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# Water-Deficit Stress Tolerance Differs between Two Locoweed Genera (*Astragalus* and *Oxytropis*) with Fungal Endophytes

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Locoweeds are plants of the genera *Astragalus* and *Oxytropis* (Fabaceae family) and are toxic to cattle, sheep, and horses. The toxic property of locoweeds is due to the alkaloid swainsonine (SWA), which is synthesized by an endophytic fungus *Alternaria* spp. section *Undifilum*. Although the endophyte–locoweed complex is often considered mutualistic, empirical evidence for benefits to host plants is lacking. This study: 1) compared the growth, photosynthesis, and leaf pigment and antioxidant concentrations between endophyte-infected and endophyte-free plants under well-watered and water-deficit conditions; and 2) measured SWA to determine whether SWA concentrations are attenuated by water deficit and leaf age. Locoweed species in this study were woolly loco and silky crazyweed. Endophyte-infected and endophyte-free (by removal of seed coat) seedlings, as confirmed by DNA analyses, were grown under greenhouse conditions for 6 mo, after which plants were subjected to three 12- to 15-d water-deficit periods that created sublethal drought conditions. Results suggest that the endophyte did not influence photosynthetic gas exchange and leaf pigment concentrations. Under well-watered conditions only, endophyte-infected woolly loco plants had lower shoot and root biomass and higher concentrations of α-tocopherol than endophyte-free plants. SWA analyses revealed taxon-specific effects of water deficit, with water deficit increasing SWA concentrations in young leaves of woolly loco but not affecting SWA concentration in silky crazyweed. These results suggest that the endophyte behaves as a parasite in woolly loco plants grown under optimal but not under water-limited conditions. Further, results indicate that drought conditions elevate the toxicity of woolly loco plants. Improved understanding of endophyte-locoweed interactions and factors influencing SWA levels will contribute to the development of livestock management strategies to predict toxicity in particular locoweed populations.
**Nomenclature:** Silky crazyweed, *Oxytropis sericea* Nutt.; woolly loco, *Astragalus mollissimus* Torr.
**Key words:** Alkaloid, antioxidants, commensalism, locoism, mutualism, swainsonine.

Locoweeds are toxic plants of the genera *Astragalus* and *Oxytropis* (Fabaceae family) native to the western United States that contain the indolizidine alkaloid swainsonine (SWA) (Figure 1), an alkaloid that causes the livestock neurological disease, "locoism" (Stegelmeier et al. 1999). SWA inhibits the lysosomal α-mannosidase and Golgi mannosidase II in animals (Elbein et al. 1981), which leads to severe defects of the central nervous and reproductive systems, stillbirths, and death (James et al. 1981; McLain-Romero et al. 2004) and results in severe economic losses, as locoweeds are widely distributed across the western United States (James et al. 1992; Torell et al. 2000). SWA is synthesized by the endophytic fungus *Alternaria* spp. section *Undifilum* of the locoweeds (Baucom et al. 2012; Lawrence et al. 2016; Woudenberg et al. 2013) and is found in the leaves, stems, flowers, and seeds (Braun et al. 2003; Pryor et al. 2009; Ralphs et al. 2008). SWA concentration in plant organs can comprise from 0.0001% to 0.5% of dry mass and varies widely between *Astragalus* and *Oxytropis* taxa and varieties and between different populations of the same species (Delaney et al. 2011; Gardner et al. 2001; Ralphs et al. 2002; Vallotton et al. 2012). Additionally, SWA varies even between individual plants in the same population (Cook et al. 2009, 2011) and different organs on the same plant (Cook et al. 2012). The endophyte was detected by microscopy, culturing, and DNA analysis (polymerase chain reaction [PCR]) in plants with high levels of SWA (Braun et al. 2003; Ralphs et al. 2008). For plants with low levels of SWA, the endophyte was detected by PCR but could not be cultured (Ralphs et al. 2008). Strong correlation was found between the SWA level in a plant and the amount of SWA produced in vitro by the fungus isolated from individual plants in locoweed populations (Braun et al. 2003).

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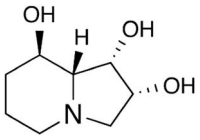


Figure 1. Swainsonine structure.

Fungal endophytes are considered nearly ubiquitous among plant species (Rodriguez et al. 2009; Saikkonen et al. 1998; Singh et al. 2011), and much of the research done on plant host–fungal endophyte relationships indicates that such relationships are mutualistic (Clay 1993; Clay and Schardl 2002; Faeth and Sullivan 2003). Many benefits conferred by endophytes are context specific, such as increased resistance to biotic stresses like herbivory (Clay and Schardl 2002) and pathogen infection (Rodriguez et al. 2009), enhancing invasion ability of the host (Casas et al. 2016), altering the host genome (Guo et al. 2015), or improved host plant tolerance to different abiotic stress conditions like high temperatures, nitrogen deficiency, and water deficit (Kannadan and Rudgers 2008; Malinkowsky and Belesky 2000; Ravel et al. 1997; Rodriguez et al. 2008). One specific mechanism by which an endophyte provides stress tolerance to its plant host is via improved oxidative stress tolerance (Hamilton et al. 2012; Rodriguez and Redman 2005; White and Torres 2010). However, other experiments have shown that fungal endophytes can be harmful or have no effect on their hosts under certain conditions (Faeth 2009; Faeth and Sullivan 2003). Also, although an endophyte may confer benefits to a plant host, this benefit may be at the expense of trade-offs with resistance to certain herbivores or pathogens, so the net effect of an endophyte to its host will be context specific (Rodriguez et al. 2009). Further complicating plant–endophyte relationships is the fact that these interactions are influenced by plant and endophyte genotypes (Faeth and Sullivan 2003).

According to a framework for understanding consequences of plant–endophyte interactions (Saikkonen et al. 1998), the symbiosis between locoweeds and *Alternaria* spp. section *Undifilum* is thought to be mutualistic, because it is vertically transmitted from one locoweed generation to another through seed (Braun et al. 2003; Ralphs et al. 2011), where it is localized in the parenchymal layers of a seed coat (Oldrup et al. 2010), and the fungus produces the alkaloid that is poisonous to livestock (Braun et al. 2003). However, previous studies could not find any antiherbivore benefits provided by the endophyte to the host plant: SWA was not a feeding deterrent of pea aphids at 0.05% of a synthetic diet (Dreyer et al. 1985). The endophyte did not seem to protect a woolly locoweed from insect herbivory by *Cleonidius* spp. (Thompson et al. 1995). Although livestock initially would avoid locoweeds, they may habituate to grazing them and even recruit other animals to eat locoweeds (Pfister et al. 2003; Ralphs et al. 1990). Since locoweed endophyte is not an effective deterrent to herbivory, it must provide other benefits to the plant to be considered a mutualist.

