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Influence of Seed Endophyte Amounts on Swainsonine Concentrations in *Astragalus* and *Oxytropis* Locoweeds

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ABSTRACT: Locoism is a toxic syndrome of livestock caused by the ingestion of a subset of legumes known as locoweeds endemic to arid and semiarid regions of the western United States. Locoweeds contain the toxic alkaloid swainsonine, which is produced by the endophytic fungi *Undifilum* species. Two chemotypes of plants can coexist within toxic populations of locoweeds: chemotype 1 plants are defined as individuals containing swainsonine concentrations greater than 0.01% and quantitatively greater amounts of *Undifilum*, while chemotype 2 plants are defined as individuals containing less than 0.01% swainsonine and quantitatively smaller amounts of *Undifilum*. To elucidate the mechanisms that govern chemotypes, the amount of *Undifilum* in seeds/embryos was manipulated, thus altering subsequent swainsonine concentrations in three locoweed species: *Astragalus mollissimus*, *Astragalus lentiginosus*, and *Oxytropis sericea*. Chemotype 1 seeds that were fungicide-treated or had the seed coat removed resulted in plants with swainsonine concentrations comparable to those in chemotype 2 plants. Conversely, embryos from seeds of chemotypes 1 and 2 that were inoculated with the endophyte resulted in plants with swainsonine concentrations comparable to those of chemotype 1 plants. This reproducible interconversion between the two swainsonine chemotypes suggests that the quantity of endophyte present in the seed at the time of germination is a key determinant of the eventual chemotype. Additionally, this is the first report of the inoculation of locoweeds with the endophyte *Undifilum* species.

KEYWORDS: *locoweed, swainsonine, endophyte, Undifilum, qPCR, inoculation*

INTRODUCTION

Locoism is a plant-induced disease in animals grazing the semiarid and arid western United States first observed and described by cattlemen of the 1800s.[1] Locoism results from prolonged grazing of specific leguminous plants in the *Astragalus* or *Oxytropis* genera,[2] which are generally called locoweeds. Locoweeds represent only a small percentage of the species in the *Astragalus* or *Oxytropis* genera as many *Astragalus* species contain nitro-toxins or are selenium-accumulators, and many other members of both genera are nontoxic.[3] Livestock afflicted with locoism suffer from neurological issues, reproductive problems,[3] and weight loss.[4] Swainsonine was identified as the toxin causing clinical signs similar to those of locoism in the Australian legume *Swainsona canescens*[5] and later identified as the toxin in locoweeds by Molyneux and James.[6] Additionally, other plants contain swainsonine and poison animals, including some *Ipomoea*, *Sida*, and *Turbina* species in South America and Africa.[7−10]

An investigation of the fungi associated with locoweeds resulted in the discovery and characterization of the endophyte *Embellisia*,[11,12] which was later named *Undifilum oxytropis*.[13] Additionally, other species of *Undifilum* are present in *Astragalus lentiginosus* and *A. mollissimus*.[14] *Undifilum* spp. are responsible for swainsonine synthesis[11,15] and, therefore, the toxicity of locoweeds.[16] *Undifilum* spp. can be detected in all plant parts but do not appear to grow outside of the living plant as mycelia or conidia.[17,18] *Undifilum* spp. are vertically transmitted from one generation to the next via hyphae in the seed coat.[19,20] Removing the seed coat from *O. sericea* results in plants with no detectable swainsonine and thus blocks the vertical transmission of the endophyte.[19]

Swainsonine concentrations vary greatly among *Astragalus* and *Oxytropis* locoweed species, varieties, populations, and individuals within the same population.[17,18,21,22] Two swainsonine chemotypes can be detected in wild populations of *Astragalus* and *Oxytropis* species. Chemotype 1 plants are defined as individuals containing swainsonine concentrations greater than 0.01% and would pose considerable risk to grazing livestock. Alternatively, chemotype 2 plants are defined as individuals containing less than 0.01% where swainsonine is typically not detected in the plant, and such plants would likely pose little risk to grazing livestock.[17,18,23] Additionally, chemotype 1 plants contain greater amounts of *Undifilum* spp. than chemotype 2 plants.[17,18] In the populations of the locoweed species surveyed, chemotype 1 plants generally represent greater than 85% of the plants.[17,18]

