| Date Received |
| --- |
| For Administrative Use Only |

Agriculture Funding Consortium

# Full Research Project Final Report Form

- All sections must be completed.
- One electronic copy and one signed original copy are to be forwarded to the lead funding agency as per the investment agreement.
- A detailed statement of expenses incurred during the course of the project must be submitted along with this report.
- For any questions regarding the preparation and submission of this report, please contact the representative of the lead funding agency.

**Members of the Agriculture Funding Consortium**

- Alberta Crop Industry Development Fund Ltd. (ACIDF)
- Alberta Livestock and Meat Agency Ltd. (ALMA)
- Alberta Innovates Bio Solutions (AI-Bio)
- Alberta Barley Commission (ABC)
- Alberta Beekeepers
- Alberta Canola Commission (ACPC)
- Alberta Chicken Producers (ACP)
- Egg Farmers of Alberta (EFA)
- Alberta Milk (AM)
- Alberta Pork (AP)
- Alberta Pulse Growers Commission (APGC)
- Potato Growers of Alberta (PGA)
- Alberta Wheat Commission (AWC)
- Western Grains Research Foundation (WGRF)

## Section A: Project overview

1. Project number: 2012F028R
2. Project title: Evaluation of the toxicity of the secondary metabolites produced by *Leptosphaeria maculans*
3. Research team leader: Xiujie (Susie) Li
4. Research team leader's organization: Alberta Innovates - Technology Futures
5. Project start date (MM/DD/YYYY): April 1, 2012
6. Project completion date (MM/DD/YYYY): March 31, 2014
7. Project final report date (MM/DD/YYYY): June 30, 2014

## Section B: Non-technical summary (max 1 page)

China's 2009 import ban on blackleg disease-infected canola seed, caused by the fungus *Leptosphaeria maculans*, resulted in significant economic losses for Canadian farmers. *L. maculans* produces a secondary metabolite, sirodesmin PL, which is structurally related to the potent mycotoxin gliotoxin. Mycotoxins are primarily of concern due to their ability to cause toxicities, including infertility and even death in farm animals. Moreover, humans may be exposed to mycotoxins through the food chain, with resulting health damage, including cancer.

Before this study, no reports were available on sirodesmin PL contamination levels and sirodesmin PL toxicity to animals or humans. However, given export market concerns, research targeted toward determining the risk of *L. maculans* toxicity in Alberta canola is warranted.

In this study we found that sirodesmin PL has comparable toxicity to gliotoxin, however, no sirodesmin PL was found in canola products. This includes canola seeds collected from blackleg infected fields including seeds with very high levels of *L. maculans* contamination. No sirodesmin PL was detected in canola oil or canola meal obtained from processors or retailers. We conclude that the likely reasons for this include low levels of blackleg contamination in seed from commercial fields, combined with low levels of toxin production by many isolates. Put colloquially, seed contamination levels are low, and even if seed is contaminated, it is not necessarily contaminated with fungal strains that produce appreciable amounts of toxin. We therefore reason that sirodesmin PL contamination does not present a serious risk to the Canadian canola industry. Moreover, we predict that sirodesmin PL levels in Canadian harvested canola seed is unlikely to increase over time, even though pathogen populations appear to be adapting to the resistance genetics of Canadian *B. napus* cultivars, possibly leading to more frequent blackleg epidemics in the future. The toxin production of the isolates we examined was not associated with virulence; some of the most virulent isolates (towards recent *B. napus* cultivars) also produced the lowest levels of toxin.

This result serves to assure the canola industries that at present, Alberta canola and its oil and meal products are free of Sirodesmin PL which gives them confidence in canola products for export. It also assures consumers and livestock industries that Alberta canola products are safe for human consumption and for use as feed for livestock production. We anticipate that the result will bring economic benefit to canola growers.

# Section C: Project details

### 1. Project team (max ½ page)

Dr. Xiujie (Susie) Li contributed to the project coordination, planning, and reporting, she also contributed to the method development for sirodesmin PL purification and produced enough sirodesmin PL for toxicity study and to be used as standard for determining the sirodesmin PL levels in varies survey samples. Mr. Ralph Lange contributed to bin survey and obtaining samples from growers and 20/20 seed lab and report review. Dr. Jian Yang contributed to *L. maculans* strain selection, seed spiking, and reviewing of the report. Dr. Hugh Semple and Mr. Jeff Bechard conducted the toxicology study and reviewed the report.

### 2. Background (max 1 page)

Canola is the third major crop in Alberta, and a main cash crop for farmers. Blackleg is devastating and one of the most economically important diseases for rapeseed/canola (*Brassica napus* and *Brassica rapa*) (Gugel and Petrie, 1992; Howlett, 2004) that results in significant yield loss. Blackleg disease is caused by the fungus *Leptosphaeria maculans* (Bokor, 1972; Barbetti, 1975; Howlett et al., 2001) which produces metabolites including sirodesmins, phomalirazine, and depsipeptide phomalides (Pedras and Seguin-Swartz, 1992), but sirodesmin PL is the major phytotoxin produced by the fungus (Ferezou et al., 1977). Levels of sirodesmin

PL production vary among isolates of *L. maculans* (Soledade et al. 1998). Sirodesmin PL belongs to the family of epipolythiodioxopiperazine (ETP), a class of secondary metabolites characterized by the presence of a highly reactive disulphide-bridged diketopiperazine ring synthesised from two amino acids (Gardiner et al., 2005). Gliotoxin produced by *Aspergillus fumingatus* was the first EPT reported and is the best characterized. Gliotoxin is known to be immunosuppressive and it is considered a serious health hazard to immunocompromised patients (Latgé, 1999) by causing cytotoxicity and cell death through apoptosis and necrosis (DeWitte and Bols, 2005). Feeds contaminated with gliotoxin are potentially toxic to other animals (Pena et al., 2010), as in aspergillosis in turkeys (Richard et al., 1996) and camel death as reported by Gareis and Wernery (1994), Gareis and Wernery also indicated that other secondary metabolites from the ETP family may function as mycotoxins as well.