Environmental conditions have long been known to increase toxicity of endophyte-infected grasses (Rodriguez et al. 2009; Saikkonen et al. 2006), and in some cases locoweed (Oldrup et al. 2010). Water stress–inducing media and low pH increased SWA and biomass production of in vitro grown silky crazyweed seedlings and SWA concentration in cultured fungus isolated from the plant (Oldrup et al. 2010). Under greenhouse conditions, water deficit did not alter biomass and SWA concentration in silky crazyweed plants, but SWA concentration in woolly loco increased 1.6-fold after exposure to three 14-d water-deficit periods (Vallotton et al. 2012). Additionally, soil nitrogen levels, optimum for growth, did not alter SWA concentration in locoweed taxa that varied in endophyte content (Delaney et al. 2011). Since locoweed poisoning of livestock is a significant problem in Mongolia and Tibet (Zhao et al. 2009), western China (Li and Nan 2007; Wang et al. 2006), the western United States (Fox et al. 1998), and other parts of the world, understanding of locoweed biology, the nature of the ancient relationship between locoweeds and the fungus (Creamer and Baucom 2013), and the factors that influence SWA levels is essential for the development of management strategies that benefit from predictions of toxicity of particular plant populations under specific conditions.

In this study, we compare woolly loco and silky crazyweed plants with and without the fungal endophyte under well-watered and water-limited conditions in the greenhouse. We compare these two genera because each produces a high level of SWA and each has a different growth pattern, with woolly loco a short-lived perennial and silky crazyweed a long-lived perennial (Fox et al. 1998). For the first time in locoweed research, we use greenhouse-grown, endophyte-free plants produced by manual seed coat removal to test the following hypotheses: (1) the fungus provides benefit to its host plant regardless of watering conditions, thus plants with the endophyte will always have greater biomass and improved oxidative stress tolerance



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compared with endophyte-free plants; 2) plants with the endophyte will have improved oxidative stress tolerance and greater biomass than endophyte-free plants under water deficit, but not under well-watered conditions; and 3) endophyte-free plants grown from embryos will not produce SWA, but SWA concentration in plants with the endophyte is influenced by water deficit.

## Materials and Methods

**Plant Material and Growth Conditions.** Woolly loco and silky crazyweed seeds were collected from Farley, NM (36.24°N, 104.07°W, elev. 1,848 m) and east of Maxwell, NM (36.48°N, 104.16°W, elev. 2042 m), respectively. All seeds were surface sterilized by successive submersion in 70% ethanol for 1 min, de-ionized water for 30 s, and 10% bleach for 2 min, and rinsed in sterile de-ionized water for 5 min. Air-dried seeds were scarified with sandpaper and soaked in water for 30 min. Plants with and without endophyte were grown from seeds with and without coat, respectively (Oldrup et al. 2010). Seed coat and inner membrane were removed using sterile tweezers, and the resulting embryos were surface sterilized with 1% bleach solution for 30 s and rinsed in sterile de-ionized water. Seeds with coats and embryos were germinated on water-moistened filter paper in petri dishes at room temperature, and transferred after germination to prehydrated Jiffy-7 Peat® pellets (Ferry-Morse Seed, Fulton, KY 42041). Seedlings were grown in the greenhouse for 2 to 3 wk and then transferred to 10 by 10 cm (0.9-L) square pots containing a 3:1:1 mixture of soil (Metro-Mix® 300, Sun Gro Horticulture, Nogales, AZ 85621), perlite (Therm-O-Rock West, Chandler, AZ 85226), and sand (Pakmix, Dixon, CA 95620). After 3 mo, all plants were transferred into 16.5-cm (1.9-L) round pots containing the same soil mixture and grown in the greenhouse under natural light conditions at 16/35 C minimum/maximum temperature. All plants were watered regularly and fertilized biweekly with 20-20-20 (N-P-K) Peters Professional® All Purpose Plant Food (Spectrum Group, United Industries, St. Louis, MO 63114), using a solution of 2 g of fertilizer in 1 L of water.

**Experimental Design and Water-Deficit Treatment.** All plants established in the greenhouse were tested to confirm presence or absence of endophyte DNA and arranged in a randomized complete block design of six blocks for each of two experiments that were conducted from December to May (Experiment 1) and from February to June (Experiment 2), respectively. Each block had 8 plants: one endophyte-infected (E+) and one endophyte-free (E−) plant for each water treatment (well watered and water deficit) and each taxon (woolly loco and silky crazyweed), for a total of 96 plants. Plants were subjected to three 12- to 15-d water-deficit periods (WDP) to create sublethal drought conditions, wherein well-watered plants were watered to field capacity (600 to 700 ml $pot^{-1}$) when soil was still moist, while water-deficit plants were watered at 50% field capacity (ca. 300 ml $pot^{-1}$) only when soil appeared dry and the plants were wilted. Each WDP was followed by a 2-wk recovery period when all plants were watered to full capacity and fertilized. Water potential was measured on young and mature leaves at the end of WDP1 and WDP2, and samples of 6 to 15 young and mature leaves were collected for water content and SWA analysis at the end of each WDP and final recovery period. Young leaves were defined as leaves expanded to ca. 80% of full size, and mature leaves were defined as the oldest green leaves that were not senescing.

**Water Potential and Leaf Water Content Measurement.** One randomly selected young leaf and one randomly selected mature leaf were harvested from each plant and kept in a plastic bag in a cooler with ice for up to 1 h prior to being measured. Leaf water potential ($\Psi$) was measured using a pressure chamber (PMS Instrument, Albany, OR 93722) and compressed nitrogen. Leaf water content was calculated as:

$$(FW - DW)/FW \times 100\,\% \quad [1]$$

where FW was fresh leaf weight (leaf weight obtained within 1 h after harvest) and DW was dry leaf weight (weight after the leaf was dried at 65 C for 72 h).

**Biomass Measurement.** Plants were harvested after the final recovery period and separated into shoots and roots. Roots were defined as the tissue below the soil surface and were washed using a hydropneumatic elutriation system (Gillison's Variety Fabrication, Benzonia, MI 49616). Roots were assessed for presence or absence of nodules to confirm the endophyte did not induce additional nitrogen assimilation as reported in Delaney et al. (2011). Nodules were separated from roots. Shoots, roots, and nodules were dried at 65 C for 96 h and weighed for calculation of shoot dry mass (including



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leaves removed for SWA measurements), root dry mass (including nodules), total dry mass (shoot dry mass + root dry mass), and root-to-shoot ratio (total dry root mass/total dry shoot mass).

