Pathogen Diversity and Biological Control of Verticillium Wilt of Tomato

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Declaration

I, Hazim Sabah Al-Hamadani, declare that the ideas, experimental work, results, analyses, and conclusion reported in this dissertation are entirely my own efforts, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted for any other award.

Signature of candidate

May 2021
Date

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Abstract

Verticillium dahliae is an important soil-borne pathogen that attacks a wide range of hosts, resulting in economic crop losses worldwide. With mounting concerns over pesticide use within current consumer markets, there is a need to search for alternative control options. One such approach is the development of biocontrol agents. This was the purpose of this study, which explored the effect of biological control strategies for tomato Verticillium wilt caused by *V. dahliae* using antagonistic fungi, either alone or in combination with or without salicylic acid (SA).

Pathogenicity of four strains of *V. dahliae* included DAR 33757 from cotton, DAR 31890 from tomato, DAR 44537 from potato and DAR 81260 from olive, and these were assessed on the tomato Grosse Lisse cultivar. Among these strains, *V. dahliae* DAR31890 from tomato was significantly more virulent on tomato. Therefore, this strain was selected in all subsequent experiments.

Potential antagonistic fungi were isolated from cotton roots and soil that adhered to the roots (rhizosphere soil) and evaluated as potential biocontrol agents (BCAs) against *V. dahliae*. The greatest suppression of fungal activity towards the pathogen was recorded by isolates 2 and 7, which were isolated from rhizosphere soil and subsequently identified as *Trichoderma harzianum* and an *Aspergillus* sp.

In vitro, these antagonists inhibited microsclerotia (MS) germination and mycelial growth of *V. dahliae*, reduced disease severity and promoted tomato plant growth in the glasshouse experiments. Thus, these two fungi were chosen as BCAs for further experiments in the next experiments.

Hoagland nutrient solution plus tomato root exudates significantly stimulated MS germination compared to water agar only, and also increased the fungal weight of the pathogen and antagonistic fungi in liquid culture experiments. Of the root exudate compounds tested, amino acids, organic acids, and sugars significantly stimulated MS germination compared with the water agar treatments.

Dipping tomato seedling roots in spore suspensions of *T. harzianum* or *Aspergillus* sp. prior to inoculation of stems with *V. dahliae* significantly reduced disease severity and increased tomato growth parameters. The results also showed that soluble protein concentration was significantly increased in tomato leaves treated with BCAs.

SA at concentrations of 0.5, 1 and 2 mM *affected* mycelial growth of *V. dahliae, T. harzianum* and *Aspergillus* sp. Tomato seed germination and seedling vigour were enhanced at 0.5 mM concentration of SA and significantly reduced at 2 mM concentration.

The combination of SA and BCAs enhanced activities of these fungi against *V. dahliae*. The combination of SA and BCAs significantly reduced disease severity and enhanced shoot dry weight of tomato plants more than using either antagonists or SA alone.

Overall, this study provides insight into the efficacy of *T. harzianum* and *Aspergillus* sp. isolates for biological control of *V. dahliae*. Also, a combination of BCAs and SA can improve the consistency of biological control against plant pathogens more than using them individually.

Chapter 1. Introduction

1.1 Overview of Verticillium wilt in tomatoes

Tomato is a major vegetable crop widely consumed around the world. It is utilized in a diverse range of cuisines, whether enjoyed fresh or processed in sauces, pastes, soups, juices, concentrates, and other products (Bergougnoux 2014, Çolak et al. 2020). Tomatoes have considerable nutritional and health benefits for the body and are a good source of nutrients, vitamins, mineral, and antioxidants (Vélez-Terreros et al. 2021). Tomato plants are impacted by range of natural enemies including insects, mites, fungi, bacteria and viruses. Various pathogens infect tomatoes and cause serious diseases (Yuqing et al. 2018, Liu & Wang 2020, Prasad et al. 2020), including the *fungus Verticillium dahliae,* which causes Verticillium wilt. Verticillium wilt is one of the most important disease in tomatoes, causing wilt symptoms and potentially leading to the death of the entire plant during the advanced stages of infection (Kumar et al. 2018, Sheu et al. 2023).

Verticillium wilt has become a serious problem in many plant species in numerous countries around the world, being reported to affect over 400 plant species (Ziazia et al. 2021). Hosts include several economically important crops such as those in the Solanaceae (e.g. potato, tomato, eggplant), Cucurbitaceae (e.g. melons, cucumber), Brassicaceae (cabbage, Brussel sprouts), Rosaceae (e.g. strawberry), Asteraceae (e.g. sunflower) and Malvaceae (cotton) (Kong et al. 2022).

1.2 Managing Verticillium wilt

Several methods have been employed to mitigate the effect of Verticillium wilt, such as using resistant cultivars, fungicides, crop rotation, root exudates, biological control, and induced systemic resistance (Trapero et al. 2013, Benouzza et al. 2021, Ramírez-Gil & Morales-Osorio 2021, Zhao et al. 2023). *Resistant cultivars are a favourable strategy for the control of plant pathogens, particularly Verticillium wilt, due to their efficiency, low cost (once developed) and limited environmental impact. integrating of resistant cultivars into a disease management approach is often the most promising strategy of controlling Verticillium wilt* (Song et al. 2020, Devi et al. 2021, Valverde et al. 2023). *The use of fungicides is challenging due to increasing concerns about health and environmental side effects. Additionally, the endophytic life cycle of the pathogen renders it largely inaccessible to fungicide applications* (Wei et al. 2019). *While crop rotations can be beneficial for some diseases, especially Verticillium wilt, they should be viewed as a preventative rather than a curative disease management option. This is because microsclerotia can survive in the soil for long periods, and the pathogen has a wide host range. Therefore, crop rotation is generally less effective compared to other*

disease management methods (Wheeler et al. 2019). *However, controlling Verticillium wilt in plants remains challenging using resistant cultivars, fungicides, and crop rotation due to the structure of microsclerotia and its long-term survival with host plants.*

Root exudates are substances released into the soil by plant roots, including amino acids, sugars, organic acids, peptides, vitamins, enzymes, fungal stimulators, nucleotides, inhibitors and attractants and many miscellaneous compounds (Tan et al. 2013, Zhang et al. 2014, Vishwakarma et al. 2017a). These compounds serve as essential nutrients for soil organisms, including both pathogens and biological control agents. López-Moral et al. (2023) found that root exudates from olive plants significantly induced microsclerotia germination of *V. dahliae* compared to the control treatment (without exudates). Fernández et al. (2017) *discovered that media containing gluconic acid from tomato root exudates increased growth of Trichoderma asperellum strain T34.* Therefore, understanding the interactions between root exudates and biocontrol agents may help to identify improved control methods.

Biological control utilises living organisms such as fungi, bacteria and insects against plant pests (Fontana et al. 2021, Lahlali et al. 2022). These organisms have different mechanisms for affecting plant pathogens including competition, antagonism and mycoparasitism (Ghorbanpour et al. 2018, Köhl et al. 2019). Many studies have reported the use of biological control agents to inhibit *V. dahliae* either directly via mycoparasitism, or indirectly through competition for nutrients and space, or biochemical effects due to antibiotics promoting plant growth and stimulating plant defences. Results of Kong et al. (2022) showed that volatile organic compounds produced by *T. koningiopsis* T2 significantly inhibited mycelium growth of *V. dahliae* and reduced disease severity in cotton and tobacco. Results of Zhang et al. (2023) indicated that volatile compounds produced by *Bacillus* sp. T6 strain inhibited *V. dahliae* spore germination and mycelial growth. Azabou et al. (2020) demonstrated that *B. velezensis* OEE1 significantly decreased Verticillium wilt of olive *induced* by *V. dahliae*. Results of Li et al. (2014) showed that the non-volatile substances produced by *Penicillium simplicissimum* completely inhibited *V. dahliae* growth.

Trichoderma species are known to be excellent biological control agents against plant pathogens that cause significant losses to many economic crops. Studies indicated that a great number of fungal strains that used in biological control programs belong to the genus *Trichoderma* (Ghazanfar et al. 2018, Benouzza et al. 2021, Thakur 2021). This fungus has achieved remarkable success in reducing the infection of *V. dahliae* on different crops such as cotton (Kong et al. 2022), olive (Reghmit et al. 2021), tomato (Benouzza et al. 2021), and eggplant (Bilginturan & Karaca 2021).

In addition to these roles and functions that biological control agents are known to enact, they are also potentially involved in stimulating plant defence mechanisms. Induced resistance is one such

plant defence reaction that either gives some plant varieties resistance to pathogens or *tolerance* to infection. This is because of chemical compounds that include proteins and phytoalexins that inhibit the growth of pathogens (Pagán & García-Arenal 2018, Ninkuu et al. 2022). These compounds increase within the plant due to the influence of biotic or abiotic agents, such as fungi, bacteria, on expression levels of salicylic acid and jasmonic acid (Deenamo et al. 2018, Stempien et al. 2020, Kaur et al. 2022).

Although, biological control is a promising alternative, it is not widely used in tomato production. Combining it with other non-conventional control methods may improve its effectiveness against Verticillium wilt in tomatoes. For example, induced resistance is a common mechanism of biocontrol (Romera et al. 2019), and resistance inducers like salicylic acid are effective against some diseases (Salwan et al. 2023).

1.3 Research questions

The objective of this study was to examine the research gaps related to the effects of biological control agents against Verticillium wilt in tomatoes, evaluate the relevant modes of action and to understand potential strategies for control. The aim was to answer the following research questions:

1. Do the isolated fungi have the ability to control *V. dahliae* and stimulate plant defences against Verticillium wilt in tomato?
2. Do root exudates affect the germination and growth of the pathogen *V. dahliae* and potential of fungal isolated (antagonist fungi)?
3. Does the combination of fungal isolates and salicylic acid improve the control of *V. dahliae* compared with individual treatments?

Chapter 2. Literature review

Scientists are looking for a new strategy for managing of tomato Verticillium wilt that is effective and not harmful to the environment or human health. Biological control is an alternative to chemical control, safer and ecologically acceptable in the management of plant diseases. Previous studies have demonstrated that interaction of biological control agents (BCA) with other chemical compounds has wide range of effectiveness against plant pathogen. However, there are no or few studies that speak of the role of chemical compounds in improving biological control in managing of tomato Verticillium wilt. This chapter will review previous work on Verticillium wilt, disease management, and the role of BCAs in controlling plant pathogens through various mechanisms such as inducing plant defence against pathogen attack.

2.1 Tomato plant

Tomato (*Solanum lycopersicum* L.) is one of the most important economic crops in the world and belongs to the Solanaceae family. It is believed that the original habitat of this crop is Central America, where it had great importance for the ancient Incas and Aztecs (Dorais et al. 2001). During the 16th century, tomato was introduced to Europe and later spread to the Mediterranean area. Breeding development has focused on four key areas: (1) high yielding varieties (2) resistant varieties (3) long shelf-life and (4) taste and nutritive quality (Alsamir 2019). Tomato is grown either in the field or under greenhouse conditions, and it is an essential source of phytochemicals and nutrients such as potassium, lycopene, iron, folate, and vitamin C (Bhowmik et al. 2012). Furthermore, tomato is produced for either fresh fruit or processed food industries while preserving its nutritional value (Bhatia et al. 2004, Heuvelink 2018). There are several health benefits for tomato and their products in a diet, this is attributed to their antioxidant content (Ali et al., 2020). According to Food and Agriculture Organization Statistics FAOSTAT (2021) The top three tomato-producing countries were China (67.5 Mt), India (21.1 Mt) and Turkey (13.1 Mt), and Asia was the largest tomato producing region (Figure 2.1).

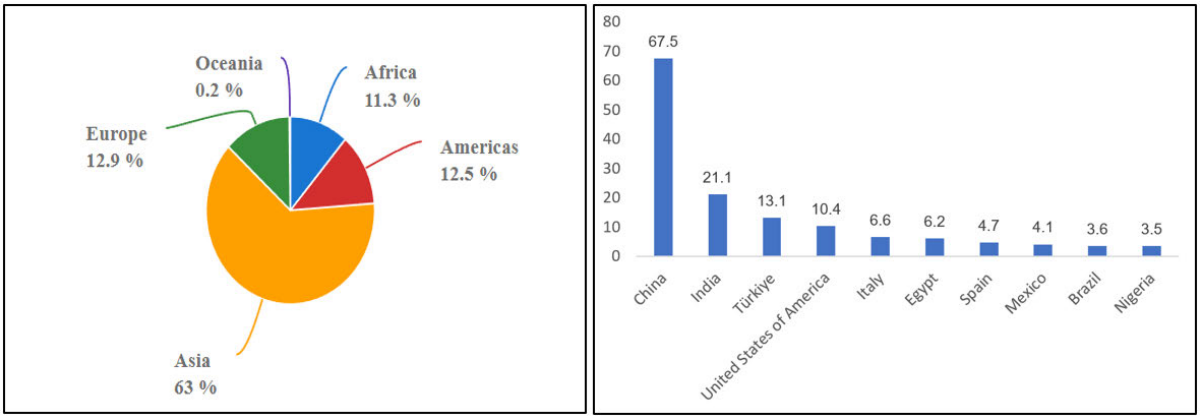


Figure 2.1 Tomato production by region (left) and the top tomato-producing countries globally in 2021 (right). (FAOSTAT, 2021).

It is known that different types of pathogens, including fungi, bacteria, viruses are capable of inducing disease in tomato plants. One of the major problems in tomato cultivation is Verticillium wilt disease caused by *V. dahliae* (Kleb.), that causes wilting disease in many important economic crops (Jabnoun-Khiareddine et al. 2009b).

2.2 Verticillium wilt

Verticillium wilt is an important vascular wilt disease that affects plants in many countries of the world (Zhou et al. 2013, Zhang et al. 2014, Xiu-hua et al. 2015). It is now known to infect many plant hosts including trees, shrubs, ornamentals, weeds, and other herbaceous plants. Also, it is a major factor in determining the production for many agricultural crops, including cabbage, chili pepper, cotton, eggplant, tomato, potato, and watermelon (Bhat & Subbarao 1999). This disease causes a significant loss in the quality and quantity of the crop. It sometimes leads to death of the entire plant or to severe damage before harvest date. In China, Verticillium wilt led to losses of 30% in cotton production between 1970 and 1980 (Bugbee & Sappenfield 1970). In 1961, about 580,000 bales of cotton were lost due to Verticillium wilt in the United States (Albers 2013). *V. dahliae* and *V. albo-atrum* (in the broad sense) cause losses of billions of dollars annually to crops worldwide. The losses in potato yields rose to 50% measuring the natural situation 10 – 15%, while in lettuce crops, losses have reached 100% (Klosterman et al. 2009). Verticillium wilt of alfalfa is also very important, especially in North America, Europe, and Japan. In the United State and Canada Verticillium wilt causes losses in alfalfa yield of up to 60% (Calpas 1991). Many studies indicated that the members of the ascomycete genus *Verticillium* are the main cause of Verticillium wilts (Hanson 2000, Negahi 2013, Zhang et al. 2013, Inderbitzin & Subbarao 2014). The taxonomic history of *Verticillium* is long. In 1816, the first species of *Verticillium* was found and about 190 species have been described. The taxonomy of *Verticillium* has changed and nowadays we distinguish ten *Verticillium* species, *V.*

dahliae, *V. albo-atrum*, *V. alfalfae*, *V. nonalfalfae*, *V. klebahnii*, *V. nubilum*, *V. tricorpus*, and *V. zaregamsianum V. isaacii*. Five new *Verticillium* species *are* described as new species *V. alfalfa*, *V. nonalfalfae*, *V. klebahnii*, *V. zaregamsianum* and *V. isaacii*. In the new taxonomy, *V. albo-atrum* is split into three species: *V. albo-atrum*, *V. alfalfae* and *V. non-alfalfae*. Both *V. dahliae* and *V. non-alfalfae* (but not *V. albo-atrum*) are pathogenic on tomato (Inderbitzin et al. 2011, Inderbitzin & Subbarao 2014).

V. dahliae is a soil-borne pathogen that can lead to plant vascular disease and significant economic loss on over 400 plants species worldwide, including economically important crops (Song et al. 2020). The first isolation of *V. dahliae* was from dahlia plants and identified as a new species by Klebahn in 1913 (Vermeulen 2020). In tomato plants, isolates of *V. dahliae* were designated as race 1, and non-race 1, using differential lines of tomato with and without the *Ve* gene responsible for resistance. However, the race 2 of *V. dahliae* broke the *Ve* resistance (Bubici & Cirulli 2008). *In 2017, two tomato cultivars, Aibou and Ganbarune-Karis, were shown to be resistant to some non-race 1 strains of V. dahliae. Isolates of V. dahliae that were non-pathogenic for these cultivars were termed race 2, while isolates pathogenic to Aibou and Ganbarune-Karis cultivars were termed race 3* (Acharya et al. 2020). Bender and Shoemaker (1984) reported that in 1978, about 56% of 108 tomato fields in Western North Carolina affected by Verticillium wilt. The author also indicated that 89 of 96 *V. dahliae* isolates were race1, and 7 were race 2. According to Karagiannidis et al. (2002), *V. dahliae* reduced tomato fresh and dry weight (28 and 35%, respectively), and plant height by 14%. Results of (Ashworth Jr et al. 1979) demonstrated that increase of *V. dahliae* inoculum density caused increasing in tomato infection rate, and restriction of root growth. *Plants infected* by the fungus *V. dahliae* exhibited a reduction in the rate of photosynthesis, a smaller leaf area, and stomatal conductance (Buhtz et al. 2017). Bollig et al. (2013) found that leaf photosynthesis rates were impacted, and root length diminished in *Verticillium*-infected tomato plants. Tomato plants inoculated with *V. dahliae* showed yellowing at leaf margins, necrotic spots, followed by wilt and leaf drop (Mazzotta et al. 2022). *V. dahliae* is the most common species that causes damage to the tomato crop and will be the focus of this thesis.

2.2.1 Morphology of V. dahliae

In 1816, Nees von Esenbeckin erected the genus *Verticillium* based on morphological characters, and more than 190 species were described under the genus *Verticillium* (Zare et al. 2004). *Verticillium* belongs to the phylum Ascomycota and the sexual stage is unknown or yet to be discovered (Klosterman et al. 2009). Species of *Verticillium* can be differentiated morphologically by the types of resting structures they form. *V. albo-atrum*, *V. alfalfae* and *V. nonalfalfae* form resting mycelium.

V. dahliae form MS. *V. isaacii* form chlamydospore and microsclerotium. *V. klebahnii* form chlamydospore and MS. *V. longisporum* form microsclerotium. *V. nubilum* form chlamydospore (Inderbitzin et al. 2011). According to Yu et al. (2016) *Verticillium* isolates were divided into two groups depending on microsclerotial shape and conidial length. The first group is fit the description of *V. dahliae*, colony of *V. dahliae* originally *appears* white or a light cream colour, but eventually become black with the formation of MS (Figure 2.2). Mycelium is abundant fluffy and septate, multinucleate, hyaline, and branched. Conidia *are* cylindrical to oval hyaline, unicellular, and borne singly on whorl conidiophore, measuring < 6.0 µm in length. MS are spherical and compact, black or dark brown, and measuring 20 – 200 µm in diameter. Phialides are sharp at the apices, and the short branching is grouped in whorls. The second group fit the description of *V. longisporum*, colonies are whitish to grey. Mycelium is sparse aerial, hyaline, and cylindrical to ellipsoid, measuring < 6.5 µm in length. MS are elongate to irregularly spherical, measuring 30 – 250 µm in diameter.

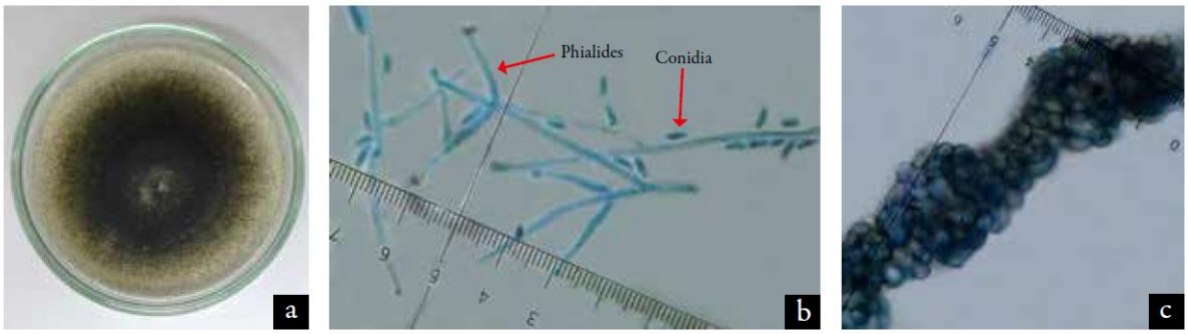


Figure 2.2. Colony of *Verticillium dahliae* after 15-days on PDA (a), Conidia and phialides (40X) (b) and MS (40X) (c) (Leon-Ttacca et al. 2019).

2.2.2 Life cycle of V. dahliae

The life cycle of *V. dahliae* is usually similar in most plants. During the winter season, the fungus remains in plant debris and soil as MS (Chawla 2011). Soesanto and Termorshuizen (2001) indicated that MS could withstand the extreme temperatures, as *is* able to survive within a wide range of temperature from10 - 30 ºC, although with low viability at 10 and 30 ºC. MS can remain in the soil for up to 14 years, until MS are stimulated to germinate by the presence of root exudates (Klosterman et al. 2009, Aljawasim 2014). There are three major stages during the life cycle of Verticillium wilt fungi: infection, colonisation, and invasion.

During the infection stage, hyphae *begin* to grow toward the roots of plants because of the exudation of materials produced such as amino acids, organic acids and sugars that stimulate the germination of pathogenic fungi (Schroth & Hildebrand 1964). Following germination of MS, the hyphae begin to penetrate the plant through the ruptured epidermal root cells, root tips, lateral roots, or wounds.

After that, hyphae grow through the cortex of the plant intercellularly and intracellularly, eventually the fungus begins to produce conidia which colonise the xylem (Smith 1979, Hiemstra & Harris 1998, Albukhari 2015).

The second stage is colonisation; mycelium of the fungus begins to produce asexual conidia which remain within xylem tissue. Eventually, the fungus begins to attack the plant, and it secretes toxins and enzymes working on the decomposition of cellular walls of plant (Carder et al. 1987, Dobinson et al. 1997). The mycelia and conidia clog the vascular tissue, which prevents transport of nutrients to the vegetative part of the plant. Xylem tissues become congested with gums, gels and tyloses. These structures are produced in the plant as a response to the invading pathogens. At the end, inhibiting the transport of nutrients and water lead to closed stomata, yellowing and wilting of leaves, and plant death (El-Zik 1985).

Finally, the fungus begins to invade all plant tissues, and continues to infect other neighbouring plants through the formation of large numbers of fungal MS. By the end of the growing season or after the host dies, the fungus is able to survive as MS in the soil or on plant debris lasting for many years (Agrios 2005).

2.2.3 Symptoms

Wilt symptoms appear at different times. However, most shrubs and trees show wilt symptoms in summer. Symptoms in some of the Midwest areas of the USA may show in early March (spring) and in late November (autumn) (Smith 1979). Recognising external symptoms often is difficult because wilt symptoms are similar to those expressed by Fusarium wilt, early blight and bacterial canker so it is often confused with all these diseases. *Verticillium* affects the plant uniformly and wilt symptoms do not appear just on one side. Yellowish blotches and brown veins may appear on the lower leaves, and finally brown dead spots. The brown spots are light brown and not dark brown to blackish that caused by early blight (Horticulture Diagnostic Laboratory 2018). Wilt symptoms usually appear in a V- shape on lower leaves or leaflets with yellowing at leaf margins in a fan-shape. As symptoms progress, some brown necrotic spots appear on infected plants leaves and leaf veins become brown (McAvoy 2018). Verticillium wilt also has effects on growth traits in plants such as lateral branching, plant length, and concentration of substances in roots, stems, leaves (Xiao & Subbarao 2000). Cross-section of the stem in a *Verticillium*-infected plant shows a discoloration, which occurs due to increase in the rate of some toxic compounds such as melanin in the xylem vessel cell walls and vascular tissues which prevent water from reaching leaves and plant branches (Yadeta & Thomma 2013). While *Fusarium* attacks plant of all ages causing wilting and dying of lower leaves with loss of green colour in the leaf midrib, often on one side of the plant, and the plant often dies before reaching

fruiting stage. When the lower portion of the stem is cut lengthwise, uniform brown discoloration appears in the woody tissue (Chitwood-Brown et al., 2021).

2.3 Management of Verticillium wilt

2.3.1 Chemical control

Chemical compounds such as pesticides are very important for control of plant pathogens through speed and direct impact (Miyake et al. 2005, Bruck 2009, Hirooka & Ishii 2013). On the other hand, their potential harmful influence on the environment and human health and misuse of agrochemicals led to a decline in use and the prohibition of many pesticides in agriculture (Rowe & Powelson 2002, Pal & Gardener 2006, Goicoechea 2009). Also, some chemicals are very expensive (Compant et al. 2005). For example, chloropicrin, methyl bromide and ethylene bromide have a high impact on *V. dahliae* population in the soil, but those chemicals were costly and can only be justified for control in high-value crops (Mapope 2005). In addition, methyl bromide and similar fumigants are no longer used because of their effects on the ozone layer (Taylor 1994). According to Sonmez et al. (2006) increase of using fungicides that have copper (Cu) caused decreased tomato yields, fruit number and dry weight of the root system. Although some fungicides like trifloxystrobin, azoxystrobin and prochloraz have a significant effect on Verticillium wilt, there are no recommended specific fungicides for Verticillium wilt on any crop (Kurt et al. 2003, Bubici et al. 2006). The overall challenges of managing soil borne disease has led to the need for integrated approaches that do not use chemical application, like biocontrol or systemic resistance for disease management.

2.3.2 Resistant cultivars

The use of resistant plants to control plant disease is a form of biological control. Over the last decades, selection methods were used to obtain resistant plants rather than breeding methods. *When* the disease occurs, the resistant plants survive until the end of season and produce seeds for planting in the following seasons. Plant breeding in order to obtain disease-resistant plants gave a scientific basis for subsequent studies. In the early 1900s, Rowland H. Biffin from Cambridge University in England showed that resistance in some wheat cultivars to rust diseases was inherited according to Mendel 's laws, which is controlled by Mendelian genes. These are sometimes known as resistance genes (*R* genes) or major genes because of *their* major role in plant resistance. After this finding, many breeding programs worked to incorporate resistance genes into many important economic crops (Keane & Brown 1997). It is possible to *explore* resistance-related genes from various perspectives such as the cell wall, transcription factors, pattern recognition receptors (PRRs), enzymes, and salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Song et al. 2020).

Several plant diseases resistance (*R*) genes have been cloned and assigned to classes based on structural features. For example, *PRS2* and *RPM1* resistance genes of *Arabidopsis thaliana* against *Pseudomonas syringae* (Bent et al. 1994). *N* gene from tobacco against tobacco mosaic virus (Whitham et al. 1996). *L6* gene from flax against *Melamspora lini* (Lawrence et al. 2007). *RPP5* gene from *Arabidopsis* against *Peronospora parasitica* (Parker et al. 1993). *Xa21* gene from rice against *Xanthomonas* (Peng et al. 2008). In tomatoes, *Pto* genes *change by confer* resistance to *Pseudomonas syringae* (Thilmony et al. 1995). *LRR* gene against *Cladosporium fulvum on tomato* (Jones et al. 1994). The *Ve1* gene conferring resistance to race 1 isolates of *V. dahliae* and *V. albo-atrum* (Acharya et al. 2020).

Tomato *Ve* encodes surface receptors that called receptor-like protein (RLPs) class, which typically *consists* of an extracellular leucine-rich repeat domain. The RLPs class encompasses proteins that play an important role in disease resistance, such as *Ve1* and *Ve2* genes *confer* resistance against *V. dahliae* and *V. albo-atrum*. Cf resistance proteins that confer resistance against the leaf mould pathogen *C. fulvum* (Kruijt et al. 2005).

There have been many papers on testing of resistance of tomato varieties to *V. dahliae* (Acharya et al. 2020, Song et al. 2020, Chavarro-Carrero et al. 2021, Udriște et al. 2022). According to Fradin et al. (2009), *Ve1* provides resistance in tomato plants against race 1 of *V. dahliae* and *V. albo-atrum*. Usami et al. (2017) reported that although some tomato cultivars containing the *V2* locus such as Aibou and Ganbarune-Karis were resistant to *V. dahliae* race 2, some other isolates of *V. dahliae* race 2 can overcome this resistance and these are referred to as race 3. This type of breakdown of resistance is likely to be a continuing problem with the use of single gene resistance. The *Ve1* gene codes for a cell surface-like receptor which gives resistance to race 1 isolates of *V. dahliae* by recognising the Ave1 effector of the pathogen and that this resistance can be overcome by non-race 1 strains of *V. dahliae* (Acharya et al. 2020).

Although there are ongoing research projects into *Verticillium* resistance, the development of new tomato varieties and the current varieties do not provide a substantial increase in resistance. Also, some tomato varieties were resistant to *V. dahliae*. However, other isolates of *V. dahliae* overcame this resistance. This type of breakdown in the resistance leads to continuation of the problem.

2.3.3 Biological control

Biological control means using living organisms such as fungi, bacteria, and insects against plant pests and diseases (Prajapati et al. 2020). These natural enemies achieved great successes in this area and have created a kind of natural balance (Hajieghrari et al. 2010). It is believed that the first reference in the scientific literature to the term biological control dates to the article published by the

German plant pathologist Carl von Tubeuf in 1914 entitled “Biologische Bekampfung von Pilzkrankheiten der Pflanzen". Since then, many biocontrol products have been found to be effective against plant diseases (Prajapati et al. 2020). The terms "biological control" or its synonym name "biocontrol" have been used in different fields of biology such as entomology and plant pathology. In the entomology field, the term biological control or biocontrol refers to the use of predatory insects, microbial pathogens and entomopathogenic nematodes to suppress the different populations of insects. As for plant pathology, it refers to suppressing the diseases by microbial antagonists in addition to control of weed populations by use of host-specific pathogens (Pal & Gardener 2006).

A biological control agent is an organism, such as an animal (e.g., beneficial nematodes), insect (e.g., arthropod predators), microorganisms (e.g., bacteria, fungi, and viruses) (Van Lenteren et al. 2020) that is used to control a pest species including plant pathogens.

Beneficial bacteria such as *Pseudomonas* and *Bacillus* use a large variety of mechanisms to protect plants from pathogens. According to Manikandan et al. (2010) the combination of seed treatment, seedling dip and soil drenching of *P. fluorescens* liquid formulation recorded the minimum disease incidence of Fusarium wilt on tomato, and increased fruit yield compared to untreated control under glasshouse and field conditions. Previous studies conducted by Mavrodi et al. (2012) revealed that *Pseudomonas* strains significantly reduced disease symptoms of both *Rhizoctonia solani* AG-8 and *Pythium ultimum* on wheat crop. Zhang et al. (2020) found that volatile organic compounds (VOCs) from *Bacillus* significantly reduced the colony size and mycelial penetration of *Aspergillus solani in vitro*, and inhibited the conidia germination, and the lesion areas, *in vivo*. Results of Khedher et al. (2015) showed that *B. subtilis* V26 significantly inhibited *R. solani* growth in vitro, and suppressed of potato root canker and black scurf tuber colonisation compared to control treatments under greenhouse conditions.

Fungal biological control agents play a significant role in controlling plant diseases throughout the world. According to Hassine et al. (2022), *culture* filtrates of *Penicillium* sp. and *Gliocladium* spp. significantly reduced mycelial growth and spore germination of *Colletotrichum coccodes* that causes anthracnose disease on tomato. Results of Ehrlich (1987) revealed that co-inoculation of non-aflatoxigenic strains of *A. parasiticus* with aflatoxigenic strains substantially reduced the production of aflatoxins in corn under in vitro conditions. A study conducted by Abbas et al. (2011) demonstrated that soil inoculation of non-aflatoxigenic strain of *A. flavus* with aflatoxigenic strain mixture significantly reduced aflatoxins contamination in corn.

Trichoderma species are widely used in agriculture to control various plant diseases affecting crops. Integrated use of both *T. harzianum* and Carbendazim significantly reduced wilt incidence in eggplant caused by *F. oxysporum* f. sp. *melongenae* (Jamil & Kumar 2022). Six *Trichoderma* isolates strongly

inhibited mycelial growth of *Alternaria solani* in dual culture tests, and reduced early blight symptoms in greenhouse conditions (Metz & Hausladen 2022). Nofal et al. (2021) demonstrated that *T. atrovirde* had a significant inhibition against *F. oxysporum* and improved tomato growth, as well as the increase in phenol content in the plants. Similarly, the dual culture technique showed that *T. asperelloides* Ta41 had a high antagonistic activity against *R. solani* Rs33, and lowest disease index under greenhouse conditions (Heflish et al. 2021).

Biological control is usually environmentally safe and has been considered a viable alternative method to manage plant disease (Heydari & Pessarakli 2010). Because the conventional methods are not enough for controlling Verticillium wilt, there is great potential for BCAs to manage this disease (Deketelaere et al. 2017). BCAs can protect plants against Verticillium wilt through different mechanisms include mycoparasitism, antibiosis, competition for nutrients and infection sites, plant growth promotion, and induced resistance (Deketelaere et al. 2017, Prajapati et al. 2020). The modes of action for the different antagonists against Verticillium wilt are shown in Table 2.1.

Table 2.1. Mode of action of selected biological agents against Verticillium wilt. 1VOCs: volatile compounds. 2DAMP: damage associated molecular pattern. Source (Deketelaere et al. 2017).

Genus antagonist	Reduced germination of inoculum	Plant growth promotion	Competition for infection sites/space/nutrients	Induced resistance	Antibiosis in vitro	Mycoparasitism in vitro
BACTERIA						
Bacillus	X (iturins)	X	X	X (iturins)	X	X
Paenibacillus	X	X		X	X	X
Streptomyces	X (prodiginines)	X	X	X	X	X
Pseudomonas		X	X	X	X	X
Serratia	X	X				X
FUNGI						
Pythium	X	X				X
Fusarium	X (VOCs)[1]	X	X	X (DAMP release)[2]	X	X
Trichoderma	X	X			X	X
Verticillium		X	X	X (DAMP release)		
Talaromyces	X				X	X
Penicillium		X		X (DAMP release)	X	X
Muscodor	X (VOCs)					
Gliocladium	X	X			X	X
Mycorrhizae		X		X		

Mycoparasitism

Mycoparasitism is the ability of fungal antagonistic agents to parasitise other fungi for utilising them as food. Mycoparasitism has been widely used for the biological control of plant pathogens.

