Liriodenine, Early Antimicrobial Defence in *Annona diversifolia*

Iván De la Cruz-Chacón[a], Alma Rosa González-Esquinca[a,*],
Patricia Guevara Fefer[b], and Luis Felipe Jímenez Garcia[c]

[a] Laboratorio de Fisiología y Química Vegetal, Facultad de Ciencias Biológicas, Universidad de Ciencias y Artes de Chiapas, Libramiento Norte Poniente s/n. Col. Lajas Maciel, C. P. 29032, Tuxtla Gutiérrez, Chiapas, México. Fax: +52-961-10-12-894. E-mail: aesquinca@unicach.mx
[b] Departamento de Ecología y Recursos Naturales, Facultad de Ciencias, Universidad Nacional Autónoma de México, Cd. de México, Distrito Federal, México
[c] Departamento de Biología Celular, Facultad de Ciencias, Universidad Nacional Autónoma de México, Cd. de México, Distrito Federal, México

* Author for correspondence and reprint requests

Z. Naturforsch. **66 c**, 377–384 (2011); received November 25, 2010/March 26, 2011

Annonaceae aporphine alkaloids, of which liriodenine is the most abundant, have not been extensively studied from a biological standpoint. The goal of this study was to investigate the role of liriodenine in antimicrobial defense during early developmental stages in *Annona diversifolia*. The fungi *Rhizopus stolonifer* and *Aspergillus glaucus*, which are responsible for seed deterioration, were isolated during imbibition, and their antifungal activity was determined by diffusion, macrodilution, and metabolic inhibition assays using purified liriodenine and alkaloid extracts obtained from embryos, radicles, and roots at early developmental stages. The presence of liriodenine in extracts was quantified by high-performance liquid chromatography. Purified liriodenine and alkaloidal extracts inhibited both fungi, and there was a positive relationship between extract activity and amount of liriodenine contained therein. The quantity of liriodenine present in extracts suggests its importance in controlling other phytopathogens.

Key words: Alkaloid, Annonaceae, Early Development

Introduction

Plants are not able to move in search of a more suitable environment for growth and development. As such, they are often subjected to environmental stress caused by biotic or abiotic factors. This fact is probably one of the reasons why plants have acquired and perfected over thousands of years of evolution defense mechanisms that allow them to respond to external attacks, and therefore live in very different environments (Dixon, 2001; Hartmann, 2007).

Damage caused to plants by invading pathogens (bacteria, fungi, viruses, and viroids) or by herbivory frequently leads to metabolic responses aimed at the survival of the species. Such responses require the allocation of energy resources to build up stores of defense substances, involving a complete biosynthetic machinery based on a process of differentiation (Luckner, 1980; Wink, 2010). For example, *Manduca sexta* herbivory of *Nicotiana* leaves and flowers increases the amount of alkaloids in new leaves and flower nectar (Baldwin, 1999; Adler *et al*., 2006).

Liriodenine (Fig. 1) is a benzylisoquinoline alkaloid (oxoaporphine) that has antibacterial (Hufford *et al.*, 1975, 1980; Villar *et al.*, 1987; Nissanka *et al.*, 2001; Rahman *et al.*, 2005; Wirasathien *et al.*, 2006), antifungal (Hufford *et al*., 1980; Nissanka *et al.*, 2001; Rahman *et al.,* 2005), antiprotozoal (Waechter *et al.*, 1999; Wirasathien *et al.*, 2006), and cytotoxic (Chen *et al.*, 1997; De Siqueira *et al.*, 1998; Chang *et al.*, 2004; Wirasathien *et al.*, 2006) properties. To date, liriodenine has demonstrated activity against 22 phytopathogenic agents, mostly *in vitro* and seven *in situ* (Table I), which suggests

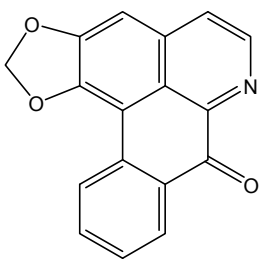

Fig. 1. Chemical structure of liriodenine.

Table I. Liriodenine activity on phytopathogenic organisms.

