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Iridoid glycosides from fruits reduce the growth of fungi associated with fruit rot

Susan R. Whitehead[1,2,*], *Jordan Tiramani*[1] *and M. Deane Bowers*[1,3]

[1] *Department of Ecology and Evolutionary Biology, University of Colorado Boulder, Boulder, CO 80309, USA*
[2] *Department of Entomology, Cornell University, Ithaca, NY 14853, USA*
[3] *Museum of Natural History, University of Colorado Boulder, Boulder, CO 80309, USA*
*Correspondence address. Department of Entomology, 2130 Comstock Hall, Ithaca, NY 14853, USA.
Tel: +1-434-841-1512; Fax: +1-607-255-0939; E-mail: susan.whitehead@cornell.edu

Abstract

Aims

Ripe, fleshy fruits generally function as rewards to attract mutualistic seed dispersers, but many fruits also contain high concentrations of toxic secondary metabolites. These compounds may serve a variety of adaptive roles in seed dispersal or as a defense against non-dispersing seed predators or pathogens. We tested the effects of iridoid glycosides from fruits of a hybrid bush honeysuckle, *Lonicera* × *bella*, on the growth of two pathogenic fungal strains associated with fruit rot, *Alternaria tenuissima* and *Aspergillus tubingensis*.

Methods

Fungi were isolated from field-collected *L.* × *bella* fruits and identified using molecular techniques. Their growth rates were assessed *in vitro* in the presence of varying concentrations of pure loganin, one of the most abundant iridoid glycosides in fruits, as well as fruit extracts containing a mix of at least seven different iridoid glycosides.

Important Findings

Loganin had strong dose-dependent negative effects on the growth of both fungi. Extracts from fruits had no effect on *Aspergillus* but a strong antifungal effect on *Alternaria* that increased with fruit ripening. Total iridoid glycoside concentrations in extracts were not good predictors of variation in fungal growth, but several individual compounds had significant negative effects. Although iridoid glycosides have primarily been studied as antiherbivore defenses in leaves, these results indicate that they can also function to reduce the growth of fungi associated with fruit rot.

Keywords: Caprifoliaceae, fruit rot, fruit secondary metabolites, generalized defense, *Lonicera*, plant–pathogen interactions

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INTRODUCTION

The primary function of fleshy fruits is to attract mutualist animals, which provide critical seed dispersal services in exchange for the nutritional rewards provided by fruits. Yet, in addition to nutritional rewards, many ripe, fleshy fruits contain secondary metabolites that can be deterrent or even highly toxic to consumers (Beckman 2013; Herrera 1982; Levey *et al.* 2007). The occurrence of secondary metabolites in fruits has historically been described as an evolutionary paradox: why would a structure that functions to attract consumers contain toxic metabolites (Cipollini and Levey 1997a; Heim de Balsac 1928)? Although in some cases fruit secondary metabolites may occur primarily as a pleiotropic consequence of their defensive role in other plant parts (Ehrlen and Eriksson 1993; Whitehead and Poveda 2011), in other cases the compounds in fruits are unique to fruit flesh or occur in higher concentrations in fruits than in other tissues, suggesting that fruit secondary metabolites serve an important adaptive role (Whitehead and Bowers 2013). A number of hypotheses have been proposed to explain how fruit secondary metabolites may increase plant fitness (Cipollini and Levey 1997a), and a few have gained some empirical support (reviewed in Cipollini 2000; Levey *et al.* 2007). For example, fruit secondary metabolites have been shown to provide association cues for mutualists (Hodgkison *et al.* 2007; Lomascolo *et al.* 2010), deter less effective vertebrate seed dispersers (Filardi and Tewksbury 2005; Levey *et al.* 2006), or alter the gut retention time of seeds in vertebrate guts (Baldwin and Whitehead 2015; Murray *et al.* 1994; Wahaj *et al.* 1998; Tewksbury *et al.*

2008). In addition to these effects on vertebrate consumers, fruit secondary metabolites may play an important role in the defense of fruits against non-dispersing invertebrate seed predators and microbial pathogens (Cipollini and Stiles 1993; Cipollini and Levey 1997b; Cazetta *et al.* 2008; Schaefer *et al.* 2003; Tewksbury *et al.* 2008).

A number of studies have suggested that the defense of fruits against pests, and fungal pathogens in particular, is likely one of the primary functions of secondary metabolites in fruits (Cipollini and Stiles 1992; Cipollini and Levey 1997b; Cazetta *et al.* 2008; Tewksbury *et al.* 2008). Fungal pathogens have a large impact on plant fitness—they can spread rapidly once they are introduced to a plant and directly reduce seed viability and reproductive output (Herrera 1982). The production of antifungal secondary metabolites in fruits may lead to trade-offs between attraction of seed dispersers and defense against pests (Cipollini *et al.* 2004; Cazetta *et al.* 2008; Schaefer *et al.* 2003), or, alternatively, certain secondary metabolites may specifically reduce fungal attack with minimal effects on vertebrate dispersers (Struempf *et al.* 1999; Tsahar *et al.* 2002). However, research on the bioactivity and defensive function of fruit secondary metabolites against fungal pathogens has been mostly limited to a few study systems and classes of compounds (Levey *et al.* 2007), and additional research is necessary to understand whether the antifungal role of fruit secondary metabolites is ecologically and evolutionarily relevant across a broad range of systems.

