In Vitro Environmental Stresses for Enhancing Withanolides Production in *Physalis angulata* L.

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Abstract. Production of secondary metabolites through in vitro culture can be increased by providing environmental stress conditions. Therefore, the objective of this study was to determine the effect of in vitro environment stress by the addition of elicitor on the withanolides production of shoots culture of *P. angulata*. Two weeks old shoot culture of *P. angulata* was elicited in either MS medium + biotic elicitor (25, 75, and 125 mg/L chitosan) or abiotic (PEG 2.5, 3, and 5%) for two weeks. The control was MS0 medium without the addition of growth regulators or elicitor. The results of HPLC analysis showed that chitosan elicitor had better effect than PEG. Chitosan was more effective in increasing withanolide content than PEG with a little inhibition of growth. Elicitation with PEG slightly increased withanolide with more inhibiting shoot growth. This result shows that stress manipulation in the in vitro environment through elicitation is potential strategy to improve withanolides production from shoot culture of *P. angulata*.

Keywords: HPLC, medicinal plants, shoot culture, withanolides

1. Introduction

Physalis angulata L. (Ciplukan) is an herbaceous plant belonging to the eggplant family or Solanaceae. This plant extract has been widely consumed by people with asthma, hepatitis, malaria, rheumatism and dermatitis [1]. Apart from being an important alternative source of leishmanidal agents [2, 3, 4] *P. angulata* is also an antibacterial [5, 6], antiproliferative, anti-inflammatory [7], immunomodulators [8], antioxidants [9] and has antidiabetes effect [10], lowering blood pressure and reducing menopausal symptoms (hypertension, depression and anxiety etc) [11]. The metabolites compound in *P. angulata* which is biologically active promotes health effect called withanolides.

Withanolides are secondary compounds mainly produced by genera from Solanaceae, especially subfamily Solanoideae. *Physalis* is one of the main contributors to the structure of withanolide besides genera *Jaborosa* Juss., *Datura* L. and *Withania* Pauq [12]. Plant secondary metabolites are derivatives of primary metabolites that do not play a role in plants growth, development and reproduction. Secondary metabolite compounds are synthesized to performed special function under certain condition and depend on physiological conditions and stages of plant development. Fluctuations in environmental conditions cause plants to have defense strategies to successfully complete their life cycle. In other words, plant secondary metabolites are produced in response to environmental stress.

The chemical composition of secondary metabolites profiles in medicinal plants was affected by shifting environmental condition [13]. The environmental stress plays a role in directing the metabolism to regulate the production of active constituents in medicinal plants [13]. Individual environmental stress can selectively increase some secondary metabolites in plants. This eventually caused difficulties in standardizing medicinal raw materials which are an important stage in the production of herbal medicines.

Plant secondary metabolites are generally synthesized in small amounts which depend on physiological stage of plants. Fluctuations in environmental conditions can induce change in secondary metabolites profile. These constraints make plant cells / tissue culture as an alternative platform for the production of plant secondary metabolites under the controlled condition. Some strategies are carried out to maximize secondary metabolites produced through plant cell cultures, including manipulation of culture conditions by providing stress through elicitation [14]. Plant cell culture with elicitation of target compounds is a promising alternative to obtain valuable metabolites compared to extraction of plants that grow in nature [15]. Elicitation is an effective method for increasing the production of secondary metabolites that involves exogenous addition of elicitors (abiotic or biotic) in the growth medium which consequently triggers stress response [16, 17]. Elicitors are compounds that are applied in small amounts to stimulate secondary compounds related to all types of plant defenses.

Plant organs that grow in vitro are capable of producing secondary compounds whose synthesis patterns resemble intact plants [18]. In addition, secondary metabolites produced by organ culture is more stable [19]. In vitro shoot culture of *W. somnifera* produced Withanolide A in higher amount than those of the aerial parts of field grow plants [20]. Therefore the effect of environmental stress through elicitor application on the biosynthesis capability of secondary compounds in *P. angulata* shoot culture needs to be identified. The aim of this study was to determine the effect of elicitation on shoot growth and withanolides production of in vitro *P. angulata* plants.

