°C, under continuous light for 16 h/d). After 4 weeks, plantlets were subcultured into the same medium for elicitor treatment.

Elicitor treatment

Elicitation was carried out with either yeast extract, methyl jasmonate, chitosan or salicylic acid at various concentrations. Elicitors were added to 14 day-old whole plant cultures, which were harvested after elicitor treatment at 3, 6 and 9 days. Each experiment was done in triplicate.

Sample preparation and plumbagin analysis

Whole plants were dried at 50°C for 48 h and ground until powder. Dried powder samples (30 mg) were extracted with 0.5 ml methanol four times using an ultrasonic bath for 15 min each time. After filtration and evaporation to dryness, the residues were reconstituted in 1 ml methanol. Plumbagin contents were determined by competitive ELISA using a monoclonal antibody against plumbagin as follows Sakamoto et al. (2008). The absorbance at 405 nm was measured using a microplate reader (MRX revelation, Dynex Technologies). All reactions were carried out at 37°C.

Statistical analysis

Results were reported as the mean ± standard deviation (SD) and analyzed by the Bonferroni multiple test. Differences with $P < 0.01$ were considered as significant.

RESULTS AND DISCUSSION

Results revealed that certain elicitors affect the production of plumbagin from *D. indica* whole plant cultures. Various types and concentrations of elicitors were added to the regenerated plants after 14 days of culture to examine their effect on plumbagin accumulation. Figure 1 shows the effect of methyl jasmonate on plumbagin production. Production of plumbagin in methyl jasmonate treated cultures was significantly increased at day 6 and slightly decreased at day 9 of elicited time. Methyl jasmonate at 50 µM gave the highest concentration of plumbagin (0.92±0.04 mg/g dry wt), which was double of the control (0.50±0.01 mg/g dry wt). These results are similar to the observation of Ziaratnia et al. (2009) and Putalun et al. (2010) which reported the enhancement of 7-methyljuglone accumulation in *D. capensis* by jasmonic acid and plumbagin level in *D. burmanii* by methyl jasmonate, respectively.

The effect of yeast extract on plumbagin production was shown in Figure 2. Application of 0.5 mg/ml yeast extract at day 6 of exposure time did effectively induce the accumulation of plumbagin (2.69±0.03 mg/g dry wt.), amount representing 5.4-fold greater concentration. In addition, the effect of yeast extract on plumbagin accumulation decreased when increasing elicitor concentration. Thus, yeast extract at 0.5 mg/ml was considered as the most effective treatment for enhancement of plumbagin. This result showed the greatest on plumbagin production which was higher than the wild plant (1.00±0.06 mg/g dry wt.) and previous report on *in vitro* shoot of *D. burmanii* (1.50±0.1 mg/g dry wt.) (Putalun et al., 2010). Yeast elicitor is one of the most commonly used elicitor for enhancement of the secondary metabolites. Yeast extract was known to effectively bind to receptors on the plant cells. It induced the synthesis of phenylalanine ammonium lyase activity and enhanced phenylpropanoids accumulation in plant cell cultures of *Cistanche deserticola* (Cheng et al., 2005). However, not all plant species response to this elicitor. Yeast is the effective elicitor on plumbagin production in *Drosera* species but not in *Plumbago* species (Chuntaratin, 2006). The effect of salicylic acid (50 to 200 µM) on plumbagin production is shown in species (Chuntaratin, species

