Identification and Characterization of *Trichoderma* species from Vineyards in British Columbia and Studies on Their Potential Use as Biological Control Agents Against the Grapevine Trunk Disease Botryosphaeria Dieback

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submitted by Jinxz Pollard-Flamand in partial fulfillment of the requirements of

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Abstract

One of the most important biotic factors limiting grapevine health around the world is grapevine trunk disease (GTD). Fungal pathogens responsible for GTD infect vines mainly through pruning wounds and cause an overall decline and eventual death of the plant. Currently, there are no chemical fungicides nor biological control agents (BCA) registered in Canada for the control of GTD. Species of *Trichoderma* are capable of promoting grapevine health and can also protect vines by actively antagonizing plant pathogenic fungi, including GTD pathogens, through several modes of action. Accordingly, the objectives of this research were i) to identify *Trichoderma* spp. from the Okanagan Valley in British Columbia (BC) by multi-locus gene sequencing and ii) to screen for isolates that can be used as BCA against *Diplodia seriata* and *Neofusicoccum parvum*, two of the most prevalent GTD fungi found in BC. In total, 29 *Trichoderma* isolates were obtained from grapevines in BC. Molecular analyses of the internal transcribed spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA (rDNA) and a partial sequence of the translation elongation factor 1-alpha gene allowed the identification of seven species, including *T. asperelloides*, *T. atroviride*, *T. canadense*, *T. harzianum*, *T. koningii*, *T. tomentosum*, and *T. viticola*. Among these, *T. canadense* and *T. viticola* are novel species to the scientific community. The antagonistic abilities of *Trichoderma* species against *D. seriata* and *N. parvum* were screened *in vitro* via dual culture assays and best performing isolates were tested *in plantae* under greenhouse controlled conditions via detached cane assays alongside commercial pruning wound protectants. The species that performed best under greenhouse conditions were selected to further testing under field conditions in a Merlot vineyard. *Trichoderma*-based treatments from BC provided >90% reduction of infection when pruning wounds where challenged with the pathogens 1d, 7d, 21d, and 60d post-treatment in the field. Overall, *Trichoderma* isolates from BC performed similar or better when compared against

commercial products. This research represents the initial steps required for the development and registration of local BCA for management of GTD in Canada.

Lay Summary

Grapevine trunk diseases (GTD) are caused by many different fungi. These fungi infect grapevines when spores land on open wounds and invade the wood of the plant. GTD are a major threat to grape production because they reduce yield, fruit quality, and will eventually kill the vine. Biocontrol agents are organisms that can be used to control plant pests due to their naturally antagonistic behavior. Several *Trichoderma* spp. have been shown to actively parasitize GTD fungi while contributing to the health of the plant. This study tested the biocontrol abilities of 26 *Trichoderma* isolates found in British Columbia against two important GTD fungi under laboratory, greenhouse and natural field conditions. Among them, isolates from three different species were able to provide more than 90% control for up to 60 days post-treatment against two important GTD fungi. *Trichoderma* isolates from BC performed similar or better when compared against commercial products.

Preface

This research project was developed primarily by myself and Dr. Úrbez-Torres with revisions and guidance provided by all members of the research committee.

For the study relating to identification and characterization of *Trichoderma* spp. from BC, Dr. Úrbez-Torres and I were primarily responsible for experimental design. Significant contributions were made by Julie Boulé (SuRDC) in the performance of the detached cane assays which evaluated local *Trichoderma* spp. and she was responsible for the performance of the detached cane assays evaluating the commercial products. I was primarily responsible for the DNA extraction, PCR amplification, and sequencing. I also helped Dr. Úrbez-Torres in compiling *Trichoderma* spp. sequences for subsequent phylogenetic analysis which was performed primarily by himself. The experimental design of the characterization studies was primarily my responsibility with guidance from Dr. Úrbez-Torres. I was primarily responsible for performing characterization experiments including determining optimum temperature for mycelial growth, optimum temperature for conidial germination, and the dual culture antagonism assay.

For the field studies evaluating *Trichoderma* spp. under field conditions, I helped with experimental design and field work and fungal re-isolations was conducted as a joint effort with help from undergraduate students, Julie Boulé (SuRDC), Melanie Walker (SuRDC), and Dr. Úrbez-Torres.

I was primarily responsible for all the data analysis and writing of the thesis with guidance from supervisors Dr. Hart and Dr. Úrbez-Torres.

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Chapter 1: Introduction

Grapevines, *Vitis vinifera* L. and *Vitis* spp., are one of the most extensively grown and economically important fruit crops worldwide. North America, with 0.43 million ha, is the fourth largest grape producer after the European continent (3.55 million ha), Asia (2.04 million ha) and South America (0.53 million ha) (FAOSTATS 2019). Grape-producing countries benefit tremendously from the major economic impact that grape and wine industries provide, regardless of their size. In Canada for instance, where only about 14,000 ha of grapevines are cultivated, the grape and wine industry generated over 37,000 full-time equivalent jobs and contributed over CAD$9 billion to the national economy in 2015 (Rimerman 2017). British Columbia (BC), with over 5,000 ha cultivated, is the second largest grape region in Canada after Ontario. In 2015, BC grape and wine industry alone contributed over CAD$2.77 billion of overall economic impact. An average bottle of wine sold in BC generates $48.17 economic impact, the highest among the Canadian provinces. More than 90% of grape production in BC is located throughout the Okanagan (84.1%) and Similkameen (6.4%) Valleys in the southern interior of the Province but other wine regions include the Fraser Valley, Kamloops, Vancouver Island, and the Gulf Islands (Bremmer 2014). Estimated at one million annual visitors, wine tourism is significant and growing throughout BC, particularly in the Okanagan Valley. Though BC is still considered a young and emerging wine growing region, its distinct combinations of soils and climate makes it suitable for producing a broad range of *V. vinifera* cultivars and high quality award winning wines.

However, this success also comes with challenges. Grapevine cultivation generates substantial production costs due to i) the high initial financial investment for vineyard establishment, ii) the costly annual vineyard operations required for production, and iii) the delay of up to three years that takes a vineyard to produce a full crop. Moreover, grapes are known to host the widest variety of pathogens of any woody agricultural plant (Martelli 1997; Wilcox et al. 2015); and

thus, a significant amount of the production costs are associated with intense pest and disease management programs, which include cultural practices, the cost of chemical and/or biological control products, and their application (Cooper et al. 2012). For example, grape growers in California spent over USD$189 million in 2011 to control one disease alone, grapevine powdery mildew caused by the fungus *Erysiphe necator* Schwein (Sambucci et al. 2014). Among all bacterial, fungal and viral diseases affecting grapes nowadays, grapevine trunk diseases (GTD) are considered the most important biotic factor reducing yield and limiting vineyard lifespan worldwide due to their complexity and narrow number of effective control strategies (Bertsch et al. 2013).

1.1 Grapevine trunk diseases

Grapevine trunk diseases occur wherever grapes are grown and are caused by a wide range of taxonomically unrelated fungal pathogens. Currently, over 130 fungal species belonging to 34 genera have been associated with different GTD (Gramaje et al. 2018). Grapevine trunk diseases include Petri disease and black foot disease, primarily affecting young vineyards (< 6 year-old) and Botryosphaeria dieback, esca, Eutypa dieback, and Phomopsis dieback affecting mostly mature vineyards (> 6 year-old). Petri disease is primarily caused by *Phaeomoniella chlamydospora* W. Gams, Crous, M.J. Wingf. & Mugnai and several fungi in the genus *Phaeoacremonium* W. Gams, Crous & M.J. Wingf. (Wilcox et al. 2015). The abovementioned fungi along with several basidiomycete species in the genera *Fomitiporia* Murrill, *Fomitiporella* Murrill, *Inocutis* Fiasson & Niemelä, *Inonotus* P. Karst., and *Phellinus* Quél. are also involved in the esca disease complex (Gubler et al. 2015). Black foot disease is primarily caused by fungi in the genera *Campylocarpon* Halleen, Schroers & Crous and *Ilyonectria* Diederich and they are found associated with necrotic tissues in roots and the basal end of rootstocks (Agusti-Brisach and Armengol, 2013). Several species in the family Botryosphaeriaceae Theiss. & P. Syd and Diatrypaceae Nitschke cause Botryosphaeria and Eutypa dieback, respectively (Úrbez-Torres,

2011; Rolshausen et al. 2015). *Diaporthe ampelina* (Berk. & M.A. Curtis) R.R. Gomes, Glienke & Crous (formely *Phomopsis viticola* Sacc.) is the main fungal pathogen causing Phomopsis dieback (Úrbez-Torres et al. 2013a).

With the exception of fungi causing black foot disease, which are soil-borne and infect grapevines primarily through wounds in the roots and openings at the basal end of the rootstock in grafted vines or scion in self-rooted vines, most fungal pathogens causing GTD are air-borne. Depending on the fungal species, grapevine pathogens responsible for Botryosphaeria dieback, Eutypa dieback, esca, and Phomopsis dieback release ascospores or conidia from respective perithecia or pycnidia embedded in the bark and/or on the surface of dead grapevine wood (Gramaje et al. 2018). Additionally, many of the fungi known to cause GTD have been also reported to cause cankers and dieback symptoms in many different woody perennial crops (Carter 1991; Gramaje et al. 2016). Accordingly, it is has been demonstrated that some of these hosts can serve as a source of inoculum primarily when near vineyards. Ascospores and conidia are released under favorable environmental conditions, which are primarily associated with rain events and/or high relative humidity (RH) along with temperatures above freezing, which also favor spore germination (Úrbez-Torres et al. 2010a, 2010b). Spores are then ejected from perithecia or pycnidia and spread by wind or rain-droplets within and/or between vineyards. Spores then land on susceptible pruning wounds to germinate and start colonizing new xylem vessels and pith parenchyma cells. Grapevines can be affected by one or more GTD at the same time since individual vines can be infected with different pathogens due to the multiple infection opportunities throughout a season and over the years. Overall, GTD cause stunting, chlorotic foliage, wilting of leaves or entire shoots, general dieback, wood necrosis, and perennial cankers (Gramaje et al. 2018). Many review articles are currently available with detailed descriptions on the specific symptomatology caused by each GTD, including black foot (Agusti-Brisach and Armengol, 2013; Halleen et al. 2006), Petri disease and esca (Mostert et al.

2006; Mugnai et al. 1999), Botryosphaeria dieback (Úrbez-Torres, 2011), Eutypa dieback (Carter, 1991), and Phomopsis dieback (Úrbez-Torres et al. 2013).

It is well-accepted that GTD represent one of the major threats to the future economic sustainability of viticulture, causing significant economic losses due to reduced yields, increased crop management costs for cultural and chemical preventive measures, and shortened life span of vineyards (Bertsch et al. 2013; Gramaje et al. 2016, 2018; Kaplan et al. 2016). Productivity is reduced over time by death of the spurs, canes, and/or cordons. The economic importance of GTD has been shown in several studies reported from around the world. In Italy, studies conducted at the end of the 1990s reported about 15% of the young vines in Sicily with symptoms of decline and high mortality within the first year of planting (Sidoti et al. 2000). Esca incidence has reached up to 80% in many mature vineyards of southern Italy (Romanazzi et al. 2009). It has been estimated that approximately 12% of French vineyards are economically unviable due to esca disease, leading to estimated losses of €1 billion (Lorch 2014). In California, annual economic losses due to Botryosphaeria and Eutypa dieback have been estimated at $USD260 million per year (Siebert 2001). In South Australia, yield losses of 1,500 kg per ha were estimated when 47% of Shiraz vines were affected by Eutypa dieback, leading to losses of AUD$2,800 per ha (Wicks and Davies 1999). More recently, GTD incidence and consequent plant mortality has also been reported to be rising throughout Chinese vineyards (Yan et al. 2013).

1.1.1 Botryosphaeria dieback

Chamberlain et al. (1964) reported *Diplodia mutila* (Fr.) Mont. (as *Sphaeropsis malorum* Berk.) to be isolated in about equal proportions, either alone or together with *D. ampelina* from lesions on trunks and stubs in vineyards in Ontario, Canada. Additionally, the same study confirmed

that spores of *D. mutila* could infect freshly cut grapevine stubs and be re-isolated from the resulting lesions completing Koch's postulates. Therefore, this study can be considered the first evidence of a Botryosphaeriaceae species as a grapevine trunk pathogen. Currently, Botryosphaeria dieback is the most prevalent and widespread GTD in vineyards around the world (Úrbez-Torres, 2011) and it has been associated with 26 botryosphaeriaceous taxa in the genera *Botryosphaeria* Ces. & De Not., *Diplodia* Fr., *Dothiorella* Sacc., *Lasiodiplodia* Ellis & Everh., *Neofusicoccum* Crous, Slippers & A.J.L. Phillips, *Neoscytalidium* Crous & Slippers, *Phaeobotryosphaeria* Speg., and *Spencermartinsia* A.J.L. Phillips, A. Alves & Crous (Gramaje et al 2018). Among these, species in the genera *Lasiodiplodia* and *Neofusicoccum* were shown through pathogenicity studies to be the most virulent on grapevines (Úrbez-Torres et al., 2009; Úrbez-Torres et al., 2008; van Niekerk et al., 2004). Symptoms of Botryosphaeria dieback may vary depending on the causal organism involved but generally includes bud necrosis, delayed spring growth, stunted shoots, dark and sunken lesions of infected wood, and wedge shaped cankers that can be observed via in cross-sections of spurs, cordons and/or trunks (Úrbez-Torres, 2011).

Fungi responsible for causing Botryosphaeria dieback overwinter in fruiting bodies (pycnidia) embedded in the bark of infected vines and, under optimum environmental conditions of relative humidity (RH) and temperature, get discharged to the environment (Úrbez-Torres et al. 2015). Spores are then spread short distances by the splash of rain droplets or large distances by wind (Amponsah et al. 2009; Úrbez-Torres et al. 2010). Conidia landing on pruning wounds can germinate and infect the vine by colonizing the vascular woody tissues, including phloem and xylem. Spore release of Botryosphaeriaceae spp. depend on climatic conditions and therefore, their abundance in a certain region at any particular point of the year will rely on the local climate. For example, in California it was found that most Botryosphaeriaceae spores (>60%) were trapped following rain events in the Winter months from December to February, remained

low in Fall and primarily absent during summer months (Úrbez-Torres et al. 2010). In contrast, spore trapping studies conducting in New Zealand showed spore release of Botryosphaeriaceae spp. to occur the entire year, with highest spore release during the growing season (Amponsah et al. 2009).

1.2 Available management strategies against grapevine trunk diseases

Grapevine trunk diseases management is notoriously complex due to the varied modes of infection and high diversity of pathogens. In addition, since the prohibition or phase out of some effective systemic chemicals such as sodium arsenite and the benzimidazol fungicide benomyl in the early 2000s (Decoin, 2001), GTD management is focused on development and implementation of prophylactic strategies to prevent infection. Infection by GTD pathogens can happen at any point from the grapevine propagation process in the nursery to the mature vineyard (Gramaje et al. 2018). GTD pathogens have been isolated and identified in healthy-looking mother vines and rootstocks as well as in water and on equipment used at different steps in the propagation process through the use of molecular detection methods and fungal isolation from suspected sources (Gramaje and Armengol 2011). As an example to understand the importance of these infection portals, it has been shown in New Zealand nurseries that infection by *P. chlamydospora* could increase from 40% to 70% as a result of nursery processing, resulting in infected material being distributed to vineyards which can significantly compound losses downstream (Whiteman 2004).

It has been proposed that the best approach to mitigate the impact of GTD is the implementation of an effective integrated pest management (IPM) strategy, which must include a wide range of management practices to limit sources of inoculum and to protect vines and cuttings from infection at every major point of susceptibility (Bertsch et al. 2013). IPM in nurseries often includes extensive sanitation practices to reduce localized inoculum sources,

such as thoroughly cleaning cold storage facilities and hydration tanks, implementation of hot water treatment (HWT) where nursery material is exposed usually to 50 °C for 30 minutes to control endogenous and exogenous pests already present in nursery cuttings, and chemical or biological control dips to protect nursery cuttings from future infection (Gramaje et al. 2009, Sawant and Sawant 2008, Fontaine et al. 2016).

1.2.1 Cultivar resistance

Similar to other diseases, the most cost-efficient management strategy for the control of GTD fungal infections could be the existence of GTD resistant cultivars (Gramaje et al. 2018). However, it has been shown that there is currently no cultivar resistant to any trunk disease pathogen although some cultivars have been shown to be more tolerant than others (Eskalen et al., 2001; Gramaje et al., 2010; Martínez-Diz et al. 2019; Rolshausen et al. 2015; Úrbez-Torres and Gubler 2009). This has been attributed in part to the difference in dimension of the xylem vessels of the different cultivars (Pouzoulet et al. 2017).

1.2.2 Cultural practices

In young and mature vineyards, IPM continues in the form of cultural practices such as vineyard sanitation, which consists mainly in the removal of pruning debris and infected parts (spurs, cordons, and trunks) from vineyards, which has been shown to be a significant source of GTD inoculum (Elena and Luque, 2015). Cultural practices also include remedial vine surgery, a technique that manages the infection of aerial wood without the need to replant the vine completely. Remedial surgery removes the infected part of the vine (usually trunk) and subsequently retrains new shoots from the base to form a new vine (Sosnowski et al. 2011). Pruning wounds in mature vineyards are the most important portal for infection by air-borne spores of Botryosphaeria dieback, esca, Eutypa dieback, and Phomopsis dieback pathogens and studies have focused on the development of control measures to prevent pruning wound infections (Gramaje et al. 2018). Studies in California have shown that by pruning late in winter,

after major spore releases triggered by rainfall occurs during the months of December and January, can significantly reduce infection (Úrbez-Torres et al. 2009). Late pruning can be a difficult task to manage for large scale vineyards which may rely on beginning pruning early in the season to allow enough time to finish before bud break occurs. To avoid pruning wound infection in large scale vineyards during periods of high spore loads early in the season, the practice of double pruning can be employed. Double pruning involves first non-selectively pruning canes to a uniform height of about 30 to 45 cm above spur positions before the growing season starts to allow for a more final pruning down to a two bud spur later in the season when there is less rain and temperatures are higher (Weber et al. 2009). Infections of pruning wounds will still occur; however, because GTD pathogens can only colonize pruning wounds at a rate of a few centimeters per month, the infected portions of the canes will be removed during final pruning, which is expedited significantly as a result of the initial pre-pruning (Weber et al. 2009). Essentially, it is possible to reduce the number of grapevine pruning wound infections through appropriate vineyard management practices; however, chemical fungicides and biocontrol agents have also been shown to be capable of providing control of grapevine pruning wound infections and can provide growers with another tool to help mitigate losses to GTD.

1.2.3 Chemical fungicides

Chemical fungicides have been shown to be effective in managing GTD pathogens when applied properly; however, public concern as to their safety has resulted in restricting the availability of some effective control products against GTD in grape growing countries around the world. For example, sodium arsenite was one of the main control methods for esca in France; however, it was banned in the early 2000s due to health concerns and cost (Zanzotto and Morroni, 2016). Another example is benomyl, effective against a wide range of plant pathogens, including GTD (Moller and Kasimatis 1980), but was phased out in Europe and North America in 2001 due to significant human, animal and environmental health impacts

(Decoin 2001). Though still used in some countries, there have been reports questioning the efficacy of benomyl products in California since losses to Eutypa dieback continue despite its wide-spread use (Halleen et al. 2006). Furthermore, carbendazim, another effective product against GTD have been also recently banned in Europe in most viticulture areas (Mondello et al. 2018).

The inefficacy of chemical fungicides in the management of GTD lies in the fact that they are caused by a wide range of taxonomically unrelated fungi which, as a group, respond to any one fungicide at varying degrees (Gramaje et al., 2012). Furthermore, it is typical that if a synthetic fungicide includes only one active ingredient, it will exhibit only one mode of action which lends well to the development of resistant strains of GTD that may be selected for if only one fungicide is depended upon (Gramaje et al. 2018). For these reasons, it is important to have several synthetic fungicides available from different chemical families that exhibit different modes of action against all of the GTD pathogens relevant to the region so as to avoid the development of resistances (Gramaje et al. 2018). Studies have evaluated the efficacy of different synthetic fungicides in the management of GTD and although this work has historically focused on pruning wound protection against *E. lata* (Moller and Kasimatis, 1980), studies have since evaluated the same or similar synthetic fungicides for the control of *P. chlamydospora*, *Phaeacremonium* spp., *Phomopsis* spp., and Botryosphaeriaceae spp. *in vitro* and/or *in plantae* (Bester et al. 2007; Halleen et al. 2010; Sosnowski et al. 2008).

Alternative synthetic fungicides like tebuconazole and flusilazole, as well as certain gel and paint wound sealing products, which sometimes contain synthetic fungicides, have been developed and are used widely to protect pruning wounds. These chemicals have been shown to be effective for up to 14 days post application (Mondello et al. 2018); however, they require re-application if it rains soon after being applied, and pruning wounds can be susceptible to

infection by GTD pathogens for up to 12 weeks depending on the time of pruning so more than one application may be needed which significantly increases production costs (Halleen et al. 2006; Úrbez-Torres and Gubler, 2011). The use of synthetic agro-chemicals is estimated at about 250,000 tonnes per year in Europe alone, 72% of which are fungicides (Woo et al. 2014). This heavy dependence on chemical pesticides is unsustainable, especially with a growing world population that is estimated to hit 9.7 billion by the year 2050 (Guzman-Guzman et al. 2019).

1.2.4 Biological control

A biological control agent (BCA) is an organism selected based on its intrinsic antagonistic nature towards a particular pest, or set of pests, and can function as an effective and sustainable alternative to chemical pesticides (Wilson 1997). The biocontrol of fungal grapevine diseases, including GTD, has been thoroughly reviewed in the past few years (Compant and Mathieu, 2016; Gramaje et al., 2018; Mondello et al., 2018). Experiments on the biological control of GTD, specifically as pruning wound protectants, date back to 1991 when Ferreira et al. were able to obtain 100% control of *E. lata* infection by treating fresh pruning wounds with a suspension of *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 applied four hours before being challenged with 500 spores of *E. lata*. In this study, *B. subtilis* was compared against two concentrations of the fungicide benomyl, and although the benomyl treatments did provide a high degree of control, *B. subtilis* was the only treatment to provide 100% control when treated canes were evaluated nine months post-treatment. Since then, 29 different bacterial species have been evaluated as potential biocontrol agents against GTD (Mondello et al. 2018). Several studies have also evaluated the biocontrol potential of several fungi, primarily *Cladosporium herbarum* (Pers.) Link and *Fusarium lateritium* Nees:Fr. with mixed results (Munkvold and Marois, 1993; Rolshausen and Gubler, 2005). Better results on controlling GTD pathogens have shown the use of either endophytic fungi such as *Clonostachys rosea* (Link) Schroers, Samuels,

Seifert & W. Gams (Silva-Valderrama et al. 2020) and *Pythium oligandrum* Drechsler (Yacoub et al., 2016) or both rhizospheric fungi and bacteria (Álvarez-Pérez et al. 2017). Most recently, biocontrol studies against GTD fungi have expanded to the use of natural compounds, including garlic, plant and seaweed extracts, inorganic salts and/or ozonated water among many others (Ayres et al. 2017; Calzanaro et al., 2014; Cobos et al., 2015; Romeo-Olivan et al. 2019). However, the use of these compounds is still at the first stages of development and further field studies are required to determine their potential as biocontrol products. Among all these BCA, fungal species within the *Trichoderma* Pers. genus are by far the most studied worldwide to be used as root dips, soil drench applications and/or pruning wound protectants for the control of GTD fungi with the most promising results for further characterization and development into registered commercial products (Compant and Mathieu, 2016; Gramaje et al. 2018; Mondello et al. 2018).

1.2.4.1 *Trichoderma*: a case study

Fungal species in the genus *Trichoderma* are asexual ascomycetes that are widely used in agriculture worldwide as beneficial plant symbionts capable of promoting plant growth while actively antagonizing plant pathogenic fungi (Woo et al. 2014). *Trichoderma* spp. are ubiquitous in soil all over the world and are often the most isolated fungi from soil with 10^1-10^3 culturable propagules per gram of soil in most temperate and tropical forests (Harman et al. 2004). The genus was established in 1794 with four species, of which only *Trichoderma viride* Pers. remains. In 2017, 287 species of *Trichoderma* had been identified through morphological and molecular identification techniques, mainly DNA sequencing and analysis of the 5.8S ribosomal DNA internal transcribed spacer region (ITS1-5.8S-ITS2), part of the nuclear translation elongation factor gene (*TEF1*-α), and the second largest RNA polymerase II subunit (*RPB2*) (Zhu et al. 2017). In the past, identification based on phenotypic characteristics such as morphology and growth rates at different temperatures were not sufficient to differentiate

between divergent species as a result of phenotype overlaps (Chaverri and Samuels, 2003). Since the advent of molecular identification techniques, *Trichoderma* spp. have been identified as the asexual stage of the *Hypocrea* Fr. and the phylogenetic relationships between species in the genus have been elucidated (Bisset 1992; Chaverri and Samuels, 2003; Samuels et al. 2010).