Phytopathogen	Plant disease		Sensibility		Reference
Agrobacterium tumefaciens (b)	Crown gall	10 μg/disc	IC	*In vitro*	Khan *et al.* (2002)
Aspergillus flavus	Yellow mold	400 μg/disc	IH: 17 mm	*In vitro*	Rahman *et al*. (2005)
Aspergillus fumigatus	Grain storage mold	25 μg/mL	MIC	*In vitro*	Hufford *et al.* (1980)
Aspergillus niger ATCC 16888	*Aspergillus* crown rot	3.1 μg/mL	MIC	*In vitro*	Hufford *et al.* (1980)
Aspergillus niger	*Aspergillus* crown rot	400 μg/disc	IH: 15 mm	*In vitro*	Rahman *et al*. (2005)
Aspergillus versicolor	Grain storage mold	400 μg/disc	IH: 28 mm	*In vitro*	Rahman *et al*. (2005)
Botrytis fabae	Gray mold	38 μg/mL	B	*In situ*	Hufford *et al.* (1980)
Cladosporium cladosporioides	*Hormodendrum* ear rot	2 μg; 100 μg	IC	*In vitro*	Monteiro *et al*. (2007)
Cladosporium sphaerospermum	*Cladosporium* rot	100 μg	IC	*In vitro*	Monteiro *et al*. (2007)
Cryptococcus neoformans		12.5 μg/mL	MIC	*In vitro*	Zhang *et al*. (2001)
Erysiphe polygoni	Powdery mildew	300 μg/mL	B	*In situ*	Wu (2006)
Fomitopsis pinicola	Brown crumbly rot	2 μg/mL	MIC	*In vitro*	Wu (2006)
Gloeophyllum trabeum	Wood rots	2 μg/mL	MIC	*In vitro*	Wu (2006)
Helminthosporium teres	Barley brown patches	38 μg/mL	B	*In situ*	Wu (2006)
Laetiporus sulphureus	Brown cubical rot	2 μg/mL	MIC	*In vitro*	Wu (2006)
Lenzites betulina	Wood decay	0.76 μg/mL	MIC	*In vitro*	Wu (2006)
Phytophora infestans (p)	Potato late blight	300 μg/mL	A	*In situ*	Hufford *et al.* (1980)
Plasmopora viticola (p)	Downy mildew of grapes	38 μg/mL	B	*In situ*	Hufford *et al.* (1980)
Puccinia graminia-tritici	Stem rust, cereal rust	300 ppm	B	*In situ*	Hufford *et al.* (1980)
Pyricularia oryzae	Rice leaf blast	38 μg/mL	B	*In situ*	Wu (2006)
Trametes versicolor	Wood rot	3.5 μg/mL	MIC	*In vitro*	Khan *et al.* (2002)
Xanthomonas campestris (b)	Leaf spots, blights	10 μg/disc	IC	*In vitro*	Khan *et al.* (2002)
Syncephalastrum racemosum	Black soft rot	3.1 μg/mL	MIC	*In vitro*	Hufford *et al.* (1980)

A, 97 to 100% disease control; B, 90 to 96% disease control; IH, inhibition halo; IC, inhibitory concentration; MIC, minimum inhibitory concentration; b, bacteria; p, protozoon.

that it likely participates in plant defense responses.

The presence and abundance of liriodenine in several Annonaceae family species has been reported (Guinaudeau *et al*., 1994; Bentley, 2002, 2003, 2004, 2005, 2006). In particular, liriodenine is rich in *Annona diversifolia* roots of adult specimens, and is present in early germination stages in endosperm and rootlets, as well as in roots and stems of seedlings (González-Esquinca *et al.*, 2005). A seedling with two to six leaves at approximately 30 to 50 days of age has about the same amount of liriodenine as an adult plant (0.2% of the plant dry weight) (González-Esquinca, 2001; De la Cruz-Chacón and González-Esquinca, 2011). This early production indicates that it is a substance of biological significance during early development. Furthermore, during seed storage and imbibition for germination *in vitro*, seeds are often attacked by fungi that manage to completely break down the endosperm, inflicting damage on the embryo (rudimentary in Annonaceae). However, if seeds are able to germinate, the fungal population decreases as the embryo develops, a phenomenon that coincides with the emergence and subsequent increase of liriodenine. This suggests that the presence of liriodenine may inhibit the growth of fungal pathogens. To investigate a possible defense role of liriodenine, we evaluated the activity of purified liriodenine and alkaloidal extracts from *Annona diversifolia* embryo radicles in early developmental stages, seedling radicle, and roots against fungi that are phytopathogenic for this species.

