

EFFECTS OF METHYL-JASMONATE ON 9-METHOXYCANTHIN-6-ONE CONTENT IN *EURYCOMA LONGIFOLIA* (TONGKAT ALI) ROOT CULTURE

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Abstract

Eurycoma longifolia is a flowering plant from the Simaroubaceae family and it has been identified as one of the most intriguing medicinal plants in Malaysia. In the present study, the production of 9-methoxycanthin-6-one, an alkaloid compound was determined with various methyl-jasmonate (MeJA) concentrations using root culture via liquid system. Quantification of 9-methoxycanthin-6-one was confirmed by using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Quantitative analysis using HPLC displayed highest concentration of 9-methoxycanthin-6-one content in the absence of MeJA treatment (control) followed by increasing concentrations of MeJA (1, 10 and 100 µM). Microanatomical analysis using Scanning Electron Microscope (SEM) has shown that root hair morphology of *E. longifolia* does not change significantly, whereas roots hair displayed rough surfaces with increases MeJA concentrations. Therefore, MeJA is not a suitable elicitor to increase 9-methoxycanthin-6-one compound in *E. longifolia*'s root culture.

Key words: *E. longifolia*, Root culture, 9-methoxycanthin-6-one, MeJA, SEM.

Introduction

There are many species that are reported as medicinal plants and *Eurycoma longifolia* (locally known as Tongkat Ali) is one of the most popular tropical folk medicine in Southeast Asian countries such as Malaysia, Indonesia and Vietnam. Almost all parts of *E. longifolia* have been used traditionally for medicinal purpose and taken as supplements. In Malaysia, it is well-known among various ethnic groups for treating disease and enhancing health and as it is sometimes referred as 'Malaysian ginseng' (Ng & Jagananth, 2000).

The gradual disappearance of this plant is caused by the indiscriminate collection of the taproot as the raw material for drug preparations (Sobri *et al.*, 2005). The roots of this plant are used as folk medicine for the treatment of aches, persistent fever, tertian malaria, sexual insufficiency, as health supplements (Perry & Metzger, 1980), dysentery and glandular swelling (Darise *et al.*, 1983) and antibacterial activity (Monica *et al.*, 2013). Alkaloids are important secondary plant metabolites obtained from higher plants and are useful in drug preparations. Compound 9-methoxycanthin-6-one is an important secondary metabolite that has shown significant cytotoxicity against human lung cancer (A-549) and human breast cancer (MCF-7) cell lines (Kuo *et al.*, 2003). It is believed that 9-methoxycanthin-6-one could possibly possess anti-proliferative effect in ovarian cancer cells (Nurhanan *et al.*, 2002) and also as a potential antitumor compound found in callus cultures (Rosli *et al.*, 2009).

In recent years, *In vitro* techniques have been increasingly used in the conservation of threatened plants and this trend is likely to continue as more species face the risk of extinction (Sarasan *et al.*, 2006; Hussain *et al.*, 2011; Abbassi *et al.*, 2011). Limited supply of plant materials for the recovery of bioactive compounds has stimulated the demand to develop alternative *In vitro* methods for the production of active compounds from cell

cultures (Monica *et al.*, 2011, 2012; Hussain *et al.*, 2013). The successful production of 9-methoxycanthin-6-one using *In vitro* cultures of *E. longifolia* has a potential in large-scale production (Rosli *et al.*, 2009). Being a recalcitrant plant, it has a low percentage of germination and it takes longer time to germinate due to the indiscriminate collection of taproot from the forest for the usage of raw material in the health-related and supplement preparing industries. The plant needs to be rapidly produced on a commercial scale to comply with the needs of the herbal and pharmaceutical industry (Sobri *et al.*, 2005; Hussain *et al.*, 2014).

The accumulation of 9-methoxycanthin-6-one compound in *E. longifolia* can be affected by several factors like compositions of media use and also physical factors of the plant (Rosli *et al.*, 2009). Previously, several factors that affecting the production of 9-methoxycanthin-6-one (Rosli *et al.*, 2009) and its distribution and quantification from *In vivo* plants and callus from the *In vitro* culture of *E. longifolia* (Maziah *et al.*, 2011) were reported. However, no reports on the production of 9-methoxycanthin-6-one from the root culture obtained via *In vitro* system. Therefore, the aim of this study was to determine the effects of MeJA on the production of 9-methoxycanthin-6-one supported with SEM analysis and HPLC analysis by using root cultures of *E. longifolia* before and after the treatment being introduced.

Materials and Methods

Plant material and sterilization of explants: Mature *E. longifolia* seeds were collected and washed with tap water for 5 minutes. Then, the seeds were surface sterilized using a few drops of Tween-20 followed by washing under running water for 30 min. After the initial washing, the seeds were added to 30% (v/v) Clorox® (Sodium hypochlorite, 5.25%) regular bleach for 15 min and washed 3 times in sterile distilled water.