Previously, we proposed three mechanisms that may explain the origin of chemotype 2 plants in *A. lentiginosus*, *A. mollissimus*, and *O. sericea*. First, plants with no or very little detectable swainsonine concentrations may have arisen due to imperfect transmission of a critical amount of the endophyte to the seed or seedling[24] that is required for the plants to be colonized and produce concentrations of swainsonine characteristic of chemotype 1 plants. In this regard, we have reported that seeds of a chemotype 1 plant result in progeny that are either chemotype 1 or 2, thus demonstrating imperfect transmission.[20] Second, the genotype of the endophyte may

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yet be different between plants that differ in their swainsonine accumulation. Recent work does not support this suggestion, as we have reported that the Internal Transcribed Spacer (ITS) of the rDNA region of *Undifilum* spp. is identical between plants of chemotypes 1 and 2.[18] Third, low swainsonine (chemotype 2) plants may suppress endophyte growth and thus alkaloid production due to a plant genotype by endophyte interaction similar to that observed in grasses.[25,26]

To further investigate these possibilities, the objective of this study was to determine if the difference in swainsonine concentrations between chemotypes is due to the amount of *Undifilum* in germinating seeds or results from a plant-by-endophyte interaction whereby the growth of the endophyte is suppressed by the host plant. Specifically, we addressed the following questions: (1) What are the swainsonine concentrations and endophyte amounts in chemotype 1 and chemotype 2 seeds? (2) Can plants derived from chemotype 1 seeds have swainsonine concentrations similar to those of chemotype 2 plants by reducing the amount of *Undifilum* in chemotype 1 seeds? (3) Can plants derived from chemotype 2 seeds have swainsonine concentrations similar to those of chemotype 1 plants by inoculating chemotype 2 embryos with *Undifilum*?

MATERIALS AND METHODS

Plant Material. Seeds previously collected from chemotype 1 (swainsonine concentrations >0.01%) and chemotype 2 (swainsonine concentrations <0.01%, usually not detected) plants from populations of *Astragalus mollissimus* var. *earleii* (Fort Davis, Texas, N 30° 33′ 30.2″, W 103° 51′ 40.5″), *Astragalus lentiginosus* var. *wahweapensis* (Wahwah Valley, Utah, N 38° 23′ 45.6″, W 113° 17′ 28.66″), and *Oxytropis sericea* var. *sericea* (Park Valley, Utah, N 41° 54′ 15.4″, W 113° 20′ 54.9″) were used for all experiments described below.[22] Individual seeds collected from chemotype 1 and chemotype 2 plants were analyzed for swainsonine and endophyte amounts. Seeds were treated with 3 different methods to qualitatively reduce the amount of *Undifilum* inoculum: (1) removal of the seed coat, (2) fungicide treatment of seeds, and (3) heat treatment. Additionally, embryos were inoculated with *Undifilum* cultures to expose embryos of each chemotype to similar amounts of endophyte. Plants derived from the above-mentioned seeds/embryos were grown in a greenhouse with a 16 h photoperiod and day/night temperatures of 25 °C/20 °C. Plants were grown in cone-tainers (Stuewe and Sons Inc., Tangent, Oregon) with a 3:1 sand/peat mixture. All above ground parts of the plant were harvested and frozen. Subsequently, the harvested plants were freeze-dried and ground (1-mm screen). Swainsonine and DNA were extracted from this plant material for further analyses.

Control Plants. Seeds derived from chemotype 1 and chemotype 2 plants of each species were scarified and planted, and the resulting plants (n = 10−14 plants per chemotype per species) were grown in a greenhouse for 4 months representing control plants.

Seed Coat Removal. Surface sterilized seeds derived from chemotype 1 plants of each species were scarified and imbibed in sterile water for 10 min. Subsequently, the seed coat and any other layers of material were removed from the embryo to reduce the initial amount of *Undifilum* inoculum. The embryos were then planted, and the resulting plants (n = 8−12 plants per species) were grown in a greenhouse for 8 months.

Fungicide Treatment. Seeds derived from chemotype 1 plants of each species were scarified and imbibed overnight in a 0.9% pyraclostrobin (BASF, Research Triangle Park, NC) solution to reduce the initial amount of *Undifilum* inoculum. Pyraclostrobin, a strobilurin class fungicide, has a mode of action effective against fungal species (*Alternaria* spp.) closely related to *Undifilum*.[13] Subsequently, seeds were potted, and the resulting plants (n = 10 plants per species) were grown in a greenhouse for 8 months.

Heat Treatment. Seeds derived from chemotype 1 plants of each species were incubated at 60 °C for 11 days in an oven (similar treatment was effective in grasses) to reduce the initial amount of *Undifilum* inoculum. Subsequently, seeds were scarified and potted, and the resulting plants (n = 9−10 plants per species) were grown in a greenhouse for 8 months.

