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M.Sc. Thesis

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DEVELOPMENT OF A SUSTAINABLE
BIOFUNGICIDE AGAINST PLANT
PATHOGENS USING *Trichoderma*
harzianum CULTIVATED ON AGRO-
INDUSTRIAL WASTE

M.Sc. THESIS

SUBMITTED TO THE DEPARTMENT OF BIOENGINEERING
AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF ABDULLAH GUL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

By

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I hereby declare that all information in this document has been obtained in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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M.Sc. thesis titled “Development of A Sustainable Biofungicide Against Plant Pathogens Using *Trichoderma harzianum* Cultivated on Agro-Industrial Waste” has been prepared in accordance with the Thesis Writing Guidelines of the Abdullah Gül University, Graduate School of Engineering & Science.

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ABSTRACT

DEVELOPMENT OF A SUSTAINABLE BIOFUNGICIDE
AGAINST PLANT PATHOGENS USING *Trichoderma*
harzianum CULTIVATED ON AGRO-INDUSTRIAL WASTE

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Agricultural practices are crucial for many industries, particularly for food production. Agricultural crops face various challenges throughout the supply chain, from cultivation to consumption, resulting in up to 80% crop loss each year. Several solution strategies have been developed to minimize crop loss. However, chemical products, which are commonly used to combat phytopathogens, exhibit harm to living organisms, nature, water resources, and soil structure. The use of biological alternatives is promoted to eliminate the side effects of chemicals. *Trichoderma harzianum* is a well-known biocontrol agent with its antagonistic activities through mycoparasitism, competition for nutrients and space, and the production of antimicrobial secondary metabolites, while simultaneously promoting plant growth. In this study, the antifungal activities of *T. harzianum* against five phytopathogens, which are *Fusarium solani*, *Rhizoctonia solani*, *Aspergillus welwitschiae*, *Colletotrichum coccodes*, and *Botrytis cinerea*, were demonstrated. Additionally, the effects of both non-volatile and volatile organic compounds produced by *T. harzianum* were tested against these phytopathogens. The strongest inhibitory activity was 96.76% against *C. coccodes*, similarly there were promising antagonistic activity against other phytopathogens. Its growth-promoting properties were also assessed, and indole-3-acetic acid and siderophore production was observed. Moreover, the cultivation of *T. harzianum* was conducted on a medium based on apple pomace for a sustainable and environmentally friendly approach, contributing to the circular economy and valorization of the agro-industrial waste. These findings highlight the potential of *T. harzianum* as an eco-friendly alternative to chemical fungicides and as a plant growth-promoting agent in sustainable agriculture.

Keywords: Biofungicide, T. harzianum, Apple Pomace, Phytopathogens, Mycoparasitism

ÖZET

TARIMSAL-ENDÜSTRİ ATIKLARI ÜZERİNDE YETİŞTİRİLEN *Trichoderma harzianum* KULLANILARAK BİTKİ PATOJENLERİNE KARŞI SÜRDÜRÜLEBİLİR BİR BİYOFUNGİSİT GELİŞTİRİLMESİ

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Tarım uygulamaları, birçok endüstri için özellikle de gıda üretimi için hayati öneme sahiptir. Tarımsal ürünler, yetiştirme sürecinden tüketime kadar olan tedarik zinciri boyunca her yıl %80 ürün kaybıyla sonuçlanabilen çeşitli zorluklarla karşılaşmaktadır. Ürün kaybını en aza indirmek için çeşitli çözüm stratejileri geliştirilmiş olsa da yaygın olarak kullanılan kimyasal ürünler canlılara ve çevreye zarar vermektedir. Kimyasalların yan etkilerini ortadan kaldırmak için biyolojik alternatiflerin kullanımı önemle teşvik edilmektedir. *Trichoderma harzianum*, mikoparazitizm, besin ve alan için rekabet avantajı ve antimikrobiyal sekonder metabolit üretimi gibi antagonistik aktiviteleri ile bitki gelişimini destekleyici özellikleri sayesinde iyi bilinen bir biyokontrol ajanıdır. Bu çalışmada, *T. harzianum*'un, *Fusarium solani*, *Rhizoctonia solani*, *Aspergillus welwitschiae*, *Colletotrichum coccodes* ve *Botrytis cinerea* olmak üzere beş fitopatojene karşı antifungal aktiviteleri ortaya konulmuştur. Ayrıca, *T. harzianum* tarafından üretilen uçucu ve uçucu olmayan organik bileşiklerin söz konusu patojenler üzerindeki etkileri değerlendirilmiştir. En güçlü inhibisyon %96,76 ile *C. coccodes*'e karşı gözlemlenmiştir, benzer şekilde diğer fitopatojenlere karşı da umut verici antagonistik aktivitesi bulunmuştur. Bitki gelişimini teşvik edici özellikleri de incelenmiş ve indol-3-asetik asit ile siderofor üretimi gözlemlenmiştir. Bunun yanı sıra, *T. harzianum*'un inkübasyonu için sürdürülebilir ve çevre dostu bir yaklaşım olarak, ayrıca tarımsal-endüstri atıklarının değer kazanımına ve döngüsel ekonomiye katkı sağlayan elma posası bazlı bir besiyeri ortamı oluşturulmuştur. Elde edilen bulgular, *T. harzianum*'un çevre dostu bir biyofungisit ve biyogübre adayı olarak tarımda kullanılabilirliğini desteklemektedir.

Anahtar kelimeler: Biyofungisit, *T. harzianum*, Elma Posası, Fitopatojenler, Mikoparazitizm

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LIST OF ABBREVIATIONS

AP	Apple Pomace
BCA	Biocontrol Agent
BOD	Biochemical Oxygen Demand
CAS	Chrome Azurol Sulphonate
COD	Chemical Oxygen Demand
CWDE	Cell Wall Degrading Enzyme
EU	European Union
g	Gram
GC-MS	Gas Chromatography–Mass Spectrometry
GRAS	Generally Recognized As Safe
IAA	Indole-3-Acetic Acid
ISR	Induced Systematic Resistance
kg	Kilogram
LAB	Lactic Acid Bacteria
mg	Milligram
mL	Milliliter
μL	Microliter
nm	Nanometer
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PGPR	Plant Growth-Promoting Rhizobacteria
PIRG	Percent Inhibition of Radial Growth
PVK	Pikovskaya Medium
RPM	Revolutions Per Minute
RSM	Response Surface Methodology
SD	Standard Deviation
SSF	Solid State Fermentation
VOC	Volatile Organic Compound
w/w	Weight/Weight



To my lifelong beloved Ömer

Chapter 1

Introduction

Agriculture has played a crucial role in the survival of people since ancient times, and now it is still significant according to the United Nations Sustainable Development Goals, the second article is “Zero Hunger”. Zero Hunger can be achieved by providing food security, improving nutrition, and promoting agricultural sustainability [1]. There are challenges to achieve agricultural productivity, since many consequences of climate change like temperature changes, rainfall patterns directly affect food insecurity, which is heavily dependent on agricultural activities. These changing global patterns give rise to reduction in crop yield, growing season change, and dependence on chemicals. Furthermore, climate change disrupts food distribution systems [2].

Given these issues, it can be understood that malnutrition, hunger, and food shortages are among the biggest challenges to be addressed nowadays. Currently, a quarter of the people in the world faces malnutrition, resulting in a huge burden on the healthcare system and on the economy. Therefore, developing sustainable, safe, eco-friendly, and efficient agricultural systems has gained importance. Crucially, effective farming techniques are the key to reducing the adverse effects of these challenges on agricultural productivity [2,3].

Biotechnological approaches have been promising for advancing many industries, including agriculture. Biotechnological solutions are required to reduce the reliance on chemicals that disrupt soil integrity and quality. Bio-based materials offer effective solutions to agricultural problems while protecting living organisms and nature from the harmful effects associated with chemicals [4].

Biopesticides are known as microorganisms and naturally occurring molecules that present significant effects to control plant diseases. Their active agents range from fungi, bacteria, viruses, actinomycetes, and yeasts. *Trichoderma* spp. are among the most

extensively studied fungal species due to their potent antifungal activities; thus, they are among the well-known and widely used biofungicides [4].

1.1 Agricultural Issues

It is an undeniable fact that nutrition is essential for all living beings to maintain a healthy life. According to Maslow (1970), human needs are organized into a hierarchy, depicted as a pyramid with five levels. At its base are physiological needs, including breathing, food, water, shelter, clothing, and sleep, which must be met before higher-level needs can be fulfilled. These basic requirements are essential for good health, making nutrition one of the most basic human rights [5]. Although food is the most basic need and the demand is increasing by leaps nowadays, the challenges such the unstable and growing population, the consequences of climate change, problems in water resources and land use give rise to a huge inadequacy of food [6]. Agriculture is the main source of food since the efficient use of land takes an important place for maintaining the food production capacity. Implementing innovative and sustainable agricultural practices can enhance productivity and resilience, ensuring a stable food supply. Beyond food production, agriculture plays a vital role in the economies of low- and middle-income countries by providing employment and supporting livelihoods. Thus, strengthening agricultural systems is vital for ensuring food security and boosting economic development, yet it comes with numerous challenges [7].

The main causes of agricultural issues include environmental challenges, infrastructure constraints, economic barriers, policy and governance issues, social and educational barriers, and biological problems. Environmental challenges consist of climate change, unavailability of resources like water inadequacy, and loss of biodiversity. All these results in huge problems in agriculture practices [8]. Infrastructure constraints and economic barriers with insufficient access to technology and financial supply make the adoption of modern agricultural techniques difficult, resulting in farms being highly susceptible to environmental effects. Policy and governance issues include the inconsistent regulations lead to the renunciation from investment and hinder innovations, and insufficient support from government institutions prevents the access of farmers to training and courses for recent techniques and information. On the other hand,

educational barriers also limit the applications of good agricultural practices since many farmers are not aware of the precautions and technologies for fighting against agricultural issues [9]. Furthermore, the challenge likely to be the biggest cause of agricultural problems can be biological. Biological problems can vary in a wide range from viruses to insects. Additionally, biological problems account for an undeniable part of the total agricultural issues. According to the statistics of the Food and Agriculture Organization, it is reported that biological threats cause a reduction of about 20-40% of the total crop yield every year in the world [10].

1.1.1 Biology-Caused Agricultural Issues

The causes of biological agricultural issues vary in a wide range. Generally, living organisms are responsible for these issues faced in the field. Among them, pest species pose one of the most significant threats to agriculture. Any species of pest are unwanted by the farmers due to severe damage to crops, forestry, and stored foods directly. It is estimated that approximately 30% of crop loss is caused by pests each year globally. Depending on the crop species, this number can unfortunately increase up to 90%. The most common pest groups known as enemies of farmers are nematodes, snails, mites, insects, and rodents. Besides their direct physical damage, they are capable of transmitting pathogen viruses, bacteria, or fungi to infect the living things in the environment. Moreover, the lethal effect of a pest often varies depending on the crop species. For instance, grains such as maize are particularly vulnerable to mites [11]. Furthermore, aphids take a major place in the group of pests, and they can lead to serious plant damage by digesting the sap and transmitting the viral vectors to the plants that cause many plant illnesses. Also, they have target plant species like other pest species. However, they can quickly evolve according to changes in plant quality since they are looking for their exclusive needs and habitats found in their target plants. Moreover, their adaptation skills are not restricted, and they can develop resistance to pesticides. These contribute to distinct genetic diversity of aphids. Knowledge about their evolutionary mechanism is therefore essential to develop efficient management strategies against them [12]. The severity of pests is a clear fact, but the death of plants is not limited by only pests. Another major biological threat in agriculture is the presence of fungal pathogens, leading to severe crop diseases and further reduce yields.

The fungal species are generally saprophytic and required for the recycling of nutrients, but some of them called phytopathogens are known for their harm to the plants. The toxins produced by phytopathogens cause severe diseases in plant species, including root and stem rots, vascular wilt, and black spot [13,14]. Moreover, their pathogenic mechanism consists of many other factors. During the metabolism of pathogens, some molecules, such as metabolites, enzymes, and toxins, are produced. When these molecules interact with the plants, the pathogenicity of the fungi occurs. Each molecule has different mechanisms of action. For instance, carbohydrate-active enzymes consisted of cell wall degrading enzymes (CWDEs) of phytopathogenic fungi directly interact with the host, and the cell wall structure of the host depends on these enzymes since the general composition of plant cell walls consists of carbohydrate-based molecules, including cellulose, pectin, and lignin [15]. Mainly, CWDEs degrade the cell walls of the host plants, and the invasion and colonization of the fungi become faster. Chitinase, cellulase, xylanase, β -galactosidase, and pectinase are among the most well-known CWDEs [14-18]. *Rhizoctonia solani* is a phytopathogen, producing a variety of CWDEs and its damages to roots and radicles is reported by many studies. Additionally, the extent of damage increases with the higher concentration of these enzymes, especially cellulases, which give major harm to the plant by softening tissues and breaking down the cell wall composition [14]. Besides these enzymes, other molecules, notably toxins, play a significant role in infecting plants [14,19,20]. Particularly, the small secreted proteins are found among the most crucial effectors, and there is no doubt about their role in the fungal infection [15]. These toxins induce a variety of visible symptoms on the plants, including leaf spotting and yellowing, wilting, necrosis, chlorosis, and inhibition of growth. The damage mechanism of toxins starts with the permeability increase of the cell membrane of the host, dysfunctionalities in metabolism and loss of physiological functions [14,15].

The consequences of these fungi-caused severe plant diseases result in approximately 70% of total crop loss in the world, and this huge crop loss causes hundreds of billions of dollars each year [14]. Only in United States, phytopathogenic fungi are responsible for an estimated \$20 billion in crop losses each year. Moreover, the crop loss caused by fungal pathogens is more than the loss caused by insects. [21]. One of the most well-known and most destructive plant diseases is root rot disease. The severity of crop loss can range from small yield loss to complete field loss. This depends on the environmental conditions, the specific phytopathogen, and host susceptibility. Certain

plants, including legumes, can be more prone to diseases since they appear to be primary hosts for the pathogens. The causes of rot disease are generally known as fungi and oomycetes, while bacteria and viruses can also cause root rots [22]. Different species of living organisms play various roles in agricultural fields, infect numerous crops, and result in varying crop loss. Table 1. 1 summarizes the common phytopathogens and their consequences.

Table 1. 1 Common pathogen species and diseases caused by them

	Pathogen species	Common Name	Affected Crops	Symptoms	Reference
I N S E C T S	<i>Myzus persicae</i>	Peach-potato aphid	Carrot, lettuce, potato, cucumber, wheat	Leaf curling, yellowing of leaves	[23]
	<i>Helicoverpa armigera</i>	Tomato fruit worm	Tomato, cotton, maize, chickpea	Defoliation	[24]
	<i>Bemisia tabaci</i>	Whitefly	Tomato, cucumber, eggplant, pepper	Yellowing of leaves	[25]
	<i>Leptinotarsa decemlineata</i>	Colorado potato beetle	Potato	Defoliation	[26]
F U N G I	<i>Rhizoctonia solani</i>	Root rot fungus	Potato, sugar beet, legume, cabbage	Root rot, damping off in seedlings	[22]
	<i>Aspergillus welwitschiae</i>	Black mold	Onion, grape	Black spores on crop and toxin production potential	[27]
	<i>Fusarium solani</i>	Root rot fungus	Potato, legume	Root rot	[22]
	<i>Botrytis cinerea</i>	Gray mold	Tomato, cucumber, pepper	Rots and gray mold on fruit	[28]
	<i>Colletotrichum coccodes</i>	Black dot	Potato, tomato	Lesion, black dot	[29]
	<i>Aspergillus niger</i>	Black mold	Date fruit, onion, peanut	Black spores on crop	[30]
	<i>Fusarium oxysporum</i>	Fusarium wilt	Cotton, banana	Wilting, yellowing, defoliation	[31]
B A C T E R I A	<i>Xanthomonas campestris</i>	Bacterial spot	Cabbage, broccoli	Black veins, lesions on leaf	[32]
	<i>Pseudomonas syringae</i>	Bacterial blight	Tomato, broccoli	Necrosis, fruit speck	[33,34]
	<i>Ralstonia solanacearum</i>	Bacterial wilt	Potato, tomato	Wilting, brown rot	[35]
	<i>Erwinia amylovora</i>	Fire blight	Apple, pear	Shoot infection	[36,37]

N E M A T O D E S	<i>Meloidogyne</i> spp.	Root knot nematode	Tomato, potato, carrot, coffee	Formation of galls	[38]
	<i>Heterodera</i> spp.	Cereal cyst nematode	Barley, oat, wheat	Cyst formation, root distortion	[39]
	<i>Pratylenchus</i> spp.	Lesion nematode	Wheat, rice, potato, cotton, sugar beet	Necrosis, root vascular disruption	[40]
	<i>Bursaphelenchus xylophilus</i>	Pine wilt nematode	Pine trees	Rapid wilting, weakened chemical defense	[41]
O O M Y C E T E S	<i>Phytophthora infestans</i>	Late blight pathogen	Potato, tomato	Lesion, foliage, tuber infection	[42]
	<i>Phytophthora cactorum</i>	Crown rot pathogen	Apple, strawberry	Root, stem, crown and fruit rot and damping off	[43]
	<i>Aphanomyces euteiches</i>	Root rot pathogen	Bean, pea	Yellowing of root tissue, root rot	[44]

1.2 Solution Strategies

With advancements in agriculture, the number of solutions developed to address the various agricultural problems has also increased. Numerous products are utilized to protect plants from pests, fungi, bacteria, oomycetes, viruses, and adverse environmental conditions. Agrochemicals are widely applied in agricultural fields. In particular, pesticides, which are chemical formulations primarily used in agriculture play a crucial role in safeguarding plants from pests, weeds, and various plant diseases [45]. Moreover, fungicides, a specific type of commonly used pesticides, can inhibit or kill the fungi and controls the crop damage caused by the fungi. According to the European Union (EU) Pesticides Database, 147 out of the 496 approved pesticides are classified as fungicides. Surprisingly, 46% of all pesticides sold in the EU belong to fungicides, highlighting the agricultural industry's strong reliance on their use. This dependence continues to grow, reflecting an increasing trend in fungicide application [46]. However, increased use of these chemical substances in agriculture results in harmful consequences not only for humans but also for ecosystems, animals, and pollinators [45-47]. In humans, exposure to pesticides can lead to a variety of health abnormalities, which depends on the type of pesticides, exposure time, dosage, contact method, and health status of individuals.

Mainly, dermatological, respiratory, endocrine, carcinogenic, and gastrointestinal effects are reported. For instance, the studies revealed that triazine is one of the classes of chemical pesticides associated with endocrine disorders. In addition, there is a connection between triazine and oxidative stress, cytotoxicity, and dopaminergic effects [45]. In Italy alone, approximately 600 agrochemical-related acute poisoning cases were annually reported between 2013 - 2015 [46]. Moreover, pesticides can also change the microbial diversity of the soil and negatively affect the plant-associated microorganisms, leading to poor soil quality [47]. Furthermore, pesticide residues found on the crops cannot be completely removed by washing or peeling, and this led to the implementation of the strict regulations on agrochemical usage. Not only are the application dosages restricted based on the classes of pesticides, but also dangerous and harmful substances are banned [45], [46]. The EU has decreased the number of approved pesticides by nearly half, reducing the total to nearly 500 over the past 25 years [46].

The global recommendation to reduce the use of chemical pesticides has led to increased interest in alternative approaches. New solution strategies are developed and evaluated every day to minimize the health risks of these chemicals. It is reported that using safer alternatives instead of chemicals is one of the most urgent topics in agricultural practices [45,47]. Moreover, the EU encourages the use of less harmful products instead of chemicals in agriculture with the European Pesticide Regulation (EC) No. 1107/2009. At this point, biological solutions have emerged as the ideal alternatives. These biological solutions consist of many beneficial microorganisms that can act as biofertilizers and biocontrol agents by presenting plant growth-promoting and antimicrobial activities against phytopathogens. These microorganisms can belong to bacterial or fungal species, and they present their activities when they are applied to seeds, leaves, or soil of the plant. Their effectiveness is associated with various mechanisms such as the production of biologically active secondary metabolites, enzymes, and other bioactive compounds. For instance, some of the abilities of microorganisms, including nitrogen fixation, plant hormone production, phosphate solubilization, and siderophore production, promote plant growth. These microorganisms are called plant growth-promoting rhizobacteria (PGPR), and the most common PGPR include the genera *Azotobacter*, *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Rhizobium*. Moreover, some microorganisms can produce antimicrobial molecules such as organic acids, antibiotics, and enzymes that can inhibit the growth and spread of phytopathogens [47]. The most common microorganisms

used in agriculture as biocontrol agents are *Pseudomonas fluorescens*, *Bacillus megaterium*, *Bacillus subtilis*, *Trichoderma harzianum*, and *Trichoderma viride* [48-52].

