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PRODUCTION OF 9-METHOXYCANTHIN-6-ONE IN ELICITED *EURYCOMA LONGIFOLIA* HAIRY ROOT

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The hairy root of *Eurycoma longifolia* has been established by infection with *Agrobacterium rhizogenes* strain A4, to meet the herbal industry demand for the 9-methoxycanthin-6-one compound, an anti-cancer agent. In this study, 9-methoxycanthin-6-one was found in control and elicited cultures of *E. longifolia* hairy root. Two elicitors, methyl jasmonate and salicylic acid, were applied at different concentrations to the cultures and their effects on production of 9-methoxycanthin-6-one and growth of hairy root were studied. Methyl jasmonate at concentration of 0.1 mM elicited the highest production of 9-methoxycanthin-6-one after exposure for 168 hours in both 4- and 10-week-old hairy root cultures when compared with the unelicited control and salicylic acid. While 0.1 mM salicylic acid was sufficient to induce a high production of the targeted compound in 4-week-old cultures, the 10-week-old cultures required 2.0 mM salicylic acid to produce high amounts. Elicitation with methyl jasmonate and salicylic acid at higher concentrations caused degradation of the hairy root biomass.

Keywords: Anti-cancer agent, alkaloid, exponential phase, methyl jasmonate, salicylic acid, tissue culture

INTRODUCTION

Eurycoma longifolia, also known as *tongkat ali* in Malaysia, *pasak bumi* in Indonesia and *cay ba binh* in Vietnam, is an angiosperm from the family Simaroubaceae. This plant species is found in the lowlands up to 1200 m above sea level, and favours sandy soil (Patahayah et al. 2011). Mature plants can grow up to 12 m, with a characteristic straight stem and few branches. In 2012, the species was listed as one of five valuable medicinal plants in Malaysia under the Agricultural National Key Economic Area initiatives. *Eurycoma longifolia* has traditionally been used to treat ulcers and malaria, but is now mainly been taken to boost male libido. The root extract, which exhibits cytotoxic effects on the ovarian cancer cell line CaOV-3, has potential benefits for women's health (Nur-Hanan et al. 2005).

Accompanying the rise in popularity of this species as a medicinal plant are concerns that existing wild stocks in local forests are being overharvested. Uncontrolled harvesting of the wild plant by local traditional and modern practitioners, and pest and disease outbreaks in cultivated stocks, are among major issues that threaten the ability of wild and cultivated *E. longifolia* to sustainably meet market demand for the raw material (Patahayah et al. 2011, Nor Hasnida et al. 2012). Medicinal value of a herb is expressed by the activity of its secondary metabolites; thus, mass production of the secondary metabolites in a controlled environment could help meet the demand for these plant phytochemicals. For *E. longifolia*, secondary metabolites produced through tissue culture—specifically hairy root culture—could supplement secondary metabolites obtained from whole root extracts, and help meet the industrial demand for these plant pharmaceuticals.

Among the secondary metabolites present in *E. longifolia* extracts, alkaloids and quassinoids are dominant. The alkaloid compounds 9-methoxycanthin-6-one, canthin-6-one and 9-hydroxycanthin-6-one have been studied both in-vivo and in-vitro for biological activity and production (Nor Hasnida 2008, Bhat & Karim 2010, Maziah et al. 2011). Biological studies

on the *E. longifolia* root extracts have shown its potential as anti-malarial (Chan et al 1986), anti-proliferative (Syamsul et al. 2004) and anti-cancer (Thu et al. 2017) agents. The 9-methoxycanthine-6-one compound exhibited cytotoxic activity against several cancer cell lines such as breast, lung, ovary and oral cells (Kuo et al. 2003, Nur-Hanan et al. 2005). The 9-methoxycanthin-6-one compound was discovered in large quantities in the taproot of intact plants of *E. longifolia* (Maziah et al. 2011), and was also present in roots of tissue-cultured plantlet (Nor Hasnida et al. 2012) as well as in callus (Maziah et al. 2011) and hairy root cultures (Nor Hasnida 2008). In the present study we focused on the production of 9-methoxycanthin-6-one in hairy root culture of *E. longifolia*.

