

Effects of Sequential and Simultaneous Double Elicitation on Aloe-Emodin Production in Cassia Tora Root Cultures

Palin Teptat

Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand, 90110

Pimpimon Tansakul

Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand, 90110. Phytomedicine and Pharmaceutical Biotechnology Research Excellence Center, Faculty of Pharmaceutica

Athip Sakunphueak (✉ athip.s@psu.ac.th)

Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand, 90110 <https://orcid.org/0000-0001-8788-3544>

Research

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Abstract

Cassia tora root cultures were established to study anthraquinone production and as an alternative source of anthraquinone. In this study, several plant defence-based techniques were used to enhance aloe-emodin production in *Cassia tora* root cultures. Elicitation and the initial optimisation of exposure time and culture age suggested that 100 mg/L yeast extract was the most efficient elicitor, with the highest efficiency during early to mid-stationary growth phase. The elicited root produced approximately 2.7-2.9 folds-improvement in aloe-emodin content when compared with the control. The efficiency of the elicitor tended to increase along with the exposure time. Extending the elicitation period to the end of culture cycle (9-10 days) increased the production up to 6.21 times (5.740 ± 0.584 mg/gDW). Simultaneous double elicitation and sequential double elicitation also showed their effectiveness on aloe-emodin production. Aloe-emodin production was improved up to 9.09 times higher than the control by a simultaneous add-on of 50 mg/L chitosan with 100 mg/L yeast extract. Meanwhile, sequential elicitation of 50 mg/L chitosan for 24 hours prior to elicitation by yeast extract improved the production up to 11.27 times higher than the control. Both treatment styles exhibited a particular advantage toward the extracellular accumulation, which might facilitate the use of two-phase culture or in conjunction with other enhancing techniques.

1. Introduction

Plants have profound impact on human lives. One important role is supplying pharmaceutically-value compounds. For examples, khellin from *Ammi visnaga* is a lead compound of the asthma prophylactic drug, sodium cromoglycate. Paclitaxel from *Taxus* sp. as well as vinblastine and vincristine from *Catharanthus roseus* have been used as chemotherapy for various types of cancer (Rungsung W et al., 2015). Aloe-emodin is an anthraquinones reportedly produced by *Cassia tora* (Kim YM et al., 2004) and has been studied as a candidate for an anticancer drug for decades (Özenver N et al., 2018). Other than the laxative effect which is the hallmark of anthraquinones, the compound has been reported to exhibit cytotoxic activity toward various cancer cell lines without causing undesirable effects on normal cells (Liu K et al., 2012; Pecere T et al., 2000). Apart from the pharmaceutical investigation, the development of an aloe-emodin supply source is undeniably a vital part of drug research and development, since the commercial price of aloe-emodin is still relatively high. Moreover, the chemical synthesis might not be cost-effective due to the involvement of extreme reaction conditions, heavy metal catalysts, and a series of reactions (Franck H-G, 1988).

Plant tissue cultures have become an alternative source of natural products. They have been widely used, not only as a model to study secondary metabolite production, but also as an industrial scale production source of some naturally occurring drugs (Bringi V et al., 2012). In spite of this, the major drawback of natural sources is low production level, since secondary metabolites are mainly produced as defence response against stress conditions (Wilson SA and Roberts SC, 2012). Elicitation is a current technique to increase secondary metabolite production. It is based on triggering plant defence systems by using biotic or abiotic stress. The efficiency of elicitation depends on various factors, such as plant species, elicitor

concentration, exposure time and growth stage. Optimisation of these factors reportedly improves the elicitation outcome (Naik PM and Al-Khayri JM, 2016).

Chitosan and yeast extract are well-known fungal elicitors. The studies in *Cassia* sp. as well as other species have consistently suggested their effectiveness in anthraquinone production (Malerba M and Cerana R, 2018; Hassen O and Chang T, 2017; Chetri SK et al., 2015; Abdel-Rahman IAM et al., 2013; Vascosuelo A., et al., 2003). Moreover, a number of studies have suggested the advantages of using more than one enhancing technique in combination. Elicitation has been reported to synergistically work with many techniques, for example, precursor feedings (Sivanandhan G et al., 2014) and two-phase cultures (Byun SY et al., 1992). Simultaneous double elicitation is another technique to improve elicitation efficiency. It is based on the synergistic effect of two or more elicitors. The combination of methyl jasmonate and chitosan was reported to exhibit a dual elicitation effect (Al-Gendy AA and Lockwood GB, 2005).

Primed state is an underlying defence protection suggested to be established in plants after interaction with pathogens. Plants with primed states are more sensitised to stress than usual and are able to produce more intense defence responses (Malerba and Cerana, 2018; Jia X et al., 2016; Fitza KNE et al., 2013; Pastor V et al., 2013; Po-wen C et al., 2013; Prakongkha I et al., 2013; Povero G et al., 2011; Paulert R et al., 2010; M and Cerana R 2008; Katz VA et al., 1998). Because of this characteristic of the primed state, we assumed that performing elicitation in primed plants should induce the extra production level of secondary metabolites.

Our previous study showed that *C. tora* root cultures were able to be used as a potential alternative source of aloe-emodin (Sakunphueak A et al., 2013). In order to improve the yield of aloe-emodin in *C. tora* root cultures, effects of chitosan and yeast extract elicitation were investigated in this study. The factors that affect the outcome of elicitation were optimised and 2 treatment patterns including simultaneous double elicitation and sequential double elicitation of both elicitors were studied.

