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Potential role for saccharopine reductase in swainsonine metabolism in endophytic fungus, *Undifilum oxytropis*

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ABSTRACT

Locoweed plants in the southwestern United States often harbour a slow-growing endophytic fungus, *Undifilum oxytropis* (Phylum: *Ascomycota*; Order: *Pleosporales*), which produces a toxic alkaloid, swainsonine. Consumption of *U. oxytropis* by grazing animals induces a neurological disorder called locoism for which the toxic alkaloid swainsonine has been reported to be the causal agent. Little is known about the biosynthetic pathway of swainsonine in endophytic fungi, but previous studies on non-endophytic ascomycetous fungi indicate that pipecolic acid and saccharopine are key intermediates. We have used degenerate primers, Rapid amplification of cDNA ends (RACE)-PCR and inverse PCR to identify the gene sequence of *U. oxytropis* saccharopine reductase. To investigate the role of this gene product in swainsonine metabolism, we have developed a gene deletion system for this slow-growing endophyte based on our recently established transformation protocol. A strain of *U. oxytropis* lacking saccharopine reductase had decreased levels of saccharopine and lysine along with increased accumulation of pipecolic acid and swainsonine. Thus, saccharopine reductase influences the accumulation of swainsonine and its precursor, pipecolic acid, in *U. oxytropis*.

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Introduction

Locoweeds (*Astragalus sp.* and *Oxytropis sericea*) are perennial flowering plants found frequently in the rangelands of the western United States, Asia, and South America (Kingsbury 1964; Molyneux & James 1982; James & Nielson 1988; Cook *et al.* 2009). Consumption of locoweeds by cattle, sheep, and horses induces a neurological condition termed locoism (James & Panter 1989). The etiological agent of locoism, swainsonine (1, 2, 8-trihydroxyindolizidine), is produced by fungal endophytes that reside within the locoweeds (Braun *et al.* 2003). The common endophyte of the *O. sericea* Nutt. Locoweed was recently classified as *Undifilum oxytropis*, belonging to the phylum *Ascomycota* and order *Pleosporales* (Cook *et al.* 2009; Graham *et al.* 2009; Pryor *et al.* 2009).

Undifilum oxytropis can be isolated from stems, seeds, and leaves of locoweed plants (Ralphs *et al.* 2002; Braun *et al.* 2003). The fungus is transmitted from one generation to the next through the seed coat (James & Panter 1989) (Kingsbury 1964). When *U. oxytropis* grown in pure culture was fed to rats symptoms of locoism were induced (McLain-Romero *et al.* 2004).

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Swainsonine, an alkaloid, is known to be produced by three groups of fungi, *U. oxytropis* (Braun *et al.* 2003; Cook *et al.* 2009; Pryor *et al.* 2009; Mukherjee *et al.* 2010), *Rhizoctonia leguminicola* (Smalley *et al.* 1962), and *Metarhizium anisoplae* (Sim & Perry 1997). A water-soluble trihydroxyindolizidine compound, swainsonine was first isolated from the Australian legume *Swainsona canescens* (Colgate *et al.* 1979). It inhibits lysosomal α-mannosidase, which is involved in the catabolism of glycoproteins (Harris *et al.* 1988) and golgi α-mannosidase II (Broquist 1985) and this activity has been shown to be the cause of locoism in cattle (Sim & Perry 1997). Swainsonine also has potential therapeutic benefits. It can inhibit growth and movement of tumour cells (Hino *et al.* 1985) and can prevent the processing and expression of cell surface complex oligosaccharides in tumour cells (Tulsiani *et al.* 1990).

The biochemical pathway for swainsonine production has been partially characterized in *R. leguminicola* and *M. anisopliae* (Wickwire *et al.* 1990; Sim & Perry 1997; Naranjo *et al.* 2004). In another ascomycete, *Penicillium chrysogenum*, which does not produce swainsonine, saccharopine oxidase acts on saccharopine to produce 1-piperideine6-carboxylic acid (P6C) that in turn, leads to the formation of L-pipecolic acid in presence of pipecolate oxidase (Naranjo *et al.* 2004). Saccharopine reductase (EC number 1.5.1.10) converts P6C to form saccharopine, thereby impacting the accumulation of saccharopine and L-lysine in the cell (Naranjo *et al.* 2004). In *R. leguminicola*, pipecolic acid is formed by the catabolism of L-lysine that can lead to the synthesis of the alkaloids slaframine and swainsonine (Wickwire *et al.* 1990).

