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Detection of Insect Juvenile Hormone III and Its Precursors from *in Vitro* Plantlets of *Cyperus aromaticus*

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Juvenile hormone III (JH III) is one of the five closely related sesquiterpenoid compounds important in the regulation of an insect physiological processes in both the developing and the mature insect. JH III and its biosynthetic intermediates, farnesol and methyl farnesoate were detected in the *in vitro Cyperus aromaticus* plantlets and the mature plants growing in the field. A higher amount of methyl farnesoate (52.7%) and farnesol (31.4%) were found to be present in the *in vitro* plantlets as compared to the field grown mother plants which contained lower methyl farnesoate (19.5%) and farnesol (14.8%). On the other hand, the mature mother plant (4 months old) contained a higher percentage of JH III than the *in vitro* plantlets. The estimated content of JHIII analyzed by gas chromatography indicated that the amount of JHIII in the 10 weeks old *in vitro* plantlets and the four months old mature plants of *C. aromaticus* was 0.076 μg/g and 0.085 μg/g respectively. This study indicated that *in vitro* culture system could be a potential tool for the production of this natural derived bio-insecticide and could provide a material source for further investigations of the biosynthesis of JH III in *C. aromaticus.*

Keywords: Cyperus aromaticus, micropropagation, gas chromatography-mass spectroscopy, insect juvenile hormone III, farnesol, methyl farnesoate

Insect juvenile hormones were recognized as regulators for metamorphosis and other physiological processes in all insect species. It is also required by the adults of many insect species for several reproductive functions such as ovarian development, yolk synthesis and maturation of eggs in females, pheromone function, and accessory reproductive gland development in males (Bede et al., 1999a).

The juvenile hormones are a family of five closely related sesquiterpenoid compounds important in the regulation of insect development and reproduction. Juvenile hormone III (JH III) was found to be the most ubiquitous of the juvenile hormones (JHs) (Schooley et al., 1984). Since the discovery of JHs, there has been much interest in using JHs as insect control agents. Many natural products from plant and animal extracts have been tested for JH activity on various insect species (Baker et al., 1984; Schwaritz et al., 1998). Several Juvenile Hormone mimics have been found in plants, but none of these compounds closely resembles the JHs in chemical structure (Browers, 1991).

Toong et al. (1988) had successfully isolated insect JH III, methyl-10 R, 11-epoxy-3,7,11-trimethyl 2E,6E-dodecadienoate and its metabolic precursor in insects, methyl farnesoate, from *Cyperus iria* and *C. aromaticus* plants (Cyperaceae). The plant JH III was found to have the same 10 R configuration as that previously determined for JH III secreted by insect tissue. The finding was further confirmed by Bede et al. (1999b), when JH III, farnesol and methyl farnesoate were identified from callus and cell suspension cultures of *C. iria*. These findings suggested the possibility of a similar JH III biosynthesis pathway in *Cyperus* species and insect.

The tissue culture studies of the *Cyperus* species are limited, because these plants are grown naturally as weeds and are considered to have no economic importance. However, the discovery of JH III in *C. iria* and *C. aromaticus* has attracted attention. The generation of undifferentiated plant cell cultures in *Cyperus* genera was first reported by Fisher (1977), who successfully established callus and suspension cultures of *C. rotundus* on Schenk and Hildebrandt (SH) basal medium in the presence of the auxin growth regulator naphthalene acetic acid, NAA. Tissue culture of *C. iria* L. was carried out to investigate the biosynthetic pathway of JH III in this plant (Reynolds et al., 1990). In the present study, an *in vitro* culture system was developed to micropropagate the *C. aromaticus* and to determine whether JH III could be produced from these *in vitro* plantlets. The JH III, farnesol and methyl

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farnesoate present in the *in vitro* plantlets were compared with the field grown mother plants using gas chromatography mass spectroscopy (GC-MS).

