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ARTICLE *in* BIOCONTROL · APRIL 2009

Impact Factor: 1.69 · DOI: 10.1007/s10526-008-9180-y

BioControl (2009) 54:459–466
DOI 10.1007/s10526-008-9180-y

Activity of quinolizidine alkaloids from three Mexican *Lupinus* against the lepidopteran crop pest *Spodoptera frugiperda*

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Received: 23 March 2006 / Accepted: 10 June 2008 / Published online: 27 June 2008

Abstract Bitter lupins (*Lupinus* spp.) are not used as a protein source because of their toxicity. However, they may have alternative uses as potential sources of natural insecticides. Quinolizidine alkaloids (QA) of three Mexican *Lupinus* species (Fabaceae): *L. montanus* (HBK), *L. stipulatus* (Agardh) and *L. aschenbornii* (Schauer), were analyzed by capillary Gas Chromatography-Mass Spectrometry. Sparteine was found in high amounts in both *L. montanus* and *L. aschenbornii* while the major alkaloids in *L. stipulatus* extract were aphylline and an epiaphylline-like compound. Alkaloid extracts were tested for their insecticidal activity using larvae of the Fall Armyworm, *Spodoptera frugiperda* (Smith); (Lepidoptera, Noctuidae) as a model pest. We compared LD_{50} values and mean weight of caterpillars fed with alkaloid extracts of the three species studied with those of sparteine, a widespread QA found in various lupin species. Extracts of *L. montanus* and *L. aschenbornii* were found to be as effective as sparteine and extracts *L. stipulatus* were found to be the most toxic against the larvae of *S. frugiperda*. This suggests that the various QA act differently on caterpillars, and could be used to control *Spodoptera* populations.

Keywords *Lupinus montanus* · *Lupinus stipulatus* · *Lupinus aschenbornii* · Sparteine · Insecticidal activity · Botanical insecticides

Abbreviations

GC-MS	Gas Chromatography coupled with Mass Spectrometry
LD_{50}	Lethal Dose killing 50% of individuals
MS	Mass Spectrometry
QA	Quinolizidine Alkaloid
RI	Kovats Retention Index

Introduction

Lupinus is one of the largest genera of the family Fabaceae. Around 500 species have been described worldwide, most of them occurring in America, with only 12 in Europe and North Africa. More than 100 lupin species grow in Mexico (Bermúdez-Torres 1998). Only few are used as potential protein crops: *Lupinus mutabilis* Sweet, a South American species,

Handling editor: Samantha Cook.

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and the European *Lupinus angustifolius* L., *Lupinus albus* L. and *Lupinus luteus* L. Other species are also cultivated, mainly for ornamental uses, such as *Lupinus polyphyllus* Lindley (Gross 1982; IENICA (Interactive European Network for Industrial Crops and their Applications) 2002). The remaining species of this large genus have been poorly studied, mainly because of their bitterness and/or toxicity. Considering the high diversity of this genus in Mexico, an exploratory study was started by Mexican research institutes to search for some alternative uses for their endemic species.

Quinolizidine alkaloids (QA) are secondary compounds typical of lupins (Kinghorn and Balandrin 1984). They are produced in large amounts and serve as chemical defense against pathogens and herbivores (Wink 1988). The secondary chemistry of Mexican *Lupinus* species has not been studied in detail to date, but it is potentially interesting, because of the high diversity of the both the taxa and the alkaloid structures they produce (Bermúdez-Torres 1998).

QA show a wide range of biological activities: they can inhibit the multiplication of viruses (Wink 1987), the proliferation of bacteria (Wink 1984a; Tyski et al. 1988; De la Vega et al. 1996) and the growth of certain fungi (Wink 1984a; Wippich and Wink 1985). Some allelopathic (phytotoxic) effects of QA have been described, including the inhibition of the growth of competing plants (Wink 1983; Wink and Twardowski 1992; Múzquiz et al. 1994). They can also deter a number of herbivores (nematodes, caterpillars, beetles, aphids, locusts, snails, rabbits and cows) but also pollinators such as bees (Gegear et al. 2007). Some are directly toxic or mutagenic (Wink 1984b, 1994). Deterrent or toxic effects of QA such as sparteine, lupanine, cytisine and 13-tigloyloxylupanine against phytophagous insects have been evaluated in some detail on some Lepidoptera. Several authors suggest that acetylcholine receptors and Na^+/K^+ channels are modulated by these compounds (Paolisso et al. 1985; Korcz et al. 1987; Wink 1992, 1993).