2. Material and Methods

2.1. Plant materials

Three weeks old multiplied shoots derived from nodes explants were subcultured to MS0 medium to increase the height of single shoot. After one week the shoots with a height of ± 0.5 cm are ready to subculture in the elicitation medium.

2.2. Shoot culture in in vitro environmental stress induced by elicitor

Elicitation treatments on shoot culture referred to previous report with some modifications [21]. Single shoots were subcultured on MS medium supplemented with either biotic elicitor (chitosan 25, 75 and 125 mg / L) or abiotic (PEG 2.5, 3, and 5%). The control was hormone free MS medium. The shoots cultured in elicitation or control medium were incubated under continuous light condition (600 lux) at 23 ± 2 ºC for two weeks. The shoots fresh weight was weighed at the initial stage (week 0) and the end (week 2) of the period of elicitation. Shoot height and leaf number were measured /counted at the age of 0, 1 and 2 weeks. Subsequently, shoots were prepared for analysis of withanolides secondary compounds.

2.3. HPLC analysis of secondary compounds withanolides

2.3.1. Sample preparation.

Two weeks old shoot from control and elicitation medium treatment were dried in oven at 70°C for 72 hours. The dried sample was weighed as much as 0.5 g, then crushed with pestle mortar. The mashed sample was added to 25 ml of p.a methanol, stirred until homogeneous and left for 30 minutes. Then sonification with the sonicator was carried out for 45 min at room temperature. The solution was concentrated using a rotary evaporator. The concentrated extract produced was reconstituted with 10

ml methanol, then strain. The solution filtrate is ready to be injected. This step was carried out according to [22] with modification.

2.3.2. Preparation of solution.
Standard, sample, and mobile phase solutions (eluents) were filtered with PTFE membrane and degassing. The standard and sample solutions were filtered again with cellulose nitrate membrane on the HPLC injector syringe before being injected into HPLC.

2.3.3. Identification of Withanolides by HPLC.
HPLC analysis, referred to [22] with modification was carried out with Shimadzu with the SPD M20-A Photo Diode Array detector. The analysis was determined at 35 °C in the Shim-pack VP ODS 5 DSm column, 150 x 4.6 mm. The mobile phase with the isocratic method used acetonitrile and water with ratio 60:40. The analysis was carried out at a wavelength of 215 nm with a flow rate of 1 ml / min for 50 minutes.

3. Result and Discussion

3.1. Effect of in vitro environmental stress on shoot growth
The results showed that Physalis shoots cultured on MS medium + 75 mg/L chitosan for 2 weeks were still able to increase in height (6.5 ± 2.6 cm). However, the 125 mg / L chitosan produced lower shoot height than control (5.4 ± 2.9 cm) and only reached 4.9 ± 3.4 cm (Figure 1A). In contrast, the addition of PEG in all concentrations inhibited growth which resulting in shorter shoots (1.4 - 1.6 cm) than controls (5.4 ± 2.9 cm) (Figure 1B).

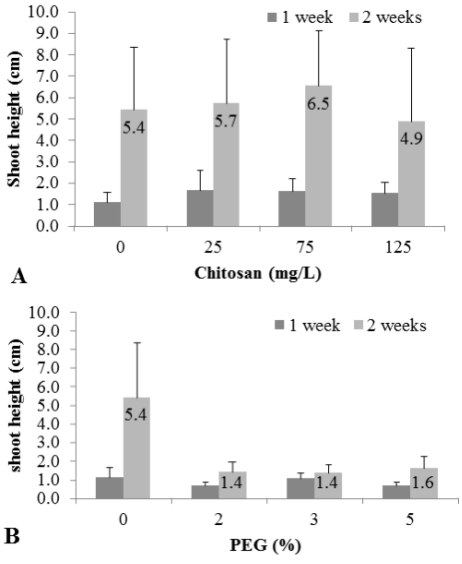

Figure 1. Effect of elicitor on the shoot height of in vitro *P. angulata* L.