Sirodesmin PL and gliotoxin are structurally very similar. Research by Gardiner et al. (2004) has demonstrated the homologues of gene clusters, responsible for the biosynthesis of an ETP, between *L. maculans* and *A. fumigatus*. Sirodesmin PL is claimed to be a mycotoxin due to its antibacterial activity (Boudart 1989), however, there is a lack of information regarding the toxicity of sirodesmin PL to humans and animals.

In 2009 China banned Canadian canola seed from entering the country due to concerns over blackleg disease transmission. China imported 2.9 Mt of canola in 2008-2009, decreased to 2.25 Mt in 2009-10 and further reduced imports to1.6 Mt in 2010-11, decreases which translate into significant losses for the Canadian canola industry. Negotiations and activities underway since 2009 are resulting in the re-establishment of market access. Science-based evidence illustrating that mycotoxin contamination of canola seed is not a concern or is under control would assist this process. Environment Canada has expressed concern over sirodesmin PL toxicity (in correspondence concerning regulation of *L. maculans* fungal inoculum for field nursery applications). Our lack of knowledge regarding the toxicity and importance of sirodesmin PL may limit our ability to apply for *L. maculans* field testing under the New Product Notification provisions of the Canadian Environmental Protection Act. This in turn would limit use of *L. maculans* in field trials, thus stifling the development of new cultivars and disease control strategies.

## 3. Objectives and deliverables (max 1 page)

Objectives of this project include:

- Produce sirodesmin PL by selecting virulent isolates of *L. maculans* and culturing these isolates to produce their secondary metabolite, and purifying the metabolites
- Determine the toxicity of sirodesmin PL in cells and animals
- Determine the sirodesmin PL contamination level in Alberta's canola seed, oil and meal by bin survey and sampling from Alberta oilseed processing industries

Deliverables of this project include:

- The acute toxicity and cytotoxicity data of sirodesmin PL
- The level of contamination of sirodesmin PL in Alberta canola seeds and products
- Reports
- publication

## 4. Research design and methodology (max 4 pages)

4.1. Culture of virulent type *Leptosphaeria maculans*

Virulent isolates of *L. maculans* were selected and cultured on V8 agar (20% V8-juice, 0.75 g $CaCO_3$, 100 mg streptomycin sulfate, 40 mg Rose Bengal, 15 g agar) at 24±3°C for 20 days. Pycnidiospores were collected by flooding the plates with sterile distilled water, filtered through sterile nylon mesh, centrifuged at 454 ×g, re-suspended in sterile water and stored at -20°C (Soledade et al. 1998). Cultures were prepared by inoculating 10% V8 liquid medium in flasks with pycnidiospore suspension at 1 x $10^7$ spores/mL of a 20-day-old culture of the fungus on V8 agar plates. Flasks were incubated at 22°C, 12/12h light/darkness on a shaker at 130 rpm for seven days. The liquid culture was used for toxin extraction.

4.2. Extraction and purification of sirodesmin PL.

The purification of sirodesmin PL was performed as described by Soledade and Yu (2009). The *L. maculans* culture was centrifuged to remove fungal cells. The culture broth was then extracted with ethyl acetate (EtOAc) and concentrated to dryness by $Na_2SO_4$ with a rotary evaporator and purified with flash column chromatography (FCC). Sirodesmin PL was first fractionated on a silica column with 100% hexane followed by 10%, 20% and 30% ethyl acetate. Fraction containing sirodesmin PL from the first column were collected and further purified on another silica column with 100% dichloromethane followed by 1% and 2% methanol.

4.3. Sirodesmin PL determination

High performance liquid chromatography (HPLC) was used to determine the sirodesmin PL production in different isolates. A Beckman HPLC system (Mississauga ON, L5N 6V8) equipped with auto-sampler, photo diode-array detector and a fraction collector was used. *L. maculans* extract was run through an Agilent Prep-C18 Scalar column (5 µm particle size silica, 4.6 x 150 mm). Twenty microliters of sample was injected to the column and linear gradient elution with 25% acetonitrile in water to 100% acetonitrile over 35 min at a flow rate of 1.0 mL per minute was used (Soledade et al., 2000). Wavelength of 220 and 240 nm were used to detect the sirodesmin PL peak. Peak at the retention time of 13.93 min was collected and sent to University of Alberta Mass-Spectral lab for structural confirmation.

4.4. Screening for sirodesmin PL production by different *L. maculans* isolates

Seventeen *L. maculans* isolates collected in Canada from various locations with different virulence were used to detect the level of sirodesmin PL production and to investigate the relationship between virulence and sirodesmin PL production. The most productive isolates were selected.