Elicitation enhances production of secondary metabolite in plants and is used to study in-vivo and in-vitro production pathways (Ramirez-Estrada et al. 2016). During elicitation, plant cells receive stress signals that activate various responses, including defence mechanism activation, which leads to the production of secondary metabolites (Naik & Al-Khayri 2016). Previous studies have obtained optimum elicitation results by manipulating the combination of type of elicitor used, concentration, time of elicitation and exposure duration (Naik & Al-Khayri 2016). Methyl jasmonate and salicylic acid are common elicitors that have been studied for their effects in other plant species. These elicitors act as signalling molecules in secondary metabolite production, plant defence systems and gene expression (Ramirez-Estrada et al. 2016). The present study evaluates the effects of methyl jasmonate and salicylic acid on 9-methoxycanthin-6-one production in *E. longifolia* hairy root culture.

MATERIALS AND METHODS

Hairy root culture of *Eurycoma longifolia*

Eurycoma longifolia hairy root maintained at the Tissue Culture Laboratory of the Forest Research Institute Malaysia were used in this study. This hairy root resulted from the transformation of *E. longifolia* roots with *Agrobacterium rhizogenes* strain A4 (Nor Hasnida 2008). The culture medium used was Murashige and Skoog (MS) basal medium with pH adjusted to 4.9 and autoclaved at 121 °C for 15 min. Each flask was inoculated with 0.2 g hairy root tips. The cultures were incubated in the dark on an orbital shaker (110 rpm) at 22 ± 2 °C. The hairy root cultures were harvested every two weeks until week 12, and oven dried at 37 °C to constant weight. Three replicates of both fresh and dry weights were measured for each harvest. Growth curve of the hairy root culture was plotted from the dry-weight data.

Elicitation

Elicitation at two culture ages was conducted based on the hairy root growth curve. The early and late exponential phases were determined at 4 and 10 weeks respectively. Hairy root cultures used for this experiment were prepared using an approximate inoculum size of 0.2 g hairy root in 50 mL of MS basal medium under the same culture conditions described above. Methyl jasmonate and salicylic acid were separately dissolved in ethanol and filter-sterilised through a PTFE membrane filter with a 0.25 µm pore size. Elicitor concentrations tested were 0.1, 1.0 and 2.0 mg L^{-1} for both methyl jasmonate and salicylic acid. Hairy root culture without the addition of elicitor was used as control. Each concentration was tested for 24, 96 and 168 hours on 4- and 10-week-old hairy root cultures. The dry weights of all samples were recorded.

Extraction and HPLC quantification of 9-methoxycanthin-6-one

Dried hairy root samples were pulverised into fine powder. Methanol extraction was conducted at a ratio of 50 mg root powder:1 mL solvent. Crude compound was extracted in a water bath sonicator and filtered through membrane filter with 0.45 µm pore size, prior to HPLC analysis. For the detection of 9-methoxycanthin-6-one in the culture medium, 1 mL of culture medium was filtered through membrane filter with 0.45 µM pore size and added into the HPLC vial. The HPLC mobile phase was set at 0.1% (v/v) formic acid mixed with distilled water and 0.1% (v/v) formic acid mixed with acetonitrile, with a flow rate of 1.0 mL min^{-1}, injection volume of 10 µL and a detection wavelength of 366 nm. As for the stationary phase, a reverse phase C18 column (4.6 mm × 250 mm, 5 µm silica particle size, pore size 100 Å) was used (Nor Hasnida et al. 2012). The content of 9-methoxycanthin-6-one in the

samples was determined based on the standard curve developed using 9-methoxycanthin-6-one pure compound. From the HPLC profile, 9-methoxycanthin-6-one was identified as the peak located at retention time 27 ± 0.9 min for week 4 and 25 ± 0.9 min for week 10. Each was referred to their respective control and standard as well as UV spectrum for the identification of 9-methoxycanthin-6-one. The evaluation of 9-methoxycanthin-6-one content from dried root and culture media of *E. longifolia* were performed with three replicates each. Statistical analysis of data using mean and standard error was calculated.

