DISSERTATION

MUTUALISMS RELATION TO SWAINSONINE IN *OXYTROPIS* FROM THE UNITED STATES AND CHINA

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JOSE RODOLFO VALDEZ BARILLAS ENTITLED MUTUALISMS RELATION TO SWAINSONINE IN OXYTROPIS FROM THE UNITED STATES AND CHINA BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

MUTUALISMS RELATION TO SWAINSONINE IN *OXYTROPIS* FROM THE UNITED STATE AND CHINA

Swainsonine producing *Oxytropis* can establish mutualisms with dinitrogen fixing bacteria and endophytic fungi. Dinitrogen fixation facilitates the growth of *Oxytropis* species in low nitrogen soil, while sustaining the fungal-plant symbiosis. Contributions from dinitrogen fixation in *Oxytropis sericea* development and swainsonine synthesis were studied in a greenhouse experiment. The role of *Oxytropis* mutualisms in swainsonine production was also tested beyond *O. sericea* by studying swainsonine producing *Oxytropis* from China. For the greenhouse study it was hypothesized that fixed dinitrogen is used by the fungal endophyte during the synthesis of swainsonine inside *Oxytropis*. It was also hypothesized that dinitrogen fixing *Oxytropis* growing under nitrogen stress conditions can allocate fixed nitrogen toward plant biomass and still sustain swainsonine synthesis by the fungal endophyte. In a second study, it was hypothesized that endophytic fungal and rhizobial mutualisms in *Oxytropis* from the United States and China are similar. It was also hypothesized that alkaloid similarities in *Oxytropis* from both continents could be explained by similar fungal endophyte hosted by *Oxytropis* from the US and Chinese. ^{15}N-enrichment on dinitrogen fixing and non dinitrogen fixing *O. sericea* was detected in ^{15}N-swainsonine produced by non-dinitrogen fixers. Low ^{15}N-swainsonine was detected in dinitrogen fixers as a result of ^{14}N incorporation. These results suggest the fungal endophyte is a nitrogen sink. Non-dinitrogen fixing *O. sericea* with no fungal endophyte had greater biomass than non-dinitrogen fixers with fungal endophyte. Non-dinitrogen fixers with fungal endophyte

produced similar levels of swainsonine, but no increase in biomass. Dinitrogen fixers with fungal endophyte had greater biomass than non-dinitrogen fixers with fungal endophyte. Older dinitrogen fixers produced more swainsonine than non dinitrogen fixers, increased plant growth and fungal biomass. Results from the second study suggest that dinitrogen fixing *Oxytropis* from China produced swainsonine in association with a fungal endophyte that is 99% similar to the fungal endophyte in *Oxytropis sericea* from the United States. These studies suggest that dinitrogen fixation increases the ecological niche of *Oxytropis* in both continents and sustains the plant-fungal symbiosis, as well as swainsonine production. Fungal symbiosis and dinitrogen fixation are old mutualisms that have been maintained by *Oxytropis* populations in both continents.

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This dissertation is dedicated to

My son Mateo James

Who has been a source of inspiration since the day he was born

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Chapter 1

Swainsonine production in *Oxytropis sericea* is affected by differences in nitrogen contribution from N_2-fixation vs. soil nitrogen

Introduction

More than 90% of legume species are capable of producing dinitrogen fixing nodules in association with *Rhizobium* (Sprent 2002). The nitrogen supplied by root nodules enables legumes to sustain growth and colonize soils with low nitrogen. Legumes can also allocate additional resources from dinitrogen fixation toward defenses, reproduction, increasing biomass, or to cope with environmental stressors. Additional resources credited to the association with *Rhizobium* can often facilitate symbiotic associations with other microbial organisms that could potentially benefit the legume's fitness (O'dell and Trappe 1992, Bordeleau and Prévost 1994, Denison et al. 2003). Legume-microbial associations, besides dinitrogen fixing bacteria, include symbiosis with below and aboveground endophytic fungi. Such associations may act as indirect nitrogen sinks for nutrients or plant defenses in exchange of photosynthates (Lyones et al. 1990, Divon et al. 2005, Hobbie and Hobbie 2006).

A potential model to study legume-microbial symbiotic associations is *Oxytropis sericea*, an herbaceous legume capable of producing dinitrogen fixing nodules in symbiosis with *Rhizobium* when growing in soils with low nitrogen (Valdez Barillas et al. 2007). *Oxytropis sericea* also hosts an endophytic fungus in its aboveground tissues. The endophytic fungus is responsible for the synthesis of swainsonine, which is present the leaves and reproductive structures of *O. sericea.* Swainsonine is an alkaloid with important medical applications and has been studied for its toxicity to animals that frequently graze on *O. sericea.*

Swainsonine production in *O. sericea* is extremely variable within and among populations (Gardner et al. 2001, Ralphs et al. 2002, Ralphs et al. 2008), making it difficult to determine the accurate mean levels of plant swainsonine in a given population. It is not clear which factors contribute to swainsonine variability, however a study by Oldrup (2005) reported that pH and water induces an increase of swainsonine by the fungus. Another study by Valdez Barillas et al. (2007) reported that nitrogen fixing *O. sericea* could have greater swainsonine content than non-nitrogen fixing individuals when growing in soils with low nitrogen. Because swainsonine is a nitrogen-based secondary metabolite produced by an endophytic fungus, a better understanding on how nitrogen cycling is linked to the synthesis of swainsonine may help explain the nature of the alkaloid variability in *O. sericea.*

Nitrogen uptake by roots and N_2 fixation

Nitrogen assimilation in plants is affected by nitrogen dynamics in the soil and mechanisms of root N absorption. During the process of nitrogen assimilation, the plant's internal N demand is more important than soil nitrogen availability (Kronzucker et al. 1998, Touraine et al. 2001, Dunbabin et al. 2003, and Lambers et al. 2006). To cope with soil nitrogen dynamics leguminous species have developed a sophisticated root system that optimizes nitrogen uptake and is capable of hosting symbiotic N_2-fixing bacteroids inside root nodules. The root system is capable of scavenging for scattered patches of water and nitrogen sources (Dunbabin et al. 2003), and dinitrogen fixation gives legumes access to dinitrogen (NH_3^-) reduced by bacteroids when soil nitrogen is unavailable.

Soil nitrogen is generally taken up by roots as nitrate or ammonium. Both ions are either stored in the vacuoles of root cells, moved from the cytosol of the root cell to the apoplasm, or transported via the xylem to the shoots as ions or amino acids (Touraine et al. 2001). The uptake of nitrogen is dependent upon root and shoot demand. The internal factors that regulate such demand include carbon dioxide, photosynthetic flux density, amino acids, organic acids, cytokinins, and fluctuations in pH mediated by carbohydrates such as malate (Meyer and Stitt 2001, Von Wirén et al. 2001). However, when soil nitrogen is unavailable nitrogen fixation provides an alternate supply for plant shoot growth during each developmental stage. Thus, allowing the plant to allocate and relocate nitrogen toward reproduction, defenses, or storage (Kim et al. 1993, Lodwig et al. 2003, Scharff et al. 2003, Lee et al. 2006, and Valdez Barillas et al. 2007).

Inside the root nodule, dinitrogen fixing bacteria stop cell division and differentiate into bacteroids. Bacteroids shut down ammonium assimilation in response to the dicarboxylic acids such as malate, and amino acids such as glutamate supplied by the plant. Reduced dinitrogen is then exported as aspartate and ammonium from nodules to the plant cytosol (Lodwig et al. 2003). The exported ammonium is transferred to glutamate molecules, supplied by the plant, to form glutamine. Glutamine is either exported to the shoots via xylem, or its amide is transferred to aspartate from the nodules to form asparagine. Asparagine is transported from the nodules to photosynthetic tissues via xylem, where it is then catabolized by deamination back to aspartate before entering the chloroplast (Mills et al. 1980, Azevedo et al. 2006). The aspartate that enters the leaves is used inside the mesophyll chloroplasts as a precursor during the synthesis of

amino acids, such as methionine, isoleucine, threonine, and lysine (Morot-Gaudry et al. 2001, Azevedo et al. 2006).

Alkaloid and amino acid distribution in legumes

Amino acid feedbacks in legumes can have an effect on the synthesis and distribution of defensive compounds such as alkaloids, and osmoregulators during drought stress. For instance, lysine is a protein-bound and basic amino acid that is synthesized via L-diaminopimelate during photosynthesis (Mills et al. 1980). Lysine is catabolized in leaves, floral organs, and seeds by the enzymes ketoglutarate reductase (LKR), saccharopine dehydrogenase (SDH), and lysine decarboxylase (LD) (Moulin et al. 2006). The first enzyme (LKR) produces Saccharopine, the second enzyme (SDH) produce glutamate and alpha- aminoadipate-semialdehyde, which produce pidperideine-6-carboxylate (P6C) by spontaneous cyclization. The third enzyme (LD) produces cadaverine.

During alkaloid synthesis in white lupines, lysine, cadaverine, and P6C are used as the precursor of hydroxylupanine and lupanine (Golebiewski and Spenser 1987). These two alkaloids are synthesized in the leaves and transported via phloem to the rest of the plant (Lee et al. 2006). Alkaloids can also be stored in plant vacuoles. For example, alkaloids such as pyrrolizidines are stored in plant vacuoles in their N-oxide form (Narberhaus et al. 2004), or in the case of quinolines, alkaloids can be transported across the cell membrane to the cytoplasm by an ABC transporter and from the cytoplasm into a vacuole through a H^+/alkaloid antiporter for storage (Otani et al. 2005).

During drought or salt stress glutamate and ornithine produce proline, which is an important osmoregulator, energy-reducing sink, nitrogen storage compound, a hydroxyl-radical scavenger, and regulator of redox potential (Morot-Gaudry et al. 2001). The reduction of P6C by the enzyme pipecolate dehydrogenase yields L-pipecolate, which is also an important osmoregulator (Moulin et al. 2006).

The distribution and concentrations of amino compounds in legumes can be affected by the rates of dinitrogen fixation and nitrogen uptake from roots. For example, a study by Parsons and Baker (1996) showed that nodulated white lupines (*Lupinus albus*) increased glutamate and aspartate, and decrease asparagine concentrations in both phloem and xylem sap when dinitrogen fixation was suppressed. Plants given nitrate had higher levels of asparagine and aspartate in xylem sap and amino acids levels where higher in the phloem sap. Nitrate uptake from roots also decreased nodule growth and activity more so than ammonium. Ammonium fed plants, conversely had higher glutamine concentration in both xylem and phloem sap. Therefore, high asparagine levels in the xylem are indicative of root uptake of nitrate, reduced by the GS/GOGAT (Glutamate synthase/ Glutamine synthetase) system. Root nodules are a storage sink for asparagine during dinitrogen fixation (Lodwig et al. 2003), and export most of the ammonium in the form of glutamine via the xylem (Parson and Baker 1996).

Fungal endophytes and plant nitrogen physiology

Besides soil nitrogen dynamics, the internal demand for nitrogen is also affected by environmental stress, drought and herbivory stress, and plant-microbial interactions (Watson and Poland 1999, Wyka and Galen 2000, Mandyam and Jumpponen 2005,

Waller et al. 2005). For example, in plant-fungal interactions the fungus is capable of altering the physiology of the plant to its advantage (Rodrigues Costa Pinto et al. 2000, Schulz et al. 2002, Dakora 2003, and Waller et al. 2005). These types of interactions occur both aboveground or below ground, and range from parasitic to mutualistic.

Although the effects of parasitic or pathogenic bacteria, and fungi have been reported in economically important plant species, an emerging field of research has been studying the effects of mutualistic associations on the internal physiology of the host plant (Lyon et al. 1990, Parsons and Baker 1996). The effects on plant internal nitrogen have been extensively studied in N_2-fixing plants, and to a lesser degree in mycorrhizal plants and cool-season grasses infected by fungal endophyte in the aboveground tissue. Even less research has been conducted on non-grass species infected by mycorrhizal and dark septate fungal endophytes (Currah and Van Dyk 1986, O'dell and Trappe 1992, Bills 1996, Wilson 2000, Faeth and Fagan 2002, Braun et al. 2003, Arnold et al. 2003, Kucht et al. 2004, Mandyam and Jumpponene 2005, and Steiner et al. 2006).

Rather than being the exception, plant-fungal mutualistic interactions are more common than pathogenic interactions (Saar et al. 2001, Arnold et al. 2003, Chapaval Pimental et al. 2006). Mutualistic fungal endophytes have been reported to defend their host plant from pathogenic fungi, enhance drought and salinity stress tolerance, and can be responsible for the synthesis of toxic and medicinal secondary metabolites in the aboveground tissue of their host plant (Clay and Schardl 2002, Arnold et al. 2003, Braun et al. 2003, McLain-Romero et al. 2004, Waller et al. 2005, Verma et al. 2007). Thus, suggesting a linkage between nutrient acquisition and secondary metabolite production by the fungal endophyte, which alters the physiology of the infected tissue in the host

plant (Kulkarni and Nielsen 1986, Tan et al. 2001, Divon et al. 2005, Howlett 2006, Divon and Fluhr 2007).

Fungal infections can change nitrogen concentration inside and outside the infected tissue of the host plant, either in the form of a nitrogen starvation response by the plant to the fungus or as a result of alterations in the apoplasm attributed to the fungus. Compatible biotrophic and endophytic fungi have adapted to utilize the physiological changes triggered by the plant, in the apoplasm of the infected area, to their advantage. For instance, the infection of Tall fescue (*Festuca arundinaceae*) by the fungal endophyte *Acremonium ceonophialum* resulted in higher ammonium, greater glutamine synthetase activity, and decreased nitrate concentration in the infected tissue. Alkaloid production also increased in infected plants fertilized with 10mM of NH_4SO_4 (Lyon et al. 1990). The infection of tomato plants with the compatible fungus *Cladosporium fulvum* resulted in a two to five fold increase in amino acid concentration in the infected apoplasm, compared to incompatible infections (Solomon and Oliver 2001). When studying the response from a fungus growing under nitrogen and carbon starvation conditions, the tomato infecting fungus *Fusarium* oxysporum f.sp. *lycopersici* over expressed three amino acid permeases, an uricase , a peptide transporter, and showed down regulation of nitrate reductase (Divon et al. 2005).

The changes on nitrogen concentration in fungal infected plants are also a function of fungal uptake of nutrients from the apoplasm and the efflux of secondary metabolites to the apoplasm. Most fungi have a higher preference for amino acids with high N:C ratios such as alanine, serine, aspartic acid and glutamic acid (Hasija 1970). For example, Stemphylium botryosum possess two transport systems for nitrogen. One is an L-

asparagine/glutamine specific membrane protein (permease) that works during nutrient starvation. The second is a non-specific permease that actively transports amino acids such as leucine, lysine arginine and phenylalanine (Breiman and Barash 1976). In another fungal endophyte study, *Acremonium coenophialum* preferred arganine, asparagine, cysteine, glutamine, proline, and serine as N sources for biomass, and lysine, tryptophan, and alanine did not support fungal growth (Kulkarni and Nielsen 1986). The flux of basic amino acids such as lysine and arganine are also mediated by lysine specific and non-specific permeases (Hillenga et al. 1996, Fujii et al. 2002, Struck et al. 2004).