Exotic bush honeysuckles (*Lonicera* spp., Caprifoliaceae) are ornamental shrubs that are considered invasive in much of the eastern and mid-western United States (USDA 2015). One of the most problematic species is *Lonicera* × *bella* Zabel., a hybrid of *L. tatarica* L. and *L. morrowii* A. Gray that occurs across a broad range of habitat types and crowds out native shrubs, reducing biodiversity and impacting a range of native species (Barnes and Cottam 1974). It produces large numbers of berries that are dispersed primarily by birds (McCay *et al.* 2009), although white-tailed deer may also be important as seed dispersers (Vellend 2002; SRW personal communication). The berries remain on the plant late into winter, and have been shown to be an important component of bird diets later in the season once more nutritious fruits have disappeared (White and Stiles 1992).

Fruits of *Lonicera* × *bella* contain two important classes of secondary compounds, iridoid glycosides (IGs) and phenolics (Cipollini *et al.* 2008; Jurikova *et al.* 2012; Whitehead and Bowers 2013). Here, we focus on IGs, a diverse group of monoterpene-derived compounds that have numerous biological activities (Dinda and Debnath 2013), including antifungal effects (Graikou *et al.* 2002; Van der Sluis *et al.* 1983). Their ecological role in antiherbivore defense is well-established (Bowers 1991; Dobler *et al.* 2011), but their importance as a defense against fungal pathogens has been less studied. The few studies that have examined the effects of IGs on ecologically relevant fungi have shown mixed results depending both on the identity of the fungus and the chemical form (glycoside or aglycone) of the compounds (Biere *et al.* 2004; Baden and Dobler 2009; Marak *et al.* 2002), with some fungi even benefiting from the presence of IGs (Marak *et al.* 2002). A diverse mixture of at least 12 individual IGs occur in *Lonicera* fruits, the most common of which are (in order of abundance): secologanin, secoxyloganin, sweroside, morroniside, loganin, kingiside and loganic acid (Whitehead and Bowers 2013). Total IG concentrations in fruits are high, ~2- to 3-fold higher than the concentrations in leaves (Whitehead and Bowers 2013) and also highly variable (ranging from 2 to 20% dry weight in ripe fruits), providing an excellent opportunity to test for how variation in secondary metabolites influences fungal growth and fruit defense. Past work has shown that total IG concentrations and some individual IGs, specifically loganin and one unidentified minor compound, are negatively correlated with damage from fungi in natural populations (Whitehead and Bowers 2013). However, experimental bioassays are necessary to confirm the bioactivity of IGs against specific fungal strains that are associated with *Lonicera* fruit rot.

This study addresses three questions related to the IGs in *L.* × *bella* fruit and their potential antifungal effects: (i) Does loganin, one of the primary IG components in *L.* × *bella* fruits, reduce fungal growth *in vitro*? (ii) Do the complex mixtures of IGs found in *L.* × *bella* unripe and ripe fruits reduce fungal growth *in vitro*? and (iii) Does the variation in total or individual IG concentrations across *L.* × *bella* individuals predict variation in fungal growth?

MATERIALS AND METHODS

Study organism and site

Lonicera × *bella* is a hybrid deciduous perennial shrub that is considered invasive throughout much of North America (Barnes and Cottam 1974; USDA 2015). Its parental species are *L. tatarica* and *L. morrowii*, both of which are popular landscape and ornamental species. The hybrid is thought to have arisen repeatedly in cultivation and wherever the two parental species co-occur in the introduced range (Barnes and Cottam 1974). In our study area of Colorado, establishment has been relatively recent, with the first herbarium records occurring in the early 1970s (COLO 2011). Field collections of fruits used in this study were obtained from Skunk Canyon (39.98611 N, −105.27660 W), located in southwest Boulder County, Colorado. The study site can be characterized as dry and rocky foothills grassland and *L.* × *bella* is abundantly distributed along trails and in riparian areas.

Fruit collection

Fruits were field-collected from *L.* × *bella* shrubs for use in IG analysis and the preparation of extracts for fungal bioassays (described below). In mid-June, 25 plants were haphazardly selected from the study site and ~50 unripe fruits were collected from each plant. Each plant was tagged and

similar numbers of ripe fruits were collected from the same individuals in early August. Fruits were collected from multiple branches throughout the shrub to obtain a representative sample. After collection, fruits were immediately transported to the laboratory and oven-dried for 72 h at 50°C (as is typical for IG analysis; see Whitehead and Bowers 2013 and references therein).

Fungal isolation and identification

In mid-August a third collection of overripe, nearly rotting fruits was made from a selection of 10 plants from the study population and used to isolate fungal strains for use in bioassays (described below). Fruits were collected in sterile bags and brought immediately back to the laboratory, where fungi were isolated from fruit pulp using methods modified from Cipollini and Levey (1997b). Fruits were removed from sterile bags and immersed in a solution of 20% ethanol and 0.5% sodium hypochlorite for 2 min to sterilize the fruit surface. Each fruit was then cut in half using a sterile blade and the two halves were placed face down on agar plates. Four plates were prepared per plant. The composition of the agar used to grow the fungi was prepared to mimic the nutrient composition of ripe *Lonicera* fruits, as reported in Witmer (1996, 1999) and (Tanaka and Tanaka 1998). The 2% agar solution contained 6.6% sugars (2:2:1 glucose:fructose:sucrose), 0.18% lipids (1:1 corn oil:peanut oil), 0.048% protein (soy protein isolate) and 2.15% cellulose (Cipollini and Levey 1997b). After several days of incubation at 28°C, individual fungal colonies resulting from fruit cultures were sorted by morphological type and further isolated into pure cultures. The two most common fungal morphotypes from fruit cultures (see Results) were chosen for use in bioassays, isolated in pure cultures, and identified using molecular techniques.