1.2.1 *Trichoderma* spp.

Trichoderma spp. belong to the genus of fungi in the family of *Hypocreaceae* [53]. These fungi have been recognized for their significance for centuries, with the first report by Persoon in 1794 [54]. They commonly colonize plant roots and are predominantly found in the rhizosphere [53,55]. The main morphological characteristics of *Trichoderma* spp. include rapid growth, repetitive branching, and dense, velvety conidia with a yellowish-green appearance. Due to the morphological similarities among *Trichoderma* isolates, identification through morphological observation alone is challenging, making species-level determination more critical and essential [53]. In earlier studies, these challenges led to the belief that only a single species, *T. viride*, existed. The first identification protocol for distinguishing species was developed by Rifai in 1969 [56]. According to studies by Rifai (1969), *Trichoderma* strains can be categorized into nine aggregates by morphological characteristics based on the term species aggregate [57]. However, it is proposed by later studies that within the species, there were similar characteristics based on the morphology and conidiophore structure, *Trichoderma* is therefore categorized into 5 sections including *Trichoderma*, *Saturnisporium*, *Pachybasium*, *Hypocreanum*, and *Longibrahmatum* [53,58]. Additionally, recent studies conducted in 2013 indicated that there are more than 200 phylogenetically determined *Trichoderma* species [59].

Trichoderma spp. have gained popularity due to their biocontrol potential in agricultural fields for the last seven decades. Recent research has shown that *Trichoderma* spp. produce antimicrobial compounds, including secondary metabolites that can inhibit the growth of phytopathogenic organisms and serve as effective biocontrol agents (BCA) against various plant diseases [53,55,56,60]. In the 1930s, Weindling revealed that *T. lignorum* had a mycoparasitic effect on phytopathogen *Rhizoctonia solani*. The mycelial growth of *T. lignorum* inhibited the mycelial growth of *R. solani*. Furthermore, the concept of mycoparasitism was defined by Weindling as the parasitic mechanism of a fungus on another one [61]. In light of this discovery, many studies have been conducted

so far to examine the action mechanisms of the genus *Trichoderma* as a BCA [60]. There are two types of action mechanisms underlying *Trichoderma* spp. antifungal activities: direct and indirect action mechanisms as represented in Figure 1. 1. The direct action mechanisms target the pathogens responsible for diseases through mycoparasitism, competitive advantage, and antagonistic effects, while the indirect action mechanisms enhance the plant health by improving the immune system and inducing systemic resistance (ISR) [62,63].

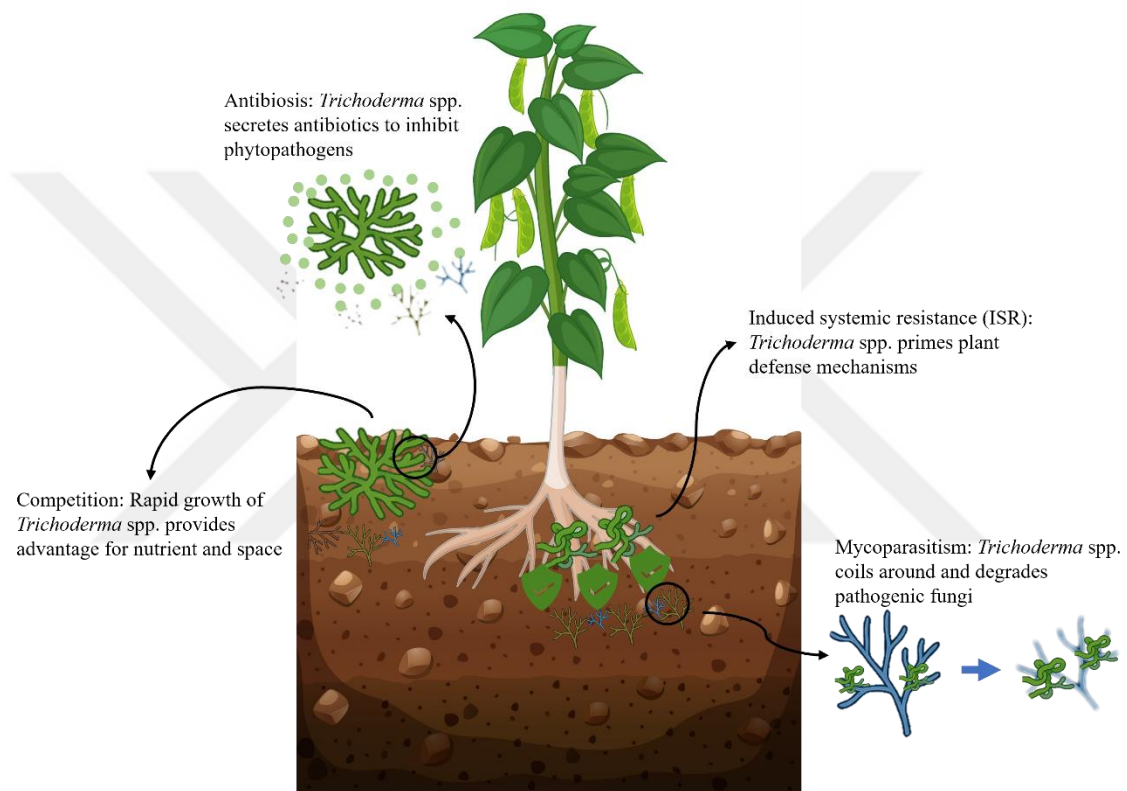


Figure 1. 1 Schematic representation of the mechanisms by which *Trichoderma* spp. promotes plant growth and protects against phytopathogens. These include antibiosis (secretion of antimicrobial compounds), competition for nutrient and space in the rhizosphere, mycoparasitism (direct attack on pathogenic fungi), and ISR (induction of plant immune response).

1.2.1.1 Direct Action Mechanism of *Trichoderma* spp.

Trichoderma spp. exhibit remarkable antimicrobial effects against phytopathogens, making them too significant to overlook. The majority of these antimicrobial effects are occupied by the direct action mechanisms, especially mycoparasitism, which is one of their most crucial characteristics [63,64].

Mycoparasitism occurs in four phases. Initially, a chemical signal attracts the antagonistic fungus. After this stimulus, a specific recognition supervened, after which *Trichoderma* spp. attacks by coiling around the hyphae of the pathogen. Finally, lytic enzymes are secreted to degrade the cell wall of the host [63].

Moreover, a significant advantage of *Trichoderma* spp. lies in their competitive abilities. Several factors in the soil influence the growth of microorganisms, including the availability of space for colonization and essential nutrients such as carbon, nitrogen, and minerals. Organisms seeking to establish themselves in the soil require these factors for optimal growth. *Trichoderma* spp. have a great ability to grow quickly by taking up more space due to their rapid growth and efficient uptake of nutritional elements. BCAs can outcompete phytopathogens by occupying their potential infection sites and controlling pathogenic activities, thereby effectively controlling plant diseases [65].

The nature of the soil can inhibit the germination of fungi. *Trichoderma* spp. benefit from a natural soil phenomenon known as soil fungistasis, a concept first introduced by Dobbs and Hinson [66]. Soil fungistasis is provided by the characteristics of the soil and the microbial load of the soil. The microorganisms found in the soil microbiome can suppress the spread and growth of some fungal species. As an instance, they can inhibit the hyphal growth of the fungi and force sporulation, which can further enhance the biocontrol efficiency of *Trichoderma* spp. [67].

1.2.1.2 Indirect Action Mechanism of *Trichoderma* spp.

There is a strong interaction between *Trichoderma* spp. and the plants. In addition to the impacts of *Trichoderma* spp. on the plant diseases, they can colonize in the root of the plants and provide benefits to them, including improvement in plant growth, nutrient availability, crop production, and disease resistance [65].

BCAs including *Trichoderma* spp. exhibit plant root colonization. An interaction is observed between plants and *Trichoderma* spp. physically. The hyphae of *Trichoderma* spp. surround the root cortex. Moreover, this interaction is an example of a symbiotic relationship, since *Trichoderma* spp. take place in a nutritious habitat, and the plants gain resistance to the diseases [65].

The close proximity between *Trichoderma* spp. and the plant roots causes direct molecular crosstalk. As the close interaction between *Trichoderma* spp. and plant continues, different types of compounds that have elicitor effects are produced by *Trichoderma* spp.. Such compounds can be classified as enzymatically active proteins, defense-reaction inducing gene products, and low-molecular-weight compounds released because of enzymatic activity. The effect of compounds produced by *Trichoderma* spp., acting as elicitors, helps plants to express genes related to plant defense and response systems, and as a result, plant growth is promoted, availability of nutrients and root system development are enhanced. The development of the plant with *Trichoderma* spp. increases the space available for proper *Trichoderma* spp. colonization by increased available nutritious area, eventually the indirect action mechanisms between plants and *Trichoderma* spp. against phytopathogens increase [64].

1.3 Agro-Industrial Wastes

Microorganisms play the leading role in microbiological research and require appropriate culture conditions for proper maintenance. The primary requirement of microorganisms is a nutrient-rich culture medium for cultivation, typically containing carbon and nitrogen sources along with essential minerals, varying by the species. For each species, there are specialized commercial culture media for optimum growth. However, the high cost of these commercial culture media poses a significant challenge, particularly for large scale industrial applications [68].

Waste management has become a critical issue in recent decades, driven by population growth and increasing human activities. Furthermore, it is reported that inefficient waste management strategies pose significant risks to human health and public welfare [69,70]. As urbanization and industrialization accelerate, the need for effective environmental and sustainable management strategies has become a priority, especially in developed countries [70].

It is known that agro-industrial wastes are very promising resources for microbial cultivation due to their rich nutritional contents [68]. Considering the diverse

geographical and ecological conditions of Türkiye, it is seen that it is well-suited for agriculture of various crops. Moreover, it is possible to produce 75 out of 138 fruit families in Türkiye and our country is among the native regions of apple production. Every region in Türkiye has suitable soil and climate for apple production. Globally, apples are one of the economically important crops, with Türkiye ranking fourth in production, contributing 4.14% to the total yield. According to 2012 data, approximately 65 million tons of apples are produced in the world [71].

1.3.1. Apple Pomace

During industrial processing, approximately 25% of apples remain as pomace, resulting in a utilization rate of only 75% in industries such as juice production. Despite the limited utilization of apple, apple pomace (AP) also possesses a nutrient-rich composition, making it a valuable resource for various applications. Additionally, its high water content renders it particularly suitable for fermentation. However, when AP is not properly valorized and is instead discarded in fields, it contributes to environmental pollution due to its susceptibility to fermentation by undesirable microorganisms and its high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), which is approximately 300 g/kg. This elevated COD level signifies that the introduction of AP into soil or water leads to excessive oxygen consumption, thereby disrupting the ecosystem. Furthermore, the microbial fermentation of AP in soil or water, driven by its high BOD, results in substantial oxygen depletion, ultimately harming aquatic ecosystems [72].

Beyond mitigating environmental contamination caused by spoilage microorganisms, the valorization of AP offers a cost-effective substrate for the controlled fermentation of microorganisms, facilitating the production of high value-added products [72,73]. A wide range of industrial enzymes, biofuels including bioethanol and biogas, antibiotics, and bioplastics can be produced in large quantities by various microorganisms. The transformation of industrial waste into valuable products aligns with the principles of the circular economy, where upcycling contributes to a more sustainable business model [74]. For instance, cellulase, one of the most widely used enzymes across various industries, is primarily produced by fungal species. Many of these fungi can be cultivated on agro-industrial waste to generate high value-added products. This process

serves as a compelling example of converting a low-value byproduct into a beneficial and essential resource [73].

1.3.2 Solid State Fermentation of Agro-industrial Waste

Solid-state fermentation (SSF) is a technique used for cultivating microorganisms on agro-industrial waste materials. SSF defines fermentation on a solid substrate with minimal free water while maintaining sufficient moisture to support microbial growth. From past to present, SSF has served as the foundation for numerous fermentation techniques, establishing the fundamental principles of the fermentation process. Additionally, SSF offers several advantages, particularly in the valorization of agro-industrial waste. These benefits include reduced energy requirements and lower wastewater generation. Moreover, SSF is considered as an environmentally friendly approach as it facilitates the recycling of solid waste in nature. However, proper microorganisms, suitable substrates, and optimum fermentation conditions are important for the successful development of bioprocesses in SSF. Fungi and yeast cells are generally considered as proper microorganisms for SSF since they require less water than bacterial cells. However, many bacterial cells can be successfully cultured by SSF [75]. Since ancient times, lactic acid fermentation has been used for the protection of foods and enhancing their shelf life. Lactic acid bacteria (LAB) are considered GRAS (Generally Recognized As Safe) and are used to ferment many types of foods. For instance, soybeans are fermented by LAB in SSF. This process enhances the nutritional composition of soybeans and increases their shelf life. Furthermore, SSF improves the organic acid production of LAB compared to spontaneous fermentation by maintaining the desired conditions throughout the process [76].

The substrate selection is the key part of the SSF since it is the source of nutrients and physical support of cultivation. The substrate should provide adequate water activity and oxygen transfer for microorganisms, while being suitable for intended application. The substrate selection can be based on disposal, value-addition, or production of a specific product. Additionally, proper microorganisms are selected based on the purpose and the substrate [75]. As an example of value-addition by SSF, the nutritional profile of the legumes or cereals can be enriched by the microorganisms with SSF. *Rhizopus* spp. are used to ferment chickpeas by SSF, and it is observed that SSF significantly improves

nutritional characteristics and increases the protein content of chickpeas with the enzymes produced by *Rhizopus* spp. [77]. As an example of the production of a specific product by SSF, many commercial enzymes are produced by special microorganisms. The substrate is selected according to the requirements of the microorganism and fermented by SSF. According to the study of Chutmanop et al. (2008), the rice bean, which is an agro-industrial substrate, is used for the cultivation of *Aspergillus oryzae* for protease production, which is a widely used enzyme in numerous industries, including detergent, food, and dairy industries [78].

1.4 Objectives and Scope of the Thesis

In this study, it is aimed to evaluate the antifungal characteristics and plant growth-promoting properties of *T. harzianum* that was cultivated using agro-industrial waste. The potential of using *T. harzianum* as a biofungicide instead of chemicals was highlighted. The ecological impacts, advantages, benefits, action mechanisms, and inhibitory capabilities of biological alternatives were emphasized. Furthermore, valorizing agro-industrial waste raised awareness for circular economy and proper waste management strategies.

An agro-industrial waste was utilized for the cultivation of *T. harzianum* instead of a commercial culture medium. The nutritional composition of AP was analyzed, and its valorization for cultivation was explored.

Moreover, *in vitro* assays were performed to observe the inhibitory effects against phytopathogens. A dual culture assay, a non-volatile compound assay, and a volatile organic compound assay were carried out to examine the antagonistic activity of *T. harzianum*. Chemical assays were performed to analyze the phosphate solubilization, siderophore production, and indole-3-acetic acid production abilities of *T. harzianum*.

To sum up, the increasing demand for sustainable agricultural has intensified interest in microbial biocontrol agents and biofertilizers derived from low-cost resources. *T. harzianum*, a well-known fungal species with strong antagonistic and plant growth-promoting abilities, offers considerable potential in this context. In the present study,

apple pomace, which is an abundant agro-industrial waste, was explored and optimized as an alternative culture medium to support the growth and metabolite production of *T. harzianum*. The antifungal efficacy and metabolites of *T. harzianum* was assessed against key phytopathogens using dual culture and metabolite-based inhibition assays. Additionally, plant-growth promoting properties, including indole-3-acetic acid (IAA) production, siderophore production, and phosphate solubilization, were evaluated to determine its biofertilizer potential. Plant and pepper experiments were conducted to validate its performance under practical conditions. These findings demonstrate the feasibility of converting organic waste into a valuable microbial product for sustainable crop protection and productivity enhancement.



Chapter 2

Materials and Methods

2.1 Species and Culture Conditions

Trichoderma harzianum was used as a biocontrol agent. *Rhizoctonia solani*, *Fusarium solani*, *Aspergillus welwitschiae*, *Botrytis cinerea*, and *Colletotrichum coccodes* were used as phytopathogenic fungal species. These phytopathogens were kindly provided by Tarımsal Araştırmalar ve Politikalar Genel Müdürlüğü (TAGEM). Potato Dextrose Agar (PDA) (Condalab) and Potato Dextrose Broth (PDB) (Liofilchem) were used for the general cultivation of the fungal species. Apple pomace (AP) was kindly provided by a local fruit juice company in Türkiye for the cultivation of *T. harzianum*. The culture conditions were 28°C for all fungal species and 120 RPM for agitation if needed.

2.1.1 Spore Suspension Preparation of *Trichoderma harzianum*

The spores on the surface of the 7-day grown *T. harzianum* Petri dishes were aseptically collected with isotonic buffer. The isotonic buffer was added onto the spores at the amount of nearly 5 mL depending on the spore concentration on the Petri dish. After pouring the isotonic buffer, the spores were scraped using a sterile microscope slide as a spatula. The spores were in a centrifuge tube and vortexed to mix spores with buffer. After mixing, the solution was filtered with fine mesh sieve to separate the spores from mycelia and hyphal structures. The concentration of the obtained spore suspension was determined by serial dilution and spore count on the Petri dish [79,80]. The spore suspension was stored at +4°C for further use.

2.2 Alternative Culture Media Optimization

2.2.1 Analysis of Physicochemical Properties of Apple Pomace

The physical and chemical properties and nutritional composition of raw AP were analyzed [73]. Moisture content was determined according to the weight changes by heating the environment with controlled temperature using Thermogravimetry Differential Thermal Analysis (TG/DTA) (PerkinElmer, Diamond TG/DTA). pH was measured using a pH meter (Mettler Toledo InPro3100). Nitrogen, carbon, hydrogen, and protein contents were examined using an Elemental Analysis machine (Thermo Fisher Scientific). The sulfur value was determined using X-ray fluorescence (XRF) spectrometry (PANalytical Axios Advanced). Cellulose and hemicellulose values were calculated using a Soxhlet extraction machine. For the ash determination, the ash oven was used (Protherm PLF 120/7). Sugar contents were analyzed by High-Performance Liquid Chromatography (Agilent 1260). Mineral contents were measured using an Inductively Coupled Plasma – Mass Spectrometer (Agilent 7500A).

2.2.2 Enrichment of Apple Pomace

The availability of nutrition and enrichment of the nutritional profile of AP were optimized for the optimum growth of *T. harzianum*. The culture medium groups consisting of AP were listed in Table 2. 1. Before adding the other components, the AP were dry heated in the oven at 70°C until they completely dried to provide a standard moisture content. Response surface methodology (RSM) was used for the adjustment of ratios of the additional sources. The ammonium sulfate as a nitrogen source was used between 0-2% (w/w), sodium chloride as a salt source was used between 0-2% (w/w), while water content was added in between 30-200% (w/w) [81]. For positive control, potato dextrose broth (PDB) was added to AP in different ranges.

Additional nitrogen sources and salts were added in to experimental groups, which include 10 g of AP. The moisture was adjusted by the water content in 3 different ratios. RSM was used to form culture media optimization groups by using MATLAB (Table 2.

1). Experiments for each group conducted in triplicates except the 13th group which is the center position for the RSM experiment and conducted in six replicates for statistical significance. In addition to experimental groups, 3 different control groups were prepared using the same ratio of AP with the addition of varying amounts of PDB (30%, 115%, and 200%). All culture media groups were autoclaved at 121°C for 15 minutes. After autoclaving, they were placed into the sterile Petri dishes. *T. harzianum* was inoculated to the culture media at 10⁶ spores per gram for each AP group. The Petri dishes were incubated at 28°C. After 1 week of incubation, 1 g of culture was taken for growth analysis.