**Swainsonine Analysis.** Leaf samples were dried at 65 C for 72 h and ground with a mortar and pestle. SWA was extracted from 0.1 g of ground tissue using 2% acetic acid and chloroform, purified from acetic acid fraction with Dowex 50WX8-100 ion-exchange resin, and eluted with 1 M ammonium hydroxide (Gardner et al. 2001). An aliquot of SWA extract was dried under nitrogen, redissolved in 50% methanol, and analyzed by direct-infusion tandem mass spectrometry following methods described by Delaney et al. (2011) and using an Agilent 1100AS autosampler (Agilent Technologies, Santa Clara, CA 95051) and a linear quadrupole ion-trap mass spectrometer (LTQ, ThermoFisher, San Jose, CA 95134) operating in selected ion mode. The solvent system was 50/50 acetonitrile/water with 0.1% formic acid at a flow rate of 200 µl $min^{-1}$. Collection of a single tandem mass spectrum for each analysis was triggered by the observation of the SWA [M + H] + ion (*m*/*z* 174.11) and served for compound identification. Separate calibration was performed for the ranges from 5 to 100 ng $ml^{-1}$ and from 100 to 5,000 ng $ml^{-1}$; the response was linear for each range, and the detection limit was 1 µg $g^{-1}$ dry leaf. Quantitation was performed by integration of the SWA elution profile using the Xcalibur software package (ThermoFisher, San Jose, CA 95134).

**Leaf Gas-Exchange Measurements.** Leaf gas-exchange measurements were taken 2 wk after the final recovery period for Experiment 1 only, due to an equipment malfunction during Experiment 2. Young leaf tips (ca. 7 to 10 leaflets) were placed in the conifer chamber attached to an infrared gas analyzer–based photosynthesis system (LI-6400, LI-COR, Lincoln, NE 68504) as described previously (Delaney et al. 2011; Ratnayaka et al. 2003). Net photosynthesis, stomatal conductance, transpiration, vapor pressure deficit, internal $CO_2$ concentration, and leaf temperature were measured between 9:00 AM and 11:00 AM under ambient light (photosynthetically active radiation approximately 1,400 ± 200 µmol $m^{-2}$ $s^{-1}$) at a flow rate of 400 µmol $s^{-1}$ and an internal $CO_2$ concentration of 400 µmol $mol^{-1}$. Each measured leaf was excised, and its area was determined using a LI-COR LI-3000 leaf area meter to allow for adjustment of the gas exchange parameters of the leaf area.

**Leaf Pigments and Antioxidant Compounds.** Pigments and antioxidants were extracted with acetone from the leaflets pooled from several young leaves. Extracts were prepared and analyzed according to Ratnayaka et al. (2003) using high-performance liquid chromatography (Agilent 1100, Agilent Technologies, Palo Alto, CA 95051) with a Spherisorb OSD-1 (5-µm particle size, 250 by 4.6 mm inner diameter) reverse-phase column protected by an Alltima All Guard C-18 guard column (7.5 by 3 mm inner diameter; Grace Davison Discovery Sciences, Deerfield, IL 60015), and managed by Chemstation A.08.03 software. The mobile phase was acetonitrile:methanol:Tris-HCl (0.05 M, pH 7) (857:96:43 v/v/v) followed by methanol:ethyl acetate (68:32 v/v). Chlorophylls and carotenoids were detected at 440 nm using a photodiode array detector, and α-tocopherol was detected using a fluorescence detector with excitation at 295 nm and emission at 340 nm. The relative de-epoxidation state of the xanthophyll cycle pigments was estimated by the xanthophyll cycle conversion ratio,

$$(Z+A)/(V+Z+A) \quad [2]$$

where Z, A, and V are zeaxanthin, antheraxanthin, and violaxanthin, respectively. Standards of chlorophyll *a* and *b*, β-carotene, lutein, Z, α-tocopherol (Sigma-Aldrich, St. Louis, MO 63103), A, and V (DHI Water and Environment, Horsholm, Denmark) were used for identification and quantitation.

**DNA Isolation, Polymerase Chain Reaction, and Restriction Digestion.** Following DNA extraction using three to five apical leaflets from young leaves (DNeasy Plant Mini Kit, Qiagen, Valencia, CA 20874), DNA quantity and integrity were evaluated by agarose gel electrophoresis. PCR reactions for the detection of fungal endophyte were performed with OR1 and the internal transcribed spacer (ITS) 5 primers and DNA polymerase (GoTaq® Flexi, Promega, Madison, WI 53711) as described by Ralphs et al. (2008). The reactions were run in a DNA thermal cycler (PerkinElmer, Waltham, MA 02451) using the following profile: 94 C for 3 min, followed by 30 cycles of 94 C for 45 s, 48 C for 60 s, and 72 C for 60 s. PCR products were digested with the restriction endonuclease Ava II (Promega, Madison, WI 53711) (Ralphs et al. 2008). PCR products and restriction fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.



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**DNA Sequencing.** PCR products were purified (QIAquick PCR Purification Kit, Qiagen, Valencia, CA 91355) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA 94404) and 50 µM of the ITS 5 primer. The reactions were purified on Performa® DTR Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD 20877) and run on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA 94404). The sequence alignments were generated using ClustalW with default parameters (Chenna et al. 2003; Thompson et al. 2004), and database searches were performed with BLAST (available on the National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov).

**Statistical Analysis.** The main effects of taxon, seed coat treatment, water treatment, leaf age, and their interactions were analyzed using PROC GLM in SAS (SAS Institute, Cary, NC 27513). Significant means were separated by LS Means ($P \leq 0.05$). Levene's tests for homogeneity of variance indicated equal variances between experiments for all response variables, and there were no interactions; therefore, response variables were pooled across experiments. For α-tocopherol responses to seed coat and water treatments, severe violations of normality assumptions compelled nonparametric methods for data analysis. Accordingly, these data were analyzed using Friedman's analysis of variance by ranks. Pearson's coefficient was determined for the relationship between leaf water content and water potential.