Naranjo *et al.* reported that the disruption of the *lys7* gene, which encodes for saccharopine reductase in *P. chrysogenum*, results in the accumulation of large amounts of pipecolic acid (Naranjo *et al.* 2004). Pipecolic acid is known to ultimately impact swainsonine production. Thus, saccharopine reductase, although involved in formation of saccharopine, may play a role in the metabolism of swainsonine and lysine, two major end products of the lysine metabolic pathway in the alkaloid producing fungus *U. oxytropis*. However, little is known about the biosynthetic pathway of swainsonine produced by *U. oxytropis* despite its potential importance.

The aim of this study was two-fold; first to identify the gene sequence of *U. oxytropis* saccharopine reductase and second to disrupt the function of saccharopine reductase using our established transformation system (Mukherjee *et al.* 2010) and evaluate the resulting levels of biochemical products of the pathways related to swainsonine metabolism. We report the identification of the saccharopine reductase gene sequence from *U. oxytropis* and that disruption of the gene led to high accumulation of P6C, swainsonine and pipecolic acid, along with a decrease in the levels of saccharopine and lysine suggesting the involvement of saccharopine reductase in the swainsonine and lysine metabolic pathways.

Materials and methods

Strains, media, and culture condition

Undifilum oxytropis was cultured from leaves of *Oxytropis sericea* (white locoweed), which was collected from Green River, WY, USA (hereafter referred to as isolate 25-1 of *U. oxytropis*). Intact plant samples were pressed and dried for subsequent isolation and culturing of the endophyte. The tissues were surface sterilized for 30 s in 70 % ethanol, followed by 3 min in 20 % bleach, and then 30 s in sterile water. Tissues were dried on sterile paper towels and plated on water agar media. Plates were stored at room temperature (25 °C) for future use. Fungal hyphae were transferred to potato dextrose agar (PDA) plates and grown at room temperature for at least 14 d. Hyphae from the recovered endophytes were transferred onto PDA plates and maintained at 18 °C (Ralphs *et al.* 2008; Mukherjee *et al.* 2010). The 25-1 isolate described above has been preserved as desiccated mycelia and stored at both 4 °C and −80 °C. The pressed *Oxytropis sericea* 25-1 specimen was stored at room temperature at the New Mexico State University-Center for Natural History Collections (NMSU-CNHC).

Nucleic acid isolation from Undifilum oxytropis

Fungal genomic DNA was extracted using the DNeasy Plant Mini Kit following the manufacturer's protocol (Qiagen, Valencia, CA, USA). *Undifilum oxytropis* DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and analyzed on a 1 % agarose gel. The purified DNA was used for PCR, cloning, and sequencing as described below.

Degenerate PCR

Saccharopine reductase coding sequences from *Magnaporthe grisea* (nts 1350-2303, accession number: AF144424) and *Penicillium chrysogenum* (nts 395-1348, accession number: XM_002564566) were aligned and degenerate primers sacred_seq-F and sacred_seq-R (Table 1) were designed from the alignment. *Undifilum oxytropis* DNA was amplified using PCR and the degenerate primers. PCR was performed using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). The resulting PCR product was cloned in pGEMTeasy cloning vector (Promega, Madison, WI, USA) and sequenced using Li-Cor 4200 Global IR 2 system at the New Mexico State University Molecular Biology Sequencing Facility.

Rapid amplification of cDNA ends (RACE)-PCR

RACE-PCR was used to obtain additional 3′ sequence of saccharopine reductase. One μg of total RNA was extracted using the Plant RNeasy kit (Qiagen, Valencia, CA, USA). The isolated RNA was treated according to manufacturer's instructions of the First Choice RLM-RACE-PCR kit (Ambion, Austin, TX, USA) and processed to amplify the cDNA ends. The amplified product was cloned into a pGEMTeasy cloning vector (Promega, Madison, WI, USA) and the product was sequenced using universal M13 forward and reverse primers using the Li-Cor system as described above.

Inverse PCR

Inverse PCR was used to obtain sequence of the 5′ end of saccharopine reductase. Five μg of *Undifilum oxytropis* DNA was digested with 10 units of *Sal*I (Promega, Madison, WI, USA).

Table 1 – Primers used in this study to amplify saccharopine reductase and screen for disruption mutants.

