Iridoid and secoiridoid glycosides in a hybrid complex of bush honeysuckles (Lonicera spp., Caprifoliaceae): Implications for evolutionary ecology and invasion biology

Susan R. Whitehead *, M. Deane Bowers

Ecology and Evolutionary Biology and Museum of Natural History, University of Colorado at Boulder, UCB 334, Boulder, CO 80309, USA

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ABSTRACT

Interspecific hybridization among non-native plant species can generate genotypes that are more reproductively successful in the introduced habitat than either parent. One important mechanism that may serve as a stimulus for the evolution of invasiveness in hybrids is increased variation in secondary metabolite chemistry, but still very little is known about patterns of chemical trait introgression in plant hybrid zones. This study examined the occurrence of iridoid and secoiridoid glycosides (IGs), an important group of plant defense compounds, in three species of honeysuckle, Lonicera morrowii A. Gray, Lonicera tatarica L., and their hybrid Lonicera × bella Zabel. (Caprifoliaceae), all of which are considered invasive in various parts of North America. Hybrid genotypes had a diversity of IGs inherited from both parent species, as well as one component not detected in either parent. All three species were similar in that overall concentrations of IGs were significantly higher in fruits than in leaves, and several compounds that were major components of fruits were never found in leaves. However, specific patterns of quantitative distribution among leaves, unripe fruits, and ripe fruits differed among the three species, with a relatively higher allocation to fruits in the hybrid species than for either parent. These patterns likely have important consequences for plant interactions with antagonistic herbivores and pathogens as well as mutualistic seed dispersers, and thus the potential invasiveness of hybrid and parental species in their introduced range. Methods established here for quantitative analysis of IGs will allow for the exploration of many compelling research questions related to the evolutionary ecology and invasion biology of these and other related species in the genus Lonicera.

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1. Introduction

Hybridization between plant species has been implicated as an important mechanism that can underlie the evolution of invasiveness (Ellstrand and Schierenbeck, 2000; Schierenbeck and Ellstrand, 2009). A large source pool of genetic variation in hybrid genotypes provides increased raw material on which natural selection can act, potentially leading to evolutionary novelty in life history, morphology, phenology, or secondary metabolite chemistry that can make some hybrid populations better adapted to new environments (e.g. Geiger et al., 2011; Oberprieler et al., 2010; Schweitzer et al., 2002). However, despite the importance of interspecific hybridization in invasion biology, plant evolution, and the structuring of ecological communities (Barton, 2001; Hegarty and Hiscock, 2005; Martinsen et al., 2001; Schierenbeck and Ellstrand, 2009; Whitham et al., 1999), there are still many unanswered questions about patterns of trait introgression in hybrids, particularly for secondary metabolite chemistry (Orians, 2000). Hybrids can differ chemically from the parental species both qualitatively and quantitatively—they may have chemical compounds typical of one or both parents, fail to express certain compounds produced by parents, or have novel compounds not typical of either parent (Cheng et al., 2011; Orians, 2000; Orians and Fritz, 1995; Rehill et al., 2006; Rieseberg and Ellstrand, 1993). Because plant chemistry has important consequences for species interactions and, therefore, the reproductive success of plants (Coley and Barone, 2001; Eisner and Meinwald, 1995), a better understanding of the chemical variation among hybrids may provide important insights into why certain hybrids become invasive in introduced ranges while others never establish viable populations (Ellstrand and Schierenbeck, 2000; Fritz, 1999; Fritz et al., 1999; Strauss, 1994; Whitney et al., 2006).