Endophyte Cultures. Endophyte cultures were obtained from chemotype 1 seeds of *Astragalus lentiginosus*, *A. mollissimus*, and *Oxytropis sericea*. Cultures were obtained from seeds by the followings means: seeds were bisected, and the seed halves were surface sterilized in a mixture of 70% ethanol, 30% bleach, and 0.01% Triton X-100 (Promega, Madison, WI) solution for 5 min, followed by a second step of 30% bleach and 0.01% Triton X-100 for 5 min, and a third step wherein the seed halves were rinsed two times in sterile water. Seed halves were placed on potato dextrose agar (Becton, Dickinson and Company, Sparks, MD) and placed in the dark at 25 °C for 2−3 weeks. Subsequently, subcultures were prepared and grown for 2−3 weeks for use in the inoculation procedure. All *Undifilum* cultures used in the inoculation procedure were no older than 2 months and had not been subcultured more than one time.

Inoculation. Seeds derived from plants of chemotypes 1 and 2 were scarified and surface sterilized using the procedure described above. After the seeds had been rinsed, the seed coat was removed as described above, and the embryos were placed on plates corresponding to a single *Undifilum* source culture derived from the same species. The embryos were germinated while physically touching *Undifilum* cultures to simulate the proximity of endophyte hyphae that would be encountered by an embryo with an endophyte-laden seed coat. Embryos were left in contact with cultures for 7 days in a growth chamber with a 16 h photoperiod at a constant temperature of 24 °C (n = 4 to 9 plants per chemotype per species). Seedlings were removed from the agar plates, transferred to cone-tainers (Stuewe and Sons, Tangent, Oregon), and allowed to grow in a greenhouse for 4 months.

Swainsonine Extraction. Swainsonine detection and concentration were measured using a modification of a previously published procedure.[27] In brief, a measured quantity (50 mg) of dried plant material or a single seed was placed in a 2 mL screw-cap microcentrifuge tube. The ground plant material was extracted in 1.5 mL of 2% acetic acid for 18 h. After extraction, the samples were centrifuged, and 0.05 mL of extract was added to 0.95 mL of 20 mM ammonium acetate in a 1 mL autosampler vial. Single seed extractions were performed on a smaller scale to account for the mass of the seed. Samples were then analyzed by LC-MS as described by Gardner et al.[21] The detection limit of swainsonine was 0.001% of dry weight using this extraction procedure. In cases where swainsonine concentrations were lower than the detection limit (treatments: remove seed coat, fungicide application, and the chemotype 2 controls), swainsonine was concentrated for increased detection capabilities using the solid phase extraction (SPE) procedure on Strata-X-C 33 μm, cation mixed-mode polymeric sorbent columns, 30 mg (Phenomenex, Torrance, California, USA), used by Gardner and Cook.[27] The resulting dried extract obtained from the solid phase extraction was dissolved in 0.1 mL of water and again analyzed by LC/MS as described previously.[21]

DNA Extraction. DNA was extracted from lyophilized, ground plant material (~20 mg) or a single seed using the DNEasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Extractions were performed according to the manufacturer's instructions. DNA was quantitated with an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Quantitative PCR. The fungal endophyte was quantitated by qPCR as described in Cook et al.[28] using a CHROMO 4 quantitative PCR detector (Bio-Rad Laboratories Inc., Hercules, CA). The limit of quantitation was 0.2 pg endophyte DNA per ng total DNA. The primers used were ITS 5 (5′ GGA AGT AAA AGT CGT AAC AAG G 3′)[29] and OR1a (5′ GTC AAA AGT TGA AAA TGT GGC TTG G 3′), which amplify the ITS region of the rDNA region. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). A subset of PCR products were verified to be *Undifilum* via restriction enzyme digest, agarose gel electrophoresis, and sequencing.[28]

