Interaction between arbuscular mycorrhizal fungi and Penicillium pinophilum for growth promotion and tolerance to Verticillium wilt disease in lettuce and tomato

A PhD Thesis

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Interaction between arbuscular mycorrhizal fungi and *Penicillium pinophilum* for growth promotion and tolerance to *Verticillium* wilt disease in lettuce and tomato

レタスとトマトにおける生育促進効果とバーティシリウム萎凋病害抵抗性に対するアーバスキュラー菌根菌と *Penicillium pinophilum* の相互作用

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要旨（日本語）
本研究では、トマトとレタスにおける、*Penicillium pinophilum* EU0013 とアーバスキュラー菌根菌との相互作用について，成長促進と *Verticillium dahliae* による萎凋性病害への耐性の観点から調査を行った．これらの有益な微生物の作用に関与する有効性とメカニズムを確認するために下記のような一連の実験を単独の微生物接種状態，または二重接種状態により実施した。*P. pinophilum* EU0013 によって生成されるいくつかの二次代謝産物の測定も行った．第 2 章では、*in-vitro* での *P. pinophilum* の特徴付けにより、リン酸塩可溶化、シデロフォア、および有機酸生産能力を有することが確認された．レタスとトマトの両方で、共接種により菌根菌と *P. pinophilum* のコロニー形成が増加し、微生物間に相互利益的な働きがあることが示唆された。レタスには単一の接種が適していることが示され，宿主の成長応答は土壌、接種材料、および真菌コンソーシアムの組成に依存していた。第 3 章では、5 つのトマト品種における果実のファイトケミカルの指標と必須微量元素に対する *P. pinophilum* の影響を報告した。 *P. pinophilum* の効果による果実バイオマスの増加とファイトケミカルおよび微量元素量は品種間で異なった．特に 2 つの品種において、*P. pinophilum* がファイトケミカルおよび微量元素濃度の調節を介してトマト果実の栄養補助食品の価値を改善する能力を実証した．第 4 章では、*P. pinophilum* と AM 菌が 2 つのトマト品種の生長と萎凋病に対する耐性に及ぼす影響が報告された。この実験で，*P. pinophilum* による *V. dahliae* の *in vitro* 成長阻害が示された。 AM 菌と *P. pinophilum* の両方が、SOD 遺伝子発現と宿主の生理学的パラメーターの変調を介して、病気の発生率を低下させ、植物の成長指数を高めた。 AM 菌類で処理されたグループでは果実のバイオマスの増加が観察され，*P. pinophilum* で処理されたグループは病徴が減少した。ただし、AM 菌と *P. pinophilum* の共接種は、単一の接種と比較した場合、宿主の成長に対する改善効果に一貫性があるわけではなかった。第 5 章では、AM 菌と *P. pinophilum* を接種した後のレタスの成長と萎凋病に対する耐性を評価した。 *P. pinophilum* の接種により、レタスの成長指数が改善され、病徴が軽減され、病原体の増殖が抑制され、活性酸素種 (ROS) が減少し、生理学的数値が改善した。AM 菌は、根から茎への *V. dahliae* の増殖も阻害したが、*P. pinophilum* 接種下においても、レタスの成長を改善しなかった。これは、トマトよりも敏感な植物であるレタスには、単回接種が適していることを示す.第 6 章では、コムギイノキュラムの *P. pinophilum* EU0013 の二次代謝産物について，液 Liquid Chromatography Time-of-Flight Mass Spectrometry (LC-TOFMS)を使用して陽イオンおよび陰イオン代謝物を分析した。128 の代謝物の存在が示唆され、*P. pinophilum* は、フェノール化合物、アルカロイドとステロイド、脂肪酸と脂質、遍在する抗酸化物質であるコエンザイム 10 などのさまざまな代謝物を生成していた。結論として、*P. pinophilum* EU0013 は、*V. dahliae* によるトマトとレタスの病徴を抑制するため、またトマトの果実品質の改善し生育促進するために，AM 菌と組み合わせて使用することが推奨される。

GENERAL ABSTRACT (English)