MATERIALS AND METHODS

Production of *in Vitro* Plantlets

Mature field grown plants of *C. aromaticus* were collected from Batu Uban, Penang, Malaysia. The young shoots near the base of the rhizomes were used as explants. The explants were washed with "Good Maid", a commercial detergent, and rinsed under running tap water for 30 min. They were then surface sterilized using 95% ethanol (v/v) for 20 sec followed by immersion in 1.0 g L^{-1} mercuric chloride solution for 10 min. After rinsing three times with sterile distilled water, the explants were immersed in 300 mg L^{-1} of sodium dichloroisocyanurate (NaDCC) solution for 24 hr with continuous agitation by placing on a rotary shaker (B Braun Certomat®) at 120 rpm. After again rinsing three times with sterile distilled water, the explants were inoculated on solid Murashige and Skoog (1962) (MS) medium supplemented with 1.0 g L^{-1} of potassium permanganate and 100 mg L^{-1} kanamycin for one week. The aseptic explants that were established were used for subsequent experiments. The aseptic explants were cultured into the proliferation medium for *C. aromaticus* formulated earlier (Chan, 2000). The proliferation medium was consisted of a modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with three times the concentration of MS micronutrient, iron and vitamins supplemented with 1.0 mg L^{-1} 6-benzylaminopurine (BA) and 0.5 mg L^{-1} 3-indolebutyric acid (IBA). The medium was adjusted to the desired pH between 5.7-5.8 with 1 M NaOH or 1 M HCl before autoclaving at 121 °C for 13 minutes with a pressure of 1.05 kg cm^{-2}. The multiple shoots formed on the proliferation medium were separated singly and subcultured onto the proliferation medium every 3 weeks to obtain more shoots. Individual shoots were separated from the multiple shoots and were then cultured on the modified MS medium without any growth regulators for root induction and the production of *in vitro* plantlets. The plantlets were maintained in the basic MS medium for 10 weeks before they were harvested for the preparation of extracts and determination of JH III. The cultures were placed in the culture room with a temperature regulated at 25±2°C under continuous lighting with cool white fluorescent tubes at an intensity of 30 μmol m^{-2} s^{-1}.

Preparation of Extracts

The 10-week old *in vitro* plantlets and 4-months matured field grown *C. aromaticus* before flowering were used for the preparation of plant extracts. The whole plants were chopped finely and dried under white fluorescent tubes at an intensity of 30 μmol m^{-2} s^{-1} until constant weight. A dried weight of 25.0 g of *in vitro* plantlets and mother plants were steam distilled separately. The distillates were extracted with chloroform and were then evaporated in a rotary evaporator to approximately 0.5 ml and dried with nitrogen gas. The concentrated extracts were further dissolved in 10 ml of chloroform and filtered using 0.25 μm syringe filter before analysis for JH III, methyl farnesoate and farnesol.

Detection of JH III, Farnesol and Methyl Farnesoate in Plant Extract Using Gas Chromatography

Samples were analyzed by capillary GC using a polar phase 30×0.25 mm Supelcowax 10 column. Two minutes after 1.0 μl of samples were injected onto a split injector, the oven and injector temperatures were increased by 10.0°C min^{-1} from 50°C to a final temperature of 230°C. The nitrogen carrier gas linear flow velocity was maintained at 1.0 mL min^{-1}. A 0.5 mg L^{-1} solution of 70% pure standard JH III (Sigma, USA) was also analyzed by GC under the same conditions. The GC chromatographs were detected by a flame ionization detector.

Gas Chromatography-Mass Spectroscopy

The same conditions of GC were applied to Hewlett Packard 5890 Series II GC-MS analysis. The helium carrier gas linear flow velocity was maintained at 1.0 ml min^{-1} while the GC-MS transfer line temperature was maintained at 230°C. JH III was identified based on retention time and comparison of its spectra (18-300 amu) to that of standard JH III after separation on a polar phase 30×0.25 mm Supelcowax 10 column connected with an electron ionization mode mass spectrometer. The detection of farnesol and methyl farnesoate were compared and confirmed by the mass spectrometer library search.