Table 2. 1 The culture media groups by RSM for *T. harzianum* cultivation

Groups	Components (% w/w) for 10 g of dried AP
1	115 mL dH ₂ O
2	2 g sodium chloride + 115 mL dH ₂ O
3	2 g ammonium sulfate + 115 mL dH ₂ O
4	2 g ammonium sulfate + 2 g sodium chloride + 115 mL dH ₂ O
5	1 g sodium chloride + 30 mL dH ₂ O
6	1 g sodium chloride + 200 mL dH ₂ O
7	2 g ammonium sulfate + 1 g sodium chloride + 30 mL dH ₂ O
8	2 g ammonium sulfate + 1 g sodium chloride + 200 mL dH ₂ O
9	1 g ammonium sulfate + 30 mL dH ₂ O
10	1 g ammonium sulfate + 200 mL dH ₂ O
11	1 g ammonium sulfate + 2 g sodium chloride + 30 mL dH ₂ O
12	1 g ammonium sulfate + 2 g sodium chloride + 200 mL dH ₂ O
13	1 g ammonium sulfate + 1 g sodium chloride + 115 mL dH ₂ O
C1	30 mL PDB
C2	115 mL PDB
C3	200 mL PDB

2.3 Analysis of Antifungal Activities of *T. harzianum*

2.3.1 Dual Culture Assay

T. harzianum, *F. solani*, *R. solani*, *A. welwitschiae*, *B. cinerea*, and *C. coccodes* were inoculated into PDA plates and incubated for 7 days. After the incubation, the 7-day grown 7.7 mm fungi plugs were aseptically excised using an inverted sterile 1000 μL pipette tip and inoculation loop. After that, they were placed on opposite sides of Petri dishes and 1 cm away from the PDA Petri dish border as described in the study of Sánchez-Montesinos et al. (2021) [82]. Triplicates and negative control groups were prepared for the assay. Only the pathogen plug was placed on one side of PDA, 1 cm away from the Petri dish border for the negative control. Then, all plates were incubated at 28°C. The radial growth of the pathogen was recorded, and the percentage inhibition of radial growth (PIRG) was calculated according to the Figure 2. 1.

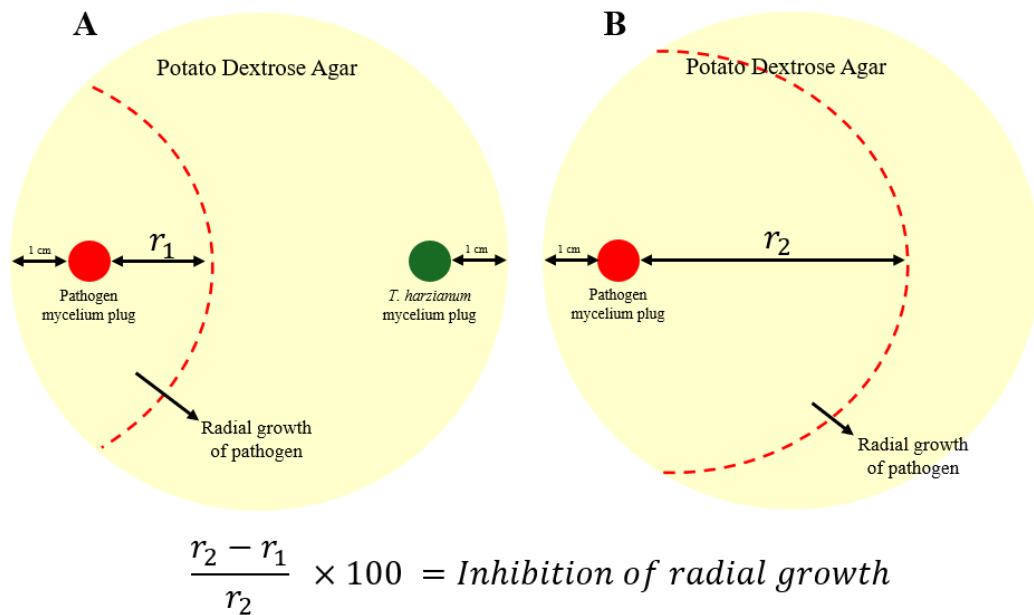


Figure 2. 1 Dual culture assay to evaluate the radial growth inhibition of the pathogen by *T. harzianum*. A: Treatment group: *T. harzianum* and the pathogen inoculated on opposite sides of the same Petri dish to observe direct antagonistic interaction. B: Negative control group: Petri dish inoculated only with the pathogen.

2.3.2 Non-volatile Compound Assay

The 1 mL spore suspension of *T. harzianum* was inoculated into 200 mL of PDB and incubated at 28°C with continuous shaking at 120 RPM for seven days. Following incubation, the culture was centrifuged at 10,000 RPM for 20 minutes. The resulting supernatant was filtered using a 0.22 µm syringe filter. The filtered culture filtrate was then mixed with PDA at a 1:1 ratio before solidification at approximately 50°C. For the negative control groups, distilled water was used in place of the culture filtrate. For each group, three Petri dishes were prepared. Pathogen mycelium plugs were placed at the center of Petri dishes in triplicate and incubated at 28°C. The radial growth of the pathogen was recorded, and the percentage inhibition of radial growth (PIRG) was calculated according to the Figure 2. 2 [83].

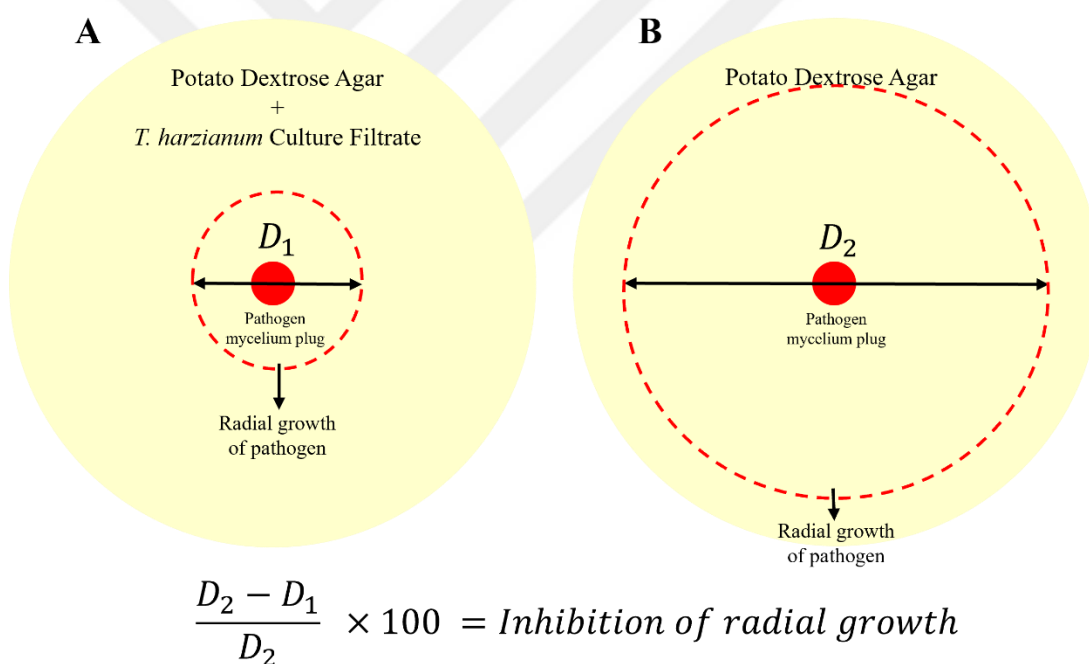


Figure 2. 2 Non-volatile compound assay to evaluate the radial growth inhibition of the pathogens by *T. harzianum*. A: Pathogen inoculated on the treatment group: PDA medium supplemented with *T. harzianum* culture filtrate (1:1, v/v). B: Pathogen inoculated on the negative control group: PDA medium without *T. harzianum* filtrate. In both plates, a mycelial plug of the pathogen was placed at the center to assess radial growth.

2.3.3 Volatile Organic Compound Assay

PDA plates were prepared for two-compartment Petri dishes for the VOC assay of *T. harzianum*. Three Petri dishes were prepared for each group. 7-day-old mycelium plugs were aseptically excised using inverted sterile 1000 μ L pipette tips. *T. harzianum* mycelium plug was inoculated to one side of the compartments with the help of sterile inoculation loops, and the phytopathogen plug was inoculated to the other side of the compartment, as shown in the Figure 2. 3. After that, the Petri dishes were sealed with parafilm to prevent the evaporation of VOCs. After the incubation, the radial growth of the pathogen was recorded, and the percentage inhibition of radial growth (PIRG) was calculated according to the Figure 2. 3 [84].

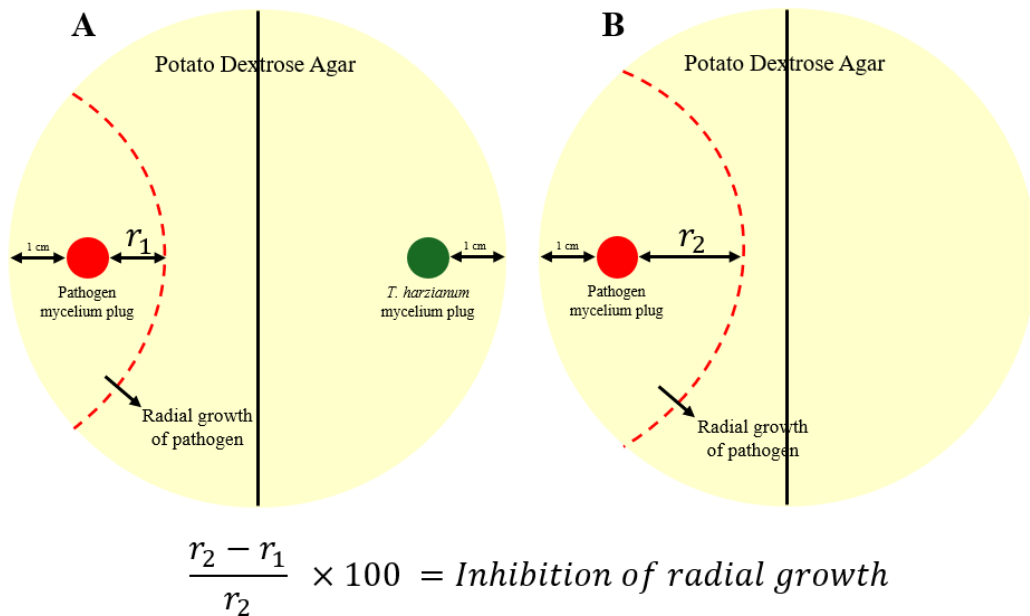


Figure 2. 3 Volatile organic compound (VOC) assay to evaluate the radial growth inhibition of the pathogen by *T. harzianum*. A: Treatment group: *T. harzianum* and the pathogen co-inoculated on opposite sides of the same Petri dish to allow VOC-mediated interaction. B: Negative control group: Petri dish inoculated only with the pathogen.

2.4 Analysis of Plant Growth Promoting Properties of *T. harzianum*

2.4.1 Indole-3-Acetic Acid (IAA) Production

The presence of indole-3-acetic acid (IAA) was determined colorimetrically using the Salkowski reagent. The Salkowski reagent was prepared using 15 mL concentrated H₂SO₄ added to sterile 25 mL distilled water slowly. Then, 750 µL of sterile 0.5 M FeCl₃.6H₂O was added to it. The culture filtrate of *T. harzianum* and the Salkowski reagent were put into a tube in a 1:2 ratio as described in the study of Das et al. (2019) [85]. For the negative control group, sterile PDB was added into the Salkowski reagent with the same ratio. The color changes in the tubes were observed. The pinkish-red color indicates the indole-3-acetic acid production by the *T. harzianum*.

2.4.2 Siderophore Production

A chrome azurol sulphonate (CAS) assay was performed for the indication of siderophore production by *T. harzianum* [86]. CAS media (Solarbio) was prepared according to the instructions and autoclaved at 121°C for 15 minutes. Separately, microbiological agar was prepared based on the final liquid volume adjusted to 1.5% agar concentration and autoclaved at 121°C for 15 minutes. After autoclaving, they were mixed and poured onto Petri dishes. Seven-day grown *T. harzianum* mycelium plugs were placed in the center of the Petri dishes and incubated at 28°C. In addition, a siderophore producing bacteria, identified as *B. thuringiensis*, was used as a positive control. During the incubation, the Petri dishes were observed whether there would be a clear zone around the mycelium plug. The clear zone formation indicates that the *T. harzianum* is able to produce siderophore.

2.4.3 Phosphate Solubilization

Pikovskaya medium (PVK) was prepared to assess the phosphate solubilization ability of *T. harzianum*. The medium was formulated using the ingredients listed in Table 2. 2. The medium was autoclaved at 121°C for 15 minutes and poured onto Petri dishes. Seven-day grown *T. harzianum* mycelium plugs were placed in the center of the Petri dishes and incubated at 28°C. A species of PGPR, identified as *Pseudomonas* sp., was used as a positive control. The PVK agar contains insoluble phosphate sources, and if *T. harzianum* can solubilize them, a clear zone will form around the mycelium plug, indicating phosphate solubilization activity [87].

Table 2. 2 Pikovskaya's medium composition

Components	Amount (g/L)
Yeast extract	0.5
Dextrose	10
Calcium phosphate	5
Ammonium sulphate	0.5
Potassium chloride	0.2
Magnesium sulphate	0.1
Manganese sulphate	0.0001
Ferrous sulphate	0.0001
Agar	15

2.5 Plant Experiments of *T. harzianum*

2.5.1 Pot Experiments

A greenhouse environment was developed with maintained temperature as 28°C, 60% humidity and 12-hour period of red-blue light for a controlled climate chamber for the growth of wheat (*Triticum aestivum*). The wheat seeds were germinated at 28°C for 24 hours between wet cottons. After germination, twelve wheat seeds were inoculated into approximately 200 g of soil, that was autoclaved at 121°C for 15 minutes in

consecutive 2 days, for each group. *T. harzianum* was inoculated at the same time in the amount of 10^8 for each pot with 1 mL of potato dextrose broth (PDB), and all pots were irrigated. After 2-days of incubation, the phytopathogens were inoculated into the pots in the amount of 10^6 for each pot. The pots were irrigated daily. After 3 weeks, the roots and weights of the plants were analyzed. The experimental setup was adapted with modifications from the study of Sánchez-Montesinos et al. (2021) to align with the experimental conditions of this research [82].

2.5.2 Pepper Fruit Experiments

The peppers (*Capsicum annuum*) were washed and sterilized with 3% hypochlorite for 15 seconds, then rinsed with distilled water. Three peppers were used for each group. Two wounds were formed on the surface of the pepper as an inoculation spot and 100 μ L of 10^6 spore/mL of *T. harzianum* spore suspension was inoculated to each spot in treatment groups. 100 μ L of 10^5 spore/mL of pathogen spore suspensions were inoculated to relevant group. For the control, 100 μ L of sterile distilled water was inoculated. The peppers were incubated at 28°C with 60% humidity. The severity of symptoms was observed daily. The method was adapted with modifications from the studies of Yadav et al. (2023) and Kim et al. (2023) to align with the experimental conditions of this research [88,89].

Chapter 3

Results

3.1 Alternative Culture Media Optimization

3.1.1 Analysis of Physicochemical Properties of Apple Pomace

The physicochemical properties of apple pomace were analyzed, and the analysis results are found in Table 3. 1. The findings revealed that its composition presents a valuable potential substrate for biological applications. The moisture content was found $75.31 \pm 1.25\%$, indicating a high-water content, that enables efficient fermentation without water addition for microbial growth and enzymatic activity. The pH of the sample (3.62 ± 0.2) suggests an acidic nature which can be selective for the growth of many microorganisms. However, *Trichoderma* spp. can grow effectively in a wide range of pH (pH 2.0-6.0), with optimal growth typically observed at pH 4.0. Moreover, the optimum growth rates of different strains can vary at certain pH values [90]. This pH value indicates that the sample is suitable for *Trichoderma* spp. cultivation.

The total carbon content (47.28%) is relatively high, indicating that AP is a promising organic carbon source. The total nitrogen content (1.88%) is lower but shows that there are some nitrogenous compounds necessary for microbial metabolism. Moreover, the protein content (11.75%) further suggests a fair nutritional value for maintaining microbial growth. However, these values can be enriched with additional nitrogen sources for optimal growth.

Additionally, the presence of complex polysaccharides, including cellulose (24.19%) and hemicellulose (30.11%), supports the apple pomace as an alternative culture media for optimal growth of fungal species. The values of mineral content were also consistent with the apple pomace analysis in the literature. These properties present

a promising culture medium for microorganisms. The enrichments could help to increase the obtained biomass if needed [73].

Table 3. 1 Physicochemical Properties of Apple Pomace

Content	Values
Moisture (%)	75,31±1,25
pH	3,62±0,2
Total solid (%)	21,05±0,75
Volatile solid (%)	1,61±0,11
Suspended solid (%)	1,21±0,15
Total carbon (%)	47,28
Total nitrogen (%)	1,88
Hydrogen (%)	6,95
Sulfur (%)	0,012
Cellulose (%)	24,19
Hemicellulose (%)	30,11
Protein (%)	11,75
Ash (%)	0,66±0,03
Reducing sugars (Glucose, fructose, sucrose) (%)	0,092±0,001
Minerals	
Phosphorus (mg/kg)	1.077±10
Potassium (mg/kg)	26,7±6.5
Calcium (mg/kg)	39,2±1.2
Sodium (mg/kg)	707,0±5.0
Magnesium (mg/kg)	35,6±1.0
Copper (mg/kg)	1,5±0.2
Zinc (mg/kg)	1,9±0.1
Manganese (mg/kg)	0,13±0.03
Iron (mg/kg)	9,6±1.0

3.1.2 Alternative Culture Media Optimization of Apple Pomace

The potential of apple pomace for *T. harzianum* growth was investigated. The spore suspension of *T. harzianum* was used for inoculum into AP at 10^6 spore/g. The *T. harzianum*-inoculated Petri dishes to be used for spore suspension preparation were incubated for 7 days at 28°C (Figure 3. 1). After proper growth, the spores were collected to be inoculated into AP groups. The spore concentration of spore suspension was 10^{10} spore/mL, and the spore suspension was examined under microscope (Figure A. 1). For the AP inoculum, the spore concentration was adjusted to 10^6 spore/g.



Figure 3. 1 Seven-day-old *T. harzianum* culture grown on PDA medium in a Petri dish.

After 7 days of incubation, the growth of *T. harzianum* on AP (Figure A. 2) was analyzed by serial dilution and plate count of biomass. Additionally, the mycelial growth of *T. harzianum* was observed daily, and denser growth was recorded in Group 10, indicating that there is a positive correlation between moisture content and growth rate. The lower growth rates were observed in the groups that had lower moisture content and higher salt amount. Furthermore, according to the averages of the spore count of each group (Table 3. 2), an RSM graph was created using MATLAB, as shown in Figure 3. 2. The results of RSM showed that the ammonium sulfate has a significantly small coefficient based on the polynomial equation. The presence of ammonium sulfate showed a minimal improvement in growth. However, the presence of sodium chloride presented a negative impact on the development. The RSM graph and equation obtained from the results indicated that the highest spore count could be obtained in 143.33% water content

in addition to dried AP (Figure 3. 2). However, the spore count of this optimum group on the seventh day of incubation (1.5×10^{10}) revealed that Group 10 had slightly higher biomass (3.8×10^{10} spore/g) (Table 3. 2). Although the RSM-derived equation suggested that ammonium sulfate had a limited effect on growth, its inclusion did enhance the growth rate. Therefore, the use of ammonium sulfate can be evaluated based on a balance between cost and performance expectations. Moreover, the compositional analysis of AP seen in Table 3. 1 showed that it contains adequate levels of protein and sodium, along with a high moisture content of 75%. These findings suggested that AP alone holds strong potential as a cultivation substrate.

Dhillon et al. (2012) also utilized apple pomace (AP) as a substrate for the cultivation of the fungal species *A. niger* to produce cellulase and hemicellulase enzymes. The study involved supplementing AP with various inducers; however, the addition of certain inducers, including methanol and ethanol, led to lower enzyme yields compared to the group cultivated solely on AP. In contrast, other inducers enhanced enzyme production to varying extents. The study emphasized that the relationship between the cost of inducers, the rate of enzyme production, and overall yield plays a critical role in determining the economic feasibility of using AP as a low-cost substrate [73].