RESULTS AND DISCUSSION

Eurycoma longifolia hairy root growth curve

Hairy root growth exhibited a lag phase from 0 to 2 weeks, then entered an exponential phase in week 4 with a maximum dry weight of 360 mg obtained in week 10 followed by a decrease in growth (Figure 1). Hairy root growth in our study extended over longer duration than the 3 weeks maximum recorded by Lim et al. (2011) for cell suspension cultures of *E. longifolia* callus in MS medium (pH 5.7). In their study, cell fresh weight increased daily until week 2 and decreased thereafter, with maximum fresh weight obtained after two weeks. Cell suspension and hairy root cultures of *E. longifolia* evidently require different cultivation periods to obtain maximum growth. Hairy roots have potential to grow in extended duration.

Effect of elicitors

Growth of elicited Eurycoma longifolia hairy root

Methyl jasmonate and salicylic acid had similar effects on hairy root growth in 4- and 10-week-old cultures, with dry weight increment negatively correlated to elicitor concentration (Figures 2 and 3). At 4 weeks, elicitation using both methyl jasmonate and salicylic acid significantly reduced hairy root dry weight, while the reduction of growth following elicitation at 10 weeks was not significant. Methyl jasmonate (0.1 and 1.0 mM) and salicylic acid (0.1 mM) elicitation at 10 weeks showed no growth inhibition compared with control, 24 hours after exposure, but the lowest dry weight was obtained from methyl jasmonate 2.0 mM at 168 h and salicylic acid, 2.0 mM at 24 hours. High methyl jasmonate concentrations, i.e. 2.0 mM showed decreased in biomass as early as at 24 hours elicitation. This trend was similar with salicylic acid at concentrations 1.0 and 2.0 mM where the hairy root biomass decreased at 24 hours. Hairy root elicitation with methyl jasmonate (0.1 and 1.0 mM) and salicylic acid (0.1 and 1.0 mM) showed that the biomass was decreasing over increasing time of exposure indicating inhibition was correlated with exposure time and elicitor concentration. Previous studies that applied elicitors in concentrations from 0.01 mM to 10 mM have reported different effects. For example, the response of 2.0 mM methyl jasmonate to *Scopolia parviflora* adventitious root culture was similar with our finding where

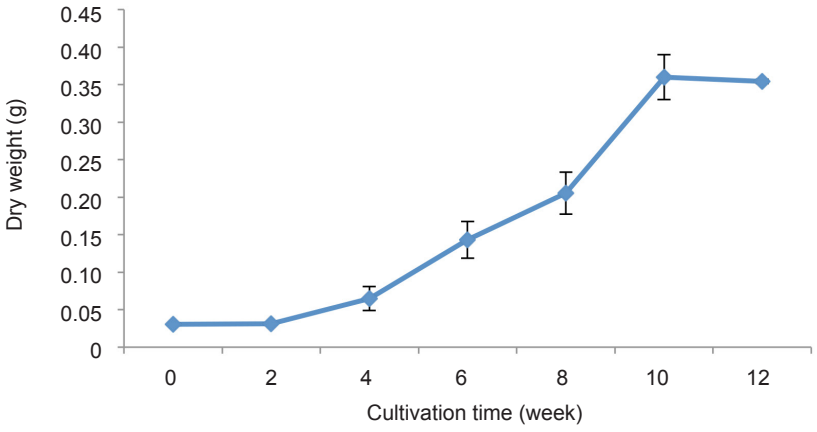

Figure 1 Growth curve of *Eurycoma longifolia* hairy root culture in Murashige and Skoog basal media with pH 4.9

