Localization of endophytic *Undifilum* fungi in locoweed seed and influence of environmental parameters on a locoweed in vitro culture system

Erik Oldrup, Jennifer McLain-Romero, Anna Padilla, Andrew Moya, Dale Gardner, and Rebecca Creamer

Abstract: Endophytic *Undifilum oxytropis* (Q. Wang, Nagao & Kakish) Pryor, Creamer, Shoemaker, McLain-Romero, & Hambleton found within toxic locoweeds (*Astragalus* and *Oxytropis* spp.) produces the alkaloid swainsonine, which is responsible for locoism in grazing animals. We sought to determine the location of *U. oxytropis* within locoweed seed, develop endophyte free plants, and assess the influence of environmental stresses on locoweed and endophyte cultures. *Undifilum* was identified within the parenchyma layers in seeds of *Astragalus lentiginosus* M.E. Jones using light microscopy and polymerase chain reaction. *Astragalus lentiginosus* and *Oxytropis sericea* Nutt. seedlings produced in embryo culture without seed coats did not contain swainsonine or fungus. Plants produced from whole seed contained *U. oxytropis* in both foliage and root tissues. When the in-vitro cultured plants of *O. sericea* and *U. oxytropis* cultures were subjected to environmental stresses including high temperature, low and high pH media, nutrient deficient media, and polyethylene glycol (PEG) amended media to simulate water deficit, both dry mass and swainsonine levels were affected. Swainsonine levels were greatest for *O. sericea* and *Undifilum* cultures in PEG or hydrochloric acid amended media. Plants grown in PEG-amended media had significantly greater dry mass, while *Undifilum* grown in PEG-amended media had lower dry mass than other treatments.

Key words: Undifilum, fungal endophyte, locoweed, seed transmission.

Résumé : L'endophyte *Undifilum oxytropis* (Q. Wang. Nagao & Kakish) Pryor, Creamer, Shoemaker, McLain-Romero & Hambleton, que l'on retrouve chez les plantes locogènes (*Astragalus* et *Oxytropis* spp.), produit l'alcaloïde de Swainson, responsable du locoïsme chez des animaux herbivores. Les auteurs ont cherché à déterminer la localisation de la substance responsable dans la semence, ont développé des plantes sans endophytes, et ont évalué l'influence des stress environnementaux sur les plantes locogènes et les cultures d'endophytes. Ils ont identifié l'*Undifilum* dans les couches parenchymatiques de l'*Astragalus lentiginosus* M.E. Jones, à l'aide de la microscopie photonique et de la réaction de polymérisation en chaîne. Les plantules de l'*A. lentiginosus* et de l'*Oxytropis sericea* Nutt., produites à partir de cultures d'embryons sans téguments, ne contiennent ni le champignon ni la swainsonine. Par contre, les plantes produites à partir de graines entières contiennent l'*U. oxytropis* dans leurs tissus foliaires et racinaires. On affecte les poids secs des plantes et leurs teneurs en swainsonine, lorsqu'on soumet des plants de l'*O. sericea* et l'*U. oxytropis* cultivés in vitro à des stress environnementaux, incluant une température élevée, des milieux à haut et faible pH, des déficiences en nutriments, ainsi qu'un apport en polyéthylène glycol (PEG) pour simuler un déficit en eau. On obtient des teneurs en swainsonine plus importantes chez l'*O. sericea* et l'*U. oxytropis* lorsqu'on apporte dans leurs milieux de culture du PEG ou de l'acide chlorhydrique. Les plantes cultivées en présence de PEG montrent des poids secs plus élevés, alors que l'*U. oxytropis* montre des poids secs plus faibles en présence du même traitement.

Mots-clés : Undifilum, endophyte fongique, plante locogène, transmission par la semence.

[Traduit par la Rédaction]

Introduction

Toxic locoweed plants (*Astragalus* and *Oxytropis* spp.) contain the alkaloid swainsonine, (1,2,8-trihydroxyoctahydroindolizidine; Molyneux and James 1982), which inhibits α-mannosidases (Dorling et al. 1980; Winkler and Segal

Received 23 July 2009. Accepted 26 February 2010. Published on the NRC Research Press Web site at botany.nrc.ca on 7 May 2010.

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1984). Locoweed-poisoned grazing animals exhibit symptoms of locoism, including lack of muscular coordination, difficulty eating and drinking, changes in certain blood enzymes, and vacuolation of organs (James et al. 1981; James and Panter 1989; Ralphs et al 1988; Stegelmeier et al. 1995).

Locoweeds are toxic perennial flowering plants in the Fabaceae family, found in arid and semiarid regions in the western USA (Allred 1991), China (Li and Wang 2003), and Peru (Ramirez Luque 2006). The plants are prolific seed producers, and seed can remain viable after many years in the soil (James et al. 1999). The distribution and populations of locoweeds vary annually, owing to environmental conditions (James et al. 1968). In preliminary studies, Purvines and Ralphs (1999) found that high precipitation appeared to increase the growth of locoweed plants, while below-normal precipitation caused an increase in swainsonine levels. Many *Astragalus* species, such as *A. crassicarpus* Nutt. and *A. arenarius* L. are considered non-toxic (Allred 1991), and some populations of *Oxytropis lambertii* Pursh have been shown to contain very low levels (<0.001%) of swainsonine (Ralphs et al. 2002). These non-toxic species and (or) populations are classified as milkvetches and are considered excellent forage for cattle.

Swainsonine was originally isolated from the legume *Swainsona canescens* (Benth.) F. Muell. in Australia (Colgate et al. 1979), and has also been isolated from *Ipomoea carnea* Jacq. in Brazil (Molyneux et al. 1995), and from locoweeds. The fungus *Undifilum oxytropis* (Q. Wang, Nagao & Kakish) Pryor, Creamer, Shoemaker, McLain-Romero, & Hambleton (Pryor et al. 2009) can be isolated from locoweeds (Braun et al. 2003) and produces swainsonine in culture, as well as in planta. Swainsonine has also been isolated from the fungi *Rhizoctonia leguminicola* Gough & E.S. Elliot (Schneider et al. 1983; Harris et al. 1988) and *Metarhizium anisopliae* (Metschn.) Sorokin (Hino et al. 1985).

Undifilum oxytropis grows endophytically in locoweeds, causing no obvious symptoms to the plants (Braun et al. 2003). The exact nature of the symbiosis has not yet been determined. *Undifilum oxytropis* grows very slowly in culture (<0.2 $mm \cdot d^{-1}$), produces two hyphal types (a compact torulose basal layer and a surface layer of elongated filamentous aerial hypae), and can produce ellipsoid conidia with 2–4 transepta under certain conditions. *Undifilum oxytropis* has been isolated from leaves, stems, seed, and flowers of toxic locoweed species including *Oxytropis sericea* Nutt., *O. lambertii*, *Astragalus mollissimus* Torr., and *A. lentiginosus* M.E. Jones collected from the field (Braun et al. 2003). The fungus was isolated from the flowers, stems, and leaves of *O. kansuensis* Bunge (Wang et al. 2006), *A. wootonii* Sheldon, *A. pubentissimus* Torr. & A. Gray, and several varieties of *A. mollissimus* and *A. lentiginosus* (Ralphs et al. 2008). *Undifilum oxytropis* has not been found to sporulate on living plant tissues.

The toxic effect of *Undifilum oxytropis* on rats is indistinguishable from that caused by toxic locoweeds (McLain-Romero et al. 2004). Rats that fed on locoweed or the *Undifilum* endophytes gained less weight than alfalfa-fed rats, and showed intracellular vacuolation in renal, pancreatic, and hepatic tissues. Although the locoweed endophytes were initially thought to be species of *Alternaria*, later taxonomic studies suggested that they were most closely related to *Embellisia*. Further characterization has shown the fungi to belong to a new genus, *Undifilum* (Pryor et al. 2009). The other characterized species of *Undifilum*, *U. bornmuelleri* Pryor, Creamer, Shoemaker, McLain-Romero, & Hambleton (P. Magnus) sporulates on vetch leaves and has not been reported to be a seed pathogen.