2. Materials And Methods

2.1 Establishment of *C. tora* root cultures

The root cultures were established from *C. tora* plantlets. The root explants were excised and cultured in Gamborg's B5 media supplemented with 0.1 mg/L 1-naphthaleneacetic acid and 1.0 mg/L kinetin. The root cultures were generated from the root explants, separated from the original root and subcultured every 21 days until homogenous. The roots were cultured under sterile and dark conditions at 25 ± 2 °C with continuous shaking at 60 rpm. The homogenous root cultures were subsequently supplied as plant material for the studies (Sakunphueak A et al., 2013).

2.2 Preparation of elicitors

Chitosan: the stock solution of chitosan was prepared from low molecular weight chitosan (50–190 kDa) (Sigma-Aldrich) with 75–85% degree of deacetylation. Chitosan powder was dissolved in a small amount of 2% acetic acid with sonication. The solution was then adjusted to the final volume with distilled water, and sterilized by autoclaving (121 °C, 15 min).

Yeast extract: the stock solution of yeast extract (Himedia) was prepared by dissolving yeast extract powder in distilled water. The solution was sterilized by autoclaving (121 °C, 15 min).

2.3 Elicitation and optimisation of the affecting factors

2.3.1 Optimisation of elicitor type and concentration

The optimisation of elicitor type and concentration was performed in 12-day old *C. tora* root cultures. Chitosan was added to the culture media to make a final concentration of 10, 50, 100 and 250 mg/L, while yeast extract was added to make a final concentration of 10, 50, 100 and 1000 mg/L. Sterilized-distilled water at the same volume as the elicitor solution was added to make the control. Each elicitor was left to contact with the root cultures for 24 hours. The elicited root cultures and the media were then harvested and collected for the extraction processes.

2.3.2 Optimisation of exposure time

The 12-day old root cultures were elicited with the optimised elicitor from 2.3.1, then left to contact with the elicitor for 12, 24, 36 and 48 hours. The control was treated with sterilized-distilled water at the same volume as the elicitor solution. Subsequently, the elicited root cultures and the media were harvested and collected for the extraction processes.

2.3.3 Optimisation of culture age

The optimised elicitor from 2.3.1 was used to elicit the 9, 12, 16 and 18 days-old *C. tora* root cultures, under the optimised elicitation period suggested by 2.3.2. The root cultures at the same age were treated with sterilized-distilled water at the same volume as the elicitor solution to make the control group. Then, the elicited root cultures and the media were harvested and collected for the extraction processes.

2.4 Simultaneous double elicitation and sequential double elicitation

Simultaneous double elicitation was performed in the 12 days-old *C. tora* root cultures, using a combination of various concentrations of chitosan (10, 50 and 100 mg/L) along with 100 mg/L of yeast extract. Sequential double elicitation was performed by pre-treating the 12-day old root cultures with chitosan for 24 hours prior to the 100 mg/L yeast extract elicitation. Chitosan was added into the culture media to make a final concentration of 10, 50 and 100 mg/L. After 24 hours, yeast extract at 100 mg/L was added into the pre-treated root cultures. The code of each treatment group is described in Table 1.

Table 1
Code of treatment group in simultaneous double elicitation and sequential double elicitation experiments

Code	Treatment		
	Day 12	Day 13	Day 21
Control	-	-	Harvested
C10	10 mg/L chitosan	-	Harvested
C50	50 mg/L chitosan	-	Harvested
C100	100 mg/L chitosan	-	Harvested
Y100	100 mg/L yeast extract	-	Harvested
DC10Y100	10 mg/L chitosan and 100 mg/L yeast extract	-	Harvested
DC50Y100	50 mg/L chitosan and 100 mg/L yeast extract	-	Harvested
DC100Y100	100 mg/L chitosan and 100 mg/L yeast extract	-	Harvested
PC10Y100	10 mg/L chitosan	100 mg/L yeast extract	Harvested
PC50Y100	50 mg/L chitosan	100 mg/L yeast extract	Harvested
PC100Y100	100 mg/L chitosan	100 mg/L yeast extract	Harvested
List of Figure Captions			

The treated root cultures were maintained until the end of culture cycle, then harvested. The root cultures and the media were collected for the subsequent extraction processes.

2.6 Extraction of aloe-emodin

2.6.1 Intracellular aloe-emodin

The root cultures were dried at 50 °C for 24–48 hours and ground into powder. The root powder (100 mg) was extracted with 5% HCl in methanol (30 mL) at 70–75 °C using reflux apparatus. The extract was then concentrated by vacuum evaporator at 40 °C and reconstituted with methanol (10 mL) before being subjected to HPLC quantitative analysis of aloe-emodin (Sakunphueak A et al., 2013).

2.6.2 Extracellular aloe-emodin

The culture media were acidified to pH 2 using 1N hydrochloric acid, to switch the protonated aloe-emodin to the deprotonated form. The acidified media subsequently underwent the liquid-liquid extraction

with ethyl acetate. Then, the organic solvent part was collected and concentrated by vacuum evaporation at 40 °C. The extract was reconstituted in methanol and subjected to the HPLC analysis (Sakunphueak A et al., 2013).

