8-20-2024 10:00 AM

Influence of Ginsenosides on the Pathogenicity of Ilyonectria

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

Abstract

Commercial cultivation of American ginseng (*Panax quinquefolius* L.) suffers from ginseng replant disease (GRD) when planted in a former ginseng garden. GRD typically manifests as a severe root-rot due to fungus *Ilyonectria mors-panacis*. Ginsenosides released to the soil by ginseng are thought to contribute to GRD but, to be effective, ginsenosides need to be present in the soil. Using soils collected from commercial ginseng gardens, I show that ginsenosides accumulate for the first three and a half years of cultivation but decline in the fourth year. They are largely gone within one-year post-harvest, suggesting that they are not direct contributors during GRD. Instead, I revealed that *Ilyonectria* spp., specifically *I. rufa*, increase in virulence on ginseng roots when pre-exposed to ginsenosides *in vitro*, suggesting ginsenosides may contribute to establishing the GRD state. Additionally, while various *Ilyonectria* isolates were able to metabolize select ginsenosides, this did not correlate with virulence.

Keywords

Ginseng Replant Disease, GRD, root rot, ginsenosides, triterpenoids, fungal pathogen, Traditional Chinese Medicine, *Ilyonectria*, liquid chromatography mass spectrometry

Summary for Lay Audience

Ginseng is a valuable plant grown for its use in Traditional Chinese Medicine (TCM). American ginseng is grown commercially in North America and yields more than $100 million in sales each year. Ginseng growers face a big challenge known as ginseng replant disease (GRD). GRD is characterized by root rot disease caused by the fungus *Ilyonectria mors-panacis* when ginseng is cultivated in soil previously used for growing the crop. GRD is thought to be helped by ginsenosides, the chemical compounds produced by ginseng that give the roots their value in TCM. This is because ginsenosides can increase the growth of the fungus *Ilyonectria mors-panacis*. To have an effect on *Ilyonectria mors-panacis* growth in a ginseng garden, ginsenosides have to be present in the soil. In my thesis work, I found that ginsenosides accumulate in ginseng soils over the first three and a half years of cultivation. Then the levels drop by the time of harvest. Ginsenoside levels drop further after harvest and are barely present in the soil a year later. Since ginsenosides do not exist in the soil for a long time, they cannot be a direct cause of GRD. To understand how ginsenosides relate to GRD, I next studied how *Ilyonectria mors-panacis* and related species change when they were exposed to ginsenosides in our lab. I found that a species similar to *Ilyonectria mors-panacis*, known as *Ilyonectria rufa*, became more harmful to ginseng after it was exposed to ginsenosides. My research suggests that some *Ilyonectria* fungi may become more harmful when exposed to ginsenosides in the soil while ginseng is being grown. This makes them more able to infect ginseng the next time it is planted in the same garden.

Co-Authorship Statement

Sections 2.1 – 2.4: Rabas, A., **Colo, A.,** Kaberi, K., Ivanov, D., & Bernards, M.A. (2024). The Properties of Ginsenosides in Ginseng Garden Soil: Accumulation, Persistence and Behaviour. (Submitted to *Plant & Soil*)

Rabas, A. - investigation, validation, writing, review, and editing. Ginsenosides were extracted from ginseng garden soils for four time points.

Colo, A. - investigation, validation, writing, review, and editing. Approximately 30% of work on this manuscript was conducted by myself. Ginsenosides were extracted from ginseng garden soils for six time points.

Kaberi, K. - methodology, investigation, validation, writing, review, editing. Ginsenosides were extracted from ginseng garden soils for nine time points.

Ivanov, D.A. - conceptualization, methodology, writing, review, editing

Bernards, M.A. - conceptualization, supervision, funding acquisition, writing, original draft.

Acknowledgments

I am deeply grateful for the individuals who provided me with immense support these past couple years while completing my MSc at Western University. The past six years of my undergraduate and graduate studies here have been an incredible experience, enriched by the people I have met.

First and foremost, I would like to express how thankful I am for my supervisor, Dr. Mark Bernards, whose encouragement and support have been instrumental in my academic journey. My love for research began at the end of third year in my undergrad, when I started an undergraduate summer research internship (USRI) in Dr. Bernards' lab studying ginseng. As I completed this USRI, subsequently my fourth-year thesis project, and a second USRI in his lab, my joy for research grew which was definitely fueled by Mark. I then knew I wanted to continue in research and was very pleased when Mark accepted me into the Master's degree program. These past two years have flown by, but I have grown significantly as a research scholar under his mentorship.

I would also like to extend my gratitude to my lab colleagues, many whom I have grown to be close with. Lorena Yeung and Jessica Sinka, for all of the laughs, support, and immense knowledge you both provided me with. Andrew Rabas, for being my ginseng companion and always providing a helping hand. Mina Esfandiari, for your kindness and support. Andrea Ong and Madeleine Tran, for helping me collect chlorophyll fluorescence data during your USRI/USRA and for being terrific lab mates. And an honorable mention to 4th floor NCB member, Eleanor Khochaba, for making me laugh a bit too much and being a great travel buddy. Eleanor, Lorena, and Jessica, our conference trips to Montreal and Winnipeg will be memories I cherish.

My sincere thanks to Dr. Sean Westerveld and Amy Fang Shi (OMAFRA) for your prompt willingness for us to sample ginseng fields and collect ginseng seeds and roots for my studies. My project would not have been possible without it. And further gratitude to my advisory committee, Dr. Greg Thorn and Dr. Vera Tai, for your knowledge on ginseng, sequencing, and microbes. Greg, I attribute my love for fungi now to the knowledge that you have taught me on fungi from my undergrad and Masters. And a

thanks to my funding sources: NSERC, Canadian Agricultural Partnership (CAP), and Western University.

Lastly, to my best friend of many years, Emma Bobier. Thank you for all of the picnics, plant-trips, beach and camping adventures, and overall support of me since high school. And of course, my greatest appreciation to my mom, Lidija, who has always provided me with love and support, and has taught me so many skills. I am very grateful for all of the hikes and camping trips we have taken to relieve the stress of grad school. You have consistently been encouraging of my career-path and I always appreciate it. And to my cat Ollie, for being the sweetest little companion who encouraged me to take breaks while writing my thesis.

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List of Abbreviations

A260/A280 = ratio of absorbance at 260 nm and 280 nm

A260/A230 = ratio of absorbance at 260 nm and 230 nm ANOVA = analysis of covariance

ARD = Apple Replant Disease

CCFC = Canadian Collection of Fungal Cultures

CITES = Convention of International Trade in Endangered Species of Wild Fauna and Flora

CNS = central nervous system

CTAB = cetyltrimethylammonium bromide

DAOMC = Canadian Collection of Fungal Cultures

EDTA = ethylenediaminetetraacetic acid

ESI = electrospray ionization

ESW = Environmental Sciences Western Field Station

F_v/F_m = variable fluorescence/maximum fluorescence

GRD = Ginseng Replant Disease

HIS = histone H3

HKY = Hasegawa–Kishino–Yano

HPLC = high pressure liquid chromatography

LCMS = liquid chromatography-mass spectrometry m/z = mass-to-charge ratio

MAFFT = Multiple Alignment using Fast Fourier Transform

MEGA = Molecular Evolutionary Genetics Analysis

nrRNA-ITS = nuclear ribosomal RNA-internal transcribed spacer

OGGA = Ontario Ginseng Grower's Association

OMAFRA = Ontario Ministry of Agriculture, Food and Rural Affairs

PCR = polymerase chain reaction

PDA = potato dextrose agar

PPD = 20(S)-protopanaxadiols

PPT = 20(S)-protopanaxatriols

PVDF = polyvinylidene fluoride

QQ = quantile-quantile

SARA = Species at Risk Act

SDS = sodium dodecyl sulfate

SPE = solid phase extraction

TAFC = N,N0 ,N00-triacetylfusarinine C

TEF = translation elongation factor 1

TCM = Traditional Chinese Medicine

ToF = Time of Flight

TUB = partial β-tubulin

Vcap = capillary voltage

1 Introduction

1.1 American ginseng: history and endangerment

American ginseng (*Panax quinquefolius* L.) of the family Araliaceae, is a slow-growing herbaceous perennial grown commercially for its highly valued roots and is used in Traditional Chinese Medicine (TCM) (Proctor & Bailey, 1987). American ginseng is one of the three most common and economically valuable *Panax* species amongst 17 identified *Panax* spp.; the other two common species being *P. ginseng* C.A. Mey. (Asian ginseng) and *P. notoginseng* (Burk) F.H. Chen (Chinese ginseng) (Zhang et al., 2020). In Canada, American ginseng is grown in Ontario (the primary producer), Quebec, and British Columbia, whereas in the United States, American ginseng is primary grown in Wisconsin (Charron & Gagnon, 1991; Hou, 2019; Punja, 2011). Ginseng and its medicinal applications were first described in "Shen-Nong-Ben-Cao-Jing" (Shen-Nong-Herbal Classic) edited in approximately 200 AD (Shou-Zhong, 1746). French Jesuit priest Jartoux, in China, published a paper speculating the occurrence of ginseng in North America. Consequently, French Jesuit priest Joseph Lafitau who had read the paper, found American ginseng near Montreal in 1716 (König, 2020; Hou, 2019). Asian ginseng had previously been used for its health benefits in TCM to treat headaches, digestion, and fever; however, American ginseng demand increased in China due to its differing effects from that of Asian ginseng (*Panax ginseng*). The former has been described as providing a "yin" effect due to its calm and cooling effects, compared to the latter producing "yang" warming and stimulating effects (Shou-Zhong, 1746; Westerveld & Shi, 2021).

Due to its high demand but low availability in the wild, American ginseng cultivation began in the late 1800s. As demand increased, American ginseng was subject to over-harvesting, poaching, and habitat destruction, resulting in the endangerment of the plant in 2003, as listed by the Species at Risk Act (SARA) (S.C. 2002, c.29). Consequently, Canadian CITES (Convention of International Trade in Endangered Species of Wild Fauna and Flora) permits are required to harvest or cultivate American ginseng (Government of Canada, 2022). In Canada, Ontario is the major exporter of American ginseng and contributed approximately $275 million in revenue in 2015; however, this

value had declined to $133 million in 2023, likely due to the difficulties associated with growing the crop (Government of Canada, 2024).

1.2 Cultivation of American ginseng

To mimic conditions for wild American ginseng grown in deciduous and mixed forest regions, cultivated American ginseng is grown in sandy-loam soils shaded by a synthetic shade cloth to allow 30% sunlight (Charron & Gagnon, 1991; Proctor & Bailey, 1987). Cultivated ginseng requires three to four years of growth for roots to reach marketable size, in contrast to the longer duration of at least nine years for wild grown American ginseng (Beyfuss, 1999; Proctor & Bailey, 1987). Raised soil beds covered by a layer of straw mulch typically of wheat or barley are often used to prevent damage from cold winter weather and to maintain soil moisture in the summer (Punja, 2011). Seeds harvested in late August from the third and fourth years of growth are first cold-stratified (~2°C) across an 18–20-month (two winters) stratification period prior to germination.

Cold-stratified seeds are planted in raised, well-drained, sandy-loam soil beds of pH 5.0 to 6.5 in early September. An umbel-shaped inflorescence develops during the third and fourth years of growth and one to three red berries develop within a pericarp during an 8–12-week period beginning in early spring (Anderson et al., 1993; Carpenter & Cottam, 1982; Charron & Gagnon, 1991). Below ground, American ginseng is composed of an elongated taproot and rhizome, and aboveground an aerial stem grows to comprise a whorl of palmately-compound leaves (Government of Canada, 2002). Ginseng morphology is often described as first-year seedlings possessing one leaf with three leaflets, second-year two-leaved juveniles, and three-to-four-year adults consisting of three to five leaves (Anderson et al., 1993; Carpenter & Cottam, 1982; Charron & Gagnon, 1991; Proctor et al., 2003).

1.3 Replant disease

Replant disease, also termed ‘soil sickness’, occurs in several crops as the result of repeated planting of the same crop in the same field leading to damage in the subsequent growth seasons when the crops are planted. The disease has been extensively explored in

apples (Braun, 1991; Hoestra, 1968) and to a lesser extent in other fruits including grapevines (Waschkies et al., 1993, 1994), peach (Bent et al., 2009), cherry (Mai, 1978), pear (Mai, 1978), and plums (Sewell & Wilson, 1975).

The causal agents for replant diseases are species-specific and are composed of biotic and abiotic factors. Biotic factors often include pathogens such as fungi, oomycete, and nematodes, while abiotic factors often include soil pH and soil composition. For instance, apple replant disease (ARD) is characterized by brown-black root discolouration and reduced growth, yield, and flavour (Mazzola & Manici, 2012). Causal agents contributing to ARD include oomycete pathogens Pythium and Phytophthora and fungal pathogens *Cylindrocarpon* and *Rhizoctonia* (Mazzola, 1998). In addition, the plant-parasitic nematode *Pratylenchus penetrans* (Cobb) Filipjev & Schuurmans-Stekhoven contributes to ARD (Jaffee, 1982; Kanfra et al., 2022). Lastly, abiotic factors such as unbalanced nutrition, low pH, and poor drainage may exacerbate ARD formation (Hoestra, 1968; Mazzola & Manici, 2012).

In addition to several pome and stone fruits, newly planted ginseng grown in a former ginseng garden is often subject to ginseng replant disease (GRD). As proposed by Westerveld and Shi (2021), GRD is defined as the "lingering negative effects of ginseng cropping on subsequent plantings, regardless of the interval and beyond normal pathogen carryover. It is primarily expressed as increased wilting of tops and root rot due to *Ilyonectria mors-panacis*". GRD is characterized by a decrease in crop quality and quantity and dark brown discolouration due to root rot (Campeau et al., 2003; Rahman & Punja, 2005). Specifically, infection begins with darkening of the fibrous root tips progressing into the taproot and eventually into the root tips and lateral roots and causing blackened shriveled roots (Ministry of Agriculture, Food and Rural Affairs, 2015).

Aboveground symptoms are not present until significant infection is in the roots, often leading to disintegrated roots, giving GRD the interchangeable name of 'disappearing root rot' disease (Farh et al., 2018; Westerveld & Shi, 2021). Root rot associated with GRD should not be confused with damping-off disease or rusty root disease. Damping-off disease is characterized by the wilting of leaves and collapsing of the shoot, leading to

plant death likely induced by *Pythium* spp., and is a large threat at the seedlings stage (Reeleder & Brammall, 1994). In contrast, rusty root disease is characterized by reddish-brown areas on the surface of the root and crown of the tap root that can be wiped off (Hildebrand, 1935; Reeleder et al., 2002; Reeleder & Brammall, 1994). Both rusty root and root rot disease have been attributed to *Cylindrocarpon destructans* (Zinssm.) Scholten (reclassified as *Ilyonectria mors-panacis* (A.A. Hildebr.) A. Cabral & Crous) (Hildebrand, 1935; Reeleder et al., 2002; Reeleder & Brammall, 1994).

In Ontario, replant gardens may yield zero or negligible amounts of ginseng due to GRD (Ontario, 2024). Unfortunately, GRD has been reported in replant gardens that had not been used to grow ginseng for up to 80 years prior (Ontario, 2024; Westerveld & Shi, 2021). Suitable garden sites with sandy-loam soils and appropriate environmental conditions are decreasing in availability, and consequently a major concern for commercial ginseng growers is the shrinking area of available land; this has caused approximately 45% of Ontario ginseng growers to leave the industry in the past five years (Ontario, 2024; Westerveld & Shi, 2021).

1.4 Cause and prevention of ginseng replant disease

The primary cause of root rot due to GRD in American ginseng is the fungal pathogen *Ilyonectria mors-panacis* Hildebrand, 1935; Punja, 1997; Reeleder et al., 2002; Reeleder & Brammall, 1994; Westerveld et al., 2023). Damage caused by *I. mors-panacis* can allow for infection by other fungi, consequently exacerbating the disease (Ministry of Agriculture, Food and Rural Affairs, 2015). Other diseases that may be present include *Phytophthora* blight and root rot (Bobev et al., 2003), *Alternaria* leaf blight (Neils et al., 2021), *Rhizoctonia* crown rot (Punja, 1997), *Botrytis* blight (Punja, 1997), caused by *Phytophthora cactorum* (Lebert & Cohn) J. Schröt., *Alternaria panax* Whetzel, *Rhizoctonia solani* J.G. Kühn, and *Botrytis cinerea* Pers., respectively (Westerveld & Shi, 2021). Rusty root rot caused by *Ilyonectria robusta* (A.A. Hildebr.) A. Cabral & Crous has been implicated in Asian ginseng although it was previously speculated that infection by *I. robusta* on American ginseng may not be due solely to disease but may instead be the result of incompatible interactions between ginseng roots and *I. robusta* (Farh et al., 2018; Guan et al., 2020; Jiang et al., 2023).

It was recently determined that several *I. robusta* isolates displayed similar lesion size areas on roots as some *I. mors-panacis* isolates, but were significantly smaller than other *I. mors-panacis* isolates used by Behdarvandi et al. (2023). Amongst the *I. mors-panacis* isolates used in the study, there was a varying degree of virulence when examining root lesion sizes (Behdarvandi et al., 2023). Previously, *Pythium irregulare* Buisman (now *Globisporangium irregulare* (Buisman) Uzuhashi, Tojo & Kakish.) and *Fusarium solani* (Mart.) Sacc were thought to be the primary pathogens of GRD as they have been previously isolated from ginseng garden soils and when tested *in vitro*, were pathogenic towards American ginseng (Ivanov & Bernards, 2012; Ivanov et al., 2016; Nicol et al., 2003). However, *I. mors-panacis* appears to be the primary pathogen of GRD as it is consistently isolated from rotted ginseng roots (Punja, 1997; Reeleder & Brammall, 1994; Westerveld & Shi, 2021).

Aside from root rot due to *I. mors-panacis*, several other factors may exacerbate GRD. Root wounds created by insects and farming tools, can help the pathogen enter into the root more readily (Rahman & Punja, 2005). Soil pH can also contribute to root rot if it decreases to <5, as opposed to the optimal pH ranging from 5.5–6 (Hankins, 2009; Rahman & Punja, 2005). Furthermore, application of iron chelate (FeNaEDTA) to wounded roots and foliar application of iron can increase the production of polyphenol oxidase, an enzyme involved in root rot, and subsequently increase root rot (Rahman & Punja, 2006). Younger roots, particularly two-year old roots, are more susceptible to root rot likely due to their incomplete development (Rahman & Punja, 2005).

To date, no appropriate management method to prevent or treat GRD exists. Fumigation of raised beds with chloropicrin or metam sodium improves yield with some disease still present at harvest. However, fumigation is being phased out due to its detrimental effects to the environment and human health (Westerveld, 2013; Westerveld et al., 2023). Soil solarization may also improve yield for the first two years of ginseng cultivation, but does not produce significant yields of marketable roots in third year of cultivation (Rabas, 2021; Westerveld et al., 2023). Nonetheless, fumigation and solarization-treated replant gardens yield lower than industry averages (Westerveld et al., 2023). Currently available fungicides in Canada (fludioxonil and captan) suppress GRD but do not prevent or

appropriately treat the disease (Ministry of Agriculture, Food and Rural Affairs, 2021. Fungicides propiconazole, benomyl, and fluzinam can reduce *Ilyonectria* root rot in fields; however these fungicides have not been tested in replant gardens (Ziezold et al., 1998). Furthermore, repetitive benomyl application in the early 1970s has resulted in benomyl-resistance in several fungi (Damicone, 2017). Methods such as the application of organic amendments or biocontrol using antagonistic bacteria or fungi, have not been identified but would likely be insufficient in preventing or managing GRD (Westerveld & Shi, 2021).

