Exploring the biocontrol potential of *Streptomyces* isolates against *Pythium violae*, a dominant carrot pathogen

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Exploring the biocontrol potential of Streptomyces isolates against Pythium violae, a dominant carrot pathogen

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# Table of Contents

**Chapter 1 – Introduction** .................................................................................................................................................. 1

  - Carrot production in the USA and Kern County, California ............................................................. 1
  - Cavity spot disease of carrots .............................................................................................................. 1
  - Current control of cavity spot disease ............................................................................................... 4
  - *Streptomyces*, an unusual bacterial genus known for its production of antimicrobial compounds .......................................................................................................................... 6

**Literature Cited** ......................................................................................................................................................... 9

**Chapter 2 - Exploring the biocontrol potential of *Streptomyces* isolates against *Pythium violae*, a dominant carrot pathogen** .................................................................................................................................................. 13

  - Abstract ...................................................................................................................................................... 13
  - Introduction ............................................................................................................................................... 14

**Materials and Methods** ............................................................................................................................................... 17

  - Isolation of oomycetes from carrot cavity spot lesions ................................................................ 17
  - Genetic characterization of isolated oomycetes ........................................................................... 18
    - Genomic DNA extraction ................................................................................................................. 18
    - Amplification, sequencing, and analysis of genomic fingerprint regions .................................. 18
  - Maintenance and long-term storage of *Pythium* species ............................................................ 20
  - Pathogenicity test fulfilling Koch’s postulates .......................................................................... 21
    - Root inoculation test ....................................................................................................................... 21
    - Soil assay ........................................................................................................................................ 22
  - Metalaxyl test ....................................................................................................................................... 23
  - Antibiosis assays .............................................................................................................................. 23
Genetic characterization of *Streptomyces* inhibitors ................................................................. 25

Genomic DNA extraction ........................................................................................................... 25

Amplification, sequencing, and analysis of genomic fingerprint region .................................. 26

Multilocus Sequence Analysis (MLSA) .................................................................................... 26

*Streptomyces* against *Streptomyces* challenge assays ....................................................... 27

Results and Discussion .......................................................................................................... 28

Three *Pythium* strains isolated from diseased carrots ........................................................ 28

*Pythium* survives long-term storage with rye grains and hemp seeds ................................ 29

Koch’s postulates confirm *P. violae* pathogenicity ................................................................. 30

Metalaxyl inhibits the growth of *P. violae* ............................................................................ 32

Four *Streptomyces* strains display a strong antagonistic effect against *P. violae* and other pathogens .......................................................................................................................................................... 33

*Streptomyces* inhibitors interspecific interaction ................................................................ 36

Conclusion .................................................................................................................................. 37

Literature Cited .......................................................................................................................... 38

Chapter 3 - *Pythium spinosum*, a potential new carrot cavity spot pathogen ..................... 65

Abstract ...................................................................................................................................... 65

Introduction ............................................................................................................................... 66

Materials and Methods ............................................................................................................ 67

Isolation of cavity spot pathogens ........................................................................................... 67

Genetic characterization of isolated pathogens ...................................................................... 68

Genomic DNA extraction ........................................................................................................ 68
Amplification, sequencing, and analysis of genomic fingerprint regions .................. 68

Pathogenicity test fulfilling Koch’s postulates .............................................................. 70

Root inoculation test ................................................................................................... 70

Soil assay ..................................................................................................................... 71

Metalaxyl test .............................................................................................................. 72

Results and Discussion ................................................................................................ 73

*Pythium spinosum* was isolated from carrot cavity spot lesions .............................. 73

Koch’s postulates confirms *P. spinosum* pathogenicity ......................................... 74

Metalaxyl inhibits the growth of *P. spinosum* ....................................................... 75

Conclusion ................................................................................................................... 76

Literature Cited ............................................................................................................ 78

**Chapter 4 - Conclusion** ......................................................................................... 86

Literature Cited ............................................................................................................ 89
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Abstract

The United States is among the largest carrot (*Daucus carota*) producing countries in the world and California ranks first with a production of over 85% of all carrots grown in the US. Carrot cavity spot is a common disease of carrots worldwide, which reduces the carrot quality causing significant economic losses for the growers. In Kern County, California, the disease is mainly caused by the fungal-like, plant pathogenic oomycete, *Pythium violae*. The disease is difficult to manage because current chemical control is becoming unreliable due to microbial degradation and increased resistance of the pathogens. Hence, a biocontrol organism that naturally inhibits the pathogen would provide a solution and reduce the need for harmful anti-oomycete chemicals. *Streptomyces* is one of the dominant bacterial genera in soil with over 500 species. It is renowned for the production of an exceptional arsenal of natural products with important agricultural, medical, and biotechnological applications, including antimicrobial products.

Oomycetes were isolated from typical cavity spot lesions on diseased carrot tissue collected from the Bakersfield area and were identified via amplification and sequence analysis of two fingerprint regions coding for the cytochrome C oxidase subunit 2 (*cox2*) and the internal transcribed spacer (ITS). Pathogenicity of the isolated strains was confirmed through a root inoculation test and a soil assay. In addition to isolating *P. violae* strains, two *P. spinosum* strains were shown to cause cavity spot lesions. This is the first time that this *Pythium* species is linked to carrot cavity spot.

Out of a collection of 153 previously isolated *Streptomyces* strains obtained from diverse soils in Kern County, CA four isolates were obtained that were able to strongly inhibit the growth of *P. violae* strains *in vitro* indicating their potential as biocontrol agents. These strong inhibitors were identified as *Streptomyces spectabilis, Streptomyces chartreusis, Streptomyces cavourensis,* and either *Streptomyces taurus* or *Streptomyces hawaiiensis* via amplification and multilocus sequence
analysis of five housekeeping genes including 16S rRNA, *atpD*, *recA*, *rpoB*, and *trpB*. The target range of these *Streptomyces* species was explored by testing them against other isolated oomycete and fungal pathogens including *Pythium spinosum*, *Phytophthora helicoides*, *Fusarium oxysporum*, *Fusarium falciforme*, *Fusarium solani*, *Sclerotium rolfsii*, and *Sclerotinia sclerotiorum*. These species showed a strong antagonistic effect against most of these pathogens. The selected strong *Streptomyces* inhibitors will enable us to put forward a sustainable approach for control of cavity spot as well as other important carrot diseases.

**Key words:** *Pythium violae*, *Streptomyces*, isolation, identification, pathogenicity, antibiosis, biocontrol
List of Tables

Chapter 2

Table 2.1. Primers used in this study ................................................................. 44

Table 2.2. Identification of *Streptomyces* strain GM18-46 through MLSA based on top 3 hits obtained from BLAST results .................................................................................. 45

Table 2.3. Identification of *Streptomyces* strain GM18-72 through MLSA based on top 3 hits obtained from BLAST results .................................................................................. 46

Table 2.4. Identification of *Streptomyces* strain GM18-83 through MLSA based on top 3 hits obtained from BLAST results .................................................................................. 47

Table 2.5. Identification of *Streptomyces* strain GM18-150 through MLSA based on top 3 hits obtained from BLAST results .................................................................................. 48

Chapter 3

Table 3.1. Primers used in this study ........................................................................ 81
List of Figures

Chapter 2

Figure 2.1. Lesions on freshly harvested carrots used for the isolation of cavity spot pathogens ................................................................. 49

Figure 2.2. Gel electrophoresis confirming the successful amplification of the cox2 gene (581 bp) and the ITS region (949 bp) of an isolated oomycete ................................................................. 50

Figure 2.3. Subcultured oomycete identified as Pythium violae on CMA plate showing actively growing mycelium. This picture was taken at 5 dpi ................................................................. 51

Figure 2.4. Pythium violae stored for long term storage at both room temperature (23°C) and 18°C under three different conditions: a) sterile deionized water, b) sterile deionized water containing sterilized rye grains, and c) sterile deionized water containing sterilized hemp seeds ................................................................. 52

Figure 2.5. Root inoculation test confirming the pathogenicity of the P. violae strains: a) negative control (CMA), b) positive control (P. violae strain from WSU), and c) locally isolated P. violae strain. Results were scored at 5 dpi ................................................................. 53

Figure 2.6. Soil assay confirming the pathogenicity of the P. violae strains as shown by the typical cavity spot lesions that formed on the carrot tissue (indicated by the arrows): a) negative control (non-inoculated), b) positive control (P. violae strain from WSU), and c) locally isolated P. violae strain. The severe lesions resembling root rot on top part of the carrots were caused by a combination of P. violae and a contamination of a pathogenic fungus, Fusarium ................................................................. 54

Figure 2.7. Sensitivity of the locally isolated P. violae strain to metalaxyl (Ridomil Gold 480 SL): a) P. violae growth on CMA with no metalaxyl added and b) P. violae growth on CMA plate containing 3 μl of metalaxyl. Pictures were taken at 4 dpi ................................................................. 55

Figure 2.8. Sensitivity of the P. violae strain from WSU to metalaxyl (Ridomil Gold 480 SL): a) P. violae growth on CMA with no metalaxyl added and b) P. violae growth on CMA plate containing 3 μl of metalaxyl. Pictures were taken at 4 dpi ................................................................. 56

Figure 2.9. Key to score the strength of the antibiosis of Streptomyces strains toward Pythium violae. The strength of antibiosis is determined by the size of the inhibition zone separating the Pythium mycelium from the Streptomyces colony ........................................ 57

Figure 2.10. Antibiosis assays showing the final selection of four strong Streptomyces strains (GM18-46, GM18-72, GM18-83, and GM18-150) that strongly inhibited the growth of a) locally isolated P. violae strain and b) a P. violae strain obtained from WSU.
Pictures were taken at 5 dpi of *P. violae* on plates that were already inoculated with *Streptomyces* strains 10 days before ........................................ 58

Figure 2.11. Phenotypes of locally isolated oomycete (on CMA) and fungal (on SabDex) pathogens at 5 dpi including a) *Pythium spinosum*, b) *Phytophthymus helicoides*, c) *Fusarium falciforme*, d) *Fusarium solani*, e) *Fusarium oxysporum*, f) *Sclerotium rolfsii*, and g) *Sclerotinia sclerotiorum* .................................................. 59

Figure 2.12. Antibiosis assays showing the inhibitory effect of strong *Streptomyces* inhibitors (GM18-46, GM18-72, GM18-83, and GM18-150) against the locally isolated oomycete pathogens: a) *Pythium spinosum* and b) *Phytophthymus helicoides*. Pictures were taken at 5 dpi of the oomycete pathogens on plates that were already inoculated with *Streptomyces* strains 10 days before ........................................ 60

Figure 2.13. Antibiosis assays showing the inhibitory effect of strong *Streptomyces* inhibitors (GM18-46, GM18-72, GM18-83, and GM18-150) against the locally isolated fungal pathogens: a) *Fusarium falciforme*, b) *Fusarium oxysporum*, c) *Fusarium solani*, d) *Sclerotium rolfsii*, and e) *Sclerotinia sclerotiorum* .................................................. 61

Figure 2.14. Colony characteristic of the strong *Streptomyces* inhibitors (GM18-46, GM18-72, GM18-83, and GM18-150) shown on three different media (PMA, ISP-4, and CMA) at 7 dpi ................................................................. 62

Figure 2.15. Antibiosis assays showing the inhibitory effect of the *Streptomyces* inhibitors against each other on a) CMA and b) PMA. The *Streptomyces* inhibitors were streaked together on the plates and their growth was evaluated at 7 dpi ......................... 63

Figure 2.16. Antibiosis assays showing the inhibitory effect of the *Streptomyces* inhibitors against each other on a) CMA and b) PMA. Each *Streptomyces* inhibitor was streaked in the middle of the plate. Ten days later, the other three inhibitors were streaked perpendicular and close to the middle streak. Their growth was determined at 4 dpi .................................................. 64

Chapter 3

Figure 3.1. Subcultured oomycete identified as *Pythium spinosum* (isolated from the Bakersfield area) on CMA plate showing actively growing mycelium (5 dpi). The *P. spinosum* strain isolated from the Riverside area looked similar on CMA plate .................. 82

Figure 3.2. Root inoculation test confirming the pathogenicity of the *P. spinosum* strains: a) negative control (CMA), b) positive control (locally isolated *P. violae* strain), c) *P. spinosum* strain isolated from Bakersfield area, and d) *P. spinosum* strain isolated from Riverside area. Results were scored at 5 dpi .............................................................. 83
Figure 3.3. Soil assay confirming the pathogenicity of the *P. spinosum* strains a) negative control (non-inoculated) b) *P. spinosum* strain isolated from Bakersfield area, c) *P. spinosum* strain isolated from Riverside area ................................................................. 84

Figure 3.4. Sensitivity of the *P. spinosum* strain isolated from the Bakersfield area towards metalaxyl (Ridomil Gold 480 SL): a) *P. spinosum* growth on CMA with no metalaxyl added and b) *P. spinosum* growth on CMA plate containing 3 µl of metalaxyl (4 dpi). The *P. spinosum* strain isolated from the Riverside area had the similar results ....... 85

Appendix A. Multiple sequence alignment of the *Pythium* species DNA sequences ............... 91
Chapter 1

Introduction

Carrot production in the USA and Kern County, California

The United States of America is among the largest carrot (*Daucus carota*) producing countries in the world. Within the U.S., the highest carrot producing states include California, Texas, Washington, Michigan, Florida, Colorado, and Wisconsin. California ranks first with a production of over 85% of all carrots grown in the United States. Carrots are grown year-round in four main production areas of California: the southern San Joaquin Valley and the Cuyama Valley; the southern desert; the high desert; and the central coast (Nunez et al. 2008) and exported to the rest of the country as well as abroad.

Among all the major carrot production areas in California, Kern County (San Joaquin Valley) is known to have 60% of the overall production. Kern County is well known for growing a wide variety of agricultural crops ranging from tree nuts and fruits to a tremendous assortment of vegetables with carrots as the main commodity. Carrots are planted from December to March to be harvested from May to July, and from July to September to be harvested from November to February (Nunez et al. 2008) and in 2019 production accounted for $336,151,000, ranking carrots as the sixth most important commodity produced after grapes, almonds, pistachios, citrus, and milk (2019 Kern County Agricultural Crop Report).

Cavity spot disease of carrots

Carrots can be affected by a variety of diseases that reduces both their yield and market value (Davis and Nunez 2007). Several of these diseases are related to seedborne pathogens which are found worldwide, such as Alternaria leaf blight and bacterial leaf blight that can affect up to 100%
of the acreage in a particular region where such carrot seeds are sown. Other important carrot
diseases include root knot caused by nematodes leading to forking and stubbing of the roots and
gall formation on the taproot and secondary roots, bacterial soft rot caused by *Erwinia carotovora*,
and root dieback caused by *Pythium* species leading to forking and stubbing of roots.