Embellisia spp. have been found infecting seed (Simmons 1983): *Embellisia eureka* E.G. Simmons was isolated from *Medicago* seed, *E. planifunda* E.G. Simmons was isolated from surface-sterilized wheat seed, and *E. abundans* E.G. Simmons was isolated from *Dianthus* and *Hordeum* seeds (Agarwal and Sinclair 1987). Other Pleosporaceae, such as *Alternaria*, are well-known plant pathogens, many of which are seed-transmitted. Thirty-one species of *Alternaria* spp. have been reported to be seed-borne pathogens of major crops.

Several studies have shown that the presence of *U. oxytropis* in locoweeds can be correlated with locoweed toxicity (Braun et al. 2003; Gardner et al. 2004; Wang et al. 2006). Furthermore, a study that compared the swainsonine levels in 16 populations of *O. lambertii* plants with ability to culture the fungus *U. oxytropis* from the plants, showed that a high swainsonine content was correlated with endophyte presence (Gardner et al. 2004). A survey of *Astragalus* and *Oxytropis* spp. locoweeds from throughout the western USA, showed that high swainsonine levels were found in plants from which *U. oxytropis* was cultured and the fungus was detected by PCR (Ralphs et al. 2008).

Management of locoweeds to prevent livestock poisoning focuses mainly on the plant, which is difficult because swainsonine content in locoweed plants is highly variable (Ralphs et al. 1988; Smith et al. 1992). Knowledge of how environmental factors affects the toxicology of the plants and the fungus is essential for efficacious management of the plants. In addition, localization of the fungus within the plant could provide information on how the fungus is transmitted between plants.

The objectives of this research were to determine the location of *Undifilum* within seed tissues, and establish a plant-culture system to grow locoweeds free of their fungal endophyte. In addition, the role of environmental factors on swainsonine production was studied in planta and in cultured fungus.

Materials and methods

Isolation of fungus from seed tissues

Locoweed seed lots collected between 1968 and 2001 from Utah, Arizona, and Nevada (Table 1) by members of the United States Department of Agriculture Poisonous Plants Research Laboratory, Logan, Utah, were tested for the presence of viable *Undifilum* by culturing of fungal isolates. After field collection, the seeds were stored at room temperature in envelopes. The seeds were scarified with sand-paper (medium grit) and soaked in sterile water for approximately 30 min, and then the seed coats and membrane layers were removed aseptically and placed onto potato dextrose agar (PDA) plates. The remaining embryos were also

Table 1. *Undifilum oxytropis* cultured from locoweed seed tissues.

	Successful isolations per seed tissue tested	
Seed collection location and date	Embryo	Seed coat
Astragalus lentiginosus		
Henry Mt., UT 1968	0/15	7/15
Winslow, AZ, 1970	0/15	7/15
Henry Mt., UT, 1978	0/15	8/15
Jena, NV 1978	0/15	1/3
Oxytropis sericea		
Raft River Mt., UT, 1978	0/15	4/5
Raft River Mt., UT, 1979	0/15	13/15
Raft River Mt., UT, 1992	0/15	13/15
Raft River Mt., UT, 2001	0/15	4/5

placed onto PDA plates. The agar plates were maintained at room temperature, and after one week they were examined with a dissecting microscope. Fungal growth on the PDA plates was identified as *Undifilum* by comparative morphology with reference samples and testing by polymerase chain reaction (method described below).

In-vitro culture of locoweeds

Seeds used for the production of in-vitro-culture plants were provided by M. Ralphs (Logan, Utah). *Oxytropis sericea* seed was collected from Raft River Mt., Utah, in 1984, and *Astragalus lentiginosus* seed was collected from Henry Mt., Utah, in 1978. Seeds were scarified with fine grit sandpaper, and soaked for 15 min in sterile deionized water. They were then surface sterilized using 10% bleach (0.6% hypochlorite) for 30 s, followed by 70% ethanol for 30 s. The seeds were then rinsed in sterile distilled water for 30 s, dried on sterilized paper towels for 10 h, and transferred onto water agar plates. Since these seeds possessed seed coats, this treatment was referred to as "with coat".

"Embryo only" treatments were produced by removing the seed coat and residual parenchymatous tissue with two sterile forceps, after scarification of the seeds. The extracted embryos were then surface sterilized with 10% bleach for 30 s, followed by 10% ethanol for 30 s, and then rinsed in sterile distilled water for 30 s prior to placing them onto water agar, and allowing them to germinate in a Percival model E 36L growth chamber for 7 d at 28 °C with 550 micromoles·m^{-2}·s^{-1} PAR continuous light. After one week, all of the germinated plants (derived from whole seed or embryos) were transferred into magenta boxes (75 mm × 75 mm × 100 mm) containing approximately 100 mL ULTO media (Reyes-Vera et al. 2004). The plants were incubated for 30 d and then transferred to fresh ULTO media for an additional 30 d.

ULTO medium (Reyes-Vera et al. 2004) contains: 2.7 g Murashige and Skoog major salts (ammonium-free) (Sigma M2909), 4.4 g casein hydrolysate, 12.6 mg phloroglucinol, 100 mg glutamic acid, 0.575 g proline, 100 mg thiamine HCl, 25 mg pyridoxine HCl, 12.5 mg inositol, 5.5 g agar, and 1 L deionized water. If necessary, the pH of the media was adjusted to 5.5. PEG-ULTO was prepared as before, with the addition of 32 g polyethylene glycol 8000. For high pH ULTO and low pH ULTO, the media was prepared as before, and adjusted to pH 6.5 with NaOH or to pH 4.5 with HCl, respectively. For N-deficient ULTO, 10 mL MS Base (Sigma M0529) and 0.056 g potassium phosphate were substituted for the MS major salts. For P-deficient ULTO, 10 mL MS Base, 0.095 g calcium nitrate, and 0.035 g potassium sulfate were substituted for the MS major salts. For K-deficient ULTO, 10 mL MS base and 0.095 calcium nitrate were substitute for the MS major salts. The vitamins thiamine, pyridoxine, and inositol were filter sterilized and added to the autoclaved media. Agargel (Sigma) was added at 5 g·L^{-1} for all plant media.

Plant treatments

Oxytropis sericea plants produced from whole 1984 Raft River Mt. seed were subjected to eight different treatments: baseline control, PEG, high temperature, low pH, high pH, nitrogen deficient, potassium deficient, and phosphorus deficient. Each treatment included a 30 d growth period at baseline parameters and a 30 d treatment phase, for a total of 60 d. The experimental design was a one-way treatment structure in a completely randomized design. All experiments were conducted in a Percival® model E-36L growth chamber with the specification mentioned above. The data were analyzed for all treatments using SAS performing an ANOVA and LSD test using proc GLM (SAS Institute Inc. 2002–2003). The experiments had 10 replications for every treatment and were repeated three times.

For the 30 d growth period, plants were maintained on ULTO media in magenta boxes (1 seedling per box) at 28 °C. After the 30 d growth period, plants were transferred to the 30 d treatment phase (baseline control, PEG-ULTO, high temperature, low pH, high pH, nitrogen deficient, potassium deficient, or phosphorus deficient). For the baseline control, plants were transferred to fresh ULTO media pH 5.5 and incubated at 28 °C for 30 d. The PEG-ULTO consisted of ULTO containing 32 g·L^{-1} polyethylene glycol 8000 (PEG). This was calculated to produce an osmotic stress (water deficit) equal to –1.86 MPa (Gill et al. 2001) compared with the baseline control level of –0.30 MPa. For the high temperature treatment, plants were grown on ULTO at 32 °C for the treatment period. For pH treatments, the pH of the ULTO was adjusted to 4.5 for the low pH treatment, by addition of hydrochloric acid, and to 6.5 for the high pH treatment, by addition of potassium hydroxide, prior to autoclaving. The nutrient-deficient treatments consisted of Murashige and Skoog salts modified to be deficient in nitrogen, phosphorus, or potassium, with the addition of 0.056 g·L^{-1} monobasic potassium phosphate to the nitrogen-deficient media, 0.95 g·L^{-1} calcium nitrate and 0.035 g·L^{-1} potassium sulfate to the phosphorus-deficient media, and 0.025 g·L^{-1} calcium nitrate to the potassium-deficient media, to maintain the other nutrients.