2.7 Quantitative analysis of aloe-emodin

The aloe-emodin content was analysed using a HPLC system (Shimadzu, Japan), with photodiode array detector, and an autosampler. The separation was conducted in a reversed-phase TSKgel® ODS-80Tm C-18 column (5 µm, 150 mm x 4.6 mm i.d.). The gradient mobile phase consisted of 70% methanol and 30% aqueous acetic acid (2% v/v). The injection volume was 10 µL. The separation was performed with a flow rate of 1 mL/min and the quantitative wavelength was set at 266 nm. The relative concentration of aloe-emodin was calculated from the calibration curve of aloe-emodin standard and expressed in terms of mg per gram of the dry root powder weight (mg/gDW) (Sakunphueak A et al., 2013).

2.8 Statistical analysis

The data analysis was performed through the application PSPP (version 1.2.0), using one-way ANOVA and the post-hoc Tukey HSD test. The confidence interval was set to be 95 or 99% ($p < 0.05$ or 0.01).

3. Results And Discussion

3.1 Elicitation

3.1.1 Effects of elicitor type and concentration

Chitosan and yeast extract have been frequently reported as successful elicitors for enhancing anthraquinone production in many plants, such as *C. angustifolia* (Chetri SK et al., 2015), *C. bicapsularis* (Abdel-Rahman IAM et al., 2013), *Rudgea jasminoides* (Oliveira MDC et al., 2007), *Oldenlandia umbellata* (Krishnan RS and Siril EA, 2016), *Rubia akane* (Jin JH et al., 1999), *R. tinctorum* (Vascosuelo A et al., 2003) and *Myristica fragrans* (Iyer I et al., 2011). In this study, the un-elicited root cultures began the production of aloe-emodin on day 12 of the cycle and reached the peak level on day 20. The varied concentrations of yeast extract and chitosan were used to enhance the production. The elicitation period was initially set at 24 hours to avoid cytotoxicity caused by long-time contact with the elicitor. The elicitation effect was found to be highly influenced by the type and concentration of elicitor. At 10 mg/L, both elicitors failed to enhance the production. However, their effects gradually improved as the concentration increased. At 50 mg/L of chitosan, the production level was elevated, but still not better than the control. In comparison, 50 mg/L of yeast extract significantly increased the production by 1.68 times when compared with the control (0.391 ± 0.024 mg/gDW). The peak performance of chitosan was observed at the concentration of 100 mg/L. It increased aloe-emodin production from the control by 1.18 times (0.276 ± 0.007 mg/gDW). While, the peak effect of yeast extract was also observed at 100 mg/L but it yielded a higher level of aloe-emodin than chitosan did (Fig. 1). Yeast extract at 100 mg/L was the most effective elicitor, increasing aloe-emodin production up to 2.71 times (0.633 ± 0.048 mg/gDW) when

compared with the control. Nevertheless, the concentration-dependent manner only applies to the particular range of concentrations. At relatively high concentrations, the effects of chitosan and yeast extract were dropped from their peak level. Furthermore, these elicitors differently affected the accumulation in both intracellular and extracellular compartments. Yeast extract promoted a comparable accumulation in both compartments, whereas chitosan had a distinct advantage toward the intracellular accumulation (Fig. 1).

The results suggested that *C. tora* root cultures were more susceptible to yeast extract than to chitosan. Interestingly, similar findings were also observed in *C. angustifolia* (Chetri SK et al., 2015) and *C. bicapsularis* (Abdel-Rahman IAM et al., 2013). Yeast extract showed potential to enhance *Cassia* sp. anthraquinone production, as well as other acetate-malonate-derived anthraquinones (Krishnan RS and Siril EA, 2016; Oliveira MDC et al., 2007). Meanwhile, chitosan was reportedly efficient in *Rubiaceae* (Vascosuelo A., et al., 2003; Jin JH et al., 1999) and other species producing Rubia-typed anthraquinones (Iyer I et al., 2011). According to current knowledge on the plant defence system, it might be assumed that the different preference for chitosan and yeast extract is contributed by the different type and number of defence components expressed in the two genera.

Normally, plants respond to pathogen stress through the induction of pattern-triggered immunity (PTI). The epitope part of pathogen or the so-called pathogen-associated molecular pattern (PAMP) is recognized by a specific pattern-recognizing receptor (PRR) on plant plasma membrane. The interaction between PAMP and PRR will trigger a variety of signalling pathways and pathogenesis-related genes. Consequently, immediate defence responses are triggered (Bigeard J et al., 2015; Mazzotta S and Kemmerling B, 2011; Povero G et al., 2011; Jones JDG and Dangl JL, 2006), frequently characterized as stomatal closure (Gudesblat GE et al., 2009), cell wall lignification (Lee MH et al., 2019), callose accumulation (Pastor V et al., 2013), hypersensitivity responses (Balint-Kurti P, 2019) and secondary metabolite production (Piasecka A et al., 2015). Chitin is a structural component of the fungal cell wall which can be recognized by a plant defence system. During plant-pathogen interaction, chitin is believed to be cleaved by plant chitinases into an oligosaccharide. The chitin oligosaccharide is recognized by plant specific PRR called “chitin elicitor receptor kinase 1 (CERK1)” which is cooperated by “chitin elicitor receptor binding protein (CEBiP)” (Yin H et al., 2016). However, some pathogens have evolved mechanisms to shield themselves from plant defence systems. One of the mechanisms is turning chitin into its deacetylated derivative, chitosan, which plant chitinases exhibited less affinity on. Some studies suggested a compound lack of a specific PRR in plants (Yin H et al., 2016). Therefore, chitosan is naturally supposed to be less efficient than chitin (Sanchez-Vallet A et al., 2015; Vander P et al., 1998). According to the results, we assumed that the defence mechanism of *C. tora* root cultures might not be sophisticated enough to efficiently respond to chitosan. It might have chitinases with less affinity on chitosan or consist of less compatible defence components. Meanwhile, yeast extract is a mixture of fungal cell components. It could contain many components that can trigger the defence system. Thus, yeast extract showed to be more efficient than chitosan at the same concentration. Furthermore, chitosan was suggested to negatively affect the growth and cause overstressed conditions in root tissue (Lopez-

Moya F et al., 2017; Overvoorde P et al., 2010). This might be another influence that leads to the lower elicitation efficiency of chitosan in *C. tora* root cultures.

