Plant Cell Reports (1999) 19:20–25

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Production of insect juvenile hormone III and its precursors in cell suspension cultures of the sedge, *Cyperus iria* L.

Received: 30 October 1998 / Revision received: 11 February 1999 / Accepted: 3 March 1999

Abstract Juvenile hormones (JHs) are sesquiterpenoids that regulate metamorphosis and reproduction in most insect species. There has been one report of an insect JH in plants: JH III, methyl-10*R*,11-epoxy-3,7,11-trimethyl 2*E*, 6*E*-dodecadienoate, has been identified in two sedge species, *Cyperus iria* L. and *C. aromaticus* (Ridley) Mattf and Kük. This is the first report of callus and cell suspension cultures derived from *C. iria*. Farnesol and methyl farnesoate, two biosynthetic intermediates of JH III in insects, as well as JH III have been identified in suspension culture cell extracts by gas chromatography-mass spectroscopy. These cultures thus provide a useful in vitro model to investigate the biosynthesis of JH III in the sedge, *C. iria*.

Key words *Cyperus iria* L. · Juvenile hormone III · Methyl farnesoate · Callus · Suspension culture

Abbreviations *2,4-D* 2,4-Dichlorophenoxyacetic acid · *B_5 medium* Gamborg's medium · *GC-MS* Gas chromatography-mass spectroscopy · *JH* Juvenile hormone · *MF* Methyl farnesoate · *MS medium* Murashige and Skoog medium · *SH medium* Shenk and Hildebrandt medium

Introduction

Juvenile hormones (JHs) are a group of structurally related sesquiterpenoids that regulate insect development and reproduction (Gilbert et al. 1996). Six naturally occurring insect JHs have been isolated (Bede and Tobe 1999; and references therein) which share common structural features of a methyl ester moiety on C-1 and an epoxide function. Of these compounds, JH III, methyl-10*R*,11-epoxy-3,7,11-trimethyl 2*E*, 6*E*-dodecadienoate (Fig. 1), appears to be ubiquitous and in many insect orders is the only JH present (Tobe and Feyereisen 1983). In immature insects, these hormones are required for the maintenance of juvenile characteristics (Tobe and Stay 1985). In adult female insects, JHs regulate reproductive processes such as vitellogenin uptake and oocyte maturation (Tobe and Stay 1985).

In insects, the isoprenoid skeleton of JHs is synthesized through the mevalonate pathway (Monger 1985). A phosphatase or pyrophosphatase catalyzes the removal of the pyrophosphate group from the sesquiterpenoid intermediate farnesyl diphosphate (Fig. 1). Successive oxidation of farnesol to the aldehyde (farnesal) and carboxylic acid (farnesoic acid) is catalyzed by one or two NAD^+-dependent dehydrogenase(s) (Baker et al. 1983). In the cockroach, *Diploptera punctata*, methylation of farnesoic acid generates the methyl ester, methyl farnesoate (MF), followed by an epoxidation at C-10, C-11 to produce JH III (Cusson et al. 1991).

One defensive strategy of plants against insect herbivory is the production of secondary metabolites that interfere with insect physiology (Rosenthal and Janzen 1979; Rosenthal and Berenbaum 1991). It has been suggested that it is difficult for insects to evolve counteradaptive strategies to phytochemicals which act at the same physiological targets and in the same manner as their endogenous hormones (Bowers 1991). In fact, there are many examples of plants that produce phytoecdysteroids, compounds which are structurally identical to or which mimic the insect moulting hormone, 20-hydroxyecdysone (Adler and Grebenok 1995). However, there has been only one report of the identification of an insect JH from plants: JH III and MF have been isolated from the sedges *Cyperus iria* L. and *C. aromaticus* (Ridley) Mattf and Kük (Toong et