Methods and Materials

Plant material

In September 2007, roots were collected along with 10,000 seeds of *A. diversifolia* in Delegación

de Copoya, Chiapas, Mexico. The reference specimen (352) was deposited in the Eizi Matuda Herbarium, Universidad de Ciencias y Artes de Chiapas, Tuxtla Gutiérrez, Chiapas, México.

Developmental stages evaluated

A. diversifolia at five stages of early development was obtained: unimbibed seeds, day 1 of imbibition, days 2 and 10 of germination (radicles of 1 and 5 cm, respectively), and 50-day-old seedlings (with six leaves). To this end, 500 seeds were randomly selected for each assay and germinated using the technique of germination between paper (Ministerio de Agricultura, 1976). To reach the desired stage of development, seeds were placed within Conviron™ series CMP4030 plant growth chambers (Winnipeg, Canada) and maintained at 24–28 °C with 12 h of light at an intensity of 500 μmol/(m^2 s), achieved using white light from fluorescent lamps, and relative humidity between 65% and 75%. For each stage, embryos, radicles, and roots were carefully separated, and 10 g (fresh weight) plant material, or 2 g for embryos, were used for alkaloid extraction. Tests were conducted in quintuplicate.

Total alkaloid extraction from plant organs

Plant material was dried at room temperature. After being ground thoroughly, samples (10 g) were wetted with a saturated solution of Na_2CO_3 (35 mL) and then allowed to dry for 48 h at room temperature. Alkaloids were extracted with $CHCl_3$ (250 mL) under constant agitation for 1 h, subjected to filtration to obtain the chloroform phase, and then washed with distilled water. The chloroform extracts were extracted with 1 M HCl (125 mL), and then the aqueous phase was alkalized to pH 9.5 with a saturated Na_2CO_3 solution, after which it was extracted again with $CHCl_3$ (60 mL). Excess water was removed with anhydrous Na_2SO_4. To obtain a total alkaloid extract, the solvent was evaporated *in vacuo* at 25 °C. The alkaloid mixture gave a positive reaction with Dragendorff reagent and was subsequently analysed by high-performance liquid chromatograpy (HPLC). All extractions were performed in quintuplicate.

Liriodenine source and identification

Liriodenine was isolated from roots of *A. diversifolia* as previously described (De la Cruz-Chacón and González-Esquinca, 2011). A purified sample of liriodenine was identified by Dr. Mariano Martínez Vázquez, Instituto de Química, Universidad Nacional Autónoma de México (UNAM), Cd. de México, Distrito Federal, México.

Quantification of liriodenine in extracts

Liriodenine was quantified by liquid chromatography using a Perkin Elmer HPLC system, Series NCI 200 (Norwalk, CT, USA) and a Spheri-5 RP-18 reverse phase column (100 x 4.6 mm; 5 μm particle diameter). The mobile phase consisted of water/methanol (30:70, isocratic), and the flow rate was 1 mL/min; the column temperature was maintained at 30 °C. UV detection was conducted at 254 nm. A calibration curve was obtained by analysing a series of stock solutions containing 100, 50, 25, 6.25, 3.13, 1.56, 0.78, 0.39 μg/mL of liriodenine. The curve was linear in this range, with an r value of 0.99 and r^2 = 0.98. The assay conditions were determined using the Perkin Elmer Turbochrom work platform. Extracts were dissolved at a concentration of 1 mg/mL, and then 20 μL of this solution was analysed by HPLC. All liriodenine peaks were compared to its characteristic absorption bands [248 (log ε 4.47), 272 (log ε 4.41), 310 (log ε 3.91), and 414 nm (log ε 4.01)]. The absorption spectra were obtained using a Thermo Electron Corporation Genesys 10 UV spectrophotometer (Madison, WI, USA).

Isolation of fungi from A. diversifolia seeds

Ten seeds that were contaminated with fungi during the process of imbibition were selected. Fungal samples were obtained with a sterile microbiological loop, and then cultured in culture plates on BD BIOXON™ Sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) (Cuatitlan Izcalli, Estado de México, México) in a RIOSSA™ (Cd. de México, Distrito Federal, México) incubator for 7–8 d at 30 °C. The most abundant colonies were selected and then used for seven consecutive reseedings in both types of culture media to isolate and purify the microorganisms. The isolated fungi were inoculated on SDA to obtain strains, which were stored in glycerol and/or liquid paraffin at 4 °C until use. *Rhizopus stolonifer* and *Aspergillus glaucus* were identified at the phytosanitary laboratory "Grupo de Estudios Moleculares aplicados a la Biología" (GeMBio), Centro de Investigación Científica de

Yucatán (CICY), Mérida, México, using morphometric keys from PDA cultures. Specimens were registered with the identification keys GeMBio NACF0010 and NACF0011. For activity assays, fungi were reseeded twice consecutively in SDA and incubated at 37 °C for intervals of 24 to 72 h before use.