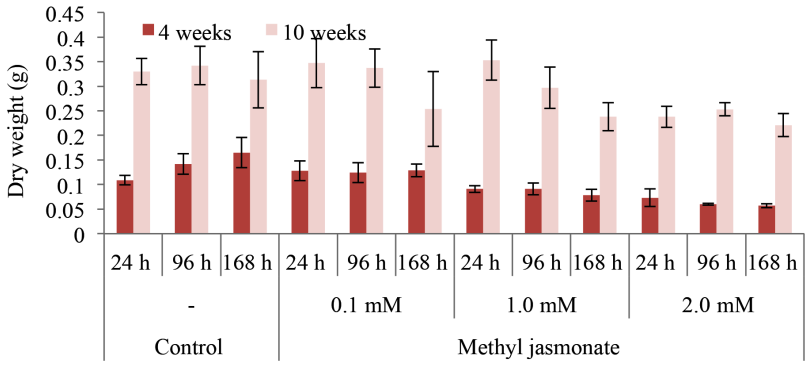

Figure 2 Effect of methyl jasmonate on hairy root growth at 4 and 10 weeks over time (hours); error bars represent ± standard errors; n = 3

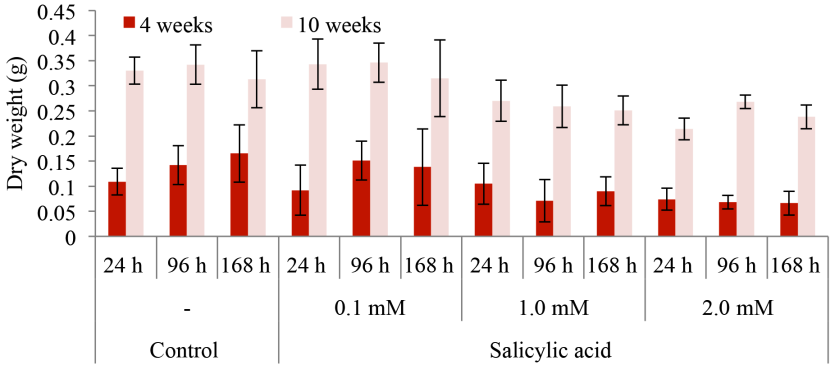

Figure 3 Effect of salicylic acid on hairy root growth at 4 and 10 weeks over time (hours); error bars represent ± standard errors; n = 3

the repression of root growth occurred after 24 hours and severely damaged after 72 hours. Salicylic acid however did not show negative effects on the *S. parviflora* root when treated with 2.0 mM at 72 hours. (Kang et al. 2004). In the present study, elicitation using these two signalling compounds also affected hairy root morphology in both 4- and 10-week-old cultures. Hairy root turned to brown colour after elicitation, while the root colour of the controls remained light yellow (Figure 4).

Severe inhibition of hairy root growth when elicited at week 4 as the culture entered an exponential growth phase was likely due to redirection of cell activity and resources from active division to defence against the elicitor and production of secondary metabolites (Natanael et al. 2014). Similarly, in this study, although biomass decreased, 9-methoxycantin-6-one increased in elicited hairy root compared with untreated root in 4-week-old cultures, supporting the idea that elicitation during early exponential phase affects cell growth. For example, the hairy root biomass treated with 0.1 mM methyl jasmonate after 168 hours decreased to 0.057 g compared with 0.165 g in control for the same time. However, the production of 9-methoxycanthin-6-one increased up to three fold compared with control. Methyl jasmonate and salicylic acid are signalling compounds involved in plant defence response (Smetanska 2008, Zhou & Wu 2006). During exponential growth, the primary metabolites in plants are used for biomass production instead of to produce secondary metabolite (Ramirez-Estrada et al 2006). The application of exogenous methyl jasmonate probably induced specific signalling in cells, depressing the primary metabolism and activating the secondary metabolism (Kang et al. 2004). However the specific mechanism of methyl jasmonate elicitation in *E. longifolia* hairy root is still unknown.