To date, species in the *Trichoderma* genus are one of the most widely studied organisms for use in biocontrol products employed as biopesticides and biofertilizers (Pascale et al. 2017). A review was conducted in 2007 and found over 60% of registered biopesticides used for all crop protection world-wide were based on *Trichoderma* spp. (Kottb et al. 2015). In 2010, there were 21 strains of *Trichoderma* used in agriculture with over 250 *Trichoderma* products on the market in 2014 (Woo et al. 2014). The products that were certified consisted mainly of strains from eight *Trichoderma* spp., including *T. asperellum* Samuels, Lieckf. & Nirenberg*, T. atroviride* P. Karst., *T. gamsii* Samuels & Druzhin.*, T. hamatum* (Bonord.) Bainier, *T. harzianum* Rifai*, T. polysporum* (Link) Rifai*, T. virens* (J.H. Mill., Giddens & A.A. Foster) Arx, and *T. viride* (Woo et al. 2014).

1.2.4.2 *Trichoderma* species as pathogens

Although species in *Trichoderma* are generally recognized as universal plant symbionts, some species are known to be economically important pathogens causing primarily green mold in the mushroom production industry (Park et al. 2006; Samuels et al. 2002; Zhu et al. 2017). This is important to note because in addition to being the second largest grape producing province in Canada, BC is also the second largest mushroom producing province with over 47.3 million kilograms produced in 2017 with a farm gate value of $CAD173.8 million (Beaulieu-Fortin 2019). In addition to this, one strain of *T. viride* was shown to exhibit pathogenic behavior towards *Pinus nigra* seedlings (Li Destri Nicosia et al, 2015), and several strains are also well-known pathogens of lemon fruit causing a post-harvest disease called Trichoderma rot (Dodd et al.

2010). Although only isolated from immunocompromised individuals, *Trichoderma* has also been shown to cause opportunistic infections in humans and animals (Kredics et al. 1999; Kuhls et al. 2003; Samuels 1996; Zhu et al. 2017).

1.2.4.3 *Trichoderma* species as biocontrol agents

Trichoderma-based BCA have been researched for over 30 years on different crops for different applications and are some of the most studied BCA used in agriculture today. In classic studies, wild strains would be isolated from an environmental niche that somehow reflected the conditions under which the BCA was to be effective so as to select for genetic populations that may have genes of interest (Hadar, 1984). Since then, *Trichoderma* strains that have proven effective for different purposes have been amalgamated to produce improved genetically modified progeny via protoplast fusion (Stasz et al., 1988). *Trichoderma*-based treatments come in the form of wettable powders that contain viable granules of the active BCA, or extractions of *Trichoderma* secondary metabolites. The wettable powders can be applied at each stage of the plant's growth from seed treatments, soil drenches, dipping treatments of nursery cuttings, and/or aerial sprays (Gramaje et al., 2018; Mondello et al., 2018; Woo et al., 2014).

Mechanisms of direct antagonism include direct competition for the same environmental niche, which in grapevines can include xylem and parenchyma. In addition, *Trichoderma* spp. antagonize via antibiosis including the secretion of cell wall degrading enzymes (CWDEs) such as chitinases and antagonistic secondary metabolites like nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), which can cause lysis and hyphal swelling in the prey (Guzman-Guzman et al. 2019). In addition to antagonism of plant pests, *Trichoderma* spp. can also act as effective plant symbionts capable of colonizing the rhizosphere and phyllosphere of a plant often leading to increased plant growth, greater drought resistance, increased nutrient

uptake, and the induction or priming of a plant's immune response which can further protect a plant from plant pathogens (Guzman-Guzman et al. 2019).

1.2.4.4 *Trichoderma* species and grapevines

Trichoderma products have been shown to be effective against a wide range of fungal grapevine diseases including powdery mildew, downy mildew caused by *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, grey mold caused by *Botrytis cinerea* Pers., grapevine anthracnose caused by *E. ampelina* (de Bary) Shear, and GTD (Compant and Mathieu, 2016; Gramaje et al., 2018; Mondello et al., 2018; Pertot et al. 2017; Sawant & Sawant 2008). Modes of application of *Trichoderma* products on grapevines vary depending on the intended control. Treatments include dipping grapevine cuttings in spore suspensions at both soaking and grafting stages in the nursery process, soil drenches for root protection from black foot fungi, aerial sprays by tractor-driven sprayers to protect from foliar diseases, and targeted sprays or drop inoculation of pruning wounds against GTD aerial spore inoculum (Gramaje et al., 2018). In addition to the biocontrol ability of *Trichoderma* spp., it has also been shown that *T. harzianum* strain T22 or even just extracts of its secondary metabolite 6-pentyl-α-pyrone effectively increased the quality and yield of *V. vinifera* cv. Sangiovese (Pascale et al. 2017). Clearly, it would be ideal to identify and characterize a single *Trichoderma* isolate that might express all of these beneficial traits. In accordance with this aim,it is common to see *Trichoderma*-based products composed of more than one strain of the same species or from different species (Mondello et al. 2018; Woo et al. 2014).

Available studies on the use of *Trichoderma* spp. and/or *Trichoderma*-based biocontrol products against GTD is shown in Table 1.1. The first biocontrol studies using *Trichoderma* spp. on grapevines were conducted in Italy against esca (Bisiach et al. 1996; Di Marco et al. 2000) followed by studies in South Africa for the control of black foot and Petri disease (Fourie et al.

2001) or for Eutypa dieback in Australia (John et al. 2004). However, some of these first studies reported inconclusive results, primarily because efficacy results were based solely on the observation and recording of external plant symptoms (Bisiach et al. 1996; Di Marco et al. 2000). However, since then, many different *Trichoderma*-based formulations have been screened against a wide range of different GTD fungi under controlled laboratory and/or greenhouse conditions but also under natural field conditions with very promising results (Table 1.1).

The most common method for *in vitro* assessment of biocontrol abilities of *Trichoderma* spp. is via dual culture antagonism assays which allow for high-throughput screening in a short period of time and can enable the study of microscopic interactions between the fungi (John et al. 2004; Mutawila et al. 2015; Reis et al. 2019; Silva et al. 2020). *In plantae* studies conducted in greenhouses and/or glass-houses have been performed to investigate the effect of *Trichoderma* on several GTD in nursery settings using either detached cane assays or potted vines (Mondello et al. 2018; Reis et al. 2019; Úrbez-Torres et al. 2020). Field studies under natural conditions in which plants are treated and then artificially inoculated on either shoots, pruning wounds or roots, are the final and most informative stage of experimental screening yielding the most relevant data with respect to registration and commercial application in the vineyard (Reis et al. 2019). However, only about half of the studies currently available include in field testing of *Trichoderma*-base products (Table 1.1).

Additionally, the effectiveness of *Trichoderma*-based products has been shown to vary with respect to time of application post-pruning, grapevine cultivar, as well as the GTD organism that they are tested against (Mondello et al. 2018; Mutawila et al. 2011a, 2016a; Reis et al. 2019). Some studies rely on the presence of natural inoculum, whereas others employ artificial

inoculation to varying levels which make them difficult to compare among each other (Reis et al. 2019).

Finally, most of the studies on *Trichoderma* control of GTD are based on a small set of isolates in the form of commercial products used in other crops or previously identified BCA strains (Table 1.1). This may present a problem when adopting a BCA product from another climate or ecosystem as it has been reported that the effectiveness of a *Trichoderma*-based BCA can vary between *in vitro* and *in situ* studies performed under different environmental conditions (Heydari and Pessarakli 2010; Verma et al. 2007). In recent years, studies evaluating the potential of local grapevine endophytic *Trichoderma* isolates have increased in popularity (Berlanas et al. 2018; Marraschi et al. 2019; Santos et al. 2016; Úrbez-Torres et al. 2020).

Table 1.1. Summary of studies evaluating the biocontrol activity of Trichoderma spp. against GTD fungi.

		Grapevine Trunk Diseases Screened[a]						Experiments				
***Trichoderma* spp. and (strain)**	**Trade name**	**BF**	**PD**	**ES**	**BD**	**ED**	**Petri**	**in vitro**	**greenhouse**	**field**	**nursery**	**Reference**
*T. asperelloides, T. asperellum, T. harzianum, T. koningiopsis**					X			X				Marraschi et al. 2019
T. asperellum and *T. gamsii*	Remedier			X						X		Aloi et al. 2015
T. asperellum and *T. gamsii*	Remedier			X						X		Reggiori et al. 2014
T. atroviride					X		X			X	X	Berbegal et al. 2020
T. atroviride (USPP-T1 & USPP-T2), *T. harzianum* (ECO 77)			X	X	X	X				X		Mutawila et al. 2011b
T. atroviride (USPP-T1 & USPP-T2), *T. harzianum* (ECO 77)				X	X	X	X	X		X		Mutawila et al. 2015
T. *atroviride* (USPP-T1 & USPP-T2), *T. harzianum* (ECO 77)				X	X	X	X	X				Mutawila et al. 2016b
T. atroviride SC1							X				X	Pertot et al. 2016a
T. atroviride SC1 and T. atroviride I-1237		X		X						X		Martínez-Diz et al. 2020
T. atroviride					X			X	X			Pintos et al. 2012
*T. atroviride, T. guizhouense, T. harzianum, T. koningiopsis, T. longibrachiatum, T. paratroviride, T. paraviridescens, T. sp., T. spirale**					X	X		X	X			Úrbez-Torres et al. 2020
T. harzianum	RootShield						X		X		X	Di Marco and Osti, 2007
T. harzianum	Trichopel-R	X					X		X		X	Fourie et al. 2001
T. harzianum	Trichoflow -T	X					X				X	Fourie and Halleen, 2006
T. harzianum	T-77 & Trichoseal		X	X	X	X				X		Halleen et al. 2010
T. harzianum	Trichoflow -T						X			X	X	Halleen and Fourie, 2016
T. harzianum						X		X				John et al. 2004
T. harzianum	Trichoseal, Vinevax								X	X		John et al. 2005
T. harzianum						X			X	X		John et al. 2008
T. harzianum	Trichostar			X	X		X	X				Kortekamp et al. 2012
T. harzianum, *T. asperellum, T. viride, T. atroviride*		X						X	X			Santos et al. 2016
T.harzianum + *T. atroviride* (USPP-T1 & USPP-T2)	Biotricho, Vinevax, ECO 77		X	X	X	X	X	X		X		Kotze et al. 2011
T. harzianum (T39) and *T. longibrachiatum*	Trichodex						X		X	X	X	Di Marco et al. 2004
T. harzianum , T. polysporum						X		X				Christen et al. 2005

		Grapevine Trunk Diseases Screened[a]						Experiments				
***Trichoderma* spp. and (strain)**	**Trade name**	**BF**	**PD**	**ES**	**BD**	**ED**	**Petri**	**in vitro**	**greenhouse**	**field**	**nursery**	**Reference**
T. harzianum, Trichoderma spp. + *Gliocladium*	Trichoflow -T						X		X	X	X	Fourie and Halleen, 2004
Trichoderma spp.		X								X	X	Berlanas et al. 2018
Trichoderma sp. (T154)*				X				X	X	X		Carro-Huerga et al. 2020
Trichoderma sp.					X		X	X				Silva et al. 2020
T. viride				X						X		Bisiach et al. 1996

[a]Grapevine trunk disease abbreviations: BF = Black Foot, PD = Phomopsis Dieback, ES = Esca, BD = Botryosphaeria Dieback, ED = Eutypa Dieback, Petri = Petri Disease.
*Studies that evaluated *Trichoderma spp.* isolated from grapevines.

1.3 Grapevine trunk diseases in Canada

The Canadian grape and wine industry is relatively young compared to other wine regions and studies surveying the prevalence and etiology of GTD in Canada have only recently begun to emerge with a focus in the province of BC (Úrbez-Torres et al. 2014a, Úrbez-Torres et al. 2014b). However, some preliminary survey work in the provinces of Ontario and Québec has been conducted within major studies determining the status of several GTD pathogens in the North East grapevine regions of the USA (Lawrence et al. 2017; Petit et al. 2011; Travadon et al. 2013). Field surveys conducted in BC between 2010 and 2014 showed GTD symptoms to occur in >90% of vineyards with up to 54% incidence in a single vineyard (Úrbez-Torres et al. 2014a). To date, over 40 different GTD pathogens associated with black foot, Botryosphaeria dieback, esca, Eutypa dieback, Petri disease, and Phomopsis dieback have been identified in BC (Úrbez-Torres et al. 2014a, 2014b, Úrbez-Torres and O'Gorman 2016). This demonstrates the need to develop and implement control strategies that will mitigate the impact that these disease have on grapevine health and thus, support an economically important and growing industry. However, contrary to the rest of grape-growing regions in the world, there are no control products, chemical or biological, currently registered and available to growers for GTD management in Canada. With the growing trend of organic and sustainable viticulture practices in the BC grape and wine industry, the identification and characterization of local BCA for their potential control of GTD is an industry priority to help mitigate further economic losses to both the BC and the Canadian grape and wine industry.

1.4 Research objectives

Can *Trichoderma* spp. collected from grapevines in the Okanagan Valley in BC be used as biocontrol agents against grapevine trunk diseases?

> **H_0**: *Trichoderma* spp. collected from grapevines in the Okanagan Valley in BC have no control activity against GTD when used as pruning wound protectants.

H_1: *Trichoderma* spp. collected from grapevines in the Okanagan Valley in BC have control activity against GTD when used as pruning wound protectants.

Objectives to Address Research Hypothesis

1) Identify and characterize *Trichoderma* spp. collected from vineyards in the Okanagan Valley by means of morphological, biological, and molecular studies.
2) Assess the potential of *Trichoderma* spp. as biocontrol agents against Botryosphaeria dieback fungi under controlled greenhouse and natural field conditions

Chapter 2: Identification and Characterization of *Trichoderma* species from vineyards in British Columbia and assessment of their potential use as biocontrol agents against fungal pathogens causing Botryosphaeria dieback

2.1 Introduction

Domesticated grapevine (*Vitis vinifera* L.), with over 6.9 million ha under production in 2019, is one of the most cultivated fruit crops worldwide (FAO, 2021). Grapes, consumed fresh and processed as juice or wine, are an economically important contributor to the region in which they are grown no matter its size. For instance, the Canadian grape and wine industry, with just over 14,000 ha cultivated, contributed over CAD$9 billion to the national economy through sales, tourism, and jobs in 2015 (Rimerman, 2017). Despite of the regional and/or national economic success created by grape and wine industries around the world, grapevines host the highest number of pathogens of any agricultural crop (Martelli, 1997; Wilcox et al. 2015). Accordingly, disease control practices are among the main contributors of grapevine production costs (Sambucci et al. 2014). Grapevine diseases, if unmanaged, can cause substantial untenable economic losses due to yield reduction and plant mortality (Atallah et al. 2012; Fuchs et al. 2021; Kaplan et al. 2016; Naidu et al. 2014). As a result of all the diseases affecting grapevines, viticulture accounts for 35% of all pesticides used worldwide, which exceeded 4 million tons in 2018 (Compant & Mathieu, 2016; FAO, 2021).

Among all diseases known to affect *V. vinifera*, grapevine trunk diseases (GTD) are considered one of the most destructive and economically important (Bertsch et al. 2013; Gramaje et al. 2018). Grapevine trunk diseases are caused by a wide range of taxonomically unrelated fungi primarily in the Ascomycota and include black foot disease, Botryosphaeria dieback, esca, Eutypa dieback, Petri disease, and Phomopsis dieback (Gramaje et al. 2018). With the exception of black foot disease, which is caused by several soil-borne fungi mainly belonging to

the genus *Ilyonectria* (Agustí-Brisach and Armengol, 2013), the rest of GTD fungi infect grapevines primarily via air-borne spores (ascospores or conidia) through pruning wounds (Rolshausen et al. 2010; Rooney-Latham et al. 2005; Úrbez-Torres, 2011; Úrbez-Torres et al. 2013). As GTD fungi colonize the vascular tissue of the vine, symptoms including wood necrosis, dark streaking of xylem vessels and/or perennial cankers can be observed in cross sections done in spurs, cordons and/or trunk. Overtime, and as a consequence of restriction of the vascular system, grapevines may show reduce or lack of spring growth, poor vigor and progressive dieback, which result in yield losses and eventual death of the plant (Gramaje et al. 2018).

Apart from renewal surgery, in which infected parts of the vine are surgically removed (Sosnowski et al. 2011), no other curative treatment is currently available for vines infected by GTD. As a result, GTD are considered one of the most important biotic factors limiting vineyard lifespans worldwide (Bertsch et al. 2013). In the absence of curative treatments, intensive preventative control measures are recommended in both grapevine nurseries and commercial vineyards (Gramaje et al. 2018). Since wounds made every year during the pruning season are the main point of infection for GTD fungi, protection of pruning wounds has been, and still is, the most commonly used and researched control strategy. Pruning wound protection to manage GTD fungi is not a new approach and studies using this principle have been conducted since early 1900s (Reddick, 1914; Pine, 1957; Willison *et al.* 1964; Moller and Kasimatis, 1980). Historically, pruning wound protection against GTD fungi has been focused on the use of synthetic chemicals and thus, a large list of products have been screened in both nurseries and commercial vineyards and many of them are currently available (Gramaje and Armengol, 2011; Gramaje et al. 2018; Mondello et al. 2018; Úrbez-Torres, 2011). However, as observed in many other agricultural crops, chemical fungicides are becoming highly restricted/regulated, or even prohibited due to concerns related to high toxicity to humans, animals and/or the environment

as well as resistance development by pathogens. This, along with higher consumer demands for more sustainable agriculture production systems, has caused viticulture practices to experience important changes in the last two decades (Compant and Mathieu, 2016). As a result, it is now well-accepted that an integrated pest management approach (IPM), in which the responsible use of synthetic chemicals along with cultural practices and the use of biological control agents (BCA) and/or organic products, is the best option to be implemented in order to minimize development and spread of diseases, including GTD (Bertsch *et al.* 2013). Among all these management options, both research and usage of BCA as an alternative to control GTD has significantly increased in the last years (Mondello *et al.* 2018). A significant amount of research is currently available on the use of either generalist (*Bacillus* spp., *Pseudomonas* spp., *Streptomyces* spp., *Trichoderma* spp., and *Clonostachys* spp.) or specialist (*Agrobacterium* spp., *Ampelomyces* spp., *Cloniothyrium* spp., *Fusarium* spp., and *Aspergillus* spp.) BCA for the management of a wide broad of plant pathogens in a large range of crops (Woo et al. 2014). Among all these genera, species within *Trichoderma* are by far the most widely studied organisms to be used as BCA against many different pathogens, including GTD fungi (Woo et al. 2014; Costadone and Gubler, 2016; Mondello et al. 2018). A review conducted in 2007 found over 60% of registered fungal BCA to be *Trichoderma*-based (Verma et al. 2007).

Grapevines, primarily cultivated in southern Ontario's Niagara Peninsula and in the Okanagan Valley in south-central British Columbia (BC), are an emerging crop in Canada. Though still relatively young grape growing regions, GTD have been identified in BC, Québec, and Ontario (Chamberlain et al. 1964; Lawrence et al. 2017; Petit et al. 2011; Travadon et al. 2015; Úrbez-Torres *et al.* 2014a, 2014b). However, contrary to most grape growing countries around the world, there are currently no registered control products, neither synthetic chemicals nor BCA, for the management of GTD in Canada. This highlights the urgent need to generate data and develop strategies to help manage these devastating diseases to support an economically

important and growing industry in Canada. In addition, with the growing trend of developing a more sustainable grape and wine industry in BC there is also an important need to provide biological control alternatives against GTD fungi. Accordingly, the main objectives of this study were to i) identify and characterize by means of morphological and molecular studies *Trichoderma* spp. locally found in BC vineyards and ii) determine their potential as BCA against the botryosphaeriaceous taxa *Diplodia seriata* and *Neofusicoccum parvum*, two of the most prevalent and widespread GTD fungi infecting grapevines in BC (Úrbez-Torres and O'Gorman, 2016).

2.2 Materials and methods

2.2.1 *Trichoderma* isolates and grapevine trunk disease pathogens used in this study

A total of 29 *Trichoderma* isolates, obtained from field surveys and vineyard visits conducted in the Okanagan Valley between 2011 and 2018 were selected for this study (Table 2.1). *Trichoderma* isolates were primarily isolated from grapevine roots and the basal end of the rootstock in both young and mature vineyards as described by Úrbez-Torres et al. (2014b). Pure cultures were obtained by hyphal tip on potato dextrose agar (DIFCO™, Detroit, MI) (PDA) and *Trichoderma* isolates were stored at the Summerland Research and Development Centre (SuRDC), Summerland (BC) fungal collection as colonized PDA agar plugs in glass vials (15-20 plugs/vial) containing double-autoclaved distilled water and stored at 4 ºC until used. When needed, isolates were retrieved from the collection and revived by plating 3 to 5 colonized agar plugs into fresh 90 mm-diameter PDA Petri plates. Plates were wrapped in Parafilm and incubated in the dark at 23 °C until colonies were observed. Single colonies were obtained by replicating the edge of an active growing colony into new PDA plates.

Two GTD pathogens, *Diplodia seriata* (SuRDC-1050) and *Neofusicoccum parvum* (SuRDC-1025) were selected for this study. Both pathogens were isolated from grapevine cankers

showing Botryosphaeria dieback symptoms in the Okanagan Valley in field surveys conducted between 2010 and 2013 (Úrbez-Torres and O'Gorman, 2016). Isolates were stored and revived from the SuRDC fungal collection as described above. Both pathogens were selected as they represent two of the most prevalent GTD fungi isolated from symptomatic grapevines in BC. In addition, both isolates were proven to be pathogenic and highly virulent from Koch's postulates completed in 2015 (Úrbez-Torres and O'Gorman, 2016).

Table 2.1. *Trichoderma* isolates used in this study and GenBank accession numbers.