Table 3. 2 *T. harzianum* spore count of prepared AP groups on 7th day of incubation

Groups	Components (% w/w)	Spore count (spore/g)
1	115 mL dH ₂ O	1.1×10^{10}
2	2 g sodium chloride + 115 mL dH ₂ O	8.7×10^8
3	2 g ammonium sulfate + 115 mL dH ₂ O	1.8×10^{10}
4	2 g ammonium sulfate + 2 g sodium chloride + 115 mL dH ₂ O	9.4×10^8
5	1 g sodium chloride + 30 mL dH ₂ O	1.6×10^5
6	1 g sodium chloride + 200 mL dH ₂ O	4.2×10^9
7	2 g ammonium sulfate + 1 g sodium chloride + 30 mL dH ₂ O	9.3×10^4
8	2 g ammonium sulfate + 1 g sodium chloride + 200 mL dH ₂ O	9.6×10^8
9	1 g ammonium sulfate + 30 mL dH ₂ O	5.3×10^8
10	1 g ammonium sulfate + 200 mL dH ₂ O	3.8×10^{10}
11	1 g ammonium sulfate + 2 g sodium chloride + 30 mL dH ₂ O	5.3×10^3
12	1 g ammonium sulfate + 2 g sodium chloride + 200 mL dH ₂ O	1.3×10^8
13	1 g ammonium sulfate + 1 g sodium chloride + 115 mL dH ₂ O	8.7×10^8
C1	30 mL PDB	1.6×10^7
C2	115 mL PDB	2×10^{10}
C3	200 mL PDB	2.4×10^{11}

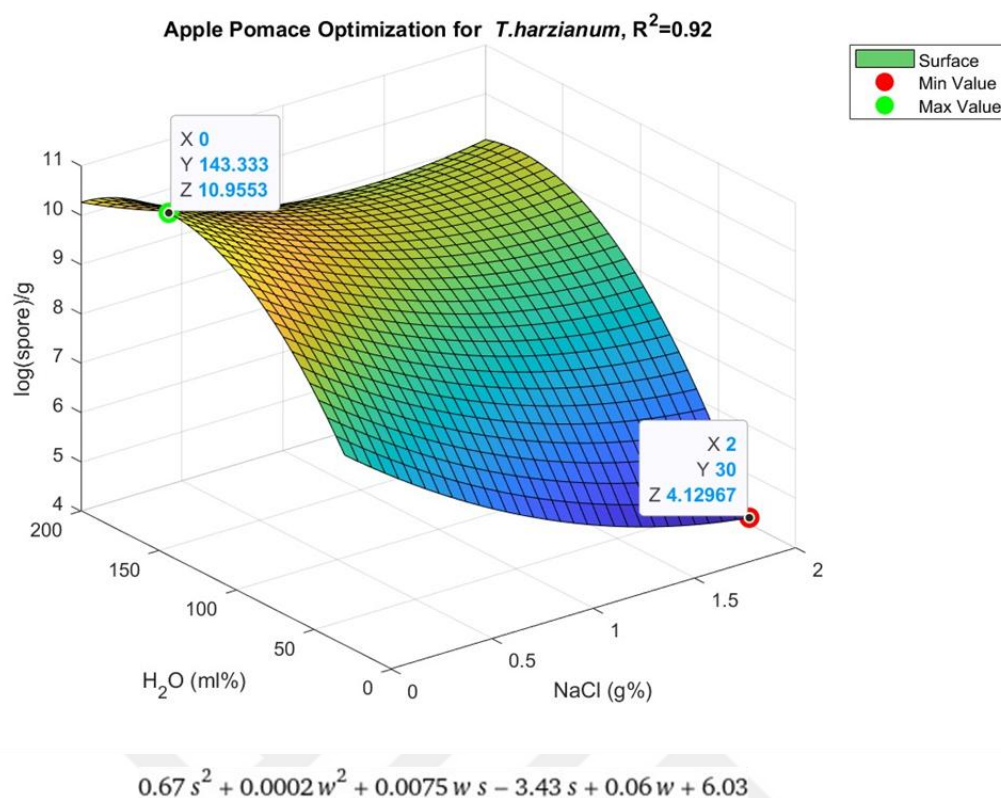


Figure 3. 2 Response Surface Methodology (RSM) plot illustrating the effect of NaCl and H₂O concentrations on spore yield of *T. harzianum* in apple pomace-based culture media. The graph indicates the predicted maximum (green) and minimum (red) spore yields.

3.2 Analysis of Antifungal Activities of *T. harzianum*

3.2.1 Dual Culture Assay

The antagonistic potential of *T. harzianum* against five phytopathogens, which are *F. solani*, *R. solani*, *A. welwitschiae*, *C. coccodes*, and *B. cinerea*, was evaluated using the dual culture assay. The results revealed that *T. harzianum* showed strong antifungal activity against all tested phytopathogens.

The radial growth inhibition rates of *T. harzianum* on tested phytopathogens were different. Antagonistic activity was assessed by measuring the radial growth of phytopathogens on the Petri dishes in the presence and absence of *T. harzianum*. The percentage of inhibition was calculated according to the equation based on the radial

growth of negative control and the *T. harzianum* treatment group. Each phytopathogen exhibited varying inhibition in growth as shown in Figure 3. 3, Figure 3. 4, Figure 3. 5, Figure 3. 6 and Figure 3. 7. The highest inhibition was observed against *C. coccodes*, with a mean inhibition rate of 96.76%, while the lowest antagonistic activity was recorded against *A. welwitschiae* with 60.9% at the end of 7 days of incubation, indicating strong competitive and mycoparasitic behavior of *T. harzianum*. Numerous studies also support these strong inhibition percentages. For instance, the study of Asran-Amal et al. (2010) revealed that *T. harzianum* inhibited the growth of *F. solani* and *R. solani* at 76.5% and 59.2%, respectively. They stated that *T. harzianum* significantly reduced the growth of these phytopathogens [91].

The antifungal activity of *T. harzianum* on *F. solani* was shown in Figure 3. 3 and Figure A. 3. The radial growth of *F. solani* in the absence of *T. harzianum* was observed in Figure 3. 3A, exhibiting an extensive radial growth on the surface of the Petri dish whereas in the presence of *T. harzianum* shown in Figure 3. 3B, a significant inhibition of phytopathogen growth was observed. Mycoparasitism and competitiveness properties of *T. harzianum* inhibited the growth of *F. solani*. *T. harzianum* exhibited a superior growth rate and nutrient uptake efficiency, enabling it to rapidly colonize the available surface area in the Petri dish and effectively suppress the radial growth of the phytopathogen. The inhibition rate of *T. harzianum* against *F. solani* was found to be 86.38% (Table 3. 3), indicating strong antagonistic activity. The study of Awad-Allah et al. (2022) reported that *T. harzianum* and *T. viride* suppressed the mycelial growth of *F. solani* by 78% and 61.2%, respectively. Their findings revealed that *Trichoderma* isolates have a significant potential to inhibit the *Fusarium* wilt disease on cherry tomatoes with their antagonism mechanisms including mycoparasitism, nutrient and space competition, and antibiosis. Additionally, they observed that *Trichoderma* species also promoted the growth of the cherry tomatoes [92]. Chen et al. (2021) tested seven *Trichoderma* isolates against *F. oxysporum*. Similarly, the strongest antagonistic activity (72.77%) was observed in the isolate identified as *T. harzianum* [93].

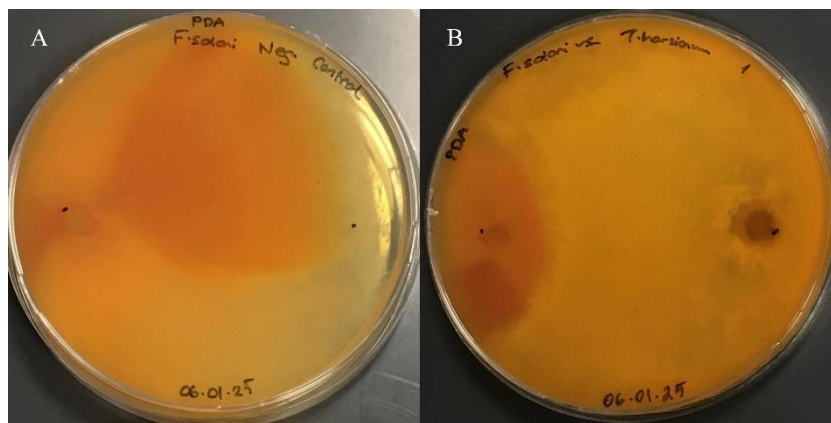


Figure 3. 3 Dual culture assay of *T. harzianum* against *F. solani* on PDA medium. A: Negative control plate showing the growth of *F. solani* alone. B: Co-culture plate showing antagonistic interaction between *T. harzianum* and *F. solani*. *T. harzianum* was inoculated on the opposite side of the pathogen, resulting in visible inhibition of *F. solani* mycelial growth.

The antagonistic activity of *T. harzianum* on *R. solani* was presented in Figure 3. 4 and Figure A. 4. The radial growth of *R. solani* was quantitatively assessed on Petri dishes in negative control and *T. harzianum* treatment groups. Figure 3. 4A illustrated the radial growth of *R. solani* in the absence of *T. harzianum*, occupying the majority of the Petri dish, whereas Figure 3. 4B showed the smaller radial growth in the presence of *T. harzianum*. Similar to *F. solani*, the mycoparasitic characteristics and competitiveness of *T. harzianum* inhibited the growth of *R. solani* effectively. The calculated inhibition rate against *R. solani* was 91.07% (Table 3. 3), highlighting a strong antifungal and antagonistic potential of *T. harzianum*. In a study, the *T. harzianum* isolate also showed a similar inhibition (68.75%) against *R. solani* that caused rice sheath blight disease. The researchers demonstrated the high antagonistic activity of *T. harzianum* isolates, suggesting that competition and production of volatile and non-volatile secondary metabolites are the reasons behind this strong inhibition [94]. A consistent result was obtained from the study of Salman and Boyraz. They performed dual culture assay against *R. solani* with 69 *Trichoderma* spp., which were isolated from varying soil and plant samples. The PIRG values were between 8% and 89% based on the isolate. The highest inhibition (89%) was observed with the *Trichoderma* isolate belonging to *T. virens* [95].

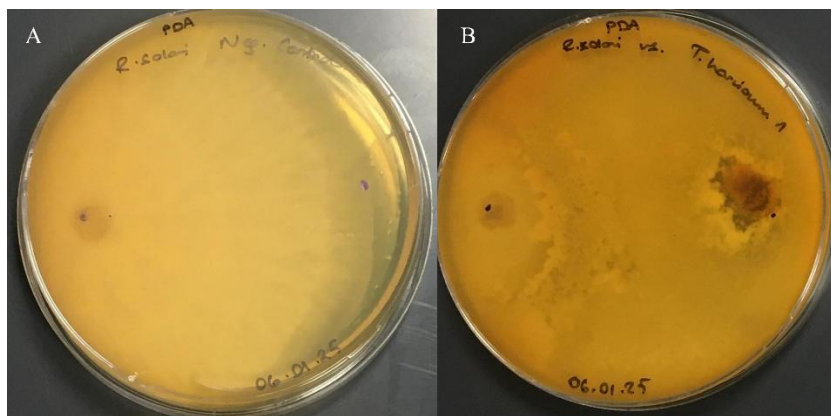


Figure 3. 4 Dual culture assay of *T. harzianum* against *R. solani* on PDA medium. A: Negative control plate showing the growth of *R. solani* alone. B: Co-culture plate showing antagonistic interaction between *T. harzianum* and *R. solani*. *T. harzianum* was inoculated on the opposite side of the pathogen, resulting in visible inhibition of *R. solani* mycelial growth.

Figure 3. 5 and Figure A. 5 presented the antagonistic interaction between *T. harzianum* and *A. welwitschiae*. The radial growth of *A. welwitschiae* was measured in both negative control and dual culture conditions. In the absence of *T. harzianum*, *A. welwitschiae* occupied more space compared to the *T. harzianum*-inoculated Petri dish. However, *T. harzianum* showed a relatively lower inhibition rate, 60.9% against *A. welwitschiae*, when compared to other tested phytopathogens (Table 3. 3). Nevertheless, the observed inhibition rate remained highly promising for potential application in agricultural disease management. The study of Voloshchuk et al. (2024) suggested that specifically the antibiosis property of isolates of *Trichoderma* spp. highly inhibited the mycelial growth of *Aspergillus flavus* and *Aspergillus parasiticus* by ranges of 40-45%, 38-42%, respectively [96]. The results of the study of Alsaedi and Alani (2014) were also consistent with these inhibition percentages. They tested two isolates of *Trichoderma* spp. against *Aspergillus* sp.. After one week of incubation, 43.9% and 46.32% PIRG were observed with two *Trichoderma* spp. isolates, respectively [97].

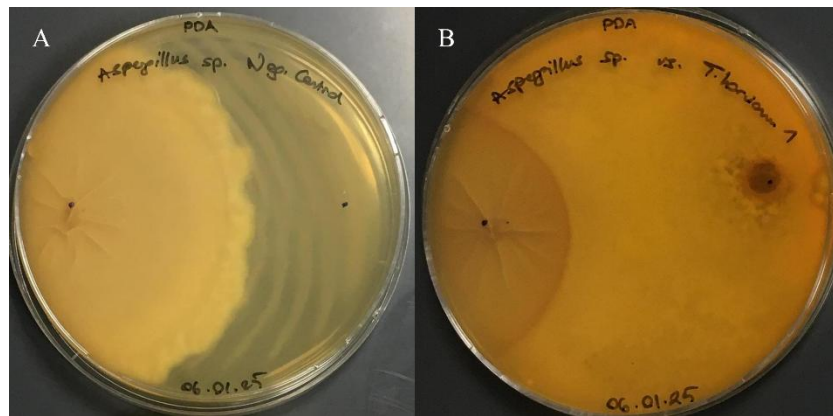


Figure 3. 5 Dual culture assay of *T. harzianum* against *A. welwitschiae* on PDA medium. A: Negative control plate showing the growth of *A. welwitschiae* alone. B: Co-culture plate showing antagonistic interaction between *T. harzianum* and *A. welwitschiae*. *T. harzianum* was inoculated on the opposite side of the pathogen, resulting in visible inhibition of *A. welwitschiae* mycelial growth.

As shown in Figure 3. 6 and Figure A. 6, *T. harzianum* demonstrated highly significant inhibitory activity against *C. coccodes* with 96.76% radial growth inhibition (Table 3. 3), reflecting strong biocontrol potential. *C. coccodes* occupied nearly half of the Petri dish under negative control condition (Figure 3. 6A). In contrast, dual culture with *T. harzianum* resulted in substantial inhibition of radial growth of *C. coccodes* (Figure 3. 6B). A similar result with this study was observed in the study of Costa et al. (2021). *T. piluliferum* was analyzed against *C. musae*, and a great inhibition was observed compared to the control. The overgrowth of *T. piluliferum* covered the all surface of Petri dish, mentioning that *T. piluliferum* received the best evaluation with 100% inhibition based on Bell's classification [98]. In a study, it was observed that 20 isolates of *Trichoderma* spp. suppressed the mycelial growth of *C. nymphaeae* at varying ranges from 50% to 61.3%, suggesting the strong antagonistic activity of *Trichoderma* spp. on the pathogen due to its mycoparasitism activity and hyphal coiling formation on the pathogen, disrupting the cellular structure [99].

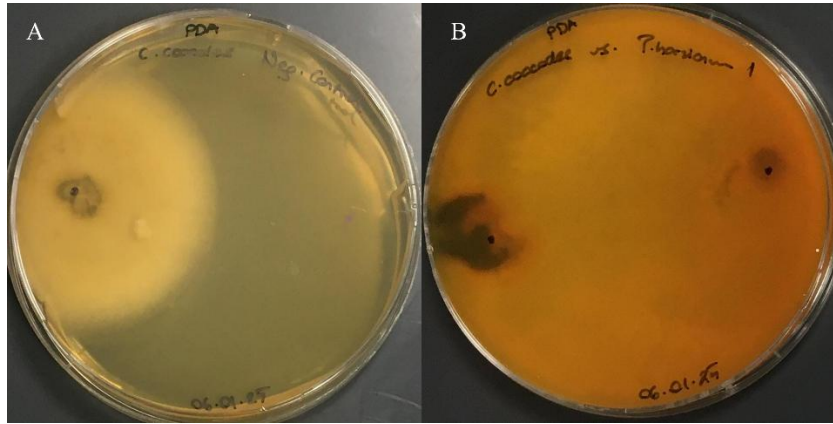


Figure 3. 6 Dual culture assay of *T. harzianum* against *C. coccodes* on PDA medium. A: Negative control plate showing the growth of *C. coccodes* alone. B: Co-culture plate showing antagonistic interaction between *T. harzianum* and *C. coccodes*. *T. harzianum* was inoculated on the opposite side of the pathogen, resulting in visible inhibition of *C. coccodes* mycelial growth.

Figure 3. 7 and Figure A. 7 showed the antifungal activity of *T. harzianum* on *B. cinerea*. In the untreated negative control Petri dish (Figure 3. 7A), *B. cinerea* showed a larger radial growth compared to *T. harzianum* treated Petri dish (Figure 3. 7B). The rapid radial growth of *T. harzianum* inhibited the expansion of *B. cinerea* on the Petri dish on a major scale. The inhibition was 85.94% (Table 3. 3), indicating strong antagonistic performance. Yuan et al. (2023) investigated the antagonistic activity of eight isolates of *Trichoderma* spp. against *B. cinerea*. The isolate that showed the highest inhibition had the 59.7% inhibition rate due to its competition advantage with rapid growth [100]. A recent study obtained a consistent result with this study. The antagonistic activity of *T. harzianum* against *B. cinerea* was analyzed by dual culture assay. After 6 days of incubation, 62.05% PIRG was observed, suggesting a strong inhibitory effect of *T. harzianum* against *B. cinerea* [101].

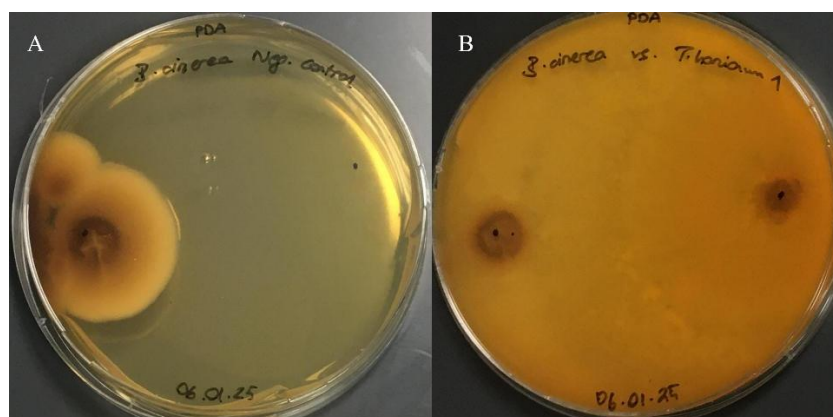


Figure 3. 7 Dual culture assay of *T. harzianum* against *B. cinerea* on PDA medium. A: Negative control plate showing the growth of *B. cinerea* alone. B: Co-culture plate showing antagonistic interaction between *T. harzianum* and *B. cinerea*. *T. harzianum* was inoculated on the opposite side of the pathogen, resulting in visible inhibition of *B. cinerea* mycelial growth.

Table 3. 3 Inhibition of radial growth (%) of phytopathogens by *T. harzianum* in dual culture assay

Phytopathogen	Radial Growth Inhibition (%) $\pm$ SD
<i>F. solani</i>	86.38 $\pm$ 0.7
<i>R. solani</i>	91.07 $\pm$ 0.6
<i>A. welwitschiae</i>	60.9 $\pm$ 0.6
<i>C. coccodes</i>	96.76 $\pm$ 4.1
<i>B. cinerea</i>	85.94 $\pm$ 2.7

3.2.2 Non-volatile Compound Assay

The antifungal activities of non-volatile compounds of *T. harzianum* were also analyzed against five phytopathogens. The culture filtrate of *T. harzianum* was mixed with PDA for the treatment groups, while only PDA was used in negative control groups. At the end of 7 days of incubation, varying ranges of radial growth inhibition were observed in each phytopathogen, as shown in Figure 3. 8, Figure 3. 9, Figure 3. 10, Figure 3. 11 and Figure 3. 12, indicating that the culture filtrate of *T. harzianum* includes bioactive non-volatile compounds with antagonistic effects.