## Results and Discussion

**Endophyte Detection in Plants.** This study was conducted on two toxic locoweed taxa, silky crazyweed and woolly loco, that typically have the fungal endophyte and the high concentration of SWA produced by that endophyte. Plants were grown from whole seeds and embryos (remaining after seed coats were removed) and tested for endophyte presence using PCR with the primers that allow amplification of the ITS region of ribosomal DNA of *Alternaria* spp. section *Undifilium*, the endophyte commonly found in locoweeds (Braun et al. 2003; Pryor et al. 2009). DNA amplification for plants grown from whole seeds produced a single amplicon of approximately 580 base pairs that was successfully cut into two fragments (approximately 200 and 380 bp) with restriction endonuclease *Ava*II confirming presence of the fungal endophyte (unpublished data). Fragments from six randomly selected, whole-seed plants from each woolly loco and silky crazyweed were sequenced, and the sequences were found to have 99% to 100% similarity with a sequence from *Undifilum* spp. that was determined by Ralphs et al. (2008). The plants from embryos (seed coats removed) did not have detectable amounts of the endophyte DNA. Plants grown from embryos consistently demonstrated a very weak single band of similar size, 580 bp (unpublished data); however, sequence analysis showed very high similarity of this PCR product to the sequences from plant genomes, especially legume genomes, and not to fungal genomes. All woolly loco and 90% of silky crazyweed plants grown from whole seeds had the endophyte DNA, while all silky crazyweed and 90% of woolly loco plants grown from embryos were endophyte-free. Approximately 10% of woolly loco plants grown from embryos had the endophyte, possibly due to incomplete removal of a seed coat, and were excluded from the experiment.

**Water-Deficit Stress.** Plants subjected to water-deficit treatment were wilted and had dull, light-colored leaves most of the time during WDPs, as well as more dry or fallen leaves than well-watered plants at the end of each WDP. However, during the recovery periods, the majority of the water-deficit plants grew well and produced new leaves. In contrast, well-watered plants had brighter color and never wilted. At the end of each WDP, water potential was consistently lower in woolly loco than in silky crazyweed and in water-deficit than in well-watered plants of both taxa (Figure 2) but was not affected by leaf age and the endophyte, regardless of water treatment.

In addition to water potential, water content of young and mature leaves was measured after WDP1 and WDP2. A positive correlation between leaf water content and water potential (correlation coefficient = 0.71, $R^2 = 0.5$, $P < 0.0001$) found in WDP1 and WDP2 suggested that water content can be used as an indicator of leaf water status. Therefore, only leaf water content was measured after WDP3 and the final recovery period. Water-deficit plants had lower leaf water content than well-watered plants after all three WDPs, while there was no difference between leaf water content of well-watered and water-deficit plants after the final recovery period (unpublished data). Leaf water content was always higher in silky crazyweed than in respective treatments of woolly loco; however, consistent with water potential results, presence of



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Figure 2. Leaf water potential of endophyte-infected (E+) and endophyte-free (E−) woolly loco and silky crazyweed plants under well-watered (WW) and water-deficit (WD) conditions. Data were averaged across two experiments and leaf age, young and mature. Bars (mean ± SE; $n = 12$) above same letters are not significantly different.

the endophyte did not affect water content regardless of water treatment. Thus, leaf water potential and water content data combined with visual observations clearly indicated that plants receiving limited amounts of water during each of three WDPs experienced water deficit, but there was no water-deficit stress after the final recovery period. Our results demonstrating higher water potential and water content in silky crazyweed leaves confirm the findings of Vallotton et al. (2012) that indicated that silky crazyweed is more tolerant to water-deficit stress than woolly loco.

**Plant Growth: Endophyte and Water Effect.** We did not detect any differences between endophyte-infected and endophyte-free plants for most of the experimental parameters in these greenhouse studies. For both plant taxa, the percent germination was similar for seeds and embryos (65%), and seedlings from seeds and from embryos had the same survival rate (ca. 100%). Root nodules were found on 77% of woolly loco and 17% of silky crazyweed plants, with similar frequency in endophyte-infected and endophyte-free plants (unpublished data). Total weight of the dried nodules from individual plants varied from 0.02 to 3.04 g for woolly loco and from 0.01 to 0.86 g for silky crazyweed, and there was no correlation between nodule weight and SWA level or association between nodule presence and SWA level in a plant (unpublished data). Similarly, in Delaney et al. (2011), the percentage of plants with root nodules was unrelated to SWA or nitrogen level, with more nodules detected on woolly loco than silky crazyweed plant roots.

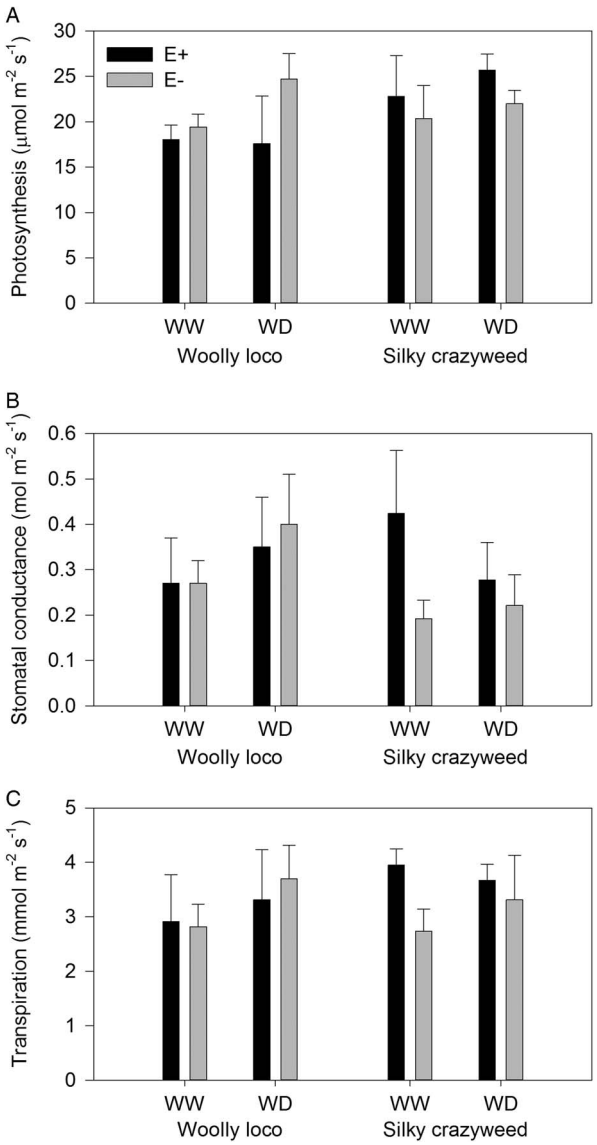


Figure 3. Leaf gas-exchange parameters of endophyte-infected (E+) and endophyte-free (E−) woolly loco and silky crazyweed plants 2 wk after recovery from well-watered (WW) and water-deficit (WD) conditions during Experiment 1. Values are means ± SE; $n = 12$.