Species	Isolate[a]	Substrate	Country	ITS[c]	*TEF1*[d]
Trichoderma albofulva	*CBS 114787 (T) = G.J.S. 01-234*	*Hypoxylon*	Thailand	-	DQ846668
Trichoderma appalachiense	*G.J.S. 97-243*	decorticated wood (*Pinus* sp.)	GA, USA	-	DQ307503
Trichoderma asperelloides	*G.J.S. 04-116 (T)*	soil	Vietnam	GU198301	GU248412
T. asperelloides	IBLF-908	n/a	Brazil	KJ646886	KP262478
T. asperelloides	**SuRDC-1442**[b]	*V. vinifera*	BC, Canada	MZ161800	MZ189381
T. asperelloides	**SuRDC-1444**	*V. vinifera*	BC, Canada	MZ161801	MZ189382
T. asperelloides	**SuRDC-1449**	*V. vinifera*	BC, Canada	MZ161802	MZ189383
T. asperelloides	**SuRDC-1453**	*V. vinifera*	BC, Canada	MZ161803	MZ189384
Trichoderma asperellum	*CBS 433.97 (T) = ATCC204424*	sclerotia of *Sclerotinia minor*	MD, USA	X93981	AF456907
T. asperellum	G.J.S. 90-7	soil	Vietnam	GU198317	EU338333
T. asperellum	G.J.S. 05-328	soil	Cameroon	GU198318	EU248627
Trichoderma atroviride	*CBS 142.95 (T)*	gallery of beetle	Slovenia	MH862505	AY376051
T. atroviride	G.J.S. 98-134	culture from holotype	-	AF456913	AF456887
T. atroviride	**SuRDC-1424**	*V. vinifera*	BC, Canada	MZ161784	MZ189365
T. atroviride	**SuRDC-1428**	*V. vinifera*	BC, Canada	MZ161785	MZ189366
T. atroviride	**SuRDC-1432**	*V. vinifera*	BC, Canada	MZ161786	MZ189367
T. atroviride	**SuRDC-1433**	*V. vinifera*	BC, Canada	MZ161787	MZ189368
T. atroviride	**SuRDC-1436**	*V. vinifera*	BC, Canada	MZ161788	MZ189369
T. atroviride	**SuRDC-1438**	*V. vinifera*	BC, Canada	MZ161789	MZ189370
T. atroviride	**SuRDC-1440**[b]	*V. vinifera*	BC, Canada	MZ161790	MZ189371
Trichoderma austrokoningii	*CBS 119080*	n/a	New Zealand	-	KJ871163.1
Trichoderma caerulescens	*CBS 130011*	*Erica arborea*	Spain	NR_134432.1	JN715615.1
Trichoderma caribbaeum	*CBS 119093*	n/a	Guadeloupe	-	KJ665443.1
Trichoderma composticola	*CBS 133497*	*Vitis vinifera*	Greece	-	KC285631.1
Trichoderma canadense	***SuRDC-1422***[b]	*V. vinifera*	BC, Canada	MZ161796	MZ189377
T. canadense	**SuRDC-1435**	*V. vinifera*	BC, Canada	MZ161797	MZ189378
T. canadense	**SuRDC-1450**	*V. vinifera*	BC, Canada	MZ161798	MZ189379
T. canadense	**SuRDC-1451**	*V. vinifera*	BC, Canada	MZ161799	MZ189380
Trichoderma dingleyae	*CBS 119056*	n/a	New Zealand	-	KJ665467.1

Species	Isolate[a]	Substrate	Country	ITS[c]	*TEF1*[d]
Trichoderma dorothea	*ICMP 16288 = G.J.S. 99-202*	*Nothofagus*	New Zealand	-	DQ307536.1
Trichoderma erinaceum	*DAOM 230019*	soil	Thailand	-	AY750880.1
Trichoderma gamsii	*DAOM 233985*	n/a	South Africa	-	KJ871264.1
Trichoderma hamatum	*DAOM 167057*	spruce forest soil	Canada, QC	NR_134371.1	EU279965.1
Trichoderma harzianum	DAOM 222149	n/a	n/a	AY605748.1	AY605791.1
T. harzianum	DAOM 233352	n/a	n/a	EF392760.1	EF392752.1
T. harzianum	**SuRDC-1423**	*V. vinifera*	BC, Canada	MZ161805	MZ189386
T. harzianum	**SuRDC-1425**	*V. vinifera*	BC, Canada	MZ161806	MZ189387
T. harzianum	**SuRDC-1426**	*V. vinifera*	BC, Canada	MZ161807	MZ189388
T. harzianum	**SuRDC-1437[b]**	*V. vinifera*	BC, Canada	MZ161808	MZ189389
T. harzianum	**SuRDC-1439[b]**	*V. vinifera*	BC, Canada	MZ161809	MZ189390
T. harzianum	**SuRDC-1441**	*V. vinifera*	BC, Canada	MZ161810	MZ189391
T. harzianum	**SuRDC-1447**	*V. vinifera*	BC, Canada	MZ161811	MZ189392
T. harzianum	**SuRDC-1448**	*V. vinifera*	BC, Canada	MZ161812	MZ189393
Trichoderma hispanicum	*CBS 130540*	n/a	Spain	-	JN715659.1
Trichoderma intricatum	*CBS 119059 = G.J.S. 97-88*	bark of very rotten wood	Thailand	-	G.J.S. 97-88
Trichoderma koningii	BMCC:LU1333	n/a	New Zealand	-	KJ871263
T. koningii	*G.J.S. 89-122*	decorticated wood	USA	AY380902.1	AY376045.1
T. koningii	DAOM 167645	n/a	n/a	EU280128.1	EU280017.1
T. koningii	**SuRDC-1443**	*V. vinifera*	BC, Canada	MZ161794	MZ189375
T. koningii	**SuRDC-1446[b]**	*V. vinifera*	BC, Canada	MZ161795	MZ189376
Trichoderma koningiopsis	*G.J.S. 93-20 = CBS 119075*	branch	Cuba	DQ313140.1	DQ284966.1
T. koningiopsis	DAOM 179516	n/a	n/a	EU280126.1	EU280012.1
T. koningiopsis	BMCC:LU624	n/a	New Zealand	-	KJ871194
Trichoderma martiale	*CBS 123052*	*Theobroma cacao*	Brazil	-	EU248618.1
Trichoderma neokoningii	*CBS 120070*	*Moniliophthora roreri*/ *Theobroma cacao*	Peru	-	KJ665620.1
Trichoderma nothescens	*CBS 134882*	bark	Australia	-	DQ307512.1
Trichoderma ochroleucum	*CBS 119502*	n/a	Europe, North America	-	FJ860659.1
Trichoderma ovalisporum	*CBS 113299 = Dis70a*	*Banisteriopsis*	Ecuador	-	AY376037.1
Trichoderma paratroviride	*CBS 136489*	*Phillyrea angustifolia*	Spain	-	KJ665627.1
Trichoderma paraviridescens	*CBS 119321*	*Fagus sylvatica*	Austria	-	DQ672610.1
Trichoderma petersenii	*CBS 119507*	n/a	Europe, North America	-	FJ860670.1
Trichoderma samuelsii	*CBS 130537*	*Moniliophthora roreri* / *Theobroma cacao*	Peru	-	JN715654.1
Trichoderma scalesiae	*CBS 120069*	*Scalesia pedunculata,* trunk endophyte	Ecuador	-	DQ841726.1
Trichoderma sempervirentis	*CBS 133498*	*Acer sempervirens*	Greece	-	KC285632.1

Species	Isolate[a]	Substrate	Country	ITS[c]	*TEF1*[d]
Trichoderma sp.	DAOM 233834	n/a	New Zealand	-	KJ871191.1
Trichoderma strigosellum	*CBS 102817*	n/a	Columbia	-	JQ425705.1
Trichoderma strigosum	*DAOM 166121*	n/a	USA	-	EU280019.1
Trichoderma taiwanense	*CBS 119058*	bark	Taiwan	-	DQ284973.1
Trichoderma theobromicola	*Dis 85f*	Herrania	Peru	DQ109525.1	EU856321.1
Trichoderma tomentosum	*DAOM 178713*	n/a	USA	MH862417.1	KJ871247.1
T. tomentosum	DAOM 195050	n/a	n/a	AY605717.1	AY605761.1
T. tomentosum	DAOM 229898	n/a	n/a	AY605737.1	AY605780.1
T. tomentosum	**SuRDC-1431**	*V. vinifera*	BC, Canada	MZ161804	MZ189385
Trichoderma trixiae	*CBS 134702*	*Picea abies*	Germany	-	DQ672606.1
Trichoderma valdunense	*CBS 120923*	n/a	Europe	-	FJ860717.1
Trichoderma vinosum	*ICMP 16294*	*Nothofagus menziesii*	New Zealand	-	AY376047.2
Trichoderma viridarium	*CBS 132568*	*Carpinus betulus*	Italy	-	KC285658.1
Trichoderma viride	CBS 119327	*Picea abies,* wood	Austria	-	DQ672617.2
T. viride	*CBS 119325*	*Pinus sylvestris,* wood	Czech Replublic	-	DQ672615.2
T. viride	CPK 1996	*Acer pseudoplatanus*, wood	UK	-	DQ672614.2
T. viride	CPK 2001	*Picea abies*, wood	Austria	-	DQ672619.2
Trichoderma viridescens	*CBS 433.34*	rotten apples	UK	AF456922	AF456905
Trichoderma viridialbum	*CBS 133495*	*Descurainia bourgeauana*	Spain	-	KC285706.1
Trichoderma virilente	*CBS 132569*	*Fraxinus ornus*	Croatia	-	KC285692.1
Trichoderma viticola	**SuRDC-1427**[b]	*V. vinifera*	BC, Canada	MZ161791	MZ189372
T. viticola	**SuRDC-1429**	*V. vinifera*	BC, Canada	MZ161792	MZ189373
T. viticola	**SuRDC-1430**	*V. vinifera*	BC, Canada	MZ161793	MZ189374
Protocrea farinosa	C.P.K. 2472	*Skeletocutis nivea/ Fraxinus excelsior*	Austria	EU703914.1	EU703892.1

[a] Isolate numbers in bold represent *Trichoderma* isolates from British Columbia used in this study. Isolate numbers in italics and underline represent ex-type specimens. CBS (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands), DAOM (Department of Agriculture Ottawa Mycology, Ottawa, ON, Canada), SURDC (Pacific Agri-Food Research Centre Fungal Collection, Summerland, BC, Canada). ATCC (American Type Culture Collection, Manassas, VA, USA).
[b] *Trichoderma* isolates used in the detached cane assay trial.
[c] ITS: Internal Transcribed Spacer
[d] TEF1-α: Translation elongation factor 1-α
n/a: Not available

2.2.2 Molecular characterization of *Trichoderma* isolates from BC

2.2.2.1 DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from pure cultures by collecting 0.1g aerial mycelia from actively growing colonies on PDA using the Power Soil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) following manufacturer's instructions. Polymerase Chain Reaction (PCR) was used to amplify the internally transcribed spacer region (ITS), including the 5.8S ribosomal gene using ITS1 and ITS4 primers (White *et al.* 1990). In addition, a partial sequence of the translation elongation factor 1 alpha gene (*TEF1*) was amplified using primers TEF71F and TEF997R (Braithwaite *et al.* 2017). PCR reactions consisted of 13.85 µL NF H2O, 2 µL 10X Buffer, 2 µL dNTPs (2.0 mM), 0.4 µL Blotto 10% w/v, 0.25 µL forward primer (20 µM), 0.25 µL reverse primer (20 µM), 0.25 µL DreamTaq (ThermoFisher Scientific, Canada), and 1 µL sample DNA. PCR amplifications were performed in a GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, CA) following the conditions described by White et al. (1990) for ITS and Braithwaite et al. (2017) for *TEF1*. All PCR products were purified using a QIAquick PCR purification Kit (QIAGEN Inc., Valencia, CA) and both strands of the ITS and *TEF1* were sequenced using a 8-capillary AB 3500 genetic Analyzer Sanger Sequencer (Foster City, CA) at the SuRDC.

2.2.2.2 Phylogenetic analyses

Forward and reverse nucleotide sequences from each *Trichoderma* isolate were assembled, proofread, and edited using Lasergene SeqMan Pro version 9.1.1.4 (DNASTAR Inc., Madison, WI). Consensus sequences were then compared with those available in the GenBank database by using the Basic Local Alignment Search Tool (BLASTn) function and homologous sequences with high similarity were recorded. ITS and *TEF1 Trichoderma* spp. from BC were separately aligned with published GenBank sequences, including ex-type specimens when available (Table 2.1), using the computer software BioEdit Sequence Alignment Editor Version 7.1.3.0 (Hall,

1999). Alignments were inspected visually and manually edited in BioEdit. A concatenated ITS and *TEF1* dataset was used to conduct a multi-locus sequence analyses in MEGA-X (Kumar *et al.* 2018). The best-fit substitution model was analyzed in MEGA-X to be used in the phylogenetic evolutionary analyses. In addition, two separate phylogenetic studies including *TEF1* sequences from different species groups were conducted. All datasets were analyzed using two different optimality search criteria, maximum likelihood (ML) method and Tamura-Neil model with 1,000 replicates to asses robustness and maximum parsimony (MP) with the bootstrap test (1,000 random additional sequence replicates) and the Tree-Bisection-Regrafting (TBR) algorithm. Phylogenetic trees with the greatest log likelihood were selected. *Trichoderma* sequences from BC were deposited into GenBank and isolates are maintain at the SuRDC fungal collection and at the Department of Agriculture Ottawa Mycology Collection (DAOM), Ottawa (ON).

2.2.3 Morphological characterization of novel *Trichoderma* species

Isolates SuRDC-1422 and SuRDC-1430, representing novel identified *Trichoderma* spp. in this study, were grown on PDA Petri plates at room temperature for 14 days in order to describe colony morphological characteristics, including colony growth and type and mycelium color using the color charts of Rayner (1970). Pictures of colonies were taken after three, seven, and 14 days using a Nikon D700 60mm. Morphological characteristics of microscopic structures such as mycelium, conidia, conidiophores, and phialides were noted after 7 days incubation on PDA at rom temperature in the under laboratory lighting conditions. Conidial suspensions were made by adding 1 mL sterile distilled water amended with Tween 20 (~1 drop per 300 mL) and gently scraping the aerial mycelia to suspend conidia. The suspension was then filtered through a sterile cotton ball before mounting on microscope slides. Reproductive structures were harvested using sterile forceps and mounted on microscope glass-slides. Structures were observed and photographed using Zeiss Axio Imager.M2 with a Zeiss AxioCam MRm at 40x

magnification using bright field and differential interference contrast. Photographs were taken using the program Zeiss ZenPro. Fifty conidia were measured per species. Length and width of conidia, phialides and conidiophores were measured using the ImageJ software. Phialide widths were measured at the widest part of the flask base. The maximum and minimum Feret diameters were measured for each conidium and the means were calculated for each isolate.

2.2.4 Optimum mycelial growth temperature study

At most, two isolates per *Trichoderma* sp. identified from the molecular studies were selected to determine their optimum temperature for radial mycelial growth. Seven mm colonized plugs were taken from the edge of six day-old actively-growing *Trichoderma* colonies on PDA. One plug per isolate was placed at the edge of a 90 mm Petri dish with a black line drawn through the center of the bottom of the plate containing 20 mL of PDA pipetted with a 50 mL disposable pipette and a powerpette plus pipette controller (Jencons Scientific, USA). Petri plates were Parafilmed and placed in their respective incubators at eight temperatures from 5 to 40°C with 5°C interval in the dark. Radial growth of each isolate was measured after four days incubation. Each isolate/temperature combination was performed in triplicate and the entire experiment was repeated. A two sample T-test was used to test for statistical differences between the first and the repeated experiment to determine if data from separate experiments could be pooled for analyses. The mean radial mycelial growth and standard error for each temperature/species combination was calculated. Statistical analyses were performed using R (R Core Team, 2021).

2.2.5 Dual culture antagonism assay

A total of 29 isolates were selected to determine the antagonistic capability of *Trichoderma* spp. identified in BC against the botryosphaeriaceous fungi *D. seriata* (SURDC-1050) and *N. parvum* (SURDC-1025) by conducting a dual culture antagonism assay (DCAA), adapted from those described by Bell et al. (1982) and Haidar et al. (2016). Briefly, colonized mycelial plugs (7 mm diameter) of each *Trichoderma* and pathogen were obtained from the edge of active growing

five day-old colonies on PDA and placed opposite to each other at the edge of 90 mm diameter Petri dishes containing 20 mL PDA. *Diplodia seriata* and *N. parvum* isolates were individually grown separately without presence of *Trichoderma* as positive controls. Colonies were incubated in the dark at 23 ºC for a total of seven days. Colonies were left to grow for two days before the pathogen colony radii was measured for the next 5 days at the same time each day. Measurement was performed from the edge of the plate to the edge of the colony or to the most distinguishable border between the *Trichoderma* spp. and the pathogen. Colonies were removed from incubators for no more than one hour each day for measurement of colony radii. All pairings were performed in triplicate and the experiment was repeated. The percent of mycelium inhibition was calculated using the formula:

$$\textbf{Percent Inhibition (\%)} = \frac{B-A}{B} * 100$$

Where A is the radius of pathogen mycelial growth co-inoculated with *Trichoderma* and B is the radius of the pathogen mycelial growth alone in the control plate measured on the seventh day post-inoculation. The experimental data from the DCAA were first subjected to an analysis of variance (ANOVA) followed by Tukey's test (P=0.05) to determine if there was a statistical difference between the first and second experimental repeat to determine if data could be pooled. Pooled data were then subjected to ANOVA followed by Tukey's test (P=0.05) to test for differences between the isolates. The standard error was calculated for all mean values. All statistical analysis were performed using R.

2.2.6 Detached cane assays

2.2.6.1 *Trichoderma* and pathogen inoculum preparation

Trichoderma inoculum from each isolate used in this experiment was obtained from four day-old colonies grown on PDA at 23 °C with a photoperiod 12:12 h per day under UV light (Phillips UVB TL 20W/12RS bulb). Plates were flooded with sterile distilled water (SDW) containing

0.05% Tween 20 and surface scrapped with a sterile metal spatula. Water was then filtered through one layer of autoclaved 25 µm pore diameter Miracloth (Melk Millipore) to remove mycelium fragment. Spore suspension of each *Trichoderma* isolate was adjusted to $1x10^6$ spores/ml with a haemocytometer. Similarly, *D. seriata* and *N. parvum* conidia were obtained from pycindia formed on colonies growing on PDA in the dark at 25°C under UV light (12:12 h) placed at 80 cm distance from the plates. Pycnidia formed after 4-5 weeks and conidia were harvested by adding 1-2 ml of SDW amended with a drop of Tween 20, then gently scraping the upper layer of the PDA to detach the pycnidia from the agar using a sterile metal spatula. Suspension containing pycnidia was transfer onto a sterile mortar and pestle and grinded to release spores from pycnidia. Suspension was then filtered through one layer of Miracloth into a 20 mL glass Pyrex test tube. Tube was vortex and spore concentration adjusted to $1x10^5$ spores/ml for both *D. seriata* and *N. parvum* using a haemocytometer.

2.2.6.2 *Trichoderma* species detached cane assay

The antagonistic activity of the best performing isolates from the dual culture experiment was evaluated *in planta* under controlled greenhouse conditions against *D. seriata* (SURDC-1050) and *N. Parvum* (SURDC-1025) via detached cane assay (DCA) modified from Ayres et al. (2014). Chardonnay dormant canes were collected from one experimental vineyard block located at the SuRDC. Canes were cut into two nodes canes (~ 20 cm length) and placed vertically through holes made in Styrofoam treys floating on water tables filled with tap water in a greenhouse. Temperature and relative humidity were monitored in the greenhouse over the duration of the experiment using one Hygrochron I-button (IButtonLink, LLC, Whitewater, WI, USA). The water level was maintained throughout the experiment by adding tap water; however, the total water was not exchanged. Care was taken to ensure that the bottoms of the canes were submerged in water for the duration of the experiment. Canes were then pruned ~4 cm above the upper bud to simulate a fresh pruning wound. A total of 270 canes were prepared per

treatment. Within 3 h after pruning, 180 canes (pruning wounds) per treatment were each inoculated with 50 µL of $1x10^6$ spores/ml (50,000 spores/wound) of each *Trichoderma* spp. A total of 90 canes per treatment were left untreated for positive and negative controls. In each treatment, 30 canes each (3 replicates of 10 repetitions each) were challenged 24 h, 7 d and 21 days after treatment with 50 µL of $1x10^5$ conidia/ml (5,000 spores/wound) of either *D. seriata* or *N. parvum* to determine how long *Trichoderma* activity last on the pruning wound. Positive controls included 30 non-treated but inoculated canes with 5,000 spores/wound of *D. seriata* or *N. parvum* each at 24 h, 7 d, and 21 d after pruning. In addition, 30 canes per treatment were left non-treated/non-inoculated to serve as negative controls to determine if natural infections happened in the collected canes from the experimental vineyard. Canes from the different treatments were randomized across the Styrofoam treys. Cuttings were maintained in the greenhouse and collected five weeks after each of the inoculation times.

Roots and leaves were removed from collected canes before they were prepared for re-isolation of the inoculated pathogens. Fungal re-isolations started by first shaving the bark around the pruning wound, then flame sterilizing the surface of the cane with 95% ethanol at which point ~1 mm tissue from the surface of the cane was discarded and ten pieces of tissue ~0.5 cm^2 were plated on PDA amended with 1 mg/ml tetracycline (Sigma-Aldrich, St. Louis) (PDA-tet). Plates were incubated for up to 10 days at 23 °C in the dark. If a plate yielded either *D. seriata* or *N. parvum*, the corresponding cane was rated as colonized by the pathogen. Treatment efficacy was based on the mean percent recovery (MPR) of *D. seriata* and *N. parvum* from treated canes and data is presented as mean percent disease control (MPDC), which was calculated as the reduction in MPR as a proportion of the inoculated control according to the formula (100 × [1 – (MPR treatment/MPR control)]) (Pitt et al. 2012).

The binary (infected or not-infected) data produced from the DCA assay were subjected to ANOVA followed by Tukey's HSD post-hoc comparison test to determine if there were significant statistical differences between the experimental means (P = 0.05). All statistical analyses were performed using R.

2.2.6.3 Commercial products detached cane assay

Commercial products (synthetic chemicals and BCA) were also included in a separate DCA to test their effectiveness in protecting pruning wounds against artificial infection by *D. seriata* and *N. parvum* (Table 2.2) and were analyzed as in 2.2.6.2.

Table 2.2. *Trichoderma* isolates from BC and commercial fungicides and biocontrol products included in the greenhouse detached cane experiments.

Trade Name	Active Ingredients	Application Rate	Manufacturer
GreenSeal™ Ultra	tebuconazole (10g/L) in paint	n/a	Omnia Nutriology (Auckland, NZ)
Bio-Tam 2.0	*T. asperellum+T. gamsii* (2%)	455g/3.8L	Isagro Inc. (MO, USA)
Mettle® 125 ME	tetraconazole	125g/L	Isagro Inc. (MO, USA)
SuRDC-1422	*T. canadense*	$1x10^6$ conidia/mL	n\a
SuRDC-1427	*T. viticola*	$1x10^6$ conidia/mL	n\a
SuRDC-1437	*T. harzianum*	$1x10^6$ conidia/mL	n\a
SuRDC-1439	*T. harzianum*	$1x10^6$ conidia/mL	n\a
SuRDC-1440	*T. atroviride*	$1x10^6$ conidia/mL	n\a
SuRDC-1442	*T. asperelloides*	$1x10^6$ conidia/mL	n\a
SuRDC-1446	*T. koningii*	$1x10^6$ conidia/mL	n\a

2.2.6.4 Identity confirmation of re-isolated *Trichoderma* species

In order to confirm that the *Trichoderma* spp. re-isolated from the treated canes were the same species inoculated as the initial treatment, pure cultures of the re-isolated *Trichoderma* spp. were obtained by hyphal tipping (one representative isolate per treatment). Total genomic DNA was extracted as previously described. PCR amplification using ITS1/ITS4 primers was followed by Sanger sequencing in one direction only using the ITS1 primer. DNA sequences were edited as previously described and aligned with the ITS sequence of each original *Trichoderma* isolate obtained for the phylogenetic analyses to confirm match in MEGA-X.

2.3 Results

2.3.1 Molecular characterization of *Trichoderma* isolates from BC

PCR amplifications of the ITS and *TEF1* regions generated, respectively, bp fragment products of 555 to 624 and 705 to 889. The two gene (*ITS*+*TEF1*) combined analyses included 53 nucleotide sequences and there were a total of 1454 positions in the final dataset. *Protocrea farinosa* isolate C.P.K. 2472 was used as outgroup for both ML and MP analyses (Jacklitsch and Voglmayr, 2015). The evolutionary analyses by ML method and Tamura-Nei model yielded the highest log likelihood tree (-7365.70), which is shown in Figure 2.1. A discrete Gamma distribution was used as best fit model to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.2770)) based on the Akaike Information Criterion (AIC) analyses in MEGA-X. Maximum parsimony analyses of combined ITS and *TEF1* taxa yielded 9 most parsimonious trees (length = 1195) with similar topology to the highest log likelihood tree. Consistency index, retention index and composite index from the MP analyses resulted, respectively, in 0.696768, 0.942667 and 0.691026. Bootstrap branch support (ML/MP) determined by 1,000 replicates is shown in Figure 2.1.

ML and MP analyses from the combined ITS and *TEF1* dataset revealed that *Trichoderma* isolates from vineyards in BC were divided into seven distinct phylogenetic clades corresponding to seven different species (Figure 2.1). Seven isolates strongly clustered (99/100 ML and MP bootstrap values, respectively) with the type specimen of *T. atroviride* CBS142.95. Similarly, one, two, four, and eight *Trichoderma* isolates from vineyards in BC clustered, respectively, with the type specimens of *T. tomentosum* DAOM 178713 (73/98), *T. koningii* G.J.S. 89-122 (99/100), *T. asperelloides* G.J.S. 04-116 (100/100), and *T. harzianum* isolates DAOM23352 and DAOM222159 from GenBank (100/100) in distinct clades (Figure 2.1). Three isolates from BC (SuRDC-1427, SuRDC-1429, and SuRDC-1430) clustered together in a well-

supported clade (98/99) closely related to the *T. viride* clade but representing a single lineage that was not associated with a type or non-type isolate. Similarly, four isolates (SuRDC-1422, SuRDC-1435, SuRDC-1450, and SuRDC-1451) clustered together in a well-supported clade (100/100) closely related to the *T. koningiopsis* clade but representing a single lineage that was neither associated with a type or non-type isolate (Figure 2.1). Therefore, these isolates were thought to represent two possible novel phylogenetic species hereinafter identified as *Trichoderma viticola* and *Trichoderma canadense*.

Two separate *TEF1* phylogenetic analyses were conducted to further support the discovery of the abovementioned novel phylogenetic species. The first analyses included 28 nucleotide sequences, including *T. viticola* isolates from BC and there were a total of 1642 positions in the final dataset. *Trichoderma viticola* isolates from BC were compared against closely related type specimens representing phylogenetic species in the *viride*/*viridescens* clade as previously described by Braithwaite et al. (2017). *Trichoderma paratroviride* isolate CBS136489 was selected as outgroup. ML analyses and Hasegawa-Kashino-Yano model yielded the highest likelihood tree (-5903.77), which is shown in Figure 2.2. A discrete Gamma distribution was used as best fit model to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.2670)) as previously described in MEGA-X. Maximum parsimony analyses of these taxa yielded four most parsimonious trees (length = 717) with similar topology to the highest log likelihood tree. Consistency index, retention index and composite index from the MP analyses resulted, respectively, in 0.603104, 0.761651 and 0.571504. Bootstrap branch support (ML/MP) determined by 1,000 replicates is shown in Figure 2.2. Isolates SuRDC1427, SuRDC-1429, and SuRDC-1430 formed a strongly supported single lineage (100/91) closely related to *T. viride* isolates as shown in the combined ITS+*TEF1* analyses confirming *T. viticola* as a novel species.