Cavity spot of carrots is a soilborne disease caused by certain *Pythium* species (Davis and
Nunez 2007). In California and Washington, cavity spot is of particular concern because this
chronic disease affects about 50% of the overall acreage and can cause complete crop losses in an
area. It is particularly difficult to manage because it usually goes unnoticed until the quality of the
crops is evaluated at harvest (Davis and Nunez 2007).

At first, *Pythium* species were classified as fungi based on their filamentous growth, but
were later classified as oomycetes, a phylogenetic lineage of fungus-like eukaryotic
microorganisms. *Pythium* species are mostly found in cultivated soils with the capability of
surviving in soil and plant debris for years. These micro-organisms contain cell walls composed
of cellulose, have coenocytic hyphae, and produce zoospores in their sporangia with two types of
flagella that provide motility in water (Schroeder *et al.* 2013). Many *Pythium* species are plant
pathogens that generally attack immature tissue of different plant parts, mainly root tips. They
have a broad host range and cause many different diseases, such as damping-off, stem rot, root rot,
root dieback, and cavity spot (Davis and Nunez 2007, Schroeder *et al.* 2013).

Cavity spot was first described in 1961 by Guba *et al.* on carrot and parsnip roots in
Massachusetts, USA. Since then, the disease has been spotted in almost all carrot producing
regions around the world (Hiltunen and White 2002). First, the disease was thought to be a
physiological disorder since there was no success in finding a causal organism (Guba *et al.* 1961).
A significant breakthrough was finally made in 1984, during a greenhouse study, when it was
discovered that cavity spot could be reduced with the application of one of three different fungicides including metalaxyl, fosetyl-Al, and propamocarb known to control oomycetous fungi (Hiltunen and White 2002). Soon after, it was confirmed by Groom and Perry (1985) that the disease was indeed caused by members of the phylum of Oomycota, or water molds, and more specifically a diverse range of *Pythium* species which varied according to the geographical area. Some of these species isolated from different areas around the world include *P. violae*, *P. sulcatum*, *P. ultimum*, *P. irregulare*, *P. cryptirregulare*, *P. dissotocum*, *P. coloratum*, *P. sylvaticum*, *P. aphanidermatum*, *P. intermedium*, and *P. rostratum* (Allain-Boulé *et al.* 2004). Even though many different species of *Pythium* have been reported to cause cavity spot, it has been determined that the slower growing species *P. violae* and *P. sulcatum* are the main causal agents (Vivoda *et al.* 1991, White 1988).

As the cavity spot pathogens infect the carrot roots, symptoms characterized by the appearance of small sunken, brown-black, circular to elliptical or irregularly shaped lesions are formed (Vivoda *et al.* 1991, Hiltunen and White 2002). The lesions darken over time rupturing the periderm and extending rapidly over time, both sideways and inwards of the surface infecting the carrot tissue, but no more than 10-12 cell layers deep (White 2003). The open cavity exposes the underneath carrot tissue leaving it vulnerable to secondary infections (Hiltunen and White 2002). *Pythium* spp. have been observed to easily penetrate the carrot tissue both intercellularly and intracellularly causing a severe tissue damage due to the production of a wide range of destructive enzymes including cellulase, pectin lyase, pectate lyase, polygalacturonase, and pectin methylesterase (Zamski and Peretz 1996). Hence, the lesions are a result of a fracture between two layers of the collapsed cells (Cooper *et al.* 2004). *P. violae* penetrates the tissue and can grow for about 3-4 days before the host recognizes the invasion (Zamski and Peretz 1995, Cooper *et al.*
2004). The plant cells around the infection site produce lignin and other phenols to stop the pathogens from spreading (Zamski and Peretz 1996). Overall, the cavity spot disease does not necessarily reduce crop yield, but carrots with even one or two visible lesions reduce their marketability as they are rejected for both the fresh as well as the processing market (Hiltunen and White 2002, Davis and Nunez 2007).

**Current control of cavity spot disease**

Several different methods are applied to control and reduce the disease incidence, ranging from specific cultural practices such as regularly monitoring the crop and adjusting irrigation schedules to applying certain fungicides and biocontrol agents (White 2003, Davis and Nunez 2007). It is recommended that fields with a history of cavity spot must be avoided for future carrot production. Additionally, carrots should be grown on raised beds to prevent excess moisture retention in the soil favoring the reproduction and spread of pathogenic *Pythium* sp. (Allain-Boulé *et al.* 2004, Davis and Nunez 2007).

Application of fungicides before and after planting carrots have shown to reduce the disease (Nunez *et al.* 2008). The most common fungicide that has been routinely used is metalaxyl (generally sold as Ridomil, Syngenta Crop Protection), which is also used for control of other diseases caused by oomycetes (Saude *et al.* 2014). At first, metalaxyl was quite efficient in controlling the disease; however, its consistency started lacking over time (Hiltunen and White 2002, White 2003, Allain-Boulé *et al.* 2004). Metalaxyl has been reported to fail to control the disease in organic soils due to binding of the active ingredient to organic matter leading to a reduction of its efficacy (White 2003). Metalaxyl loses persistence in soil and becomes less effective through microbial degradation. Moreover, several strains have developed resistance to
the fungicide (Davison and McKay 1999, Hiltunen and White 2002). Even though metalaxyl is currently not as effective as it has been in the past, it is still the standard fungicide used to control cavity spot disease (Joe Nunez, personal communication). Good results are only obtained when used on sites where either it has never been used before or where it is not as frequently used (Davison and McKay 1999). Besides metalaxyl, there are some other fungicides, such as mefenoxam, an isomer of metalaxyl, fosetyl-Al, propamocarb, and phosphorous acid that are currently being used for control of cavity spot disease and other plant diseases caused by common oomycetes. Similar to metalaxyl, these fungicides are also becoming unreliable over time with inconsistent results (White 2003).

In addition to the application of fungicides, biological agents are also used to control the disease. For several years now, microbial antagonists have been used as biological agents to control crop diseases caused by soilborne and airborne bacterial and fungal pathogens. This approach reduces the traditional disease control methods using harmful fungicides, pesticides, and insecticides (Olanrewaju and Babalola 2018; Vurukonda et al. 2018). Some biocontrol methods have been tested to control cavity spot, but none of these biocontrol agents have been commercialized. For example, the plant growth-promoting bacteria *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Serratia proteamaculans* have been studied to be used as seed treatments against cavity spot pathogens but with varying results based on organism and cultivar (White 2003). Several soil inhabiting actinomycetes, isolated from carrot rhizosphere, have been successfully tested both *in vitro* and *in vivo* against *Pythium coloratum*, one of the cavity spot pathogens (El-Tarabily et al. 1997). Along with certain bacteria, the oomycete *Pythium oligandrum*, a non-pathogenic oomycete, has also shown antagonism against soilborne pathogens, including pathogenic *Pythium* species, through mycoparasitism and production of antimicrobial
compounds. While colonizing the rhizosphere of plants, a *Pythium oligandrum* strain was shown to reduce *Pythium dissotocum*, one of the causal agents of cavity spot disease (Vallance *et al.* 2009).

Yet, even though several microbial agents showed the ability to inhibit one or more of the cavity spot pathogens, they are currently not being used in California. With biocontrol agents, chances for success increase even more when local organisms are used that are adapted to the environmental conditions in which they will be expected to function. To date, no studies have focused on exploring the properties of local microbes as biocontrol agents for cavity spot in California.

Streptomyces, an unusual bacterial genus known for its production of antimicrobial compounds

The soil represents one of the richest and most densely populated microbial environments on earth. It consists of a large number of bacteria, fungi, and other microbes which interact with each other (Jousset *et al.* 2014). Antagonistic interactions among microbial species are well-known and many bacterial species have been reported to be able to actively inhibit the growth of eukaryotic microbes such as fungi (Jousset *et al.* 2014).

Actinomycetes, a diverse family of Gram-positive filamentous bacteria are notorious for their production of a wide diversity of secondary metabolites. These natural products include antibiotic and antifungal agents that inhibit the growth of other microbes (van der Meij *et al.* 2017).

*Streptomyces*, an actinomycete, is one of the most dominant genera present in soil and the largest bacterial genus with over five hundred species. *Streptomyces* species have been discovered to produce secondary metabolites inhibiting the growth of a diverse range of microbes present in
soil (Chater et al. 2009). According to Streptomyces genome studies, Streptomyces species are known to have large linear chromosomes containing several secondary metabolite biosynthesis gene clusters; thus, providing these bacteria with an immense capacity to produce antimicrobial agents (Nett et al. 2009). In the life cycle of Streptomyces, these compounds are produced during the stationary phase by multienzyme complexes (nonribosomal peptide synthases, Harir et al. 2018). Over two-thirds of the antibiotics known to date originate from Streptomyces species, including vancomycin, erythromycin, and tetracycline. In addition, Streptomyces species also produce compounds that have applications as anticancer, antifungal, and immunosuppressive drugs such as amphotericin B, mitomycin C, ivermectin, and rapamycin (Nett et al. 2009).

Several species of Streptomyces have emerged as biocontrol agents that are safe alternatives to chemical agents (Olanrewaju and Babalola 2018, Vurukonda et al. 2018) for the control of phytopathogens. Moreover, several species can secrete hydrolytic enzymes such as chitinases, glucanases, proteases, and lipases that can lyse the cells of devastating fungal pathogens (Compant et al. 2005). The bacteria themselves as well as their purified metabolites are used as soil or foliar treatments (Rey & Dumas 2016; Vurukonda et al. 2018). For example, a number of Streptomyces species have been successfully tested as biocontrol agents against some very common and harmful soilborne fungal pathogens including Rhizoctonia solani, Fusarium sp., Sclerotium rolfsii, Fusarium oxysporum, and Sclerotinia sclerotiorum (Olanrewaju and Babalola 2018). Another study reports eight Streptomyces isolates as successful inhibitors of the destructive oomycetes Phytophthora medicaginis and Phytophthora sojae causing root rot on alfalfa and soybean (Xiao et al. 2002).

The overall purpose of this study is to discover promising local isolates of Streptomyces that can be used as biocontrol agents against the oomycete Pythium violae, which is the dominant
carrot cavity spot pathogen in California and responsible for significant losses for the local
growers. The cavity spot disease is difficult to manage and current chemical pesticides are
becoming unreliable. Hence, the search for a biocontrol organism is of crucial importance to reduce
the need for harmful pesticides but also in general to enable growers to prevent disease and save
their crops. To our knowledge, no studies have explored the properties of local microbes as
biocontrol agents for cavity spot in California. The use of local microbes has the advantage that
the microbes are adapted to the environmental conditions in which they are expected to function,
hereby increasing the chance of success in disease control. Therefore, this study aims to put
forward a selection of locally isolated *Streptomyces* isolates able to strongly inhibit the growth of
*P. violae* to initiate the formulation of a sustainable approach for cavity spot control of carrots,
major vegetable crop produced in Kern County.
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Chapter 2

Exploring the biocontrol potential of Streptomyces isolates against Pythium violae, a dominant carrot pathogen

Abstract

The United States is a dominant producer of carrots worldwide with over 85% of carrots grown in California alone, and Kern County accounts for 60% of this production. Cavity spot is one of the severe diseases of carrots caused by several oomycete pathogens of the genus Pythium with Pythium violae as the dominant species in California. The disease is difficult to manage since current chemical control is becoming unreliable due to increased resistance of the pathogens. The bacterial genus Streptomyces is renowned for the production of a broad range of natural products including antimicrobial products known to inhibit oomycete and fungal pathogens. Therefore, I explored the biocontrol potential of 153 local Streptomyces strains obtained from diverse soils in Kern County for their ability to inhibit the growth of P. violae in vitro. To accomplish this, various P. violae strains were isolated from diseased carrots collected from both conventional and organic fields in the Bakersfield area and the strains were identified via amplification and DNA sequencing of two fingerprint regions (cox2 and ITS). Pathogenicity of the isolated strains was confirmed through a root inoculation test and a soil assay. The locally isolated Streptomyces strains were tested against P. violae through an antibiosis assay and four Streptomyces isolates were selected displaying a strong antagonistic effect against P. violae. These bacteria were identified via amplification and multilocus sequence analysis (MLSA) of five housekeeping genes (16S rRNA, atpD, recA, rpoB, and trpB) as Streptomyces spectabilis, Streptomyces chartreusis, Streptomyces cavourensis, and Streptomyces tuirus or Streptomyces hawaiensis. These isolates were also tested against isolated
oomycete and fungal pathogens including *Pythium spinosum*, *Phytophthora helicoides*, *Fusarium oxysporum*, *Fusarium falciforme*, *Fusarium solani*, *Sclerotium rolfsii*, and *Sclerotinia sclerotiorum*. The isolates showed a strong antagonistic effect against most of these pathogens. Based on these results, we propose these four *Streptomyces* inhibitors as promising candidates for the biocontrol of carrot cavity spot and other severe carrot diseases.

**Key words:** *Pythium violae*, isolation, *in vitro* antibiosis, *Streptomyces*, PCR, DNA sequencing, pathogenicity, biocontrol

**Introduction**

The United States of America is among the largest carrot (*Daucus carota*) producing countries in the world. Within the U.S., the highest carrot producing states include California, Texas, Washington, Michigan, Florida, Colorado, and Wisconsin. California ranks first with a production of over 85% of all carrots grown in the United States and within California, Kern County (San Joaquin Valley) accounts for 60% of this production (Nunez *et al*. 2008).

Carrots can be affected by a variety of diseases that reduces both their yield and market value. Several of these diseases are very common and severe affecting the overall carrot production worldwide, such as cavity spot, black root rot, crown rot, soft rot, southern blight, root knot disease (Davis and Nunez 2007).

Cavity spot of carrots is a soilborne disease caused by oomycetes, particularly a diverse range of *Pythium* species, including *P. violae*, *P. sulcatum*, *P. ultimum*, *P. irregulare*, *P. cryptoirregulare*, *P. dissotocum*, *P. coloratum*, *P. sylvaticum*, *P. aphanidermatum*, *P. intermedium*, and *P. rostratum* (Allain-Boulé *et al*. 2004). Even though many of these *Pythium*
species are reported from around the world to cause cavity spot, it has been determined that the slower growing species *P. violae* and *P. sulcatum* are the main causal agents (Vivoda *et al.* 1991, White 1988). In California, *Pythium violae* is the dominant causal agent of cavity spot disease. The disease affects over 50% of the overall acreage resulting in diseased carrots that are rejected by both the fresh and processing market. This causes a great economic loss for the agricultural sector (Davis and Nunez 2007).