Exotic bush honeysuckles (Lonicera spp., Caprifoliaceae) are some of the most problematic invasive species in the eastern and mid-western United States (Nyboer, 1992; Webster et al., 2006). Most species fruit in abundance and are thought to be dispersed primarily by birds (Bartuszevige and Gorchov, 2006; Ingold and Craycraft, 1983), although white-tailed deer may also be important...
as dispersers (Vellend, 2002; Whitehead, personal observation). Their introduction and spread have led to altered plant communities
and reduced native plant diversity in many areas (e.g. Collier et al., 2002; Woods, 1993), which may be due to competitive
(Gorchov and Trisel, 2003) or allelopathic effects (Cipollini et al., 2008). High densities of honeysuckle shrubs can also have cascading
effects in ecosystems, including alteration in resource availability
for birds (Bartuszevige and Gorchov, 2006; Ingold and Craycraft,
1983), declines in amphibian communities due to high levels of
allelochemicals produced by the plants (Watling et al., 2011), and
even increased disease risk for humans through indirect effects
on deer populations that serve as reservoirs for parasites and
pathogens (Allan et al., 2010). Some of the most invasive species include Lonicera tatarica L., Lonicera morrowii A. Gray, and their
hybrid progeny Lonicera × bella Zabel, which form hybrid swarms
throughout much of the introduced range (Barnes and Cottam,
1974; Nyboer, 1992; Webster et al., 2006). The hybrid species ap-
ppears to be more successful in North America than either parent, as
evidenced by the wide variety of habitats that the hybrid inhabits;
its higher abundance relative to the parent species, and the high
frequency of hybrid individuals that exhibit morphological traits
intermediate to the parents (Barnes and Cottam, 1974; Whitehead,
personal observation).

The L. × bella hybrid complex provides an intriguing system
for phytochemical research. A comparison of secondary metabolites
produced in parental and hybrid species would add an important
new component to a growing literature on the chemical conse-
quences of hybridization and establish analytical methods that will
allow researchers to address many questions related to the evolu-
tionary ecology and invasion biology of these species. The phyto-
chemistry of Lonicera has been previously investigated due to the
importance of various species in traditional pharmacopeias, and
the genus contains at least two classes of secondary compounds
with known ecological and economic importance: iridoid and
secoiridoid glycosides (IGs) and phenolics (Chen et al., 2007; Cipollini
et al., 2008; Ikeshiro et al., 1992; Li et al., 2003; Song et al., 2006;
Svobodova et al., 2008; Wang et al., 2003; Zadernowski et al.,
2005). This study focuses on IGs, which are an important class
of plant defensive compounds found in over 50 plant families
(Bowers, 1991), but have not been previously investigated in the
and Ikeshiro et al. (1992) have provided initial descriptions of six
IGs in fruits and leaves of L. morrowii, and L. tatarica.

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The importance of various species in traditional pharmacopeias,
and its parental species L. tatarica and L. morrowii; and (3) compare
the composition and concentration of IGs among leaves, unripe
fruits, and ripe fruits in these three species. Results are discussed
in the context of their potential implications for the evolution of
chemical traits in hybrid genotypes, species interactions with her-
vibores and seed dispersers, and invasion biology.

2. Results and discussion

Leaf and fruit samples of L. morrowii, L. tatarica, and L. × bella
were obtained from the living collections at the Arnold Arboretum
of Harvard University (Cambridge, MA, USA). L. morrowii was
originally wild-collected from the Honshu Provenance in Japan in
1984, L. tatarica was wild-collected from Tajikistan in 1978, and
the hybrid species, L. × bella, is of cultivated origin and was re-
ceived at the arboretum in 1919 from Boston, MA (BG-BASE,
2011). In addition, samples were collected from three wild popula-
tions of L. × bella growing near Boulder, CO, USA. The identification
and quantification of IGs was carried out using gas chromatogra-
phy with mass spectrometry detection (GC–MS).

Six major IG components (on average representing 89.1% of
the estimated total IGs) were identified by comparison to authentic
reference standards (Table 1). One other presumably related major
component (Unknown c, 10.0% of estimated total IGs) and six min-
or components (totaling <1% of estimated total IGs) were also de-
tected and provisionally characterized as IGs based on
characteristic fragmentation patterns in mass spectra as described
in detail in Inouye et al. (1976) and Popov and Handjieva (1983).
Although there is no spectral peak associated with the molecular
ion for silylated iridoids, several peaks associated with the agly-
cone portion of the molecule are very informative, and, in combi-
nation with peaks originating from the sugar moiety, served as a
means for positive identification of previously characterized IGs.