Fungal treatments

Undifilum oxytropis cultures isolated from 1984 Raft River Mt. *O. sericea* seed coats were also tested with eight different treatments: baseline, PEG-ULTO, high temperature, low pH, high pH, nitrogen deficient, potassium deficient, and phosphorus deficient. Each culture was allowed a 30 d growth period and a 30 d treatment phase for a total of 60 d. The fungus was grown in the same growth chamber as the plants. There were restrictions in randomization and tests

were run for chamber effects. The data were analyzed for all treatments using the SAS program performing ANOVA and LSD tests using the general linear model (SAS Institute Inc. 2002–2003).

Undifilum oxytropis colonies were started 3 months prior to the experiment. To begin the experiment, one plug was taken from the outer portion of a fungal culture grown on PDA, using a size 00 cork borer, and placed into liquid ULTO media in 250 mL flasks containing 100 mL media, covered with aluminum foil, and incubated for the 30 d growth period in the growth chamber at 28 °C without shaking. Then fungal cultures were then transferred for the 30 d treatment phase (baseline, PEG-ULTO, high temperature, low pH, high pH, nitrogen deficient, potassium deficient, and phosphorus deficient). These treatments were the same as described above for the plants except that no agar was added to the media. The experiments had 10 replications for every treatment, and were repeated three times.

Swainsonine extraction and detection

After the 30-day treatment phase, plant height and width measurements were taken at the maximum points. To assay *O. sericea* and *U. oxytropis* for swainsonine, they were removed from growth media with sterile tweezers and scalpels, respectively, dried at 38 °C for 16 h, and dry mass was measured. After grinding the samples to a fine powder with liquid nitrogen, swainsonine was extracted using the methods outlined in Gardner et al. (2001). Samples were analyzed for swainsonine concentration by liquid chromatography – mass spectrometry (LC/MS). The LC/MS system consisted of an HP 1100 binary solvent pump and autosampler, a Betasil C18 reversed phase HPLC column (100 mm × 2 mm, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and a Finnigan LCQ mass spectrometer. Final swainsonine concentrations were converted from grams of swainsonine to percent swainsonine per gram of dry mass.

Microscopy of locoweed seeds and roots

For the sectioning and embedding of seeds, whole seeds of *A. lentiginosus* and *O. sericea* were soaked overnight in sterile water. The seeds were then separated into seed coat and embryos, or whole seeds were cut in half. The seed coat, embryos, and seed halves were placed in separate tubes containing a formaldehyde fixation solution (0.1 mol·L^{-1} phosphate-buffered, 4% formaldehyde pH 6.8), and incubated overnight at room temperature. Seeds were embedded in paraffin and serial sectioned to 10–15 μm thickness, as described by Fuchs (1999). The sections were stained using 0.05% trypan blue in acidic glycerol (glycerol–water–HCl, 10:8:1, *v*/*v*) and analyzed using a Nikon Optiphot microscope.

For the analysis of seed components, seeds of *A. crassicarpus* (a non-toxic milkvetch) and *A. lentiginosus* were soaked in sterile water for 1–2 h, and stained by soaking in trypan blue at 100 °C for 10 min. Seed coats of stained seeds were dissected into three separate layers consisting of the outer seed coat layer (palisade or macrosclerids), the middle seed coat layer (parenchyma), and the inner seed coat layer (crushed parenchyma or endosperm) and placed onto slides.

A dual-staining procedure was performed on roots using trypan blue and Sudan IV (0.3 g Sudan IV in 74 mL of 95% ethanol plus 24 mL of deionized water). Roots were initially washed by soaking in 2.5% KOH for 3 min at 121 °C, and quickly rinsed with water. Root tissue acidification was performed by soaking the roots in 1% HCl solution for 3 min, followed by rinsing in water. Roots were stained by placing them in trypan blue for 2 min at 121 °C, rinsed in an 80% ethanol solution, and destained in acidic glycerol (glycerol–water–HCl, 5.5:45:5, *v*/*v*) for 3 min. For the second stain, the roots were then placed in Sudan IV stain for 3 min at 121 °C, and destained in water for 2 min. Twenty-one root sections were mounted on slides and viewed using a Nikon Optiphot transmitted light microscope.

PCR detection of *Undifilum*

Total DNA extractions were prepared from embryo-only cultured locoweed foliar or root tissues, whole seed-grown *O. sericea*, mycelia of *U. oxytropis* and *Alternaria japonica*, and seed tissues. *Alternaria* was used for comparison, since it is a related member of the Pleosperaceae, even though it is not a seed pathogen. DNA extractions were performed using lysis buffer and phenol–chloroform extraction (Braun et al. 2003). An additional phenol–chloroform extraction was used for extraction of DNA from seeds.

ITS5 (White et al. 1990) and OR1 (Ralphs et al. 2008) primers were utilized for PCR. These primers amplify the rDNA ITS regions containing ITS1 and ITS2 and the intervening 5.8S rDNA. Amplifications were carried out in 50 μL reactions containing 27.5 μL sterile deionized water, 5 μL 10× buffer (100 mmol·L^{-1} Tris-HCl, pH 8.3, 500 mmol·L^{-1} KCL, 0.01% gelatin), 2 μL 10 mmol·L^{-1} dNTPs, 2.5 μL each of 5 mmol·L^{-1} primers, 5 μL 25 mmol·L^{-1} $MgCl_2$, 0.25 μL *Taq* polymerase. Amplification of DNA from plants and fungi was carried out in a Perkin-Elmer Model 480 thermal cycler (Norwalk, Connecticut) with the following parameters: 94 °C for 30 min, then 30 cycles consisting of 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 45 s. A final extension of 72 °C for 10 min followed. Samples were analyzed by electrophoresis on a 1.5% agarose gel. Since the PCR product from both *Undifilum* and *Alternaria* cultures were equal in length, the PCR products were digested with *Ava*II. Samples that produced bands of approximately 580 bp were then digested with *Ava*II restriction enzyme at 37 °C for 1 h. The amplicons from *U. oxytropis* cultures and locoweed plants are cleaved by the restriction enzyme, while amplicons from *Alternaria* cultures are not cleaved.

Amplifications were done in triplicate from three separate experiments. Sequences of selected amplicons were determined by cloning into a PGEM-EZ vector and sequencing with an ABI sequencer, as described in Braun et al. (2003).

Results

Seed culturing and microscopic localization

Undifilum was cultured from seed coats, but never from embryonic tissue (Table 1). The fungus was cultured from 48% of the *Astragalus* and 78% of the *Oxytropis* seed coats. *Undifilum* was cultured from all seed lots. This was comparable with the 80% isolation of *Undifilum* from scarified

Table 2. Microscopic examination of *Astragalus lentiginosus* and *A. crassicarpus* seeds and roots.

	A. lentiginosus		*A. crassicarpus*	
Plant part tested	No. examined	With fungus (%)	No. examined	With fungus (%)
Whole seeds	50	68	25	0
Sectioned seed embryos	25	0	25	0
Sectioned seed coats	25	44	NT	—
Roots	25	64	5	0

Note: NT, not tested.

whole *O. sericea* 1984 Raft River Mt. seed and a seed germination rate of 86 ± 3%. No other fungi were consistently cultured from the seeds.