Fungal endophyte alkaloids in dinitrogen fixing hosts

An example of plant-microbial mutualism in legumes is the tripartite interaction between the herbaceous legumes *Oxytropis sericea*, a fungal endophyte, and dinitrogen fixing bacteria (Valdez Barillas et al. 2007). *Oxytropis sericea* can establish a simultaneous symbiosis with a dark septate endophytic fungus that depends on nutrients from the apoplasm in the aboveground tissue of the plant. In exchange for nutrients, the endophytic fungus produces the alkaloid swainsonine, which often acts as a toxic defensive compound against animals that graze on *O. sericea* (Braun et al. 2003, McLain-Romero et al. 2004). The fungal endophyte acts as an indirect nitrogen sink for plant defenses, and the symbiosis with its host is indirectly sustained by dinitrogen fixation when soil nitrogen is limited (Valdez Barillas et al. 2007).

It has been hypothesize that swainsonine in *Oxytropis sericea* is synthesized during the catabolism of lysine by the fungal endophyte via L-Pipecolic acid. Nitrogen fixing *O. sericea* plants infected with a fungal endophyte can have a greater content of swainsonine

than non-dinitrogen fixing plants when soil nitrogen is limited. The individual effects of root nitrogen uptake and dinitrogen fixation have been reported in a previous study (Valdez Barillas et al. 2007), where *O. sericea* were fertilized with Miracle-Gro (15 mM NH_4 and 23 mM $(NH_2)_2CO$. A second group of *O. sericea* was treated with *Rhizobium* (NH_2-fix rate ≈ 0.024 mmoles/L air/hour) and 0.18mM NH_4NO_3. Mean swainsonine content (0.153 %, SE= 0.026) in Miracle-Gro grown *O. sericea* was not greater than *Rhizobium* treated plants (0.175%, SE=0.028). However, shoot biomass (2.32g, SE=0.302) was three times greater than *Rhizobium* treated plants (0.894g, SE=0.099).

It is still uncertain if the fungal endophyte is directly using fixed dinitrogen, soil nitrogen, or both, to synthesize swainsonine when soil nitrogen is limited. It also remains unclear if swainsonine levels in the plant are affected by the source of nitrogen (dinitrogen fixation vs. root nitrogen) used by the fungus. Therefore, this study intended to determine if the nitrogen acquired through dinitrogen fixation is used by the fungal endophyte to synthesize swainsonine vs. the nitrogen acquired from the soil. It also aimed to determine if competetition between plant and endophytic fungus for fixed dinitrogen vs. root nitrogen could explain differences in swainsonine production. For this study we proposed the following hypotheses: 1) Nitrogen from dinitrogen fixation contributes more to swainsonine synthesis by the fungal endophyte than root nitrogen in *O. sericea* treated with Rhizobium. 2) Aboveground biomass and swainsonine production will be greater in rhizobium treated than untreated O. sericea. 3) Fungal endophyte biomass and swainsonine will be greater in rhizobium treated than untreated O. sericea.

Materials and Methods

Plant material

Scarified seeds from *Oxytropis sericea* were provided by the USDA/ARS Poisonous Plant Laboratory at Logan, Utah (USA). The seed material was collected in a mountain grassland community on September 2002 at (N 41° 54' 15.4", W 113° 20' 54.9") Raft River, Utah, where *O. sericea* has historically been a dominant perennial forb during population outbreaks. The Rhizobium strains 118H2 and 118B2 were provided by Lipha Tech (Milwaukee, Wisconsin, USA) and prepared in a milled sedge peat as carrier. Strain 118B2 was isolated from *Oxytropis foliolosa* collected in Matanuska Dike Rd., Alaska 1962. Strain 118H2 was isolated from Oxytropis riparia grown in Greenhouse, 1963. Plants were grown at the University Greenhouse at Colorado Stat University (Fort Collins, Colorado, USA) from May through September 2007. Plant material was analyzed at the USDA/ARS Poisonous Plant Laboratory for swainsonine and fungal DNA (PCR) analysis.

Plant growth

Two scarified seeds of *O. sericea* were germinated in each of 180 plastic cones (D40 Deepots, Stuewe and Sons, Corvallis, Oregon, USA) filled with sand. After germination, seedlings were thinned to one per cone. All 180 plants were watered regularly with a nutrient solution described by Valdez Barillas et al. (2007), for 6 weeks (0.72mmol/L NH_4NO_3). Six weeks after germination, *O. sericea* seedlings started receiving the same nutrient solution with a lower nitrogen concentration (0.14 mmol/L NH_4NO_3) to induce nitrogen stress. Twelve weeks after germination, 60 seedlings that had grown 5-8 leaves were inoculated with *Rhizobium* strain 118H2 and another 60 with strain 118B2. The

remaining sixty seedlings were not inoculated. Four weeks after inoculation with *Rhizobium*, thirty seedlings treated with strain 118H2, thirty seedlings treated with strain 118B2, and thirty Rhizobium untreated seedlings received the same nutrient solution (0.14mmol/L NH_4NO_3) but 80% was ^{14}N-NH_4NO_3 and 20% was ^{15}N-NH_4NO_3 enriched. The final experimental layout included thirty (20%)^{15}N-NH_4NO_3 + Rhizobium untreated control seedlings, thirty (20%) ^{15}N-NH_4NO_3 treated seedling, thirty Rhizobium118B2 strain treated seedlings, thirty Rhizobium 118H2 strain treated seedlings, thirty 118B2 + (20%) ^{15}N-NH_4NO_3 treated seedlings, and thirty 118H2 + (20%) ^{15}N-NH_4NO_3 treated seedlings.

Two weeks after the ^{15}N enrichment, four seedlings from each treatment were harvested each week for five weeks. Before harvest, each sample was measure for average shoot length and number of shoots. Weekly collected samples were oven dried at 56° C for 72 hours. At the end of the experiment the dried material from each individual samples (N=120) was weighted to estimate biomass, and was homogenized with mortar and pestle (using liquid nitrogen) for swainsonine analysis. Ten samples from each treatment (N=60) where not harvested. The sixty unharvested plants were analyzed for quantitative fungal endophyte DNA and swainsonine concentration (% of dry weight)

Swainsonine analysis

Sample extractions for swainsonine and the amino acid analysis were performed following the methods published by Gardner et al. (2001). Detection of swainsonine was confirmed by comparison of GC retention time and mass spectrum to that of a standard swainsonine sample $(TMS)_3$ derivative (from *Astragalus lentigninosus*). The mass

spectrometer was assigned to integrate the peak for the derivatized swainsonine fraction of mass 260 for each sample. Atom (^{15}N) % excess was calculated as the ratio of mass 260 + 1 to mass 260 corrected for the control ratio.

Fungal endophyte analysis

Fungal DNA extraction and qRT-PCR analysis were performed at the USDA/ARS Poisonous Plant Laboratory by Dr. Daniel Cook following the methods published by Cook et al. (2008, in press) and Ralphs et al. (2008). Forty seven 25 week old *O. sericea*, which included samples from each Rhizobium and ^{15}N-NH_4NO_3 treatment were harvested. DNA was extracted from plant material (~20mg) using DNEasy Plant Mini Kit (Qiagen Inc., Valencia, CA) and quantified with the ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). The PCR primers used were described in studies published by Braun et al. (2003) and Ralphs et al.(2008).

Fungal DNA amplification and detection were assessed using a Bio-Rad CHROMO4 real-time PCR detector (Bio-Rad Laboratories Inc., Hercules, CA). Thermal cycling conditions were as follows, an initial cycle for 7 min at 95°C, 40 cycles of 15 sec at 95°C, 30 sec at 58°C, 40 sec at 72°C, and a plate read. This was followed by a melting profile to determine the purity of the reaction products where the temperature was raised from 55°C to 90°C in 0.2°C increments, held for 2 sec at each temperature, and a plate read at each temperature. The PCR product was run on a 1.0% agarose gel containing ethidium bromide at 118 volts for 20 minutes and visualized under UV illumination. The amplified PCR product was approximately 580 base pairs (bp).

For the quantification of the fungal endophyte, a standard curve was constructed from DNA extracted from a pure culture of the endophytic fungus. Each analysis included three replicate reactions for each DNA quantity. Each analysis also included a non-template control reaction, in which water was substituted for the DNA to confirm the reagents were free from contaminating template DNA. For each fungal and plant sample for the standard curves, three replicate reactions were run in 25 µl reactions containing the amount of DNA (0.01- 30 ng fungal DNA and 1 - 100 ng plant DNA) indicated on the standard curve. For each plant sample tested, three replicate reactions were run in 25 µl reactions containing 50 ng (5 µl of a 10ng / 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 of the forward and reverse primer.

Data analysis

A two-way ANOVA with contrasts (PROC GLM, SAS 9.1) was used to determine the effects of ^{15}N enrichment and Rhizobium treatment on swainsonine concentration, swainsonine $^{15}N/^{14}N$ ratios, shoot length, number of shoots, and shoot biomass per samples collected at six different time points (18 to 22 weeks post germination). For the swainsonine concentration data, studentized residuals with more than 2 deviations where considered outliers. Swainsonine data were analyzed with all values (N=126) and without samples with swainsonine values below 0.010 % (N=94) to normalize the data. Treatment effects were within and among week post germination. Multiple comparisons among Rhizobium and ^{15}N enrichment treatment effects were estimated using a LSD test ($P<0.05$) and linear contrast. A one-way ANOVA was used to determine effects of

Rhizobium treatment on fungal endophyte biomass and swainsonine concentrations. Multiple comparisons among Rhizobium treatment effects were estimated using a Tukey test ($P<0.05$) and linear contrast. The correlation between variables was also estimated to determine any relationship due to treatments effects.

Results

$^{15}N/^{14}N$ swainsonine ratios

Based on $^{15}N/^{14}N$ swainsonine ratios in *O. sericea* (18 to 22 weeks post germination), the ^{15}N-NH_4NO_3 (20%^{15}N-NH_4NO_3 + 80% ^{14}N-NH_4NO_3) treatment had a significant effect on swainsonine ^{15}N enrichment ($F_{1, 89} = 18.74$, $P<0.0001$). The ^{15}N enrichment was also significantly affected by the *Rhizobium* treatment ($F_{2, 89} = 7.47$, $P=0.0001$). Significant ^{15}N enrichment was measured in plants treated only with ^{15}N-NH_4NO_3 (17 of 20) and plants treated with Rhizobium + ^{15}N-NH_4NO_3 (11 of 40). Significant enrichment included all plants with a $^{15}N/^{14}N$ swainsonine ratio (M+1/M: 260 +1/ 260) greater than 0.240 (Table 1). All $^{15}N/^{14}N$ ratios ranged from 0.23 to 0.28 in plants treated only with 15N- NH_4NO_3, and from 0.22 to 0.24 in all the other treatments. All untreated control plants (N=20) and the standards from *A. lentiginosus* (N=4) had $^{15}N/^{14}N$ swainsonine ratios below 0.240. Plants treated with ^{15}N-NH_4NO_3 had significantly greater ^{15}N enrichment compared to other treatments ($t= 6.23$, $P=0.0001$) (Table 1). No significant differences in ^{15}N enrichment were detected between Rhizobium 118H2 and Rhizobium 118H2 + ^{15}N-NH_4NO_3 treatments (Table 1). However, differences in ^{15}N enrichment ($t=2.26$ $p=0.026$) were found between Rhizobium 118B2 and Rhizobium 118B2 + ^{15}N-NH_4NO_3 treatments (Table1). Plants treated with Rhizobium 118B2 had the lowest ^{15}N enrichment (Table1). Differences in ^{15}N enrichment between ^{15}N-NH_4NO_3 treated plants and other treatments were detectable at 20 weeks ($F_{5, 18} = 7.52$, $P=0.0006$), 21 weeks ($F_{5, 18} = 3.77$, $P= 0.016$), and 22 weeks ($F_{5, 12} = 3.06$, $P= 0.052$) post germination (Figure 1).

Changes in $^{15}N/^{14}N$ swainsonine ratios were explained by different variables among treatments. For example, in plants treated with ^{15}N- NH_4NO_3, changes in swainsonine content had 18% correlation with $^{15}N/^{14}N$ ratios compared to the number of shoots (30%). In plants treated with Rhizobium + ^{15}N- NH_4NO_3, changes in swainsonine content had a 30% correlation with $^{15}N/^{14}N$ ratios. Shoot biomass in untreated controls and plants treated with Rhizobium had a 30% correlation with changes in $^{15}N/^{14}N$ ratios.

Swainsonine content and plant measurements

Swainsonine content was extremely low (<0.0001 %) in 15-20% of plants in all treatments except the untreated controls (40%). High swainsonine content (≥ 0.100 %) was detected in 30% of plants in all treatments, except the untreated controls (40%). Untreated controls had greater variability in swainsonine concentrations than other treatments. Part of this variability was associated with differences between ^{15}N treated and untreated controls. Therefore to asses *Rhizobium* effects on swainsonine content and plant measurements, statistical comparisons were made within non-enriched plants separately from ^{15}N enriched plants, because an unintended ^{15}N treatment effect on the plants was suspected.

Swainsonine content was lower in 19 week old untreated controls compared to 18 week old plants (Figure 2). Shoot biomass was greater and number of shoots and shoots length were also lower in 19 week old untreated controls compared to 18 week old untreated controls (Figures 2,3,4, and 5). During the first two weeks, average swainsonine concentrations in ^{15}N treated plants were above 0.100 %. During the third week swainsonine concentrations increased in all treatments, particularly the untreated

controls (Figure 2). Interestingly, shoot biomass, number of shoot, and shoot length of 19 and 20 week untreated controls, Rhizobium, and Rhizobium + ^{15}N treated plants was greater than 18 or 21 week old plants (Figures 2, 3, 4, and 5). Twenty two week old Rhizobium + ^{15}N and ^{15}N enriched plants had greater ^{15}N enrichment compared to other treatments (Figure 1). Differences in swainsonine, shoot length, and shoot biomass between untreated controls and Rhizobium treated plants were greater in 22 week old plants compared to younger plants (Figures 2, 3, and 4). Swainsonine was also significantly greater in 22 week old Rhizobium treated vs. untreated controls ($F_{5,12}=5.63$, $P=0.0189$). Overall, shoot biomass, shoot length, and number shoots was significantly greater in Rhizobium treated plants compared to untreated controls (Table 1).

Changes in swainsonine content in *O. sericea* were explained by different variables among treatments. For example, in plants treated with Rhizobium 118B2, 30 % of the changes in swainsonine content were correlated with number of shoots and 40% with shoot length. In plants treated with Rhizobium 118B2 + ^{15}N- NH_4NO_3, 30% of the changes in swainsonine content were correlated with number of leaves.

Swainsonine and fungal DNA content

Based on R^2 values, 40% of changes in swainsonine content in *O. sericea* were explained by fungal DNA content (pg/ng). When outliers were removed from the data, based on studentized residuals with more than 2 deviations from the mean, 58% of the changes in swainsonine concentration were explained by fungal biomass. Fungal DNA values below 20 pg/ng explained 80% of changes in swainsonine content (n=12). The R^2 for fungal DNA values above 20 pg/ng was reduced significantly because of high

swainsonine variability, and the relationship between fungal DNA and swainsonine content becomes non-linear (Figure 7B and C). In untreated controls (n=5), 80% of the changes in swainsonine were explained by fungal DNA content. In Rhizobium 118H2 treated plants, 50% swainsonine changes were correlated with fungal DNA content. High levels of swainsonine production are associated with plant biomass. Higher swainsonine at lower fungal DNA content was found in Rhizobium treated plants compared to controls (Figure 7A).