For molecular identification, mycelia were scraped directly off culture plates and DNA was extracted from each sample using SoilMaster™ DNA Extraction Kits (MO BIO Laboratories, Inc.). We amplified the internal transcribed spacer region of the rRNA operon (ITS1 and ITS2, including 5.8S) using the ITS1F and ITS4 primer pair (Begerow *et al.* 2010; Tedersoo *et al.* 2011). Amplification was performed in a 100 µl reaction tube containing 25 µl Master Mix (Applied Biosystems AmpliTaq Gold 360), 22µl PCR grade water, 1µl 10µM ITS1F primer, 1µl 10µM ITS4 primer and 1µl DNA template. The thermal cycle consisted of 35 cycles using the following temperature program: 95°C for 10 min, 94°C for 1 min (denaturing), 55.5°C for 2 min (annealing), 72°C for 2 min (extension), 72°C for 10 min (final extension). PCR products were examined using electrophoresis to check for size and purity and submitted to Functional Biosciences (Madison, WI) for sequencing. Fungi were identified as *Alternaria tenuissima* (Nees & T. Nees: Fr) Wiltshire (hereafter *Alternaria*) and *Aspergillus tubingensis* Mosseray (hereafter *Aspergillus*) by searching for similar sequences using the NCBI BLAST database (see Results).

Extraction and quantification of iridoid glycosides

Methods for extracting and quantifying IGs were modified from previously published studies (Bowers and Stamp 1993) and are described in detail in Whitehead and Bowers (2013). The oven-dried unripe and ripe fruits were ground to a fine homogenous powder using a mortar and pestle, first removing all seeds to leave only fruit pulp and skin. From each sample of unripe and ripe fruit (N = 25 ripe samples, N = 25 unripe samples), 200 mg subsamples were extracted in methanol for 24 h, filtered and the extract evaporated to dryness. Samples were then resuspended in distilled water and partitioned three times against equal amounts of diethyl ether. The ether fraction was discarded and the water fraction, containing mostly IGs and sugars, was evaporated to dryness. The remaining residue was then resuspended in 1 ml of methanol, aliquots of 12.5 µl were removed for chemical analysis, and aliquots of 500 µl were reserved for fungal bioassays (described below). An internal standard (phenyl-β-D-gluco-pyranoside) was added to the aliquot for chemical analysis at a concentration of 0.5 mg/ml and IGs were derivatized to their trimethylsilyl analogs using Tri-Syl-Z (Thermo Scientific). Quantities of IGs were determined based on the total ion current using an HP Agilent 6890N gas chromatograph (GC) coupled with an Agilent 5975C mass spectrometer (MS). Six major IG components (loganin, secologanin, loganic acid, sweroside, secoxyloganin and morroniside, together representing ~95% of the estimated total IG concentration) were quantified based on comparison to authentic reference standards. Quantities of one additional IG component (kingiside), for which a reference standard was unavailable, were estimated based on the response factor for the internal standard. All IGs were quantified as % dry weight and converted to % wet weight for ease of comparison with concentrations in the agar growth media used in fungal assays (see below). Conversions assumed fruits contained an average water content of 66% in unripe fruits and 74% in ripe fruits, estimated from an independent set of collections from 33 individual *L.* × *bella* plants (SRW, unpublished data).

Fungal growth assays

To test the effects of IG's on fruit-associated fungi, bioassays were conducted that tested fungal growth rates on media that were supplemented with either serial dilutions of loganin or one of the 50 extracts from unripe or ripe fruits. Although loganin represents only 2–5% of the total IGs in fruits (see Results), we chose to focus on this compound because it was the only identified compound that correlated with reduced fungal growth in past work (Whitehead and Bowers 2013), and it is found in numerous plant species and is an important biosynthetic precursor to many other IGs (Dinda and Debnath 2013). To prepare supplemented plates, fruit-mimic agar was prepared as described above and poured into 60 mm sterile glass plates. Once these solidified, a smaller batch of agar was

prepared and placed in a water bath at 90°C until the temperature equilibrated. Aliquots (4.5 ml) were taken from the hot agar with a sterile pipette and quickly transferred to a test tube containing 500 µl of IG solution in sterile deionized water. This mixture was vortexed and poured over the surface of plate, creating a uniform layer of IG-supplemented agar ~3 mm thick on top of the plate. This procedure minimized the quantities of IGs required for bioassays, and we never saw any visible evidence of the substrate mycelium of the fungi growing beyond this depth.