Both chitosan and yeast extract elicitions exhibited a concentration-dependent manner until reaching the peak level and then started to decline. This phenomenon might be explained in term of defence capacity limitation. High elicitor concentration may exceed the available number of PRRs as well as the capacity of other defence components. Moreover, overstressed conditions caused by high concentrations of pathogen elicitors might be toxic to the plant cell and decrease the overall production (Overvoorde P et al., 2010).

3.1.2 Effects of exposure time and culture age

In order to further optimise the elicitation conditions, exposure time and culture age were subsequently optimised. Our result was consistent with previous reports in *Rudgea jasminoides* (Oliveira MDC et al., 2007) and *R. cordifolia* (Ghatge S et al., 2014), suggesting that longer exposure time produced better elicitation efficiency. The highest production was observed when the root cultures were exposed to the elicitor for 48 hours. The aloë-emodin production was significantly increased from the control up to 2.93 times (0.713 ± 0.010 mg/gDW).

The culture age was another affecting factor to optimise. Without elicitation, the aloë-emodin production was drastically increased from the middle of the stationary phase to the decline phase of the growth cycle. Aloë-emodin accumulation started on day 12, gradually increased until reaching the peak level on day 20, and then plummeted after day 24. According to the production pattern, the optimisation of culture age was specifically performed during the aforementioned period – day 9, 12, 16 and 18 represented lag phase, exponential phase, late linear phase and early stationary phase of growth. Interestingly, the 100 mg/L yeast extract elicitation showed to be effective at every studied culture age. Treatment on day 12 promoted the highest elicitation benefit, with the 2.16 time-improvement in aloë-emodin production (0.1802 ± 0.012 mg/gDW) but elicitation on this day affected the root cultures health. Meanwhile, the treatment on day 16 did not show significant change when compared with the control. Interestingly, elicitation on day 18 showed negative impact on aloë-emodin production, actually lowering the aloë-emodin content to 55% of the control. (Fig. 3). The result indicated that the elicitation was the most efficient during the exponential growth phase. However, it was too early to harvest the root cultures immediately after the 48 hours contact period (day 14) because the root cultures were not fully grown. Thus, we decided to perform elicitation on day 12 and harvest the elicited root cultures at the end of the culture cycle (day 21) in the following experiments.

3.2 Simultaneous double elicitation on aloë-emodin production

In the present study, 10, 50 and 100 mg/L chitosan in combination with 100 mg/L yeast extract were used to elicit the root cultures on day 12. The aloë-emodin levels were observed at the end of the culture cycle (day 21). Without chitosan, 100 mg/L yeast extract elicitation (Y100) increased the end cycle aloë-

emodin production 6.21 times (5.740 ± 0.584 mg/gDW). It was found that 10 and 50 mg/L chitosan in a combination with 100 mg/L (DC10Y100 and DC50Y100) increased the production 8.82 times (8.139 ± 0.241 mg/DW) and 9.09 times (8.389 ± 0.400 mg/gDW), respectively. In contrast, a combination of 100 mg/L chitosan and 100 mg/L yeast extract (DC100Y100) overstressed the root cultures and caused the aloe-emodin content dropped when compared with the other treatment groups (Fig. 4). With the presence of a relatively low concentration of chitosan, 100 mg/L yeast extract elicitation enhanced aloe-emodin production better than as a single elicitor. Meanwhile, the presence of the relatively high concentration of chitosan might have competitively consumed the defence resources and negatively affected the viability of the root cultures. Therefore 100 mg/L chitosan and 100 mg/L yeast extract failed to make a synergistic elicitor duo. This part of study suggested that simultaneous double elicitation of 10 mg/L chitosan and 100 mg/L yeast extract were the most effective conditions for enhancing aloe-emodin production in *C. tora* root cultures.

Double elicitation is a current technique to improve the efficiency of elicitation. It is based on the synergistic effect of two or more elicitors. Chitosan has been reported to synergistically work with many types of elicitors such as jasmonic acid and fungal oligosaccharide. Recently, a few studies have suggested that chitosan is involved in jasmonic acid-signalling pathway (Jia X et al, 2018; Yin H et al., 2013; Povero G et al., 2011). The combination of chitosan and methyl jasmonate exhibited a synergistic effect on glucotropaeolin production in *Farsetia aegyptia* (Al-Gendy AA and Lockwood GB, 2015). Interestingly, the same pathway was consistently reported to be involved with yeast extract treatment. The increase of intermediate, precursors and catalytic enzymes involved in jasmonic acid biosynthetic pathways were reportedly observed following yeast extract elicitation (Yaguchi T et al., 2017; Hasanloo T et al., 2009). Thus, chitosan and yeast extract would bi-laterally enhance the aloe-emodin production through the jasmonic acid-signalling pathway and create a new synergistic elicitor duo.