4.5. Cytotoxicity study of sirodesmin PL

The cytotoxicity study of Sirodesmin PL was conducted on standard BALB/c 3T3 cell line with real-time cell electronic sensing (RT-CES) cytotoxicity assay method (Xing et al., 2005; Xing et al., 2006). Sirodesmin PL toxin was dissolved and diluted in dimethyl sulfoxide (DMSO). BALB/c 3T3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing penicillin and streptomycin following the standard protocol. The cells were incubated at 37 °C in a humidified 7% $CO_2$ atmosphere. The treatment medium contained either the test compound, positive control, or vehicle. The Cell Index (CI) in each

test well was automatically determined and recorded by the RT-CES system hourly for 24 h. The CI values were recorded by RT-CES system at 24, 48 and 72 hours for signs of toxicity. In the parallel experiments, the same toxins were tested using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by following the standard protocol endorsed by National Institute of Environmental Health Sciences (NIEHS).

4.6. Mammalian toxicity of sirodesmin PL

The acute toxicity of sirodesmin PL in rats was evaluated in this study. A sighting study was performed to determine the dose to be used in the study. The study comprised three groups of 5 female Sprague Dawley rats given a single oral gavage administration of test article (5 mL/kg) at dose levels of 0, 25, and 50 mg/kg. The animals were observed for 14 days following test article administration and were necropsied on study day 15. The animals were regularly monitored for any signs of ill health or reaction to treatment. Body weights were measured on study days 1, 4, 8, 11, and 15 and food consumption was recorded daily. Blood samples were collected for clinical pathology on study days 2 and 15. On completion of the 14 day observation period, animals were euthanized and subjected to necropsy, with a selected list of tissues being weighed and/or preserved. Histological examination was performed on the designated tissues from all animals in the top dose and vehicle control. Only those tissues identified as target organs were evaluated in the low dose group.

4.7. Survey

4.7.1. Canola seeds survey

Canola seeds in blackleg infected fields were collected to evaluate the occurrence of sirodesmin PL contamination of harvested canola seed in Alberta. Six representative farms in Alberta were selected including Field #1038 in Wetaskiwin, #1044 in Wetaskiwin, #1030 in Vegreville, #1025 in AITF Vegreville, #1029 in Minburn, and #1033 in Innisfree. These fields were identified as blackleg infected fields by disease survey in 2013. The seeds from these fields were collected in September 2013. 2005 Westar seeds were included as a blackleg susceptible variety. DH12075, clean canola seeds, were used as control and sample for spiking. Bin samples were collected from the fields, #1030, #1025, #1029 and #1033, five months after harvest. Nine samples with known percentage of blackleg disease infection kindly provided by 20/20 seed labs were also included in the study.

4.7.2. Canola oil and meal

Only one sample of canola oil and meal was obtained from Alberta canola processing industries, a “super de-gummed oil” from Archer Daniels Midland (Lloydminster).Other companies refuse to provide samples due to concerns over the impact of finding sirodesmin PL on their products. Five more canola oil samples were purchased from grocery stores and farmer’s market, they are, canola oil from Mountain Farming of Strathmore Alberta, Canola Harvest from Richardson, no name brand canola oil, Safeway brand canola oil and Scarpone’s organic expeller pressed canola oil.

4.8. Preparation of blackleg infested canola seeds

Fifty grams of canola seeds (cv. Westar) was soaked in 80 mL water in a flask overnight, excess water was drained and then the seeds were autoclaved at 121°C for 30 min. After 24 h at room temperature, the seeds were autoclaved again. A 7-day-old culture of a virulent

isolate of *L. maculans*, Leroy #127 isolated in 1990 was flooded and pycnidiospores were collected. A 5 mL aliquot of the spore suspension was inoculated to the flask, mixed well and incubated at room temperature for 8 days. Infested seeds were air-dried in a biohazard cabinet and then stored at 4°C until used. Autoclaved seeds (50 g) received sterile distilled water was used as a control.

4.9. Extraction of sirodesmin PL from seeds and oil samples

For seed samples, whole canola seed was ground with a Wiley mill to reduce the size of particles to pass through a No. 20 sieve. Fifty grams of meal was weighed to a 500 mL flask and 250 mL of diethyl ether was added. The flasks were then put on a shaker for 30 min at 150 rpm. After extraction, the content was filtered through Whatman No.1 filter paper to collect the filtrates. 50 mL of extract was collected and air dried. Acetonitrile was then added to the extract and vortexed for 1 min on high. The mixture was centrifuged for 5 min at 4,000 rpm, the top layer was then collected for sirodesmin PL analysis.

For oil samples, diethyl ether was added directly to the canola oil, the mixture was then put on a shaker at 150 rpm for 30 min. After extraction, the contents were filtered into vacuum flask using ceramic Buchner funnel and Whatman No. 1 filter paper. Dry the extract with compressed air for 1 hr. After drying, acetonitrile was added to the dried extract the content was then vortex for 1 min on high and centrifuged for 5 min at 4,000 rpm. The top layer was collected for sirodesmin PL analysis.

Seed and oil samples were spiked with 20 µL of 90.74% pure sirodesmin PL to determine the percentage recovery.

4.10. Sirodesmin PL analysis in survey samples

Ultra high performance liquid chromatography (UHPLC) was used to detect the sirodesmin PL content in survey seeds and oil samples. The system is an Agilent 1290 Infinity Quaternary LC System equipped with quaternary pump, auto-sampler, UV Diode Array Detector (DAD). Four microliters of extracted sample was injected into a Zorbax Eclipse Plus C18 column (2.1x150 mm and particle size of 1.8 micron). The mobile phase consisted of acetonitrile and water. A linear gradient was used. Total run time was 11min including 1 min of 20% acetonitrile, 6 min of 20-80% acetonitrile, 4 min of 80% acetonitrile at the flow rate of 0.5 mL per minute. Three levels of purified sirodesmin PL were used to make a standard curve for quantitation.