Discussion

Effects of root nitrogen uptake and N_2- fixation on $^{15}N/^{14}N$ swainsonine ratios

The contribution from root nitrogen uptake vs. dinitrogen fixation in the synthesis of swainsonine by fungal endophyte in *O. sericea* was determined using a low concentration (0.14mM) of 20% enriched ^{15}N- NH_4NO_3. The range of $^{15}N/^{14}N$ ratios in this study (0.22-0.28) were within ratios estimated for other legumes. For example soybean ratios= 0.15-0.20, common bean ratios= 0.25-0.30, chick pea= 0.10-0.15, and cowpea= 0.20-0.25 (Danso et al. 1993). Effective swainsonine enrichment in ^{15}N- NH_4NO_3 treated plants, was confirmed by a significant increase ($t=2.26$ $p=0.026$) in $^{15}N/^{14}N$ ratios compared to untreated controls and *Rhizobium* treated plants. ^{15}N enrichment in *Rhizobium* treated plants was slightly greater than untreated controls, but significantly lower than ^{15}N-NH_4NO_3 treated plants ($t=4.80$, $P<0.0001$) (Figure 1). The effect of dinitrogen fixation on $^{15}N/^{14}N$ ratio in *Rhizobium* + ^{15}N- NH_4NO_3 treated plants, suggests that through dinitrogen fixation, atmospheric dinitrogen (^{14}N) is utilized by the fungal endophyte during the synthesis of swainsonine in *O. sericea* grown under low nitrogen. The fungal endophyte has access to nitrogen resources that come from both root uptake and the dinitrogen fixing nodules. Based on the results from this study, fixed dinitrogen can be incorporated into the synthesis of swainsonine by the endophyte more readily than root nitrogen. Nitrogen fixing nodules deliver nitrogen to be transported to the shoots via xylem in the form of amino acids with a high N:C ratio such as Asparagine and Glutamine (Hasija 1970). Nitrogen taken up from the soil by the roots has to satisfy the root internal demand for nitrogen before the shoot demand can be supplied, when soil

available nitrogen is limited (Kronzucker et al. 1998). In addition, the root system in *O. sericea* is the largest and most important N and C sink, more so than shoot growth (Wyka 2000, Wyka and Galen 2002).

To correct for the effect of low swainsonine (<0.010%) on $^{15}N/^{14}N$ ratios, samples with low swainsonine were removed (n=30). Differences in $^{15}N/^{14}N$ ratios among *Rhizobium* treated plants and untreated controls became smaller, and differences between plants treated only with ^{15}N-NH_4NO_3 and all other treatments became larger (Table 1 and. Table 2). The lowest ^{15}N enrichment was measured in plants treated with *Rhizobium* 118B2 ($^{15}N/^{14}N$ ratios = 0.22-0.23, n=12) (Table 2). *O. sericea* treated with *Rhizobium* 118B2 have been reported to produce a mean number of nodules 29 nodules/plant (36 weeks post germination), and 50% (R^2=0.5) of mean nitrogen fixation rates were explained by the number of nodules (Valde Barillas et al. 2007). Although the methods used in this study were not focused on measuring natural ^{15}N abundance, the low ratios in this treatment could correspond to a minor dilution on the natural abundance (0.3663% ^{15}N) as a result of increased ^{14}N incorporation from dinitrogen fixation.

Differences in $^{15}N/^{14}N$ ratios among treatments became greater and more significant at 20-22 weeks post germination (Figure 1). These differences may be attributed to greater enrichment and increased dinitrogen fixation through time. In the case of the untreated controls, the incorporation of nitrogen was probably slowed by the interaction between low affinity transporters vs. high affinity nitrogen transporters in the roots, which have a delayed response adjusting effluxes and influxes of NH4 and NO3 following nitrogen starvation (NH_4 or NO_3< 1mM) (Von Wiren et al. 2001).

Changes in $^{15}N/^{14}N$ swainsonine ratios could be explained by 10% (r=30) of changes on swainsonine in plants treated with *Rhizobium* + ^{15}N-NH_4NO_3. Another 10% of the changes in $^{15}N/^{14}N$ swainsonine ratios were explained by shoot biomass in *Rhizobium* and untreated controls. These results suggest that nitrogen fixation has a greater effect on changes in swainsonine content than non-nitrogen fixing plants. For instance, atmospheric incorporation varies among *Rhizobium* treated plants because of differences in dinitrogen fixation rates. Dinitrogen fixation rates based on an acetylene reduction assay in *O. sericea* treated with the same strains of *Rhizobium* averaged 12.45 mL ethylene/L air/hour (Valdez Barillas et al. 2007).

Effect of root nitrogen uptake vs. N_2-fxiation on swainsonine concentration and plant measurement

From the plants used in this study (N=120), 20% were low swainsonine producers (< 0.010 %). These 20% represent plants with low fungal endophyte compatibly, and possibly low infection and colonization by the endophyte. Low fungal infection has been correlated with low swainsonine content in wild *O. sericea* (Gardner et al. 2001) and in *O. sericea* grown from seeds treated with fungicide to suppress fungal infection (Valdez Barillas et al. 2007). However, 40% of the untreated control plants were low swainsonine producers and 40% were high swainsonine producers (>0.100 %). Untreated controls had the highest variability in swainsonine content. Low swainsonine content in these plants could be explained by low fungal infection and colonization. High swainsonine content could be explained by successful infection and colonization of the fungal endophyte, nitrogen stress, a fungal endophyte response to osmoregulatory changes inside the plant,

and nitrogen diverted for swainsonine synthesis by the fungal endophyte acting as N sink. In the case of *Rhizobium* treated plants, high swainsonine content may be an indirect effect of dinitrogen fixation on swainsonine synthesis by the fungal endophyte. Although all plant were growing under limited nitrogen (0.14mM NH_4NO_3), untreated controls had similar or greater swainsonine content compared to the *Rhizobium* treated plants, particularly in plants harvested 18 to 19 weeks post germination (Figure 2). Interestingly, low nitrogen conditions in untreated controls and *Rhizobium* treated plants did not limit the number of shoots per plant but the length of the shoots (Figure 4 and 5). A regrowth of shoot can be colonized by fungal endophyte from the meristematic tissue in the crown.

The fungal endophyte had a greater effect on nitrtogen resource allocation in untreated plants compared to *Rhizobium* treated plants. Under low nitrogen conditions plants lower the content of Asparagine and Glutamine (Hasija 1970). This mechanism increases the proportion of amino acids with low N: C ratio inside the plant such as phenylalanine, lysine, and proline. Lysine levels also increase during nitrogen stress because lysine is catabolized to synthesize the two osmoregulators proline and L-pipecolate (Moulin et al. 2006).

Plants respond to fungal infections by inducing nutrient starvation and lowering the free amino acid content in the area of infection (Solomon and Oliver 2001, Divon et al. 2005. In response to possibly higher levels of proline, alanine, phenylalanine or lysine, the fungal endophyte is forced to catabolize lysine, which may explain the increase in swainsonine in untreated controls. Untreated control plants were smaller than *Rhizobium* treated plants and remained small throughout the experiment (Figure 3). In addition,

untreated controls with low swainsonine content (<0.010%) had greater biomass (>0.150g) than high swainsonine producing plants (<0.150g) (Figure 6B). In the absence of a fungal endophyte acting as a nitrogen sink, untreated controls incorporated more nitrogen toward increasing biomass than plants with high swainsonine content and consequently greater fungal endophyte infection. This inverse relation between swainsonine content and biomass in untreated controls had a negative correlation of 30%.

Contrary to untreated control plants *Rhizobium* treated plants had lower swainsonine levels during the initial stages of nodulation. *Rhizobium* treated plants had significantly greater biomass, shoot length, and increased number of shoots during the 21 week post germination than untreated controls and ^{15}N-NH_4NO_3 treated plants (Figures 3, 4, and 5). In the *Rhizobium* treated plants, the competition for nitrogen by the fungal endophyte inside the plant was lower than the untreated controls.

To account fo the effects of increased biomass resulting from the absence of a fungal endophyte N sink, data was analyzed without outliers determined by studentized residuals with more than 2 deviations from the mean (n=17). Most outliers included samples with extremely high swainsonine content (>0.250%) or low swainsonine content (<0.001%). *Rhizobium* effects on swainsonine became significant 22 weeks after germination (Figure 2). Thus, swainsonine differences between untreated controls and *Rhizobium* treated plants increased during later stages of nodulation. This was confirmed in *O. sericea* harvested 22 and 25 weeks post germination and in *O. sericea* from another study grown and treated under the same conditions, and harvested at 36 week post germination (Valdez Barillas et al. 2007) (Figure 2 and Table 3).

Twenty weeks post germination *O. sericea* samples had elevated swainsonine content and shoot biomass, not explained by the treatments. One possible explanation to this high values could be indirectly related to solar radiation. Mean solar radiation (W/m^2) corresponding to the dates during 18^{th} and 19^{th} week post germination was significantly elevated compared to the levels during the 20^{th} week post germination (CSU weather station, Appendix B). Elevated solar radiation could have resulted in elevated photosynthetic flux and plant evapotranspiration. Increased photosynthetic activity could explain the increases in shoot biomass and an increase in nitrogen demand from the shoots (Meyer and Stitt 2001, Von Wirén et al. 2001). Increased nitrogen allocated toward shoot biomass combined with increased evapotranspiration, could have induce stress on the endophyte as a result of changes in osmoregulation and nutrient availability in the infected plant tissue. Low nitrogen availability and an increase in osmoregulators, could have increased the production of proline and lysine inside the plant. Increased lysine and proline possibly forced the endophyte to catalyze lysine via L-pipecolate to catabolized it further into proline and then to glutamate as a source of NH_4. Thus, increased concentrations of L-pipecolate and a reduction of amino acids with low N: C ratio could have increased swainsonine synthesis by the fungus.

Another contribution to increased shoot biomass and swainsonine could be related to a reduction in nitrogen allocated to flavonoids synthesis from aromatic amino acid. Flavonoids are regularly used to protect the plant cells from ultraviolet B (UV-B) radiation (Hollósy 2002). However, the polycarbonate material that makes up the walls and ceiling of the greenhouse can filter UV-B. In the absence of UV-B stress less nitrogen is devoted toward flavonoids synthesis and more nitrogen is available for plant

and fungal endophyte increase in biomass. UV-B filtering could also explain the greater mean swainsonine content detected in greenhouse grown (0.175 %, SE= 0.028) vs. outdoor wild *O. sericea* (0.04%, SE=0.005) (Valdez Barillas et. al. 2007, Ralphs et al. 2008).

Effect of root nitrogen uptake vs. N_2-fixation on swainsonine and fungal DNA content

The results from fungal DNA content were 40% correlated with the changes on swainsonine content in O. sericea (N=47). Above 20pg/ng the changes on swainsonine were highly variable in relation to fungal DNA content (Figure 7B). These results were skewed by the *Rhizobium* treatment effects and nitrogen stress in non-dinitrogen fixing *O. sericea*. Based on the $^{15}N/^{14}N$ swainsonine ratios results, the high variability in swainsonine content in plants with fungal DNA above 20pg/ng is not entirely explained by fungal DNA but by the physiological conditions inside the plants.

An increase in fungal biomass does not necessarily imply that swainsonine synthesis will increase, unless the fungal endophyte has a need to catabolize lysine, which is a poor source of nitrogen for increasing biomass. Lysine would be catabolized and incorporated into biomass by the fungal endophyte under nitrogen or drought stress conditions inside the plant, and low availability of alternative sources of nitrogen. For instance, fungal endophytes catabolize many sources of nitrogen for maintenance of regular metabolic functions, or to increase biomass. To increase biomass, fungal endophytes prefer nitrogen sources with high N: C ratios such as alanine, serine glutamine, aspartate glutamate and also ammonium (Hasija 1970).

An increase in fungal endophyte implies that the fungus has a source of amino acids with high N: C ratio in the apoplasm of the plant. Such conditions will take place during the early stages of fungal colonization and would continue if a nitrogen supply in the apoplasm remains constant. In this study, *Rhizobium* treated plants were possibly capable of maintaining such supply through dinitrogen fixation, thus, facilitating both fungal and shoot development.

In *Rhizobium* treated plants swainsonine and biomass increases in both plant and fungal endophyte, compared to untreated controls. Swainsonine increases as a result of a supply of amino acids with high N:C ratios from dinitrogen fixation and increased photosynthesis. However, when root nitrogen availability decreases and shoot demand increases, (as observed in untreated controls and *O. sericea* treated only with ^{15}N-NH_4NO_3) the fungal endophyte starts acting as N sink and will start competing for nitrogen with the plant. Such scenario could trigger nitrogen starvation and osmoregulatory responses inside the infected tissue by increasing osmoregulators such as proline or lysine to synthesis L-pipecolate and more proline in the apoplasm.

In response to the changes in physiology the endophyte could use the physiological changes to its advantage, by catabolizing the available nitrogen sources such as lysine or L-pipecolate and further catabolize them into proline as an auto osmoregulator, acetyl CoA for protein synthesis, and glutamate as a source of nitrogen. Such activity could indirectly result in an increased synthesis of swainsonine by the fungal endophyte but fungal growth rates would slow down.

Low swainsonine content is probably explained better by plants with low DNA content (< 20pg/ng) (R^2= 0.9) because of poor or slow fungal colonization, and low

nutrient competition between fungal endophyte and the plant. These plants will have greater shoot biomass than high swainsonine producers even when root nitrogen supply is low. Non-dinitrogen fixing plants with high fungal DNA and swainsonine content will have the lowest shoot biomass and high swainsonine content as result of high nitrogen stress and osmoregularoy responses during competition for resources with the fungal.

Conclusions

Based on ^{15}N-enrichemnt results the synthesis of swainsonine in *O. sericea* is affected by both soil nitrogen and dinitrogen fixation. When *O. sericea* grows in soils with low nitrogen, dinitrogen fixation allows the plant to increase shoot biomass and sustain the growth of the fungal endophyte. Non-dinitrogen fixers are affected by the fungal endophyte acting as a nitrogen sink inside the plant, which results in increased swainsonine synthesis, in some cases increased fungal endophyte biomass, but a slow or no increase in shoot biomass. During a more advanced stage of nodulation, both shoot biomass and swainsonine content are significantly greater in nitrogen fixing *O. sericea* relative to non-nodulated individuals. Changes in shoot biomass in nitrogen stressed and dinitrogen fixers are determined by increases in shoot length more so than number of shoots.

The variability of swainsonine levels in *O. sericea* populations could be explained in part by the presence of low and high swainsonine producing plants. Low swainsonine producers that do not fix nitrogen slowly increase shoot biomass in the absence or reduced effect from the fungal endophyte acting as N sink. High swainsonine producers that don't fix nitrogen could be affected by the fungal endophyte acting as N sink but, swainsonine levels could compensate the low shoot biomass. Low swainsonine producers that are both dinitrogen and non dinitrogen fixers could be more susceptible to herbivory and less likely to be grazed. Nodulated plants that result in high swainsonine production will be more toxic, more likely to be grazed, but will be more resilient to herbivory as a result of dinitrogen fixation. Dinitrogen fixing *O. sericea* are therefore less toxic and

more vulnerable to herbivory during the early growth stages. However, older dinitrogen fixing plants become less vulnerable to herbivory as swainsonine levels increase. The increase of swainsonine in older plants may be explained by a gradual increase in fungal biomass.