Application of the elicitor at week 10 when the culture was reaching the end of its exponential

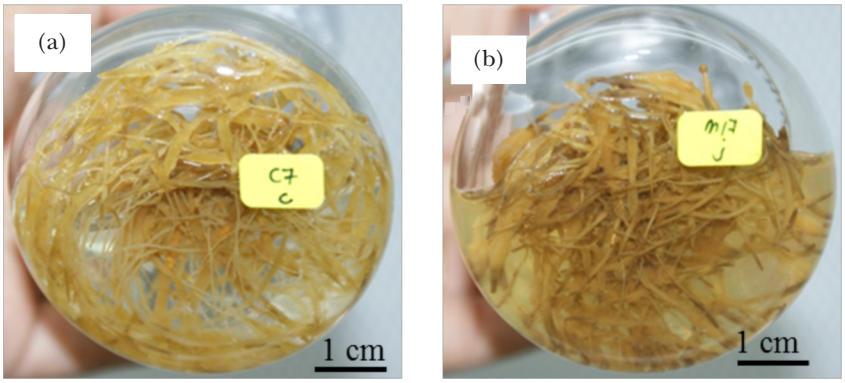

Figure 4 Morphology of *Eurycoma longifolia* hairy roots in a 10-week-old culture, (a) with no elicitation (control) and (b) elicited with 0.1 mM methyl jasmonate

growth phase did not have significant negative effect on growth. The factors causing reduction in cell growth of elicited hairy root in the present study could not be determined since we did not perform cell viability tests. Viability tests performed on a methyl jasmonate-elicited *Taxus cuspidata* cell suspension culture and supported by deep sequencing and gene expression analysis indicated that methyl jasmonate did not cause necrosis, cell membrane rupture or cell apoptosis but affected cell growth by altering cellular metabolism and affecting the cell cycle (Patil et al. 2014).

Contents of 9-methoxycanthin-6-one in dried hairy root

At both 4 and 10 weeks, 9-methoxycanthin-6-one production was active in the cultures even without elicitation as shown in the HPLC profile. Both methyl jasmonate and salicylic acid increased the production of 9-methoxycanthin-6-one in 4-week-old hairy root cultures. Elicitor concentration and duration of exposure played a major role in the production of this compound, which was highest at 0.1 mM and after 168 hours for both methyl jasmonate and salicylic acid, and decreased at higher elicitor concentrations (Figures 5 and 6). Similarly, in 10-week-old hairy root cultures, production of 9-methoxycanthin-6-one was the highest with elicitation using 0.1 mM methyl jasmonate over a longer exposure time and decreased over time at higher elicitor concentrations. At the late exponential phase, the culture entered a stationary phase with reduced cell growth activity and any unusual event could stimulate the production of secondary metabolites. There was an unidentified peak at retention time 14.559 min on the HPLC profile of the 10-week-old culture extract that was not present at 4 weeks, indicating that an unknown compound was produced in the older culture (Figure 7).

Different species have different mechanisms of action in responding to elicitors, and often more than one pathway for producing the same compound. Elicitation on different cell types from the same species may also yield different results in production of the targeted compound. However, 0.1 mM methyl jasmonate, which produced promising results in the present study, negatively affected root cell morphology of *E. longifolia* tissue culture plantlets and reduced the production of 9-methoxycanthin-6-one (Chee et al. 2015). Besides elicitor type, concentration and exposure duration when elicitation is initiated are also important factors in determining the production of compounds. The exponential phase has been found to be the best time to initiate elicitation, with early, middle and late exponential phase elicitations giving different responses (Vasconsuelo & Boland 2007).