4.11. Date analysis

All samples were triplicates. Agilent software, OpenLab CDS Chemstation Edition C.01.05 was used for data analysis on UHPLC.

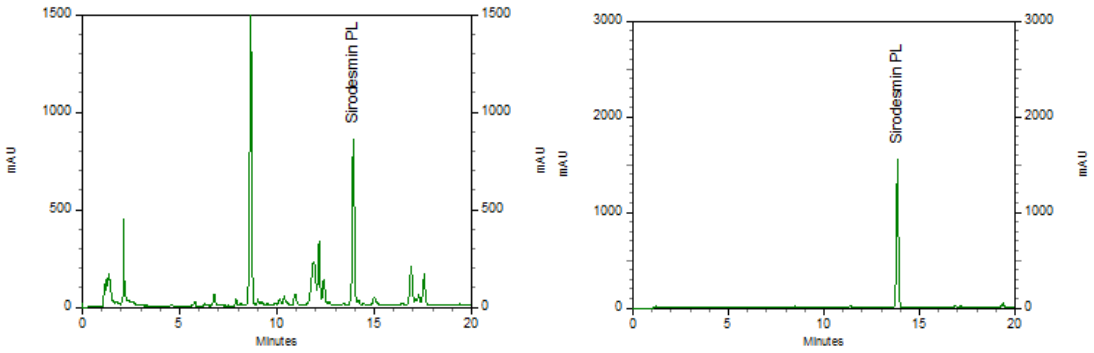
## 5. Results, discussion and conclusions (max 8 pages)

5.1. Results

5.1.1. Production of Sirodesmin PL by 23 *L. maculans* isolates

A virulent isolate, *Leptosphaeria maculans,* Leroy #127 isolated from canola was cultured using the method described by Soledade et al., (1998). Selection of this isolate

for fermentation of sirodesmin PL was based on the results of screening 17 *L. maculans* isolates, of which 4 produced no sirodesmin PL, 13 produced only low amounts of toxin (<0.1mg/L), and 5 produced moderate amount of toxin, despite confirmed virulence to canola cultivars. The production of sirodesmin PL from the isolate was confirmed by both HPLC (Figure 1) and LC-MS. Seventy liters of culture (Leroy #127) was produced and culture filtrates were collected.

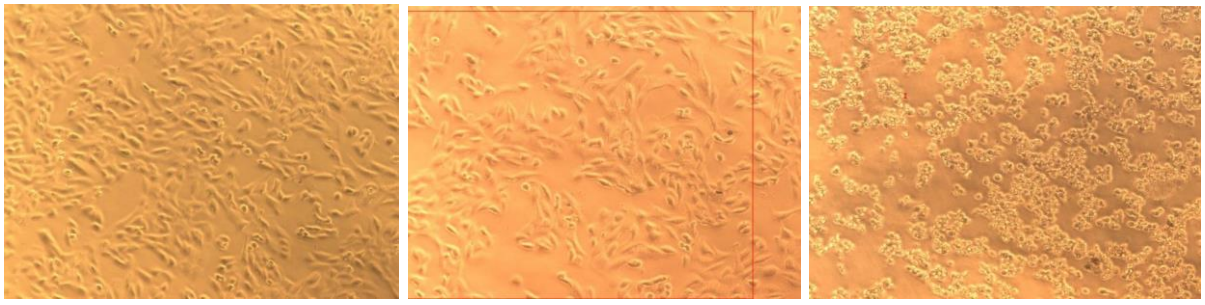



**1a** **1b**

**Figure 1**. Sirodesmin PL produced by *Leptosphaeria maculans* isolates Leroy 127, 1a, in culture filtrates; and 1b, purified sirodesmin PL

5.1.2. The cytotoxicity data of sirodesmin PL

Results showed that at low concentration, 3.9 µM, 4.8 µM, and 5.3 µM, after 24, 48 and 72 h of treatment respectively, sirodesmin PL was able to induce apoptosis to BALB/c 3T3 cells. At high concentration, 1.16 µM or higher, sirodesmin PL caused cell membrane damage and bleb appearance and mortality of the test cells. Figure 2 showed that sirodesmin PL caused decreased cell number (Figure 2 B) 4 hours after treatment, at concentration of 0.58 µM, however, the cells recovered and continued their growth after 24 hrs. At concentration of 2.32 µM, sirodesmin PL caused cell death at 24 hrs (Figure 3 C). The $LC_{50}$ of sirodesmin PL was predicted as 200.62 mg/kg.



A B C

Figure 2. Morphology of Balb/c 3T3 cells, A, Control; B, 0.58 µM of sirodesmin 4 hrs after treatment; and C, 2.32 µM of sirodesmin, 24 hrs after treatment

5.1.3. The toxicity of sirodesmin PL in rats

The acute toxicity results indicate that a single administration of sirodesmin PL to female rats by oral gavage at 50 mg/kg resulted in clinical signs of intolerance, reduced food

consumption, adverse changes in clinical chemistry parameters (indicative of acute liver toxicity), and hyperplastic changes in the bile ducts of the liver. Under the conditions of this study the no observed adverse effect level (NOAEL) was considered to be 20 mg/kg. Test article related findings at this dose were limited to clinical observations that resolved by study day 3.

Histopathological results show that a single administration of Sirodesmin PL at 50 mg/kg in female Sprague Dawley rats caused hyperplasia of the bile ducts in the liver. The severity of this finding ranged from minimal to slight and the finding was observed in all 5 treated animals. Bile duct hyperplasia was characterized by increased number of bile ducts in the portal region. The ducts were lined by flattened to cuboidal epithelium forming normal ducts. Occasionally, single to double rows of oval shaped cells forming incomplete duct structures were observed focally in some of the affected livers. In addition, the incidence and severity of mononuclear inflammatory cells infiltrating the hepatic parenchyma appeared to be increased in the livers of animals dosed at 50 mg/kg when compared to livers from animals dosed at 0 or 20 mg/kg.