3.3 Sequential double elicitation on aloe-emodin production

Sequential double elicitation was designed based on the theory of plant priming. Priming is an adaptive strategy which enhances plant defence systems. It is a consequence of plant-pathogen interaction. Priming has been suggested to confer an underlying state for long-term immune protection in plants (Shine MB et al., 2019; Shah J and Zeier 2013; Conrath U, 2006). Consistently, plants with primed states have been reported to be more sensitised to stress and able to produce more intense defence responses (Malerba M and Cerana R, 2018; Jia X et al., 2016; Fitza KNE et al., 2013; Pastor V et al., 2013; Po-wen C et al., 2013; Prakongkha I et al., 2013; Povero G et al., 2011; Paulert R et al., 2010; M and Cerana R 2008; Katz VA et al., 1998). In this part of the study, we took advantage of the primed state to further improve the efficiency of 100 mg/L yeast extract elicitation. Chitosan showed the ability to trigger defence response in the studied root cultures. Thus, it was chosen as the priming inducer. Various concentrations of chitosan were added into the culture media on day 12 in order to prime the root cultures. After 24 hours, 100 mg/L yeast extract was added to elicit the root cultures. Aloe-emodin production was measured at the end of culture cycles. It was found that suboptimal elicitor concentrations of chitosan created an entirely different priming effect. Root cultures primed with 50 mg/L chitosan (PC50Y100)

showed to be more sensitised to 100 mg/L yeast extract elicitation. However, root cultures primed with 10 mg/L chitosan (PC10Y100) were not able to elevate the aloë-emodin level when compared with 100 mg/L yeast extract elicitation alone. PC50Y100 was able to elevate the aloë-emodin production (10.401 ± 0.531 mg/gDW) up to 11.27 times higher than the control which was 1.81 times higher than using yeast extract alone. Interestingly, chitosan at 10 mg/L and 100 mg/L failed to activate the priming effect. Both root cultures primed with 10 mg/L and 100 mg/L chitosan (PC10Y100 and PC100Y100, respectively) did not improve aloë-emodin production when compared to single yeast extract elicitation (Fig. 5).

The result was consistent with many reports, suggesting that defence responses under the influence of primed states are more intense than usual. The priming effect is highly influenced by a concentration of priming inducer. As recently reported by the study in *Plumbago indica* root cultures, pretreatment with the effective elicitor concentration of chitosan (150 mg/L) failed to improve the elicitation effect of 100 mg/L yeast extract on plumbagin production (Jaisi A and Panichayupakaranant P, 2020). This strongly agrees with our study and emphasizes that an effective elicitor concentration of chitosan does not work as a priming inducer concentration for 100 mg/L yeast extract elicitation. Meanwhile, pretreatment with low concentrations of chitosan was reported to enhance the disease resistance against fungal infection in *Pinus patula* seedlings and was involved with the increased expression level of gene encoding phenylalanine ammonia lyase (PAL) (Fitza KNE et al., 2013). The improved disease resistance was suggested to be associated with the improved hydrogen peroxide level in oxidative burst (Paulert R et al., 2010). Moreover, low concentrations of systemic acquired-resistance (SAR) inducers such as benzothiadiazole, 2,6-dichloroisonicotinic acid and salicylic acid were reported to work as priming inducers in *Petroselinum crispum* cell cultures (Katz VA et al., 1998; Kauss H et al., 1992). These studies support our finding that low concentration or suboptimal concentration of elicitor is required for priming and suggests that different plant species require different types and concentrations of priming inducers.

To date, the majority of priming study have been focusing on pathogen resistance outcome. Consistent reports in term of improved pathogen resistance have made priming to the practical application for crop protection (Westman SM et al., 2019; Dwen Q et al., 2017; Xiao W et al., 2017 and Iwata M, 2001). Whereas in terms of secondary metabolites, the study is completely different and needs more intensive investigation. Recently, reports on secondary metabolite production under the influence of primed state have been from parsley cell culture-based studies, using SAR-inducers as the priming inducers (Katz VA et al., 1998; Kauss H et al., 1992) and a recent study in *P. indica* cell cultures which used methyl- β -cyclodextrin as the priming inducer (Jaisi A and Panichayupakaranant P, 2020). Our work is probably the first to report the success of using chitosan as a priming inducer to improve secondary metabolite production. It suggests a new aspect of chitosan priming, since previous reports largely focused only on the effect on disease resistance.

The precise mechanism of priming is still vague. Nevertheless, current suggestions give priority to the pre-existing accumulation of defence molecules (signalling compounds, inactive enzymes and hormones), primary metabolism remodelling and epigenetic modifications. We assumed that these intracellular