Fig. 1. Structures of major iridoid and secoiridoid glycosides from Lonicera × bella, L. morrowii, and L. tatarica.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Common Name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swerside (1)</td>
<td>Swerside</td>
<td>Inouye et al. (1976)</td>
</tr>
<tr>
<td>Secoxyloganin (2)</td>
<td>Secoxyloganin</td>
<td>Popov and Handjieva (1983)</td>
</tr>
<tr>
<td>Loganan (3)</td>
<td>Loganan</td>
<td>Inouye et al. (1976)</td>
</tr>
<tr>
<td>Loganic acid (4)</td>
<td>Loganic acid</td>
<td>Inouye et al. (1976)</td>
</tr>
<tr>
<td>Morrinoside (5)</td>
<td>Morrinoside</td>
<td>Inouye et al. (1976)</td>
</tr>
<tr>
<td>Secologanin (6)</td>
<td>Secologanin</td>
<td>Inouye et al. (1976)</td>
</tr>
</tbody>
</table>
The sugar moiety of IGs gives peaks at m/z 361 (usually the base peak), 271, 243, 217, 191, 169, 147, 129, 103, and 73, all of which were present in the spectra of all detected IGs. Ions originating from the aglycone portion of the molecules are diagnostic of individual IGs and are presented in Table 1. Characteristic IG ion fragmentation patterns are shown in Fig. 3 and include Ion B (formed from the loss of the sugar moiety from the aglycone), Ion C (arising solely from the dihydropyran ring portion), and, for secoiridoids, Ion E (formed by McLaugherty-type rearrangement of the dihydropyran ring). In a few cases, a peak was also observed due to Ion D (formed by the loss of the sugar moiety followed by the rearrangement of the TMSi group with Ion A). One unknown compound (Unknown E) appeared to be an iridoid of the oleuropane series (Bailleul et al., 1981; Calis et al., 1984; Inouye et al., 1976). By comparing the observed diagnostic m/z peaks to those that would be represented for known IGs occurring in L. morrowii, the most abundant of which were sweroside (1), secoxyloganin (2), loganic acid (3), loganic acid (4), morroniside (5), and secollogenin (6) (Fig. 1). On average, these six major components represented 89.1% of the total IGs in our samples (Table 1); however, a number of additional components were also detected. Of the three species sampled from the Arnold Arboretum, the parental species L. morrowii had the highest chemical diversity, with a total of 12 provisionally detected IGs. The chemical profiles of L. tatarica and L. × bella included only a subset of these compounds, with six individual IGs provisionally detected in L. tatarica and nine provisionally detected in L. × bella (Table 1). However, additional collections from wild populations of L. × bella in Colorado showed all of the 12 compounds detected in L. morrowii plus an additional minor unknown component not detected in either of the parental species (Table 2). Total quantities of IGs also differed among parental and hybrid species, but only for certain plant parts. Quantities were similar for all three species in leaves, but in fruits tended to be higher in the hybrid than for either parent (Fig. 2). However, this quantitative pattern among species was variable depending on the individual IG examined; for certain compounds the hybrid species contained more than either parent (e.g. secoxyloganin [2]) and for other compounds the hybrid contained less (e.g. morroniside [5]; Table 1).

Both fruits and leaves from all three honeysuckle species contained IGs, the most abundant of which were sweroside (1), secoxyloganin (2), loganic acid (3), loganic acid (4), morroniside (5), and secollogenin (6) (Fig. 1). On average, these six major components represented 89.1% of the total IGs in our samples (Table 1); however, a number of additional components were also detected. Of the three species sampled from the Arnold Arboretum, the parental species L. morrowii had the highest chemical diversity, with a total of 12 provisionally detected IGs. The chemical profiles of L. tatarica and L. × bella included only a subset of these compounds, with six individual IGs provisionally detected in L. tatarica and nine provisionally detected in L. × bella (Table 1). However, additional collections from wild populations of L. × bella in Colorado showed all of the 12 compounds detected in L. morrowii plus an additional minor unknown component not detected in either of the parental species (Table 2). Total quantities of IGs also differed among parental and hybrid species, but only for certain plant parts. Quantities were similar for all three species in leaves, but in fruits tended to be higher in the hybrid than for either parent (Fig. 2). However, this quantitative pattern among species was variable depending on the individual IG examined; for certain compounds the hybrid species contained more than either parent (e.g. secoxyloganin [2]) and for other compounds the hybrid contained less (e.g. morroniside [5]; Table 1).