The radial growth inhibition by non-volatile compound was calculated as shown in Figure 2. 2. The inhibition percentage was determined by the measurements of

phytopathogens' radial growth value differences between negative control and *T. harzianum* treatment groups. Based on these calculations, the highest radial growth inhibition was observed in *A. welwitschiae* with 75% radial growth inhibition, while the lowest inhibition was observed in *B. cinerea* with 55% (Table 3. 4). The results indicated that the non-volatile compounds of *T. harzianum* showed efficient antifungal activity across all five tested phytopathogens.

Figure 3. 8 and Figure A. 8 presented the antagonistic effects of non-volatile compounds of *T. harzianum* against *F. solani*. In the negative control Petri dish (Figure 3. 8A), including only PDA, *F. solani* occupied almost all the surface of the Petri dish. In contrast, in the Petri dish (Figure 3. 8B), including culture filtrate of *T. harzianum*, the radial growth of *F. solani* was strongly inhibited, indicating that the non-volatile compounds of *T. harzianum* had a significant antagonistic activity on *F. solani* with 61.87% (Table 3. 4) of radial growth inhibition. In a study, the radial growth inhibition rates by varying filtrate concentration (10%, 25%, and 50%) of *Trichoderma* isolates were assessed against *F. solani*. The inhibitory activity of non-volatile compounds was from 30% to 70%. This finding was consistent with the previous studies, which reported between 40% and 70% inhibition. This inhibition rate was found effective to suppress the *F. solani* mycelial growth [102].

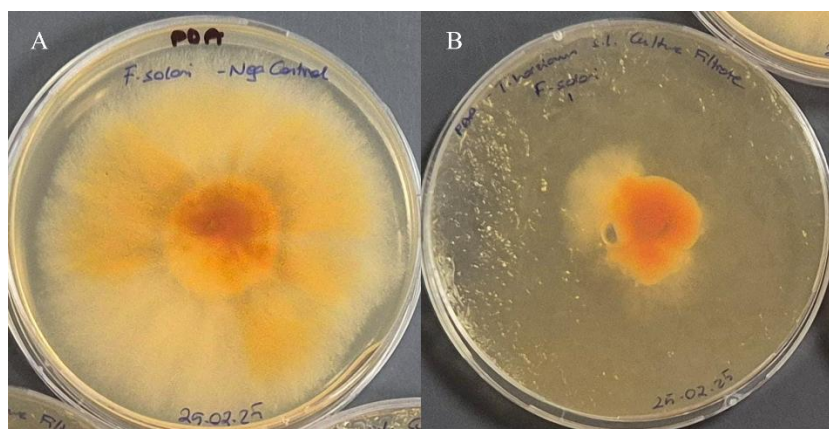


Figure 3. 8 *T. harzianum* non-volatile compound assay against *F. solani*. A: Negative control plate containing only PDA medium inoculated with *F. solani*. B: Treatment plate containing PDA supplemented with *T. harzianum* culture filtrate, showing reduced mycelial growth of *F. solani*, indicating the antifungal activity of non-volatile compounds.

As illustrated in Figure 3. 9 and Figure A. 9, *T. harzianum*'s non-volatile compounds suppressed the majority of the radial growth of *R. solani*. *R. solani* covered every point of the Petri dish in the negative control group (Figure 3. 9A) after 7 days of incubation. However, its growth was highly inhibited in the Petri dish consisting of *T. harzianum* culture filtrate (Figure 3. 9B). The percentage of radial growth inhibition was calculated as 55.63% (Table 3. 4), suggesting that non-volatile compounds of *T. harzianum* showed sufficient antagonistic activity against *R. solani*. In a study, varying ranges of culture filtrates of *T. harzianum* were assessed against *R. solani*. 25%, 50%, 75% and 100% of culture filtrates were mixed with the molten PDA. The lowest inhibition percentages were seen in the lowest concentration. In 6-days of incubation, 51.96% inhibition was observed in 50% concentration, while the mean of the inhibition percentages of the different concentrations was 51.22% [97]. In another study, different concentrations of non-volatile compounds of two *Trichoderma* isolates were also analyzed. When 50% concentration of non-volatile compound was applied, a complete inhibition of *R. solani* was observed in both *Trichoderma* spp., while lower inhibition rates were observed in lower concentrations, suggesting that the inhibitory activities of the metabolites depend on the dosage and these *Trichoderma* spp. had strong non-volatile compounds to combat *R. solani* [103].

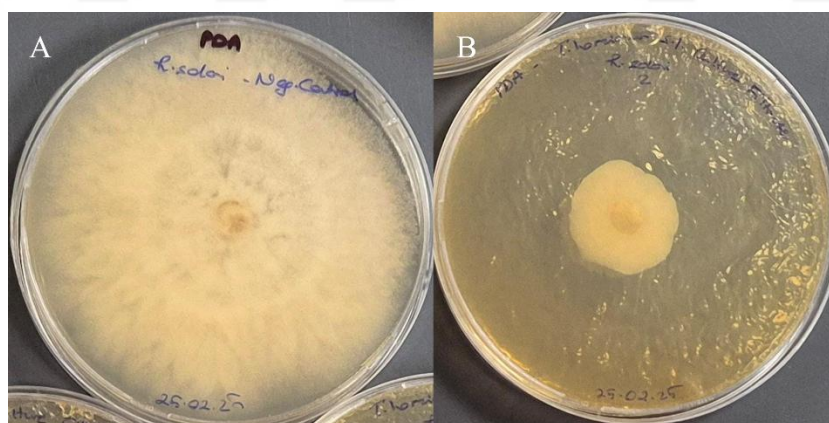


Figure 3. 9 *T. harzianum* non-volatile compound assay against *R. solani*. A: Negative control plate containing only PDA medium inoculated with *R. solani*. B: Treatment plate containing PDA supplemented with *T. harzianum* culture filtrate, showing reduced mycelial growth of *R. solani*, indicating the antifungal activity of non-volatile compounds.

The antifungal properties of non-volatile compounds of *T. harzianum* against *A. welwitschiae* was shown in Figure 3. 10 and Figure A. 10. The huge inhibition rate were seen from the differences in radial growth of *A. welwitschiae* between the negative control

Petri dish (Figure 3. 10A) and the treatment Petri dish (Figure 3. 10B). The inhibition percentage was calculated as 75% (Table 3. 4), highlighting a significant biocontrol ability of *T. harzianum* by its non-volatile compounds over *A. welwitschiae*. In a study, four different concentrations of *T. harzianum* culture filtrates were examined, 25%, 50%, 75% and 100%. The inhibition percentages of the *Aspergillus* sp. radial growth increased by the concentration rates. At the end of 6-day of incubation, 40.15% inhibition was observed in 50% concentration, while 100% concentration led to the 51.74% inhibition [97]. A similar result to this study was also obtained in a recent study. 50% concentration of culture filtrates of four *Trichoderma* isolates were tested against *A. flavus*. The inhibition rates were between 78.1% and 84.4%. The highest inhibition (84.4%) was observed in the isolate identified as *T. harzianum*. The researchers reported that the *Trichoderma* isolates had potential to suppress *A. flavus* growth due to their antimicrobial compounds, including trichomycin, antibiotics, flavonoids, and phenolic compounds, that can degrade the cell wall of pathogen and inhibit its growth [104].



Figure 3. 10 *T. harzianum* non-volatile compound assay against *A. welwitschiae*. A: Negative control plate containing only PDA medium inoculated with *A. welwitschiae*. B: Treatment plate containing PDA supplemented with *T. harzianum* culture filtrate, showing reduced mycelial growth of *A. welwitschiae*, indicating the antifungal activity of non-volatile compounds.

As seen in Figure 3. 11 and Figure A. 11, the inhibitory effect of non-volatile compounds of *T. harzianum* against *C. coccodes* presented a significant potential, with 71.77% radial growth inhibition (Table 3. 4), which was similar to the inhibition rate against *A. welwitschiae*, suggesting great potential against *C. coccodes* as well. The study of Ajith et al. (2010) was performed with four species of *Trichoderma* spp. against *C. capsici*. Different concentrations of non-volatile compounds of *Trichoderma* spp. were tested for the mycelial inhibition of *C. capsici*. *T. viride* completely inhibited the growth

of pathogen at 5% concentration, while *T. harzianum*, *T. saturnisporum*, and *T. reesei* showed inhibition by 21.83%, 44.27%, and 68.37%, respectively in 50% concentration. It was suggested that the non-volatile compounds of *Trichoderma* spp. have the potential to efficiently control anthracnose caused by *C. capsici* in bell peppers, while *T. reesei* had a stronger antagonistic activity on this strain of *C. capsici* [105]. A similar inhibition rate was observed with 20% PIRG in another study. *T. piluliferum* was analyzed against *C. musae* and inhibited the growth by 20%, suggesting a significant inhibition [98].

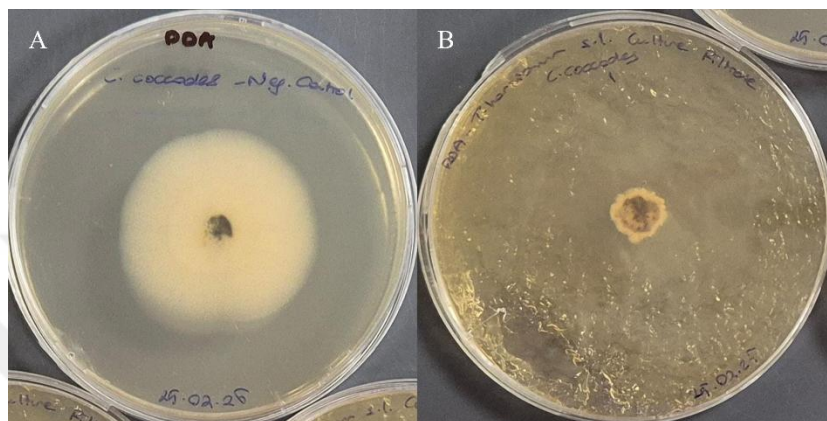


Figure 3. 11 *T. harzianum* non-volatile compound assay against *C. coccodes*. A: Negative control plate containing only PDA medium inoculated with *C. coccodes*. B: Treatment plate containing PDA supplemented with *T. harzianum* culture filtrate, showing reduced mycelial growth of *C. coccodes*, indicating the antifungal activity of non-volatile compounds.

Figure 3. 12 and Figure A. 12 represented the inhibition of radial growth of *B. cinerea* by non-volatile compounds of *T. harzianum*. The inhibition rate was calculated as 55% (Table 3. 4). Although the inhibition rate was lower than other tested phytopathogens, the result indicated a high potential for controlling the propagation of *B. cinerea*. Similar to this result, in a study, between 30% and 55% inhibition was observed against *B. cinerea* by non-volatiles molecules of *T. harzianum*. Nine *Trichoderma* spp. were tested against *B. cinerea*, and one species of *T. harzianum* and one species of *T. atroviride* showed the highest inhibition (approximately 55%). The study suggested that there is a significant reduction in the mycelial growth of the pathogen [106]. Furthermore, in another study, two *Trichoderma* spp. were tested against *B. fabae*. Varying concentrations of filtrates, 5%, 10%, 25%, and 50%, were analyzed. The increasing concentration resulted in higher inhibition rates. At 6 days incubation, *T. harzianum*

inhibited by 54.4%, while *T. album* inhibited by 68.8%, indicating an effective biocontrol activity against *B. fabae* [107].

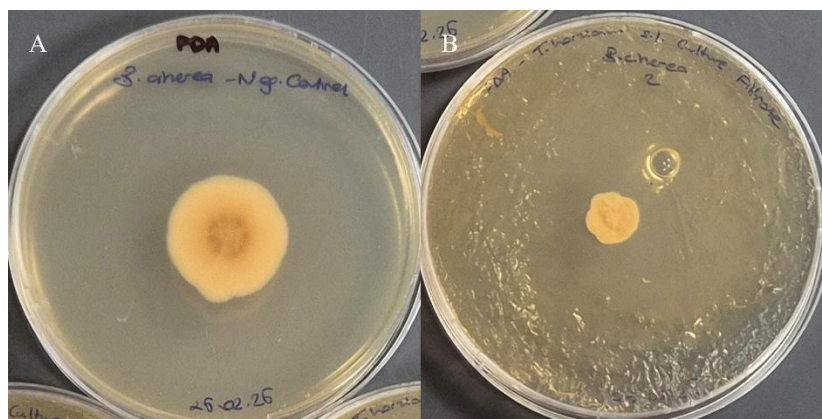


Figure 3. 12 *T. harzianum* non-volatile compound assay against *B. cinerea*. A: Negative control plate containing only PDA medium inoculated with *B. cinerea*. B: Treatment plate containing PDA supplemented with *T. harzianum* culture filtrate, showing reduced mycelial growth of *B. cinerea*, indicating the antifungal activity of non-volatile compounds.

Table 3. 4 Inhibition of radial growth (%) of phytopathogens by non-volatile compounds of *T. harzianum* in non-volatile compound assay

Phytopathogen	Radial Growth Inhibition (%) $\pm$ SD
<i>F. solani</i>	61.87 $\pm$ 5.3
<i>R. solani</i>	55.63 $\pm$ 10.79
<i>A. welwitschiae</i>	75 $\pm$ 3.5
<i>C. coccodes</i>	71.77 $\pm$ 0.4
<i>B. cinerea</i>	55 $\pm$ 2.3

3.2.3 Volatile Organic Compound Assay

The volatile organic compounds (VOCs) of *T. harzianum* were tested against phytopathogens. The fungal mycelial plugs were inoculated separately onto the Petri dishes and incubated. After 7 days of incubation, the inhibition percentages of radial growth of phytopathogens were calculated as shown in Figure 2. 3. The antagonistic activities of VOCs were not as strong as non-volatile compounds. The highest inhibition was observed against *B. cinerea* with 36.67%, while no inhibition was observed against *F. solani* and *R. solani* (Table 3. 5).

As shown in Figure 3. 13 and Figure A. 13, the presence of *T. harzianum* did not inhibit the *F. solani*, indicating the VOCs of *T. harzianum* have no inhibitory effect on *F. solani*. In the literature, varying ranges of inhibition percentages were observed. As an example, the study of Chakraborty (2008) assessed five isolates of *Trichoderma* spp. against *F. solani*. The VOC assay findings revealed that five isolates showed between 44.4% and 78.22% PIRG against *F. solani*. However, it was found that non-volatile compounds were more effective than VOCs. In the non-volatile assay, two of the isolates showed 100% inhibition [108]. Similar findings were obtained in a recent study, Oliveira et al. (2021) tested four isolates of *Trichoderma* spp. against *F. oxysporum*. The highest inhibition (42.5%) was observed by *T. longibrachiatum*, while the lowest inhibition (19.2%) was observed by *T. asperelloides*, suggesting that the VOCs of *Trichoderma* spp. inhibit the growth of the pathogen with their efficient antifungal metabolites [109].

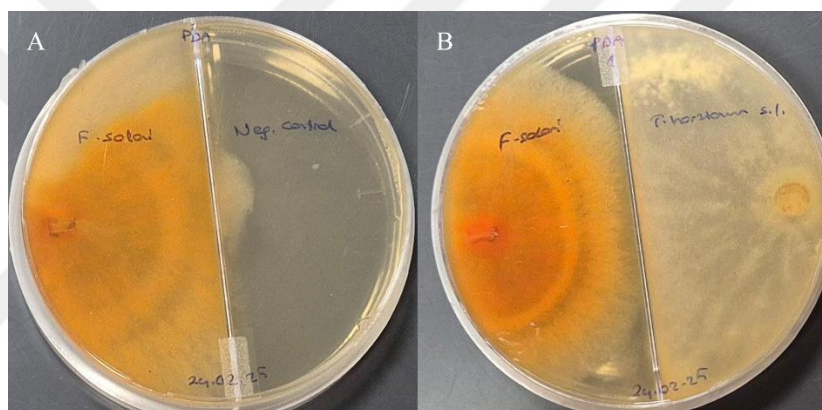


Figure 3. 13 Volatile compound assay to evaluate the potential effect of *T. harzianum* against *F. solani*. A: Negative control: Petri dish inoculated only with *F. solani*. B: Treatment group: *T. harzianum* and *F. solani* co-inoculated on opposite sides of the same Petri dish to allow exposure to volatile organic compounds.

Similarly, VOCs of *T. harzianum* could not show antagonistic activity against *R. solani*, as shown in Figure 3. 14 and Figure A. 14. In a study, the VOCs of *T. harzianum* were tested against five different phytopathogens. The highest mycelial growth inhibition was observed against *Rhizoctonia* sp. by 53.66% at seventh day of incubation. However, the mycelial growth inhibition was around 18% against *Verticillium* sp. and *Pythium* sp.. These findings suggested that the isolate of *T. harzianum* had effective antimicrobial compounds including aromatic pyrone antibiotics against *Rhizoctonia* sp. [97]. Oliveira et al. (2021) tested four isolates of *Trichoderma* spp. also against *R. solani*. The antagonistic activities VOCs of different *Trichoderma* spp. exhibited inhibition between 23.3% and 32.5%. The highest inhibition was observed by the *T. asperelloides*, while the

lowest inhibition was observed by *T. harzianum*, suggesting that all isolates had antifungal metabolites on their VOCs, including 6-pentyl-2H-pyran-2-one (6PP), 3,4-dimethyl-1-hydroxybutanoic acid, and 2-methylpropan-1-ol, that can inhibit the mycelial growth of the pathogens. However, some of the species can have more antagonistic activity on specific pathogens based on their antifungal metabolites [109].

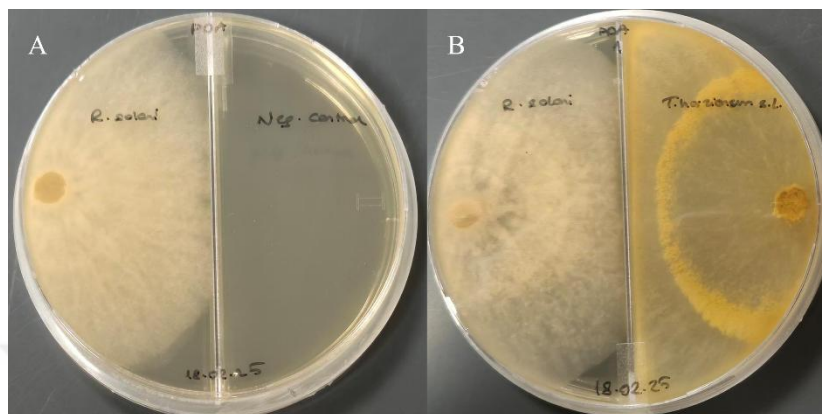


Figure 3. 14 Volatile compound assay to evaluate the potential effect of *T. harzianum* against *R. solani*. A: Negative control: Petri dish inoculated only with *R. solani*. B: Treatment group: *T. harzianum* and *R. solani* co-inoculated on opposite sides of the same Petri dish to allow exposure to volatile organic compounds.

The radial growth of *A. welwitschiae* was inhibited at the rate of 15.2% (Table 3. 5), as shown in Figure 3. 15 and Figure A. 15. This rate indicated that the VOCs of *T. harzianum* have mild inhibitory effects on *A. welwitschiae*. The study of Alsaeedi (2014) was also investigated the inhibitory effects of VOCs of *T. harzianum* against *Aspergillus* sp.. Over seven days of incubation, it was observed that mycelial growth of *Aspergillus* sp. was inhibited by 39.3%, indicating an effective activity of VOCs on *Aspergillus* sp. [97]. A consistent result was observed in the study of Abouna et al. (2024). They tested four *Trichoderma* isolates against *A. flavus*. The VOC assay results were between 29.5% and 49.9% PIRG, and the isolate that showed the highest rate was identified as *T. harzianum*. Furthermore, the inhibition rates of VOC assay were also lower than non-volatile and dual culture assay, but overall findings indicated that *Trichoderma* spp. had a great biocontrol potential against *A. flavus* [104].