changes might be the factors that discriminate the priming effect of different concentrations of chitosan in this study. (Tugizimana F et al, 2018; Espinas N et al., 2016; Zhu QH et al., 2016; Rojas CM et al., 2014; Conrath U, 2011; Jaskiewicz M et al., 2011). Chitosan at 10 mg/L might be insufficient to induce significant aloë-emodin production itself or to sensitise the root cultures. Thus, the production in the PC10Y100 was purely contributed by the effect of yeast extract. Likewise, 50 mg/L was insufficient for the significant elicitation in a 24 hours period, but it might be intense enough to trigger PTI and induce the root cultures to the primed state. The case of PC100Y100, which 100 mg/L chitosan worked poorly as priming inducer, could be explained in term of primary metabolism remodelling. It has been suggested that plants lose resources and energy during PTI induction due to metabolic changes, affecting functions required to maintain normal growth and living (Schwachtje J et al., 2018; Rojas CM et al., 2014). Furthermore, the balance of jasmonic acid and salicylic acid signalling pathways were also reported to be interfered, following PTI induction (Schwachtje J et al., 2018). In order to compensate the imbalance and supply the alternative resources for normal living as well as further defence responses, plants need to process primary metabolism remodelling (Schwachtje J et al., 2018; Rojas CM et al., 2014). Single elicitation of 100 mg/L chitosan significantly increased the aloë-emodin production. This implies that 100 mg/L chitosan was able to trigger PTI and lead to the depletion of cellular resources. Thus, the root cultures primed with 100 mg/L chitosan probably need some appropriate interval to process primary metabolism remodelling for the subsequent responses to 100 mg/L yeast extract. According to the result, it might be assumed that 24 hours was not enough time for the root cultures to fully process primary metabolism remodelling. Thus, priming with 100 mg/L chitosan for 24 hours prior to 100 mg/L yeast extract elicitation (PC100Y100) did not promote further improvement over the single treatment of 100 mg/L chitosan (C100). Furthermore, PAMPs have been known to promote negative effects on plant health. Chitosan, especially at high concentrations, was reported to negatively affect root growth and development (Overvoorde P et al., 2010), as well as interfere with expression patterns of phytohormones and cause overstress in root tissues (Lopez-Moya et al., 2017). Thus, exposing the relatively high concentration of chitosan following by yeast extract might cause root culture death, and possibly be involved with lower aloë-emodin production.

4. Conclusion

In conclusion, sequential double elicitation and simultaneous double elicitation were successful in elevating aloë-emodin production in *C. tora* root cultures. Yeast extract was the most appropriate elicitor, whereas chitosan was found to be a priming inducer when used in combination with yeast extract.

Abbreviations

C

chitosan

D

simultaneous double elicitation

P

sequential double elicitation

Y

yeast extract

PAL

phenylalanine ammonia lyase

PTI

pattern-triggered immunity

PAMP

pathogen-associated molecular pattern (PAMP)

PRR

pattern-recognizing receptor (PRR)

Declarations

6.1 Ethics approval and consent to participate

Not applicable

6.2 Consent for publication

Not applicable

6.3 Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

6.4 Competing interests

The authors declare that they have no competing interests

6.5 Funding

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6.6 Authors' contributions

PaT performed all plant tissue cultures and HPLC analysis. AS and PiT responded the data analysis and interpretation. Authors read and approved the final manuscript.

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Figures

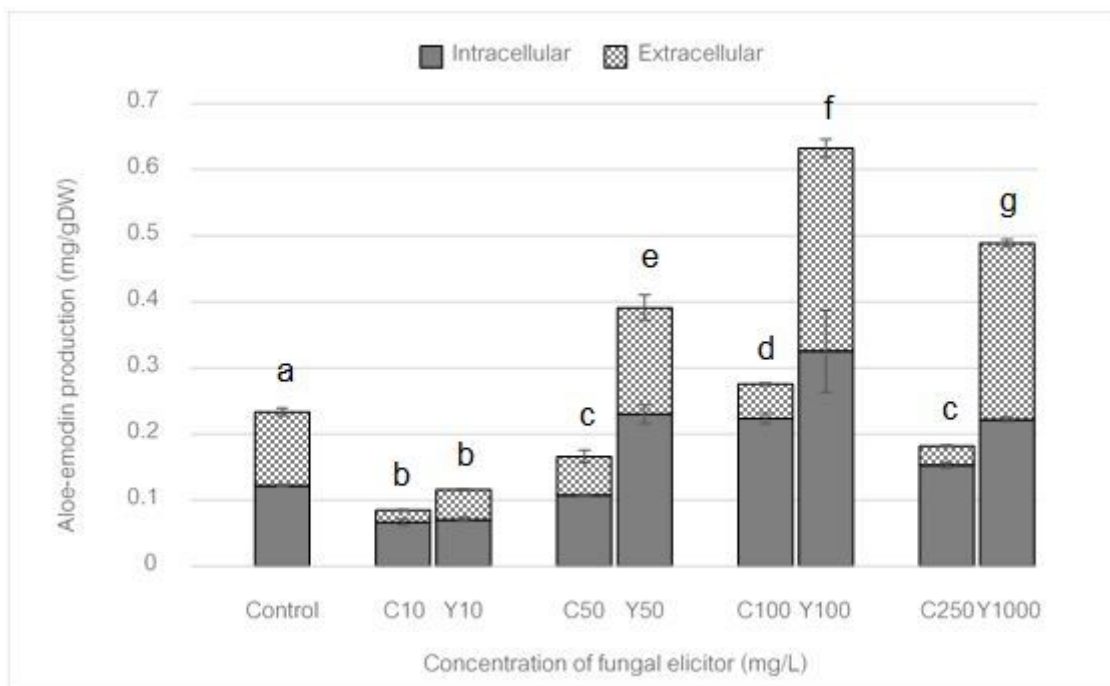


Figure 1

Effect of chitosan and yeast extract concentration on aloe-emodin production in *C. tora* root cultures. Data are displayed in term of mean \pm SD (n = 6). The different letters on the top of the bars represent significant differences (p < 0.05).

