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Transfer of quinolizidine alkaloids from hosts to hemiparasites in two *Castilleja–Lupinus* associations: analysis of floral and vegetative tissues

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Abstract

Many hemiparasites, including several members of the *Castilleja* genus (Scrophulariaceae), obtain secondary compounds from their host plants. Both *Castilleja miniata* in subalpine Colorado and *C. indivisa* in central Texas have reduced herbivory when obtaining alkaloids from the hosts *Lupinus argenteus* and *L. texensis* (Fabaceae), respectively. However, pollinators were not deterred from visiting *Castilleja* parasitizing alkaloid-containing hosts. To determine if alkaloids are present in all tissues of plants parasitizing lupins, we analyzed floral tissue as well as leaves of both *Castilleja* species. Leaves, bracts, calices, corollas, gynoecium and nectar of both *Castilleja* species were examined for quinolizidine alkaloid presence using a Dragendorff reagent, and alkaloids were identified in vegetative tissue and nectar by capillary GLC and GLC-MS. Lupanine and alpha-isolupanine were the principal alkaloids in *C. indivisa* parasitizing *L. texensis*, while principal alkaloids of *C. miniata* parasitizing *L. argenteus* were 5,6-*iso*-dehydrolupanine, alpha-isolupanine, thermopsine, and 17-oxolupanine. Except for 17-oxolupanine, which was probably synthesized by biotransformation in the parasite, all other alkaloids correspond to those present in the host plants. Alkaloids were present in the leaves of both *Castilleja* species, and in the bracts, calices and gynoecium of some plants, but never in the corollas. Alkaloids from *L. texensis* and *L. argenteus* were not detected in nectar of either *Castilleja* species. The presence of alkaloids in

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leaves and outer floral tissue of both *Castilleja* species, but not nectar, may explain why alkaloid uptake and storage affected herbivores but not pollinators.

Keywords: Parasitic plants; Herbivory; Pollination; Toxic nectar; Mass spectrometry; Quinolizidine alkaloids; Secondary compounds; Dragendorff reagent

1. Introduction

Hemiparasitic plants provide a unique opportunity to study chemically mediated interactions between plants, herbivores and pollinators. The transfer of alkaloids via root parasitism from host plants to hemiparasites has been documented in several genera, including *Cuscuta* (Convolvulaceae), *Viscum* (Loranthaceae), *Castilleja*, *Pedicularis*, *Orobanche* and *Orthocarpus* (Scrophulariaceae) (Wink et al., 1981; Stermitz and Harris, 1987; Arslanian et al., 1990; Schneider and Stermitz, 1990; Boros et al., 1991; Baeumel et al., 1992; Mead et al., 1992; Stermitz and Pomeroy, 1992; Martin Cordero et al., 1993; Wink and Witte, 1993). Because alkaloids can be transferred to all tissues of the hemiparasite, including floral tissue and nectar (Marko and Stermitz, 1997), alkaloid uptake has the potential to affect ecological interactions with both herbivores and pollinators of the hemiparasite. Evidence for effects of alkaloid uptake on pollinators as well as herbivores has been examined in two systems in the field. *Castilleja miniata* parasitizing *Lupinus argenteus* experienced reduced herbivory compared to *C. miniata* parasitizing non-alkaloid hosts, but pollinators did not discriminate between *C. miniata* parasitizing different hosts (Adler, in press). Similarly, *Castilleja indivisa* had a higher alkaloid content and received less herbivory when grown with bitter (high-alkaloid) compared to sweet (low-alkaloid) near-isogenic lines of *Lupinus albus* (Adler, 2000). *C. indivisa* parasitizing bitter hosts also received more pollination as a consequence of reduced herbivory (Adler et al., in press). The observation that pollinators are not deterred by alkaloid uptake in either *Castilleja* species suggests either that alkaloids were not present in the nectar of these plants, or that pollinators could not detect alkaloids.

In this study we identify the alkaloids transferred from *Lupinus* to *Castilleja* in two systems, and test for the presence of alkaloids in leaves, bracts, calices, corollas, gynoecium, and nectar of *Castilleja* to determine whether alkaloids have the potential to influence pollinators as well as herbivores. *Castilleja miniata* Douglas (Scrophulariaceae) and *Lupinus argenteus* Pursh (Fabaceae) are long-lived perennials from subalpine Colorado (Weber and Wittman, 1996), while *Castilleja indivisa* Engelm. and *Lupinus texensis* Hook are annuals endemic to Texas (Loughmiller and Loughmiller, 1984). Both *Castilleja* species have specialized herbivores that consume inflorescences and seeds. *C. miniata* is damaged by the plume moth *Amblyptilia* (*Platyptilla*) *pica* Walsingham (Lange, 1950; McCoy and Stermitz, 1983; Roby and Stermitz, 1984; Stermitz et al., 1986) and *C. indivisa* is damaged by the tortricid moth *Endothenia hebesana* Walker (L.S. Adler, personal observation). Broad-Tailed and