Subsequently, the procedure was repeated with 15% and 5% Clorox® for 10min and 5 min according to previous paper (Rosli *et al.*, 2009).

Induction of seed germination: The seed coat and the outer layer were removed to obtain the embryo with the size of 10mm. The embryo was inoculated in MS (Murashige & Skoog, 1962) salts with 30g/L sucrose, 5 mg/L (w/v) kinetin and solidified with 2.75 g/L (w/v) Gelrite. The embryos were germinated at 26±2°C with 16 hours of photoperiod (Philips TLD, 36 W; 150µmol.m⁻².s⁻¹) for 2 months.

Elicitation with MeJA: *E. longifolia* plantlets were obtained from the semi-solid medium and cultured into liquid MS salts with 30g/L (w/v) and 5 mg/L (w/v) kinetin. The pH of the medium was adjusted to 5.70 prior to autoclaving at 121°C for 15 min. Total of 20 mL liquid MS medium were supplemented with different concentrations of MeJA (control, 1, 10 and 100 µM) in 150 mL conical flasks. Control culture contained 20 mL of liquid MS medium in the absence of MeJA. Three replicates were used for each MeJA concentration. Plantlets with the MeJA treatments were allowed to grow at 26±2°C with 16 h of photoperiod (Philips TLD, 36 W; 150µmol.m⁻².s⁻¹) at the speed of 60 rpm. After four weeks, roots from the plantlets were harvested and the fresh and dry weights were determined.

Preparation of 9-methoxycanthin-6-one: Standard curve for 9-methoxycanthin-6-one compound was obtained by preparing a dilution of 9-methoxycanthin-6-one stock solution from lowest to highest ppm and filtered prior to HPLC analysis.

Extraction of 9-methoxycanthin-6-one from roots of *Eurycoma longifolia*: The roots of *E. longifolia* were harvested and dried in an oven at 45°C for 48 hours before being pulverised using mortar and pestle. The extraction procedure used for extracting 9-methoxycanthin-6-one was reported by Maziah *et al.* (2011) with minor modifications. Total of 0.02 g dried root samples was ground using mortar and pestle and mixed with 20 mL of solvent (CH₃OH : CHCl₃ at 4:1) and incubated in ultra-sonic bath for 15 min. The homogenate was centrifuged at 12000 g for 10 min at 4°C. The supernatant was used for thin layer chromatography (TLC) analysis. Qualitative determinations of 9-methoxycanthin-6-one concentrations were carried out by comparison with the standard compound. A quantitative determination of the 9-methoxycanthin-6-one concentration was carried out using high performance liquid chromatography (HPLC) analysis.

Analysis of 9-methoxycanthin-6-one using thin layer chromatography (TLC): The protocol for analysis of 9-methoxycanthin-6-one in *E. longifolia* using TLC was modified from Maziah *et al.* (2011). The TLC plate was modified with the vertical side length of 5 cm and horizontal side length of 4 cm. The volume of the samples needed to be spotted on the TLC plate was

optimized to 1.2 µL. The samples were spotted on a TLC plate and were ran with 8:2 (CHCl₃: CH₃OH) solvent. TLC was performed on aluminum sheets coated with silica gel 60 F₂₅₄ (Merck catalogue no. 1.05554) and developed with 25 DC-Kieselgel 60 F₂₅₄. Compound 9-methoxycanthin-6-one was detected using fluorescence emission in UV chamber model CM-10 under irradiation of UV lamp (365 nm). Rf values of 9-methoxycanthin-6-one were calculated.

Analysis of 9-methoxycanthin-6-one using high performance liquid chromatography (HPLC): The protocol for analysis of 9-methoxycanthin-6-one using HPLC was used and modified based on Chan & Choo (2001). The crude extract of *E. longifolia* was analysed with an Agilent Technologies 1120 Series HPLC (USA), model G4290 comprising E2 Chrom Elite Compact, a manual injector with 20 µL volume injection, UV detector, a quaternary pump and vacuum degasser. Reversed-phase separations were carried out using 4.6x250 mm I.D. Nova Pak C18 60A steel cartridge column; fitted at 5 micron used at a flow rate of 0.9 mL/min at room temperature. The mobile phase consisted of a mixture of acetonitrile and distilled-deionised water at 30:70 ratios. The total running time was 30 min and the detection of 9-methoxycanthin-6-one compounds was monitored at 350 nm. The reference solution of 9-methoxycanthin-6-one was prepared by dissolving in methanol (HPLC grade). The concentration of identified compounds was determined by comparing the Retention factor and peak area of external standard method.

Microanatomical analysis on roots of *Eurycoma longifolia* using SEM: The protocol on micro-anatomical analysis on roots of *E. longifolia* was done by freeze-drying method. The fresh explants were blot to remove any gel attached and moist explants were ensured by placing them on the wet filter paper. Placed them in freeze dryer after an hour of osmium fixing in fume hood and finally went for viewing under SEM.