Name	Sequence (5′–3′)	Purpose
Sacred_seq-F	GTYAACGACGAYGCCGCCCTCGACG	Saccharopine reductase sequencing
Sacred_seq-R	CCGTCCTTGTTCTCGATCTCGAAC	
5′RACE_sacred_seq-F	TCGAGGGCGGCGTCGTTCGT	Saccharopine reductase sequencing
5′RACE_sacred_seq-R	CGATCTCGTTCATGACGGTGATGC	
3′RACE_sacred_seq-F	TAGGTTCGAGATCGAGAACAAGGACGA	Saccharopine reductase sequencing
3′RACE_sacred_seq-R	CAAGTGCCTCGTCGACATTGGTTTCCT	
3′UTR_seq-R	TCACAAGACAACCACCTTACAAAG	
Inverse-PCR_sacred-F	TGGGTTCAGGCTTCGTGACCA	Saccharopine reductase sequencing
Inverse-PCR_sacred-R	CCGTATTTCTGCTAGAGCTCCTTC	
Ppd-EGP_BsaAI-F	ATATACGTGTCGTCCTTGTTCTCGATCTCGAA	Cloning of saccharopine reductase
Ppd-EGP_BsaAI-R	TAAAACGTCATACCCCAACCGTGACTCCACA	into the vector Ppd-EGFP
Ppd-EGP_SgrAI-F	ATTGAGGCCACGCGACAAAATGCGTAACCAGG	
Ppd-EGP_SapI-R	AATGCTCTTCATCAACGACGACGCCGCCCTC	
Sacred_mutant_setA-F	AAGCGGCTGACGTTTTCATTTAGT	Set A primers for mutant screening
HygB_mutant_seta-R	CAATCGCGCATATGAAATCACGCC	
Sacred_mutant_setB-F	TCCACGGCACCGTTATCAAGTCGCAATCCG	Set B primers for mutant screening
HygB_mutant_setB-R	GCGGTGAGTTCAGGCTTTTTCATATCGATG	

Digested DNA was separated on a 1 % agarose gel, excised from the gel and purified using an agarose gel DNA extraction kit (Roche Applied Science, Indianapolis, IN, USA). The digested DNA (0.1 μg) was diluted to a concentration of 0.5 μg mL^{-1} with ligation buffer (50 mM Tris HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM adenosine triphosphate and 10 μg mL^{-1} gelatin). The ligation was carried out using T4 DNA ligase (Fermentas, Glen Burnie, MD, USA) for 16 h at 4 °C. PCR primers Inverse-PCR_sacred-F and Inverse-PCR_sacred-R were used for amplification (Table 1) of 0.1 μg circularized DNA. The temperature profile for PCR was 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 5 min (Ochman *et al.* 1988). The each PCR product was gel-purified using a PCR purification kit (Roche Applied Science, Indianapolis, IN, USA) and sequenced at the NMSU Molecular Biology Sequencing Facility.

Southern blotting

Genomic DNA (5 μg) was purified from *Undifilum oxytropis* cultures grown in PDA medium and digested with EcoRI and PstI. Digested DNA was separated on a 0.8 % agarose gel, blotted onto positively charged Hybond nylon membrane (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) using the alkaline-transfer method (Sambrook & Russell 2001) and hybridized to a 1 kb digoxigenin (DIG)-labelled probe corresponding to 320-1320 nts of the *U. oxytropis* saccharopine reductase gene. DNA probe preparation, hybridization, washing, and visualization of the blots were performed in accordance with manufacturer's instructions (Roche Applied Science, Indianapolis, IN, USA).

Cloning and gene disruption

Transformation of *Undifilum oxytropis* was performed using a construct derived from the pPd-enhanced green fluorescent protein (EGFP) vector (Suzuki *et al.* 2000) previously used in this fungus (Mukherjee *et al.* 2010). pPd-EGFP contains the Hygromycin B phosphotransferase gene driven by the *Aspergillus nidulans* trpC-promoter. A 723 bp fragment of *U. oxytropis* saccharopine reductase gene (nts 350-1073) was PCR-amplified using forward and reverse primers (Ppd-EGP_SgrAI-F and Ppd-EGP_SapI-R) (Table 1) carrying *Sap*I and *Sgr*AI sites (for cloning into the downstream end of the HygB cassette) in their 5′ ends. A 719 bp long fragment of the *U. oxytropis* saccharopine reductase gene (nts 1130-1843) was PCR-amplified using forward and reverse primers (Ppd-EGP_BsaAI-F and Ppd-EGP_BsaAI-R) (Table 1) carrying *Bsa*AI (for cloning into the upstream end of the HygB cassette) sites in their 5′ ends. *Sap*I, *Sgr*AI, and *Bsa*AI restriction enzymes were chosen based on presence of restriction sites in the HygB resistance cassette (nucleotide position, downstream 7093 bp and 6698 bp for *Sap*I and *Sgr*AI, upstream 3153 bp for BsaAI, respectively). The PCR-amplified fragments from the saccharopine reductase gene and pPd-EGFP were digested with the specific restriction enzymes and ligated resulting in a manipulated vector with saccharopine reductase genes on both sides of the HygB cassette. This construct, Ppd-EGFP-sacred, was digested by *Alw*NI (nt position 7625 of the Ppd-EGFP) and *Bst*XI (nt position 2920 of the Ppd-EGFP) to create a linearized construct (linear fragment) and used to transform *U. oxytropis*.