Rufous hummingbirds (*Selasphorus platycercus* and *S. rufus*) pollinate *C. miniata* (Adler in preparation), and Black-chinned hummingbirds (*Archilochus alexandri*) pollinate *C. indivisa*. Bumblebees and occasionally other insects pollinate *C. indivisa* (Adler, 2000), while no insect pollinators were observed visiting *C. miniata* (L. S. Adler, personal observation). Thus, these two *Castilleja* species have different life history strategies and grow in different environments, but both experience substantial herbivory from inflorescence-feeding microlepidopterans and are often pollinated by hummingbirds.

Here we report the transfer of specific quinolizidine alkaloids from *Lupinus argenteus* to *Castilleja miniata*, and from *Lupinus texensis* to *Castilleja indivisa*. In addition, in both *Castilleja* species we tested leaves and floral tissue, including bracts, calices, corolla, gynoecium, and nectar, for the presence of alkaloids to determine if alkaloid uptake has the potential to influence pollination as well as herbivory in these species.

2. Materials and methods

2.1. Alkaloid presence in leaves and floral tissue

Indian paintbrush individuals obtain quinolizidine alkaloids when parasitizing lupins, but do not possess these alkaloids when parasitizing other hosts. The presence of alkaloids in Indian paintbrush was determined using a Dragendorff reagent (Harborne, 1984; Stermitz et al., 1989). Alkaloid presence indicated that Indian paintbrush were parasitizing lupins, as no other host at these field sites produced alkaloids that yielded a positive reaction to Dragendorff reagent. If alkaloids were not detected, Indian paintbrush plants were considered to be parasitizing non-lupin hosts.

Ten *C. miniata* plants growing near *L. argenteus* plants were collected from the northwest slope of the upper road at Emerald Lake, Gunnison County, Colorado on July 15, 1996. Tissue was divided into leaves, bract/calyx, petals/androecium, and gynoecium. Two *C. indivisa* plants growing near *L. texensis* plants were collected from the Lady Bird Johnson Wildflower Center (4801 LaCrosse Ave., Austin, TX 78739) on May 8, 1997. Eight additional *C. indivisa* plants growing near *L. texensis* plants were collected from entrance yard of the Stengl Reserve, Bastrop County, Texas, on May 20, 1997. These sites are approximately 100 km apart. Tissue was divided into leaves, bracts, calices, corolla, and gynoecium. The androecium was not analysed due to the small amounts of tissue. Alkaloids were extracted from fresh tissue in the field by grinding in microcentrifuge tubes in a few drops of 1 M Na_2CO_3, adding about 0.5 ml of 2:1 $CHCl_3$–MeOH, and stirring with a small stirring rod. The chloroform layer was then spotted on Whatman #1 filter paper (Whatman International Limited, Springfield Mill, England) (Stermitz et al., 1989). The presence of alkaloids was detected using a colorimetric assay with a Dragendorff reagent (Harborne, 1984).

2.2. Plant sources for GLC and GLC-MS analysis

L. argenteus and *C. miniata*: On August 19, 1997, whole flowering *L. argenteus* were collected from the northwest slope of the upper road at Emerald Lake, Gunnison County, Colorado and divided into aboveground and belowground material. Aboveground parts of flowering *C. miniata* were collected from the northwest slope between the upper and lower road, and were divided into vegetative (stems and leaves) and reproductive (inflorescences) material. Voucher specimens were deposited at the Herbarium of the Rocky Mountain Biological Laboratory: LSA 1, 3, 5 are *C. miniata*, and LSA 2, 4, 6 are *L. argenteus*.

C. indivisa: On May 20, 1997, *C. indivisa* were collected from the roadside just north of the entrance to the Lady Bird Johnson Wildflower Center, 4801 LaCrosse Avenue, Austin, TX, USA. The alkaloids of *L. texensis* were not analyzed, as previous research has shown that the alkaloid profile of this species is relatively consistent and comprised mostly of two major components, lupanine and alpha-isolupanine (Stermitz and Pomeroy, 1992; Wink et al., 1995).