Statistical analysis: Statistical analyses were conducted using SPSS for Windows Version 10.0. The data were compared by one-way analysis of variance (one way ANOVA) followed by Tukey's test to find the differences between treatment means at 95% (p<0.05) significance level of the sample.

Results and Discussion

***In vitro* root cultures of *Eurycoma longifolia*:** In the first part of the study, *E. longifolia* seeds were germinated in basal semi-solid MS medium supplemented with kinetin (5 mg/L) until the seeds germinated into plantlets in two months time. Once the roots reach 5 cm in length, the plantlets were then cultured in basal liquid MS medium with three different concentrations of MeJA treatments which are 1, 10 and 100 µM with control for 30 days. After 30 days, the roots of *E. longifolia* which achieve nearly 15cm used in the following extraction step.

Water content of MeJA treated *In vitro* root cultures of *Eurycoma longifolia*: Samples were harvested and dried in the oven at 45°C for 48 hours before being ground using a mortar and pestle. Prior to the analysis of 9-methoxycanthin-6-one for TLC and HPLC studies, fresh weight and dry weight of the roots were measured to determine the water content of the roots for different concentrations of MeJA. Figure 1 shows the differences in fresh weight and dry weight of the roots on different concentrations of MeJA treatment. For control, the fresh weight of the roots was 1.00±0.22 g and the dry weight was 0.12±0.02 g. However for 100 µM treatment, the fresh and dry weights of the roots were recorded to be 0.44±0.29 g and 0.04±0.02 g.

Figure 2 shows the water loss in the roots of *E. longifolia* after drying. MeJA treatment at 100 µM of the root sample produced the highest percentage of water loss at 91.06% compared to control with 88.19%. The water

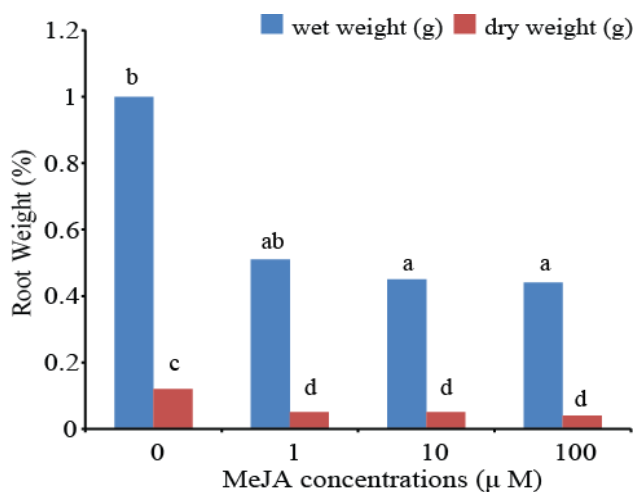


Fig. 1. Wet weight (g) and dry weight (g) of *E. longifolia* roots at different concentrations (in µM) of MeJA treatment. Means with different letter are significantly different ($p < 0.05$).

Qualitative analysis on TLC: In this study, thin layer chromatography was used to separate and identify 9-methoxycanthin-6-one in different concentrations of MeJA treatment in the roots of *E. longifolia* using solvent mixture of methanol and chloroform. Figure 3 shows TLC separation of standard 9-methoxycanthin-6-one and crude extracts from four treatments of *E. longifolia* roots. Standard 9-methoxycanthin-6-one under ultra violet (UV) chamber at 365 nm was shown as light blue band as well as the four treatments of *E. longifolia* and the Rf value of the alkaloid was 0.93. It was found that 9-methoxycanthin-6-one was detected in all different concentrations of MeJA treatment in the root samples. The quantity spotted on the TLC plate was 1.2 µL each, after conducting optimization of the volume of extract spotted on TLC plate.

Quantification of 9-methoxycanthin-6-one from roots by HPLC: HPLC analyses suggest that the retention time of 9-methoxycanthin-6-one as standard is 31.36 minutes. 9-methoxycanthin-6-one was detected in each of the treated *E. longifolia* in the retention time between 31 to 33 mins. A standard curve [$y = 472397x$, $R^2 = 0.9975$] of 9-methoxycanthin-6-one was used to quantify the levels of later compound in the control and treated root cultures as

loss seen in root cultures of *E. longifolia* subjected to MeJA treatment has similarity with that of pLAI α -deficient plants which displayed higher levels of jasmonic acid and MeJA tend to lose water more quickly than wild type plants (Yang *et al.*, 2012). According to Creelman & Mullet (1995), MeJA induces peptides in plants that shared similar homology with late embryogenesis proteins that will induce water stress or ABA (Abscisic acid). Soybean leaves that have been stressed by MeJA causes water deficit by allowing them to lose 15% of their fresh weight. MeJA seems to alter the metabolism of strawberry plants rendering the tissue with the ability to withstand water stress (Wang, 1999). However, it was recently reported that MeJA treatment did not affect the rate of water loss, respiration rate and softening rate of mango *Tommy Atkins* under subsequent storage at low temperature (7°C) and 5 days of shelf life (20°C) (Gonzalez *et al.*, 2000).