Table 1. Swainsonine and Endophyte Amounts in Chemotype 1 and Chemotype 2 Seeds from *Astragalus lentiginosus*, *Astragalus mollissimus*, and *Oxytropis sericea*[a]

	chemotype 1				chemotype 2			
		swainsonine (%)				swainsonine (%)		
species	N	range	mean	SE	N	range	mean	SE
Astragalus lentiginosus	16	0.28–0.72	0.52[a]	0.04	8		nd[b]	
Astragalus mollissimus	8	0.72–1.05	0.88[a]	0.04	4	nd–0.009	0.003[b]	0.002
Oxytropis sericea	8	0.03–0.08	0.05[a]	0.01	4		nd[b]	
		endophyte (pg/ng total DNA)				endophyte (pg/ng total DNA)		
species	N	range	mean	SE	N	range	mean	SE
Astragalus lentiginosus	16	1.1–10	4.7[a]	0.6	8		<0.2[b]	
Astragalus mollissimus	8	0.2–0.8	0.4[a]	0.1	4		<0.2[b]	
Oxytropis sericea	8	8.1–15.8	12.2[a]	0.8	4		<0.2[b]	

[a]Statistical comparisons made within a species. Different superscript letters between chemotypes represent significance at $P < 0.05$.

Sequencing. PCR products were prepared for sequencing with the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The ITS region (ITS1–5.8S–ITS2) was sequenced using both the forward and reverse primers used for amplification. DNA sequencing was performed at the Genomics Core Facility, Center for Integrative Biology, Utah State University, Logan, UT.

Data Analysis. The number of seeds and plant samples per treatment within each species ranged from 4 to 14. Samples with endophyte amounts below the limit of quantitation (0.2 pg/ng total DNA) were assigned a value of 0.1 pg/ng for graphical and statistical purposes. Endophyte amounts (pg/ng of total DNA) were transformed to a natural log for statistical comparisons; untransformed values are shown in graphs and tables unless otherwise noted. Statistical comparisons (swainsonine and endophyte amounts) were only made within a species. A one-way ANOVA was performed using Sigma Stat 3.1 with a posthoc test of significance using a Bonferroni correction. A *p*-value of <0.05 was considered statistically significant. All data are presented as the mean ± standard error.

RESULTS AND DISCUSSION

Swainsonine and Endophyte Content in Seeds. Mean swainsonine concentrations in seeds derived from a chemotype 1 plant were 0.52 ± 0.04% in *A. lentiginosus*, 0.88 ± 0.04% in *A. mollissimus*, and 0.05 ± 0.01% in *O. sericea* (Table 1). Mean endophyte amounts in seeds derived from a chemotype 1 plant were 4.7 ± 0.6 pg/ng in *A. lentiginosus*, 0.4 ± 0.1 pg/ng in *A. mollissimus*, and 12.2 ± 0.8 pg/ng in *O. sericea* (Table 1). Mean swainsonine concentrations in seeds derived from a chemotype 2 plant were not detected in *A. lentiginosus*, 0.003 ± 0.002% in *A. mollissimus*, and not detected in *O. sericea* (Table 1). Endophyte amounts in seeds derived from a chemotype 2 plant were less than 0.2 pg/ng in *A. lentiginosus, A. mollissimus*, and *O. sericea*, respectively (Table 1). Mean swainsonine concentrations and endophyte amounts were greater in seeds derived from chemotype 1 plants than in chemotype 2 plants in all three species ($P < 0.001$) (Table 1).

These results with seeds are consistent with previous reports of the swainsonine and endophyte amounts in plant material of chemotypes 1 and 2. Previously, we identified two chemotypes from individual populations of toxic *Astragalus* and *Oxytropis* locoweeds: chemotype 1 consisted of plants containing swainsonine concentrations greater than 0.01%, and chemotype 2 consisted of plants with swainsonine concentrations less than 0.01% (generally not detected or concentrations near 0.001%).[17,18] Swainsonine concentrations were greater in the seeds of the two *Astragalus* species but similar in seeds of *O. sericea* when compared to reports for other plant parts of the species investigated.[17,18] Additionally, greater amounts of *Undifilum* are associated with chemotype 1 plants than with chemotype 2 plants, which are also consistent with the seed data reported here.[17,18]

Swainsonine and Endophyte Content in Control Plants. Mean swainsonine concentrations in plants derived from nontreated chemotype 1 seeds were 0.24 ± 0.02%, 0.41 ± 0.03%, and 0.14 ± 0.01% for *A. lentiginosus*, *A. mollissimus*, and *O. sericea*, respectively (Figure 1). Mean endophyte amounts in plants derived from nontreated chemotype 1 seeds were 71.0 ± 7.8 pg/ng in *A. lentiginosus*, 13.7 ± 2.3 pg/ng in *A. mollissimus*, and 4.2 ± 1.4 pg/ng in *O. sericea* (Figure 2). Mean swainsonine concentrations in plants derived from nontreated chemotype 2 seeds were 0.0003 ± 0.0001% for *A. lentiginosus*, 0.0009 ± 0.0006% for *A. mollissimus*, and 0.0005 ± 0.0002% for *O. sericea* (Figure 1). The endophyte was detected by PCR in all plants derived from nontreated chemotype 2 seeds, but amounts were less than 0.2 pg/ng in *A. mollissimus*, *A. lentiginosus*, and *O. sericea* (Figure 2). Swainsonine and endophyte amounts were different between plants derived from chemotype 1 and chemotype 2 seeds ($P < 0.001$) (Figures 1 and 2).