The total concentration of IGs was significantly higher in unripe and ripe fruits than in leaves, but unripe and ripe fruits were not different from each other (F(2,28) = 23.95, p < 0.0001; Fig. 2). This pattern supports the predictions of optimal defense theory, which suggests that fruits should be well defended due to their high reproductive value for the plant (McKey, 1974, 1979; Rhodes and Cates, 1976; Zangerl and Rutledge, 1996). However, it is notable that levels of IGs in fruits do not necessarily diminish with ripening, as one might expect for compounds that could be potentially toxic to seed dispersers. In fact, for the dominant compound in fruits of L. × bella, secoxyloganin (2), concentrations were substantially higher in ripe fruits than in unripe fruits collected from the same branch at the same time (Table 2).

It is also notable that there was considerable qualitative and quantitative variation in IGs within individuals and species. Fruits and leaves were sampled from 2–6 branches of each individual, and often compounds detected in one collection were not detected in other branches from the same shrub, especially for minor components (Table 1). This suggests that abiotic and/or biotic factors that

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**Table 1**

<table>
<thead>
<tr>
<th>Compound identification</th>
<th>RT</th>
<th>MS</th>
<th>Lonicera morrowii</th>
<th>Lonicera × bella</th>
<th>Lonicera tatarica</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leaves</td>
<td>Unripe</td>
<td>Ripe</td>
<td>Leaves</td>
</tr>
<tr>
<td>Unknown A</td>
<td>26.01</td>
<td>165, 139</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Unknown B</td>
<td>28.87</td>
<td>301, 165, 139</td>
<td>0.03b</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sweroside</td>
<td>29.38</td>
<td>179</td>
<td>0.21b</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Secoxyloganin</td>
<td>29.77</td>
<td>297, 165, 139</td>
<td>0.07b</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Loganic</td>
<td>30.11</td>
<td>283, 165, 139</td>
<td>0.06b</td>
<td>3.36</td>
<td>3.47</td>
</tr>
<tr>
<td>Unknown C</td>
<td>30.46</td>
<td>428, 139, 197</td>
<td>0.06</td>
<td>0.08b</td>
<td>0.06</td>
</tr>
<tr>
<td>Unknown D</td>
<td>31.56</td>
<td>329, 165, 139</td>
<td>ND</td>
<td>0.47</td>
<td>ND</td>
</tr>
<tr>
<td>Unknown E</td>
<td>31.77</td>
<td>281</td>
<td>ND</td>
<td>0.06b</td>
<td>ND</td>
</tr>
<tr>
<td>Loganic acid</td>
<td>32.05</td>
<td>341, 197, 165</td>
<td>0.35</td>
<td>0.03b</td>
<td>ND</td>
</tr>
<tr>
<td>Morroniside</td>
<td>33.62</td>
<td>388, 299, 139</td>
<td>ND</td>
<td>2.05</td>
<td>1.58</td>
</tr>
<tr>
<td>Unknown F</td>
<td>35.06</td>
<td>314, 225, 165</td>
<td>0.16</td>
<td>0.07b</td>
<td>ND</td>
</tr>
<tr>
<td>Unknown G</td>
<td>36.00</td>
<td>314, 225, 165</td>
<td>ND</td>
<td>4.42</td>
<td>3.68</td>
</tr>
<tr>
<td>Secologanin</td>
<td>41.98</td>
<td>255, 165, 139</td>
<td>3.09</td>
<td>0.30b</td>
<td>ND</td>
</tr>
<tr>
<td>Total</td>
<td>3.87</td>
<td>10.93</td>
<td>8.83</td>
<td>1.71</td>
<td>13.40</td>
</tr>
</tbody>
</table>

ND = not detected.