As shown in Table 2, the fungus was observed in 68% of the whole *A. lentiginosus* seeds, 44% of the sectioned seed coats, and 64% of the dual stained roots. The fungus was not observed in the corresponding tissues from *A. crassicarpus*. The seed consisted of a palisade or macrosclerid layer, parenchyma layer, and crushed parenchyma or endosperm layer, and an embryo (Fig. 1). The fungus was observed within the crushed parenchyma or endosperm layers (Fig. 2) and either adhering to or within the parenchyma layers of the seed coat (Fig. 3). *Undifilum* was not found within any embryonic tissues. The results of fungal growth from cultured seed tissues agreed with the microscopic observations (Table 1).

Embryo culture of locoweeds

Astragalus lentiginosus and *O. sericea* plants grown in ULT-O media from embryos (without seed coats) did not produce swainsonine. Plants germinated from whole seed produced swainsonine under the same in vitro culture conditions (Fig. 4). Plants had the typical grey–green coloration and hirsuteness expected of these species of locoweed. Plants grew large enough to fill the 75 mm × 100 mm magenta boxes (data not shown). The plants did not flower and we did not alter the environmental conditions to induce flowering. The fungus was never observed growing from plants germinated without seed coats, while plants germinated from whole seed often showed small *Undifilum* colonies surrounding the base of the plants. If plants with external fungal colonies remained in the same media for an extended time, the fungus grew over the plant and appeared to weaken the plant. Rarely, sporulation of the fungus within the magenta boxes was observed. Plants transferred to sand after 30 d of growth did not survive.

PCR detection of *Undifilum*

PCR using the ITS5/OR1 primer set amplified a band of approximately 580 bp that was cleaved with *Ava*II from locoweed seeds, plants cultured from whole seeds, and pure cultures of *U. oxytropis* (data not shown). PCR amplification produced a similar 580 bp band that was cleaved with *Ava*II from *Oxytropis sericea* seed coats and inner membranes (Fig. 5, lanes 4, 5, 7, 8), but not from embryos, (Fig. 5, lanes 3 and 6). That amplicon was identical to that of *U. oxytropis* isolate L12 (GenBank FJ357335). The nucleic acid sequence of amplicons from *Undifilum*-infected *Astragalus mollissimus* was identical to that from *A. lentiginosus* and to the sequences obtained from *Undifilum* cultures previously isolated from *A. mollissimus* (GenBank FJ486217) and *A. lentiginosus* (GenBank FJ486218). *Undifilum* was detected from the leaves and root tissues of in-vitro-cultured seedlings of *O. sericea* within 2 weeks of germination; however, the fungus itself was not readily cultured.

Swainsonine levels for plant treatments

Swainsonine levels of in-vitro-produced *O. sericea* plants grown from whole seed varied significantly among the treatments. Figure 6 shows the mean swainsonine levels for each locoweed plant treatment. The mean percentage of swainsonine for the baseline treatment was 0.007% swainsonine·(g dry mass)$^{-1}$ (DM) of plant. Swainsonine levels were highest (0.052% swainsonine·(g DM)$^{-1}$ for plants in grown in PEG-ULTO, a 7-fold increase over the baseline. Locoweed plants grown in the lower pH (4.5) media showed a 4-fold increase in swainsonine levels (0.032% swainsonine·(g DM)$^{-1}$) compared with the baseline levels. Locoweed plants with the treatments of high temperature (0.006% swainsonine·(g DM)$^{-1}$), high pH (0.007% swainsonine·(g DM)$^{-1}$), nitrogen deficiency (0.008% swainsonine·(g DM)$^{-1}$), phosphorus deficiency (0.006% swainsonine·(g DM)$^{-1}$), and potassium deficiency (0.009% swainsonine·(g DM)$^{-1}$) did not show a significant change in the amount of swainsonine produced compared with baseline levels (Fig. 6).

Swainsonine levels for fungal treatments

The levels of swainsonine in *U. oxytropis* cultures also varied significantly among the treatments, with similar trends to that found for the plants. However the levels of swainsonine in *U. oxytropis* were 3- to 5-fold greater than in the plants in comparable treatments. Figure 7 shows the mean swainsonine levels for each fungal treatment. The mean percentage of swainsonine for the dried baseline was 0.029% swainsonine·(g DM)$^{-1}$ of fungus mycelium. Levels of swainsonine were greatest in *Undifilum* grown in ULTO-PEG, with a concentration of 0.157% swainsonine·(g DM)$^{-1}$, a 5.5-fold increase over baseline fungal levels. *Undifilum* grown on the lower pH (4.5) media showed a 3.9-fold increase in swainsonine level (0.111% swainsonine·(g DM)$^{-1}$) compared with the baseline control. *Undifilum* cultured at high temperature (0.023% swainsonine·(g DM)$^{-1}$), high pH (0.031% swainsonine·(g DM)$^{-1}$), nitrogen deficiency (0.028% swainsonine·(g DM)$^{-1}$), phosphorus deficiency (0.032% swainsonine·(g DM)$^{-1}$), and potassium deficiency (0.029% swainsonine·(g DM)$^{-1}$) did not show a significant change in the amount of swainsonine produced compared with the baseline controls (Fig. 7).

Figs. 1–3. Fig. 1. Cross section of *Astragalus lentiginosus* seed coat showing layers; pa, palisade or macrosclerid layer; par, parenchyma and crushed endosperm layers; em, embryo. Scale bar = 340 µm. Fig. 2. *Undifilum* within crushed parenchyma and endosperm layers of *Astragalus lentiginosus* seed coat. Scale bar = 34 µm. Fig. 3. *Undifilum* within parenchyma layers of *Astragalus lentiginosus* seed coat. Scale bar = 25 µm.

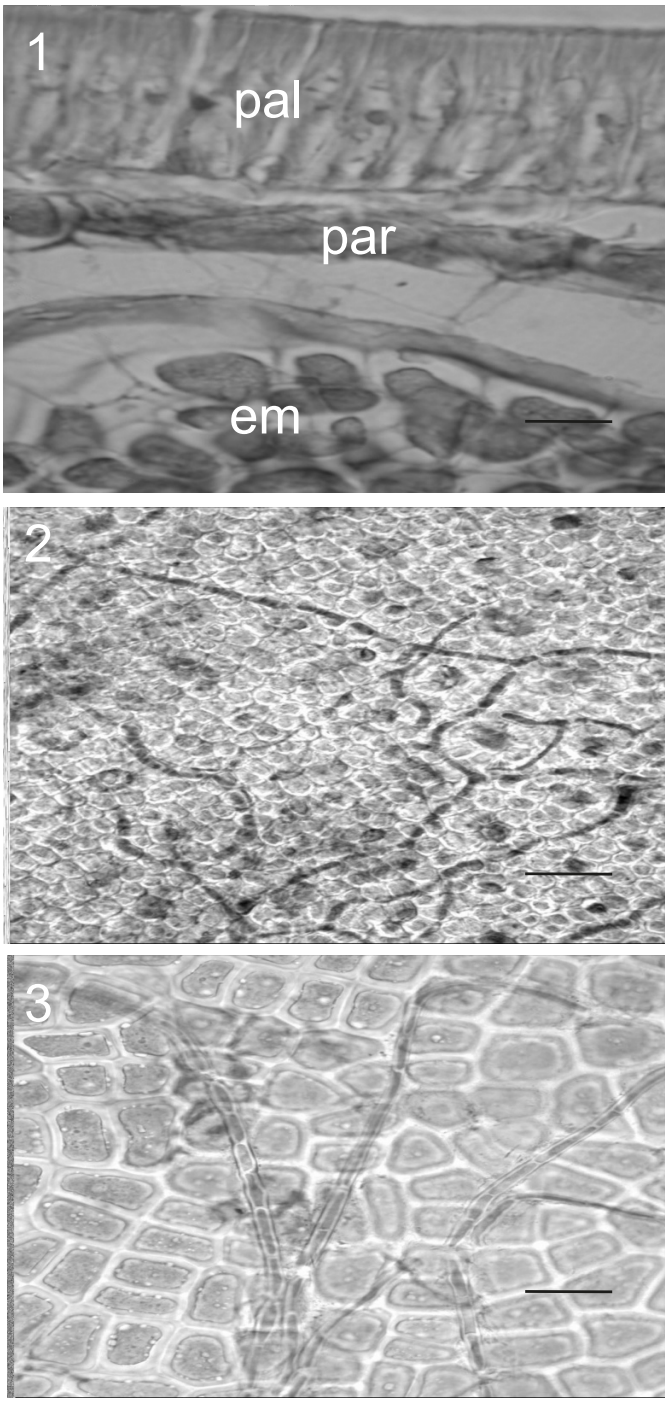


Fig. 4. Swainsonine content in embryo only *Oxytropis sericea* locoweed plants. (PBL, plant baseline treatment; EO1, plant embryo only, first replicate; EO2, plant embryo only, second replicate; EO3, plant embryo only, third replicate). For PBL, $n = 30$; for EO1, EO2, and EO3, $n = 10$. Error bar indicates SEM.

