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Quantitative PCR Method To Measure the Fungal Endophyte in Locoweeds

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A fungal endophyte (*Undifilum oxytropis*) has been implicated in the synthesis of swainsonine in *Oxytropis* and *Astragalus* species, commonly known as locoweeds. A quantitative PCR method has been developed to measure the amount of endophyte in *Oxytropis* and *Astragalus* species. The limit of quantitation was estimated to be 0.2 pg of endophyte/ng of total DNA. This method of analysis was used to quantify the amount of endophyte in 10 plants each of *Oxytropis sericea* (white point locoweed), *Astragalus mollissimus* (wooly locoweed), and *Astragalus lentiginosus* (spotted locoweed). A significant amount of individual plant variability was observed in endophyte content among individuals in all three species. In one *O. sericea* and one *A. lentiginosus* plant swainsonine concentrations were near or below the limit of detection. These plants also had the lowest amounts of endophyte when compared to the other specimens. This method will be a useful tool in further investigating the role the endophyte plays in swainsonine production in various locoweed species.

KEYWORDS: Swainsonine; locoweed; endophyte; qPCR

INTRODUCTION

Locoweed poisoning is the most widespread poisonous plant problem in the western United States (*1*, *2*). The term locoweed refers to species of *Astragalus* and *Oxytropis* (family Leguminosae) that induce the loco disease in animals, due to the presence of the toxic alkaloid swainsonine (*3*). There are 22 species of *Oxytropis* and 354 species of *Astragalus* in the United States and Canada (*4*), only 24 species of which have been verified to contain swainsonine (**Figure 1**) or have a history of causing locoism (*5*). However, locoweed intoxication is not limited to North America as there are a number of *Astragalus* and *Oxytropis* species that have poisoned animals in Asia and South America (*6*, *7*). In addition, many different plants in the world contain swainsonine, including *Swainsona canescens* in Australia and some *Ipomoea* species of South America (*8*, *9*).

The consumption of locoweed species by livestock can result in locoism, which is a chronic disease characterized by diverse sequela that include neurological changes, reproductive disturbances, and emaciation (*10*). Pathological changes are due to the actions of swainsonine, which inhibits the function of α-D-mannosidase. This subsequently leads to altered processing of oligosaccharides associated with glycoproteins and a lysosomal storage disease known as mannosidosis (*11*−*14*). Symptoms of locoism usually develop as a result of livestock grazing locoweeds for several weeks.

Recently, a fungal endophyte, *Undifilum oxytropis*, previously annotated as *Embellesia oxytropis*, found in locoweed plant species has been implicated in the synthesis of swainsonine (*15*, *16*). A positive correlation was shown to exist between swainsonine concentrations found in the plant and concentrations of swainsonine produced by the endophytic fungus cultured from the same plant when grown in culture. More recently, a survey of the major locoweeds was performed in which swainsonine concentrations were determined and the presence of the endophyte was assessed using both culturing methods and Polymerase Chain Reaction (PCR) (*17*). Results from the survey suggested that *Astragalus* locoweeds generally contain higher swainsonine concentrations than do *Oxytropis* locoweeds. Furthermore, in plants in which swainsonine was detected, the endophyte was also detected. A significant observation was that in plants with low concentrations of swainsonine ($<0.01\%$), the endophyte was detectable only via PCR and not by culturing (*17*), suggesting that PCR is more sensitive in detecting the endophyte in plant material from both *Astragalus* and *Oxytropis* species.

Quantitative PCR methods have been developed to quantify the amount of several types of plant pathogens (*18*−*20*). Recently, a quantitative-based PCR method was developed to measure the fungal endophyte *Neotyphodium* in *Lolium perenne* (*21*). Developing a method to measure the fungal endophyte of *Astragalus* and *Oxytropis* species would provide a useful tool to further understand the plant endophyte relationship as it relates to swainsonine production. The objective of this study was to develop a method to quantify the fungal endophyte in locoweed plants containing the toxin swainsonine in plant material from *Oxytropis sericea*, *Astragalus mollissimus*, and *Astragalus lentiginosus*.