Mycoparasitism depends upon the following events: Direct growth of the antagonists toward the hosts usually occurred by a chemical gradient of sugars and/ or amino acids, mutual recognition between antagonist and pathogen, lytic enzyme secretion by antagonist, antagonistic hyphae attack and coil around the host hyphae, penetration, and growth of antagonist into the host (Steyaert et al. 2003, Alizadeh et al. 2020). Various chemical compounds and enzymes can be included in these processes such as lectins, during the direct contact and recognition and cell wall-degrading enzymes, such as chitinases, proteinases, β-1,3-glucanases, and lipases, during the penetration process (Alizadeh et al. 2020). After recognition of the pathogen by antagonist, hyphae of the antagonist coils around the pathogen's hyphae and penetrate the pathogen cell and break down chitin by glucanase and chitinase. Subsequently, antagonistic hyphae release antibiotic compounds which penetrate the affected pathogen's hyphae and resynthesise the cell wall of the host inhibited by these compounds (Ghorbanpour et al. 2018). Results of Pachenari and Dix (1980) indicated that the disintegration of hyphal walls of *Botrytis allii* during parasitic *Gliocladium roseum* attack was probably due to increase β-1,3-glucanase activity. Results of Li et al. (2020b) showed that the endophytic fungus *Albifimbaria verrucaria* from wild grape significantly inhibited growth of *Botrytis cinerea* in dual culture assay and has the ability to produce chitinase.

Trichoderma spp. are widely used as biofertilisers and BCAs for plant disease. Mycoparasitism is one of the most important *Trichoderma* mechanisms to suppression plant diseases and reducing the pathogen inocula (Mukherjee et al. 2022). Microscopic observations of Benouzza et al. (2021) showed that hyphae of *T. harzianum* formed a hook or bunch around the hyphae of *V. dahliae* from where penetration took place. The author also noted that *T. atroviride* (T2) coiled around the pathogen and formed appressorium-like structures suggesting mechanical activity before penetration in the host. Results of Reghmit et al. (2021) showed that hyphae of *Trichoderma* isolates were observed coiled around mycelium of *V. dahliae* by direct confrontation, and the majority of these isolates were able to produce extracellular enzymes such as chitinases, proteases, and cellulases that demonstrated an effective potential in reducing mycelium growth of *V. dahliae in vitro*. Mycoparasitism of *Verticillium* by *Trichoderma* has been shown *in vitro*, but it is unclear how important this is *in vivo*.

Antibiosis

Antibiotics are microbial toxins or a secreted secondary metabolites with low molecular weight that is deleterious to other microorganisms at low concentrations, *are* found more in bacteria such as *Bacillus* spp. and *Pseudomonas* spp. than yeasts and fungi (Di Francesco et al. 2016, Alizadeh et al. 2020). In general, antibiosis is considered a biological process by which antagonists produce either antibiotics or another inhibitory secondary metabolite, such as one of a suite of volatiles. In both

cases, the result is inhibition of another member of the soil or plant microbial community (Gajera et al. 2013). In bacteria, particularly *Bacillus* and *Pseudomonas* numerous antibiotic metabolites, such as iturin, surfactin and fengycin, *diacetylphloroglucinol* (DAPG), pyrrolnitrin and phenazine, have been researched. In Fungi, particularly *Trichoderma* spp. are able to produce numerous antibiotic metabolites, such as hydrogen cyanide, ethylene, ketones, alcohols, and aldehydes (Tariq et al. 2020). The results of Fotoohiyan et al. (2017) suggested that metabolites produced by *T. harzianum* isolates were toxic and fungistatic against *V. dahliae* that causes Verticillium wilt on pistachio. El-Rafai et al. (2003) indicated that culture filtrate of *T. harzianum*, *T. hamatum*, *P. fluorescens* and *B. subtilis* isolates significantly decreased spore germination and germ tube length of *V. dahliae*. According to Zargar et al. (2019) two isolates of *Penicillium hordei* and *P. polonicum* were able to reduce the growth of *Colletotrichum nymphaeae* due to certain volatile and non-volatile compounds produced by these isolates. In another study, biocontrol potential of *F. oxysporum* and other isolated fungi *in vitro* was assessed against *V. dahliae* through activities of fungal cell wall degrading enzymes including chitinase, cellulase, and protease (Zheng et al. 2011). Kim et al. (1988) demonstrated that glucose oxidase extracted from culture filtrate of *Talaromyces flavus* reduced the radial growth of *V. dahliae* and MS germination Marois et al. (1984) observed that applying *T. flavus* to soil planted to cotton, tomato or eggplant caused increase in *T. flavus* populations and inhibited germination of MS of *V. dahliae*. In the same context, glucose oxidase secreted by *T. flavus* had played a major role in inhibition of *V. dahliae* hyphae and MS (Stosz et al. 1996, Murray et al. 1997). Naraghi et al. (2008) found that non-volatile extracts of *T. flavus* caused a significant inhibition in growth of *V. dahliae*. Antibiosis against *Verticillium* species has been demonstrated for several antagonists and is a common screening tool for selecting biocontrol agents using dual culture or the effect of culture filtrates on germination and growth.

Competition for nutrients and infection sites

Competition can be defined as microorganisms' challenge for available resources. For *Verticillium*, this could be particularly important, where a resource is in limited supply, competition for resources such as nitrogen, carbon, potassium, or iron, and/or infection sites in the soil and in/on the roots may be an efficient mode of action in controlling the disease (Deketelaere et al. 2017, Alshimaysawe 2018). It is expected that biocontrol agents compete for nutrients and infection sites in the rhizosphere and cortex, while some non-pathogenic strains of fungi such as *Verticillium* and *Fusarium* can also colonise the xylem and occupy the same niche as pathogenic *Verticillium* (Deketelaere et al. 2017). *Results of* Pantelides et al. (2009) *showed that the competition for nutrients or space on the root surface in eggplant are the main mechanism of action of Fusarium oxysporum against V. dahliae. A commonly cited example of competition between BCAs and Verticillium is that for iron* (Deketelaere

et al., 2017). Mulero-Aparicio et al. (2019) showed that non-pathogenic *F. oxysporum* strain FO12 is an efficient root coloniser, which could compete with *V. dahliae* in the same ecological niche on oil seed rape (*Brassica napus* L.). *Results of* Miranda-Fuentes et al. (2020) *showed that the protection conferred by B. bassiana and M. brunneum against verticillium wilt is probably the consequence of their competition with V. dahliae in the soil.* Another study included the role of *T. asperellum* T34 to control Fusarium wilt by competition for iron, Segarra et al. (2010) reported that adding iron Fe in the nutrient solution in different concentrations 1, 10, 100, and 1000 µM provided as EDTA/Fe (III), significantly reduced *Fusarium*-infected shoot by *T. asperellum* T34 at 10 µM Fe and improved plant growth promotion. Results of Gao et al. (2007) indicated that the inhibitory effects of *T. harzianum* isolate TH-1 against the pathogens of Verticillium wilt and Fusarium wilt of cotton were produced most by nutrition, space competition, and mycoparasitism. Most evidence for the role of competition has been gained for other pathogens, but it is likely to be a common component of antagonism of Verticillium wilt.

Plant growth promotion

Microorganisms in the rhizosphere play a pivotal role in promoting plant growth and protecting plants from pathogen attack by a range of mechanisms. These involve stimulation of root growth, enhancing nutrient uptake, biofertilisation, rhizoremediation, control of abiotic stress, and disease control (Mendes et al. 2013). Many saprophytic fungi, particularly isolates of *Trichoderma* species can not only protect developing roots from microbial attack in soil, but also produce a plant growth-stimulating factor (Baker 1989).

Altomare et al. (1999) found that biocontrol fungus *T. harzianum* Rifai 1295-22 was able to solubilise micronutrients and phosphate that were made available to provide plant growth. (Stewart & Hill 2014) Results of Bader et al. (2020) suggested that *Trichoderma* strains stimulated tomato plant growth through the production of phytohormones, which promoted increased leaf area, photosynthesis and uptake of phosphorus. Azarmi et al. (2011) found that three *Trichoderma* isolates including *T. harzianum* T969, *T. harzianum* T447, and *Trichoderma* sp. isolate T increased chlorophyll content, leaf area and number in tomato seedlings. According to Li et al. (2018) *T. asperellum* strain CHF78 significantly increased tomato dry weight and plant height when inoculated with or without *F. oxysporum* f.sp. *lycopersici* (FOL) compared to pathogen treatment only. The author also indicated that pre-inoculation with *T. asperellum* strain CHF78 significantly promoted nutrient uptake of K, Mg, P, and Zn as a result of reducing disease severity of Fusarium wilt. Jabnoun-Khiareddine et al. (2009b) found that fresh weights of tomato root and stem after 90 days of culture were increased more than 50% in plants treated with *Trichoderma* spp. in comparison to the untreated control. Growth

promotion of tomato by applied microbes could therefore be an important part of the improvement in plant health, due to antagonists stimulating positive responses in the associated plant.

Induced systemic resistance

Induced resistance is an enhanced level of protection in plants triggered by biological or chemical inducers against a broad range of pathogens and insect herbivores. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of induced resistance (Pieterse et al. 2014). SAR is triggered by local pathogen attack interacting with leaves, depend on two parallel pathways, one on SA, the other dependent on pipecolic acid (Pip) or N-hydroxy-pipecolic acid (Figure 2.3) (Vlot et al. 2021). The SA is the first chemical signal inducing the production of pathogenesis-related (PR) proteins. For example, PR genes code for β-1, 3-glucanases and chitinases plays an important role in preventing or reducing pathogen colonisation (Verma et al. 2019).

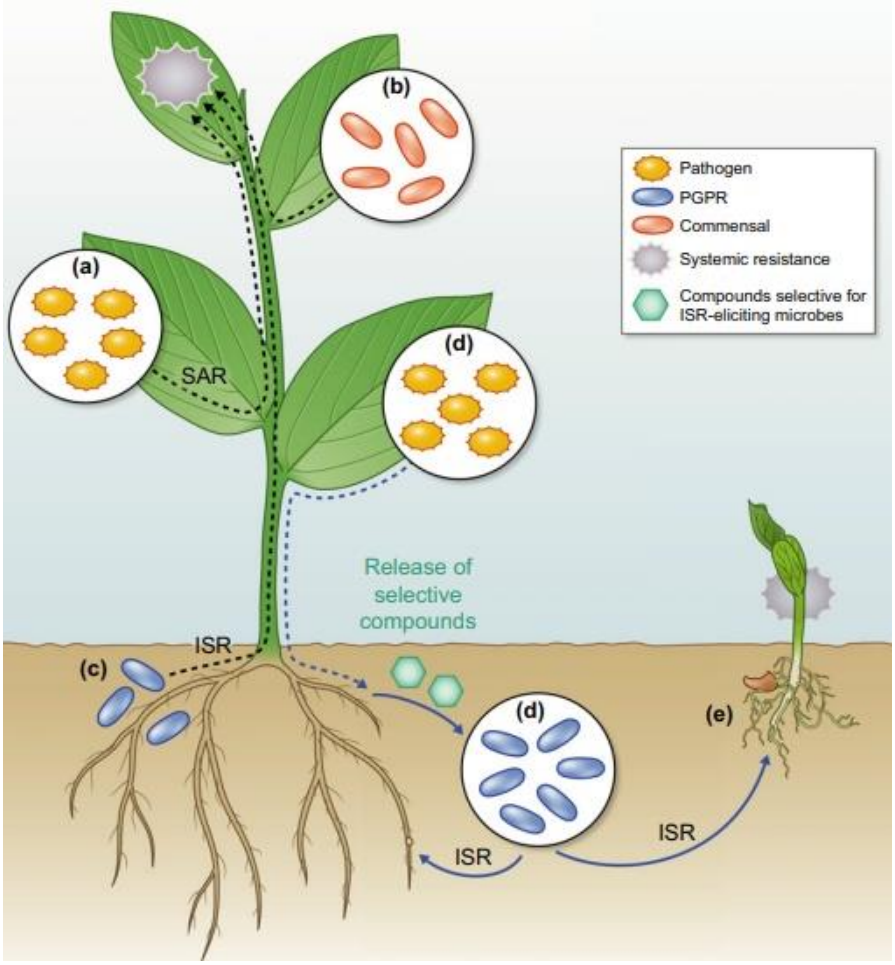


Figure 2.3. Reciprocal interactions between systemic immunity and the plant microbiome. (a) Pathogens on leaves can induce systemic acquired resistance (SAR). (b) Commensal microbes on leaves have been shown to trigger immunity. (c) Plant growth-promoting rhizobacteria (PGPR) can elicit induced systemic resistance (ISR). (d) Certain pathogenic bacteria on leaves can induce the release of compounds from plant roots into

the rhizosphere. (e) Some of these compounds selectively attract ISR-inducing microbes, which can protect successive plant generations growing on the same soil from disease. Source (Vlot et al. 2021).

Many studies have indicated that SA plays an important role in plant defence response against attack of pathogens (Ryals et al. 1996, Zhang et al. 2002, Yu et al. 2022, Kim & Lim 2023). Results of Briache et al. (2023) showed that seed pretreatment or foliar spray by SA at 1 mM and *indole acetic acid* (IAA) at 0.09 mM concentration gave the best control of *Orobanche crenata* parasitism on lentil. According to Feng et al. (2022) total phenolics content increased following foliar application of 0.5 mM SA and produced strong chemical defence responses in maize. Results of Mufti et al. (2023) revealed that SA in combination with PGPR play an important role in controlling Fusarium wilt by ISR in chickpea. Results of Saengchan et al. (2022) showed that SA at 500 µl. L^{-1} concentration significantly induced H_2O_2, peroxidase, polyphenol oxidase, and catalase activities in cassava plant.

On first encounter, plants can recognise microbial-associated molecular patterns or pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin and fungal chitin through PRRs localised on the plant cell surface. This leads to PAMP-triggered immunity (PTI). It is a form of basal resistance restricting growth of pathogen. However, pathogens have evolved to bypass this first line of defence. Therefore, plants acquired a second line of defence called effector-triggered immunity (ETI) through death of the infected and surrounding tissue fully restricting spread of the pathogen (Yu et al. 2022). Whereas ISR can be triggered by beneficial *soil-borne* microbes *including* plant growth-promoting fungi (PGPF), and plant growth-promoting rhizobacteria (PGPR), interacting with roots. and is often regulated by the hormones JA and ET (Pieterse et al. 2014).

The rhizosphere supports the activity and development of microbial community, including microorganisms capable to promote plant growth. Among the latter, PGPR and PGPF colonise roots and enhance plant growth by direct and indirect mechanisms. The *interaction of plant* with phytopathogens leads to activation plant defence mechanisms such as SAR and ISR pathways (Vacheron et al. 2013).

Many bacterial and fungal species that serve as BCAs by eliciting ISR include *Pseudomonas* spp., *Bacillus* spp., *Trichoderma* spp., *Penicillium* spp., non-pathogenic *Fusarium* and symbiotic arbuscular mycorrhizal fungi (Kloepper et al. 2004, Murthy et al. 2014, Fotoohiyan et al. 2015, Zehra et al. 2017b). The antagonist activity of these microbial species has been investigated and available as registered plant protection products for many economic crops such as tomato, potato, cotton, rice, sunflower, etc. (Zehra et al. 2021). BCAs can stimulate defence responses of plants through different pathways include the following mechanisms.

Reactive oxygen species (ROS) are key signalling molecules *that* play a crucial role control of various processes that enable the plant cells to rapidly respond to biotic and abiotic stimuli, thus contributing

to the establishment of defence mechanisms (Mittler et al. 2022). Plants under biotic or abiotic stress can produce many of ROS, including hydroxyl radical (OH), superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), etc. However, plant cell tissues are also exposed to damage because of the accumulation of ROS. Therefore, it is necessary to do an efficient scavenging of ROS by enzymatic and nonenzymatic reactions. In plants, mechanisms of enzymatic ROS scavenging dependent on peroxidase (POX), superoxide dismutase (SOD), polyphenol oxidase (PPO), glutathione peroxidase (GPX), catalase (CAT), and ascorbate peroxidase (APX), which are reducing superoxide to H_2O (Yu et al. 2022).

Bacillus cereus AR156 induced systemic resistance in *Arabidopsis thaliana* to *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) through the accumulation of callose and hydrogen peroxide and activation of SOD and POD enzymes by SA and MAPK signalling pathways (Jiang et al. 2016). The application of *Trichoderma* sp. plus plant growth regulator BR enhanced resistance to tomato grey mould caused by *B. cinerea*, through increase of peroxidase, superoxide dismutase, phenylalanine ammonia-lyase, and catalase (Li et al. 2020a). The accumulation of ROS, like H_2O_2, in tomato seedlings treated with *T. harzianum* was correlated with Fe-SOD gene (Elkelish et al. 2020). In another study, the protective activities of *T. harzianum* strain23 against tobacco mosaic virus (TMV) were associated with a significant increase in SOR scavenging enzymes PPO, CAT, and SOD, whereas nonenzymatic oxidative stress markers H_2O_2 and MDA were decreased compared to the control treatments (Abdelkhalek et al. 2022). *Aspergillus niger* boosted heat stress tolerance in sunflower through increase *of* the ROS-scavenging activities, like AAO, CAT, GR, SOD, and POD (Ismail et al. 2020).

Callose is a β-(1,3)-D-glucan polysaccharide that plays an important role in plant defence, normal growth, and development. At the site of pathogen attack, Callose is transiently deposited between the pre-existing cell wall and plasma membrane to slow pathogen spread (Wang et al. 2021b). Accumulation of callose, active oxygen, and phenylalanine lyase increased in the leaves of tobacco plants transgenic VvVe more than wild types under *V. dahliae* infection (Tang et al. 2016). *T. harzianum* triggered plant systemic defence responses by increasing of callose deposition in grapevine plants that were inoculated with *Plasmopara viticola* that causes downy mildew (Kamble et al. 2021). Beckman et al. (1982) found that Callose deposition was increased in paravascular parenchyma cells of tomato resistant cultivars than in the susceptible cultivars that were inoculated with *F. oxysporum*. Results of Hindumathy et al. (2006) showed that the elicitors derived from the cell wall of the fungus *A. niger* stimulated plant defence in pearl millet against downy mildew pathogen *Sclerospora graminicola* (Sacc.) through localised deposition of callose and lignin in the cell wall of pearl millet.

The calcium Ca^{2+} ion plays a significant role in cell development and plant immunity as well as its role of a second signal transportation for many cellular processes and diverse physiological changes. The application of *the* beneficial root-colonising fungus *Mortierella hyalina has* enhanced the aerial growth of *Arabidopsis* and activates of Ca^{2+} to resist and restrict *Alternaria brassicae* development in roots (Johnson et al. 2019). The calcium Ca^{2+} increased in *Arabidopsis* in response to bacterial flg22, NaCl, and Pep1 treatments (Cao et al. 2017). Inoculation of tomato leaf tissue with the fungus *C. fulvum* increased activation of tomato plasma membrane Ca^{2+} permeable channels and induce plant resistance that would limit or prevent cell death (Gelli et al. 1997). The elicitor EFOL-2 from the fungus *F. oxysporum* f. sp. *lycopersici* increased Ca^{2+} concentration in tomato cell and *did not* cause cell death in resistant tomato cells (Dey et al. 2010). Some studies have indicated that a *exists* between callose and Ca^{2+} (Him et al. 2001, Shikanai et al. 2022). The results of Shikanai et al. (2020) showed that the expression of genes related to cell wall and defence responses was altered, and callose concentration increased significantly in wild-type plants under low-Ca conditions, which indicated that callose is necessary to reduced cell wall damage and defence responses caused by low Ca levels.

Transcription factors are one of the major constituents of plant signalling pathways that determine the plant's response against biotic and abiotic stimuli besides their contribution to various plant processes, such as embryogenesis, nutrient deprivation, and hormone-regulated processes. Transcription factors also play a significant role in the regulation of genes associated to plant and microbe response (Sáenz-Mata et al. 2014). WRKY are proteins that regulate many processes in plants and microbe. The WRKY are denoted by 60 amino acids composed of a conserved WRKYGQK, and zinc finger-region (Rushton et al. 2010). Several transcriptional factors were involved in ISR network through SA, JA, and ET signalling *pathways* (Yu et al. 2022). According to Jiang et al. (2016) transcription factors WRKY11 and WRKY70 were involved in the regulation of rhizobacterium *B. cereus* AR156-induced systemic resistance in *Arabidopsis*, through activating the JA and SA signalling pathways. *Trichoderma* root colonisation triggered enhancement in the expression of WRKY18 and WRKY40, which activate JA-pathway responses in *Arabidopsis* (Brotman et al. 2013).

The induction of plant defence genes begins when specific receptors recognise the presence of biotic and abiotic stress. A complex regulatory network is formed as a results of the interactions that occur between genes and their products (Yu et al. 2022). The combined application of *T. harzianum* along with SA and methyl jasmonate (MeJA) increased of defence-related proteins and phenolics in tomato plants against Fusarium wilt disease (Zehra et al. 2017a). The expression of genes related to sulphur uptake and assimilation are increased in *V. dahliae* infected tomatoes during companion cropping with potato onions (*Allium cepa var. aggregatum*) (Fu et al. 2016). Results of Deng-wei et al. (2014) revealed that *StoNPR1*, a *Solanum torvum NPR1* gene increased the resistance of the transgenic potato

lines to *V. dahliae* infection, which was previously expressed in *V. dahliae*-sensitive potato. In cotton plants, *GhMLP28* gene was induced by *V. dahliae* inoculation and was responsive to defence signalling molecules, including JA, SA, and ET (Yang et al. 2015). Inoculation of *T. harzianum* TM increased the concentration of phenols, flavonoids, and lignin in tomato leave, and caused biphasic peaks as an expression of defence-related genes and secondary metabolism (Geng et al. 2022).

Secondary metabolites are compounds that play a variety of roles in the interaction of plant with its environment. Secondary metabolites are present in high concentrations in the skin of fruits and plant leaves and are involved in plants defences against biotic and/or abiotic stresses such as disease resistance, UV resistance, and pigmentation (Pusztahelyi et al. 2015, Yu et al. 2022). Antimicrobials compounds such as zealaxin A2, thymol, resveratrol, carvacrol, caulilexin C, and wyerone acid are also secondary metabolites that restrict the growth of pathogens in the apoplast (Pusztahelyi et al. 2015). High levels of zealexins were accumulated in maize stems in response to *F. graminearum* infection. Zealexin accumulation increased expression of the genes encoding *Tps6* and *Tps11*, which were among the most highly up-regulated genes (Huffaker et al. 2011).

Stomata are specialised structures surrounded by pairs of guard cells. A stoma is essential for plant survival and productivity, and *it plays* an important role in transpiration, respiration, mainly O_2 and CO_2 gas exchange, which is necessary for optimal photosynthesis. To restrict microbial invasion, there are so-called microbe-associated molecular patterns (MAMPs) such as flagellin for bacteria and chitin oligosaccharides (CTOSs) for fungi that are conserved in the whole class of microbes. Recognition of these patterns by the plant leads to stomatal defence or stomatal immunity, this reaction is a part of a plant immune response to restrict microbe's invasion (Ye et al. 2020). *T. harzianum* TM alleviated stomatal closure in tomato plants and enhanced the rate of photosynthesis and the activity of fructose 1,6-bisphosphatase (FBPase) and ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) compared with only *B. cinerea* inoculation (Geng et al. 2022). Application of mycorrhizal fungi (AMF) with compost *has* significantly improved stomatal conductance, chlorophyll fluorescence, CO_2 uptake, photosynthetic activity, and the resistance of xylem vessels of tomato plants to colonisation by *V. dahliae* in soil (Ait Rahou et al. 2021).

2.3.4 Field application of biological control agents

Field application of BCAs could prevent the development of Verticillium wilt, when applied as pre-sowing treatments. These treatments can also increase plant growth parameters. Deketelaere et al. (2020) found that dripping cauliflower seeds with different MS concentrations of *V. isaacii* Vt305 led to a satisfying biocontrol level of Verticillium wilt in the field trials. Hanson (2000) noted that inoculation of cotton seedlings at six true leaves *stage* with *V. dahliae* by stem puncture after seeds

treatment with dried preparations of *T. virens* and planting of seeds in field soil significantly reduced disease severity and improved plant length. Kaur and Vyas (2021) found that antagonistic fungal strains *Aspergillus terreus* and *T. harzianum* in combination with liquid material (biochemical formulation) significantly reduced Verticillium wilt disease and increased height and cotton yield under natural field conditions. Larena et al. (2003) found that apply *Penicillium oxalicum* at a rate of approximately 10^6–10^7 CFU/g in rhizosphere and seedbed substrate before transplanting *is* necessary for effective control of Verticillium and Fusarium wilt of tomato.

Although there has been a lot of work on selecting BCAs and examining the mechanisms of biocontrol, there have been few examples of successful application in the field and currently biocontrol is not yet used on a large scale in agriculture. As mentioned, biological control is a promising method for managing disease like *Verticillium wilt*, but there is still much that needs to be discovered about how to make it work better through improving their effectiveness of BCAs, possibly by combining with other alternative control methods such as either root exudates or amendment with abiotic factors (inducers) to stimulating microorganism population and systemic resistance in the plant.

2.3.5 Root exudates

Plant roots are an important part of the plant that grow underground and have many functions including anchoring the plant, storing food, absorbing water and minerals (Flores et al. 1999). Plant roots also secrete some chemical compounds called root exudates, which play an important role in regulation of root-microbe interactions and plant communication to the neighbouring plants (Bais et al. 2001, Canarini et al. 2019, Suarez-Fernandez et al. 2020). Root exudates are chemical compounds that have biological activity and are secreted from plant roots into the rhizosphere. The production and release of these compounds may be induced by biotic or abiotic stress. During diffusion, which depends on pH and membrane permeability, uncharged molecules and small polar molecules are transported through lipid membranes. Other compounds like amino acids, sugars, and carboxylate anions are transported through membranes from the cytoplasm of intact root cells to the soil by the aid of proteins (Badri & Vivanco 2009).

In general, these compounds are divided into two groups, organic compounds with low molecular weight such as amino acids, sugars, phenolics and organic anions, and organic compounds with high molecular weight such as pigments, proteins, mucilage and other substances (Bais et al. 2001, More et al. 2020) (Figure 2.4).

Amino acids

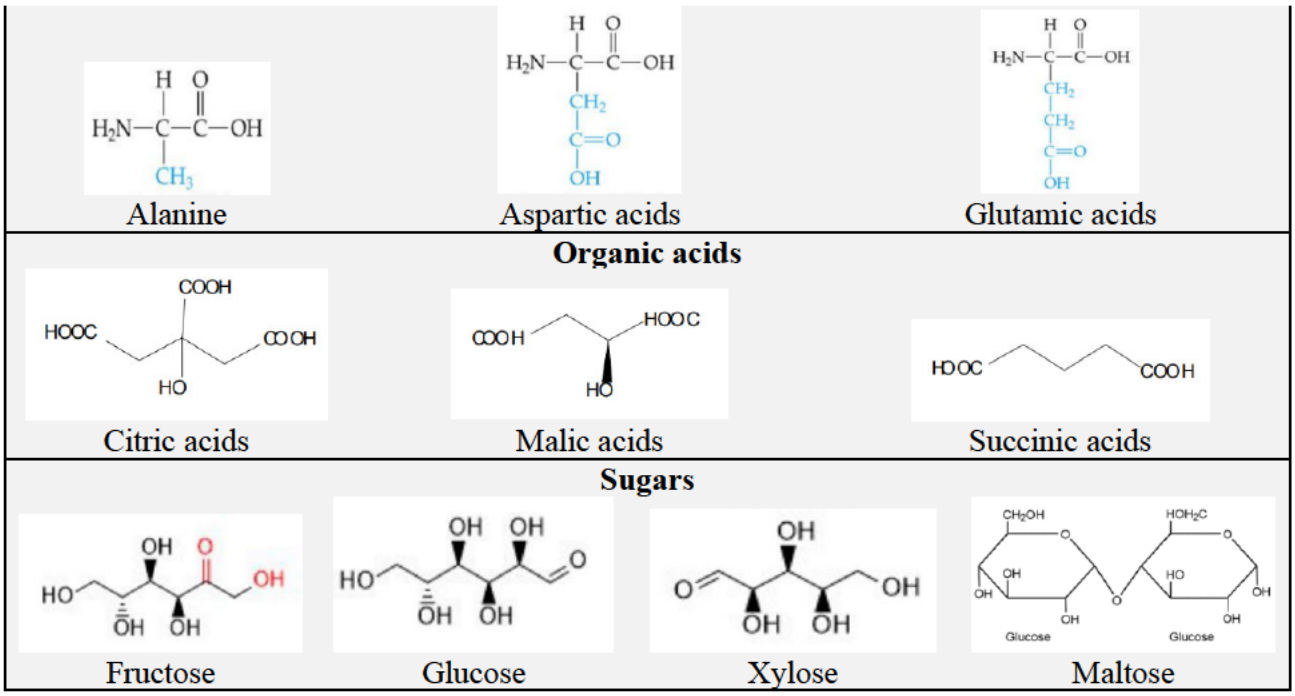


Figure 2.4. Chemical structure of some amino acids, organic acids, and sugars. Source (Xie et al. 2008, Cowsill 2012, van Laar et al. 2022).

Root exudates can have both positive and negative effects. For example, root exudates can play a major role in regulating plant growth and interaction with the other plants and microbes. According to Wu et al. (2008) the cotton root exudates can improve the growth and development of *Verticillium dahliae.* López-Moral et al. (2023) found that root exudates from oil plants (Arbequina and Picual) significantly induced conidia and microsclerotia germination of *V. dahliae.* Rahman et al. (2021) found that root exudates of the intercropping of tomato and potato onion (*Allium cepa* var. *aggregatum*) increased bioavailability of soil phosphorus (P). In particular, some plants secreted volatile compounds that have the ability to stimulate ISR in neighbouring plants (Bais et al. 2006). According to Glinwood et al. (2003) exposure of barley plants to carboline and root exudates of couch-grass (*Elytrigia repens*) caused a significant reduction in aphid infection. One of the defence methods in the *Arabidopsis* plants is by attracting the beneficial rhizobacteria *B. subtilis* by secretion of malic acid and tricarboxylic acid and recruiting these bacteria against the pathogen *P. syringae* pv. *tomato* (Rudrappa et al. 2008). Roots can regulate microbial community in rhizosphere soil through beneficial symbioses, change *of* soil properties chemically and physically and *inhibition of* growth of other plants as a type of competition (Walker et al. 2003). In contrast, microbial communities associated with roots confer specific functions to their hosts, thereby modulating plant productivity, health, and growth (Korenblum et al. 2020).

On the other hand, the negative effect of root exudates is the ability of some plants to produce phytotoxic compounds. These compounds affect seed germination, shoot growth, root growth, photosynthesis and respiration (Weir et al. 2004). Perry et al. (2005) demonstrated that using

phytotoxin and (±)-catechin in high concentrations caused inhibition of *Centaurea maculosa* seedlings. In a study on the effect of root exudates of *Conocarpus erectus* trees against some ornamental plants, results of Abdul Ameer and Al-Rekaby (2017) showed that root exudates of *Conocarpus erectus* caused inhibition of seed germination of ornamental plants. Wu et al. (2017) found that application of organic acids in the soil caused increase of hydrogen peroxide and toxins in the pathogenic fungi. Results of other experiments revealed that three organic acids citric acid, oxalic acid, and malic acid from cucumber roots stimulated mycelium growth of *T. harzianum*, which resulted in improved biomass of the plant. Naqvi and Chauhan (1980) found that 7 sugars and 12 amino acids were detected in root exudates from resistant cultivars of chilli plants. These compounds stimulated spore germination of the antagonistic fungi *T. viride* and *A. sydowii*. Furthermore, *P. fluorescens* and *T. asperellum* caused increased phenolic content in the root exudates of chickpea. In turn, wilt symptoms were reduced significantly (Kumar et al. 2017). The root exudates of maize enhanced growth of *T. harzianum*. In contrast, they inhibited sclerotia growth of the pathogen *R. solani* (Vishwakarma et al. 2017b).

Many previous studies have indicated the importance of root exudates of tomato (Steinkellner et al. 2005, Steinkellner et al. 2009, Fernández et al. 2017). Results of Tan et al. (2013) suggested that organic acid, citric acid, malic acid, fumaric acid, and succinic acid from tomato roots caused increase in root colonisation by *B. amyloliquefaciens*. In another study to evaluate the effects of the root exudates of six tomato genotypes against *Orobanche aegyptiaca*, they showed most resistance to the plant and inhibited seed germination (El-Halmouch et al. 2006). Gamliel and Katan (1992) found that bacteria and fungi showed less preference for tomato seeds and root exudates in the solarised soils that have higher rates of amino acids and amino compounds and have lower rates of sugars. Laboratory analysis revealed that citric acid increased 50% in the tomato root exudates in presence of *P. fluorescens*, whereas the rate of succinic acid decreased (Kamilova et al. 2006). Results of Tahat et al. (2010) indicated that spore germination of the arbuscular mycorrhizal fungus *Glomus mosseae* was increased with increase of root exudates of tomato plant. Root exudates from tomato that were challenged with the pathogens *F. oxysporum* f.sp. *radicis-lycopersici* (*Forl*101587), and *F. oxysporum* f.sp. *lycopersici* (*Fol* 007) resulted in enhanced potential of antagonists against pathogen (Steinkellner et al. 2008).

Root exudates are important in regulating plant-microbial interactions. Over the last several years, *researches* have provided a better understanding of how root exudates mediate communication between plants and other organisms. Yet, some questions remain unresolved including how the microorganisms present in the rhizosphere modulates exudation and root metabolism, role of root exudates in plant defence, interaction between the antagonists and plant root exudates, and how plants encourage beneficial microorganisms, and the responses that can be triggered.

2.3.6 Conclusion

Vascular wilt caused by *V. dahliae* remains a major problem in annual agricultural and horticultural plants, including economically important tomato crop. The pathogen invades the plant from the roots and spreads along the xylem vessel to other parts of the plant. The development and use of BCAs against *V. dahliae* are an appealing management strategy. So, more work is needed to discover potential BCAs for the control of Verticillium wilt based on *in vitro* assessments of antagonistic activity and potential for plant growth promotion. To utilise this kind of biological control there is a need for improvement of BCAs performance by combining it with some chemical triggers such as SA. Although there are many studies that show the significant role of SA in induction of plant defence against various pathogens, through morphological, physiological, and biochemical mechanisms, SA has not been tested with BCAs to diminish the activity of *V. dahliae* in tomato plants.