Maize is one of the major crops grown in Mexico and larvae of *Spodoptera frugiperda* (Smith) are a well-known pest of this plant. The control of this phytophagous insect is therefore economically important (Avila and Mendoza 1981). Synthetic insecticides such as organochlorides and organophosphates are used to control *Spodoptera* populations (Regnault-Roger 1997). These compounds possess the disadvantage of being non-specific in terms of toxicity (including potential toxicity to mammals), they have a long persistence in the soil and water, and furthermore, development of resistance is a potential problem. Natural compounds, such as alkaloids, derived from wild plants may represent interesting alternative bio-rational pesticides against phytophagous insects (Usmani and Knowles 2001).

The aim of this study was to characterize the QA present in three Mexican *Lupinus* species (*Lupinus montanus* HBK, *Lupinus stipulatus* Agardh and *Lupinus aschenbornii* Schauer) and to evaluate the insecticidal activities of their extracts against *S. frugiperda*, compared to the known QA sparteine.

Materials and methods

Plant material

Lupinus species were collected in the Popocatepetl-Iztaccihuatl massif between October and November 2006. The lupin species studied were harvested at different altitudes: *L. montanus* HBK (3800 m above sea-level), *L. stipulatus* Agardh (2,942 m asl) and *L. aschenbornii* Schauer (4,000 m asl). Only leaves were collected and they were dried at room temperature upon return to the laboratory.

Alkaloid extraction

Alkaloid extraction was carried out as described in Wink et al. (1995). Leaves were ground up and homogenized in 1 M hydrochloric acid. The homogenate was adjusted to pH 12 with 6 M aqueous sodium hydroxide solution. Alkaloids were extracted by solid phase extraction using Extrelut-columns® (Merck) and dichloromethane as eluent.

Characterization of alkaloids

Alkaloid extracts were separated and analyzed by Gas Chromatography-Mass Spectrometry with a Mod. HP 6890/5972 apparatus. The column type used was DB 1 30 m length, 0.25 mm i.d., 0.25 μm film thickness. The running conditions applied were a split ratio of 1:20, He carrier gas flow 1 ml min^{-1}, injector temperature was heated to 280°C. The oven

temperature was programmed as follows: 120°C, 3 min isothermal, 120–260°C with a rate of 10°C min^{-1}, then 10 min isothermal. The electron impact mass spectra were recorded at 70 eV ionization energy, scan (50–550 VMA). The Kovats index was determined by co-chromatography of a mixture of linear alkanes. Quinolizidine alkaloids were identified according to their Kovats retention indices (RI) (Wink et al. 1981, 1983), molecular ion (M^+) and the significant fragments and their relative abundance (Wink et al. 1980; Wink et al. 1981, 1982, 1983). Sparteine (100 μg ml^{-1}) was used as external standard. The amount of the alkaloid, calculated using the amount of sparteine as reference, was the weight of each compound divided by the weight of the dried material (0.5 g) from which it was derived.

Insects

Eggs of *S. frugiperda* were from a laboratory strain (CEIB, UAEM, Mexico). The eggs were placed in Petri dishes until emergence. The larvae were reared on an artificial corn/bean diet as described by Franco-Archundia et al. (2006) under laboratory conditions (20 ± 1.5°C, r. h. 70 ± 5% and 16L:8D photoperiod) until the pupal stage. Care was taken to avoid any parasitism.

Bioassays

The bioassays were carried out as described by Franco-Archundia et al. (2006), with adjustment of the amount of food given (15 ml instead of 5 ml). Artificial diet was mixed with methanol-diluted sparteine or lupin extracts (maximum value: 600 μl of methanol) at concentrations of 7, 12.5, 25, 50 and 100 μg ml^{-1}. Two negative controls were performed: one control containing only the artificial diet and another one containing 600 μl of methanol. Artificial diet (15 ml) containing respective extract/sparteine were placed in 35 ml plastic flask with a ventilated lids. A single larva was placed in each flask, 30 replicates for each treatment were carried out. Flasks were placed randomly under controlled conditions (as above) in a breeding chamber until pupation.

The insecticidal activity of extracts was determined by measuring mortality periodically through development (day 7, 10, 17, 21, 24, 28, 31 and 35). In surviving insects, we measured larval weight (day 7, 10, 17, 21 and 24). Respective LD_{50} doses were chosen for this measurement in order to better compare with mortality tests.