MATERIALS AND METHODS

Fungal Isolates. *U. oxytropis* was cultured from plant material from the following species: *O. sericea*, *A. mollissimus*, and *A. lentiginosus*.

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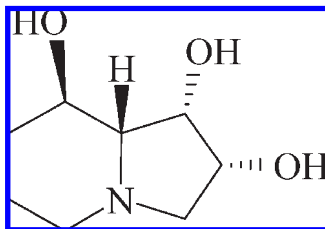

Figure 1. Chemical structure of swainsonine.

Fungal cultures were provided by Dr. Rebecca Creamer at New Mexico State University in the Department of Entomology, Plant Pathology, and Weed Science. The fungal cultures were maintained on potato dextrose agar (PDA) (DIFCO Laboratories, Detroit, MI) at room temperature.

Plant Materials. Ten plants of *O. sericea* Nuttall (white locoweed, Raft River Mountains, UT, N 41° 54′ 15.4″ W 113° 20′ 54.9″), *A. mollissimus* Torrey (wooly locoweed, Alpine, TX, N 30° 32′ 10.4″ W 103° 57′ 13.2″), and *A. lentiginosus* Douglas ex Hooker (spotted locoweed, Hanksville, UT, N 38° 11′ 36.9″ W 110° 44′ 52.5″) were collected in full flower and frozen on dry ice. Upon return to the laboratory, the plants were freeze-dried and ground. Swainsonine and DNA were extracted from this plant material for further analyses.

DNA Extraction. DNA was extracted from freeze-dried preparations of pure cultures of fungus (~20 mg) grown on potato dextrose agar and from freeze-dried ground plant material (~20 mg) using the DNEasy Plant Mini Kit (Qiagen Inc., Valencia, CA). All extractions were performed according to the manufacturer's instructions. DNA was quantified with the ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

PCR Primer Design. The PCR primers used have successfully detected the presence of the fungal endophyte, *U. oxytropis*, in *Oxytropis* and *Astragalus* species (*17*). In summary, the primers amplify the internal transcribed spacer (ITS) region and were ITS 5 (5′ GGA AGT AAA AGT CGT AAC AAG G) (*22*) and OR1 with an additional base pair at the 3′ end (5′ GTC AAA AGT TGA AAA TGT GGC TTG G 3′). Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Quantification of the Fungal Endophyte. For the quantification of the fungal endophyte, a standard curve (30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001 ng of fungal DNA) was prepared from DNA extracted from a pure culture of the endophytic fungus. A standard curve from *O. sericea* containing the fungal endophyte (100, 30, 10, 3, 1, and 0.3 ng of total DNA) was also prepared from DNA extracted from *O. sericea*. Each analysis included three replicate reactions for each DNA quantity. Each analysis also included a nontemplate control reaction, in which water was substituted for the DNA to confirm that the reagents were free from contaminating template DNA.

Fungal and plant samples for the standard curves were prepared by analyzing three replicate 25 μL reactions containing the amount of DNA (0.001−30 ng of fungal DNA or 0.3−100 ng of plant DNA) indicated on the standard curve. For each plant sample tested, three replicate reactions were performed in 25 μL reactions containing 50 ng (5 μL of a 10 ng/μL stock) of total DNA. Each reaction contained 12.5 μL of the QuantiFast SYBR Green PCR Kit master mix (Qiagen Inc., Valencia, CA) and 500 nM each of the forward and reverse primers. A standard curve was prepared by plotting the cycle threshold (Ct) against the $\log_{10}$ of the initial starting quantity of DNA. Endophyte content (per 50 ng of total DNA) was calculated from the endophyte standard curve. Endophyte amounts were expressed as picograms per nanogram of total DNA.

Amplification and detection of fluorescence were done using a Bio-Rad CHROMO4 quantitative PCR detector (Bio-Rad Laboratories Inc., Hercules, CA). Thermal cycling conditions were as follows: an initial denaturation step for 7 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 58 °C, 40 s at 72 °C, and a plate read at the end of each cycle. This was followed by a melting profile to determine the purity of the reaction products when the temperature was raised from 55 to 90 °C in 0.2 °C increments, held for 2 s at each temperature, and a plate read at each temperature.