Antifungal assays

Liriodenine antifungal activity was determined by the agar diffusion assay (Bauer *et al.*, 1966), minimum inhibitory concentration (MIC) in macrodilution (NCCLS, 1997), and glucose metabolism assay (Riesselman *et al.*, 2000; Li *et al.*, 2000). Extract activity was tested only by the glucose metabolism assay. For all three methods, we used an inoculum of $1-5 \cdot 10^6$ CFU/mL in Sabouraud dextrose broth (SDB) (Difco BD, Sparks, MD, USA) adjusted turbidimetrically to 0.1 absorbance units at 530 nm, according to a McFarland 0.5 scale, using a Thermo Electron Genesys 10 UV spectrophotometer. All determinations were made in triplicate. For the agar diffusion assay, we used Whatman No. 2 paper discs (5 mm in diameter) impregnated with 400, 200, 100, and 50 μg of liriodenine. Tests were conducted on SDA, and inhibition halos were measured after 48 h of incubation with *R. stolonifer* and after 72 h with *A. glaucus* at 37 °C.

Determination of MIC values was performed in a final volume of 2 mL of SDB at 37 °C. Liriodenine was dissolved in ethanol (Baker™, Xalostoc, Estado de México, México) at a final content of 0.5% v/v, and then evaluated at concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.25 and 1.1 μM. The cultures were incubated for 48 h at 37 °C. MIC was determined as the lowest concentration that inhibited growth.

Inhibition of the glucose metabolism was assessed by determining the amount of glucose consumed by the fungus as a source of carbon and energy after 48 h. Liriodenine was evaluated at concentrations of 100, 10, 1 μM, and extracts were evaluated at a concentration of 100 μg/mL. The assay was performed in 2 mL of SDB containing 20 mg/mL of glucose. Fungal inhibition was measured indirectly by determining the residual amount of glucose in the medium after 48 h of incubation at 37 °C. Glucose concentration was assessed by the Nelson-Somogyi method of determining reducing sugars using linear regression of a standard curve. The percentage of residual glucose for each concentration of liriodenine was calculated by comparing the glucose concentration in each assay with the glucose concentration in the culture medium, according to the following formula: % residual glucose = (assay glucose concentration/culture medium glucose concentration) · 100. The percent inhibition of glucose metabolism due to liriodenine was determined according to the following formula: % glucose inhibition = [1 – (100 – % residual glucose in each concentration/100 – % residual glucose in growth control)] · 100.

Statistical analysis

All experimental data were expressed as means ± standard deviation of five replicates (n = 5). Data were analysed by ANOVA, and further analysed by the Fisher´s least significant difference (LSD) test when significant differences were detected. A P value < 0.05 was considered significant.

Results

Liriodenine inhibited the growth of both species of fungi in all assays (Table II). The inhibitory effect of liriodenine was more significant for *A. glaucus* (MIC 100 μM) than *R. stolonifer* (MIC

Table II. Liriodenine antifungal activity in different assays.

Fungus	Assay diffusion (inhibition zone in mm)				Inhibition of glucose metabolism (%)			MIC	
	Tested concentration [μg/disc (μmol/disc)]				Tested concentration [μmol/L (μg/mL)]				
	50 (0.18)	100 (0.36)	200 (0.73)	400 (1.46)	1 (0.27)	10 (2.75)	100 (27.5)	[μmol/L]	[μg/mL]
A. glaucus	$7^{a} \pm 0.8$	$11^{ab} \pm 1.5$	$16^{b} \pm 2.1$	$26^{c} \pm 4.5$	$55^{a} \pm 5.0$	$67^{b} \pm 3.6$	$80^{c} \pm 6.0$	100.0	27.5
R. stolonifer	0 ± 0.0	$7^{b} \pm 0.6$	$10^{c} \pm 1.5$	$12^{d} \pm 0.7$	$10^{a} \pm 5.1$	$35^{b} \pm 4.7$	$55^{c} \pm 6.8$	200.0	55.1

Values in a row followed by different letters are significantly different ($P \leq 0.05$; LSD test).