Currently, the main method used to control cavity spot is the application of metalaxyl and its stereoisomer mefenoxam, systemic fungicides (Saude *et al.* 2014). However, these fungicides have become unreliable due to increased resistance of cavity spot pathogens and microbial degradation of the chemicals in the soil (Davison and McKay 1999, Hiltunen and White 2002). On the other hand, some biocontrol methods have been tested to control cavity spot, although none of these biocontrol agents have been commercialized or are currently being used to control cavity spot disease in California. For example, the plant growth-promoting bacteria *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Serratia proteamaculans* have been studied to be used as seed treatments against cavity spot pathogens but with varying results based on organism and cultivar (White 2003). Several soil inhabiting actinomycetes isolated from soils in Western Australia have been successfully tested both *in vitro* and *in vivo* against *Pythium coloratum*, one of the cavity spot pathogens (El-Tarabily *et al.* 1997). Besides certain bacteria, some strains of the oomycete *Pythium oligandrum* obtained from a collection in France also exhibited antagonism against several soilborne pathogens, especially *Pythium dissotocum*, another cavity spot pathogen (Vallance *et al.* 2009). Apart from these few studies, most studies exploring biocontrol agents have mainly focused on other *Pythium* diseases such as *Pythium* damping-off and *Pythium* root rot, two other common and severe *Pythium* diseases occurring worldwide.
*Streptomyces* is one of the most dominant bacterial genera in soil with over 500 species. These species produce a wide range of secondary metabolites with antagonistic ability against other microorganisms (Chater *et al.* 2009). Hence, several *Streptomyces* species have been put forward and even commercialized as biocontrol agents that can be used as safe alternatives to synthetic chemicals for the control of phytopathogens (Vurukonda *et al.* 2018). For example, the biofungicide Mycostop consists of a selected *Streptomyces griseoviridis* strain isolated from Finnish Sphagnum peat and is used to control diseases caused by many common phytopathogens, especially *Fusarium* species (Lahdenpera *et al.* 1991). Another registered biological fungicide, Actinovate, consists of the *Streptomyces lydicus* strain WYEC 108 originally obtained from agricultural soil in the United Kingdom. Application of this fungicide is expected to control many pathogens such as, *Fusarium, Rhizoctonia, Pythium, Phytophthora, Verticillium, Sclerotinia*, and other root decay fungi (US Environmental Protection Agency Office of Pesticide Programs – Biopesticide Registration Action Document). Moreover, several studies propose a number of *Streptomyces* species as biocontrol agents against *Rhizoctonia solani, Fusarium* sp., *Sclerotium rolfsii, Fusarium oxysporum*, and *Sclerotinia sclerotiorum* (Olanrewaju and Babalola 2018), common and harmful soilborne fungal pathogens. Some of these pathogens also cause severe diseases of carrots, such as Fusarium dry rot (*Fusarium* sp.) (Zhang *et al.* 2014), southern blight (*Sclerotium rolfsii*), and cottony rot (*Sclerotinia sclerotiorum*) (Davis and Nunez 2007). *Streptomyces* isolates were also successfully tested to inhibit the oomycetes *Phytophthora medicaginis* and *Phytophthora sojae* causing root rot on alfalfa and soybean (Xiao *et al.* 2002).

To date, no studies have explored the potential of local microbial antagonists as biocontrol agents against cavity spot in California. With biocontrol agents, chances for success increases when local organisms are used that are adapted to the environmental conditions in which they will
be expected to function. Therefore, in this study we explored the inhibitory potential of 153 locally isolated *Streptomyces* strains against *Pythium violae* and present a selection of four strong inhibitors that might act as local biocontrol agents for controlling cavity spot disease. The specific objectives of this research were to (1) explore the biocontrol potential of locally isolated *Streptomyces* strains by testing them *in vitro* against local *P. violae* strains, (2) molecularly characterize the strong *Streptomyces* inhibitors, and (3) determine the target range of the antifungal properties of the successful *Streptomyces* biocontrol agents by testing them *in vitro* against other pathogenic oomycetes and fungi causing severe plant diseases that are routinely controlled with chemical fungicides.

**Materials and Methods**

**Isolation of oomycetes from carrot cavity spot lesions**

Diseased carrots showing typical cavity spot lesions were thoroughly rinsed with tap water and cavity spot lesions were aseptically dissected from the root tissue within 24 hours of collection. The dissected tissue was placed on PARP agar, which is corn meal agar (CMA, Sigma-Aldrich) supplemented with antibacterial and antifungal chemicals including 250 mg/l ampicillin, 10 mg/l rifampicin, 10 mg/l pimaricin, and 2.5 mg/l pentachloronitrobenzene (PCNB). The plates were incubated at 23°C in the dark (White *et al.* 1993, Schrandt and Davis 1994). Four days after inoculation, the mycelial growth radiating from the carrot tissue was aseptically subcultured onto CMA plates by cutting out an agar cube (5 mm$^2$ surface area) from the edge of the mycelial growth and inoculating it upside-down onto a fresh CMA plate. Plates were incubated at 23°C in the dark for four days before being stored at 4°C.
Genetic characterization of isolated oomycetes

Genomic DNA extraction

To allow for easy collection of the oomycete mycelium, V8 broth was used. The broth was made by mixing 2.5 g CaCO$_3$ with 200 ml of V8 juice, centrifuging for 5 min at 2,000 rpm to clarify the mixture. Distilled water was added to make one liter of V8 broth. An agar cube covered with actively growing mycelium was transferred to a 250-ml Erlenmeyer flask with 25 ml of V8 broth and incubated at 23°C in the dark for 5 days without shaking. Genomic DNA was extracted from about 100 mg of mycelium using the DNeasy Plant Mini Kit (Qiagen) following the revised protocol provided by Abad and Bienapfl (2019) and with the following modifications: a prefilled tube with 0.5 mm diameter, acid washed, silica beads (Benchmark Scientific) was used instead of a Lysing Matrix A sterile screw cap tube in which the oomycete mycelium was transferred and the sample was disrupted/homogenized at 4000 beats per minute for 60 s using a BeadBug Microtube Homogenizer. The concentration of the genomic DNA was measured with a microvolume spectrophotometer (NanoDrop One Microvolume UV-Vis Spectrophotometer, ThermoFisher). The DNA was stored at -20°C until further use.

Amplification, sequencing, and analysis of genomic fingerprint regions

The identification of the isolates was performed by amplification and complete gene sequencing of the genomic fingerprint regions coding for the cytochrome C oxidase subunit 2 (cox2) located on the mitochondrial DNA and the internal transcribed spacer (ITS) spanning the ITS1, ITS2, and 5.8S gene located between the nuclear 18S rRNA and 26S rRNA gene (Choi et al. 2015, Schroeder et al. 2006). These regions were amplified via polymerase chain reaction (PCR) with specific primers for both the cox2 gene (Choi et al. 2015) and the ITS region (Schroeder et al. 2006) as listed in Table 2.1. Each 50 µl PCR reaction was set up containing 33 µl distilled water, 10 µl 5x
Herculase II reaction buffer, 1.5 µl dNTP mix, 1 µl (100 ng/µl) gDNA, 1.25 µl of both the forward and reverse primer (Table 2.1), 1 µl Herculase II fusion DNA Polymerase, and 1 µl DMSO. The reactions were run in a PCR Thermal Cycler with the following conditions: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 20 s, annealing for 20 s at the primer-pair-specific annealing temperature (Table 2.1) and extension at 72°C for 30 s. A final extension was performed at 72°C for 3 min.

The PCR reaction products were run on a 1% agarose gel, excised and extracted from the gel using the OMEGA BIO-TEK E.Z.N.A. Gel Extraction Kit. The concentration of eluted fragments was measured with a microvolume spectrophotometer (NanoDrop One Microvolume UV-Vis Spectrophotometer, Thermo Fisher Scientific, Inc.).

For the cox2 regions, the PCR products were sequenced by Laragen, Inc. (Culver City, CA) and a BLAST (Basic Local Alignment Search Tool) search was performed using the NCBI GenBank database (Altschul et al. 1990) to identify the isolates obtained from cavity spot lesions.

For the ITS regions, the eluted PCR products were cloned into the pJET1.2 vector following the CloneJET PCR Cloning Protocol (Thermo Scientific) using 50 ng of the purified PCR product. The ligation mixture was transferred to 50 µl of competent E. coli cells via heat shock transformation. Transformants were recovered by plating on selective medium (LB agar containing 100 mg/l ampicillin) and incubating at 37°C for 24 h. Ten single colonies were restreaked on fresh selective medium and their successful transformation was confirmed through a PCR colony screen. Amplification was performed with Taq DNA polymerase and specific primers for the ITS region (Table 2.1) following the protocol provided by 2.0X Taq RED Master Mix Kit (Apex BioResearch Products) except that the template DNA was replaced by part of a bacterial colony. The reaction conditions were as follows: initial denaturation at 94°C for 3 min,
followed by 30 cycles of denaturation at 92°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min. The PCR reaction products were run on 1% agarose gel to evaluate proper amplification and size of the amplified product. A positive clone was grown in LB broth containing 100 mg/l ampicillin at 37°C for 16 h while shaking. The transformed pJET1.2 vector containing the ITS region was extracted from the cultured cells using the Mini-Prep (Extraction of Plasmid) Kit and sent for sequencing with the pJET1.2 commercial primers (pJET1.2 Forward Sequencing Primer, 23-mer and pJET1.2 Reverse Sequencing Primer, 24-mer) by Laragen, Inc. (Culver City, CA). A BLAST search was performed using the NCBI GenBank (Altschul et al. 1990) to identify the isolates obtained from the cavity spot lesions.

**Maintenance and long-term storage of Pythium species**

The isolated and identified *Pythium* species from cavity spot lesions were maintained on CMA medium at 4°C for regular use. The isolates were also stored for long-term collection. For this, two different temperatures were used, each with three different conditions. Four 5-mm diameter CMA plugs containing actively growing *Pythium* mycelium were added to 10 ml sterile deionized water in a 20 ml vial containing either 6 sterilized rye grains, 6 sterilized hemp seeds, or no seeds. Loosely capped vials were stored in the dark at either room temperature (23°C) or 18°C. After a week or until mycelium growing out of the CMA plugs and seeds was noticed, the vials were tightly capped and sealed with parafilm around the cap (Abad 2019).
Pathogenicity test fulfilling Koch’s postulates

Root inoculation test

A root inoculation test (Benard 1990, Allain-Boulé et al. 2004, El-Tarabily et al. 2004) was conducted using mature carrots within 48h after harvest. The carrots were surface sterilized in 10% bleach for 2 minutes, rinsed three times in sterile distilled water, and placed in a plastic box cleaned with 70% ethanol lined with moistened sterile paper towels. Each carrot was inoculated by placing four evenly spaced 5-mm diameter CMA plugs containing actively growing mycelium of the isolated *Pythium* species on the root surface. Each plastic box contained four carrots, two inoculated with an isolated *Pythium* species, one inoculated with previously identified *Pythium violae* strain obtained from the Washington State University (WSU) serving as a positive control, and one with non-colonized CMA plugs serving as a negative control. The plastic boxes were covered to serve as a moist chamber and incubated for five days in the dark at room temperature (23°C).

The agar plugs were removed, and the carrots were thoroughly washed under tap water. To reisolate the pathogens, the same protocol was followed as described above for isolation of oomycetes from cavity spot lesions. Genomic DNA was extracted and amplification of the ITS region was done following the same protocol described above using Taq DNA Polymerase. Amplification of a single band was verified by analyzing 5 μl of the PCR reaction through agarose gel electrophoresis. Five μl of the remaining PCR products were cleaned up using the ExoSAP-IT PCR Product Cleanup Reagent protocol (Thermo Fisher Scientific, Inc.). The cleaned PCR products were sequenced by Laragen, Inc. (Culver City, CA) and a BLAST (Basic Local Alignment Search Tool) search was performed using the NCBI GenBank (Altschul et al. 1990) to confirm the identity of the re-isolated pathogens from root inoculation test. In total, the root
inoculation test was repeated three times independently to confirm the pathogenicity of the isolated *Pythium* species, one time including the re-isolation and confirmation of the pathogen identity.

*Soil assay*

Four 5-mm diameter CMA plugs of each *Pythium* isolate and the *P. violae* strain obtained from Washington State University (positive control) were transferred to an Erlenmeyer flask containing 25 ml V8 broth and incubated at room temperature (23ºC) in the dark for five days. Three cultures were prepared for each strain.

A 50/50 mix of peat moss and sand was made and batches for 5 planting pots (6 cm x 25 cm tree growing pots) were transferred to a polypropylene autoclave bag and closed well. The bags were autoclaved twice for 30 minutes with a two-day interval between the two rounds mixing the peat moss and sand mixture in the bags well between the rounds.

The V8 cultures for each isolate were pooled in a sterile beaker and mixed well using a hand mixer. The inoculum was added to a bag of sterilized peat moss and sand mix along with 250 ml deionized water to moisten the mix. The content of the bag was well mixed with a trowel to spread the inoculum through the peat moss and sand mix and divided over 5 planting pots that were cleaned with 70% ethanol. A negative control was set up with uninoculated peat moss and sand mix moistened with 350 ml deionized water and divided over 5 planting pots.

Carrot seeds were surface sterilized with 70% ethanol for 1 minute, 10% bleach for 2 minutes, and four rinses of sterile deionized water. Three carrot seeds were planted at a depth of 0.5 cm in each pot and the pots were placed under grow lights (16h photoperiod) at room temperature (23ºC). The pots were watered every day with 20 ml tap water and once per week with 20 ml Hoagland’s No. 2 Basal Salts solution (Caisson Labs). Two weeks after seed germination, seedlings were thinned leaving each pot with the strongest seedling.
During different stages of carrot growth (two and 12 weeks after seed germination), the pots were reinoculated by watering with mixed V8 cultures of the *Pythium* strains (prepared as previously described). Upon harvest (14 weeks after seed germination), carrots were uprooted and inspected for the presence of lesions characteristic of cavity spot. Similar to the root inoculation test described above, the pathogens were reisolated from cavity spots, genomic DNA was extracted, and the ITS region of each isolate was amplified and the PCR fragment was sequenced to confirm the identity of the pathogens causing the cavity spot lesions.

**Metalaxyl test**

The effect of a systemic fungicide, metalaxyl (Ridomil Gold 480 SL), was tested on the isolated *Pythium* species. Based on the field application of 20 gallons/ acre (12 µl/ petri dish), CMA plates were prepared with four different concentrations of metalaxyl mixed into 25 ml of CMA: 3, 6, 12, and 18 µl. Plates were inoculated with 5-mm diameter CMA plugs of the *Pythium* species and incubated at room temperature (23°C) in the dark for four days. The diameter of the growth of the *Pythium* mycelium was measured to determine its potential resistance towards metalaxyl. The results were compared to *P. violae* growth on regular CMA containing no metalaxyl. The metalaxyl test was performed three times to determine the consistency of obtained results.

**Antibiosis assays**

A total of 153 local *Streptomyces* isolates were screened *in vitro* against a designated *P. violae* strain. Two *Streptomyces* isolates were inoculated on opposite sides of a CMA plate. The plates were incubated at 28°C for ten days (sealed with parafilm to prevent dehydration) to allow for the colony to reach stationary phase which is characterized by the production of secondary metabolites
and spores. Then, a 5-mm diameter CMA plug from the edge of actively growing *P. violae* mycelium was inoculated upside-down in the middle of the plates. The plates were resealed with parafilm and incubated at room temperature (23°C) in the dark. The results were evaluated four days post inoculation (dpi) of *P. violae*.