RESULTS AND DISCUSSION

Production of *in Vitro* Plantlets

Each individual shoot of *C. aromaticus* was able to

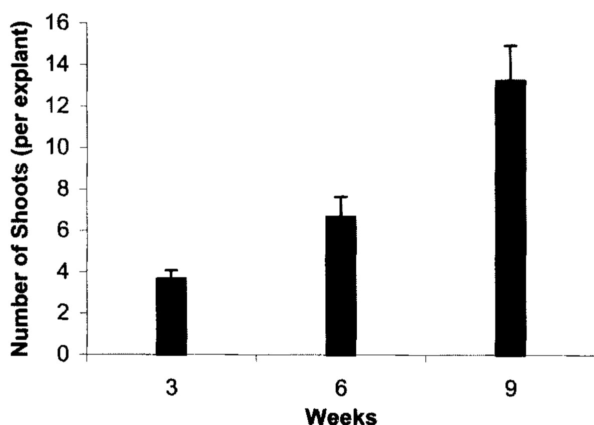

Figure 1. Formation of multiple shoots (mean number of shoots ± se, n=30) on a modified MS medium supplemented with 1.0 mg L^{-1} BA and 0.5 mg L^{-1} IBA plus three times the concentration of MS micronutrient, iron and vitamins within 9 weeks after three subcultures.

form multiple shoots when it was cultured on a modified MS medium supplemented with 1.0 mg L^{-1} BA and 0.5 mg L^{-1} IBA plus three times the concentration of MS micronutrient, iron and vitamins. An average of 13.3 shoots were produced from each shoot explant within 9 weeks after three subcultures (Fig. 1). Normal root systems were produced after 2 weeks of culturing on basic MS medium without any growth regulator.

The addition of 1.0 mg L^{-1} BA and 0.5 mg L^{-1} IBA into the modified MS medium was found to be the best combination of growth regulators for the formation of multiple shoots of C. *aromaticus*. As reported by Chan (2000), higher concentrations of MS micronutrients, chelated iron and vitamins provide optimization conditions for the plant growth and the number of shoots produced from each explant was doubled as compared to the normal content of micronutrient, iron and vitamins in the MS medium. Subculturing every three weeks induced the formation of more multiple shoots as more space was generated at each subculture for the explants to grow and produce more shoots.

Detection of JH III, Farnesol and Methyl Farnesoate

The estimated content of JH III analyzed by gas chromatography indicated that the amount of JH III in the 10 weeks old *in vitro* plantlets and the 4 months old mother plant of C. *aromaticus* was 0.076 µg/g and 0.085 µg/g respectively.

JH III and the precursors, methyl (2E, 6E) farnesoate and farnesol were detected from both the C. *aromaticus in vitro* plantlets and the mother plants. The profile of the chromatogram for *in vitro* plant extracts was compatible with the mother plants extracts (Fig. 2). The total ion chromatogram (TIC) of the mother plant extracts showed the presence of JH III at almost the same retention time compared to the standards (t_R 21.22 min). JH IIIs precursors, methyl (2E, 6E) farnesoate and farnesol were detected at retention time, t_R 16.91 min and t_R 18.23 min, respectively. The TIC of the *in vitro* plantlets extracts also produced JH III at almost the same retention time (t_R 21.15 min) as that of the standard JH III. The retention times of methyl (2E, 6E) farnesoate and farnesol generated were similar to that of the mother plants (t_R 16.88 min and t_R 18.20 min, respectively).

JH III that was detected in crude extract of the mother plant produced ion fragments of m/z (rel. int.): 266 $[M]^+$ (4), 234 (13), 206 (6), 164 (7), 135 (16), 121 (17), 81 (27), 71 (100). The ion fragments of JH III from the mother plants were compatible with the external standard with ion fragments at m/z (rel. int.): 266 $[M]^+$ (4), 234 (13), 206 (5), 164 (7), 135 (12), 121 (17), 81 (30), 71 (92). The ion fragments of JH III detected in the extract of *in vitro* plantlets were at m/z (rel. int.): 266 $[M]^+$ (4), 234 (14), 206 (7), 164 (7), 135 (13), 121 (15), 81 (30), 71 (92) and were also compatible with the external standard (Fig. 3). When compared with the standard JHIII spectrum, the mass spectrum of JHIII from the mother plants gave a 91.3% match while mass spectrum of JHIII of the *in vitro* plantlets gave 94.3% match as a confirmatory compound structure match.