Protoplast isolation and fungal transformation

Flasks containing 100 mL of potato dextrose broth (PDB, Difco Laboratories Franklin lakes, NJ, USA) were inoculated with *Undifilum oxytropis* and protoplasts were generated according to methods described previously (Mukherjee *et al.* 2010). The screening concentration of HygB used was 20 μg mL^{-1}, derived from the sensitivity of *U. oxytropis* described previously (Mukherjee *et al.* 2010). Transformants were subsequently maintained on 20 μg mL^{-1} HygB. The transformation plates were incubated for 4 d at room temperature to observe hyphal growth. Fungal cultures produced after the regeneration step were then transferred to fresh PDA-HygB containing plates. Subsequent transfer of fungal cultures was performed after 3 weeks of growth. *Undifilum oxytropis* protoplasts were regenerated on PDA plates without HygB to verify viability. Once established, stable transformants were grown on HygB-free PDA plates.

Screening for saccharopine reductase disruption mutants with PCR

Wild type and mutants (transformants) were screened for homologous recombination by PCR followed by Southern blotting. Wild type and mutant *Undifilum oxytropis* cultures were grown on PDA and DNA was isolated using a DNeasy Plant Mini Kit following the manufacturer's protocol (Qiagen, Valencia, CA, USA). PCR screening was performed using two sets of primers encompassing the cloning junctions of Ppd-EGFP-sacred. Set A forward primer (Sacred_mutant_setA-F) for screening encompassed nts 300-326 of the deciphered *U. oxytropis* saccharopine reductase gene while the reverse primer (HygB_mutant_setA-R) ended at nts 5343-5369 of the HygB cassette. Set B forward primers (Sacred_mutant_setB-F) for screening encompassed nts 4904-4930 of the HygB cassette, while the reverse primer (HygB_mutant_setB-R) encompassed nts 1874-1900 of the deciphered *U. oxytropis* saccharopine reductase gene. The expected sizes of PCR products for the *U. oxytropis* disruption mutant were approximately 2017 bp for both set A and set B. These primer sets were not expected to amplify a product from wild type *U. oxytropis*. The sequence of the amplified segment was verified from data collected at the NMSU Molecular Biology Sequencing Facility.

Screening for saccharopine reductase disruption mutants with Southern blotting

Southern hybridization was performed essentially as described above. Specifically, *Undifilum oxytropis* DNA from wild type and the disrupted colonies were digested with *Pci*I and *Pvu*II, size fractionated by electrophoresis through 0.8 % agarose, and probed with two different probes to screen for transformants. The first probe was a 1 kb saccharopine reductase-specific positive sense DIG-labelled probe corresponding to 320-1320 nts of the *U. oxytropis* saccharopine reductase gene, while the second HygB specific 1 kb probe encompasses nts 4587-5587 of the HgyB cassette. Southern hybridization was performed according to manufacturer's instructions (Roche Applied Science, Indianapolis, IN, USA).

Analysis of lysine, saccharopine, P6C, pipecolic acid, swainsonine, and α-aminoadipic acid by LC–MS (Liquid Chromatography-Mass Spectrometry)

Fungal suspension cultures of the wild type and the disruption mutant of *Undifilum oxytropis* were grown in PDA for 2 weeks at room temperature from 10 mg starting material. The fungal mass was filtered and extraction of swainsonine was performed from mycelium as described previously (Mukherjee *et al.* 2010). Dehydrated samples were then hydrated in 100 μL of water and stored at 4 °C for later analysis.