Table III. Antifungal activity of alkaloid extracts (at 100 μg/mL).

Tested extract	Inhibition of glucose metabolism (%)	
	A. glaucus	*R. stolonifer*
Embryo not imbibed	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
Embryo with 1 day imbibition	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
Seedlings with radicle of 1 cm	$29.7^{b} \pm 1.9$	$9.2^{a} \pm 3.1$
Seedlings with radicle of 5 cm	$81.8^{c} \pm 12.3$	$79.9^{b} \pm 10.1$
Roots of seedlings with six leaves	$89.2^{c} \pm 5.7$	$84.3^{b} \pm 9.9$

Values in a row followed by different letters are significantly different ($P \leq 0.05$; LSD test).

200 μM) ($P = 0.05$). For *A. glaucus*, the susceptible concentration of liriodenine was 50 μg in the diffusion assay ($P = 0.0001$) and 1 μM in the glucose metabolism assay ($P = 0.003$) while for *R. stolonifer* these concentrations were 100 μM ($P = 0.0000$) and 10 μM ($P = 0.0002$), respectively. For concentrations that had no visible effects, neither of the microorganisms was able to sporulate. The results of the glucose metabolism assay suggested that the effects of liriodenine are not immediate, or at least not within 48 h, since at all concentrations both fungi consumed a similar percentage of glucose.

For the alkaloid extracts of *A. diversifolia*, a concentration of 100 μg/mL was able to inhibit the *in vitro* glucose consumption of both fungi ($P = 0.0000$). The inhibitory effect was slight in extracts of 1-cm radicles, whereas in extracts of 5-cm radicles and seedling roots activity increased significantly by 3- and 9-fold ($P = 0.0000$; $P = 0.0000$) (Table III). The activity of these extracts was similar to that of pure liriodenine in the range of 2.75–27.5 μg/mL. The liriodenine content in 5-cm radicle extracts was in this range, while in the seedling roots the quantity was two to 25 times higher ($P = 0.0000$), which explains the increased activity of the latter (Table IV).

The chromatographic profiles of the extracts were poor, revealing between three and five compounds, including liriodenine, as the major components. The presence of these other compounds apparently did not have significant effects at the concentrations assayed, because if they were the major components of the extract (seeds with 1-cm radicles), the extract did not exhibit sufficient inhibitory activity. When the presence of liriodenine was more prominent in the extracts, antifungal activity increased (Tables III and IV). The bioassays supported this conclusion. Table II shows that at all concentrations tested, liriodenine was less active against *R. stolonifer* than *A. glaucus*. However, extracts from 5-cm radicles and seedling root extracts (Table III) each exhibited the same level of activity against both species. This would not be the case if the only active compound in the extracts was liriodenine. Furthermore, the chromatographic profile of the 1-cm radicle extract showed that liriodenine was present in minor quantities, yet there was still some inhibition of the two fungi, which was further evidence for one or more bioactive compounds in the extracts in addition to liriodenine.

The antifungal activity of *A. diversifolia* extracts, and of pure liriodenine at concentrations present in plant extracts, indicated the importance of this alkaloid as a component of extract activity. However, the results suggested that there is at least a second alkaloid, in lower portion than liriodenine, in seedling root extracts that may contribute to the antifungal activity.

Table IV. Liriodenine presence in alkaloid extracts of *Annona diversifolia*.

Developmental stage	Time [d]	Alkaloidal extract (%)	Liriodenine in extract (%)	Liriodenine content [μg/g dried tissues]	Other alkaloids (%)
Dry seeds	0	0.0	-	-	-
Seeds imbibed for 1 day	2	0.0	-	-	-
Seedlings with radicle of 1 cm	13	0.004 ± 0.00	2.84 ± 0.51	$1.13^{a} \pm 0.39$	-
Seedlings with radicle of 5 cm	24	0.34 ± 0.01	19.2 ± 2.28	$125.51^{b} \pm 22.9$	-
Seedlings with six leaves	50	0.12 ± 0.02	69.06 ± 4.22	$849.04^{c} \pm 177.58$	10.7 ± 1.9

Values in a row followed by different letters are significantly different ($P \leq 0.05$; LSD test).