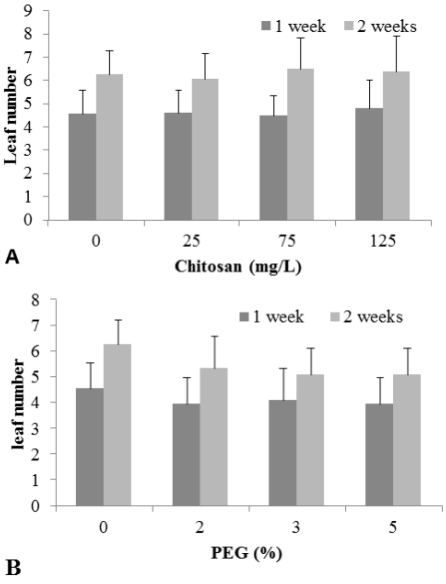

Figure 2. Effect of elicitor on the leaf number of *P. angulata* L. shoot culture

The effect of chitosan on the leaf number after two weeks of culture also showed a different pattern with the effect of PEG. The addition of chitosan with higher concentrations tended to increase the leaf number (Figure 2A), whereas addition of PEG with higher concentrations tended to decrease leaf number (Figure 2B).

The elicitor treatment of both chitosan and PEG for two weeks tended to produce lower shoots fresh weight than controls (6.3 ± 1 g) (Figure 3). However, PEG results in more decrease in shoot

fresh weight than chitosan. The shoot produced in PEG treatment had lower fresh weight (0.06 - 0.07 g) compare to those produced by chitosan (0.17 - 0.21 g).

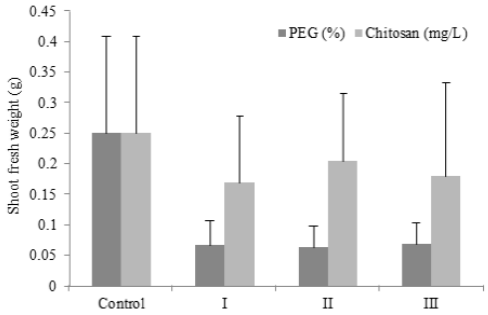

Figure 3. Effect of elicitor on the fresh weight of 2 weeks old shoot culture of *P. angulata* L.

3.2. Production of withanolides secondary compounds in environmental stress with the addition of elicitor

HPLC analysis on in vitro buds of Physalis was able to detect 32 types of Withanolides (Figure 4) compounds with varying levels between 2.76 - 51.59 μg/g DW samples (Figure 5). Nine of the 32 compounds, namely (1) Withaphysanolide A, (2) Withanolide B, (3) Withaphysalin A, (4) Withanolide A, (5) Withaferin A, (5) Withaferin A, (10) Physanolide A, (12) Physalin B, and (13) Withangulatin B presented high concentration, more than 24 μg/g DW samples. Seven withanolides compounds, namely (6) Withanolide D, (9) Dihydrowithanolide E, (14) Withangulatin E, (15) Physalin A, (16) Withangulatin I, (18) Physalin G and (20) Withangulatin A were detected between 10-20 μg / g DW samples. Meanwhile, 16 other withanolides compounds were detected only <10 μg / g DW samples.

In this study the stress induction in the culture environment through the addition of abiotic (PEG) and biotic (chitosan) elicitor to the medium for two weeks was able to increase withanolides content produced by Physalis shoot. Shoots cultured on MS medium + chitosan 75 mg/L resulted in the highest increase in production of withanolides when compared with other concentrations of both elicitors. If the concentration of chitosan was increased to 125 mg/L the production of withanolides tend to decrease. The withanolides production of shoot cultured in the three PEG elicitor concentrations tested also increased when compared with the control medium but did not exceed the levels of withanolides produced in the chitosan treatment of 25 mg/L.

Elicitors-induced stress in plant cell culture can be effective strategy to increase the yield of secondary metabolites [23]. Chitosan is a natural biopolymer that is widely found in shells of marine animals such as shrimp which can be able to stimulate plant growth and to induce tolerance to abiotic and biotic stress [24]. Chitosan induced the defense potential of plant tissues at high concentrations [25]. Adventitious root growth of adventitious root growth of Morinda citrifolia after 4 weeks of culture in medium + chitosan 2 mg/mL decreased but anthraquinones, phenolics and flavonoids production increased [26]. Liquid medium containing 15 mg/L chitosan showed the highest relative growth rate (7-fold increase) of Grammatophyllum speciosum PLBs [27].