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Figures and Tables

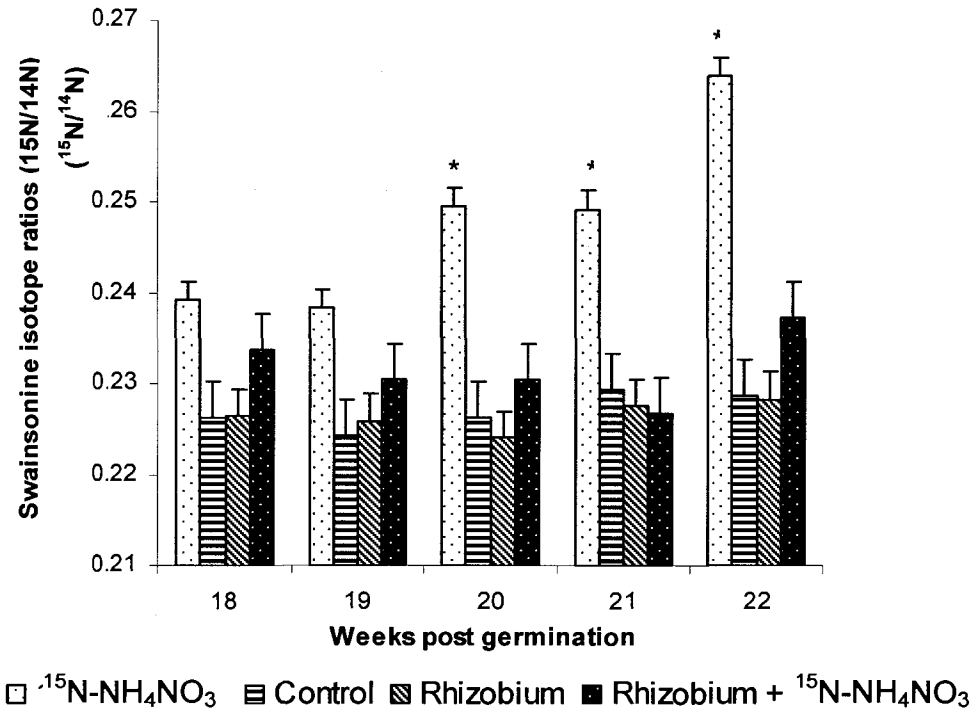

Figure 1. $^{15}N/^{14}N$ Swainsonine isotope ratios collected from 18 to 22 week old *Oxytropis sericea* treated with Rhizobium and enriched with 20% ^{15}N-$N0_3NH_4$. Bars are means; error bars indicate the standard error of the mean; Star (*) indicates significant differences among treatments within each week using an LSD test ($P < 0.05$).

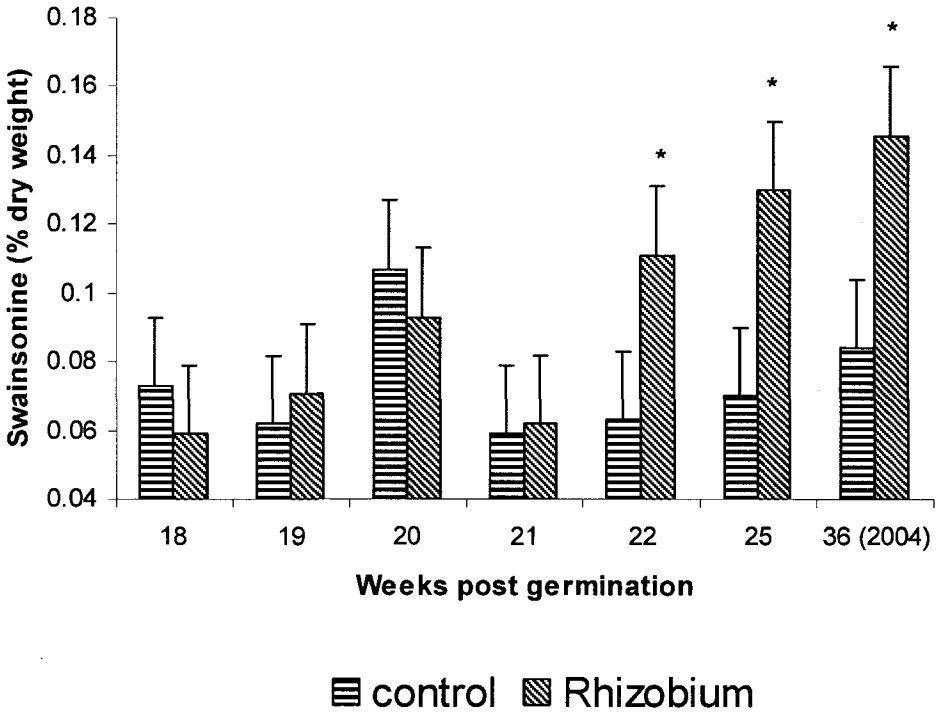

Figure 2. Differences in swainsonine concentration (% dry weight) between Rhizobium treated plants and untreated controls by week. Values from weeks 18 to 22 correspond to *O. sericea treated* with Rhizobium and harvested on September 2007. Week 36 values belong to *O. sericea* treated with Rhizobium and harvested on September 2004.Bars are means; error bars indicate standard error of the mean: Star indicates difference between treatments within each week using an LSD test ($P < 0.05$)

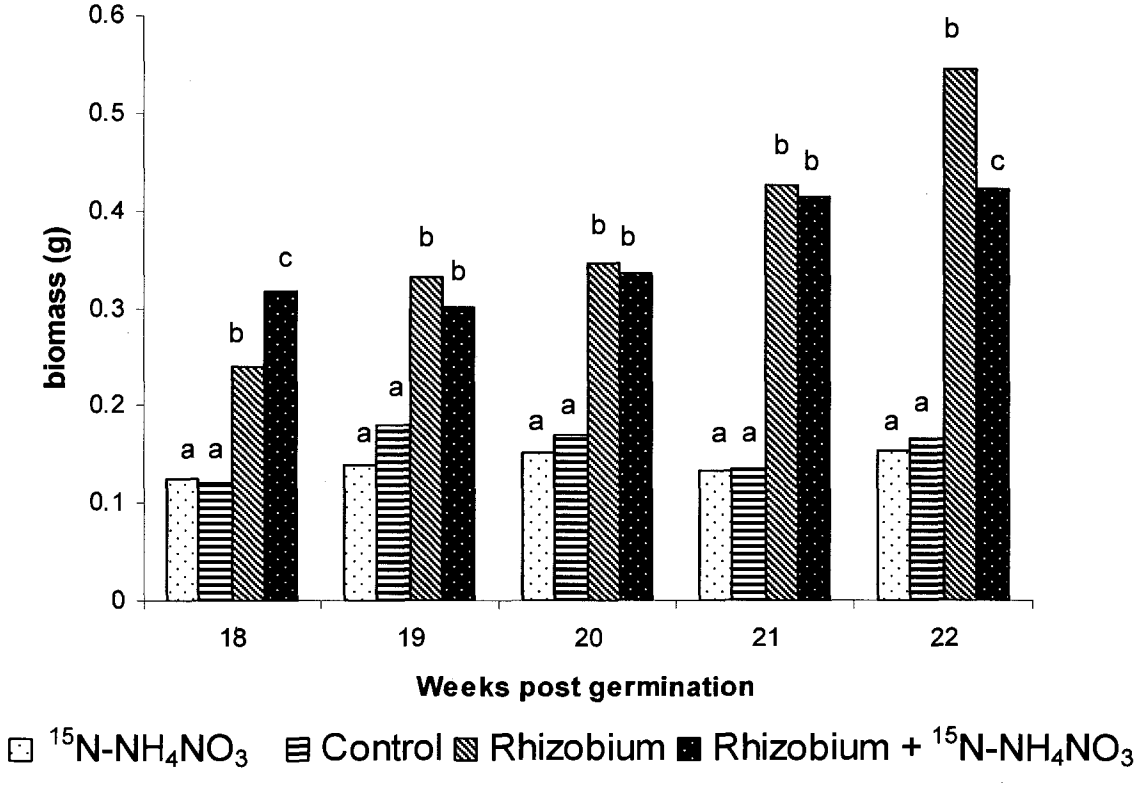

Figure 3. Differences in shoot biomass among 18 to 22 week old Oxytropis *sericea* after being treated with Rhizobium and enriched with 20% ^{15}N-NH_4NO_3. Bars are means; Letters indicate significant differences among treatments within each week using an LSD test ($P < 0.05$ for weeks 1 and 2, $P < 0.001$ for week 3, and $P < 0.0005$ for weeks 4 and 5).

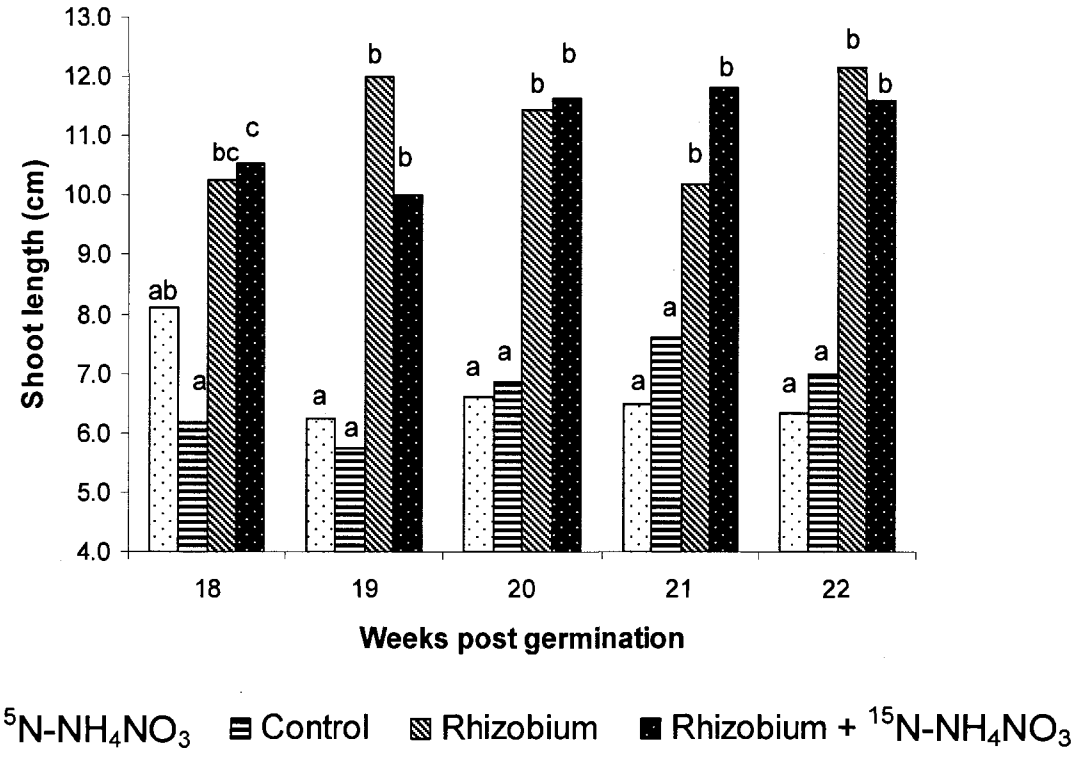

Figure 4. Differences in shoot length among 18 to 22 week old *Oxytropis sericea* treated with Rhizobium and enriched with 20% ^{15}N-NH$_4$NO$_3$. Bars are means; Letters indicate significant differences among treatments within each week using an LSD test ($P < 0.001$).

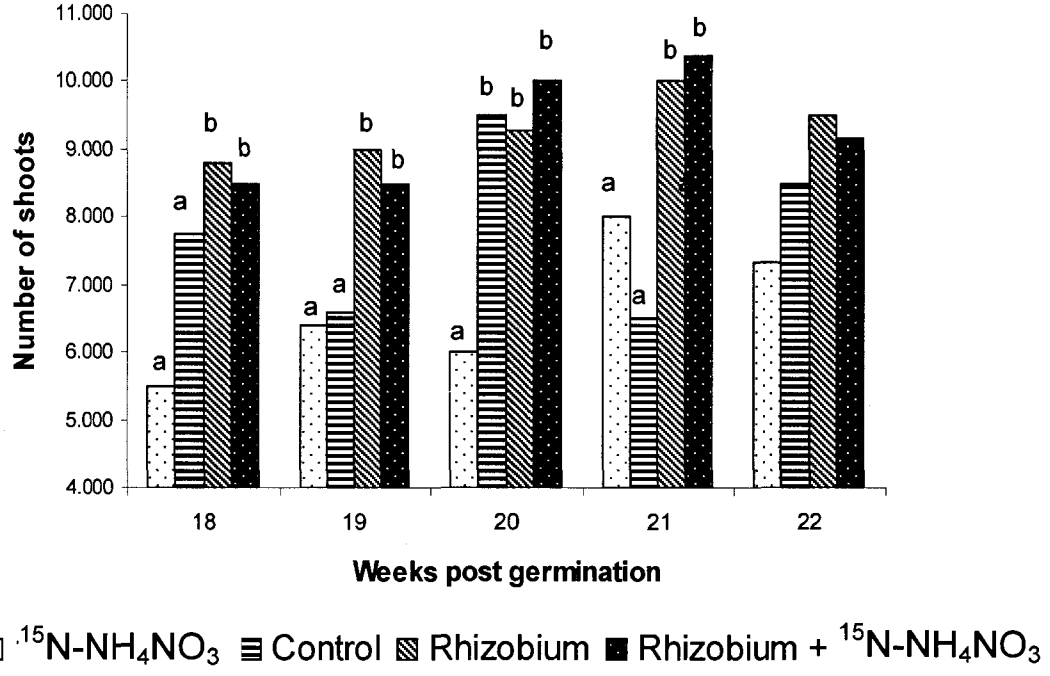

Figure 5. Differences in number of shoots among 18 to 22 week old *Oxytropis sericea* treated with Rhizobium and enriched with 20% ^{15}N-NH$_4$NO$_3$. Bars are means; Letters indicate significant differences among treatments within each week using an LSD test ($P < 0.001$).

Table 1. $^{15}N/^{14}N$ Swainsonine ratios and plant measurements from *Oxytropis sericea* treated with two strains of Rhizobium and 20% ^{15}N-NH_4NO_3.

Treatment	N	Swainsonine ratio ($^{15}N/^{14}N$)		Shoot biomass (g)		Shoot length (cm)		No. Shoots	
Rhizobium 118H2	20	0.229^{b}	(0.002)	0.426^{d}	(0.038)	12.39^{c}	(0.56)	9.42^{b}	(0.299)
Rhizobium 118H2 + ^{15}N-NH_4NO_3	20	0.224^{bc}	(0.004)	0.392^{cd}	(0.026)	12.07^{c}	(0.47)	9.85^{b}	(0.568)
Rhizobium 118B2 + ^{15}N-NH_4NO_3	20	0.231^{b}	(0.002)	0.336^{bc}	(0.023)	10.05^{b}	(0.54)	8.70^{bc}	(0.637)
Rhizobium 118B2	20	0.221^{c}	(0.003)	0.302^{b}	(0.028)	9.87^{b}	(0.33)	9.15^{b}	(0.425)
Control 1	20	0.224^{bc}	(0.004)	0.156^{a}	(0.009)	6.71^{a}	(0.28)	7.71^{ac}	(0.353)
^{15}N-NH_4NO_3	20	0.247^{a}	(0.003)	0.143^{a}	(0.006)	6.40^{a}	(0.26)	6.95^{a}	(0.294)
†Control 2	4	0.227^{b}	(0.002)						

Notes: Values presented are means of all the plant samples collected during 5 weeks; parentheses indicate standard error of the mean. Superscript letters indicate significant differences within a column using an LSD test ($P<0.05$). †Control 2: is a sample from *Astragalus lentigninosus* used as a standard for the swainsonine analysis.