Statistical analyses

Values of LD_{50} (doses at which 50% of larvae died within a defined time) were calculated using each concentration by PROBIT regression (Statistica 7.0 software) independently for each replicate as described in Legal et al. (1999). Parameters of the regression were calculated and the likelihood of convergence determined according to a quasi-Newton method. In order to reach minima of PROBIT regression functions, a search for the Rosenbrock structure was performed prior to applying this method in order to reduce weight of extreme values (Pilat et al. 1976; De Schamphelaere et al. 2004). In order to separate the 30 samples into 3 sets of 10, a random sort (repeated 50×) of 3 × 10 was performed independently on each series. Standard deviations were calculated using the three resultant LD_{50} from each treatment (Legal et al. 1999; De Schamphelaere et al. 2004).

Mean weights of caterpillars and standard errors were calculated at the various days and a Z-Mann-Whitney test (95% confidence limit, Statistica 7.0 software) was used to determine differences between treatments.

Results

Chemical analysis

Lupinus montanus extract contained sparteine (89%) as the main alkaloid, followed by an unknown compound (n.i. 1940) found previously by Meißner and Wink (1992) (6%), aphylline (2%), lupanine (1%) and alpha-sparteine (0.7%) (Table 1). The *L. aschenbornii* extract contained an alkaloid pattern similar to that of *L. montanus*: sparteine (85%), lupanine (9%), multiflorine (3%) and the same unknown compound as *L. montanus* but occurring in lower quantities (2%) (Table 1).

The profile of the *Lupinus stipulatus* extract differed substantially from that of the other two species (Table 1), containing aphylline (46%) and an epiaphylline-like compound (51%) as main alkaloids followed by lupanine (2%) and aphyllidine (1%).

Table 1 Quantity of alkaloids in μg g^{-1} dry weight in the three studied species of *Lupinus*: *L. montanus*, *L. stipulatus* and *L. aschenbornii*

Alkaloid name	Alkaloid content (μg g^{-1} dry weight)		
	L. montanus	*L. stipulatus*	*L. aschenbornii*
alpha-sparteine	5	–	–
Sparteine	640	–	780
n.i. 1940 (Meißner and Wink 1992)	47	–	20
Aphyllidine	–	7	–
Aphylline	17.6	280	–
Epiaphylline-like	–	307	–
Lupanine	9.2	11.7	86
Multiflorine	–	–	31
Total content (μg g^{-1} dry weight)	718.8	605.7	917

Table 2 List of alkaloids tentatively identified by Gas Chromatography-Mass Spectrometry and Kovats Retention Indexes (RI) in the extracts of three different *Lupinus* species: *L. montanus*, *L. stipulatus* and *L. aschenbornii*. Relative quantities indicated (%) correspond to the areas under the peaks

Alkaloid name	RI	M^+	Characteristic ions (abundance %)[a]				
			F1	F2	F3	F4	F5
alpha-sparteine	1683	234	98 (100)	137 (57)	193 (22)	234 (20)	150 (15)
Sparteine	1818	234	137 (100)	98 (90)	234 (44)	193 (25)	84 (10)
n.i. 1940 (Meißner and Wink, 1992)	1847	230	134 (100)	97 (93)	232 (30)	55 (24)	148 (20)
Aphyllidine	2054	246	98 (100)	246 (42)	136 (18)	134 (16)	110 (16)
Aphylline	2080	248	136 (100)	220 (45)	124 (40)	248 (35)	191 (20)
Epiaphylline-like	2206	248	136 (100)	246 (90)	96 (50)	122 (20)	82 (16)
Lupanine	2227	248	136 (100)	149 (60)	248 (40)	150 (34)	219 (8)
Multiflorine	2415	246	134 (100)	246 (65)	148 (20)	110 (15)	217 (5)

M^+: molecular ion. [a] Five significant fragments and their relative abundance

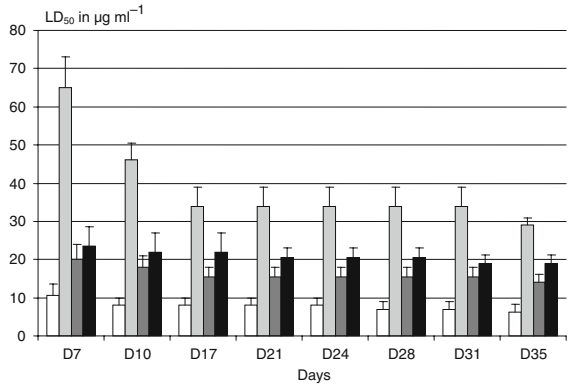

Fig. 1 LD_{50} values and standard errors at various time points (D = days) during the development of *Spodoptera frugiperda* larvae tested for each dose of sparteine/lupines extracts (n = 30). □ Sparteine, ▒ *L. montanus*, ■ *L. stipulatus*, ■ *L. aschenbornii*

Molecular weights and the main characteristic ions for all compounds are given in Table 2.