5.1.4. *L. maculans* Leroy #127 infected seeds

The amount of sirodesmin PL in canola seeds infected with *L. maculans* isolate Leroy #127 was shown in Figure 3. Results shown that 0.1, 0.2, 0.4, 0.8, 1.6% infection, (1, 2, 4, 8, and 16 severely infected seed in 1,000 clean seeds) have 0.67, 1.17, 2.53, 4.72, and 9.06 ppm of sirodesmin PL, respectively.

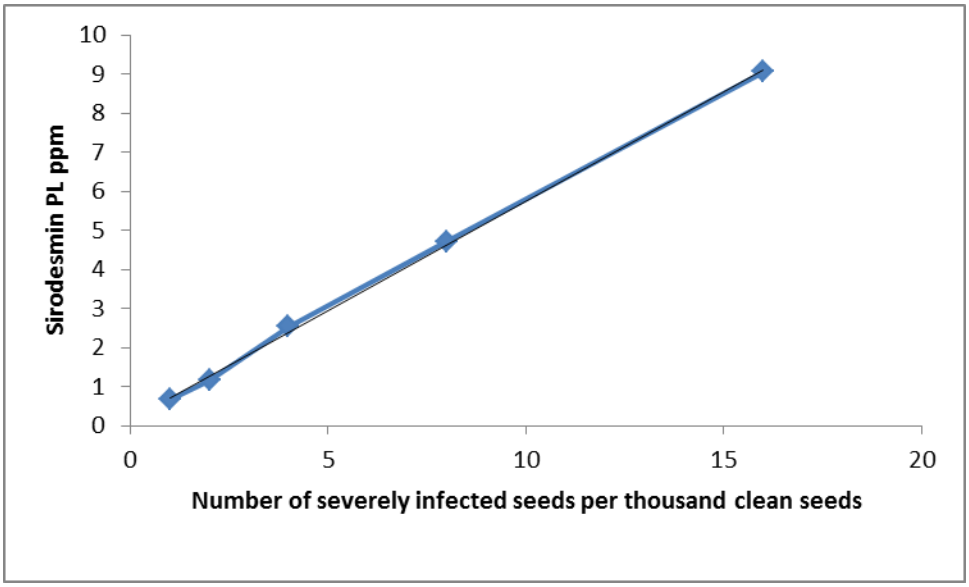



**Figure 3**. Sirodesmin PL level in relation to the percentage of infected seeds.

5.1.5. Canola seed samples

There was no sirodesmin PL detected in any of the seed samples tested (Table 1). The spiked clean sample was used to calculate the percentage recovery of the method. A 72%

recovery was achieved with this method. Same results were obtained in seed samples provided by 20/20 Seed Lab (Table 2)

**Table 1. Blackleg disease severity and sirodesmin PL content in field survey and bin survey samples**

| Cultivar | Location | Blackleg infection rate (%) | Sirodesmin PL (ppb) in field survey | Sirodesmin PL (ppb) bin survey |
|---|---|---|---|---|
| 1030 | Vegreville | 0.56 | 0 | 0 |
| 1033 | Innisfree | 1.0 | 0 | 0 |
| 1029 | Minburn | 0.69 | 0 | 0 |
| 1025 | Vegreville | 0.06 | 0 | 0 |
| Westar | Vegreville | 4.5 | 0 | NA |
| 1038 | Wetaskiwin | 2.0 | 0 | NA |
| 1044 | Wetaskiwin | 2.0 | 0 | NA |
| DH12075 | Greenhouse grown | 0 | 0 | NA |

**Table 2. Blackleg disease severity and sirodesmin PL content in seed samples provided by 20/20 Seed Lab**

| Sample ID | Variety (V)/client identifiers (CI) | Blackleg infection rate (% of seeds) | Sirodesmin PL (ppb) |
|---|---|---|---|
| 1003-225 | 6040 RR (V) | 0.1 | 0 |
| 1401-424 | BTCD30005T (CI) | 0 | 0 |
| 1304-994 | SW Wizzard (V) | 0.1 | 0 |
| 1304-1725 | Common Seed (V) | 0.4 | 0 |
| 1401-412 | 1 B042CFRHZ (CI) | 0 | 0 |
| 1401-419 | ATCP24505T (CI) | 0 | 0 |
| 1312-939 | RCCD6161C (CI) | 0 | 0 |
| 1304-1728 | Invigor 5440 (V) | 0.2 | 0 |
| 1312-937 | RCCE69001C (CI) | 0 | 0 |

5.1.6. Canola oil and meal samples

There was no sirodesmin PL detected in all the canola oil samples obtained (Table 3). The only canola meal provided by Archer Daniels Midland (Lloydminster) also showed zero content of sirodesmin PL.

**Table 3.** Sirodesmin PL content in canola oil

| Canola oil brand | Sirodesmin PL (ppb) |
|---|---|
| Mountain Farming | 0 |
| Canola Harvest | 0 |
| No name | 0 |
| Safeway | 0 |
| Scarpone's organic expeller pressed canola oil | 0 |
| Super de-gummed oil" from Archer Daniels Midland | 0 |

5.2.Discussion

In this study, sirodesmin PL was found to be toxic to rats. It is as toxic as gliotoxin. But unlike gliotoxin, no animal poisoning has been reported from eating canola products. This is a good indication that sirodesmin PL levels in canola products are too low to cause any toxicity or detoxification mechanisms may exist or both.