Chapter 3. Isolation and identification of antagonistic fungi for biological control of tomato Verticillium wilt disease

3.1 Introduction

Tomatoes play an important role in trade and agricultural production worldwide because of their high contents of essential nutrients, unique taste, health effects, and antioxidant-rich phytochemicals (Ali et al. 2020, Collins et al. 2022). The fungus *V. dahliae* is a *soil-borne* pathogen that causes Verticillium wilt disease on a wide range of plant species including vegetables, field crops and forest trees (Agrios 2005, Atallah et al. 2010, Angelopoulou et al. 2014).

V. dahliae produces structures called microsclerotia (MS), which comprise a microscopic, dense shell with thick and darkly pigmented cells, and irregular shape and size (Fradin & Thomma 2006, Vermeulen 2020). MS play an important role in the disease cycle as they are a major inoculum source and, under certain conditions, can survive in the soil for several years (Kowalska 2021). Therefore, *V. dahliae* remains a challenge to control due to its wide host range and ability to survive in soil for many years (Vermeulen 2020). Symptoms of the disease include stunted growth, wilting, yellowing, necrosis of leaves, and chlorosis of tissues. Verticillium wilt seriously affects tomato quality and yield, since heavy infection by *V. dahliae* or *V. non-alfalfae* can lead to either small fruits or zero fruit production (Pei et al. 2023). The *Ve* gene confers resistance against Verticillium wilt and, whilst widespread in tomato varieties, it does not provide complete resistance working against some races, but not others (Fradin et al. 2009). Currently, there are no commercial varieties that have complete resistance to Verticillium wilt (Pei et al. 2023).

Although chemical pesticides are commonly used to control plant diseases, their use can pose potential risks to both human health and the environment (Masi et al. 2021). In addition, most fungicides are ineffective because *V. dahliae* is a vascular pathogen and resides inside the plant for most of the disease cycle. Therefore, once the fungus enters the plant, it is very difficult to control (Vermeulen 2020).

Biological control is one of several methods that have been used to control Verticillium wilt as an alternative strategy and has received considerable attention from many researchers in recent decades (Lo 1998, Mercado-Blanco 2012, Pei et al. 2023). Fungal species have an effective role in biological control applications through mechanisms which primarily act upon plants or pathogens, including nutrient competition, mycoparasitism, the synthesis of antibiotic and hydrolytic enzymes, plant growth promotion, and the stimulation of plant defence responses (Howell 2003, Asad 2022, Manzar et al. 2022). For example, *Trichoderma* suppresses pathogen growth through competition; mycoparasitism, secreting cell wall-degrading enzymes such as ß-glucanases, chitinases, and

glucanases (Mukherjee et al. 2022); and producing antibiotics and toxins such as trichodermin, trichothecene, and a sesquiterpene (Benítez et al. 2004) which have a direct effect on the pathogens. Induced plant defence mechanisms, involving the reduction of reactive oxygen species, modifications of cell walls and cuticles, the elevation pathogenesis-related proteins, and phenolic compounds, which have indirect effect on the pathogens (Osei et al. 2021).

While biocontrol offers a potential solution with regard to controlling *Verticillium wilt*, finding suitable biological control agents (BCAs) is not always straightforward. Screening of soil, as a potentially large reservoir of different microorganisms, has potential due to the range of different microbial relationships exhibited within the soil rhizosphere or on the root surface. For example, the different types of relationships that can exist between organisms are commensalism, mutualism, neutralism, competition, parasitism, and predation (Bull et al. 2002, Nazli et al. 2020, Bahram & Netherway 2022, Baslam 2023). The number and diversity of microorganisms, both beneficial and deleterious, depends on the physical and chemical properties of the soil, as well as on the quantity and quality of root exudates, which gives shape to the structure of the root microbial community (Nazli et al. 2020). Hence, taking advantage of these fungi in biological control is considered an important step toward the use of these microorganisms to manage plant diseases as an alternative to chemical compounds.

The rhizosphere is a part of the soil surrounding a plant root that is directly influenced by root exudates and associated soil microorganisms (Ali et al. 2017b). Various compounds such as amino acids, organic acids, inorganic ions, aromatic acids, amides, aliphatic acids, sugars, volatile *compounds*, ethylene, peptides, vitamins and enzymes are exuded by plant roots and can act as chemical signals in the soil, able to attract or stimulate the growth of the beneficial microorganisms (Canarini et al. 2019, Rebolledo-Prudencio et al. 2020). Rhizosphere fungi are microorganisms found in the rhizosphere that can provide defence for roots against pathogenic attacks (Larekeng et al. 2019). For example, *Trichoderma* isolates *recovered* from rhizosphere gave excellent biocontrol and growth promotion *abilities* in *Syringa oblata* against *Syringa* powdery mildew (Liu et al. 2020). Karunasinghe et al. (2020) showed that 19 rhizosphere fungi include five isolates of *Aspergillus* species *A. insulicola* A435, *A. insulicola* A419, *A. melleus* A412, *A. terreus* A213, and *A. luchuensis* A116 gave a significant antagonistic activity against *Pythium* damping *in* cucumber.

Given that antagonistic fungi can be present in the rhizosphere and plant roots, the work reported in this chapter sought to select microbial isolates with antagonistic effects against *V. dahliae* and use them in further experiments such as root exudates or as inducers of resistance. The original intention was to use cotton as the host plant, so that BCAs were isolated from rhizosphere and cotton roots.

However, the method of pathogenicity testing on cotton was difficult to integrate with methods for testing antagonism, so tomato was used as the host crop in all experiments. The objectives were to:

1. Evaluation of the effect of four *V. dahliae* strains on the tomato cultivar Grosse Lisse by the root dipping method,
2. Determine the antagonistic activity of the isolated fungi against *V. dahliae* in dual culture screening and using an *in vitro* culture filtrate assay, and
3. Evaluation of the efficiency of the most active isolates in promoting tomato growth and reducing Verticillium wilt disease severity under glasshouse conditions.

The *V. dahliae* strains were obtained from the freeze-dried culture collection of the NSW Plant Pathology and Mycology Herbarium, Orange Agricultural Institute, NSW Department of Primary Industries.

3.2 Materials and methods

3.2.1 Isolates of pathogens

Four strains of *V. dahliae* (DAR 33757 from cotton, DAR 31890 from tomato, DAR 44537 from potato and DAR 81260 from olive) were obtained from the freeze-dried culture collection of the NSW Plant Pathology and Mycology Herbarium, Orange Agricultural Institute, NSW Department of Primary Industries (Table 3.1). These strains were maintained on plugs of potato dextrose agar (PDA) in sterile distilled water (SDW) inside small glass bottles and stored refrigerated at 4^{o} C before use.

Table 3.1. Four strains of *Verticillium dahliae* and their origins.

Isolate	Host	Location	Year isolated
DAR 33757	Cotton	Bourke, NSW	1979
DAR 31890	Tomato	Mount View, NSW	1978
DAR 44537	Potato	Albany, WA	1983
DAR 81260	Olive	Unknown, SA	2011

3.2.2 Pathogenicity test of V. dahliae strains

Preparation of microconidial inoculum

Pathogenicity was tested to select a strain that was most suitable (based on its virulence on tomato plants) for further experiments. Microconidial inoculum of the four *strains* was prepared by transferring six agar plugs (~ 0.5 cm^3) from an active colony into 250 ml potato dextrose broth (PDB) in 500 ml flasks. *All flasks were incubated at 25 ºC on a shaker at 120 rpm for 15 days.* The resultant culture for each strain was filtered through a Whatman No.1 filter paper to remove the mycelial mass. The concentration of microconidia was determined with a haemocytometer, and then adjust to 10^6 conidia /ml in SDW.

Conducting the root dipping method

Four *strains* were tested on the tomato cultivar Grosse Lisse by the root dipping method of Melouk and Horner (1974), to allow the fungal spores to directly reach the plant root surface and infect the elongation zone. In summary, tomato seeds were sown in small pots (5 cm diameter x 12.5 cm height) containing non-sterile soil mixture (Premium Potting Mix, Searles Ltd, Kilcoy QLD, and river sand at 2:1 v/v). Pots were put on benches inside a glasshouse at 25 ± 1 ºC. Tomato seedlings at 3–4 true leaves were removed from pots and their roots were washed gently under tap water to remove soil and soaked in 500 ml flasks containing 250 ml of the *Verticillium* conidial suspension (10^6 conidia/ml) for 1 hour (Ali et al., 2018). For control treatments, seedlings were soaked in SDW only. Then, seedlings were transplanted to large pots (20 x 20 cm) containing a soil mixture as outlined above. One seedling was put in each pot and four replications were used. After 6 weeks, disease severity and percent disease index (PDI) were recorded. A scale of 0 to 4 was used: 0 = leaf of healthy aspect; 1 = epinasty or wilted leaf without chlorosis; 2 = one or several slightly chlorotic bands on the leaf; 3 = chlorotic bands over the entire surface of the leaf or chlorotic bands with a necrotic centre and 4 = complete necrosis or death of the leaf (Jabnoun-Khiareddine et al. 2007). *The percent disease index (PDI) was calculated following formula* (Sultana et al. 2018),

$$\text{PDI} = \frac{\text{Sum of all disease ratings}}{\text{Total number of ratings} \times \text{Maximum grade}} \times 100$$

Stem vascular discoloration was determined by cutting the plant stem lengthwise and measuring the internal browning length (cm) inside the vascular tissue from the tap root (Rowe 1980). Growth parameters including plant height and dry weight for shoot and root systems were also determined.

3.2.3 Isolation of potential antagonistic fungi

Samples of cotton roots and soil that *adhere* to the roots were taken below the soil line 0 - 15 cm depth with the aid of a spade from different locations in fields at Boggabri in north-eastern New South Wales, Australia to obtain isolates of potential antagonists against Verticillium wilt (Zheng et al. 2011). The samples were then taken to the laboratory using cellophane bags.

Ten gram of soil samples taken from rhizosphere (soil adhering to the roots) were *suspended* in 90 ml of SDW. *One* ml of this *suspension* was transferred to tubes containing 9 ml of SDW and then a series of dilutions 10^{-3} and 10^{-4} were made. *One* ml of these dilutions *was* added to Petri dishes with 3 replicates at each dilution. Cooled autoclaved PDA containing chloramphenicol was aseptically added over the top of the diluted soil suspensions and *dishes* were incubated at 25 ± 1 ºC for 5 days (Yusnawan et al. 2019). After a 5-day period, isolates were purified, and three mycelial plugs (~ 0.5

cm^3) were placed into vials 40 ml containing 20 ml of SDW and stored at 4 ºC before use. A minimum of 4 vials were prepared for each isolate.

Cotton roots were thoroughly washed under tap water and cut into 0.5–1 cm long pieces. Segments were surface sterilised with 0.5% sodium hypochlorite for 3 min then rinsed in SDW several times. Five segments were distributed on PDA enriched with chloramphenicol (Abo-Elyousr et al. 2014) inside Petri dishes and incubated at 25 ± 1 ºC in darkness for 5 days (Yusnawan et al. 2019).

3.2.4 Determination of isolates with in-vitro antagonistic activity against tomato V. dahliae DAR 31890 strain

In a previous experiment of this chapter, the pathogenicity of four strains of *V. dahliae* was tested on tomato cultivar Grosse Lisse to select the most suitable strain (*based on its virulence on tomato plants) for further experiments.* Among these strains, the tomato strain DAR31890 was more virulent on tomatoes than the other strains examined. Therefore, this strain was chosen as the pathogen in the following experiments.

Effect of fungal isolates on mycelial growth of tomato *V. dahliae* DAR31890 strain

Fifteen isolates *recovered* from cotton roots and rhizosphere soil were evaluated for antagonism of *V. dahliae* utilising the dual culture method described by Arriagada et al. (2012). Discs (8 mm) of PDA *colonized* with *V. dahliae* were transferred to Petri dishes and incubated 3 days before treatment with *fungal isolates tested* obtained from cotton fields. *Discs* 8 mm *in* diameter from different fungal isolates *removed* from 5-day old cultures were placed opposite to *V. dahliae* on the PDA *dishes*. In controls, only *V. dahliae* was placed on one side of the Petri dish. The distance between the discs was 6.5 cm. All inoculated plates were incubated in triplicate at 25 ± 1 ºC for 7–13 days. The suppression effect for the isolates of fungi was evaluated applying the following formula: (Ma – Mb)/Ma ×100, where Ma = free mycelial growth of *V. dahliae* (control) and Mb = mycelial growth of *V. dahliae* towards the antagonist (Singh et al., 2019).

Effect of culture filtrate of fungal isolates on tomato *V. dahliae* DAR31890 strain

Six of the fungi examined in *above section* were selected for the next experiments in this chapter based on their efficiency against *V. dahliae* DAR31890 strain. These were designated as isolates 2, 5, 7, 10, 14 and 15.

Preparation of culture filtrate

The pathogenic strain of *V. dahliae* DAR31890, and six isolates of antagonistic fungi were grown separately in PDB by inoculating 100 ml of PDB in sterilised conical flasks (250 ml) to which 1ml of 10^6 conidia/ml suspension of either the pathogen or each of the antagonist isolates was added to separate flasks. All cultures were prepared in triplicate and placed on a shaker at 25 ºC at 120 rpm for

14 days. Following incubation, the culture medium was filtered through Whatman No1 filter paper then passed through 0.45 µm membrane filters to avoid any contamination and be ready for the next experiments (Raut et al. 2014). Extracts from individual culture flasks were treated as a replicate to investigate the effect of culture filtrate on MS germination and mycelial growth of *V. dahliae* DAR31890 strain.

Effect of culture filtrate on mycelial growth of tomato *V. dahliae* DAR31890 strain

Fungal isolates were tested for *their* antagonistic activity against *V. dahliae* DAR31890 strain using a culture filtrate assay as described by (Sharfuddin & Mohanka 2012). A 25 % concentration of fungal culture filtrates were prepared by placing 5 ml of sterilised filtrate into a Petri dish and then adding 15 ml of PDA, with control treatments used PDB instead of fungal filtrate. Discs (8 mm) from 7-day old *V. dahliae* colony grown on PDA were inoculated at the centre of Petri dishes. All Petri dishes in triplicates were incubated at 25 ± 1ºC for 7-13 days. Radial growth rate of the pathogen was measured and compared with the control treatments using the following formula (C1−C2)/C1 ×100, where C1 = radial growth of pathogen in control *dishes* and C2 = radial growth of pathogen in treated *dishes* (Singh et al., 2019).

3.2.5 Effect of culture filtrate on MS germination of tomato V. dahliae DAR31890 strain

Preparation of MS inoculum

The pathogen *V. dahliae* DAR31890 strain was grown on PDA covered with sterile cellophane. After 10 days of incubation at 25 ± 1 ºC, cellophane fragments with *Verticillium* (hypha, conidia, and MS) were peeled off with a spatula and transferred to a blender containing 250 ml deionised water and blended for 2–3 min. The mixture was poured through 106 µm and 45 µm sieves. This process was used to retain MS and remove other materials. MS left in the 45 µm sieve were collected in a sterile beaker containing 0.5% ethanol, which was used to surface sterilise MS for 3 min (Grunden et al. 2001). Then, MS were dropped onto sterilised filter paper until the ethanol evaporated and then transferred to a sterile beaker containing 100 ml of SDW. Finally, the suspension of MS inoculum was used in culture filtrate experiment.

Effect of culture filtrate on MS germination

Culture filtrates were tested on MS germination. Culture filtrates were prepared to a concentration of 25 % with PDA as above (3.2.4) and distributed into Petri dishes. Then, 1ml of the prepared MS suspension was distributed onto a PDA Petri dish, with three replicates established that were kept in the incubator at 25 ± 1 ºC for 7 days. Germination percentage of MS was calculated by counting approximately 250–350 in each Petri dish using a stereo microscope at x40 and determining the proportion of MS with visible germ tubes.

3.2.6 Effect of antagonists on Verticillium wilt development under glasshouse conditions

Preparation of inoculum

Inoculum of the pathogen and potential antagonists was prepared by transferring four discs of PDA with fungal cultures to flasks containing 120 ml of PDB. All flasks were shaken at 120 rpm at 25 ºC for 7–14 days. Conidial suspensions of tomato *V. dahliae* DAR31890 strain and potential antagonist isolates were prepared by filtering through sterilised Miracloth. The concentration was adjusted to 10^6 conidia/ml by using SDW and determined with a hemocytometer (Jegathambigai et al. 2009).

Seedling emergence

In order to evaluate the effect of antagonistic fungi on seedling emergence, tomato seeds (Grosse Lisse) were surface-sterilised for 1 minute with 1% sodium hypochlorite and washed two times with sterile water and then soaked in the spore suspensions of each antagonistic fungus (10^6 conidia/ml) for 60 min (Newman et al. 2002). Seeds were then transplanted to pots (16 cm diameter x 18.5 cm height) containing non-sterile soil mixture (Premium Potting Mix, Searles Ltd, Kilcoy QLD, and river sand at 2:1 v/v). Ten seeds were sown in each pot with each treatment represented by four replicates. After 7 to 14 days percent seedling emergence was recorded.

Seedling treatments

Sterilised tomato seeds (Grosse Lisse) were sown in pots (20 x 20 cm) filled with soil mixture (as above). After emergence and at 3-4 true leaves, seedlings were thinned to one per pot and treated with fungal isolates (2, 5, 7, 10, 14 and 15) by adding 200 ml of conidial suspensions at 10^6 conidia/ml to the soil around the plant (Wani & Mir 2009). After four days, 10^6/ml conidial suspensions 200 ml of the pathogen *V. dahliae* were added to the soil in the same method as above. As a control, seedlings inoculated with *V. dahliae* only (not treated with fungal isolates), and seedlings with neither fungal isolates nor pathogen were used. The fungus-free PDB, which modified with the same concentration of SDW that was added to adjust the conidial suspension of the tested fungi was added to the control treatments. Disease severity was recorded after one month of inoculation as described previously (3.2.2). Plant length and dry weight of shoot and root system were determined.

3.2.7 Identification of fungal isolates by PCR

Two fungal isolates (2 and 7) which performed well in all tests were chosen for use in further experiments. They were identified by internal transcribed spacer (ITS) sequencing (Shahid et al. 2014). The isolates were grown on PDA for 3 to 4 days then about 100 mg of fungal mycelium was scraped off with sterile surgical blades and placed into microcentrifuge tubes containing small glass beads (0.5 mm). To extract DNA, Isolate II Plant DNA Kit (Bioline) Lysis buffer PA1 was added to

the tubes which were then shaken for 30 sec at 30 Hz in a Retsch MM300 mill. DNA was then extracted following the Isolate II Plant DNA Kit manufacturer's protocol. Next, ITS sequences were amplified by PCR using master mix MyTaq (Bioline) with ITS 1, and ITS 4 primers (White et al. 1990). For the amplification process, cycling conditions included an initial denaturation at 95 °C for 60 sec, followed by 30 cycles consisting of denaturation at 95 °C for 15 s, then at 55 °C for 15 s, 72 °C for 10 s, and extension step at 72 °C for 5 min. Purified PCR products were sequenced by the Australian Genome Research Facility using the forward primer. These two fungal isolates were identified by their ITS sequence similarity to sequences in the GenBank database using BLAST search (https://blast.ncbi.nlm.nih.gov/).

3.2.8 Statistical analysis

Data were analysed by one-way or multi-factorial ANOVA, depending on the experimental design, using IBM SPSS version 25. Homogeneity of variance was checked using Levene's test, and the data for stem browning and disease severity in the glasshouse trial were log-transformed before analysis. The criterion for significance of a treatment effect was $P < 0.05$, unless otherwise stated. Tukey's HSD test was used for mean separations when treatment effects were significant.

3.3 Results

3.3.1 Pathogenicity of V. dahliae strains

Tomato root dip inoculation with four strains of *V. dahliae* was undertaken to investigate the *effect* of each strain on wilt disease severity and tomato plant parameters (plant height, dry weight of shoot and root system). *Six weeks after inoculation*, all strains caused a significant increase in disease severity compared with the control ($F_{4,15} = 19.99$, $P < 0.001$) (Figure 3.1). The highest severity of disease on tomato plants was *induced* by *V. dahliae* strain DAR 31890, initially isolated from tomato. Also, this original tomato isolate (*V. dahliae* DAR 31890) gave a significantly higher level of stem vascular discoloration compared with other tested isolates, which did not differ significantly from the control ($F_{4,15} = 8.44$, $P < 0.001$) (Figure 3.2 and Figure 3.3).

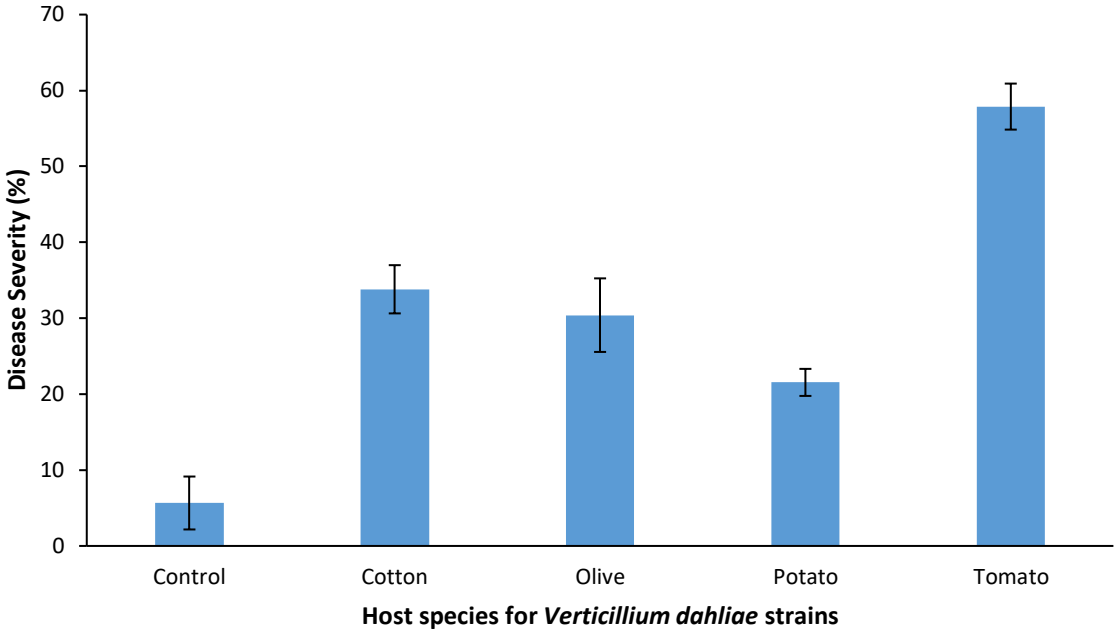


Figure 3.1. Effect of four strains of *Verticillium dahliae* (DAR33757 from cotton, DAR81260 from olive, DAR44537 from potato and DAR31890 from tomato) on % of leaves showing symptoms of wilt and expressed as disease severity (%) in tomato cultivar Grosse Lisse. Error bars show standard errors of the mean (n=4, control=uninoculated plants).

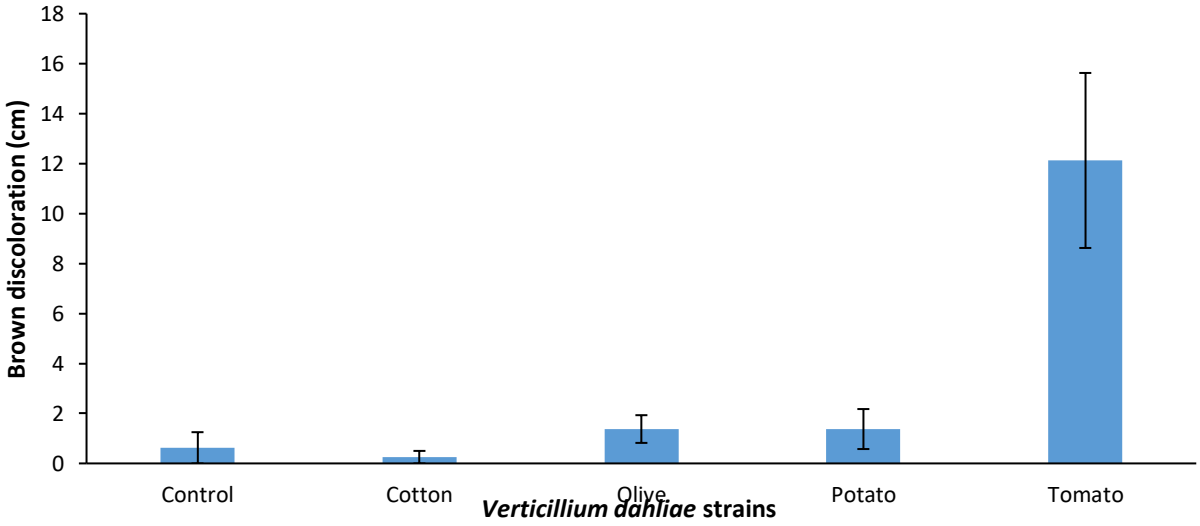


Figure 3.2. Effect of four strains of *Verticillium dahliae* (DAR33757 from cotton, DAR81260 from olive, DAR44537 from potato and DAR31890 from tomato) on stem vascular discoloration extent in tomato cultivar Grosse Lisse. Error bars show standard errors of the mean (n=4, control=uninoculated plants).

Figure 3.3. Wilt symptoms of tomato *Verticillium dahliae* DAR31890 strain in tomato cultivar Grosse Lisse. (A) yellowing of leaves. (B) Brown discoloration of the vascular tissue of the stem caused by Verticillium wilt.

The *V. dahliae* strains were significantly different from each other on plant height, with strain DAR 44537 from potato giving shorter plants than DAR 31890 from tomato ($F_{4,15} = 4.04$, $P = 0.02$) (Figure 3.4). However, none of the *V. dahliae* strains were significantly different from the control. None of the isolates showed a significant difference to the uninoculated control with regard to shoot dry weight. However, differences were observed between the potato and olive *V. dahliae* strains ($F_{4,15} = 3.46$, $P = 0.034$, Figure 3.5). There was no significant effect of *V. dahliae* strains on root dry weight ($F_{4,15} = 1.22$, $P = 0.28$) (Figure 3.6).

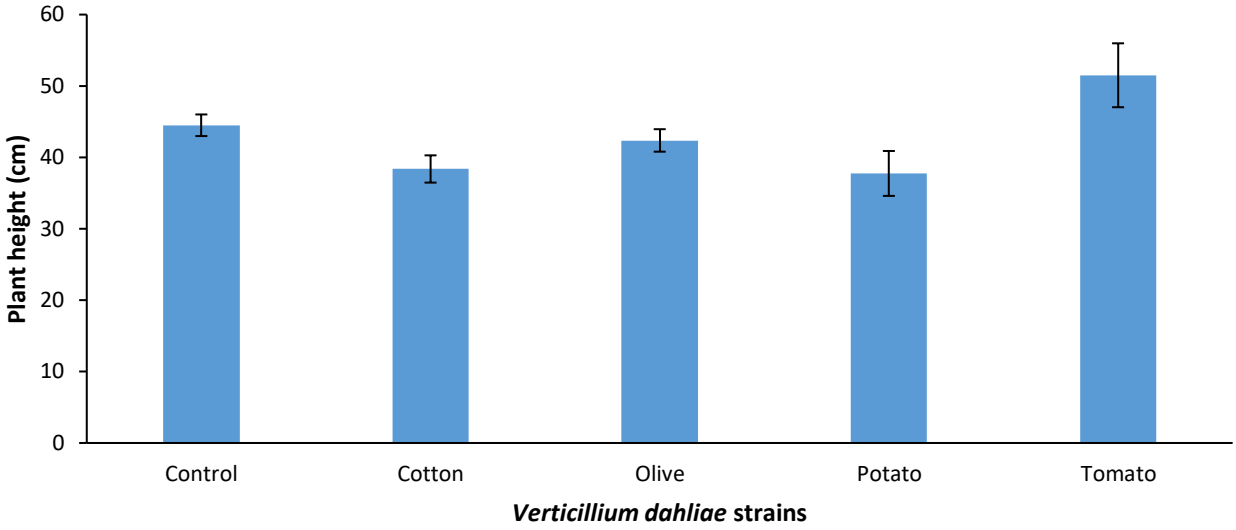


Figure 3.4. Effect of inoculation with four strains of *Verticillium dahliae* (DAR33757 from cotton, DAR81260 from olive, DAR44537 from potato and DAR31890 from tomato) on tomato cultivar Grosse Lisse height. Error bars show standard errors of the mean (n=4, control=uninoculated plants).

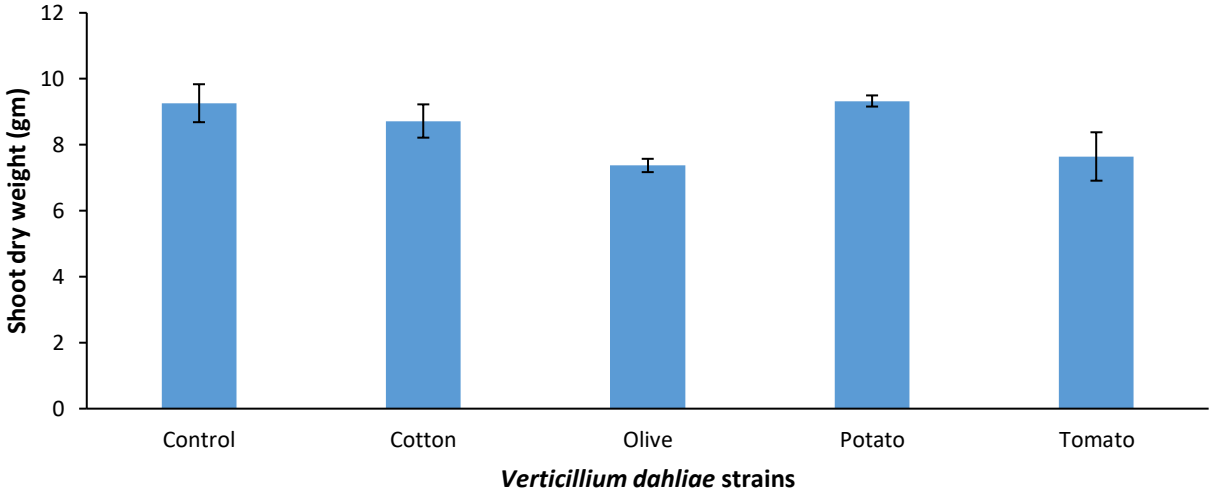


Figure 3.5. Effect of four strains of *Verticillium dahliae* (DAR33757 from cotton, DAR81260 from olive, DAR44537 from potato and DAR31890 from tomato) in tomato cultivar Grosse Lisse shoot dry weight. Error bars show standard errors of the mean (n=4, control=uninoculated plants).

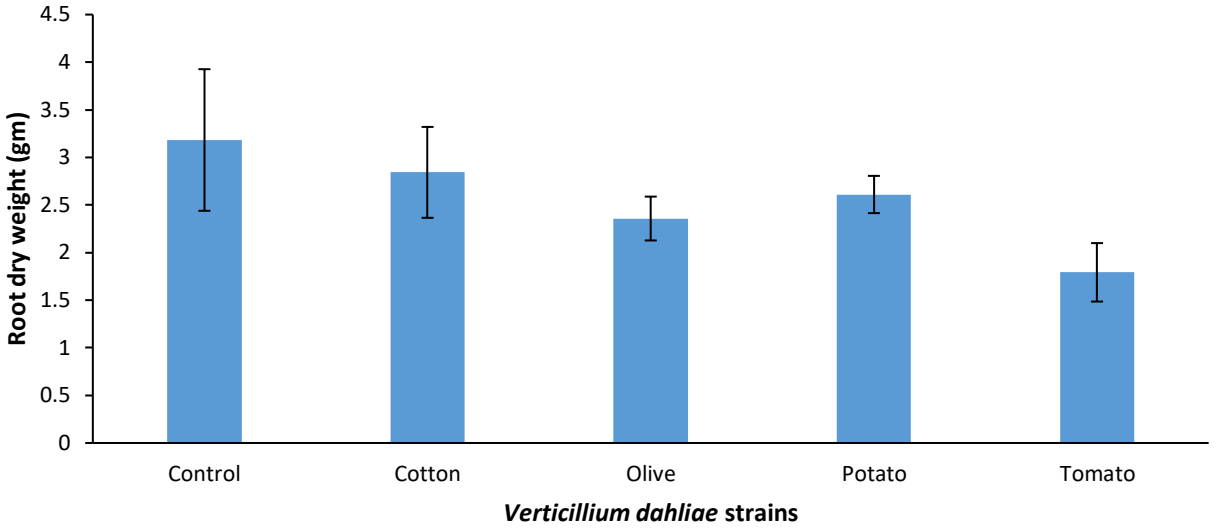


Figure 3.6. Effect of four strains of *Verticillium dahliae* (DAR33757 from cotton, DAR81260 from olive, DAR44537 from potato and DAR31890 from tomato) in tomato cultivar Grosse Lisse root dry weight. Error bars show standard errors of the mean (n=4, control=uninoculated plants).

3.3.2 Isolation of potential antagonistic fungi

A total of 15 fungi were isolated from cotton roots and rhizosphere soil (soil adhering to the roots) of heathy plants from locations in cotton fields at Boggabri in north-eastern New South Wales, Australia (Table 3.2). All the isolated fungi were serially numbered (From 1 to 15) and maintained into test tubes containing PDA and vials containing SDW. Isolates 4, 9, 11, 12, 13, and 15 were isolated from cotton roots, and 1, 2, 3, 5, 6, 7, 8, 10, and 14 were isolated from soil adhering to the roots.

Table 3.2. Fungi isolated recovered from cotton roots and rhizosphere soil of different healthy plants.

Isolate number	Cotton roots	Rhizosphere soil
1		+
2		+
3		+
4	+	
5		+
6		+
7		+
8		+
9	+	
10		+
11	+	
12	+	
13	+	
14		+
15	+	

3.3.3 Effect of fungal isolates on mycelial growth of tomato V. dahliae DAR31890 strain

In a dual culture method using the rhizosphere and soil fungal *isolates* in proximity to *V. dahliae* DAR31890 strain showed, differences *in their ability to inhibit* the pathogen mycelial growth. The difference between isolates was significant ($F_{14,30} = 8.80$, $P < 0.001$) (Figure 3.7). Most of the fungal isolates reduced mycelial growth of the pathogen *V. dahliae* markedly on PDA plates. Most isolates were not significantly different from each other, except for isolates 6 and 8, which gave significantly lower inhibition than all isolates except for 9 and 11. A representative of each morphological type of isolates, which gave high inhibition of growth, was selected for further experiments.