Table 2. $^{15}N/^{14}N$ Swainsonine ratios and plant measurements from *Oxytropis sericea* treated with two strains of Rhizobium and 20% ^{15}N-NH_4NO_3, using data without outliers

Treatment	N	Swainsonine ratio ($^{15}N/^{14}N$)		Shoot biomass (g)		Shoot length (cm)		No. Shoots	
Rhizobium 118H2	18	0.227^{c}	(0.001)	0.424^{d}	(0.035)	12.44^{c}	(0.57)	9.58^{b}	(0.309)
Rhizobium 118H2 + ^{15}N-NH_4NO_3	15	0.232^{bc}	(0.004)	0.374^{cd}	(0.029)	12.07^{c}	(0.57)	9.53^{b}	(0.682)
Rhizobium 118B2 + ^{15}N-NH_4NO_3	17	0.233^{b}	(0.002)	0.334^{bc}	(0.023)	9.97^{b}	(0.56)	9.23^{bc}	(0.656)
Rhizobium 118B2	12	0.222^{d}	(0.001)	0.320^{b}	(0.031)	10.21^{b}	(0.44)	9.83^{b}	(0.548)
Control 1	14	0.228^{c}	(0.001)	0.143^{a}	(0.008)	6.71^{a}	(0.38)	8.00^{ac}	(0.491)
^{15}N-NH_4NO_3	18	0.248^{a}	(0.003)	0.135^{a}	(0.006)	6.69^{a}	(0.41)	6.55^{a}	(0.335)
†Control 2	4	0.227^{c}	(0.002)						

Notes: Table does not include values with swainsonine content <0.01 (n=30), when considered outliers. Values presented are means of all the plant samples collected during 5 weeks; parentheses indicate standard error of the mean. Superscript letters indicate significant differences within a column using an LSD test ($P<0.05$). †Control 2: is a sample from *Astragalus lentigninosus* used as a standard for the swainsonine analysis.

Table 3. Plant measurements and swainsonine concentrations in 9 month old *O. sericea* grown in 2004 and 5 month old *O. sericea* grown in 2007 treated with two strains of Rhizobium.

Rhizobium	No. plants		No. shoots		Shoot length (cm)		Shoot biomass (g)		Swainsonine (%)	
treatment	2004	2007	2004	2007	2004	2007	2004	2007	2004	2007
118H2	13	19	22.08^{aA}	9.42^{aB}	11.77^{aA}	12.39^{aA}	0.894^{aA}	0.426^{aB}	0.175^{aA}	0.087^{aB}
			(3.358)	(0.299)	(0.662)	(0.557)	(0.099)	(0.038)	(0.028)	(0.015)
118B2	12	20	11.50^{bA}	9.15^{aA}	11.12^{aA}	9.87^{bA}	0.859^{abA}	0.302^{bB}	0.114^{bA}	0.068^{aB}
			(2.073)	(0.425)	(0.774)	(0.326)	(0.146)	(0.028)	(0.014)	(0.014)
Control	11	21	9.18^{bA}	7.71^{bA}	11.00^{aA}	6.71^{cB}	0.571^{bA}	0.156^{cB}	0.084^{bA}	0.064^{aA}
			(1.143)	(0.379)	(0.588)	(0.275)	(0.103)	(0.008)	(0.008)	(0.013)

Notes: Values presented are means; parentheses indicate standard error of the mean. Superscript lower case letters indicate significant differences within a column, and superscript upper case letters indicate significant differences between the same treatment in year 2004 and 2007 using an LSD test($P<0.05$).

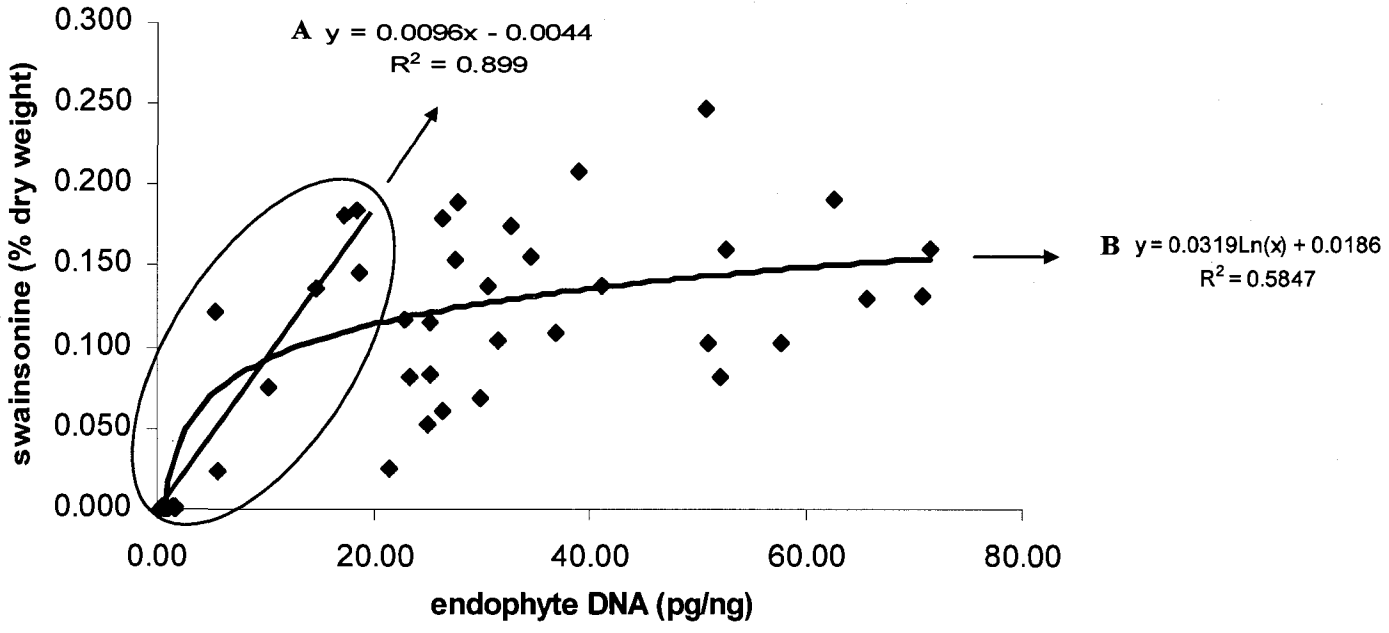

Figure 6. Endophyte DNA quantification and swainsonine content in 25 week old *Oxytropis sericea* treated with Rhizobium and ^{15}N-NH_4NO_3. **A.** Linear regression for encircled data points below 20 pg/ng (N=17). **B** Linear regression for all data points (N=44).

Appendix A. $^{15}N/^{14}N$swainsonine ratios and swainsonine concentration on 18 to 22 week old *Oxytropis sericea* treated with *Rhizobium* and ^{15}N-NH_4NO_3.

Treatment	18 weeks		19 weeks		20 weeks		21 weeks		22 weeks	
	ratio	swain	ratio	swain	ratio	swain	ratio	swain	ratio	swain
^{15}N-NH_4NO_3	0.239 (0.004)	0.052 (0.013)	0.238 (0.004)	0.113 (0.013)	0.250 (0.004)	0.108 (0.002)	0.249 (0.006)	0.057 (0.023)	0.264 (0.012)	0.058 (0.031)
Control 1	0.226 (0.003)	0.094 (0.039)	0.214 (0.015)	0.011 (0.010)	0.226 (0.002)	0.106 (0.037)	0.229 (0.000)	0.060 (0.024)	0.229 (0.000)	0.066 (0.025)
Rhizobium 118B2	0.226 (0.002)	0.049 (0.032)	0.211 (0.013)	0.067 (0.045)	0.220 (0.000)	0.101 (0.035)	0.229 (0.004)	0.042 (0.023)	0.221 (0.003)	0.088 (0.015)
Rhizobium 118B2 + ^{15}N-NH_4NO_3	0.233 (0.007)	0.051 (0.023)	0.228 (0.002)	0.074 (0.027)	0.233 (0.007)	0.066 (0.028)	0.228 (0.000)	0.060 (0.016)	0.233 (0.006)	0.099 (0.023)
Rhizobium 118H2	0.226 (0.004)	0.083 (0.024)	0.228 (0.003)	0.075 (0.037)	0.228 (0.000)	0.115 (0.025)	0.226 (0.002)	0.066 0.027)	0.236 (0.020)	0.087 (0.086)
Rhizobium 118H2 + ^{15}N-NH_4NO_3	0.234 (0.007)	0.052 (0.021)	0.233 (0.002)	0.067 (0.028)	0.231 (0.000)	0.092 (0.030)	0.212 (0.014)	0.079 (0.047)	0.241 (0.001)	0.129 (0.043)
†Control 2	0.227	0.116	n/a	n/a	0.231	0.080	0.228	0.154	0.221	0.167

Note: Values presented are means; parentheses indicate standard error of the mean. Values on ratio column are expressed in $^{15}N/^{14}N$ swainsonine ratio. Values on swainsonine column, swain=swainsonine, are expressed in % dry weight. †Control 2: is a sample from *Astragalus lentigninosus* used as a standard for the swainsonine analysis. Missing data is reporter as n/a.

Chapter 2

Mutualisms facilitate swainsonine production in *Oxytropis* species from the United States and China

Introduction

Mutualisms beyond pairwise interactions are driven by different feedbacks among symbiotic partners. Such feedbacks are affected by environmental conditions and the fitness of each partner (Beer and Simms 2000, Stanton 2003). Recent studies suggest that beneficial, neutral, and negative interactions often occur simultaneously and the net effect of the fluctuations among partner interactions determines the stability and continuity of the mutualism (Klironomos 2002, Stanton 2003, Bennett et al. 2006). For instance, plant-microbe mutualisms are sustained by the host plant, which provides photosynthates to one or more microbial partners in exchange for nutrients and feedbacks that enhance the fitness of the host plant (Bennet et al. 2006). Legumes, among mutualistic plants, are capable of hosting dinitrogen fixing bacteria, and symbiotic fungi in their roots and aboveground tissues (O'Dell and Trappe 1992, Doyle 1994, Parker et al. 1999, Provorov et al. 2002). Legume mutualisms with dinitrogen fixing bacteria and fungi have been reported in *Lupinus* and particularly in *Oxytropis* and *Astragalus,* among other legumes. Representatives from the last two genera reveal high rates of speciation and diversification in arcto-boreal, temperate arid, and semiarid regions (Wojciechowski et al. 1999, Lavin 2004). Both genera also include multiple examples of parallel biochemical and morphological adaptation to extreme environments in Eurasia, North America, and South America (Wojciechowski 2005).

High speciation and diversification in Eurasian *Astragalus* took place 12 million years ago, and in *Oxytropis* and new world *Astragalus* 4-5 million years ago (Wojciechowski 2005). Eurasian *Astragalus* and *Oxytropis* reached higher levels of

diversification as a result of East-West mountain range orientation vs. North-South in North America (Qian and Ricklefs 1999, Qian 2002, Wojciechowski 2005). Global cooling and glaciations in Eurasia and North America forced speciation and ecological adaptations to different elevations in both floras, as glaciers extended and retrieved (Qian 2002, Weber 2003). Such selective pressures were responsible for the approximately 2500 species of *Astragalus* and 300 species of *Oxytropis* in Eurasia, and 354species of *Astragalus* and 22 species of *Oxytropis* in North America (Welsh 1995, Wojciechowski et al. 1999, Welsh 2007). These ecological adaptations have been conducive to the development of multipartite interactions between dinitrogen fixing bacteria, mycorrhiza, dark septate fungi in roots and aboveground tissues (Currah and Van Dyk 1986, O'Dell and Trappe 1992, Treu et al. 1996, Laguerre et al. 1997, Braun et al. 2003, Valdez Barillas et al. 2007, Ralphs et al. 2008).

Oxytropis and *Astragalus*, also known as locoweeds, have been extensively researched for their concentration of swainsonine. Swainsonine is an indolizidine alkaloid synthesized by a fungal endophyte (*Embellisia* spp.) hosted in the aboveground tissues of some locoweeds (Braun et al. 2003, Gardner et al. 2004, Kulshreshtha et al. 2004, and Ralphs et al. 2008). Swainsonine has been identified as the responsible factor for the intoxication of animals that repeatedly graze on some locoweeds (James et al. 1981). Significant reproductive and economical losses due to livestock intoxication have been attributed to 24 species of locoweeds in the United States (Gates 1925, Kingsbury 1964, Torrel et al. 2000, Ralphs et al. 2002a). Similar reports have been made from *Astragalus* in China, where a total of 18 species of locoweeds distributed on 11,000,000 ha have been reported as harmful to livestock (Zhao et al. 2003)

Fungal endophytes from aboveground tissues have been isolated from *Astragalus adsurgens* (Li and Nan 2007) and *O. kansuensis* (Wang et al. 2006) in China. Swainsonine in Chinese locoweeds has been detected in *Astragalus strictus*, *O. ochrocephala* (Lu Xike 1993), *Oxytropis kansuensis* (Lu Xike 1993, Tong 2001), and *Oxytropis glabra* (Zheng 1995). Other studies have reported mutualism with mycorrhiza and septate fungi in the roots of North American locoweeds, which includes: *Astragalus alpinus, Astragalus applegatei, A. cottonii, A. drummondi, A. gilviflorus, A. kentrophyta, A. percinatus, A. striatus, A. vexilliflexus*, *Oxytropis campestris, O. jordalii, O. scammaniana, O. splendens,* and *O. viscida* (Currah and Van Dyk 1986, O'Dell and Trappe 1992, Treu et al. 1996, Barroetavena et al.1998).

North American locoweeds have also been studied for their ability to host dinitrogen fixing bacteria in their root nodules at various, and extreme environmental conditions (Allen and Allen 1981, Prévost et al. 1987de Faria et al. 1989, Bordeleau and Prévost 1994, Prévost et al. 2003). Nodulation and nitrogen fixation has been studied in locoweeds from China as well (Blasum 1997, Kan et al. 2007).

In a Study by Kulshreshtha et al. (2004) *Oxytropis lambertii* and *Oxytropis sericea* (New Mexico, USA), the two most important species in terms of livestock intoxication casualties and distribution, shared a high similarity coefficient (1.00) based on cpDNA analysis. Interestingly, the five varieties of *Astragalus mollissimus* also shared a high similarity coefficient (0.97), but still separated in three different clades that coincided with their geographic distribution. Results from the same study indicated that when comparing the sequences of fungal endophyte rDNA ITS 5, both species of *Oxytropis* hosted similar endophytes. In another study by Valdez Barillas et al. (2007), *Oxytropis*

sericea produced dinitrogen fixing nodules in association with *Rhizobium* strains isolated from *Oxytropis riparia* and *Oxytropis foliolosa.* These result confirmed the symbiotic compatibility and high promiscuity documented in cross inoculations between *O. campestris* and strains isolated from species of *Astragalus* and *Hedysarum* by Novikova et al. (1993). A study by Laguerre et al. (1997) on Rhizobial diversity from *Astragalus*, *Oxytropis*, and *Onobrychis,* reported that Rhizobial isolates from the same geographic region can have different 16S rDNA types, and genetically similar rhizobia may be present in soils of different continents. High symbiotic promiscuity in *Oxytropis sericea* may therefore be reflected in its ability to hybridize with *Oxytropis lambertii* and *Oxytropis campestris* (Welsh 1995).