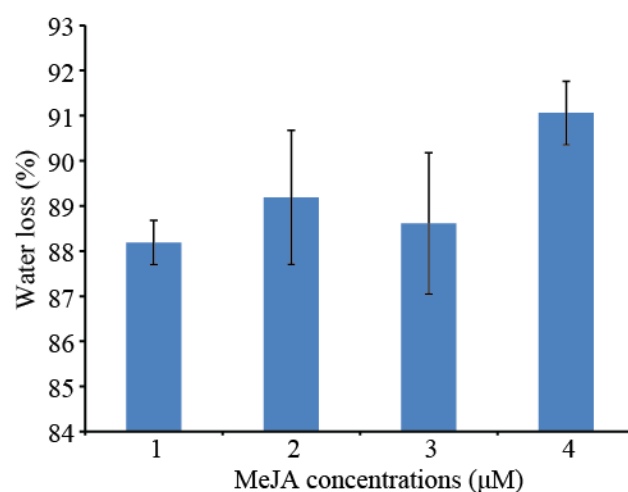


Fig. 2. Percentage of water loss (%) in *E. longifolia* at different treatments of MeJA (µM). 1: 0µM; 2: 1µM; 3: 10µM and 4: 100µM.

demonstrated in Figure 4A. Figure 4B and Table 1 show the concentrations of 9-methoxycanthin-6-one produced in different MeJA concentrations treated root. The highest concentration of 9-methoxycanthin-6-one in the root sample was present in control, at 57.17 µg/g followed by 48.69 µg/g in 1 µM of MeJA treatment, 27.78 µg/g in 10 µM of MeJA treatment and the lowest concentration of 9-methoxycanthin-6-one compound was found in the highest concentration of MeJA treatment which is 100 µM (Fig. 4C).

The results shown here are largely in contrast with the late exponential phase hairy root cultures of *Catharanthus roseus* elicited with pectinase and jasmonic acid to monitor the levels of several compounds in the indole alkaloid biosynthetic pathway (Rijhwani & Shanks, 1998). With the increasing level of jasmonic acid, the yields of ajmalicine, serpentine and horhammericine continuously increase in the hairy root cultures of *Catharanthus roseus* (Rijhwani & Shanks, 1998). Suspension cultures of *Catharanthus roseus* show increase in alkaloid production through MeJA elicitation (Jennifer, 2004) and similar results were reported on *Lithospermum erythrorhizon* (Mizukami *et al.*, 1993) and *Taxus cuspidata* (Mirjalili & Linden, 1996).

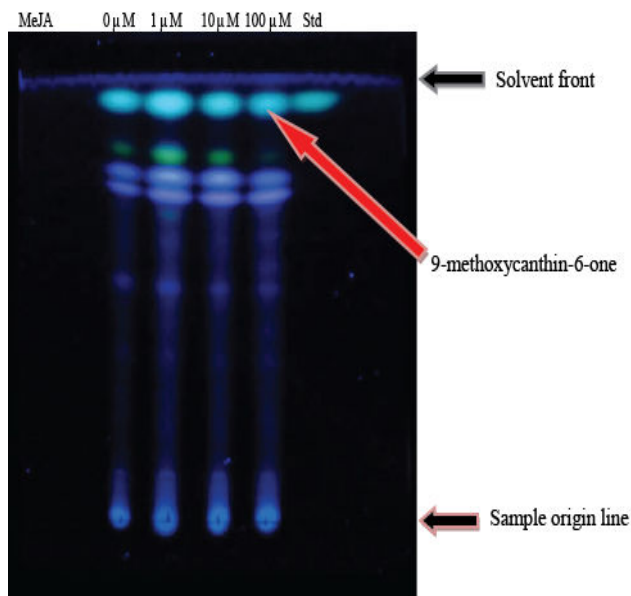


Fig. 3. Thin layer chromatogram showing the presence of 9-methoxycanthin-6-one obtained from MeJA treated root extracts as eluted on silica gel coated aluminum sheets. Pure 9-methoxycanthin-6-one was used as an authentic standard.

The effect of MeJA or any elicitor used either in the form of biotic or abiotic is dependent on a number of factors that may interact. These include the elicitor's specificity and concentrations, the duration of treatment as well as the growth stage of the culture (Holden *et al.*, 1988). Many researchers seem to be used MeJA at a concentrations of 100 μ M to increase secondary metabolite via *In vitro* cultures (Cusido *et al.*, 2002; Palazon *et al.*, 2006), with the plant cells or organs coming into direct contact with elicitors.

In this study, only the roots were directly exposed to MeJA, and 9-methoxycanthin-6-one production reduced with the increment of MeJA concentrations. This could be due to the alkaloids biosynthesis in *E. longifolia* being controlled during development and in response to stress and pathogens. According to Koda *et al.* (1996), growth inhibition by MeJA appears to be caused mainly by the disruption of cortical microtubules where a phenomenon ubiquitous in plants. Hence, in order to induce the production of 9-methoxycanthin-6-one in the roots of *E. longifolia* plantlets, precise roles and interactions of MeJA elicitor in signaling and inducing secondary metabolites should be fully understood to get a positive result. Hence, MeJA elicitor is not suitable to increase 9-methoxycanthin-6-one in the roots of *E. longifolia* plantlets.