The *Streptomyces* isolates showing mild to strong inhibition of *P. violae* were tested again through a second round of antibiosis assays (one *Streptomyces* isolate per CMA plate). Then, the strongest *Streptomyces* inhibitors were tested in a third round of bioassays as the final confirmation of their ability to strongly inhibit *P. violae*.

These selected *Streptomyces* inhibitors were also tested against other isolated oomycete and fungal pathogens. Oomycetes were isolated through pear-baiting (Erwin and Ribeiro 1996) from the pond of the Environmental Studies Area (ESA) on campus following the same protocol as described above. The fungi were originally isolated from diseased almond roots, grape roots, tomatoes, and green beans along with carrots collected from fields in Bakersfield. Within 24 hours of plant collection, the diseased plant material was thoroughly rinsed under running tap water. Parts of the diseased tissue were aseptically dissected and placed on Sabouraud Dextrose (SabDex) agar, a medium primarily used for the isolation of fungi. The plates were incubated at room temperature (23°C) in the dark for three to five days. The isolated fungi were cleaned by transferring a hyphal tip from the edge of the mycelial growth onto a new SabDex plate and incubated at room temperature (23°C) in the dark for three to five days. DNA extractions, PCR amplification of the ITS regions, and sequence analyses (ITS region) were performed following the protocols described above for *Pythium* species. Antibiosis assays were done similar to those described for *P. violae*. 
Genetic characterization of *Streptomyces* inhibitors

**Genomic DNA extraction**

The selected *Streptomyces* species were grown in 5 ml of Tryptic Soy Broth (TSB) for 2 days at 28°C while shaking (220 rpm). Cells were collected by centrifugation at 13,000 rpm for 3 min and the supernatants were removed. The pellets were washed with 1 ml of distilled water and placed at -20°C for 45 min after which the pellets were resuspended in 500 µl of TE25S buffer containing 25 mM Tris-HCl (pH 8), 25 mM EDTA (pH 8), and 300 mM sucrose. Twenty µl of 100 mg/ml lysozyme solution was added to the pellets, mixed gently, and incubated at 37°C for 1 h. Then, 5 µl of 20 mg/ml Proteinase K and 30 µl of 10% SDS was added to the pellets, mixed gently by inversion, and incubated at 55°C for 1 h while gently mixing occasionally. One hundred µl of 5M NaCl was added to the pellets and mixed thoroughly by inversion followed by 65 µl of CTAB/NaCL mix containing 0.7 M NaCl and 10% CTAB (cetyl trimethyl ammonium bromide). After mixing thoroughly by inversion, the samples were incubated at 55°C for 10 min. The samples were cooled at room temperature (23°C) for 5 min and then cooled on ice for 10 min. Five hundred µl of chloroform: isoamylalcohol (24:1) was added to the samples and mixed thoroughly by inversion. The samples were centrifuged for 10 min at 13,000 rpm. The supernatants were transferred to clean microcentrifuge tubes and cooled on ice for 10 min. One volume of isopropanol was added to the samples. The tubes were inverted 10-20 times to mix and placed back on ice for 10 min. The DNA for each sample was pelleted by centrifugation for 10 min at 13,000 rpm and the supernatants were gently poured off without dislodging the pellets. The pellets were washed with 400 µl of 70% ethanol. Using a pipet, all of the ethanol was removed without dislodging the DNA pellets and the pellets were air dried in the biosafety cabinet. The DNA for each sample was resuspended in 100 µl of clean deionized water. The concentration of the genomic DNA for each
sample was measured with a microvolume spectrophotometer (NanoDrop One Microvolume UV-Vis Spectrophotometer, ThermoFisher). The DNA for each sample was stored at -20°C until further use.

Amplification, sequencing, and analysis of genomic fingerprint region

The identification of the *Streptomyces* inhibitors was performed by amplification and complete gene sequencing of the genomic fingerprint region 16S rRNA (Guo *et al.* 2008). Taq DNA Polymerase and universal primers for the 16S rRNA gene (Table 2.1) were used to amplify the 16S rRNA coding region following the protocol provided by 2.0X Taq RED Master Mix Kit (Apex BioResearch Products). The reaction conditions were as follows: initial denaturation at 95°C for 2 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 90 sec. A final extension was performed at 72°C for 5 min.

The amplified fragments were checked for correct size and purity through agarose gel electrophoresis. The PCR products were cleaned up using the ExoSAP-IT PCR Product Cleanup Reagent protocol (Thermo Fisher Scientific, Inc.). The cleaned PCR products were sequenced by Laragen, Inc. (Culver City, CA) and a BLAST (Basic Local Alignment Search Tool) search was performed using the NCBI GenBank (Altschul *et al.* 1990) to identify the *Streptomyces* isolates.

Multilocus Sequence Analysis (MLSA)

Four housekeeping genes being *atpD*, *recA*, *rpoB*, and *trpB* (Guo *et al.* 2008, Lapaz *et al.* 2017) were amplified using specific primers (Table 2.1) and Taq DNA Polymerase following the protocol provided by 2.0X Taq RED Master Mix Kit (Apex BioResearch Products). The reaction conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing for 30 s at primer-pair-specific annealing temperatures
listed in Table 2.1 (Lapaz et al. 2017) and extension at 72°C for 90 sec. A final extension was performed at 72°C for 10 min (Guo et al. 2008).

The amplified fragments were checked for correct size and purity through agarose gel electrophoresis. The PCR products were cleaned up using the ExoSAP-IT PCR Product Cleanup Reagent protocol (Thermo Fisher Scientific, Inc.). The cleaned PCR products were sequenced by Laragen, Inc. (Culver City, CA) and a BLAST (Basic Local Alignment Search Tool) search was performed using the NCBI GenBank (Altschul et al. 1990) to identify the *Streptomyces* isolates.

**Streptomyces against Streptomyces challenge assays**

The four identified *Streptomyces* inhibitors were tested against one another through antibiosis assays. Each of the four *Streptomyces* isolates was streaked individually in the middle of a CMA and a PMA (potato mash agar - 50 g instant potato flakes and 20 g agar per liter) plate along with the other three isolates streaked perpendicular to and as close as possible but without touching the middle streak. The plates were incubated at 28°C for seven days (sealed with parafilm to prevent dehydration).

Another antibiosis assay was performed where the *Streptomyces* isolates were streaked on the plates at different times. Each of the four *Streptomyces* isolates was streaked individually in the middle of a CMA and a PMA plate. The plates were incubated at 28°C for ten days (sealed with parafilm to prevent dehydration) to allow for the colony to reach stationary phase which is characterized by the production of secondary metabolites and spores. Then, the other three *Streptomyces* isolates were streaked perpendicular to and as close as possible but without touching the initial streak. The plates were resealed with parafilm and incubated at 28°C. The results were evaluated four dpi of *Streptomyces* isolates.
Results and Discussion

Three *Pythium* strains isolated from diseased carrots

Diseased carrots were collected during the fall of 2019 from three different fields (conventional and organic fields) in the Bakersfield area with carrot cavity spot (pointed out by Joe Nunez, UC Cooperative Extension Farm Advisor for vegetable production in Kern County, CA) (Figure 2.1). Three potential *Pythium* strains were isolated from typical cavity spot lesions of carrots and grown on PARP medium. Genomic DNA of each strain was extracted followed by amplification of the genomic fingerprint regions (*cox2* and ITS).

The ITS region is the universal fingerprint region used for molecular characterization of both fungi and oomycetes. However, for this study, an additional gene *cox2* was amplified to confirm the identification of *Pythium* isolates to the species level. According to a recent study by Choi *et al.* (2015), the ITS rRNA region is not very reliable for the identification of oomycetes for two main reasons: 1) ITS-based studies for the oomycetes are often lacking due to the extremely long ITS sizes which makes it difficult to amplify and sequence these regions and 2) there are cases where the ITS region does not show much variability to differentiate two closely related species phylogenetically. Therefore, the *cox2* gene was suggested as another genomic fingerprint region yielding higher success of species identification (Choi *et al.* 2015).

Both the *cox2* gene (581 bp) and the ITS region (949 bp) were successfully amplified from genomic DNA extracted from the isolated strains (Figure 2.2). DNA sequencing and a BLAST search identified two of the isolated strains as *Pythium violae* (Figure 2.3) and one strain as *Pythium spinosum* (discussed in Chapter 3). The identification of these species was based on the highest query coverage and percent identity of NCBI GenBank sequence submissions.
A previously confirmed *P. violae* strain was obtained from Dr. Lindsey du Toit at Washington State University (WSU) to serve as a positive control in this study. Genetic characterization of this strain was performed following the same protocol as described above for local *P. violae* strains to serve as a confirmation of our procedures. DNA sequences of the isolated *Pythium violae* strains and the strain from WSU were compared through Clustal Omega Multiple Sequence Alignment (Madeira *et al.* 2019). The two locally isolated *Pythium violae* strains were 100% identical for both the *cox2* gene and the ITS region (Appendix A – Figure S1). They had a 98% similarity for the *cox2* gene and a 99% similarity for the ITS region when compared to the obtained sequences for the *P. violae* strain from WSU (Appendix A – Figure S2).

Since the two locally isolated *P. violae* strains were identical, only one of these strains was used for the rest of this study. The *P. violae* strain obtained from WSU served as a positive control throughout this study.

**Pythium survives long-term storage with rye grains and hemp seeds**

Different methods were tested for long-term storage of the *Pythium* isolates as required for the span of this study as well as to establish a collection at CSUB. *Pythium* species were stored under three different conditions (in sterilized water containing either 6 sterilized rye grains, 6 sterilized hemp seeds, or no seeds) each at two different temperatures (room temperature (23°C) and 18°C) (Figure 2.4). The viability of the *Pythium* species was determined after 6 months, 1 year, and 1.5 years of storage. *Pythium* species stored with rye grains and hemp seeds survived at both temperatures. *Pythium* species stored without seeds had unreliable results; some survived both temperatures while some did not survive at all.
It can be concluded that *Pythium* species can be stored for at least 1.5 years at both room temperature (23°C) and 18°C with both rye grains and hemp seeds. However, *Pythium* species would not survive for a long-term when stored only in water.

**Koch’s postulates confirm *P. violae* pathogenicity**

Koch’s postulates were performed to confirm the pathogenicity of the isolated *Pythium violae* strain. For the root inoculation test, freshly harvested healthy carrots were inoculated with agar disks containing active *P. violae* mycelium. The local strain formed lesions similar to those of the positive control (*P. violae* strain from WSU) on all of the inoculated sites on the carrot tissue. There were no visible lesions on carrots inoculated with CMA disks serving as negative control (Figure 2.5). The pathogens were reisolated from the affected carrot tissue and were confirmed as the *P. violae* strains used to inoculate the carrots through genetic characterization.

As described by Davis and Nunez (2007), *P. violae* is the dominant pathogen of cavity spot disease in California affecting over 50% of the overall acreage. Both *P. violae* strains used in the root inoculation test caused for the formation of lesions on healthy carrots confirming their pathogenicity. Based on the severity of the lesions formed, it can be determined that the *P. violae* strain from WSU is more virulent when compared to the local *P. violae* strain (Figure 2.5). However, the lesions formed on the carrots were different from typical cavity spot lesions found on diseased carrots harvested from a field. Although previous studies have performed the root inoculation test, it might not be a specific test to determine the specific pathogenicity of cavity spot pathogen against carrots.

Therefore, a soil assay was also performed to complete Koch’s postulates as it more closely resembles natural conditions. Carrot seeds were grown individually in tree seedling pots containing
a sterilized peat moss and sand mix (50:50) inoculated with *P. violae* mycelium (local and positive control). During different stages of carrot growth (two and 12 weeks after seed germination), the pots were reinoculated with *P. violae* mycelium. Fourteen weeks after seed germination, carrots were harvested and analyzed for the presence of cavity spot lesions. Typical cavity spot lesions were found on most of the carrots infected with *P. violae*, while uninoculated carrots did not show any lesions. However, several carrots also showed more severe lesions resembling root rot (Figure 2.6). A contamination of a fungal pathogen was suspected. Since both *P. violae* and a fungus were suspected to cause the lesions, the pathogens were reisolated from both types of lesions on the selective media PARP and SabDex for oomycetes and fungi, respectively. Both plates showed growth and genetic characterization confirmed the *P. violae* strains as the pathogens causing the typical cavity spot lesions, while a fungus from the genus *Fusarium* was isolated from the more severe necrotic lesions alongside *P. violae*. At this point, it is unclear to state whether these necrotic lesions were initiated by *P. violae* or *Fusarium*. Contamination with this fungus could have happened in several ways. For example, there might have been a possible contamination of the water that was regularly used to water the plants, contamination of a piece of equipment used during the soil assay set up, the introduction of the fungus through other organisms such as ants or bugs carrying *Fusarium* and other microbes while coming in contact with the growing carrots.

Nevertheless, not all carrots were contaminated by *Fusarium* and these carrots showed typical cavity spot lesions from which *P. violae* was successfully reisolated. Although the setup and execution of the soil assay needs to be optimized, this initial assay confirmed the pathogenicity of the isolated *P. violae* strain.
**Metalaxyl inhibits the growth of *P. violae***

A metalaxyl test was performed to determine the effect of this commonly used systemic fungicide on *P. violae* strains (local and from WSU). Agar disks containing active *P. violae* mycelium were placed on plates containing four different concentrations of metalaxyl (Ridomil Gold 480 SL) mixed into 25 ml of CMA: 3, 6, 12, and 18 µl, with 12 µl/petri dish representing the field application of 20 gallons/acre. Four dpi, the diameter of the growth of the *Pythium* mycelium was measured to determine its potential resistance towards metalaxyl. The local *P. violae* strain was sensitive to 6, 12, and 18 µl of metalaxyl with no hyphal growth from the agar disk. However, it showed resistance towards the plate containing 3 µl of metalaxyl with 3 mm hyphal growth from the agar disk. These results were compared to *P. violae* growth of 56 mm measured on regular CMA containing no metalaxyl (Figure 2.7). The *P. violae* strain from WSU was sensitive to all metalaxyl concentrations tested with no hyphal growth from the agar disk. These results were compared to *P. violae* growth of 62 mm measured on regular CMA containing no metalaxyl (Figure 2.8).

Based on the results, it can be concluded that the local strain of *P. violae* shows resistance towards a low concentration of metalaxyl (3 µl/ 25 ml CMA). Yet, this concentration is well below the recommended concentration used in the field (20 gallons/acre or 12 µl/25 ml CMA). However, the *P. violae* strain from WSU showed sensitivity to all the concentrations of metalaxyl used.