To detect compounds other than swainsonine, fungal mycelia were ground in liquid nitrogen and dehydrated samples were dissolved in 2 mL of 60 % ethanol prior to injection to the LC–MS system. The samples were incubated to evaporate ethanol, 1 mL of ultra pure water was added to the dried samples, mixed properly, and stored at 4 °C for later analysis (Moulin *et al.* 2002). The LC–MS system consisted of an HP1100 binary solvent pump, autosampler, a Betasil C18 reversed phase High Performance Liquid Chromatography column and a Micromass, Q-T of Micromass spectrometer (Acquity LC system, Waters Corporation, Milford, MA, USA). Samples were resolved through a mobile phase of 50 mM sodium acetate buffer (pH 4.2) and acetonitrile. The flow rate was 1 mL min^{-1}.

Results

Identification of saccharopine reductase gene sequence

Saccharopine reductase was identified as the enzyme of choice because no other gene sequence was available in the database from taxonomically related species that could be used to design the degenerate primers required to identify the gene sequence that encodes the enzyme. Identification of the gene sequence encoding saccharopine reductase from the endophyte *Undifilum oxytropis* was accomplished using three approaches. First, a 953 bp portion of the *U. oxytropis* saccharopine reductase genomic DNA sequence was identified using degenerate primer-based PCR. BLASTn and BLASTx comparisons revealed >90 % similarity between the deciphered *U. oxytropis* saccharopine reductase gene sequence and that of *Magnaporthe grisea* and *Penicillium chrysogenum*. Next, primers were designed from the previously deciphered genomic DNA sequence, which was used to perform RACE-PCR. The resulting cDNA sequence obtained was used to identify the stop codon, poly-A tail and 3′ untranslated region (UTR) of the *U. oxytropis* saccharopine reductase transcript (Fig 1). The length of the 3′ UTR was 124 bp. Finally; primers were designed for inverse PCR from the obtained sequences to identify the translation start codon including the 5′ UTR of the gene. The inverse PCR products revealed the 5′ segment of *U. oxytropis* saccharopine reductase including the start codon. The sequences were aligned using BLASTn and BLASTx, revealing >90 % similarity between the obtained *U. oxytropis* saccharopine reductase gene sequence and those from *M. grisea* and *P. chrysogenum*. The obtained DNA sequence of *U. oxytropis* saccharopine reductase was 1624 bp including the non-coding sequence (Fig 1). The submitted GenBank accession number for the saccharopine reductase sequence is HQ010362. PCR products of different isolates of *U. oxytropis* using same primers showed similar results (data not shown). Therefore, the sequence of saccharopine reductase identified in *U. oxytropis* is applicable to this species in general and is not strain specific.

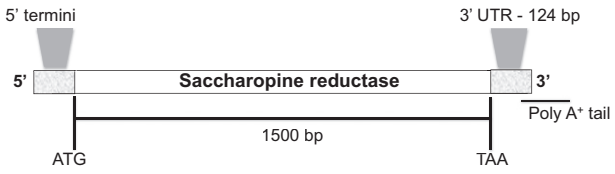

Fig 1 – Schematic diagram of the organization of the deciphered saccharopine reductase gene sequence. The open reading frame (ORF) including 3′ UTR is 1624 bp along with the poly-A tail in *Undifilum oxytropis*.

Copies of Undifilum oxytropis *saccharopine reductase*

Southern blot analysis was performed on wild type *U. oxytropis* genomic DNA to evaluate the number of copies of saccharopine reductase present. For this purpose, digested *U. oxytropis* genomic DNA was probed with an *U. oxytropis* specific probe. The single band suggested the presence of a single copy of saccharopine reductase gene in the *U. oxytropis* genome (Fig 2).

Gene disruption

Protoplast preparation and transformation were performed using techniques described previously (Mukherjee *et al.* 2010). Transformed, regenerated putative disruption mutant *Undifilum oxytropis* colonies grew on PDA plates without any observed morphological changes. Two sets of primers were used for PCR screening of the transformants: forward primers were specific to the *U. oxytropis* saccharopine reductase gene and the reverse primers were hygromycin specific. A total of 98 colonies were screened. Twelve colonies revealed positive disruption of saccharopine reductase. Amplification from the genomic DNA of one disruption mutant using set A and set B primers revealed the expected ~2 kb product as represented in Fig 3A, Lanes 2 and 4, while no product was generated with wild type *U. oxytropis* as shown in Fig 3A, Lanes 1 and 3.