IG solutions used for agar supplementation contained either purified loganin or one of the 50 extracts of unripe and ripe fruit (prepared as described above). Loganin solutions were prepared from purified loganin (obtained from Tauto Biotech, Shanghai, China) at 20 different concentrations ranging from 3.2 to 40.1 mg/ml. This procedure resulted in wet weight concentrations in the growth media (which had a density of 1.09 g/ml) between 0.03 and 0.37% for loganin and between 0.07 and 4.86% total IGs for fruit extracts. These concentrations were used as conservative estimates of IG concentrations typical of fruits in natural populations, which, in this study, ranged from ~2.77 to 12.47% wet weight for total IGs in unripe fruits and 0.12–3.09% for total IGs in ripe fruits (see Results). The variation in IG concentration in the extracts was used to address our question regarding whether natural variation in IG concentration can predict variation in fungal growth (Q3). However, it should be noted that by using natural fruit extracts we were unable to experimentally manipulate the concentrations of individual compounds, and the concentrations as well as the ratios of individual compounds may have varied among samples. Furthermore, extracts may have contained other secondary compounds, such as phenolics (Cipollini *et al.* 2008), and nonadditive interactions (Richards *et al.* 2012; Whitehead and Bowers 2014) between IGs and phenolics or among IGs or may limit our ability to detect the effects of individual compounds.

Once the IG-supplemented layers solidified, agar from each plate was cut into two strips of equal size (~50 × 15 mm wide) and placed in separate sterile plates for bioassays with the two fungal strains. Each strip was inoculated with a square plug cut from stock cultures at one end of the strip. The growth of fungal hyphae on the agar strip was measured after 48 h (*Alternaria*) or 96 h (*Aspergillus*), just prior to the hyphal growth reaching the end of the strip for the most rapidly growing sample.

Statistical analysis

To determine the effects of loganin on fungal growth rates (Q1), we used regression models with loganin concentration as the predictor variable and hyphal growth as the response, conducted separately for *Alternaria* and *Aspergillus*. Based on scatterplots that suggested a non-linear fit would be the most appropriate for the data, we used quadratic models.

To determine whether the mixtures of IGs found in unripe and ripe *L.* × *bella* fruits affect the growth of two strains of fungi (Q2), we used a one-way ANOVA with treatment (control, unripe extract or ripe extract) as the predictor and fungal growth as the response variable, followed by a Tukey *post hoc* test to compare the three treatments. This was conducted separately for *Alternaria* and *Aspergillus*. We also examined the differences in IGs between unripe and ripe fruits using a series of eight individual paired *t*-tests to compare total IGs and the seven most abundant individual IGs detected in our samples (secologanin, secoxyloganin, morroniside, loganin, kingiside, sweroside and loganic acid). To account for multiple comparisons between unripe and ripe fruits, we calculated statistical significance using a sequential Bonferroni correction to maintain a family-wise error rate of $\alpha = 0.05$. All concentration data (as a proportion of wet weight) were logit transformed prior to analysis to meet assumptions of normality (Warton and Hui 2011).

To determine whether total or individual IG concentrations in fruit extracts affected fungal growth rates (Q3), we first conducted simple linear regressions to examine the effects of total IGs on growth rates, followed by multiple regressions to examine the combined effects of the seven quantified individual compounds on growth rates. These were conducted separately for *Alternaria* and *Aspergillus* and for unripe and ripe fruits. In all regression analyses, we conducted a series of model diagnostics prior to inference, including tests for outliers, calculation of variance inflation factors (VIFs) to check for multicolinearity among predictor variables, and examination of the behavior and distribution of residuals. Although the concentrations of a number of individual compounds were significantly correlated with each other, the VIFs for the individual compounds ranged from 1.08 (loganin) to 2.38 (secologanin), suggesting multicolinearity did not have a strong impact on the variance of the estimated regression coefficients. To further validate our conclusions from the multiple regressions, we used forward and backward stepwise model simplification to ensure that significant predictor variables were robust to various model configurations (Crawley 2007).

RESULTS

Fungal identification

The two most abundant strains of fungi that we cultivated from rotting *Lonicera* × *bella* fruits were identified as *A. tenuissima* (100% query cover and *E* value = 0.0) and *A. tubingensis* (99% query cover and *E* value = 0.0). *Alternaria* was found in 33% of all fruit cultures and 80% of individual plants examined had at least one fruit infected. *Aspergillus* was present in 17% of all fruit cultures and 70% of plants had at least one fruit infected. Five other distinct morphological types were distinguished on fruits from only one or two individuals and were not isolated or identified.

Effects of loganin on fungal growth (Q1)

Pure loganin had a strong negative effect on the growth of both *Alternaria* and *Aspergillus* (Fig. 1). For *Alternaria*, the