All plant material was dried in an oven at 50°C for one week, and ground to pass through a 40 mesh screen using a Wiley Mill (Thomas Scientific, Swedesboro, NJ). All plant samples were first analyzed with GC only to determine the diversity and concentration of alkaloids (Adler et al., in press). The GC results were used to select one representative plant of each species with the highest diversity of alkaloids for further analysis with GC-MS. The plant tissues analyzed were vegetative material (stems and leaves) from *C. miniata*, roots and shoots from *L. argenteus*, and shoots from *C. indivisa*.

2.3. Nectar sources for GLC and GLC-MS analysis

Nectar was collected from *C. miniata* in the field directly downstream and adjacent to Emerald Lake, CO on July 29, 1998. A total of 182 μl of nectar was collected from 30 plants and pooled from plants with alkaloids in leaves. Nectar was collected in 5 μl microcapillary tubes (Drummond Scientific Co., Broomall, PA, USA) inserted into open flowers. Care was taken not to contaminate samples with pollen or by damaging floral tissue. Nectar was collected in the greenhouse from *C. indivisa* plants grown with 3 different *Lupinus* hosts: the native host *L. texensis*, and bitter (high-alkaloid) and sweet (low-alkaloid) near-isogenic lines of the annual *Lupinus albus*. One host was planted with each *C. indivisa* following the methods of Adler (2000). Nectar from *C. indivisa* was collected in 1 μl microcapillary tubes over several weeks and subsequently pooled. A total of 177.9 μl were collected from 14 *C. indivisa* plants grown with *L. texensis*, 210.5 μl from 11 *C. indivisa* plants grown with bitter *L. albus*, and 188.2 μl from 14 *C. indivisa* plants grown with sweet *L. albus*. For both *C. miniata* and *C. indivisa*, nectar was stored frozen in disposable 1.5 ml microcentrifuge tubes (Out Patient Services, Inc., Petaluma, CA, USA) with 100% alcohol (90% anhydrous ethanol, 5% methanol, 5% isopropanol). Samples were freeze-dried prior to analysis.

2.4. Alkaloid isolation and compound identification by GLC and GLC-MS

Alkaloids were extracted following the procedure of Wink et al. (1995). 0.5 g of dried material was homogenized in 20 ml of 0.5 N hydrochloric acid. The homogenate was adjusted to pH 12 with 6 N aqueous sodium-hydroxide solution. Alkaloids were extracted by solid-phase extraction method using ChemElute (Analytichem) and dichloromethane as eluent. The alkaloid extracts were separated and analyzed by a GLC-MS system consisting of a Carlo-Erba 4160 GC which was coupled to a Finnigan 4500 quadrupole mass spectrometer. The separation conditions were: Column type: OV 1; 30 m; 0.25 mm i.d.; 0.25 μm film thickness; split ratio 1:25; carrier gas: He; flow 1 ml/min; injector temperature: 250°C; oven temperature program: 120°C; 3 min isothermal; 120–312°C with a rate of 10°C/min; then 10 min isothermal; data system start at 138°C oven temperature. The electron impact mass spectra were recorded at 45 eV ionization energy by an INCOS datasystem. The Kovats index was determined by cochromatography with a mixture of linear alkanes and by linear interpolation between the *n*-alkane signals in the gas chromatogram. Alkaloids were identified according to their specific mass spectra and indicative RI values, determined earlier in our laboratory (Wink, 1993b; Wink et al., 1995).

Freeze dried nectar was directly taken up in 20 ml 0.5 N hydrochloric acid and processed as described above.

3. Results

3.1. Alkaloid presence in floral tissue

Field assays of two of the 10 *C. miniata* plants did not contain demonstrable alkaloids in leaves or any floral parts, indicating that these plants were not parasitizing *L. argenteus*. The eight remaining *C. miniata* plants had variable levels of alkaloids, ranging from having no detectable alkaloids in the inflorescences to having detectable alkaloids in all tissues except the corolla and androecium. In general, floral tissues that were more external, such as bracts and calices, were more likely to contain detectable alkaloids (Table 1).

One of the 10 *C. indivisa* plants did not contain detectable alkaloids. The nine remaining *C. indivisa* plants showed a pattern similar to that of *C. miniata*, except that alkaloids were never detected in the gynoecium of *C. indivisa*. Five plants had detectable alkaloids in the inflorescences and three of these also had detectable alkaloids in the calices (Table 1). Plants at both the Stengl Reserve and the Lady Bird Johnson Wildflower Center had variable levels of alkaloids in floral tissue.