Discussion

Early biosynthesis of liriodenine in *A. diversifolia* is a phenomenon similar to that seen in *Glycine max*, whose 7 day-old seedlings are able to synthesize conjugated isoflavones (daidzein and genistein) before and during infection by *Phytophthora sojae*, in quantities sufficient to inhibit the phytopathogen (Graham, 1991; Lozovaya *et al.*, 2004). This is similar to several cereal species that biosynthesize hydroxamic acids as an anticipated response to attack by aphids (*Metopolophium dirhodum*, *Rhopalosiphum maidis*, *Schizaphis graminum*, and *Sitobion avenae*). Barley, rye, and wheat seeds start producing hydroxamic acids on the second day of germination (Niemeyer, 1988).

Germination and early seedling development are critical stages of plant development that allow plants to settle effectively in an environment. It appears that the presence of secondary metabolites (*i.e.* liriodenine in *A. diversifolia*) is an important strategy during this process.

The two fungal species examined in the current study are phytopathogenic for several species of the Annonaceae family. *R. stolonifer* invades the fruit pulp of *Annona muricata* to cause brown rot in the fruits, and is considered a serious post-harvest problem (Pinto *et al.*, 2005). *A. glaucus* feeds on the seed energy reserves of different cereal species (Christensen *et al.*, 1990; Lillehoj, 1992); thus, inhibition would have beneficial effects on the developing plant.

The potency of liriodenine against *R. stolonifer* and *A. glaucus* was lower than that of other fungal phytopathogens (Table II), although at the concentrations observed in the current study (MIC values of 27.5 and 55.1 μg/mL), this compound can inhibit the majority of such microorganisms. We propose bacterial gyrase as a probable molecular target, similar to topoisomerase II, an enzyme involved in DNA replication and transcription, which was shown by Woo *et al.* (1997, 1999) to be susceptible to liriodenine.

The amount of liriodenine in 5-cm radicles and in seedling roots was about two to 14 times higher (125.51 and 849.04 μg/g dried tissue, respectively) than the *in vitro* MIC for *A. glaucus*, four to 28 times higher than the MIC for *R. stolonifer*, and 90 to 300 times higher than that of other phytopathogens. In addition, according to De la Cruz-Chacón and González-Esquinca (2011), liriodenine is present during germination in endosperm at concentrations of 0.6–36.6 μg/g tissue, and in hypocotyls and seedling stems at concentrations of 7.1–181.9 μg/g tissue. Thus, essentially the entire developing embryo would have concentrations of liriodenine equal to that required *in vitro* to achieve partial or total inhibition of fungal growth. These observations manifest energy resource allocation to early liriodenine biosynthesis and its possible role in defense mechanisms during the initial stages of *A. diversifolia* development.

The fact that non-germinated seeds are susceptible to attack by these fungi implies that secondary metabolites detected in seeds, including acetogenins, laherradurin, rolliniastatin-2, isorolliniastatin, and cherimolin-2 (González-Esquinca, 2001; Schlie-Guzmán *et al.*, 2009; Luna-Cazáres and González-Esquinca, 2010), are not sufficient barriers to the degradation of reserve material. Laherradurin and rolliniastatin-2 are found in seeds in quantities (1000 and 30 μg/g tissue, respectively) similar to that of liriodenine during germination. Given that *A. glaucus* and *R. stolonifer* are resistant to these compounds at such concentrations, and the fact that the presence of alkaloids is required for fungal inhibition, liriodenine, which is the most abundant of the alkaloids, most likely plays an important role in defense mechanisms in *A. diversifolia*, and perhaps also in other species as well. Furthermore, other bioactive compounds in addition to liriodenine most likely participate in these defenses.

Acknowledgements

We thank al programa del Doctorado en Ciencias Biológicas de la UNAM, also CONACYT and Dr. Mariano Martínez Vázquez for their help in the structural identification of liriodenine.

Adler L. S., Wink M., Distl M., and Lentz A. (2006), Leaf herbivory and nutrients increase nectar alkaloids. Ecol. Lett. **9**, 960–967.

Baldwin I. T. (1999), Inducible nicotine production in native *Nicotiana* as an example of adaptive. J. Chem. Ecol. **25**, 3–30.

Bauer A. W., Kirby W. M., Sherris J. C., and Turck M. (1966), Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin. Pathol. **45**, 493–496.

Bentley K.W. (2002), *β*-Phenylethylamines and the isoquinoline alkaloids. Nat. Prod. Rep. **19**, 332–356.