PCR products were resolved on a 1% agarose gel containing ethidium bromide at 118 V for 20 min and visualized under UV illumination. Agaraose gels were visualized and analyzed with a Kodak Image Station

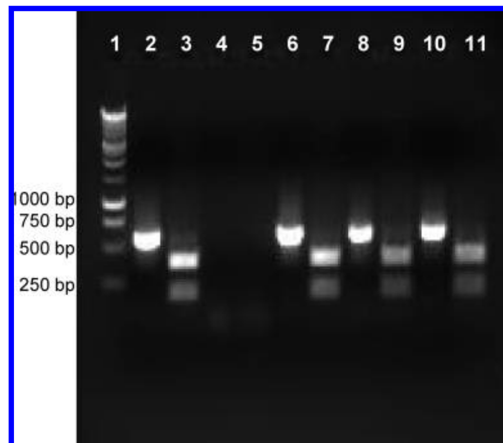

Figure 2. Agarose gel of representative qPCR analysis. Lanes: 1, 1000 bp marker; 2, *Undifilum oxytropis* (DNA from pure culture) control; 3, *Avr*II digest of lane 2 product; 4, negative control; 5, *Ava*II digest of lane 4 product; 6, *Oxytropis sericea*; 7, *Ava*II digest of lane 6 product; 8, *Astragalus mollissimus*; 9,*Ava*II digest of lane 8 product; 10, *Astragalus lentiginosus*; 11, *Ava*II digest of lane 10 product.

2000RT imager and its software (Eastman Kodak, Rochester, NY). The amplified PCR product was approximately 580 base pairs (bp). PCR products were digested with the restriction enzyme *Ava*II as a diagnostic check because this primer set may also potentially amplify *Alternaria* species. *Alternaria* species do not contain the restriction site; therefore, a digested product indicates the presence of the locoweed endophyte. Restriction enzyme digests contained 5 μL of PCR product, 0.5 μL of *Ava*II (New England Biolabs Inc., Ipswich, MA), 1 μL of NEB Buffer 4 supplied with the enzyme, and 3.5 μL of sterile water for a total volume of 10 μL. Digests were incubated at 37 °C for 1.5 h, after which the digest was heat inactivated at 65 °C for 20 min. Restriction fragments were separated by gel electrophoresis according to the conditions above. A result of two fragments with lengths of 380 and 200 bp confirmed the positive amplification of the locoweed endophyte, *U. oxytropis*.

Swainsonine Analysis. Samples were analyzed for swainsonine following the procedure described by Gardner et al. (*23*). In brief, 100 mg of plant material was extracted with chloroform and acetic acid. The acetic acid portion was passed through a cation exchange resin to retain the swainsonine, which was subsequently removed with a weak ammonium hydroxide solution. An aliquot of the final extract was then quantitatively analyzed for swainsonine by LC-MS. Swainsonine concentrations are expressed as a percent of dry matter. The detection limit of swainsonine was 0.001% of dry weight.

RESULTS AND DISCUSSION

Primer Specificity. Preliminary experiments were performed to optimize the primer concentration and annealing temperature to be used with SYBR green. The optimal reaction conditions were defined as those at which the lowest Ct value (the number of cycles for the fluorescent signal to cross a threshold) and the highest ΔRn (the background subtracted reporter fluorescence signal) were obtained. The optimal reaction conditions were 500 nM each of the forward and reverse primers and an annealing temperature of 58 °C.

A 580 bp product was amplified from a culture of the endophyte cultured from *O. sericea*, which when digested with *Avr*II resulted in two fragments with lengths of 380 and 200 bp (**Figure 2**). Similarly, a 580 bp product was amplified from *O. sericea*, *A. mollissimus*, and *A. lentiginosus* plant material, which when digested resulted in the same sized two fragments (**Figure 2**). In summary, these results demonstrate that this primer pair can be used for the detection of the endophyte in plant material of both *Oxytropis* and *Astragalus* species.