Communicated by S. Gleddie

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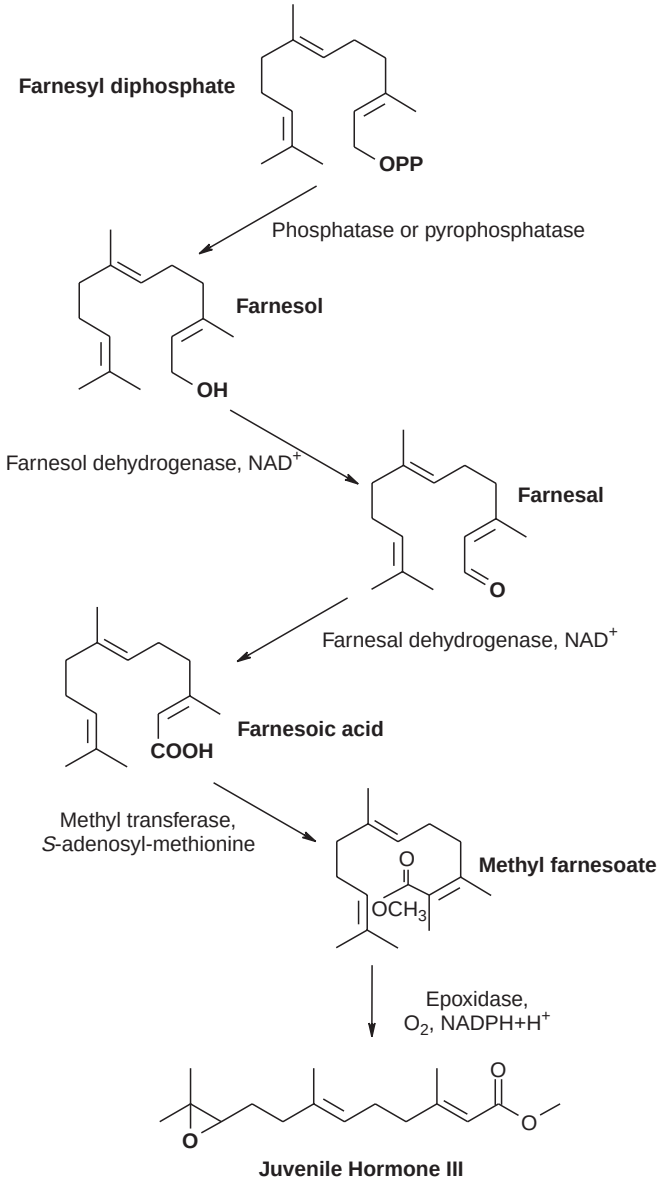

Fig. 1 Final steps of the biosynthetic pathway of JH III in the cockroach, *Diploptera punctata* (Cusson et al. 1991)

al. 1988). The objective of the research described here was to develop a cell culture of *C. iria* to permit investigation of the JH III biosynthetic pathway in the plant.

In vitro inflorescence cultures have been established for two members of the Cyperaceae, *Cyperus rotundus* L. and *Carex flacca* Schreb. (Smith 1968; Ram and Batra 1970). The aim of these studies was to investigate factors involved in floral development and did not involve the dedifferentiation and growth of plant tissue. Micropropagation of another member of the Cyperaceae, *Caustis dioica* R. Br., has also been achieved but, again, did not involve callus induction (Rossetto et al. 1992). The only report of the generation of undifferentiated cell cultures from a plant in the *Cyperus* genera was 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 naphthaleneacetic acid.

In this study, callus cultures were initiated from sterile explants of *C. iria* on a variety of tissue culture media. Once callus and suspension cultures were established, the presence of farnesol, MF, and JH III in these cells was confirmed by gas chromatography-mass spectroscopy (GC-MS).

Materials and methods

Materials

All chemicals were purchased from Sigma unless otherwise noted.

Plants

Seeds of *C. iria* were obtained from Y.C. Toong (University of Malaysia, Penang). Voucher specimens of this plant have been deposited at the Royal Ontario Museum Herbarium, Canada (TRT).

Explants

C. iria seeds were surface sterilized by submersion in 70% ethanol (Commercial Alcohols) for 1 min, followed by 5.25% sodium hypochlorite (Queen Bleach) containing a few drops of Tween 20 for 20 min and then rinsed three times with sterile distilled water. Seeds were placed on sterile, water-saturated filter paper in petri dishes (60 × 15 mm; Sarstedt). After germination, seeds were aseptically transferred to 20-ml vials (Kimble) containing the following medium: NH_4NO_3 (165 g/l), KNO_3 (190 g/l), $MgSO_4$ $7H_2O$ (37 g/l), KH_2PO_4 (17 g/l), casein hydrolysate (0.5 g/l) with 3% sucrose (BDH) and 0.8% agar (Difco-bacto). The medium was adjusted to pH 5.8 ± 0.1 with 1 M KOH (BDH) and autoclaved for 28 min at 121 °C, then poured into 20-ml vials under sterile conditions. Excised shoots and roots from the resultant aseptically grown seedlings were used as explants and cultured in plastic petri dishes under a variety of media regimes.