Abiotic elicitors give varying influence on plant growth and in the secondary metabolites production. PEG includes abiotic elicitors that induce drought stress. Drought stress is one of the physical elicitors in addition to ultrasound, light, osmotic stress, salinity, thermal stress [28]. In this study PEG inhibited the growth of Physalis shoots in vitro because PEG decreases the potential of water and thus inhibits the absorption of water and nutrients. This stress condition triggers a plant defense response by increasing the synthesis and accumulation of secondary metabolites. The current results are the first report of withanolides composition in methanol extracts of shoot of P. angulata cultured in in vitro environmental stress through elicitor application.

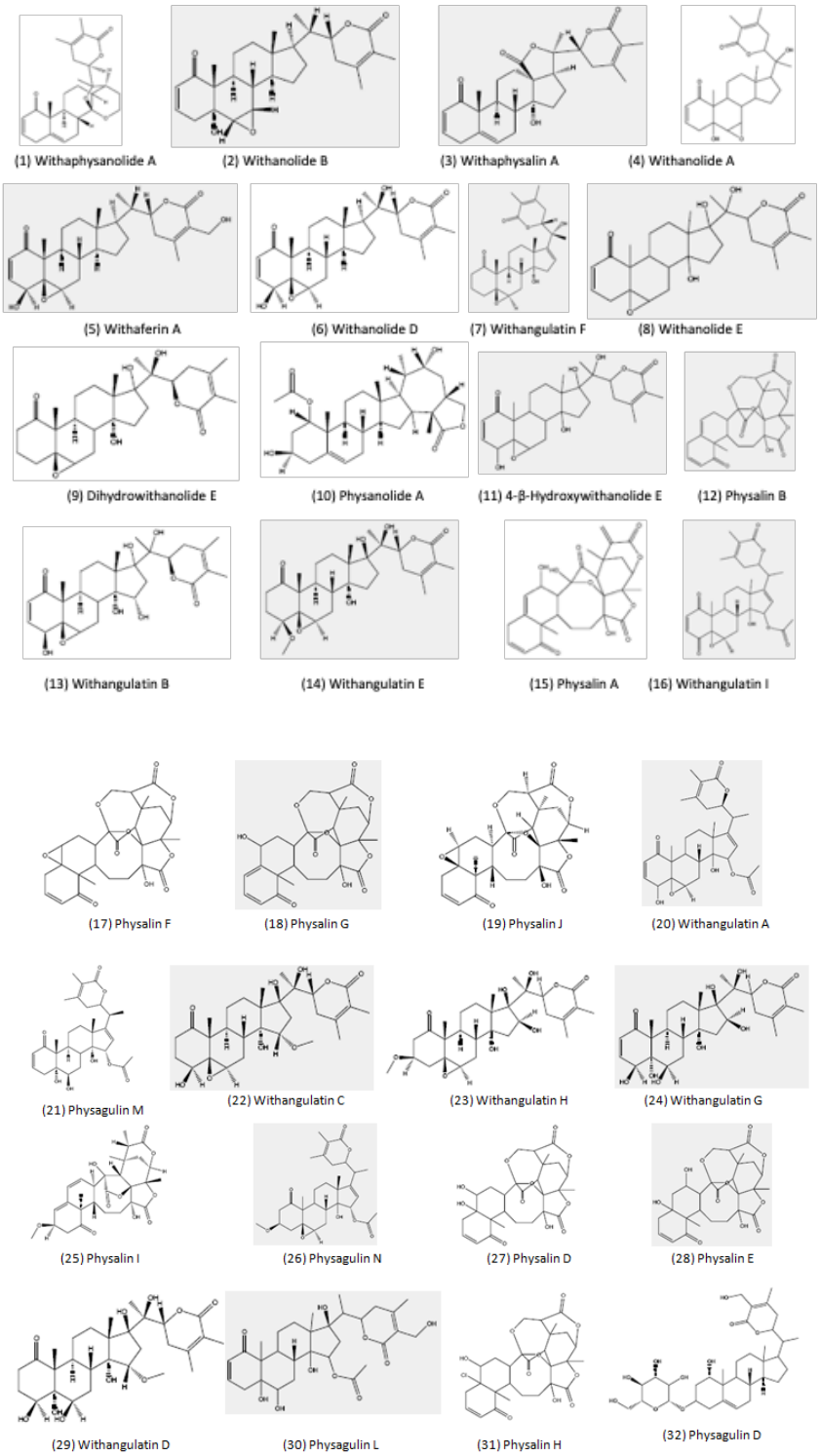

Figure 4. Chemical structures of 32 withanolides compounds isolated from shoot culture of *P. angulata* L.