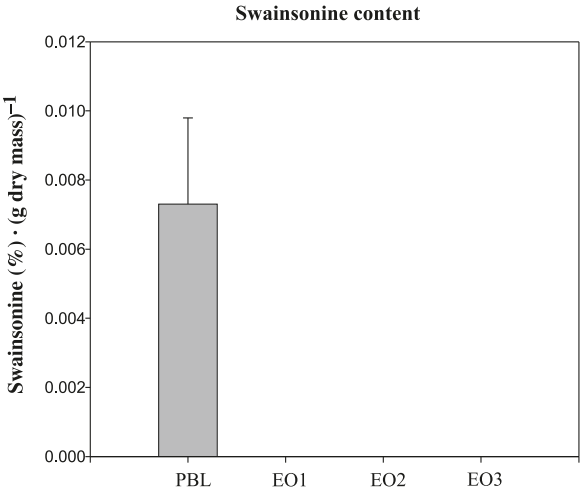
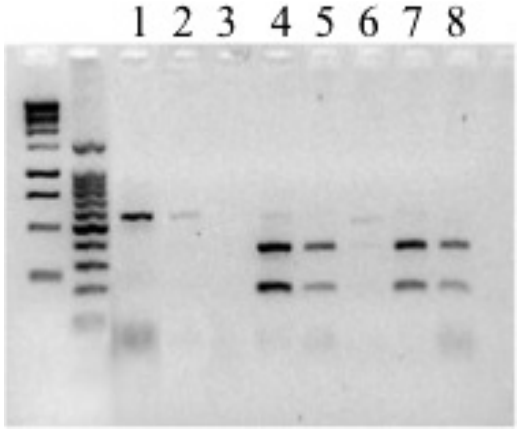


Fig. 5. Agarose gel electrophoresis of PCR with ITS5 and OR1 primers from undigested amplicon from *Alternaria japonica* (lane 1), *Ava*II digestion of amplicon from *Alternaria japonica* (lane 2), *Ava* II digestion of amplicon from *Oxytropis sericea* seed embryo (lanes 3 and 6), *Ava* II digestion of amplicon from *Oxytropis sericea* seed inner membrane (lanes 4 and 7), and *Ava II* digestion of amplicon from *Oxytropis sericea* seed coat (lanes 5 and 8).



Plant growth measurements

At the end of the 60 d growth period, observations were made on the height and width of each plant. *Oxytropis sericea* cultured in PEG-ULTO were significantly taller (4.8 cm) than any of the other treatments (Fig. 8). The heights of plants exposed to the high-temperature treatment (3.7 cm), low pH treatment (4.1 cm), high pH treatment (3.7 cm), nitrogen deficient treatment (3.5 cm), phosphorus deficient treatment (3.5 cm), potassium deficient treatment (3.5 cm), and embryo germinated (3.3 cm) plants were not significantly different from baseline plants (3.6 cm).

Locoweed plant width measurements also varied by treatment (Fig. 9). Plants grown on a low pH (4.5) media (3.7 cm) and PEG-ULTO (3.5 cm) were significantly wider than other treatments. *Oxytropis sericea* subjected to high temperature (2.8 cm) and high pH (3.1 cm) treatments were significantly narrower than the low pH plants and plants grown on PEG-ULTO, but significantly wider than the nitrogen deficient (2.1 cm), baseline (2.5 cm), phosphorus defi-

Fig. 6. Swainsonine content of *Oxytropis sericea* plants grown from whole seed after 30-day treatments. (BL, baseline treatment; DRT, PEG treatment; PH-L, low pH treatment; PH-H, high pH treatment; TEMP, high temperature treatment; N, low nitrogen treatment; P, low phosphorus treatment; K, low potassium treatment). For all values, $n = 30$; error bar indicates SEM. The same letters above each column indicate no significant difference at $\alpha = 0.05$ by LSD ($p > 0.05$).

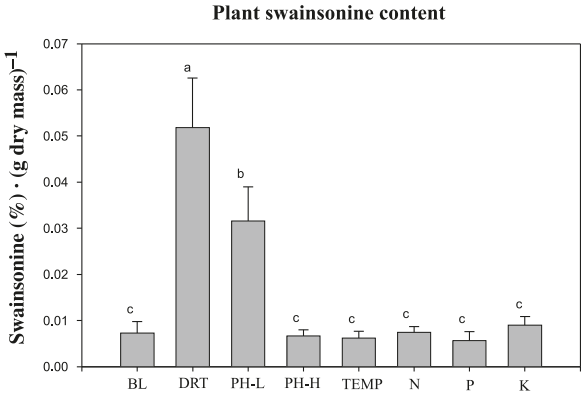
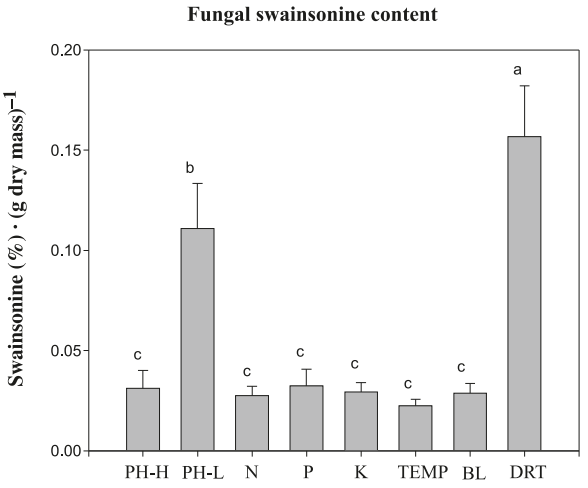


Fig. 7. Swainsonine content of *Undifilum oxytropis* cultures after 30-day treatments. (PH-H, high pH treatment; PH-L, low pH treatment; N, low nitrogen treatment; P, low phosphorus treatment; K, low potassium treatment; TEMP, high temperature treatment; BL, baseline treatment; DRT, PEG treatment). For all values, $n = 30$; error bar indicates SEM. The same letters above each column indicate no significant difference at $\alpha = 0.05$ by LSD ($p > 0.05$).



cient (2.5 cm), potassium deficient (2.4 cm) treated plants, and the embryo germinated (2.5 cm) plants.

The dry mass of the locoweed plants also varied by treatment (Fig. 10). Surprisingly, plants grown on PEG-ULTO had a significantly greater dry mass (0.355 g) than the baseline (0.146 g), low pH (0.125 g), high pH (0.129 g), nitrogen deficient (0.155 g), phosphorus deficient (0.157 g), potassium deficient (0.157 g) plant treatments, and embryo germinated (0.113 g) plants. Plants subjected to high temperature had significantly lower dry mass (0.062 g) than plants subjected to all other treatments ($\alpha = 0.05$).