Even though metalaxyl inhibits *P. violae in vitro*, its actual strength is challenged in the field as the cavity spot pathogens might show resistance over time and also due to microbial degradation of the chemicals in the soil (Davison and McKay 1999, Hiltunen and White 2002).
Four *Streptomyces* strains display a strong antagonistic effect against *P. violae* and other plant pathogens

A collection of 153 local *Streptomyces* strains was established in Fall 2018. Strains were isolated from various types of soil in the Bakersfield area. These 153 *Streptomyces* isolates were tested in *vitro* against the locally isolated *Pythium violae* for their ability to inhibit the growth of *P. violae* by plating them against *P. violae* on a petri dish (antibiosis assays). The *Streptomyces* strains were scored for their inhibition ability and the strength of their antibiosis was determined by the size of the inhibition zone (Figure 2.9). Out of a total of 18 strains that showed inhibition, four *Streptomyces* strains were selected that were able to strongly inhibit *P. violae* (Figure 2.9).

The four strong *Streptomyces* inhibitors were also tested against the *P. violae* strain obtained from WSU to determine their inhibition ability against a strain isolated from a different region. The inhibition ability of the *Streptomyces* strains was the same against both *P. violae* strains (local and from WSU) resulting in a strong inhibition zone. However, the inhibition strength of all four *Streptomyces* inhibitors was higher towards the locally isolated *P. violae* strain as compared to the *P. violae* strain from WSU based on the size of the inhibition zone (Figure 2.10).

The four strong *Streptomyces* inhibitors were also screened against other locally isolated phytopathogens important to the local as well as global agricultural industry to determine the target range of the antifungal properties of the selected *Streptomyces* isolates. Through amplification and sequencing of the ITS rRNA region, these pathogens were confirmed as *Pythium spinosum*, *Phytophthium helicoides*, *Fusarium oxysporum*, *Fusarium falciforme*, *Fusarium solani*, *Sclerotium rolfsii*, and *Sclerotinia sclerotiorum* (Figure 2.11). *Pythium spinosum* causes common plant diseases, such as damping-off, dieback and crown and root rot (Al-Sa’di *et al.* 2008, Hendricks and Roberts 2015, Sigillo *et al.* 2020). *Phytophthium helicoides* causes crown, stem,
and root rot of different plant species (Brown et al. 2019, Chen et al. 2020, Yang et al. 2013). *Fusarium* species, especially *F. oxysporum* and *F. solani* cause root and crown rot of several plants and have also been identified as the causal agents of Fusarium dry rot of carrot in China (Wang and Jeffers 2000, Zhang et al. 2014). *Sclerotium rolfsii* causes southern blight in a variety of plants including carrots and is especially important for carrot production in the southern San Joaquin Valley (Nunez et al. 2008). *Sclerotinia sclerotiorum* causes white mold or cottony rot of various plants and crops including carrots (Davis and Nunez 2007).

Based on the inhibition zone, the inhibition ability and strength of the four strong *Streptomyces* inhibitors against *P. spinosum* and *Pp. helicoides* was about the same as against *P. violae* (Figure 2.12). The inhibition effect varied with each fungal species. All *Fusarium* species showed mild to strong inhibition by all four *Streptomyces* inhibitors. Only one of the *Streptomyces* inhibitors had a strong effect on *S. rolfsii* while the other three inhibitors had no effect. *S. sclerotiorum* was strongly inhibited by two of the *Streptomyces* inhibitors while one caused a mild inhibition and one caused no inhibition of *S. sclerotiorum* (Figure 2.13).

Since there are over 500 known species of *Streptomyces*, it is difficult to identify and classify species within the genus solely based on the 16S rRNA gene sequence. Therefore, Multi Locus Sequence Analysis (MLSA) was also performed by amplification and complete gene sequencing of four housekeeping genes including *atpD, recA, rpoB*, and *trpB* (Guo et al. 2008, Lapaz et al. 2017). Based on the highest query coverage and percent identity of the first three NCBI GenBank sequence submissions obtained through a BLAST search for each housekeeping gene, the three strong inhibitors GM18-46, GM18-72, and GM18-83 were preliminary identified as *Streptomyces spectabilis, Streptomyces chartreusis*, and *Streptomyces cavourensis*, respectively (Tables 2.2 - 2.4). The identity of GM18-150 was inconclusive as there is not one species that was
commonly identified for each gene and can be either *Streptomyces taurus* or *Streptomyces hawaiiensis* (Table 2.5) (Figure 2.14). For future studies, it would be highly recommended to perform a whole genome sequencing of these *Streptomyces* species to confirm their identification.

A thorough literature study was done to determine if anything is already known about the antimicrobial activity of the identified *Streptomyces* species in general, and in particular against cavity spot pathogens. None of these four *Streptomyces* species have ever been tested against cavity spot pathogens. Therefore, this study is the first to explore the biocontrol potential of these identified inhibitors against *P. violae*, the dominant cavity spot pathogen. Yet, they have been put forward as biocontrol agents of other phytopathogens.

*Streptomyces spectabilis* has shown antimicrobial activity against several different fungal pathogens such as *Fusarium, Colletotrichum*, and *Botrytis* (Bubici 2018, Chen et al. 2018). A *Streptomyces spectabilis* strain isolated from rhizosphere of Thai medicinal plants had antagonistic activity against six plant pathogenic fungi including *Alternaria brassicicola, A. porri, Colletotrichum gloeosporioides, Fusarium oxysporum, Penicillium digitatum*, and *Sclerotium rolfsii* (Khamna et al. 2009). *Streptomyces chartreusis* has been studied as a potential biocontrol organism. A strain of *S. chartreusis* isolated from soil in India displayed antimicrobial activity against several different bacterial and fungal pathogens with the strongest inhibition of different *Fusarium* species (Dezfully and Hanumanthu 2018). An endophyte of crop plants and trees identified as a strain of *S. chartreusis* was isolated from soil in Australia and put forward as a biocontrol agent of *Rhizoctonia* causing Rhizoctonia root rot on wheat (Barnett et al. 2017). Also, *Streptomyces cavourensis* has been shown to inhibit *Colletotrichum gloeosporioides*, a fungus that infects pepper plants. The antimicrobial activity was due to the combined effect of lytic enzymes produced by *S. cavourensis* (Lee et al. 2012). The antifungal compounds from a *S. cavourensis*
strain isolated from a deep-sea sediment collected from South China Sea showed strong inhibition of the fungi *Rhizoctonia solani* and several *Fusarium* spp., especially *Fusarium oxysporum* (Pan *et al.* 2015). To our knowledge, there is no known study that has explored either *Streptomyces tuirus* or *Streptomyces hawaiiensis* as a potential biocontrol agent against other phytopathogens.

**Streptomyces inhibitors interspecific interaction**

The four strong *Streptomyces* inhibitors were also tested against one another through antibiosis assays on both CMA and PMA to determine their interspecific interaction. This was done to determine whether these inhibitors can potentially be used together as a biocontrol mixture to increase the chances of inhibition of the growth of *P. violae* and other pathogens. According to the results of the assay in which all four *Streptomyces* strains were streaked together on both CMA and PMA, the strains grew well together showing no inhibition against one another (Figure 2.15).

For the antibiosis assays where the *Streptomyces* inhibitors were streaked on both CMA and PMA at different times, the results varied. According to the results on CMA, *Streptomyces* strain GM18-46 strongly inhibited the growth of the other isolates. *Streptomyces* strain GM18-72 strongly inhibited the growth of both GM18-83 and GM18-150, but GM18-46 grew in its presence. *Streptomyces* strain GM18-83 allowed for growth of the other isolates, while *Streptomyces* strain GM18-150 strongly inhibited the growth of the other isolates (Figure 2.16). The results on PMA showed that *Streptomyces* strain GM18-46 mildly inhibited the growth of other three inhibitors. *Streptomyces* strain GM18-72 inhibited the growth of GM18-83, but both GM18-46 and GM18-150 grew in its presence. *Streptomyces* strain GM18-83 allowed for growth of the other isolates and *Streptomyces* strain GM18-150 strongly inhibited the growth of both GM18-72 and GM18-83 but allowed for the growth of GM18-46 (Figure 2.16).
Since some *Streptomyces* inhibitors showed antagonism against the other inhibitors, it might be that these species cannot be used together as a biocontrol mixture against *P. violae* and other pathogens. However, the actual interspecific interaction of these inhibitors and their inhibitory effect against *P. violae* and other pathogens can only be determined through a soil experiment in a future study.

**Conclusion**

The overall purpose of this study was to explore the biocontrol potential of local *Streptomyces* species against *Pythium violae*, a dominant carrot cavity spot pathogen. This study presents a selection of four *Streptomyces* species isolated from the Bakersfield area, CA as strong inhibitors of *P. violae*, and therefore, potential biocontrol agents of cavity spot disease. These isolates were identified as *Streptomyces spectabilis* (GM18-46), *Streptomyces chartreusis* (GM18-72), *Streptomyces cavourensis* (GM18-83), and *Streptomyces tairus* or *Streptomyces hawaiensis* (GM18-150). To our knowledge, this is the first study that showed the biocontrol potential of these selected inhibitors against *P. violae*. In addition, these species also showed inhibition of one or more other plant pathogens including *P. spinosum, P. helicoides, Fusarium spp., Sclerotium rolfsii*, and *Sclerotinia sclerotiorum* that cause severe plant diseases. Hence, they are also promising biocontrol candidates for several of these pathogens. The next step for this project would be to test the actual biocontrol potential of the *Streptomyces* species in the soil to see if and how well the bacteria can establish themselves in the soil and if they indeed can protect the plant against the pathogen.
Literature Cited


oomycetes - a comparison of the cox1 and cox2 loci. Molecular Ecology Resources 15: 1275-1288.


Hendricks, K. E. and P. D. Roberts. 2015. First report of *Pythium spinosum* as a pathogen of watermelon and in association with dieback of watermelon in Southwest Florida. Plant Health Progress 16: 77-79.


Table 2.1. Primers used in this study.

<table>
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<tr>
<th>Primer</th>
<th>Primer sequence (5’ → 3’)</th>
<th>Gene/Region</th>
<th>Annealing Temperature (°C)</th>
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<td>Cox2-RC4</td>
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<td>UN-LO28S576B</td>
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<td>27f</td>
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<td>1492r</td>
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<td>rpoBPF</td>
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Table 2.2. Identification of *Streptomyces* strain GM18-46 through MLSA based on top 3 hits obtained from BLAST results.

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<th>Percent Identity (%)</th>
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<td>16S rRNA</td>
<td><em>Streptomyces spectabilis</em> ATCC 27465</td>
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<td><em>Streptomyces spectabilis</em> NRRL 2792</td>
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Table 2.5. Identification of *Streptomyces* strain GM18-72 through MLSA based on top 3 hits obtained from BLAST results.

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<td><em>Streptomyces chartreus</em> ATCC 14922</td>
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<td><em>Streptomyces chartreus</em> NA02069</td>
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<td><em>Streptomyces chartreus</em> ATCC 14922</td>
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Table 2.4. Identification of *Streptomyces* strain GM18-83 through MLSA based on top 3 hits obtained from BLAST results.

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<td><em>Streptomyces sp.</em> JJ24</td>
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Table 2.5. Identification of *Streptomyces* strain GM18-150 through MLSA based on top 3 hits obtained from BLAST results.

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<td><em>Streptomyces tuirus JCM 4255</em></td>
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Figures

Figure 2.1. Lesions on freshly harvested carrots used for the isolation of cavity spot pathogens.
Figure 2.2. Gel electrophoresis confirming the successful amplification of the *cox2* gene (581bp) and the ITS region (949 bp) of an isolated oomycete.
Figure 2.3. Subcultured oomycete identified as *Pythium violae* on CMA plate showing actively growing mycelium. The picture was taken at 5 dpi.
Figure 2.4. *Pythium violae* stored for long term storage at both room temperature (23°C) and 18°C under three different conditions: a) sterile deionized water, b) sterile deionized water containing sterilized rye grains, and c) sterile deionized water containing sterilized hemp seeds.
Figure 2.5. Root inoculation test confirming the pathogenicity of the *P. violae* strains: a) negative control (CMA), b) positive control (*P. violae* strain from WSU), and c) locally isolated *P. violae* strain. Results were scored at 5 dpi.
Figure 2.6. Soil assay confirming the pathogenicity of the *P. violae* strains as shown by the typical cavity spot lesions that formed on the carrot tissue (indicated by the arrows): a) negative control (non-inoculated), b) positive control (*P. violae* strain from WSU), and c) locally isolated *P. violae* strain. The severe lesions resembling root rot on top part of the carrots were caused by a combination of *P. violae* and a contamination of a pathogenic fungus, *Fusarium*. 
Figure 2.7. Sensitivity of the locally isolated *P. violae* strain to metalaxyl (Ridomil Gold 480 SL):

a) *P. violae* growth on CMA with no metalaxyl added and b) *P. violae* growth on CMA plate containing 3 µl of metalaxyl. Pictures were taken at 4 dpi.
Figure 2.8. Sensitivity of the *P. violae* strain from WSU towards metalaxyl (Ridomil Gold 480 SL): a) *P. violae* growth on CMA with no metalaxyl added and b) *P. violae* growth on CMA plate containing 3 µl of metalaxyl. Pictures were taken at 4 dpi.
Figure 2.9. Key to score the strength of the antibiosis of *Streptomyces* strains toward *Pythium violae*. The strength of the antibiosis is determined by the size of the inhibition zone separating the *Pythium* mycelium from the *Streptomyces* colony.
Figure 2.10. Antibiosis assays showing the final selection of four *Streptomyces* strains (GM18-46, GM18-72, GM18-83, and GM18-150) that strongly inhibited the growth of a) locally isolated *P. violae* strain and b) a *P. violae* strain obtained from WSU. Pictures were taken at 5 dpi of *P. violae* on plates that were already inoculated with *Streptomyces* strains 10 days before.
Figure 2.11. Phenotypes of locally isolated oomycete (on CMA) and fungal (on SabDex) pathogens at 5 dpi including a) *Pythium spinosum*, b) *Phytophthium helicoides*, c) *Fusarium falciforme*, d) *Fusarium solani*, e) *Fusarium oxysporum*, f) *Sclerotium rolfsii*, and g) *Sclerotinia sclerotiorum*. 
Figure 2.12. Antibiosis assays showing the inhibitory effect of the strong *Streptomyces* inhibitors (GM18-46, GM18-72, GM18-83, and GM18-150) against the locally isolated oomycete pathogens a) *Pythium spinosum* and b) *Phytophthium helicoides*. Pictures were taken at 5 dpi of the oomycete pathogens on plates that were already inoculated with *Streptomyces* strains 10 days before.
Figure 2.13. Antibiosis assays showing the inhibitory effect of the strong *Streptomyces* inhibitors (GM18-46, GM18-72, GM18-83, and GM18-150) against the locally isolated fungal pathogens: a) *Fusarium falciforme*, b) *Fusarium oxysporum*, c) *Fusarium solani*, d) *Sclerotium rolfsii*, and e) *Sclerotinia sclerotiorum*. Pictures were taken at 5 dpi of the fungal pathogens on plates that were already inoculated with *Streptomyces* strains 10 days before.
Figure 2.14. Colony characteristic of the strong *Streptomyces* inhibitors (GM18-46, GM18-72, GM18-83, and GM18-150) shown on three different media (PMA, ISP-4, and CMA) at 7 dpi.
Figure 2.15. Antibiosis assays showing the inhibitory effect of the *Streptomyces* inhibitors against each other on a) CMA and b) PMA. The *Streptomyces* inhibitors were streaked together on the plates and their growth was evaluated at 7 dpi.
Figure 2.16. Antibiosis assays showing the inhibitory effect of the *Streptomyces* inhibitors against each other on a) CMA and b) PMA. Each *Streptomyces* inhibitor was streaked in the middle of the plate. Ten days later, the other three inhibitors were streaked perpendicular and close to the middle streak. Their growth was determined at 4 dpi.
Chapter 3

*Pythium spinosum*, a new carrot cavity spot pathogen

Abstract

Cavity spot disease is one of the most common and severe carrot diseases around the world. This disease is mainly caused by *Pythium* species with *Pythium violae* as the major causal agent, especially in California. Some other cavity spot pathogens isolated from different regions of the world include *P. sulcatum, P. ultimum, P. irregulare, P. cryptoirregulare, P. dissotocum, P. coloratum, P. sylvaticum, P. aphanidermatum, P. intermedium*, and *P. rostratum*. A new cavity spot pathogen was isolated from typical cavity spot lesions from diseased carrots obtained from two organic fields in the Bakersfield and Riverside areas of California. The isolates were identified as *Pythium spinosum* via amplification and sequence analysis of two fingerprint regions (*cox2* and ITS). Pathogenicity of *P. spinosum* was confirmed through Koch’s postulates. Both *P. spinosum* strains showed sensitivity towards metalaxyl, the systemic fungicide used to control cavity spot disease.