Insecticidal activity

The effects of the diet on *Spodoptera* larvae differed with treatment. Toxicity effects were relatively stable from day 7 except for *L. montanus* which showed a delayed action (Fig. 1).

Sparteine exhibited a consistent insecticidal activity, greater than the *Lupinus* extracts tested (Fig. 1). Notable differences occurred in LD_{50} values for *L. montanus* and *L. aschenbornii* despite similar amounts of sparteine (89% and 85%, respectively) (Table 1). The *L. aschenbornii* extract was two-fold more toxic than that of *L. montanus* (Fig. 1). The

Table 3 Mean weights (± standard errors) of *Spodoptera frugiperda* caterpillars fed on diets containing sparteine or extracts from three Lupine species. Concentrations used correspond to LD_{50} doses determined in Fig. 1

Mean Weights (mg)	Day 7	Day 10	Day 17	Day 21	Day 24
Control	5.6 (±3) a	23.0 (±12.5) b	107.4 (±62) b	213.0 (±121.9) c	264.5 (±120) a
Control methanol	4.8 (±2.2) a	18.8 (±9.0) a,b	92.0 (±53.8) b	190.0 (±90.1) c	239.9 (±98) a
Sparteine	4.6 (±1.8) a	15.8 (±7.1) a,c	74.2 (±29.4) a	132.9 (± 46) a	184.2 (±76.6) b
L. montanus	4.4 (±3.6) a	12.9 (±9.2) a,c	62.5 (±36.9) a	106.6 (73.2) a,b	167.4 (±109.8) b
L. stipulatus	3.8 (±3.1) a	9.1 (±8.4) c	79.7 (±69) a	161.6 (±128.5) d	242.8 (±120.3) a
L. aschenbornii	5.0 (±3.2) a	12.1 (± 7.1) a,c	49.5 (±34.2) c	96.7 (±64.3) b	154.1 (±108) b

Values sharing the same letter (for the same date) are not significantly different (*Z*-Mann–Whitney test)

presence of relatively high quantities of lupanine and multiflorine in *L. aschenbornii* may explain this higher efficiency.

Lupinus stipulatus treatment showed the greatest insecticidal effect of the three Lupine extracts (Fig. 1). Furthermore, effects were very fast, for a dose of 25 μg ml^{-1}, 83% of the insects had died by day 7 (data not shown).

Influence of the extracts on caterpillar growth

The variation in the weight of larvae within treatments was very high. Generally, *L. aschenbornii* elicited the greatest reduction in the weight of the larvae compared with the control, followed by *L. montanus*, then Sparteine. The weight of larvae fed diet containing *L. stipulatus* did not consistently differ significantly from our two controls (with or without methanol) (Table 3).

When sparteine or an extract rich in sparteine (*L. montanus* and *L. aschenbornii*) was added to the diet, a significant and homogeneous reduction of weight, compared with the controls, was observed from day 21 (Table 3). We noted that when these effects are dominant, caterpillars are keeping their brownish-green color during the time they survived while when toxic effects were dominant there was a fast change of color with caterpillars becoming dark brown (almost black).

We observed differences in duration of the larval stage depending on the extract/dose, however, the number of survivors was not sufficient to perform a meaningful analysis.

Discussion

The diversity of Mexican species of *Lupinus* indicates a high potential for natural alkaloid resources. These compounds can be used as insecticides either alone or in combination with other classes of pesticides such as the pyrethroids. The alkaloids have the distinct advantage of being rapidly degraded by UV and hence possess low persistence in the environment. Moreover, some are very specific as insecticides and/or deterrents as demonstrated by Wink (1992). Sparteine, lupanine, and 13-tigloyloxylupanine were investigated in detail and sparteine was found to have toxic as well as feeding deterrent properties for some Lepidoptera including *Spodoptera eridania* (Cramer), *Syntomis mogadorensis* (Blachier), *Manduca sexta* (L.), *Plutella xylostella* (L.) and *Pieris brassicae* (L.) (Wink 1992). On the other hand, some alkaloids are attractants for honeybees and may be part of their mutualistic reward (Singaravelan et al. 2005). Most Arctiinae (Noctuidae) are totally dependent on plant alkaloids for their defense strategies and even for the synthesis of their pheromones (Wink et al. 1998 and references therein).

