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Detection of swainsonine and isolation of the endophyte *Undifilum* from the major locoweeds in Inner Mongolia

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ABSTRACT

Locoweeds are *Astragalus* and *Oxytropis* species that contain the toxic alkaloid swainsonine, causing widespread poisoning of livestock in Inner Mongolia. Taxa (*Astragalus*, *Oxytropis*, *Sphaerophysa*, and *Sophora* species) suspected of causing locoism and/or poisoning in Inner Mongolia were surveyed for swainsonine and *Undifilum*, the fungal endophyte responsible for the production of swainsonine. Swainsonine was detected at concentrations greater than 0.01% in *Astragalus variabilis* and *Oxytropis glabra*. The endophyte *Undifilum* was detected by culturing and PCR in samples containing swainsonine concentrations greater than 0.01%. In some specimens of *A. variabilis* and *O. glabra* swainsonine was not detected or concentrations were less than 0.01%. In these samples the endophyte could not be cultured, but was detected by PCR. Additionally, contrary to previous reports the quinolizidine alkaloids, thermopsine, anagyrine, and lupanine, were not detected in *O. glabra* and *Oxytropis ochrocephala*, however the quinolizidine alkaloids, sophoridine, sophocarpine, and sophoramine were detected in *Sophora alopecuroides* as previously reported.

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1. Introduction

Locoweeds are poisonous plants of the *Astragalus* and *Oxytropis* genera (family Leguminosae) that contain the toxic alkaloid swainsonine, and widely grow in arid and semiarid grasslands of the world. There are 45 species of suspected locoweeds (23 species of *Astragalus* and 22 species of *Oxytropis*) (Huang et al., 2003), that grow in the main grassland region of China. Every year, locoism causes significant economic losses to the livestock industry and in recent years, locoweed populations have increased in Inner Mongolia resulting in an increased incidence in poisoning (Wu et al., 2003).

Swainsonine, a trihydroxy indolizidine alkaloid, is the primary toxin in locoweeds (Molyneux and James, 1982). The vertically transmitted fungal endophyte *Undifilum* found in locoweeds (Oldrup et al., 2010; Ralphs et al., 2011), previously described as *Embellisia* species, is responsible for the synthesis of swainsonine (Braun et al., 2003; Wang et al., 2006; Pryor et al., 2009). *Undifilum* can be detected in locoweeds using microscopy, culturing, and polymerase chain reaction (PCR), and of these, PCR is the most sensitive (Ralphs et al., 2008; Oldrup et al., 2010). Swainsonine concentrations vary greatly in

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locoweeds; for example, swainsonine may or may not be detected within varieties, populations, and/or individuals within the same population of *Astragalus* and *Oxytropis* species (Gardner et al., 2001; Ralphs et al., 2008; Cook et al., 2009b, 2011). Differences in the amount of *Undifilum* are associated with the highly variable swainsonine concentrations in *Oxytropis* and *Astragalus* (Cook et al., 2009b, 2011).

At present, little is known about the relationship between endophytes and swainsonine among locoweeds in Inner Mongolia. The major locoweeds thought to be toxic in Inner Mongolia are *Astragalus variabilis* and *Oxytropis glabra*. These two species sampled in other parts of China are reported to contain swainsonine and the fungal endophyte *Undifilum* (Yu et al., 2010). In addition, *O. glabra* and *Oxytropis ochrocephala* are reported to contain quinolizidine alkaloids, anagyrine, lupanine, and thermopsine, which may explain episodes of poisoning associated with these taxa (Yu et al., 1991; Meng et al., 1994). In addition to the two major locoweed species, *A. variabilis* and *O. glabra*, other taxa including *O. ochrocephala*, *O. glabra* var. *tenuis*, *Sophora alopecuroides*, and *Sphaerophysa salsula* were investigated as some are suspected of causing poisoning in Inner Mongolia. The objective of this research was to determine whether the above-mentioned taxa contain the toxic alkaloid swainsonine, measure its concentration, and determine the presence of the endophytic fungus, *Undifilum*, through culturing and PCR. Additionally, the presence or absence of the quinolizidine alkaloids was investigated in the taxa of interest.

2. Material and methods

2.1. Plant materials

The taxa of interest, *A. variabilis* Bunge ex Maxim, *O. glabra* DC, *O. glabra* var. *tenuis* Palib., *O. ochrocephala* Bunge, *S. alopecuroides* L., and *S. salsula* (Pall.) Taub, were sampled from herbarium specimens at Inner Mongolia Agriculture University in Hohhot, China. Samples were representative of specimens collected throughout the western rangelands in Inner Mongolia. Approximately 50 mg of plant material (leaves, flowers, and pods) was sampled from each specimen, which was subsequently ground using a Retsch MM301 mixer mill for alkaloid (swainsonine and quinolizidine) and endophyte analysis. The number of samples, species, varieties, locations and plant communities from the sampled specimens are presented in Table 1.

2.2. Isolation and identification of fungal endophytes

Fungal endophytes were isolated by using a method modified from Braun et al. (2003). Non-damaged tissues of the plant were selected and surface sterilized for 30 s in 70% ethanol, followed by 3 min in 20% bleach, and rinsed by using sterile water. Tissues were dried on sterile paper and cut into 1–3 mm segments. Each segment was pressed on potato dextrose agar (PDA) plates at room temperature, and observed for the growth of the fungal endophytes.

2.3. Swainsonine detection and quantitation

Swainsonine detection and concentration were measured using a modification of a previously published procedure (Gardner and Cook, 2011) in the following manner. A measured quantity (50 mg) of dried plant material 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 16 h with agitation. 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. Samples were analyzed by LC-MS as previously described (Gardner et al., 2001). Detection limit of swainsonine was 0.001% of dry weight using this extraction procedure.

2.4. DNA extraction

DNA was extracted from air-dried, ground plant material (~20 mg) using the DNEasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Extractions were performed according to the manufacturer's instructions. DNA was quantified with the ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

2.5. PCR primer design

The PCR primers used have successfully detected the presence of the fungal endophyte, *Undifilum* in *Oxytropis* and *Astragalus* species (Ralphs et al., 2008; Cook et al., 2009a,b, 2011). The primers used were ITS 5 (5′ GGA AGT AAA AGT CGT AAC AAG G 3′) (White et al., 1990) and OR1a (5′ GTC AAA AGT TGA AAA TGT GGC TTG G 3′) which amplify the internal transcribed spacer (ITS) region. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

2.6. PCR detection and ITS sequencing of endophyte

In brief, cultured fungal specimens were verified to be *Undifilum* using PCR methods similar to those previously published (Cook et al., 2009a). Plant material if available was verified to contain the fungal endophyte, *Undifilum*, via PCR using a Bio-Rad Dyad PCR detector (Bio-Rad Laboratories Inc., Hercules, CA). Thermal cycling conditions were as follows, an initial denaturation step for 3 min at 94 °C, followed by 30 or 40 cycles of 45 s at 94 °C, 60 s at 58 °C, and 30 s at 72 °C. A final extension of