In the present study, both methyl jasmonate and salicylic acid induced the production of 9-methoxycanthin-6-one in the 4- and 10-week-old cultures but the total amounts produced were different. Elicitation during the late exponential stage was more economical since production was greatest especially when elicited with 0.1 mM methyl jasmonate. Elicitation in the middle or late exponential phases has been suggested due to the lower inhibition effect of the elicitor on cell growth (Khosroushahi et al. 2006). At late exponential stage, the cellular system and related cellular activity are past the growth phase, hence susceptible to producing secondary metabolites

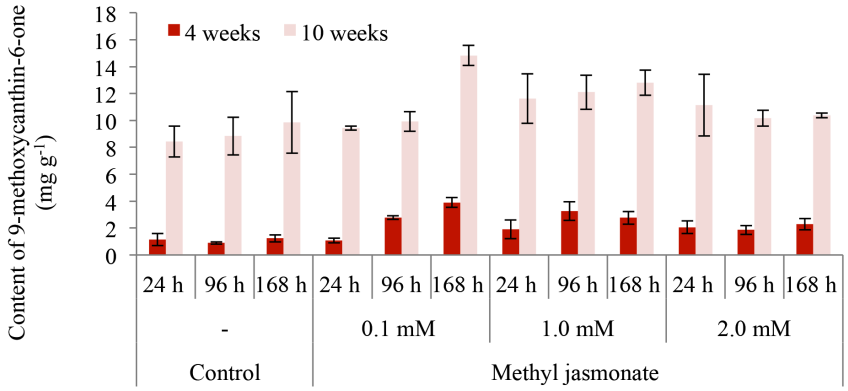

Figure 5 Effect of methyl jasmonate on 9-methoxycanthin-6-one production by 4- and 10-week-old hairy root cultures over time (hours); error bars represent ± standard errors (n = 3)

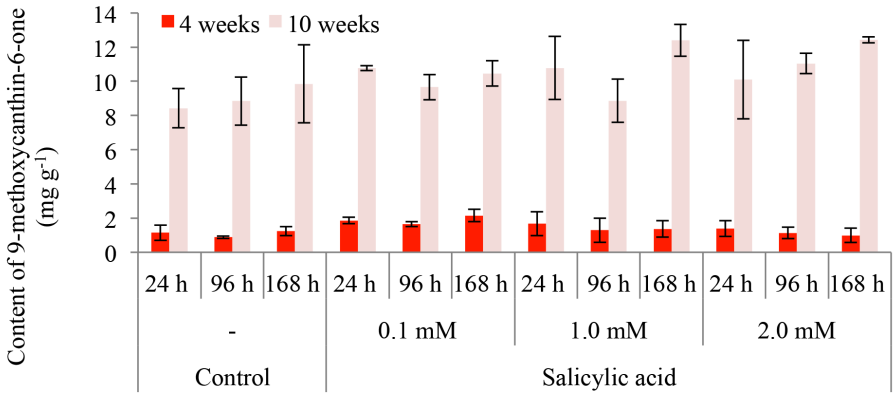

Figure 6 Effect of salicylic acid on 9-methoxycanthin-6-one production by 4- and 10-week-old hairy root cultures over time (hours); error bars represent ± standard errors; n = 3