These results are consistent with a previous report about transmission of the endophyte, and thus swainsonine, to the next generation in chemotype 1 and chemotype 2 plants. Ralphs et al.[20] reported that plants derived from seeds collected from a chemotype 1 plant give rise to principally chemotype 1 (E+) progeny and up to 15% chemotype 2 (E~) progeny to indicate imperfect transmission, while plants derived from seeds collected from a chemotype 2 plant give rise to only chemotype 2 plants.

Swainsonine and Endophyte Content in Reduced Inoculum Plants. Seed coats were removed from chemotype 1 seeds to diminish the endophyte quantity to determine if reducing the amount of *Undifilum* would result in plants with swainsonine concentrations similar to those of chemotype 2 plants. Mean swainsonine concentrations in plants derived from chemotype 1 seeds where the seed coat was removed were 0.0003 ± 0.00009% in *A. lentiginosus*, 0.0004 ± 0.0001% in *A. mollissimus*, and less than 0.0001% in *O. sericea* (Figure 1). The endophyte was detected by PCR in all *A. lentiginosus* and *A. mollissimus* plants derived from chemotype 1 seeds where the seed coat was removed, but amounts were less than 0.2 pg/ng (Figure 2A and B). The endophyte was not detected by PCR in any of the *O. sericea* plants derived from chemotype 1 seeds where the seed coat was removed (Figure 2C). Swainsonine and endophyte amounts in plants derived from chemotype 1