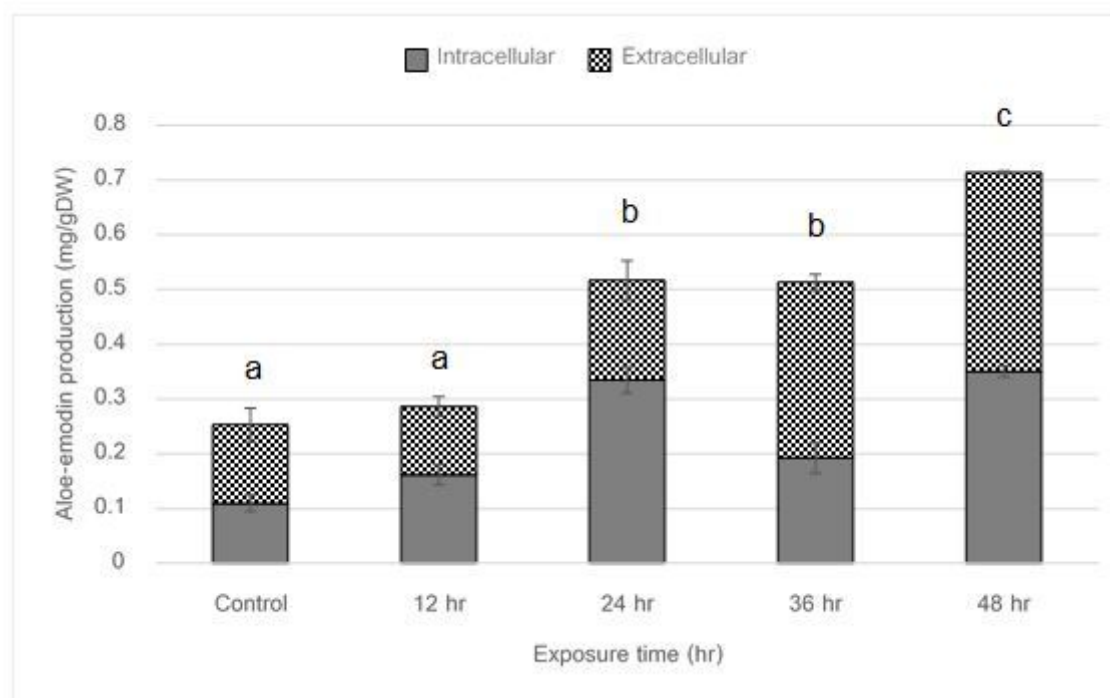


Figure 2

Effect of exposure time on aloe-emodin production in response to 100 mg/L yeast extract elicitation. Data are the mean \pm SD (n = 6). The different lowercase letters on the top of the bars represent significant differences (p < 0.01).

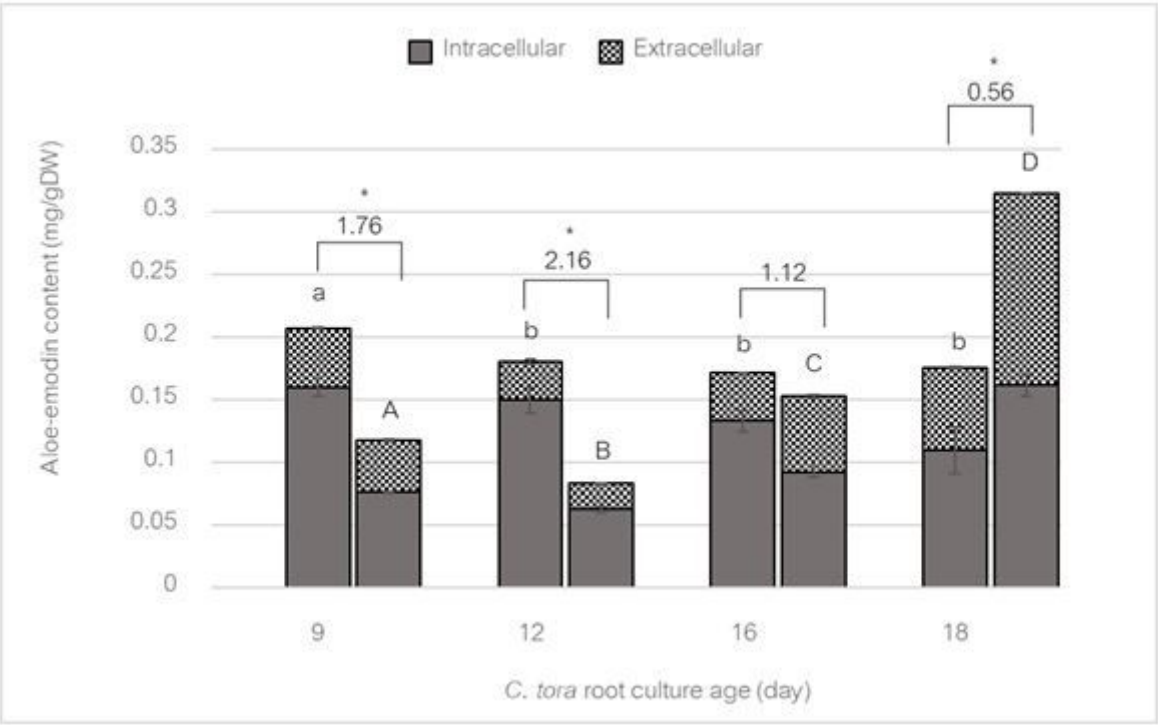


Figure 3

Effect of exposure time on aloe-emodin production in response to 100 mg/L yeast extract elicitation. Data are the mean \pm SD (n = 6). The different lowercase letters on the top of the bars represent significant differences (p < 0.01).