Among the three species studied here, *L. stipulatus* is highly insecticidal. Mortality of caterpillars fed on this extract was high (up to 80%) after day 7 (Fig. 1) suggesting fast-acting toxic effects.

Surviving caterpillars had similar weights to the controls, and these larvae remained dark brown in colouration (Table 3). Aphylline and an epiaphylline-like compound are the two main alkaloids for this species. The mode of action and metabolic targets of these compounds as yet remain unstudied. Both are promising molecules for biological control.

Lupinus montanus and *L. aschenbornii* may represent an interesting and alternative source for sparteine, which seems to act efficiently on larval control of *Spodoptera*. Delayed toxic activity demonstrated by our LD_{50} results for *L. montanus* indicates that toxicity persists up to day 17 (Fig. 1).

QA such as Sparteine are known to be stable when they are not exposed to direct UVs or in contact with micro-organisms which degrade them rapidly (Coldham et al. 2005 and references therein). These results led us to hypothesize that surviving caterpillars are able to partially reduce alkaloids as can some other families of Lepidoptera (Wink et al. 1998; Wink and Legal 2001; Wink and Theile 2002).

The differences in alkaloid composition between species indicate a broad range of defense strategies against predation. Moreover, the fact that some intraspecific variations in alkaloid composition occur with the season and the altitude at which the plants grow (Carey and Wink 1994) may explain why the proportions and sometimes even the composition of the alkaloids varies between published studies (Carey and Wink 1994; Bermúdez-Torres et al. 1999; Garcia Lopez et al. 1999).

Lupinus montanus is very rich in sparteine corroborating results of Nowacki (1963) and Garcia Lopez et al. (1999) who found that this compound represents 90% of the total amount of alkaloids in this plant (89% in our case, Table 1). Wink et al. (1995) also reported sparteine as the main alkaloid for some Palearctic species (*L. luteus*, *L. arboreus*, *L. arcticus*, *L. holosericeus* and *L. sericeus*).

The contents of our extracts of *L. stipulatus* were similar to that reported by Garcia Lopez et al. (1999) except that these authors did not find lupanine (total amount of sparteine + lupanine was <0.02%) either indicating intraspecific variation or problems with plant identification. It is interesting to note that this very toxic species grows at lower altitudes than *L. montanus* and *L. aschenbornii* and is therefore in contact with a greater diversity of phytophagous fauna. A similar alkaloid composition has been reported for two North American species: *L. caudatus* and *L. hartwegii* (Wink et al. 1995).

The species for which alkaloid composition was found to be the most variable in proportions and composition is *L. aschenbornii*. In comparison with one of our former studies (Bermúdez-Torres et al. 1999) using extracts made outside the flowering period (data not shown), the extracts used in this study were richer in sparteine and many high-molecular-weight alkaloids are missing. A complete study including these seasonal changes and elevation therefore appears necessary at least for this very high elevation species (growing at between 3,500 m and 4,500 m). These results can be compared with data obtained for *Lupinus argenteus* (Carey and Wink 1994) and various other plants (Berkov et al. 2004, 2005; Reyes-Chilpa et al. 2004).

The diversity of Mexican *Lupinus* species indicates a very rich natural resource for new bio-rational alkaloids potentially active on various pests. This study represents the starting point of a project involving the biochemical study of various species/populations of *Lupinus* in Mexico in relation with their evolutionary history. Other studies will investigate the regulation of QA production depending on biotic and abiotic conditions. Owing to their low toxicity for mammals, low remanence and relative ease of extraction/synthesis, some QAs may represent a promising alternative types of insecticides to control pest populations.

Acknowledgements Peter Winterton is thanked for his help in correcting English. This study was supported by CONACYT (26411-N) and CGPI (978038). Laura Lina is greatly thanked for providing *Spodoptera frugiperda* eggs.

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Author Biographies

Dr. Kalina Bermudez-Torres was formerly a microbiologist and was studying in Germany. She is now involved in various projects including the regulation of the production of alkaloids in Mexican wild plants.

Dr. Jorge Martinez-Herrera is a young doctor now involved in studying toxic compounds of *Jatropha curcas*. His speciality is organic chemistry.

Master Rodolpho Figueroa Brito is working since years on plant insects relationships. His main model is *Spodoptera frugiperda*. Biological regulations of pest populations represent his main activity.

Pr. Michael Wink is the dean of the University of Heidelberg and is working on numerous fields. Among these, alkaloids of *Lupinus* of the world were representing a large part of his past work.

Dr. Luc Legal is actually in sabbatical years in Mexico. He is working on evolutionary scenarios, including those between plants and insects, and ecology. His main models are among Lepidoptera.