The second *TEF1* analyses involved also 28 nucleotide sequences, including *T. canadense* isolates from BC and there were a total of 942 positions in the final dataset. *Trichoderma canadense* isolates from BC were compared against closely related type specimens representing phylogenetic species in the *viride*/*koningii* clade as previously described by Braithwaite et al. (2017). *Trichoderma austrokoningii* isolate CBS119080 was selected as outgroup. ML analyses and Hasegawa-Kashino-Yano model yielded the highest likelihood tree (-5709.96), which is shown in Figure 2.3. A discrete Gamma distribution was used as best fit model to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 3.5291)) as previously described in MEGA-X. Maximum parsimony analyses of these taxa yielded two most parsimonious trees (length = 966) with similar topology to the highest log likelihood tree. Consistency index, retention index and composite index from the MP analyses resulted, respectively, in 0.629534, 0.747795 and 0.526398. Bootstrap branch support (ML/MP) determined by 1,000 replicates is shown in Figure 2.3. Isolates SuRDC1422, SuRDC-1435, SuRDC-1450, and SuRDC-1451 formed a strongly supported single lineage (89/100) closely related to *T. koningiopsis* as shown in the combined ITS+*TEF1* analyses confirming *T. canadense* as a novel species (Figure 2.3). Additionally, this phylogenetic analyses confirmed *T. koningii* isolates from BC to cluster together in a well-supported clade (90/99) with the type as well as non-type specimens (Figure 2.3).

Figure 2.1. Phylogram of the highest log likelihood tree from an analyses of the ITS and *TEF1* combined dataset. Numbers in front and after the slash represent ML and MP bootstrap values from 1,000 replicates, respectively. Values represented by a dash (-) were less than 70% for the bootstrap analyses. Type specimens are indicated with an asterisk and *Trichoderma* isolates from vineyards in BC are indicated in bold. Bar indicates the number of substitutions per site.

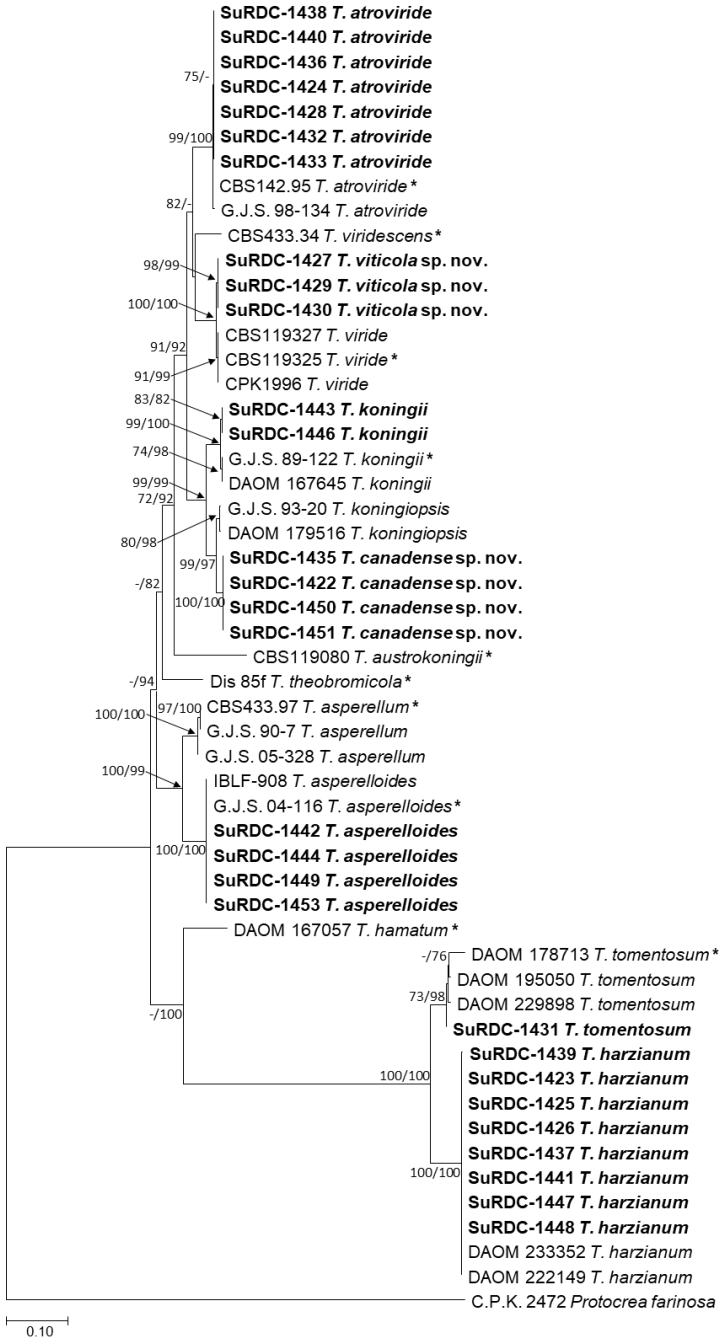


Figure 2.2. Phylogram of the highest log likelihood tree from an analyses of the *TEF1 viride/viridescens* dataset. Numbers in front and after the slash represent ML and MP bootstrap values from 1,000 replicates, respectively. Values represented by a dash (-) were less than 70% for the bootstrap analyses. Type specimens are indicated with an asterisk and *Trichoderma* isolates from vineyard in BC are indicated in bold. Bar indicates the number of substitutions per site.

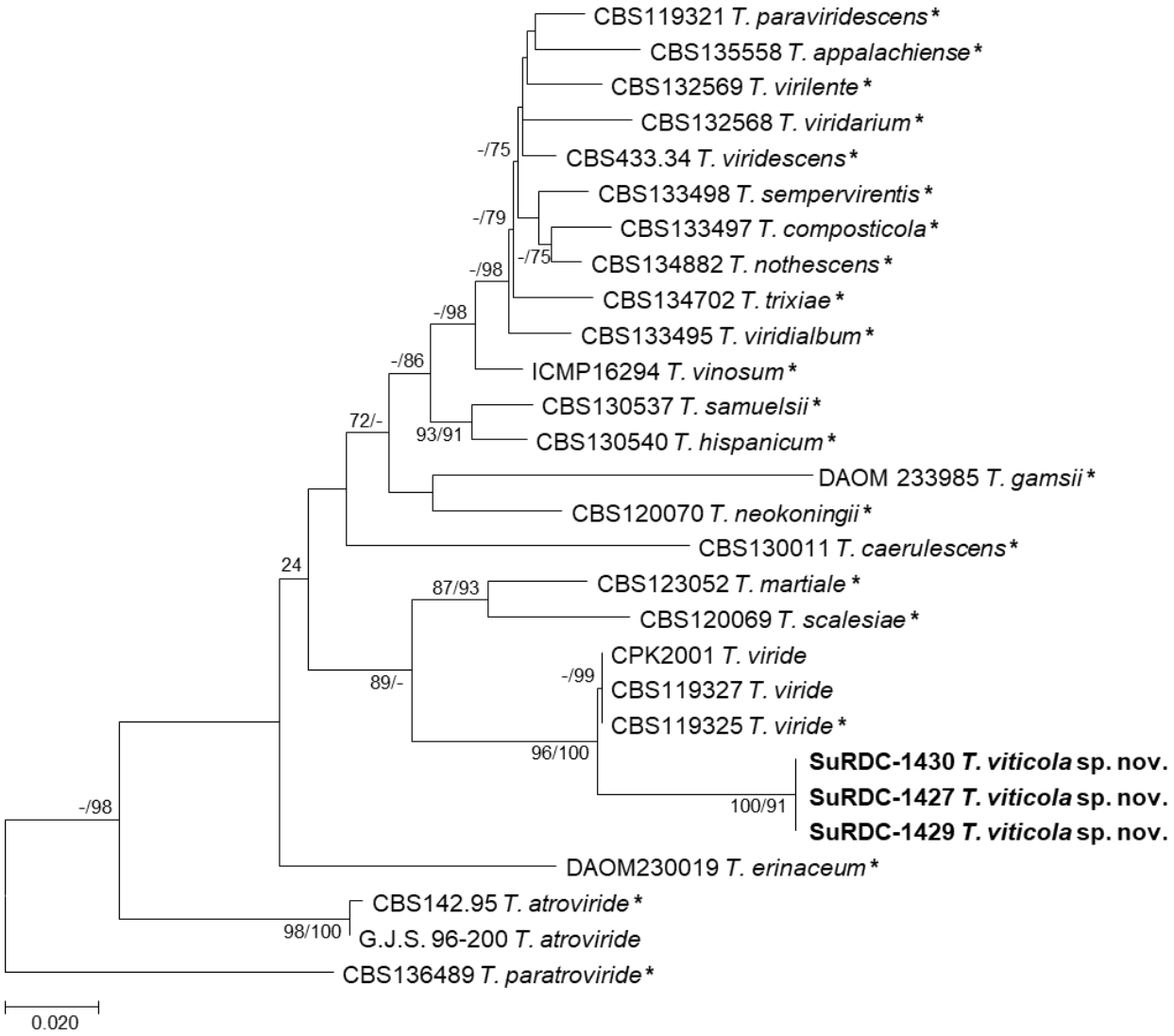
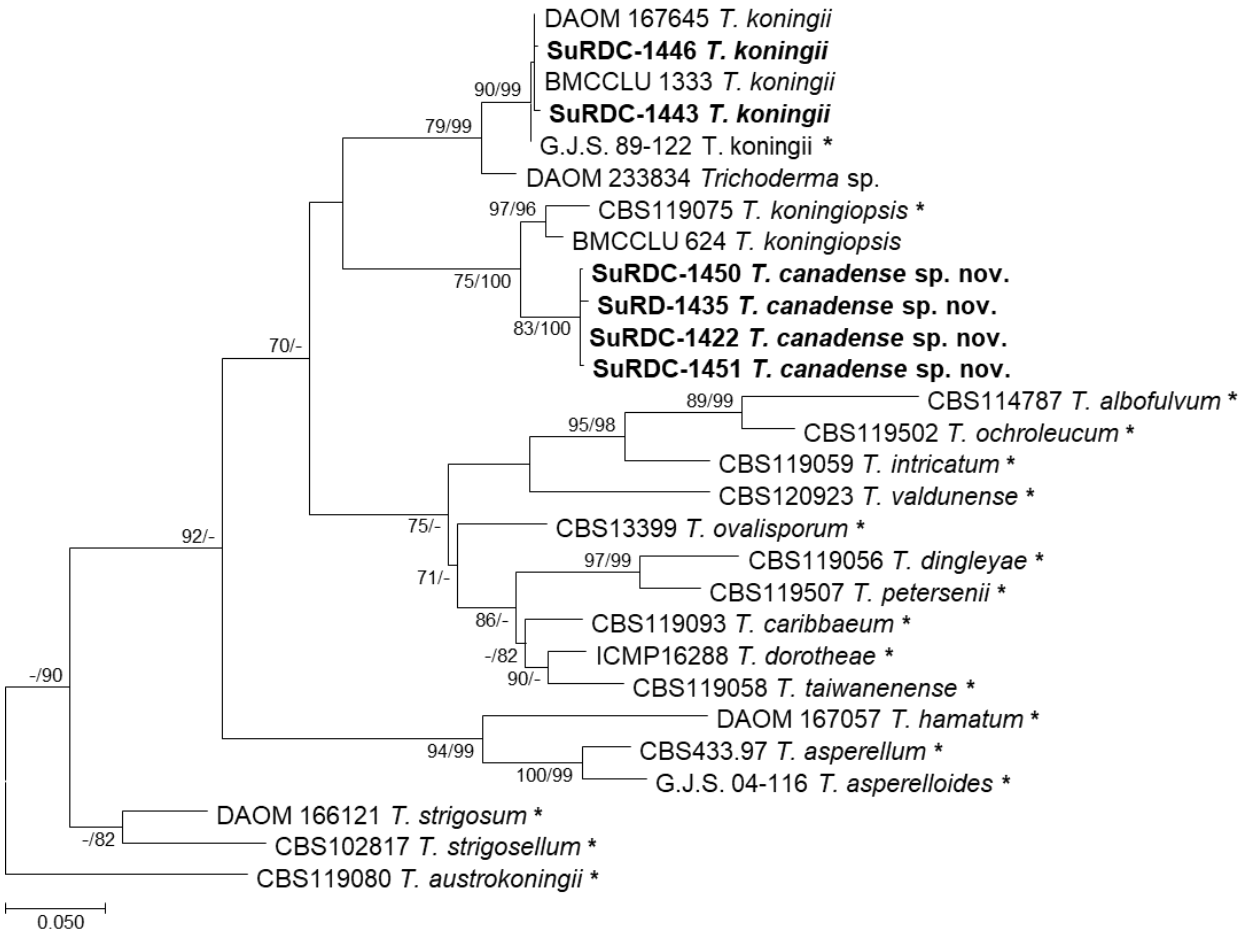


Figure 2.3. Phylogram of the highest log likelihood tree from an analyses of the *TEF1 viride/koningii* dataset. Numbers in front and after the slash represent ML and MP bootstrap values from 1,000 replicates, respectively. Values represented by a dash (-) were less than 70% for the bootstrap analyses. Type specimens are indicated with an asterisk and *Trichoderma* isolates from vineyards in BC are indicated in bold. Bar indicates the number of substitutions per site.



2.3.2 New species taxonomy

2.3.2.1 *Trichoderma canadense*

Trichoderma canadense J.R. Úrbez-Torres & J. Pollard-Flamand sp. nov.

Figure 2.4

Etymology. Named after Canada, where this species was collected.

Minimum temperature for growth 5 °C, optimum 25 °C, maximum 40 °C. On PDA after 96 h, colony radius 6.3 mm at 5 °C, 83 mm at 25 °C and 6.7 mm at 40 °C. Colonies on PDA initially white, regularly circular, distinctly zonate with dense mycelium in the centre and slightly loose at the margin. Aerial hyphae loose. No diffusing pigment (Figure 2.4). Colony turning green after 5 days in the centre and expanding gradually with time towards the margin (Figure 2.4). Conidial production noted after 7 d at (~22 °C), starting around the inoculum.

Mycelium consisting on branches, septate hyaline hyphae that occur singly 3.5-7.5 µm wide. Conidiophores on PDA mostly aggregated, but after about X days forming compact pustules up to 1.5 mm diameter (Figure 2.4). Pustules clearly observed around the inoculum after 14 days, first white, turning green with age and hyphae protruding beyond the surface 0.5-0.8 mm in diameter. Conidiophores developing predominantly as branches from the aerial mycelium, pyramidal, main axis coarse and straight (up to 137.6 µm long). Branches predominantly straight, thinner than main axis, paired at irregular intervals. Terminal cells circular to ellipsoidal. Phialides hyaline, obpyriform to langeniform, occasionally curved from terminal conidiophore branches or arising singly along the sides of the conidiophore (4.5–)9.8–10.6 (–16.6) × (2.9–)4.5–4.7(–6.5) µm, length/width ratio = 2.6 (*n*=50). Conidia cylindrical with rounded ends, often ellipsoidal or subglobose (3.9–)4.8–5.2 (–5.9) × (3.2–)3.7(–4.6) µm, length/with ratio = 1.3, average area 13.8 µm (*n*=50), thin walled, singly pale-yellow, light green in mass (Figure 2.4). Chlamydospores unobserved. Teleomorph undetermined.

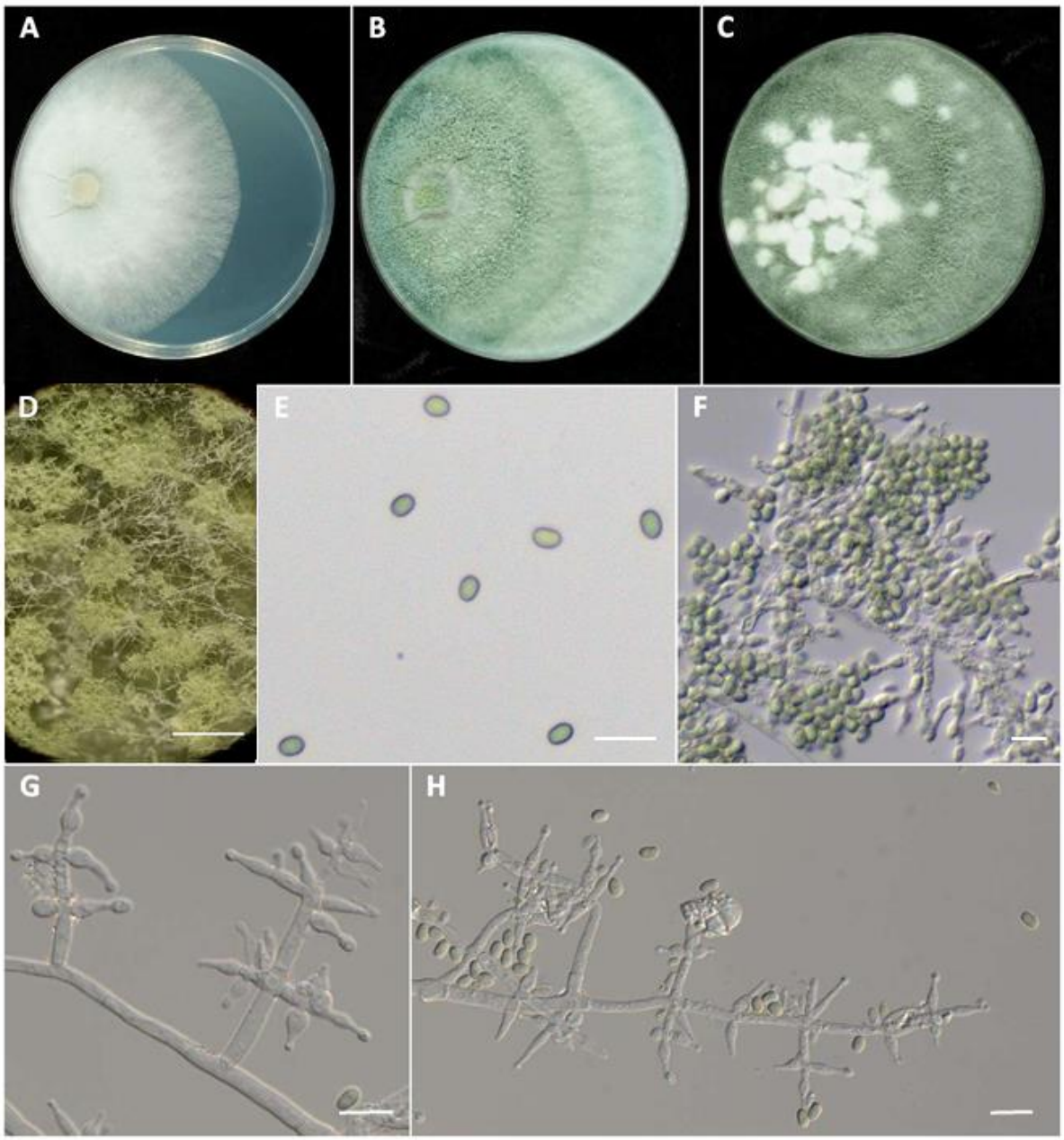
Type: Canada, British Columbia, Okanagan Valley, isolated from vascular tissue (xylem) at the basal end of 'Riparia gloire' rootsock (*Vitis riparia*) grafted onto 'Merlot' (*Vitis vinifera*), J.R. Úrbez-Torres: SuRDC-1422 .

Additional Material Examined: Canada, British Columbia, Okanagan Valley, J.R. Úrbez-Torres: isolated from vascular tissue (xylem) at the basal end of 'Riparia gloire' rootsock grafted onto Pinot Noir, SuRDC-1435, roots SuRDC-1450, and graft-union SuRDC-1451.

Sequences: SuRDC-1422 (MZ161796, ITS, MZ189377, *TEF*), SuRDC-1435 (MZ161797, ITS, MZ189378, *TEF*), SuRDC-1450 (MZ161798, ITS, MZ189379, *TEF*), SuRDC-1451 (MZ161799, ITS, MZ189380, *TEF*)

Notes: Trichoderma canadense was isolated four times from the xylem tissue of four different young grapevines in the current study. A BLAST search (NCBI GenBank) of the ITS1-5.8S-ITS2 sequences of *T. canadense* resulted in 100% homology with a large number of *T. koningiopsis* as well as *Trichoderma* sp. isolates. However, a BLAST search of the TEF1-alpha yielded between 95 and 97% homology with *T. konongiopsis* isolates, including ex-type isolate CBS119075. *Trichoderma canadense* can be readily distinguished from known *Trichoderma* spp. by phylogenetic analyses of the concatenated ITS and TEF1-alpha sequences as well as TEF1-alpha alone. Phylogenetic analyses revealed *T. canadense* to be closely related to *T. koningiopsis*.

Figure 2.4. *Trichoderma canadense* SuRDC-1422. Cultures on PDA at ~22 °C after 3 days (**A**), 7 days (**B**) and 14 days (**C**). **D** conidiation pustules on PDA after 14 days. Single conidia (**E**) and mass conidia (**F**). Conidiophores and phialides (**G** and **H**). Scale bars represent 1 mm (**D**) and 10 µm (**E, F, G, H**).



2.3.2.2 *Trichoderma viticola*

Trichoderma viticola J.R. Úrbez-Torres & J. Pollard-Flamand sp. nov.

Figure 2.5

Etymology. Named after the host it was isolated from, *Vitis vinifera*.

Minimum temperature for growth 5 °C, optimum 20 °C, maximum 40 °C. On PDA after 96 h, colony radius 9.3 mm at 5 °C, 62.3 mm at 20 °C and 6.3 mm at 40 °C. Colonies on PDA initially white, regularly circular, zonate with dense cottony mycelium in the centre becoming loose towards the margin. Aerial hyphae loose. No diffusing pigment (Figure 2.5). Colony white with dense mycelium until fully covered Petri plate and turning gradually light-green (centre) to dark-green (margin) after 3 days (Figure 2.5). Conidial production noted after 3 d at approximately 22 °C under lab lighting conditions, starting around the inoculum.

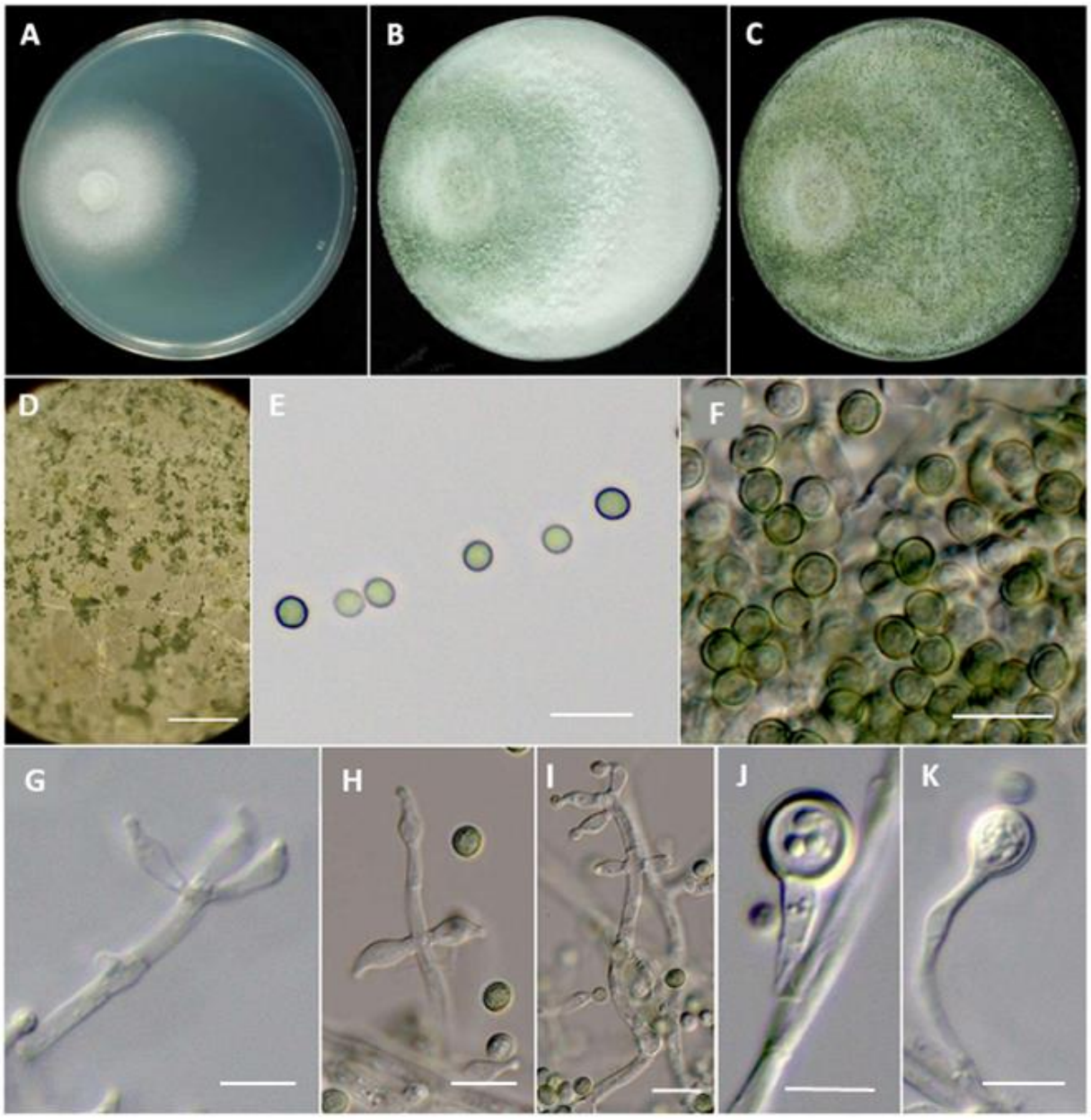
Mycelium consisting on branches, septate hyaline hyphae that occur singly 2-4 µm wide. Conidiophores on PDA mostly aggregated, forming loose pustules first becoming compact up to 0.1-0.3 mm diameter (Figure 2.5). Small and loose pustules formed around the inoculum becoming pale cream and compact after 14 days with hyphae protruding beyond the surface (Figure 2.5). Conidiophores developing predominantly as branches from the aerial mycelium, main axis coarse and straight (up to 72.2 µm long). Conidiophores primarily unbranched and often terminating in a whorl of 2 or 3 phialides (Figure 2.5). Terminal cells circular to subglobose. Phialides ampulliform to langeniform, occasionally curved and often constricted below the tip to form a narrow neck (Figure 2.5). Phialides arising singly, paired and at irregular intervals along the axis of the conidiophore (5.4–)7.7–8.1(–11.6) × (2.3–)3.1–3.3(–5.5) µm, length/width ratio = 2.4 (*n*=50). Conidia subglobose (3.9–)4.4(–5.1) × (3.3–)4.0(–4.7) µm, length/with ratio = 1.1, average area 13.2 µm (*n*=50), thin walled, granular, hyaline when young becoming light green with age, dark green in mass (Figure 2.5). Chlamydospores present, thick walled, spherical, granular, single, arising from hyphae. Teleomorph undetermined.