* Low intensity MS peak.

**Fig. 2.** Average (± SE) estimated total iridoid and secoiridoid glycoside (IG) concentration in leaves, unripe fruits, and ripe fruits of three species of Lonicera. Estimated totals include compounds 1–6 (89.1% of total) as well as Unknowns A–G that were provisionally identified as IGs. Averages are from 3–6 collections taken from a single individual of each species.
influence IG composition and concentration may be localized within a plant and that additional sampling of these and other individuals may show considerable variation and additional minor components not detected here. For the hybrid species, \(L \times bella\), a total of four different individuals were sampled, and the chemical composition was highly variable. In particular, there were four compounds present in our collections from Colorado that were not present in the collections from the Arnold Arboretum. Even within Colorado, different individuals had different compositions, e.g. secoxyloganin (2), which is the dominant IG in fruits for most individuals, was not detected here. For the hybrid species, although birds and other frugivores do tolerate ripe fruits likely have important implications for the palatability of fruits to mutualists and therefore the reproductive success of the hybrid species. Although birds and other frugivores do tolerate

\[\text{Strawberry} \times \text{Blueberry} \\rightarrow \text{Hybrid} \]

\[\begin{align*}
\text{Compounds} & : \text{Loganic acid} \\
\text{Concentration} & : \text{variable}
\end{align*}\]

**Table 2**

Comparison of iridoid and secoiridoid glycoside quantities (% dry wt) in four individuals of \(L \times bella\). Quantities represent averages from 2–6 samples taken from a single individual from each location.

<table>
<thead>
<tr>
<th>Compound Identification</th>
<th>Arnold Arboretum</th>
<th>CO-Skunk Canyon</th>
<th>CO-Gregory Canyon</th>
<th>CO-Bluebell Canyon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaves</td>
<td>Unripe</td>
<td>Ripe</td>
<td>Leaves</td>
</tr>
<tr>
<td>Unknown A</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.37</td>
</tr>
<tr>
<td>Unknown B</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.19</td>
</tr>
<tr>
<td>Sweroside</td>
<td>0.98</td>
<td>0.17</td>
<td>0.2</td>
<td>3.92</td>
</tr>
<tr>
<td>Secoxyloganin</td>
<td>0.37</td>
<td>10.75</td>
<td>13</td>
<td>0.30</td>
</tr>
<tr>
<td>Logaric acid</td>
<td>0.01</td>
<td>0.1</td>
<td>0.09</td>
<td>0.25</td>
</tr>
<tr>
<td>Morroniside</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.11</td>
</tr>
<tr>
<td>Unknown C</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.11</td>
</tr>
<tr>
<td>Unknown D</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.28</td>
</tr>
<tr>
<td>Unknown E</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.30</td>
</tr>
<tr>
<td>Loganic acid</td>
<td>0.04</td>
<td>0.06</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>Morroniside</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.07</td>
</tr>
<tr>
<td>Unknown F</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.20</td>
</tr>
<tr>
<td>Unknown G</td>
<td>ND</td>
<td>0.21</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Secologanin</td>
<td>0.31</td>
<td>1.77</td>
<td>0.14</td>
<td>3.44</td>
</tr>
</tbody>
</table>

\[\text{Total} \quad 1.71 \times 13.40 \times 13.64 \times 8.92 \times 24.38 \times 23.31 \times 8.68 \times 20.00 \times 11.21 \times 6.65 \times 9.14 \times 12.28\]

ND = not detected.

CO = Colorado.
high levels of secondary metabolites in certain fruits (Barnea et al., 1993; Cipollini and Levey, 1997b; Filardi and Tewksbury, 2005; Levey and Del Rio, 2001), the little existing evidence for the effects of IGs on birds suggests that some of these compounds can be strongly deterrent and have emetic properties (Bowers, 1980). One possible explanation for the maintenance of high levels of IGs in the ripe fruits of the hybrid may be that these compounds function to defend fruits against antagonistic seed predators and fruit pathogens, leading to longer persistence times on the plant and higher overall levels of fruit removal over time (Cipollini and Levey, 1997a; Cipollini and Stiles, 1992). Long persistence time of ripe fruit may be especially beneficial for plants in an introduced range, since often non-native fruits (including those of Lonicera) are not removed until late in the season once high-quality native fruits are no longer available (White and Stiles, 1992).