3.2. Nectar analyses

The alkaloid content of all nectars from both *C. miniata* and *C. indivisa* was less than 1 μg in the 150–200 μl of nectar collected. Although the limit of detection by

Table 1
Detectable alkaloids in leaf and floral tissues of *C. miniata* and *C. indivisa*, as determined by the use of a Dragendorff reagent (see text)[a]

C.miniata					
	Leaf	Bract/calyx	Corolla/androecium	Gynoecium	
Plant 1					
Plant 2	×	×		×	
Plant 3	×				
Plant 4	×				
Plant 5	×				
Plant 6	×			×	
Plant 7					
Plant 8	×	×			
Plant 9	×				
Plant 10	×	×			
C.indivisa					
	Leaf	Bract	Calyx	Corolla	Gynoecium
Plant 1 (WC)	×				
Plant 2 (WC)	×	×	×		
Plant 3 (ST)	×				
Plant 4 (ST)	×	×	×		
Plant 5 (ST)					
Plant 6 (ST)	×	×			
Plant 7 (ST)	×	×			
Plant 8 (ST)	×	×	×		
Plant 9 (ST)	×				
Plant 10 (ST)	×				

[a] WC = collected at the Lady Bird Johnson Wildflower Center; ST = collected at the Stengl Reserve. '×' indicates the presence of alkaloids.

GLC and GLC-MS is lower than 10 ng, quantification is not possible at amounts less than 1 μg due to the possibility of contamination from the laboratory. Traces of sparteine and multiflorine were found in nectar of *C. indivisa* parasitizing bitter *L. albus*, but alkaloids from other samples were in quantities so small that the possibility of laboratory contamination cannot be ruled out.

3.3. Whole plant analyses

The principal alkaloids of *C. miniata* were 5,6-*iso*-dehydrolupanine, alpha-isolupanine, thermopsine, and 17-oxolupanine (Table 2). With the exception of 17-oxolupanine, these alkaloids were present in concentrations very similar to those in the roots of the host *L. argenteus*. 17-Oxolupanine in *C. miniata* may be a biotransformation product of lupanine (Table 2). The composition of major alkaloids from *L. argenteus* was similar in roots and shoots, although trace quantities of some alkaloids were found only in the shoots. (Table 2). The principal alkaloid in

Table 2
Alkaloid composition and content in *Castilleja miniata* parasitizing *Lupinus argenteus*, and in the roots and shoots of *L. argenteus*[a]

Alkaloids	*C. miniata*	*L. argenteus* roots	*L. argenteus* shoots
Alpha-isosparteine			3.5%
Sparteine	×		×
Dehydrosparteine 1&2			0.3%
Ammodendrine			×
Dihydrocytisine	×		×
5, 6-*iso*-dehydrolupanine	12%	18%	7%
Alpha-isolupanine	58%	48.3%	50.5%
Lupanine	×	×	×
Dehydrolupanine	×	×	×
3-Hydroxylupanine	×	×	×
Thermopsine	29%	31.6%	22.7%
17-Oxolupanine	3%		
Ester of hydroxythermopsine?	×		
5 Unknown derivatives of aphylline and aphyllidine		×	9%
Alkaloid content: mg/g dry weight	3.09	0.47	3.26

[a] '×' indicates that only trace amounts were detected, i.e. less than 1% of total alkaloids.

C. indivisa was lupanine, with smaller quantities of alpha-isolupanine, dehydrolupanine, multiflorine and 13-hydroxylupanine (Table 3).

4. Discussion

Castilleja plants with detectable alkaloids in leaves had variable levels of alkaloids in floral tissues, based on our testing with Dragendorff reagent. Some *C. miniata* and *C. indivisa* had detectable alkaloids in floral bracts and calices, and two *C. miniata* had alkaloids in the gynoecium. However, no plants had alkaloids in the corolla and only trace amounts were found in nectar of any plant. This transfer of alkaloids to some but not all floral tissues may have important implications for the ecology of *Castilleja* hemiparasites. Calices and floral bracts are the most conspicuous parts of the inflorescence; corollas are relatively small and hidden. Adults of the most common microlepidopteran herbivores of *C. indivisa* oviposit on the outside of floral bracts and calices, and these tissues were chewed through by newly hatched larvae before inner tissues were damaged (L.S. Adler, personal observation). Therefore, alkaloids in bracts and calices might decrease herbivory by affecting early larval establishment of these caterpillars. The lack of alkaloids in nectar from either *Castilleja* species is consistent with the observation that pollinators do not discriminate against *C. miniata* or *C. indivisa* parasitizing alkaloid-containing *Lupinus* hosts.