Type: Canada, British Columbia, Okanagan Valley, isolated from vascular tissue (xylem) at the basal end of 'Riparia gloire' rootsock (*Vitis riparia*) grafted onto Pinot Meunier (*Vitis vinifera*), J.R. Úrbez-Torres: SuRDC-1430.

Additional Material Examined: Canada, British Columbia, Okanagan Valley, J.R. Úrbez-Torres: isolated from vascular tissue (xylem) at the basal end of '3309 Couderc' rootsock (*Vitis riparia* x *V. rupestris*) grafted onto New York Muscat (Muscat Hamburg x Ontario), SuRDC-1427 , basal end of 'Riparia gloire' rootsock grafted onto Pinot Meunier SuRDC-1429.

Sequences: SuRDC-1427 (MZ161791, ITS, MZ189372, *TEF*), SuRDC-1429 (MZ161792, ITS, MZ189373, *TEF*), SuRDC-1430 (MZ161793, ITS, MZ189374, *TEF*).
Notes: Trichoderma viticola was isolated three times from the xylem tissue of three different young grapevines in the current study. A BLAST search (NCBI GenBank) of the ITS1-5.8S-ITS2 sequences of *T. viticola* resulted in 100% homology with a large number of *T. viride* isolates. However, a BLAST search of the TEF1-alpha resulted in 97% homology with few *T. viride* isolates. *Trichoderma viticola* can be readily distinguished from known *Trichoderma* spp. by phylogenetic analyses of the concatenated ITS and TEF1-alpha sequences as well as TEF1-alpha alone. Phylogenetic analyses revealed *T. viticola* to be closely related to *T. viride*.

Figure 2.5. *Trichoderma viticola* SuRDC-1430. Cultures on PDA at ~22 °C after 3 days (**A**), 7 days (**B**) and 14 days (**C**). Conidiation pustules on PDA after 7 days (**D**) and 14 days (**E**). Single conidia (**F**) and mass conidia (**G**). Conidiophores and phialides (**H**-**J**). Chlamydospores (**K** and **L**). Scale bars represent 1 mm (**D**) and 10 µm (**E, F, G, H, I, J, K**).



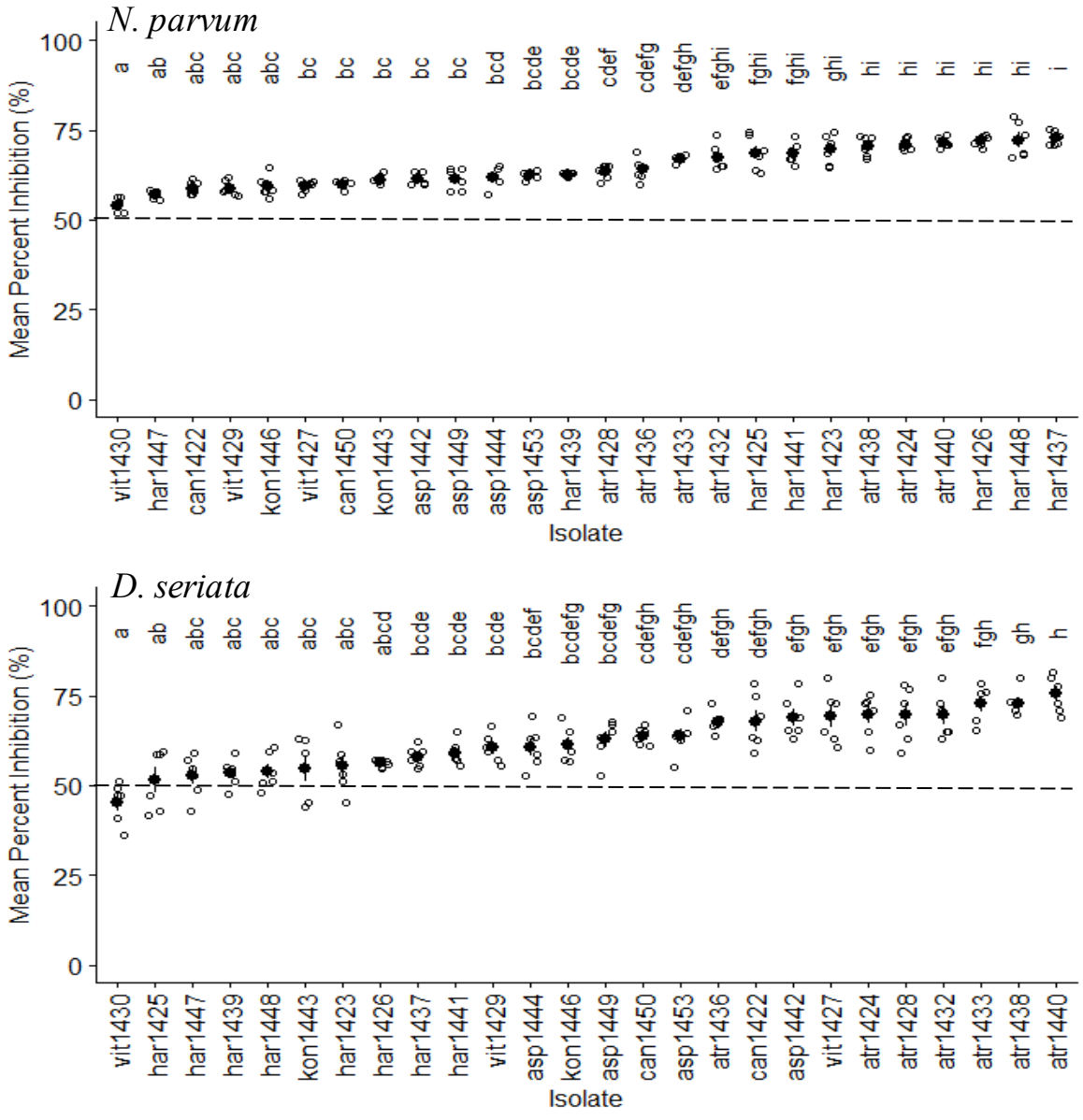
2.3.3 Optimum mycelial growth temperature study

A student T-test showed no significant difference of the means between the first and second experiments ($P < 0.05$) so data from the two experiments were pooled. All *Trichoderma* isolates from BC used in this experiment grew over the range of temperatures 10-30°C after four days incubation in PDA. All species showed growth at 35°C with the exception of *T. harzianum*, *T. tomentosum* and *T. viticola*. *Trichoderma atroviride* and *T. koningiopsis* showed no growth at 5°C and none of the isolates grew at 40°C. Optimum temperature for mycelial growth varied among species. The temperatures at which each *Trichoderma* spp. reached the maximum radial growth were 20°C for *T. atroviride* (SuRDC-1436 and SuRDC-1440) and *T. viticola* (SuRDC-1427 and SuRDC-1429), 25°C for *T. asperelloides* (SuRDC-1442 and SuRDC-1449), *T. canadense* (SuRDC-1422 and SuRDC-1451), *T. koningii* (SuRDC-1443 and SuRDC-1446), and *T. tomentosum* (SuRDC-1431), and 25-30°C for *T. harzianum* (SuRDC1423 and SuRDC1437).

2.3.4 Dual culture antagonism

A student T-test showed no significant difference of the means between the first and second experiments ($P < 0.05$) so data from the two experiments were pooled. The level of antagonism of *Trichoderma* isolates used in this experiment against GTD pathogens *D. seriata* and *N. parvum* is shown in Figure 2.6. The mean percent radial mycelial growth inhibition (MPRGI) of *D. seriata* and *N. parvum* by *Trichoderma* isolates from BC selected for this experiment ranged from 45.2% to 75.5% (Figure 2.6). The highest MPRGI values for *D. seriata* (72.6%) and *N. parvum* (75.5%) were recorded with *T. harzianum* (SuRDC-1437) and *T. atroviride* (SuRDC-1440) isolates, respectively (Figure 2.6). With the exception of *T. viticola* isolate SuRDC-1430, which showed 45.2 MPRGI when paired against *N. parvum*, the rest of the isolates showed MPRGI higher than 50% against both *D. seriata* and *N. parvum*. *Trichoderma atroviride* isolates showed the highest MPRGI against *D. seriata* and *N. parvum*. *Trichoderma harzianum* isolates overall showed lower MPRGI against *D. seriata* than *N. parvum* (Figure 2.6).

Figure 2.6. Mean percent radial growth inhibition (MPRGI) of *Trichoderma* isolates from BC against GTDs pathogens *Diplodia seriata* SuRDC-1050 and *Neofusicoccum parvum* SuRDC-1025 measured after 5 d. White dots represent data from experiment 1 and repeated experiment 2. Solid black dots represent the MPRGI for each isolate calculated from six replicates. Bars represent standard errors of the means. Dash line represents 50% inhibition threshold. Columns accompanied by the same letter were determined not to be statistically different by the Tukey-Kramer Honest Significant Difference post-hoc test (P = 0.05).



2.3.5 Detached cane assays

Results from the DCA greenhouse trial assessing selected *Trichoderma* isolates from BC are shown in Table 2.3. The average values for this DCA were determined to be 17.7°C and 38% RH for the duration of the experiment MPR of *D. seriata* and *N. parvum* from positive controls inoculated with 5,000 spores/wound were higher than 80% when wounds were inoculated 1d, 7d, and 21d after pruning (Table 2.3). *Diplodia seriata* and *N. parvum* were not isolated from non-treated and non-inoculated (natural infection) negative controls (data not shown). *Trichoderma* isolates from BC tested in the DCA provided MPDC ranging between 23 and 100% against both *D. seriata* and *N. parvum* (Table 2.3). *Trichoderma asperelloides* (SuRDC-1442) and *T. atroviride* (SuRDC-1440) provided the highest MPDC (70-100%) against *D. seriata* from day one to day 21 after treatment. The rest of the isolates provided a range of MPDC between 10 and 100% when pruning wounds were challenged with *D. seriata* one to 21 days after treatment (Table 2.3). *Trichoderma atroviride* (SuRDC-1440) provided the highest MPDC (100%) against *N. parvum* from day one to day 21 after treatment. *Trichoderma asperelloides* (SuRDC-1442) and *T. canadense* (SuRDC-1422) also provided high MPDC (77-100%) from one to 21 days after treatment. The rest of the isolates provided lower MPDC (23-88%) against *N. parvum*. All the isolates tested in the DCA trial provided MPDC higher than 50% when pruning wounds were challenged with 5,000 spores/wound of *D. seriata* and *N. parvum* 21 days after treatment with the exception of *T. koningii* (SuRDC-1446) with a MPDC of 47% for *D. seriata* (Table 2.3). With the exception of *T. viticola* (SuRDC-1427), all the isolates tested showed an increase of MPDC over time.

Results from the DCA greenhouse trial assessing selected commercial products against these same *D. seriata* and *N. parvum* isolates are shown in Table 2.4. The GreenSeal fungicide exhibited a consistent medium level of MPDC (44-58%) against *D. seriata* from one to 21 days post-treatment and a high level of control against *N. parvum* (MPDC 73-100%). The fungicide

Mettle had a relatively lower MPDC against *D. seriata* and very poor control of *N. parvum* (4-21%). The biocontrol Biotam 2.0 demonstrated medium to high effectiveness against both pathogens from seven to 21 days (58-100%). In general, MPDC values of the fungicides Mettle and GreenSeal decreased over time while MPDC values of Biotam 2.0 increased over time.

Table 2.3. Results of detached cane assay with *Trichoderma* isolates from BC under controlled greenhouse conditions. Values represent the mean percent recovery (MPR) of *D. seriata* and *N. parvum* from pruning wounds treated with *Trichoderma* isolates or with a water control and challenged with 5,000 spores of each *D. seriata and N. parvum* 1d, 7d, and 21d post treatment. Values followed by the same letters in each row are not statistically different by the Tukey-Kramer multiple comparison test (P = 0.05).

			SuRDC Isolate Number and Species						
Pathogen Inoculation time	**Calculation**	**Control**	**1422 *canadense***	**1427 *viticola***	**1437 *harzianum***	**1439 *harzianum***	**1440 *atroviride***	**1442 *asperelloides***	**1446 *koningii***
D. seriata									
1 Day	MPR	100a	33bc	77a	40c	90a	7b	30bc	77a
	MPDC[a]		67	23	60	10	93	70	23
7 Days	MPR	87a	13b	20cb	10b	43cd	0b	3b	60ad
	MPDC		85	77	89	50	100	96	31
21 Days	MPR	100a	7b	43c	3b	13b	0b	0b	53c
	MPDC		93	57	97	87	100	100	47
N. parvum									
1 Day	MPR	100a	23bc	37bd	60de	77ae	0c	20bc	43bd
	MPDC		77	63	40	23	100	80	57
7 Days	MPR	83a	0b	27bc	57ad	47cd	0b	0b	30cd
	MPDC		100	68	32	44	100	100	64
21 Days	MPR	80a	0b	13bcd	10bc	37d	0b	3bc	27cd
	MPDC		100	83	88	54	100	96	67

[a]MPDC: mean percent disease control, calculated as (100x (1-(MPR treatment/MPR control)))

Table 2.4. Results of detached cane assay for commercial chemical fungicides and biocontrol products under greenhouse controlled conditions. Values represent the MPR of *D. seriata* and *N. parvum* from pruning wounds treated with commercial pruning wound treatments or with sterile distilled water as control and inoculated with *D. seriata* and *N. parvum* spore suspension 1d, 7d, and 21d post treatment. Values followed by same letters in each row were determined not statistically different by the Tukey-Kramer multiple comparison test (P = 0.05).

Pathogen/Day of Inoculation	Calculation	Control	GreenSeal	Mettle	Bio-Tam 2.0
D. seriata					
1 Day	MPR	87b	37c	53ac	70ab
	MPDC		58	39	19
7 Days	MPR	83c	47b	63bc	13a
	MPDC		44	24	84
21 Days	MPR	80c	40b	57bc	0a
	MPDC		50	29	100
N. parvum					
1 Day	MPR	97c	0b	77ac	63a
	MPDC		100	21	34
7 Days	MPR	87c	7b	83c	37a
	MPDC		92	4	58
21 Days	MPR	73b	20a	70b	20a
	MPDC		73	5	73

2.3.5.1 Identity confirmation of re-isolated *Trichoderma* species

The ITS sequences of each representative *Trichoderma* isolate was aligned using the MUSCLE algorithm to confirm match in MEGA-X. The results of these alignments showing query cover and percentage match between inoculated isolates and the DNA extracted from the re-isolations of those treatments are compiled in Table 2.5. Results confirmed that the re-isolated *Trichoderma* isolates were indeed the same inoculated isolate.

Table 2.5. Summary of ITS alignment for confirmation of isolate identity of greenhouse detached cane assay *Trichoderma* re-isolations.

Isolate ID	*Trichoderma* sp.	Query Cover	Percent match (%)	Number of confirmation sequences
SuRDC-1422	*T. canadense*	492	100	4
SuRDC-1427	*T. viticola*	494	100	2
SuRDC-1431	*T. tomentosum*	600	100	3
SuRDC-1437	*T. harzianum*	503	100	3
SuRDC-1439	*T. harzianum*	511	100	3
SuRDC-1440	*T. atroviride*	484	100	5
SuRDC-1442	*T. asperelloides*	493	100	3
SuRDC-1446	*T. koningii*	493	100	2

2.4 Discussion

2.4.1 Phylogenetic analysis

This study represents the first attempt to characterize *Trichoderma* spp. naturally occurring on grapevines in Canada. Twenty-nine *Trichoderma* isolates were identified from grapevines in BC to the species level by sequencing the ITS gene region and partial *TEF1* gene by performing both single- and multi-locus phylogenetic analysis. In total, five known species, including *T. asperelloides*, *T. atroviride*, *T. harzianum*, *T. koningii*, and *T. tomentosum* and two novel species, *T. canadense* and *T. viticola*, were identified in this study.

Five of the species found in the present study commonly occur in different ecosystems from different regions (Farr and Rossman, 2021). For instance, *T. asperelloides*, first described in 2010 as a cryptic species within *T. asperellum*, is known to have a wide sympatric distribution and has been isolated mostly from agricultural soils in tropical countries (Samuels et al. 2010). To the best of our knowledge, this is the first report of *T. asperelloides* in Canada and is the first report from grapevines worldwide. Similarly, *T. atroviride*, *T. harzianum*, and *T. koningii* have been reported from many countries around the world from a broad range of different hosts, including grapevines (Farr and Rossman, 2021; Gonzalez and Tello, 2011; Jayawardena et al. 2018a, 2018b).

In Canada, *T. atroviride* have been reported from many different hosts and sources from Alberta, BC, Ontario (ON) and Québec (QC) provinces (Bisset, 1992). Similarly *T. harzianum* have been found from different hosts in Canada, including *Cannabis sativa* (Punja et al. 2019), *Carpinus caroliniana* (Bills and Polishook 1991), *Populus tremuloides* (Ginns 1986), *Solanum lycopersocum* (Johnston-Monje et al. 2017), and *Ulmus americana* (Kindermann et al. 1998). Contrarily, *T. koningii* has a much narrower host range in Canada and it has only been reported from *C. caroliniana* (Bills and Polishook 1991) and from mushroom compost (Samuels et al. 2006). *Trichoderma tomentosum* was first discovered in 1991 along with other 11 *Trichoderma* spp. as a species aggregate of *T. hamatum* and described from *S. lycopersicum* in BC, *Helianthus annus* in Manitoba, *Ulmus* sp. in ON, and *Pinus* sp. in QC (Bisset, 1991). Since then, the species is shown to have a broader distribution and it has been reported from Europe (Jaklitsch and Voglmayr, 2015), Guatemala (Hoyos-Carvajal et al. 2009), New Zealand (Braithwaite et al. 2017), and USA (Bisset, 1991). The present study expands the host distribution of *T. atroviride*, *T. harzianum*, *T. koningii*, and *T. tomentosum* and report these species for the first time from grapevines in Canada. In addition, to the best of our knowledge this is the first report of *T. tomentosum* from grapevines worldwide. In addition to the above species, two clades were identified which aligned closely with *T. harzianum* and *T. koningiopsis,* however, they were identified as distinct from those species based on their phylogenetic relationships and named *T. canadense* and *T. viticola*.

The phylogenetic analysis employed in this study was sufficient to support species level identification using two genetic loci, ITS and *TEF1*. It has been shown that identification of *Trichoderma* spp. based on ITS alone is insufficient as the sequence is identical or nearly identical for several species of the genus (Qiao et al. 2018). Genetic markers such as *RPB2* and *TEF1* are more informative on account of higher interspecific variations between species with *TEF1* shown to provide high resolution superior to other phylogenetic markers in this genus

(Jaklitsch et al. 2012; Jaklitsch and Voglmayr 2015; Qiao et al. 2018). It is for these reasons that a single locus tree was analyzed using *TEF1* alone, and in combination with ITS, however ITS was not analyzed alone.

The practice of analyzing multiple genetic markers for the identification of species is called genealogical concordance phylogenetic species recognition (GCPSR) (Taylor et al. 2000). Using this species recognition concept, a species is delimited when either one locus is analyzed and supported or when multiple loci are analyzed separately and do not contradict each other (Dettman et al. 2003; Taylor et al. 2000). In this sense, if another highly informative locus such as RPB2 was included in the phylogenetic analysis the clades may be separated further. For example, if *RPB2* were analyzed and contradicted the grouping of these isolates, cryptic species could potentially be delineated as was the case with the *T. asperellum* and *T. harzianum* clades when they were revisited and GCPSR was applied (Chaverri et al. 2015; Samuels et al. 2010).

2.4.2 Dual culture antagonism assay

This study evaluated the *in vitro* antagonism of 29 *Trichoderma* isolates representing four known species, *T. asperelloides, T. atroviride, T. harzianum, T. koningii* and two newly described, *T. canadense* and *T. viticola* against two Botryosphaeria dieback pathogens: *D. seriata* and *N. parvum*. The only *T. tomentosum* isolate identified in this study (SuRDC-1431) did not survive in the culture collection and therefore was not included in this assay. The two pathogens selected in this study, *D. seriata* and *N. parvum*, are among the most commonly isolated GTD fungi in BC and also represent a moderate and a highly virulent species, respectively (Úrbez-Torres 2011; Úrbez-Torres et al. 2014a, 2014b). The biocontrol abilities of *T. asperelloides, T. atroviride*, *T. harzianum,* and *T. koningii* isolates from Australia, Hungary, New Zealand, South Africa, and Spain have been tested *in vitro* specifically against both *D.*

seriata and *N. parvum* and were shown to be highly effective at inhibiting the growth of the pathogens (Mondello et al. 2018) which is in agreement with our findings using isolates from BC, Canada. In addition to DCAA, the secondary metabolites of *T. harzianum* (isolate T-77) and two *T. atroviride* (isolates UST1 and UST2) have been tested *in vitro* for inhibition of conidial germination and mycelial growth of GTD pathogens including *N. parvum* (Mutawila et al. 2016).

In the current study, we hypothesize that the mode of action *in vitro* was competition for space and overgrowth of the pathogen by the *Trichoderma* isolates as shown by the MPDC results. Whether or not this represents the full mechanism of defense is not clear because microscopic observations of BCA-pathogen interactions were not conducted in this study. It has been shown that isolates of *T. atroviride* and *T. harzianum* were capable of hyphal coiling/adhesion and hyphal swelling/disintegration when interacting *in vitro* with *N. parvum, D. seriata,* as well as with *L. theobromae* (Kotze et al. 2011).

Although *in vitro* assays are not a guarantee of BCA activity *in plantae* (Bell et al. 1982), this assay served as the first level of screening to identify which isolates exhibited the highest level of *in vitro* antagonism before investing in more advanced screening *in plantae* in the greenhouse under controlled conditions or further studies in the field under natural conditions. This was critical to avoid unnecessarily large and expensive experiments at the greenhouse level where the repetitions increase significantly to control for the increased variation intrinsic to greenhouse and field trials.

Future work could investigate the modes of action of antagonism by our isolates microscopically *in vitro* as has been explored for other effective *Trichoderma* isolates (John et al. 2005; Kotze et al. 2011) and could also test for the tolerance of effective isolates to fungicides that may affect performance in the context of a field trial or commercial application management where multiple

fungicides may be used to control other grapevine diseases and could affect optimal BCA performance as suggested by Marraschi et al. (2019).

2.4.3 Detached cane assays

The goal of the DCA experiment was to take the best performing *Trichoderma* isolates from the DCAA and test them alongside commercial products to compare their ability to colonize and protect grapevine pruning wounds from infection by *D. seriata* and *N. parvum* in the greenhouse under controlled conditions. We found in this study that some of our local *Trichoderma* isolates (SuRDC1422, SuRDC1440, and SuRDC1442) were capable of providing very high control when detached Chardonnay canes were treated immediately after pruning and were then challenged by *D. seriata* or *N. parvum* one, seven, and 21 days later. This represents the second time a DCA in the greenhouse has been used to evaluate the efficacy of grapevine endophytic *Trichoderma* spp. for the control of *D. seriata* and *N. parvum* (Úrbez-Torres et al. 2020) and is the first time evaluating *T. koningii* and *T. asperelloides* against these GTD in the greenhouse.

Previously, the effect of TIFI, a commercial *T. atroviride*-based product (Giten Biological, Tarragona, Spain) was evaluated by treating pruning wounds of potted grapevines with mycelial plugs of *T. atroviride* either before or after inoculation with one of five Botryosphaeriaceae spp., including *N. parvum* (Pintos et al. 2012). This study showed that treating with *Trichoderma* before infection resulted in reduction in pathogen recovery and was able to reduce necrosis lengths by 65.7 to 91.9%; however, the pathogen was still re-isolated from the *Trichoderma* treated potted vines from 38% of the inoculated vines (Pintos et al. 2012). It has also been shown that *Trichoderma* BCA can grow isolated in a wide range of hosts (Gams & Bisset, 1998; Hosseyni-Moghaddam & Soltani, 2014; Samuels 2005) and propagate, lasting years in the environment in some cases (John et al. 2008; Longa et al. 2009). It is for these reasons that we modified the DCA to test our isolates for effectiveness from one to 21 days post-treatment so as

to determine how long it would take them to be effective, and for how long they may remain effective.