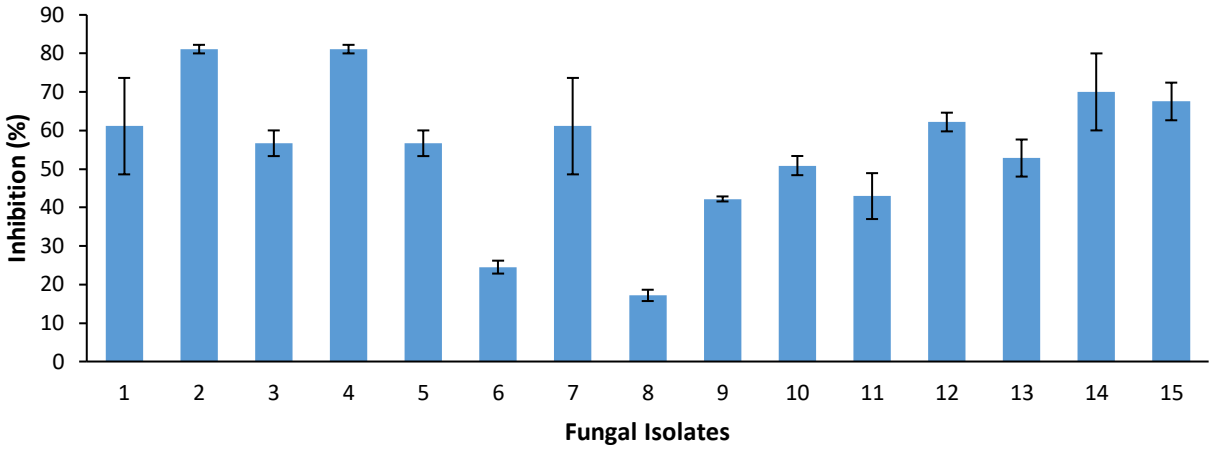


Figure 3.7. Effect of fungal isolates on mycelial growth of *Verticillium dahliae* in dual culture method. Error bars show standard errors (n=3).

3.3.4 Effect of culture filtrate of fungal isolates on mycelial growth of tomato V. dahliae DAR31890 strain

The tested culture *filtrates* of fungal isolates 2, 7, and 15 significantly decreased growth of *V. dahliae* ($F_{7,16} = 108.86$, $P < 0.001$) (Figure 3.8 and Figure 3.9). The culture filtrate of isolate 7 was the most effective compared with the control, with no growth of *V. dahliae* on the Petri dish medium.

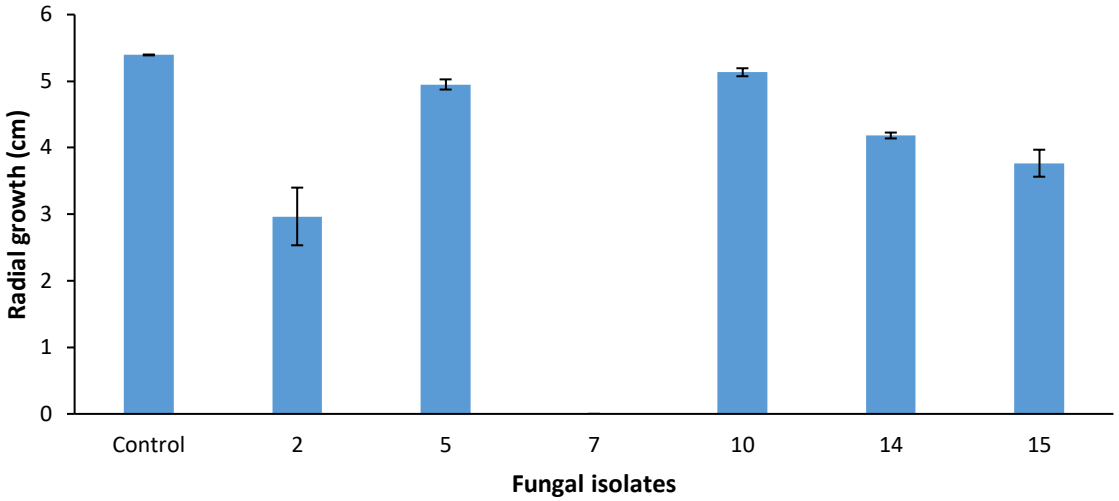


Figure 3.8. Effect of culture filtrate of fungal isolates (2, 5, 7, 10, 14 and 15) on mycelial growth of *Verticillium dahliae*. (Control=PDB only). Error bars show standard errors (n=3).

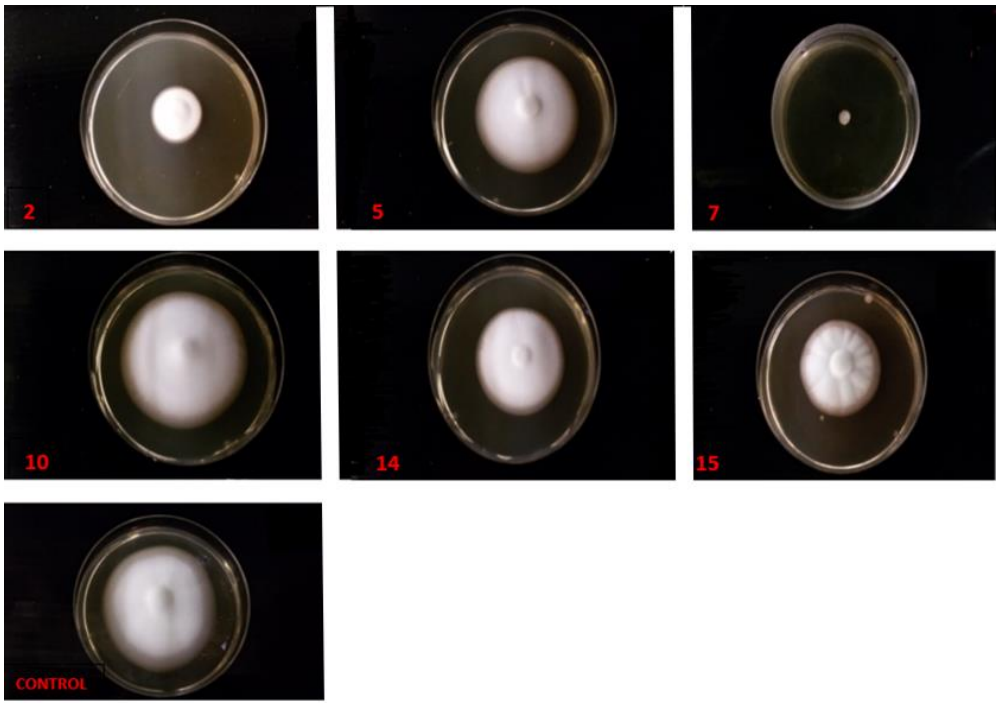


Figure 3.9. Effect of culture filtrate of fungal isolates (2, 5, 7, 10, 14 and 15) on mycelial growth of *Verticillium dahliae*.

3.3.5 Effect of culture filtrate on MS germination

MS germination was significantly decreased in all treatments that were treated with culture *filtrates* of fungal isolates, except for isolate 10 ($F_{6,14}$ = 39.48, P < 0.001) (Figure 3.10). Culture filtrate of isolates 2, 5, and 7 had significantly higher level of growth inhibition than all isolates.

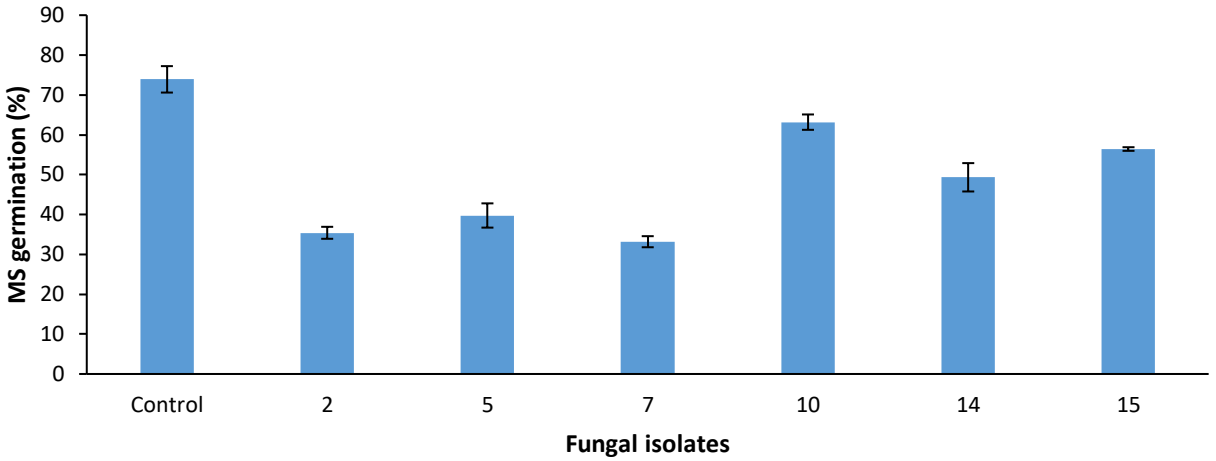


Figure 3.10. Effect of culture filtrates of fungal isolates on germination rate of *Verticllium dahliae* microsclerotia. Error bars show standard errors (n=3). (Control=PDA only).

3.3.6 Effect of fungal isolates on Verticillium wilt development under glasshouse conditions

The effect of conidial suspensions of fungal isolates on tomato percent seedling emergence is shown in Figure 3.11. There was no significant effect of fungal treatment on seedling emergence ($F_{6,21}$ = 0.62, P = 0.71).

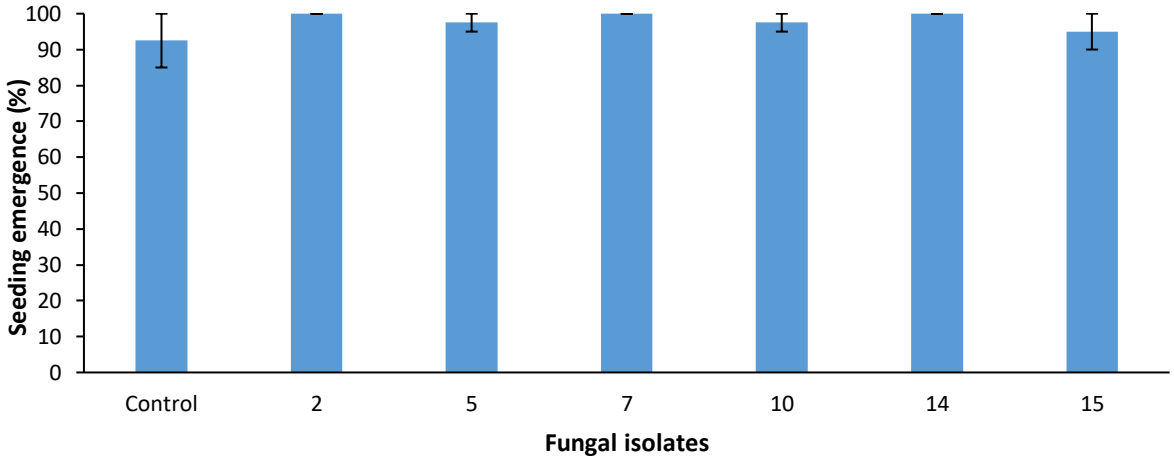


Figure 3.11. Effect of fungal isolates on tomato cultivar Grosse Lisse seedling emergence. Error bars show standard errors (n=4). Control=Tomato plant only.

Treatment with fungal isolates had a significant effect on the disease severity caused by the pathogen *V. dahliae* on tomato plants ($F_{6,21}$ = 2.80, P = 0.037) (Figure 3.12). The severity of Verticillium wilt in plants treated with Vert + 2 and Vert + 7 was significantly less than in the control treatment

(pathogen only), although there were no significant differences between the antagonist isolates, or between isolates 5, 10, 14 or 15 and the control. Treatments 2, 5, 7, 10, 14, and 15 that had not been inoculated with pathogen were excluded from statistical analysis because no symptoms were seen in plants.

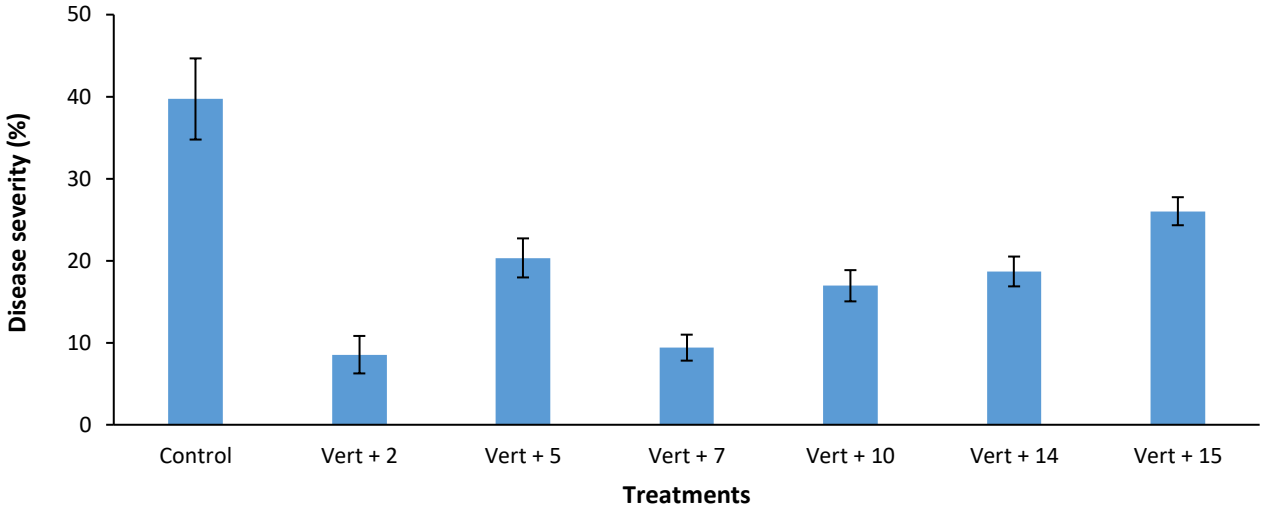


Figure 3.12. Effect of fungal isolates (2, 5, 7, 10, 14 and 15) on % of leaves showing symptoms of wilt and expressed as disease severity (%) in tomato cultivar Grosse Lisse. Error bars show standard errors (n=4). Control=Pathogen only, Vert=Verticillium dahliae.

There were significant effects of *V. dahliae* ($F_{1,42}$ = 45.57, P < 0.001), antagonists ($F_{6,42}$ = 34.84, P < 0.001), and their interaction ($F_{6,42}$ = 4.32, P = 0.002) on plant height (Figure 3.13). Inoculation with *V. dahliae* reduced the height of control plants by 23%. Antagonist isolates 2 and 7 significantly increased height in both *V. dahliae*-inoculated and uninoculated plants. There were no significant differences between the heights of *V. dahliae*-inoculated and uninoculated plants for any antagonist except isolate 5.

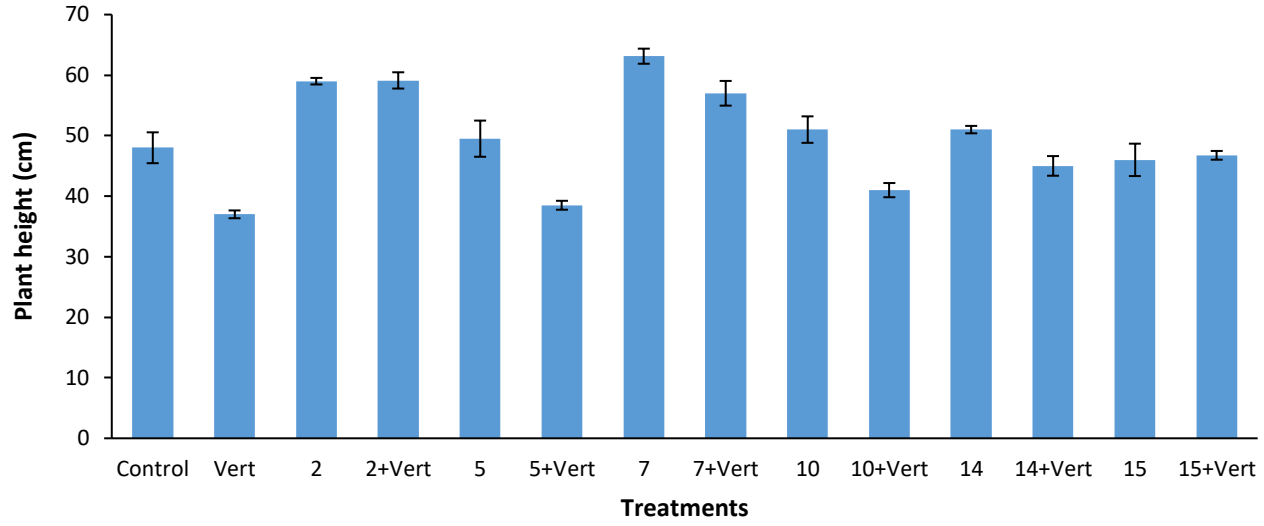


Figure 3.13. Effect of fungal isolates (2, 5, 7, 10, 14 and 15) in tomato cultivar Grosse Lisse height. Error bars show standard errors (n=4). Control=Plant only, Vert=*Verticillium dahliae*.

There were significant effects of *V. dahliae* ($F_{1,42}$ = 19.71, P < 0.001), antagonists ($F_{6,42}$ = 23.51, P < 0.001), and their interaction ($F_{6,42}$ = 4.02, P = 0.003) on shoot dry weight (Figure 3.14). Inoculation with *V. dahliae* reduced the dry weight of control plants by 36%. Antagonist isolates 2 and 7 significantly increased dry weight in both *V. dahliae*-inoculated and uninoculated plants.

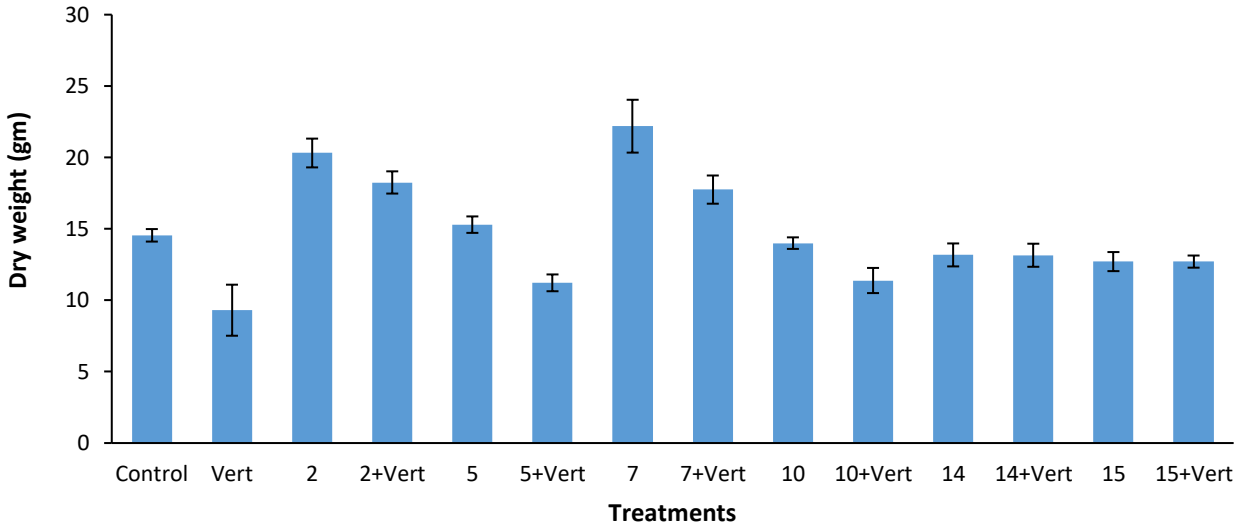


Figure 3.14. Effect of fungal isolates (2, 5, 7, 10, 14 and 15) in tomato cultivar Grosse Lisse shoot dry weight. Error bars show standard errors (n=4). Control=Plant only, Vert=*Verticillium dahliae*.

3.3.7 Identification of the most active fungal isolates

Isolates 2 and 7 consistently gave the greatest inhibition of growth and MS germination, reduction in disease severity and promotion of growth of tomato plants. These two fungal isolates were identified by their ITS sequence similarity to sequences in the GenBank database using BLAST search (Table

3.3). Isolate 2 was identified as *T. harzianum*, while isolate 7 shared approximately 97% similarity to *A. tubingensis* and a close similarity to a number of other black-spored *Aspergillus* species, including *A. niger* and *A. welwitschiae*. Isolate 7 will therefore be referred to as *Aspergillus* sp.

Table 3.3. Most similar sequence in GenBank using BLASTn search of ITS sequences of two antagonist isolates.

Isolate	Most similar accession	Similarity	Putative identification
2	KP263659	99%	*Trichoderma harzianum*
7	KP764873	97%	*Aspergillus tubingensis*

3.4 Discussion

In this chapter, the pathogenicity of four strains of *V. dahliae* - DAR 33757 from cotton, DAR 31890 from tomato, DAR 44537 from potato and DAR 81260 from olive were examined for their impact on the host plant, tomato cultivar Grosse Lisse. Further to this, some fungi from soil and cotton roots were studied for their antagonistic activity towards *V. dahliae* for their consideration as potential BCAs for Verticillium wilt on tomato.

The results indicated that all strains of *V. dahliae* caused a vascular wilt and an increase of tomato disease severity compared to the control. These results are consistent with previous studies that indicated the ability of the pathogen to infect a second host (Pantelides et al. 2009, Arslan & Dervis 2010, Bilodeau et al. 2012). This result also conformed with Jabnoun-Khiareddine et al. (2006a) who found that isolates of *V. dahliae* from potato were highly pathogenic and attacked tomato and eggplant. Yildiz et al. (2009) reported that the virulence of *V. dahliae* isolates from weed hosts in cotton fields was greater on cotton plants than on weeds. The ability of *V. dahliae* to colonise numerous host plants could be because it has a large number of genes encoding carbohydrate-active-enzymes (CWDE), phytotoxins, and cell wall degrading enzymes (Fradin & Thomma 2006, Yalin et al. 2022). Among the strains of *V. dahliae*, the strain DAR31890 from tomato was significantly more virulent on tomato than the other strains examined in this research. In the same way, brown vascular discoloration was highest in tomato stems, which were treated with the tomato isolated strain of *Verticillium* DAR31890. Although this is consistent with some level of host preference, the main aim of this experiment was to select an isolate that would be sufficiently pathogenic for further experiments. Based on the disease and pathology observed in the host plant, tomato cultivar Grosse Lisse, all subsequent experiments which investigated potential BCAs used the tomato isolated *V. dahliae* DAR31890.

Out of the 15 isolated fungi, six fungal isolates (4, 9, 11, 12, 13, and 15) were collected from cotton roots, while the other nine (1, 2, 3, 5, 6, 7, 8, 10, and 14) were collected from rhizosphere soils. Interestingly, isolates 2 and 7, which were later identified as *T. harzianum* and *Aspergillus* sp., and

performed best against tomato *V. dahliae* isolate DAR31890, were taken from the same environment (soil adhering to the roots). Similar results for isolation of antagonistic fungi from rhizosphere soil were reported by several other researchers (Jain et al. 2012, Ru & Di 2012, Kale et al. 2018, Yoo et al. 2018, Liu et al. 2020). High levels of compounds exuded by roots in the rhizosphere attract much greater numbers of microorganisms such as beneficial rhizosphere microbes than elsewhere in the soil. These microbes protect plants against pathogens mainly by means of a wide variety of mechanisms including antagonism or resource competition (Barea et al. 2005, Nihorimbere et al. 2011, Meena et al. 2017, Yin et al. 2021).

A total of 15 isolated fungi were screened for their ability to inhibit mycelial growth and the production of MS in dual culture investigation. The results demonstrated that some of the isolated fungi were able to affect the pathogen and inhibit mycelial growth and the production of MS in Petri dishes. The highest inhibition occurred in respect of isolate 2, later identified as *T. harzianum*, compared with the other isolates. These results align with Jabnoun-Khiareddine et al. (2009b) who found that three endogenous *Trichoderma* species reduced mycelial growth of *V. dahliae*. Reghmit et al. (2021) demonstrated that *Trichoderma* species significantly reduced mycelium growth of *V. dahliae* that causes olive wilt disease. These results are consistent with those obtained by Carrero-Carrón et al. (2016) who observed that all three strains of *T. asperellum* were able to overgrow and reduce the colony size of *V. dahliae* isolates in dual culture tests. According to Benouzza et al. (2021), *Trichoderma* isolate 15 significantly inhibited the mycelial growth of *V. dahliae in vitro* dual culture tests. Similarly, Morán-Diez et al. (2019) found that *T. atroviride* T11 was able to inhibit growth and overgrown colonies of *V. dahliae* on agar plates.

The inhibition shown by the antagonists may be due to the ability of antagonists to grow rapidly as a way of competing for space, nutrients, essential elements (Janisiewicz et al. 2000, Di Francesco et al. 2016), and mycoparasitic activity (Sharma et al. 2009, Abo-Elyousr et al. 2014). *Trichoderma* has the ability to parasitise other fungi since mycoparasitism is an ancestral trait of *Trichoderma* (Mukherjee et al. 2022). The mechanisms of mycoparasitism, which include directed growth of *Trichoderma* toward other fungi, attachment and coiling, and the production of a range of antifungal extracellular enzymes such as chitinolytic or glucanolytic enzymes (Howell 2003, Harman et al. 2004, Harman 2006, Rai et al. 2019). According to Benouzza et al. (2021) microscopic observation of *Trichoderma* hyphae showed parasitic behaviour against *V. dahliae.* Regragui and Lahlou (2005) referred to the ability of *T. harzianum* to release some antibiotics and enzymes like chitinase and cellulase that play a role in the degradation of cell walls of the pathogen *V. dahliae*. Microscopic observation carried out by Reghmit et al. (2021) demonstrated that hyphae of *Trichoderma* were coiled around mycelium of *V. dahliae*. The authors also reported that when the pathogen was

encircled, cell walls were observed to degrade due to the release of several lytic enzymes involved in the parasite reaction.

Moreover, *Trichoderma* isolates had the ability to produce the volatile organic compounds (VOCs) such as Octadecane, 13-Docosenamide, Undecane, Eicosane, Tetradecanoic acid, 9-Octadecenamide, and n-Hexadecanoic acid, which may affect the mycelial growth of *V. dahliae* (Reghmit et al. 2021). The detrimental effect of *Trichoderma* species may be associated with some release of inhibitory substances, such as antibiotics (Lewis & Papavizas 1987, Xiaojun et al. 2014, Fotoohiyan et al. 2017, Asad 2022).

The culture filtrate test of the antagonistic fungi 2 and 7 showed the highest effect on mycelial growth and MS germination of *V. dahliae*. The culture filtrate of isolate 7 was the most effective compared with the control. Similar results were reported by Reghmit et al. (2021) who found that *Trichoderma* filtrate at 20 % concentration was more inhibitive to *V. dahliae* than a 10 % concentration. Benouzza et al. (2021) showed that all *Trichoderma* culture filtrates significantly inhibited mycelial growth of *V. dahliae* strains V1 and V2. According to Fotoohiyan et al. (2017), culture filtrates of *T. harzianum* Tr4 and Tr12 isolates were the most effective in the growth inhibition of *V. dahliae*.

Previous studies also mentioned the potential of culture filtrates of some *Aspergillus* isolates on mycelial growth of plant pathogens. Hosen and Shamsi (2019) reported that culture filtrates of *A. flavus*, *A. niger*, and *A. fumigatus* showed 40.91, 34.09, and 54.55 %, growth inhibition of *F. merismoides* at 5, 10, and 15 % concentrations, respectively. Halo et al. (2018) showed that culture filtrates of *A. terreus* significantly reduced radial growth of *P. aphanidermatum*. Zhao et al. (2018b) showed that culture filtrate of *A. tubingensis* strain QF05 reduced grey mould on tomato fruits and plants caused by *B. cinerea*. Attia et al. (2022a) found that the extracts of *A. fumigatus* exhibited significant antifungal activity against *F. oxysporum* that causes Fusarium wilt disease in tomato.

The suppression of *V. dahliae* by culture filtrates on PDA is an indication that the antifungal property of 2 and 7 isolates may depend on different kinds of lytic enzymes and antibiotics produced by antagonists (Ghisalberti & Sivasithamparam 1991, Howell 2003, Raza et al. 2013, Nagamani et al. 2017, Reghmit et al. 2021). Elad and Kapat (1999) demonstrated that two isolates of *T. harzianum* were effective producers of chitinase, β-1,3-glucanase, and protease in a liquid culture medium. Halo et al. (2018) showed that glucan in the cell walls of *P. aphanidermatum* was affected by the glucanase enzyme produced by *A. terreus*. Furthermore, the inhibition shown by the antagonists may be due to secondary metabolites present in the fungal filtrates. Many *Trichoderma* species are the most prominent producers of secondary metabolites (Vinale et al. 2014, Zeilinger et al. 2016, Khan et al. 2020, Asad 2022). *Aspergillus* genera also produce an abundance of beneficial antimicrobial bioactive secondary metabolites (Siddiqui et al. 2004, Abdallah et al. 2014, Yu et al. 2021a, Choi &

Ahsan 2022). Attia et al. (2022a) showed that PD broth medium treated with ethyl acetate fungal extracts of *A. fumigatus* inhibited fungal growth *F. oxysporum*.

In glasshouse experiments, the results demonstrated that soil treated with suspensions of BCAs promoted tomato plant growth and decreased disease severity caused by infection with *V. dahliae*. These results agreed with those of previous studies that indicated the role of *BCAs* in control of tomato Verticillium wilt (Larena et al. 2003, Sabuquillo et al. 2006, Giotis et al. 2009, Jabnoun-Khiareddine et al. 2009b). Dutta (1981) found that some fungi isolated from tomato rhizosphere including *T. viride, Penicillium* spp. and *Gliocladium* spp., were most effective in controlling Verticillium wilt on tomato plants. Similar results for the potential of antagonistic fungi as biocontrol agents against Verticillium wilt were reported by Yuan et al. (2017) who found that the endophytic fungi *P. simplicissimum* and *T. flavus* significantly reduced Verticillium wilt disease in cotton. Patil et al. (2017) reported using a soluble powder formulation from the fungus *A. niger* (10g per kg of seed) to reduced eggplant wilt caused by *F. oxysporum* f. sp. *melongenae*. Marois et al. (1982) found that six isolates of 34 *soil-borne* fungi examined including *T. harzianum, A. alutaceus, P. lilacinus, G. virens* and *T. flavus* reduced Verticillium wilt on eggplant in glasshouse experiments.

The significant effect of antagonists may be due to their ability to rapidly colonise the rhizosphere, compete for nutrients, and secrete some antibiotics and enzymes that affect pathogens (Whipps 2001, Asad 2022). Antifungal chitinase isolated from *A. niger* inhibited the growth of *R. solani, F. culmorum,* and *F. solani* (Brzezinska & Jankiewicz 2012). Soil amendment with antagonistic fungi may provide protection to the plant by enhancing its resistance against pathogens (Nawrocka & Małolepsza 2013, Khoshmanzar et al. 2020, Yeon et al. 2022).

Moreover, the present study revealed that antagonistic fungi improved plant growth parameters (plant height and shoot dry weight) with or without the pathogen. These results aligned with Jabnoun-Khiareddine et al. (2009b) who found that fresh weights of tomato root and stem were increased *by* more than 50% in plants treated with *Trichoderma* spp. compared with control. Khoshmanzar et al. (2020) found that inoculation with *Trichoderma* isolates increased tomato shoot dry weight and root volume compared to the control treatment. These authors reported that one *T. harzianum* strain enhanced shoot potassium uptake, while Bader et al. (2020) showed that native *T. harzianum* strains from Argentina increased tomato shoot length, fresh and dry weight of shoot and roots. Kaur and Vyas (2022) indicated that the antagonistic strains of *A. terreus* and *T. harzianum* significantly reduced the symptoms of Verticillium wilt disease and promoted the growth of cotton plants. Attia et al. (2022a) demonstrated that *A. fumigatus* and *R. oryzae* significantly improved growth parameters (shoot length, root length, and number of leaves) in tomato plants.

Biological activity of secondary metabolites produced by *Trichoderma* species such as koninginin and 6-pentyl-apyrone may *act* as plant growth regulators (Vinale et al. 2008). *Trichoderma* spp. also produce organic acids, such as citric, gluconic acids that permit the solubilisation of phosphate Ion, micronutrients and mineral cations such as iron, Mn, and Mg, useful for plant metabolism (Benítez et al. 2004, Harman et al. 2004). Plants treated with *Aspergillus* may increase total phenols, free proline, POD, and PPO enzymes that play a key role in plant growth (Attia et al. 2022a). According to Dulf et al. (2015) solid-state fermentation by *A. niger* significantly increased antioxidant activity and phenolic contents in *Sambucus nigra*. Hussain et al. (2022) showed that *A. welwitschiae* significantly improved the plant growth of *Glycine max* L. by enhancing the activity of antioxidant enzymes. Abdelaziz et al. (2022) demonstrated that the endophytic *A. fumigatus* increased phenols, proline, POD, PPO, SOD and CAT enzymes activity in *Zea mays* plants.

Finally, through the results mentioned in this chapter, isolates 2 and 7 *in vitro* gave the greatest inhibition in fungal growth and MS germination, and also reduced Verticillium wilt disease incidence and promoted tomato growth under glasshouse conditions compared with the other isolates tested. These two fungal isolates were identified by ITS sequence similarity, compared to sequences in the GenBank database using a BLAST search. Isolate 2 has been identified as *T. harzianum*, while isolate 7 was identified as *Aspergillus* sp. Therefore, these two fungi were chosen as BCAs against the pathogen *V. dahliae* DAR31890 from tomato for further experiments described in the following chapters.