The mechanisms by which plant secondary compounds are transported to nectar are not well understood (Adler, in press). In an analysis of *Castilleja sulphurea* parasitizing *Delphinium occidentale*, norditerpenoid alkaloids were found in all

Table 3
Alkaloid composition and content in *Castilleja indivisa* parasitizing *Lupinus texensis*, and *L. texensis* seeds[a]

Alkaloids	*C. indivisa*	*L. texensis* (Stermitz and Pomeroy, 1992)
Sparteine	×	×
Ammodendrine	×	×
Iso-angustifoline	×	
Tetrahydrorhombifoline	×	
Angustifoline	×	
Alpha-isolupanine	5%	90% combined with lupanine
5,6-Dehydrolupanine	×	
Lupanine	86%	90% combined with alpha-isolupanine
Dehydrolupanine	3%	
Multiflorine	×	
17-Oxolupanine	2%	
N-formylangustifoline	×	
13-Hydroxylupanine	1%	×
13-Hydroxymultiflorine	×	
13-Methoxymultiflorine	×	
13-Angeloyloxymultiflorine	×	
13-Tigloyloxymultiflorine	×	
13-Tigloyloxylupanine	×	
Alkaloid content, mg/g dry weight	1.9	

[a] '×' indicates that only trace amounts were detected, i.e. less than 1% of total alkaloids.

tissues tested, including flowers, seeds and nectar (Marko and Stermitz, 1997). However, in this study, individuals of neither *Castilleja* species obtained measurable alkaloids in nectar when parasitizing *Lupinus* hosts. It would be very useful to compare transport mechanisms in *C. sulphurea* to those in *C. miniata* or *C. indivisa* to determine whether *C. sulphurea* actively transports alkaloids into nectar, or whether *C. miniata* and *C. indivisa* prevent their diffusion. Experiments with *C. sulphurea* would also be important to determine whether alkaloid uptake reduces pollination, as well as herbivory, when alkaloids are present in nectar.

The alkaloids identified in *C. miniata* and *C. indivisa* by GLC-MS are consistent with those found in previous studies. Lupanine and alpha-isolupanine made up about 90% of the alkaloids in *L. texensis* whole plants analyzed previously (Stermitz and Pomeroy, 1992), and about 83% of the alkaloids in *L. texensis* seeds (Wink et al., 1995). These two alkaloids were identified from *C. indivisa* parasitizing this host (Stermitz and Pomeroy, 1992), and comprise 91% of the alkaloids found in *C. indivisa* in the current study (Table 3). *L. argenteus* has relatively high between- and within-site variation in quinolizidine alkaloid profiles in Colorado, and several chemotypes have been described (Wink and Carey, 1994). The *L. argenteus* and *C. miniata* alkaloid profile found in this study are identical to chemotype C described previously.

The mechanism of alkaloid transport and storage may be different in hemiparasites than in their hosts. In lupins, quinolizidine alkaloids are synthesized in leaf chloroplasts. Subsequently, they are exported via the phloem to all plant parts, and accumulate in epidermal tissues and fruits (Wink, 1992; Wink, 1993b).

These alkaloids are stored in vacuoles where their concentrations can reach 200 mM (Wink, 1993a), and the uptake into vacuoles is facilitated by ATP driven alkaloid proton antiporters (Mende and Wink, 1987). *Castilleja* and other parasites that sequester quinolizidine alkaloids from their host plants may either utilize similar transporters to control alkaloid transport and storage, or rely on simple diffusion. The alkaloid distribution in *Castilleja* differs from that in lupins; all parts of a lupin flower (including pollen, petals, and carpels) contain alkaloids (Wink, 1992), whereas alkaloids were rare or absent in the more internal floral parts of *Castilleja*. Future work examining transport mechanisms is necessary to determine whether hemiparasites possess specific enzymes for transporting host-derived compounds into vacuoles.

One caveat of this study is that sample sizes are fairly small, and plants were sampled from limited locations. Although the presence of alkaloids in outer floral tissues and the absence of alkaloids in nectar are consistent with previous ecological studies, it is important to keep in mind that levels of alkaloids in floral tissues could vary between populations. We have speculated on species-level consequences of alkaloid presence or absence, but recommend more intensive between-population sampling of *Castilleja* before broad generalizations are made.

In conclusion, our results support previous research documenting the uptake of alkaloids from *Lupinus* host plants in the annual hemiparasite *C. indivisa* and the perennial *C. miniata*. We detected alkaloids in the leaves and outer floral parts of some *Castilleja* hemiparasites, but not in the nectar. These results are consistent with ecological field studies demonstrating that *Castilleja* species parasitizing alkaloid-containing *Lupinus* hosts experienced decreased herbivory but no decrease in pollination. Thus in these species, alkaloid uptake may be an additional benefit of parasitism, in that herbivory is reduced without any costs due to decreased pollination.

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