This project initially was intended to produce enough sirodesmin PL to conduct a toxicity study. One of the most virulent isolates of *L. maculans* #394 was selected. However this isolate produced a low quantity of sirodesmin PL. To save the cost of the project, a high sirodesmin PL production strain needed to be selected. Therefore, 17 isolates in AITF's possession were cultured and their production of sirodesmin PL was measured with HPLC. In this study we found that the yield of sirodesmin PL was not necessarily related to the virulence of *L. maculans*. The results also indicated that finding the high sirodesmin PL production strain of *L. maculans* in Alberta or Canada is more important in determining sirodesmin PL contamination than finding the strains that cause virulent outbreaks. High blackleg incidence does not mean high sirodesmin PL contamination. So far there is no information available on the whole spectrum of sirodesmin PL production profile in Alberta. Since the toxicity of sirodesmin PL has been confirmed, this information will be very useful in predicting future sirodesmin PL outbreaks in Alberta and Canada.

To determine the contamination level of sirodesmin PL on farm, we conducted an in-field survey as well as a bin survey from the same field where canola seeds were sampled. Since there was a low disease incidence in Alberta in 2013, seeds collected from disease survey fields may have better representation for the overall sirodesmin PL contamination levels in Alberta canola farms rather than samples from clean fields. Seeds with known percentages of blackleg infection were also kindly provided by 20/20 Seed Labs. Overall we have a good representation of samples for the survey. To determine the sirodesmin PL content in seeds infected by sirodesmin PL producer, seeds were purposely inoculated with *L. maculans* isolate Leroy #127. Sirodesmin PL was found even in 0.1% (one infected seed in 1,000 clean seeds) infected seeds. This may indicate that the *L. maculans* isolate(s) that causes blackleg disease in the survey seeds is not a high sirodesmin PL production strain(s). This is evident by the 0.4% blackleg infection observed in seeds provided by 20/20 Seed Labs showing zero sirodesmin PL content.

All the oil and meal production companies proposed in this project were contacted and an effort was made to obtain samples from these companies. However, only Archer Daniels Midland (Lloydminster) agreed to provide both canola oil and meal samples for analysis. Other companies either did not response to us or refused to provide samples over the concern that finding sirodesmin PL would have negative impact on their products. Samples from grocery stores and farmer's markets were obtained instead for this study. Three samples, a sample from Archer Daniels Midland, canola oil from Mountain Farming of Strathmore Alberta, and Canola Harvest from Richardson are claimed to use local Alberta canola seeds; no name brand canola oil, Safeway brand canola oil and Scarpone's organic expeller pressed canola oil are from distributers and we could not trace the origin of their seeds.

For all the seeds, oil and meal samples tested, no sirodesmin PL was detected, indicating that at present, Alberta canola products are free of sirodesmin PL. The reasons for this might be low sirodesmin PL in the samples, the *L. maculans* stains in Alberta in recent years is not high sirodesmin PL producers and sirodesmin PL is not stable or is easily degraded or detoxified during the process to produce oil (that might be the case for No Name brand, Safeway brand, and Scarpone's organic expeller pressed canola oil as we do not know where those seeds used for oil production were produced). The short shelf life of sirodesmin PL was evident when pure sirodesmin PL was stored. Total degradation occurred within 2 days of storage in solvent at -20°C.

## 5.3.Conclusion

In this study we found that while sirodesmin PL has comparable toxicity to gliotoxin, no sirodesmin PL was found in canola products. This includes canola seeds collected from blackleg infected fields including seeds with very high levels of *L. maculans* contamination. , No sirodesmin PL was detected in canola oil or canola meal obtained from processors or retailers. We conclude that the likely reasons for this include low levels of blackleg contamination in seed from commercial fields, combined with low levels of toxin production by many isolates. Put colloquially, seed contamination levels are low, and even if seed is contaminated, it is not necessarily contaminated with fungal strains that produce appreciable amounts of toxin. We therefore reason that sirodesmin PL contamination does not present a serious risk to the Canadian canola industry. Moreover, we predict that sirodesmin levels in Canadian harvested canola seed is unlikely to increase over time, even though pathogen populations appear to be adapting to the resistance genetics of Canadian *B. napus* cultivars, possible leading to more frequent blackleg epidemics in future. The toxin production of the isolates we examined was not associated with virulence; some of the most virulent isolates (towards recent *B. napus* cultivars) also produced the lowest levels of toxin.

It may be advisable, however to periodically sample harvested seed for sirodesmin PL contamination, to ensure toxin levels remain low or undetectable, and to alert the industry to any unexpectedly high levels of toxin. Moreover, the frequency of toxigenic *L. maculans* isolates in field populations appears to be low, but this should be confirmed. Our data suggest that sirodesmin PL production appears to be decreasing in *L. maculans* populations over time, but our sample size was too small to draw any firm conclusions, so this too should be confirmed with more extensive sampling. Formation and depletion of sirodesmin PL in harvested canola should also be examined to identify any storage conditions that either

increase or decrease toxin levels in contaminated seed. Finally, it would be useful to determine the portioning of any sirodesmin PL in contaminated seed into post-processing seed and oil fractions.

## 6. Literature cited

Provide complete reference information for all literature cited throughout the report.


Boudart, G., 1989. Antibacterial activity of sirodesmin PL phytotoxin: Application to selection of phytotoxin-deficient mutants. Applied and Environmental microbiology 55:(6)1555-1559.