Chapter 4. Effect of tomato root exudates on pathogen and antagonistic fungi

4.1 Introduction

In recent decades, the use of biological control agents (BCAs) to control plant pathogens has increased because of increasing public concern about the potential harmful effects of chemical pesticides on human health and the environment (Pal & Gardener 2006, Fernández et al. 2017, Roca-Couso et al. 2021). There are several mechanisms that BCAs use against plant pathogens including competition, parasitism, predation, and antibiosis (Deketelaere et al. 2017, Liu et al. 2021, Manzar et al. 2022). These mechanisms can be successful if the BCAs successfully establish in the rhizosphere. The rhizosphere is a different environment from bulk soil because of the accumulation of plant root exudates in this area (Singh & Mukerji 2006). Root exudates refer to a complex mixture of organic substances that are released from living plant roots in the rhizosphere. They may contain amino acids, organic acids, inorganic ions, aromatic acids, amides, aliphatic acids, sugars and volatile compounds in addition to ethylene, peptides, vitamins and enzymes (Lugtenberg et al. 1999, Uren 2000, Kamilova et al. 2006, Canarini et al. 2019, Vives-Peris et al. 2020).

In addition to being used as nutrient source for rhizosphere colonisation and microorganisms in the soil, rhizosphere compounds may play a significant role in plant-plant and plant-microbe interactions. The interactions between plant and their microbial communities in the rhizosphere can have beneficial impacts on plants, including disease suppression (Aulakh et al. 2001, Lugtenberg & Kamilova 2009, Huang et al. 2014). For example, root exudates such as long-chain fatty acids and amino acids stimulated by foliar pathogen infection can recruit some beneficial rhizosphere microbes such as *Pseudomonas* strains to help host plants to resist foliar pathogen infection (Wen et al. 2021). Shi et al. (2021) found that wheat root exudates led to an increase in the density of total bacteria and of *Pseudomonas*, *Bacillus* and *Streptomyces* spp., and significantly affected the bacterial community composition. *In vitro*, Vishwakarma et al. (2017a) showed that root exudates of sorghum and maize inhibited growth and sclerotia germination of the pathogen *R. solani* and enhanced growth of *T. harzianum*. Liu et al. (2009) reported that root exudates of grafted eggplants inhibited mycelium growth of *Verticillium dahliae*. Mol and Riessen (1995) found that potato root exudates stimulated MS germination of *V. dahliae.* According to Farley et al. (1971), soil moistened with sucrose solution increased MS germination of *V. dahliae.* Olsson and Nordbring-Hertz (1985) found that percentage of MS germination was higher in a mineral salts solution plus sucrose compared with water or mineral salts solution alone.

In tomato plants, Mazzotta et al. (2022) demonstrated that a green extract of olive leaf loaded in chitosan nanoparticles diminished the symptoms of *Verticillium wilt*, and significantly enhanced tomato plant growth. Fernández et al. (2017) noted that an increase of gluconic acid levels in the root exudates of tomato caused enhanced populations and efficiency of *T. asperellum* to control grey mould disease caused by *B. cinerea*. Steinkellner et al. (2009) observed that tomato root exudates increased microconidia germination for antagonistic *F. oxysporum* strains.

Numerous studies have demonstrated the importance of interactions between tomato plants and rhizosphere microbes through plant root secretions (Tahat et al. 2010, Tan et al. 2013, Panichikkal & Krishnankutty 2021, Raza et al. 2022), but there is little information available about the interaction between *V. dahliae*, *Trichoderma* spp., *Aspergillus* spp., and tomato root exudates. Therefore, the aim of this study was to determine the effect of tomato root exudates and some of the multiple components such as amino acids, organic acid, and sugars on the pathogen *V. dahliae* and the antagonistic fungi *T. harzianum* and *Aspergillus sp*. The study also sought to determine whether adding sugars could stimulate MS to germinate, making them more susceptible to antagonisms. The research therefore sought to identify whether adding germination stimulants could possibly increase the effect of biocontrol.

4.2 Materials and methods

4.2.1 Preparation of root exudates

Root exudates were prepared by surface-sterilising tomato seeds of cultivar Grosse Lisse with 1 % sodium hypochlorite for 1 min then washing with sterilised water three consecutive times. Sterilisation of cheesecloth, beakers, and Hoagland nutrient solution was achieved by autoclaving at 121°C for 20 minutes. Ten seeds of tomato were placed on a double layer of cheesecloth installed inside the beaker 500 ml at a level that touches the surface of Hoagland nutrient solution (200 ml), and tightly covered by sterile aluminium foil to prevent contamination. All beakers were placed in the growth chamber at 25°C with 16 h light (traditional fluorescent light) and 8 h of darkness. After 21 days, Hoagland nutrient solution with root exudates was collected and kept in the freezer for the next trials. To check microbial contamination, 1ml of root exudates and distributing it into Petri dishes containing PDA and then incubation at 25°C for 5 days to verify whether there was any contamination or not (Ali et al. 2018). The samples were stored inside the freezer until used in trials.

4.2.2 Effect of root exudates on MS germination

To evaluate the effect of root exudates on MS germination of *V. dahliae* DAR31890 strain, sterile water agar (4.5 g/50 ml water) that had been held in a water bath at 60 °C was mixed with 225 ml of

each of the root exudates (RE), Hoagland nutrient solution (HS) and Hoagland nutrient solution plus 1 g/L sucrose (HSS) to get the final concentration 20 g/L water (Alshimaysawe 2018). Then, 20 ml from each were poured into sterilised Petri dishes. Water agar was used as a control treatment. A 1 ml of MS suspension (described previously in Section 3.2.5) was added to the Petri dishes, and they were kept inside the incubator at 25°C for 7 days. Germination percentage of MS was calculated by counting approximately 250–350 in each Petri dish using a stereo microscope at x40 by counting the proportion of MS with visible germ tubes.

4.2.3 Effect of root exudates on pathogen in dual culture

The effect of root exudates on *T. harzianum* and *Aspergillus* sp. activity against the pathogen *V. dahliae* DAR31890 strain was studied in dual culture method. *Twenty* ml from each of the root exudates (RE), Hoagland nutrient solution (HS) and Hoagland nutrient solution plus sucrose (HSS) were added to the Petri dishes after amending them with agar (as above). Discs of 8 mm diameter from 5-day old cultures of the antagonistic fungi *T. harzianum* and *Aspergillus* sp. were placed on the opposite side from discs of *V. dahliae*. The distance between the discs was 6.5 cm. In control, only the pathogen *V. dahliae* was placed on one side of the Petri dishes. All inoculated plates (three replications) were incubated at 25±1°C for 6 days. The suppression effect of antagonistic fungi was evaluated with the following formula (Ng et al. 2015),

$$Inhibition = \frac{R1 - R2}{R1} \times 100$$

R1 = Radial growth of V. dahliae in the absence of the antagonists (control)

R2 = Radial growth of V. dahliae in the presence of the antagonists (treatments).

4.2.4 Effect of root exudates on growth of fungi

Root exudates (RE), Hoagland nutrient solution (HS) and Hoagland nutrient solution plus sucrose (HSS) were used to study their effect on dry weight of fungal mass for the pathogen *V. dahliae* DAR31890 strain and antagonists. *Twenty* ml from all liquid mediums were poured into sterilised tubes (150 ml) then 1ml of an approximately 10^6 conidia/ml spore suspension of the pathogen and antagonists were added to the tubes. All tubes in three replicates were placed at 25 ºC on a rotary shaker at 120 rpm for two weeks. After that, the fungal mass of all the fungi were harvested by filtering through the sterilised Whatman No1 filter paper then dried at 60 ºC in the oven for two days and weighed.

4.2.5 Effect of amino acids, organic acid, and sugars on MS germination

In this experiment, the effect of amino acids (alanine, aspartic acid and glutamic acid), organic acids (citric, malic and succinic acids) and sugars (fructose, glucose, maltose and xylose) was studied on MS germination. These compounds were chosen because they are the most abundant components of tomato root exudates (Lugtenberg et al. 1999, Kamilova et al. 2006). To get a 1 g/L concentration, 1g from each of these components was dissolved into 100 ml SDW and sterilised through Millipore filtration (0.22 µm filter) (Pedrotti et al. 1994, Hassan et al. 2015). The solutions were poured into 1000 ml flasks *containing* slightly cooled sterile water agar (20/900 ml water) and mixed gently to make a final concentration 1 g/L (Kravchenko et al. 2003). Water agar was used as a control treatment. *Twenty* ml of these concentrations individually were placed inside the Petri dishes then 1 ml of MS suspension was spread on top of the agar with gentle mixing. All plates were incubated at 25 ºC for 5 days. The results were determined by calculating the percentage of MS germination as above (4.2.2) in each of the Petri dishes.

4.2.6 Interaction between fructose and BCAs for controlling Verticillium wilt under glasshouse conditions

The addition of an MS germination stimulant combined with antagonists was tested in pots. Fructose was used because it is the most abundant sugar in tomato root exudates (Kamilova et al. 2006). Sterilised tomato seeds were planted in small pots (5 cm diameter x 12.5 cm height) filled with non-sterilised soil mixture (Premium Potting Mix, Searles Ltd, Kilcoy QLD, and river sand at: 2:1 v/v). Tomato seedlings (cv. Grosse Lisse) at 3-4 true leaves were transplanted into large pots (20 x 20 cm) containing the same soil as above (one plant per pot). After 1week conidial a suspension of 10^6 conidia/ml of bioagents *T. harzianum* and *Aspergillus* sp. were added to the pots by adding 200 ml of the suspension to each pot around the seedlings. Then, 200 ml of conidial suspension (10^6 conidia/ml) of the pathogen *V. dahliae* DAR31890 strain was added to the pots 3 days later. Fructose sugar was prepared in three concentrations 0.5, 1, and 2 g/L. Then, 300 ml of each concentration was added to the pots weekly. All pots in three replicates were put in the glasshouse at 25±1°C and under natural light. After six weeks from transplanting, disease severity was recorded as described previously (Section 3.2.2).

Tomato plant *growth* parameters (plant height, dry weight of the shoot and root system) were determined.

4.2.7 Statistical analysis

Data were analysed by ANOVA in IBM SPSS 25, using $P < 0.05$ as the criterion for significance. When appropriate, Tukey's test was used for mean separation.

4.3 Results

4.3.1 Effect of root exudates on pathogen MS germination

All treatments had significantly higher germination of MS than the water agar control ($F_{3,8} = 34.03$, $P < 0.001$, Figure 4.1). *The root exudate treatment was not significantly different from Hoagland nutrient solution.* The germination of MS in Hoagland nutrient solution plus sucrose was significantly greater than in Hoagland nutrient solution alone, showing that sucrose stimulated germination.

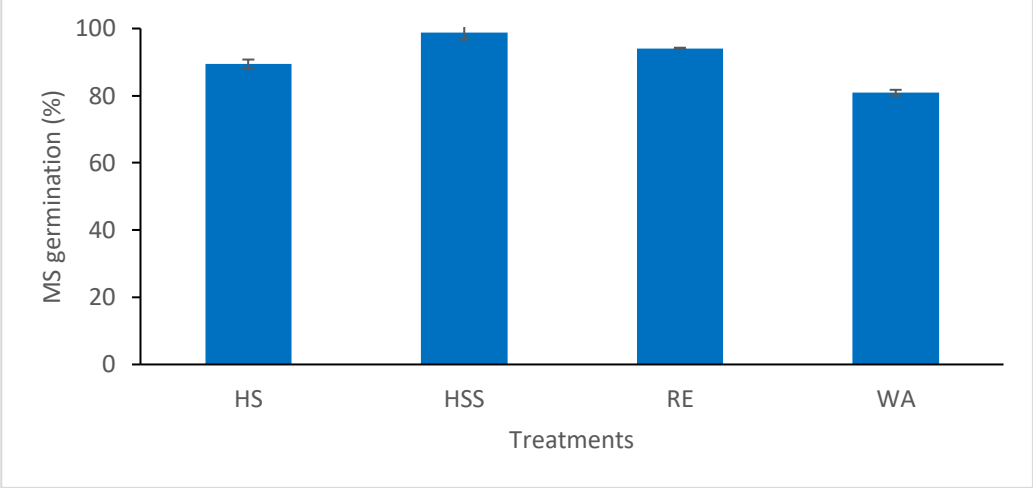


Figure 4.1. Effect of Hoagland solution (HS), Hoagland solution plus sucrose (HSS) and root exudates (RE) and water agar (WA) on MS germination of *Verticillium dahliae* DAR31890 strain. Error bars show standard errors (n=3).

4.3.2 Effect of root exudates on inhibition of pathogen growth in dual culture

There was a significant ($F_{2,12} = 26.53$, $P < 0.001$) effect of the interaction between antagonist species and treatment in inhibition of *V. dahliae* DAR31890 strain in dual culture (Figure 4.2). Results showed that there was no significant difference between the effect of root exudates and Hoagland nutrient solution on the inhibition percentage for the pathogen by either *Trichoderma* or *Aspergillus*. However, adding sucrose to HS significantly reduced the inhibition of *V. dahliae* by *T. harzianum* treatments.

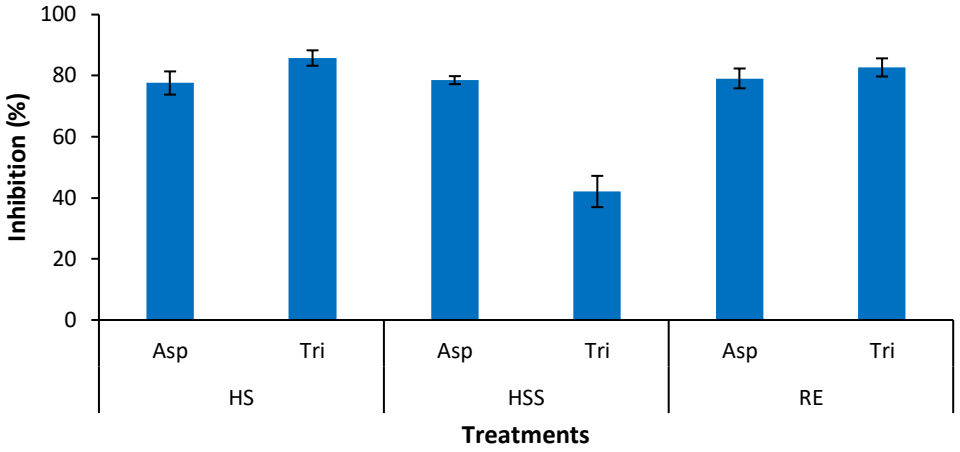


Figure 4.2. Effect of Hoagland solution (HS), Hoagland solution plus sucrose (HSS) and root exudates (RE) on the pathogen *Verticillium dahliae* DAR31890 strain in dual culture method with *Aspergillus* sp. (Asp) and *T. harzianum* (Tri). Error bars show standard errors (n=3).

4.3.3 Effect of root exudates on growth of fungi

There was a significant ($F_{4,18}$ = 6.72, P = 0.002) effect of the interaction between fungal species and treatment on growth in liquid culture. The biomass of *Aspergillus* sp. and *V. dahliae* DAR31890 strain was significantly greater in Hoagland nutrient solution plus sucrose (Figure 4.3) than in either root exudates or Hoagland nutrient solution. The biomass of *T. harzianum* did not differ significantly between treatments.

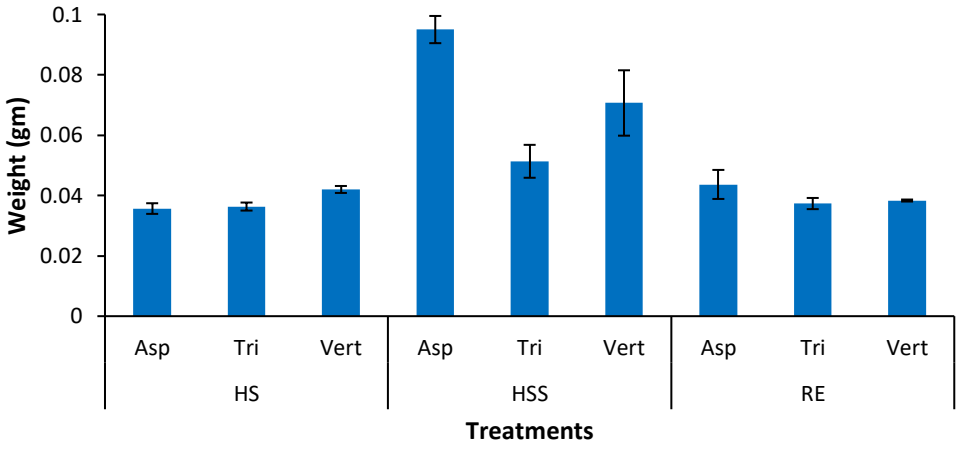


Figure 4.3. Effect of Hoagland solution (HS), Hoagland solution plus sucrose (HSS) and root exudates (RE) on the antagonists and pathogen biomass. Antagonists: *Aspergillus* sp. (Asp), *T. harzianum* (Tri) and *Verticillium dahliae* (Vert). Error bars show standard errors (n=3).

4.3.4 Effect of amino acids, organic acids, and sugars on MS germination

Data for all amino acids, organic acids and sugars were analysed together, but are presented separately in the figures for clarity. There was a significant ($F_{10, 22}$ = 19.64, P < 0.001) effect of supplement on

germination of MS. Each individual amino acid significantly stimulated MS germination compared with water agar treatment (Figure 4.4). Similarly, organic acids and sugars also significantly stimulated MS germination, except for malic acid and xylose, which were not significantly different from the water agar treatment (Figure 4.5 and Figure 4.6). Apart from xylose and malic acid, there were no significant differences between the other tested sugars, amino acids, or organic acids when they were analysed together.

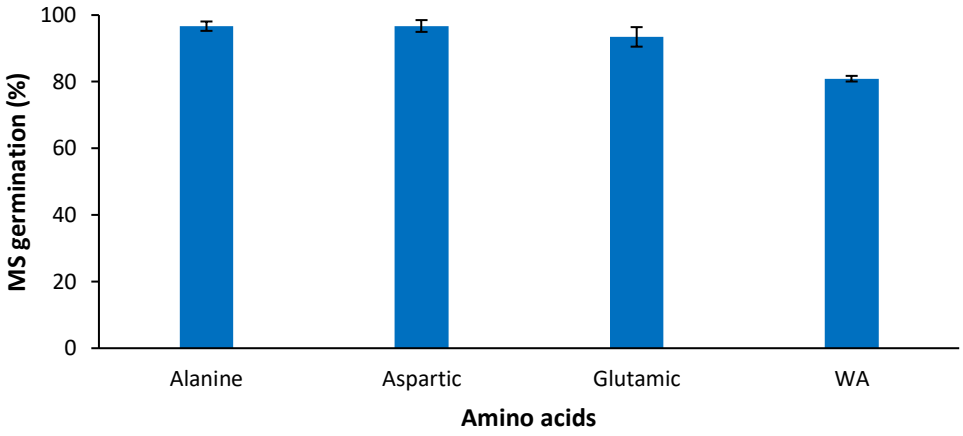


Figure 4.4 Effect of amino acids compounds (alanine, aspartic acid and glutamic acid) and water agar (WA) on MS germination of *Verticillium dahliae* DAR31890 strain. Error bars show standard errors (n=3).

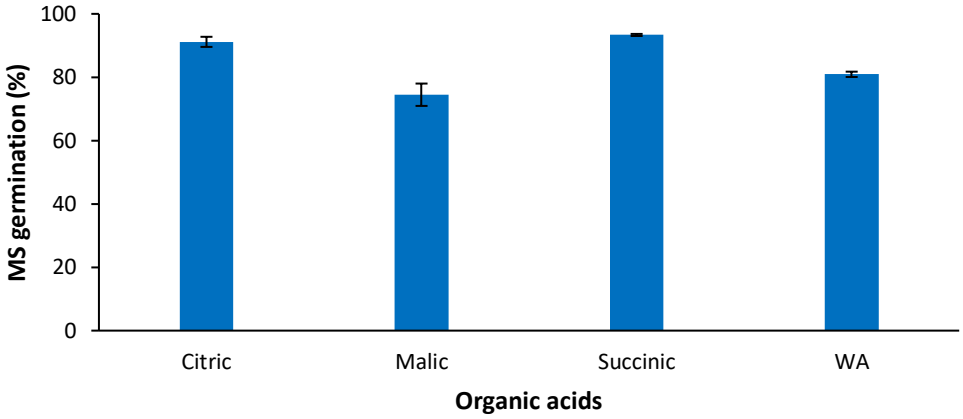


Figure 4.5. Effect of organic acids compounds (citric, malic and succinic acids) and water agar (WA) on MS germination of *Verticillium dahliae* DAR31890 strain. Error bars show standard errors (n=3).

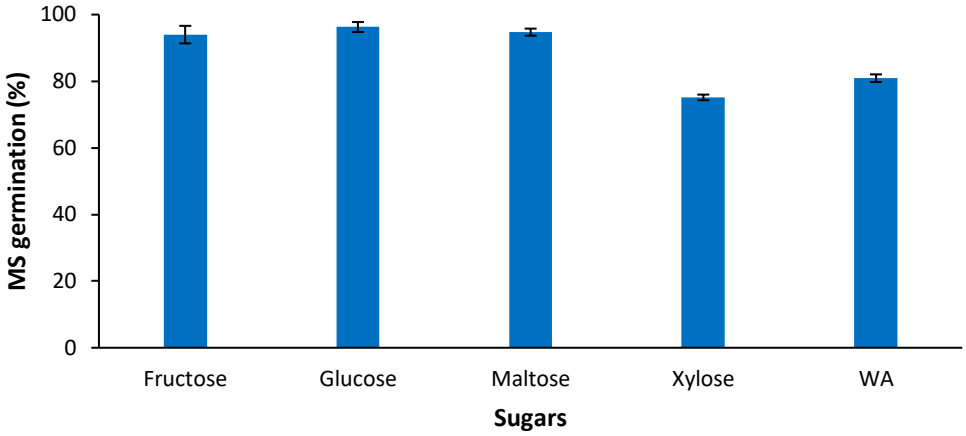


Figure 4.6. Effect of sugars (fructose, glucose, maltose, and xylose) and water agar (WA) on MS germination of *Verticillium dahliae* DAR31890 strain. Error bars show standard errors (n=3).

4.3.5 Interaction between fructose and BCAs for controlling Verticillium wilt under glasshouse condition

There was a significant ($F_{9,20}$ = 22.04, F < 0.001) effect of treatment on disease severity. The results showed that all biocontrol treatments in the presence or absence of fructose significantly reduced disease severity, compared with the pathogen only treatment, except for *Aspergillus* at 0.5 g/L fructose (Figure 4.7). None of the treatments that included fructose resulted in significantly lower disease severity than either *Aspergillus* or *Trichoderma* alone.

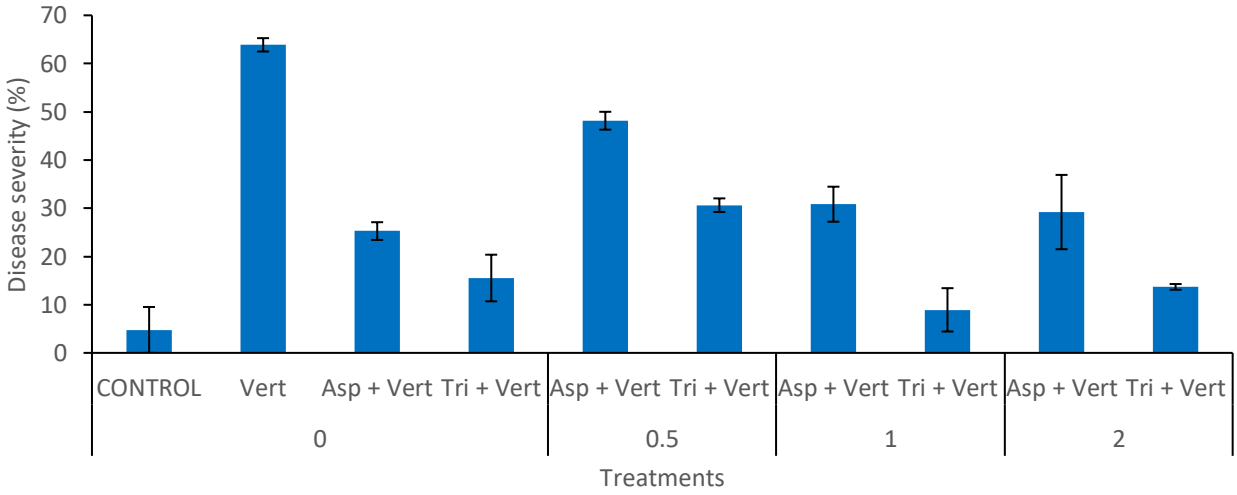


Figure 4.7. Effect of antagonists and fructose (0, 0.5, 1, and 2 g/L) on Verticillium wilt disease severity on tomato Grosse Lisse cultivar whole plants. Antagonists: *Aspergillus* sp. (Asp), *T. harzianum* (Tri) and *Verticillium dahliae* (Vert). Error bars show standard errors (n=3).

There was a significant ($F_{9,20}$ = 3.88, F = 0.006) effect of treatment on plant height (Figure 4.8). Infection with *V. dahliae* DAR31890 strain significantly reduced height relative to the uninoculated

control. However, only the treatment with *Trichoderma* plus 2 g/L fructose significantly increased height relative to the *V. dahliae* only treatment.

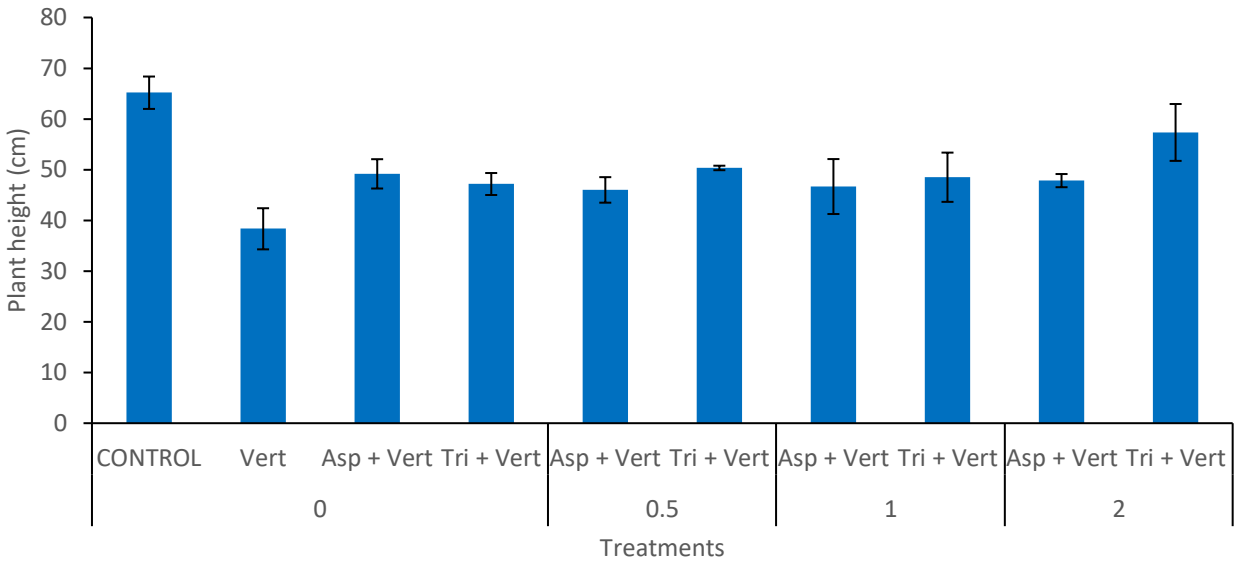


Figure 4.8. Effect of antagonists and fructose (0, 0.5, 1, and 2 g/L) on tomato Grosse Lisse cultivar height. Antagonists: *Aspergillus* sp. (Asp), *T. harzianum* (Tri) and *Verticillium dahliae* (Vert). Error bars show standard errors (n=3).

There was a significant ($F_{9,20} = 8.57$, $F < 0.001$) effect of treatment on shoot dry weight (Figure 4.9). Infection with *V. dahliae* DAR31890 strain reduced shoot dry weight by half compared with the uninoculated control. Both treatments of antagonistic fungi *T. harzianum* with 2 g/L fructose and *Aspergillus* sp. without fructose significantly increased shoot dry weight of tomato plants compared with the *Verticillium* only treatment (Figure 4.9). All other treatments were not significantly different from the *Verticillium* only treatment. However, none of the antagonist treatments had a significant effect on root dry weight ($F_{9.20} = 1.90$, $F = 0.112$, Figure 4.10).

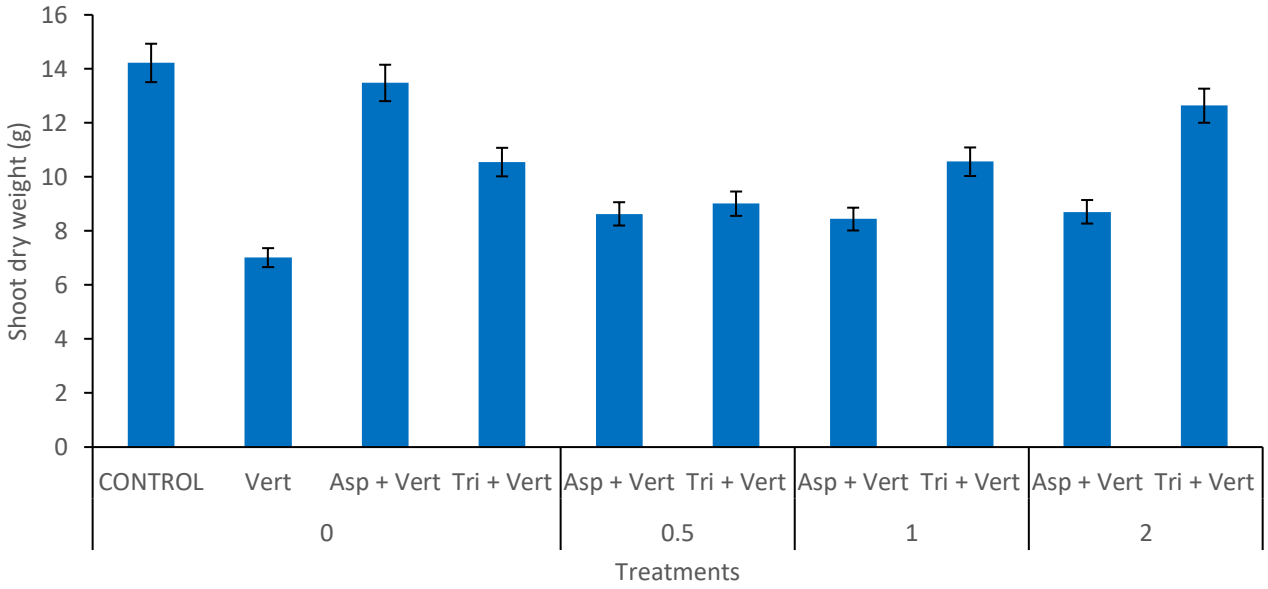


Figure 4.9. Effect of antagonists and fructose (0, 0.5, 1, and 2 g/L) on shoot dry weight of tomato Grosse Lisse cultivar. Antagonists: *Aspergillus* sp. (Asp), *T. harzianum* (Tri) and *Verticillium dahliae* (Vert). Error bars show standard errors (n=3).

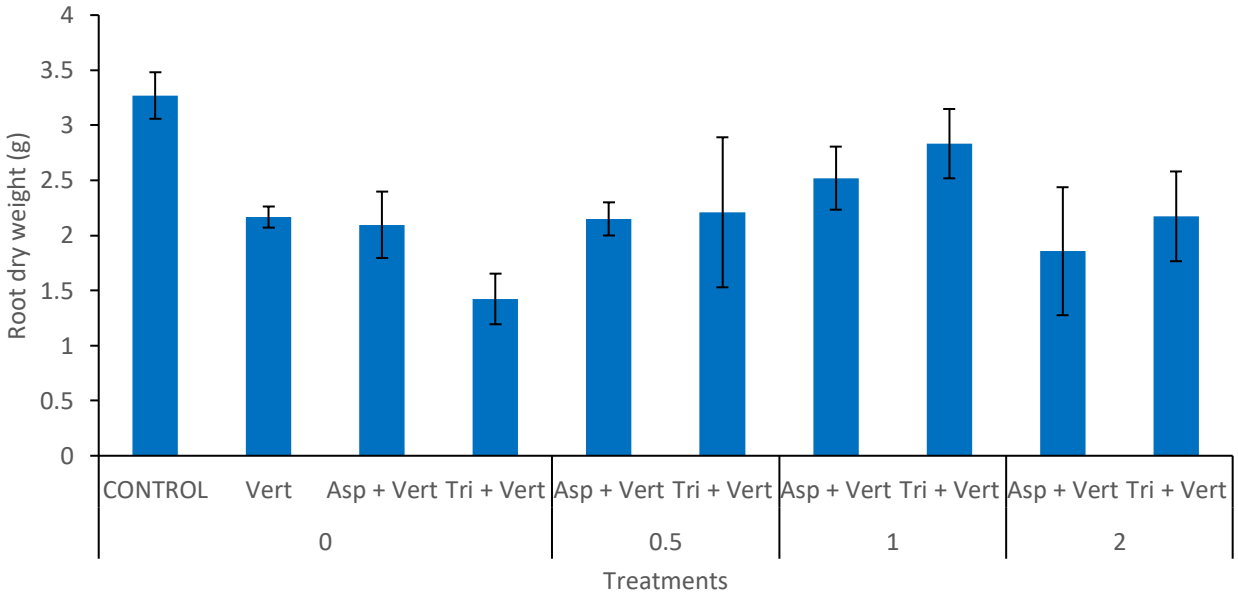


Figure 4.10. Effect of antagonists and fructose (0, 0.5, 1, and 2 g/L) on root dry weight of tomato Grosse Lisse cultivar. Antagonists: *Aspergillus* sp. (Asp), *T. harzianum* (Tri) and *Verticillium dahliae* (Vert). Error bars show standard errors (n=3).