Media

Because the appropriate nutrient conditions for the initiation of callus from *C. iria* seedlings were unknown, explants were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with the following organic compounds (values in parentheses indicate the range of concentrations tested): sucrose (2–6%), thiamine (0.1–40.0 μM), pyridoxine (0.6–6.0 μM), niacin (4.0–40.0 μM), casein hydrolysate (0.01–0.5 mg/l), and *myo*-inositol (0.1–0.6 mM). To these media, either the synthetic auxin growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5–5.0 mg/l) or a cytokinin growth regulator, kinetin (0.1–1 μM) or zeatin (0.5–50.0 μM) was added. Explants were also cultured on the following media: B_5 (Gamborg et al. 1968), SH (Schenk and Hildebrandt 1972), White's (White 1963) as well as 1/2 MS salts, supplemented with 3% sucrose, 3.0 μM thiamine-HCl, 2.4 μM pyridoxine-HCl, 4.0 μM nicotinic acid, 0.5 g/l casein hydrolysate, 0.6 mM *myo*-inositol and 2.5 mg/l 2,4-D. The pH of each medium was adjusted to pH 5.8 ± 0.1 with 1 M KOH or 1 N HCl (Caledon) and autoclaved for 28 min at 121 °C. All chemicals used for tissue culture media were obtained from Sigma, except cupric sulfate ($CuSO_4.5H_2O$) (Fisher), cobaltous

chloride ($CoCl_2.6H_2O$) (BDH). For each treatment, three replicates were prepared.

Callus culture

Under aseptic conditions, explants were placed in various orientations (vertical, horizontal) on the agar plates and the plates incubated in a growth chamber at 26 ± 2 °C under either low-intensity illumination (2800 lux) with a 16:8 (light:dark) photoperiod or in dark conditions. After 3 weeks, callus tissue was selected and grown in the same fresh medium. After a number of subcultures, the optimal conditions for growth were determined. Callus was established on three media: MS, SH, and B_5 supplemented with 3% sucrose, 3.0 μM thiamine-HCl, 2.4 μM pyridoxine-HCl, 4.0 μM nicotinic acid, 0.5 g/l casein hydrolysate, 0.6 mM *myo*-inositol, and 2.5 mg/l 2,4-D. Callus on SH and B_5 media were maintained in the light-dark cycle, while callus on MS media grew best under dark conditions. At 3-week intervals, the callus was subcultured onto fresh media.

Suspension cultures

Suspension cultures were initiated from established SH and MS callus cultures by adding the cells to 100 ml medium in 250-ml Erlenmeyer flasks. Medium for suspension cultures was prepared exactly as that for callus culture but without inclusion of agar. Suspension cultures were maintained in a shaking incubator at 26 ± 2 °C in the dark with rotary gyration at 125 rpm. Cells were subcultured at 18-day intervals (approximately 1:30 dilution).

Chemical analysis

SH suspension cells were collected by filtration and homogenized in pentane (HPLC grade, Burdick & Jackson). The organic phase was evaporated under vacuum in a rotovaporator (Buchler Instruments) to approximately 0.2 ml and added to an anhydrous sodium sulfate- (BDH, approximately 0.5 g) hydroxyapatite (BioRad, approximately 0.4 g) Pasteur pipette column that had been prewashed with 5 ml ethyl ether (HPLC grade, Burdick & Jackson) and isooctane (HPLC grade, Burdick & Jackson). After addition of sample, the columns were washed with 5 ml pentane and the sample was eluted with 5 ml pentane-ether (70:30).

Sample preparation

Extracts were further purified by thin layer chromatography on silica plates with fluorescent dye indicator (Merck) in toluene- (ACS, BDH) ethyl acetate- (HPLC grade, BDH) acetic acid (ACS, BDH) (85 ml:15 ml:4 drops) after focusing in methanol (HPLC grade, Burdick & Jackson). Bands containing MF ($R_f = 0.66$) and JH III ($R_f = 0.59$) were scraped and extracted in ether. The solvent was evaporated under nitrogen and the residue dissolved in hexane (GC-MS grade, Burdick & Jackson). The samples were further purified by silica gel chromatography. The silica gel (silica gel 60, 230–400 mesh, EM Science) was washed twice with hexane followed by three washes with acetone (HPLC grade, Burdick & Jackson) prior to slurry packing in a 20 × 1.6 cm stainless steel HPLC column fitted with 10-μm frits and 7.5-mm end fittings. The column was attached to the injector of a Varian 1400® gas chromatograph and helium was passed through the column at 30 ml/min. The oven temperature was increased to 300 °C for 24 h. Column chromatography was performed using Pasteur pipettes fitted with a glass wool plug and containing a 1-cm bed of silica gel. The silica column was then preconditioned with 2 ml hexane (GC grade, Burdick & Jackson). After application of the sample in hexane, the column was washed with 1 ml hexane. Farnesal and MF were eluted with 500 μl of 2.5% ethyl acetate (Burdick & Jackson, glass distilled) in hexane. JH III was eluted with 500 μl of 10% ethyl acetate in hexane. All eluting solvents contained 500 pg of farnesyl acetate as an internal standard.