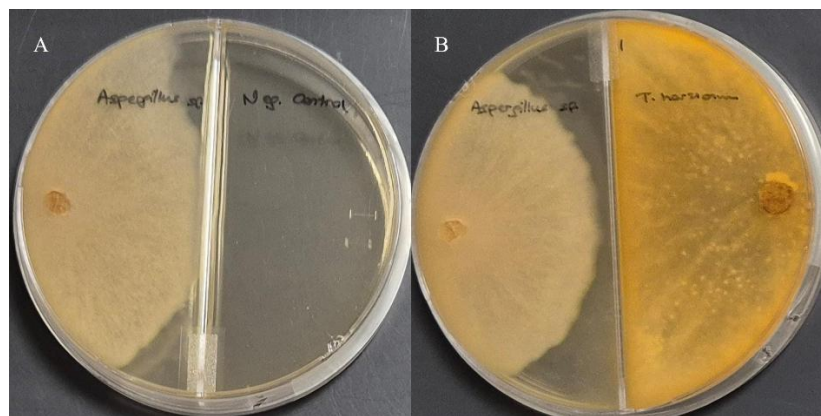


Figure 3. 15 Volatile compound assay to evaluate the potential effect of *T. harzianum* against *A. welwitschiae*. A: Negative control: Petri dish inoculated only with *A. welwitschiae*. B: Treatment group: *T. harzianum* and *A. welwitschiae* co-inoculated on opposite sides of the same Petri dish to allow exposure to volatile organic compounds.

The suppression of radial growth of *C. coccodes* was 12.85% (Table 3. 5) as shown in Figure 3. 16 and Figure A. 16, revealing that the VOCs of *T. harzianum* have antagonistic activity against *C. coccodes*. In a recent study, four isolates of *Trichoderma* spp. were evaluated for their antagonistic activity against *C. acutatum*. The volatile organic compounds (VOCs) emitted by the *Trichoderma* isolates demonstrated inhibition rates ranging from 12.82% to 47.41% on PDA plates, indicating their potential as effective BCAs. The VOC profiles of *Trichoderma* spp., both alone and in dual culture with *C. acutatum*, were analyzed using gas chromatography–mass spectrometry (GC-MS). Among the identified VOCs, ketones were the most abundant, followed by terpenes, heterocyclic compounds, and organosulfur compounds. Notably, the composition and quantity of VOCs differed between the mono- and dual-culture conditions, suggesting that *Trichoderma* spp. may alter their metabolic pathways to produce antifungal VOCs in response to environmental stimuli or the presence of pathogens [110]. Like the findings of this study, four isolates of *Trichoderma* spp. showed inhibition between 8.81% and 67% by their VOCs against *C. capsici*. The highest inhibition was observed by the VOCs of the isolate, identified as *T. harzianum*, which was found to be more effective than other isolates [105].

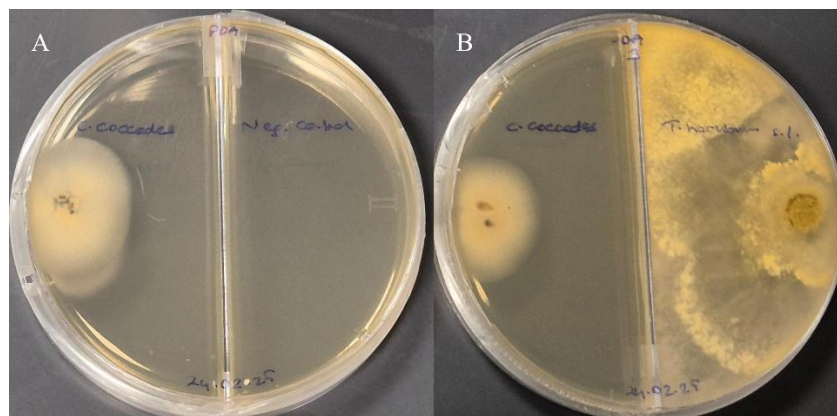


Figure 3. 16 Volatile compound assay to evaluate the potential effect of *T. harzianum* against *C. coccodes*. A: Negative control: Petri dish inoculated only with *C. coccodes*. B: Treatment group: *T. harzianum* and *C. coccodes* co-inoculated on opposite sides of the same Petri dish to allow exposure to volatile organic compounds.

Inhibition was observed against *B. cinerea*. The inhibition of radial growth of *B. cinerea* was calculated at 36.67% (Table 3. 5) according to the measurements (Figure 2. 3) of the Petri dishes shown in Figure 3. 17 and Figure A. 17, indicating that the VOCs of *T. harzianum* have better antifungal activity against *B. cinerea* than other phytopathogens. VOCs of nine *Trichoderma* spp. isolates were tested against *B. cinerea* in recent research. The inhibition rates were between 13.9% and 63%, suggesting a significant reduction. Additionally, the produced VOCs were analyzed using GC-MS. The results showed that 23 molecules produced by these *Trichoderma* spp. including alkenes, alcohols, and esters abundantly [111]. High PIRG values, varying between 64.8% and 73.78%, were also observed in a recent study that tested four *Trichoderma* spp. against *B. cinerea*, suggesting an evident inhibition. The highest inhibition (73.78%) was observed by *T. koningiopsis*. According to GC-MS analysis, the most abundant molecules were also alkenes, alkanes, and esters which are the part of a biocontrol mechanism. Additionally, the most abundant compound was identified as β -phellandrene found in many isolates of *Trichoderma* spp. [112]. In another study, relatively lower inhibition rates were observed with two *Trichoderma* spp. which were tested against *B. fabae*. In 6 days of incubation, *T. harzianum* inhibited by 39.7%, while *T. album* inhibited by 50.4%, suggesting an effective inhibitory activity on *B. fabae* [107].

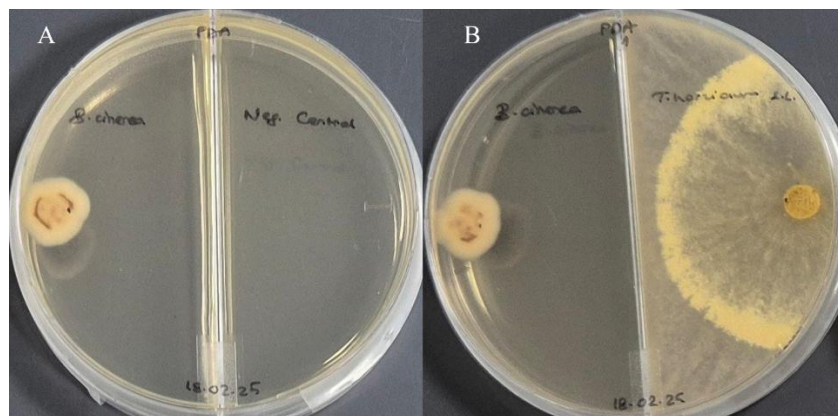


Figure 3. 17 Volatile compound assay to evaluate the potential effect of *T. harzianum* against *B. cinerea*. A: Negative control: Petri dish inoculated only with *B. cinerea*. B: Treatment group: *T. harzianum* and *B. cinerea* co-inoculated on opposite sides of the same Petri dish to allow exposure to volatile organic compounds.

Table 3. 5 Inhibition of radial growth (%) of phytopathogens by VOCs of *T. harzianum* in VOC assay

Phytopathogen	Radial Growth Inhibition (%) $\pm$ SD
<i>F. solani</i>	-
<i>R. solani</i>	-
<i>A. welwitschiae</i>	15.2 $\pm$ 6.5
<i>C. coccodes</i>	12.85 $\pm$ 9.8
<i>B. cinerea</i>	36.67 $\pm$ 5.2

3.3 Analysis of Plant Growth Promoting Properties of

T. harzianum

3.3.1 Indole-3-Acetic Acid Production

The Salkowski reagent was used to indicate the production of indole-3-acetic acid (IAA) by *T. harzianum* which is a plant hormone belonging to auxin class and playing an important role in regulating plant growth and development. The change of color from transparent yellowish to reddish pink was observed in the Salkowski reagent tube, including the culture filtrate of *T. harzianum*, after 30 minutes of incubation as shown in Figure 3. 18. This color change indicated that *T. harzianum* produces IAA. In this

colorimetric assay, the culture filtrate of *T. harzianum* culture was mixed with Salkowski reagent. A chemical reaction was observed between Fe^{3+} ions, found in the Salkowski reagent, and the IAA, produced by the *T. harzianum*, in an acidic environment of Salkowski reagent. This chemical reaction resulted in a reddish-pinkish color [113].

This assay is preferred in many studies to investigate IAA production by microorganisms. For instance, the study conducted by Vasait et al. (2023) demonstrated that an isolate of *Trichoderma* spp. produced 118 $\mu\text{g/mL}$ of IAA after 96 hours of incubation in PDB. The researchers assessed IAA production by measuring concentrations at specific incubation times, using pure IAA as a reference to generate a standard curve at 530 nm with a spectrophotometer. The highest IAA concentration was recorded at the 96th hour of incubation; beyond this point, a decline in IAA levels was observed [114]. In addition, the IAA production of two *Trichoderma* isolates were analyzed by Salkowski reagent in study of Bogumil et al. (2013). After 30 minutes of mixing the culture filtrates of two isolates and Salkowski reagent, they observed pinkish color in both, indicating production of IAA [115].

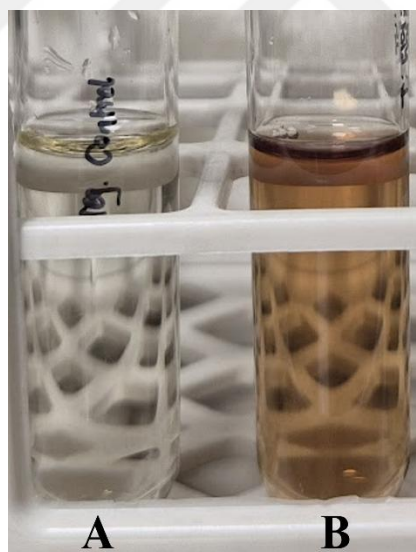


Figure 3. 18 Indole-3-acetic acid production assay. A: Negative control group without *T. harzianum* culture filtrate, B: *T. harzianum* culture filtrate added group.

3.3.2 Siderophore Production

The siderophore is an iron chelator produced by microorganisms to bind to iron found in the soil, which is not easily accessible to plants, and forms soluble complex that

facilitates its transport to the cell, thereby meeting the nutritional need of the organism for iron. The siderophore production was analyzed using Chrome Azurol Sulphonate (CAS) agar including hexadecyltrimethylammonium bromide (HDTMA), and Fe (III) solution, resulting in a blue colored agar medium. The CAS-iron complex is found in the agar, but siderophores have a high affinity to ferric ions. If siderophores are found in the environment, ferric ions are chelated, and CAS dye is released into the environment, causing a color change. The CAS agar has a dark blue color; if the microorganism produces siderophore, a halo is formed around the colony [116].

There was a small clear zone around *T. harzianum*, indicating that it produces siderophore. The bacteria isolate, a well-known PGPR called *B. thuringiensis* producing siderophore, was used as positive control. Around its colony, there was a clear zone, indicating siderophore production (Figure 3. 19). This finding is consistent with the recent study by Go et al. (2023), in which siderophore production by *Trichoderma* spp. was evaluated using CAS agar. Four *Trichoderma* isolates were tested, all of which demonstrated the ability to produce siderophores to varying extents. One isolate induced a complete color change of the CAS agar from blue to orange, whereas the others produced smaller orange zones surrounding their colony [87]. Additionally, the study of Bogumil et al. (2013) showed that two isolates of *Trichoderma* spp. produced siderophores. The CAS agar assay revealed that the produced siderophores chelated iron from CAS agar complex, resulting in an orange halo on the Petri dishes [115].

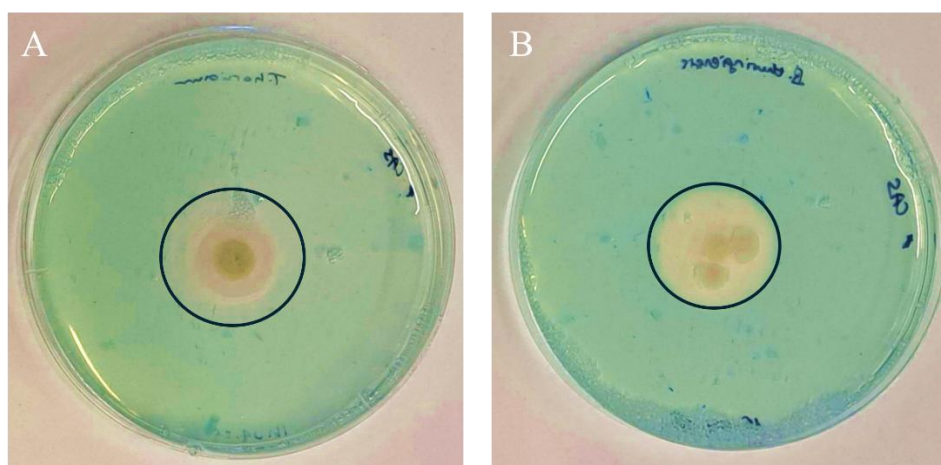


Figure 3. 19 Siderophore production assay for *T. harzianum* using CAS agar. A: *T. harzianum* inoculated CAS agar, B: Positive control with *B. thuringiensis* producing siderophore.

3.3.3 Phosphate Solubilization

Phosphate solubilization ability of microorganisms is crucial for plant growth because it can enhance the availability of phosphorus, an essential but often limiting nutrient in soils. Phosphate solubilization by microorganism transforms inaccessible phosphorus into forms that plants can use, boosting growth, and crop productivity. For the detection of phosphate-solubilizing microorganisms, Pikovskaya's (PVK) agar was used, which has tricalcium phosphate as an insoluble phosphate source. If the microorganism is able to solubilize the phosphate, a clear zone is formed around the colony. However, there was no clearance around the *T. harzianum*, meaning that it could not solubilize the phosphate. Around the colony of *Pseudomonas* sp., that can solubilize phosphate and used as a positive control, a clear zone was observed (Figure 3. 20). The studies of Go et al. (2023) analyzed the phosphate solubilization abilities of four *Trichoderma* spp. isolates by PVK agar assay. The findings showed that three of the isolates solubilized the phosphate, while one of them could not solubilize, indicating the phosphate solubilization capability depends on the strain of *Trichoderma* spp. [87]. Bogumil et al. (2013) reported that two *Trichoderma* isolated were inoculated to PVK agar for phosphate solubilization assay but neither of the isolates could solubilize the phosphate since there was no clear zone on the Petri dishes [115].

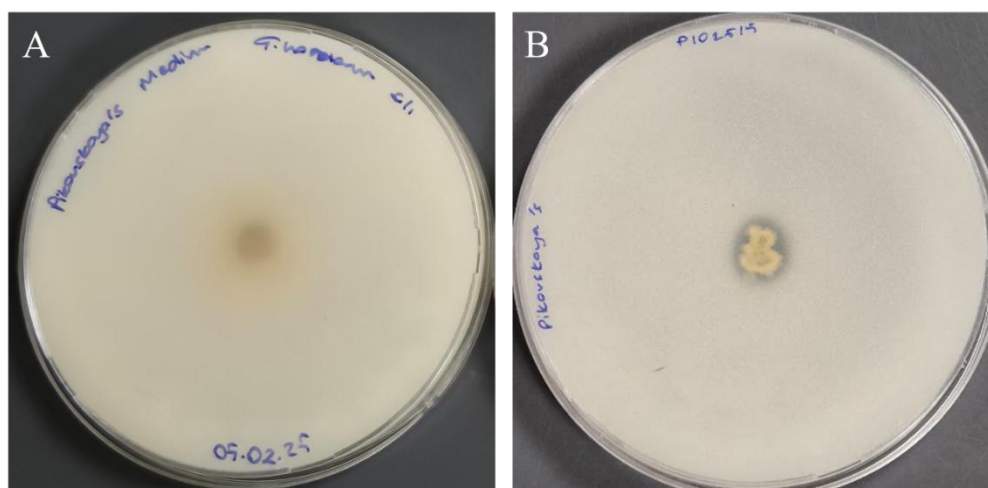


Figure 3. 20 Phosphate solubilizing assay for *T. harzianum* using PVK. A: *T. harzianum* inoculated PVK agar, B: Positive control with *Pseudomonas* sp. solubilizing phosphate.

3.4 Analysis of Plant Experiments of *T. harzianum*

3.4.1 Pot Experiment

At the end of the 3 weeks, the properties of plants were compared based on each group. The wet weight of plants, root health, and length were recorded to determine the impact of *T. harzianum* on plant growth and combating with phytopathogens. The weights of plants belonging to treatment group were higher. The plants of negative control groups were lighter. *T. harzianum* treatment groups had relatively longer root length than negative control groups (Table 3. 6). The root health was examined by observation (Figure A. 18). The plants of *T. harzianum* groups had healthier root structure. These findings revealed that *T. harzianum* had a positive impact in plant growth. Its metabolites were able to enrich plant growth, while antifungal properties inhibited the negative effects of phytopathogens on the plants. This direct plant experiment showed that the *T. harzianum* could be used as BCA to suppress the harmful activities of pathogens in the agricultural field.

The most marked effect of the treatment was observed in the *F. solani* group. *F. solani* induced severe root disease in five of the infected plants, resulting in root rot. The remaining plants in this group also exhibited significantly reduced root length and biomass compared to the corresponding *T. harzianum*-treated group, suggesting that *T. harzianum* contributed to improved root health and overall plant vigor (Table 3. 6). In the *R. solani* group, plants treated with *T. harzianum* exhibited shorter roots; a similar trend was observed in the *C. coccodes* group. However, in both cases, the treated plants displayed greater biomass, likely due to healthier root systems, whereas plants infected with the pathogens alone developed long but notably thinner roots (Table 3. 6). In the *A. welwitschiae* and *B. cinerea* groups, *T. harzianum* treatment led to both increased root length and plant weight (Table 3. 6). The roots and stems of all treated plants were much healthier, and the *T. harzianum*-inoculated plants were heavier than the plants belonging to control group, indicating that *T. harzianum* not only supported the plant growth but also suppressed the negative effects of these five pathogens in plants.

The study of Ommati and Zaker (2012) tested eight isolates of *Trichoderma* spp. against *F. solani* using potato in greenhouse conditions for 2 months. The results supported the findings of this study and indicated that all *Trichoderma* spp. showed decrease in disease incidence, particularly two of the isolates were significantly effective to reduce the wilt disease [117]. Hormoz et al. (2006) assessed *T. harzianum* against *R. solani*, *F. solani*, *C. coccodes*, and *F. oxysporum* using potato in laboratory and field experiments. The findings of this study were also consistent with previous studies, revealing that *T. harzianum* was efficient in the reduction of disease incidence. The researchers recommended the use of *T. harzianum* for disease management strategies due to their promising results [118].

Table 3. 6 The physiological properties of the plants in pot experimental groups

Groups	Root length (cm) $\pm$ SD	Plant weight (g) $\pm$ SD
Control	15,27 $\pm$ 1,65	0,16 $\pm$ 0,06
<i>T. harzianum</i>	12,54 $\pm$ 2,7	0,26 $\pm$ 0,07
<i>F. solani</i> - <i>T. harzianum</i> treatment	15,62 $\pm$ 3,49	0,22 $\pm$ 0,09
<i>F. solani</i>	7,2 $\pm$ 7,44	0,14 $\pm$ 0,06
<i>R. solani</i> - <i>T. harzianum</i> treatment	11,54 $\pm$ 2,89	0,23 $\pm$ 0,09
<i>R. solani</i>	15,83 $\pm$ 2,49	0,14 $\pm$ 0,04
<i>A. welwitschiae</i> - <i>T. harzianum</i> treatment	15,14 $\pm$ 2,98	0,2 $\pm$ 0,07
<i>A. welwitschiae</i>	11,68 $\pm$ 3,66	0,16 $\pm$ 0,05
<i>C. coccodes</i> - <i>T. harzianum</i> treatment	14,54 $\pm$ 4,28	0,28 $\pm$ 0,1
<i>C. coccodes</i>	15,25 $\pm$ 3,18	0,19 $\pm$ 0,06
<i>B. cinerea</i> - <i>T. harzianum</i> treatment	16,46 $\pm$ 2,99	0,22 $\pm$ 0,04
<i>B. cinerea</i>	11,04 $\pm$ 4,55	0,2 $\pm$ 0,11

3.4.2 Pepper Fruit Experiments

The peppers were incubated maintaining humidity to prevent the peppers from drying out. The symptoms of diseases were observed daily. On the fifth day, on the pathogen negative control groups, more severe physical damages were observed when compared with *T. harzianum* treatment groups. The peppers had lost their hardness and freshness, also had become dry and thin in structure. On the treatment groups, peppers

more protected the freshness and hard structure of the first day. The incidence of disease symptoms was suppressed by the *T. harzianum* in the treated groups (Figure 3. 21).