4.4 Discussion

In this experiment, we explored the effect of tomato root exudates on the pathogen and on the efficiency of BCAs in controlling tomato Verticillium wilt. The *in vitro* results indicated that there was a significant effect of root exudates on MS germination of the *V. dahliae* DAR31890 strain compared with water agar only. These findings were similar to research completed by Olsson and Nordbring-Hertz (1985) who found that the microsclerotial germination percentage of *V. dahliae* was

higher in a mineral salt solution plus sucrose than in mineral salts solution alone, or water. López-Moral et al. (2022) demonstrated that root exudates from olive cultivars induced MS germination of *V. dahliae* compared with the control treatment (without exudates). Ali et al. (2017a) reported that the addition of root exudates to the Hoagland nutrient solution significantly increased spore germination of the fungus *F. oxysporum* compared to water only. The positive effect of root exudates on MS germination could be due to its nutrient composition, or as an important source of carbon and energy (Vančura et al. 1977, Carvalhais et al. 2011, Alvarez et al. 2012, Mavrodi et al. 2021). If the sources of carbon differ by host genotype, this could explain some of the differences in the characteristics of the pathogen populations (Baergen et al. 1993, Mol & Riessen 1995). The results also showed that MS could well germinate in SDW. This aligns with Sarenqimuge et al. (2022) who found no significant differences in rates of MS germination of *V. longisporum* between sterile double distilled water, PDB, and glucose solution.

The results showed that adding sucrose to the Hoagland solution reduced inhibition of *V. dahliae* by *T. harzianum* in dual culture experiments. This result could be because the sucrose did not affect the growth of *T. harzianum* but gave an advantage to and stimulated mycelial growth of the pathogen. This is what occurred when adding sucrose to the Hoagland solution, which led to an increase in the fungal weight of the pathogen and antagonistic fungi. These results could be due to the fact that sucrose was the best carbon source for the pathogen and antagonistic fungi (Kravchenko et al. 2003, Grahovac et al. 2021). This was confirmed *in vitro*, when exploring the effect of some root exudate components including amino acids, organic acids and sugars on MS germination of *V. dahliae*.

There was no significant effect of root exudates on the fungal weight of the pathogen and antagonistic fungi compared with the Hoagland nutrient solution. These results could be because the concentration of root exudate in the Hoagland solution was either too high or low. This result aligned with Wang et al. (2015) who observed that high concentrations of root exudates at 400 ml/L reduced spore germination of the fungus *R. intraradices*. Hassan et al. (2015) found that high concentrations of acetic acid (at 10%) inhibited the fungal growth of *A. flavus* and *P. purpurogenum.* Furthermore, it could be that some of the compounds secreted from the roots may have a negative effect on fungal growth, despite potentially being stimulatory for MS germination, as with some organic acid components.

All of the root exudates compounds except malic acid and xylose significantly stimulated MS germination compared with the water agar treatments. These results are consistent with many studies that indicated the role of some root exudate compounds in stimulation of *Verticillium* MS germination (Olsson & Nordbring-Hertz 1985, Vidauri 1998, López-Moral et al. 2022). According to Vidauri (1998), the greatest MS germination of *V. dahliae* was recorded at 7.5 and 11.25 µg/ml amino acid

concentration. Yuxiang et al. (2008) found that alanine was the most active among amino acids compounds in stimulation of spore germination of *V. dahliae*. Congly and Hall (1976) demonstrated that the germination percentage of *V. dahliae* was higher in an aqueous sucrose solution than in salt solutions. The reason for an improvement in germination after adding root exudates compounds, including amino acids, organic acids and sugars to the culture medium, may be that these compounds were considered as an energy source, or due to the carbon content and nutrient requirements for MS germination (Singh & Mukerji 2006, Canarini et al. 2019).

The low germination of MS in malic acid and xylose could be due to the fact that some compounds, when used separately, may not be sufficient to enhance germination of MS. Therefore, MS requires another carbon source for germination. In addition, some compounds may have antifungal activities against pathogens. Barbero-López et al. (2020) demonstrated that propionic acid 1 g/L concentration significantly inhibited growth of all decay fungi.

In glasshouse experiments, it was expected that treatment with fructose might reduce disease, since MS would germinate and interact with BCAs before plant roots grew near them. However, this hypothesis was not supported by the experimental work. The results showed that adding fructose to the soil markedly reduced the effect of the antagonistic fungi against the pathogen, with similar or higher disease severity being observed. This may be due to either competition or preferential utilisation differences between the pathogen and antagonists for carbon source in rhizosphere. Sugars, including fructose, glucose, galactose, and sucrose, are all major sources of carbon and energy in the soil (Lugtenberg et al. 1999, Costa et al. 2002, Pegg & Brady 2002), though their ability to promote growth and their preferences for use by different soil organisms appears largely unexplored.

Therefore, because of the positive effect of root exudate compounds on MS germination, it appears valuable to undertake further work using these compounds to simulate MS germination and to examine their interaction with BCAs before crop planting. Also, all of the BCAs, with and without sugar, showed significantly reduced disease severity and promoted plant growth compared with the pathogen only treatment. This is possibly because protection of the plant by *Trichoderma* and *Aspergillus* depends on mechanisms other than the inhibition of pathogen growth, such as induced resistance or mycoparasitism.

Chapter 5. *Trichoderma harzianum* and *Aspergillus* sp. induced systemic resistance in tomato against *V. dahliae* DAR31890

5.1 Introduction

Tomato is ranked as the second most consumed *vegetable* crop in the world, after potato (Behiry et al. 2023). Many pathogens such as fungi, nematodes, bacteria, and viruses attack tomato plants *leading* to serious diseases and yield loss. Among these pathogens, *V. dahliae* is one of the most serious tomato *pathogen*, and causes Verticillium wilt *disease* (Jabnoun-Khiareddine et al. 2009b).

Plants under various pathogens stress can produce many of reactive oxygen species (ROS), including OH, O^{2-}, and hydrogen peroxide. Therefore, accumulation of ROS in plant tissues can lead to cellular damage and ultimately cell death (Małolepsza & Różalska 2005, Torres et al. 2006, Zehra et al. 2017b, Rodrigues & Furlong 2022). Hydrogen peroxide accumulation in leaf tissues may increase the activity of several antioxidant enzymes such as peroxidase (PO), catalase (CAT), ascorbate peroxidase (APx), guaiacol peroxidase (GPx), and superoxide dismutase (SOD), which are a major defence mechanism of plants (Xia et al. 2009, Nikraftar et al. 2013, Zehra et al. 2023).

Peroxidase enzyme is a key enzyme of the phenyl propanoic pathway, and its activity increases many times in plant tissues infected by pathogens (Ojha & Chatterjee 2012). It is widespread in plant tissues and important contributor to the defence mechanism of the host by playing an essential role in the biosynthesis of plant cell wall components, which are well known in plant defence responses to pathogens (Kalra et al. 1995). Peroxidase is a ROS source in plants; it is also linked to cell walls. This enzyme regulates and participates in ROS production during the protection of plants against external attacks, and programmed cell death (da Silva et al. 2016).

Biological control agents (BCAs) can protect plants by way of specialised mechanisms such as antagonism, mycoparasitism and secretion of enzymes (Alizadeh et al. 2020, Reghmit et al. 2021), by competition, and by stimulating plant resistance through production of some biochemical compounds by the plant that are active against pathogens (Gajera et al. 2013, Alshimaysawe 2018). During plant-microbe interactions, many metabolites including, but not limited to, low molecular weight compounds, proteins with enzymatic activity, and other secondary metabolites, such as β-1, 3 glucanase, chalcone synthase, PO, polyphenol oxidase (PPO), chitinase, and phenylalanine ammonia-lyase (PAL), trigger defence mechanisms in the plants against pathogens by increased expression of pathogenesis-related (PR) genes that reduce plant pathogen infection (Hage-Ahmed 2007, Verma et al. 2019, Yu et al. 2022). For example, when the *Trichoderma* spp. come into contact with plant roots, they produce at least three types of elicitors such as peptides, proteins and low-molecular weight compounds that elicit plant defence responses against plant pathogens (Harman et al. 2004).

Nemafree and oxalic acid formulation derived from *A. niger* increase the amount of secondary metabolites, such as total phenolics, lignin, and flavonoids in tomato plants (Yeon et al. 2021).

In the last decade, induced systemic resistance (ISR) has become a widely recognised phenomenon. Many plant growth promoting rhizobacteria (PGPR), such as *Pseudomonas* spp., *Bacillus* spp., and *Streptomyces* spp., serve as BCAs by inducing resistance responses in associated plants against a broad range of pathogens (Chowdappa et al. 2013, Fotoohiyan et al. 2015, Suresh et al. 2022). For example, Nithya et al. (2019) reported that *Pseudomonas* sp. VSMKU2 significantly increased total phenol contents activity in crop plants, and that it could be used as a bioinoculant for the management of rice sheath blight. The antagonistic *Bacillus* spp. has been found to enhance antioxidant defence activities in rice leaves and roots (Rais et al. 2017). In addition to PGPR, there are also plant growth promoting fungi (PGPF), such as members of fungal genera *Trichoderma*, *Aspergillus*, *Fusarium*, *Penicillium*, *Piriformospora*, and *Phoma* (Hossain et al. 2017). These fungi can be used as biocontrol *agents* for several pathogens and as PGPF, because they do not harm humans and animals, and are eco-friendly. Their activity is proposed to be due to the production of a series of metabolites inhibiting fungal and bacterial activities, with the advantages of fast growth, strong resistance, and simple nutrition requirements.

The antagonistic properties of *Trichoderma* strains are based on the activation of multiple mechanisms, and act as biocontrol agents against numerous classes of plant pathogens, either directly or indirectly. Recent research indicates that *Trichoderma* spp. can also induce systemic and localised resistance to a wide spectrum of plant pathogens (Harman et al. 2004, Yan et al. 2021). Zehra et al. (2023) demonstrated that the fungus *T. harzianum* enhanced tomato defence-related genes against the pathogen *F. oxysporum* f. sp. *lycopersici* by increasing PAL and PO activities. The fungus *T. harzianum* improved tomato systemic resistance against root-knot nematodes through the accumulation of flavonoids, lignin, phenols, and cellulose as well as via the activity of chitinases, amylase, β-1,3-glucanase, and proteases (Yan et al. 2021). Total phenol and flavonoid contents increased in tomato plants treated with the *T. harzianum* strain KABOFT4 compared to untreated plants (Abo-Elyousr & Marei Almasaudi 2022). According to Fotoohiyan et al. (2015) five isolates of *T. harzianum* stimulated the defence enzymes PO and PAL in pistachio seedlings against *V. dahliae*. The activities of SOD, POD, PAL, and PPO were significantly higher in eggplant leaves treated with *T. harzianum* T23 compared with a *V. dahliae* only treatment (Liu et al. 2014).

Zhao et al. (2018b) found that the fungus *A. tubingensis* had the ability to produce some chemical compounds, such as indole acetic acid, phosphate solubilisation and siderophore production that have positive effects on reducing grey mould disease of tomato while also improving plant *growth* parameters. Salas-Marina et al. (2011) demonstrated that *A. ustus* stimulated systemic resistance in

Arabidopsis thaliana against *P. syringae* and *B. cinerea* through induction of jasmonic and salicylic acid (JA and SA) and defence-related genes in the plant.

However, few studies have reported a biocontrol effect and prevention mechanism of *T. harzianum* on Verticillium wilt in tomato plants (Jabnoun-Khiareddine et al. 2009b, Naraghi et al. 2010), while there appear to be no studies regarding *Aspergillus* sp. Therefore, the aim of this study was to find out whether biocontrol agents could induce tomato plant resistance against *V. dahliae* and promote plant growth.

5.2 Materials and methods

5.2.1 Induction of systemic resistance to V. dahliae DAR31890 strain by the antagonistic fungi

To determine if BCAs T. harzianum and an Aspergillus sp. induced resistance to V. dahliae DAR31890 strain, stem inoculation of tomato with the pathogen V. dahliae was done after BCAs were applied to roots. The experimental design had two factors: roots inoculated with *T. harzianum*, *Aspergillus* sp. or uninoculated; and stems inoculated with *V. dahliae* or uninoculated. There were four replicates of each treatment.

Tomato seeds of Grosse Lisse cultivar were sown in small pots (5 x 12.5 cm), filled with soil mixture as described previously (Chapter 3, 3.2.2). Pots were put on benches inside the glasshouse at 25±1 °C. Tomato seedlings at 3-4 true leaves were removed from the pots and their roots were washed under tap water gently to remove the soil then tomato roots were dipped in the spore suspensions of 10^6 conidia/ml of *T. harzianum* or *Aspergillus* sp. for 60 min. For control treatment, roots of tomato seedlings were dipped in sterile distilled water only. Seedlings were transplanted to larger pots (20 x 20 cm) filled with a soil mixture. Tomato seedlings were inoculated with the pathogen *V. dahliae* after 7 days from transplanting (De Cal et al. 1997). *A wound approximately 0.5 cm long was made with scalpel in the stem, 2-3 cm above the soil surface.* A disc of PDA with 7-day old culture of *V. dahliae* (0.5 cm diameter) was placed on each wound. Each wound was wrapped by cotton and Parafilm. *The treatments included*

1. Control (without pathogen and antagonists)
2. *V. dahliae* only (pathogen)
3. *T. harzianum* only
4. *Aspergillus* sp. only
5. *T. harzianum + V. dahliae*
6. *Aspergillus* sp. *+ V. dahliae*

Cotton and Parafilm were removed 7 days after inoculation of the pathogen. Disease severity, brown discoloration (as described at the end of Section 3.2.2, page 28) and plant growth parameters (leaves

length, plant height, and dry weight) were determined. Leaf length was measured with ruler from the beginning of petiole to the end of the terminal leaflet for the lower leaves.

5.2.2 Determination of enzymatic activity

After three days of treatment with antagonistic fungi *T. harzianum* and *Aspergillus* sp. in the above experiment, two or three leaflets were harvested from all tomato seedlings and collected individually inside resealable pouches and brought to the laboratory. Then, a small amount of Polyvinylpolypyrrolidone PVPP and sand were added to labelled microcentrifuge tubes 1ml with 1ml 50 mM potassium acetate buffer (pH 5.0) containing 5 mM glutathione and 1mM of EDTA. Leaflets 0.2 – 0.3 g fresh weight were added to the microcentrifuge tubes and ground well with a micro pestle then all tubes were put inside ice tubes racks then centrifuged at 9,000 g for 5 min (Eppendorf Minispin Plus Centrifuge) (Dann & Deverall 2000). Supernatants were poured into new tubes and stored in the freezer until assay of enzyme activities.

Peroxidase activity was identified by the appearance of pink colour because of guaiacol oxidation with hydrogen peroxide. Guaiacol (Sigma, 50 µl, 0.02 M), 2.0 ml 0.2 M sodium phosphate buffer pH 5.8, and 0.5 ml 0.38 M were added to the disposable cuvettes 3.0 ml then 100 µl of plant extract was added. The cuvettes were inverted to mix the ingredients together before being read in the spectrophotometer (Biochrom Libra S11) for optical density at 470 nm for 5 min. Concentration of protein in crude extracts was determined by Bradford Assay with bovine serum albumin (BSA) as standard. Results of peroxidase were calculated as ΔOD_{470} mg protein $^{-1}$ min $^{-1}$ (Dann & Deverall 2000).

5.2.3 Statistical analysis

The pot trial was analysed by two-way ANOVA in IBM SPSS 25. One-way ANOVA was used for the enzyme assays. Homogeneity of variance was checked using Levene's test. As a result, the data for stem browning (Figure 5.2) were log-transformed before analysis.

5.3 Results

5.3.1 Induction of systemic resistance to V. dahliae DAR31890 strain by the antagonistic fungi.

There were significant main effects of pathogen ($F_{1,18}$ = 134.85, P < 0.001) and antagonist ($F_{2,18}$ = 32.87, P < 0.001) treatment, *and their interactions* ($F_{2,18}$ = 38.11, P < 0.001), on disease severity. The pathogen-only treatment caused wilt symptoms on 66 % of tomato leaves and 7.5 cm *extent of vascular browning* (Figure 5.1 and Figure 5.2). Pre-inoculation with BCAs *T. harzianum* and *Aspergillus* sp. significantly reduced disease severity compared with the pathogen only treatments.

The best reduction of disease severity was recorded by *T. harzianum* (Tri + Vert), which was not significantly different from the treatments that had not been inoculated with the pathogen (Figure 5.1). There were significant effects of pathogen ($F_{1,18}$ = 13.60, P < 0.002) and of the interaction between pathogen and antagonist ($F_{2,18}$ = 8.93, P < 0.002), on the logarithm of the length of browning in the stem. The treatments of *T. harzianum* (Tri + Vert) and *Aspergillus* sp. (Asp + Vert) significantly reduced brown discoloration inside the plant tissue compared with the pathogen only treatment. There was no significant difference between antagonist only treatments and the treatment that had not been inoculated with either antagonists or *Verticillium* (Figure 5.2, $F_{2,18}$ = 1.24, P = 0.31).

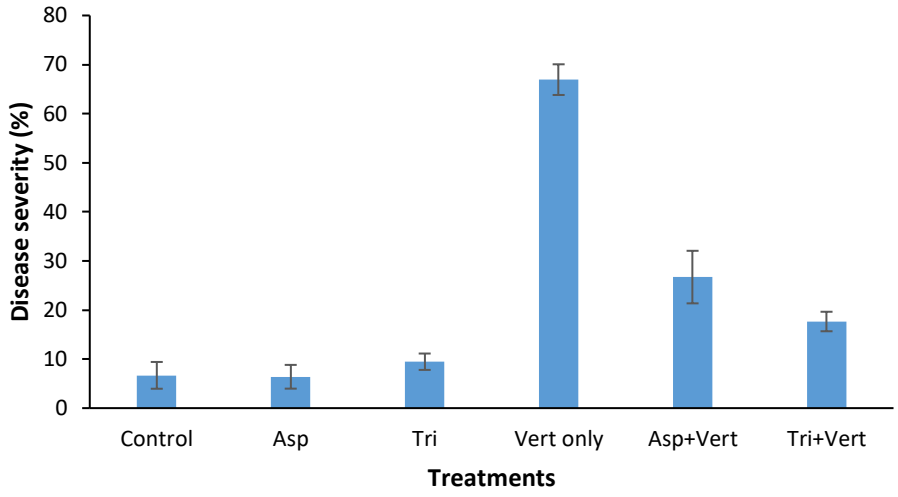


Figure 5.1. Effect of antagonistic fungi *Trichoderma harzianum* and *Aspergillus* sp. (Tri and Asp) on disease severity of tomato (Grosse Lisse) plants inoculated with the pathogen *Verticillium dahliae* DAR31890 strain (Vert). Error bars show standard errors (n=4).

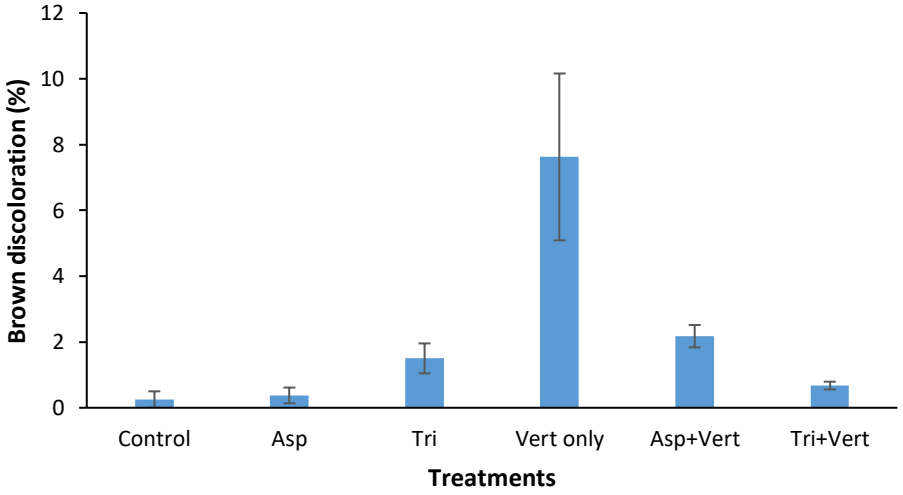


Figure 5.2. Effect of antagonistic fungi *Trichoderma harzianum* and *Aspergillus* sp. (Tri and Asp) on stem vascular discoloration extent on tomato (Grosse Lisse) plants inoculated with the pathogen *Verticillium dahliae* DAR31890 strain (Vert). Error bars show standard errors (n=4).

Inoculating tomato seedlings with *V. dahliae* significantly reduced leaf length ($F_{1,18}$ = 8.33, P = 0.01). Leaf length of tomato plant was significantly increased by inoculation with *T. harzianum* and *Aspergillus* sp. ($F_{2,18}$ = 16.11, P < 0.001). However, the interaction between the pathogen and antagonist treatments was not significant ($F_{2,18}$ = 3.25, P = 0.06) (Figure 5.3).

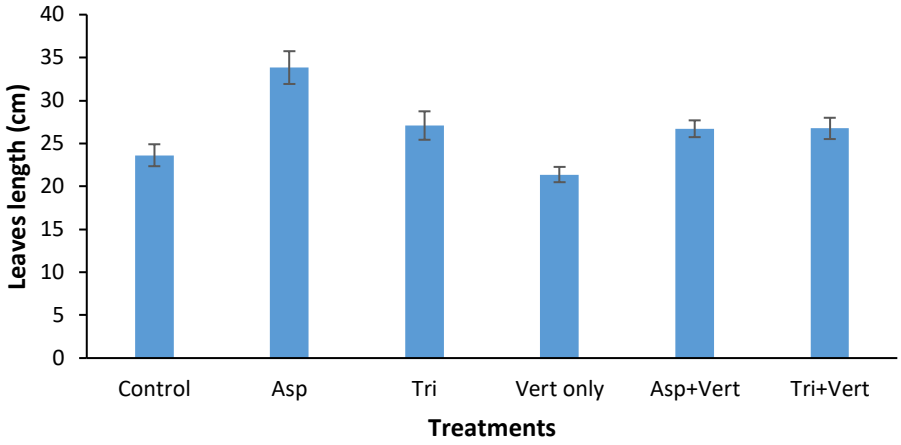


Figure 5.3. Effect of antagonistic fungi *Trichoderma harzianum* and *Aspergillus* sp. (Tri and Asp) on leaf length of tomato (Grosse Lisse) plants inoculated with the pathogen *Verticillium dahliae* DAR31890 strain (Vert). Error bars show standard errors (n=4).

The main effect of pathogen on plant height was not significant ($F_{1,18}$ = 0.48, P = 0.50). However, the effects of antagonist $F_{2,18}$ = 3.81, P = 0.04) and the interaction between pathogen and antagonist ($F_{2,18}$ = 5.25, P = 0.016) were significant. Plant height was significantly increased in the *T. harzianum* (Tri + Vert) treatment compared with pathogen only (Figure 5.4).

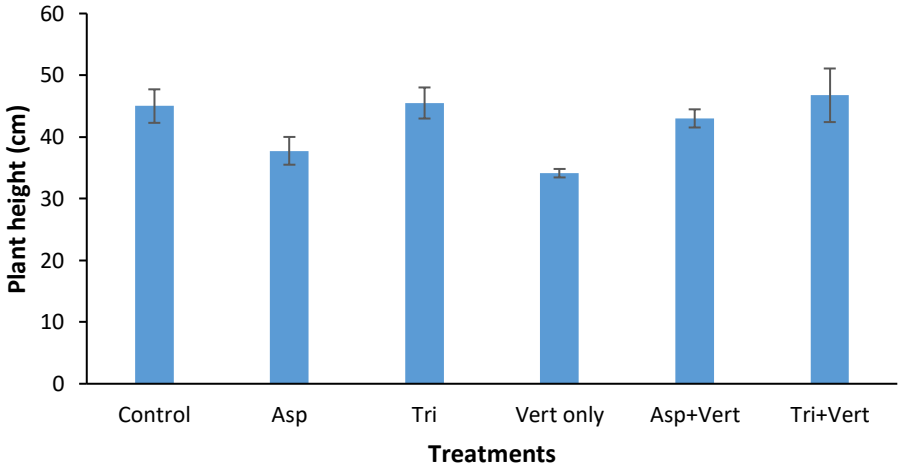


Figure 5.4. Effect of antagonistic fungi *Trichoderma harzianum* and *Aspergillus* sp. (Tri and Asp) on plant height of tomato (Grosse Lisse) plants inoculated with the pathogen *Verticillium dahliae* DAR31890 strain (Vert). Error bars show standard errors (n=4).

There was a significant effect of antagonist on shoot dry weight ($F_{2,18}$ = 17.83, P = 0.008). Dry weight of the shoots system increased significantly with *T. harzianum* treatments (Tri + Vert) compared with the pathogen only (Figure 5.5) However, the effects of pathogen ($F_{2,18}$ = 0.02, P = 0.89) and the interaction between pathogen and antagonist ($F_{2,18}$ = 2.34, P = 0.125) were not significant.

There were no significant effects of the pathogen ($F_{1,18}$ = 1.15, P = 0.30), antagonists ($F_{2,18}$ = 2.94, P = 0.08) or their interaction ($F_{2,18}$ = 1.01, P = 0.40) on root dry weight because of the greater variability of root weight measurements (Figure 5.6).

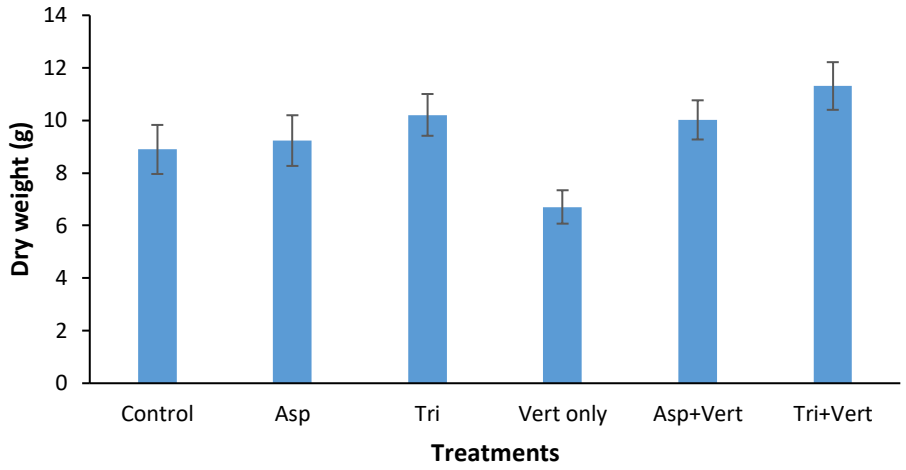


Figure 5.5. Effect of antagonistic fungi *Trichoderma harzianum*, and *Aspergillus* sp. (Tri and Asp) on shoot dry of tomato (Grosse Lisse) plants inoculated with the pathogen *Verticillium dahliae* DAR31890 strain (Vert). Error bars show standard errors (n=4).

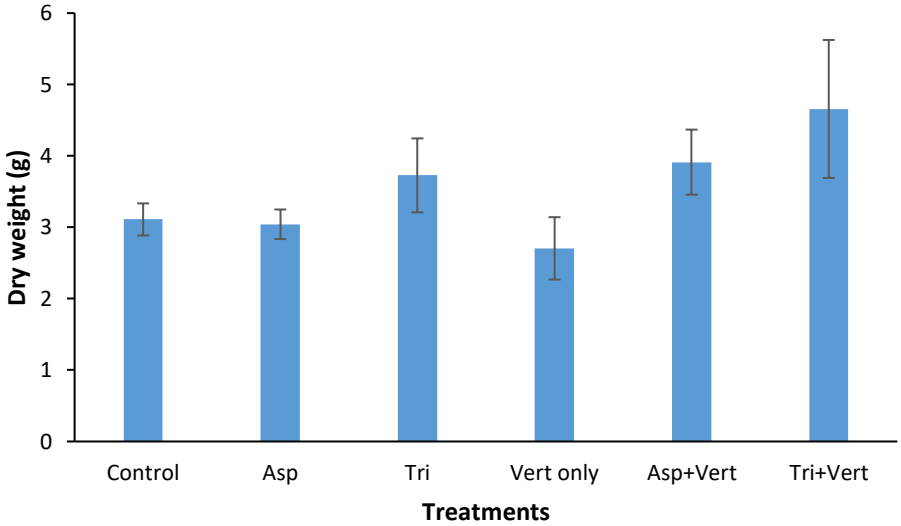


Figure 5.6. Effect of antagonistic fungi *Trichoderma harzianum* and *Aspergillus* sp. (Tri and Asp) on root dry weight of tomato (Grosse Lisse) plants inoculated with the pathogen *Verticillium dahliae* DAR31890 strain (Vert). Error bars show standard errors (n=4).

5.3.2 Determination of enzymatic activity.

The results showed that peroxidase activity in leaves of tomato plants was not significantly different ($F_{2,21}$ = 0.71, P = 0.50) in plants treated with *T. harzianum* or *Aspergillus* sp. compared with untreated control plants (Figure 5.7), but the soluble protein concentration was significantly ($F_{2,21}$ = 19.07, P < 0.001) increased in the induced plants compared with the untreated control plants (Figure 5.8).

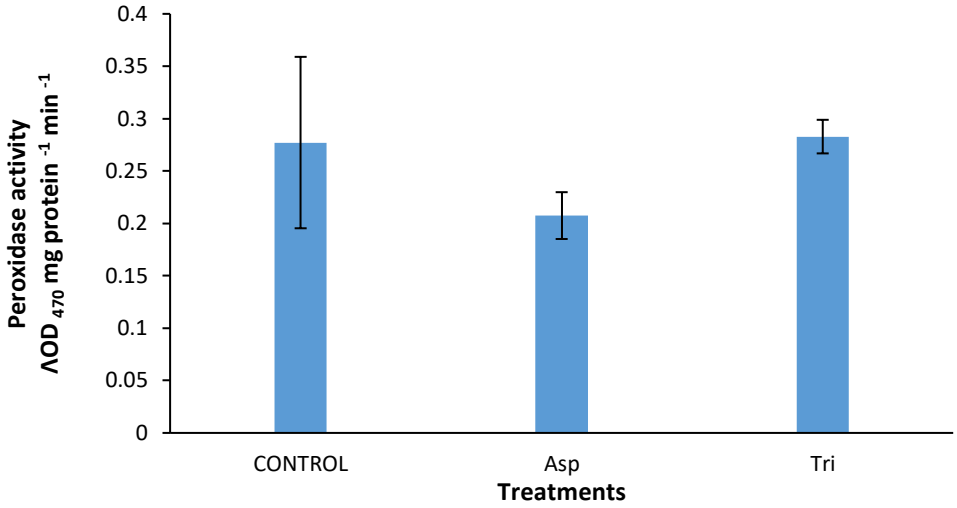


Figure 5.7. Activity of peroxidase in leaves of tomato plants (Grosse Lisse) after treatment with antagonistic fungi *Trichoderma harzianum* and *Aspergillus* sp. (Tri and Asp). Error bars show standard errors (n=8).

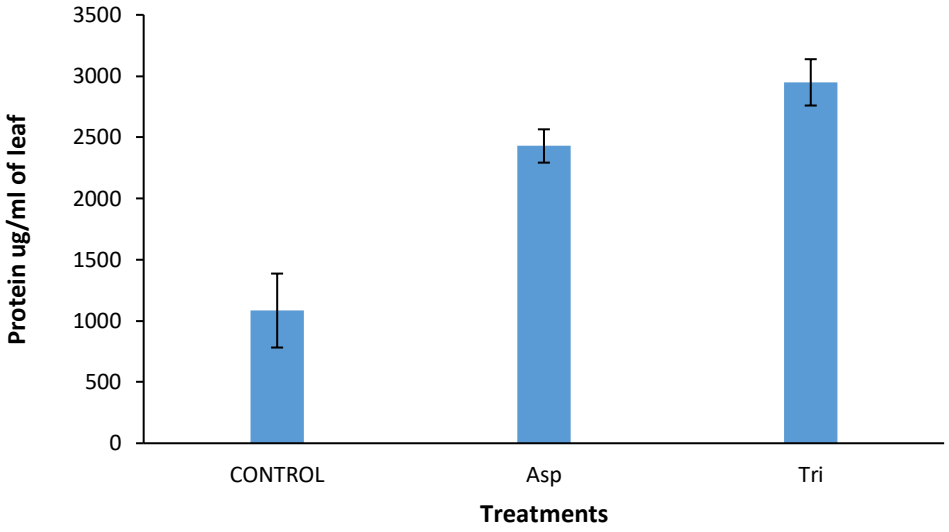


Figure 5.8. Soluble protein concentration in leaves of tomato plants (Grosse Lisse) after treatment with antagonistic fungi *Trichoderma harzianum* and *Aspergillus* sp. (Tri and Asp). Error bars show standard errors (n=8).

5.4 Discussion

While most studies have focused on direct antagonistic mechanisms between BCAs and plant pathogens, in this chapter the ability of *T. harzianum* and *Aspergillus* sp. to induce defence response

in the plant was studied, as well as determination of peroxidase activity in tomato leaves after treatment with a biocontrol agent on plant parts spatially separated from the site of *V. dahliae* inoculation.

Pre-inoculation with BCAs significantly reduced disease severity and brown discoloration of Verticillium wilt. These results are consistent with Jabnoun-Khiareddine et al. (2009b) who found that treatment of tomato seeds with *Trichoderma* species for three weeks before infection with MS of *V. dahliae* reduced Verticillium wilt severity and brown discoloration index on tomato plants. These results were also supported by Fotoohiyan et al. (2015) who found that the occurrence of wilt disease in pistachio plants inoculated with *Trichoderma* isolates seven days before infection with MS of *V. dahliae* were lower in comparison with plants inoculated with the pathogen *V. dahliae* alone. Zehra et al. (2017b) reported tomato seedlings treated with *F. oxysporum* f. sp. *lycopersici* 3 days after inoculation with *T. harzianum* and sprayed with SA and JA were protected against Fusarium wilt. Hafiz et al. (2022) demonstrated that pre-treatment of rapeseed roots with both *T. harzianum* OMG16 and *Bacillus velezensis* FZB42 restricted *V. longisporum* infection in roots, and induce *the* JA and ET hormone signal transduction pathways in rapeseed, which are very important for activating ISR. De Cal et al. (1997) found that applying the fungus *P. oxalicum* to the tomato roots before stem inoculation with the pathogen *F. oxysporum* reduced *Fusarium wilt* severity. Application of elicitors *A. fumigatus* and *R. oryzae* one week before infection with *F. oxysporum* significantly reduced Fusarium wilt disease in tomato plants (Attia et al. 2022a). In another study, Attia et al. (2022b) found that soil treatment or foliar spraying by *A. tubingensis*, both reduced the severity of *F. oxysporum* infection in pepper plants.