1.5 Ginsenosides: bioactive triterpenoid saponins

Ginsenosides are bioactive triterpenoid saponins produced by ginseng, and are the medicinal component prized in TCM (Christensen, 2009; Sun et al., 2016; Zhou et al., 2015). To date, more than 100 ginsenosides have been identified within *Panax* spp. and these differ in their profiles among species (Chen et al., 2019; Christensen, 2009; Sun et al., 2023). Ginsenosides are categorized into two groups; dammarane-type saponins featuring a tetracyclic triterpenoid skeleton and oleanane-type saponins featuring a pentacyclic triterpenoid skeleton. Dammarane-type saponins are further grouped into 20(S)-protopanaxadiols (PPD) with saccharide side chains at C-3 and C-20, while the 20(S)-protopanaxatriols (PPT) contain saccharide side chains at C-6 and C-20 and a hydroxyl at C-6. In contrast, ocotillol saponins contain a saccharide side chain at C-6 and an epoxy ring at C-20 position (Nguyen et al., 2020; Yamasaki, 2000). American ginseng predominately produces PPD- and PPT-type ginsenosides (Figure 1.1) and thus these are the ginsenosides commonly studied. PPD-type ginsenosides include Rb_1, Rd, Gypenoside XVII, and F_2, while the most common PPT-type ginsenosides include Rg_1 and Re (Court et al., 1996; Li et al., 1996; Teng et al., 2002). Other ginsenosides produced by American ginseng are Rh, Rg_2, Rh_2, although these are rarely found and are often in small amounts (Qu et al., 2009). Ginsenoside profiles differ amongst *Panax* spp. and can be used to discriminate between *Panax* spp. For instance, the Rb_1:Rg_1 ratio is approximately 10:1 in *P. quinquefolius* whereas it is between 1–3 in *P. ginseng* and *P. notoginseng* (Sun et al., 2011). In addition, ginsenoside biomarkers uniquely present in select *Panax* species, may

be used to distinguish between species. These include, PPT-type ginsenoside F_{11} in *P. quinquefolius*, Rf and Rs_1 in *P. ginseng*, and Ra_3 in *P. notoginseng* (Yang et al., 2016).

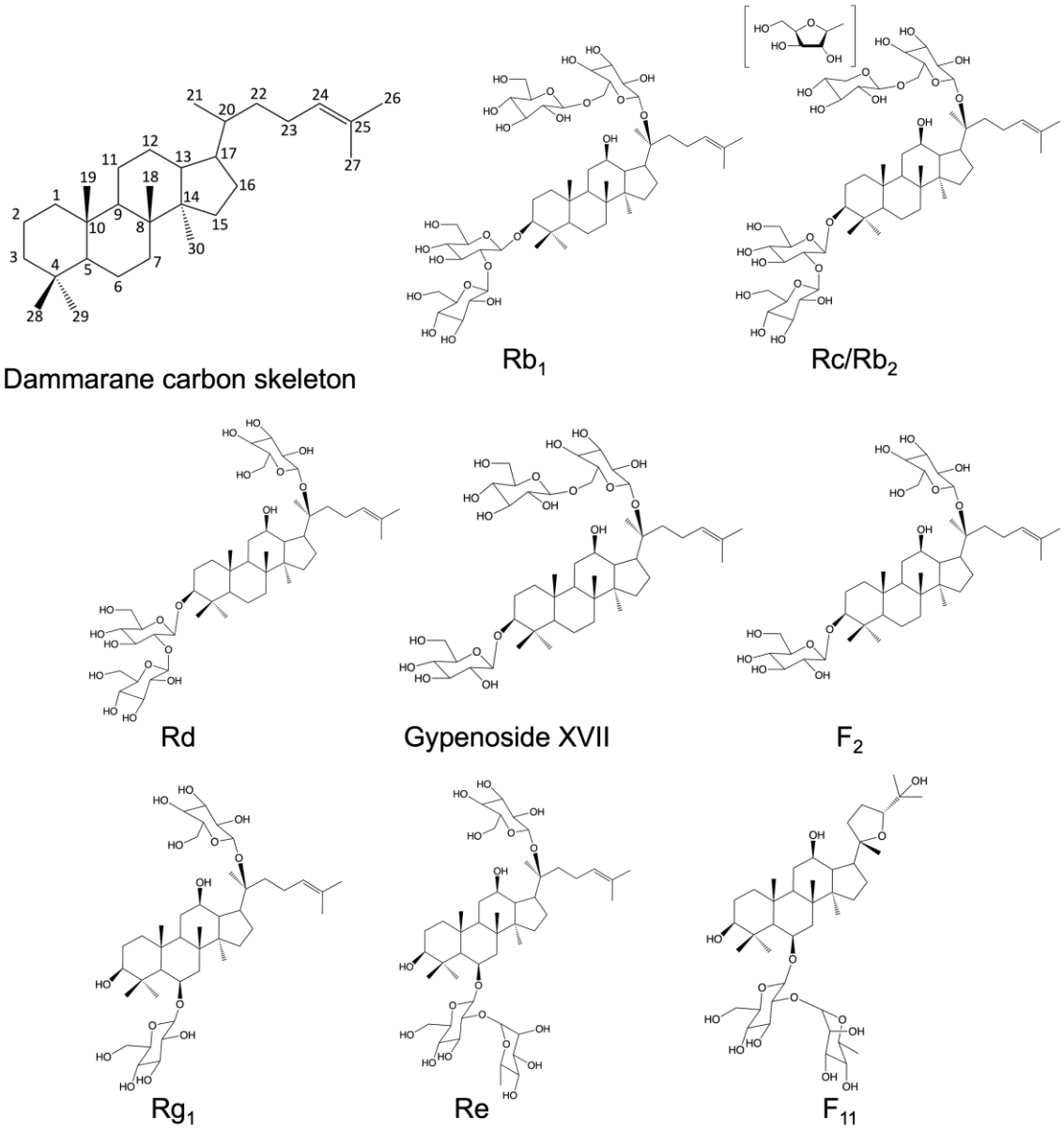

Figure 1.1. Structures of the nine ginsenosides used in my study.
Structures of dammarane-type ginsenosides 20(S)-protopanaxadiols (Rb_1, Rc/Rb_2, Rd, Gypenoside XVII, and F_2) and 20(S)-protopanaxatriols (Rg_1, Re, F_{11}) are shown. Ginsenoside quantity is dependent on the plant tissue and the age of the plant.

Ginsenosides make up approximately 3–8% of the dry weight of American ginseng roots in one-year old and four-year old plants, respectively (Court et al., 1996). In leaves, ginsenosides make up approximately 2–4% of the dry weight in four-year old plants (Li et al., 1996). In four-year old American ginseng, the quantity of ginsenosides are greatest

in fibrous roots > flower > branch root > main root > leaf > stem. In regards to American ginseng age, total ginsenoside concentration in roots increases from 1 to 5-year old roots and is mainly driven by increase in Re and Rb_1 (Li et al., 1996; Qu et al., 2009). Total concentration in leaves remain relatively stable from 1 to 5-year old plants with the exception of lower ginsenoside concentration in three-year old plants, and is mainly composed of Rd and Rb_3 (Qu et al., 2009). Ginsenoside profiles have been studied in diseased American ginseng (i.e., normal, mild, moderate, and severe disease) and it has been shown that ginsenoside abundance and profiles are relatively normal regardless of disease. Diseased roots however, are not attractive to buyers and therefore are not marketable (Yu et al., 2021).

As previously mentioned, medicinal applications of ginsenosides in TCM have been used for centuries. Research shows that specific ginsenosides may have different medicinal effects. For instance, correlations have been found between Rg_1 and Rb_1 enhancing central nervous system (CNS) activity (Chang et al., 2008), Rb_1, Rg_1, Rg_3, and Rh_2 in protective effects on neurodegeneration, Rh_2 and Rg_3 in suppressing various cancers (Chen et al., 2018; Chung et al., 2013), and Rg_1 and Rg_3 in relaxing smooth muscle (Kang et al., 1995; Kim et al., 2003). However, more research needs to be done on the potential medicinal effects in humans as many of these findings are reported in rats and mice (Xiang et al., 2008).

1.6 *Ilyonectria*: contributor to ginseng replant disease

Ilyonectria mors-panacis is an ascomycete in the Nectriaceae family that spreads through its mycelial growth and produces asexual conidia, asexual chlamydospores, and sexual ascospores (Cabral et al., 2012). For long periods, *I. mors-panacis* remains in the soil as chlamydospores to overcome harsh temperature conditions (Kang et al., 2016) and can grow in low oxygen environments (Sutherland et al., 1989). Moreover, *I. mors-panacis* and the related *I. robusta* significantly increase in abundance in *P. ginseng* garden soils during cultivation and are in the greatest abundance in the fourth year of cultivation (Tong et al., 2021). In American ginseng garden soils, *I. mors-panacis* has also been

identified in replant soils, in trace amounts in first-time harvest soils, and in soils with no ginseng previously grown (Rajsp, 2023).

The first investigation into root rot due to *I. mors-panacis* was in 1918 by Zinssmeister, who isolated *Ramularia panacicola* Zinssm. and *R. destructans* Zinssm. from ginseng root rot, both of which were later reclassified as *I. mors-panacis* (Cabral et al., 2012; Westerveld & Shi, 2021). In 1935, A.A. Hildebrand differentiated *R. mors-panacis* A.A. Hildebrand and *R. robusta* A.A. Hildebr. from *R. panacicola* (Hildebrand, 1935), the former reclassified as *I. mors-panacis* and the latter as *I. robusta* (Cabral et al., 2012). As research on GRD grew, *Cylindrocarpon destructans* (formerly *Ramularia destructans*) was the first confirmed causal agent of GRD; however, this species was also reclassified as *I. mors-panacis* (Cabral et al., 2012). In older literature, *I. mors-panacis* is identified as *C. destructans* as this reclassification occurred in 2012 (Cabral et al., 2012).

In 2012, an extensive multi-gene analysis and investigation into morphological variation on *Ilyonectria* isolates was conducted. The study focused on *Cylindrocarpon*, *Ilyonectria*, *Neonectria*, and *Ramularia* species using four gene regions including partial β- tubulin (TUB), histone H3 (HIS), translation elongation factor 1-α (TEF), and nuclear ribosomal RNA-Internal Transcribed Spacer (nrRNA-ITS), but found that HIS gene sequences were just as informative in identifying species as combining the four gene sequences (Cabral et al., 2012). As theorized, several species were distinguished from *Ilyonectria radiciola* (Gerlach & L. Nilsson) P. Chaverri & Salgado and were proven to be a species complex as members did not group monophyletically and instead clustered in species groups (Cabral et al., 2012). Consequently, twelve new taxa were delineated and the species *Ilyonectria mors-panacis* was first proposed. These twelve new taxa included *I. mors-panacis*, *I. robusta*, *I. rufa* A. Cabral & Crous, *I. pseudodestructans* A. Cabral, Rego & Crous, *I. lusitanica* A. Cabral, Rego & Crous, *I. cyclaminicola* A. Cabral & Crous, *I. venezuelensis* A. Cabral & Crous, *I. europaea* A. Cabral, Rego, & Crous, *I. panacis* A. Cabral & Crous, *I. crassa* (Wollenw.) A. Cabral & Crous, *I. gamsii* A. Cabral & Crous, and *I. liliigena* A. Cabral & Crous (Cabral et al., 2012).

Infection by *I. mors-panacis* has been attributed to other factors. Enzymes such as pectin lyase, laccases, proteases, pectinase, and cellulase have been isolated and contribute to root degradation (Pathrose, 2012; Rahman & Punja, 2005). Furthermore, virulent strains of *C. destructans*/*I. mors-panacis* have been found to produce greater amounts of pectinase and polyphenol oxidase than avirulent strains (Rahman & Punja, 2005).

Siderophores may also facilitate infection on ginseng by *I. mors-panacis*. A siderophore (N,N0 ,N00-triacetylfusarinine C (TAFC)) has been isolated from several *Ilyonectria* isolates and appears to be produced in virulent isolates but is absent in avirulent isolates (Walsh et al., 2022). Siderophores are metabolites produced during iron-depleted conditions and effectively bind iron in the environment (Haas, 2003). It is suspected that siderophores contribute to root rot as iron is a key component to many infection processes (Weinberg, 1999). It has been suggested that the application of purified recombination AfEstB, a TAFC esterase, could be used as a siderophore-targeted biocontrol of *Ilyonectria* although this has not been tested on a large-scale in ginseng fields (Walsh et al., 2022).

1.7 Ginsenoside-pathogen interactions

Ginsenosides and fungal pathogens may both contribute to GRD as previous studies have identified interactions between ginsenosides and pathogens. Ginsenosides have known antimicrobial activity and have been shown to reduce membrane integrity of microbes (Sikkema et al., 1995). It has also been shown that ginsenosides are mildly fungitoxic towards some soil-borne fungi, but show differential effects on pathogen growth (Nicol et al., 2003; Zhao et al., 2012). For instance, ginsenosides can inhibit the growth of several fungi including *Trichoderma* spp., *Alternaria panax*, *A. porri* (Ellis) Cif., *A. solani* Sorauer, *Fulvia fulva* (Cooke) Cif., *Fusarium solani*, *F. oxysporum* Schltdl., and *Aspergillus nidulans* (Eidam) G. Winter, but promote the growth of *C. destructans*/*I. mors-panacis* (Nicol et al., 2002; Zhao et al., 2012). Specifically, Zhao et al. (2012) demonstrated that PPT-type ginsenosides were inhibitory on the five nonpathogens and *C. destructans*/*I. mors-panacis*. However, PPD-type ginsenosides stimulated the growth of *C. destructans*/*I. mors-panacis* and continued to inhibit the growth of the five nonpathogens. On the other hand, growth rate of *I. robusta* and *I. leucospermi* L.

Lombard & Crous was significantly reduced in the presence of PPT- and total ginsenosides and not affected by PPD-type ginsenosides (Farh et al., 2016). Ginsenosides Gypenoside XVII and F_2 inhibit hyphal growth of *C. destructans*/*I. mors-panacis* and nonpathogens of ginseng (*Fusarium graminearum* Schwabe, *Exserohilum turcicum* (Pass.) K.J. Leonard & Suggs, *Phytophthora megasperma* Drechsler, and *Pyricularia oryzae* Cavara) (Wang et al., 2018).

Oomycete growth is also affected by ginsenosides. The growth of *Globisporangium irregulare* and *Phytophthora cactorum* has been shown to increase when exposed to ginsenosides (Nicol et al., 2003). A correlation between the pathogenicity of *G. irregulare* and the metabolism of ginsenosides was shown for a group of *G. irregulare* accessions isolated from a range of host plants across a wide geographical range (Ivanov & Bernards, 2012). That is, as pathogenicity increased, the ability to deglycosylate ginsenosides increased (Ivanov & Bernards, 2012). Furthermore, plate assays with *G. irregulare* and select ginsenosides were examined and showed contrasting effects. Notably, mycelial growth was enhanced on plates with crude ginsenoside extract from roots and with Rb_1 whereas, a zone of inhibition by *G. irregulare* resulted when grown in the presence of F_2 (Ivanov et al., 2016).

The metabolism of ginsenosides by some fungal and oomycete pathogens of ginseng has also been explored. When *I. mors-panacis* was cultured with ginsenosides, it metabolized PPD-type ginsenosides (Rb_1, Rb_2, Rc, and Rd), but not PPT-type ginsenosides (Re and Rg_1) (Wang et al., 2018). Similarly, *I. mors-panacis* significantly metabolized only PPD-type ginsenosides by approximately 13% (Farh et al., 2016). In contrast, *I. robusta* and *I. leucospermi* significantly metabolized both PPD- and PPT-type ginsenosides. However, *Panax* ginseng roots infected with *I. mors-panacis* have significantly reduced PPD- and PPT-type ginsenoside concentrations. Other fungal species, such as *I. robusta*, significantly increased PPD- and PPT-type ginsenoside concentrations in infected P. ginseng roots. In *I. leucospermi* infected *P. ginseng* roots, PPD-type ginsenoside concentrations were significantly increased (Farh et al., 2016).

Lastly, when *G. irregulare* was cultured with ginsenosides, nearly all PPD-type ginsenosides (Rb_1, Rb_2, Rc, Rd, and some Gypenoside XVII) were metabolized into the minor ginsenoside F_2 via deglycosylation (Yousef & Bernards, 2006). Overall, ginsenosides, have antimicrobial effects on some fungi (Farh et al., 2016; Wang et al., 2018; Zhao et al., 2012) increased growth of others (Farh et al., 2016; Wang et al., 2018), and stimulate chemotropism or chemotaxis in oomycetes (Ivanov et al., 2016).

To date, the presence of ginsenosides in soil during American ginseng cultivation has not been investigated extensively. This analysis is critical to understand the ginsenoside-pathogen interactions that may be occurring in ginseng garden soil during cultivation and post-harvest that may contribute to conditions in which GRD can be established. In Sanqi ginseng (*Panax notoginseng*) cultivated soils, ginsenosides have been measured in one-, two-, and three-year bulk soils with no prior ginseng cultivation and found to gradually increase from one to three years of cultivation (~2, ~4, ~6 µg/g soil, respectively) (Yang et al., 2015). In regard to American ginseng, ginsenosides have been detected in rhizosphere soil when collecting root-associated soil during ginseng cultivation (Nicol et al., 2003), but this is not representative of what is occurring during cultivation in bulk soil. The persistence of ginsenosides post-harvest has been previously investigated in soils from two Ontario ginseng fields (one near Harrow and the other near Vineland) (Kaberi, 2021). While trace amounts of ginsenosides were found in soils one-month post-harvest, and approximately one- and two-years post-harvest, there were no measurements of ginsenosides from the same soils during cultivation. Consequently, it remains unclear how the measured amounts of ginsenosides relate to how much was present during cultivation.

1.8 Research objectives and hypotheses

To better understand ginsenoside-pathogen interactions, a comprehensive analysis of ginsenosides in ginseng garden soils during ginseng cultivation, and their subsequent persistence post-harvest, is critical to understanding how ginsenosides may contribute to GRD-potential in ginseng garden soils. My analysis will provide insight into which ginsenosides accumulate in the soil during American ginseng cultivation, and which

ginsenosides remain in newly harvested ginseng garden soils, that may be interacting with known pathogens, such as *I. mors-panacis*. I hypothesize that ginsenosides will accumulate across four years of ginseng cultivation and persist in relatively low abundance during the first-year post-harvest. As mentioned, ginsenosides have been measured in Sanqi ginseng soils but, only for one–three years of cultivation, not four (Yang et al., 2015). The persistence of ginsenosides has been previously investigated in two Ontario ginseng fields, but these trace amounts of ginsenosides found post-harvest could not be compared to ginsenoside abundance during ginseng cultivation in the same fields. It is critical to track the accumulation of ginsenosides across ginseng cultivation and in the same fields post-harvest, to determine if ginsenosides persist in soil and ultimately understand whether they may directly contribute to GRD. The first objective (1) of this research project was to complete the measurement of ginsenoside accumulation in soils planted with ginseng for the first time, across four years of cultivation and measure ginsenoside persistence in soils post-harvest. The measurement of ginsenoside accumulation was initiated by Karina Kaberi in her MSc work, but only encompassed the first two years of cultivation (Kaberi, 2021). I will complete measurements for the third and fourth years of cultivation as well as for the first-year post-harvest.

Previously, the virulence of *Cylindrocarpon destructans*/*I. mors-panacis* was examined on American ginseng (Seifert et al., 2003), but several isolates studied have since been reclassified and, consequently, an investigation on the virulence of *Ilyonectria* isolates on American ginseng is necessary. As part of this research project, I aimed to confirm the species identity of the isolates used in this study, received as *I. mors-panacis*, by determining the sequence of a portion of their HIS gene and placing these in a phylogeny together with sequences of known reference strains. Knowledge of species identification of the *Ilyonectria* isolates used in this study is critical to have a comprehensive understanding of findings from subsequent objectives.

It is known that *I. mors-panacis* is present in trace amount in non-replant ginseng garden soils and both *I. mors-panacis* and *I. robusta* significantly increase in abundance during

ginseng cultivation (Rajsp, 2023; Tong et al., 2021). However, root rot is greater in GRD gardens than first-time planted gardens (Rajsp, 2023). I hypothesize that exposure to ginsenosides prior to inoculation of ginseng roots will alter the virulence of low virulent *Ilyonectria* isolates toward American ginseng. My second objective (2) was to determine disease severity in ginseng roots inoculated with different isolates of *Ilyonectria* with and without previous exposure to ginsenosides.

It is evident from previous literature that various fungal and oomycete pathogens metabolize some ginsenosides. A positive correlation has been observed between the metabolism of ginsenosides and virulence on ginseng by *G. irregulare* (Ivanov & Bernards, 2012); however, it is not clear if specific *Ilyonectria* species or isolates metabolize ginsenosides equally effectively. I hypothesized that different *Ilyonectria* isolates will metabolize ginsenosides differently. I predict that the isolates with the most efficient metabolism of ginsenosides will display greater virulence towards American ginseng. My third objective (3) was to determine whether different isolates of *Ilyonectria* metabolize ginsenosides differently and correlate the findings with virulence results from objective 2.

Overall, completion of my objectives will establish the degree to which ginsenosides accumulate in ginseng garden soil, whether they persist beyond harvest, and what role they may play in determining the virulence of *Ilyonectria*. If *Ilyonectria* develops increased virulence post-exposure to ginsenosides, it may provide insight as to why root rot is prevalent in ginseng replant soils even though *I. mors-panacis* was likely present in the garden soil prior to the cultivation of the first ginseng crop.