Similarly, Yadav et al. (2023) reported that *T. harzianum* and *T. asperellum* treated peppers showed insignificant disease symptoms caused by *C. truncatum*, while significant lesions were observed in the untreated peppers. These *Trichoderma* isolates suppressed the disease incidence of *C. truncatum* strongly. The researchers suggested that *Trichoderma* spp. demonstrated strong potential in enhancing plant growth, reinforcing structural defenses, and activating defense-related gene expression in peppers against anthracnose [88]. Consistent symptoms were also observed in the study of Kim et al. (2023). They investigated the curative and preventive properties of *Trichoderma* isolates against *C. acutatum*, anthracnose causing agent on peppers. The preventive method exhibited a much stronger efficacy but both preventive and curative methods by *Trichoderma* treatment suppressed disease severity significantly, revealing the excellent biocontrol potential of *Trichoderma* isolate to combat anthracnose [89].

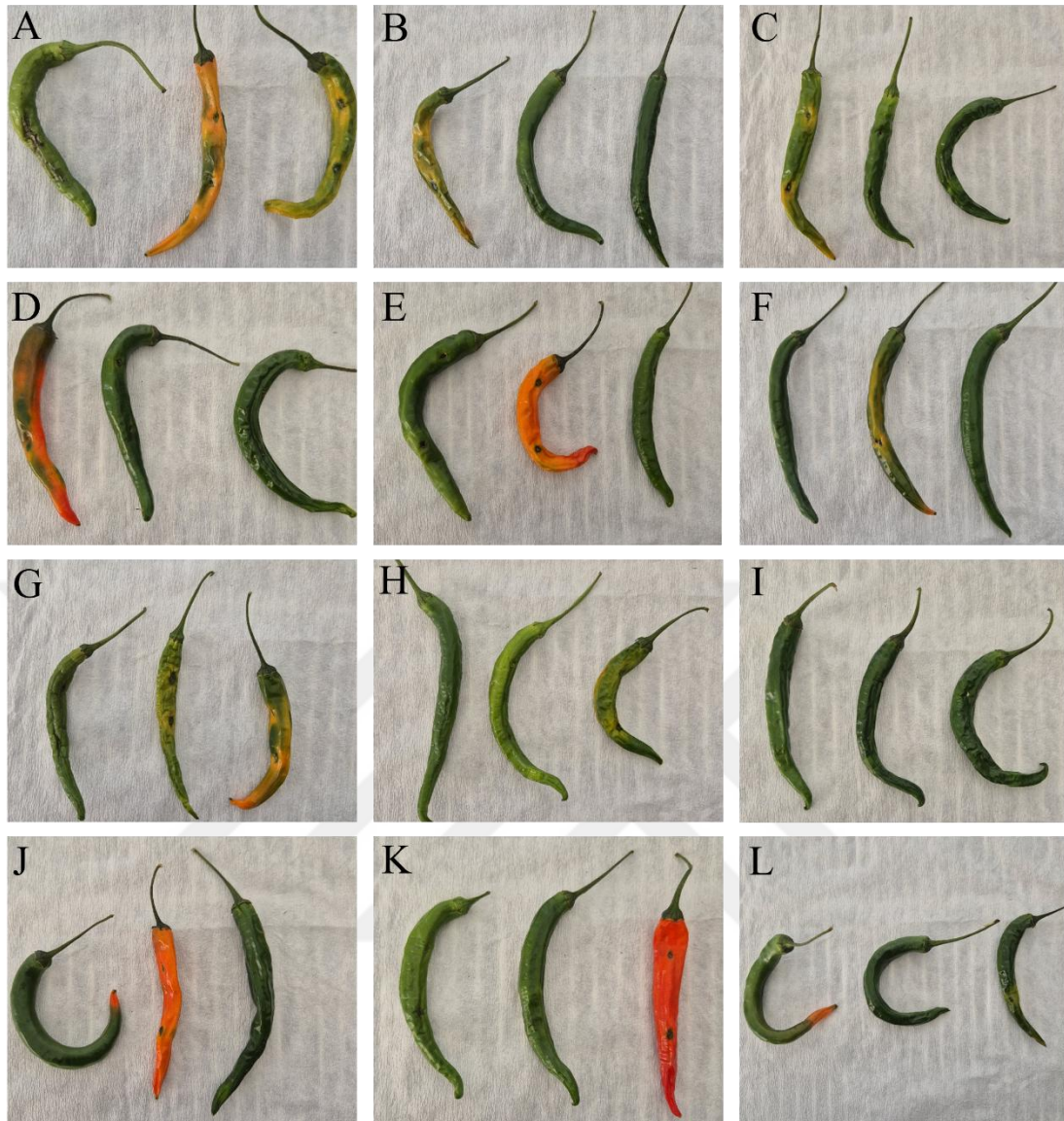


Figure 3. 21 Pepper experiment results on 5-days of incubation. A: *F. solani* negative control, B: *R. solani* negative control, C: *A. welwitschiae* negative control, D: *F. solani* treatment, E: *R. solani* treatment, F: *A. welwitschiae* treatment, G: *C. coccodes* negative control, H: *B. cinerea* negative control, I: Control group, J: *C. coccodes* treatment, K: *B. cinerea* treatment, L: *T. harzianum* group.

Chapter 4

Conclusions and Future Prospects

4.1 Conclusions

Agricultural problems hold great importance for sustainable food production. The consequences of the agricultural problems show themselves in many dimensions of life. Particularly, billions of dollars are lost annually. Agricultural losses are of such magnitude that they cannot be ignored. The majority of the problems are caused by biological factors. Many kinds of organisms, including bacteria, fungi, nematodes, and insects, are responsible for the crop loss.

There are several ways to eliminate these agricultural problems. Chemical products are commonly used to combat pathogens. However, their side effects are nonignorable. The soil structure, quality, and its natural flora are affected by chemicals in the long term. The water resources are also contaminated, and the chemical residues on the crops give rise to health concerns. In addition, pathogens can gain resistance to these chemicals over time, resulting in lower efficiency. The governments strongly recommend stopping the use of these chemicals and promoting the use of biological alternatives instead. Raising awareness about the environment and public health encourages the use of biofungicides.

T. harzianum, with its well-known antifungal properties, represents a promising candidate for biofungicides. This study has demonstrated the antifungal properties of *T. harzianum* against five phytopathogens that are frequently encountered in problematic agricultural fields. Moreover, the results revealed that the inhibitory activities of *T. harzianum* are strong. Significant antagonistic effects were observed, with inhibition rates reaching up to 96%. The antifungal properties are attributed to distinct action mechanisms, including competitiveness for nutrients and space, mycoparasitism, and the

antimicrobial secondary metabolites. The observed inhibition rates against the common phytopathogens show that *T. harzianum* is promising for agricultural applications.

Moreover, all the cultivation of *T. harzianum* was performed using apple pomace instead of a commercial and expensive culture medium. This approach contributes to the circular economy and valorization ideas of the industrial waste that has abundantly accumulated in nature.

To sum up, in this study, apple pomace, which is a nutrient-rich agro-industrial by-product, was optimized as a low-cost culture medium for the growth of *T. harzianum*. The antifungal activity of both the fungal culture and its metabolites was evaluated through dual culture assay, non-volatile and volatile metabolite analysis against commonly faced phytopathogens. In addition to its biocontrol potential, its plant growth-promoting properties were assessed via IAA production, siderophore production, and phosphate solubilization assays. To validate its efficacy under real conditions, both pot experiments and experiments on pepper were conducted. This comprehensive approach not only demonstrates the biocontrol and biofertilizer potential of *T. harzianum* cultured on waste-derived media but also supports sustainable and environmentally friendly agricultural practices.

4.2 Societal Impact and Contribution to Global

Sustainability

The development of environmentally friendly biocontrol agents such as *T. harzianum* instead of harmful chemicals is one of the crucial steps toward sustainable agricultural practices. For reducing crop loss due to phytopathogenic organisms in agricultural fields, it is important to emphasize and encourage the use of *T. harzianum* as a biological alternative to synthetic fungicides, also offering a strategy to reduce the chemical residues caused by chemical agricultural products on the surface of crops, soil, and nature. The demand for these chemicals should be immediately reduced since they lead to several environmental and health concerns, including soil and water contamination, reduced soil quality, negative impacts on non-target organisms, and the

development of fungicide-resistant pathogen strains. On the other hand, *T. harzianum* presents a biological disease management strategy with proven antagonistic properties and minimum side effects, providing more sustainable agricultural practices.

In addition to biocontrol capabilities, an upcycling strategy is offered in this study. An agro-industrial waste was valorized to cultivate *T. harzianum* instead of using a commonly used commercial culture medium. This cost-effective waste-derived media component contributes to the circular economy and waste valorization techniques. Beyond the conversion of industrial waste to a high value-added product for biocontrol agent production, this valorization is a significant example of sustainability by reducing the waste accumulation in the environment.

Considering the United Nations Sustainable Development Goals (SDGs) (<https://sdgs.un.org/goals>), the decrease in chemical use, providing a biological alternative solution, and valorization of an abundant industrial waste contribute to multiple dimensions of global sustainability. The use of *T. harzianum* as a biofungicide follows SDG 2: Zero Hunger by providing a safe and sustainable agricultural application with reduced crop loss and preventing the health concerns and environmental harms of chemical products. Additionally, minimizing the use of chemicals reduces the environmental footprint and contamination of soil and water sources by contributing to SDG 13: Climate Action. Moreover, the valorization of apple pomaces for biofungicide production emphasizes SDG 12: Responsible Consumption and Production by providing the conversion of waste to a value-added product, reflecting a circular economy.

4.3 Future Prospects

This thesis demonstrated the antagonistic effects of *T. harzianum* against five phytopathogens in laboratory conditions and revealed its promising potential for use in agricultural fields. For further applications, these lab-scale studies can be turned into field experiments to observe the efficacy of *T. harzianum* in varying conditions of the open field, including changing temperatures, soil structures, and humidity that can be faced in actual agricultural areas. Particularly, *T. harzianum* should be applied to the perennial

plants for analysis of its antifungal activity and plant growth-promoting properties. Greenhouse or field experiments using perennial plants can be performed to observe it.

Moreover, pilot-scale fermentation should be optimized for commercial biofungicide production by using apple pomace. Beyond apple pomace, other abundant industrial waste can also be utilized as sustainable, low-cost culture media. Following the fermentation, formulation processes can be developed for stable biofungicide during the shelf life for commercialization.

Co-culture approaches can be developed to improve the antagonistic activity of *T. harzianum*. Other beneficial microorganisms, including PGPRs, are also inoculated with *T. harzianum* to enhance its field performance and broaden its spectrum of control. Furthermore, understanding the molecular principles, including transcriptomic and metabolomic analyses, of the action mechanisms of *T. harzianum* can provide more comprehensive insights.

To sum up, further studies on agricultural field experiments, scale up, and formulation developments are significant, especially on the way to commercialization. Additionally, extensive molecular studies can give more ideas about the principles behind action mechanisms.

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APPENDIX

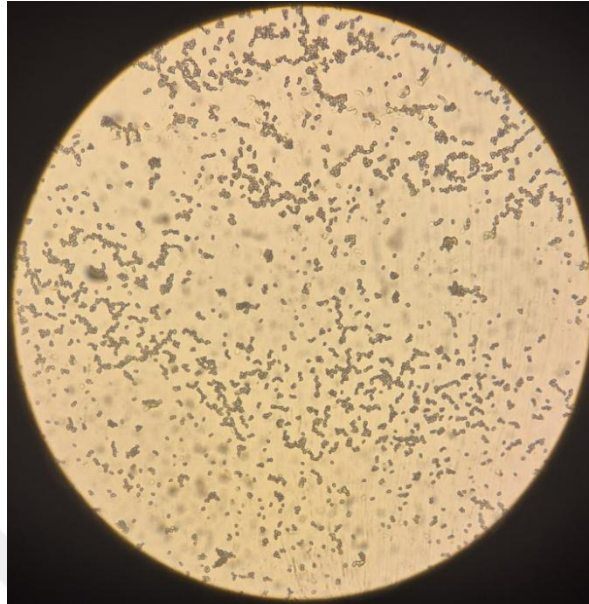


Figure A. 1 Microscope image of spore suspension of *T. harzianum* (40X magnification and 10X ocular lens).

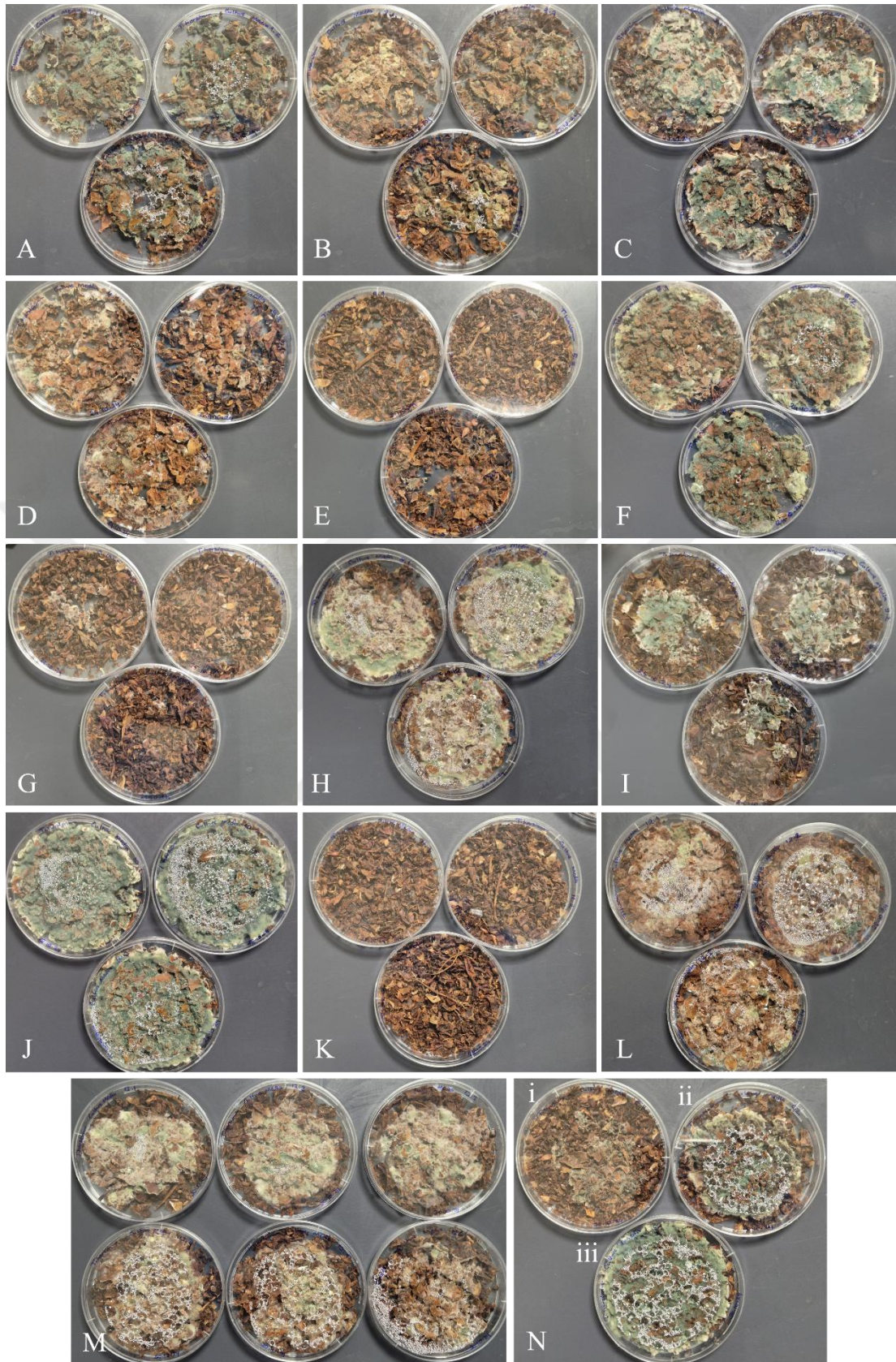


Figure A. 2 *T. harzianum* growth in apple pomace groups. A: Group 1, B: Group 2, C: Group 3, D: Group 4, E: Group 5, F: Group 6, G: Group 7, H: Group 8, I: Group 9, J: Group 10, K: Group 11, L: Group 12, M: Group 13, N: Control groups (i: C1, ii: C2, iii: C3).

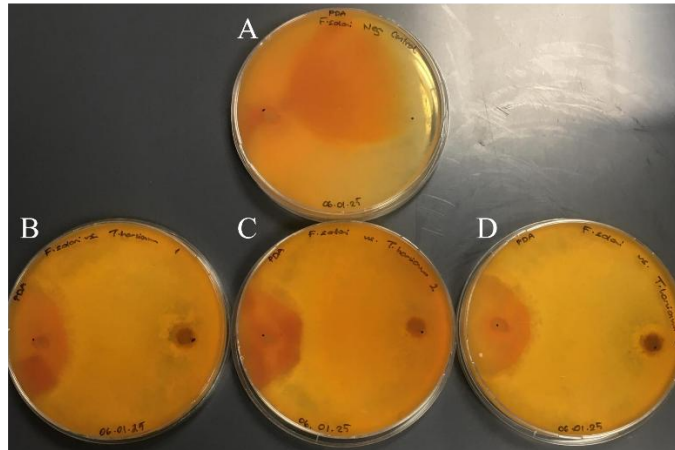


Figure A. 3 *F. solani* and *T. harzianum* dual culture assay. A: Negative control; B, C and D: Triplicate of *T. harzianum* treatment group.

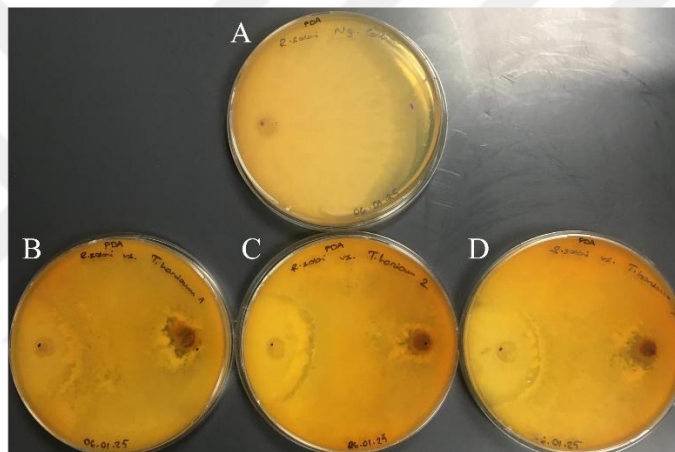


Figure A. 4 *R. solani* and *T. harzianum* dual culture assay. A: Negative control; B, C and D: Triplicate of *T. harzianum* treatment group.

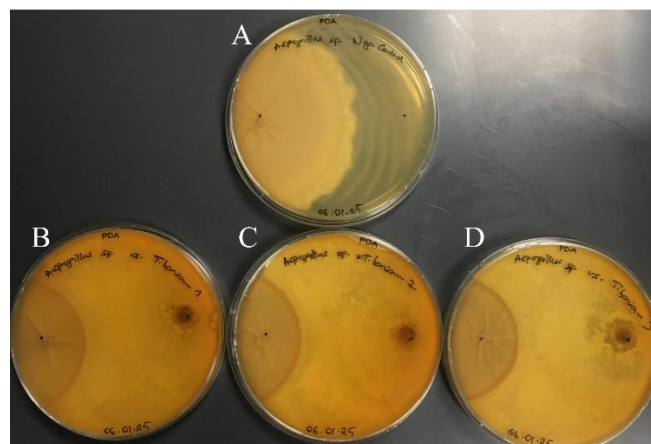


Figure A. 5 *A. welwitschiae* and *T. harzianum* dual culture assay. A: Negative control; B, C and D: Triplicate of *T. harzianum* treatment group.