On the other hand, in our research pre-inoculation with BCAs enhanced tomato plant growth parameters (including plant height, leaf length, and dry weight for shoot system). The results aligned with Zehra et al. (2017b) who found that inoculation of tomato plants with *T. harzianum* and chemical inducers SA and MeJA three days before infection with *F. oxysporum* f. sp. *lycopersici* increased tomato shoot length, root length, and stem diameter compared to a control treatment. These results also align with Nzanza (2012) who found that *T. harzianum* and arbuscular mycorrhizal fungi (AMF) enhanced plant growth and increased tomato root and shoot length. Carrero-Carrón et al. (2016) reported that strains of *T. asperellum* significantly increased olive plant height compared with *V. dahliae* infected plants. Bilginturan and Karaca (2021) showed that *T. atroviride* in combination with the bacteria *P. koreensis* and *B. subtilis* stimulated plant growth and yield values of eggplant compared to plants treated with the pathogen *V. dahliae*. The results are also consistent with Chowdappa et al. (2013) who found that inoculation of tomato seeds with *T. harzianum* and *B. subtilis* led to significant increases in leaf area, fresh weight and length for shoots and roots system. Javed et al. (2020) showed that *A. terreus* significantly enhanced tomato plant growth. Inoculation of tomato

plants with *A. flavus* enhanced their fresh weight and plant length in comparison to untreated plants (Abdel-Motaal et al. 2020).

Contact of BCAs with roots may stimulate plant defences, resulting in reduced disease severity and brown discoloration as a result of delayed pathogen movement within BCAs treated plants (De Cal et al. 1997). This may be due to increased activity of enzymes such as PO, PAL and CHS by BCAs in tomato seedlings infected with *V. dahliae*, which are involved in the polymerisation of proteins and precursors of lignin or suberin in plant cell walls, thus building a physical barrier that can prevent penetration of pathogens in cell walls or movement through vessels (Fotoohiyan et al. 2015, Patel & Saraf 2017, Kumar et al. 2021). Brotman et al. (2013) showed that genes responsible for plant defence are up-regulated during *Trichoderma* colonisation such as ATPSK2, involved in organ morphogenesis and cell proliferation, and ANAC081, known to be associated with increased leaf size and biomass traits.

The results also showed that enzyme activity of peroxidase in leaves of tomato plants was markedly increased in tomato leaf extracts treated with *T. harzianum* but was not significant compared with untreated control treatments. This may be because the data was only from a single point in time. However, soluble protein concentration was significantly increased in tomato leaves treated with BCAs. These results are consistent with Elkelish et al. (2020) who found that pre-treatment with *T. harzianum* increased accumulation of soluble protein and other compounds such as proline, flavonoids, anthocyanin, and sugars in tomato seedlings. Our results are also supported by Houssien et al. (2010) who found that combining *T. harzianum* with salicylic acid (SA) increased soluble protein concentrations in tomato plants. Yu et al. (2021b) demonstrated the tomato seedlings treated by *T. asperellum* TaspHu1 significantly increased the soluble protein content and soluble sugar content in tomato. Hussein et al. (2021) reported that soluble proteins significantly increased in tomato plants treated with different concentrations of *T. harzianum* (10^5 and 10^7 spores/mL). It is worth noting that application of *A. ochraceus* individually or under saline stress conditions led to enhancement in the soluble protein contents of barley plants (Badawy et al. 2021). The inoculation of *A. japonicus* increased the nutritional quality of phenolic, flavonoids, soluble sugars, proteins, and lipids in soybean and sunflower plants in comparison to endophyte-free plants (Hamayun et al. 2018).

Several studies indicate that the activation of peroxidase and other enzymes by biocontrol agents plays a crucial role in the resistance of plants to pathogenic attack (Yedidia et al. 1999, Sreedevi et al. 2011, Madhavi et al. 2018). Chowdappa et al. (2013) have showed that systemic resistance in tomato seedlings against early and late blight infection was significantly enhanced through induction of growth hormones and defence enzymes including PO, PPO, and superoxide by *B. subtilis* OTPB1 and *T. harzianum* OTPB3. Yadav et al. (2021) reported that enhancing the activity of defence-related

enzymes such as PO, POX, PPO, CAT, and PAL by *T. harzianum*, *T. asperellum* and *Paenibacillus dendritiformis* increased plant resistance against anthracnose caused by *Colletotrichum truncatum*.

The enhanced peroxidase activity by BCAs may contribute to induced resistance by increasing the concentration of free radicals and by helping to generate hydrogen peroxide, which is considered toxic to diverse microbial pathogens (Hammerschmidt et al. 1982).

Based on the present study, it can be concluded that the pre-application of antagonists *T. harzianum* and *Aspergillus* sp. before *V. dahliae* infection significantly decreased subsequent disease severity, improved growth and development of host plants, and enhanced total soluble proteins. This enhancement of peroxidase enzymatic activity suggests that application of BCAs may enhance tomato plant capacity to protect themselves against subsequent pathogen infection through the stimulation of plant defences. Consequently, this study provided further knowledge of biological control management of Verticillium wilt disease of tomato plants, based on activation of plant defence (systemic resistance).

Chapter 6. Improvement of biocontrol of Verticillium wilt of tomato by addition of salicylic acid

6.1 Introduction

V. dahliae is a soil-borne fungus that can contribute to vascular disease of several plants, including tomato, and result in significant economic loss. Therefore, various methods have been used to control this pathogen including biocontrol which offers a potential non-chemical means of controlling plant diseases. In this context, antagonistic fungi are an exciting and rapidly evolving field of research (Moosavi & Zare 2020), and there is increasing interest in exploiting fungal BCAs against plant pathogens due to well-known biological control mechanism such as fungal parasitism, antibiosis, competition for nutrients and space, as well as the induction of defensive responses in plants (Harman 2006). Fungal BCAs have gained wide acceptance due to their relatively broad spectrum in terms of disease control (Verma et al. 2007). However, the practical application of BCAs often fails to provide complete disease control in the field or under greenhouse conditions (Alabouvette et al. 2006). To address this issue, researchers have attempted to use BCAs in combination with other additives such as chemical products e.g., fungicides; in combination with physical methods e.g., solarization or steam sterilization; and alongside agronomical practices such as the enhancement of suppressive soils (Spadaro & Gullino 2005). Several studies carried out on a large scale showed that combining BCAs with fungicides resulted in higher efficacy and persistence against plant pathogens (Bhai & Thomas 2010, Widmer 2019, Sánchez-Montesinos et al. 2021, Zhang et al. 2021). However, fungicide use has been gradually reduced, due to its negative impacts on human health and on the environment (Zhang et al. 2021). There is no specific fungicide available to control Verticillium wilt (Fradin & Thomma 2006). There are also alternative methods of disease control such as host resistance (Bakade et al. 2022). Therefore, it is necessary to identify alternatives to fungicides to improve efficiency of BCAs.

One of the modern alternatives to fungicides to improve the efficiency of BCAs is to use plant growth elicitors. Elicitors are chemical compounds from biotic sources including carbohydrates, *fungi*, plant growth promoting rhizobacteria, hormones such as salicylic acid (SA), jasmonic acid, gibberellic acid, ethylene, abscisic acid, auxin, and peptide hormones, and abiotic sources include drought, salt, light, and temperature (Bari & Jones 2009, Thakur et al. 2019). The combination of BCAs with elicitors may provide more consistent control levels against plant pathogens than of BCAs or any the components used alone (Giotis et al. 2012).

Among of these elicitors, SA has been found to be active as an antimicrobial agent in several trials as inducers of plant disease resistance (Abdel-Monaim 2013). *SA is a hormone present in plants in the form of free phenolic acid, associated with various functions in the plants and acts as an inducer of defence responses against pathogens* (Raskin 1992, Tian et al. 2007, Hayat et al. 2013). Jabnoun-Khiareddine et al. (2015) found that SA and chitosan could be used as potential inducers of systemic acquired resistance to successfully control tomato phytopathogenic fungi, including *V. dahliae*. Mahesh et al. (2017) found that priming of seeds with SA pre-sowing led to a significant increase in eggplant growth and inducing resistance against *V. dahliae*. Taheri et al. (2021) demonstrated that *SA improved growth and resistance of olive plants through increased phenol and superoxide dismutase activity in plant roots.* Seed treatment with SA significantly induced systemic resistance in chickpea against Fusarium wilt caused by *F. oxysporum f. sp. ciceri* (Sarwar et al. 2005).

SA is among the additives widely used with antagonists for the control of plant diseases (Qin et al. 2003, Rajkumar et al. 2008, Zehra et al. 2017b, Lyousfi et al. 2021). Abdel-Monaim (2013) reported that biocontrol agents *Trichoderma viride* and *Bacillus megaterium* combined with SA were more effective than use of them individually for controlling *F. oxysporum*, *F. solani*, *Rhizoctonia solani* and *Macrophomina phaseolina* that caused root rot and damping off of faba bean plants. According to Ojha and Chatterjee (2012), *T. harzianum* in combination with SA enhanced the activities of peroxidase and polyphenol oxidase in tomato plants against *F. oxysporum*. In addition, Houssien et al. (2010) reported that the combination of *T. harzianum* with SA enhanced tomato defences against *F. oxysporum* f.sp. *lycopersici.*

Thus, the aim of this study was to: (i) evaluate the *in vitro* activity of SA against the pathogen *V. dahliae* DAR31890 strain and antagonistic fungi *T. harzianum* and *Aspergillus* sp.; and (ii) determine SA efficiency alone or in combination with BCAs against Verticillium wilt, and to elucidate their impacts on tomato growth, under glasshouse conditions.

6.2 Materials and methods

6.2.1 Effect of SA on mycelial growth of the pathogen and antagonistic fungi

The effect of SA on mycelial growth of *V. dahliae* DAR31890 strain, *T. harzianum* and *Aspergillus* sp. was studied in culture. Three aqueous solutions of SA were prepared at concentrations 0.5, 1.0 and 2.0 mM, *based on 0.5 mM= 0.069 g/L, 1 mM= 0.138 g/L, and 2 mM= 0.276 g/L* of potato dextrose agar (PDA). About 20 ml of PDA medium amended with three concentrations of SA was poured into each Petri dish. Discs (8 mm diameter) from 5 day old cultures of either the antagonistic fungi or the pathogen were placed in the centre of the Petri dishes. All Petri dishes in three replicates were incubated at 25±1ºC with each treatment removed once the control plates had reached full growth (9

cm diameter) (Jabnoun-Khiareddine et al. 2015). After incubation, inhibition effect of SA was determined by following formula (Gaigole et al. 2011),

$$Inhibition = \frac{R1 - R2}{R1} \times 100$$

where *R1* = radial growth of the pathogen or antagonistic fungi in the control plate, and *R2* = radial growth of the pathogen or antagonistic fungi in the treatment plates.

6.2.2 Effect of SA on tomato seed germination and seedling vigour

Surface sterilized tomato seeds Grosse Lisse cultivar (3.2.6) were soaked in three concentrations of SA 0.5, 1.0 and 2.0 mM by placing 250 seeds in 50 ml of each SA concentration in the sterilized tubes. In control treatments, tomato seeds were soaked in sterile distilled water. All tubes were placed on a rotary shaker at 25°C for 3 hours. Ten seeds were placed on moistened filter paper discs in Petri dishes (Mahesh et al. 2017). Three replicates were used for each concentration. The Petri dishes were put in the incubator at 25°C for 12 days. Seed germination percentage was calculated by following formula,

$$G = \frac{Sgerm}{Stotal} \times 100$$

G = seed germination (%), *Sgerm* = number of seeds germinated, and *Stotal* = total number of seeds used. A germination test was used to estimate seedling vigour, which is the extent of damage to seeds (Zhao et al. 2016),

$$VI = G \times (Rt + Sht)$$

where *VI* = the vigour index, *G* = seed germination (%), *Rt* = mean root length, and *Sht* =mean shoot length.

6.2.3 Effect of SA on Verticillium wilt in glasshouse experiments

Tomato seeds which had been treated with three concentrations of SA (0.5, 1.0 and 2.0 mM) for 3 hours were sown in small pots (5 x 12.5 cm) filled with soil mixture (Premium Potting Mix, Searles Ltd, Kilcoy QLD, and river sand at: 2:1 v/v). The seedlings at 3-4 true leaves were transplanted into large pots (20 x 20 cm) filled with the same soil (single plant/pot). *After five days from transplanting into large pots*, all pots were inoculated with 200 ml of conidial suspension 10^6 conidia/ml of the pathogen *V. dahliae* DAR31890 strain except control treatments, which used water instead of the inoculum (Mahesh et al. 2017). *Conidial suspension was added to the soil around the plant*. Four replicates were used for each treatment. Disease severity was measured as described previously in

3.2.2. Seed germination and tomato plant *growth* parameters (plant height, dry weight of the shoot and root system) were determined.

6.2.4 Efficacy of combination between SA and biocontrol agents on Verticillium wilt

Effect of biocontrol agents *T. harzianum* and *Aspergillus* sp. in combination with SA on tomato Verticillium wilt was studied under glasshouse conditions. *Tomato seeds, which had been soaked at 0.5 mM SA concentration for 3 hours* (Mahesh et al. 2017), were sown in pots (5 x 12.5 cm) filled with non-sterilised soil (as 6.2.3). Seedling at 3-4 true leaves were transplanted in large pots containing 2 kg of soil (single plant/pot). A 200 ml volume of conidial suspension (10^6 conidia/ml) of *T. harzianum* and *Aspergillus* sp. were added to the pots three days *after transplanting in large pots.* Inoculum with the pathogen *V. dahliae* (200 ml of conidial suspension 10^6 conidia/ml) occurred 4 days after the treatment with antagonists. *The treatments included*

1. Control (without pathogen, antagonists and SA)
2. *V. dahliae* only
3. *T. harzianum* + *V. dahliae*
4. *Aspergillus* sp. + *V. dahliae*
5. SA + *V. dahliae*
6. *T. harzianum* + SA + *V. dahliae*
7. *Aspergillus* sp. + SA + *V. dahliae*

Four replicates were used for each treatment. As described above, disease severity and plant parameters were determined after six weeks following the application of the pathogen.

6.2.5 Statistical analysis

Data were analysed by ANOVA in IBM SPSS 25. Homogeneity of variance was tested with Levene's test. Data for the effect of concentration of SA on inhibition of mycelial growth (Figure 6.1) were log-transformed before analysis. Tukey's test was used for mean separation in ANOVA tests. The effect of SA on germination was analysed using the Kruskal-Wallis test.

6.3 Results

6.3.1 Effect of SA on mycelial growth of the pathogen and antagonists

There were significant effects of concentration of SA ($F_{2,18}$ = 10.0, P = 0.001) and fungus ($F_{2,18}$ = 24.11, P < 0.001) on inhibition of growth, but the interaction between these factors was not significant ($F_{4,18}$ = 1.34, P = 0.29). The results showed that SA significantly reduced mycelial growth of the fungi, and the degree of inhibition increased with an increase in the concentration (Figure 6.1 and

Figure 6.2). Among the SA concentrations, 2 mM concentration of SA showed the highest percentage of mycelial growth inhibition. Also, significantly higher inhibition percentage of mycelial growth was recorded in *V. dahliae* DAR31890 strain treatment compared with *T. harzianum* and *Aspergillus* sp. treatments.

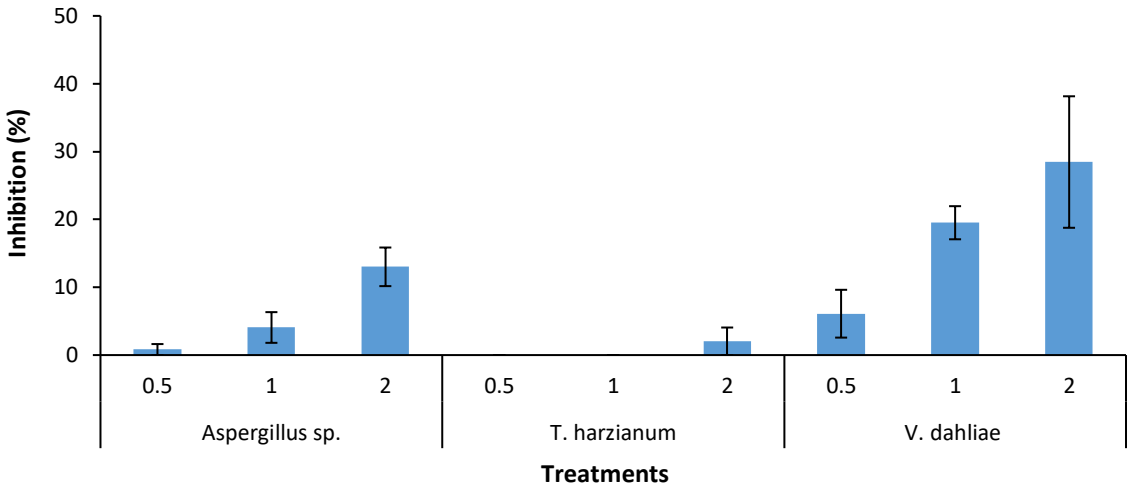
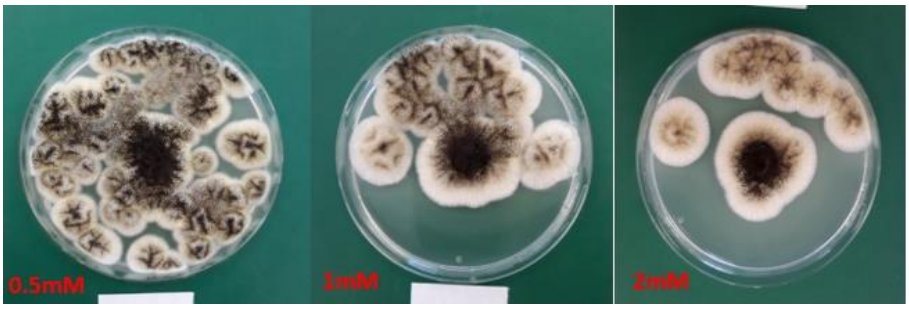
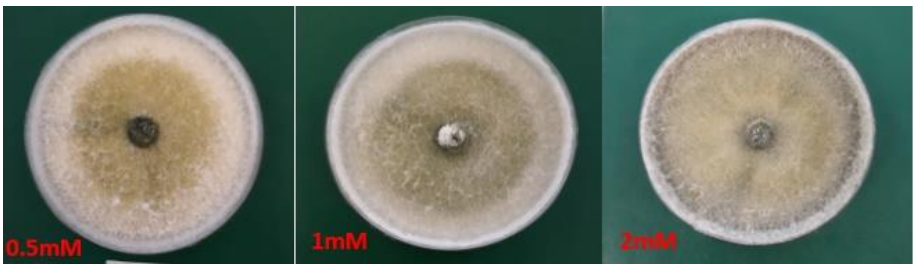


Figure 6.1. Effect of salicylic acid concentrations (0.5, 1.0 and 2.0 mM) on inhibition of mycelial growth of the pathogen and antagonists. Error bars show standard errors (n=3).

Aspergillus sp.



Trichoderma harzianum



Verticillium dahliae

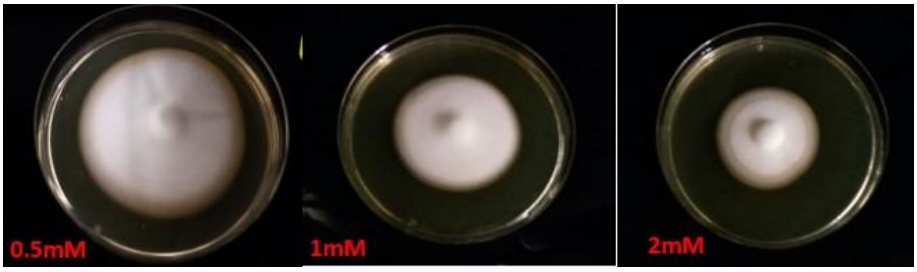


Figure 6.2. Effect of salicylic acid concentrations (0.5, 1.0 and 2.0 mM) on inhibition of mycelial growth of the pathogen and antagonists.

6.3.2 Effect of SA on tomato seed germination and seedling vigour

The effect of SA at different concentrations (0.5, 1 and 2 mM) on seed germination was represented in Figure 6.3. Based on the Kruskal-Wallis test, SA concentration had a significant effect on germination ($H = 9.67$, $P = 0.022$). Germination was significantly lower at 2 mM SA than for the control or other SA treatments, which did not differ from the control.

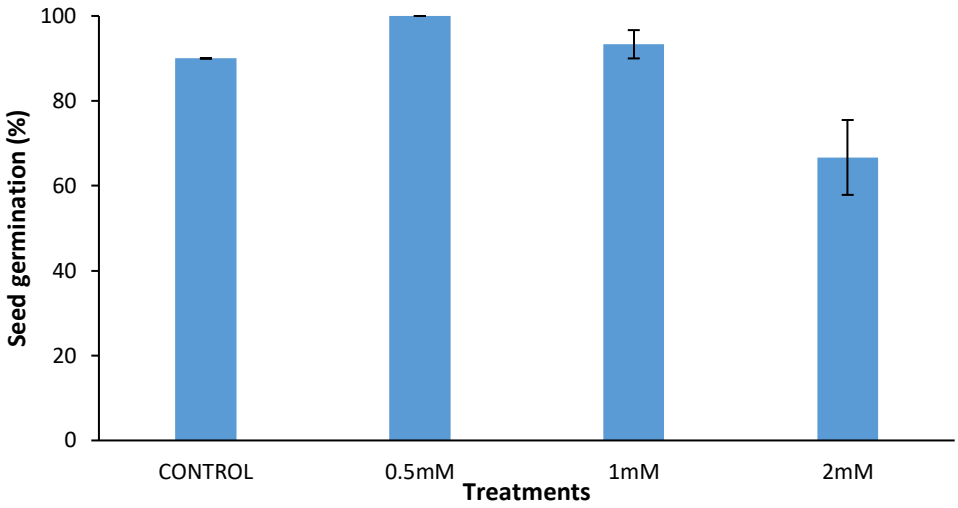


Figure 6.3. Effect of salicylic acid concentrations (0.5, 1.0 and 2.0 mM) on tomato (Grosse Lisse) seeds germination. Error bars show standard errors (n=3).

Seedling vigour was also significantly ($F_{3,8} = 36.55$, $P < 0.001$) affected by SA concentrations (Figure 6.4 and Figure 6.5). The seedling vigour was significantly increased at 0.5 mM concentration of SA compared with the control treatment. However, seedling vigour at 2 mM was significantly reduced compared with the control treatment.

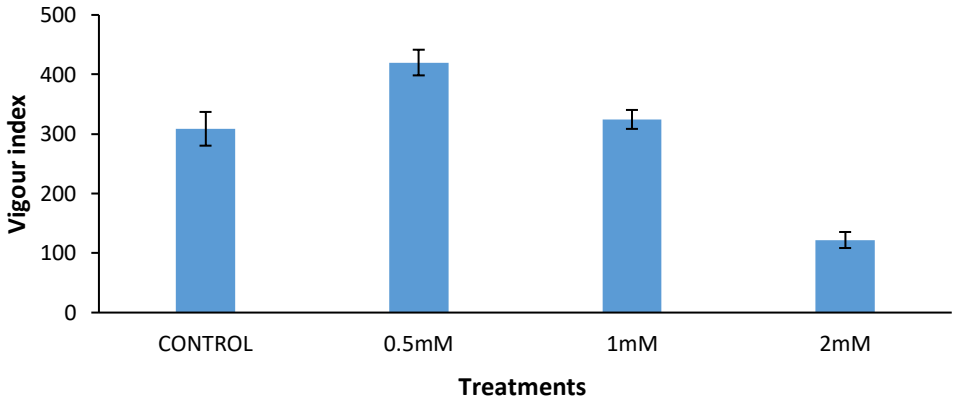


Figure 6.4. Effect of salicylic acid concentrations (0.5, 1.0 and 2.0 mM) on tomato (Grosse Lisse) seedling vigour. Error bars show standard errors (n=3).

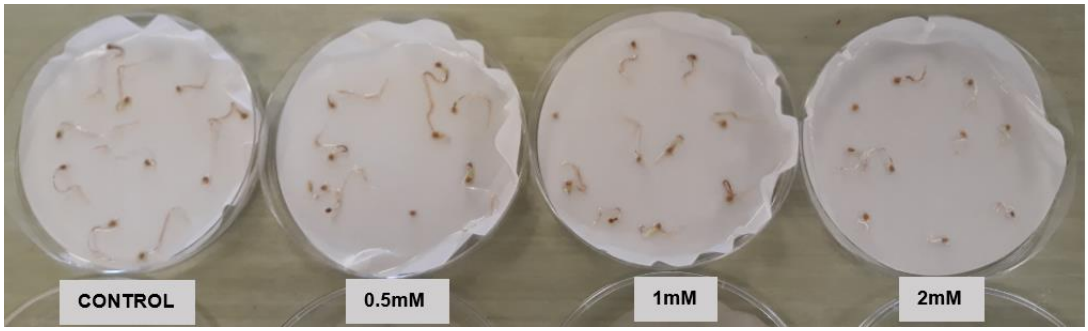


Figure 6.5. Effect of salicylic acid concentrations (0.5, 1.0 and 2.0 mM) on tomato seedling vigour.

6.3.3 Effect of SA on Verticillium wilt in glasshouse experiments

The effect of soaking tomato seeds with 0.5, 1, and 2 mM concentrations of SA for 3h before planting was evaluated on disease severity and plant parameters. The effect of treatment was significant ($F_{4,15}$ = 10.48, P < 0.001). The results showed that the severity of disease was significantly reduced at 2 mM concentration of SA compared with the pathogen only treatment (Figure 6.6).

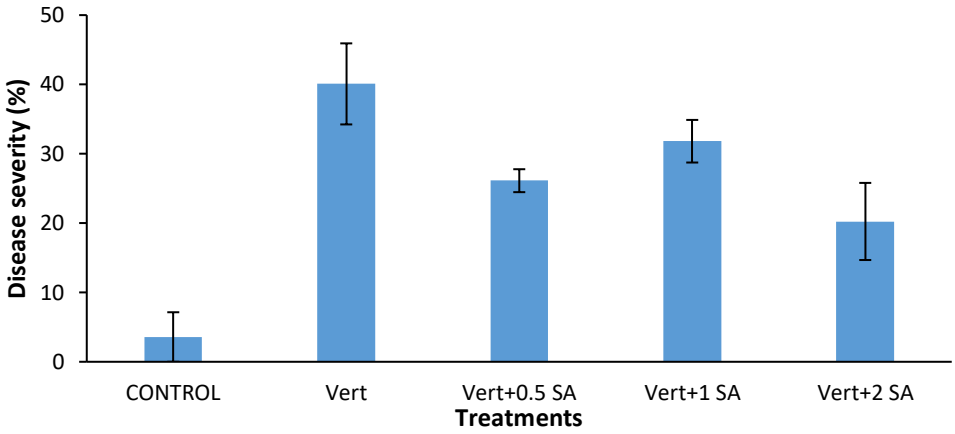


Figure 6.6. Effect of salicylic acid concentrations (0.5, 1.0 and 2.0 mM) on tomato (Grosse Lisse) disease severity. Error bars show standard errors (n=4).

The treatments significantly affected plant height ($F_{4,15}$ = 9.13, P = 0.001) and shoot dry weight ($F_{4,15}$ = 5.00, P = 0.009). Tomato seeds primed with 0.5 mM concentration of SA showed a significant increase in plant height compared with the pathogen only treatment (Figure 6.7). Also, shoot dry weight was significantly increased at 0.5 mM compared with 2 mM concentration of SA (Figure 6.8), but there was no significant difference between all treatments in their effect on root dry weight ($F_{4,15}$ = 1.57, P = 0.23) (Figure 6.9).

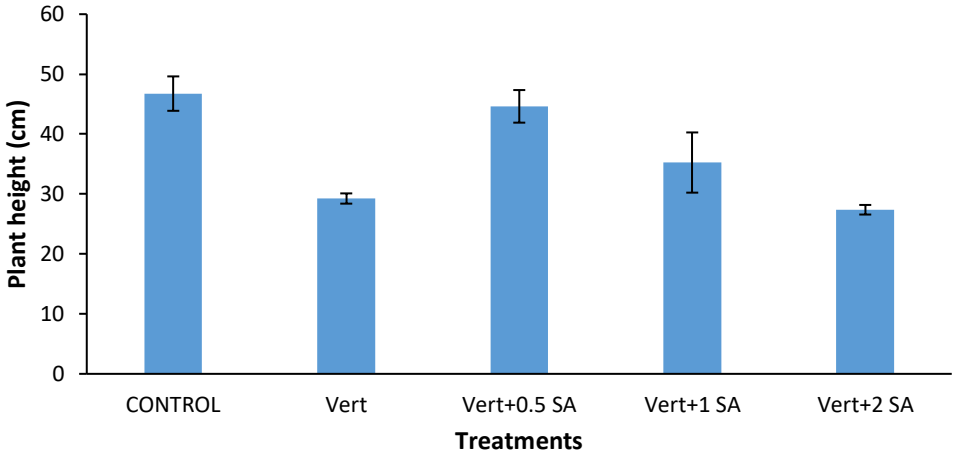


Figure 6.7. Effect of salicylic acid concentrations (0.5, 1.0 and 2.0 mM) on tomato (Grosse Lisse) plant height. Error bars show standard errors (n=4).

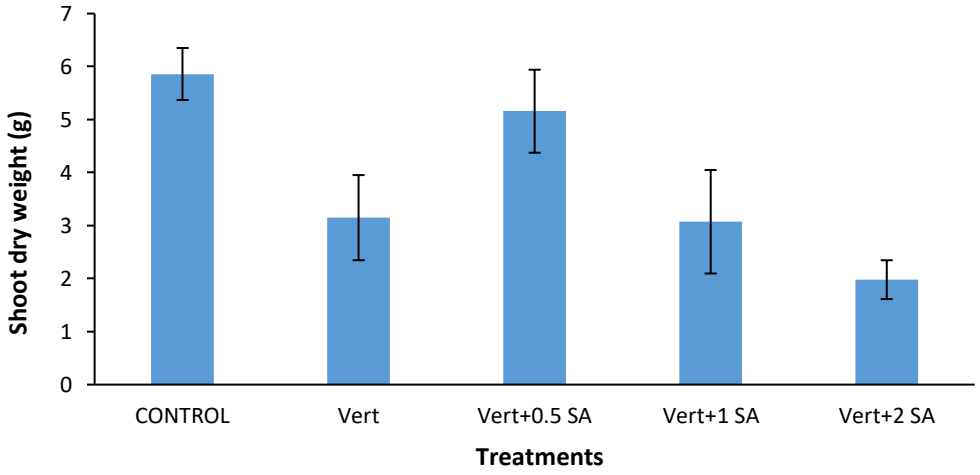


Figure 6.8. Effect of salicylic acid concentrations (0.5, 1.0 and 2.0 mM) on tomato (Grosse Lisse) shoot dry weight. Error bars show standard errors (n=4).

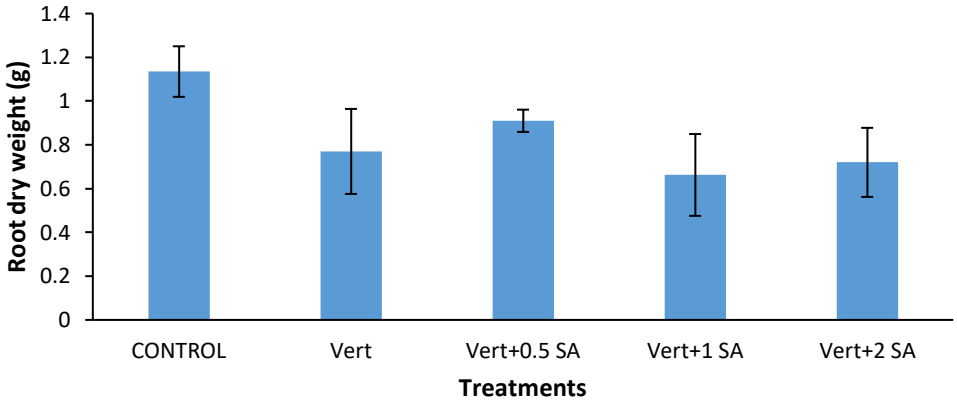


Figure 6.9. Effect of salicylic acid concentrations (0.5, 1.0 and 2.0 mM) on tomato (Grosse Lisse) root dry weight. Error bars show standard errors (n=4).

6.3.4 Efficacy of combination between SA and biocontrol agents on Verticillium wilt

There was a significant effect of treatment on disease severity ($F_{6,21} = 14.11$, $P < 0.001$). The results in Figure 6.10 showed that the combination between 0.5 mM SA and each antagonistic fungus significantly reduced disease severity compared with the control (pathogen only), antagonists by themselves or SA by itself.

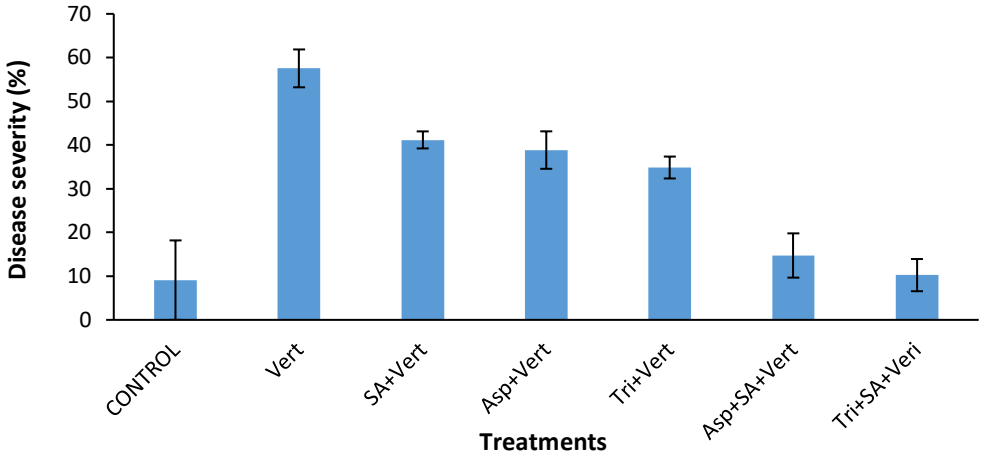


Figure 6.10. Effect of the combination between salicylic acid (0.5 mM) and antagonists on tomato (Grosse Lisse) disease severity. Error bars show standard errors (n=4).

The results showed that there was no significant difference between all treatments on plant height compared with the pathogen only treatments ($F_{6,21} = 0.97$, $P = 0.47$; Figure 6.11).