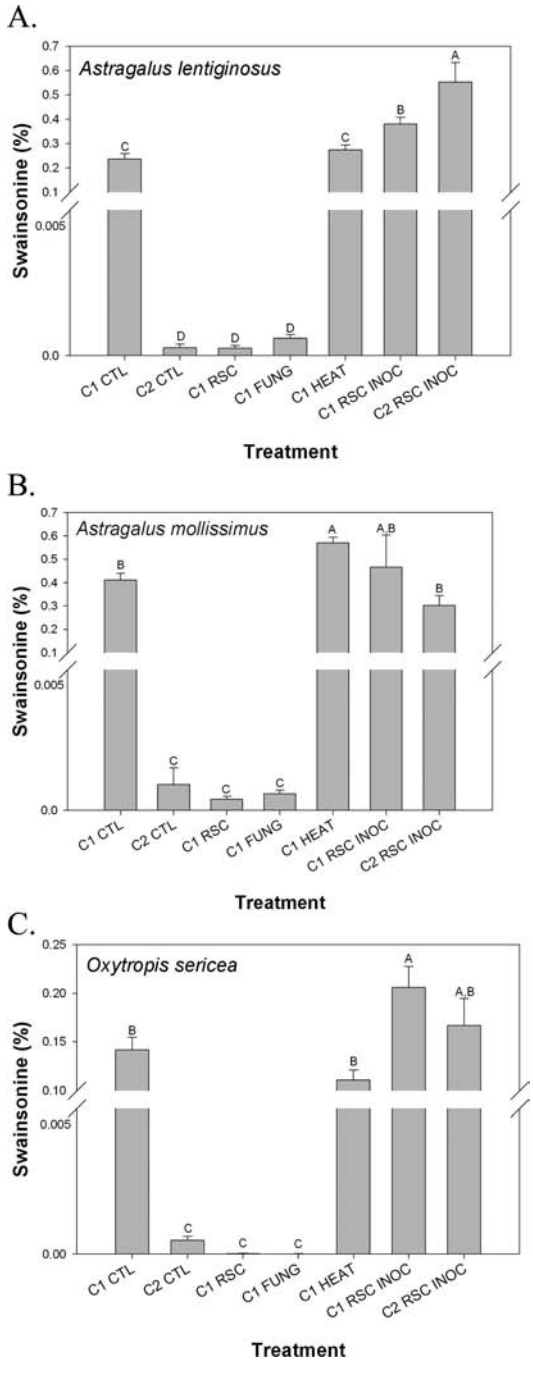

Figure 1. Swainsonine concentrations (%) in plants: (A) *Astragalus lentiginosus*; (B) *Astragalus mollissimus*; and (C) *Oxytropis sericea*. Mean swainsonine concentrations ± standard error from plants representing each treatment: C1 CTL (plants derived from control chemotype 1 seeds), C2 CTL (plants derived from control chemotype 2 seeds), C1 RSC (plants derived from chemotype 1 seeds where the seed coat was removed), C1 FUNG (plants derived from fungicide treated seeds), C1 HEAT (plants derived from heat treated seeds), C1 RSC INOC (plants derived from endophyte inoculated chemotype 1 embryos), and C2 RSC INOC (plants derived from endophyte inoculated chemotype 2 embryos). The Y axis shows a break from 0.005% swainsonine to 0.1% in *Astragalus lentiginosus, Astragalus mollissimus*, and *Oxytropis sericea*. Different letters above each bar represent significance at $P < 0.05$.

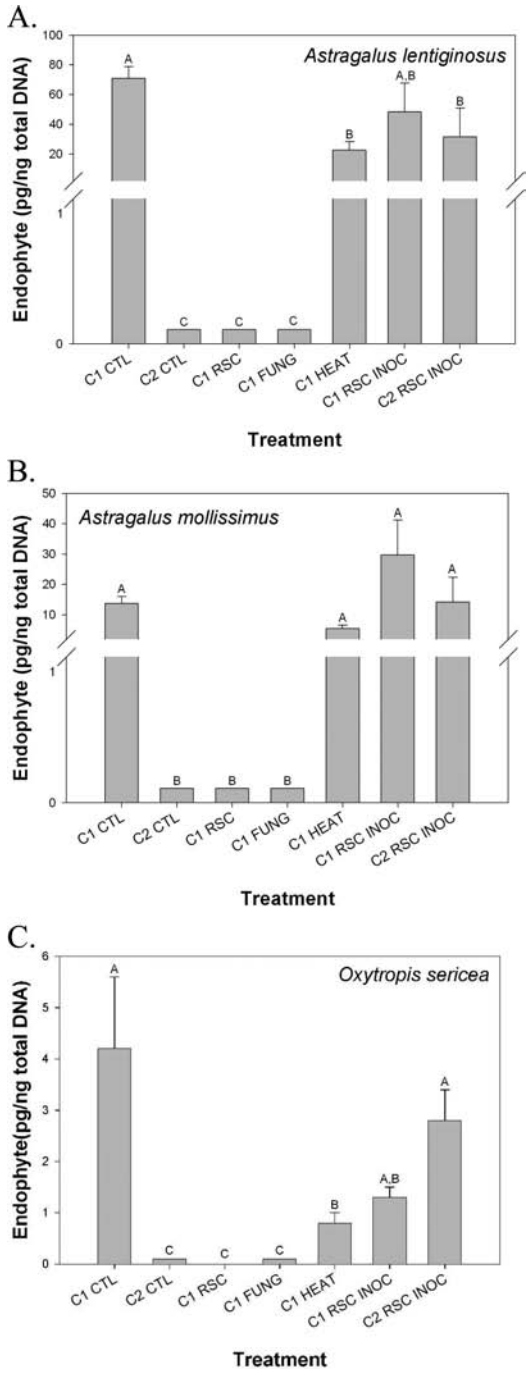

Figure 2. Endophyte amounts (pg/ng total DNA) in plants: (A) *Astragalus lentiginosus*; (B) *Astragalus mollissimus*; and (C) *Oxytropis sericea*. Mean endophyte amounts ± standard error from plants representing each treatment: C1 CTL (plants derived from control chemotype 1 seeds), C2 CTL (plants derived from control chemotype 2 seeds), C1 RSC (plants derived from chemotype 1 seeds where the seed coat was removed), C1 FUNG (plants derived from fungicide treated seeds), C1 HEAT (plants derived from heat treated seeds), C1 RSC INOC (plants derived from endophyte inoculated chemotype 1 embryos), and C2 RSC INOC (plants derived from endophyte inoculated chemotype 2 embryos). The Y axis shows a break in endophyte content from 1 to 2 pg/ng total DNA in *Astragalus lentiginosus* and *Astragalus mollissimus*. Different letters above each bar represent significance at $P < 0.05$.

seeds where the seed coat was removed differed ($P < 0.001$) from those in plants derived from chemotype 1 seeds but did not differ ($P = 1.0$) from those in plants derived from chemotype 2 seeds (Figures 1 and 2).