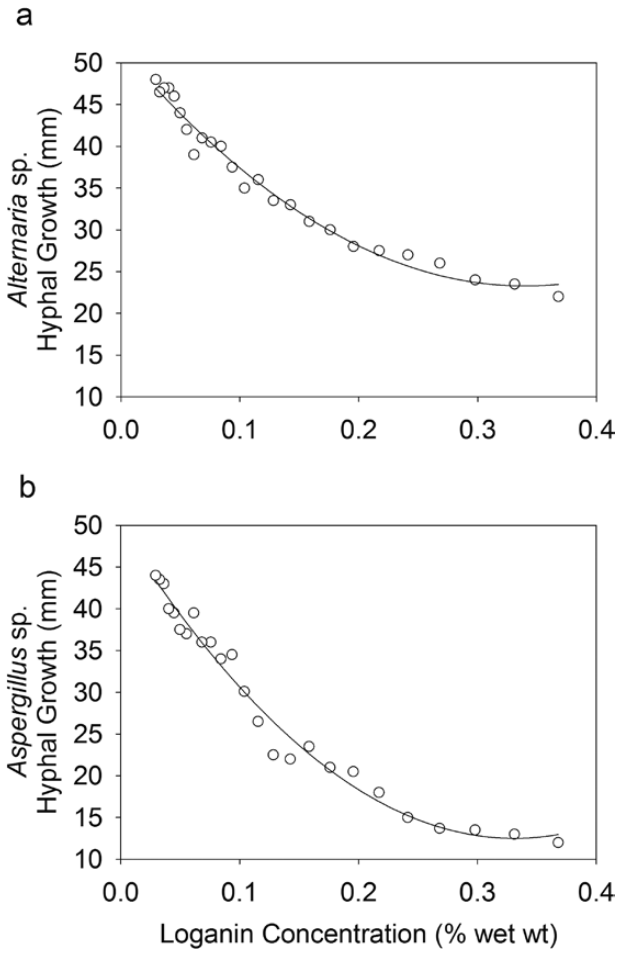

Figure 1: effects of loganin concentration on the hyphal growth of *A. tenuissima* (**a**) and *A. tubingensis* (**b**) on supplemented agar over 48 h (a) or 96 h (b).

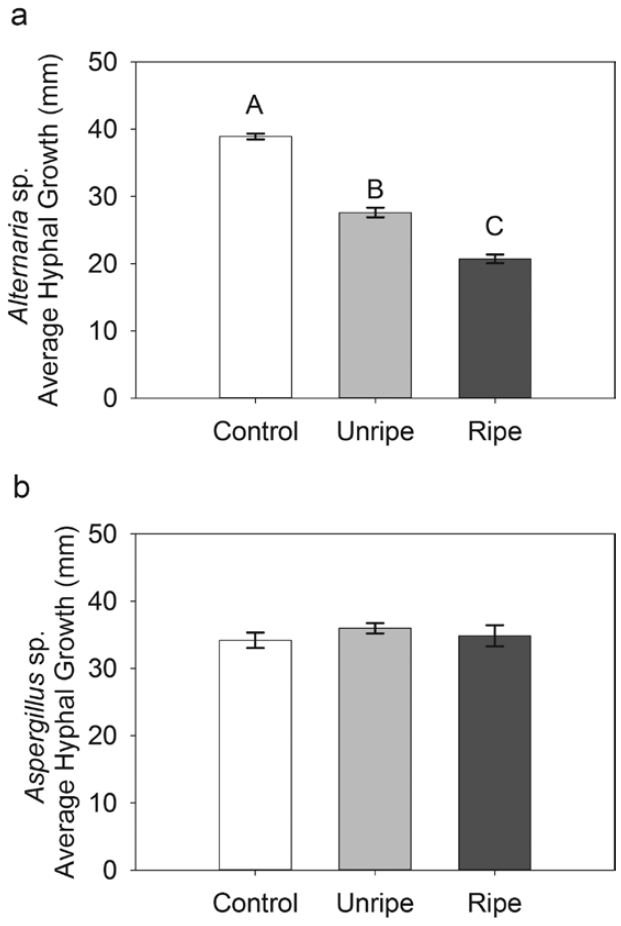

Figure 2: average (±SE) hyphal growth of *A. tenuissima* (**a**) and *A. tubingensis* (**b**) on agar supplemented with unripe or ripe fruit extracts from *Lonicera* × *bella* relative to unsupplemented controls. Bars that do not share a letter are significantly different in Tukey *post hoc* tests for multiple comparisons.

relationship between growth (y) and loganin concentration (x) was modeled as $y = -166.83x + 245.41x^2 + 51.63$; this model described 98% of the variation in fungal growth ($F_{2,22} = 563.9$, $N = 25$, $P < 0.001$, Adjusted $R^2 = 0.98$). For *Aspergillus*, the relationship between growth (y) and loganin concentration (x) was modeled as $y = -224.70x + 339.34x^2 + 49.71$; this model described 98% of the variation in fungal growth ($F_{2,22} = 535.5$, $N = 25$, $P < 0.001$, Adjusted $R^2 = 0.98$).

Effects of fruit extracts on fungal growth (Q2)

For *Alternaria*, we found that IG extracts from both unripe and ripe fruits decreased fungal growth rates *in vitro* ($F_{2,53} = 254.5$, $P < 0.001$) and the antifungal effects were stronger for ripe fruit extracts compared to unripe ($P < 0.001$ for all *post hoc* comparisons; Fig. 2a). For *Aspergillus*, we found no effect of IG extract treatment on fungal growth ($F_{2,53} = 0.55$, $P = 0.58$; Fig. 2b). For both experiments, a number of trials were excluded from the analysis due to contamination of the growth media with other microbes. For *Alternaria*, our final dataset included 18 trials with unripe extracts, 16 trials with ripe extracts and 22 control trials. For *Aspergillus*, our final dataset included 18 trials with unripe extracts, 18 trials with ripe extracts and 20 control trials. The average total IG concentration was ~5-fold higher in unripe fruits relative to ripe fruits (7.21 and 1.38% w.w., respectively; Fig. 3). However, the relative changes in concentration between unripe and ripe fruits varied considerably among compounds (Fig. 3). In particular, the most abundant compound in unripe fruits, secologanin, decreased over 10-fold with ripening, whereas other compounds stayed relatively constant (e.g. secoxyloganin, sweroside).