Bentley K. W. (2003), *β*-Phenylethylamines and the isoquinoline alkaloids. Nat. Prod. Rep. **20**, 342–365.

Bentley K. W. (2004), *β*-Phenylethylamines and the isoquinoline alkaloids. Nat. Prod. Rep. **21**, 395–424.

Bentley K. W. (2005), *β*-Phenylethylamines and the isoquinoline alkaloids. Nat. Prod. Rep. **22**, 249–268.

Bentley K. W. (2006), *β*-Phenylethylamines and the isoquinoline alkaloids. Nat. Prod. Rep. **23**, 444–463.

Chang H. C., Chang F. R., Wu Y. C., and Lai Y. H. (2004), Anti-cancer effect of liriodenine on human lung cancer cells. Kaohsiung J. Med. Sci. **20**, 365–371.

Chen K. S., Wu Y. C., Teng C. M., Ko F. N., and Wu T. S. (1997), Bioactive alkaloids from *Illigera luzonensis*. J. Nat. Prod. **60**, 645–647.

Christensen C. M., Meronuck R. A., and Sauer D. B. (1990), Moisture content, invasion by *Aspergillus glaucus*, and germ discoloration in blends of corn of different initial moisture contents. Plant Dis. **74**, 985–988.

De la Cruz-Chacón I. and González-Esquinca A. R. (2011), Liriodenine alkaloid in *Annona diversifolia* during early development. Nat. Prod. Res. (in press).

De Siqueira J. M., Bomm M. D., and Gomes P. N. F. (1998), Estudo fitoquímico de *Unonopsis lindmanii* – Annonaceae, biomonitorado pelo ensaio de toxicidade sobre *Artemia salina* Leach. Quím. Nova **21**, 557–559.

Dixon R. A. (2001), Natural products and plant disease resistance. Nature **411**, 843–847.

González-Esquinca A. R. (2001), Contribución al estudio del género *Annona* (Annonaceae). Análisis fitoquímico de tres especies del estado de Chiapas. Ph. D. thesis, Universidad Nacional Autónoma de México, Distrito Federal, México.

González-Esquinca A. R., Luna-Cazáres L. M., and De la Cruz Ch. I. (2005), The alkaloid liriodenine during germination of *Annona diversifolia* Saff. XVII. International Botanical Congress, International Association of Botanical and Mycological Societies, July 17 to 23, 2005, Vienna, Austria.

Graham T. L. (1991), Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. Plant Physiol. **95**, 594–603.

Guinaudeau H., Leboeuf M., and Cavê A. (1994), Aporphine alkaloids V. J. Nat. Prod. **57**, 1033–1135.

Hartmann T. (2007), From waste products to ecochemicals: Fifty years research of plant secondary metabolism. Phytochemistry **68**, 2831–2846.

Hufford C. D., Funderburk M. J., Morgan J. M., and Robertson L. W. (1975), Two antimicrobial alkaloids from heartwood of *Liriodendron tulipifera* L. J. Pharm. Sci. **64**, 789–792.

Hufford C. D., Sharma A. S., and Oguntimein B. O. (1980), Antibacterial and antifungal activity of liriodenine and related oxoaporphine alkaloids. J. Pharm. Sci. **69**, 1180–1183.

Khan M. R., Kihara M., and Omoloso A. D. (2002), Antimicrobial activity of *Michelia champaca*. Fitoterapia **73**, 744–748.

Li R. K., Elie C. M., Clayton G. E., and Ciblak M. A. (2000), Comparison of a new colorimetric assay with the NCCLS broth microdilution method (M-27A) for antifungal drug MIC determination. J. Clin. Microbiol. **38**, 2334–2338.

Lillehoj E. B. (1992), Aflatoxin: genetic mobilization agent. In: Mycotoxins in Ecological Systems, Handbook of Applied Mycology, Vol. 5 (Bhatnagar D., Lillehoj E. B., and Arora D. K., eds.). Marcel Dekker, New York, pp. 1–22.

Lozovaya V. V., Lygin A. V., Li S., Hartman G. L., and Widholm J. M. (2004), Biochemical response of soybean roots to *Fusarium solani* f. sp. glycines. Infection Crop Sci. **44**, 819–826.

Luckner M. (1980), Expression and control of secondary metabolism. In: Secondary Plant Products. Encyclopedia of Plant Physiology (Bell E. A. and Charlwood B. V., eds.). Springer Verlag, New York, pp. 23–50.