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Treating chemotype 1 seeds with the fungicide pyraclostrobin was also used to diminish the endophyte quantity to determine if reducing the amount of *Undifilum* would result in plants with swainsonine concentrations similar to those of chemotype 2 plants. Mean swainsonine concentrations in plants derived from pyraclostrobin-treated chemotype 1 seeds were 0.0001 ± 0.00004% in *A. lentiginosus*, 0.0006 ± 0.0001% in *A. mollissimus*, and less than 0.0001% in *O. sericea* (Figure 1). The endophyte was detected by PCR in all *A. mollissimus*, *A. lentiginosus*, and *O. sericea* plants derived from pyraclostrobin-treated chemotype 1 seeds, but amounts were less than 0.2 pg/ng (Figure 2). Swainsonine and endophyte amounts in plants derived from pyraclostrobin-treated chemotype 1 seeds differed ($P < 0.001$) from those in plants derived from chemotype 1 seeds but did not differ ($P = 1.0$) from those in plants derived from chemotype 2 seeds (Figures 1 and 2). Seed fungicide treatment generally reduced swainsonine and endophyte in subsequent plants as effectively as seed coat removal for all three taxa.

Treating grass seeds with heat is effective at destroying vertically transmitted endophytic fungi.[30] Mean swainsonine concentrations in plants derived from heat-treated chemotype 1 seeds were 0.27 ± 0.02%, 0.57 ± 0.02%, and 0.11 ± 0.01% for *A. lentiginosus*, *A. mollissimus*, and *O. sericea*, respectively (Figure 1). Mean endophyte amounts in plants derived from heat-treated chemotype 1 seeds were 22.6 ± 5.7 pg/ng in *A. lentiginosus*, 5.5 ± 1.1 pg/ng in *A. mollissimus*, and 0.8 ± 0.2 pg/ng in *O. sericea* (Figure 2). Swainsonine and endophyte amounts in plants derived from heat-treated chemotype 1 seeds are consistent with those in chemotype 1 plants (Figures 1 and 2). This data suggests that *Undifilum* species are not sensitive to heat unlike the grasses where heat is an effective tool to destroy vertically transmitted endophytic fungi.[30] We speculate that this may be due to differences in the endophyte associated with each plant (*Neotyphodium* versus *Undifilum*) or possibly the hard seed coat covering *Astragalus* and *Oxytropis* seeds.

Plants derived from seed coat removal or fungicide treatment of chemotype 1 seeds showed swainsonine concentrations consistent with those of chemotype 2 plants. These results suggest that these treatments altered the amount of endophyte in contact with the embryo thus altering the chemotype of the plant. These results support the hypothesis that the amount of *Undifilum* in a germinating seed is critical to determining whether the developing plant will have swainsonine concentrations consistent with those of a chemotype 1 or 2 plant.

Swainsonine and Endophyte Content in Inoculated Plants. Embryos of each chemotype were inoculated with the corresponding *Undifilum* species from each plant species to determine if embryos of seeds of chemotypes 1 and 2 could yield plants with swainsonine concentrations similar to those of chemotype 1 plants. Chemotype 1 embryos were successfully inoculated for each species with mean swainsonine concentrations of 0.38 ± 0.03%, 0.47 ± 0.14%, and 0.21 ± 0.02% for *A. lentiginosus*, *A. mollissimus*, and *O. sericea*, respectively (Figure 1). Mean endophyte amounts in plants derived from inoculated chemotype 1 embryos were 48.4 ± 19.4 pg/ng in *A. lentiginosus*, 29.8 ± 11.6 pg/ng in *A. mollissimus*, and 1.3 ± 0.2 pg/ng in *O. sericea* (Figure 2). Chemotype 2 embryos were successfully inoculated with mean swainsonine concentrations of 0.55 ± 0.08%, 0.30 ± 0.04%, and 0.17 ± 0.03% for *A. lentiginosus*, *A. mollissimus*, and *O. sericea*, respectively (Figure 1). Mean endophyte amounts in plants derived from inoculated chemotype 2 embryos were 31.5 ± 9.3 pg/ng in *A. lentiginosus*, 14.2 ± 8.1 pg/ng in *A. mollissimus*, and 2.7 ± 0.6 pg/ng in *O. sericea* (Figure 2). Additionally, the endophyte was sequence verified in a group of selected samples from each species of plant.