2 Materials and Methods

2.1 Ginseng garden soil sampling locations

Three independent commercial gardens with no known prior ginseng cultivation history, located in Norfolk County, Ontario, Canada (Figure 2.1), were used in this study. Each garden was seeded in August 2018 in raised soil beds. Soil was sampled (see section 2.2) in the fall (Oct/Nov) of 2018 and at four time points each year, for four years, until ginseng was harvested in October 2022 (Table 2.1). To examine ginsenoside persistence in these soils, we collected soil from sites 1 and 3 (n = 5 per site) in 19 L buckets (Home Depot) on April 21, 2023 to be able to resample from these soils for a year post-harvest. Site 2 soil was not available for use. Buckets were transferred to the Environmental Sciences Western Field Station (ESW), Ilderton, Ontario on May 4, 2023, to remain undisturbed for one year (Table 2.1). Buckets, with holes drilled in the bottom (15 per bucket, ~2 cm diameter) were placed in the ground up to their rim, ~95 cm apart from each replicate of the same site and surrounded by fencing to prevent disturbance (Figure 2.2).

Figure 2.1. Map displaying locations of three ginseng garden sites (1, 2, 3) in Norfolk County, Ontario.
Google maps image of Norfolk Country, Ontario (red-dotted line). Image accessed August 2024. Scale bar displays 20 km distance.

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Table 2.1. Time period and soil collection dates for sites 1, 2, and 3.
Soils collected during planting, year 1 (2019), year 2 (2020), year 3 (2021), year 4 (2022), and harvest, were collected in Norfolk County, Ontario. Soils resampled during persistence time period, were collected at Environmental Sciences Western Field Station (ESW). Ginseng was harvested in early October and soils were collected in late October.

Time Period		Soil Collection Date
Planting[a]	**Oct/Nov**	October 23, 2018
Year 1 (2019)[a]	**Apr/May**	April 25, 2019
	Jun/Jul	June 27, 2019
	Aug/Sep	September 19, 2019
	Oct/Nov	October 29, 2019
Year 2 (2020)[a]	**Apr/May**	May 6, 2020
	Jun/Jul	June 25, 2020
	Aug/Sep	August 24, 2020 (Site 1, 3), September 1, 2020 (Site 2)
	Oct/Nov	October 23 (Site 1, 3) and 25, 2020 (Site 2)
Year 3 (2021)[b]	**Apr/May**	May 5, 2021 (Site 2, 3), May 6, 2021 (Site 1)
	Jun/Jul	June 28, 2021
	Aug/Sep	August 26, 2021 (Site 1, 3), September 3, 2021 (Site 2)
	Oct/Nov	November 4, 2021
Year 4 (2022)[c]	**Apr/May**	April 29, 2022
	Jun/Jul	July 6, 2022
	Aug/Sep	August 23, 2022
Harvest[c]	**Oct/Nov**	October 27, 2022
Persistence[c]	**Apr/May**	May 4, 2023 (Site 1, 3)
	Oct/Nov	October 23, 2023 (Site 1, 3)

[a]Ginsenosides extracted and analyzed by Karina Kaberi (former MSc student).
[b]Ginsenosides extracted and analyzed by Andrew Rabas (current PhD student).
[c]Ginsenosides extracted and analyzed, and whole dataset analysis by Anka Colo.

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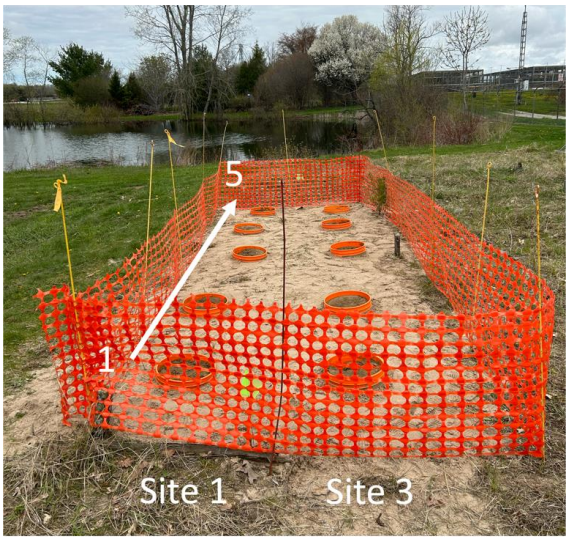

Figure 2.2. Arrangement of soil samples to monitor soil ginsenoside persistence. Soil used to examine persistence of ginsenosides in soil was collected in 19 L pails and placed in the ground at the Environmental Sciences Western field station, Ilderton, ON**.** Soil samples from site 1 are displayed on the left, replicates 1–5 from front to back. Soil samples from site 3 are displayed on the right, replicates 1–5 from front to back.

2.2 Soil sampling

At each garden site, three soil cores (~2.5 cm diameter, 30 cm deep) from each of five sampling areas were collected. At each site, sampling areas started at the edge of the garden (northeast for sites 1 and 2, southeast for site 3), through the laneway to the 7th bay, and in the field from the 5th post. Starting at the 5th post, soils were sampled from the middle bed in the bay opposite the post for five sequential posts, yielding five sampling areas (Figure 2.3). At each site, control soil samples were collected starting opposite the 7th bay of each site, sampled at every 1.5 posts along the edge of the field for 5 sampling areas. Garden soil was collected using a LaMotte 1055 galvanized steel soil sampler and control soil was collected using a trowel. Both samplers were cleaned between each sampling area using paper towel and sterilized with 20% bleach (1% sodium hypochlorite) prior to departing the site. Soils were collected into plastic bags (Ziploc® Brand Freezer Bags Medium), transported in a cooler to Western University and stored at

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-20°C until analyzed. Prior to extraction, soils were dried in 50 mL polypropylene tubes (Falcon, Fisher Scientific) at 60°C for 7 days (Isotemp Oven, Fisher Scientific). Soils were sieved using a 35 mesh (500 μm) (Fieldmaster) to remove debris.

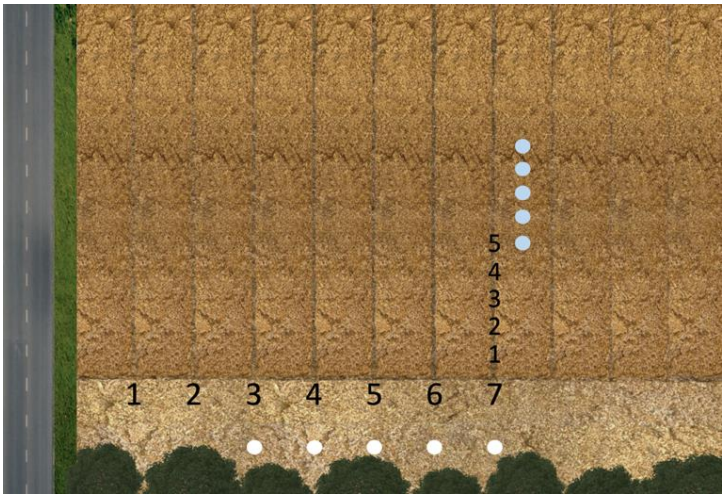

Figure 2.3. Schematic of soil sampling sites with a ginseng garden.
Horizontal numbers represent individual bays. Vertical numbers represent posts. Blue circles located in the middle of ginseng bed represent ginseng garden sampling areas. White circles represent non-ginseng garden (control) sampling areas outside the ginseng garden beds. Three soil cores were collected and pooled from each sampling area to form a replicate.

2.3 Extraction of ginsenosides

Ginsenosides were extracted from soil using an optimized ginsenoside extraction protocol developed by Kaberi (Kaberi, 2021). Briefly, 5 g dried soils were weighed into 50 mL polypropylene tubes (Falcon, Fisher Scientific) and extracted with 20 mL of MeOH (80%) (OptimaTM for HPLC, Fisher ChemicalTM) on a gyratory shaker (175 rpm) for one hour. Extractions were repeated twice more, once for one-hour extractions and once overnight (at least 18 hours). At the end of each extraction, samples were centrifuged (1700 × g, 5 min) and the supernatant collected. Extracts were pooled into 100 mL round bottom flasks, dried to aqueous using rotary evaporation (Rotavapor R-100®, Buchi, Delaware, US) and transferred to 15 mL polypropylene tubes (Falcon, Fisher Scientific). Samples were purified and concentrated using HyperSep™ C-18 200 mg solid phase extraction (SPE) columns (Product Number 60108-303, Thermo Scientific™). Prior to

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use, SPE columns were washed with 3 mL of MeOH (100%) and equilibrated with 6 mL of deionized water. Samples were then loaded onto equilibrated SPE columns, and columns were washed with 3 mL of MeOH (30%). Ginsenosides were eluted with 4 × 1 mL of MeOH (100%) and the eluents pooled and dried under N_2 at 60°C (50 port RapidVap® Vertex Nitrogen Dry Evaporator). Dried ginsenoside residues were reconstituted in 25–50 µL of MeOH (50%).

2.4 Identification and quantitation of ginsenosides using liquid-chromatography mass-spectrometry

A composite ginsenoside mixture solution (0.02 mg/mL) of ten ginsenosides (Rg_1, Gypenoside XVII, F_{11}, F_2, Rd, Rb_2, Rb_3, Rc, Rb_1 and Re) was prepared using MeOH (100%) as a chromatographic standard for liquid chromatography mass spectrometry (LCMS). Prior to LCMS analysis, ginsenoside standards and soil extract samples (25 µL) were diluted with an equal volume of internal standard avenacoside-A (25 µL, 0.625 ng/mL or 0.05 µg/mL in water) (SMB00251, Sigma Aldrich) in HPLC vials fitted with microvolume inserts. For each LCMS worklist, triplicate injections of a solvent blank (20% MeOH) were followed by the ginsenoside standard mixture solution, samples, and the same ginsenoside standard mixture solution used at the beginning of the worklist to ensure consistency.

An Agilent 1260 liquid chromatograph coupled with a 6230 ToF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), fitted with a Dual Spray ESI (electrospray ionization) was used to quantify ginsenosides from soil (adapted from Ivanov et al. (2016)). Samples (2 µL) were injected onto a C8 column (Zorbax Eclipse Plus column, 2.1 × 50 mm, 1.8 µm, Agilent Technologies, Santa Clara, CA, USA) at a flow rate of 0.400 mL/min. Automated internal calibration was conducted using reference ions 121.0508 and 922.0096 m/z during each run. Samples were eluted with a gradient of acetonitrile (CH3CN) in H_2O using solvent A (0.1% HCO_2H, 1 mg/L NaOAc) and solvent B (90% CH_3CN, 0.1 % HCO2H, 1 mg/L NaOAc). Elution gradient was as follows: 20% B in A (1 min), linear gradients to 35% B (2 min) and 100% B (6 min) and held at 100% B (1 min) before returning to initial conditions. The column was

equilibrated at start conditions (20% B) for 12 minutes between runs for a total sample run time was 23 minutes. The eluent entered the mass spectrometer through an ESI source with a N_2 sheath gas flow of 12 L/min at 300°C, a nebulizer pressure of 45 psi, and the fragmentor voltage set to 120 V with a Vcap of 4500 V. Ginsenosides, as their Na^+- adducts $[M + Na]^+$, were detected and quantified in positive ion mode. For planting–harvest soils, calibration curves were used and created by Kaberi (2021) (See Appendix; Supplementary Table 1). New calibration curves were created to quantify ginsenosides in soils sampled for persistence measurements (See Appendix; Supplementary Table 1). Calibration curves were created using ginsenoside standards (Rg_1, Gypenoside XVII, F_{11}, F_2, Rd, Rb_2, Rb_3, Rc, Rb_1 and Re) (Biopurify Phytochemicals Ltd., Chengdu, China) serially diluted with 50% MeOH. Data were analyzed using (Agilent MassHunter Qualitative Analysis software, version B.10.0). Extracted ion chromatographs were generated from total ion chromatographs by inputting m/z values of interest (See Appendix; Supplementary Table 1) and setting quant ion m/z to symmetric (m/z) ± 0.1000 (tolerance), and integrated peak areas and retention times were recorded.

2.5 Selected *Ilyonectria* isolates and species confirmation using Sanger sequencing

Twelve independent *Ilyonectria* isolates (received as *I. mors-panacis*) were obtained from the Canadian Collection of Fungal Cultures (CCFC, Ottawa). Isolates were originally collected from various host plants and geographic regions (Table 2.2). To confirm the identity of the *Ilyonectria* species in this thesis, Sanger sequencing of fungal histone H3 (HIS) gene was used.

Table 2.2 Twelve *Ilyonectria* isolates selected from Canadian Collection of Fungal Cultures (CCFC, Ottawa, Canada).
Ilyonectria species number used for identification. DAOMC code provided by CCFC. *Ilyonectria* isolates were isolated from a host plant and in a specified location, stated by CCFC

***Ilyonectria* Species Number**	**DAOMC Code**	**Host Plant**	**Location**
I.sp.1	226721	*Pseudotsuga menziesii*	Canada, British Columbia
I.sp.2	251609	*Picea glauca*	Canada, Quebec
I.sp.3	251610	*Panax quinquefolius*	Canada, Ontario
I.sp.4	150670	*Poa pratensis*	Canada, Alberta
I.sp.5	251608	*Pseudotsuga menziesii*	Canada, British Columbia
I.sp.6	139398	*Prunus cerasus*	Canada, Ontario
I.sp.7	251602	*Panax quinquefolius*	Canada, Ontario
I.sp.8	251603	*Panax quinquefolius*	Canada, Ontario
I.sp.9	230337	*Panax* sp.	Japan, Nagano Prefecture
I.sp.10	230338	*Panax* sp.	Japan, Nagano Prefecture
I.sp.11	234582	*Panax quinquefolius*	Canada, Ontario
I.sp.12	251601	*Panax quinquefolius*	Canada, Ontario

Individual *Ilyonectria* isolates were grown in potato dextrose broth (20 mL) (PDA, R454312, Remel, Fisher Scientific) on a gyratory shaker (150 rpm) for one week. DNA was isolated using a modified cetyltrimethylammonium bromide (CTAB) and chloroform extraction and ethanol precipitation (Bainbridge et al., 1990; Lee et al., 1988; Moller et al., 1992). Briefly, 100 µL of mycelial suspension was ground using a mortar and pestle in liquid nitrogen and transferred to 1.7 mL microcentrifuge tubes. Next, 400 µL of buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 3% SDS) and 4 µL of Proteinase K (20 mg/mL) (PB0451, Bio Basic) were added, and the mixture incubated (65°C, 45 min) while mixing every 5–10 min. Samples were centrifuged (17 000 × g, 5 min) and 400 µL of supernatant transferred to clean 1.7 mL tubes. RNAse A (2 µL, 20 mg/mL) (CAS No. 9001-99-4, Product No.R5500, Millipore Sigma) was added and the samples incubated in a dry block (37°C, 15 min). Next, 180 µL of 5 M NaCl (pre-heated to 65°C) and 65 µL of 10% CTAB (pre- heated to 65°C) were added and the samples incubated for a further 10 min at 65°C. Next, 650 µL of SEVAG (chloroform: isoamyl alcohol (24:1)) was added, the samples gently mixed, and incubated at -20°C for 45 min. Samples were centrifuged at 4°C (15 min, 17 000 × g) and the aqueous phase (top 450 µL) transferred to autoclaved

1.7 mL tubes containing 15 µL of NaOAc (3 M) and mixed. Cold ethanol (1 mL, 100%) was added, and the samples mixed and centrifuged (10 min, 10 000 × g). The supernatant was decanted, and the pellet washed twice with 1 mL of ethanol (70%), centrifuging (5 min, 10 000 × g) to collect the pellet each time. DNA pellets were dried and dissolved in 50 µL of warm $T_{10}E_{0.1}$ (10 mM Tris-HCl, 0.1 mM EDTA) (60°C).

Since the fungal HIS gene sequence provides the same species level identification as the combination of HIS, internal transcribed spacer (ITS) of the nrRNA gene operon, partial β- tubulin (TUF), and translation elongation factor 1-α (TEF) (Cabral et al., 2012), the HIS gene sequence alone was used in this thesis. To amplify HIS gene (~650 bp) using polymerase chain reaction (PCR), forward primer (CYLH3F; 5'-AGG TCC ACT GGT GGC AAG-3') and reverse primer (CYLH3R; 5'-AGC TGG ATG TCC TTG GAC TG-3') were used (Crous et al., 2004). In each PCR sample, up to 4 µL of DNA template and sufficient MasterMix were added to create a final volume of 25 µL. MasterMix solutions (21 µL) consisted of 12.5 µL Accustart II ToughMix DNA Taq Polymerase (containing MgCl2, dNTPs, Taq DNA polymerase) (QuantaBio, MA, USA), 6.0 µL molecular grade water, 1.25 µL forward primer (CYLH3F; 5 µM), and 1.25 µL reverse primer (CYLH3R; 5 µM). PCR was carried out using a Biometra Tadvanced thermocycler (Montreal Biotech Inc.) with the following conditions: denaturation at 94°C for 4 min, followed by 30 cycles of denaturation, annealing, and extension, where each cycle consisted of 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, and a final extension of 72°C for 7 min. HIS gene amplification was confirmed using 1.5% gel electrophoresis stained with SafeView Classic DNA Dye (Applied Biological Materials Inc.).

Following gel electrophoresis, PCR products were purified using Qiagen MinElute PCR Purification Kit (ID: 28004, QIAGEN®). DNA concentration (ng/µL), A260/A280, and A260/A230 were examined using a spectrophotometer (Nanodrop OneC Spectrophotometer, Thermo Scientific, De, USA). Lastly, purified PCR products were sequenced with CYLH3 (HIS) primers (Crous et al., 2004) using Sanger sequencing at Robarts Research Institute (London, Ontario). Forward and reverse reads were aligned (~650 bp) using SnapGene software (Version 6.2.2, SnapGene®, San Diego, California)

and consensus sequences (400 bp) were used for species identification using Basic Local Alignment Search Tool (BLAST) (National Library of Medicine, National Center for Biotechnology Information, NCBI).

2.6 Phylogenetic analysis of *Ilyonectria* species

All twelve *Ilyonectria* isolates used in this thesis were compared to 33 other *Ilyonectria* isolates with varied species identity using HIS gene sequence data from GenBank. In total, 45 *Ilyonectria* isolates were aligned using MAFFT multiple sequence alignment software (version 7; Katoh & Standley, 2013). Then, using Molecular Genetics Analysis (MEGA, version 11; Tamura et al., 2021), sequences were trimmed to 400 bp, and compared using a Maximum Likelihood phylogenetic tree (HKY model) computed using 1000× bootstrap analysis.

2.7 Ginseng seedling production

One-year old American ginseng seedlings were prepared by Andrew Rabas (PhD student, Bernards; as described in Ivanov et al., 2016). Twice stratified American ginseng seeds were obtained by Dr. Sean Westerveld (Ontario Ministry of Agriculture, Food and Rural Affairs, OMAFRA) and Amy Shi (Ontario Ginseng Grower's Association, OGGA) and kept in a cold room (4°C) until use. Seedlings were planted in 5% bleach (0.25% sodium hypochlorite)-sterilized 4-inch plant pots filled with 600 g of PRO-MIX soil (Mycorrhizae General Purpose Plant Growing Medium) (four seeds per pot). Seedlings were grown in a growth chamber for four weeks with the following conditions: 25°C, 60% humidity, 16h/8h light/dark cycle. Seedlings were watered approximately once a week.