The ion fragments from the methyl (2E, 6E) farnesoate detected in mother plants were m/z (rel. int.): 250 $[M]^+$ (4), 207 (7), 136 (11), 121 (22), 114 (33), 81 (25), 69(100) and farnesol were at m/z (rel. int.): 222 $[M]^+$ (1), 161 (3), 136 (9), 121 (8), 81 (28), 69 (100). When compared with the Wiley database library, the mass spectrum of methyl (2E, 6E) farnesoate from the mother plants gave a 97% match as well as a confirmatory compound structure match, while mass spectrum of farnesol gave 95% match as well as a confirmatory compound structure match.

Ion fragments from the methyl (2E, 6E) farnesoate detected in the *in vitro* plantlets were m/z (rel. int.): 250 $[M]^+$ (6), 207 (10), 136 (11), 121 (24), 114 (33), 81 (25), 69 (100) and farnesol at m/z (rel. int.): 222 $[M]^+$ (1), 161 (3), 121 (9), 81 (31), 69 (100). The mass spectrum of methyl (2E, 6E) farnesoate and farnesol were compared with Wiley database library and both gave 94% match as well as confirmatory compound

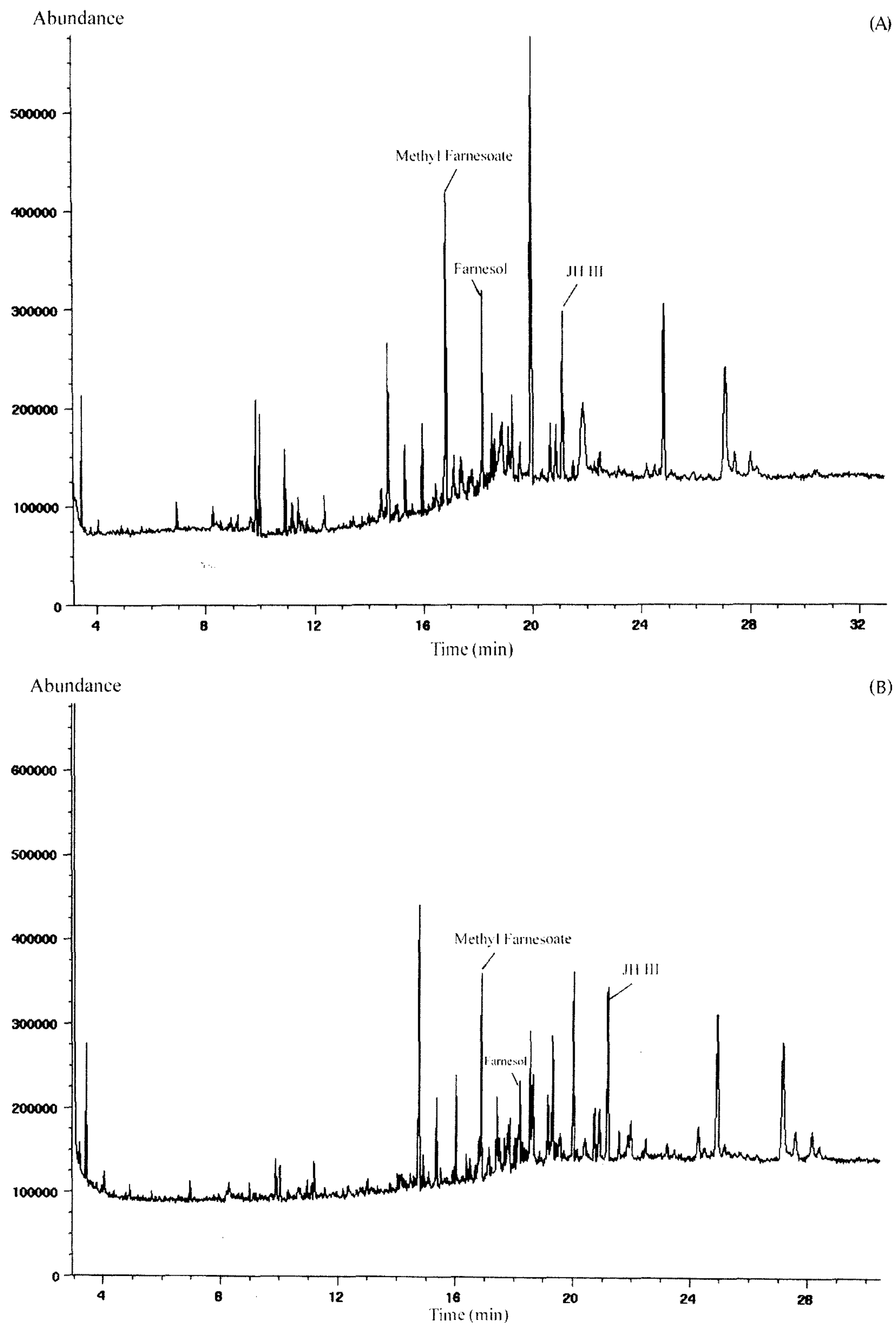

Figure 2. Total Ion chromatogram showing detection of farnesol, methyl farnesol and JH III from (A) the *in vitro* plantlets of *C. aromaticus* and (B) the mother plants of *C. aromaticus*.