Considering the high level of symbiotic compatibility in some North American *Oxytropis* species, taking into account the reports on swainsonine production in Chinese *Oxytropis* and *Astragalus*, and the report on fungal endophyte isolated from the swainsonine producing *Oxytropis kansuensis*, we studied the similarities between locoweed mutualisms and plant toxicity in both countries. For this study, we hypothesized that the mutualistic symbionts and swainsonine levels in some locoweed species growing in arid and semi-arid ecosystems of China are similar to those from the United States. We tested this hypothesis by studying locoweed populations from Inner Mongolia, China, to: 1) determine swainsonine levels in leaves, 2) isolate fungal endophytes from leaves 3) compare swainsonine levels and fungal endophytes between locoweeds from both countries, and 4) search for dinitrogen fixing root nodules .

Material and Methods

Study site description

Sites location, elevation and plant community of collected material are shown in Table 1. All sites were selected based on previous reports on *Oxytropis* species distribution in Western Inner Mongolia. Each site represents a plant community where livestock intoxication with *Oxytropis* has been reported. The vegetation types at each collecting site included: the desert steppe of Yjinhuolo, desert of Wushen, the sandy shrublands of Eutoke Qi, desert of Alxa Zuo, and the salty irrigated low land near Linhe.

All collection sites in Inner Mongolia were located in arid and semiarid regions. The dates of the field study coincided with part of the summer monsoon season; therefore, soil moisture was greater on sites at lower elevations. Soil texture in all sites was sandy, with the exception of the Yijinhuolo desert steppe, which had sandy loam and sandy areas. All sites had 30-50% coarse soils. Elevation among sites was similar (1300-1380m), and the lowest elevation corresponded to the salty low irrigated lands (1044m) (Table 1).

Sampling and preparation of collected plant material

Oxytropis glabra was collected at lower basins or between sand dunes. *Oxytropis glabra* population in the desert steppe and sandy shrublands was found in association with *O. gracillima.* Populations in both desert sites were found in association with *Oxytropis aciphylla.*

Plant material from the three species of *Oxytropis* (*O. glabra*, *O. aciphylla*, and *O. gracillima*) was collected from one or two populations in each site. Plant material from one species of Astragalus (A. variabilis) was collected from on population in one site. Plant material was collected between July 28 and August 4 of 2005.Twenty to thirty individual plants from each species were randomly selected keeping a 50m distance within species, and three subsamples were collected from the above ground tissues of each plant. A subsample of flowers and leaves was prepared for swainsonine analysis, a second subsample of flowers and leaves was prepared for fungal endophyte isolation, and a third subsample was prepared in silica gel for fungal endophyte DNA PCR analysis. Plant material for swainsonine analysis and fungal isolations were oven dried for at 52° C for 72hrs.

For the assessment of dinitrogen fixing nodules, one or two plants from each species neighboring a harvested individual were dug around the roots to search for nodules. Active nodules were confirmed by the presence of leghemoglobin. Nodules were prepared in 80% ethanol for morphological analysis using a dissecting microscope.

Swainsonine analysis and sample preparation

Oven dried plant material was ground through a 1-mm^2 grid. Swainsonine extraction and analysis (% dry weight) were followed as describe by Gardner et al (2001). In brief, 100mg of plant material from each sample were weighed into a 15mL labeled test tube with screw cap. A 5mL 2% acetic acid and 4mL chloroform aliquot was added to each test tube. After overnight mechanical rotation, samples were centrifuged. The upper layer was passed through an ion exchange resin. Swainsonine was eluted from the ion

exchange resin with 5mL of ammonium hydroxide. Swainsonine was evaporated under N_2 flow and resuspended in 100μL aliquots for liquid chromatography-mass spectrometry. Standards for this analysis were generated form *Astragalus lentiginosus* material previously assayed for swainsonine concentration.

Swainsonine data analysis

Swainsonine concentration for each species was compared among populations using two sets of data. One set of data included all samples with swainsonine concentration <0.001%, these samples were 50% or the entire data set. A second data set included samples with swainsonine concentration >0.001%. Swainsonine means among populations were compared using one-way ANOVA (PROC GLM, SAS 9.1) to determine location effect on swainsonine. Multiple comparisons among populations were made using LSD and linear contrast ($P<0.05$).

Fungal DNA extraction from plant samples and PCR analysis

Plant genomic DNA of *Oxytropis* was extracted by CTAB method for PCR amplification and suspended in 50μL of sterile water. The PCR reaction mixture contained a final concentration of 1μL of 10 μM dNTP's (Promega, Madison, WI, USA), 6 μL of 25mM $MgCl_2$, 2.5μL of 5μM ITS 5 primer, 0.25μL GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA), and10μL 5x green GoTaq Flexi buffer. Primers used to amplify the internal transcribed spaces (ITS) region were OR1 (5' GTC AAA AGT TGA AAA TGT GGC TTG 3') and ITS 5 (White et al. 1990). Conditions for PCR were 94°C for 3min flowed by 30cycles of 94°C for 45 sec, 48°C for 1min, and

72°C for 30 sec with a final extension at 72°C for 5 min. The PCR product was analyzed in 1.8% agarose gel with ethidium bromide and run at 100V for 1.5hr.

To differentiate between *Alternaria* and *Embellisia*, all positive amplifications were digested with 5µL of the restriction enzyme *AvaII* (Promega, Madison, WI, USA), 1µL buffer C supplied with enzyme, and sterile water for a total of 10µL. After incubation for 1hr at 37°C, the restriction fragments were run through and Agarose gel using the previously mentioned conditions. Two bands (200 and 380 bp) indicated *Embellisia*, one bands indicated *Alternaria.*

Isolation and culturing of the fungal endophyte

Leaves and stems from plant subsamples containing fungal DNA were surface sterilized 30sec in 70% ethanol, 3 min in 20% bleach, and 30sec in sterile water. Tissues were plated into 15% water agar media and store at room temperature. Endophyte fungal growth was transferred to potato dextrose agar (PDA) plates and stored at room temperature.

Fungal endophyte RNA isolation from cultures and sequencing

Approximately 1cm^3 of culture mycelia was placed in a 1.5ml microcentrifuge tube, dried with liquid nitrogen and ground with a plastic drill bid. Ground material was homogenized in with 700µL of lysis buffer and incubated at 65°C for 1hr. After incubation, 700µL of phenol:chloroform (1:1) were added. Samples were vortex briefly and centrifuged at 12,000rpm for 15 min. The upper layer was transferred to new tube and extracted a second time with phenol:chloroform. The upper layer was transferred to a

new tube and received 700μL for chloroform and was centrifuged at 12,000rpm for 8 min. The upper layer was precipitated with 20μL of 3M NaOAc and 300μL of isopropanol. Tubes were centrifuged at 14,000rpm for 7 min. The upper layer was gently decanted and pellet was washed with 70% ethanol. Ethanol was decanted and tubes were allowed to air dry on paper towels. The RNA pellets were resuspended in 200μL of sterile water. RNA purification was conducted using the Geneclean II kit (Bio 101) and sequenced using method described by Nishiguchi et al. (2004).

The sequence of the fungal endophyte ribosomal RNA for ITS4 and ITS5 from *Oxytropis glabra* was compared using a pairwise alignment with the sequence of the fungal endophyte ribosomal RNA for ITS4 and ITS5 (*Embellisia* sp. L12, AY228650) from *Oxytropis sericea* (Braun et al. 2003). Pairwise alignment was used to determine regions of local similarity and the evolutionary relationship between the two sequences using Basic Local Alignment Search Tool (BLAST) program (National center of biotechnology information) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, April 2008). The BLAST program compared the fungal endophyte nucleotide sequence to a sequence database and calculated the statistical significance of best matches.

Results

Swainsonine concentration and endophyte infection in leaves of Oxytropis

Swainsonine was detected in 50% of *Oxytropis glabra* from all sites. The Alxa desert population had the lowest fungal endophyte infection per plant (1/15), followed by the northern sandy shrubland population (Eutoke Qi1), and the Wushen desert population (Wushen) (5/15) (Table 2). Greater fungal endophyte infections per plant were found in the desert steppe (Yjinhuolo) (10/15), the southern sandy shrubland (Eutoke Qi2) (11/15), and the salty low land (Linhe) (13/15) populations. Swainsonine concentration was significantly different among *O. glabra* populations ($F_{5,84} = 4.79$, P= 0.0007). Such significant differences are attributed to the Alxa desert population, which included only one plant with swainsonine >0.001%. Significant differences were found between the Wushen desert population and all treatments (t=2.5, P=0.035) (Table 2). When swainsonine concentration was compared without including samples with swainsonine concentration <0.001%, differences in swainsonine concentration among populations was less significant than the previous analysis ($F_{5,45}$= 5.52, P=0.043). Significant differences were also found between all populations and the Wushen population (t=2.17 P=0.0351) (Table 3). Swainsonine was also detected (0.11%) in samples of *Astragalus variabilis* pooled together (n=15), which corresponded to the Alxa population. Swainsonine was not detected in samples of *Oxytropis aciphylla* or *Oxytropis gracillima* from all populations.

Fungal endophyte comparison between O. glabra and O. sericea

Isolated fungal cultures from *Oxytropis glabra* populations (Figure 3A-3F) and *A. variabilis* (Figure 3A and 3B) had similar growth rates compared to fungal isolates from *Oxytropis sericea* (Figure 3G). Fungal morphology was slightly different between fungal cultures from the Wushen desert population (Figure 3C), and the Yijinhuolo desert steppe population (Figures 3E and 3F). However, conidial morphology was similar among all cultures and differences between melanized (dark) and hyaline (white) mycelia among cultures is common. The conidial morphology among isolated fungal endophyte from *O. glabra* and *A. variabilis* is similar to descriptions reported for *O. sericea* (Braun et al. 2003), *Astragalus adsurgens* (Li and Nan et al. 2007), and *O. kansuensis* (Wang et al. 2006).

Based on the rDNA ITS5 PCR amplification, all populations of *Oxytropis glabra* had samples with fungal endophyte (Figure 4). The highest infection was found in *O. glabra* from Sandy Shrublands populations (10/10) and the lowest was found in the Desert population (1/5) (Figure 5). To confirm if the fungal endophyte form *O. glabra* belonged to the genus *Embellisia* or *Alternaria*, amplified PCR from each sample that gave a positive hit or a band (Figure 4) was digested with enzyme *AVII*. The enzyme *AVII* digestions confirmed that all the fungal endophyte isolates from *O. glabra* populations collected in the Linhe salty low land, Eutoke Qi and 2 sandy shrublands, and the Wushen desert, are related to *Embellisia* sp (Figure 5). However, not all isolates from *O. glabra* collected at the Alxa desert (3/4) and the Yijinjuolo desert steppe (4/5) (Figure 3E) were all *Embellisia* (Figure 5).

The similarities observed among fungal endophyte cultures were confirmed when comparing the ITS5 sequences alignment between a fungal isolate from an *O. glabra* collected at Salty low land (Linhe) and the fungal isolate from *O. sericea* collected at River Raft, Utah, USA. Both sequences had 99% similarity and were different by only one base pair (Figure 5). When constructing a phylogenetic tree comparing the fungal isolate from *O. glabra* sequence to the 10 best matches from the NCBI database, using BLAST program, both fungal isolates cluster together, and are phylogenetically closer to *Alternaria* than other related genera from the Pleosporaceae family (Figure 6).

Assessment of dinitrogen fixing root nodules on Oxytropis

Nodules were collected from *Oxytropis glabra* and *Oxytropis gracillima* at all sites. Both species produce indeterminate nodules, and all collected nodules were actively fixing dinitrogen, which was assessed by the presence of leghemoglobin. Nodules were morphologically similar in both species and averaged 0.3cm to 0.8cm in length (Figure 8). Nodules in both species were found in secondary roots at 15 to 30cm below the surface. Active nodules where also observed on *Hedysarum mongolicum,* which was also found in association with *O. glabra* at the desert steppe and sandy shrubland sites. Nodules where observed on *Astragalus variabilis*, reported as a swainsonine producer (Zhao et al. 2006), which grows in association with *Oxytropis glabra* in the desert sites of Alxa. Nodules were not observed on *Oxytropis aciphylla.*

Discussion

The results from this study confirmed that swainsonine was present in *O. glabra* from all vegetation communities and *A. variabilis* from Alxa desert. Active root nodules were found on swainsonine producing *O. glabra, O. gracillima* and on *A. variabilis*. Fungal endophytes were isolated from all swainsonine producing *O. glabra* and *A. variabilis*. Therefore, nitrogen-fixing *Oxytropis* and *Astragalus* from Inner Mongolia, that produce swainsonine, are infected with endophytic fungi. Mutualisms between *Oxytropis* from Inner Mongolia and the United States are highly similar. Mutualisms in both continents are associated with plant toxicity.

The presence of swainsonine in *Oxytropis* and *Astragalus* from China confirms earlier findings on *Oxytropis* and *Astragalus* from the United States, which attribute the synthesis of swainsonine in these plants to a fungal endophyte related to the genus *Embellisia* (Braun et al. 2003 and Ralphs et al. 2008). Undetectable levels of swainsonine in *O. glabra* are related to lack of fungal endophytes. The presence and absence of a fungal endophyte, and the presence and absence of dinitrogen fixing root nodules could have important implications on the survival and the production of swainsonine in *Oxytropis* and *Astragalus*. Such implications have been studied in *O. sericea* and discussed in the first chapter of this dissertation.

The differences observed between swainsonine production and percentage of endophyte infection within and among populations of *Oxytropis* in this study could be explained by environmental conditions more so than genetic differences among populations. When comparing swainsonine concentrations among countries, swainsonine

concentrations in different species of *Oxytropis* are similar, even when growing in different plant communities and types of vegetation (Appendix A). Interestingly differences do occur among genera. For instance, the *Oxytropis* population at the Alxa desert is subject to dryer conditions and therefore greater drought stress than other populations of *Oxytropis*. Under similar dry conditions *Astragalus* (*Astragalus variabilis*) species from Alxa desert have greater swainsonine production compared to *Oxytropis* from Alxa desert. These results concur with studies comparing *Oxytropis* and *Astragalus* in the United States, where *Astragalus* species produce greater levels of swainsonine (Ralphs et al. 2008) possibly as a result of better compatibility between endophyte and plant. *Astragalus* species have greater diversity and display greater specificity in ecological adaptations and geographic speciation than *Oxytropis* (Kulshreshtha et al. 2004).

Swainsonine production and fungal endophyte in locoweeds, China vs. US

Another important aspect of *Oxytropis* and *Astragalus* is that both share similarities in fungal endophytes and alkaloids production, within and among continents. Although phylogenetic differences occur among genera, as a result of selective adaptation, symbiotic phylogenies are more similar among some species and different among others across genera. For example, *Oxytropis* are a monophyletic group, which branched from *Astragalus* more than 4 million years ago (Wojciechowski 2005). Both new world *Oxytropis* and *Astragalus* underwent selective diversification during the same period (~4million years ago, Wojciechowski 2005). However, *Oxytropis* rate of speciation (22 sp) was slower than *Astragalus* (354 sp) in North America (Welsh 2007), and in Eurasia

as well (300sp of *Oxytropis* vs. 2000sp of *Astragalus*, Wojciechowski et al. 1999). Although selective pressures have been acting on these genera since the cooling period of the late Tertiary and the Quaternary glacial Maxima, the fungal endophyte mutualistic associations were not drastically differentiated as the host plant population was. It appears that certain host plant species, with greater symbiotic compatibility, were more successful than other species among the genera. The similarity between fungal endophytes found in *Oxytropis* from both continents suggests that the fungal symbiosis was present before speciation occurred. Perhaps because the endophyte is vertically transmitted through seed, the symbiotic fungal populations in both continents did not undergo speciation.