**Key words:** new pathogen, cavity spot, *Pythium*, amplification, pathogenicity, Koch’s postulates
Introduction

Cavity spot of carrot (Daucus carota) is one of the most severe and chronic diseases worldwide. Cavity spot is a soilborne disease caused by Pythium species belonging to a group of microorganisms called oomycetes or water molds (Davis and Nunez 2007). These pathogens infect carrot roots forming small sunken, brown-black, circular to elliptical or irregularly shaped lesions on the surface of carrots (Vivoda et al. 1991, Hiltunen and White 2002). There is a diverse range of Pythium species that infect carrots around the world and all species have been confirmed as primary pathogens of cavity spot based on the geographical regions they were isolated from. Some of these species isolated include P. violae, P. sulcatum, P. ultimum, P. irregulare, P. cryptoirregulare, P. dissotocum, P. coloratum, P. sylvaticum, P. aphanidermatum, P. intermedium, and P. rostratum (Allain-Boulé et al. 2004). Even though many different species of Pythium have been reported to cause cavity spot, it has been determined that the slower growing species P. violae and P. sulcatum are the main causal agents (Vivoda et al. 1991, White 1988).

In California, cavity spot is mainly caused by P. violae as the dominant pathogen affecting about 50% of the overall acreage (Davis and Nunez 2007). In a recent study, cavity spot pathogens were isolated from diseased carrots collected from both conventional and organic fields in the Bakersfield area. These pathogens were molecularly characterized and identified to the species level. Based on their identification, P. violae was the main pathogen isolated. However, another species, P. spinosum, was isolated twice from cavity spot lesions from organic fields located in different regions in California (Bakersfield and Riverside). This species has not been associated with cavity spot disease. Therefore, in this study we performed Koch’s postulates through a root inoculation test and a soil assay to determine the pathogenicity of P. spinosum. Both tests had
promising results showing that *P. spinosum* is a cavity spot pathogen. The specific objective of this research was to confirm *P. spinosum* as a new cavity spot pathogen.

**Materials and Methods**

**Isolation of cavity spot pathogens**

Diseased carrots showing typical cavity spot lesions were thoroughly rinsed with tap water and cavity spot lesions were aseptically dissected from the root tissue within 24 hours of collection. The dissected tissue was placed on PARP agar, which is corn meal agar (CMA, Sigma-Aldrich) supplemented with antibacterial and antifungal chemicals including 250 mg/l ampicillin, 10 mg/l rifampicin, 10 mg/l pimaricin, and 2.5 mg/l pentachloronitrobenzene (PCNB). The plates were incubated at 23°C in the dark (White *et al.* 1993, Schrandt and Davis 1994). Four days after inoculation, the mycelial growth radiating from the carrot tissue was aseptically subcultured onto CMA plates by cutting out an agar cube (5 mm² surface area) from the edge of the mycelial growth and inoculating it upside-down onto a fresh CMA plate. Plates were incubated at 23°C in the dark for four days before being stored at 4°C.

Along with the isolation of local cavity spot pathogens, a previously isolated *Pythium spinosum* strain was received from Dr. Cassandra Swett at University of California, Davis. This strain was isolated from diseased carrots showing typical cavity spot lesions obtained from an organic field in the Riverside area. Through genetic characterization, the strain was identified as *P. spinosum*. This received *P. spinosum* strain was used along with the locally isolated *P. spinosum* strain throughout this study.
Genetic characterization of isolated pathogens

Genomic DNA extraction

To allow for easy collection of the oomycete mycelium, V8 broth was used. The broth was made by mixing 2.5 g CaCO₃ with 200 ml of V8 juice, centrifuging for 5 min at 2,000 rpm to clarify the mixture. Distilled water was added to make one liter of V8 broth. An agar cube covered with actively growing mycelium was transferred to a 250-ml Erlenmeyer flask with 25 ml of V8 broth and incubated at 23°C in the dark for 5 days without shaking. Genomic DNA was extracted from about 100 mg of mycelium using the DNeasy Plant Mini Kit (Qiagen) following the revised protocol provided by Abad and Bienapfl (2019) and with the following modifications: a prefilled tube with 0.5 mm diameter, acid washed, silica beads (Benchmark Scientific) was used instead of a Lysing Matrix A sterile screw cap tube in which the oomycete mycelium was transferred and the sample was disrupted/homogenized at 4000 beats per minute for 60 s using a BeadBug Microtube Homogenizer. The concentration of the genomic DNA was measured with a microvolume spectrophotometer (NanoDrop One Microvolume UV-Vis Spectrophotometer, ThermoFisher). The DNA was stored at -20°C until further use.

Amplification, sequencing, and analysis of genomic fingerprint regions

The identification of the isolates was performed by amplification and complete gene sequencing of the genomic fingerprint regions coding for the cytochrome C oxidase subunit 2 (cox2) located on the mitochondrial DNA and the internal transcribed spacer (ITS) spanning the ITS1, ITS2, and 5.8S gene located between the nuclear 18S rRNA and 26S rRNA gene (Choi et al. 2015, Schroeder et al. 2006). These regions were amplified via polymerase chain reaction (PCR) with specific primers for both the cox2 gene (Choi et al. 2015) and the ITS region (Schroeder et al. 2006) as listed in Table 3.1. Each 50 µl PCR reaction was set up containing 33 µl distilled water, 10 µl 5x
Herculase II reaction buffer, 1.5 µl dNTP mix, 1 µl (100 ng/µl) gDNA, 1.25 µl of both the forward and reverse primer (Table 3.1), 1 µl Herculase II fusion DNA Polymerase, and 1 µl DMSO. The reactions were run in a PCR Thermal Cycler with the following conditions: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 20 s, annealing for 20 s at the primer-pair-specific annealing temperature (Table 3.1) and extension at 72°C for 30 s. A final extension was performed at 72°C for 3 min.

The PCR reaction products were run on a 1% agarose gel, excised and extracted from the gel using the OMEGA BIO-TEK E.Z.N.A. Gel Extraction Kit. The concentration of eluted fragments was measured with a microvolume spectrophotometer (NanoDrop One Microvolume UV-Vis Spectrophotometer, Thermo Fisher Scientific, Inc.).

For the cox2 genes, the PCR products were sequenced by Laragen, Inc. (Culver City, CA) and a BLAST (Basic Local Alignment Search Tool) search was performed using the NCBI GenBank database (Altschul et al. 1990) to identify the isolates obtained from cavity spot lesions.

For the ITS regions, the eluted PCR products were cloned into the pJET1.2 vector following the CloneJET PCR Cloning Protocol (Thermo Scientific) using 50 ng of the purified PCR product. The ligation mixture was transferred to 50 µl of competent E. coli cells via heat shock transformation. Transformants were recovered by plating on selective medium (LB agar containing 100 mg/l ampicillin) and incubating at 37°C for 24 h. Ten single colonies were restreaked on fresh selective medium and their successful transformation was confirmed through a PCR colony screen. Amplification was performed with Taq DNA polymerase and specific primers for the ITS region (Table 3.1) following the protocol provided by 2.0X Taq RED Master Mix Kit (Apex BioResearch Products) except that the template DNA was replaced by part of a bacterial colony. The reaction conditions were as follows: initial denaturation at 94°C for 3 min,
followed by 30 cycles of denaturation at 92°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min. The PCR reaction products were run on 1% agarose gel to evaluate proper amplification and size of the amplified product. A positive clone was grown in LB broth containing 100 mg/l ampicillin at 37ºC for 16 h while shaking. The transformed pJET1.2 vector containing the ITS region was extracted from the cultured cells using the Mini-Prep (Extraction of Plasmid) Kit and sent for sequencing with the pJET1.2 commercial primers (pJET1.2 Forward Sequencing Primer, 23-mer and pJET1.2 Reverse Sequencing Primer, 24-mer) by Laragen, Inc. (Culver City, CA). A BLAST search was performed using the NCBI GenBank (Altschul et al. 1990) to identify the isolates obtained from the cavity spot lesions.

**Pathogenicity test fulfilling Koch’s postulates**

*Root inoculation test*

A root inoculation test (Benard 1990, Allain-Boulé et al. 2004, El-Tarably et al. 2004) was conducted using mature carrots within 48h after harvest. The carrots were surface sterilized in 10% bleach for 2 minutes, rinsed three times in sterile distilled water, and placed in a plastic box cleaned with 70% ethanol lined with moistened sterile paper towels. Each carrot was inoculated by placing four evenly spaced 5-mm diameter CMA plugs containing actively growing mycelium of the isolated *Pythium spinosum* on the root surface. Each plastic box contained four carrots, two inoculated with an isolated *Pythium spinosum* strain, one inoculated with previously isolated and identified *Pythium violae* strain serving as a positive control, and one with non-colonized CMA plugs serving as a negative control. The plastic boxes were covered to serve as a moist chamber and incubated for five days in the dark at room temperature (23°C).
The agar plugs were removed and the carrots were thoroughly rinsed under tap water. To reisolate the pathogens, the same protocol was followed as described above for isolation of oomycetes from cavity spot lesions. Genomic DNA was extracted and amplification of the ITS region was done following the same protocol described above using Taq DNA Polymerase. Amplification of a single band was verified by analyzing 5 µl of the PCR reaction through agarose gel electrophoresis. Five µl of the remaining PCR products were cleaned up using the ExoSAP-IT PCR Product Cleanup Reagent protocol (Thermo Fisher Scientific, Inc.). The cleaned PCR products were sequenced by Laragen, Inc. (Culver City, CA) and a BLAST (Basic Local Alignment Search Tool) search was performed using the NCBI GenBank (Altschul et al. 1990) to confirm the identity of the re-isolated pathogens from root inoculation test. In total, the root inoculation test was repeated three times independently to confirm the pathogenicity of the isolated P. spinosum strains, one time including the re-isolation and confirmation of the pathogen identity.

Soil assay

Four 5-mm diameter CMA plugs of Pythium spinosum strains and a previously isolated and identified P. violae strain (positive control) were transferred to an Erlenmeyer flask containing 25 ml V8 broth and incubated at room temperature (23°C) in the dark for five days. Three cultures were prepared for each strain.

A 50/50 mix of peat moss and sand was made and batches for 5 planting pots (6 cm x 25 cm tree growing pots) were transferred to a polypropylene autoclave bag and closed well. The bags were autoclaved twice for 30 minutes with a two-day interval between the two rounds mixing the peat moss and sand mixture in the bags well between the rounds.

The V8 cultures for each isolate were pooled in a sterile beaker and mixed well using a hand mixer. The inoculum was added to a bag of sterilized peat moss and sand mix along with 250
ml deionized water to moisten the mix. The content of the bag was well mixed with a trowel to spread the inoculum through the peat moss and sand mix and divided over 5 planting pots that were cleaned with 70% ethanol. A negative control was set up with uninoculated peat moss and sand mix moistened with 350 ml deionized water and divided over 5 planting pots.

Carrot seeds were surface sterilized with 70% ethanol for 1 minute, 10% bleach for 2 minutes, and four rinses of sterile deionized water. Three carrot seeds were planted at a depth of 0.5 cm in each pot and the pots were placed under grow lights (16h photoperiod) at room temperature (23°C). The pots were watered every day with 20 ml tap water and once per week with 20 ml Hoagland’s No. 2 Basal Salts solution (Caisson Labs). Two weeks after seed germination, seedlings were thinned leaving each pot with the strongest seedling.

During different stages of carrot growth (two and 12 weeks after seed germination), the pots were reinoculated by watering with mixed V8 cultures of the *Pythium* strains (prepared as previously described). Upon harvest (14 weeks after seed germination), carrots were uprooted and inspected for the presence of lesions characteristic of cavity spot. Similar to the root inoculation test described above, the pathogens were reisolated from cavity spots, genomic DNA was extracted, and the ITS region of each isolate was amplified. The PCR fragments were sequenced to confirm the identity of the pathogens causing the cavity spot lesions.

**Metalaxyl test**

The effect of a systemic fungicide, metalaxyl (Ridomil Gold 480 SL), was tested on the isolated *Pythium spinosum* strains. Based on the field application of 20 gallons/ acre (12 µl/ petri dish), CMA plates were prepared with four different concentrations of metalaxyl mixed into 25 ml of CMA: 3, 6, 12, and 18 µl. Plates were inoculated with 5-mm diameter CMA plugs of the *P.*
*Pythium spinosum* strains and incubated at room temperature (23°C) in the dark for four days. The diameter of the growth of the *Pythium* mycelium was measured to determine its potential resistance towards metalaxyl. The results were compared to *P. spinosum* growth on regular CMA containing no metalaxyl. The metalaxyl test was performed three times to determine the consistency of obtained results.

**Results and Discussion**

*Pythium spinosum* was isolated from carrot cavity spot lesions

Two *Pythium* strains were isolated from typical cavity spot lesions on carrots from two organic fields in the Bakersfield and Riverside area of California. Genomic DNA of each strain was extracted followed by amplification of the genomic fingerprint regions (*cox2* and ITS).