2.8 Pathogenicity assay of *Ilyonectria* isolates in American ginseng

To test the pathogenicity of *Ilyonectria* isolates on American ginseng, one-year old American ginseng seedlings were inoculated with each *Ilyonectria* isolate using an *in*

vivo inoculation method adapted from Reeleder & Brammall (1994) and Ivanov & Bernards (2012). Twelve *Ilyonectria* isolates were tested (twelve treatments) along with one control. Five replicates per treatment (twelve treatments with *Ilyonectria* and one treatment as control) were used. *Ilyonectria* isolates were grown on potato dextrose agar (R454312, Remel, Fisher Scientific) in Petri plates (100 mm × 15 mm, Fisher Scientific) using a 1.2 cm diameter plug of an *Ilyonectria* isolate that had previously been grown for three plate transfers on PDA. Isolates were grown for 2–3 weeks in the dark at room temperature, to approximately 7 cm diameter. PDA plates without *Ilyonectria* were used for control samples. First, 590 g of three times autoclaved Bomix® construction sand was weighed into a 500 mL beaker. *Ilyonectria* cultures (7 cm diameter) were placed into the beaker with sand, and thoroughly mixed using a spatula. Next, 100 mL of ultrapure water was added to the sand-culture mixture and mixed again. One autoclaved paper towel was placed into the bottom of a 4-inch pot that had been previously sterilized with 5% bleach (0.25% sodium hypochlorite) solution for 24 hours, and the sand-culture mixture was added to the pot. A 3-inch-deep hole was created in the center of the sand and a single one-year old American ginseng seedling was placed inside. American ginseng seedlings in pots were placed in metal dissection trays (three pots per tray) and placed on a table beneath a shaded window facing south-east to mimic ginseng garden growth conditions and rotated along the table every 3–4 days. Seedlings were watered (75 mL) with Hoagland's nutrient solution (Hoagland & Arnon, 1950) into metal trays when needed (approximately twice a week). Hoagland's nutrient solution consisted of 2 ml/L $MgSO_4$ (1M), 4 mL/L KH_2PO_4 (1M), 10 mL/L KNO_3 (1M), 10 mL/L $Ca(NO_3)_2$ (1M), 2 mL/L Fe-EDTA 3.35 g/L EDTA, 2.50 g/L $FeSO_4 \cdot 7H_2O$), and 2 mL/L micronutrients. Micronutrients consisted of 2.86 g/L H_3BO_3, 1.81 g/L $MnCl_2 \cdot 4H_2O$, 0.11 g/L $ZnCl_2$, 0.074 g/L $CuSO_4 \cdot 5H_2O$, 0.029 g/L $Na_2MoO_4 \cdot 2H_2O$).

2.8.1 Pathogenicity assay: chlorophyll fluorescence

Chlorophyll fluorescence (F_v/F_m, as a measure of photochemical efficiency of photosystem II) was recorded using a chlorophyll fluorometer (Opti-Sciences OP30p+). Chlorophyll fluorometer settings were as follows; power: 40%, saturation: 60%, width:

1.0 s. Chlorophyll fluorescence measurement clips were placed on the second (middle) leaf of each seedling and leaves were allowed to dark-adapt for 30 minutes. Measurements were taken starting at 12:30 pm every day for 28-days.

2.8.2 Pathogenicity assay: measure of disease severity using disease severity index

On the final day (28th day) of the experiment, American ginseng seedlings were examined for plant health using a five-point disease severity index (Table 2.3) adapted from (Ivanov & Bernards, 2012; Reeleder & Brammall, 1994). Seedlings were scored using a single-blind study by three participants. Averages were calculated per replicate and standard error was calculated within each treatment amongst replicates.

Table 2.3. Five-point disease severity index established for American ginseng. Disease severity (1–5) and symptoms displayed ranging from control to severe disease. Table was adapted from Reeleder and Brammall (1994) and Ivanov and Bernards (2012) to now include symptoms 'green and wilted leaves' for disease severity scores 4–5.

Disease Severity		Symptoms
1	Control	–No discolouration of leaves (green), may have some small discoloured spots –Leaves turgid/firm and not wilted –Firm/turgid stem attached to root (not damped off) –Main root with no discolouration (whitish) with secondary and tertiary roots intact
2	Mild disease	–No discolouration of leaves (green), may have some small discoloured spots –Leaves turgid/firm and not wilted –Stem not firm/turgid but not dampened off –Main root with no discolouration (whitish) with secondary and tertiary roots intact
3	Medium disease	–Discolouration of leaves (yellowish/brown) in more than half of visible surface –Leaves turgid/firm and not wilted –Stem not firm/turgid but not dampened off –Main root with no discolouration (whitish) with secondary and tertiary roots intact
4	High disease	–Discolouration of leaves (yellowish/brown) and leaves wilted OR green and leaves wilted, in more than half of visible surface –Stem not firm/turgid but not dampened off –Tap root with no discolouration (whitish) with secondary and tertiary roots intact
5	Severe disease	–Discolouration of leaves (yellowish/brown) and leaves wilted OR green and leaves wilted, in more than half of visible surface –Stem dampened off or detached near main root –Main root exhibits discolouration (orange/brown)

2.9 Growing *Ilyonectria* on ginsenoside-infused PDA media

Prior to testing the pathogenicity of *Ilyonectria* isolates with and without previous exposure to ginsenosides, each *Ilyonectria* isolate was grown on PDA (R454312, Remel, Fisher Scientific) infused with crude ginsenoside extract (1 mg/mL) in Petri dishes (100 mm × 15 mm, Fisherbrand). The crude ginsenoside mixture used was previously extracted from three-year old American ginseng roots by Dr. Dimitre Ivanov according to Nicol et al. (2002). To prepare 25 (20 mL) plates, 500 mg of dried crude ginsenoside extract (~75% ginsenosides by weight) was placed in a 50 mL polypropylene tube (Falcon, Fisher Scientific) and 25 mL of sterile ultrapure water was added. The solution was vortexed until dissolved and filtered using polyvinylidene fluoride (PVDF) syringe tip membrane filters (0.2 µM, Sarstedt Filtropur S, PES, 83.1826.001) into a new 50 mL polypropylene tube. Using a 1 L Erlenmeyer flask, 19.5 g of PDA and 500 mL of ultrapure water was added and autoclaved. Following autoclaving, once PDA solution was cooled (~35°C), crude ginsenoside extract solution was added and mixed using a stir rod. Then, 20 mL of PDA-ginsenoside extract solution (1 mg/mL) was aliquoted per petri plate (100 mm × 15 mm, Fisherbrand) and the plates wrapped in aluminum foil and stored at 4°C until use.

2.10 *In vivo* inoculation of American ginseng roots using *Ilyonectria* with and without exposure to ginsenosides

To test the pathogenicity of *Ilyonectria* isolates with and without previous exposure to ginsenosides, two-year old American ginseng roots (obtained from Dr. Sean Westerveld, OMAFRA) were inoculated as described by Behdarvandi (2020) and Walsh et al. (2022). Six *Ilyonectria* isolates differing in virulence, as previously established from pathogenicity assays (Section 2.8), including four low virulence and two high virulence isolates, with and without previous exposure to ginsenosides (12 treatments with *Ilyonectria*) were tested. To generate cultures with prior ginsenoside exposure, isolates

were grown for four plate transfers on ginsenoside-infused PDA plates (1 mg/mL) prior to use.

Inoculum was prepared by growing the six *Ilyonectria* isolates used (I.sp.1, I.sp.2, I.sp.3, I.sp.4, I.sp.6, I.sp.11) on PDA with and without ginsenosides, as appropriate, for 11-days. All tools were autoclaved twice with 24 hours between each autoclave period and inoculations were done in a laminar flow hood. First, using a sterile razor, lateral roots were removed from two-year old American ginseng roots and roots were shortened to ~7.5 cm by cutting from the bottom of the root, if necessary. Roots were then soaked in 70% ethanol (10 min), wiped with sterile paper towel to remove any debris, placed in a second bath of 70% ethanol (2 min), then 20% bleach (1% sodium hypochlorite) (5 min), and lastly water (2 min). A fresh batch of ethanol and bleach was used for every ~20 roots. Roots were air dried for a few minutes prior to inoculation. Then, using a sterling silver pin (Petite Pearlized Pins, Loops & ThreadsTM), one 4 mm diameter *Ilyonectria* agar plug was pinned onto the surface of one root, approximately 5 mm deep. Lastly, each root was placed into one 50 mL glass bottle or 50 mL polypropylene tube (Figure 2.4a). All samples were placed in a large plastic container previously wiped with ethanol. Paper towels soaked in water were placed inside the container to create a humid environment. At 1-day-post-infection and 16-days-post-infection, images of each ginseng root with a ruler in the frame, were taken. As controls, ginseng roots were inoculated with PDA infused with ginsenoside extract and PDA with no ginsenoside extract. Five replicates were used per treatment (six treatments with *Ilyonectria* and two control treatments) (totaling to 14 groups and 70 samples).

Using photos taken 16-days-post-infection, lesions were measured free-hand using ImageJ (version 1.54g; Java 13.0.6 [64-bit]). A lesion was determined by a dark brown/black discolouration. First, a scale was set using ImageJ on the ruler in each image. A free-hand trace was drawn surrounding a lesion where each root was inoculated, and area (mm^2) was recorded (Figure 2.4b).

Figure 2.4. Two-year old American ginseng root inoculation.
Root inoculated with PDA plug at (a) 1-day-post-infection, in a 50 mL polypropylene tube and at (b) 16-days-post-infection beside a ruler. Yellow tracing used to calculate lesion area using ImageJ.

2.11 *In vivo* metabolism of ginsenosides by *Ilyonectria* with and without exposure to ginsenosides

To test the capacity of each *Ilyonectria* isolate to metabolize ginsenosides, the same six *Ilyonectria* isolates (I.sp.1, I.sp.2, I.sp.3, I.sp.4, I.sp.6, I.sp.11) previously grown with and without ginsenosides were used in *in vitro* liquid culture assays as described by Yousef & Bernards (2006). *Ilyonectria* isolates were grown for ten plate-transfers on ginsenoside-infused PDA plates (exception of I.sp.11 for six plate-transfers). Five replicates were used per twelve treatment groups (six *Ilyonectria* isolates grown with and without ginsenosides) and per two control groups (N = (12 + 2) × 5 = 70). First, 800 mL of Czapek-Dox minimal media without sugar (pH 7.6) (3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g KCl, and 0.01 g $FeSO_4 \cdot 6H_2O$, per litre; BD Difco) was prepared. Crude ginsenoside extract (100 mL of 8 mg/mL) was filter sterilized using PVDF syringe tip

membrane filters (0.2 µM, Sarstedt Filtropur S, PES, 83.1826.001) into the Czapek-Dox minimal medium once cooled following autoclaving, to create a final concentration of 1 mg/mL crude ginsenoside extract. Then, 10 mL of 1 mg/mL crude ginsenoside broth media was transferred to Erlenmeyer flasks (50 mL) and an 8 mm diameter plug (edge of PDA culture) of 12-day old cultures were placed inside of each flask. Cultures were incubated at room temperature in the dark, without shaking, for 7 days. On the 8th day, ginsenosides were recovered by filtering culture medium through nylon membranes (0.45 µM, Whatman polyamide membrane filters (NL17)) to remove mycelia. Recovered spent-ginsenoside medium were concentrated using SPE columns (HyperSep™ C-18 200 mg SPE columns (Product Number 60108-303, Thermo Scientific™) and analyzed by LCMS as described in 2.3–2.4.

2.12 Statistical analyses

All graphing and statistical analyses were conducted using GraphPad prism (version 9.5.1, Boston, Massachusetts USA) unless otherwise indicated. Normality and homoscedasticity were tested using a normality QQ plot and homoscedasticity plot.

2.12.1 Ginsenoside accumulation and persistence in ginseng garden soils

Due to a non-normal distribution, average nmol ginsenosides/g soil were transformed into $\log_{10}$((nmol ginsenosides/g soil) + 1)) to obtain a normal distribution. Transformed nmol ginsenosides/g soil were analyzed using a Friedman test with a Dunn's post-hoc test to compare to 'planting' as control.

2.12.2 *Ilyonectria* pathogenicity assay

One replicate from I.sp.4, I.sp.8, and I.sp.11, were removed due to accidental mechanical death of the plant (I.sp.4) or outliers in the data (I.sp.8, I.sp.11). Average F_v/F_m measurements on the 28th-day were analyzed using a one-way ANOVA with a Dunnett's multiple comparisons post-hoc test. Average disease severity indices on the 28th-day were analyzed using a one-way ANOVA with a Dunnett's multiple comparisons post-hoc test.

2.12.3 *In vivo* inoculation of Americana ginseng roots using *Ilyonectria* with and without exposure to ginsenosides

Several replicates were removed due to root rot not caused by *Ilyonectria* inoculation, resulting in 3–5 replicates per treatment. These replicates include Control (without ginsenoside exposure, 1); with ginsenoside exposure, 2); I.sp.1 (without ginsenoside exposure, 1; with ginsenoside exposure, 1); I.sp.2 (without ginsenoside exposure, 1; with ginsenoside exposure, 2); I.sp.3 (with ginsenoside exposure, 2); I.sp.4 (without ginsenoside exposure, 1; with ginsenoside exposure, 1); I.sp.6 (without ginsenoside exposure, 1; with ginsenoside exposure, 2); I.sp.11 (without ginsenoside exposure, 1; with ginsenoside exposure, 2). Due to a non-normal distribution, average lesion size was transformed into log10(lesion area (mm2) + 1) to obtain a normal distribution. Transformed lesion areas were analyzed using a two-way ANOVA with a Šidák's post-hoc test.

2.12.4 *In vivo* metabolism of ginsenosides by *Ilyonectria* with and without exposure to ginsenosides

Average amounts of each ginsenoside (Rg_1, Re, F_{11}, Rb_1, Rc/Rb_2, Rd, Gypenoside XVII, and F_2) relative to control (1.0) remaining after 7 days were calculated for each *Ilyonectria* isolate (I.sp.1, I.sp.2, I.sp.3, I.sp.4, I.sp.6, I.sp.11) with and without prior exposure to ginsenosides. One replicate of I.sp.2 with ginsenoside exposure was removed due to sample loss. Average ginsenosides amounts (mg) were analyzed using a two-way ANOVA with a Tukey's post-hoc test.

3 Results

3.1 Ginsenoside accumulation and persistence in ginseng garden soils

The accumulation of ginsenosides (Rg_1, Re, F_{11}, Rb_1, $Rc/Rb_2/Rb_3$, Rd, Gypenoside XVII, F_2) was tracked in the soils of three commercial ginseng gardens over the course of four years (i.e., from planting to harvest), and their persistence was measured during the first post-harvest year (Figure 3.1). A Friedman test revealed a significant difference in log*10*((nmol ginsenosides/g soil) + 1) between each time period within each site ($P<0.0001$, Site 1 Friedman statistic = 67.28, Site 2 Friedman statistic = 46.65, Site 3 Friedman statistic = 57.30). In general, all three sites revealed the same trends in ginsenoside accumulation, with the amounts of ginsenosides increasing from below detection to 8.4 ± 5.7 nmol/g soil (Site 2) to 26.1 ± 13.4 nmol/g soil (Site 3) to 34.6 ± 27.1 nmol/g soil (Site 1), over the first three and a half years. This equates to between 7 and 32 μg/g soil. In the late summer and fall of year four, however, the amounts of ginsenosides in ginseng garden soils decreased, relative to peak accumulation in the late summer of the previous year (See Appendix; Supplementary Table 2). Ginsenosides were not found in any of the control soil samples from any of the three sites, at any time during the four-year collection period (data not shown). Post-harvest, there was little further recovery of ginsenosides from garden soil when left undisturbed over winter and throughout the following summer (Figure 3.1).

In ginseng garden soils, protopanaxadiols accumulated more than protopanaxatriols with an average of approximately 5–16 times greater over the course of the four-year cultivation cycle and post-harvest. Amongst the protopanaxadiols, Gypenoside XVII and F_2 were the most commonly found, while Rb_1 was the least commonly found PPD ginsenoside. In contrast, amongst the protopanaxatriols, F_{11} was the most common, while Rg_1 and Re were relatively equal (data not shown).

A version of this chapter has been accepted for publication (Rabas, A., Colo, A., Kaberi, K., Ivanov, D.A. and Bernards, M.A. (2024) The Properties of Ginsenosides in Ginseng Garden Soil: Accumulation, Persistence and Behaviour. Plant & Soil (in press)).

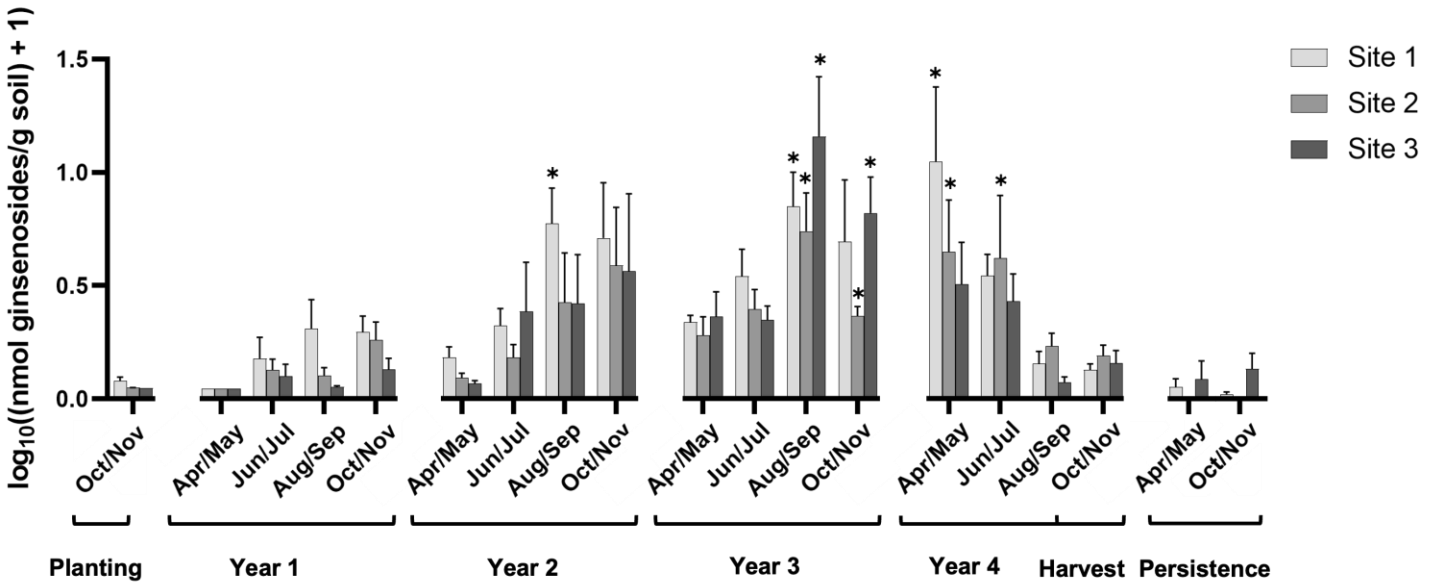


Figure 3.1. Accumulation and persistence of ginsenosides in ginseng garden soils.
The accumulation of ginsenosides (log_{10} ((nmol ginsenosides/g soil) + 1) in the soils of three commercial ginseng gardens was assessed in soils collected between planting and harvesting. Values are calculated as mean ± SE. Garden soils (n = 5) were collected in the fall of the year of planting (October/November), and throughout each year of cultivation at four time points (April/May, June/July, August/September, October/November). The persistence of ginsenosides in soils post-harvest was assessed in soils collected from two of the three commercial garden sites at two time points (April/May, October/November)) following the year of harvest (Persistence). Ginsenosides were identified by LCMS (as described in Section 2.4). Statistical analysis using a Friedman's test for each site (Site 1, Site 2, Site 3) with Dunn's post-hoc test, was used to compare log_{10}((nmol ginsenosides/g soil) + 1) at Planting (control) to log_{10}((nmol ginsenosides/g soil) + 1) at each time point (*P<0.05).

A version of this chapter has been accepted for publication (Rabas, A., Colo, A., Kaberi, K., Ivanov, D.A. and Bernards, M.A. (2024) The Properties of Ginsenosides in Ginseng Garden Soil: Accumulation, Persistence and Behaviour. Plant & Soil (in press)).

3.2 Selected *Ilyonectria* isolates species confirmation

Twelve *Ilyonectria* isolates that differed in their species identification, virulence, host plant and location they were isolated from (Table 3.1) were selected from the Canadian Collection of Fungal Cultures (CCFC). The twelve *Ilyonectria* isolates were identified as *Ilyonectria mors-panacis* from CCFC; however, several of the isolates had been re-identified as other *Ilyonectria* species (Cabral et al., 2012). Additionally, several of the isolates had been examined previously for their virulence on American ginseng; but, this study was conducted in 2003 (Seifert et al., 2003). To confirm species identification and virulence, the twelve *Ilyonectria* isolates were genotyped and examined for virulence towards American ginseng. The morphology of each of the 12 strains grown on PDA without ginsenosides, and of a subset grown on PDA supplemented with ginsenosides, is shown in Figure 3.2.