This study investigated the interactions between *Penicillium pinophilum* EU0013 and arbuscular mycorrhizal fungi, for growth promotion and tolerance to *Verticillium dahliae* induced wilt disease, in tomato and lettuce. Series of experiments were conducted to ascertain the efficacy and mechanisms involved in the action of these beneficial microbes, via single and dual inoculation partnerships, including the determination of some secondary metabolites produced by *P. pinophilum* EU0013, for the first time. In chapter two, the characterization of *P. pinophilum in vitro* confirmed it possesses phosphate solubilizing, siderophore and organic acid producing abilities. In both lettuce and tomato, mycorrhizal and *Penicillium* root colonization were increased during co-inoculation indicating a reciprocal fungal stimulation between the microbes. It was shown that single inoculation was better for lettuce, as the host growth response was dependent on the soil, inoculum material, and fungal consortium composition. In chapter three, the effects of *P. pinophilum* on fruit phytochemical indices and essential trace elements in five tomato cultivars is reported. Increases in fruit biomass and modulation of phytochemicals by *P. pinophilum* varied between cultivars, as it demonstrated the ability to improve the nutraceutical value of tomato fruits via modulations of phytochemicals and trace element concentrations, particularly in two cultivars. In chapter four, the effect of *P. pinophilum* and an AM fungus on the growth and tolerance of two tomato cultivars to *Verticillium* wilt, is reported. This experiment showed the *in-vitro* growth inhibition of *V. dahliae* by *P. pinophilum*. Both AM fungi and *Penicillium* reduced diseases incidence, and boosted plant growth indices, via regulation of SOD gene expression and host physiological parameters. Increases in fruit biomass by AM fungi treated groups

was observed, while *P. pinophilum* treated groups showed reduced disease symptoms of *V. dahliae*. However, the co-inoculation of AM fungi and *P. pinophilum* did not consistently improve host growth when compared to single inoculation, and the mechanisms of protective effect of the inoculants against *V. dahliae* was observed to be cultivar dependent. In chapter five, I evaluated lettuce growth and tolerance to *Verticillium* wilt after inoculation with an AM fungus and *P. pinophilum. P. pinophilum* inoculation improved growth indices, reduced disease symptoms, inhibited pathogen proliferation, reduced reactive oxygen species (ROS) and improved physiological parameters in lettuce. However, the AM fungi did not improve lettuce growth under disease stress, even in its partnership with *P. pinophilum*, although it also inhibited *V. dahliae* proliferation from roots to stem. This again showed that lettuce being a more delicate plant than tomato, is better suited for single inoculation. In chapter six, an evaluation of the secondary metabolites of *P. pinophilum* EU0013 from a wheat inoculum, was performed using Liquid Chromatography Time-of-Flight Mass Spectrometry (LC-TOFMS). Cationic and anionic metabolites of control and inoculated samples were analyzed. The result indicated 128 metabolites present, with varying metabolites produced by *P. pinophilum* such as phenolic compounds, alkaloid and steroids, fatty acids and lipids, and coenzyme 10, which is a ubiquitous antioxidant. In conclusion, *P. pinophilum* EU0013 is recommended for use against *Verticillium* wilt in tomato and lettuce, alongside improvement in fruit quality and growth promotion of tomato, in partnership with AM fungi.

CHAPTER ONE
General Introduction

1.1 Background

Vegetables are vital to the general good health of humans, providing essential vitamins and minerals, dietary fiber, and phytochemicals, thus reducing risk from diseases and other medical conditions (Oguntibeju et al., 2013). Their successful production in field and greenhouses depend substantially on pathogen control and proper plant nutrition. To promote plant growth and fight diseases, chemical fertilizers and pesticides are widely used in vegetable production, but their availability, affordability, and safety are important issues confronting farmers in many countries. To reduce the environmental and human health concerns raised by these fertilizers and pesticides, non-chemical means of growth promotion and disease control are required and beneficial for global food security and quality of human health (FAO, 2020). Plant growth-promoting microorganisms (PGPM) could play a huge role in this regard, as they are more eco-friendly than chemical methods, and often combine growth-promotion, biotic and abiotic stress tolerance, and nutrient enrichment aspects (Ibiang et al., 2020). However, the wide-spread adoption of a PGPM in agricultural production will require many important questions to be answered, such as its effects on the target host, mechanisms of action, interaction with other microorganisms, impact of soil/cultivation/inoculum conditions, consistency of outcomes, etc. (see Fig. 1.1). Also, as a way of optimizing the outcomes, the use of dual inoculation or mixed inocula consisting of