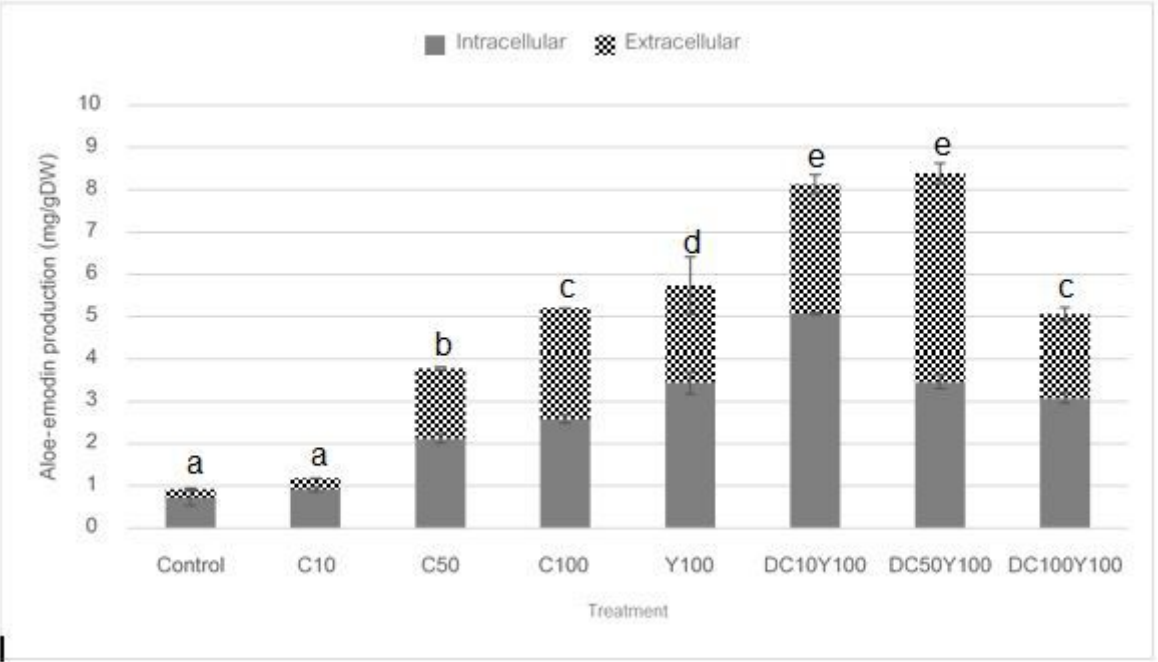


Figure 4

Aloe-emodin production in response to simultaneous double elicitation using chitosan and yeast extract. Data are the mean \pm SD (n = 6). The different lowercase letters on the top of the bars represent significant differences (p < 0.05)

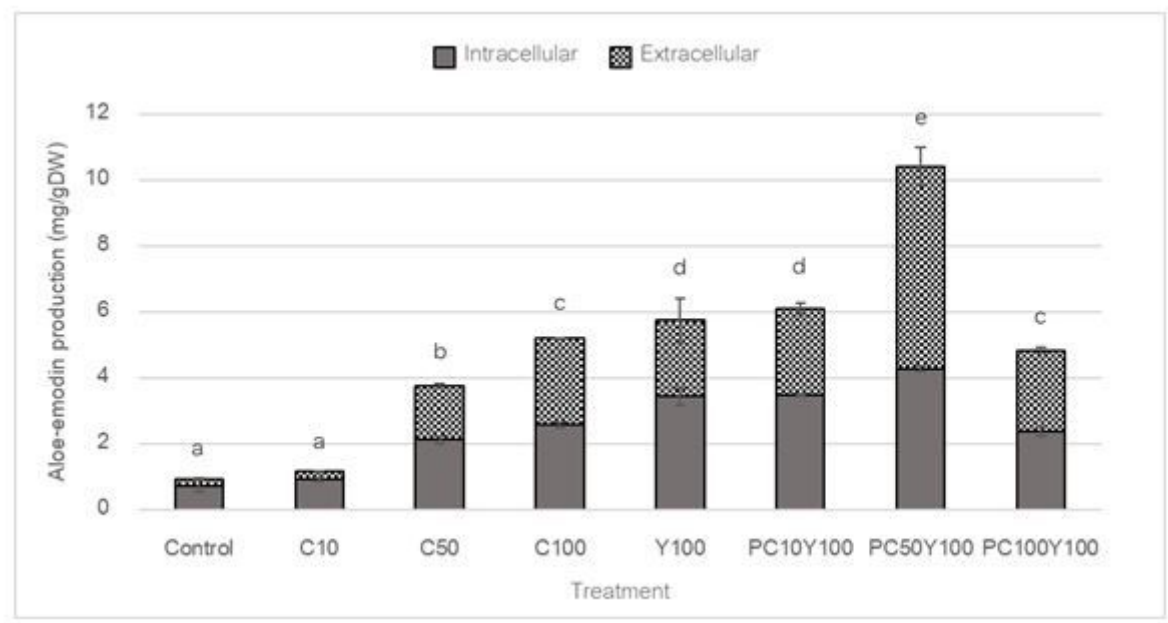


Figure 5

Aloe-emodin production in response to sequential double elicitation using chitosan and yeast extract. Data are the mean \pm SD (n = 4-6). The different lowercase letters on the top of the bars represent significant differences (p < 0.05).

Supplementary Files

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