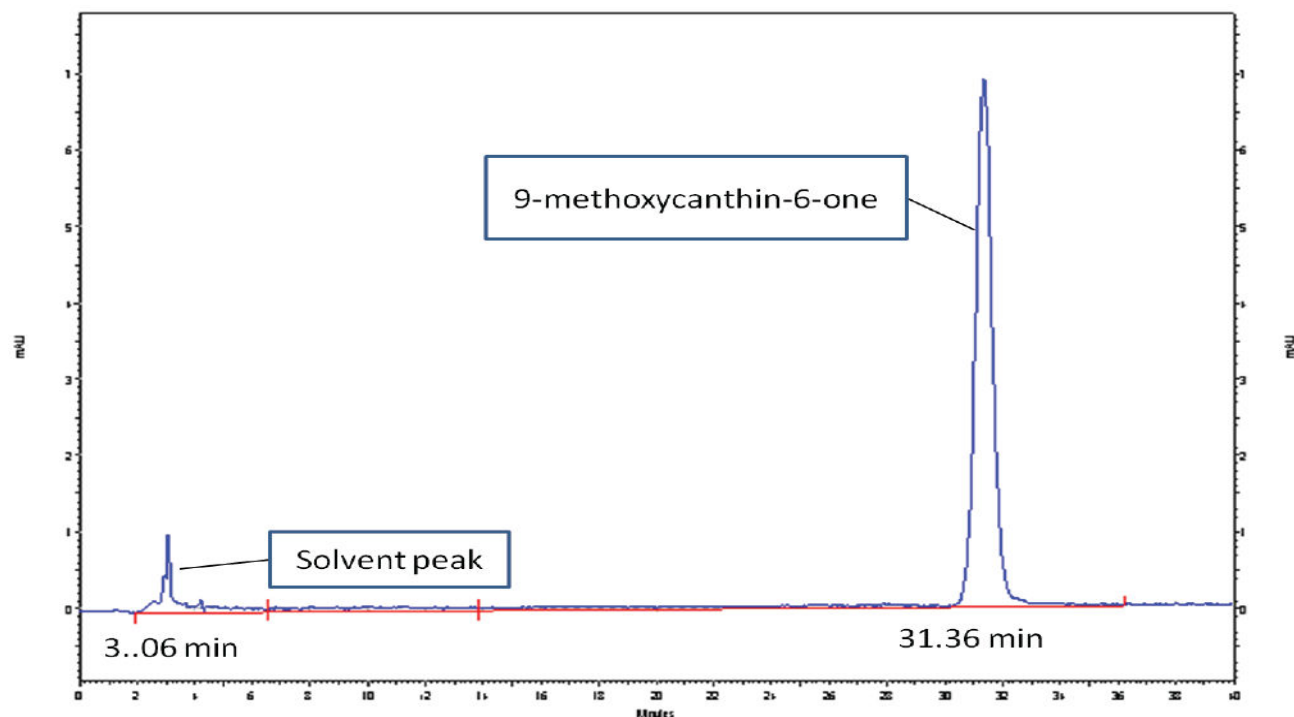


Fig. 4A. HPLC chromatogram showing elution of 25ppm of 9-methoxycanthin-6-one as standard at retention time 31.36 min as detected at 350 nm.

Micro-anatomical analysis on roots of *Eurycoma longifolia* using scanning electron microscope (SEM): The scanning electron microscope (SEM) micro-anatomical analysis was conducted on fresh root cultures of *E. longifolia* treated with different concentrations of MeJA. This analysis was done to determine the surface and root hair morphology on *E. longifolia* when elicited with MeJA. In this study, the result shows that the surface underneath the root hair is smooth and no physical damage was seen for

samples without MeJA treatment whereas rough surface was seen on the surface of roots with MeJA treatment at all tested concentrations (Fig. 5). On the other hand, the root hair was not affected even in the presence of MeJA treatment (Fig. 6). Hence, MeJA elicitation shows surface morphology damage on the roots of *E. longifolia*. Recently, Bhavani *et al.* (2014) reported on histology somatic embryos of *E. longifolia* in relevance in *Agrobacterium rhizogenes*-mediated transformation system.