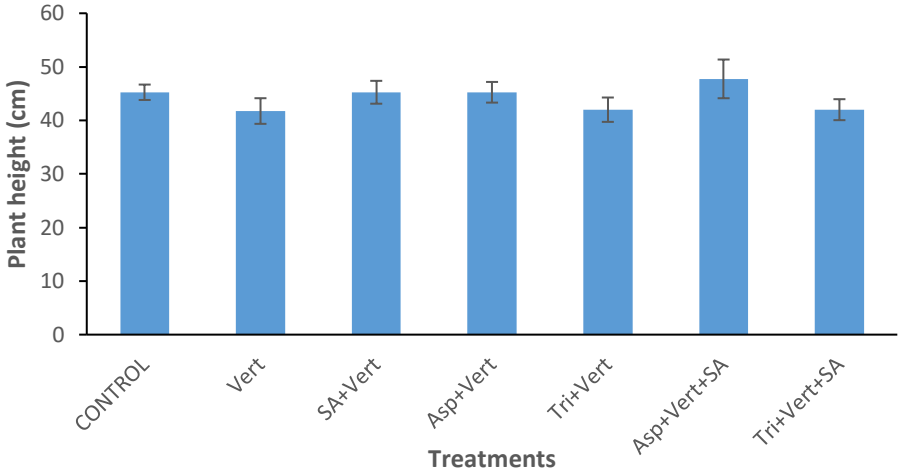


Figure 6.11. Effect of the combination between salicylic acid (0.5 mM) and antagonists on tomato (Grosse Lisse) height. Error bars show standard errors (n=4).

Treatments significantly affected shoot dry weight ($F_{6,21} = 19.36$, $P < 0.001$) and root dry weight ($F_{6,21} = 7.38$, $P < 0.001$). Shoot and root dry weight were significantly increased in treatments that included SA and antagonistic fungi compared with the pathogen only treatment (Figure 6.12 and Figure 6.13). The combination of *Trichoderma* and SA had significantly greater shoot and root dry weight than

either the *Trichoderma* or SA treatments alone. Combining *Aspergillus* with SA gave significantly higher shoot and root dry weight than SA alone, but the increase compared with *Aspergillus* alone was not significant.

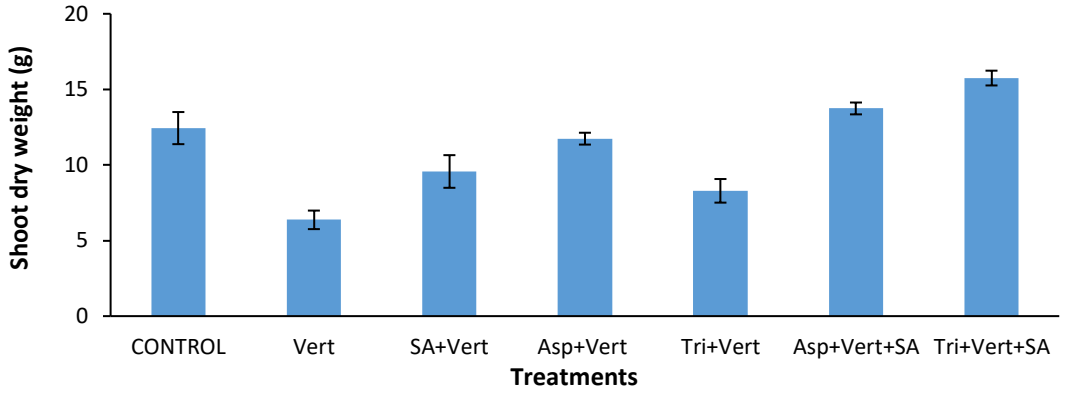


Figure 6.12. Effect of the combination between salicylic acid (0.5 mM) and antagonists on tomato (Grosse Lisse) shoot dry weight. Error bars show standard errors (n=4).

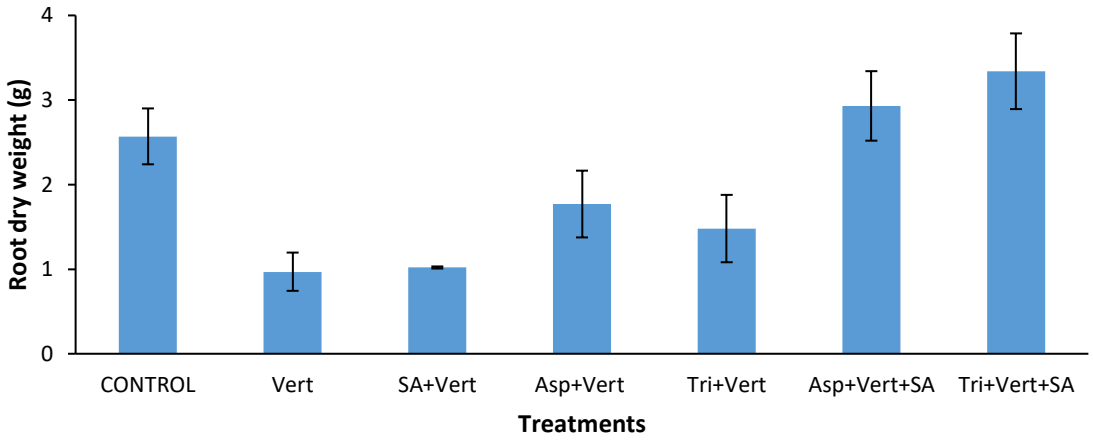


Figure 6.13. Effect of the combination between salicylic acid (0.5 mM) and antagonists on tomato (Grosse Lisse) root dry weight. Error bars show standard errors (n=4).

6.4 Discussion

In this chapter, the possibility of improvement of biological control against *V. dahliae* DAR31890 strain through using BCAs and SA in combination was investigated. The effect of three concentrations of SA including 0.5, 1 and 2 mM were studied on mycelial growth of the pathogen *V. dahliae* DAR31890 strain and the antagonistic fungi *T. harzianum* and *Aspergillus* sp. and effect of these concentrations on tomato Verticillium wilt under glasshouse conditions.

In vitro data revealed that mycelial growth of the pathogen and antagonists was significantly reduced with increased SA concentration. These results are consistent with previous studies indicating that the inhibitory effect of SA on mycelial growth increased linearly with increasing SA concentrations

(Ozgonen et al. 2001, Qin et al. 2003, Yu et al. 2007, Singh & Singh 2017, Kumar & Bains 2018, Lyousfi et al. 2021). Previous studies have shown that phenolic compounds demonstrate strong antifungal activity (McCue et al. 2000, Medina et al. 2006, Hussin et al. 2009, Arslan & Dervis 2010, Berne et al. 2020), and SA was classified as a hormone present in plants in the form of a free phenolic acid. Therefore, it can be said that the effect of SA on mycelial growth of the pathogen and antagonists may be due to phenolic compounds (Wu et al. 2008, Dong et al. 2010, Panahirad et al. 2014). The inhibitory effect of phenolic compounds may lead to inactivation of intracellular and extracellular enzymes, cytoplasmic membrane damage, and granulation of cytoplasm (Dambolena et al. 2012, Xu et al. 2018). Neto et al. (2015) showed that SA at 2.5 mM caused damage to the conidia plasma membrane of *P. expansum* after 30 minutes of contact with the phenolic compound. Moreover, fungal growth of pathogens and antagonists appears to be pH dependent. Therefore, raising SA concentration may increase the pH value, and thus negatively affect fungal growth (Wheeler et al. 1991, Kredics et al. 2004, Xing et al. 2017, Li et al. 2021).

The results also showed that seed germination and seedling vigour were enhanced at 0.5 mM concentration of SA, and significantly reduced at 2 mM concentration. The results of this study were consistent with several other studies where seed priming with low SA concentrations improved germination rates and seedling vigour (Dolatabadian et al. 2009, Yang et al. 2016, Zanganeh et al. 2020). Mahesh et al. (2017) found that 0.5 mM concentration of SA significantly enhanced eggplant (*Solanum melongena*) seed germination and seedling vigour compared with a control treatment. Similarly, Ghoohestani et al. (2012) found that pre-treatment of tomato seeds by SA at 150 mg/L improved germination and vigour indices in saline conditions, while Rajjou et al. (2006) showed that SA at 0.5 mM concentration substantially improved vigour and seed germination of *A. thaliana* under salt stress conditions. These authors also noted that seed germination was significantly inhibited above 1 mM SA concentration. Anaya et al. (2018) showed that the maximum rate of seed germination of *Vicia faba* of 70% was reached with 0.25 mM and 0.5 mM SA after 5 days under control treatment.

Seed priming with SA may regulate different physiological pathways in plants, which in turn may aid plant growth and development (Rajjou et al. 2006, Ashraf et al. 2010, Singh & Gautam 2013). The improvement in seed germination and seedling vigour may be due to the significant modulation of SA in uptake and metabolism of mineral elements (Gunes et al. 2007, Yusuf et al. 2013, Es-sbihi et al. 2020). For example, pre-sowing seed treatment with 0.5 mM SA significantly increased Fe, cysteine, and chlorophyll content in maize, and conferred tolerance to Pb toxicity in plants (Zanganeh et al. 2020). In research conducted by Chakraborty (2021) on the effect of SA on induction of growth vigour of tomato plant, the author found that trichome density and chlorophyll content in tomato seedlings improved with SA treatment. The enhancement of plant growth at low SA concentrations

could be because SA caused increased cell division at the apical meristem of seedling roots (Ghoohestani et al. 2012). On the other hand, the negative effect of high SA concentrations on seed germination and plant growth is potentially due to oxidative stress, which is either induced by SA or increased superoxide dismutase activity and H_2O_2 accumulation as a result of increased Cu and Zn activities (Rao et al. 1997, Yusuf et al. 2013, Galviz-Fajardo et al. 2020), or it may be that high concentrations of SA inhibit α-amylases in the aleurone layers thought to be essential for seed germination, which are tightly regulated by gibberellic acid and abscisic acid (Xie et al. 2007, Carrera-Castaño et al. 2020)

In the glasshouse experiments, the results demonstrated that 2 mM concentration of SA had reduced Verticillium wilt disease severity, as observed in other studies that have indicated a role for SA in inhibiting plant diseases (Spletzer & Enyedi 1999, Edgar et al. 2006, Mandal et al. 2009, Panahirad et al. 2014). Jabnoun-Khiareddine et al. (2015) found that SA applied as a soil drench at 10 mM concentration had reduced tomato Verticillium wilt and improved plant height and fresh weight for the shoot and root system. Similar effects were reported by Mahesh et al. (2017) who showed that seed priming with SA for 3 hours significantly reduced eggplant Verticillium wilt incidence under greenhouse conditions. Sorahinobar et al. (2022) found that seed priming with 1 mM and 2 mM SA showed a higher level of resistance against *F. graminearum* that causes wheat seedling blight. Gao and Zhang (2013) reported that SA at 0.2 mM induced SAR to *Physalospora piricola* that causes pear ring rot disease.

However, plant height, shoot and root dry weight were significantly increased at 0.5 mM concentration of SA compared with a pathogen only treatment. These results aligned with Madany et al. (2020) who found that seed priming with SA significantly increased the shoot length and dry biomass as well as the root length and dry biomass in tomato seedlings. Chakma et al. (2021) reported that interactions between seed priming of SA and soil moisture regime significantly increased the height, leaf area and shoot dry weight in tomato plants. Mahesh et al. (2017) also found that 0.5 mM concentration of SA significantly increased growth parameters including plant height, number of leaves, and shoot fresh and dry weight in eggplant seedlings. Coşkun et al. (2021) reported that SA at 0.5 and 1mM concentrations significantly improved plant parameters (leaf numbers, shoot diameter, root fresh weight, shoot fresh weight, and shoot dry weight for pepper (*Capsicum annuum* L.).

Seed priming with SA before sowing may enhance diverse cellular, biochemical responses (Conrath et al. 2002), or regulate plant response to disease by inducing antimicrobial defence compounds (phytoalexins), or by activation of defence related enzymes such as ß-1,3-glucanase and chitinase (Mahesh et al. 2017). Further, Sorahinobar et al. (2022) have reported that increased ROS activity

after SA treatment can be correlated with increased levels of H_2O_2 related to the induction of hypersensitive responses (HR), which is a kind of programmed cell death at the site of infection with the pathogens. Seeds treated with SA could show increases in the activities of peroxide, polyphenol oxidase and phenylalanine ammonia-lyase which are associated with plant resistance by oxidation and deposition of phenolic compounds in the plant cell wall (Ojha & Chatterjee 2012, Thakker et al. 2013, Narasimhamurthy et al. 2019). Further, application of SA regulates various physiological pathways such as increasing the chlorophyll content in plants, which in turn help plant growth and development (Houssien et al. 2010, Hayat et al. 2013).

As a result, higher concentrations of SA provided better control of disease, but also reduced plant vigour and growth. This may be due to oxidative stress caused by SA. So, the lowest concentration of SA 0.5 mM was chosen to test in combination with the antagonists.

SA was used as a foliar spray in most previous studies. In this study, seeds were soaked in an aqueous solution of SA for 3 hours before planting. The results showed that the combination of SA with *T. harzianum* and *Aspergillus* sp. was more efficient in reducing disease severity of the *V. dahliae* DAR31890 strain and increasing plant growth parameters (root and shoot dry weight) than using them individually. The results of this study are in line with Coşkun et al. (2021) who found that the applications of SA and arbuscular mycorrhizal fungi had positive contributions to control *V. dahliae* that causes Verticillium wilt on pepper, and enhanced plant growth parameters. Houssien et al. (2010) found that the combination of SA and *T. harzianum* enhanced tomato plant defences against Fusarium wilt. These results also are consistent with those obtained by Zehra et al. (2017b) who found that the combination of *T. harzianum* and SA reduced tomato infection by *F. oxysporum* and significant increase in dry weight of shoot and root. Naserinasab et al. (2011) found that SA and *T. harzianum* decreased tomato infection by nematode *M. javanica* when they were used together. The results of El-Khallal (2007) showed that the combination of SA with jasmonic acid and arbuscular mycorrhizal fungi significantly reduced tomato infection by *F. oxysporum*.

Plants pre-treated with SA and the BCAs *T. harzianum* and *Aspergillus* sp. together may become primed to respond faster and show stronger activation of defence responses against *V. dahliae* compared with control plants (Zehra et al. 2017a). *This could be due to a higher induction of defence proteins and enzymes in plants that were pre-treated with SA and BCAs* (Zehra et al. 2017b). Several studies have shown that plant root exudates act as chemo-attractants of BCAs (Huang et al. 2014, Lombardi et al. 2018, Zhou et al. 2019, Vives-Peris et al. 2020, Clocchiatti et al. 2021). Therefore, *the improvement in plant health by SA may involve increased root exudates, thus increasing the efficiency of BCAs.*

Finally, it may be concluded that seed priming with SA, following *treatments* by BCAs could provide promising integrated alternatives and more efficiency than using them alone in suppressing the *V. dahliae* DAR31890 strain by including a variety of defence and antagonism mechanisms.

Chapter 7. General discussion

The principal aim of this thesis was to study the effect of isolated fungi on growth and activity of the *V. dahliae* DAR31890 strain, and to test whether biological control of Verticillium wilt in tomato could be enhanced by combining with other treatments, especially root exudates and inducers of systemic resistance. This was completed by first obtaining biological control agent (BCA) isolates that could be used against Verticillium wilt disease caused by *V. dahliae*, screening for dual culture, antagonistic activity using a culture filtrate assay *in vitro*, and evaluating the efficiency of these isolates in promoting tomato growth and reducing Verticillium wilt disease incidence under glasshouse conditions. The effect of tomato root exudates and some of the multiple components such as amino acids, organic acids, and sugars was studied on the pathogen *V. dahliae* and the antagonistic fungi. The aim of this study was also to evaluate of efficiency of BCAs on plant growth promotion as well as detection of tomato defence-related enzymes induced by BCAs. The final chapter presented the *in vitro* activity of salicylic acid (SA) against the pathogen *V. dahliae* and antagonistic fungi to determine the efficiency of SA alone or in combination with BCAs against Verticillium wilt under glasshouse conditions.

7.1 Key findings

7.1.1 Isolation and identification of antagonistic fungi for biological control of tomato Verticillium wilt disease (Chapter 3)

A pathogenicity test was completed, involving four strains of *V. dahliae* including DAR 33757 from cotton, DAR 31890 from tomato, DAR 44537 from potato and DAR 81260 from olive, tested on the tomato Grosse Lisse cultivar. The results indicated that all strains of *V. dahliae* caused vascular wilt and increase in tomato disease severity. This could be because this pathogen was able to infect a wide range of plant hosts (Woolliams 1966, Pantelides et al. 2009, Acharya et al. 2020). Bhat and Subbarao (1999) showed that some strains of *V. dahliae* presented differential pathogenicity on various hosts. However, strain DAR31890 from tomato was more virulent on tomato than the other strains. Therefore, this strain was chosen for further investigations in all remaining experiments.

In this study, fifteen fungal isolates were recovered from cotton roots and rhizosphere soil (soil adhering to the roots) in fields at Boggabri, Australia. Although this isolation was not from a field used to cultivate tomato, the results demonstrated that some of the isolated fungi were able to reduce mycelial growth of tomato *V. dahliae* DAR31890 strain in dual culture experiments. Previous studies have indicated the potential efficiency of BCAs isolated from non-host crop or natural habitats against various plant pathogens (Tsahouridou & Thanassoulopoulos 2002, Islam et al. 2014, Syamsia et al.

2021, Xue et al. 2021). There is a high probability that fungi with a similar role in BCAs are also widely present in the natural ecosystems (Islam et al. 2014). Benouzza et al. (2021) found that 15 *Trichoderma* isolates from olive trees significantly reduced mycelial growth of tomato *V. dahliae* in a dual culture test. These effects were due to the ability of the antagonistic fungi to directly parasitize the pathogen, cause antibiosis, and compete for space and nutrients (Rajani et al. 2021, Zhao et al. 2022). Experiments with culture filtrates produced from these fungi indicated that there was a suppressive effect on growth and germination of microsclerotia (MS). This suppression was due to volatile and non-volatile compounds that were able to degrade the cell walls of the pathogen (Naraghi et al. 2010, Raut et al. 2014, Latz et al. 2018).

In glasshouse experiments, where the isolates from the *in vitro* culture work were used to inoculate tomato plants and the supporting soil, suppression of tomato wilt was observed. The highest suppression was recorded by isolates 2 and 7, which were later identified as *T. harzianum* and an *Aspergillus* sp., respectively. Interestingly, isolates 2 and 7 were isolated from same place (rhizosphere soil). These results aligned with several previous studies that indicated the importance of rhizosphere soil due to its richness in microbes (Hawkes et al. 2007, Lambers et al. 2009, Mendes et al. 2014, Zhao et al. 2018a, Xu et al. 2022). Higher microbial populations exist in rhizosphere soil than in bulk soil since various compounds such as amino acids, sugars, vitamins, and organic chemicals are exuded by plant roots (Nichols et al. 1997, Manoharachary & Mukerji 2006). In the case of root diseases, the pathogen must interact with the rhizosphere fungi before entering the root tissues. Therefore, antagonism may occur between these fungi and pathogen, affecting its advancement (Whipps 2001, Chapelle et al. 2016, Ali et al. 2017b). Dutta (1981) found that some fungi isolated from tomato rhizosphere including *T. viride, Penicillium* spp. and *Gliocladium* spp. were most effective in controlling Verticillium wilt on tomato plants. According to Mao et al. (2020) the strain *T. hamatum* MHT1134, which was isolated from rhizosphere soil of pepper fields had the largest antagonistic effect against Fusarium wilt.

In addition, four *Aspergillus* spp. isolates significantly decreased the disease severity index of *F. oxysporum* f. sp. *melonis* with an increase of shoot and root dry weights (Boughalleb-M'Hamdi et al. 2018). *A. niger* NBC001, which was isolated from a soybean rhizosphere, significantly suppressed cyst nematode in the field (Na et al. 2021).

Therefore, given both the plate and glasshouse inhibition that isolates 2 and 7 showed toward *V. dahliae* DAR31890 and given that there exist several sources of evidence for *T. harzianum* and *Aspergillus* sp. to offer pathogen control, these two isolates were selected as BCAs for all subsequent experiments. As these two antagonists are not closely related, they were suitable for testing with

regards to whether any interactions with root exudates or in combination with resistance inducers were general or specific to particular biocontrol agents.

7.1.2 Effect of tomato root exudates on pathogen and antagonistic fungi (Chapter 4)

Root exudates participate in a variety of interactions within the root zone (rhizosphere) such as attracting beneficial or parasitic microorganisms (Lugtenberg & Kamilova 2009, Huang et al. 2014), and play an important role in the process of infection of tomato by *V. dahliae* (Subba-Rao & Bailey 1961). We studied the effect of tomato root exudates and some root exudate components including certain amino acids, organic acids, and sugars known to be found in the root exudates of tomato on the biology of *V. dahliae* DAR31890 strain and on the efficiency of antagonistic fungi *T. harzianum* and *Aspergillus* sp.

The results indicated that all treatments (Hoagland nutrient solution, Hoagland nutrient solution plus sucrose, and root exudates in Hoagland nutrient solution), gave significantly higher MS germination than the water agar control. MS germination in Hoagland nutrient solution plus sucrose was significantly greater than in the other solutions. Adding sucrose to the Hoagland solution reduced inhibition of *V. dahliae* by *T. harzianum* in the dual culture experiments and increased the biomass of *Aspergillus* sp. and *V. dahliae* in the liquid culture experiment. This may be because sucrose is an important carbon source, thus increasing competition between the pathogen and antagonistic fungi (Kravchenko et al. 2003, Grahovac et al. 2021). On the other hand, using some of the root exudate compounds individually, such as some amino acids, organic acids and sugars, significantly stimulated MS germination of *V. dahliae* compared with the water agar treatments.

López-Moral et al. (2023) showed that dissolved organic carbon in an exudate solution significantly induced *V. dahliae* MS germination in comparison with a control where exudates were absent. Similar results were reported by Vidauri (1998) who found that the greatest MS germination of *V. dahliae* occurred when amino acids were added at 7.5 and 11.25 µg/ml. Krigsvold et al. (1982) found that the percentage of MS germination of *Cylindrocladium crotalariae* was significantly higher in root exudations containing high levels of carbon. According to Ruan et al. (1995) flavonoids in root exudates stimulated spore germination of *F. solani*. Root exudates can be loosely classified into two groups: low molecular weight compounds including amino acids, organic acids, sugars, phenolics, and secondary metabolites; and high molecular weight compounds including proteins and complex carbohydrates. These compounds stimulate growth and *act* as chemo attractants of microbes (Bais et al. 2006, Lombardi et al. 2018, Upadhyay et al. 2022).

In glasshouse experiments, attempting to make the pathogen more sensitive to antagonism by adding fructose to stimulate MS germination was not successful. Sugars are considered one of the major

sources of carbon (Campbell et al. 1997, Preston-Mafham et al. 2002, Wang et al. 2021a), so adding fructose to the soil markedly reduced efficiency of BCAs against *V. dahliae*. This confirms the findings of laboratory experiments which indicated that adding sucrose to the solution stimulated MS germination and reduced inhibition of *V. dahliae* by antagonistic fungi. This could be due to competition between the pathogen and other microorganisms for carbon sources in rhizosphere. However, that reduction did not affect the activity of the antagonistic fungi. This may be because these fungi depend on other mechanisms such as mycoparasitism or induced resistance.

Based on the results presented in Chapter 4, it can be concluded that root exudates and some individual compounds, especially sucrose, stimulated MS germination. Therefore, future research may explore the use of different compounds to simulate MS germination, providing further opportunities for biological control of the pathogen before crop planting, since MS become more susceptible to antagonism if they must germinate.

7.1.3 Trichoderma harzianum and Aspergillus sp. induced systemic resistance in tomato against V. dahliae DAR31890 (Chapter 5)

Pre-inoculation of tomato roots (using a method of dipping the plant roots) with *T. harzianum* and *Aspergillus* sp. significantly reduced disease severity of the *V. dahliae* DAR31890 strain, and improved plant growth. Previous studies have indicated that *Trichoderma* and some genera of *Aspergillus* elicit plant growth promotion and induction of resistance against a wide range of pathogens. De Meyer et al. (1998) demonstrated that addition of *T. harzianum* T39 to the soil 7 days prior to *B. cinerea* inoculum significantly reduced the intensity of grey rot in lettuce, tomato and pepper. According to Behiry et al. (2023), *T. pubescens* significantly enhanced tomato development and induced systemic resistance (ISR) against *R. solani*. Djonovic et al. (2007) showed that inoculation of maize roots by *T. virens* gave systemic protection of leaves inoculated with *C. graminicola*. Potting mix amended with *T. hamatum* T382 and *Rhizoctonia* P9023 isolate 2 weeks prior to inoculation with *B. cinerea* provided protection for geranium against botrytis blight (Olson & Benson 2007).

The fungus *A. niger* also has the ability to stimulate the activity of four defence-related enzymes in tomato in order to manage pathogens (Goswami et al. 2019). Jin et al. (2019) reported that *A. niger* NBC00 is an effective biocontrol agent against cyst nematode *H. glycines* via stimulation of soybean plant defences. Mahapatra et al. (2015) showed that fungal elicitors from *A. flavus* and *A. parasiticus* triggered systemic acquired resistance in maize against *A. flavus* and *A. parasiticus*, and enhanced growth and development of plant. According to Syamsia et al. (2021) six endophytic fungi including *Daldinia eschscholtzii, Sarocladium oryzae, Rhizoctnia oryzae, Penecillium allahabadense, and*

Aspergillus foetidus promoted cucumber growth by stimulating secondary metabolites. El-Maraghy et al. (2020) showed that pre-treatment with plant growth promoting fungi (PGPF) strains including *A. flavus*, *A. niger*, *P. citrinum*, *P. chrysogenum*, and *T. koningiopsis* stimulated ISR against wilt disease associated with *R. solani*. According to Naziya et al. (2019) 70 fungi isolates including 16 isolates belonged to *Aspergillus* sp. showed significant protection against anthracnose disease caused by *C. capsici* in chilli, and significantly enhanced seed and plant growth parameters.

In the enzymatic activity assay, the results showed that, after dipping tomato seedling roots in the spore suspensions of biocontrol agents, peroxidase activity was not increased in tomato leaves although soluble protein concentration was significantly increased. Wang et al. (2020) revealed that four *T. asperellum* isolates significantly increased the soluble protein level in poplar leaf. According to Kaur and Kumar (2020) the average total soluble protein content was significantly enhanced in mung bean leaf when the soil was treated with *Trichoderma* and other antagonists. Oligosaccharide of a strain of *A. niger* significantly increased soluble protein, as well as phenolic content and peroxidase activity in *Taxus chinensis* leaves (Li et al. 2003).

According to Moradi et al. (2012) soluble proteins and β-1, 3-glucanas activity were higher in chickpea cultivars after inoculation with *B. subtilis*, *T. harzianum* compared to the uninoculated control. Ahmed (2017) found that *Brevibacillus formosus* strain DSM 9885 and *Brevibacillus brevis* strain NBRC 15304 increased soluble proteins levels in potato leaves and enhanced resistance to brown leaf spot that caused by *Alternaria alternata*. Also, the highest increase in soluble proteins and soluble sugar content were determined in tomato plants due to treatment with *Trichoderma* spp. (Yeon et al. 2022).

In the same vein, El-Maraghy et al. (2020) demonstrated that *Aspergillus* and other isolated fungi from wheat rhizosphere soil activated the pathogenesis-related gene, plant defensive chitinase, β-1, 3-glucanase, and increased plant-specific defensive proteins against *R. solani*. In addition, soluble protein contents of barley plants increased as a result of application of *A. ochraceus* individually or under salinity stress (Badawy et al. 2021).

Based on this study and other data, the ability of *T. harzianum* and *Aspergillus* sp. to inhibit the growth of the *V. dahliae* DAR31890 strain, to enhance plant growth, and to induce the soluble protein content in tomato leaf supports the application of *T. harzianum* and *Aspergillus* sp. as potential bioagents for managing Verticillium wilt disease in tomato and other crops.

7.1.4 Improvement of biocontrol of Verticillium wilt of tomato by the addition of salicylic acid (Chapter 6)

In this experiment, we planned to investigate the possibility of improvement of biological control against the *V. dahliae* DAR31890 strain by combining BCAs with the abiotic elicitor salicylic acid (SA). SA is one of the most important phenolic compounds which is extensively distributed in plants, and is a defence-related plant hormone that acts as a regulatory signal to mediate plant defences against various stresses (Huang et al. 2021).

In vitro, the results showed that the inhibitory effect of SA on mycelial growth of the pathogen and BCAs, and on tomato seed germination and seedling vigour increased with increasing SA concentrations. High concentrations of SA have been associated with inhibition of several fungi in other studies (Kumar & Bains 2018, Lyousfi et al. 2021). In addition to the observation of inhibition, the concentration of SA required in media or seed treatments have been reported as similar to the 2 mM concentrations observed to offer similar inhibition in this study (El-Mergawi & Abd El-Wahed 2020, Palve et al. 2022).

The negative effect of SA at high concentrations may be due to the accumulation of phenolic compounds (Wu et al. 2008, Dong et al. 2010, Panahirad et al. 2014), to an increase in hydrogen peroxide levels as a result of increased Cu, Zn activities, or to oxidative stress which is induced by SA (Yusuf et al. 2013). Increased seedling vigour at low concentrations of SA could be because SA caused increasing cell division at the apical meristem of the seedling roots (Ghoohestani et al. 2012).

In glasshouse experiments, seed treatments with a low concentration 0.5 mM of SA significantly reduced disease severity and increased tomato growth parameters. This may be related to SA stimulating plant defences and enhancing enzymes activity in the plants (Ojha & Chatterjee 2012, Narasimhamurthy et al. 2019).

Amendment of biological control with SA led to enhanced activity of BCAs against *V. dahliae* DAR31890 strain more than using each alone. This study showed that the combination of BCAs with SA significantly reduced disease severity of *V. dahliae* and enhanced shoot and root dry weight of tomato plants. This is consistent with results obtained by Houssien et al. (2010) who found that the combination of SA and *T. harzianum* enhanced plant defences of tomato against Fusarium wilt. According to Jafarbeigi et al. (2020) tomato plants treated with *Trichoderma*, SA, and β-aminobutyric acid (BABA), either individually or in combination, significantly enhanced plant defences against *B. tabaci*.

Tomato treated with *T. harzianum*, *Aspergillus* sp., and SA together showed a faster response and stronger activity for cellular defence after pathogen challenge compared to individual treatments. Considerable improvement in plants treated with BCAs and SA in the current study could be linked

as a defence response stimulated against *V. dahliae* invasion in tomato plants. Therefore, reduced disease incidence in the plant could be indicative of either increased fungal control of the pathogen via competition, antagonism or further up regulation of the plants ISR pathways.

7.2 Conclusion

The results of each chapter supported the goals of this study, which were to examine the research gaps related to the effects of BCAs against Verticillium wilt in tomato plants; to evaluate the relevant modes of action; and to understand potential strategies for control. Isolated *T. harzianum* and *Aspergillus* sp. strains from a cotton rhizosphere soil gave the greatest *in vitro* inhibition of *V. dahliae* DAR31890 growth and MS germination, and reduced Verticillium wilt disease incidence and promoted tomato growth under glasshouse conditions when compared with the other isolates. Therefore, the first aim of this study was achieved by selecting effective biocontrol agents with different characteristics, for use in subsequent experiments. Tomato root exudates and some of the identified components of tomato exudates, such as amino acids, organic acids, and sugars, significantly stimulated MS germination. This supports the possibility of controlling verticillium wilt after stimulating MS germination. Pre-treatment with BCAs was significantly beneficial in preventing *V. dahliae*, promoting plant growth and enhancing total soluble proteins. Combining the induction of systemic resistance via SA treatment with BCAs provided better control than either treatment in isolation, suggesting a promising method for improving management of the disease. To conclude, the use of *T. harzianum* and *Aspergillus* sp. isolates alone or in combination with SA can control the Verticillium wilt disease caused by *V. dahliae*, and at the same time promote tomato plant growth. This work presents a new approach for the management of Verticillium wilt disease of tomato plants depends on SA as inducer and suppression of the fungal pathogenicity by applying *T. harzianum* and *Aspergillus* sp. as BCAs.

7.3 Suggestions for future studies

The screening resulted in a selection of two interesting biocontrol candidates *T. harzianum* and *Aspergillus* sp. *against* the *V. dahliae* DAR31890 strain. These two fungi were isolated from cotton rhizosphere soil. Therefore, it would be useful to conduct further research on beneficial fungi from different crops (non-host) and to investigate their efficiency against pathogens. However, while these two fungi proved very useful in laboratory and greenhouse experiments, further work is needed to test their effectiveness in the field.

There was no significant effect of root exudates on fungal growth and weight of pathogens and antagonists compared to the Hoagland nutrient solution. This may be because the concentration ratio

of root exudates in the Hoagland solution may be high or low. Therefore, it will be necessary to carry out more laboratory studies to identify the concentration ratio of root exudates and their effect on BCAs.

However, some root secretion compounds had stimulated MS germination when used individually. It is experimentally difficult to measure the concentration of compounds that fungi are exposed to in the root zone. However, stimulation of MS by some root secretion compounds in combination with BCAs is considered to have potential to control *V. dahliae*. Stimulation of MS germination to break the resistant dormant phase, and then killing them before infection of the plant by some biotic or abiotic factors could be completed through application of germination stimulants at different times before planting the crop. More work is needed to identify compounds which stimulate germination without giving the pathogen an advantage over the antagonist.

It is evident that plant defences were stimulated after three days of treatment with BCAs through an increase of soluble protein concentrations in the leaves of tomato seedlings. Therefore, it may be helpful to apply BCAs to plants before contact with the pathogen, to give more chance for these plants to stimulate their defences against the pathogens. Further work needs to be completed on how to apply the BCAs in the field to achieve this. For example, seedlings could be treated before transplanting, or BCAs could be applied at the time of sowing of direct seeded tomato.

The combination of SA with BCAs was more efficient than using them alone. This work therefore presents a new approach for the management of biological control of Verticillium wilt disease of tomato plants, whereby the defence of the plant against the pathogen is activated using SA as inducer, combined with suppression of the fungal pathogenicity by applying *T. harzianum* and *Aspergillus* sp. as BCAs. Therefore, the study suggests that there is considerable potential for exploiting SA and BCAs for the management of Verticillium wilt, particularly in cultivars of tomato, and this potential may be better understood by conducting wider experiments and testing other inducers of resistance such as benzothiadiazole. In other crops, benzothiadiazole is often applied to seed, so more work could be completed on application of resistance inducers to seeds or transplants of tomato.

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