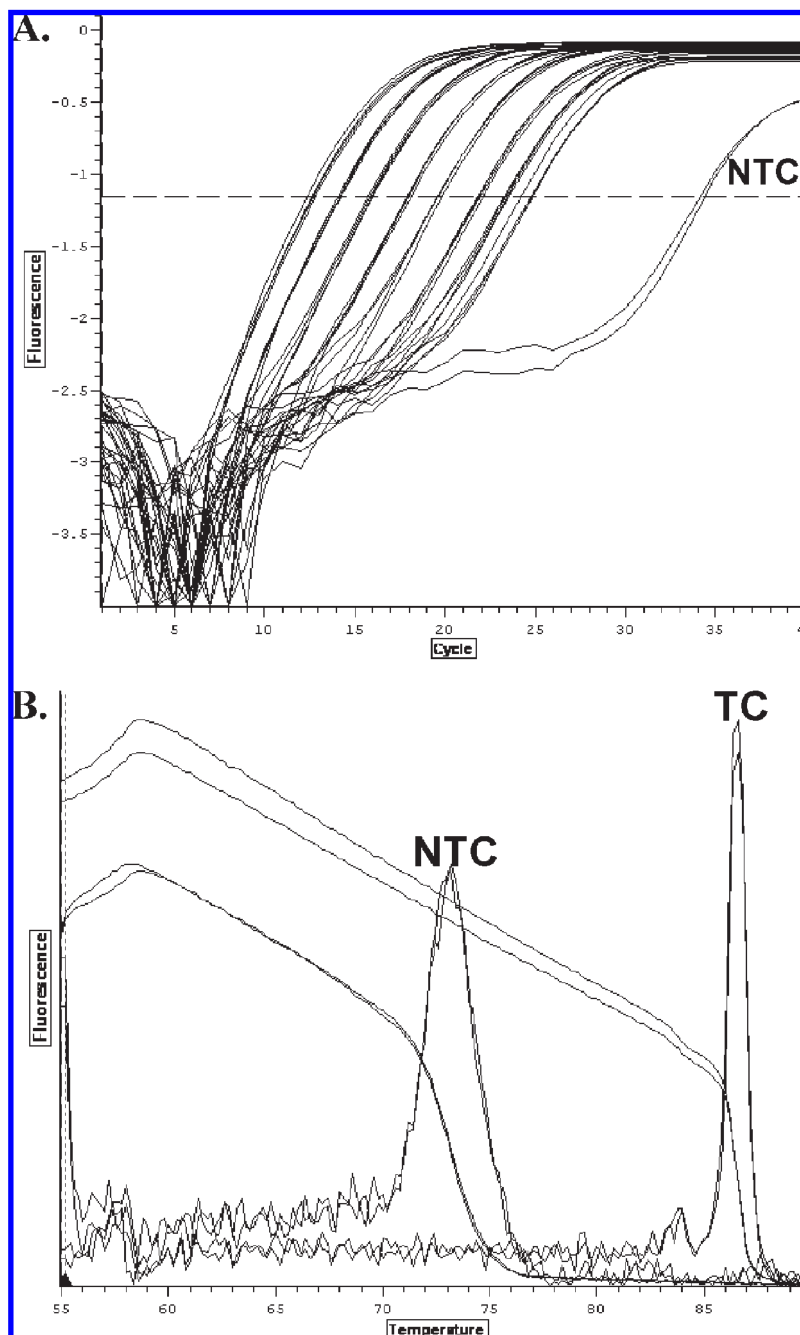

Figure 3. (**A**) qPCR analysis of DNA from a pure culture of the endophyte from *Oxytropis sericea* at eight DNA concentrations (30, 10, 3, 1, 0.3, 0.1, 0.03, and 0.01 ng) amplified with the primer set. Nontemplate control is also shown. (**B**) Melting curve analysis of qPCR of the nontemplate control reaction and DNA from a pure culture of the endophyte from *O. sericea* (template control reaction).