Ferezou, J. P., C. Riche, A. Quesneau-Thierry, C. Pascard-Billy, M. Barbier, J. F. Bousquet, and G. Boudart. 1977. Structure de deux toxines isolees de cultures du champignon Phoma lingam Tode. Nouv. J. Chim. 1: 327-334.

Gareis, M., and Wernery, 1994. Determination of gliotoxin in samples associated with cases of intoxication in Camels. Mycotoxin Research. 10: (1) 2-8.

Gardiner DM, Cozijnsen AJ, Wilson LM, Pedras MS, Howlett BJ. 2004. The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus Leptosphaeria maculans. Mol Microbiol. 53 (5): 1307-18.

Gardiner, D.M., P. Waring, B.J. Howlett, 2005. The epipolythiodioxopiperazine (ETP) class of fungal toxins: distribution, mode of action, functions and biosynthesis. Microbiology-Sgm 151:121-1032.

Gugel, R.K. and G.A. Petri, 1992. History, occurrence, impact and control of blackleg of rapeseed. Canadian Journal of Plant Pathology. 14:36-45.

Hewlett, B.J. 2004. Current knowledge of the interaction between Brassica napus and *Leptosphaeria maculans*. Canadian Journal of Plant Pathology. 26:245-252.

Latge, J.P., 1999. *Aspergillus fumigatus* and aspergillosis. Clin. Microbiol. Rev. 12:310-350 Pardo J, Urban C, and Galvez EM., 2006. The mitochondrial protein Bak is pivotal for gliotoxin-induced apoptosis and a critical host factor of *Aspergillus fumigatus* virulence in mice. J Cell Biol. 174:509–519

Pena, G.A., C.M. Pereyra, M.R. Armando, S.M. Chiacchiera, C.E. Magnoli, J.L. Orlando, A.M. Dalcero, C.A.R. Rosa, and L.R. Cavaglieri, 2010. *Aspergillus fumigatus* toxicity land gliotoxin levels in feedstuff for domestic animals and pets in Argentina. Letters in Applied microbiology. 50:77-81.

Poiret B, Kollmann A, Bousquet JF. 1985. Antifungal activity of sirodesmin PL and two natural analogs isolated from *Phoma lingam* (Tode) Desm. and antagonistic action of zinc. Agronomie sciences des productions vegetales et de l'environnement. 5: (6)533-538.

Soledade, M., C. Pedras and Corinne J. Biesenthal. 1998. Production of the host-selective phytotoxin phomalide by isolates of *Leptosphaeria maculans* and its correlation with sirodesmin PL production. Can. J. Microbiol. 44: 547-553.

Soledade, M, Pedras C, Seguin-Swartz GS. 1992. The blackleg fungus: phytotoxins and phytoalexins. Can J Plant Pathol. 14: (1) 67-75.

Soledade, M., C. Pedras, and Y. Yu, 2009. Mapping the sirodesmin PL biosynthetic pathway — A remarkable intrinsic steric deuterium isotope effect on a 1H NMR chemical shift determines β-proton exchange in tyrosine. Canadian Journal of Chemistry. 87: (4) 556-562.

Stanzani M, Orciuolo E, Lewis R, et al., 2005. *Aspergillus fumigatus* suppresses the human cellular immune response via gliotoxinmediated apoptosis of monocytes. Blood.105:2258–2265.

Sugui JA, Pardo J, and Chang YC., 2007. Gliotoxin is a virulence factor of *Aspergillus fumigatus*: gliP deletion attenuates virulence in mice immunosuppressed with hydrocortisone. Eukaryot Cell. 6:1562–1569.

**7. Benefits to the industry (max 1 page)**

a) Describe the impact of the project results on Alberta's agriculture and food industry (results achieved and potential short-term, medium-term and long-term outcomes).

1. This project provides first-hand information of the safety or toxicity of the canola products infected with blackleg fungus *Leptosphaeria maculans*. Sirodesmin PL is not produced by all the *L. maculans* strains. If a field is infected with *L. maculans.* It does not necessarily mean sirodesmin PL present. This information will benefit both canola producers.

2. There was no information available regarding the safety of the blackleg fungal metabolite sirodesmin PL before. However the toxic effect of gliotoxin which belongs to the same toxin family and has structure similarity with sirodesmin PL was reported. Now that we know that sirodesmin PL is as toxic as gliotoxin, this result will raise the awareness of canola producers.

3. Even though there is blackleg disease in Alberta, there is zero sirodesmin PL found even in the most infected field. This result will protect the market value of the products and result in increased exports.

4. This project is the first research done regarding the mammalian toxicity of metabolites of blackleg fungus. As such, it proactively prepares Alberta for any issues or concerns raised regarding the product in the future. For example, the generated data on sirodesmin PL toxicity will be useful if any outbreaks of toxicosis are attributed in future to canola oil or meal, and data on toxin absence in canola products will be useful in trade negotiations or to address safety and health concerns.

b) Quantify the potential economic impact of the project results (*e.g.*, cost-benefit analysis, potential size of market, improvement in efficiency, etc.).

According to Canola Council of Canada, Canadian-grown canola contributes $19.3 billion to the Canadian economy each year, including more than 249,000 Canadian jobs and $12.5 billion in wages. Alberta is one of the main canola producers in Canada. The results of this project provide comfort to canola consumers and will further boost the export of canola.