In China 18 species of locoweeds distributed on 11,000,000 ha have been reported as harmful to livestock (Zhao et al. 2003). From those 18 species, 3 (*O. glacialis, O. kansuensis*, and *O. glabra*) are highly cosmopolitan and responsible for significant issues with livestock intoxication attributed to swainsonine. The range of *O. glacialis* extends across the alpine populations in the Tibetan Himalayan region and west of the Qinghai-Tibetan plateau. At lower elevations, *O kansuensis* extends across the eastern Qinghai-Tibetan plateau, merging into the Loess plateau, including the Sichuan basin and the Gansu province. Following the north east Arid and semi-arid regions of Inner Mongolia, *O. glabra* is the swainsonine producer. In North America, out of the 24 swainsonine producing locoweeds, *Oxytropis sericea* is by far the most cosmopolitan extending North-South (vs. East West in China) from Texas to South Western Canada. Furthermore, *O. sericea* hybridizes with *O. lambertii* and *O. campestris*, which extends its range of influence. In some areas the hybrids (spotted locoweeds) and O. lambertii are

infected with a similar endophyte and produce swainsonine (Ralphs et al. 2002, Braun et al. 2003, Ralphs et al. 2008).

Rhizobial symbiosis and distribution of swainsonine producing locoweed

It is also important to consider the role of rhizobial symbioses into the equation, since active root nodules were found in plants from each population of swainsonine producing *Oxytropis*. Swainsonine was detected and root nodules where present in O. glabra individuals growing in different vegetation communities. The presence of O. glabra in different ecosystems could be related to symbiotic promiscuity. For example, *O. glabra* can establish symbiotic associations with strains related to *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium* (Kan et al. 2007). Symbiotic promiscuity could in part explain the distribution of *O. glabra* across the Qinghai plateau and the Inner Mongolian Loess plateau. According to Laguerre et al. (1997), rhizobia isolates from the same geographic region can have different 16S rDNA types, and genetically similar rhizobia may be present in soils of different continents. *Oxytropis* from North America show a similar story but with different geographic distribution oriented North-South as a result of North-South mountain barriers. North American *Oxytropis* are nodulated by *Rhizobium* and *Mesorhizobium* which includes Alaska, western Canada and North West of the United States ecotypes (Prévost et al. 2003, Laguerre et al. 1997). Symbiotic promiscuity has allowed species such as *O. sericea* to increase its ecological niche by its ability to associate with strains found in Alaskan or Canadian soils (Valdez Barillas et al. 2007).

Astragalus species have greater symbiotic specificity with a group of strains that includes *Bradyrhizobium*, *Rhizobium* and *Mesorhizobium* (Laguerre et al. 1997). For

instance, some *Astragalus* species are capable of fixing dinitrogen under extreme environmental conditions in symbiosis with *Bradyrhizobium* (Bordeleau and Prévost 1994). Greater symbiotic specificity with strains adapted to extreme conditions could explain the smaller distribution of *A. variabilis* to only one population, but higher swainsonine content that O. sericea when growing in arid conditions.

Conclusion

Oxytropis species form the United States and China have both undergone diversification and speciation independently, however, mutualistic associations have been conserved. *Oxytropis sericea* in the United States have maintained a symbiosis that perhaps came from a common ancestor with *Oxytropis* from China. Such highly conserved symbiosis with fungal endophytes is probably maintained through vertical transmission. Fungal endophyte in *Oxytropis* from both continents are highly similar and synthesize the same indolizidine alkaloid swainsonine. Dinitrogen fixing *Oxytropis* are capable of colonizing areas disturbed by heavy grazing or agriculture, and high symbiotic promiscuity could explain the ability in these genera to increase its ecological niche. *Oxytropis* and *Astragalus* in both continents share similar mutualisms, however, *Astragalus* share a similar fungal endophyte with Oxytropis. *Astragalus* species in both continents are capable of producing greater swainsonine levels than *Oxytropis* when growing in similar plant communities and climate conditions.

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Tables and Figures

Table 1. Vegetation type, name and location of each study site

Vegetation type	Site	Jurisdiction	Coordinates	Elevation (m)
Desert	Alxa Zuo	Alxa league	40° 9' 17"N 104° 40' 54"E	1335
Sandy Shrubland	Eutoke Qi1	Ordos prefecture-level city	38° 23' 58"N 107° 40' 18"E	1351
Sandy Shrubland	Eutoke Qi2	Ordos prefecture-level city	38° 8' 15"N 107° 37' 16"E	1327
Salty low land	Linhe	Bayanur league	40° 48' 36"N 107° 18' 19"E	1028
Desert	Sand management Wushen	Ordos prefecture-level city	38° 58' 55"N 109° 7' 32"E	1311
Desert Steppe	SW of Chen ling Yijinholo	Ordos prefecture-level city	39° 19' 55"N 109° 48' 7"E	1369

Table 2. Mean swainsonine concentrations in *O. glabra* at each study site

Vegetation type	Site	N	N of plants infected	Swainsonine (% dry weight)	Swainsonine (%) Max - Min
Desert	Alxa Zuo	15	1	0.0008 (0.0008)	0.012 0
Sandy Shrubland	Eutoke Qi1	15	5	0.037^{a} (0.007)	0.098 0
Sandy Shrubland	Eutoke Qi2	15	11	0.033^{a} (0.006)	0.073 0
Salty low land	Linhe	15	13	0.048^{a} (0.006)	0.071 0
Desert	Wushen	15	5	0.026^{b} (0.012)	0.159 0
Desert Steppe	Yijinholo	15	10	0.053^{a} (0.006)	0.074 0

Note: Plants with undetectable levels of swainsonine (<0.001%) were included in this table. Values in swainsonine column are means. Parentheses indicate standard error of the mean. Max= refers to maximum swainsonine value. Min= refers to minimum swainsonine value. N= refers to total number of plants analyzed. Superscript letters indicate significant difference using an orthogonal linear contrast estimate ($P < 0.05$).

Table 3. Swainsonine concentration in Oxytropis glabra at each study site

Vegetation type	Site	N	N of plants infected	Swainsonine (% dry weight)	Swainsonine (%) Max - Min
Desert	Alxa	15	1	0.012	n/a n/a
Sandy Shrubland	Eutoke Qi1	15	5	0.051[a] (0.021)	0.098 0.015
Sandy Shrubland	Eutoke Qi2	15	11	0.045[a] (0.003)	0.073 0.033
Salty low land	Linhe	15	13	0.056[a] (0.003)	0.071 0.031
Desert	Wushen	15	5	0.078[b] (0.021)	0.159 0.044
Desert Steppe	Yijinholo	15	10	0.053[a] (0.006)	0.074 0.006

Note: Plants with undetectable levels of swainsonine (<0.001%) were not included in this table. Values in swainsonine column are means. Parentheses indicate standard error of the mean. Max= refers to maximum swainsonine value. Min= refers to minimum. Superscript letters indicate significant difference using an orthogonal linear contrast estimate ($P < 0.05$).

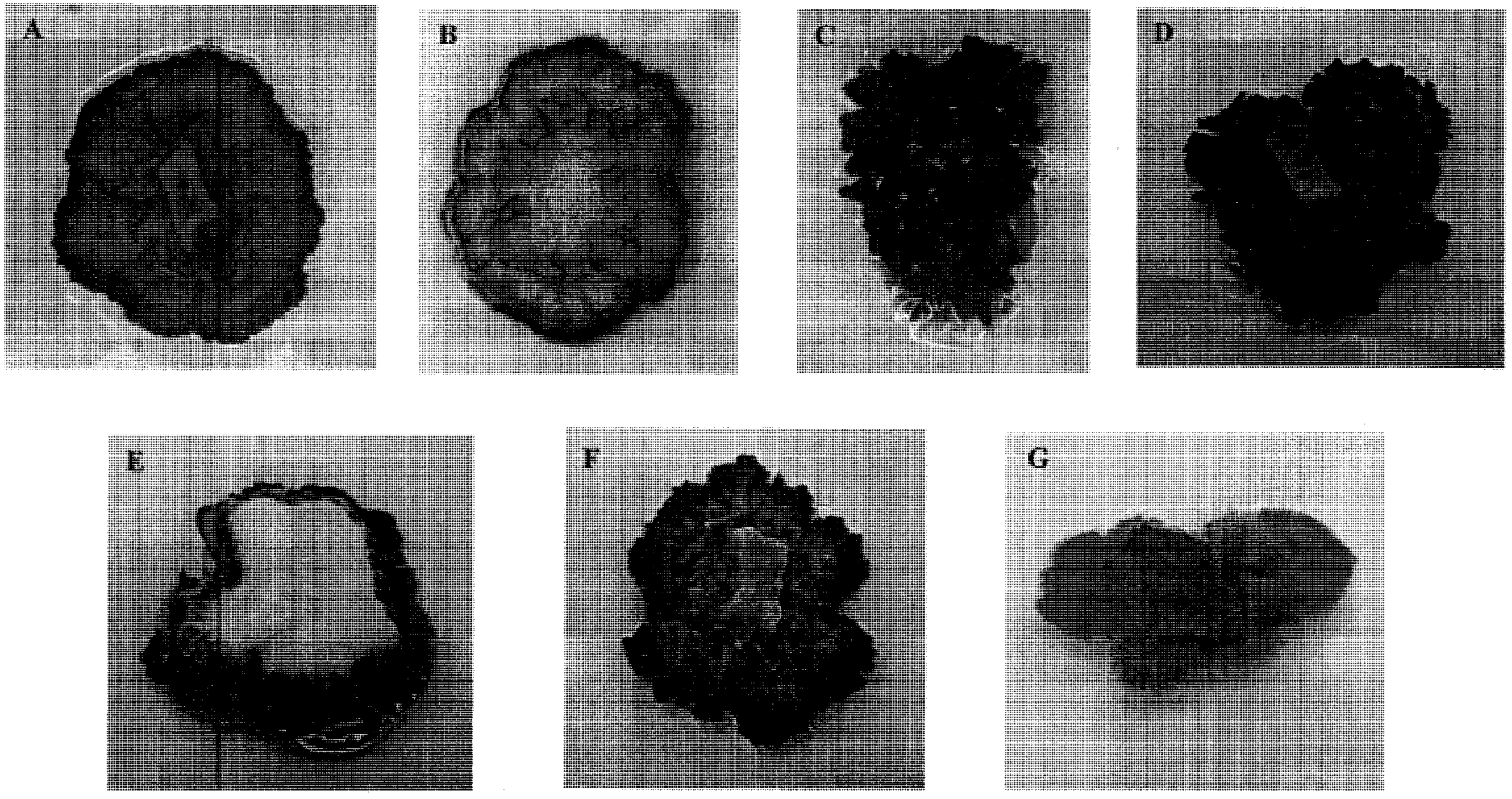

Figure 1. Photographs of fungal endophyte cultures isolated from different locoweeds. **A. and B.** Fungal cultures isolated from *Astragalus variabilis* (desert in Alxa). **C.** Fungal culture isolated from *Oxytropis glabra* (desert in Wushen). **D.** Fungal culture isolated from *Oxytropis glabra* (Salty low irrigated land in Linhe). **E and F** Fungal culture isolated from *Oxytropis glabra* (desert steppe Yijinhuolo).**G.** Fungal culture isolated from *Oxytropis sericea* (Raft River Mountains, Utah, USA).

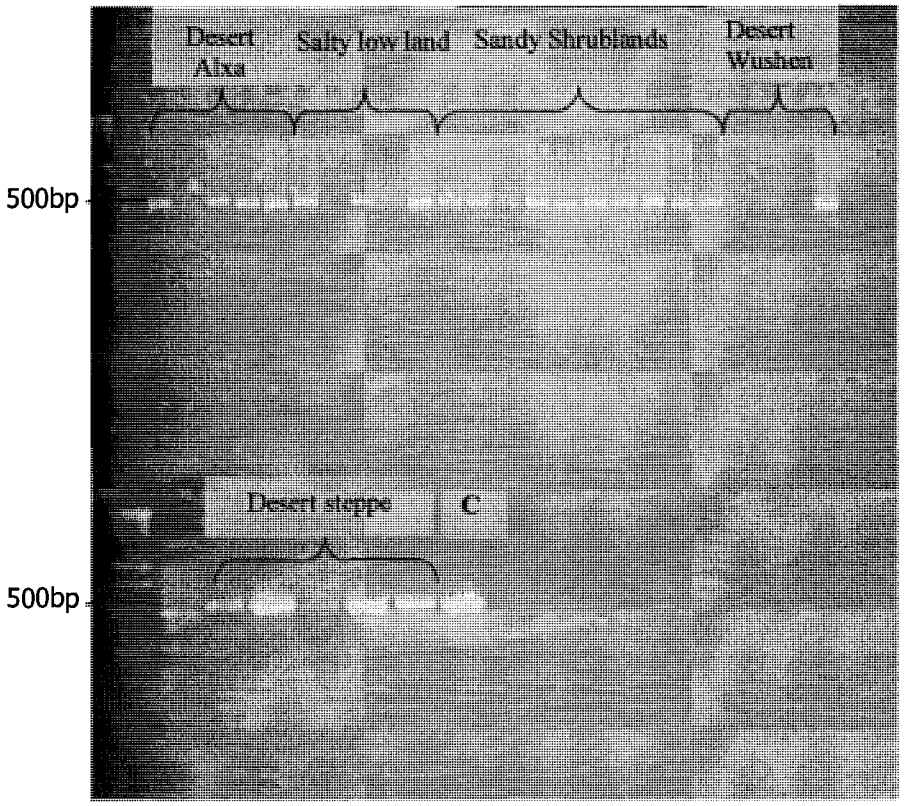

Figure 2 PCR amplification of fungal endophyte rDNA ITS5 in *Oxytropis glabra* collected from six sites in four vegetation types. Bands correspond to 500bp. C = refers to DNA ITS5 primers control.