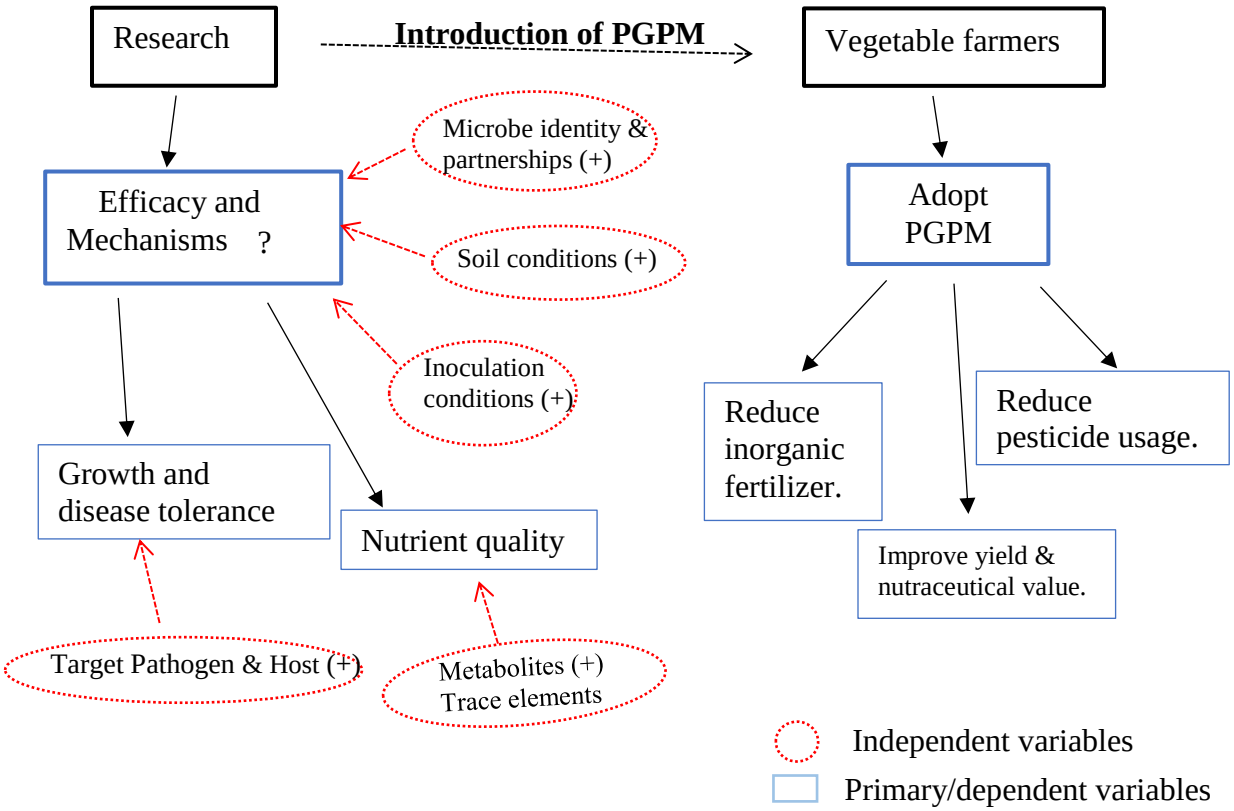

Fig 1.1. Conceptual framework of the study showing the issues relating to the adoption of PGPM for sustainable utilization in vegetable production.

two or more species (consortia) might be used to engender synergistic effects that confer a wider range of benefits to the host (Zhang et al., 2012; Meena et al., 2018). PGPM includes many groups of fungi, such as arbuscular mycorrhiza (AM) and endophytes. The endophytic fungus *Penicillium pinophilum* EU0013 isolated from eucalyptus roots (Teshima and Sakamoto, 2006) was reported to improve seed germination, plant growth, and *Fusarium* disease tolerance in tomato (Alam et al., 2011a; Alam et al., 2011b). It is a potential active ingredient in the formulation of commercial inoculum consisting of one or more PGPM, or even a consortium of non-target beneficial fungi, but knowledge on its partnership with AM fungi is limited, neither its action against *Verticillium* disease. The elucidation of physiological, molecular, and rhizosphere-based mechanisms and their interactions is therefore crucial for closing the knowledge gap. Adoption of *P. pinophilum* for improving growth, fruit quality/nutraceutical value and biocontrol of both *Verticillium* and *Fusarium* diseases will be of benefit to farmers, consumers, and the environment.