This is the first report of inoculating locoweed plants with the swainsonine-producing endophyte, *Undifilum*. Using this inoculation method, we show that embryos of chemotypes 1 and 2 of a given locoweed species can be inoculated with the corresponding *Undifilum* species with the resulting plants having swainsonine concentrations consistent with chemotype 1 plants. This suggests that the amount of swainsonine in chemotype 2 plants is not the result of a plant−endophyte interaction whereby the plant suppresses the growth of the endophyte and thus alkaloid production as was reported in ryegrass.[26] If a plant−endophyte interaction were responsible for the swainsonine concentration in chemotype 2 plants, one would expect the resulting plants from inoculated chemotype 2 embryos to have low or nondetectable swainsonine concentrations. These results are consistent with the hypothesis that the amount of *Undifilum* in a germinating seed is critical in determining the amount of swainsonine in the resulting plant.

Inoculating embryos of chemotypes 1 and 2 with *Undifilum* cultured from a chemotype 1 plant does not eliminate the possibility that chemotype 2 plants are harboring a genetically distinct, less potent variant of the endophyte, one less inclined to, or less capable of, rampant colonization of its host. *Undifilum* species can be detected by PCR in chemotype 2 plants[17,18] but cannot be cultured from chemotype 2 plants,[22] thus making it difficult to directly test this hypothesis. Sequencing of the *Undifilum* ITS of the rDNA region amplified from chemotype 1 and chemotype 2 plants showed no sequence differences associated with each chemotype.[18] Although the possibility of a genetically distinct, less potent variant of endophyte merits consideration, it is unlikely as we can detect no differences between the endophyte in each chemotype, and the two chemotypes can be interconverted through manipulating the amount of endophyte present at seed germination.

The three locoweed species investigated, *A. lentiginosus*, *A. mollissimus*, and *O. sericea*, showed similar responses in swainsonine and endophyte amounts to the treatments manipulating endophyte amounts. The one exception was the detection of the endophyte in plants derived from chemotype 1 seeds where the seed coat was removed. In *O. sericea*, the endophyte was not detected, while in *A. lentiginosus* and *A. mollissimus*, the endophyte was detected. This may be due to differences between the endophyte−embryo associations in the three species, or it may be due to simply the sensitivity of the PCR assay. We suggest that it is likely due to the sensitivity of the PCR assay as *O. sericea* plants contain less endophyte than *A. lentiginosus* and *A. mollissimus* chemotype 1 control plants.

The three locoweed species investigated, *A. lentiginosus*, *A. mollissimus*, and *O. sericea*, showed trends in swainsonine and endophyte amounts similar to those previously observed. Swainsonine concentrations from the three species were greater when grown in the green house compared to those of field plants;[22] however, the relative rank order is similar (*A. mollissimus* ≥ *A. lentiginosus* > *O. sericea*). Endophyte amounts were highly variable among the three species; however, similar trends in endophyte amounts among the three species were observed in green house grown plants by Ralphs et al.[20] Differences in swainsonine concentrations among the three species could be due to plant genotype, endophyte amount, and/or *Undifilum* species present. Swainsonine concentrations

are reported to be influenced by endophyte amount in *O. sericea*[31] and by *Undfilum* isolate.[11] Cook et al.[31] reported that endophyte and swainsonine amounts are correlated in chemotype 1 plant parts representing different phenological stages. Braun et al.[11] reported that *Undifilum* isolates from locoweed species that contain more swainsonine produced more swainsonine in culture than isolates from plants with less swainsonine.

A swainsonine concentration of 0.001% has been suggested as a conservative threshold of toxicity.[24] Plants derived from fungicide-treated seed had swainsonine concentrations below this toxicity threshold. Fungicide application to locoweed plants may decrease swainsonine concentrations and/or may disrupt the transmission of the endophyte to subsequent generations, thus altering locoweed toxicity. Additionally, due to the internal growth habits of *Undifilum* species, it is likely that low swainsonine chemotype 2 plants rarely encounter sufficient amounts of externally derived *Undifilum* in natural environments to become chemotype 1 plants. These hypotheses as well as others merit further investigation to better describe the locoweed−endophyte relationship.

In conclusion, we suggest that successful/robust/rampant colonization of endophyte and subsequent swainsonine concentrations within locoweed plant species appears to be governed by some critical mass of endophyte present during seed germination. This is supported by the reproducible interconversion between the two swainsonine chemotypes through methods used to manipulate the amount of endophyte present at seed germination. Lastly, an *Undifilum* inoculation method was developed, which will allow further investigation of endophyte host specificity within populations, varieties, species, and genera.

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Notes

The authors declare no competing financial interest.

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