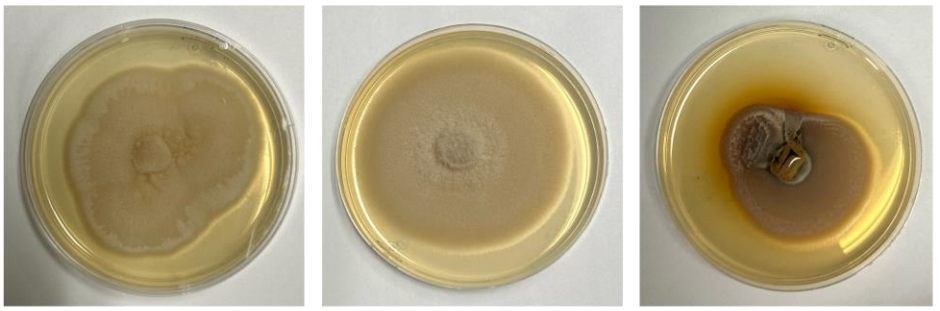
The histone H3 (HIS) genes from the twelve *Ilyonectria* isolates were sequenced using histone H3 (HIS) primers (Cabral et al., 2012). Using sequences obtained from Sanger sequencing, sequences of each of the twelve *Ilyonectria* isolates (See Appendix; Supplementary Data 1) were identified based on the reference sequence with the highest query cover and percent identification in BLASTn. As further confirmation, a 400-base consensus sequence was obtained for each of the twelve *Ilyonectria* isolates and used to create a phylogenetic tree together with sequences of 33 other reference strains, obtained from GenBank (Figure 3.3). Sequences of *I. mors-panacis*, *I. rufa* and *I. robusta* each clustered into a unique clade, and all 12 isolates used in this study grouped into the appropriate clade associated with their species prediction from Cabral et al. (2012). The single isolate of *I. pseudodestructans* used in the analysis was placed on a branch by itself.

A version of this chapter has been accepted for publication (Rabas, A., Colo, A., Kaberi, K., Ivanov, D.A. and Bernards, M.A. (2024) The Properties of Ginsenosides in Ginseng Garden Soil: Accumulation, Persistence and Behaviour. Plant & Soil (in press)).

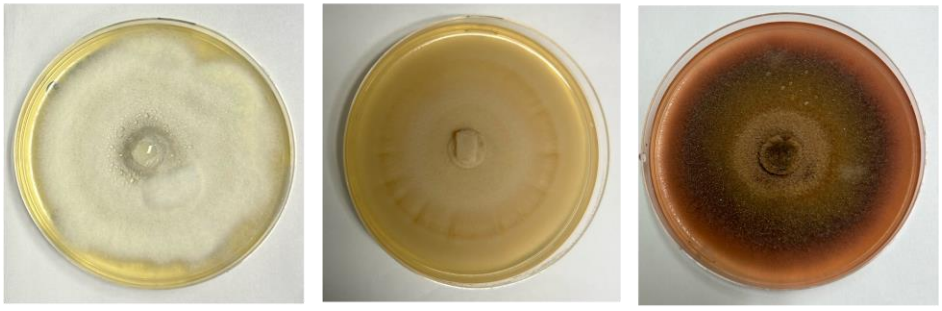
Table 3.1. Twelve *Ilyonectria* isolates selected from Canadian Collection of Fungal Cultures (CCFC, Ottawa, Canada).
Ilyonectria species number used as simple identification. *Ilyonectria* species were confirmed using histone H3 gene sequencing and phylogenetic analysis with sequences of known reference strains (Figure 3.3). Virulence was confirmed by *in vivo* inoculation of American ginseng (Figure 3.4). Metadata for the host plant and geographical origin of each isolate are stated in Table 2.3.

***Ilyonectria* Species Number**	***Ilyonectria* Species**	**Virulence**
I.sp.1	*I. rufa*	Low
I.sp.2	*I. rufa*	Low
I.sp.3	*I. mors-panacis*	Low
I.sp.4	*I. mors-panacis*	Low
I.sp.5	*I. rufa*	Low
I.sp.6	*I. robusta*	High
I.sp.7	*I. mors-panacis*	High
I.sp.8	*I. mors-panacis*	High
I.sp.9	*I. mors-panacis*	High
I.sp.10	*I. mors-panacis*	High
I.sp.11	*I. mors-panacis*	High
I.sp.12	*I. mors-panacis*	High

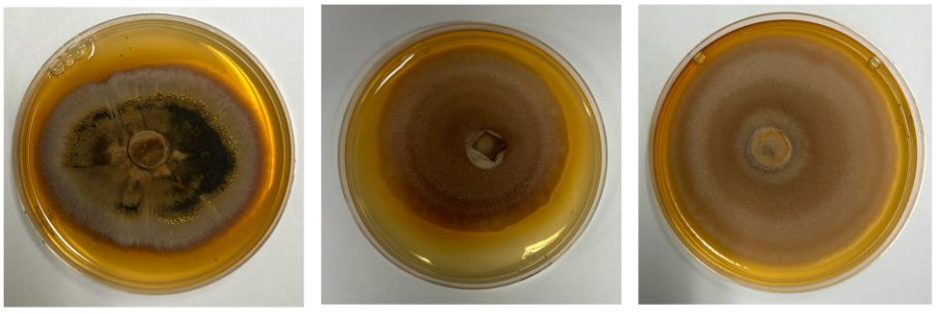
(A)



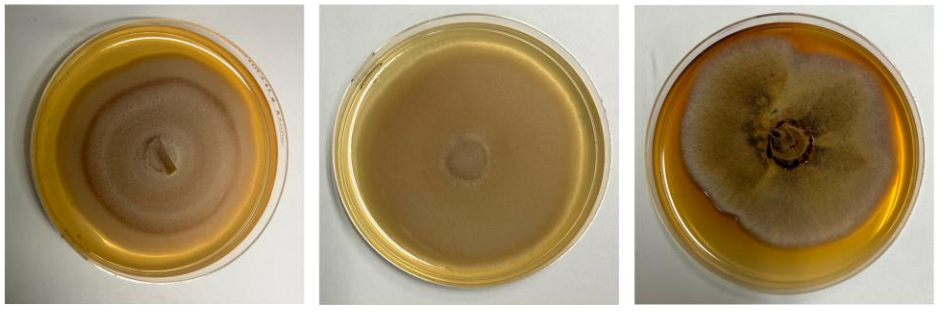
I.sp.1 I.sp.2 I.sp.3



I.sp.4 I.sp.5 I.sp.6



I.sp.7 I.sp.8 I.sp.9



I.sp.10 I.sp.11 I.sp.12

(B)

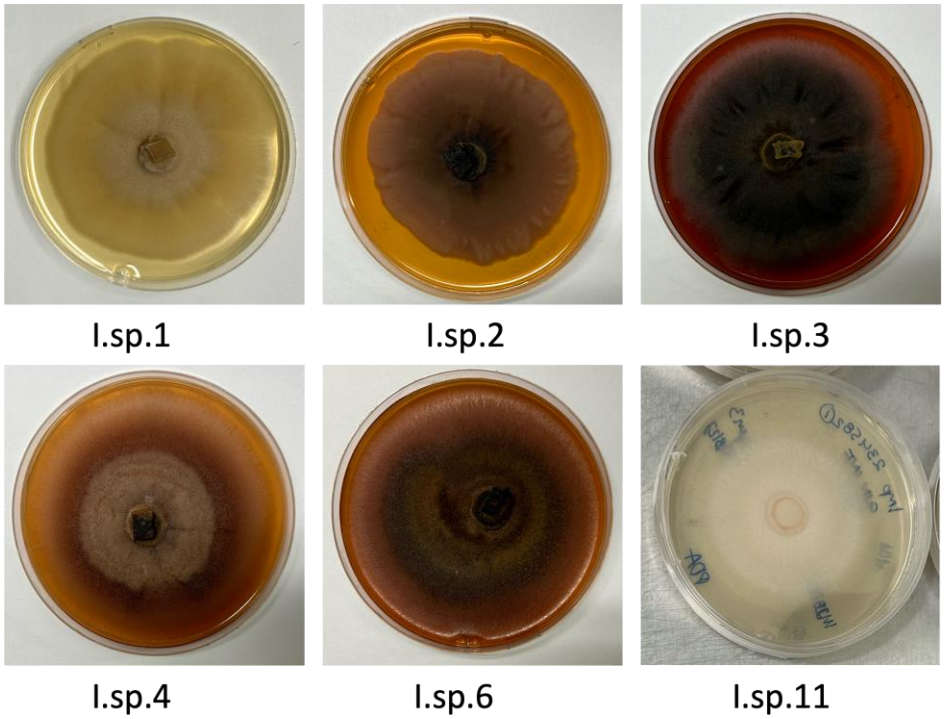


Figure 3.2. Growth of *Ilyonectria* isolates on potato dextrose agar (PDA) plates with and without ginsenoside supplement.

(A) Twelve *Ilyonectria* isolates (I.sp.1–I.sp.12) were grown for approximately 2 weeks on PDA plates (20 mL) not supplemented with ginsenosides. (B) A subset of six isolates (I.sp.1, I.sp.2, I.sp.3, I.sp.4, I.sp.6 and I.sp.11) were grown for 2 weeks on PDA plates (20 mL) supplemented with ginsenosides (1 mg/mL) for twelve plate transfers (exception of I.sp.11 for 4 plate-transfers).

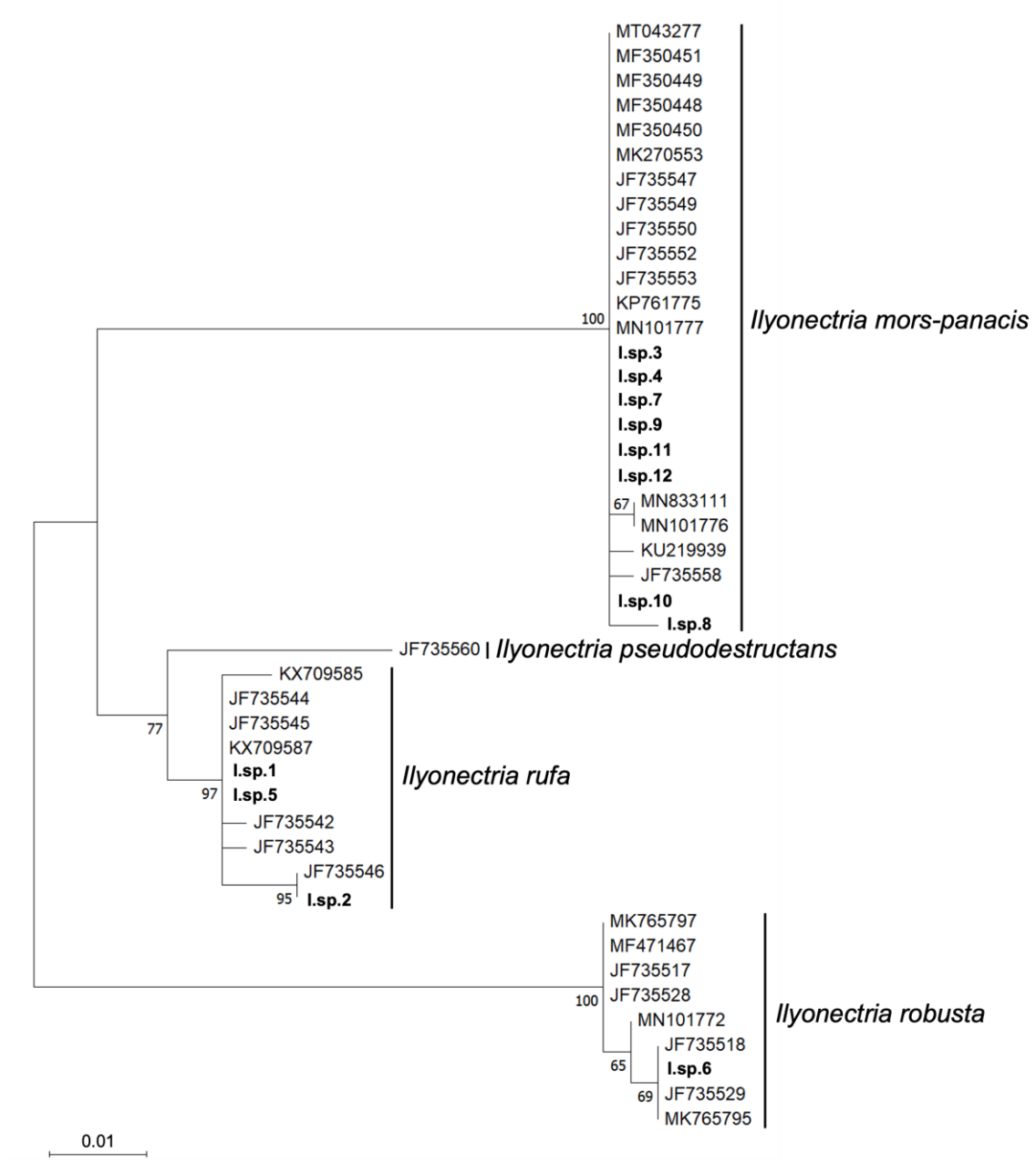


Figure 3.3. Phylogenetic analysis of *Ilyonectria* species.
A Maximum Likelihood estimation (HKY model) phylogenetic tree, based on sequences from histone H3 (HIS) fungal region (400 bp) was constructed using MEGA 11 after alignment in MAFFT. *Ilyonectria* isolates used in this study (I.sp.1–I.sp.12) are bolded. Remaining *Ilyonectria* isolates were identified by GenBank® accession numbers. Node support values (percent) are displayed from a 1000× Bootstrap analysis.

3.3 Pathogenicity assay: chlorophyll fluorescence and disease severity

To test the pathogenicity of each of the twelve *Ilyonectria* isolates, an *in vivo* pathogenicity assay was conducted. Average F_v/F_m was plotted against days post infection for each *Ilyonectria* isolate used to infect one-year old American ginseng seedlings (Figure 3.4). In general, F_v/F_m began to decline for ginseng plants inoculated with several of the *Ilyonectria* isolates, but this was not significant until the 28th-day-post-infection, ($P<0.0001$, $F(12,49) = 5.124$; one-way ANOVA). Dunnett's post-hoc test analysis revealed that while the average F_v/F_m for each of *I. rufa* I.sp.1 ($P>0.9999$), I.sp.2 ($P=0.9954$), and I.sp.5 ($P=0.2144$), and *I. mors-panacis* I.sp.3 ($P=0.7938$), I.sp.4 ($P=0.5465$), were not statistically significant compared to the F_v/F_m of the control, the average F_v/F_m for *I. robusta* I.sp.6 ($P=0.0076$), and *I. mors-panacis* I.sp.7 ($P=0.0051$), I.sp.8 ($P=0.0044$), I.sp.9 ($P=0.0014$), I.sp.10 ($P=0.0015$), I.sp.11 ($P=0.0028$), and I.sp.12 ($P=0.0014$) were statistically significant compared to F_v/F_m of the control. Although statistically insignificant, five *Ilyonectria* isolates were grouped into "low" virulence (*I. rufa*; I.sp.1, I.sp.2, I.sp.5, *I. mors-panacis*; I.sp.3, I.sp.4) due to some effect on F_v/F_m of American ginseng. Seven *Ilyonectria* isolates were grouped into "high" virulence (*I. robusta*; I.sp.6, *I. mors-panacis*; I.sp.7, I.sp.8, I.sp.9, I.sp.10, I.sp.11, I.sp.12) as these isolates yielded statistically significant average F_v/F_m when compared to average F_v/F_m of the control treatment, on the 28th-day-post-infection (See Appendix; Supplementary Table 3).

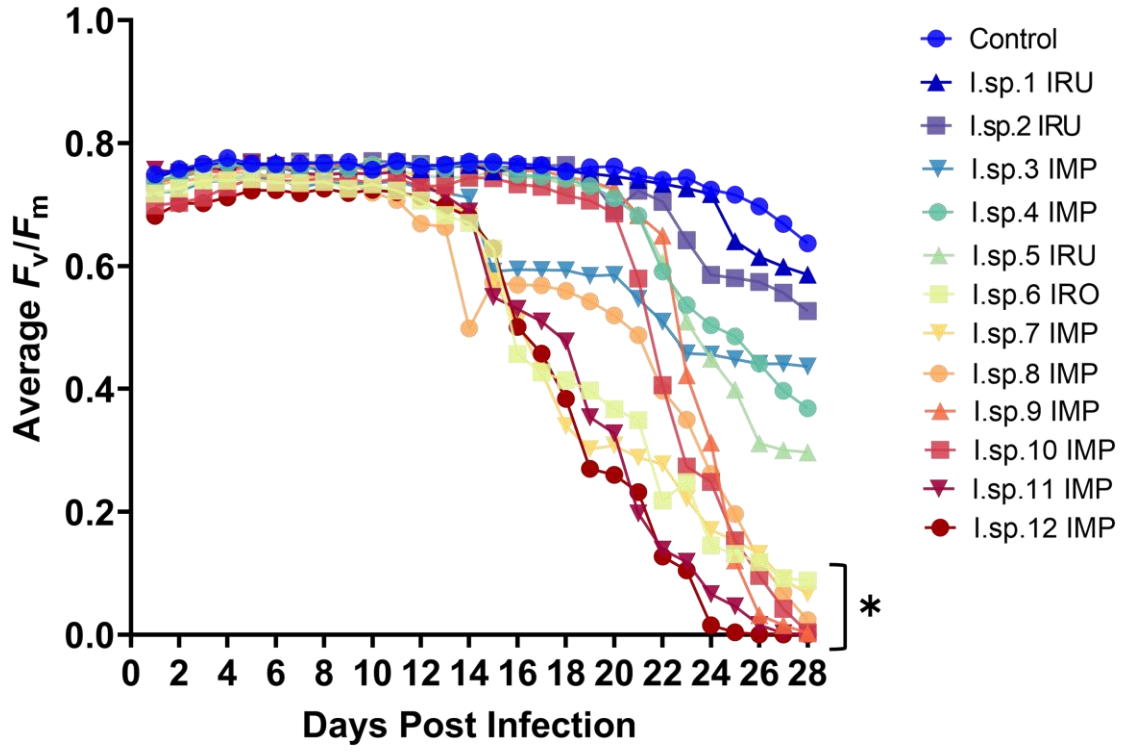


Figure 3.4. Virulence analysis of twelve *Ilyonectria* spp. Daily F_v/F_m measurements of one-year old American ginseng seedlings inoculated with one of twelve *Ilyonectria* isolates or non-inoculated (control) for 28-days were made post-inoculation.
The daily average F_v/F_m (n = 4–5) of each *Ilyonectria* isolate are plotted. Error bars were removed for clarity. Statistical analysis was performed on measurements at 28-days-post-infection using one-way ANOVA with Dunnett's post-hoc test. The data grouped with a bracket yielded significantly different F_v/F_m values from control F_v/F_m values (*P<0.05). IRU = *Ilyonectria rufa*. IMP = *Ilyonectria mors-panacis*. IRO = *Ilyonectria robusta*.

To further support F_v/F_m measurements of one-year old American ginseng seedlings inoculated with various *Ilyonectria* isolates or control, disease severity was examined by assessing damage to the roots of the seedlings. A one-way ANOVA revealed a significant difference in average disease severity on the 28th day of the experiment, of American ginseng seedlings inoculated with each of twelve *Ilyonectria* isolates or control (water & PDA)-inoculated roots (P=0.0006, F(12,49) = 3.6; one-way ANOVA) (Figure 3.5). Using a Dunnett's post-hoc test the average disease severity of roots inoculated with *I. rufa* isolates I.sp.1 (P=0.9641), I.sp.2 (P=0.3812), I.sp.5 (P=0.4580), and *I. mors-panacis* isolates I.sp.3 (P=0.3812), and I.sp.4 (P=0.6321) were not significantly different from

average disease severity in control (water & PDA)-inoculated roots. By contrast, the average disease severity of roots inoculated with I. robusta I.sp.6 (P=0.0041), and *I. mors-panacis* isolates I.sp.7 (P=0.0020), I.sp.8 (P=0.0441), I.sp.9 (P=0.0302), I.sp.10 (P=0.0083), I.sp.11 (P=0.0019), and I.sp.12 (P=0.0010) was significantly greater than average disease severity in control (water & PDA)-inoculated roots. These data further support the categorization of five *Ilyonectria* isolates as "low" virulence (*Ilyonectria rufa*; I.sp.1, I.sp.2, and I.sp.5, and *I. mors-panacis*; I.sp.3 and I.sp.4) and the other seven *Ilyonectria* isolates as "high" virulence (*Ilyonectria robusta*; I.sp.6, and *I. mors-panacis*; I.sp.7–I.sp.12) (Table 3.1).