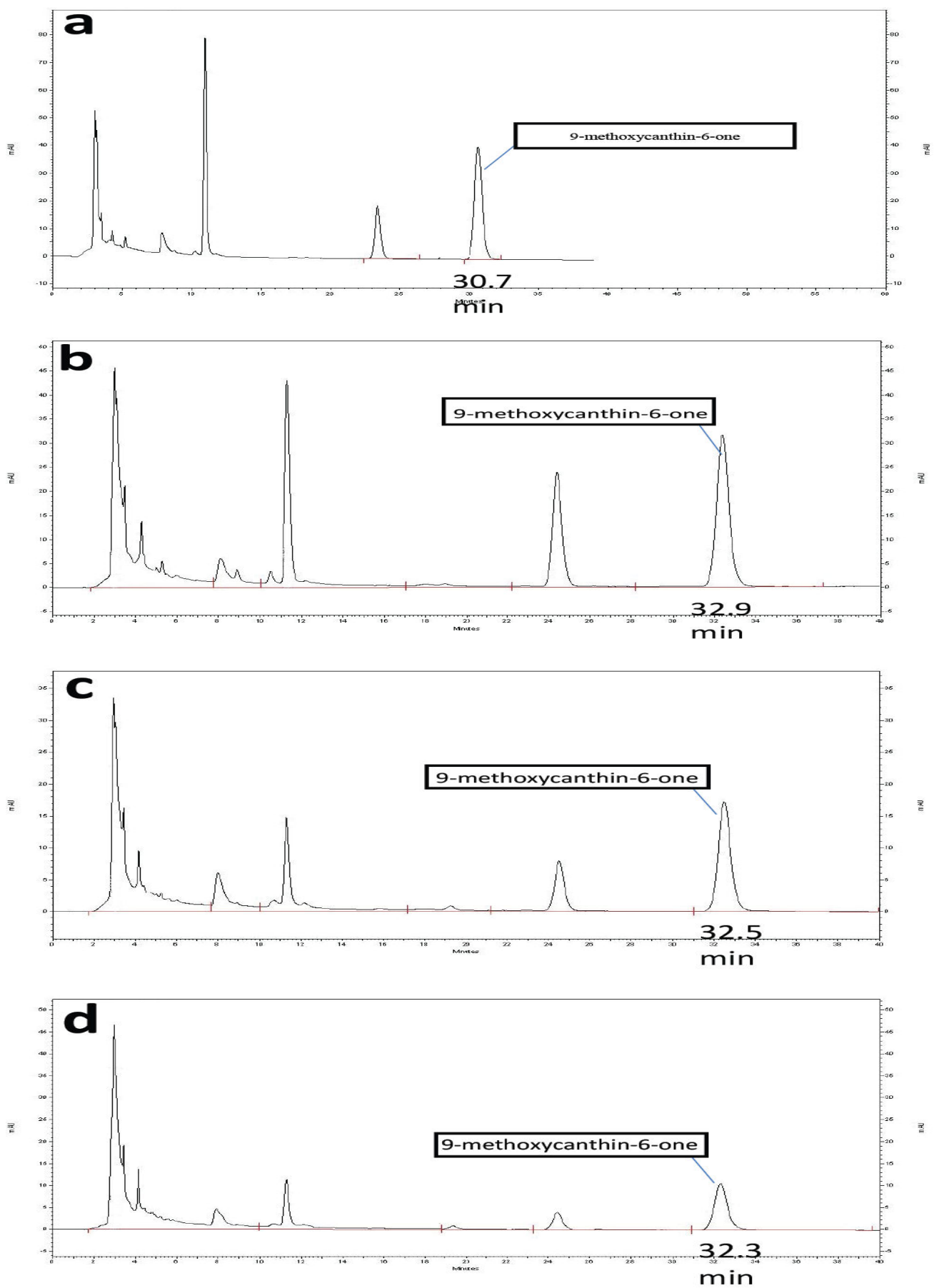
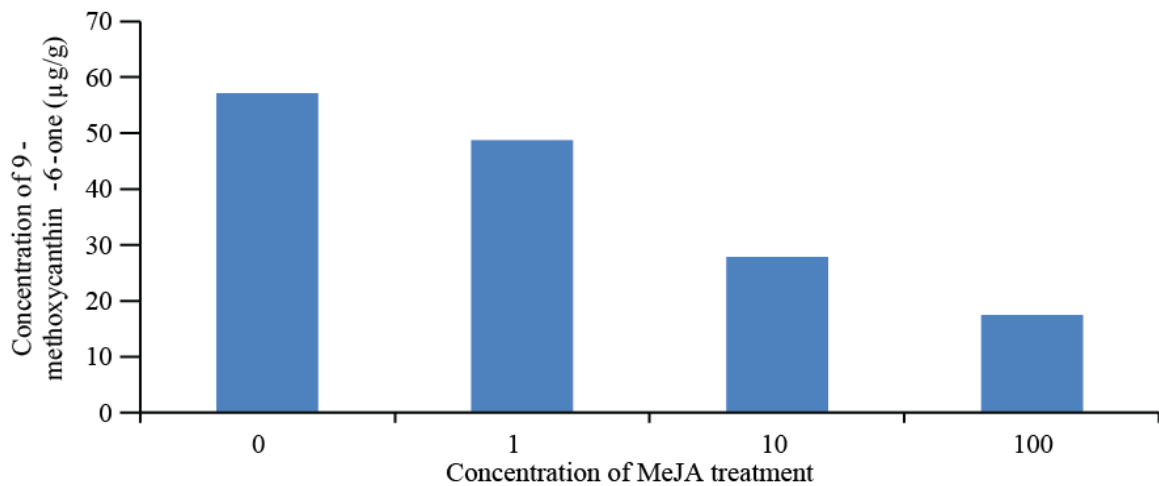