3. Conclusions

This study demonstrates that the hybrid honeysuckle, *L. × bella*, exhibits a complex composition of IGs that is variable among individuals and reflects contributions from both parental species as well as some patterns of distribution that are not typical of either parent. Concentrations of IGs are higher in fruits than in leaves for both parents and the hybrid species, and in the hybrid species concentrations remain high even in ripe fruits. These patterns have important implications for plant defense as well as mutualistic interactions with seed dispersers, and may influence the reproductive success of the hybrid species. The characterization of compounds and methods for quantitative analysis established in this study will provide essential tools for future work in this system, which offers an excellent model within which ecologists and evolutionary biologists can explore questions related to the chemical ecology of plant/herbivore and fruit/frugivore interactions, the patterns of trait introgression during hybridization, and the invasion biology of a widespread and problematic group of non-native shrubs in North America.

4. Experimental

4.1. Plant material

Samples of leaves, unripe fruits, and ripe fruits were obtained from the living collections of the Arnold Arboretum (Harvard University, Cambridge, MA, USA) for all three species examined in this study: *L. tatarica* (Acc. #299–78), *L. mollowii* (Acc. #525–84), and *L. × bella* (Acc. # 10087 and its asexual propagule, Acc. #392–92). Dried specimens of these accessions are also available from the Herbarium of the Arnold Arboretum at Harvard University. To obtain material for chemical analysis, two to six branches were clipped from each individual (depending on availability) when the fruits on that plant were in mid-ripening (between June 26 and July 12, 2011), thus obtaining leaves, unripe, and ripe fruits from the same branch at the same time.

Additional samples of *L. × bella* were collected during July 2008 in the same manner from three populations occurring near Boulder, CO, in Bluebell Canyon (39.99135 N, −105.28568 W), Gregory Canyon (39.99727 N, −105.2940 W), and Skunk Canyon (39.98611 N, −105.27660 W). Identification as *L. × bella* was confirmed by two local authorities (Tim Hogan and Dina Clark, Herbarium COLO, University of Colorado Museum of Natural History). Voucher specimens from the Colorado collections used in this study were deposited at the Herbarium COLO at the University of Colorado (Acc. #’s 543191, 543192, and 543193). Plant samples were field-collected as clippings of entire branches and stored in coolers until they arrived at the laboratory (within 24 h of collection). For each sample, leaves, unripe fruits, and ripe fruits were separated, retaining a minimum of 20 leaves, 10 unripe, and 10 ripe fruits. Only fruits that were completely unripe (green) or completely ripe (red or orange) were retained for analysis; those at intermediate stages were discarded. All samples were then oven-dried for 48 h at 50 °C (as is typical for IG analysis; see Gardner and Stermitz, 1988; Jamieson and Bowers, 2010; Lampert and Bowers, 2011; Quintero and Bowers, 2011b).

4.2. Extractions of IGs

Methods for extraction of IGs were modified from previously published studies (Bowers and Stamp, 1993; Gardner and Stermitz, 1988). The dried fruits and leaves were ground to a fine homogenous powder using a mortar and pestle, first removing all seeds from the fruit samples by grinding fruits through a wire mesh strainer with the pestle. Seeds were discarded, leaving only pulp and skin, which was further ground to a fine powder in the mortar and pestle. For chemical analysis, aliquots (25 mg) were taken from fruit samples and aliquots (50 mg) were taken from leaf samples, weighed to the nearest 0.01 mg. A smaller mass of fruit material (25 mg) was used because preliminary analyses showed high levels of IGs in fruit samples, leading to overloaded chromatograms. Each sample was placed in a test tube with MeOH (5 ml), tightly capped, vortexed, and left overnight for extraction. All samples were then filtered, and the extracts evaporated to dryness. Extracts were then re-suspended in H2O (3 ml), and an internal standard (phenyl-β-D-glucopyranoside) was added to each. Samples were then partitioned three times against equal volumes of Et2O. The Et2O fractions were discarded, and the H2O fractions, containing mostly IGs and sugars, were evaporated to dryness. Each residue was then re-suspended in MeOH (1 ml) and left overnight to allow complete dissolution of IGs. Samples were then vortexed and aliquots (100 μl) were transferred to micro-inserts for GC vials and evaporated to dryness at 50 °C.