The ITS region is the universal fingerprint region used for molecular characterization of both fungi and oomycetes. However, for this study, an additional gene *cox2* was amplified to confirm the identification of *Pythium* isolates to the species level. According to a recent study by Choi et al. (2015), the ITS rRNA region is not very reliable for the identification of oomycetes for two main reasons: 1) ITS-based studies for the oomycetes are often lacking due to the extremely long ITS sizes which makes it difficult to amplify and sequence these regions and 2) there are cases where the ITS region does not show much variability to differentiate two closely related species phylogenetically. Therefore, the *cox2* gene was suggested as another genomic fingerprint region yielding higher success of species identification (Choi et al. 2015).

Both the *cox2* gene and the ITS region were successfully amplified from genomic DNA extracted from the isolated strains. DNA sequencing and a BLAST search identified both strains
as *Pythium spinosum* (Figure 3.1). The identification of these species was based on the highest query coverage and percent identity of GenBank sequence submissions in BLAST.

DNA sequences of the isolated *P. spinosum* strains were compared through Clustal Omega Multiple Sequence Alignment (Madeira *et al.* 2019). Both strains had a 97% similarity for the *cox2* gene but were 100% identical for the ITS region (Appendix A – Figure S3).

*P. spinosum* is a soilborne pathogen that causes some very common plant diseases. For example, damping-off affecting greenhouse cucumbers in Oman was attributed to *P. spinosum* (Al-Sa’di *et al.* 2008), crown and root rot of greenhouse cucumbers in Italy (Sigillo *et al.* 2020), dieback of watermelon in Southwest Florida (Hendricks and Roberts 2015), root rot of Chili (*Capsicum annuum*) in Pakistan (Nawaz *et al.* 2016), and root and crown rot of Ginseng in the USA (Qi *et al.* 2021).

**Koch’s postulates confirms *P. spinosum* pathogenicity**

Koch’s postulates were performed to confirm the pathogenicity of the isolated *Pythium spinosum* strains. For the root inoculation test, freshly harvested healthy carrots were inoculated with agar disks containing active *P. spinosum* mycelium. The isolated strains formed lesions similar to those of the positive control (local *P. violae* strain) on all of the inoculated sites on the carrot tissue. There were no visible lesions on carrots inoculated with CMA disks serving as negative control (Figure 3.2). The pathogens were reisolated from the affected carrot tissue and through genetic characterization, they were confirmed as the *P. spinosum* strains used to inoculate the carrots.

Both *P. spinosum* strains used in the root inoculation test caused for the formation of lesions on healthy carrots confirming their pathogenicity. Based on the similarity in lesion formation by the locally isolated *P. violae* (positive control), it can be concluded that *P. spinosum*
is a possible new cavity spot pathogen. However, the lesions formed on the carrots were different from typical cavity spot lesions found on diseased carrots harvested from a field. Although previous studies have performed the root inoculation test, it might not be a specific test to determine the pathogenicity of cavity spot pathogen.

Therefore, a soil assay was also performed to complete Koch’s postulates that more closely resembled natural conditions. Carrot seeds were grown individually in tree seedling pots containing a sterilized peat moss and sand mix (50:50) inoculated with \textit{P. spinosum} mycelium. During different stages of carrot growth (two and 12 weeks after seed germination), the pots were reinoculated with \textit{P. spinosum} mycelium. Fourteen weeks after seed germination, carrots were harvested and analyzed for the presence of cavity spot lesions. Lesions were found on most of the carrots infected with \textit{P. spinosum}, but these lesions were smaller in size compared to typical cavity spot lesions seen on diseased carrots harvested from the carrot fields. This same observation has also been reported before by Mike Davis (Professor Emeritus – UC Davis, personal communication). The uninoculated carrots did not show any lesions (Figure 3.3). Pathogens were reisolated from the lesions and genetic characterization confirmed the \textit{P. spinosum} strains as the pathogens causing the typical cavity spot lesions.

Hence, the soil assay confirmed the pathogenicity of \textit{P. spinosum} resulting in cavity spot lesions on the carrots. To our knowledge, this is the first time that \textit{P. spinosum} is linked to carrot cavity spot.

**Metalaxyl inhibits the growth of \textit{P. spinosum}**

A metalaxyl test was performed to determine the effect of this commonly used systemic fungicide on \textit{P. spinosum} strains. Agar disks containing active \textit{P. spinosum} mycelium were placed on plates
containing four different concentrations of metalaxyl (Ridomil Gold 480 SL) mixed into 25 ml of CMA: 3, 6, 12, and 18 µl, with 12 µl/petri dish representing the field application of 20 gallons/acre. Four days post inoculation (dpi), the diameter of the growth of the *Pythium* mycelium was measured to determine its potential resistance towards metalaxyl. Both *P. spinosum* strains were sensitive to all metalaxyl concentrations tested with no hyphal growth from the agar disk. These results were compared to *P. spinosum* growth of 83 mm measured on regular CMA containing no metalaxyl (Figure 3.4).

Based on the results, it can be concluded that both strains of *P. spinosum* (from the Bakersfield and Riverside areas) showed sensitivity to all the concentrations of metalaxyl confirming the inhibitory strength of metalaxyl in controlling this newly discovered cavity spot pathogen. This is in line with a study that showed high sensitivity to metalaxyl of twenty-four strains of *P. spinosum* isolated from infected cucumbers in Oman (Al-Sa’di et al. 2008). Even though metalaxyl has been shown to inhibit *P. spinosum in vitro* through different studies, its actual strength would be challenged in the field where pathogens are showing resistance over time and also due to microbial degradation of the chemicals in the soil (Davison and McKay 1999, Hiltunen and White 2002). Moreover, the *P. spinosum* strains used in this study were isolated from organic fields and synthetic pesticides like metalaxyl are not permitted to be used in organic farming.

**Conclusion**

A great number of *Pythium* species have been studied and identified as carrot cavity spot pathogens around the world. Through this study, another *Pythium* species isolated from typical cavity spot lesions on carrots from two organic fields in California was discovered as a new causal agent of
cavity spot disease. This *Pythium* species was molecularly characterized as *Pythium spinosum*. The pathogenicity of *P. spinosum* was also determined through a root inoculation test and a soil assay. To our knowledge, there is no known study to date that has associated *P. spinosum* with cavity spot disease.
Literature Cited


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Hendricks, K. E. and P. D. Roberts. 2015. First report of *Pythium spinosum* as a pathogen of watermelon and in association with a dieback of watermelon in Southwest Florida. Plant Health Progress 16: 77-79.


on growth of *Pythium violae* from carrot. Plant Disease 78: 335-338.


### Tables

Table 3.1. Primers used in this study.

<table>
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<th>Primer</th>
<th>Primer sequence (5’ → 3’)</th>
<th>Gene/Region</th>
<th>Annealing Temperature (°C)</th>
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<td>50</td>
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Figure 3.1. Subcultured oomycete identified as *Pythium spinosum* (isolated from the Bakersfield area) on CMA plate showing actively growing mycelium (5 dpi). The *P. spinosum* strain isolated from the Riverside area looked similar on CMA plate.
Figure 3.2. Root inoculation test confirming the pathogenicity of the *P. spinosum* strains: a) negative control (CMA), b) positive control (locally isolated *P. violae* strain), c) *P. spinosum* strain isolated from the Bakersfield area, and d) *P. spinosum* strain isolated from the Riverside area. Results were scored at 5 dpi.
Figure 3.3. Soil assay confirming the pathogenicity of the *P. spinosum* strains a) negative control (non-inoculated) b) *P. spinosum* strain isolated from the Bakersfield area, c) *P. spinosum* strain isolated from the Riverside area.
Figure 3.4. Sensitivity of the *P. spinosum* strain isolated from the Bakersfield area towards metalaxyl (Ridomil Gold 480 SL): a) *P. spinosum* growth on CMA with no metalaxyl added and b) *P. spinosum* growth on CMA plate containing 3 µl of metalaxyl (4 dpi). The *P. spinosum* strain isolated from the Riverside area had the similar results.
Chapter 4

Conclusion

A variety of plant pathogens cause severe diseases that not only reduce crop yield but also affect their marketability. Over the years, there has been an increase in plant diseases due to new emerging bacterial, fungal, and viral pathogens, especially on agricultural lands planted to monocultures (Nazarov et al. 2020). The similar genetic and physical environments of agro-ecosystems create a selective environment for the rapid emergence of new plant pathogens that affect plants at different stages of growth (Nazarov et al. 2020). These pathogens also increase disease incidence as they evolve to become more virulent overcoming crop resistance and pesticides thus becoming a threat to agricultural sustainability and food security (McDonald and Stukenbrock 2016). A single pathogen can be a causal agent of several plant diseases while a single plant species can be infected by several phytopathogens resulting in the same disease. For example, a new cavity spot pathogen *Pythium spinosum* was discovered through this study adding to the list of many different *Pythium* species known to cause carrot cavity spot disease around the world (Allain-Boulé et al. 2004). *P. spinosum* is already known to infect a variety of plants causing damping-off, dieback and crown and root rot (Al-Sa’di et al. 2008, Hendricks and Roberts 2015, Sigillo et al. 2020).

To control the existing and new emerging phytopathogens causing severe diseases, farmers and growers mainly rely on synthetic fungicides. However, these fungicides are becoming unreliable as pathogens are showing resistance over time and also due to microbial degradation of the chemicals in the soil (Davison and McKay 1999, Hiltunen and White 2002). Also, the excessive use and misuse of synthetic fungicides can be detrimental to the environment and health. Due to
these concerns and an increase in the awareness of the people regarding healthy food and environment, there is a need for a safe alternative to synthetic chemicals used in the fields to control plant diseases (Gerhardson 2002, Suprapta 2012).

Biological control is a method that uses microorganisms known to produce secondary metabolites with antagonistic activities against plant pathogens (Suprapta 2012). Many researchers nowadays are focusing on biological control using microbial antagonists, not only as a safe alternative strategy, but also as a sustainable approach to control plant diseases. However, it is very important to understand the interactions between antagonists and pathogens to determine the capability of antagonists to be used as biocontrol agents (Nega 2014). The use of local microbial species as biocontrol agents has the advantage that these microbes are adapted to the environmental conditions in which they are expected to function, hereby increasing the chance of successfully controlling the pathogen populations. Also, the selection of biocontrol agents that are highly effective against pathogens would allow for better chances of disease control if applied in high densities once or several times during a growing season also known as augmentative biological control (Köhl et al. 2019).

As considered in this study, the aim was to explore the biocontrol potential of local Streptomyces isolates against Pythium violae and select the most effective Streptomyces inhibitors as promising biocontrol agents to control cavity spot disease. Streptomyces is known to produce secondary metabolites with inhibitory ability against other microorganisms especially phytopathogens (Chater et al. 2009). Several Streptomyces species have been put forward as biocontrol agents that can be used as safe alternatives to synthetic chemical pesticides used to control phytopathogens (Vurukonda et al. 2018). In addition to these biocontrol agents, this study being the first of its kind to explore the inhibitory effect of local Streptomyces isolates against P.
violae puts forward four *Streptomyces* species as promising biocontrol candidates of cavity spot disease and other severe plant diseases.
Literature Cited


Hendricks, K. E. and P. D. Roberts. 2015. First report of *Pythium spinosum* as a pathogen of watermelon and in association with a dieback of watermelon in Southwest Florida. Plant Health Progress 16: 77-79.


Appendix A

CLUSTAL O(1.2.4) multiple sequence alignment
Sequences (1:2) Aligned. Score: 100

cox2 gene:

Pv-1    GGCAATGGTGGTTTCAAGATCCCTGCAACACCTGTATGGAAGGATTATTAACTTTCCATC  60
Pv-2    GGCAATGGTGGTTTCAAGATCCCTGCAACACCTGTATGGAAGGATTATTAACTTTCCATC  60

Pv-1    ATGATTTAATGTTTTTTTTAATTGTTGTAACTGTTTTTGTTTGCTGGATGTTATTTAGAG  120
Pv-2    ATGATTTAATGTTTTTTTTAATTGTTGTAACTGTTTTTGTTTGCTGGATGTTATTTAGAG  120

Pv-1    TTATTACTCTTTTTGATGAAAAAATAAATAAAATACCTTCAACTTATGTACATGGTTGCTA  180
Pv-2    TTATTACTCTTTTTGATGAAAAAATAAATAAAATACCTTCAACTTATGTACATGGTTGCTA  180

Pv-1    CATTGGCTTTTATTATATCGCATCCTATCCAGCTTTAATTTTAATATTATACCTTTAAAATTA  240
Pv-2    CATTGGCTTTTATTATATCGCATCCTATCCAGCTTTAATTTTAATATTATACCTTTAAAATTA  240

Pv-1    TAGGTAGTCAATGGTATTGGAGTTATGAATATTCTGATAATTTAGAATTTTCGGATGAAC  300
Pv-2    TAGGTAGTCAATGGTATTGGAGTTATGAATATTCTGATAATTTAGAATTTTCGGATGAAC  300

Pv-1    CTTTAATTTTTGATAGTTATATGGTACAAGAAAATGATTTAGAAATTGGTCAATTTAGAC  360
Pv-2    CTTTAATTTTTGATAGTTATATGGTACAAGAAAATGATTTAGAAATTGGTCAATTTAGAC  360

Pv-1    GTCCAGGTCGTTTAAATCAAACTTCTATGTTTATTAAAAGAGAAGGTGTTTTTTATGGTC  420
Pv-2    GTCCAGGTCGTTTAAATCAAACTTCTATGTTTATTAAAAGAGAAGGTGTTTTTTATGGTC  420

Pv-1    AATGTAGCGAAATTTGTGGAATAAATCA  480
Pv-2    AATGTAGCGAAATTTGTGGAATAAATCA  480

Pv-1    CAGCTTCAGATGTTTACCTATTCAGTTGCAATACCTTCTTTAGGTATTAAATTAGATGCTT  540
Pv-2    CAGCTTCAGATGTTTACCTATTCAGTTGCAATACCTTCTTTAGGTATTAAATTAGATGCTT  540

Pv-1    GTCCAGGTCGTTTAAATCAAAACTTCTATGTTTATTAAAAGAGAAGGTGTTTTTTATGGTC  600
Pv-2    GTCCAGGTCGTTTAAATCAAAACTTCTATGTTTATTAAAAGAGAAGGTGTTTTTTATGGTC  600

Pv-1    AATGTAGCGAAATTTTGAGAATTCACAATGTCATTATGCTTATTGTCATTATGCTTATTGTC  628
Pv-2    AATGTAGCGAAATTTTGAGAATTCACAATGTCATTATGCTTATTGTCATTATGCTTATTGTC  628
CLUSTAL O(1.2.4) multiple sequence alignment
Sequences (1:2) Aligned. Score: 100