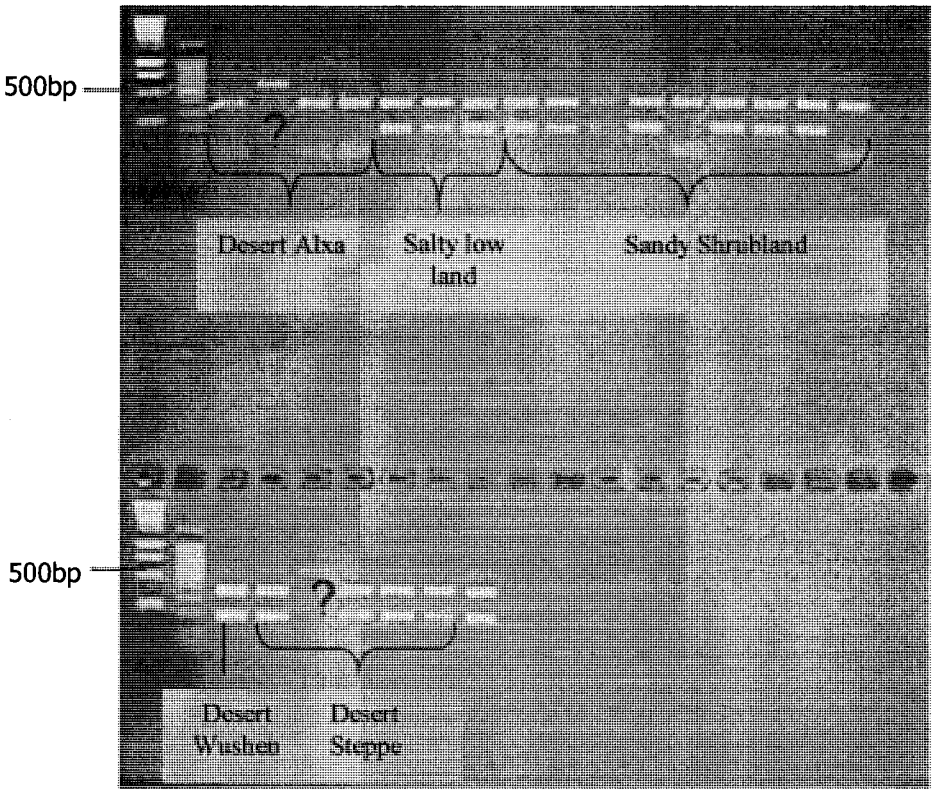

Figure 3 PCR amplification of fungal endophyte rDNA ITS5 digested with enzyme AVII. Upper band correspond to 200bp and lower band correspond to 380bp. Doubles bands indicate that fungal endophyte is related to *Embellisia* sp. Question marks indicate possible *Alternaria* sp.

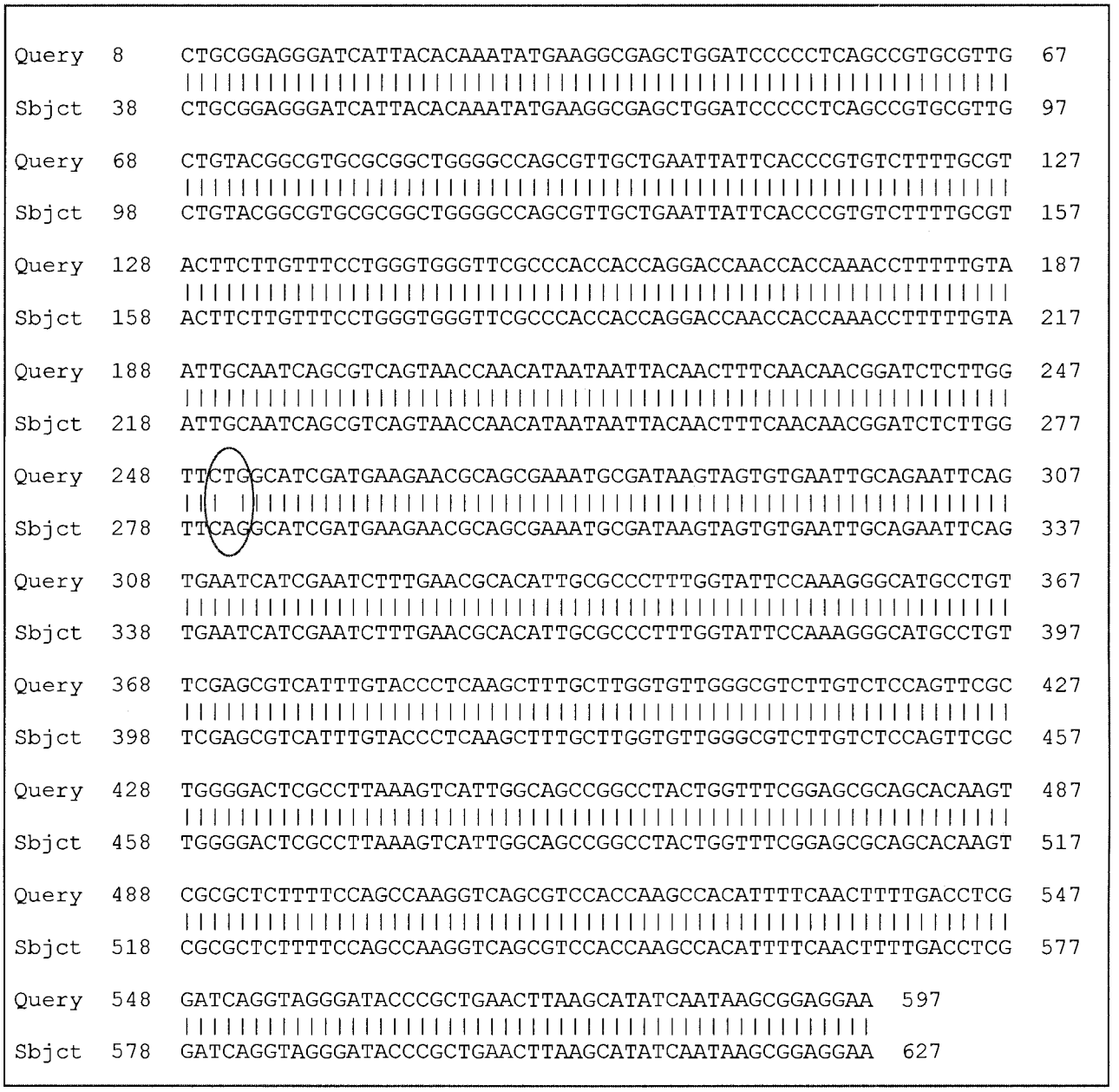

Figure 4. Ribosomal RNA ITS5 sequence alignments. Query, corresponds to Embellisia sp. L12 sequence (NCBI accession #=AY228650) from *Oxytropis Sericea* collected at salty low irritation land (Linhe, Inner Mongolia, China).Sbjct, corresponds to fungal endophyte from *Oxytropis glabra.* Sequences have 99% (589/590bp) similarity and differ by one base pair (base pair= 252). Oval encircles the mismatch base pair.

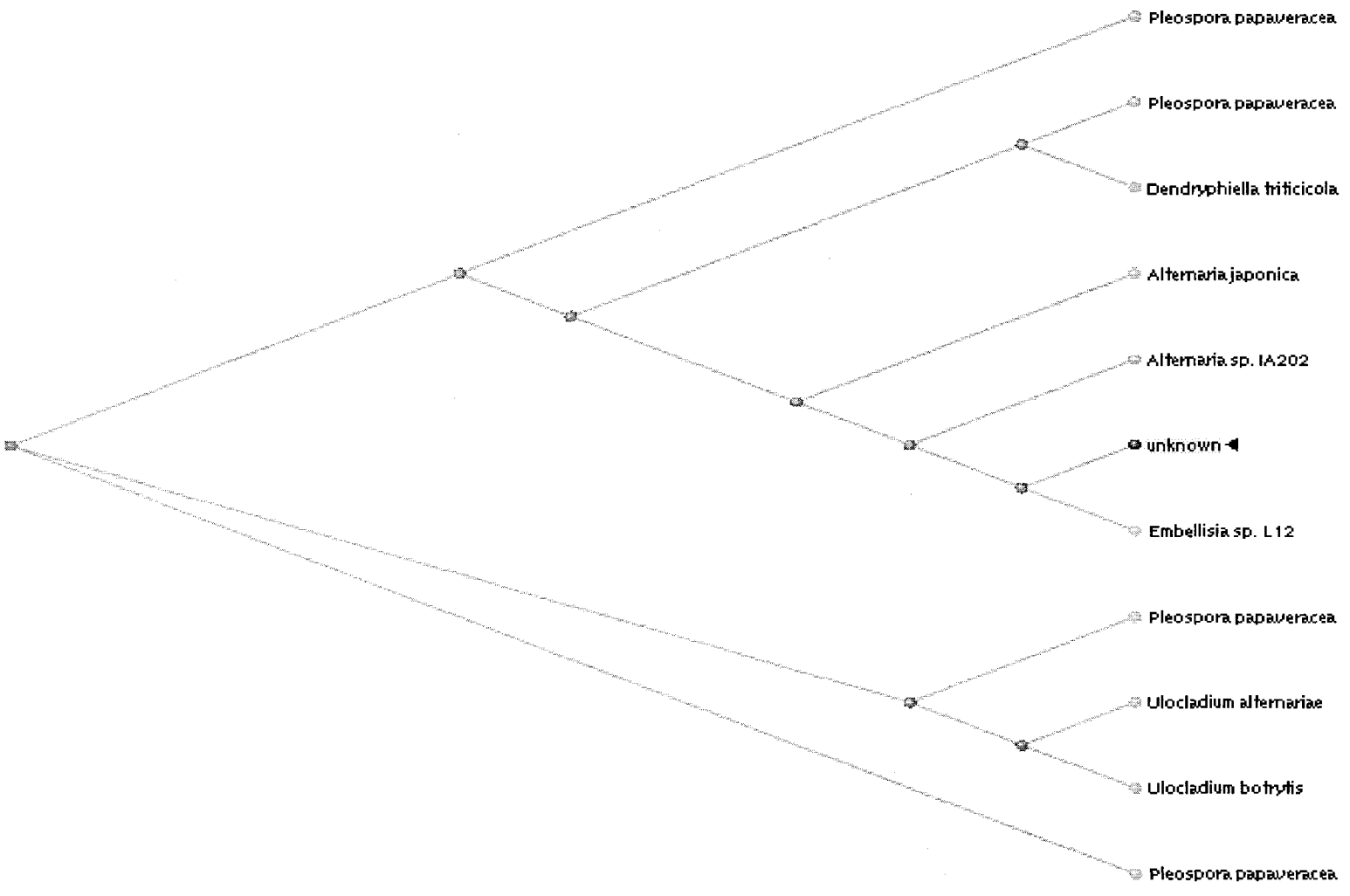

Figure 5. Neighbor joining phylogenetic tree based on Juke Cantor Method using pair wise comparison values between unknown fungal endophyte from *Oxytropis glabra* and the 10 closets DNA sequences. Nodes correspond to evolutionary distances. Unknown label corresponds to fungal endophyte form *Oxytropis glabra.* Tree was generated using BLAST program from NCBI Genetic data base (2008).

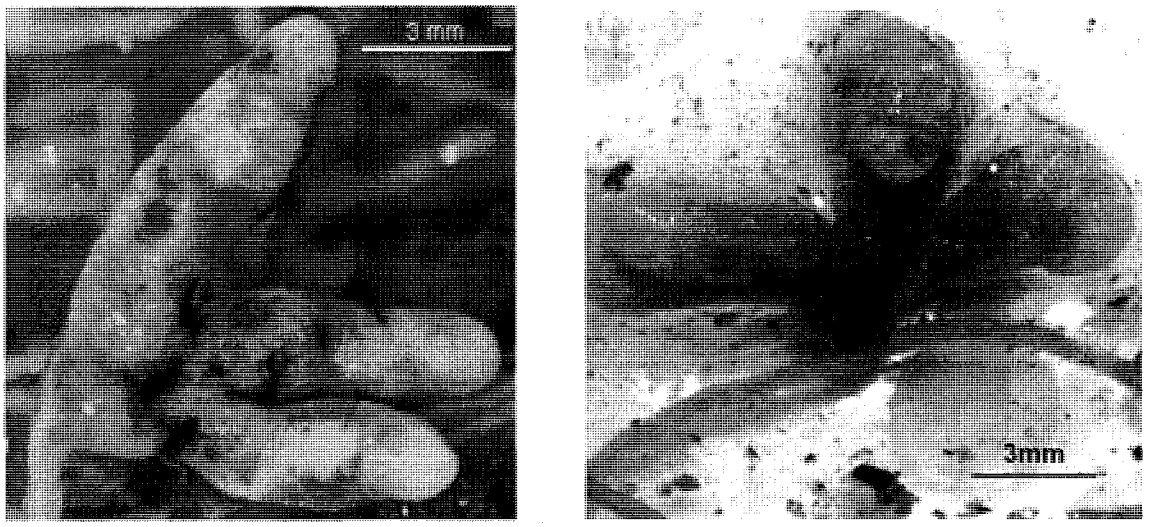

Figure 6. Photographs of dinitrogen fixing indeterminate root nodules from *Oxytropis*. Left photograph corresponds to nodule from *Oxytropis sericea* inoculated with *Rhizobium* strain 118B2. Right photograph corresponds to nodule from *Oxytropis glabra* collected in Inner Mongolia, China.

Appendix A. Swainsonine concentration in locoweed populations from the United States and China

North America (U.S.)			Inner Mongolia (China)		
Site/ elevation	**Host plant/ swainsonine%**	**Plant community**	**Site/ Elevation**	**Host plant/ swainsonine%**	**Vegetation type**
Des Moines, NM 2200m	*Oxytropis sericea* 0.03 - 0.21	Southern Mixed Prairie Blue grama-little bluestem	Linhe, Bayanur 1028m	Oxytropis glabra 0.031 - 0.071	Salty low irrigated land
Virginia Dale, CO 2000m	*Oxytropis sericea* 0 - 0.18	Mixed grass prairie	Yjinjuolo, Ordos 1369m	Oxytropis glabra 0 - 0.074	Desert Steppe
Raft River, UT 3000m	*Oxytropis sericea* 0 - 0.12	Alpine sage brush	Eutoke Qi, Ordos 1330m	Oxytropis glabra 0033 - 0.073	Sandy shrubland
Kanab, UT 1421m	*Oxytropis lambertii* 0 - 047	Desert shrub	Wushen, Ordos 1311m	Oxytropis glabra 0 - 0.159	Desert
Flagstaff, AZ ~2100m	*Oxytropis lambertii* 0.022-0.106	Pinyon/ juniper woodland	Alxa Zuo 1335m	Oxytropis glabra 0 - 0.012	Desert

Apendix B. Productivity and composition of vegetation community at each *Oxytropis glabra* collection site. Vegetation measurements and species composition were provided by Dr. Zhao Mengli from Agricultural University of Inner Mongolia in Hohot, China.

Vegetation Type	Populations name	O. glabra cover (%)	O. glabra frequency (%)	O. glabra biomass (g/m^2)	Forbs biomass (g/m^2)	Grasses biomass (g/m^2)	Weeds biomass g/m^2)	Number of species	Total vegetation cover (%)	Representative species
Desert Steppe	SW Cheng ling Yijinholo	25	27	111.25	97.5	245.08	158.68	18	137	*Carex duriuscula* *Puccinellia distans* *Artemisia ordosica* *Ixeris denticulate* *Leymus secalinus*
Desert Sand management center	MU US sand management WuShen banner	33	33	121.86	59.88	230	163.09	24	72	*Hedysarum mongolicum* *Leymus secalinus* *Glaux maritima*
Sandy shrubland	Eutoke Qian banner	82	84	1242.36	94.04	952.74	92	9	98	*Achnatherum splendens* *Leymus secalinus* *Glaux maritima*
Sandy shrubland	Eutoke Qian banner	61	63	510.68	80.36	577.68	275.03	10	80	*Phragmites dustralis* *Leymus secalinus* *Suaeda glauca*
Desert	Ba Yan Nao Er in Alxa Zuo banner			495.22	17.25	595.36	380.13	11	110	*Cenchrus calyculatus* *Carex duriuscula*
Salty low land	LinHe			147.7	246.06	366.18	155.24	15	86	*Leymus secalinus* *Glaux maritima*