1.2 Soil-plant-microbe interactions

The soil serves as the home of different groups of microbes and a source of structure and nutrients to plants. Plants interact with the soil and microorganisms of diverse kinds such as bacteria, fungi, protists, (see Fig. 1.2) etc., which could be endophytes, mycorrhiza, pathogens, saprophytes, amongst others (Brader et al., 2017). Varying types of interaction exist (see Fig. 1.3). Some interactions benefit the plants, while others are harmful to them. These could take place with microbial associations in the roots as well as in the shoots of plants (see Fig 1.4) as shaped by millions of years of evolution (Dolatabadian, 2021).

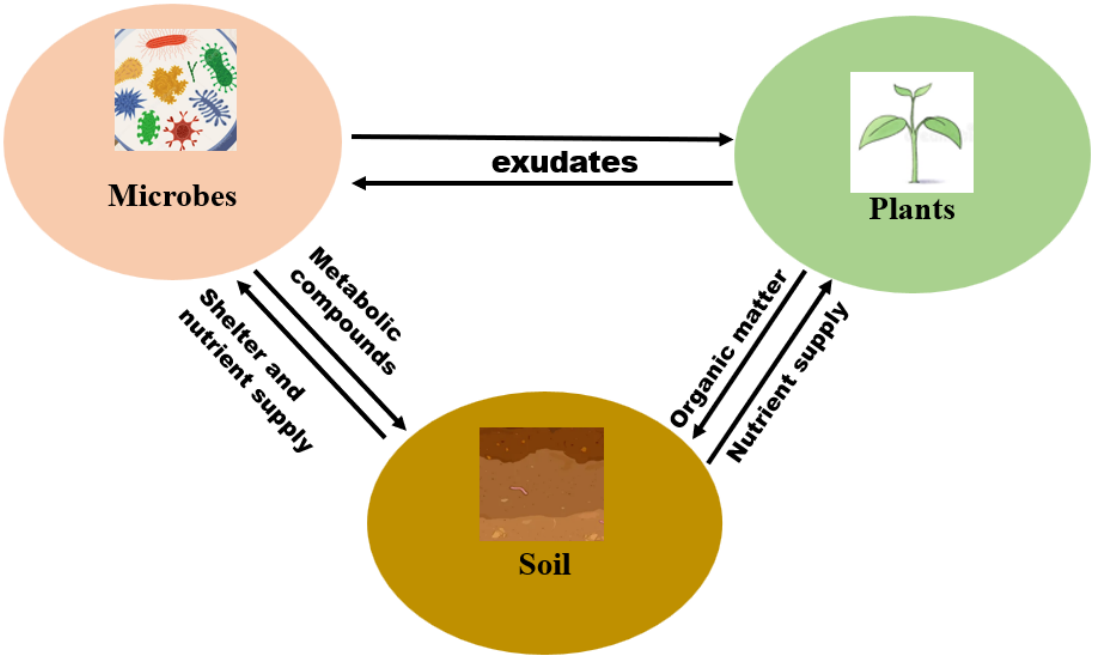

Fig. 1.2. Soil-plant-microbe interactions

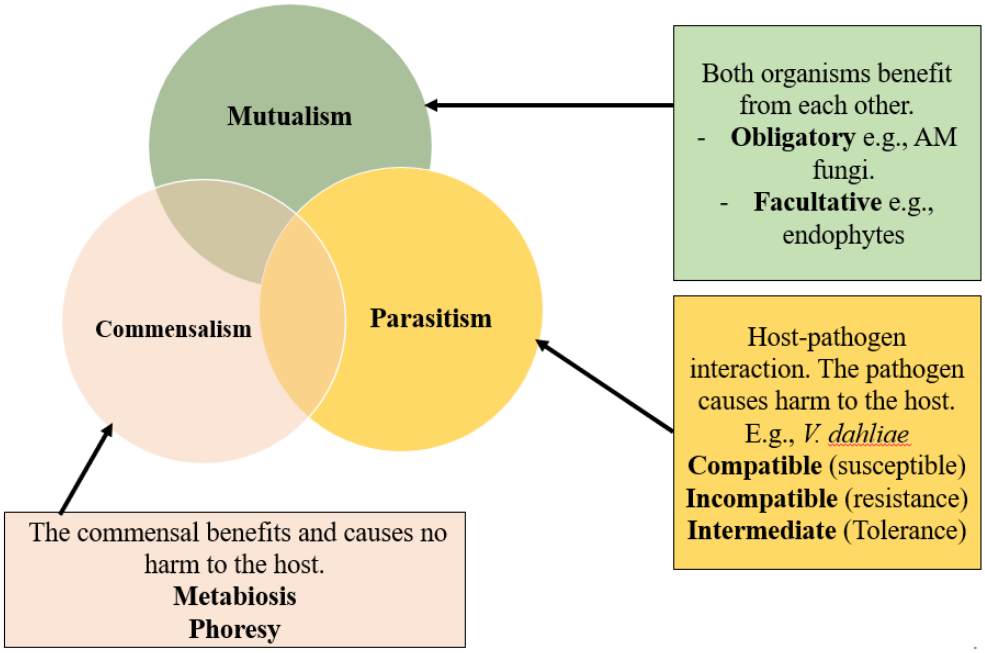

Fig. 1.3. Types of interactions

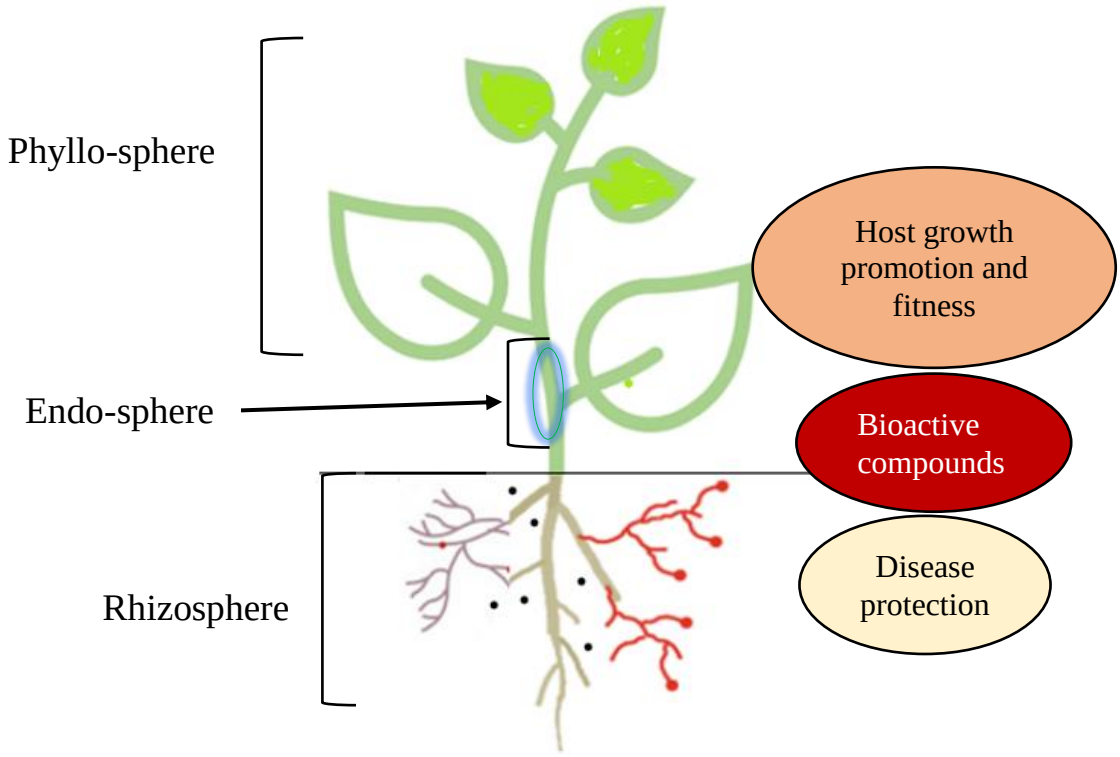

Fig. 1.4. Plant - microbe interaction illustration showing benefits and spheres of association with the host.

The roots of plants produce exudates of different chemical composition which establishes colonization by these microbes. These exudates include phytohormones, secondary metabolites, sugars, organic acids, fatty acids, amino acids, enzymes, and they help plants communicate with its surrounding environment in different ways. Some of the exudates regulates plant growth and development, some induce resistance against pathogens, while others send signals to attract beneficial microbes, which in most cases form symbiotic association with plants and enhance nutrients acquisitions (Ibiang, 2020). In fact, many

microorganisms maintain a significant interaction with plants to complete their life cycle. Some examples of microorganisms which interacts with plants include bacteria, actinomycetes, viruses, protists, and fungi and they could be found in different parts of the plants (Gopal and Gupta, 2016). Fungi play a fundamental role in the ecosystem. They act as symbionts, decomposers, or parasites of plants. They reproduce sexually and asexually through spore formation. Fungi differ in their mode of behavior which is mainly dependent on the substrate available for their proliferation. The fungi used in this study include both pathogenic and beneficial, and they are discussed in the succeeding sections.