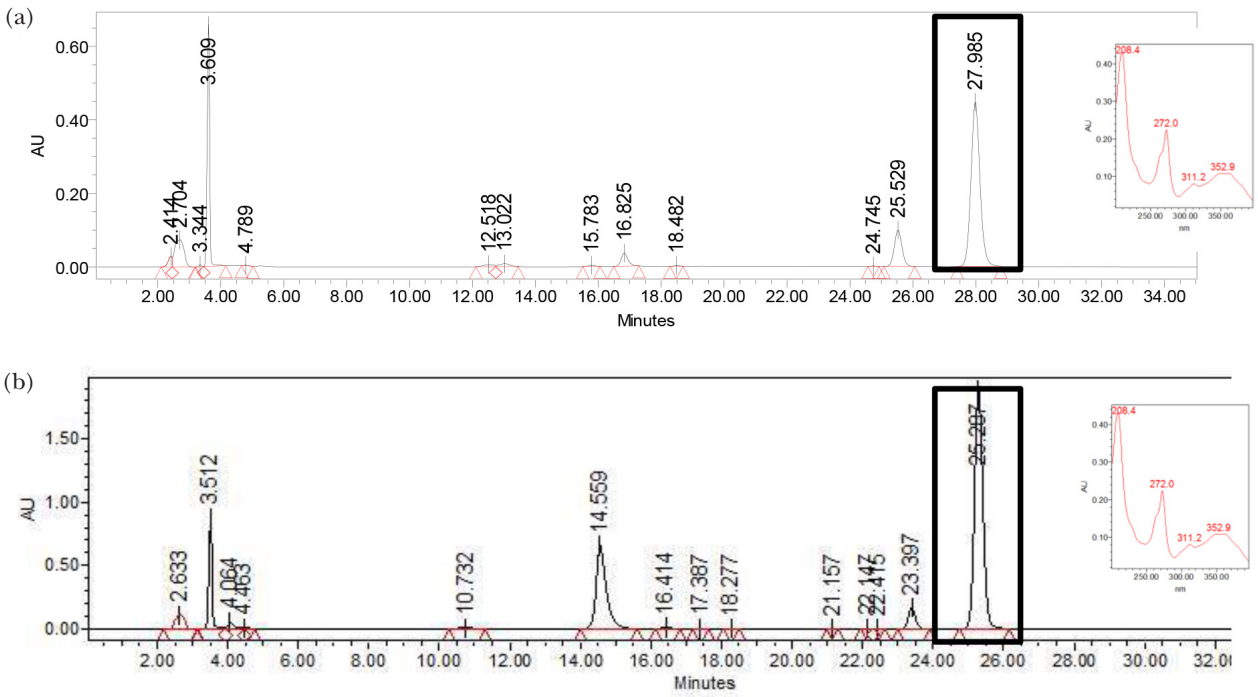

Figure 7 HPLC profile of elicited *E. longifolia* hairy root cultures elicited with 0.1 mM methyl jasmonate at (a) 4 and (b) 10 weeks; peaks for 9-methoxycanthin-6-one are highlighted

particularly when triggered by outside factors (James et al. 2008). Our findings support the previous results that the suitable age of culture for elicitation is during the late exponential growth phase (Savitha et al. 2006, Kuźma et al. 2009).

Plant susceptibility to certain elicitors and the cascade of plant defence reactions induced by elicitors vary among plant species. However, as the same elicitor can induce a defence response in different plant species, those plants may have common receptors for that elicitor (Ferrari 2010). A defence reaction is initiated with the interaction between the elicitor and plant membrane cell receptor. From there, myriad defence reactions occur in the cytosol, followed by the induction of defence gene expression and secondary metabolite accumulation (Ferrari 2010, Zhang et al. 2012, Ramirez-Estrada et al. 2016). The time it takes for secondary metabolites to be produced after defence activation depends on the plant species as well as elicitor type and concentration used. In the present study, the cultures produced the highest amounts of 9-methoxycanthin-6-one when exposed to methyl jasmonate at a concentration of 0.1 mM at 168 hours after elicitation. In *Taxus cuspidata* hairy root culture, 0.1 mM methyl jasmonate successfully induced the production of paclitaxel from day 1 with maximum production achieved at 14 days after elicitation (Kim et al. 2009).

Secretion of 9-methoxycathin-6-one into culture medium

Traces of 9-methoxycanthin-6-one produced from the secretory activity of the hairy root cells were detected in the culture medium of both 4- and