Fig. 8. Height of *Oxytropis sericea* plants after 30-day treatments. (BL, baseline treatment; DRT, PEG treatment; PH-L, low pH treatment; PH-H, high pH treatment; TEMP, high temperature treatment; N, low nitrogen treatment; P, low phosphorus treatment; K, low potassium treatment; EMBR, plants germinated without seed coats and grown under baseline conditions). For all values, $n = 30$; error bar indicates SEM. The same letters above each column indicate no significant difference at $\alpha = 0.05$ by LSD ($p > 0.05$).

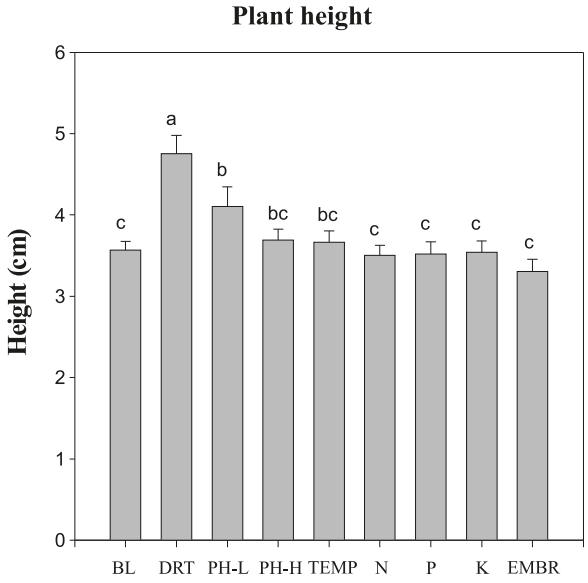
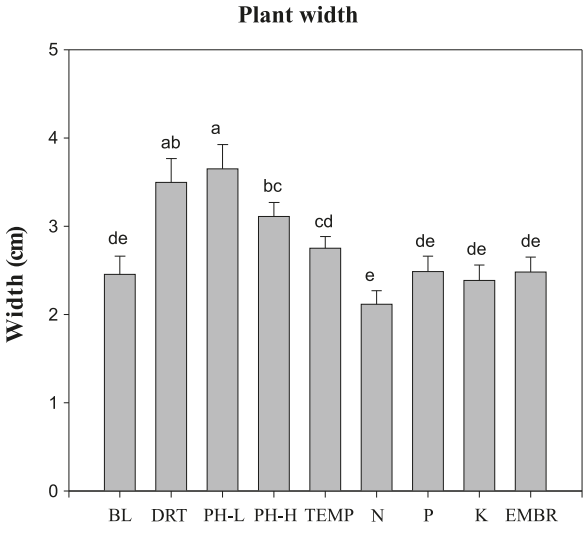


Fig. 9. Width of *Oxytropis sericea* plants after 30-day treatments. (BL, baseline treatment; DRT, PEG treatment; PH-L, low pH treatment; PH-H, high pH treatment; TEMP, high temperature treatment; N, low nitrogen treatment; P, low phosphorus treatment; K, low potassium treatment; EMBR, plants germinated without seed coats and grown under baseline conditions). For all values, $n = 30$; error bar indicates SEM. The same letters above each column indicate no significant difference at $\alpha = 0.05$ by LSD ($p > 0.05$).



Fungal dry mass

Fungal dry mass varied with treatment, but with different trends than the plant dry mass. The baseline fungus (0.514 g) and the potassium deficient cultures (0.417 g) had

Fig. 10. Dry mass of *Oxytropis sericea* plants after 30-day treatments. (BL, baseline treatment; DRT, PEG treatment; PH-L, low pH treatment; PH-H, high pH treatment; TEMP, high temperature treatment; N, low nitrogen treatment; P, low phosphorus treatment; K, low potassium treatment; EMBR, plants germinated without seed coats and grown under baseline conditions). For all values, n = 30; error bar indicates SEM. The same letters above each column indicate no significant difference at $\alpha = 0.05$ by LSD ($p > 0.05$).

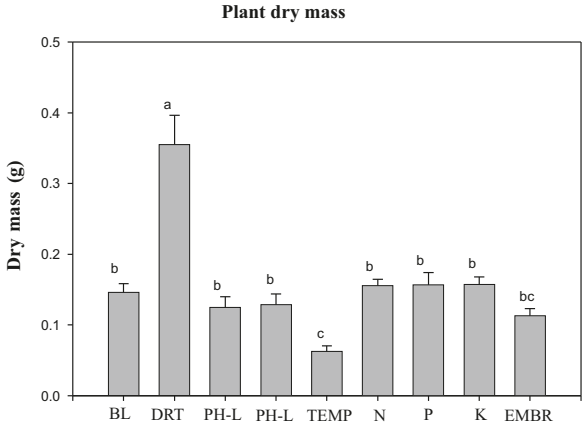
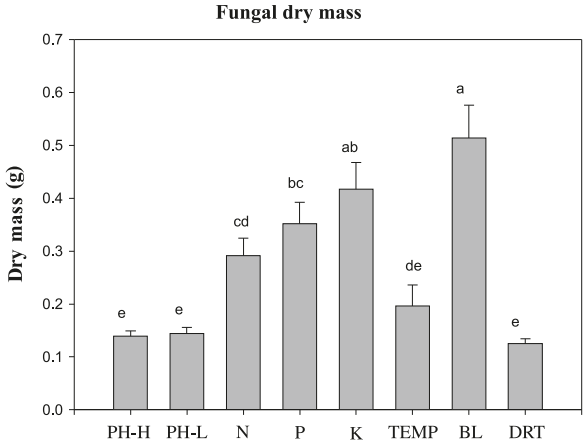


Fig. 11. Dry mass of *Undifilum oxytropis* after 30-day treatments. (PH-H, high pH treatment; PH-L, low pH treatment; N, low nitrogen treatment; P, low phosphorus treatment; K, low potassium treatment; TEMP, high temperature treatment; BL, baseline treatment; DRT, PEG treatment). For all values, n = 30; error bar indicates SEM. The same letters above each column indicate no significant difference at $\alpha = 0.05$ by LSD ($p > 0.05$).



a similar mass, which was greater than for the rest of the treatments (Fig. 11). The nitrogen deficient (0.292 g) and phosphorus deficient (0.352 g) cultures weighed significantly less than the baseline and potassium deficient cultures, but significantly more than the cultures exposed to the high temperature (0.196 g), low pH (0.145 g), high pH (0.140 g), or PEG-ULTO (0.125 g) treatments ($\alpha = 0.05$).

Discussion

This study shows that the *Undifilum* endophytes of locoweed are found within the coat and not the embryo of locoweed seed. This was shown by PCR, culturing, and microscopy. The embryo culture results also support this conclusion, since only plants that were germinated from seed with coats contained swainsonine. These results agree with our previous experiments (Braun et al. 2003), which showed that the fungus can be cultured from seed, and are consistent with our predictions that the endophytes could be maternally transmitted.

Although the seed used in this study was not recently collected, locoweed seed is known to remain viable for up to 40 years in the soil (James et al. 1999). The *O. sericea* seed used for in-vitro cultures had a germination percentage of 86%, and of the viable seed, 80% contained *Undifilum*. Obviously seed lots from different locoweed species, different locations, and different years will have different germination rates, swainsonine levels (Molyneux et al. 1989), and levels of *Undifilum* infection.

Our discovery of endophytes within the parenchyma layers of locoweed seed was similar to that for fungi infecting soybean seed:, e.g., *Alternaria alternata* (Fr.) Keissl. was found infecting all layers of the soybean seed coat (Kunwar et al. 1986). Similarly, *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore and *Cercospora sojina* Hara were found infecting only seed coat tissues of soybean (Kunwar et al. 1985).

The embryo-culture experiments demonstrated that a locoweed germinated without its seed coat does not contain swainsonine or *Undifilum oxytropis*. This supports our previous research that the endophyte is responsible for swainsonine production in locoweed plants. Our prior results (Braun et al. 2003) had hypothesized that the endophyte at least partially controlled swainsonine production, and these results strengthen that hypothesis. Although they looked identical to the toxic *Undifilum*-containing plants, the plants grown from embryos did not survive well outside of the tissue culture environment. We did not alter the environmental conditions to induce flowering, so whether the plants could produce viable endophyte-free seed is not known.