1.3 Arbuscular mycorrhizal fungi

Mycorrhizal symbiosis is an ancient symbiotic association that is established between plants roots and mycorrhizal fungi. Different mycorrhizal fungi exist but, the arbuscular mycorrhizal (AM) fungi have been intensively studied due to their important role in agriculture (Smith and Read, 2008; Bonfante and Genre, 2010). They are obligatory and colonizes about 80% of plants. AM fungi provide mineral nutrients, confer protection to host against biotic and abiotic stresses in exchange for photosynthate. Plant roots releases the exudate - strigolactone, which serve as a signal molecule to attracts resting mycelium of AM fungi in the surrounding environment (Lopez-Raez et al., 2011). AM fungi in response releases its own signal molecule called “myco factor” which is also recognized by the roots and stimulate an increased production of Ca^{2+} in the root cells (Dowarah et al., 2021). AM fungi colonizes the root cortex and establishes the arbuscles, which are responsible for nutrients exchange between the roots and fungi (See Fig. 1.5). Furthermore, an external mycelium is formed which extends wider into the

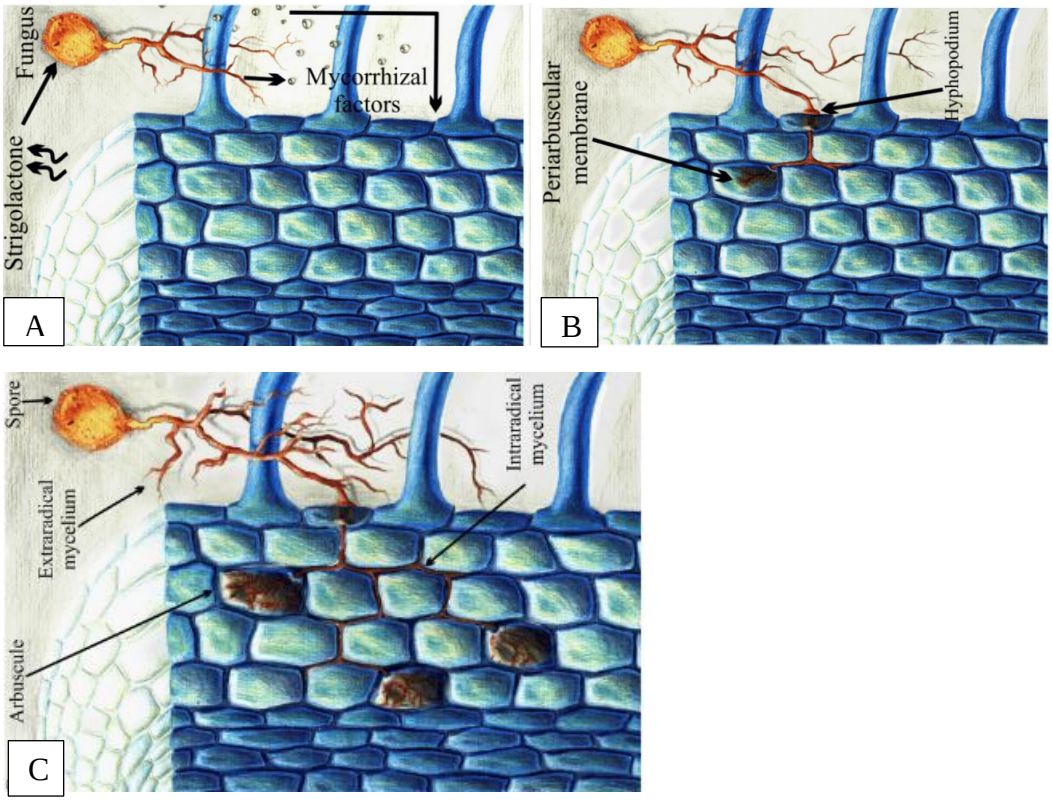

Fig. 1.5. Illustration of arbuscular mycorrhizal root colonization (Vergara et al., 2019).

rhizosphere and serve to enhance nutrients (especially phosphate) and water uptake in plants (Smith and Read, 2008). This often results to increase in growth, higher nutrients availability and disease resistance in plants (Parniske, 2008; Bonfante and Genre, 2010). Due to their benefits during this symbiosis, AM fungi are considered as biofertilizers and biocontrol agents. However, the benefits to AM-colonized plants often stem from the increased phosphate (P) nutrition. Thus, the fertility of the soil in terms of P nutrition is an important consideration. Obviously, understanding the best conditions for AM application and action is beneficial.