## 8. Contribution to training of highly qualified personnel (max ½ page)

Ms. Wendi Dmytriw, a Lab technician is trained to producing sirodesmin PL from *L. maculans* cultures, she also learned how to extract sirodesmin PL from this culture.
Mr. Rodney Werezuk, a lab technician, was trained to extract sirodesmin PL from seeds samples, oil and meal samples, he also gain knowledge and experience on detecting sirodesmin PL on (High Performance Liquid Chromatography) HPLC and (Ultra High performance Liquid Chromatography) UHPLC and data analysis with software.

## 9. Knowledge transfer/technology transfer/commercialisation (max 1 page)

This project obtained knowledge of the toxic property of the sirodesmin PL produced by blackleg pathogen *L. maculans* and this knowledge will be provided to the canola industry

The outputs of the major project components will be distributed and utilized as follows:

• We have selected isolates, produced the sirodesmin PL, and examined the acute and cytotoxicity of sirodesmin PL. Isolates with defined levels of toxin production will be submitted to the Canadian national culture collection to facilitate future work on *L. maculans* toxins by other researchers. Since this project has also generated the largest amount of sirodesmin PL produced to date, we will serve as source of toxin for future research. The data generated will be compiled into reports and refereed publications (e.g. Canadian Journal of Plant Pathology) To ensure uptake by persons and organizations involved in international trade and canola safety and marketing, we will forward reports to the Canola Council of Canada (via ACPC or directly). The Canola Council is working with the federal government in mitigating the Chinese trade restrictions, and is also a central point of information for the entire canola industry.

• Data generated by the survey and evaluation of meal and oil for toxin contamination was included in this report, and will also be communicated in scientific journals. The results of analyses will also be communicated back to our collaborators in the survey, e.g. terminal owners and the canola processors. As for the toxicity studies, we will also communicate results with the Canola Council, for the reasons stated above. We made and will continue make presentations in grower and industry meetings.

We have generated poster presentations titled" Evaluation of Sirodesmin PL Produced by *Leptosphaeria Maculans*" and "Evaluation of the toxicity and occurrence of sirodesmin PL in vitro and in rapeseed products" in international conferences.

## Section D: Project resources

1. **Statement of revenues and expenditures:**
   a) **In a separate document certified by the organization's accountant or other senior executive officer, provide a detailed listing of all cash revenues to the project and expenditures of project cash funds.** Revenues should be identified by funder, if applicable. Expenditures should be classified into the following categories: personnel; travel; capital assets; supplies; communication, dissemination and linkage; and overhead (if applicable).

      Please see the attached report.

   b) **Provide a justification of project expenditures and discuss any major variance (*i.e.*, ± 10%) from the budget approved by the funder(s).**

There was $30,977.19 over the proposed budget spent on labor due to the unexpected difficulties to select a good isolate for sirodesmin PL production in the first year and overhead charge which was covered by AITF. The spending on supplies listed on the statement $4,824.85 was the fund spend by our lab however, the $15,175.15 which also proposed for supplies was spend by toxicology group for obtaining animals and supplies used for the toxicology study which is included in the "technical services". The total travel expense is $5,073.54, which included $1,402.88 from the expenditure report and $3,370.66 will be deducted from the outstanding fund from ACIDF to cover the travel expense occurred in May 2014.

**2. Resources:**

Provide a list of all external cash and in-kind resources which were contributed to the project.

| **Total resources contributed to the project** | | |
|---|---|---|
| **Source** | **Amount** | **Percentage of total project cost** |
| **Agriculture Funding Consortium** | $60,000 | 27.89% |
| Other government sources: Cash | $95,136.30 | 44.22% |
| Other government sources: In-kind | | % |
| Industry: Cash | $60,000 | 27.89% |
| Industry: In-kind | | % |
| **Total Project Cost** | $215,136.30 | 100% |

| **External resources (additional rows may be added if necessary)** | | |
|---|---|---|
| **Government sources** | | |
| Name (only approved abbreviations please) | Amount cash | Amount in-kind |
| ACIDF | $60,000 | |
| Alberta Innovates - Technology Futures | $95,136.30 | |
| **Industry sources** | | |
| Name (only approved abbreviations please) | Amount cash | Amount in-kind |
| ACPC | $60,000 | |
| | | |

## Section F: Suggested reviewers for the final report

Provide the names and contact information of four potential reviewers for this final report. The suggested reviewers should not be current collaborators. The Agriculture Funding Consortium reserves the right to choose other reviewers. Under *Section 34* of the *Freedom of Information and Protection Act (FOIP)* reviewers must be aware that their information is being collected and used for the purpose of the external review.

**Reviewer #1**
Name: Soledade Pedras
Position: Professor
Institution: University of Saskatchewan
Address: 110 Science Place, Saskatoon SK, S7N 5C9
Phone Number: 306 966 4772
Fax Number:
Email Address: s.pedras@usask.ca

**Reviewer #2**
Name: Ting Zhou
Position: Research Scientist
Institution: Agriculture Agri-Food Canada
Address: 93 Stone RD W, Guelph Ontario, N1G 5C9
Phone Number: 519 780 8036
Fax Number:
Email Address: ting.zhou@agr.gc.ca

**Reviewer #3**
Name: Trevor Smith
Position: Professor
Institution: University of Guelph
Address: 50 Stone Road East, Building #70, Guelph Ontario, N1G 2W1
Phone Number: 519 824 4120
Fax Number:
Email Address: tsmith@uoguelph.ca

**Reviewer #4**
Name: Randy Kutcher
Position: Associate professor
Institution: University of Saskatchewan
Address: Rm 3C02 Ag Bldg - 51 Campus Drive
Phone Number: 306 966-4951
Fax Number:
Email Address: randy.kutcher@usask.ca