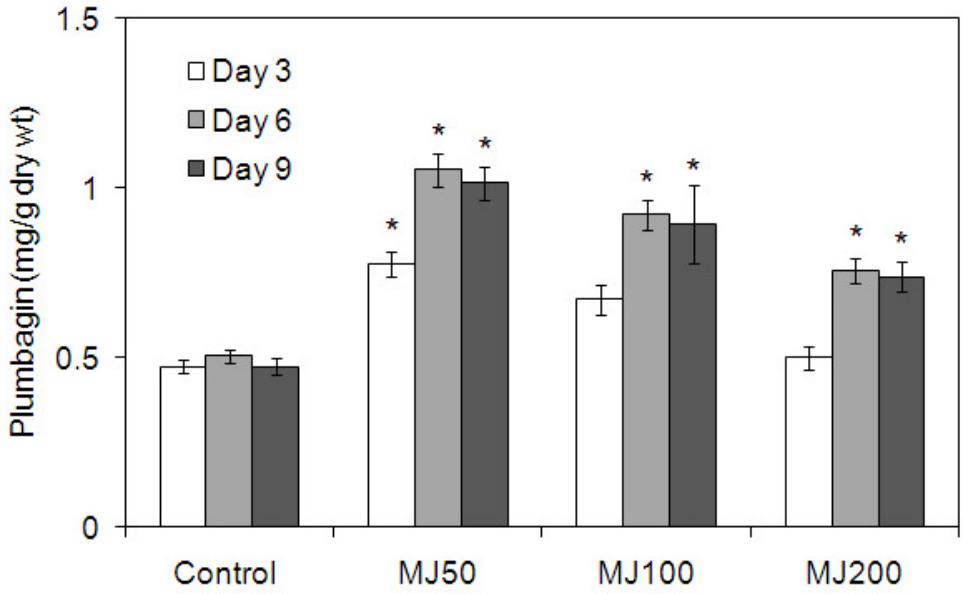

Figure 1. Effect of methyl jasmonate (MJ) on plumbagin production in plant cultures. Methyl jasmonate (50, 100 and 200 µM) was added to 28 day-old plant cultures and cultivated at 25°C with light 16 h/day for a further 3, 6 and 9 days. The values are expressed as mean ± SD. *Indicates statistical significances at the $P < 0.01$ level.

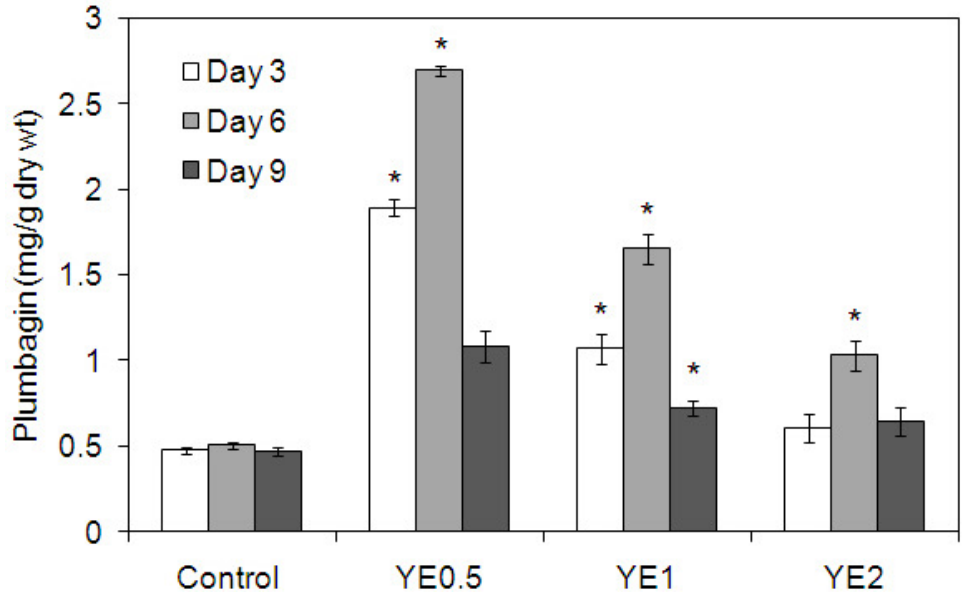

Figure 2. Effect of yeast extract (YE) on plumbagin production in plant cultures. Yeast extract (0.5, 1 and 2 mg ml^{-1}) was added to 28 day-old plant cultures and cultivated at 25°C with light 16 h/day for a further 3, 6 and 9 days. The values are expressed as mean ± SD. *Indicates statistical significances at the $P < 0.01$ level.

(Chuntaratin, 2006). The effect of salicylic acid (50 to 200 µM) on plumbagin production is shown in Figure 3. Most of the salicylic acid treated plants showed significant increasing effect on the production of plumbagin at 3 days of treatment. No differences in plumbagin content were observed at day 6 and 9. The addition of 100 µM salicylic acid induced the highest production of plumbagin (1.31± 0.05 mg/g dry wt.), which was 2.7-fold in