A DCA was first developed by Ayres et al. (2011) to test the colonization of detached canes by *E. lata* over the course of three months as well as to test the efficacy of grapevine pruning wound protection products in controlling *E. lata*. This method eliminates the need for potted vines used in other studies (Pintos et al. 2012) and allows for inexpensive, high-throughput screening of pruning wound products *in plantae*, which can yield rapid results (Úrbez-Torres et al. 2020), especially when compared against field trials which most often span multiple years at which point they may still yield inconclusive results (Di Marco et al 2000). In this regard, this experiment served as an intermediate level of screening *Trichoderma* for their GTD control as they are challenged *in plantae* vs. *in vitro*, although the effects of natural weather and ecological pressures are still not included as in field trials (Reis et al. 2019).

In previous studies, inoculum dose used to evaluate the efficacy of fungicides to protect pruning wounds from GTDs has varied greatly (10-100,000 conidia or ascospores) (Ayres, 2011; Elena et al., 2015; Kotze et al., 2011; Pitt et al., 2012; Reis et al. 2019; Rolshausen et al., 2010). In order to ensure robust recovery of the pathogens from the positive controls, wounds were challenged with 5,000 conidia of either pathogen which far exceeds the natural pressure that might be present in the field (Gramaje et al. 2018). It was for this reason as well that we chose a Chardonnay cultivar as it is known to be more susceptible to infection by GTD fungi, including Botryosphaeriaceae spp. than other cultivars (Travadon et al. 2013). With the combination of a high inoculum pressure and a cultivar that is known to be susceptible, we were able to achieve high recovery of the pathogens from the positive controls in the detached cane assays in this study (73-100%). This is important to note because the value of MPDC reported in this study is

relative to the recovery of the pathogen and further supports the high degree of biological control observed in this study.

For example, although *T. harzianum* and *T. atroviride* isolates have previously provided 82% and 92% control of *D. seriata* and *N. parvum* respectively, the pathogens were only recovered 38% and 22% in the positive controls respectively. In our DCA experiment, we achieved 80-100% recovery of the pathogens in our positive controls and still SuRDC-1440 was able to achieve 93-100% reduction of the pathogen recovery when challenged 24 hours post-treatment. SuRDC-1440 also demonstrated 100% MPDC of both *D. seriata* and *N. parvum* seven and 21 days post-treatment with high recovery in the positive controls (73-100%). This is interesting because BCA such as *Trichoderma* are known to require time to colonize pruning wounds before they are highly effective (Kotze et al. 2011). A recent study showed similar results in a greenhouse experiment using identical inoculum doses and experimental design and screening *Trichoderma* isolates from different hosts from Italy against *D. seriata* and *N. parvum* demonstrating the robust and repeatable nature of this evaluation system (Úrbez-Torres et al. 2020). These results highlight the rapid colonization exhibited by the three best performing local *Trichoderma* isolates, including *T asperelloides* (SuRDC-1442), *T. atroviride* (SuRDC-1440) and *T. canadense* (SuRDC-1422), and indicate that these isolates could potentially provide immediate GTD control on pruning wounds similar to chemical fungicides but with longer lasting and increasing effects. In general, local and commercial *Trichoderma* treatments became more effective when the time between treatment and inoculation increased whereas the MPDC of GreenSeal and Mettle were either consistent or decreased over time. This is important to note as it indicates that GreenSeal and Mettle may require re-application every month whereas the *Trichoderma* treatment results indicate they may require only one application although further testing is required over longer periods of time to confirm this.

In order to fully quantify the effectiveness of these *Trichoderma* isolates from BC, further screening is required in the field to evaluate their potential control under natural environmental conditions. Similar to the DCA experiments, we need also to obtain data regarding how long it will take for these *Trichoderma* spp. to protect the pruning wound and for how long they will remain effective, which will have important implications on developing a proper management strategy against GTD fungi in the field (Harman 2000).

Chapter 3: Field evaluation of *Trichoderma* species from British Columbia to assess their potential biological control activity against Botryosphaeria dieback fungal pathogens

3.1 Introduction

Grapevine trunk diseases (GTD) are a complex causing a broad range of different vascular and foliar symptoms depending on the fungi involved (Gramaje et al. 2018) and include black foot and Petri diseases, causing decline in young vineyards (Agusti-Brisach and Armengol, 2013; Mugnai et al. 1999) and Botryosphaeria dieback (Úrbez-Torres, 2011), esca (Mugnai et al. 1999), Eutypa dieback (Rolshausen et al. 2015), and Phomopsis dieback (Úrbez-Torres et al. 2013) causing decline in mature vineyards. Currently, over 130 different fungi from 34 genera have been associated with the different GTD but not all fungi have been confirmed as causal agents by completion of Koch's postulates (Gramaje et al. 2018). It is well-recognized that the main portal of infection for most GTD fungi is through pruning wounds. Fungal spores (ascospores or conidia) are released from fruiting bodies (perithecia or pycnidia) present on infected vines within or from adjacent vineyards and/or from alternate hosts surrounding vineyards by wind-blown or rain-splashed (Gramaje et al. 2018). Spores then land on susceptible pruning wounds and, under favourable conditions, proceed to colonize the woody tissues leading to dieback symptoms and eventual vine death (Fontaine et al. 2016; Gramaje et al. 2018; Mondello et al. 2018).

Grapevine trunk diseases are currently considered one of the most important biotic factors reducing yield and limiting vineyard lifespans worldwide causing untenable economic loses (Siebert et al. 2001; Wicks and Davis, 1999; Kaplan et al. 2016; Gramaje et al. 2018). Currently, there are no synthetic fungicides with a curative effect for GTD infected vines and thus, the only currently available curative option is remedial surgery which involves surgically removing infected parts of the vine and subsequently retraining new shoots from the base to

form a new vine (Savocchia et al. 2014; Sosnowski et al. 2011). However, remedial surgery cannot always be performed, it is costly, and requires highly skilled workers to be completed (Epstein et al. 2008). Therefore, with the absence of curative treatments, control of GTD primarily employs strategies to prevent infection, the most effective and widely implemented of which focus on pruning wound protection (Pitt et al. 2012; Rolshausen et al. 2010; Sosnowski et al. 2013). Protection of pruning wounds against GTD by means of synthetic fungicide products is not new and many studies evaluating a large range of active ingredients have been published (Gramaje et al. 2018; Mondello et al. 2018). However, interest in developing more sustainable agriculture production systems to respond to an increasing demand of organically and/or sustainable grown products, has recently caused viticulture practices to experience important changes, including the wide adoption of integrated pest management (IPM) approaches adapted in most viticulture regions worldwide (Compant and Mathieu, 2016). As a result of this shift in cultural practices, interest in the use of biological control agents (BCA) and/or organic products to control grapevines diseases and pests, including GTD has significantly grown in the last two decades (Compant and Mathieu, 2016; Mondello et al. 2018).

A broad number of generalist (*Bacillus* spp., *Pseudomonas* spp., *Streptomyces* spp., *Trichoderma* spp., and *Clonostachys* spp.) and specialist (*Agrobacterium* spp., *Ampelomyces* spp., *Cloniothyrium* spp., *Fusarium* spp., and *Aspergillus* spp.) BCA have been studied and screened for the management of many different plant pathogens in a large range of crops, including grapes (Woo et al. 2014; Compant and Mathieu, 2016). Among them, *Bacillus*, *Fusarium*, and *Trichoderma* spp. have been investigated as pruning wound protectants against GTD fungi (Costadone and Gubler, 2016; Gramaje et al. 2018; Mondello et al. 2018). However, species of *Trichoderma* are by far the most widely studied organisms to be used as BCA against plant pathogens, including GTD fungi. A review conducted in 2007 found over 60% of registered fungal BCAs were *Trichoderma*-based (Verma et al. 2007). *Trichoderma*, now widely

used in agriculture around the world, is a well-studied genus that has recognized potentials for diverse applications, including industrial enzyme producers, general BCA, and bio-fertilizers (Guzman-Guzman et al. 2019; Woo et al. 2014). Nevertheless, a review of BCA for the management of fungal plant pathogens identified, among several research gaps, the need to further explore the optimization of biocontrol products based on the development of locally acclimatized BCA strains that may be better adapted to the environmental conditions of the region of interest and thus, obtain better performance against the targeted pathogen/host in the region (Heydari & Pessarakli, 2010). In addition, in order to measure the true efficacy of BCA, for instance *Trichoderma* spp. used as pruning wound protectants against GTD fungi, experiments need to be conducted under natural environmental conditions relevant to the ecosystem they will be deployed in as climate can vary significantly from region to region, which may affect the behavior of any BCA.

Field surveys conducted between 2010 and 2015 showed GTD to be widely spread through both young and mature vineyards in British Columbia (BC) with over 40 different GTD fungi identified (Úrbez-Torres et al. 2014a, 2014b; Úrbez-Torres and O'Gorman, 2016). Similar to many other viticulture regions around the world, GTD appear to play also an important role in plant health in BC. However, contrary to other countries, there are currently no chemical nor biocontrol products commercially registered in Canada against GTD. Accordingly, the objectives of this study were to further characterize three *Trichoderma* spp. from BC for their ability to germinate under different temperatures and to assess their biocontrol capacity under both greenhouse and natural field conditions and to compare their performance against both chemical and *Trichoderma*-based commercial pruning wound protection products. Furthermore, the ability of two local *Trichoderma* spp. to survive long-term on grapevine pruning wounds and propagate endophytically into the grapevine pruning wounds after application was investigated.

3.2 Materials and methods

3.2.1 *Trichoderma* and pathogen inoculum preparation

For the greenhouse detached cane assay (DCA), *Trichoderma* inoculum from each selected isolate, including *T asperelloides* (SuRDC-1442), *T. atroviride* (SuRDC-1440) and *T. canadense* (SuRDC-1422) was obtained from four day-old colonies grown on PDA at 23 °C with a photoperiod 12:12 h per day under UV light (Phillips UVB TL 20W/12RS bulb). For the field trials, *Trichoderma* inoculum was obtained from 14 day-old colonies grown on PDA at ~23 °C on the laboratory bench top under lab lighting next to a window. For both greenhouse DCA and field studies, plates were flooded with sterile distilled water (SDW) containing 0.05% Tween 20 (Fisher Scientific) and the surface was scrapped with a sterile metal spatula. The conidial suspension of each *Trichoderma* isolate was adjusted to $1x10^6$ conidia/ml with a haemocytometer before the solutions were combined in equal parts to achieve three dual combination treatments, including BC-1 (SuRDC-1422 + SuRDC-1440), BC-2 (SuRDC-1422 + SuRDC-1442), BC-3 (SuRDC-1440 + SuRDC-1442), and one triple combination treatment, BC-4 (SuRDC-1422 + SuRDC-1440 + SuRDC-1442), all of which had a final concentration of $1x10^6$ conidia/ml.

For pathogen inoculum, *D. seriata* and *N. parvum* conidia were obtained from pycindia formed on colonies growing on PDA in the dark at 25°C under UV light (12:12 h) placed at 80 cm distance from the plates. Pycnidia formed after 4-5 weeks and conidia were harvested by adding 1-2 ml of SDW amended with a drop of Tween 20, then gently scraping the upper layer of the PDA to detach the pycnidia from the agar using a sterile metal spatula. The suspension containing pycnidia was transferred onto a sterile mortar and pestle and ground to release the conidia from the pycnidia. This suspension was then filtered through one layer of Miracloth into a 20 mL glass Pyrex test tube. The tube was vortexed and conidial concentration was adjusted

to $1x10^5$ conidia/ml (5,000 conidia applied per wound) to be used for greenhouse experiments and $4x10^4$ conidia/ml (2,000 conidia applied per wound) for use in field experiments for both *D. seriata* and *N. parvum* using a haemocytometer.

3.2.2 Optimum temperature conidial germination study

T asperelloides (SuRDC-1442), *T. atroviride* (SuRDC-1440) and *T. canadense* (SuRDC-1422) isolates were selected to determine the optimum temperature for conidial germination by evaluating percent germination after five, eight, 10, 12, and 24 hrs incubation at 10, 15, 20, 25, and 30 °C. Methods were adapted from Schubert et al. (2010) and Úrbez-Torres et al. (2010) and the following protocol was developed. Mycelial plugs of the respective *Trichoderma* isolates were plated on 90 mm PDA plates and grown on the benchtop ~23 °C for two days. Plates were then Parafilmed and placed again on the bench top where they were incubated for 13-14 days under laboratory lighting conditions next to a window that let in sunlight to produce conidia. Conidial suspensions were made by adding 1 mL sterile distilled water amended with Tween 20 (~1 drop per 300 mL) and gently scraping the aerial mycelia to suspend conidia. The suspension was then filtered through a sterile cotton ball before it was serial diluted to a concentration of $1x10^5$ conidia per mL using a hemocytometer. Ninety mm diameter Petri plates containing 20 mL of PDA amended with tetracycline (0.1mg/mL) were prepared and labeled the day before use for the germination study, and left in the laminar flow cabinet to dry overnight. All suspensions were made up at 6:30 PM the night before and were stored in a 4 °C refrigerator until plating the next morning. At 5:30 AM of the next day, the suspension was vortexed before a treatment of one 10 µL drop of $1x10^5$ conidia per mL suspension was plated in the center of each plate. All plating was completed within 30 minutes at which point 5 plates per *Trichoderma* spp. were placed in each of the five incubators set to temperatures of 10, 15, 20, 25, and 30 °C. Two I-buttons were placed inside two separate treated plates and were incubated with the other plates in each of the five incubators and set to measure absolute humidity and temperature

every five minutes. Plates were left unsealed and were removed in triplicate at 5, 8, 10, 12, and 24 h post-treatment and stored in the cold at 4 °C for no longer than 2 hours while microscopy measurements were performed. To evaluate the percent germination of the plated conidia, 10 µL of calcofluor (1000mg/mL) was added to a glass cover slip before it was placed on top of the conidia for observation. The conidia were observed and photographed using a Zeiss Axio Imager.M2 with a Zeiss AxioCam MRm at 20x magnification with an excitation frequency of 365 nm observed through a DAPI filter. Photographs were taken using Zeiss ZenPro. At least 50 conidia were randomly counted from each replicate resulting in at least 150 conidia counted per species x temperature x incubation time combination. Conidia were considered germinated when the germ tube length exceeded the diameter of the spore (Schubert et al. 2010). The entire experiment was repeated. The mean percent conidial germination and the standard errors were calculated for each experimental combination. The binomial germinated vs. non-germinated data from each experiment was analysed separately via general linear model with a logit link treating isolate, temperature, time, and all their interactions as fixed effects. Tukey-Kramer honest significant difference multiple pairwise comparison tests were used to compare times within temperatures at the 5% significance level (Úrbez-Torres et al. 2010). All analyses were performed using R (R Core Team, 2021).

3.2.3 Detached cane assays

3.2.3.1 *Trichoderma* combination treatments against *D. seriata* and *N. parvum*

Combinations of the best performing *Trichoderma* isolates obtained from DCA conducted in chapter 2 and described in 3.2.1. were assessed to determine if treatments containing a mix of *Trichoderma* spp. could increase the mean percent disease control (MPDC) of *D. seriata* and *N. parvum* compared to treatments using a single species. For each treatment, conidial suspensions were made to a total of $1x10^6$ conidia/ml using equal proportions of each isolate present in the selected combination. The experimental design was conducted following the

same methodology used for the DCA described in 2.2.5.2. Briefly, Chardonnay dormant canes were collected from one experimental vineyard block located at the SuRDC. Canes were cut into two nodes canes (~ 20 cm length) and placed vertically through holes made in Styrofoam treys floating on water tables filled with tap water in a greenhouse. Temperature and relative humidity were monitored in the greenhouse over the duration of the experiment using one Hygrochron I-button (IButtonLink, LLC, Whitewater, WI, USA) and average values were determined to be 18°C and 38% RH over the duration of the experiment. The water level was maintained throughout the experiment by adding tap water; however, the total water was not exchanged. Care was taken to ensure that the bottom of the canes were submerged in water for the duration of the experiment. Canes were then pruned 4 cm above the upper bud to simulate a fresh pruning wound. A total of 270 canes were prepared per treatment. Within 3 h after pruning, 180 canes (pruning wounds) per treatment were each inoculated with 50 µL of $1x10^6$ spores/ml (50,000 spores/wound) of each *Trichoderma* spp. A total of 90 canes per treatment were left untreated for positive and negative controls. In each treatment, 30 canes each (3 replicates of 10 repetitions each) were challenged 24 h, 7 d and 21 days after treatment with 50 µL of $1x10^5$ conidia/ml (5,000 spores/wound) of either *D. seriata* or *N. parvum* to determine how long *Trichoderma* activity last on the pruning wound. Positive controls included 30 non-treated but inoculated canes with 5,000 spores/wound of *D. seriata* or *N. parvum* each at 24 h, 7 d, and 21 d after pruning. In addition, 30 canes per treatment were left non-treated/non-inoculated to serve as negative controls to determine if natural infections happened in the collected canes from the experimental vineyard. Canes from the different treatments were randomized across the Styrofoam treys. Cuttings were maintained in the greenhouse and collected five weeks after each of the inoculation times. Fungal re-isolations were performed on the canes as in 2.2.5.2.

3.2.3.2 Commercial products against *N. parvum* and *D. seriata*

Two commercial products GelSeal and T-77, a tebuconazole amended wound sealant and a *T. atroviride-based* BCA, respectively were also included in separate DCA following manufacturer's instructions (Table 3.1) with their own positive and negative controls to test for product effectiveness in the greenhouse before further evaluation in the field. Experimental design was identical to the DCA described in section 2.2.5.2. Briefly, Chardonnay dormant canes were collected from one experimental vineyard block located at the SuRDC. Canes were cut into two nodes canes (~ 20 cm length) and placed vertically through holes made in Styrofoam treys floating on water tables filled with tap water in a greenhouse. Temperature and relative humidity were monitored in the greenhouse over the duration of both experiments using one Hygrochron I-button (IButtonLink, LLC, Whitewater, WI, USA) and average values were determined to be 20°C and 50% RH over the duration of the GelSeal experiment and 25°C and 33% RH over course of the T-77 experiment. The water level was maintained throughout each experiment by adding tap water; however, the total water was not exchanged. Care was taken to ensure that the bottoms of the canes were submerged in water for the duration of the experiments. Canes were then pruned 4 cm above the upper bud to simulate a fresh pruning wound. Within 3 h after pruning, pruning wounds were treated with GelSeal or T-77, depending on the DCA. Canes from the different treatments were randomized across the Styrofoam treys. Cuttings were maintained in the greenhouse and collected five weeks after each of the inoculation times.

In each treatment, 30 canes each (3 replicates of 10 repetitions each) were challenged 24 h, 7 d and 21 days after treatment with 50 µL of $1x10^5$ conidia/ml (5,000 spores/wound) of either *D. seriata* or *N. parvum* to determine how long the activity of GelSeal and T-77 last on the pruning wound. Positive controls included 30 non-treated but inoculated canes with 5,000 spores/wound of *D. seriata* or *N. parvum* each at 24 h, 7 d, and 21 d after pruning. In addition, 30 canes per

treatment were left non-treated/non-inoculated to serve as negative controls to determine if natural infections happened in the collected canes from the experimental vineyard. Fungal re-isolations were performed on the canes as in 2.2.5.2..

The data from each experiment was fitted to a logistic regression which is a general linear model specific for binomial data with a logit link. In order to determine the significance of the fixed effects of pathogen, time of inoculation, treatment and their interactions, a three-way analysis of deviance was performed which is approximately χ^2-distributed. This was followed by Tukey-Kramer's Honest Significant Difference comparison post-hoc test to determine if there were significant statistical differences between the means across treatments for each pathogen and within each evaluation time. All analyses were performed using R.

Table 3.1. List of pruning wound treatments used in this study.

Trade Name	Active Ingredients	Manufacturer	Application Rate	Application Method
BC-1	*T. canadenses* + *T. atroviride*	n/a	$1x10^6$ conidia/mL	Drop-Inoculation, 50 µL
BC-2	*T. canadenses* + *T. asperelloides*	n/a	$1x10^6$ conidia/mL	Drop-Inoculation, 50 µL
BC-3	*T. atroviride* + *T. asperelloides*	n/a	$1x10^6$ conidia/mL	Drop-Inoculation, 50 µL
BC-4	*T. canadense* + *T. atroviride* + *T. asperelloides*	n/a	$1x10^6$ conidia/mL	Drop-Inoculation, 50 µL
GelSeal	Tebuconazole	Omnia Specialities Australia Pty Ltd	30mL GelSeal/100L of water	Sprayed
T-77	*T. atroviride* strain 77-b	Plant Health Products (Pty) Ltd, South Africa	Manufacturer's instructions	Sprayed until run-off
Vitiseal	Acrylic Copolymer	VitiSeal International, LLC., USA	Manufacturer's instructions	Sprayed until run-off
SuRDC-1440*	*T. viticola*	n/a	$1x10^6$ conidia/mL	Sprayed until run-off
SuRDC-1442*	*T. harzianum*	n/a	$1x10^6$ conidia/mL	Sprayed until run-off

***Treatments evaluated for depth of colonization and endurance trial only**

3.2.4 Screening of *Trichoderma* species and commercial products against *D. seriata* and *N. parvum* in a Merlot vineyard

In order to evaluate and compare the *Trichoderma* treatments BC-1, BC-2, and BC-3 and commercial pruning wound protection products GelSeal, T-77, and VitiSeal, under natural climatic conditions, a field trial was conducted in an 18 year-old 'Merlot' vineyard at the Summerland Research and Development Center (SuRDC), Summerland, BC. Vines in this block were trained in a bilateral cordon, spur-pruned in a vertical shoot positioning system and vineyard maintenance practices such as fertilization, irrigation, and pest control were performed as standard for the region.

According to the dates presented in table 3.2, each year vines were pruned before bud break down to three bud canes so that the surface of the pruning wound lay flat for drop treatment with the different products and drop inoculation with pathogens via micropipette. *Trichoderma* treatments BC-1, BC-2, and BC-3 were tested in the field using 50 µL aliquots of 1×10^6 spores per mL (50,000 spores per wound). Commercial products GelSeal, T-77 and VitiSeal were used as industry standard controls and were applied according to manufacturer's instructions (Table 3.1). *Trichoderma* and commercial product treatments were applied within 3 h after pruning (Table 3.2). In the 2019 field trial, immediately following treatment, the vineyard block experienced a heavy rain and so the treatments were re-applied the next day. In 2020, vines received one treatment. Pruning wounds were then challenged 1 d, 7 d, 21 d, and 60 d post-treatment using 50 µL aliquots of 4×10^4 spore/ml suspensions (2,000 spores per wound) of either *D. seriata* or *N. parvum*. Positive controls were non-treated but inoculated with the same amount of spores of *D. seriata* or *N. parvum* 1 d, 7d, 21, d and 60 d after pruning. Negative controls were pruned, left untreated, and exposed only to natural disease pressure to determine the base levels of pathogen incidence in the field. Treatments were arranged in a randomized block design to control for variation within the vineyard. Each treatment x pathogen x timing

combination was repeated on five vines and all canes per vine were treated. Treatments were applied to the same vines from one year to the next. Five weeks after each inoculation time with the pathogen, at least 10 canes per vine were collected and stored at 4 °C until they were re-isolated as described in 2.2.6.2. Briefly, fungal re-isolations started by first shaving the bark around the pruning wound, then flame sterilizing the surface of the cane with 95% ethanol at which point ten pieces of tissue ~0.5 cm^2 were taken from the surface of the pruning wound plated on PDA amended with 1 mg/ml tetracycline (Sigma-Aldrich, St. Louis) (PDA-tet). Plates were incubated for up to 10 days at 23 °C in the dark. If a plate yielded either *D. seriata* or *N. parvum*, the corresponding cane was rated as colonized by the pathogen. Treatment efficacy was based on the mean percent recovery (MPR) of *D. seriata* and *N. parvum* from treated canes and data is presented as mean percent disease control (MPDC), which was calculated as the reduction in MPR as a proportion of the inoculated control according to the formula (100 × [1 – (MPR treatment/MPR control)]).

The data from each experiment was fitted to a logistic regression which is a general linear model specific for binomial data with a logit link. In order to determine the significance of the fixed effects of pathogen, time of inoculation, treatment and their interactions, a three-way analysis of deviance was performed which is approximately χ^2-distributed. This was followed by Tukey-Kramer's Honest Significant Difference comparison post-hoc test to determine if there were significant statistical differences between the means across treatments for each pathogen and within each evaluation time. All analyses were performed using R.

Table 3.2. Pruning, treatment, inoculation, and sample collection dates for the field trials conducted in 2019 and 2020 growing seasons.