4.3. Identification and quantification of IGs using GC–MS

IGs were converted to their trimethylsilyl (TMSI) analogs by adding Tri-Sil-Z derivatizing reagent (Thermo-Scientific, Waltham, MA, USA) (100 μl) to the evaporated sample and heating for 20 min at 70 °C in a mineral oil bath. After derivatization, each sample (0.2 μl) was injected onto an HP Agilent 6890N GC coupled with an Agilent 5975C inert mass selective detector with an ion source of 70 eV at 230 °C and equipped with a DB-1MS capillary column (30 m × 0.25 mm i.d., 0.1 μm film thickness; Agilent Technologies, Santa Clara, CA, USA). Ultra-pure He was used as carrier gas at a flow rate of 2 ml min⁻¹, a split flow ratio of 100:1, and a front inlet temperature of 275 °C. The following oven conditions were employed: initial temperature 180 °C, initial hold time 1 min; ramp 1: 5 °C min⁻¹ to 200 °C, hold time 11 min; ramp 2: 2 °C min⁻¹ to 260 °C, hold time 0 min; ramp 3: 30 °C min⁻¹ to 320 °C, hold time 0 min; for a total run time of 48 min. These oven conditions were modified from previously described methods (Gardner and Stermitz, 1988) to ensure adequate peak resolution of IGs while minimizing the run time for each sample. A blank sample (Tri-Sil-Z only) was run after every five samples to ensure there was no carryover between runs. Data were recorded and processed using MSD ChemStation software (version D.02.00.275).

IGs were identified by comparisons of retention times and mass spectra with authentic standards, including sweroside (1), secoglyconin (2), loganic (3), loganic acid (4), morroniside (5), and secoxyloganin (6). Secoglyconin (2) was provided by Søren R. Jensen (Technical University of Denmark), morroniside (5) was purchased from Tauto Biotech Co., Ltd. (Shanghai, China), and all other standards were purchased from Indofine Chemical Company.
(Hillsborough, NJ, USA). On average, the IGs identified using reference standards represented 89.1% of the total IGs in our samples, and included all major components except for Unknown G.

Estimated quantities of all compounds were based on peak areas in total ion current chromatograms. For individual IGs for which reference standards were available (1–6), a six-point calibration curve ($R^2 = 0.997$) was created with concentrations ranging from 0.01–5 mg/mL. Secologanin (6) was observed as two well-resolved peaks that occurred in a regular ratio of 1:1.2; this occurred for both authentic standards and plant extracts containing secologanin (6). These likely represent stereoisomers; however their fragmentation patterns in mass spectra are indistinguishable and thus could not be definitively differentiated in the scope of this study. Thus, to determine total quantities of secologanin (6), the sum of the two peak areas was used; this method gave excellent linearity ($R^2 = 0.997$) in a six-point calibration curve with known concentrations of authentic secologanin (6). To approximate concentrations of IGs for which no reference standards were available, a response factor equivalent to that of our internal standard was assumed.

4.4. Statistical analysis

To determine whether IG concentrations differed among leaves, unripe fruits, and ripe fruits, a one-way analysis of variance was employed using the statistical software JMP v. 8 (JMP, 2009), with tissue type specified as a fixed effect and individual plant and branch as nested random effects included in the error term. This was followed by a Tukey HSD post hoc test for multiple comparisons to determine pairwise differences among tissue types. Data were arcsin-square root transformed prior to analysis to meet assumptions of normality. Meaningful statistical comparisons of the three species were not possible since all samples of the parent species, L. tatarica and L. morrowii, were obtained from a single individual of each species at the Arnold Arboretum.

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