Gas chromatography

Samples were analyzed by capillary GC using a 30 m × 0.25 mm (internal diameter) SE30 (apolar phase; Alltech) analytical column connected to the cool-on-column injector with a 10-cm length of 0.5-mm (internal diameter) deactivated fused silica. Two minutes after sample injection onto a cool-on-column injector (–60 °C), oven and injector temperature were increased by 7.5 °C/min until a final temperature of 180 °C was reached. The helium carrier gas linear flow velocity was 18 cm/s. Samples were also analyzed by GC on a polar 30 m × 0.25 mm Carbowax 20 M column (Alltech), under the same conditions as above. Standards were obtained from Sigma, with the exception of methyl farnesoate that was chemically synthesized from farnesol according to Latli and Prestwich (1991). Recoveries of standards after chromatographic procedures were in excess of 80% for farnesol and 85% for MF and JH III.

Gas chromatography-mass spectroscopy

Compounds were identified based on retention time and comparison of their spectra (60–350 amu) to standards after separation on a 30 m × 0.25 mm analytical DB5-MS column (J & W) interfaced to a Finnigan Matt ITS40 ion trap mass spectrometer operated in the chemical ionization mode. The initial injector temperature was 40 °C which after the first 120 s, increased at 170 °C per minute until 270 °C was reached. The initial column temperature was 40 °C which after 5 min increased at 5 °C per min to 210 °C. The helium carrier gas linear flow velocity was 24 cm/s and the GC-MS transfer line temperature was 230 °C. Isobutane (Matheson, 99.9%) was the reagent gas used for chemical ionization.

Results and discussion

Cell cultures

Two to 3 weeks after initiation, undifferentiated tissue protuberances were visible from the sterile explants. The orientation of the explants did not seem to affect callus formation. After 3 months, callus cultures were established on MS, B_5, and SH media, each supplemented with 3% sucrose, 3.0 μM thiamine, 2.4 μM pyridoxine, 4.0 μM nicotinic acid, 0.5 g/l casein hydrolysate, 0.6 mM *myo*-inositol, and 2.5 mg/l of the synthetic auxin growth regulator 2,4-D. These cultures produced compact friable callus. Whitish cells formed on the MS medium and knobby and gray-yellow callus predominated on the B_5 and SH media. Initially, callus growth appeared better on the B_5 and SH plates, which may reflect the lower ammonia concentrations in these media. Establishment of callus was unsuccessful on White's medium. Callus cultures have been maintained for over 5 years on SH and MS media.

From the callus cultures, cell suspension cultures were initiated in SH and MS media. Cells in both media were elongated and ovoid. Cells grown in MS media were creamy white and grew rapidly in small clusters, whereas the cells in the SH media were yellow, formed

larger clumps and exhibited slower growth. A typical growth curve was observed in these cell suspension cultures: there was an initial lag phase, followed by the logarithmic growth phase and the cells entered the stationary phase 18 days after subculturing. High levels of the auxin, 2,4-D, were necessary for initiation and maintenance of the tissue culture; in fact, root formation was induced when these cultures were transferred to auxin-free media. Suspension cell cultures in these media have been maintained for over 4 years.