1.4 Endophytes and their mode of action as biocontrol agents

Endophytes are microorganisms that live within plant cells for a part or an entire life cycle without causing harm. They penetrate and live in different parts of plant such as roots, leaf, and stem (Yadav, 2018). Endophytes presence in a host helps to promote their growth through nutrient cycling, regulation of phytohormones and protection against biotic and abiotic stress. (Andreozzi et al., 2019; Shen et al., 2019: Ibiang and Sakamoto, 2022). There is an increase in the study of endophytes due to their ability to produces many bioactive compounds (Waqas et al., 2014). These bioactive compounds are relevant in medicine, agricultural and industrial sectors. They are used to make natural products such as antioxidants and can be used as antimicrobial, anticancer, and biocontrol agents (Gouda et al., 2016; Yadav, 2018). As biocontrol agents, they prevent the penetration of the host root by a pathogen or reduce the establishment of pathogen structures and disease symptoms. They deploy a range of mechanisms against phytopathogen such as antibiosis, parasitism, activation of defense response, competition for nutrients, infection site, and photosynthates

(Glick, 2015; Ruano-Rosa, and Mercado-Blanco, 2015). Some of these mechanisms are discussed in the subsections below.

1.4.1 Antibiosis

Antibiosis refers to the suppression of microbial growth and proliferation by the action of biomolecules or bioactives. Endophytes are known to secrete many bioactive compounds some of which possess antimicrobial characteristics, which suppresses the proliferation of phytopathogens. (Gunatilaka, 2006). They could be antifungal or antibacterial and include terpenes, phenols, flavonoids, quinones, peptides, steroids, etc. (Lugtenberg et al., 2016). Some researchers have reported that secretion of antimicrobial compounds is triggered by the presence of different microbes occupying the same rhizosphere. These secretions can be from host plant or the endophyte. However, there are also reports which indicates that both host plants and endophytes use different pathways to enhance secretion of these antibiotic compounds to suppress the growth of phytopathogens. (Kusari et al., 2012, Ludwig-Muller, 2015).

1.4.2 Competition for space and nutrients

Endophytes colonizes many parts of plants and as such prevent phytopathogens from occupying the same space. Competition for space and nutrients is a very strong mechanisms they use against phytopathogens since their colonization is usually both local and systemic (Latz et al., 2018). This mode of action involves preventing access to nutrients and space to the pathogen, hence preventing their activities and/or minimizing their spread and impact. Competition mechanisms work side by side with other biocontrol mechanisms. Arnold et al.,

(2003) reported that the application of endophytes mixture on leaves of plant reduced disease incidence and some strains of the endophytes were observed to produce metabolites.

1.4.3 Siderophore production

Siderophores are low molecular weight compounds with high affinity for iron (Fe). Many endophytes produce these molecules to chelate Fe and make them available to plants while starving the pathogens (Yadav, 2018). During the process of chelating Fe, other nutrients are made available, hence providing more nutrition and fitness to plants, and suppressing the effect of the pathogen. In addition, since Fe is essential for other physiological processes, such as respiration, the potential overall negative impact of pathogen is minimized.

1.4.4 Phytohormones production

Endophytes produce phytohormones which regulates plant growth and development. They promote and protect plant through the secretion of different plant hormones such as Indole acetic acid (IAA), gibberellic acids (GA), and cytokinin (Khan et al. 2014). The production of these hormones alters plant physiology, morphology, and developments. IAA are the most common plant hormones and act as effector molecule between endophytes and plants. They are responsible for lateral root formation, root and shoot elongation, cell division, apical dominance, and responses to pathogen (Sun et al. 2014, Aloni et al. 2006). As a result of lateral root formation, increases in nutrients absorption is enhanced for the host plant, which in turn increases plant biomass (Contreras-Coornejo et al. 2009).