At 2 wk after the final recovery period, gas-exchange parameters did not differ greatly between taxa (Figure 3). Net photosynthesis did not differ between taxa regardless of water deficit and was at levels measured previously in these taxa (Delaney et al. 2011; Vallotton et al. 2012). However, there was a taxon main effect for stomatal conductance ($P = 0.0026$) and transpiration ($P = 0.0056$), most likely because silky crazyweed has a smaller leaf surface boundary layer with trichomes parallel to leaf surface as compared with woolly loco, which has trichomes perpendicular to the leaf surface (Fox et al. 1998; Vallotton et al. 2012). Gas-exchange parameters were not affected by endophyte



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Table 1. Leaf pigment in endophyte-infected (E+) and endophyte-free (E−) woolly loco and silky crazyweed plants under well-watered and water-deficit conditions.[a]

| Taxon | Endophyte | Chlorophyll *a* | Chlorophyll *b* | β-carotene | Lutein | Xanthophyll cycle conversion ratio |
|---|---|---|---|---|---|---|
| | | μg $g^{-1}$ dw[b] | μg $g^{-1}$ dw | μg $g^{-1}$ dw | $g^{-1}$ dw | (Z + A)/(V + Z + A)[b] |
| Woolly loco | E− | 3,156 ± 214 | 1,232 ± 76 | 592 ± 47 | 13.04 ± 0.76 | 0.14 ± 0.01 |
| | E+ | 3,092 ± 190 | 1,186 ± 70 | 580 ± 34 | 13.43 ± 0.64 | 0.17 ± 0.01 |
| Silky crazyweed | E− | 3,230 ± 178 | 1,309 ± 68 | 586 ± 34 | 13.26 ± 0.65 | 0.16 ± 0.02 |
| | E+ | 3,051 ± 179 | 1,318 ± 76 | 507 ± 34 | 12.42 ± 0.59 | 0.12 ± 0.01 |

[a] Data were averaged across two experiments and across water-deficit and well-watered plants. Values are mean ± SE, $n = 12$.
[b] Abbreviations: dw, dry weight; Z, zeaxanthin; A, antheraxanthin; V, violaxanthin.

or interactions between endophyte and taxon. These results suggest that the endophyte did not alter the taxa's ability to recover from water deficit, as similarly reported in Vallotton et al. (2012) for silky crazyweed and woolly loco plants grown with their endophyte under lathhouse conditions.

Interestingly, concentrations of leaf pigments (chlorophyll *a* and *b*, β-carotene, and lutein, as well as the xanthophyll cycle conversion ratio, in young leaves after the final recovery period), did not differ between woolly loco and silky crazyweed and were not influenced by the endophyte or previous water deficit (Table 1). Leaf pigment and xanthophyll cycle results for locoweed in this study were inconsistent with recent evidence that endophytes increase antioxidant stress responses in some species (Hamilton et al. 2012; White and Torres 2010). However, the results of the current study agreed with previous research that found similar levels of leaf pigments and antioxidants between woolly loco and silky crazyweed plants grown with nitrogen supplementation high enough to enhance plant growth and low enough to induce antioxidant protective systems (Delaney et al. 2011). Thus, our data suggest that the young leaves recovered from the water-deficit stress regardless of endophyte. This result was also consistent with the visual appearance (color and size) of the new leaves growing during the recovery periods in all plants.

Regardless of water treatment, silky crazyweed had lower shoot biomass and similar root biomass compared with woolly loco, as has been seen in previous common-garden studies (Delaney et al. 2011; Vallotton et al. 2012) (Figure 4A and B; total biomass not shown). Consequently, root-to-shoot ratio was 1.7-fold higher in silky crazyweed than in woolly loco, suggesting that more carbon was allocated to roots and less to shoot growth in silky crazyweed. Since silky crazyweed is a slower-growing but longer-lived perennial (Fox et al. 1998; Vallotton et al. 2012), this reserved carbon may improve plant regrowth after overwintering or stress conditions. Presence of the endophyte did not affect root or shoot biomass of silky crazyweed regardless of water treatment or biomass of woolly loco under

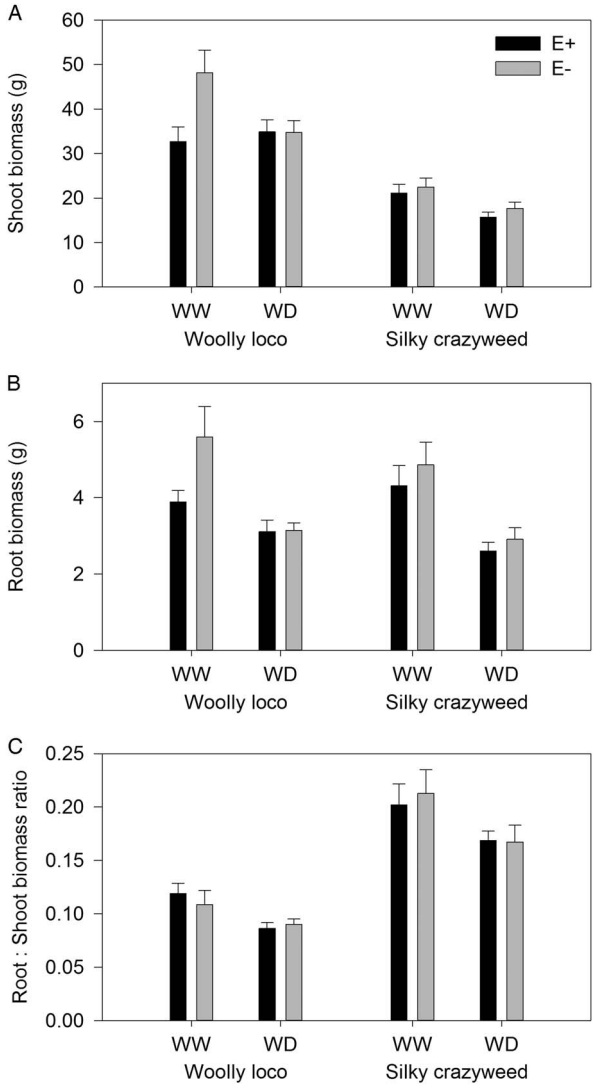


Figure 4. (A) Shoot and (B) root biomass and (C) root-to-shoot ratio of endophyte-infected (E+) and endophyte-free (E−) woolly loco and silky crazyweed plants under well-watered (WW) and water-deficit (WD) conditions. Values are means ± SE; $n = 12$.