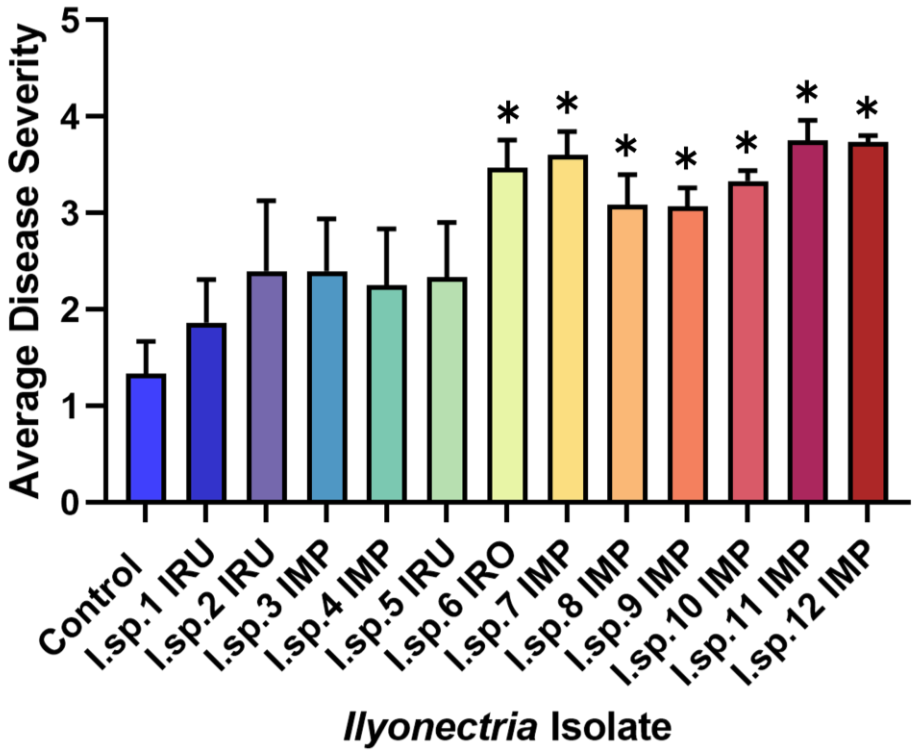


Figure 3.5. Root assessment of one-year old American ginseng seedlings inoculated with various *Ilyonectria* isolates.
The average disease severity of one-year old American ginseng seedlings (n = 4–5) inoculated with one of twelve *Ilyonectria* isolates or non-inoculated (control) was assessed 28-days-post-infection using a disease severity index. Values were calculated as mean ± SE. Data were analyzed using one-way ANOVA with Dunnett's post-hoc test. Disease severity estimates that differed significantly from uninoculated control roots are marked with an asterisk (*P<0.05). IRU = *Ilyonectria rufa*. IMP = *Ilyonectria mors-panacis*. IRO = *Ilyonectria robusta*.

3.4 *In vivo* inoculation of American ginseng roots using *Ilyonectria* with and without exposure to ginsenosides

To examine the impact of ginsenosides on the pathogenicity of *Ilyonectria* isolates toward American ginseng, a root bioassay was conducted using *Ilyonectria* isolates with and without previous exposure to ginsenosides *in vitro*. I used four low virulence isolates (*I. rufa*; I.sp.1, I.sp.2., *I. mors-panacis*; I.sp.3, I.sp.4) and two high virulence isolates (*I. robusta*; I.sp.6, *I. mors-panacis*; I.sp.11). Using photos taken 16-days-post-infection, the size of dark brown/black lesions formed on roots at the site of inoculation were measured free-hand using Image-J software and plotted as the $\log_{10}$(lesion size (mm^2) + 1) (Figure 3.6). A two-way ANOVA revealed significance differences in average $\log_{10}$(lesion size (mm^2) + 1) on American ginseng roots inoculated with *Ilyonectria* isolates with and without previous exposure to ginsenosides in our lab ($P<0.0001$, $F(6,38)=7.7$; two-way ANOVA). In pairwise comparisons between *Ilyonectria* isolates with and without previous exposure to ginsenosides, average $\log_{10}$(lesion size (mm^2) + 1) was significantly greater for I.sp.2 previously exposed to ginsenosides ($P<0.0001$) using a Šidák's post-hoc test. With the exception of I.sp.1, which trended toward larger lesion size after exposure to ginsenosides ($P=0.0743$), no other isolate showed a significant change in virulence after exposure to ginsenosides (See Appendix; Supplementary Table 4). These data suggest that prior exposure to ginsenosides can increase the virulence of *I. rufa* towards American ginseng roots. It should be noted that although average $\log_{10}$(lesion size (mm^2) + 1) was not significantly different when comparing treatment groups for I.sp.4 (two-way ANOVA with Šidák's; $P=0.3310$), when comparing $\log_{10}$(lesion size (mm^2) + 1) to the control for each applicable treatment group (control inoculated with PDA plug and control inoculated with PDA plug infused with ginsenosides), I.sp.4 without ginsenoside exposure was not significantly greater (two-way ANOVA with Dunnett's; $P=0.3193$). However, ginseng roots inoculated with I.sp.4 previously exposed to ginsenosides, displayed significantly greater average $\log_{10}$(lesion size (mm^2) + 1) ($P=0.0146$) (See Appendix; Supplementary Table 4).

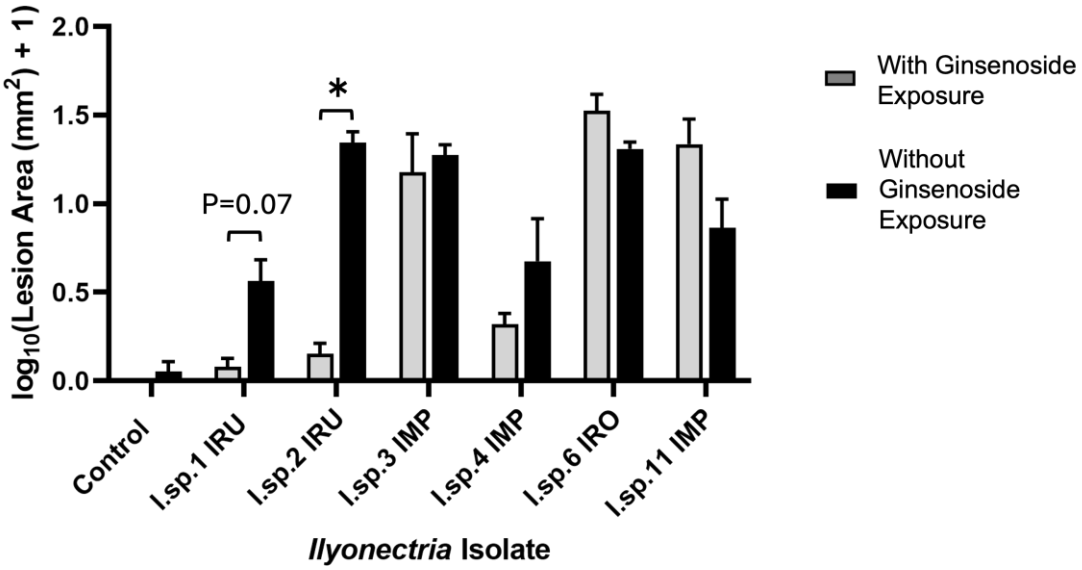


Figure 3.6. Impact of prior exposure to ginsenosides on virulence of *Ilyonectria* spp. toward ginseng.
Lesion size, plotted as $\log_{10}$(lesion area (mm_2) + 1), on two-year old American ginseng roots (n = 3–5) was measured after inoculation with one of six *Ilyonectria* isolates (IRU; I.sp.1, I.sp.2, IMP; I.sp.3, I.sp.4, I.sp.11, IRO; I.sp.6) with and without previous exposure to ginsenosides or water (Control). Lesions were measured using Image-J, 16-days-post-infection. Values were calculated as mean ± SE. Statistical analysis using two-way ANOVA with Šidák's post-hoc test, was used to compare differences between lesion size caused by the same isolate with or without prior ginsenoside exposure. *P<0.0001. IRU = *Ilyonectria rufa*. IMP = *Ilyonectria mors-panacis*. IRO = *Ilyonectria robusta*.

3.5 *In vivo* metabolism of ginsenosides by *Ilyonectria* with and without exposure to ginsenosides

To examine the difference in the metabolism of ginsenosides by *Ilyonectria* isolates an *in vitro* metabolomic assay was conducted using *Ilyonectria* isolates (*I. rufa*; I.sp.1, I.sp.2, *I. mors-panacis*; I.sp.3, I.sp.4, I.sp.11, *I. robusta*; I.sp.6) with and without previous exposure to ginsenosides. On the 7^{th} day, minimal broth supplemented with ginsenosides and incubated with *Ilyonectria* isolates, was collected, filtered, and analyzed

by LCMS. The PPT-type ginsenosides (Rg_1, Re, F_{11}) and PPD-type ginsenosides (Rb_1, Rc/Rb_2, Rd, Gypenoside (G) XVII, F_2) were assessed. Measurements were normalized by calculating the amount of ginsenosides (mg) relative to control (1.0) (Figure 3.7). Control replicates from treatments 'without ginsenoside exposure' and 'with ginsenoside exposure' were combined to a total of 10 replicates as there was no significant difference (two-way ANOVA, $F(8,72) = 0.1956$, $P=0.9907$) between treatment groups for control samples when comparing individual ginsenosides ($P>0.9999$) and total ginsenosides ($P=0.7777$). Amounts of individual ginsenosides in control samples were also measured (See Appendix; Supplementary Table 5). An unidentified ginsenoside (sodium adduct mass: 807.4865 m/z, retention time: 6.87 min) was detected in total ion chromatograms as an abundant peak from select *Ilyonectria* isolates, that was not present in control samples (Figure 3.8). While the amounts of some ginsenosides were greater, and some lower, in media recovered from isolates with and without prior exposure to ginsenosides, there were no overall trends between amounts of ginsenosides (mg) relative to control and *Ilyonectria* isolates. No apparent trends were detected amongst *Ilyonectria* species either. Ginsenoside profiles differed between I.sp.1 and I.sp.2 (*I. rufa*) and between I.sp.3, I.sp.4, and I.sp.11 (*I. mors-panacis*).

Using a two-way ANOVA with a Dunnett's post-hoc test, all *Ilyonectria* isolates (I.sp.1, I.sp.2, I.sp.3, I.sp.4, I.sp.6, I.sp.11) metabolized Rb1 relative to control with the exception of I.sp.2 with prior exposure to ginsenosides ($P=0.4771$). Furthermore, F_2 was metabolized by all *Ilyonectria* isolates with and without prior exposure to ginsenosides. Relative to control, several *Ilyonectria* isolates (I.sp.2, I.sp.3, I.sp.4, I.sp.6, I.sp.11) bio-transformed pre-existing ginsenosides into Gypenoside (G) XVII, but I.sp.1 (without ginsenoside exposure; $P=0.3662$, with ginsenoside exposure; $P=0.8832$) and I.sp.6 with prior exposure to ginsenosides ($P=0.4937$), did not bio-transform significantly greater amounts of ginsenosides compared to the abundance of ginsenosides present in broth of control treatments. No *Ilyonectria* isolates significantly metabolized PPT-type ginsenosides. Select *Ilyonectria* isolates (I.sp.2, I.sp.3, I.sp.4, I.sp.6) bio-transformed pre-existing ginsenosides into PPT-type ginsenosides and yielded significantly greater amounts of ginsenosides relative to control. Specifically, I.sp.2 without exposure to ginsenosides, had significantly higher levels of Rg_1 ($P=0.0206$) and F11 ($P<0.0001$), as

did I.sp.3 with exposure to ginsenosides (P=0.0081) and I.sp.4 without exposure to ginsenosides (P=0.0182). I.sp.4 with exposure to ginsenosides also had higher levels of F_{11} (P<0.0001), as did I.sp.6 without exposure to ginsenosides (P<0.0001).

Using a two-way ANOVA with a Šidak's post-hoc test to compare treatment groups (with and without previous exposure to ginsenosides), only I.sp.2 had significantly lower amounts of total ginsenosides (P=0.0058) and PPD-type ginsenosides (P<0.0001) when comparing with and without previous exposure to ginsenosides, suggesting there was a change in metabolism following an increase in virulence when previously exposed to ginsenosides (See Section 3.4). No significant difference in amounts of PPT-type ginsenosides was observed amongst *Ilyonectria* isolates when comparing treatment groups (See Appendix; Supplementary Table 6).

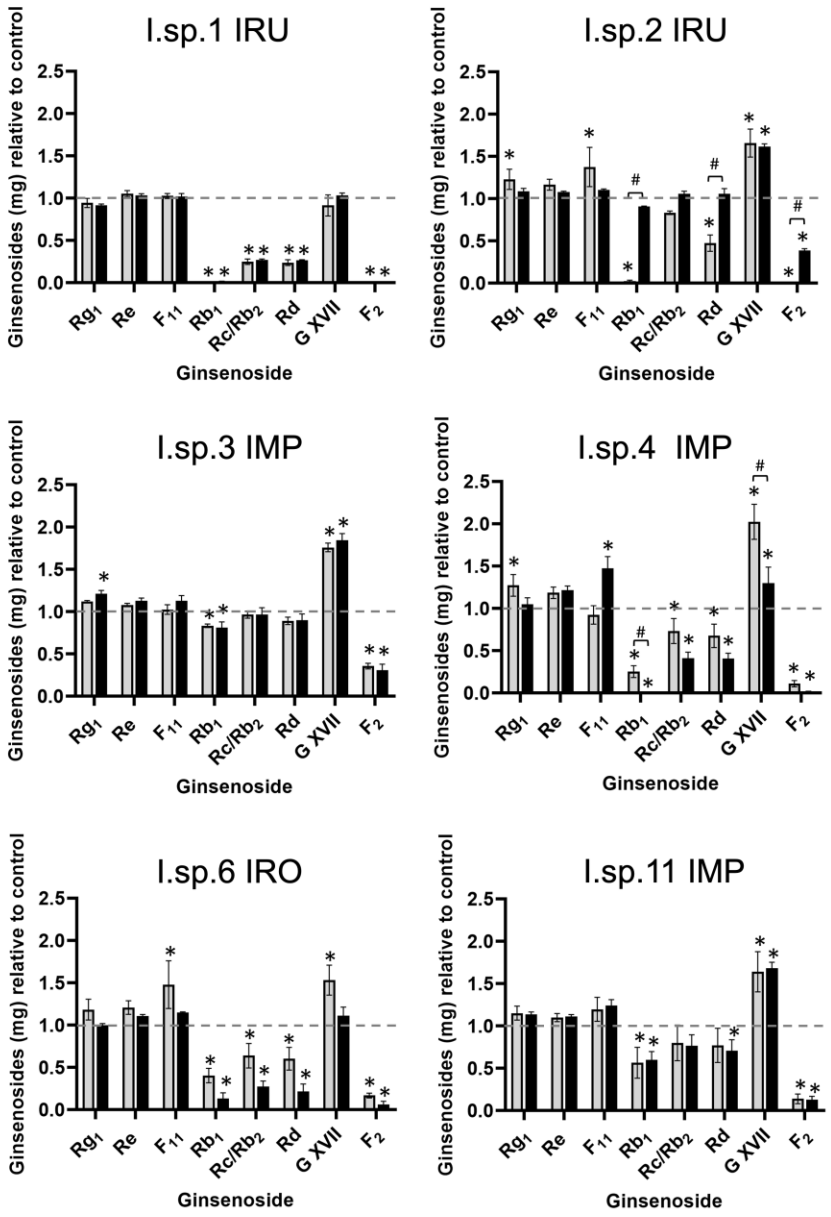

I.sp.1 IRU
I.sp.2 IRU
I.sp.3 IMP
I.sp.4 IMP
I.sp.6 IRO
I.sp.11 IMP
Ginsenosides (mg) relative to control
Ginsenoside
Rg1
Re
F11
Rb1
Rc/Rb2
Rd
G XVII
F2
Without Ginsenoside Exposure
With Ginsenoside Exposure

Figure 3.7. Recovery of individual ginsenosides from culture media incubated with *Ilyonectria* spp.
Ilyonectria isolates (IRU; I.sp.1, I.sp.2, IMP; I.sp.3, I.sp.4, I.sp.11, IRO; I.sp.6) (n = 4–5) were incubated in broth supplemented with ginsenosides (1 mg/mL) for 7 days. Ginsenosides were recovered from the media and analyzed by LCMS (as described in 2.11). Ginsenosides 20(S)-protopanaxatriols (Rg_1, Re, F_{11}) and 20(S)-protopanaxadiols (Rb1, Rc/Rb_2, Rd, Gypenoside (G) XVII, F_2) were assessed. Values were calculated as mean ± SE. Statistical analysis using two-way ANOVA with Dunnett's post-hoc test (*P<0.05) and Šidak's post-hoc test (#P<0.05). IRU = *Ilyonectria rufa*. IMP = *Ilyonectria mors-panacis*. IRO = *Ilyonectria robusta*.

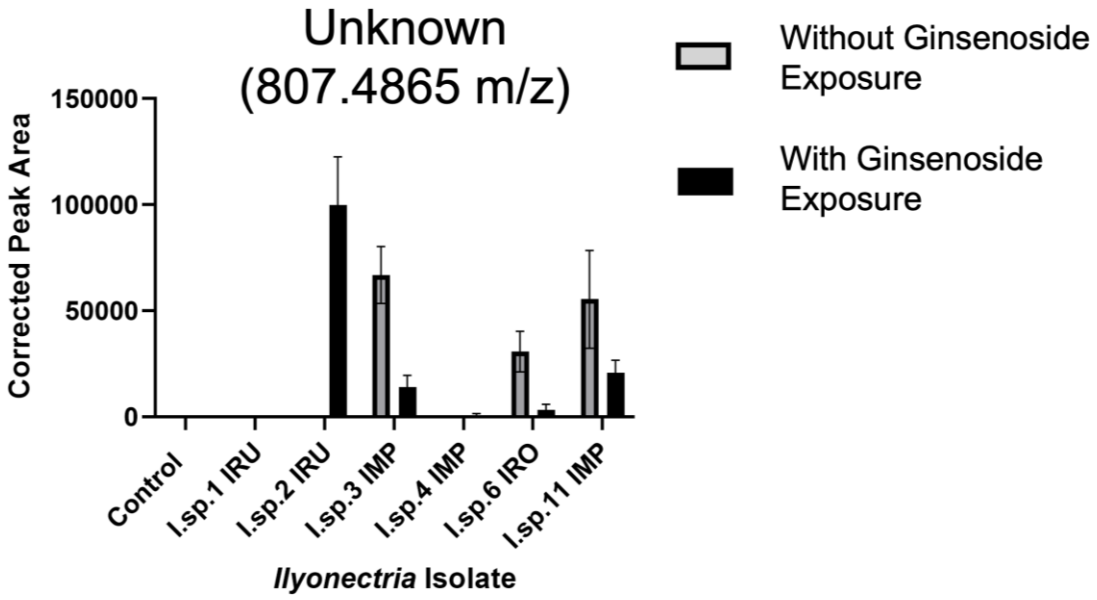


Figure 3.8. Recovery of unknown ginsenoside from culture media incubated with *Ilyonectria* spp.
An unidentified ginsenoside (sodium adduct mass: 807.4865 m/z, retention time: 6.87 min) was occasionally recovered from culture media incubated with *Ilyonectria* spp. (IRU; I.sp.1, I.sp.2, IMP; I.sp.3, I.sp.4, I.sp.11, IRO; I.sp.6) that was not recovered from culture media without incubation with *Ilyonectria* (control). IRU = *Ilyonectria rufa*. IMP = *Ilyonectria mors-panacis*. IRO = *Ilyonectria robusta*.

4 Discussion

4.1 The impact of ginsenosides in ginseng garden soil

Ginsenosides have known and complex effects on soil microbes including inhibiting the growth of several fungi while promoting the growth of others including the primary GRD pathogen *I. mors-panacis* (Luo et al., 2020; Miao et al., 2023; Nicol et al., 2002; Zhao et al., 2012). Investigating the abundance of ginsenosides during ginseng cultivation and the persistence of ginsenosides post-harvest is critical to understanding the role that ginsenosides may play in the establishment of GRD.