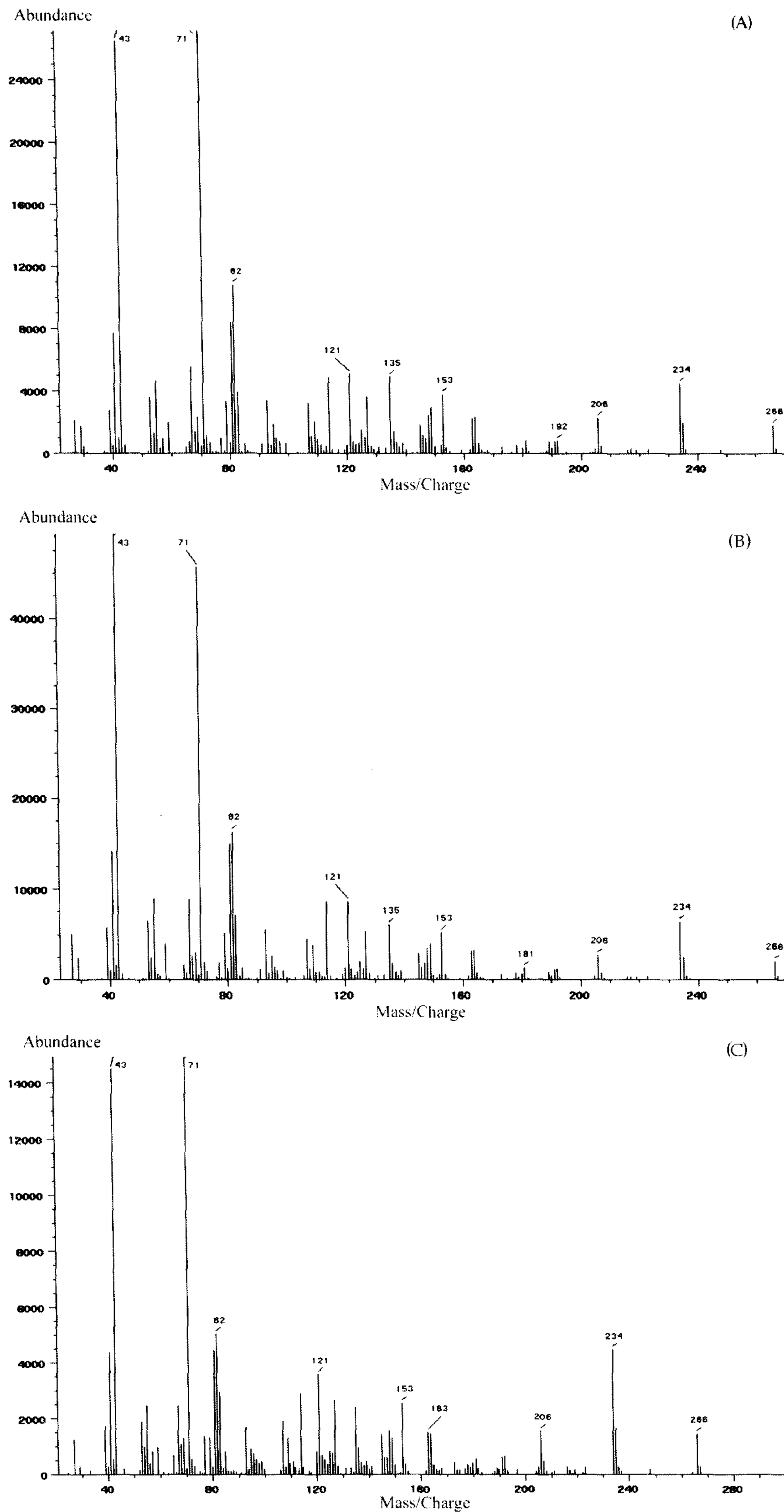

Figure 3. Mass spectra of JH III from the mother plant of *C. aromaticus* (A), standard (B), and the *in vitro* plantlets of *C. aromaticus* (C).

structure match.

The results hence showed that the ion fragments of JH III from mother plant and *in vitro* plantlets were compatible with the external standards. The main ion fragmentation generated by JH III could also be discovered in *C. iria* suspension cell extract with mass spectrometer under chemical ionization mode (m/z 71, 81, 121, 135) (Bede et al., 1999b). This showed that JH III from both field grown and *in vitro* plantlets of *C. aromaticus* has the same 10 R configuration as that previously determined for JH III secreted by insect tissue and from *C. iria*.

Methyl (2E, 6E) farnesoate and farnesol, the immediate biosynthetic precursors of JH III in insect, were also present in the field grown and *in vitro* cultured *C. aromaticus* crude extracts. The main ion fragments present in mass spectral of methyl (2E, 6E) farnesoate (m/z 69, 114, 121) and farnesol (m/z 69, 81, 136) were also detected by Baker et al. (1990) in hemipteran, *Oncopeltus fasciatus* utilizing GC-MS with selected ion monitoring (SIM). The isolation of these biosynthetic intermediates parallel to the finding in *C. iria* (Bede et al., 1999b) and again suggest that the late steps in the JH III pathway for both the plant and insect may be similar.

The presence of very clear peaks and spectra on farnesol, methyl (2E, 6E) farnesoate and JH III from the crude extracts of both the *in vitro* plantlets and their mother plants indicated high levels of these compounds contained in the *C. aromaticus*. A higher percentage of methyl (2E, 6E) farnesoate and farnesol was found to be present in the 10 weeks old *in vitro* plantlets while the mature mother plant (4 months