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water-deficit conditions. However, in well-watered conditions, endophyte-infected woolly loco plants had 30% lower shoot and root biomass (Figure 4A and B). For woolly loco, Friedman rank-sum tests indicated that α-tocopherol levels differed between endophyte-infected (E+) and endophyte-free (E−) plants under well-watered conditions ($\chi^2_1 = 6.0$, $P = 0.01$; Figure 5), but α-tocopherol levels did not differ between endophyte types under water-deficit conditions ($\chi^2_1 = 0.33$, $P = 0.56$). For silky crazyweed, α-tocopherol levels did not differ between endophyte types under well-watered ($\chi^2_1 = 0.33$, $P = 0.56$) and water-deficit conditions ($\chi^2_1 = 1.0$, $P = 0.32$). Because SWA is not involved (see discussion below), these results on woolly loco suggest that the endophyte behaves as a parasite under optimal but not under water-limited conditions and do not support the hypothesis that locoweed–endophyte interaction is a commensal relationship in which the fungus gains resources from the plant, but the host plant is unaffected (Creamer and Baucom 2013). A similar conclusion can be inferred from a previous replacement series experiment that indicated that endophyte-free woolly loco plants were more competitive than endophyte-infected plants when grown under well-watered conditions in a greenhouse (Schutte et al. 2014). The lack of harmful effect of the endophyte on woolly loco under water-deficit conditions may be due to a negative effect of water deficit on the endophyte itself; it has been reported that fungal biomass decreased when it was cultured alone on water deficit–inducing medium (Oldrup et al. 2010).

Water deficit decreased shoot and root biomass of all silky crazyweed plants regardless of endophyte by 23% and 40%, respectively, and shoot and root biomass of endophyte-free woolly loco plants by 28% and 44%, respectively (Figure 4). Root biomass was only reduced 20% in endophyte-infected woolly loco, and although water deficit did not decrease shoot biomass of endophyte-infected woolly loco plants, this biomass was not higher than in endophyte-free plants. Overall, despite some decrease, biomass did not have a dramatic response to water deficit, suggesting that these locoweed taxa are well adapted to growth in water-limited conditions, and even though they sustained some injury during water-deficit periods, they were able to resume growth when conditions became favorable. These results are consistent with the observations that the plants shed many of their leaves when they were stressed but produced new leaves during recovery periods, probably mobilizing

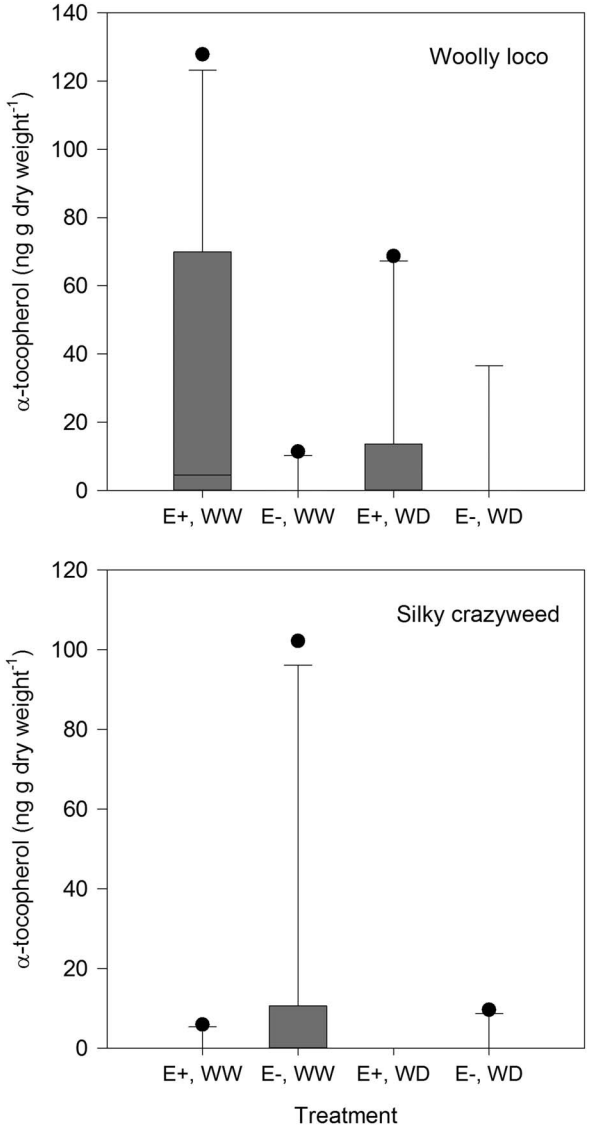


Figure 5. Box plots for α-tocopherol in endophyte-infected (E+) and endophyte-free (E−) woolly loco and silky crazyweed plants under well-watered (WW) and water-deficit (WD) conditions. The natural lower limit on α-tocopherol data (0 ng g dry weight$^{-1}$), combined with very low α-tocopherol concentrations for specific plants, produced boxes that did not extend above the *x*-axis. Where present, upper ends of boxes are 75th percentiles, box midlines are median values, and ends of bars are the 90th percentiles. Filled dots represent observations greater than the 90th percentile of the data.

resources stored in the roots, which could explain why root-to-mass ratio in water-deficit plants of both taxa was lower than in well-watered plants (by 22% and 18% in woolly loco and silky crazyweed, respectively; Figure 4).

Previous studies found a positive effect of simulated water deficit on biomass of silky crazyweed seedlings grown under sterile conditions (Oldrup et al. 2010) but a lack of water-deficit effect on biomass of four locoweed taxa, including



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woolly loco and silky crazyweed grown in a lathhouse (Vallotton et al. 2012). The differences between those reported here and published results could be explained by differences in experimental conditions and degree of water-deficit stress. The first study (Oldrup et al. 2010) was conducted on seedlings grown on polyethylene glycol (PEG)-containing media in closed magenta boxes (approximately 100% relative air humidity), and the second study (Vallotton et al. 2012) used field-collected locoweed transplants growing in soil in a lathhouse and receiving measured amounts of water. In this study, seedlings were grown in soil in the greenhouse environment with leaf water potential consistently lower in plants subjected to water-deficit treatment. As in Vallotton et al. (2012), silky crazyweed consistently maintained higher water potential than woolly loco, supporting the idea that silky crazyweed is more drought tolerant than woolly loco, regardless of endophyte presence.