Secondary metabolites

Pooled extracts of suspension culture cells were analyzed by GC-MS. JH III and two of its biosynthetic precursors in insects, MF and farnesol, were detected (Fig. 2a–c). The MF and JH III content of individual cell suspension cultures (100 ml, day 18) and of callus cultures (day 21) were analyzed (Table 1). These compounds accounted for less than 0.1% of the detectable ions in the samples. Farnesol was also present, but in amounts too low to quantify. The total MF in suspension cultures (cells and media) was higher from the SH than in MS media. For both media, approximately equal amounts of MF were detected in both the cells and the media. Callus cultures grown on MS plates produced more MF than suspension cultures. This was reversed for the SH callus and suspension cell cultures. The average amount of MF detected in these SH suspension cultures was approximately 5 ng/g fresh weight. This is much higher than the quantity of JH III

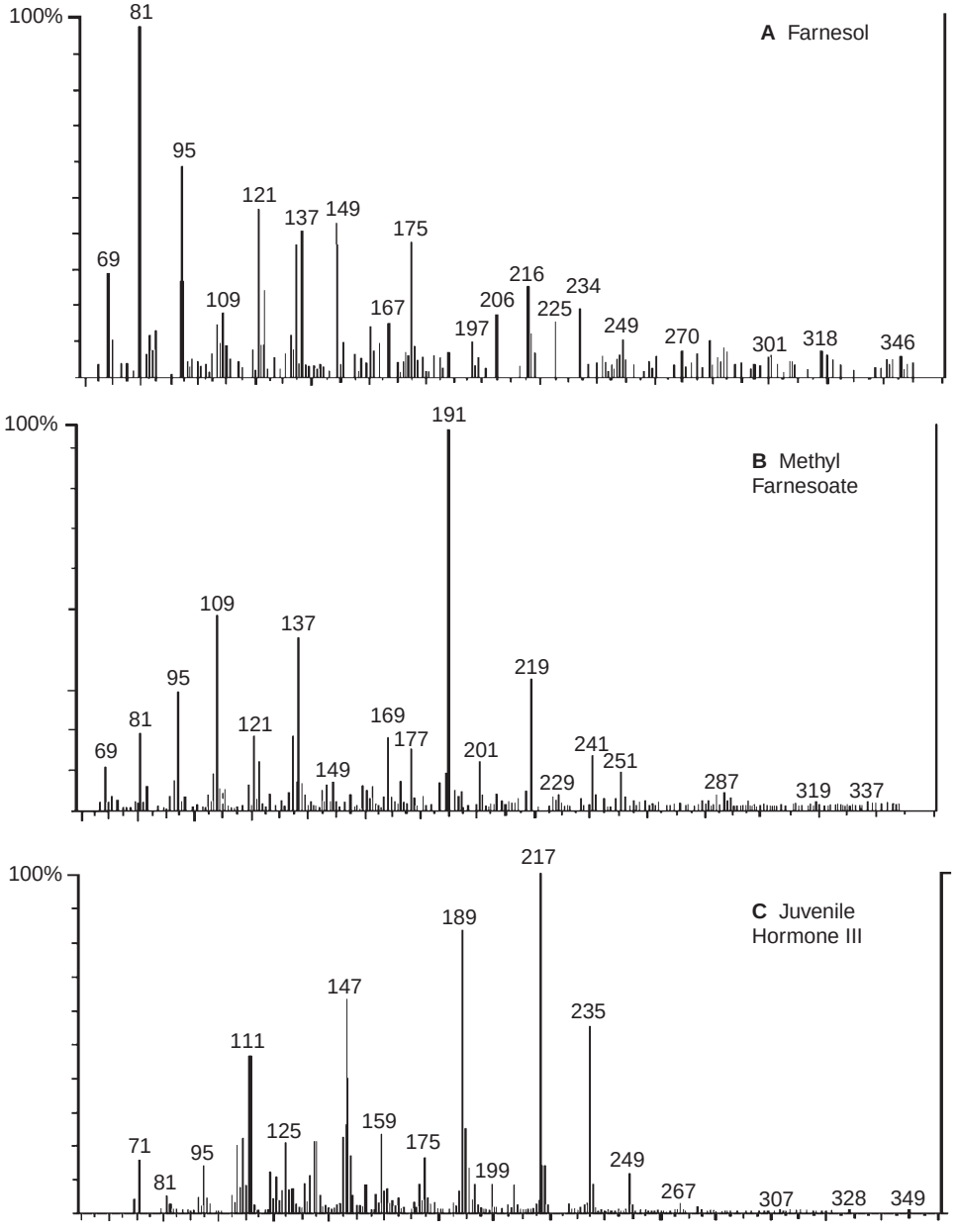

Fig. 2A–C Identification of JH III and its biosynthetic precursors in the extract from *Cyperus iria* suspension culture cells. **A** Mass spectrum of farnesol. **B** Mass spectrum of MF. **C** Mass spectrum of JH III