Luna-Cazáres L. M. and González-Esquinca A. R. (2010), Susceptibility of complete bacteria and spheroplasts of *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* to rolliniastatin-2. Nat. Prod. Res. **24**, 1139–1145.

Ministerio de Agricultura (1976), Reglas Internacionales para ensayos de semillas. Instituto nacional de semillas y plantas de vivero. Dirección General de la Producción Agraria, reprinted for the SARH, Distrito Federal, México.

Monteiro M. C. M., Leptokarydis I. H., Silva G. H., da Silva V. C., Bolzani V. S., Young M. C. M., and Lopes M. N. (2007), Constituintes químicos isolados dos caules de *Michelia champaca* L. (Magnoliaceae). Eclet. Quím. **32**, 13–18.

NCCLS (National Committee for Clinical Laboratory Standards) (1997), Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard, NCCLS document M27-A. National Committee for Clinical Laboratory Standards, Wayne, PA, USA.

Niemeyer H. M. (1988), Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones): defence chemicals in the Gramineae. Phytochemistry **27**, 3349–3358.

Nissanka A. P., Karunaratne V., Bandara B. M., Kumar V., Nakanishi T., Nishi M., Inada A., Tillekeratne L. M., Wijesundara D. S., and Gunatilaka A. A. (2001), Antimicrobial alkaloids from *Zanthoxylum tetraspermum* and *caudatum*. Phytochemistry **56**, 857–861.

Pinto A. C. de Q., Cordeiro M. C. R., De Andrade S. R. M., Ferreira F. R., De C. Filgueiras H. A., Alves R. E., and Kinpara D. I. (2005), In: *Annona* Species. Taxonomy and Botany (Williams J. T., ed.). International Centre Underutilised Crops, University of Southampton, Southampton, UK, pp. 3–16.

Rahman M. M., Loppa S. S., Sadik G., Harun-Or-Rashid, Islam R., Khondkar P., Alam A. H., and Rashid M. A. (2005), Antibacterial and cytotoxic compounds from the bark of *Cananga odorata.* Fitoterapia **76**, 758–761.

Riesselman M. H., Hazen K. C., and Cutler J. E. (2000), Determination of antifungal MICs by a rapid susceptibility assay. J. Clin. Microbiol. **38**, 333–340.

Schlie-Guzmán M. A., García-Carrancá A., and González-Esquinca A. R. (2009), *In vitro* and *in vivo* antiproliferative activity of laherradurin and cherimolin-2 of *Annona diversifolia* Saff. Phytother. Res. **23**, 1128–1133.

Villar A., Mares M., Rios J. L., Canton E., and Gobernado M. (1987), Antimicrobial activity of benzylisoquinoline alkaloids. Pharmazie **42**, 248–250.

Waechter A. I., Cavé A., Hocquemiller R., Bories C., Muñoz V., and Fournet A. (1999), Antiprotozoal activity of aporphine alkaloids isolated from *Unonopsis buchtienii* (Annonaceae). Phytother. Res. **13**, 175–177.

Wirasathien L., Boonarkart Ch., Pengsuparp T., and Suttisri R. (2006), Biological activities of alkaloids from *Pseuduvaria setosa*. Pharm. Biol. **44**, 274–278.

Woo S. H., Reynolds M. C., Sun N. J., Cassady J. M., and Snapka R. M. (1997), Inhibition of popoisomerase II by liriodenine. Biochem. Pharmacol. **54**, 467–473.

Woo S. H., Sun N. J., Cassady J. M., and Snapka R. M. (1999), Topoisomerase II inhibition by aporphine alkaloids. Biochem. Pharmacol. **57**, 1141–1145.

Wink M. (2010), Introduction: Biochemistry, physiology and ecological functions of secondary metabolites. In: Biochemistry of Plant Secondary Metabolism, 2nd ed. (Wink M., ed.). Wiley-Blackwell, Oxford, UK, pp. 1–19.

Wu C. C. (2006), Study on antifungal compounds of *Michelia formosana*. Master Thesis, School of Forestry and Resource Conversion, National Taiwan University, Taipei, Taiwan.

Zhang Z., ElSohly H. N., Jacob M. R., Pasco D. S., Walker L. A., and Clark A. M. (2001), New sesquiterpenoids from the root of *Guatteria multivenia.* J. Nat. Prod. **65**, 856–859.