To differentiate between single targeted integration and multiple integration events, Southern blotting was performed. Using a HygB specific probe, four colonies showed multiple integration of the Ppd-EGFP-sacred cassette. Fig 4A, Lane 1 represents the signal detected for one of the *U. oxytropis* multiple integration colonies.

Three of the disruption mutant colonies possessed single integrations of the Ppd-EGFP-sacred cassette. Fig 4A, Lane 3 represents the signal detected for one of the *U. oxytropis* single successful disruption mutant colonies. A 2 kb PCR product revealed that the linear hygromycin resistant gene cassette was able to replace a segment of the saccharopine reductase gene. Sequence analysis of PCR products during the screening process indicated successful disruption of the saccharopine reductase gene in the *U. oxytropis* genome. Southern blot experiments with the hygromycin resistant gene specific probe

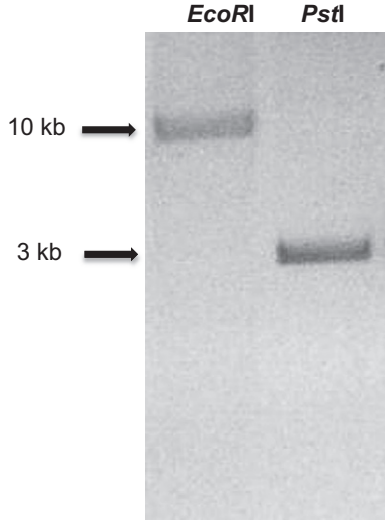

Fig 2 – Southern blot analysis performed to detect number of copies of saccharopine reductase gene in *U. oxytropis*. Briefly the genomic DNA was digested with EcoRI and PstI, transferred to Hybond membrane and probed with saccharopine reductase-specific probe.

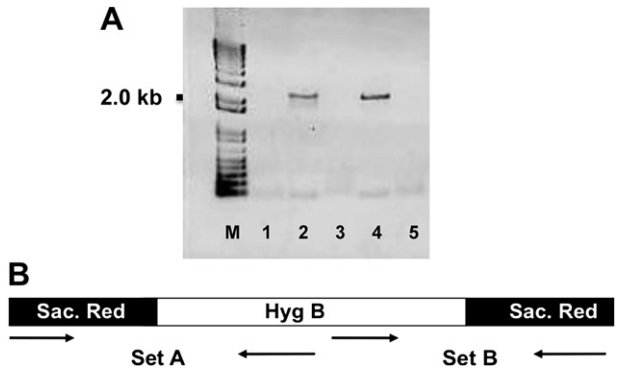

Fig 3 – (A) PCR based screening of a saccharopine reductase disruption mutant in *Undifilum oxytropis*. DNA from wild type *U. oxytropis* was amplified with primer sets A and B (Lanes 1 and 3) while DNA from *U. oxytropis* disruption mutant was amplified with Primer sets A and B (Lanes 2 and 4); expected band size: 2017 bp. Primer sets A and B as defined in the Materials and methods section were used to screen. Lane 5: negative (no template) control for PCR (no DNA in reaction). Lane M: 1 kb Plus ladder (Invitrogen). (B): Schematic representation of the location of primers A and B on the disruption *U. oxytropis* mutant.

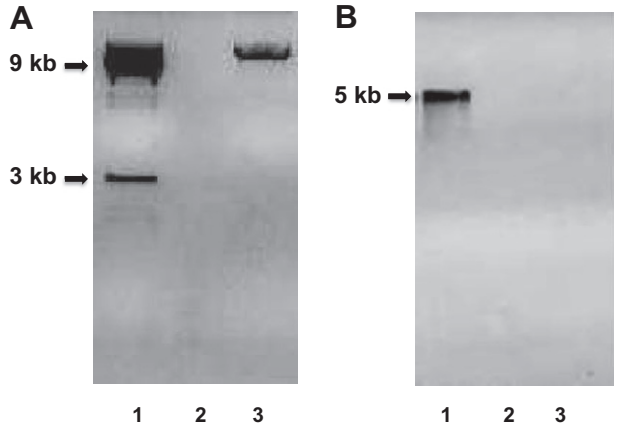

Fig 4 – (A) Screening of single and multiple integrations using Southern blot in saccharopine reductase disruption mutants. Lane 1: *Undifilum oxytropis* mutant colony with multiple integration, Lane 2: wild type *Undifilum oxytropis* colony, Lane 3: *Undifilum oxytropis* mutant with single disruption. Probe used: HygB specific probe. (B): Southern blot screening to decipher disruption of saccharopine reductase in *Undifilum oxytropis*. Lane 1: wild type *Undifilum oxytropis* colony, Lane 2: *Undifilum oxytropis* mutant colony with multiple integration, Lane 3: single *Undifilum oxytropis* mutant with single disruption. Probe used: *Undifilum oxytropis* saccharopine reductase-specific probe.