Table 1 Identification of compounds from *Cyperus iria* cell suspension and callus cultures. MF and JH III amounts (pg/g fresh weight) were measured in *C. iria* cell suspension cultures (individual flasks, day 18) and in callus cultures (day 21) by GC-MS. The data represent the content of combined cell and media extracts from individual flasks of cell suspension cultures and of the cell extract of the callus culture, comparing the amount produced in MS or SH media

	Suspension culture		Callus culture	
	MS	SH	MS	SH
MF	54.4	5012.1	1070.9	133
JH III	125.6	80.19	1.72	3.2

produced by these cultures ($\sim$0.08 ng/g fresh weight). The quantities of JH III produced in SH or MS suspension cultures were similar and approximately fifty times greater than the amounts found in the respective callus cultures. Again, little difference was seen in the amount of JH III in the cells or media. The differences in MF and JH III production between callus and suspension cultures and between SH and MS media reflect the commonly observed effects of factors such as the nature of the explant, the degree of differentiation, light and nutrients on the biosynthesis of plant secondary metabolites in vitro (Charlwood and Charlwood 1991; Banthorpe 1994).

JH III biosynthetic pathway

At present, little is known about the biosynthesis of JH III in the sedge, *C. iria*. In insects, the sesquiterpenoid backbone is synthesized through the classical mevalonate pathway (Monger 1985). In plants, this also appears to be the predominant pathway of isoprenoid biosynthesis, particularly of higher sterols (Bach 1995). However, in bacteria and in the thylakoids of cyanobacteria, a unique pathway synthesizes the isoprene C_5 units through the condensation of a pyruvate-derived activated acetaldehyde with a triose phosphate derivative, followed by skeletal rearrangement (Rohmer et al. 1993). Recently, evidence for this pathway has been found in higher plants (Bach 1995 and references therein; Eisenreich et al. 1996). It is not known which of these pathways provides the sesquiterpenoid backbone of JH III in *C. iria*.

MF, the immediate biosynthetic precursor of JH III in the insect, has been identified in four *Cyperus* species: *C. iria*, *C. monophyllus*, *C. pilosus* and *C. serotinus* (Iwamura et al. 1978, 1979; Komai et al. 1981; Toong et al. 1988). Another biosynthetic intermediate, farnesol, has also been isolated from the roots of the latter three species (Iwamura et al. 1978, 1979; Komai et al. 1981). These common intermediates found in both the plant and insect suggest that the late steps in the JH III pathway may be similar.

Cell suspension cultures provide a rapidly growing, relatively homogeneous biomass that is ideal for the investigation of metabolic pathways and could provide an in vitro model to elucidate the pathway of JH III biosynthesis in the sedge, *C. iria*. One problem with this approach is the frequent absence of secondary metabolite production in undifferentiated tissue. This may be attributed to changes in carbon fluxes in rapidly dividing cells and in gene expression and enzyme activity in these cells, as well as to the absence of morphological differentiation which may be required for enzyme activity or for the sequestration of secondary metabolites, particularly cytotoxic compounds (Charlwood and Charlwood 1991; Banthorpe 1994).

C. iria cell suspension cultures are able to produce the secondary metabolites farnesol, MF, and JH III. These compounds are also found in the insect JH III biosynthetic pathway suggesting that the plant and insect pathways may be similar. Therefore, these cell suspension cultures provide an excellent model to investigate the JH III biosynthetic pathway in *C. iria* and to study its regulation. Future work will focus on increasing the yield of these compounds in the SH suspension cultures and the investigation of their biosynthetic pathway.

Although there are many examples of phytochemicals that interfere with insect physiological processes (Rosenthal and Janzen 1979; Rosenthal and Berenbaum 1991), the selective pressures driving the evolution of these compounds are unclear (Rhoades 1979; Futuyma and Keese 1992). It has been argued that many secondary metabolites that presently play a protective role against insect herbivores originally evolved as a response to vertebrate herbivory (Hay and Steinberg 1992). However, the production in *C. iria* of JH III, a compound that specifically disrupts endocrine-related processes in insects, may be an example of a phytochemical that has evolved to protect the plant against insect herbivory. Although it is tempting to speculate that this is the case, the biological role of this compound in the plant is unknown at present.

Acknowledgements We are grateful to Y.C. Toong for providing the *Cyperus iria* seeds as well as to P. Bowser, C. Garside, P. Koladich and L. Rogers for critical reading of this manuscript and insightful comments. This research was supported by grants from the National Sciences and Engineering Research Council of Canada (SST).

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