Quantitative PCR analysis using 10 ng of template DNA isolated from pure cultures of the endophyte from *O. sericea*, *A. mollissimus*, and *A. lentiginosus* resulted in very similar Ct values (see Supporting Information). These results demonstrate a similar response from equal quantities of DNA isolated from cultures of the endophyte from the three plant species.

Amplification Efficiency, Limit of Quantification, and Limit of Detection. The amplification efficiency and sensitivity were determined using serial dilutions of DNA isolated from the endophyte cultured from *O. sericea*. In **Figure 3** the real time PCR profile (Figure**3A**) and melting curve (**Figure 3B**) are shown. The amplified PCR product was detected in a linear range from 0.01 to 30 ng of initial quantities of endophyte DNA (**Figure 3A**). The equation of the standard curve was $y = -3.54x + 17.9$. The coefficient of determination was 0.996 ($p < 0.001$), indicating a high correlation between the amount of endophyte DNA and the corresponding Ct values. The slope of the standard curve was −3.54, indicating an amplification efficiency of 91.6%. The limit of detection of the method was 0.001 ng (data not shown); however, this was not in the linear range of the standard curve. The melting temperature of the PCR product was 86.4 °C as determined from the disassociation analysis on the melting curve (**Figure 3B**). The nontemplate control resulted in amplification of no visible PCR products as seen on the agarose gel in **Figure 2**; however, a nonspecific product was amplified with a melting temperature of 73.2 °C as determined from the disassociation analysis on the melting curve in the nontemplate reaction (**Figure 3B**). This product was observed only in the melting curve

Table 1. Swainsonine and Endophyte Contents in *Oxytropis* and *Astragalus* Species[a]

	O. sericea		*A. mollissimus*		*A. lentiginosus*	
plant	swainsonine (%)	endophyte (pg/ng)	swainsonine (%)	endophyte (pg/ng)	swainsonine (%)	endophyte (pg/ng)
1	0.089	61.2	0.378	7.4	0.197	26.6
2	0.085	9.5	0.301	7.9	0.195	3.3
3	0.079	10.9	0.280	1.4	0.193	10.8
4	0.075	14.4	0.248	4.4	0.174	4.1
5	0.074	4.3	0.245	5.1	0.172	8.9
6	0.071	14.5	0.240	2.8	0.157	9.0
7	0.051	34.9	0.231	3.4	0.139	6.9
8	0.047	5.9	0.209	3.2	0.13	11.8
9	0.042	14.6	0.203	1.0	0.111	3.4
10	0	0.2	0.199	3.8	0.002	0.5
mean	0.061	17.1	0.253	4.0	0.147	8.5
correlation	$r = 0.40, p = 0.24$		$r = 0.68, p = 0.03$		$r = 0.50, p = 0.14$	

[a] Data represents 10 plants of *O. sericea*, *A. mollissimus*, and *A. lentiginosus* each analyzed for swainsonine and endophyte content separately. Quantitative PCR assays with SYBR Green were run in triplicate. PCR reactions contained 50 ng of total DNA.

analysis in the nontemplate control reactions and not in any reactions that contained template.

This method will be used to quantify the endophyte from a plant sample consisting of plant and fungal DNA; therefore, we determined the amplification efficiency of the primer pair using DNA isolated from *O. sericea* plant material. The amplified PCR product from the *O. sericea* plant material was detected in a linear range from 0.3 to 100 ng of total DNA. The 0.3 ng total DNA sample contained approximately 6.3 pg of endophyte DNA/50 ng of total DNA as calculated from the standard curve, which was just below the quantitative limit of the endophyte standard curve (10 pg). Hereafter, the endophyte content in all samples was normalized to picograms of endophyte DNA per nanograms of total DNA. The equation of the standard curve was $y = -3.79x + 23.7$. The coefficient of determination was 0.998 ($p < 0.001$), indicating a high correlation between the amount of endophyte DNA and the corresponding Ct values in plant material. The slope of the standard curve was −3.79, indicating an amplification efficiency of 83.6%. The desired product was amplified with similar amplification efficiencies using both DNA from a pure endophyte culture and DNA isolated from plant material. These results suggest that the plant DNA interferes minimally with amplification of the PCR product specific to the endophyte.