support the single disruption of saccharopine reductase. No signal was detected from wild type *U. oxytropis* in the Southern blot using a Hyg B specific probe (Fig 4A, Lane 2). Screening of the disruption mutants using a saccharopine reductase-specific probe revealed no signal (Fig 4b, Lanes 2–3) as compared to unaltered *U. oxytropis* where a single band was observed (Fig 4B, Lane 1).

Biochemical analysis

Swainsonine, pipecolic acid, α-aminoadipic acid, saccharopine, lysine, and P6C content were measured by LC–MS (Naranjo *et al.* 2004) in both wild type *Undifilum oxytropis* and disruption mutants. Saccharopine reductase disrupted mutant fungal strains exhibited an increase in concentration of swainsonine and pipecolic acid and a decrease in saccharopine and lysine level, but no difference in α-aminoadipic acid between wild type and mutant was detected. No swainsonine was detected in the growth media when tested. Twelve individual saccharopine reductase disruption and wild type *U. oxytropis* colonies were tested (Table 2). Due to the lack of an available P6C chemical standard, only relative quantitative data was obtained for this compound by mass-spectrometry (Fig 5A). Chromatographic plots revealed a higher accumulation of P6C in the disruption mutants (Fig 5C) as compared to wild type strain where a low concentration of P6C was detected (Fig 5B). We propose a possible pathway (Fig 6) showing increasing and decreasing intermediates in lysine-swainsonine metabolic pathway in *U. oxytropis* based on our chromatographic measurements.

Previous studies indicated that P6C might be formed by non-enzymatic cyclization of α-aminoadipic acid semialdehyde, which is unstable for detection using chromatographic methods (Sim & Perry 1997). The standard for α-aminoadipic acid semialdehyde is not also available commercially, so this intermediate compound was not measured.

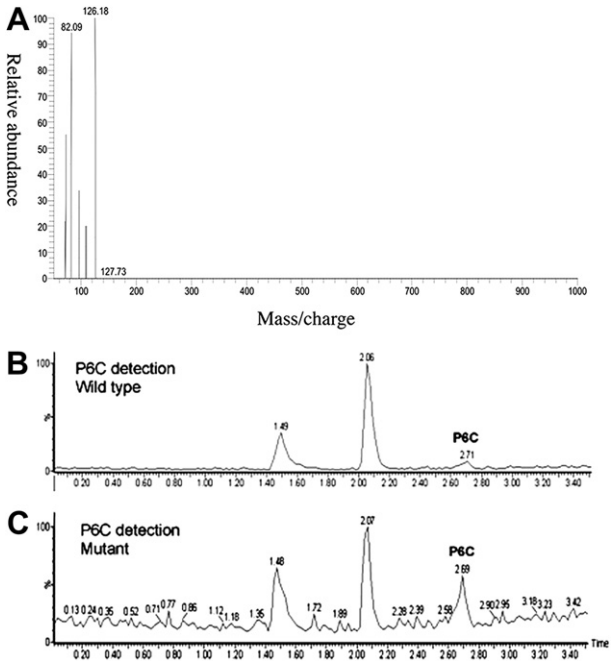

Fig 5 – (A) Mass spectrometric detection of P6C in single saccharopine reductase disruption mutant *Undifilum oxytropis*; X-axis represents mass:charge ratio while Y-axis represents relative abundance of the compounds. (B). Chromatographic representation of P6C in wild type *Undifilum oxytropis*; X-axis represents time in minutes while Y-axis represents relative abundance of the compounds. The peak represents the retention time of P6C (2.71). (C). Chromatographic representation of P6C in single saccharopine reductase disruption mutant *Undifilum oxytropis*; X-axis represents time in minutes while Y-axis represents relative abundance of the compounds. The peak represents the retention time of P6C (2.69).