Undifilum was detected more readily with PCR than by culturing. The ability to detect *Undifilum* by PCR while unable to culture the fungus was also observed with *Undifilum* endophytes from *Astragalus mollisimus* Torr. var. *thompsoniae* (S. Watson) Barneby and *A. amphioxys* A. Gray (Ralphs et al. 2008); however, the amount of swainsonine detected was very low. For *Undifilum* to be successfully amplified from seeds, an additional phenol–chloroform extraction was required, compared with amplification from fungal cultures, suggesting that there may be inhibitors to PCR detection found within seeds.

We were able to detect the fungus from leaves and root tissues of in-vitro cultured locoweed seedlings within 2 weeks of germination. The detection of fungus from root tissues was somewhat surprising because we have not isolated *Undifilum* from the roots of field-collected plants (Braun et al. 2003). Locoweed roots are normally nodulated in field conditions (Valdez Barillas et al. 2007), and we did not add nodulating bacteria to our embryo culture system. Thus, within the embryo culture-system conditions, the fungus may have been able to infect the roots, while in the field, bacteria may replace or compete with the fungi for space within the roots.

Growth of *O. sericea* plants and swainsonine levels in those plants increased when cultured in a water stress-inducing media. The biomass of *Undifilum*-infected *O. sericea* plants grown on PEG-ULTO was approximately double that

of the other plant treatments in this study. Embryo-germinated plants grown on PEG-ULTO resulted in half the biomass of *Undifilum*-infected *O. sericea* grown on PEG-ULTO. This suggests that the endophyte may help plant growth in this stress environment or, the plant may prefer an environment with lower available water. Swainsonine levels in the PEG-ULTO treated plants were still significantly lower than in field-collected plants, which have been reported as high as 0.705% (Achata 2009) for *O. sericea*. There are many possible reasons for the very low levels of swainsonine in the in vivo cultured plants compared with field plants, including the very young age and small size of the cultured plants compared with field plants, differences in the growing environments, and differences in the genetics of the fungal isolate infecting the plants.

Undifilum grown in a medium with pH of 4.5 had an increase in swainsonine concentration (0.1109%) compared with the baseline treatment (0.0287%). These results show similar trends to *Metarhizium anisopliae*, which produces greater concentrations of swainsonine in a medium containing pH 7.0 than pH 9.0 (Sim and Perry 1995). *Undifilum* grew best at pH of 5.5, and better at a temperature of 28 °C than 32 °C. Comparison of the dry mass of the fungal cultures show that the baseline and potassium deficient cultures produced similar dry mass, which was greater than that for the other treatments, suggesting that these were optimal growing conditions. The amount of fungus (dry mass) produced was not positively correlated with swainsonine production. PEG-ULTO and low pH culture treatments produced the highest *Undifilum* swainsonine concentrations while the fungal dry mass for the two treatments produced the lowest averages compared with the other treatments.

The relationship between *U. oxytropis* and its locoweed plant hosts has not been well-defined. The locoweed plant appears to provide a haven for the endophytic fungus and seed dissemination. The data in this study show that embryo-germinated plants had a similar biomass, height, and width as baseline plants, but did not produce swainsonine. The locoweed plants did not appeared to be harmed by the presence of the endophyte. The endophyte produces swainsonine, which is theorized to deter herbivory, suggesting that this may be a mutualistic association, with both organisms benefiting from the association when the plants are healthy and not stressed.

This research supports the hypothesis that control of locoweed toxicity can only be achieved by controlling the *Undifilum* fungi within the locoweeds. Our results showed that environmental conditions cause variation in swainsonine levels in cultured *O. sericea* and *U. oxytropis*. These results were achieved in a controlled environment with a single stress applied for each treatment. Studies on the influence of multiple environmental conditions on mature plant growth and swainsonine levels would be beneficial to relate the stresses to a natural environment. Using the results found in this study and understanding the physiological relationship of the plant and endophytic fungus could provide better management strategies and options for reducing toxicity of locoweed plants.

Acknowledgements

We thank Mike Ralphs (Logan, Utah) for providing locoweed seed, advice, and encouragement. We also thank David Graham for locoweed seed. We recognize the financial support of the New Mexico State University Agricultural Experiment Station, USDA-CSREES Special Grant No. 59-5428-1-327, NSF-ADVANCE Institutional Transformation Program (NSF0123690), and the Minority Access to Research Careers program (NIH GM07667-29).

References

Achata, J.A. 2009. Variation in Undifilum DNA and swainsonine content in locoweed. M.S. thesis, New Mexico State University, Las Cruces, N.M.

Agarwal, V.K., and Sinclair, J.B. 1987. Principles of seed pathology. CRC Press, Boca Raton, Fla.

Allred, K.W. 1991. Locoweeds and livestock poisoning. New Mexico Coop. Ext. Ser. Pub. 400:B-15 .

Braun, K., Romero, J., Liddell, C., and Creamer, R. 2003. Production of swainsonine by fungal endophytes of locoweed. Mycol. Res. **107**(Pt 8): 980–988. doi:10.1017/S095375620300813X. PMID:14531620.

Colgate, S.M., Dorling, P.R., and Huxtable, C.R. 1979. A spectroscopic investigation of swainsonine: an alpha-mannosidase inhibitor isolated from *Swainsona canescens.* Aust. J. Chem. **32**: 2257–2264. doi:10.1071/CH9792257.

Dorling, P.R., Huxtable, C.R., and Colegate, S.M. 1980. Inhibition of lysosomal alpha-mannosidase by swainsonine, an indolizidine alkaloid isolated from *Swainsona canescens*. Biochem. J. **191**(2): 649–651. PMID:6786280.

Fuchs, J.T. 1999. Characterization of the relationship of *Meloidogyne incognita* in association with chile, yellow nutsedge and purple nutsedge. M.S. thesis, New Mexico State University, Las Cruces, N.M.

Gardner, D.R., Molyneux, R.J., and Ralphs, M.H. 2001. Analysis of swainsonine: extraction methods, detection, and measurement in populations of locoweeds (*Oxytropis* spp.). J. Agric. Food Chem. **49**(10): 4573–4580. doi:10.1021/jf010596p. PMID: 11599990.

Gardner, D.R., Romero, J., Ralphs, M.H., and Creamer, R. 2004. Correlation of an endophytic fungus (*Alternaria* spp.) with the presence of swainsonine in Lambert locoweed (*Oxytropis lambertii*). *In* Poisonous plants and related toxins. *Edited by* T. Acamovic, C.S. Stewart and T. Pennycott. Oxford University Press, Cary, N.C. pp. 32–38.

Gill, P.K., Sharma, A.D., Singh, P., and Bhullar, S.S. 2001. Effect of various abiotic stresses on the growth, soluble sugars and water relations of sorghum seedlings grown in light and darkness. Bulg. J. Plant Physiol. **27**: 72–84.

Harris, C.M., Schneider, M.J., Ungemach, F.S., Hill, J.E., and Harris, J.E. 1988. Biosynthesis of the toxic indolizidine alkaloids slaframine and swainsnonine in *Rhizoctonia leguminicola*: Metabolism of 1-hydroxyindolizidines. J. Am. Chem. Soc. **110**(3): 940–949. doi:10.1021/ja00211a039.

Hino, M., Nakayama, O., Tsurumi, Y., Adachi, K., Shibata, T., Terano, H., Kohsaka, M., Aoki, H., and Imanaka, H. 1985. Studies of an immunomodulator, swainsonine. I. Enhancement of immune response by swainsonine in vitro. J. Antibiot. (Tokyo), **38**(7): 926–935. PMID:3161855.