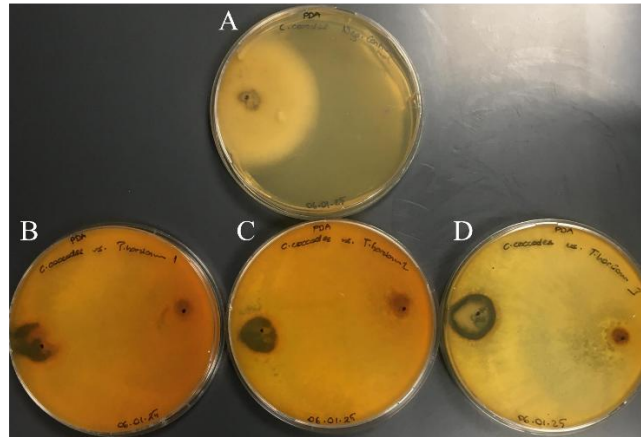


Figure A. 6 *C. coccodes* and *T. harzianum* dual culture assay. A: Negative control; B, C and D: Triplicate of *T. harzianum* treatment group.

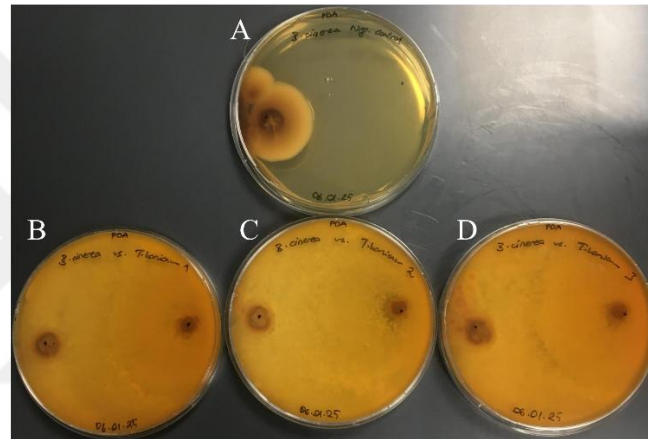


Figure A. 7 *B. cinerea* and *T. harzianum* dual culture assay. A: Negative control; B, C and D: Triplicate of *T. harzianum* treatment group.

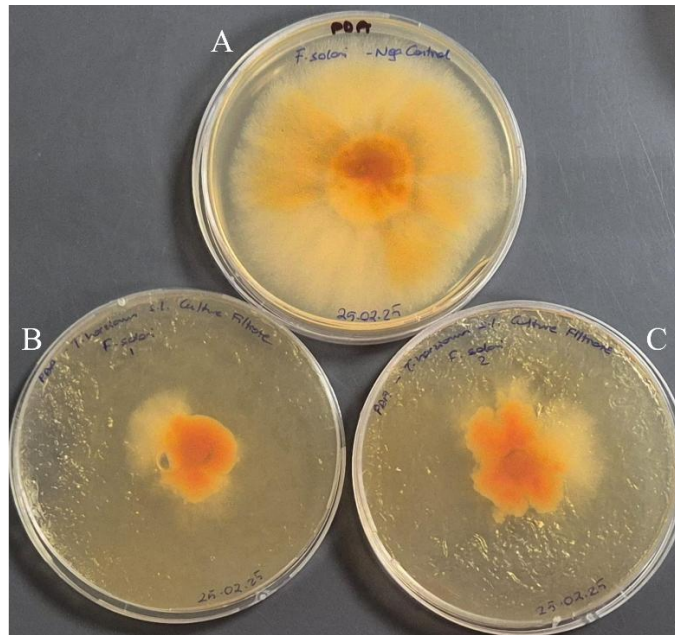


Figure A. 8 *F. solani* non-volatile compound assay. A: Negative control, B and C: Duplicate of *T. harzianum* culture filtrate added group.

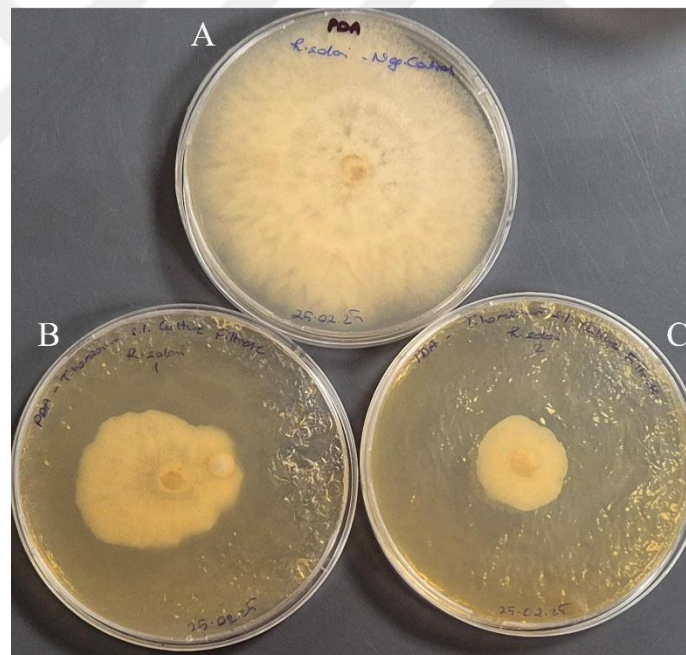


Figure A. 9 *R. solani* non-volatile compound assay. A: Negative control, B and C: Duplicate of *T. harzianum* culture filtrate added group.

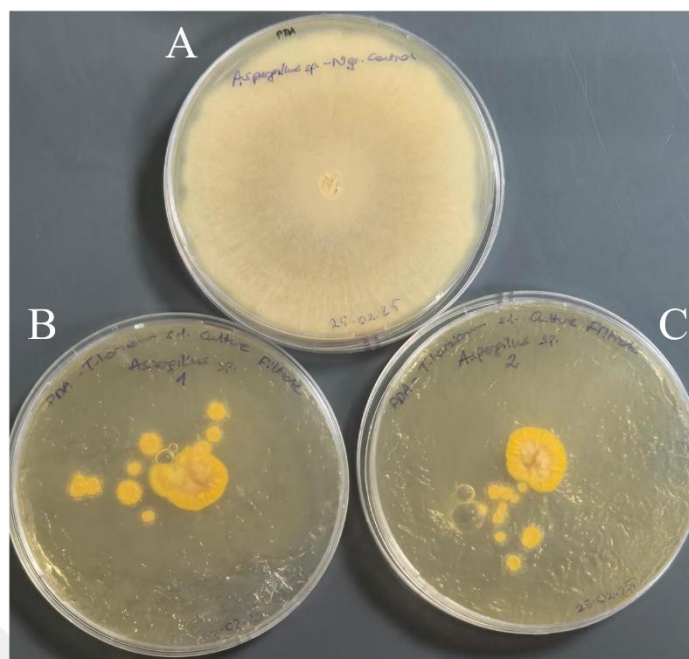


Figure A. 10 *A. welwitschiae* non-volatile compound assay. A: Negative control, B and C: Duplicate of *T. harzianum* culture filtrate added group.

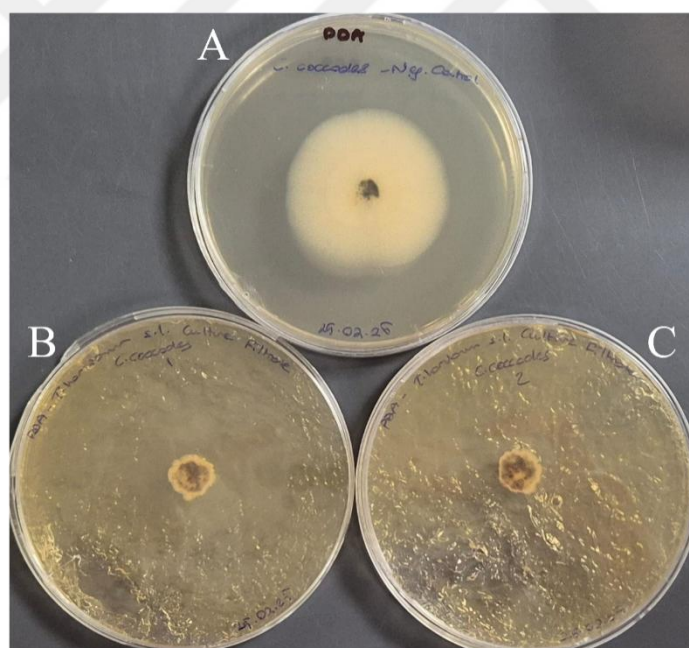


Figure A. 11 *C. coccodes* non-volatile compound assay. A: Negative control, B and C: Duplicate of *T. harzianum* culture filtrate added group.

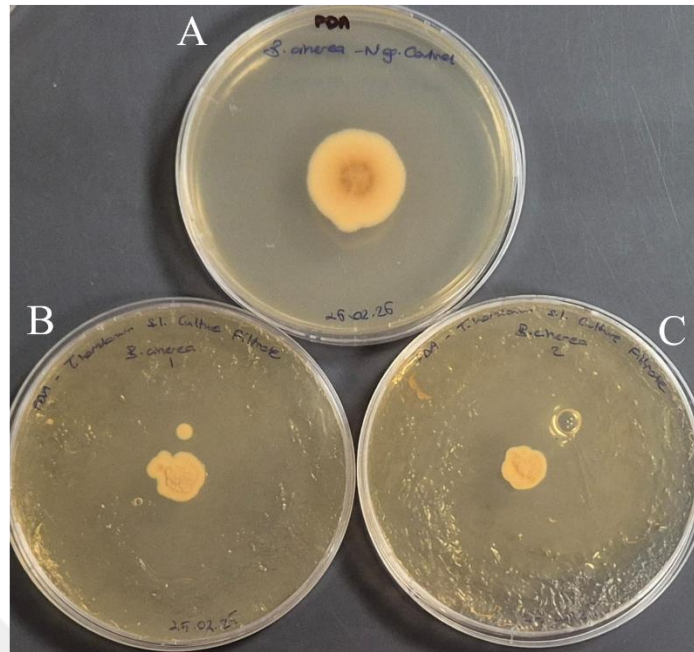


Figure A. 12 *B. cinerea* non-volatile compound assay. A: Negative control, B and C: Duplicate of *T. harzianum* culture filtrate added group.

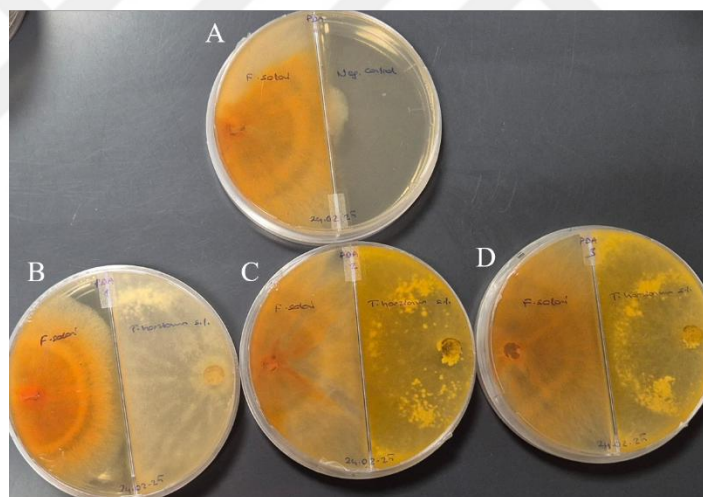


Figure A. 13 *F. solani* VOC assay. A: Negative control, B, C and D: Triplicate of *T. harzianum* inoculated group.

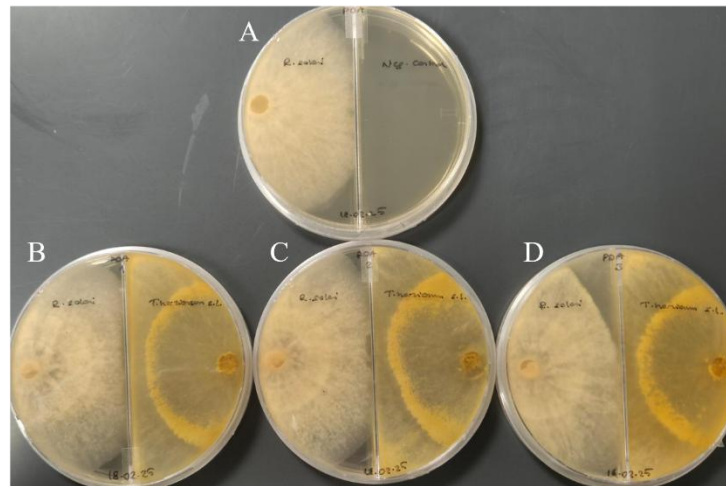


Figure A. 14 *R. solani* VOC assay. A: Negative control, B, C and D: Triplicate of *T. harzianum* inoculated group.

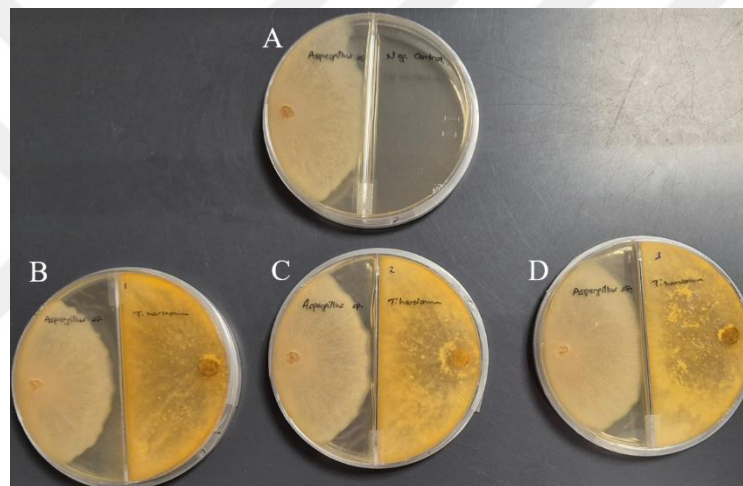


Figure A. 15 *A. welwitschiae* VOC assay. A: Negative control, B, C and D: Triplicate of *T. harzianum* inoculated group.

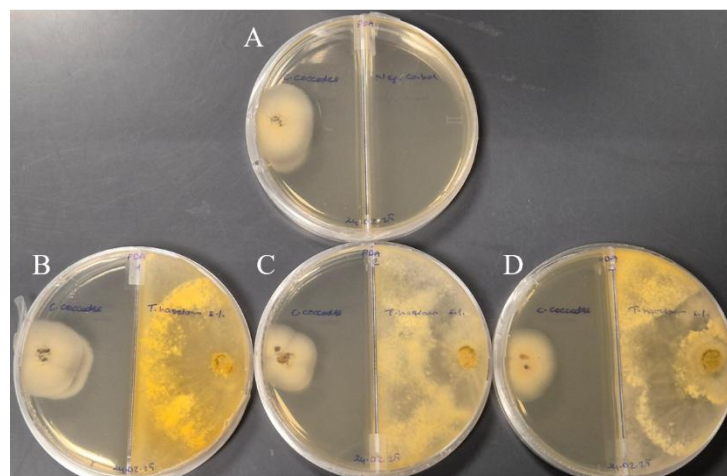


Figure A. 16 *C. coccodes* VOC assay. A: Negative control, B, C and D: Triplicate of *T. harzianum* inoculated group.

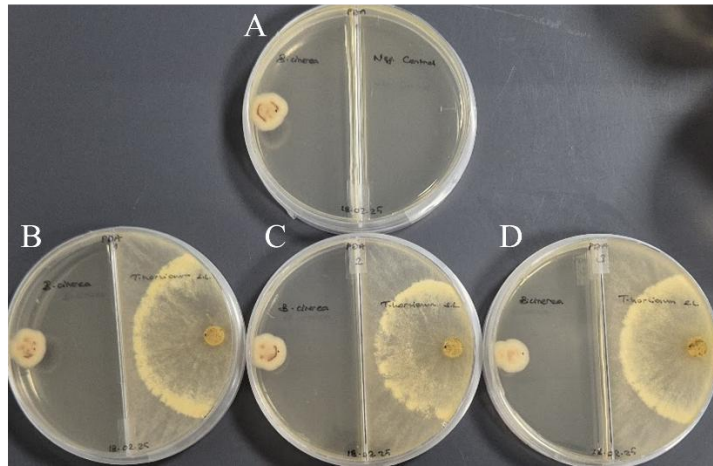


Figure A. 17 *B. cinerea* VOC assay. A: Negative control, B, C and D: Triplicate of *T. harzianum* inoculated group.

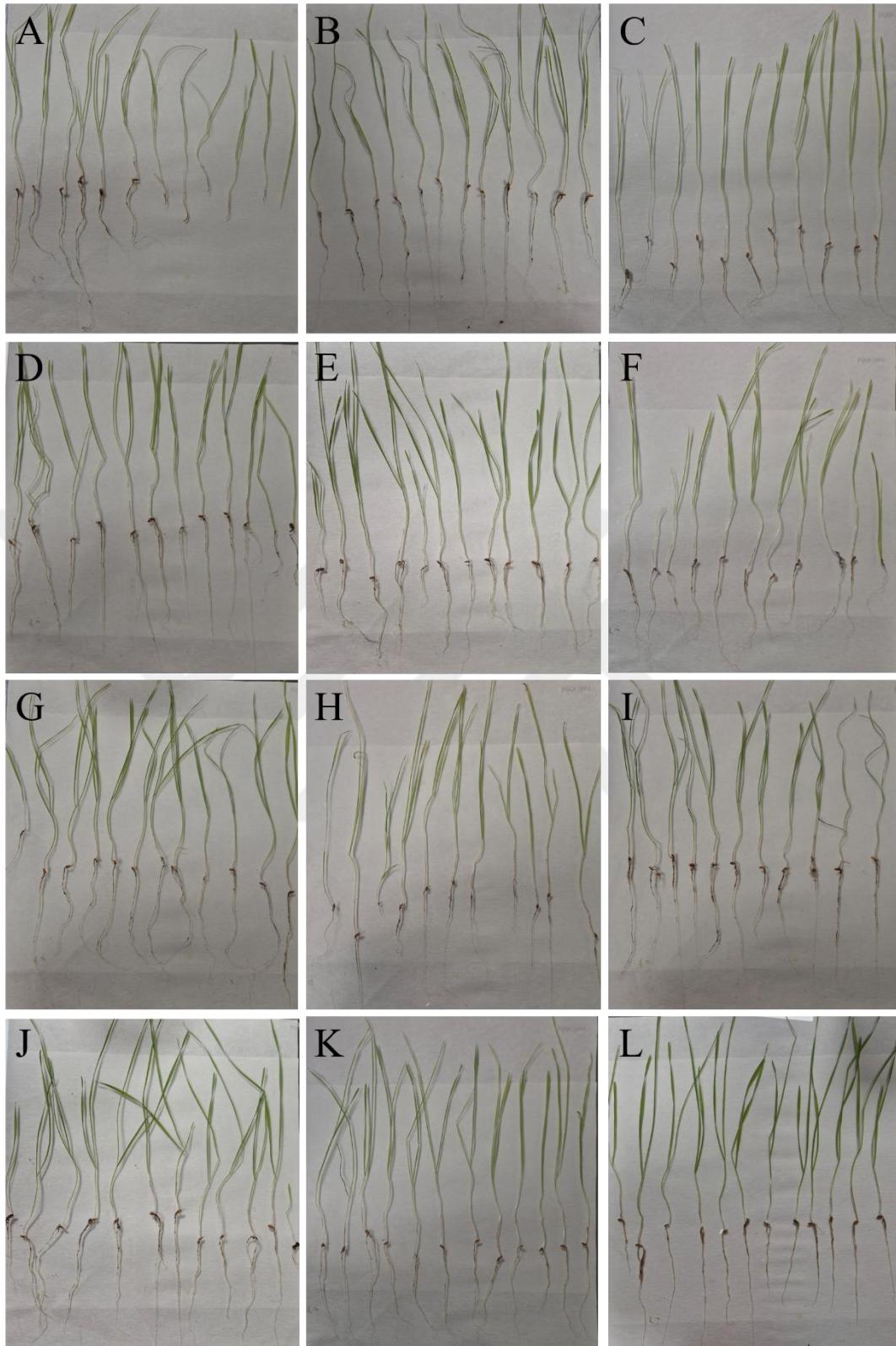


Figure A. 18 Plant experiment results. A: *F. solani* negative control, B: *R. solani* negative control, C: *A. welwitschiae* negative control, D: *F. solani* treatment, E: *R. solani* treatment, F: *A. welwitschiae* treatment, G: *C. coccodes* negative control, H: *B. cinerea* negative control, I: Control group, J: *C. coccodes* treatment, K: *B. cinerea* treatment, L: *T. harzianum* group.

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