Relationship between IGs and fungal growth (Q3)

There were no relationships between total IGs in extracts and fungal growth for either *Alternaria* (Unripe: $R^2 = 0.03$, $P = 0.47$; Ripe: $R^2 = 0.09$, $P = 0.20$) or *Aspergillus* (Unripe: $R^2 = 0.15$, $P = 0.09$; Ripe: $R^2 < 0.001$, $P = 0.97$). In multiple regressions examining the relationships between individual IGs and fungal growth, we found that two compounds in ripe fruit extracts, sweroside and loganin, had negative effects on *Alternaria* (Fig. 4; Table 1) and one compound in unripe fruit

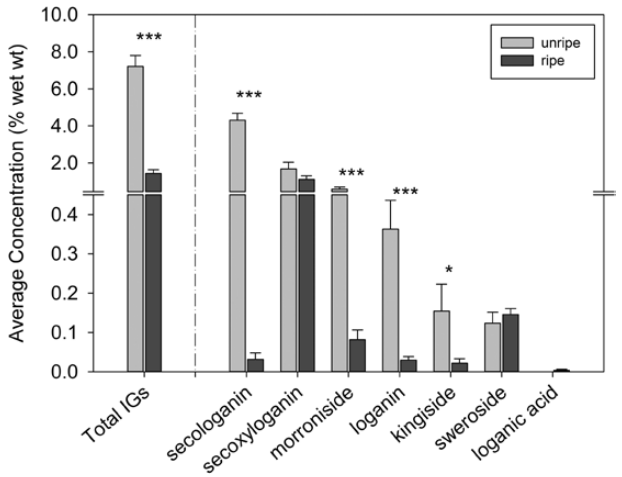

Figure 3: average (±SE) concentration of total iridoid glycosides (IGs) and seven individual IGs in unripe and ripe fruits of *Lonicera* × *bella*. Stars show significant differences between unripe and ripe fruits in multiple comparisons using *P* values corrected to maintain a family-wise error rate of α = 0.05 (*$P < 0.05$, ***$P < 0.001$).

extracts, secoxyloganin, had a negative effect on *Aspergillus* (Fig. 4; Table 1).

DISCUSSION

The role of secondary metabolites in plant defense has been studied primarily in leaves, but fruits can also contain diverse mixtures and high concentrations of deterrent or toxic compounds (Whitehead and Bowers 2013). Our previous research showed that the fruits of *Lonicera* × *bella* contain IGs at 2- to 3-fold higher concentrations than leaves, and certain compounds correlate with reduced fungal damage in natural populations, suggesting an adaptive role for fruit secondary metabolites (Whitehead and Bowers 2013). Here we used controlled experiments *in vitro* to test whether IGs can reduce the growth rates of two strains of fungi associated with rotting fruit, *Alternaria* and *Aspergillus*. We found that loganin, one of the primary IGs found in *Lonicera* fruit, has strong antifungal effects that reduced the hyphal growth of both fungal species in a dose-dependent manner (Fig. 1). For *Alternaria*, IG-rich fruit extracts reduced fungal growth and ripe fruit extracts had stronger antifungal effects than unripe extracts (Fig. 2). There were no effects of the extracts on *Aspergillus*. Overall, our results clearly show that IGs, which have primarily been studied in the context of their deterrent effects on insect leaf herbivores, can reduce the growth of fungi associated with fruit rot. However, the effectiveness of IGs as antifungal agents and their relative importance in determining the outcome of plant–pathogen interactions likely varies depending on the specific blends of compounds and fungal species involved.

Both fungal strains identified from *L.* × *bella* fruits in Colorado are generalist opportunistic plant pathogens.