ITS region:

Pv-1  CGTAACAAGATTTCCGTAAGTGAACCTGCCGAAAGGATCATTACCACAACCTAAAACCTTC  60
Pv-2  CGTAACAAGTTCTCCCGTAAAGCAGACCTGCCGCAGATATTACCACAACCTAAAACCTTC  60
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Pv-1  CACGTGAAACTCGTAAGTAATAGATTCAGTGCCCTGTGCGGAGGAGGAACGAAGGTTGGTCT  120
Pv-2  CACGTGAAACTCGTAAGTAATAGATTCAGTGCCCTGTGCGGAGGAGGAACGAAGGTTGGTCT  120
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Pv-1  CTGCGCTGTGGCGGAGGAGGAACGAAGGTTGGTCTTGTGTGTGCACAGCAATGTGTGTGT  180
Pv-2  CTGCGCTGTGGCGGAGGAGGAACGAAGGTTGGTCTTGTGTGTGCACAGCAATGTGTGTGT  180
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Pv-2  GCGGGACTGGCTGATCTATTTTTTTCAAACCCCATACAAAATGACTGATTTATACTGTGA  240
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Pv-1  GAACGAAAGTTCTTGCTTTTAACTAGATAACAACTTTCAGCAGTGGATGTCTAGGCTCGC  300
Pv-2  GAACGAAAGTTCTTGCTTTTAACTAGATAACAACTTTCAGCAGTGGATGTCTAGGCTCGC  300
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Pv-1  ACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATTGCAGAATTCA  360
Pv-2  ACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATTGCAGAATTCAGTGAGT  360
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Pv-1  CATCGAAATTTTGAACGCATATTGCACTTCCGGGTTCGTCCTGGAAGTATGTCTGTATCA  420
Pv-2  CATCGAAATTTTGAACGCATATTGCACTTCCGGGTTCGTCCTGGAAGTATGTCTGTATCA  420
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Pv-1  GTGTCCGTAAATCAAACTTGCCTTTCTTTCTG TGTAGTCAGGAGAAGATGAGTGCAGA  480
Pv-2  GTGTCCGTAAATCAAACTTGCCTTTCTTTCTGTGTAGTCAGGAGAAGATGAGTGCAGA  480
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Pv-2  TAAAACGACACGATCTTTCTACTGTGTTACTGAGAAGTGCGACGCTCGAATGCGGTGGTCTT  540
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Pv-1  CGGATCGCTGCGTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  600
Pv-2  CGGATCGCTGCGTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  600
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Pv-1  GGTATGTTATGCTTCGGCTGTACAATGTTGCGTGATTGGGAGTGGAATTCGTTTGTGCCT  660
Pv-2  GGTATGTTATGCTTCGGCTGTACAATGTTGCGTGATTGGGAGTGGAATTCGTTTGTGCCT  660
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Pv-1  TGAGGTGTACTGACGGTTGTGTGCTTGAACTGGAGGTTGTTGTGTAGTAGAGTGTTGTGT  720
Pv-2  TGAGGTGTACTGACGGTTGTGTGCTTGAACTGGAGGTTGTTGTGTAGTAGAGTGTTGTGT  720
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Pv-1  TAAAACGACACGATCTTTCTACTGTGTTACTGAGAAGTGCGACGCTCGAATGCGGTGGTCTT  780
Pv-2  TAAAACGACACGATCTTTCTACTGTGTTACTGAGAAGTGCGACGCTCGAATGCGGTGGTCTT  780
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Pv-1  CGGATCGCTGCGTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  840
Pv-2  CGGATCGCTGCGTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  840
***************************************************************
Figure S1. DNA sequence comparison of \textit{P. violae} strains for both \textit{cox2} gene and ITS region through Clustal Omega Multiple Sequence Alignment. Both Pv-1 and Pv-2 represent the locally isolated strains of \textit{P. violae}.
CLUSTAL O(1.2.4) multiple sequence alignment
Sequences (1:2) Aligned. Score: 98

cox2 gene:

Pv-1  
GGCAATGATTTTTCAAGATCCTGCAACACTGTATGGAAGTATTATTAACTTCCATC  

Pv-C  
GGCAATGATTTTTCAAGATCCTGCAACACTGTATGGAAGTATTATTAACTTCCATC  

Pv-1  
ATGATTAAATTAAAAATTTTTTTTATGTTGCAACTCTTGTGCTGATTTATTTAGAG  

Pv-C  
ATGATTAAATTAAAAATTTTTTTTATGTTGCAACTCTTGTGCTGATTTATTTAGAG  

Pv-1  
TTATTACTCTCTCTGATGAAATTATTTGTTTTGGTTTGCTGGATGTTATTTAGAG  

Pv-C  
TTATTACTCTCTCTGATGAAATTATTTGTTTTGGTTTGCTGGATGTTATTTAGAG  

Pv-1  
TTATTGAAATTATTTGGACTACTATACCAGCTTTAATTTTTATCTATAGCTATTCCAT  

Pv-C  
CTATTGAAATTATTTGGACTACTATACCAGCTTTAATTTTTATCTATAGCTATTCCAT  

Pv-1  
CATTTGCTTTATTATATTCAATGGATGAAGTAATTGATCCTATTATTACTTTAAAAGTTA  

Pv-C  
CATTTGCTTTATTATATTCAATGGATGAAGTAATTGATCCTATTATTACTTTAAAAGTTA  

Pv-1  
TAGGTAGTCAATGGGATTTATGAAATTATCTGATATTTTGAATTTTTCCGATGAGAC  

Pv-C  
TAGGTAGTCAATGGGATTTATGAAATTATCTGATATTTTGAATTTTTCCGATGAGAC  

Pv-1  
CTTTAATTTTTGATAGTTATATGGTACAAGAAAATGTATTAGAAATTGGTCAATTTAGAC  

Pv-C  
CTTTAATTTTTGATAGTTATATGGTACAAGAAAATGTATTAGAAATAGGTCAATTTAGAC  

Pv-1  
TTTTAAGAATTAAAATCTGTTTCTCAACTAATAGCTATTAGCTATTATTATTAATTA  

Pv-C  
TTTTAAGAATTAAAATCTGTTTCTCAACTAATAGCTATTAGCTATTATTATTAATTA  

Pv-1  
CAAGTTACGATGTTTCTACATCTCATTTAGTGATTAATTTTTAGCTATTAGCTATTATTATTAATTA  

Pv-C  
CAAGTTACGATGTTTCTACATCTCATTTAGTGATTAATTTTTAGCTATTAGCTATTATTATTAATTA  

Pv-1  
GTCCTAGGCGTTTTAAATCAACTTCTATGTTTTATTTAAAGAGAGGTTTTTTATGGTC  

Pv-C  
GTCCTAGGCGTTTTAAATCAACTTCTATGTTTTATTTAAAGAGAGGTTTTTTATGGTC  

Pv-1  
AATGTAACCAATTTGTGGAATAAATCA  

Pv-C  
AATGTAACCAATTTGTGGAATAAATCA

94
CLUSTAL O(1.2.4) multiple sequence alignment

Sequences (1:2) Aligned. Score: 99

ITS region:

Pv-1  CGTAAACAAGGTCTGCGATGGAAGCTCGCGGAACAGGCTTCAATTACACACCTAAACACTTTCTC  60
Pv-C  CGTAAACAAGGTCTGCGATGGAAGCTCGCGGAACAGGCTTCAATTACACACCTAAACACTTTCTC  60

Pv-1  CACGTGAACCGTCAAGTATATGGTCTCAGAGTGCCTCTGCTCAGAGTGCCTCAGAGTCC  120
Pv-C  CACGTGAACCGTCAAGTATATGGTCTCAGAGTGCCTCTGCTCAGAGTGCCTCAGAGTCC  120

Pv-1  CTGCGGCTTGAGGAGGAGGAACGAAGGTTGGTCTTGTGTGTACAGAATGTGTGTGT  180
Pv-C  CTGCGGCTTGAGGAGGAGGAACGAAGGTTGGTCTTGTGTGTACAGAATGTGTGTGT  180

Pv-1  GCGGGACTGGCTGATCTATTTTTCAAACCCCATACAAAATGACTGATTTATACTGTGA  240
Pv-C  GCGGGACTGGCTGATCTATTTTTCAAACCCCATACAAAATGACTGATTTATACTGTGA  240

Pv-1  TAAAACGACACGATCTTTCTACTGTTACTGAGAAGTGCGACGCTCGAATGCGGTGGTCTT  300
Pv-C  TAAAACGACACGATCTTTCTACTGTTACTGAGAAGTGCGACGCTCGAATGCGGTGGTCTT  300

Pv-1  TGTAAGTCTCTGCTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  360
Pv-C  TGTAAGTCTCTGCTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  360

Pv-1  CGGATCGCGTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  420
Pv-C  CGGATCGCGTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  420

Pv-1  GTGTCCGTAAATTCAAACTTGCCTTTCTTTTTCTGTGTAGTCAGGAGAAGATGAGTGCAGA  480
Pv-C  GTGTCCGTAAATTCAAACTTGCCTTTCTTTTTCTGTGTAGTCAGGAGAAGATGAGTGCAGA  480

Pv-1  TGTAAGTCTCTGCTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  540
Pv-C  TGTAAGTCTCTGCTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  540

Pv-1  TAAAACGACACGATCTTTCTACTGTTACTGAGAAGTGCGACGCTCGAATGCGGTGGTCTT  600
Pv-C  TAAAACGACACGATCTTTCTACTGTTACTGAGAAGTGCGACGCTCGAATGCGGTGGTCTT  600

Pv-1  CGGATCGCGTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  660
Pv-C  CGGATCGCGTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  660

Pv-1  GTGTCCGTAAATTCAAACTTGCCTTTCTTTTTCTGTGTAGTCAGGAGAAGATGAGTGCAGA  720
Pv-C  GTGTCCGTAAATTCAAACTTGCCTTTCTTTTTCTGTGTAGTCAGGAGAAGATGAGTGCAGA  720

Pv-1  TGTAAGTCTCTGCTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  780
Pv-C  TGTAAGTCTCTGCTGCAGTTGTCGGGGTCCTCGGCGTGAACATATGGAGGAAGCCTCGATTCGC  780

Pv-1  GATGTACAGCGACGCCACTTCATTTTGTTGTGGGTAGAGGACGTCTATTTGGGAAAGAAG  840
Pv-C  GATGTACAGCGACGCCACTTCATTTTGTTGTGGGTAGAGGACGTCTATTTGGGAAAGAAG  840
Pv-1      TAGTATCGGCAGCTTTTGGTAGTTGAAGGTAGTATCTCAATTGGACCTGATATCAGACAA  900
Pv-C      TAGTATCGGCAGCTTTTGGTAGTTGAAGGTAGTATCTCAATTGGACCTGATATCAGACAA  900
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Pv-1      GACTACCCGCTGAACTTAAGCATATTAATAAGCGGAGGAAAAGAAAC  947
Pv-C      GACTACCCGCTGAACTTAAGCATATTAATAAGCGGAGGAAAAGAAAC  947
******************************************************************************

**Figure S2.** DNA sequence comparison of *P. violae* strains for both *cox2* gene and the ITS region through Clustal Omega Multiple Sequence Alignment. Pv-1 represents the locally isolated *P. violae* strain and Pv-C represents the *P. violae* strain from WSU.
CLUSTAL O(1.2.4) multiple sequence alignment
Sequences (1:2) Aligned. Score: 97

cox2 gene:

Ps         GGCAAATGGTTTTCAAGATCCTGCAACCCCCAGTTATGGAAGGTATAATTAACTTTTACC 60
Ps-SL      GGCAAATGGTTTTCAAGATCCTGCAACCCCCAGTTATGGAAGGTATAATTAACTTTTACC 60

Ps         ACGATTTAATGTTTTTTTTATTTATCTGTTGATATTTTATTAGAG 120
Ps-SL      ACGATTTAATGTTTTTTTTATTTATCTGTTGATATTTTATTAGAG 120

Ps         TTATTACCTTTTTTGATGAAAAAATAAAATACCCCTCTCTACTGTTGACATGTGCA 180
Ps-SL      TTATTACCTTTTTTGATGAAAAAATAAAATACCCCTCTCTACTGTTGACATGTGCA 180

Ps         CTATTGAAATTATTTGGACATCAGTACCTGCTTTAATTTTATTAACTGTAGCTGTACCTT 240
Ps-SL      CTATTGAAATTATTTGGACATCAGTACCTGCTTTAATTTTATTAACTGTAGCTGTACCTT 240

Ps         CATTTGCTTTATTATATTCTATGGATGAAGTTATTGATCCAATTATTACTTTAAAAGTAA 300
Ps-SL      CATTTGCTTTATTATATTCTATGGATGAAGTTATTGATCCAATTATTACTTTAAAAGTAA 300

Ps         TAGGAATCTGAAATGGTATTGGAGTTATGAATATTCTGATAATTTAGAATTTTCTGATGAC 360
Ps-SL      TAGGAATCTGAAATGGTATTGGAGTTATGAATATTCTGATAATTTAGAATTTTCTGATGAC 360

Ps         CTTTAATTTTTGATAGTTATATGGTGCAAGAAAATGATTTAGAAATAGGTCAATTTAGAC 420
Ps-SL      CTTTAATTTTTGATAGTTATATGGTGCAAGAAAATGATTTAGAAATAGGTCAATTTAGAC 420

Ps         TTTTAGAAGTATGTAATTTCATGTTAGTATGTTCCAACATATATAGCTATATTAGATATATTA 480
Ps-SL      TTTTAGAAGTATGTAATTTCATGTTAGTATGTTCCAACATATATAGCTATATTAGATATATTA 480

Ps         CAGCTTCAAGATGTTTTACATTCAATGCGCTATACCTTTTTGATATAAATTAGATGCTTT 540
Ps-SL      CAGCTTCAAGATGTTTTACATTCAATGCGCTATACCTTTTTGATATAAATTAGATGCTTT 540

Ps         GTCCAGGTCGTTTAAATCAAGACAACTTCATCTGTTTATGAAAAGAAGAGGATATTTTATGGCC 600
Ps-SL      GTCCAGGTCGTTTAAATCAAGACAACTTCATCTGTTTATGAAAAGAAGAGGATATTTTATGGCC 600

Ps         AATGTAGCGAAATTTTGATGGAATTAAATCA 628
Ps-SL      AATGTAGCGAAATTTTGATGGAATTAAATCA 628

*************** ** **********************************
CLUSTAL O(1.2.4) multiple sequence alignment
Sequences (1:2) Aligned. Score: 100

**ITS region:**

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**Figure S3.** DNA sequence comparison of *P. spinosum* strains for both *cox2* gene and the ITS region through Clustal Omega Multiple Sequence Alignment. Ps represents the *P. spinosum* strain isolated from the Bakersfield area and Ps-SL represents the *P. spinosum* strain isolated from the Riverside area.