To date, ginsenosides have not been measured in American ginseng bulk soil during cultivation nor in the same soils post-harvest. Ginsenosides have been measured in Sanqi ginseng (*Panax notoginseng*) bulk soil for one–three years of cultivation during which a gradual increase in ginsenosides concentrations was observed, but measurements post-harvest were not conducted (Kaberi, 2021; Yang et al., 2015). Examining the abundance of ginsenosides in bulk soil cultivated with American ginseng is a novel approach as ginsenoside patterns in soil may differ amongst species of *Panax* cultivation, as observed in Asian and American ginseng roots (Qu et al., 2009; Samukawa et al., 1995). This analysis is more informative in understanding the role ginsenosides may play in GRD formation because, post-harvest ginsenoside concentrations can be compared relative to cultivation. If ginsenosides do not persist, they are likely not direct contributors because they are not present in soils when ginseng is planted again and instead establish conditions for GRD to occur. Additional time points within each cultivation year are required to have a better understanding of patterns of ginsenoside accumulation in soils, in contrast to the three time points studied by Yang et al. (2015).

My data showed that ginsenosides accumulate in ginseng garden soil during the first three and a half years of cultivation (between 7 and 32 μg/g soil), before showing signs of decline. This is similar to the findings of Yang et al. (2015), who found that ginsenosides accumulated steadily over three years of continuous ginseng growth, reaching nearly 6

μg/g soil (Yang et al., 2015). Moreover, these results were similar to the pattern of ginsenoside concentration over time in Asian ginseng roots (Samukawa et al., 1995) but dissimilar to previously reported ginsenoside concentration in American ginseng roots (Qu et al., 2009). That is, Samukawa et al. (1995) reported an annual increase in ginsenosides in Asian ginseng roots over the first three years of cultivation, a decline during the fourth year, followed by increases in years five and six. In contrast, Qu et al. (2009) reported a gradual increase in ginsenoside concentration from 1–5-year-old American ginseng roots. It should be noted that neither study measured the same suite of ginsenosides as I did. Notably, Gypenoside XVII and F_2, the two most abundant ginsenosides measured in my study, were not measured by Samukawa et al. (1995) or Qu et al. (2009). However, the proportions of Gypenoside XVII and F_2 were relatively consistent throughout cultivation and do not explain the difference in patterns amongst studies. In my study, ginseng flowers were removed during the third and fourth years of cultivation. Although not previously investigated, the removal of flowers may result in altered biosynthesis of ginsenosides and subsequently, decrease the production or exudation of ginsenosides from ginseng roots as observed in my study. It is not known when ginseng flowers were removed in the studies by Samukawa et al. (1995) and Qu et al. (2009), therefore flower removal affecting ginsenoside production is merely a speculation. Similarly to Qu et al. (2009), Dai et al. (2020) also revealed that total ginsenosides in Asian ginseng roots increased from ages 2–4; however, ginseng roots were not collected from the same field when examining ages 2–4, and my research shows that peak ginsenosides concentrations vary amongst fields (~8–35 nmol/g soil).

In regard to the persistence of ginsenosides in ginseng garden soils, unfortunately, Yang et al. (2015) did not report ginsenoside levels beyond year three of Asian ginseng cultivation. Based on my analysis, ginsenosides do not persist in ginseng garden soils for long and return to near non-detectable levels approximately one-year post-harvest. Nonetheless, my data suggests ginsenosides are not a direct cause of GRD and instead, may contribute to the establishment of conditions in which GRD occurs. Furthermore, I found PPD-type ginsenosides accumulated more than PPT-type ginsenosides with an average of approximately 5–16 times greater over the course of the four-year cultivation

cycle and post-harvest. Since it has been shown that PPD-type ginsenosides stimulate the growth of *I. mors-panacis* (Zhao et al., 2012) and *I. mors-panacis* accumulates significantly in the fourth year of *P. ginseng* cultivation (Tong et al., 2021), these results suggest that *I. mors-panacis* inoculum may similarly increase during the four-year cultivation of American ginseng, especially when ginsenosides reach peak concentrations in third year. When *I. mors-panacis* inoculum is substantial, older ginseng roots may be robust enough to not be very susceptible to infection by *I. mors-panacis*; however, in subsequent plantings, newly emerged or young seedlings may be very susceptible to *I. mors-panacis* pre-existing in the soil.

In addition to *I. mors-panacis*, other fungi have been shown to accumulate in later years of ginseng cultivation. Fungal pathogens *Plectosphaerella*, *Cladosporium*, and *Alternaria* and saprophytic fungi *Talaromyces* and *Saitozyma* accumulated during the fifth year of Asian ginseng cultivation, perhaps due to the use of ginsenosides as a carbon source and subsequently increasing the abundance of these fungal pathogen (Jin et al., 2022). It has been documented that *Fusarium* and *Monographella* utilize ginsenosides as a carbon source, particularly ginsenosides Rb_1, Rg_1, and Rd; however, other microorganisms likely utilize ginsenosides as a carbon source (Luo et al., 2020). Possibly due to the utilization of ginsenosides as a carbon source by microorganisms, in ginseng garden soils during cultivation, Rb_1 was the least abundant relative to other PPD-type ginsenosides, and Rg_1 abundance was low relative to other PPT-type ginsenosides. *In vitro* exposure to ginsenosides can also enhance the growth of other fungal pathogens including *Fusarium*, *Monographella*, *Gibberella*, and *Neocosmospora*, and oomycete pathogens *Globisporangium irregulare* (previously *P. irregulare*) and *Phytophthora cactorum* (Luo et al., 2020; Miao et al., 2023; Nicol et al., 2003). Soil fungal diversity has been also shown to decrease with aged Asian ginseng soil (Jin et al., 2022, Xiao et al., 2016). These differences in fungal communities are likely due to the accumulation of ginsenosides during ginseng cultivation, perhaps leading to the increase of several fungal pathogens in these soils, as noted above.

Since *Ilyonectria* root-rot is more prevalent during subsequent plantings of ginseng even though *Ilyonectria* is present in soils during the first cultivation of ginseng (Tong et al., 2021), I investigated whether previous exposure to ginsenosides enhances *Ilyonectria* virulence. To understand if the pathogenicity of *Ilyonectria* on ginseng increases following ginsenoside exposure, twelve *Ilyonectria* isolates were screened for their pathogenicity towards American ginseng. Of the twelve, all but one of the seven isolates (*I. mors-panacis*; I.sp.7 – I.sp.12) previously isolated from ginseng (with the exception of *I. mors-panacis* I.sp.3) displayed a high virulence toward ginseng, while the remaining ones did not. This suggests that prior exposure to ginseng (and ginsenosides) may lead to higher *Ilyonectria* virulence and thus could help explain why *Ilyonectria* root rot is more prevalent during subsequent plantings of ginseng. To determine whether prior exposure to ginsenosides enhances *Ilyonectria* virulence, six *Ilyonectria* isolates (i.e., four low virulent; *I. rufa*; I.sp.1, I.sp.2, *I. mors-panacis* I.sp.3, I.sp.4, and two high virulent; *I. robusta*; I.sp.6, *I. mors-panacis* I.sp.11) were used. By comparing the virulence of *Ilyonectria* isolates on American ginseng roots following growth on ginsenoside-infused plates (four plate transfers), I observed that two isolates of *I. rufa* (I.sp.1 and I.sp.2), previously isolated from gymnosperms, showed increased in virulence on American ginseng. When comparing ginseng roots inoculated with *Ilyonectria* isolates with and without prior exposure to ginsenosides in my lab, ginseng roots inoculated with I.sp.1 previously exposed to ginsenosides yielded significantly greater lesion sizes compared to ginseng roots inoculated with I.sp.1 not previously exposed to ginsenosides ($P<0.0001$). However, ginseng roots inoculated with I.sp.2 yielded lesion areas that trended towards significant increases when previously exposed to ginsenosides in my lab ($P=0.07$). In my lab, each isolate was exposed to a high dose of ginsenosides for several months, well beyond the abundance typically present in ginseng garden soils. For example, the highest amount of ginsenosides detected in soils was an average of 0.0316 mg/g dried soil (from the early collection in fourth year at cultivation in site 1). Whereas in this study, *Ilyonectria* was supplemented with 1 mg/mL of ginsenosides. Thus, the experimental dose of the ginsenosides were 30-fold greater than the highest amount present in soils.

Regarding I.sp.3 and I.sp.4 (*I. mors-panacis*), these isolates were previously found to be low virulent isolates when tested on intact American ginseng seedlings but caused disease when tested on detached American ginseng roots. This result was somewhat surprising for isolate I.sp.3 since it was previously isolated from American ginseng.
Isolate I.sp.4, on the other hand was previously isolated from *Poa pratensis* (Kentucky bluegrass) and not American ginseng, but both I.sp.3 and I.sp.4 were confirmed in this study to be *I. mors-panacis*. Various factors differ between the two bioassays that may have led to these results. For example, different-aged ginseng (one-year old seedings and two-year-old detached roots) were used, and the pin used to hold the inoculum plug in place on detached American ginseng roots provided direct entry for infection.
Additionally, it is not uncommon for fungi to lose their virulence following continuous subculturing on media (Butt et al., 2006). All twelve isolates tested were previously isolated in approximately 1970's–1990's (Cabral et al., 2012), suggesting that some isolates may have lost some virulence. On the contrary, it is also clear that many isolates were able to retain their virulence despite being isolated decades prior, suggesting that *Ilyonectria* in ginseng garden soils could retain virulence and contribute to GRD decades later.

The apparent increase in virulence of *Ilyonectria* isolates exposed to ginsenosides led me to believe that virulence may be related to the ability to metabolize ginsenosides.
Such a correlation has been found for the ginseng pathogen *Globisporangium irregulare* (previously *Pythium irregulare*), an oomycete (Ivanov & Bernards, 2012). The metabolism of ginsenosides by the six *Ilyonectria* isolates grown previously with and without ginsenosides was examined and surprisingly, there was no correlation between virulence and metabolism of ginsenosides. All *Ilyonectria* isolates (*I. rufa*; I.sp.1, I.sp.2, *I. mors-panacis*; I.sp.3, I.sp.4, *I. robusta*; I.sp.6) were able to metabolize select ginsenosides and all *Ilyonectria* isolates, with the exception of I.sp.2, the isolate that had increased its virulence when exposed to ginsenosides, metabolized significant amounts of PPD- type ginsenoside Rb_1. Rb_1 can be converted into ginsenosides Gypenoside XVII, Rd, and F_2 by the removal of one or two glucose groups at the C-3 and C-20 positions (Figure 4.1), typically through the action of extracellular glycosidases (Zeng et al., 2021).

Gypenoside XVII was found to accumulate in the culture media of several *Ilyonectria* isolates, regardless of whether they had prior exposure to ginsenosides, likely from the deglycosylation of Rb_1. The decrease in the amounts of Rb_1 does not exactly equate to the amount of Gypenoside XVII accumulating and, therefore, Rb_1 is likely also being degraded into Rd and F_2 (which may also be further metabolized), or broken down into other, unknown breakdown products. For instance, an unidentified compound (sodium adduct mass: 807.4865 m/z, retention time: 6.87 min) was detected in spent media from some *Ilyonectria* isolates and was not present in control samples. The sodium-adduct mass of this unknown compound is the same as F_2 albeit with a later retention time, suggesting two possible structures that could be derived from the deglycosylation of Rb_1, Gypenoside XVII, Rd, and Rc/Rb_2 (Figure 4.1). This unidentified ginsenoside accumulated the most in the I.sp.2 exposed to ginsenosides and in some other *Ilyonectria* isolates; however, there was no correlation between the presence of the novel ginsenoside, prior exposure to ginsenosides, or virulence.

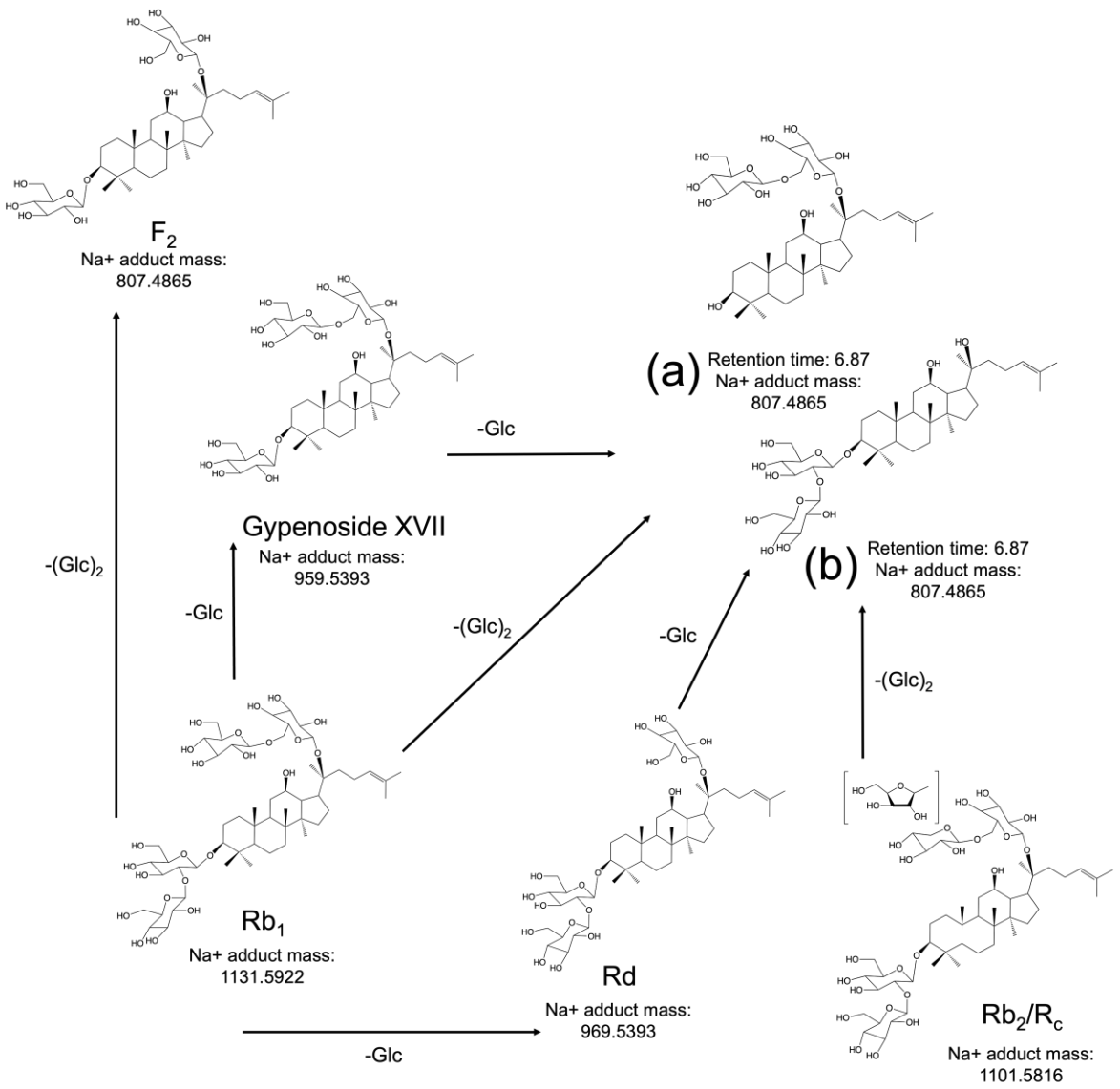

F2
Na+ adduct mass:
807.4865
Gypenoside XVII
Na+ adduct mass:
959.5393
Rb1
Na+ adduct mass:
1131.5922
Rd
Na+ adduct mass:
969.5393
(a)
Retention time: 6.87
Na+ adduct mass:
807.4865
(b)
Retention time: 6.87
Na+ adduct mass:
807.4865
Rb2/Rc
Na+ adduct mass:
1101.5816
-Glc
-(Glc)2
-Glc
-(Glc)2
-Glc
-(Glc)2
-Glc

Figure 4.1. Hypothetical degradation of known 20(S)-protopanaxadiol ginsenosides into an unidentified ginsenoside after deglycosylation by *Ilyonectria* isolates.
Ginsenosides (Rb_1, Gypenoside XVII, Rd, Rb_2/R_c) may be deglycosylated by extracellular glycosidase(s) from *Ilyonectria* isolates into an unidentified ginsenoside (Na^+ adduct mass: 807.4865 m/z, retention time: 6.87 min). The unknown compound has the same m/z as F_2, but a different retention time. Two possible structures, designated (a) and (b), consistent with the known compound m/z are shown. Loss of sugars from the C-3 position of ginsenosides Rb_1 and Gypenoside XVII would yield structure (a). Loss of sugars from the C-20 position of ginsenosides Rb_1, Rd, and Rb_2/R_c would yield structure (b). Noted in this diagram is the possible degradation of Rb_1 into F_2, Gypenoside XVII, Rd, and Rb_2/Rc. The degradation of Gypenoside XVII, Rd, and Rb_2/Rc into F_2 is not indicated, but is possible as well.

All *Ilyonectria* spp. isolates used in my study were able to metabolize select PPD-type ginsenosides. This is consistent with the metabolism of PPD-type ginsenosides, but not PPT-type ginsenosides, reported earlier (Wang et al., 2018). When combining these results with what is now known about ginsenosides in ginseng garden soils, it is evident that *I. mors-panacis* may be metabolizing PPD-type ginsenosides and subsequently, increasing in growth during ginseng cultivation. Since many *I. mors-panacis* isolates were able to metabolize Rb_1, it may be that they quickly metabolize Rb_1 in ginseng garden soils converting it into several other ginsenosides (Gypenoside XVII, Rd, and F_2). This may provide some explanation for the low abundance of Rb_1 in ginseng garden soils and the relatively high abundance of Gypenoside XVII and F_2.

Regarding *I. rufa,* this species was first identified as a species within the *I. radicicola* species complex in 2012 (Cabral et al., 2012). Due to its recent identification, our knowledge of *I. rufa* is limited and it may have been previously examined in other studies investigating *Ilyonectria* and ginseng. It is possible that past studies on *C. destructans*/*I. mors-panacis*, were examining other species of *Ilyonectria* instead, such *I. rufa*. From my research, it has become abundantly clear that the morphology of the *Ilyonectria* isolates used, is variable amongst species and not consistent with previous studies utilizing the same isolates.

When Cabral et al. (2012) examined several *Ilyonectria* isolates, they described the phenotype of several isolates that were also used in my research. Surprisingly, the phenotype of several of the isolates that I used differed from that reported by Cabral et al. (2012) despite being identified by sequencing as the same species. For instance, I.sp.1 (*I. rufa*) (DAOMC 226721, CPC 13536) was described as rosy-buff to cinnamon on PDA, with aerial mycelium buff to rosy-buff or pale luteus in the center. In my research, I consistently observed I.sp.1 as pale or muted yellow with no rose or cinnamon colour apparent. I.sp.2 (*I.rufa*) (DAOMC 251609, 94-1628) was similarly described by Cabral et al. (2012), but instead when grown in the absence of ginsenosides in our lab, the colour of I.sp.2 was similar to I.sp.1, but when it was grown in the presence of ginsenosides, it was a chestnut or sienna tone that turned the PDA media orange. Similarly, I.sp.3 (*I.*

mors-panacis) (DAOMC 251610, CBS 120365) and I.sp.4 (*I. mors-panacis*) (DAOMC 150670, CPC 13534) were also described differently by Cabral et al. (2012). However, the description of I.sp.6 (*I. robusta*) (DAOMC 139398, CPC 13532) by Cabral et al. (2012) was similar to what was observed in my study. Lastly, I.sp.11 (*I. mors-panacis*) (DAOMC 234582, CBC 120367), one of the most virulent strains in my study, was described as chestnut to sienna on PDA by Cabral et al. (2012), but I found it to be consistently a pale-muted yellow colour. Rahman & Punja (2005) noticed that highly aggressive *I. mors-panacis*/*C. destructans* strains were dark to rust brown-coloured while the less aggressive strains were beige to light brown-coloured on PDA. This observation was refuted in my study as it was clear that *I. rufa* and *I. mors-panacis* changed their morphology drastically and could not be identified to the species level or have their virulence predicted by their morphology.

4.2 Conclusion and future directions

Overall, my research provides an explanation as to why *Ilyonectria* root rot is more prevalent during subsequent plantings of ginseng and can partially be attributed to its exposure to ginsenosides during the first cultivation of ginseng. It is already known that *I. mors-panacis* can remain in soils for long-periods of time as chlamydospores, to overcome winters and other sub-optimal environmental conditions (Kang et al., 2016). Therefore, *I. mors-panacis* may be present in ginsenoside-altered virulent form as chlamydospores, in former ginseng garden soils and ready for when ginseng is planted again, even years later.