Reproducibility of the Extraction Procedure and Quantitative PCR Assay. The reproducibility of the method was investigated by preparing four DNA extracts from each of the three species of interest, *O. sericea*, *A. mollissimus*, and *A. lentiginosus*, and determining the endophyte content in each sample by running a qPCR assay of each sample in triplicate. The same procedure was repeated the next day. The Ct values demonstrated the reproducibility of the DNA extraction, sample preparation, and the qPCR assay for *O. sericea* (see Supporting Information). These results demonstrate that there is low intraday and interday variation. Endophyte content was calculated from the standard curve and is expressed as picograms per nanogram of total DNA. The *O. sericea* sample contained 35.1 pg of endophyte DNA/ng of total DNA with a standard deviation of ±7.0 pg (relative standard deviation of ±19.9%). Comparable results regarding reproducibility were obtained for *A. mollissimus* and *A. lentiginosus* (data not shown). In addition, the reproducibility of the assay was assessed by running an internal control representing a single DNA extraction in triplicate with each set of samples over an extended period of sample analyses. The relative standard deviation was found to be ±13.5%, calculated from the standard deviation of a control sample of *O. sericea* ($x = 36$ pg ± 4.9 pg, $n = 18$).

Determination of Endophyte Content in Plant Samples. As proof of principle, this quantitative PCR method was used to investigate the endophyte content in 10 samples of *O. sericea*, *A. molissimus*, and *A. lentiginosus* and to correlate those quantities to swainsonine (**Table 1**). Swainsonine concentrations were also determined for each sample (**Table 1**). Swainsonine concentrations in *O. sericea*, *A. mollissimus*, and *A. lentiginosus* were similar to those reported by Ralphs et al. (*17*). Furthermore, the endophyte was detected in all 30 samples even though swainsonine was not detected in all of the samples. These data suggest three conclusions. First, the amount of endophyte detected in individual plants is highly variable. The amount of endophyte detected within the same species ranged from 0.2 to 61.2 pg/ng of total DNA in *O. sericea*, from 1.0 to 7.9 pg/ng of total DNA in *A. mollissimus*, and from 0.5 to 26.6 pg/ng of total DNA in *A. lentiginosus*. Second, plants with concentrations of swainsonine below or near the detection threshold (<0.001%) have lower endophyte amounts when compared to other plants within the same species that have higher swainsonine concentrations. For example, plant 10 of *O. sericea* and plant 10 of *A. lentiginosus* both contained low or no detectable swainsonine concentrations and had low endophyte amounts when compared to plants within the species that contained more swainsonine. Third, in *A. mollissimus* a significant positive correlation ($r = 0.68$, $p = 0.03$) exists between swainsonine concentrations and endophyte amounts. However, in *O. sericea* the correlation was $r = 0.40$ ($p = 0.24$), and in *A. lentiginosus* the correlation was $r = 0.50$ ($p = 0.14$). The discrepancy between species merits further investigation with a greater number of plants. Interestingly, it has been demonstrated in the *Neotyphodium* perennial rye grass symbiosis that the content of some secondary compounds produced by the fungal endophyte such as peramine and lolitrem B are positively correlated with endophyte amounts, but not others, such as ergovaline (*21*).

In conclusion, a qPCR method has been developed to quantify the fungal endophyte in *O. sericea*, *A. molissimus*, and *A. lentiginosus*. Using this method it will be possible to further investigate the role the endophyte plays in swainsonine production in various locoweed species. Furthermore, the method outlined in this study should be useful in investigating the endophyte as it relates to swainsonine in other *Astragalus* and *Oxytropis* spp. not tested directly in this study. It is anticipated that this method will lead to a greater knowledge of the factors controlling the occurrence and concentration of swainsonine in locoweed species, thus potentially leading to methods for the prevention of locoweed poisoning in livestock.

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Supporting Information Available: Supplemental Tables 1 (mean Ct values of fungal isolates from *Oxytropis* and *Astragalus* species) and 2 (repeatability of the extraction method). This material is available free of charge via the Internet at http://pubs.acs.org.

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