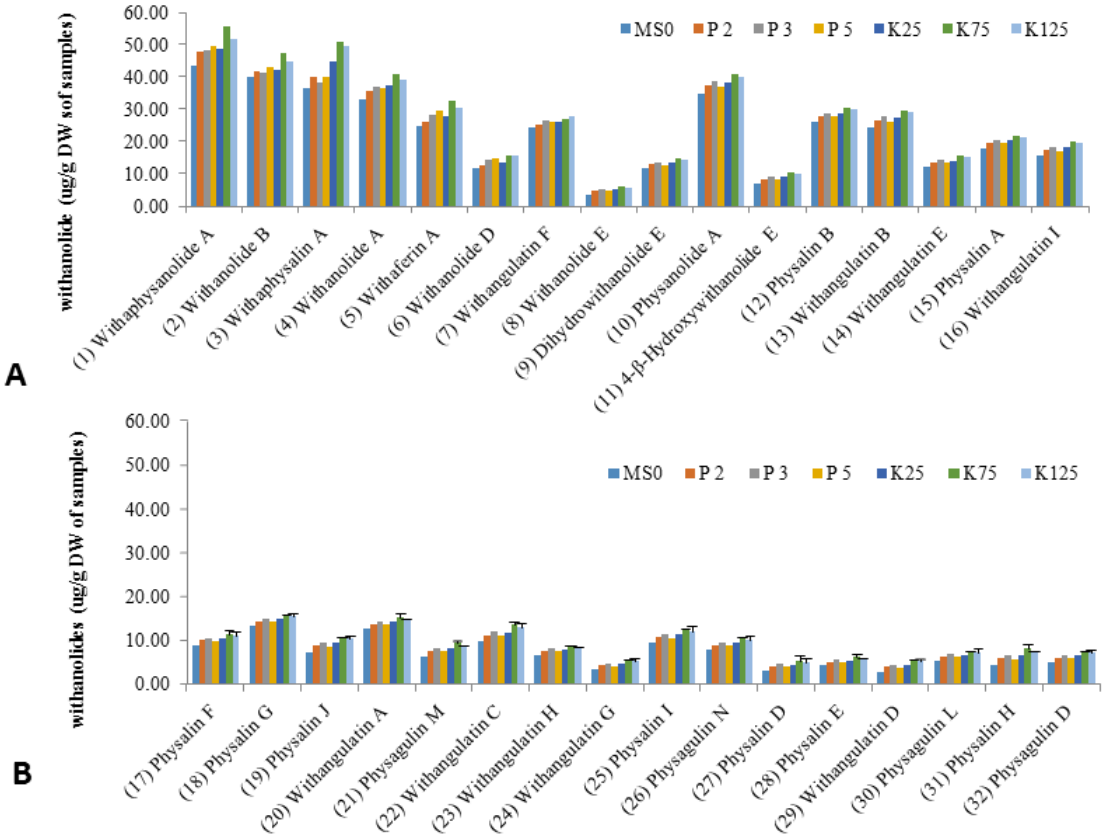

Figure 5. Effect of elicitor on withanolides content of 2 weeks old shoot culture of *P. angulata* L. A). Withanolides compound with RT= 21.465 – 29.646 min. B). Withanolides compound with RT= 29.652 – 36.812 min. Note: P = Poly Ethylene Glycol (PEG) (%), K = Chitosan (mg/L)

4. Conclusion

This study indicates that chitosan elicitor is more prospective than PEG because chitosan increases the content of the compound withanolides more with a smaller level of growth resistance. Whereas the PEG elicitor further inhibits the growth of shoots with less increase in the content of withanolides. Application of elicitor in in vitro technique can be used to enhance production of withanolides from *P. angulata* plant. Optimization of chitosan concentration needs to be tried to determine the optimal accumulation of withanolides.

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