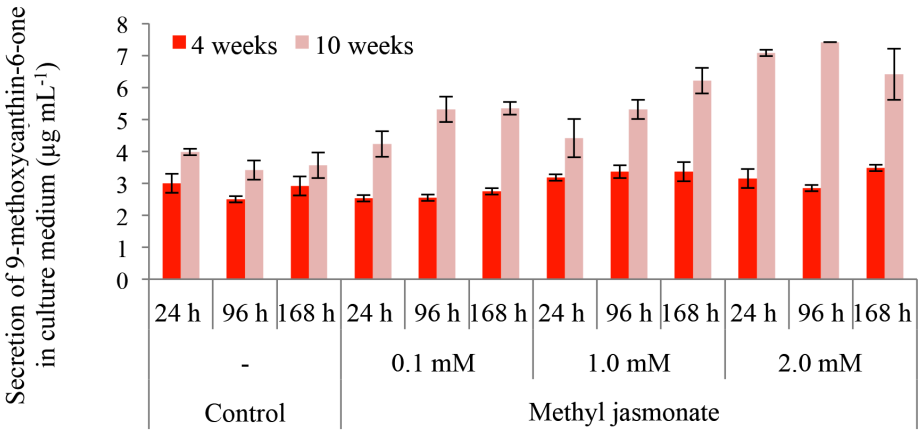

Figure 8 Effect of methyl jasmonate on secretion of 9-methoxycanthin-6-one by 4- and 10-week-old hairy roots into the culture medium over time (hours); error bars represent ± standard errors; n = 3

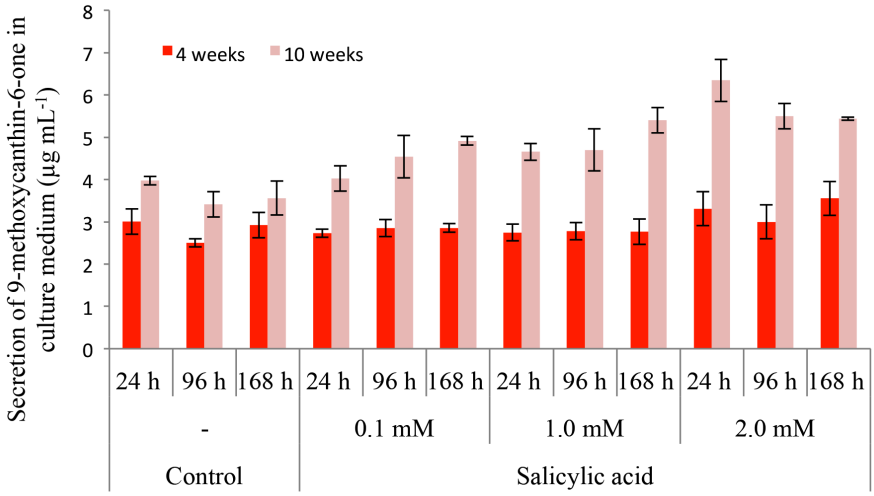

Figure 9 Effect of salicylic acid on secretion of 9-methoxycanthin-6-one by 4- and 10-week-old hairy roots into the culture medium over time (hours); error bars represent ± standard errors; n = 3

10-week-old controls (Figures 8 and 9). Contents of 9-methoxycanthin-6-one in the culture media of elicited 4- and 10-week-old cultures were higher than those in the controls, with the 10-week-old elicited culture medium containing 1.5 to 2 times more 9-methoxycanthin-6-one than the 4-week-old elicited culture medium. The concentration of the elicitor is an important factor influencing the amount of secondary metabolites released by cells into the culture medium, e.g. methyl jasmonate enhanced the release of secondary metabolites directly into the medium in hairy root cultures of *Vitis vinifera* (Hosseini et al. 2017) and *Rubia tinctorum* (Perassolo et al. 2017). These studies are important because of the ability to obtain targeted secondary metabolites from culture media without harvesting the hairy root adds value in their large-scale production.

CONCLUSIONS

The present study showed that methyl jasmonate and salicylic acid successfully induced the production of 9-methoxycanthin-6-one in *E. longifolia* hairy root. Its production could potentially be maximised through optimal manipulation of elicitor concentration, timing and duration of the elicitation. Our findings can be used in further experiments exploring the feasibility of producing 9-methoxycanthin-6-one in a bioreactor. Upscaling the production of secondary metabolite in a bioreactor would necessitate the testing of combinations of the parameters influencing the production process for optimal output.

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