	Pruning/Treatment Dates			Inoculation Dates				Cane Collection Dates			
Year	**Pre-Prune[a]**	**Pruned[b]**	**Treated**	**Day 1**	**Day 7**	**Day 21**	**Day 60**	**Day 1**	**Day 7**	**Day 21**	**Day 60**
2019	April 8	April 10	April 11	April 12	April 18	May 2	June 10	May 16	May 23	June 6	July 16
2020	March 12	May 6	May 7	May 8	May 14	May 28	July 9	June 16	June 19	July 3	August 10

[a] Vineyard pruned to 4 to 5 node canes (~40 cm length)
[b] Final cut made flat above the upper bud 24h before treatment

3.2.5 *Trichoderma* pruning wound distance colonization trial in a Chardonnay vineyard

The ability of two local *Trichoderma* spp. to survive long-term on grapevine pruning wounds and propagate endophytically into the grapevine pruning wounds after application was investigated in a 12 year-old experimental Chardonnay vineyard located at the SuRDC, Summerland, BC. Vines in this block were trained in a bilateral cordon, spur-pruned in a vertical shoot positioning system and vineyard maintenance practices such as fertilization, irrigation, and pest control were performed as standard for the region. On April 24th, 2019 three rows were pruned leaving four node canes and the wound surface was left flat for *Trichoderma* spp. inoculation just below the fourth node. The following day, single species conidial suspension of 1x10^6 conidia/mL of *T. atroviride* (SuRDC-1440) and *T. asperelloides* (SuRDC-1442) were applied to pruning wounds using spray bottles previously sterilized in 10% bleach overnight. Sterile distilled water amended with Tween 20 was applied to run-off as negative control. Treatments were arranged in a randomized block design with three rows spread across the block to control for variation within the vineyard. Each treatment x month of collection combination was repeated on three vines and all canes per vine were treated.

One (May 2019), two (June 2019), three (July 2019), four (August 2019), and 10 (February 2020) months post-treatment at least 10 canes from each treated vine were collected for a total of 30 canes per treatment x timing combination (90 canes per month). Collected canes were stored at 4 °C in plastic bags each containing one rep of one treatment until fungal re-isolations

were performed. To determine the depth of colonization, the method of John et al. (2008) was adapted. The bark from the entire cane was removed and 2 cm pieces were taken up to a distance of 10 cm representing five evaluation depths (0-2 cm, 2-4 cm, 4-6 cm, 6-8 cm, and 8-10 cm). These pieces were flame sterilized, quartered longitudinally, plated on PDA amended with tetracycline (0.1mg/mL), and left to incubate in the dark at 23 °C for 5-10 days. If any one of the plates yielded *Trichoderma*, that section was considered to be colonized by that fungus.

3.3 Results

3.3.1 Optimum temperature conidial germination study

Conidial germination of three *Trichoderma* spp. was evaluated after five, eight, 10, 12, and 24 hours incubation at 10, 15, 20, 25, and 30°C. The data from the original and the repeat experiment were analyzed as two separate general linear models. The effects of isolate, time, and temperature were highly significant in both experiments (Table 3.3). In both experiments, the temperature at which 50% germination was reached in the shortest time period was 30°C for all isolates, with SuRDC-1422, SuRDC-1440, and SuRDC-1442 reaching >50% germination at eight, 10, and 10 h, respectively. None of the isolates showed germination at 10°C over the 24 hour period of evaluation although the conidia of each isolate did swell in size by the 24 hour evaluation (Figure 3.1). Each isolate exhibited germination after 24 hours at 15°C incubation with a high germination rate of 87 and 91% for SuRDC-1422 and lower germination rates of 18-26% and 13-16% for SuRDC-1440 and SuRDC-1442 respectively between experiments. For SuRDC-1422, SuRDC-1440, and SuRDC-1442 there was 3-11%, 0%, and 0% germination respectively after 12 hours incubation at 20 °C, however, after 24 hours at 20 °C, all isolates reached 100% germination. At 25 °C incubation, SuRDC-1422 began germinating fastest with 1-6% germination after 8 hours reaching 100% germination at 12 hours. SuRDC-1440 and SuRDC-1442 followed a similar trend at 25 °C incubation and began germinating after 10 hours reaching 100% germination at 24 hours in both experiments (Figure 3.1).

Figure 3.1. Effect of temperature on the conidial germination *Trichoderma* spp. from grapevines from BC after 5, 8, 10, 12, and 24 hours incubation. The first and repeat experiments were analyzed separately. Means represent the percent conidial germination of at least 150 conidia and error bars represent standard error. Means within the same temperature with the same letter are not significantly different at the 0.05 level. Dotted lines represent 50% germination.

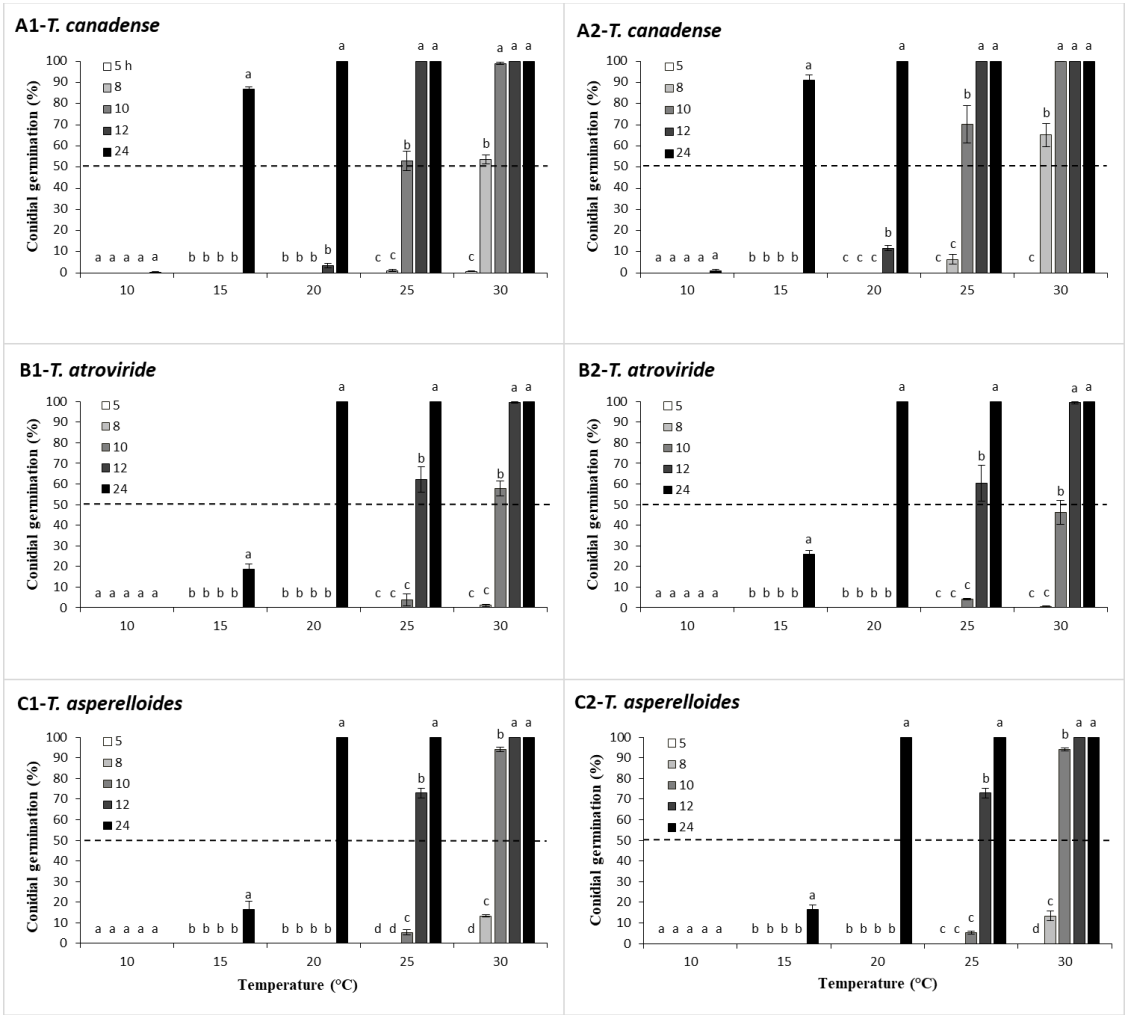


Table 3.3. Analysis of fixed effects of isolate, temperature, time and their interactions on conidial germination of three *Trichoderma* spp. using two general linear models to analyse the original and the repeat data separately.

Experiment	Effect	Num df	Deviance	Pr(<Chi)
Experiment 1	Temperature	4	4197.8	< 2.2e-16
	Time	4	8588.6	< 2.2e-16
	Isolate	2	888.4	< 2.2e-16
	Temperature*Time	16	1.5	1.0000
	Isolate*Temperature	8	60.1	4.381e-10
	Isolate*Time	8	7.0	0.5403
	Isolate*Temperature*Time	32	0.4	1.0000
Experiment 2	Temperature	4	3777.4	<2e-16
	Time	4	7708.9	<2e-16
	Isolate	2	1151.9	<2e-16
	Temperature*Time	16	5.1	0.9952
	Isolate*Temperature	8	151.2	<2e-16
	Isolate*Time	8	6.5	0.5883
	Isolate*Temperature*Time	32	1.4	1.0000

3.3.2 Detached cane assays

I-button temperature and humidity average values were determined to be 18°C and 38% RH over the duration of the experiment. The DCA, evaluating the local *Trichoderma* combinations and the commercial products GelSeal and T-77 were analyzed separately as they were conducted at different times (Table 3.4, 3.5, 3.6). There was a high recovery of *D. seriata* and *N. parum* in the non-treated but inoculated positive controls from one to 21 days post-pruning in all three DCA (Table 3.4, 3.5, 3.6). Recovery of *D. seriata* was generally lower across the experiment (53-87%) compared with *N. parvum* inoculated canes (73-100%). There was no recovery of either *D. seriata* or *N. parvum* from the negative controls in any of the three DCA. Every treatment combination (BC-1, BC-2, BC-3, BC-4) was able to achieve between 90 and 100% MPDC against *D. seriata* and *N. parvum* from one to 21 days post-treatment (Table 3.4).

The commercial *Trichoderma*-based BCA T-77 was capable of providing a high level of control against both pathogens from one to 21 days according to the Tukey-Kramer multiple

comparison test (P = 0.05). T-77 showed 84-86% MPDC against both pathogens one day post-treatment and it achieved 100% and 91% MPDC against *D. seriata* and *N. parvum* respectively 21 days post-treatment (Table 3.5). The wound sealing commercial fungicide GelSeal was able to provide 100% MPDC against *N. parvum* from one to seven days post-treatment however the control dropped to 77% at 21 days post-treatment (Table 3.6). The control of GelSeal against *D. seriata* was statistically lower when compared against *N. parvum* seven days post-treatment (P > 0.05). At one and 21 days post-treatment, the control by GelSeal against *D. seriata* was not statistically different to the control against *N. parvum* according to the Tukey-Kramer multiple comparison test (P = 0.05) (Table 3.6).

Table 3.4. Results of detached cane assays under controlled conditions at Summerland Research and Development Center. Values represent the MPR of *D. seriata* and *N. parvum* from pruning wounds treated with experimental *Trichoderma* treatments wound treatments or with a sterile distilled water control, inoculated with 2 different pathogens 1, 7, and 21 days post treatment. Values followed by same letters in each row were determined not statistically different by the Tukey-Kramer multiple comparison test (P = 0.05).

Pathogen/Day of Inoculation	Calculation	Control	BC-1	BC-2	BC-3	BC-4
D. seriata						
1 Day	MPR	70a	0b	0b	0b	0b
	MPDC[a]		100	100	100	100
7 Day	MPR	73a	0b	0b	0b	0b
	MPDC		100	100	100	100
21 Day	MPR	80a	0b	0b	0b	0b
	MPDC		100	100	100	100
N. parvum						
1 Day	MPR	87a	3b	0b	0b	7b
	MPDC		97	100	100	93
7 Day	MPR	90a	0b	0b	0b	0b
	MPDC		100	100	100	100
21 Day	MPR	100a	0b	0b	0b	0b
	MPDC		100	100	100	100

Table 3.5. Results of detached cane assays using Chardonnay grape canes under controlled greenhouse conditions at Summerland Research and Development Center. Values represent the mean percent infection of 30 pruning wounds treated with T-77 or with a water control, inoculated with two different pathogens one, seven, and 21 days post treatment. Values within the same day of inoculation followed by same letters were determined not statistically different by the Tukey-Kramer multiple comparison test (P = 0.05).

		Pathogen Inoculated	
Day of Inoculation	Calculation	*N. parvum*	*D. seriata*
1 Day	MPR Untreated Control	83a	73a
	MPR T-77 Treated	13b	10b
	MPDC	84	86
7 Day	MPR Untreated Control	93a	67b
	MPR T-77 Treated	7c	13c
	MPDC	93	80
21 Day	MPR Untreated Control	77a	53a
	MPR T-77 Treated	7b	0b
	MPDC	91	100

Table 3.6. Results of detached cane assays using Chardonnay canes under controlled greenhouse conditions at Summerland Research and Development Center. Values represent the mean percent infection of 30 pruning wounds treated with GelSeal or with a water control, inoculated with two different pathogens one, seven, and 21 days post treatment. Values within the same day of inoculation followed by same letters were determined not statistically different by the Tukey-Kramer multiple comparison test (P = 0.05).

		Pathogen Inoculated	
Day of Inoculation	Calculation	*N. parvum*	*D. seriata*
1 Day	MPR Untreated Control	97a	87a
	MPR GelSeal Treated	0b	17b
	MPDC	100	81
7 Day	MPR Untreated Control	87a	77a
	MPR GelSeal Treated	0c	33b
	MPDC	100	57
21 Day	MPR Untreated Control	73a	53ab
	MPR GelSeal Treated	17c	37bc
	MPDC	77	31

3.3.3 Screening of *Trichoderma* species and commercial products against *D. seriata* and *N. parvum* in a Merlot vineyard

The first and repeated field trials which evaluated three local *Trichoderma* treatments (BC-1, BC-2, BC-3) and three commercial pruning wound protection products GelSeal, T-77, and VitiSeal for their abilities to protect grapevine pruning wounds from infection by *D. seriata* and *N. parvum* at one, seven, 21, and 60 days post-treatment in a Merlot vineyard were analyzed separately. In the 2019 field trial, analysis of deviance found that the effect of pathogen and pathogen interacting with treatment were significant ($P < 0.05$). The individual effects of time of inoculation, treatment, and the two-way interaction of time of inoculation and treatment, and the three way interactions of pathogen, time of inoculation, and treatment were highly significant ($P < 0.001$) (Table 3.7). In the 2020 field trial, the individual effects of time of inoculation and treatment were significant ($P < 0.001$) (Table 3.7). Across both years, *Trichoderma* spp. were recovered from pruning wounds at rates of 68-100% (Table 3.8, 3.9)

As a general trend, as time between pruning and inoculation increased, MPR of both pathogens decreased in the positive controls (Table 3.8, 3.9). When treated canes were inoculated with *D. seriata* and *N. parvum* one day post-treatment and collected for re-isolation five weeks later, treatments BC-1, BC-2, BC-3, GelSeal, and T-77 provided between 75 and 100% MPDC overall for both years (Table 3.8, 3.9). VitiSeal exhibited lower control at this time of evaluation in both years for both pathogens with MPDC values between 20 and 62% (Table 3.8, 3.9). In the 2020 field trial, VitiSeal provided control against *D. seriata* that was not statistically different from treatments BC-1, BC-2, BC-3, and T-77 according to the Tukey-Kramer HSD multiple comparison test ($P = 0.05$).

When treated canes were inoculated with *D. seriata* and *N. parvum* seven days post-treatment and collected for re-isolation five weeks later, treatments BC-1, BC-2, BC-3, GelSeal, and T-77

provided over 80% MPDC overall for both years and the treatment MPR values were determined not significantly different at each time against each pathogen for both years according to the Tukey-Kramer HSD multiple comparison test (P = 0.05). In the 2019 field trial, VitiSeal provided control against *N. parvum* that was not significantly different when compared to the other treatments. However, VitiSeal control of *D. seriata* was statistically lower than the other treatments when challenged seven days post-treatment in 2019 (P = 0.05) exhibiting the lowest MPDC at 70% with MPR at 22% (Table 3.8).

When treated canes were inoculated with *D. seriata* and *N. parvum* 21 days post-treatment and collected for re-isolation five weeks later, all treatments provided over 80% MPDC overall for both years and the treatment MPR values were determined not significantly different at each time against each pathogen for both years according to the Tukey-Kramer HSD multiple comparison test (P = 0.05) (Table 3.8, 3.9).

When treated canes were inoculated with *D. seriata* and *N. parvum* 60 days post-treatment and collected for re-isolation five weeks later, treatments BC-1, BC-2, BC-3, T-77, and GelSeal provided between 68 and 100% MPDC overall for both years and the treatment MPR values were determined not significantly different at each time against each pathogen for both years according to the Tukey-Kramer HSD multiple comparison test (P = 0.05). VitiSeal provided equivalent control against both pathogens for both years except that in the 2019 field trial, MPR from VitiSeal treated vines was not statistically different from the positive control (P = 0.05) (Table 3.8).

Table 3.7. Analysis of fixed effects of pathogen inoculated, time of inoculation, pruning wound treatment, and their interactions on MPR of *D. seriata* and *N. parvum* using two general linear models to analyse the original and the repeat data separately.

Experiment	**Effect**	Num df	Deviance	Pr(<Chi)
2019 Field Trial	Pathogen	1	2083.6	0.0236393
	Time	1	1997.8	<2e-16
	Treatment	6	1249.1	<2e-16
	Pathogen*Time	1	1247	0.1495040
	Pathogen*Treatment	6	1231.2	0.0148702
	Time*Treatment	6	1203	8.73e-05
	Pathogen*Time*Treatment	6	1177.1	0.0002263
2020 Field Trial	Pathogen	1	2017.8	0.75285
	Time	1	1926.0	<2e-16
	Treatment	6	1436.8	<2e-16
	Pathogen*Time	1	1436.5	0.60560
	Pathogen*Treatment	6	1431.8	0.58987
	Time*Treatment	6	1419.5	0.05478
	Pathogen*Time*Treatment	6	1411.3	0.22262

Table 3.8. Percentage of canes of cv. Merlot yielding *Diplodia seriata* or *N. parvum* in the 2019 field trial Summerland Research and Development Center, Summerland, British Columbia, Canada. Values represent the MPR of *D. seriata* and *N. parvum* from pruning wounds treated with experimental *Trichoderma* treatments wound treatments (BC-1, BC-2, BC-3), GelSeal, T-77, VitiSeal or with no treatment, inoculated with 2 different pathogens 1, 7, 21, and 60 days post treatment. Data are means of 50 replicates and bars denote standard error. Values followed by same letters in each row were determined not statistically different by the Tukey-Kramer multiple comparison test (P = 0.05).

Pathogen/Day of Inoculation	Calculation	Control	BC-1	BC-2	BC-3	T-77	GelSeal	Vitiseal
D. seriata								
1 Day	MPR	90a	4cd	21c	0d	0d	2d	72b
	MPDC		96	77	100	100	97	20
	%*Trichoderma*		98	81	100	96		
7 Day	MPR	72a	4c	0c	0c	2c	4c	22b
	MPDC		94	100	100	97	94	70
	%*Trichoderma*		96	91	100	94		
21 Day	MPR	48a	4b	0b	0b	5b	2b	4b
	MPDC		92	100	100	90	95	92
	%*Trichoderma*		94	100	100	88		
60 Day	MPR	24a	4b	6b	0b	4b	0b	2b
	MPDC		82	75	100	83	100	91
	%*Trichoderma*		94	88	90	92		
N. parvum								
1 Day	MPR	82a	0c	4c	4c	10c	0c	34b
	MPDC		100	95	95	88	100	59
	%*Trichoderma*		100	98	100	90		
7 Day	MPR	60a	2b	4b	2b	4b	0b	14b
	MPDC		97	93	97	93	100	76
	%*Trichoderma*		100	96	94	94		
21 Day	MPR	50a	0b	0b	0b	4b	0b	4b
	MPDC		100	100	100	92	100	92
	%*Trichoderma*		100	100	100	98		
60 Day	MPR	21a	0b	2b	0b	2b	4b	9ab
	MPDC		100	90	100	90	81	59
	%*Trichoderma*		90	92	95	94		

Table 3.9. Percentage of canes of cv. Merlot yielding *Diplodia seriata* and *N. parvum* in the 2020 field trial Summerland Research and Development Center, Summerland, British Columbia, Canada. Values represent the MPR of *D. seriata* and *N. parvum* from pruning wounds treated with experimental *Trichoderma* treatments wound treatments (BC-1, BC-2, BC-3), T-77, GelSeal, Vitiseal, or with no treatment inoculated with 2 different pathogens 1, 7, 21, and 60 days post treatment. Data are means of 50 replicates and bars denote standard error. Values followed by same letters in each row were determined not statistically different by the Tukey-Kramer multiple comparison test (P = 0.05).

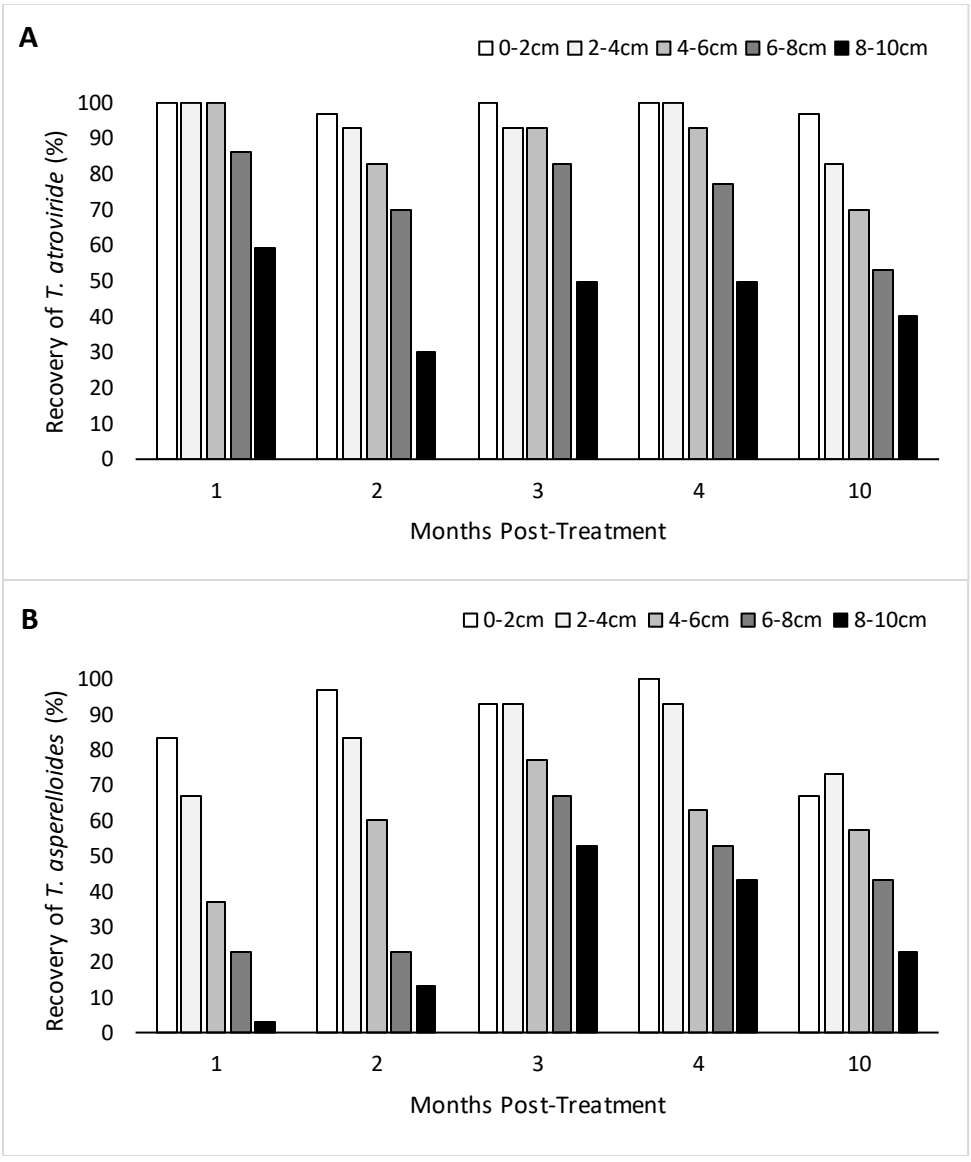
Pathogen/Day of Inoculation	Calculation	Control	BC-1	BC-2	BC-3	T-77	GelSeal	Vitiseal
D. seriata								
1 Day	MPR	68a	14bc	10bc	6bc	12bc	4c	26b
	MPDC		79	85	91	82	94	62
	%*Trichoderma*		90	86	100	84		
7 Day	MPR	56a	10b	6b	4b	8b	2b	6b
	MPDC		82	89	93	86	96	89
	%*Trichoderma*		84	84	92	88		
21 Day	MPR	40a	4b	0b	0b	6b	2b	6b
	MPDC		90	100	100	85	95	85
	%*Trichoderma*		84	90	86	82		
60 Day	MPR	20a	0b	0b	2b	0b	6b	4b
	MPDC		100	100	90	100	70	80
	%*Trichoderma*		80	80	82	74		
N. parvum								
1 Day	MPR	72a	12b	12b	2b	8b	2b	36c
	MPDC		83	83	97	89	97	50
	%*Trichoderma*		90	90	98	90		
7 Day	MPR	66a	4b	2b	6b	4b	4b	8b
	MPDC		94	97	91	94	94	88
	%*Trichoderma*		92	86	84	92		
21 Day	MPR	38a	2b	6b	4b	4b	0b	4b
	MPDC		95	84	89	89	100	89
	%*Trichoderma*		90	88	90	90		
60 Day	MPR	24a	2b	0b	0b	0b	0b	2b
	MPDC		92	100	100	100	100	92
	%*Trichoderma*		68	94	88	74		

3.3.4 *Trichoderma* pruning wound distance colonization trial in a Chardonnay vineyard

Trichoderma atroviride (SuRDC-1440) and *T. asperelloides* (SuRDC-1442) as well as a sterile distilled water negative control were applied to Chardonnay pruning wounds and the canes were collected and fungi were re-isolated at five depths, 0-2 cm, 2-4 cm, 4-6 cm, 6-8 cm, and 8-10 cm at five evaluation times including one, two, three, four, and 10 months post-treatment. The mean percent recovery for each treatment x depth x time combination was calculated and the data was graphed for each isolate (Figure 3.2) but the negative controls were not graphed and the data was not analyzed further.