James, L.F., and Panter, K.E. 1989. Locoweed poisoning in livestock. *In* Swainsonine and related glycosidase inhibitors. *Edited by* L F. James, A.D. Elbein, R.J. Molyneux and C.D. Warren. Iowa State University Press, Ames, Iowa. pp. 23–38.

James, L., Bennet, K.L., Parker, K.G., Keeler, R.F., Binns, W., and

Lindsay, B. 1968. Loco plant poisoning in sheep. J. Range Manage. **21**(6): 360–365. doi:10.2307/3896082.

James, L.F., Bennet, K.L., Parker, K.G., Keeler, R.F., Binns, W., and Lindsay, B. 1981. Syndromes of *Astragalus* poisoning in livestock. J. Vet. Med. Assoc. **178**: 146–150.

James, L.F., Panter, K.E., Stegelmeier, B.L., Ralphs, M.H., Pfister, J.A., and Gardner, D.R. 1999. *Astragalus* and *Oxytropis* poison livestock with different toxins. *In* Locoweed research. *Edited by* T.M. Sterling and D.C. Thompson, New Mexico Coop. Ext. Ser. Res. Rep. **730:** 10–11.

Kunwar, I.K., Singh, T., and Sinclair, J.B. 1985. Histopathology of mixed infections by *Colletotrichum truncatum* and *Phomopsis* spp. or *Cercospora sojina* in soybean seeds. Phytopathology, **75**(4): 489–492. doi:10.1094/Phyto-75-489.

Kunwar, I.K., Manandhar, J.B., and Sinclair, J.B. 1986. Histopathology of soybean seeds infected with *Alternaria alternata.* Phytopathology, **76**(5): 543–546. doi:10.1094/Phyto-76-543.

Li, J.K., and Wang, J.H. 2003. The present situation and prospect of the study on locoweed in China. Agric. Sci. China, **2**: 687–698.

McLain-Romero, J., Creamer, R., Zepeda, H., Strickland, J., and Bell, G. 2004. The toxicosis of *Embellisia* fungi from locoweed (*Oxytropis lambertii*) is similar to locoweed toxicosis in rats. J. Anim. Sci. **82**(7): 2169–2174. PMID:15309966.

Molyneux, R.J., and James, L.F. 1982. Loco intoxication: indolizidine alkaloids of spotted locoweed (*Astragalus lentiginosus*). Science, **216**(4542): 190–191. doi:10.1126/science.6801763. PMID:6801763.

Molyneux, R.J., James, L.F., Panter, K.E., and Ralphs, M.H. 1989. The occurrence and detection of swainsonine in locoweeds. *In* Swainsonine and related glydosidase inhibitiors. *Edited by* L.F. James, A.D. Elbein, R.J. Molyneux and C.D. Warren. Iowa State University Press, Ames, Iowa. pp. 100–117.

Molyneux, R.J., McKenzie, R.A., O'Sullivan, B.M., and Elbein, A.D. 1995. Identification of the glycosidase inhibitors swainsonine and calystegine B_2 in Weir vine (*Ipomoea* sp. Q6 [aff. calobra]) and correlation with toxicity. J. Nat. Prod. **58**(6): 878–886. doi:10.1021/np50120a009. PMID:7673932.

Pryor, B.M., Creamer, R., Shoemaker, R.A., McLain-Romero, J., and Hambleton, S. 2009. *Undifilum*, a new genus for endophytic *Embellisia oxytropis* and parasitic *Helminthosporium bornmuellerii* on legumes. Botany, **87**(2): 178–194. doi:10.1139/B08-130.

Purvines, J., and Ralphs, M.H. 1999. Drought-stressed locoweed contains more swainsonine. *In* Locoweed research. *Edited by* T.M. Sterling and D.C. Thompson, New Mexico Coop. Ext. Ser. Res. Rep. **730:** 34–35.

Ralphs, M.H., James, L.F., Nielsen, D.B., Baker, D.C., and Molyneux, R.J. 1988. Cattle grazing Wahweap milkvetch in southeastern Utah. J. Anim. Sci. **66**(12): 3124–3130. PMID:3230074.

Ralphs, M.H., Welsh, S.L., and Gardner, D.R. 2002. Distribution of locoweed toxin swainsonine in populations of *Oxytropis lambertii*. J. Chem. Ecol. **28**(4): 701–707. doi:10.1023/A:1015228507950. PMID:12035920.

Ralphs, M.H., Creamer, R., Baucom, D., Gardner, D.R., Welsh, S.L., Graham, J.D., Hart, C., Cook, D., and Stegelmeier, B.L. 2008. Relationship between the endophyte *Embellisia* spp. and the toxic alkaloid swainsonine in major locoweed species (*Astragalus* and *Oxytropis*). J. Chem. Ecol. **34**(1): 32–38. doi:10.1007/s10886-007-9399-6. PMID:18060459.

Ramirez Luque, A.C. 2006. Efecto del consumo de "garbancillo" (*Astragalus dilinghamii* J.F. Macbride) en ratas Holtzman de laboratorio [Effects of the consumption of 'garbancillo' (*Astragalus dillinghamii* J.F. MacBride) in Holtzman lab rats]. B.S. thesis, Universidad Nacional Agraria La Molina, Peru.

Reyes-Vera, I., Hubstenberger, J., and Barrow, J. 2004. Hyperhydricity reversal and clonal mass propagation of four-winged salt bush (*Atriplex canescens* (Pursh) Nutt.) cultivated in vitro. *In* Vitro Cell. Develop. Biol.-Plant **40**:55-A.

SAS Institute Inc., 2002–2003 SAS/STAT User's Guide, Version 9.1. SAS Institute Inc., Cary, N.C.

Schneider, M.J., Ungemach, F.S., Broquist, H.P., and Harris, T.M. 1983. (1*S*,2*R*,8*R*,8α*R*)-1,2,8-trihydroxyoctahydroindolizine (swainsonine), an α–mannosidase inhibitor from *Rhizoctonia leguminicola.* Tetrahedron, **39**(1): 29–32. doi:10.1016/S0040-4020(01)97625-2.

Sim, K.L., and Perry, D. 1995. Swainsonine production by *Metarhizium anisopliae* determined by means of an enzymatic assay. Mycol. Res. **99**(9): 1078–1082. doi:10.1016/S0953-7562(09)80776-4.

Simmons, E.G. 1983. An aggregation of *Embellisia* species. Mycotaxon, **18**: 216–241.

Smith, G.S., Allred, K.W., and Kiehl, D.E. 1992. Swainsonine content of New Mexican locoweeds. Proceedings, Western Section, Amer. Soc. Anim. Sci. **43**: 405–407.

Stegelmeier, B.L., Molyneux, R.J., Elbein, A.D., and James, L.F. 1995. The lesions of locoweed (*Astragalus mollissimus*), swainsonine, and castanospermine in rats. Vet. Pathol. **32**(3): 289–298. doi:10.1177/030098589503200311. PMID:7604496.

Valdez Barillas, J.R., Paschke, M.W., Ralphs, M.H., and Child, R.D. 2007. White locoweed toxicity is facilitated by a fungal endophyte and nitrogen-fixing bacteria. Ecology, **88**(7): 1850–1856. doi:10.1890/06-0728.1. PMID:17645031.

Wang, Q., Nagao, H., Li, Y.L., Wang, H.S., and Kakishima, M. 2006. *Embellisia oxytropis*, a new species isolated from *Oxytropis kansuensis* in China. Mycotaxon, **95**: 255–260.

White, T.J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* PCR protocols: a guide to methods and applications. *Edited by* M.A. Innis, D.H. Gelfand, J. Sninsky and T.J. White, Academic Press, San Diego, Calif. pp. 315–352.

Winkler, J.R., and Segal, H.L. 1984. Inhibition by swainsonine of the degradation of endocytosed glycoproteins in isolated rat liver parenchymal cells. J. Biol. Chem. **259**(3): 1958–1962. PMID:6198327.