**Swainsonine Concentration: Endophyte and Water Effects.** SWA concentration in the leaves was strongly influenced by presence of the endophyte and moderately affected by plant taxon, leaf age, water treatment, and sampling time (Figures 6A and 7A). Endophyte-infected silky crazyweed and woolly loco plants contained up to 0.3% and 0.4% SWA, respectively. In contrast, SWA concentrations were near detection limits (0.001%) in endophyte-free plants, regardless of water treatment (Figures 6B and 7B), confirming our DNA evidence that the endophyte had essentially been removed with seed coat removal. In endophyte-infected plants, SWA concentration decreased by 20% with leaf age in woolly loco but increased by 90% in silky crazyweed, suggesting taxon-specific regulation of SWA production and/or SWA degradation. Water deficit moderately increased SWA concentration (by 25% to 30%) in mature and young leaves of endophyte-infected woolly loco after all three WDPs and in mature leaves

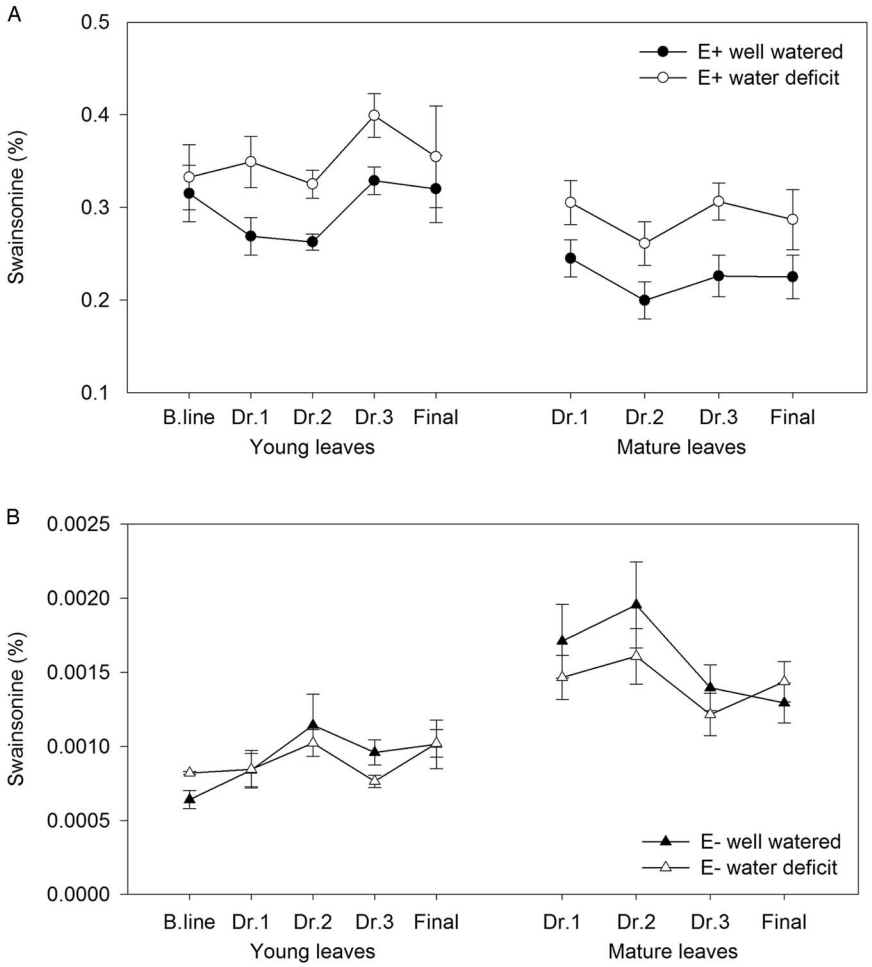


Figure 6. Swainsonine content of young and mature leaves of (A) endophyte-infected (E+) and (B) endophyte-free (E−) woolly loco plants under well-watered (WW) and water-deficit (WD) conditions (B.line, baseline; Dr., drought period). Swainsonine content was measured as percent of dry leaf weight. Values are means ± SE; $n = 12$.



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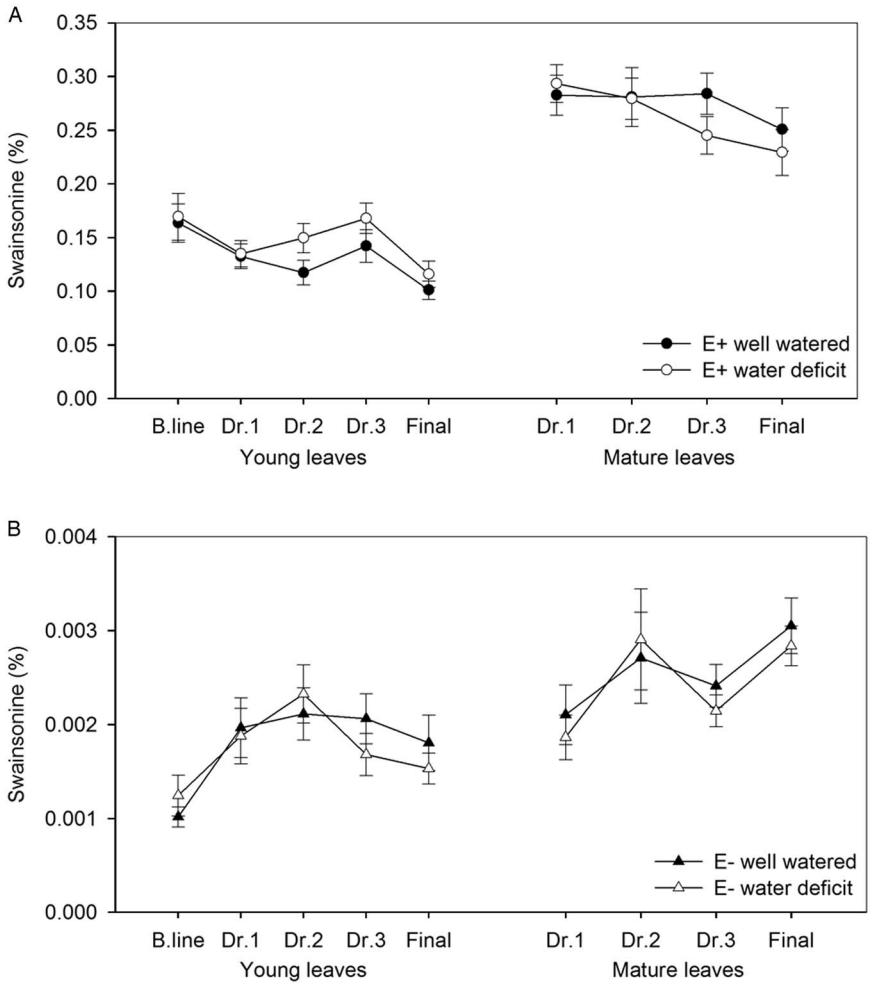