Trichoderma atroviride (SuRDC-1440) was isolated from >90% of Chardonnay canes at every evaluation time and colonized up to 10 cm below the pruning wound within a month of treatment (Figure 3.2a). *T. asperelloides* (SuRDC-1442) was isolated at slightly lower rates of over 70% of canes from one to ten months pot-treatment however the depth of colonization was overall lower and it took three months to reach >50% *Trichoderma* recovery at the 8-10cm depth (Figure 3.2b). *Trichoderma* was not recovered from any of the negative control canes except for at the 0-2cm depth two and three months post-treatment at 23 and 17 % respectively. *Trichoderma* was also recovered four months post-treatment at less than 5% at depths of 0-2cm and 2-4cm.

Figure 3.2. Extent of colonization of Chardonnay canes at depths of 0-2cm, 2-4cm, 4-6cm. 6-8cm, and 8-10cm below the pruning wounds by BCAs (A) *Trichoderma atroviride* (SuRDC-1440), (B) *Trichoderma asperelloides* (SuRDC-1442), and (C) sterile distilled water amended with Tween 20 as negative control at evaluation points of one, two, three, four, and 10 months after hand-spray application on fresh pruning wounds. The x-axis denotes months post-application where one month post-application corresponds with the month of May. The y-axis denotes mean percent recovery of *Trichoderma* from canes at the different depths (n =30).



3.4 Discussion

Our objective was to characterize *Trichoderma* spp. from BC for their ability to perform as potential BCA in BC vineyards. Using both greenhouse and natural field conditions we compared their performance against commercial products and evaluated the ability of these isolates to survive long-term on grapevine pruning wounds.

In the first study, three *Trichoderma* spp. from BC were tested for their ability to germinate under different temperatures. Subsequently, the biocontrol capacity of these isolates as well as two commercial pruning wound protection products were assessed under greenhouse conditions in order to determine which treatments performed well enough to warrant in field trials at a larger scale under natural field conditions. We then evaluated the biocontrol capacity of the best performing treatments, as well as the disease control of three commercial pruning wound protection products under field conditions. Finally, the ability of these local *Trichoderma* BCAs to survive long-term on grapevine pruning wounds and propagate endophytically into the grapevine pruning wounds after application was investigated.

3.4.1 Optimum temperature conidial germination study

In order to gain insight into the temperature gradients at which these three *Trichoderma* spp. are active the three best performing isolates from chapter two were tested for their germination capacity at five degree intervals from 10-30°C on PDA. All isolates exhibited some degree of germination from 15-25 °C after 24 hours incubation and all isolates exhibited an optimum germination temperature at 30°C (the temperature at which conidial germination reached 50% in the shortest time) (Úrbez-Torres et al. 2010). Although none of the isolates germinated at 10 °C, the swelling of the conidia after 24 hours indicated they could potentially germinate at this temperature given more incubation time.

To the best of our knowledge, with respect to *T. asperelloides* and *T. canadense*, this represents the first time these *Trichoderma* spp. were evaluated for percent conidial germination with respect to temperature and time. The results for *T. atroviride* (SuRDC-1440) from our study are consistent with those from another study which evaluated the effects of relative humidity, and pH on conidial germination for a different *T. atroviride* isolate (T-15603.1) on modified low-nutrient agar at five degree intervals from 10-30°C at 3, 6, 12, 24, and 36 hours (Schubert et al. 2010). That study reported that the maximum germination rate was obtained between 25-30°C when water activity of the media was high (0.998) with an acidic pH of 5 (Schubert et al. 2010). Although on different types of media, this is in agreement with our study, where *T. atroviride* (SuRDC-1440) germinated optimally at 30°C with water activity of the PDA media left unmodulated via the replacement of water with glycerol as in the other experiment and so it was assumed to be high.

The results of our study demonstrate that all three *Trichoderma* spp. identified from grapevines in BC have the capacity to germinate within 24 hours under high humidity conditions with high nutrient availability in the dark from 15-30°C and began to activate within 24 hours at 10°C under the same conditions. In the BC disease management context, these results may suggest that these species may become biologically active within 24 hours when average temperatures reach 15°C or higher and potentially as low as 10°C although it will take longer for them to become effective. In BC, these temperatures are not reached usually until early spring, which may limit early application of these BCA. Also, judging by the evidence from other studies that indicate lower levels of germination and mycelial growth when water is limited, (Begoude et al. 2007; Schubert et al. 2010), this could inform management strategies to maintain higher levels of moisture during and immediately following treatment with *Trichoderma*-based BCA.

With respect to percent conidial germination, further studies could investigate the long-term germination and/or survival at low temperatures to identify how early Canadian growers might be able to apply them as products in the field especially in climates where pruning is performed in the colder months of the season (December to February). Future work could also optimize the production and storage of *Trichoderma* inoculum by testing for the optimum media-type, pH, temperature, light, humidity, and subsequent storage conditions in order to produce the highest quality inoculum for treatment as has been investigated for *T. atroviride* LU132 (Daryaei et al. 2020). Finally, it could be of interest to investigate conidial germination of these three promising BCA at lower water activities in order to gain more insight into what conditions are most favourable for BCA applications especially considering the dry climate of the Okanagan Valley where these BCAs may be applied.

3.4.2 Detached cane assays

The goal of these DCA experiments was to take the best performing *Trichoderma* isolates from the previous DCA performed in chapter two in order to determine if combination treatments incorporating two or three *Trichoderma* isolates could offer higher MPDC of *D. seriata* and *N. parvum*. In addition, the two separate DCA were performed using two commercial pruning wound protection products not yet registered in Canada to test their abilities to protect pruning wounds from infection by *D. seriata* and *N. parvum* in the greenhouse under controlled conditions.

This study demonstrated that even when *D. seriata* and *N. parvum* were recovered at high rates from non-treated positive controls from one to 21 days post-treatment, local *Trichoderma*-based treatments and commercial products GelSeal and T-77 were capable of providing a very high level of disease control against both pathogens, except GelSeal was less effective against *N. parvum*. It is hypothesized that these treatments may perform better if challenged with a lower,

more natural pest pressure which would be found in the field (Elena et al. 2016; Gramaje et al. 2018).

This represents the third time a DCA in the greenhouse has been used to evaluate the efficacy of grapevine endophytic *Trichoderma* spp. for the control of *D. seriata* and *N. parvum* (Úrbez-Torres et al. 2020) and is the first time evaluating combination treatments of *T. canadense, T. asperelloides,* and *T. atroviride* against these GTD causing fungi in the greenhouse. The significance of this uniquely designed DCA was discussed in chapter two (2.4.4) and therefore will not be discussed further. This study evaluated treatments that combined species of *Trichoderma* since the majority of *Trichoderma*-based BCA products on the market contain more than one species in hopes of increasing the overall robustness of our treatments (Woo et al. 2014). As remarked previously for the single isolate treatments in chapter two, in order to fully quantify the effectiveness of these combination *Trichoderma* treatments from BC, further screening is required in the field to evaluate their potential for disease control under natural environmental conditions (Harman 2000).

3.4.3 Screening of *Trichoderma* species and commercial products against *D. seriata* and *N. parvum* in a Merlot vineyard

We evaluated three local *Trichoderma* treatments (BC-1, BC-2, BC-3) and three commercial pruning wound protection products GelSeal, T-77, and VitiSeal for their ability to protect grapevine pruning wounds from infection by *D. seriata* and *N. parvum* at one, seven, 21, and 60 days post-treatment in a Merlot vineyard in Summerland, BC over two years. This represents the first study to compare the efficacy of local and commercial BCA against commercial pruning wound sealing products in Canada and is the first field study to evaluate the efficacy of pruning wounds from one to 60 days post-treatment.

Our study found that the three local *Trichoderma* treatments (BC-1, BC-2, and BC-3) were capable of providing reductions of pathogen incidence of up to 100% from one to 60 days post-treatment. In particular, BC-3 provided over 80% MPDC from one to 60 days post-treatment against both *D. seriata* and *N. parvum* over two seasons. These local treatments provided equal to or greater control than the commercial pruning wound treatments GelSeal, T-77, and VitiSeal. Specifically, GelSeal and T-77 provided greater than 80% control against both *D. seriata* and *N. parvum* over two seasons.

This is in agreement with the results from a South African study which found reductions in recovery of *D.seriata* and *N. parvum* of 80% and 85% respectively when canes treated with *T. atroviride* (USPP-T1) were compared with non-treated canes artificially inoculated seven days post-treatment (Kotze et al. 2011). This South African study evaluated the ability of these treatments to control artificial inoculations seven days post-treatment to allow the BCA treatments to colonize the wounds and become effective. Although the MPDC values are similar between our study and that of Kotze et al. (2011), the absolute reduction of MPR observed was greater in our study. This is because at seven days post treatment, our study observed higher recovery in the non-treated but inoculated controls for *D. seriata* and *N.* parvum, with MPR values of 56-72% and 60-66% respectively between the two years compared with MPR values of *D. seriata* and *N. parvum* 38% and 22% respectively (Kotze et al. 2011). This means that although the MPDC relative to the positively inoculated vines is reported as 80-85%, the absolute reductions of MPR are 32% and 18% (Kotze et al. 2011). In this regard, our results show absolute reductions in MPR of *D. seriata* and *N. parvum* of 52-72% and 58-64% at seven days post-treatment between the two years. Kotze et al. (2011) also tested T-77 for its control and observed absolute reductions in MPR of *D. seriata* and *N. parvum* 23% and 15% where as our study observed absolute reductions of 48-70% and 56-62% for each pathogen respectively. This represents the highest reductions of disease incidence of these two pathogens by

Trichoderma-based BCA on pruning wounds in the field to date. The reason for the higher recovery of *D. seriata* and *N. parvum* observed in our study could be the result of the shorter incubation time of five weeks in our study compared with the 8 month incubation period employed in the South African study which might be observing lower recoveries due to lower survival rates of the pathogens in the tissues sampled.

Our study also demonstrates that these high levels of control can be achieved using *Trichoderma*-based treatments even when treated canes are artificially inoculated just 24 hours post-treatment but also remain effective 21 and 60 days post-treatment as well. In disagreement with our results, a recent study in Spain reported that commercial *Trichoderma*-based products were not capable of providing statistically significant reductions of *D. seriata* recovery when treated vines were compared with non-treated vines artificially inoculated 24 hours post-treatment. Reasons for this disagreement are likely the result of low colonization of pruning wounds by *Trichoderma* (5-14%) compared with the high colonization of pruning wounds in our study (68-100%), which could be due to the different *Trichoderma* strains used, application rate, the climate at application, and/or different affinities by *Trichoderma* for different grapevine cultivars, as hypothesized by the authors (Martínez-Diz et al. 2020; Mutawila et al. 2011a).

The *Trichoderma*-based products used in the Spanish study, Esquive (*T. atroviride* I-1237) and Vintec (*T. atroviride* SC1) were hand-sprayed on wounds until run-off at application rates of $4x10^7$ CFU/mL and $1x10^7$ CFU/mL, respectively, which is a much higher application rate than our study considering that we treated canes with drop-inoculation with a specific volume of 50µL per wound corresponding to 50,000 conidia per wound and $1x10^6$ CFU/mL. In this case, even with conidial viability of more than 85% as reported by the authors (Martínez-Diz et al. 2020), the application rates of their study were higher compared to our study. The mean temperatures in the week after application were under 10 °C for this Spanish study as well as for our study

according to the Government of Canada weather data report for the area. This is important to note because although our isolates did not exhibit germination at 10 °C *in* vitro within 24 hours, this shows that these isolates can still protect pruning wounds when applied and challenged within 24 hours in the field by artificial inoculation below 10 °C. This same study from Spain found high levels of control of *D. seriata* when canes were treated with a wound sealing paste combined with tebuconazole and were artificially inoculated with *D. seriata* 24 hours post-treatment (Martínez-Diz et al. 2020) which is in agreement with our findings for GelSeal.

When comparing GelSeal with VitiSeal in our study, it was found that when canes were challenged 24 hours post-treatment, GelSeal consistently provided higher control than VitiSeal treatments against both pathogens over both years tested however at seven, 21 and 60 days the control provided by VitiSeal increased. This could be the result of insufficient setting of the VitiSeal wound sealant at 24 hours post-treatment. Therefore the discrepancy between GelSeal and VitiSeal protection 24 hours post-treatment could therefore be due differences in sealant formulation or could be the result of the amended tebuconazole fungicide which might be responsible for the immediate protection as the sealant sets and becomes effective.

The results of our study demonstrate that local and commercial *Trichoderma*-based products as well as commercial pruning wound sealants can provide high control of two economically important GTD pathogens in the field and provides the first field data in Canada to support the registration grapevine pruning wound protectants. There was similar control provided by *Trichoderma*-based treatments and GelSeal over the 60 day evaluation period. Although GelSeal and *Trichoderma*-based treatments are both amenable to the economic application method of tractor driven sprayers (Gramaje et al. 2018) GelSeal may only be applied during dormancy before bud-break and requires that workers not enter the vineyard for 24 hours post-application (GelSeal product label) whereas *Trichoderma*-based treatments may be applied to

the whole vine even after bud-break although further studies would be required to confirm the effectiveness of this method of application with these specific BCA treatments in this region.

Our study demonstrates that T-77, based on a South African strain of *T. atroviride* was capable of providing control of *D. seriata* and *N. parvum* that was comparable to the control provided by combination treatments based on local isolates of three different *Trichoderma* spp. and that all the *Trichoderma*-based BCAs tested are capable of long-term protection of Merlot pruning wounds. The hypothesis that locally isolated *Trichoderma* spp. may offer higher control based on acclimatization was therefore determined to be false in the case of T-77 compared with locally screened isolates and found instead that the screening process was the most relevant factor in determining which *Trichoderma* strains can act as effective BCA in the Okanagan. As a result, our study identified four *Trichoderma*-based BCA treatments that are highly effective in protecting grapevine pruning wounds from infection by *D. seriata* and *N. parvum* from one to 60 days post-treatment.

3.4.4 *Trichoderma* pruning wound distance colonization trial in a Chardonnay vineyard

The ability of two local *Trichoderma* isolates to survive long-term on grapevine pruning wounds and colonize endophytically into the grapevine cane after application was tested in the field. Overall we found both isolates tested were highly capable pruning wound colonizers, surviving on the surfaces of the pruning wounds and at distances below the pruning wound of up to 10 cm from one to ten months post-treatment under field conditions.

Application by hand-sprayer of *T. atroviride* (SuRDC-1440) and *T. asperelloides* (SuRDC-1442) resulted in a high recovery of *Trichoderma* from pruning wounds up to 10 months post-treatment and colonization of the canes up to 10 cm below the pruning wound. These results are similar to a study performed by John et al. (2008) where they found that applying Trichoseal (*T.*

atroviride), *T. harzianum* AG1 as a conidial suspension (10^9 conidia per ml), or *T. harzianum* AG1 in Trichoseal nutrient base, led to a high degree of colonization of pruning wounds from 0-10cm below the pruning wound after three months incubation in the field. It is important to note differences in conidial concentrations between the two experiments (10^6 conidia per mL vs. 10^9 conidia per mL) as well as the fact that in the John et al. (2008) experiment, canes were also artificially inoculated with mycelial plugs of *E. lata* two and seven days post-treatment and the experiment was performed on Shiraz vine cuttings in the glasshouse where as our study was performed on Chardonnay vines in the field under natural conditions.

Our study is unique in that we followed persistence of our BCA over 10 months. Such long-term evaluation is critical for understanding the endophytic nature of these BCA with respect to depth and time will enable growers to apply these treatments only when necessary, possibly once per year or less if applied at the appropriate time of the year and immediately after pruning as demonstrated in this experiment. In this study, grapevine canes were pruned artificially long just below the fourth bud to allow re-isolation of *Trichoderma* up to 10 cm though canes are normally pruned much shorter to a two bud spur. This indicates that under standard viticulture conditions, these BCA may be capable of colonizing down into the spur and possibly into the cordon of the vine within few months of application.

We were able to recover *Trichoderma* in the negative controls from the surfaces of the pruning wounds. This may be due to aerosolization of *Trichoderma* inoculum by the hand-sprayer compared with targeted drop inoculation but could also be the result of dispersal from treated to non-treated pruning wounds via wind and rain. While this study showed rapid and deep colonization of Chardonnay vines in the field using local *Trichoderma* isolates, we were unable to determine the nature of the relationship with host. Future studies could investigate the long-term health and productivity of treated vines with respect to non-treated vines in order to ensure

a mutually symbiotic relationship exists between the *Trichoderma* isolates and the grapevines. For example, a previous study has shown that applications of *Trichoderma* either as foliar sprays or root drenches can significantly improve the yield and quality of grapes (Pascale et al. 2017). A similar study could be performed with these isolates and could investigate several modes of application including tractor-driven sprayer and targeted hand-sprayers.

Our results demonstrate that both *Trichoderma* spp. are capable of long-term colonization of Chardonnay pruning wounds under field conditions and are capable of rapidly propagating endophytically down into the vines.

Chapter 4: Conclusion

This study aimed to elucidate the potential of *Trichoderma* spp. collected from grapevines in the Okanagan Valley in BC to be used as biocontrol agents against grapevine trunk diseases. Therefore, the objectives of this study were 1) to identify and characterize *Trichoderma* spp. collected from grapevines in the Okanagan Valley by means of morphological, biological, and molecular studies, and 2) to assess the potential of *Trichoderma* spp. as biocontrol agents against locally relevant Botryosphaeria dieback pathogens under controlled greenhouse and natural field conditions.

4.1 Identify and characterize Trichoderma species collected from vineyards in the Okanagan Valley by means of morphological, biological, and molecular studies

Through multi-locus phylogenetic analysis, this study identified seven *Trichoderma* spp. isolated from grapevines in BC, including *T. asperelloides, T. atroviride, T. harzianum, T. koningii, T. tomentosum* and two novel to the scientific community named *T. canadense* and *T. viticola* described here for the first time. This is the first report of *T. asperelloides* in Canada and its first report from grapevines worldwide. This study also expands the host distribution of *T. atroviride*, *T. harzianum*, *T. koningii*, and *T. tomentosum* and reports these species for the first time from grapevines in Canada. In addition, to the best of our knowledge this is the first report of *T. tomentosum* from grapevines worldwide. The six *Trichoderma* spp. that survived best in culture were characterized by studying their optimum temperature for mycelial growth as well as their biocontrol capacity against *D. seriata* and *N. parvum* via DCAA. The best performing isolates of *Trichoderma* spp. tested in the DCAA were then further screened in the greenhouse under controlled conditions before testing in the field in order to focus time and resources.

The three best performing isolates belonging to *T. asperelloides* (SuRDC-1442), *T. atrovoride* (SuRDC-1440), *T. canadense* (SuRDC-1422), were tested for the ability of their conidia to germinate across a temperature range relevant to the climate of the Okanagan Valley. It was

found optimum germination temperature for all three *Trichoderma* spp. to be at <90% RH and 30°C with high levels of germination observed within 24 hours at 15°C. This represents the first time conidial germination has been evaluated against temperature and time for *T. asperelloides* and *T. canadense* and the results from *T. atroviride* agree with a similar study that evaluated a different strain of the same species (Schubert et al. 2010). Future characterization should investigate the ability of these *Trichoderma* spp. to germinate under lower temperatures for longer time periods to help identify the earliest point in the season at which these BCA may be effective when applied.

4.2 Assessment of potential Trichoderma species as biocontrol agents against Botryosphaeria dieback fungi under controlled greenhouse and natural field conditions

4.2.1 Detached cane assays under greenhouse conditions

The DCA performed under greenhouse conditions served as a high-through-put and low-cost intermediate test for the control of Botryosphaeria Dieback pathogens *D. seriata* and *N. parvum*. Unlike the *in vitro* DCAA, the DCA are more biologically relevant to the control of Botryosphaeria Dieback on grapevine pruning wounds which successfully helped to identify the *Trichoderma* spp. treatments that were the most effective *in vivo* and not only *in vitro* before investing more time and resources evaluating them on a larger scale in the field on mature vines. These DCA also helped to test commercial products for their suitability in protecting pruning wounds from *D. seriata* and *N. parvum* before they were used in the comparative field trial. This represents the second time DCA have been implemented to test grapevine pruning wound products for the control of *D.seriata* and *N. parvum* with respect to time from one to 21 days post-treatment (Úrbez-Torres et al. 2020) and is the first study to evaluate *T. koningii* and *T. asperelloides* against these GTD in the greenhouse. These DCA helped to identify three locally developed treatments based on dual combinations of *T. asperelloides* (SuRDC-1442), *T. atroviride*, and *T. canadense* (SuRDC-1422) as well as two commercial products GelSeal and T-77 that were deemed suitable for comparative testing in the field.

4.2.2 Field evaluation of *Trichoderma* species

4.2.2.1 Screening of *Trichoderma* species and commercial products against *D. seriata* and *N. parvum* in a Merlot vineyard

In order to assess the best performing *Trichoderma*-based pruning wound treatments identified in the DCA experiments, they were tested in the field and compared with commercial pruning wound sealant treatments for their efficacy in controlling *D. seriata* and *N. parvum* from one to 60 days post-treatment. We identified four *Trichoderma*-based treatments and one fungicide amended wound sealing agent not yet registered in Canada that provided high control of Botryosphaeria Dieback pathogens in the field from one to 60 days post treatment over two growing seasons. This represents the first study to observe these levels of control in the field using *Trichoderma*-based BCA treatments against these particular GTD pathogens after just 24 hours post-treatment and up to 60 days post-treatment. This also represents the first study in Canada to evaluate *Trichoderma*-based BCA for the treatment of GTD in th field.

The results of this study provide the industry with pragmatic solutions to prevent Botryosphaeria Dieback infection with demonstrated effectiveness in high disease pressure scenarios. Our results suggest that application of BC-1, BC-2, BC-3, T-77, or GelSeal immediately after pruning in the months of March and April (before bud-break) can significantly reduce infections by Botryosphaeria Dieback pathogens, *D. seriata* and *N. parvum*, from one to 60 days post-treatment which covers the period of highest susceptibility. Under natural disease pressure, it is hypothesized that these treatments would provide even higher control (Gramaje et al. 2018). Future studies could investigate the control provided by these treatments using tractor driven sprayers and could expand the understanding of these products by testing them on different grapevine cultivars and with other GTD pathogens to identify the breadth of the control provided by these treatments in other locally relevant infection scenarios.

4.2.2.2 *Trichoderma* pruning wound distance colonization trial in a Chardonnay vineyard

This study offers preliminary results indicating that two of the best performing *Trichoderma* spp. isolates are capable of propagating endophytically into the pruning wounds of Chardonnay canes on mature vines in the field and are able to survive long-term through the Winter season. This represents the first study to evaluate the colonization depth of *Trichoderma* spp. in grapevine canes with respect to time. Future work in this area should repeat this design to increase the reliability of this single year trial and could include more long-term study to evaluate the limits of how extensively grapevines may be colonized by these *Trichoderma* spp..

4.3 A Future Promising for Grapevines and Grim for GTD

The objectives of this research were successfully completed. Seven *Trichoderma* spp. were identified and characterized and locally isolated *Trichoderma* spp. were shown to exhibit very high control of Botryosphaeria Dieback pathogens, *D. seriata* and *N. parvum*, from one to 60 days post treatment in the field. These BCA treatments performed as well or better than chemical and BCA commercial pruning wound protectants. The success of these locally developed *Trichoderma*-based BCA treatments is attributable to the multi-level screening process which identified successful isolates, first *in vitro* via DCAA, followed by DCA under controlled conditions to identify the three isolates that were also successful *in plantae*, before the final confirmative challenge in the field. Since there are currently no products, chemical or BCA, registered for the control of GTD on grapevine pruning wounds in Canada, this study provides the BC industry the initial fundamental research required to register pruning wound products with demonstrated effectiveness against two GTD pathogens relevant to the area. Future work should concentrate on evaluating the effects of these BCA on long-term vine health and determine the effects on fruit yield and quality to ensure that these treatments are beneficial to the whole plant system before work is done to register them as BCA for agricultural use.

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