Both low and high virulence *Ilyonectria* isolates were able to metabolize select ginsenosides, thus the ability to metabolize ginsenosides does not correlate with the pathogenicity of *Ilyonectria* on American ginseng. This contrasts with oomycete *G. irregulare*, whose virulence on American ginseng correlates with the ability to metabolize ginsenosides (Ivanov & Bernards, 2012). It may be that *I. mors-panacis* is isolated from infected American ginseng roots more than other *Ilyonectria* species perhaps due to our lack of knowledge regarding other *Ilyonectria* species, such as *I. rufa*, or due to the competition between these species in the soil. For instance, when *G. irregulare* is in close proximity to F_2, it swims away from F_2 resulting in a zone of

inhibition when grown in close proximity to F_2 *in vitro*, but does not display this zone of inhibition when exposed to Re, Rb_1, and crude ginsenoside extract from ginseng roots, (Ivanov et al., 2016). It has been recently shown that PPD-type ginsenosides are less mobile in soil than PPT-type ginsenosides therefore, PPD-type ginsenosides likely accumulate closer to ginseng roots (Andrew Rabas, unpublished data). The accumulation of PPD ginsenosides near roots may attract *Ilyonectria* towards the roots. When in close proximity to roots, *I. mors-panacis* may display a differing chemotropism than other *Ilyonectria* isolates and inoculate ginseng roots more readily. The chemotropism of *I. mors-panacis* towards each PPD-type ginsenoside should be investigated in the future.

Since the metabolism of ginsenosides is not correlated with *Ilyonectria* virulence, there are likely other factors involved in the difference in virulence as well as increase in virulence following exposure to ginsenosides. Not dismissing the fact that the two assays used in my study differed in several factors; I.sp.3 and I.sp.4 were not highly virulent when I examined the pathogenicity of these isolates on intact American ginseng seedlings in sand, but large lesions where produced when inoculation of ginseng roots was facilitated by a wound on detached roots. Highly virulent isolates may be able to infect ginseng roots more readily via enzymes that contribute to root cell wall degradation, such as pectin lyase, laccases, proteases, pectinase, and cellulase (Pathrose, 2012; Rahman & Punja, 2005). In the future, an investigation into the extracellular enzymes produced by low and high virulent isolates, with and without previous exposure to ginsenosides, should be explored.

In addition to investigating extracellular enzymes, the microbiome of ginseng garden soils should be assessed in concert with assessing ginsenoside accumulation. There are clear effects to soil microbiome during ginseng cultivation, such as an increase in *I. mors-panacis* inoculum (Tong et al., 2021), a decrease in fungal diversity (Jin et al., 2022, Xiao et al., 2016) and an increase in soil-borne fungal pathogens (Jin et al., 2022). However, it is not known if the changes in ginsenoside accumulation during cultivation influence the differences in soil microbiome. Studies that have measured ginsenoside accumulation in ginseng garden soils reveal different patterns of accumulation (Samukawa et al., 1995; Yang et al., 2015) thus, correlations with changes in soil microbiomes cannot be

confidently made in unrelated studies (Jin et al., 2022, Xiao et al., 2016). The bacterial microbiome in the same soils used in my study has been assessed for the first three of four years of cultivation and a decrease in bacterial diversity has been reported (Wan, 2023), similarly to Xiao et al. (2016) who found a decrease in microbial diversity with an increase in cultivation years. The soil mycobiome also needs to be assessed to better understand the impact of ginsenoside accumulation on fungi in bulk soils and what effects this may have on the establishment of GRD. Nonetheless, my research has provided some insight to the effects that ginsenoside accumulation and persistence may pose on *Ilyonectria* spp. and their virulence and as to why *Ilyonectria* root rot is more prevalent during subsequent plantings of ginseng. That is, it can be partially attributed to an increase in virulence by *Ilyonectria* spp. following exposure to ginsenosides during the first cultivation of ginseng.

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Appendices

Supplementary Table 1. Calibration curve equations calculated for nine ginsenosides.
Calibration curves created for individual ginsenosides using a linear regression (y = mx+b) and R^2 calculated.

Ginsenoside	Mass $[M+Na]^+$	Calibration Curve Equation (pmol/inj)[a]	R^2 Value[a]	LOD[a] (pmol/ 2 µL)	Calibration Curve Equation (µg/mL) [b]	R^2 Value[b]	LOD[b] (µg/mL)
Rb_1	1131.5922	Y = 39581x - 1351	0.9959	0.0154	Y = 8117.1x + 3470.3	0.9990	0.7111
Rb_2	1101.5816	Y = 25131x - 3875	0.9994	0.0354	Y = 9211.9x - 615.71	0.9994	0.0175
Rc	1101.5816	Y = 21183x - 4117	0.9979	0.0354	Y = 17308x + 5401.2	0.9931	0.0901
Rd	969.5393	Y = 20303x - 912.2	0.9993	0.0245	Y = 6813.8x + 4757.8	0.9970	0.2799
Gyp XVII	969.5393	Y = 28697x - 9423	0.9939	0.0483	Y = 15446x - 3204.4	0.9962	0.1358
F_2	807.4865	Y = 30077x - 3.829	0.9997	0.0030	Y = 35115x - 1498.1	0.9975	0.1090
F_{11}	823.4814	Y = 28062x - 4014	0.9965	0.0969	Y = 109033x - 11132	0.9989	0.0251
Rg_1	823.4814	Y = 26139x - 2070	0.9997	0.0178	Y = 21982x + 16020	0.9951	0.0818
Re	969.5393	Y = 25967x - 2488	0.9999	0.0315	Y = 16315x + 858.67	0.9996	0.1063

[a] Calibration curve equation used to quantify ginsenosides in soils from Planting–Harvest periods.

[b]Calibration curve equation used to quantify ginsenosides in soils from Persistence period.

Supplementary Table 2. Statistical analysis of measured ginsenosides (Rg_1, Re, F_{11}, Rb_1, Rc/Rb_2, Rd, Gypenoside XVII, F_2) in ginseng garden soils in sites 1, 2, and 3. Friedman test with Dunn's multiple comparisons. Log_{10}((nmol ginsenosides/g soil) + 1) measured at each time period were compared to planting (October/November) as a control.

Site 1

Time Period		Dunn's Adjusted P Value
Planting	**Oct/Nov**	/
Year 1 (2019)	**Apr/May**	>0.9999
	Jun/Jul	>0.9999
	Aug/Sep	>0.9999
	Oct/Nov	>0.9999
Year 2 (2020)	**Apr/May**	>0.9999
	Jun/Jul	>0.9999
	Aug/Sep	0.0359
	Oct/Nov	0.1258
Year 3 (2021)	**Apr/May**	>0.9999
	Jun/Jul	0.1061
	Aug/Sep	0.0201
	Oct/Nov	0.206
Year 4 (2022)	**Apr/May**	0.0245
	Jun/Jul	0.0892
	Aug/Sep	>0.9999
Harvest	**Oct/Nov**	>0.9999
Persistence	**Apr/May**	>0.9999
	Oct/Nov	>0.9999

Site 2

Time Period		Dunn's Adjusted P Value
Planting	**Oct/Nov**	/
Year 1 (2019)	**Apr/May**	>0.9999
	Jun/Jul	>0.9999
	Aug/Sep	>0.9999
	Oct/Nov	0.2335
Year 2 (2020)	**Apr/May**	>0.9999
	Jun/Jul	>0.9999
	Aug/Sep	0.7213
	Oct/Nov	0.1639
Year 3 (2021)	**Apr/May**	0.2773

	Jun/Jul	0.0635
	Aug/Sep	0.0021
	Oct/Nov	0.0424
Year 4 (2022)	**Apr/May**	0.0092
	Jun/Jul	0.0424
	Aug/Sep	0.4543
Harvest	**Oct/Nov**	>0.9999

Site 3

Time Period		**Dunn's Adjusted P Value**
Planting	**Oct/Nov**	/
Year 1 (2019)	**Apr/May**	>0.9999
	Jun/Jul	>0.9999
	Aug/Sep	>0.9999
	Oct/Nov	>0.9999
Year 2 (2020)	**Apr/May**	>0.9999
	Jun/Jul	>0.9999
	Aug/Sep	0.7753
	Oct/Nov	0.5113
Year 3 (2021)	**Apr/May**	0.4426
	Jun/Jul	0.3288
	Aug/Sep	0.0047
	Oct/Nov	0.0134
Year 4 (2022)	**Apr/May**	0.2415
	Jun/Jul	>0.9999
	Aug/Sep	>0.9999
Harvest	**Oct/Nov**	>0.9999
Persistence	**Apr/May**	>0.9999
	Oct/Nov	>0.9999

Supplementary Data 1. FASTA format sequences of *Ilyonectria* isolates used in phylogenetic analysis (section 3.3).
Raw FASTA format sequences of *Ilyonectria* isolates (I.sp.1–I.sp.12) were obtained by amplifying HIS region using Sanger sequencing (section 2.5). *Ilyonectria* isolates were identified as described in sections 3.2–3.3. DAOMC code provided by CCFC. *Ilyonectria* isolate isolated from host plant and location, stated by CCFC.

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TCCCTCTTCGAGGACACC
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CTGGTGAGTTTTCCCCTGTGCCGACCTGACCGTCTCGTCTTCTGCTCGACGCGTCTTGCAGCATCGCACATACCCAACGT
CTTCCTCACATACTGACATCACACAGCCCGCAAGAGCGCCCCCTCTACCGGTGGTGTCAAGAAGCCTCACCGCTACAAG
CCCGGTACCGTCGCTCTCCGTGAGATTCGACGATACCAGAAGTCGACCGAGCTTCTCATCCGCAAGCTCCCCTTCCAGC
GTCTGGTAAGCATCATCCATGCCCACACCCAACGTGCCACATCAAGCATCCACTCTAACGCACCCGACACAGGTCCGTG
AGATCGCCCAGGACTTCAAGAGCGACCTTCGCTTCCAGTCCTCCGCCATCGGCGCCCTCCAGGAGTCCGTCGAGTCCTA
CCTTGTCTCCCTCTTCGAGGACACCAACCTGTGCG

Supplementary Table 3. Statistical analysis of virulence analysis of twelve *Ilyonectria* spp. on American ginseng seedlings.
One-way ANOVA with Dunnett's post-hoc test was performed on measurements at 28-days-post-infection compared to control chlorophyll fluorescence F_v/F_m values (A) or disease severity (B).

(A) Chlorophyll Fluorescence

Ilyonectria Isolate	Dunnett's Adjusted P Value
I.sp.1	>0.9999
I.sp.2	0.9954
I.sp.3	0.7938
I.sp.4	0.5465
I.sp.5	0.2144
I.sp.6	0.0076
I.sp.7	0.0051
I.sp.8	0.0044
I.sp.9	0.0014
I.sp.10	0.0015
I.sp.11	0.0028
I.sp.12	0.0014

(B) Disease Severity

Ilyonectria Isolate	Dunnett's Adjusted P Value
I.sp.1	0.9641
I.sp.2	0.3812
I.sp.3	0.3812
I.sp.4	0.6321
I.sp.5	0.4580
I.sp.6	0.0041
I.sp.7	0.0020
I.sp.8	0.0441
I.sp.9	0.0302
I.sp.10	0.0083
I.sp.11	0.0019
I.sp.12	0.0010

Supplementary Table 4. Statistical analysis of virulence of six *Ilyonectria* isolates with and without previous exposure to ginsenosides, on American ginseng roots. Two-way ANOVA with (A) Šidak's post-hoc test was performed on $\log_{10}$(lesion area (mm^2) + 1) of each isolate comparing treatment groups (without and with ginsenoside exposure), and with (B) Dunnett's post-hoc test was performed on $\log_{10}$(lesion area (mm^2) + 1) of each isolate with each treatment group (without and with ginsenoside exposure) compared to applicable control (without and with ginsenoside exposure).

(A)

Ilyonectria Isolate	Treatment	Šidak's Adjusted P Value
Control	Without Ginsenoside Exposure	>0.9999
	With Ginsenoside Exposure	
I.sp.1	Without Ginsenoside Exposure	0.0743
	With Ginsenoside Exposure	
I.sp.2	Without Ginsenoside Exposure	<0.0001
	With Ginsenoside Exposure	
I.sp.3	Without Ginsenoside Exposure	0.9986
	With Ginsenoside Exposure	
I.sp.4	Without Ginsenoside Exposure	0.331
	With Ginsenoside Exposure	
I.sp.6	Without Ginsenoside Exposure	0.8967
	With Ginsenoside Exposure	
I.sp.11	Without Ginsenoside Exposure	0.1365
	With Ginsenoside Exposure	

(B)

Ilyonectria Isolate	Treatment	Dunnett's Adjusted P Value
I.sp.1	Without Ginsenoside Exposure	0.9953
	With Ginsenoside Exposure	0.0592
I.sp.2	Without Ginsenoside Exposure	0.8969
	With Ginsenoside Exposure	<0.0001
I.sp.3	Without Ginsenoside Exposure	<0.0001
	With Ginsenoside Exposure	<0.0001
I.sp.4	Without Ginsenoside Exposure	0.3193
	With Ginsenoside Exposure	0.0146
I.sp.6	Without Ginsenoside Exposure	<0.0001
	With Ginsenoside Exposure	<0.0001
I.sp.11	Without Ginsenoside Exposure	<0.0001

With Ginsenoside Exposure	0.0022

Supplementary Table 5. Amount of individual ginsenosides recovered from culture media without incubation with *Ilyonectria* (control).
Ginsenosides from control culture media were recovered 7-days-post-incubation, and individual ginsenosides were measured (mean ± SE). Ginsenosides are ordered from highest to lowest amounts.

Ginsenoside	Mean ± SE
Rb_1	1.504 ± 0.042
Gypenoside XVII	0.712 ± 0.038
Re	0.629 ± 0.017
F_2	0.416 ± 0.027
Rc/Rb_2	0.164 ± 0.010
F_{11}	0.138 ± 0.008
Rd	0.090 ± 0.006
Rg_1	0.089 ± 0.003

Supplementary Table 6. Statistical analysis of metabolism of ginsenosides by *Ilyonectria* isolates with and without previous ginsenoside exposure.

Two-way ANOVA with (A) Dunnett's post-hoc test and (B) Šidak's post-hoc test was performed on relative amounts of ginsenosides compared to control (1.0) of each ginsenoside (Rg_1, Re, F_{11}, Rb_1, Rc/Rb_2, Rd, Gyp. XVII, F_2), 20(S)-PPT (protopanaxatriols), 20(S)-PPT (protopanaxadiols), and total ginsenosides.

(A)

Ilyonectria Isolate	Treatment	Two-Way ANOVA			Dunnett's Adjusted P Value										
		P Value	F Value	SS (Type III)	Rg_1	Re	F_{11}	Rb_1	Rc/Rb_2	Rd	Gyp. XVII	F_2	20(S)-PPT	20(S)-PPD	Total
I.sp.1	Without Ginsenoside Exposure	<0.0001	(20, 176) = 28	8.9	0.6448	0.6701	0.9145	<0.0001	<0.0001	<0.0001	0.3662	<0.0001	0.8202	<0.0001	<0.0001
	With Ginsenoside Exposure				0.4475	0.8983	0.9633	<0.0001	<0.0001	<0.0001	0.8832	<0.0001	0.9688	<0.0001	<0.0001
I.sp.2	Without Ginsenoside Exposure	<0.0001	(20, 187) = 21	11	0.0206	0.1181	<0.0001	<0.0001	0.1165	<0.0001	<0.0001	<0.0001	0.0411	<0.0001	0.0001
	With Ginsenoside Exposure				0.5260	0.5922	0.4225	0.4771	0.7619	0.7401	<0.0001	<0.0001	0.5558	0.9718	0.9094
I.sp.3	Without Ginsenoside Exposure	<0.0001	(20, 187) = 17	6	0.1781	0.4636	0.9237	0.0457	0.8454	0.2490	<0.0001	<0.0001	0.5039	0.9993	0.9563
	With Ginsenoside Exposure				0.0081	0.1369	0.1388	0.0199	0.8605	0.2922	<0.0001	<0.0001	0.1079	0.9972	0.8530
I.sp.4	Without Ginsenoside Exposure	<0.0001	(20, 187) = 21	15	0.0182	0.1392	0.6977	<0.0001	0.0212	0.0043	<0.0001	<0.0001	0.2575	0.0115	0.1322
	With Ginsenoside Exposure				0.8629	0.0757	<0.0001	<0.0001	<0.0001	<0.0001	0.0089	<0.0001	0.0431	<0.0001	<0.0001
I.sp.6	Without Ginsenoside Exposure	<0.0001	(20, 187) = 13	10	0.1609	0.1017	<0.0001	<0.0001	0.0019	0.0006	<0.0001	<0.0001	0.0415	0.0046	0.1223
	With Ginsenoside Exposure				0.9996	0.5142	0.3009	<0.0001	<0.0001	<0.0001	0.4937	<0.0001	0.5473	<0.0001	<0.0001

I.sp.11	Without Ginsenoside Exposure	<0.0001	(20, 187) = 8.3	7.9	0.3559	0.6349	0.1857	0.0007	0.1732	0.1048	<0.0001	<0.0001	0.5170	0.1458	0.4340
	With Ginsenoside Exposure				0.4178	0.5614	0.0811	0.0018	0.0988	0.0298	<0.0001	<0.0001	0.4330	0.2094	0.5480

(B)

***Ilyonectria* Isolate**	**Treatment**	**Two-Way ANOVA**			**Šidak's Adjusted P Value**										
		P Value	**F Value**	**SS (Type III)**	**Rg_1**	**Re**	**F_{11}**	**Rb_1**	**Rc/Rb_2**	**Rd**	**Gyp. XVII**	**F_2**	**20(S)-PPT**	**20(S)-PPD**	**Total**
I.sp.1	Without Ginsenoside Exposure	0.8821	(10, 77) = 0.50	0.038	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.3923	>0.9999	>0.9999	0.9996	>0.9999
	With Ginsenoside Exposure														
I.sp.2	Without Ginsenoside Exposure	<0.0001	(10, 88) = 11	3.5	0.9202	0.9982	0.1606	<0.0001	0.4082	<0.0001	>0.9999	0.0085	0.9701	<0.0001	0.0058
	With Ginsenoside Exposure														
I.sp.3	Without Ginsenoside Exposure	0.8839	(10, 88) = 0.50	0.063	0.9212	0.999	0.807	>0.9999	>0.9999	>0.9999	0.9437	0.9995	0.9932	>0.9999	>0.9999
	With Ginsenoside Exposure														
I.sp.4	Without Ginsenoside Exposure	<0.0001	(10, 88) = 5.0	2.5	0.7576	>0.9999	0.0025	0.6024	0.2648	0.5255	<0.0001	0.9994	0.9998	0.1629	0.6135
	With Ginsenoside Exposure														
I.sp.6	Without Ginsenoside Exposure	0.8445	(10, 88) = 0.56	0.32	0.9316	0.9996	0.2894	0.571	0.179	0.1214	0.0703	0.9993	0.9888	0.4625	0.6389
	With Ginsenoside Exposure														

I.sp.11	Without Ginsenoside Exposure	>0.9999	(10, 88) = 0.037	0.028	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
	With Ginsenoside Exposure														

Curriculum Vitae

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Academic Conferences:
Colo, A., & Bernards, M.A. (2024). Do Ginsenosides Alter the Pathogenicity of *Ilyonectria*? Plant Canada. Winnipeg, MB, Canada. [Oral Presentation]

Colo, A., Ong, A., Tran, M., & Bernards, M.A. (2023). Pathogenicity of *Ilyonectria mors-panacis* on American ginseng Using Chlorophyll Fluorescence Measurements. Canadian Society of Plant Biologists, Eastern Regional Meeting. Montreal, QC, Canada. [Poster]

Colo, A., Ong, A., Tran, M., & Bernards, M.A. (2023). Pathogenicity of *Ilyonectria mors-panacis* on American ginseng Using Chlorophyll Fluorescence Measurements. Canadian Pest Management Conference. Guelph, ON, Canada. [Poster]

Colo, A., Ong, A., Tran, M., & Bernards, M.A. (2023). Pathogenicity of *Ilyonectria mors-panacis* on American ginseng Using Chlorophyll Fluorescence Measurements. Western Biology Graduate Research Forum. London, ON, Canada. [Poster]

Ginseng Grower Meetings:
Colo, A.., & Bernards, M.A. (2024). Do Ginsenosides Alter the Pathogenicity of *Ilyonectria*? Ginseng Replant Working Group. Simcoe, ON, Canada. [Oral Presentation]

Colo, A., & Bernards, M.A. (2023). A Bacterial Microbiome Analysis of Solarized Ginseng Garden Soils. Ginseng Replant Working Group. Simcoe, ON, Canada. [Oral Presentation]