Figure 7. Swainsonine content of young and mature leaves of (A) endophyte-infected (E+ ) and (B) endophyte-free (E– ) silky crazyweed plants under well-watered (WW) and water-deficit (WD) conditions (B.line, baseline; Dr., drought period). Swainsonine content was measured as percent of dry leaf weight. Values are means ± SE; $n = 12$.

after final recovery, although this release from SWA production was not the reason for the increase in root or shoot biomass in woolly loco. In contrast, water deficit did not affect SWA concentration in silky crazyweed, further suggesting the difference in regulation of SWA level in these two taxa. Although SWA concentrations slightly fluctuated over the course of the experiment in all plants, such changes with time occurred in well-watered and in water-deficit plants and likely resulted from the influence of some environmental factors and/or phenological stage. Our results agree with the earlier report (Vallotton et al. 2012) that greater SWA concentrations were detected after water-deficit treatments in woolly loco but not in silky crazyweed. However, another study (Oldrup et al. 2010) found water deficit increased SWA in silky crazyweed and fungal cultures due to increased SWA synthesis rather than greater fungal biomass. The discrepancy between Oldrup et al. (2010) and our results can be explained by different methods for imposing water deficit and plant age: PEG-containing growth medium was used by Oldrup et al. (2010) to simulate water deficit in small seedlings grown in sterile magenta boxes, whereas greenhouse-grown plants and restricted watering were used in our study. It is also possible that water deficit in our experiments was not strong enough to influence SWA level in silky crazyweed, although it was sufficient to decrease plant mass.

Since water-deficit and well-watered endophyte-infected woolly loco plants had the same biomass, the observed 30% increase of SWA concentration in water-deficit plants most likely would create a higher overall toxicity of woolly loco plants under drought conditions. Thus, drought conditions could further elevate the already high toxicity of woolly loco plants typically containing 0.1% to 0.5% SWA, an amount that considerably exceeds the conservative critical toxic level of <0.001% (Cook et al. 2011). In addition to this, woolly loco plants are likely to be more toxic when



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more young leaves are present, whereas silky crazyweed plants probably are more toxic when they have more old leaves. In contrast, endophyte-free plants are not likely to pose significant risk to grazing livestock because of very low SWA levels of 0.001% to 0.003%, regardless of moisture conditions. Overall, despite some increase in SWA concentration under drought conditions, the effect of drought on locoweed toxicity is not as strong as the effect of endophyte presence. Other studies also have shown that SWA concentration in different locoweed taxa depends on the presence of the endophyte and genotype of the host plant rather than environmental conditions (Cook et al. 2013; Delaney et al. 2011; Ralphs et al. 2011; Vallotton et al. 2012).

**Nature of the Locoweed–Endophyte Complex.** Mutualism is considered to be the most likely type of symbiosis when an endophyte is vertically transmitted (Saikkonen et al. 1998). A mutualistic relationship among locoweed taxa and their endophytes could also be supported by high frequency of endophyte-infected plants in at least some locoweed populations (Cook et al. 2009, 2011, 2012; Ralphs et al. 2002; this study). Previous work on grasses demonstrated enhanced growth of endophyte-infected plants (Clay 1993), although a more recent meta-analysis suggests endophytes do not affect grass performance ubiquitously (Saikkonen et al. 2006). In our greenhouse experiments, however, where the endophyte was physically removed and plants were successfully grown in the greenhouse, the endophyte did not have any positive effect on growth of two locoweed taxa or induction of stress-response pigments or antioxidants. For woolly loco under well-watered conditions, the endophyte-reduced biomass was associated with biochemical indicators of oxidative stress. These results may indicate that despite diverting nutrients away from the plant for fungal metabolism and SWA production, the cost of harboring the endophyte is not great enough for the plant to eliminate the fungus, because there is a low cost of the endophyte, as suggested for several locoweed taxa (Creamer and Baucom 2013; Delaney et al. 2011; Vallotton et al. 2012). It is possible, though, that the fungus may benefit the plant via another mechanism, one not leading to biomass increase. It is also possible that some yet unidentified conditions or specific stage of plant development could improve plant fitness, fecundity, or competitiveness to ensure locoweed plant persistence. This role may enable woolly loco to tolerate the moderate decrease in biomass caused by the fungus.

It is possible that the relationship between the endophyte and locoweeds evolved from a purely parasitic or beneficial one to a dynamic complex that alternates between harmful and beneficial depending on the plant's environment. Both genera, *Oxytropis* and *Astragalus*, have representative species in China, and both have relationships with *Alternaria* spp. (Wang et al. 2006), and another novel species has been implicated as a pathogen of *A. adsurgens* (Li and Nan 2007). The discovery of related species of fungi interacting in different ways with both *Oxytropis* and *Astragalus* species raises interesting questions about the evolution of the complex between locoweeds and their endophytes. It is possible this relationship has developed differently between the fungus and the two plant genera.

Ralphs et al. (2011) found that *Alternaria* spp. section *Undifilum* endophyte is maternally transmitted to most of the progeny in several locoweed taxa. In our study, all woolly loco and a majority of silky crazyweed plants grown from intact seeds had the endophyte, indicating a high rate of endophyte-infected plants in the populations where the seeds were collected. However, very low endophyte and SWA concentrations and a low rate of endophyte-infected plants found in some other locoweed varieties and populations (Delaney et al. 2011; Ralphs et al. 2002, 2008; Vallotton et al. 2012) suggest that the relationship between a locoweed host and its endophyte may be a dynamic one that is affected by both plant and fungal genotype and, possibly, by environmental factors. The geographical spread of locoweeds is such that different taxa and even different populations from the same taxon grow in very different environments and are exposed to many stressors (Kulshrestha et al. 2004). The differences in frequency of endophyte-infected plants and concentration of SWA among locoweed taxa, varieties, and populations could be explained by differences in plant and endophyte genotypes (Braun et al. 2003; Cook et al. 2013) and different interactions between locoweeds and their endophytes under varying environmental conditions.

It is still not clear whether locoweeds benefit from the endophyte under some environmental conditions and whether loss of the fungus is associated with a decrease in fungal virulence or a stronger defense system against fungal infection in certain locoweed populations. To fully elucidate the relationship between the locoweed endophyte and different locoweed taxa, further studies need to be conducted on endophyte-infected and endophyte-free locoweeds grown under different environmentally stressful



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conditions and high pathogen and herbivore loads and at different stages of plant life cycle. Studies of the variability of vertical transmission of the fungus in distinct locoweed taxa and varieties and in individual populations from different geographical regions could also give insight into the dynamics of the relationship between the fungus and locoweeds and help to develop measures for control of locoweed poisoning.

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