old) contain a higher percentage of JH III but a relatively low percentages of methyl (2E, 6E) farnesoate and farnesol (Table 1). Bede et al. (1999a) also found in their studies that JH III levels increased steadily from two week old immature plants of *C. iria* until the plant was three months old when it was near the flowering stage. At flowering, a dramatic decrease of JH III was observed in all the plant tissues and JH III levels subsequently increased again until it reached maturity. Our result also showed that the JH III were actively synthesized in the young *C. aromaticus*, as shown in the 10 weeks old *in vitro* plantlets and reached a higher level in the mature mother plants before flowering stage. These also indicated that the JH III played an important role in protecting the plant from the attack of insects at different growth stages of *C. aromaticus*. The presence of JH III may also contribute to the aggressive and invasive nature of this weed species.

The present study indicated that the *in vitro* culture system could be an alternative method for the production of JH III from *C. aromaticus*. The field grown *C. aromaticus* will take 4 months to reach maturity while *in vitro* plantlets could be mass-produced within 10 weeks. This *in vitro* culture system will be a more rapid and effective way for mass propagating *C. aromaticus* and provide the material source for the production of JH III and its precursors and for future further research.

Table 1. Relative percentage amounts of farnesol, methyl farnesoate and JH III from *in vitro* plantlets and mother plants of *C. aromaticus* analyzed by GC.

Plant Materials	Relative % of compounds		
	Farnesol	Methyl Farnesoate	Juvenile Hormone III
Mother Plants	14.80	19.48	77.02
In vitro Plantlets	31.41	52.65	33.41

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