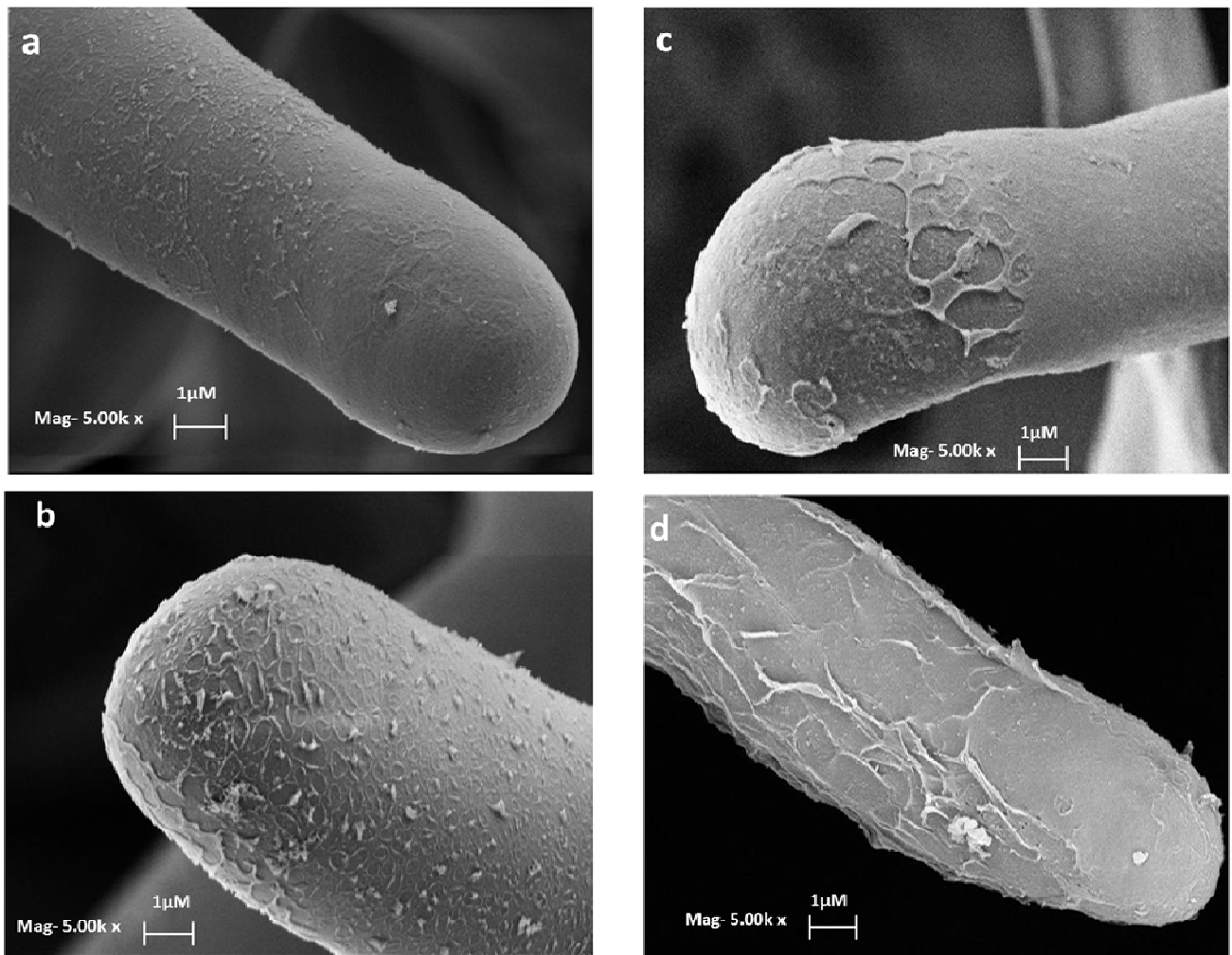