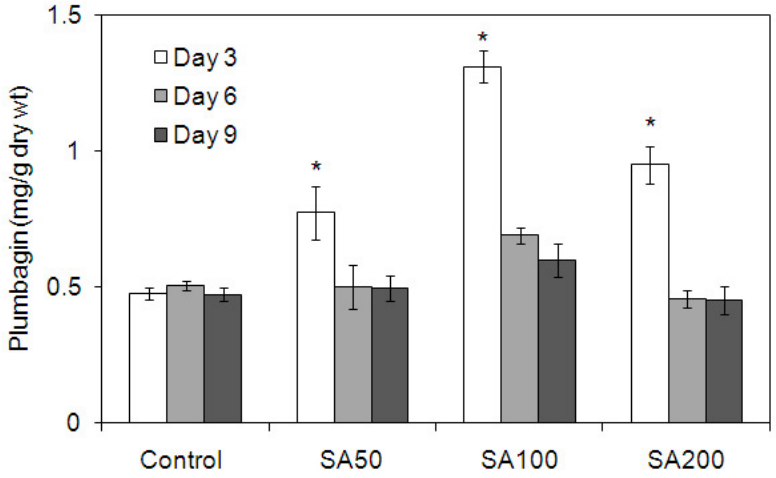

Figure 3. Effect of salicylic acid (SA) on plumbagin production in plant cultures. Salicylic acid (50, 100 and 200 µM) was added to 28 day-old plant cultures and cultivated at 25°C with light 16 h/day for a further 3, 6 and 9 days. The values are expressed as mean ± SD. *Indicates statistical significances at the $P < 0.01$ level.

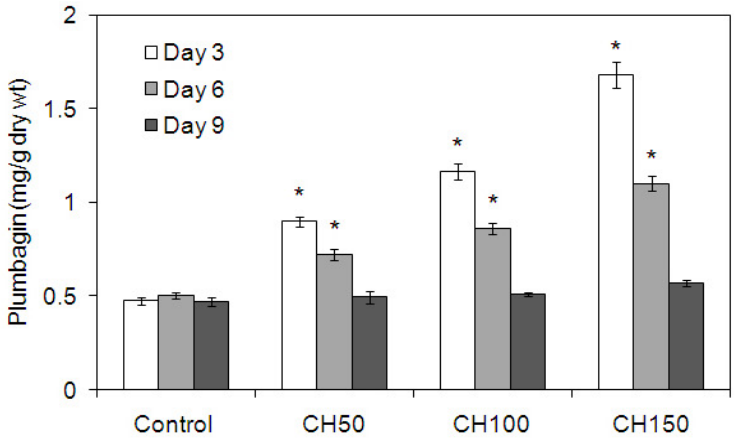

Figure 4. Effect of chitosan (CH) on plumbagin production in plant cultures. Chitosan (50, 100 and 150 mg l^{-1}) was added to 28 day-old plant cultures and cultivated at 25°C with light 16 h/day for a further 3, 6 and 9 days. The values are expressed as mean ± SD. *Indicates statistical significances at the $P < 0.01$ level.

comparison with non-elicited plants. These findings were in tune with Ziaratnia et al. (2003) and Putalun et al. (2010) who reported the similar effect of salicylic acid in *D. capensis* and *D. burmanii*, respectively. Salicylic acid treatment resulted in the activation of phenylpropanoids synthesis and secretion, in the apoplast of phytoalexins compounds with antipathogen action (Krasavina, 2007). In addition after 6 day of treatment with salicylic acid, the *in vitro* plants were changed to brown, the culture media was changed to yellow and plumbagin content was decrease as the control. Therefore, it seems likely that salicylic acid damaged the plants and caused to leak of compounds into media. However, the plumbagin in media was not found. It has been suggested that yellowing coloration could also be due to other phenolic compounds as a response to elicitor-induced stress (Zhao et al., 2001).

Influence of chitosan (50 to 150 mg/l) on plumbagin production was shown in Figure 4. A direct correlation between chitosan concentration and the plumbagin yield was observed. Chitosan at 150 mg/l gave the highest content of plumbagin (1.68±0.07 mg/g dry wt.), which was 3.5-fold of the control. Chitosan remarkably elicited plumbagin production on day 3 and decreased when increasing the elicitation time. In contrast to this finding, Putalun et al. (2010) reported that plumbagin accumulation in *D. burmanii* was stimulated at 6 day of chitosan.

This might be for several reasons such as response time to different plant species as well as its sorption potential (Shabani et al., 2009; Orlita et al., 2008).

In conclusion, this study indicated that different elicitors had different effect on plumbagin production from *D. indica* whole plant cultures. The maximum concentration of plumbagin achieved was 2.69±0.03 mg/g dry wt treated with 0.5 mg/ml yeast extract for 6 days which was 5.4 and 2.7-fold higher than in control plants (0.50±0.01 mg/g dry wt.) and wild plants (1.00±0.06 mg/g dry wt.), respectively. The optimum incubation time for elicitation was 6 days for yeast extract and methyl jasmonate whereas salicylic acid and chitosan was 3 days. Plumbagin content from our investigation showed a higher level than that found in previous study on *in vitro* shoot of *Drosera* species (Putalun et al., 2011). Furthermore, *D. indica* show a rapid growth compared with other species. Our findings indicate that the application of elicitors allows the high-plumbagin production in *in vitro* culture of *D. indica*.

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