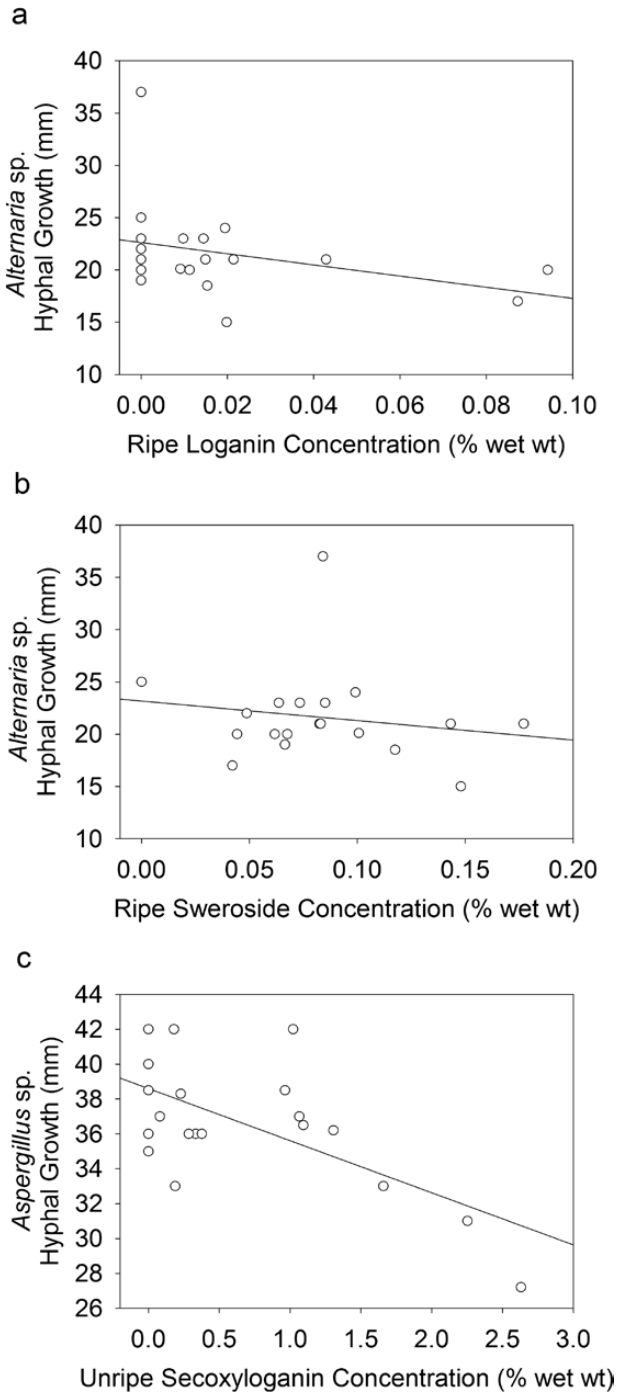

Figure 4: relationship between individual iridoid glycosides in extracts and fungal growth. Loganin (**a**) and sweroside (**b**) concentrations in ripe fruit extracts predicted the growth of *A. tenuissima* over 48 h. Secoxyloganin (**c**) in unripe fruit extracts predicted the growth of *A. tubingensis* over 96 h.

A. tenuissima is a cosmopolitan and common pathogenic endophyte. It has been isolated from at least 346 host plant species and causes leaf spot and wilt diseases as well as fruit rot (Blodgett and Swart 2002; Farr and Rossman 2015; Howard and Albregts 1973). It can often act as a latent pathogen, with

Table 1: results from linear models showing the effects of individual IGs in fruit extracts on the growth rate of two strains of fungi

	Alternaria			*Aspergillus*		
Model coefficients	Estimate	*t*	Pr (>|*t*|)	Estimate	*t*	Pr (>|*t*|)
Ripe fruits[a]						
Intercept	24.79	16.76	<0.001***	33.47	6.34	<0.001***
Secologanin	18.89	1.96	0.08	8.21	0.21	0.84
Secoxyloganin	−0.99	−1.23	0.25	−1.62	−0.64	0.53
Morroniside	7.84	1.43	0.18	1.70	0.08	0.94
Loganin	−29.40	−2.52	0.03*	9.02	0.18	0.86
Kingiside	−23.42	−0.80	0.44	27.13	0.69	0.50
Sweroside	−24.23	−2.31	0.04*	35.55	0.93	0.37
Loganic acid	−44.83	−0.81	0.44	46.42	0.21	0.84
Unripe fruits[b]						
Intercept	24.31	6.91	<0.001***	40.25	14.69	<0.001***
Secologanin	0.51	0.50	0.63	0.36	0.45	0.66
Secoxyloganin	0.53	0.55	0.59	−3.02	−3.97	0.002**
Morroniside	3.78	1.37	0.19	−3.22	−1.50	0.16
Loganin	−1.05	−0.16	0.88	−5.67	−1.09	0.29
Kingiside	−3.22	−0.83	0.42	−0.67	−0.21	0.84
Sweroside	−1.55	−0.12	0.91	8.03	0.78	0.45

[a]Overall model fit for *Alternaria*: $F_{7,10} = 3.30$, $P = 0.043$, adjusted $R^2 = 0.49$, residual error = 1.76 on 10 df; overall model fit for *Aspergillus*: $F_{7,16} = 0.37$, $P = 0.91$, adjusted $R^2 = 0.24$, residual error = 7.97 on 16 df.
[b]Overall model fit for *Alternaria*: $F_{6,14} = 0.66$, $P = 0.69$, adjusted $R^2 = -0.12$, residual error = 3.77 on 14 df; overall model fit for *Aspergillus*: $F_{6,13} = 2.85$, $P = 0.05$, adjusted $R^2 = 0.37$, residual error = 2.93 on 13 df.

disease symptoms only occurring following insect feeding or other wounding (Blodgett and Swart 2002; Shortt *et al.* 1982). *A. tubingensis* is part of the *A. niger* aggregate (black rot fungi), which are among the most ubiquitous saprophytic fungi in soils and decaying plant material (Abarca *et al.* 2004; Varga *et al.* 2004). They are common causal agents of post-harvest rot in agricultural commodities but can also cause infection in living plant tissues following wounding (Logrieco *et al.* 2003; Varga *et al.* 2004). Due to the recent introduction of *Lonicera* × *bella* to the western USA (Barnes and Cottam 1974), it is likely that highly generalized opportunistic colonizers such as these two species are the most important pathogens in the system.