Fig. 4B. HPLC chromatogram showing elution of 9-methoxycanthin-6-one from *E. longifolia* treated with different MeJA treatments (a) control, (b) 1 μ M, (c) 10 μ M, and (d) 100 μ M.

Table 1. Analysis on the concentration of 9-methoxycanthin-6-one in *Eurycoma longifolia* roots ($\mu\text{g/g}$)

Concentrations of MeJA (μM)	Peak area (au)	Peak height (mV)	9-methoxycanthin-6-one concentration (unit)	Retention time (min)
Control	27007611	681586	57.17	30.7
1	22999808	530047	48.69	32.9
10	13123529	289853	27.78	32.5
100	8234206	179389	17.43	32.3

Fig. 4C. Effect of MeJA treatment on concentrations of 9-methoxycanthin-6-one produced in root cultures of *E. longifolia*.Fig. 5. SEM photomicrographs of surfaces of *E. longifolia* from roots treated with different concentrations of MeJA. (a) control, (b) 1 μM , (c) 10 μM and (d) 100 μM .

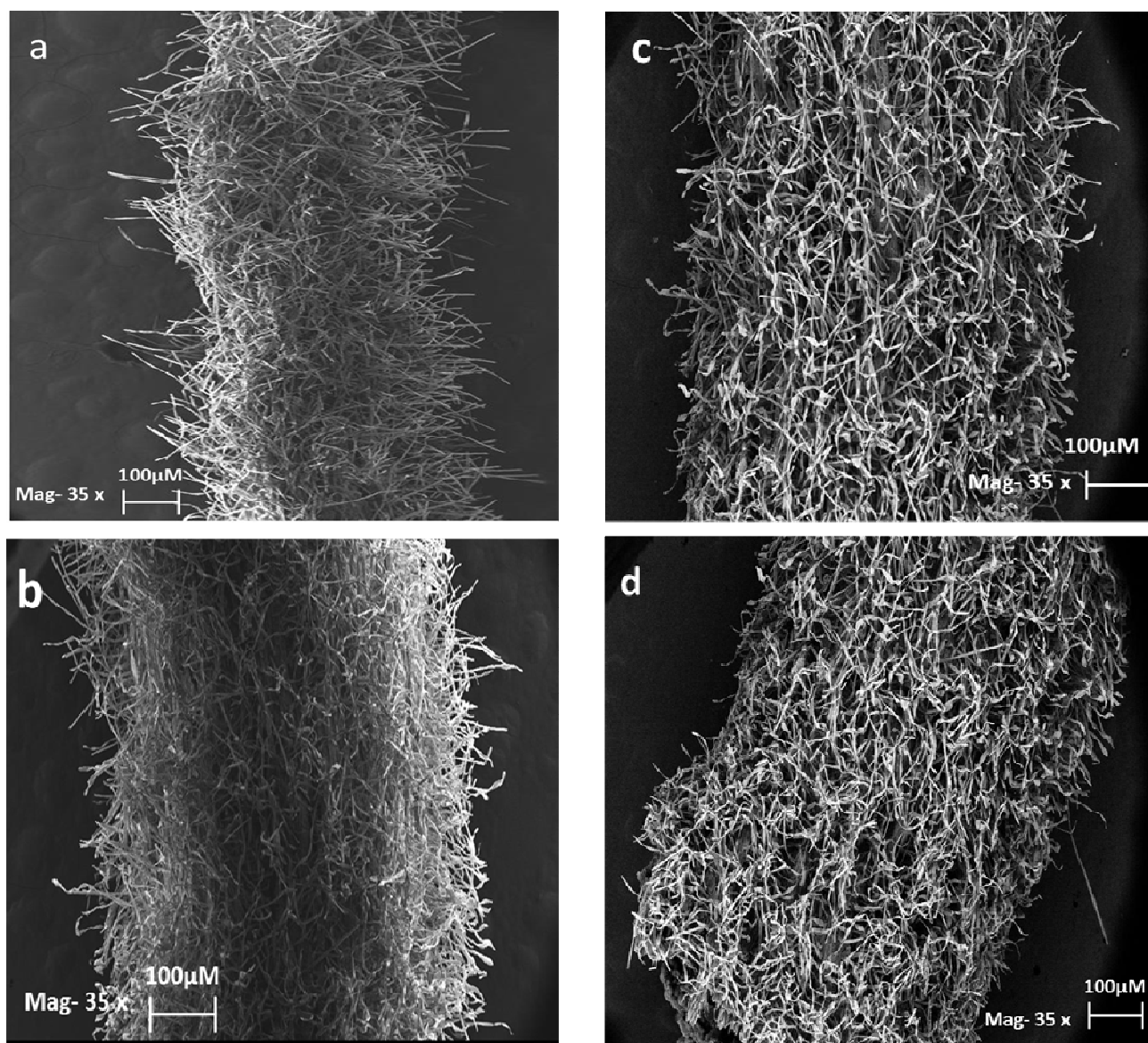


Fig. 6. SEM photomicrographs of surfaces of *E. longifolia* from roots treated with different concentrations of MeJA. (a) control, (b) 1 μ M, (c) 10 μ M and (d) 100 μ M.

Conclusion

Qualitative analysis on TLC revealed that 9-methoxycanthin-6-one was present in all treatments. Based on HPLC analysis, highest 9-methoxycanthin-6-one compound was obtained in the absence of MeJA elicitor. Water loss rate was highest at 100 μ M MeJA compared to the control treatment. Hence, MeJA is not a suitable elicitation compound to induce the production of 9-methoxycanthin-6-one in root cultures of *E. longifolia*.

Acknowledgements

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