Discussion

In order to examine the function of saccharopine reductase, we have modified our recently developed transformation protocol for *Undifilum oxytropis* (Mukherjee *et al.* 2010) to develop a specific gene deletion method for a slow-growing toxin-producing endophyte. Disruption of saccharopine reductase led to the accumulation of P6C, pipecolic acid (a precursor of swainsonine), and swainsonine. However, the levels of saccharopine and lysine decreased upon disruption of saccharopine reductase. The impact of inoculation of the saccharopine reductase-deleted endophyte in plant host and the level of different biochemical intermediates were not tested because a functional inoculation system is not yet available for this system.

It has been previously reported that pipecolic acid is converted to lysine through P6C and saccharopine (Kinzel & Bhattacharjee 1979). Biochemical pathways for swainsonine production have been partially characterized in *Magnaporthe anisopliae* and *Rhizoctonia leguminicola*. As reported in an earlier publication (Sim & Perry 1997), different pathways may be involved or active during the formation of swainsonine, and saccharopine reductase has an impact on swainsonine synthesis through this pathway. Naranjo *et al.* also reported that a *lys7* (saccharopine reductase) disruption in *Penicillium chrysogenum* caused elevated accumulation of pipecolic acid and P6C (Naranjo *et al.* 2004). While these two studies investigated fungal saccharopine reductase, the two fungal organisms in each

Table 2 – Levels of biochemical intermediates in wild type *Undifilum oxytropis* and saccharopine reductase disruption mutant of *Undifilum oxytropis*.

Compound	Wild type ± 1 SE (μg mL^{-1})[a,b]	Disruption mutant ± 1 SE (μg mL^{-1})[a,b]
Swainsonine	0.50 ± 0.02	4.2 ± 0.01
Pipecolic acid	0.30 ± 0.08	5.5 ± 0.02
Saccharopine	0.35 ± 0.08	>0.1 ± 0.03
Lysine	0.50 ± 0.05	0.32 ± 0.02
α-aminoadipic acid	0.45 ± 0.02	0.52 ± 0.05

a Starting tissue 10 mg of *Undifilum oxytropis* (dry weight).
b $n = 12$ for all sets of measurements.

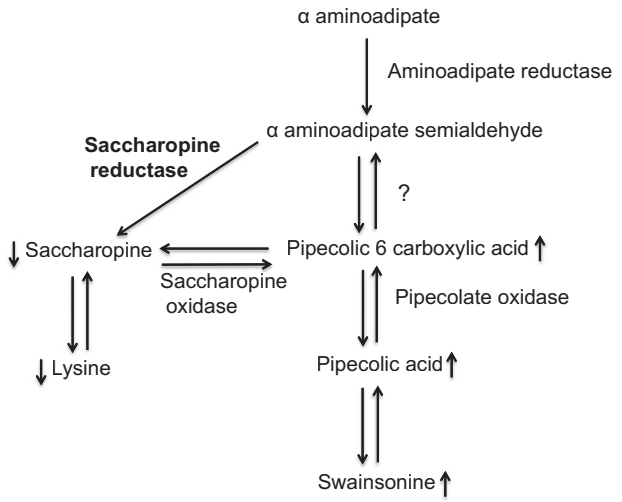

Fig 6 – Impact of saccharopine reductase deletion on the lysine biosynthetic pathway in *Undifilum oxytropis* along with the possible location of the enzyme.

study differ as *P. chrysogenum* does not produce swainsonine but *U. oxytropis* does produce this compound. This study reveals that saccharopine reductase influences the swainsonine metabolic pathway along with the formation of saccharopine and lysine in *U. oxytropis*.

We showed that deletion of the saccharopine reductase gene affects the accumulation of P6C and pipecolic acid in *U. oxytropis*. These results open questions about how swainsonine is synthesized from lysine and how degradation of swainsonine occurs to produce saccharopine, the precursor of lysine. Identification of the gene sequence of saccharopine reductase has facilitated the characterization of the role of this enzyme while the establishment of a transformation system using an ascomycete vector opens a new avenue to further characterize and manipulate different enzymes in the pathway. The establishment of a gene knock out methodology in *U. oxytropis* will now permit a more detailed study of this system to decipher other intermediates in the swainsonine pathway.

Swainsonine is an important inhibitor of mannosidase II and is a potent antitumour agent with immunomodulatory activity (Mohla *et al.* 1989), so further understanding of the underlying biomolecules that lead to the formation of swainsonine will expand our knowledge of the biosynthesis of a compound with natural pharmaceutical value as well as contributing to the understanding of the roles of secondary metabolites in plant–fungal interactions. Furthermore, this may permit the development of fungal strains unable to produce functional toxin, thus alleviating field toxicity problems.

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