Bioassays with loganin clearly showed that this IG can inhibit the growth of both *Alternaria* and *Aspergillus* across the range of concentrations typical of *Lonicera* fruit (Fig. 1). These results are in contrast to previous work by Marak *et al.* (2002a), which found anti-fungal effects of IGs from *Plantago lanceolata* only in the presence of plant-derived β-glucosidases that convert IGs to their aglycone forms. That study showed that without β-glucosidases, IGs were actually utilized as a carbon source and enhanced fungal growth for both a specialist and generalist fungal pathogen of *P. lanceolata*. Furthermore, Marak *et al.* (2002a) found that the aglycone forms only inhibited the growth of a specialist, but not a generalist fungal pathogen. One important factor that may help explain the discrepancy between this past work and the results presented here is the extent to which the fungal species studied produce their own β-glucosidases that may cleave glucose from the aglycone portion of the molecule and thereby increase toxicity. Many fungi, including *A. tenuissima* and some species of *Aspergillus* do produce β-glucosidases (Shrestha *et al.* 2015), but additional research is necessary to understand the extent to which IGs are metabolized by these fungal-derived enzymes. The nutritional environment of the interactions may also explain some variation in the bioactivity of IGs. We used a growth medium that mimicked the nutrient content of *Lonicera* fruit, where sugar concentrations were high (6.6%) relative to those used in previous studies (~1.75%; Marak *et al.* 2002), and thus glucose was not likely to be a limiting factor for fungal growth under these conditions. Clearly, the bioactivity of IGs and their ecological importance in plant–pathogen interactions can depend on a variety of factors, including the specific structures of the compounds involved, the manner and extent to which the compounds are metabolized by the fungi, and the nutrient environment in which the interactions occur.

For *Alternaria*, we found a negative overall effect of IG-rich fruit extracts on hyphal growth (Fig. 2), and two individual compounds in ripe fruits, loganin and sweroside, were associated with reduced fungal growth. However, the total IG concentration in the extracts was not a good predictor of bioactivity. In fact, ripe fruit extracts, which contained roughly 4-fold lower total IG concentrations (Fig. 3), had increased antifungal effects relative to unripe fruit extracts (Fig. 2). This may be due to the presence of other compounds in extracts, such as phenolics, that were not quantified in this study, or due

to differences in the relative concentrations of IGs in unripe versus ripe extracts. The bioactivity of phenolics may provide the most likely explanation for the increase in antifungal activity in ripe fruits, since phenolics have known antifungal activity (Grayer and Harborne 1994) and can also increase with fruit ripening in some *Lonicera* species (Ochmian *et al.* 2012). However, IGs or the interaction between phenolics and IGs may also play a role. Not all IGs decreased in concentration with fruit ripening. Wet weight concentrations of secoxyloganin and sweroside were not different in unripe and ripe fruits, and sweroside even trended towards increased levels in ripe fruits (Fig. 3). These variable changes with ripening could be simply due to non-adaptive, passive differences in the rate at which different compounds are metabolized. Alternatively, developmental control of IGs could be an adaptive response to changing selective pressures in different phenological stages. Interestingly, sweroside was one of only two individual IGs in ripe fruit extracts that was a significant predictor of fungal growth rates (Fig. 4; Table 1). One explanation for our results is that an increased risk of attack from fungal pathogens in ripe fruits compared to unripe has selected for the maintenance of certain IGs in ripe fruits, such as sweroside, that provide increased chemical defense against fungi. But regardless of the chemical mechanism, an interesting implication of our results is that ripe fruits, which are generally increasing in attractiveness and nutritional quality in order to attract dispersal agents, may still be able to simultaneously maintain or even increase defenses against pathogens.

For *Aspergillus*, we found no overall antifungal effect of IG-rich fruit extracts, and secoxyloganin in unripe extracts was the only compound associated with reduced hyphal growth (Fig. 4; Table 1). Although these results are seemingly in contrast to the clear negative effects of loganin when tested in isolation, loganin represented only 5% of the total IGs in unripe extracts and 2% in ripe extracts. Other IGs in the extracts may have no effects, negative effects or even positive effects on growth, and their effects in combination can also depend on synergistic or antagonistic interactions among compounds (Richards *et al.* 2012; Whitehead and Bowers 2014). Extracts may also have contained other primary or secondary metabolites that influenced fungal growth and masked any potential negative effects of individual IGs. Additional tests with purified IGs, tested alone and in combination, would help disentangle the potential factors influencing the growth of this fungi on *Lonicera* fruit.

Overall, the very clear negative effect of loganin on fungal growth demonstrated in this study supports the hypothesis that IGs that occur in fleshy fruits can function at least in part to defend fruits against fungi. However, the limited role of IGs in explaining variation in antifungal properties of fruit extracts suggests that other chemical defenses not quantified in this study may potentially play an equal or even larger role in mediating interactions with fruit-associated fungi in *Lonicera*. Antifungal properties of IGs in unripe and ripe fruits likely combine with other mechanisms to explain variation in interactions between *Lonicera* fruits and fungal pathogens. Furthermore, the antifungal functions of IGs in fruits do not preclude other ecologically relevant roles for the same compounds. Thus, future studies should continue with a holistic approach to understanding the ecological functions and evolutionary origins of fruit secondary compounds. IGs provide an excellent example of 'generalized defense compounds' (Biere *et al.* 2004; Krischik *et al.* 1991) that can simultaneously mediate interactions with the multitude of herbivores, natural enemies and pathogens with which plants interact in natural environments.

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