1.4.5 Induced defense response in plant

Endophytes induce defense response in plants by regulating their immunity. They act as microbial elicitors which stimulate signals that begins chemical defense in plants (Malik et

al., 2020). Some chemical elicitors such as salicylic acid, benzothiadiazole, benzoic acids are known to produce phenolics in host which promotes plant defense system (Heimpel and Mills, 2017). Defense response in plants can be stimulated by root colonization of PGPM, pest and pathogens, and could be induced systemic resistance (ISR) or systemic acquired resistance (SAR). ISR is associated to PGPM while SAR is associated to pests and pathogens infectivity (Romera et al., 2019). ISR and SAR can be differentiated by their elicitors and or signaling pathways. The mechanisms by which microbial elicitors induces resistance are not well understood, but many reports have shown that they do so through the recognition of distinctive receptors by plants. These receptors known as pattern recognition receptors (PRRs) are able to recognize exudates or structures present on the surface of microbes referred to as pathogen or microbe - associated molecular patterns (PAMPs and MAMPs) (Boller and He, 2009). Upon an attack, plant poses a self-derived response called the damage-associated molecular pattern (DAMPs), then the PAMPs induces defense against the pathogen and enhance host resistance (Villena et al., 2018). PAMP-triggered immunity (PTI) tracks the invading pathogen and is the first defense of pattern recognition (Dodds and Rathjen, 2010). The pathogen uses virulence effector molecules to subdue plant immune system by restraining the host from detecting its presence using PTI, thereby establishing infection in the plant. This process leads to the effector-triggered immunity (ETI) (Meena and Swapnil, 2019). ETI comprises of intracellular nucleotide binding sites and leucine-rich repeat domains and serves as the secondary defense. On achievement of a second defense by the host, the receptor proteins moderate the recognition of the particular pathogen's effector molecules, and this will send a signal towards the defense genes (Zhou and Zhang 2020). PTI

and ETI usually induce resistance in host situated away from the point of infection, which triggers defense in other plant tissues that were not infected, hence they are systemic in nature.

1.5 *Verticillium* wilt disease

Verticillium wilt disease is caused by *Verticillium dahliae*, a soil borne pathogenic fungus which affects many crops. *V. dahliae* has a wide host range of over 200 plant species and can last in the soil for a long period (Sun et al., 2014). A small, secreted protein called hydrophobin, have been reported to be responsible for the formation of the resting structure - microsclerotia. This structure is formed in the absence of a host and can survive for as long as a decade in the soil. Plant infectivity begins with the germination of the microsclerotia upon receiving signals from root exudates, with the mycelium infecting the tips of the roots. Symptoms in plants are observed two to three weeks after infection depending on the host (Ibiang et al., 2021). The fungal pathogen continues its spread in an acropetal order all through the vascular tissues of the plant giving rise to conidiospores that continue the colonization cycle. *Verticillium*-plant interaction results in either susceptibility, resistivity, or tolerance interaction. In susceptible interaction, there's a successful establishment of the pathogen which results in symptoms expression. In resistance interaction, plant poses a defense against pathogen resulting in an incompatible interaction, while in host tolerance, plants can suppress disease expression and show less symptoms. Common disease symptoms are yellowing/chlorosis of the lower leaves, necrotic lesions at the edges of the leaves, and browning of the vascular tissues (Witzel et al., 2017). Disease intensity has been attributed to the mass of microsclerotia present in the soil. However, environmental conditions are also a major determinant of symptoms expression. Different methods such as host resistance, soil

fumigation, and the use of biocontrol agents have been used to combat *Verticillium* wilt disease.

1.6 Objectives of study

1 To investigate the partnerships between *Penicillium pinophilum* and AM fungi for growth promotion in lettuce and tomato under varying soil and inoculum substrate conditions.

2 To evaluate the effect of *Penicillium pinophilum* on fruit quality indices in different tomato cultivars.

3 To evaluate the effect of inoculation of AM fungi and *Penicillium pinophilum* on the tolerance to *Verticillium dahliae* in tomato and lettuce.

4 To characterize some secondary metabolic compounds produced by *Penicillium pinophilum*.

CHAPTER TWO

Performance of tomato and lettuce to arbuscular mycorrhizal fungi and *Penicillium pinophilum* EU0013 inoculation varies with soil, culture media of inoculum, and fungal consortium composition

2.1. Abstract

The context-dependent nature of plant-microbe interaction in the rhizosphere is of significance in terms of the consistency of beneficial microbe effects in inoculated plants. A series of experiments testing the effect of inoculation with arbuscular mycorrhizal fungi (AMF) and a root-associated fungus, *Penicillium pinophilum* EU0013, on the growth and root colonization of tomato and lettuce, is reported. Three AMF species (*Rhizophagus irregularis, Rhizophagus intraradices,* and *Claroideoglomus etunicatum*) and *Penicillium* (inoculum from corn and wheat media) were used in AMF-plant, *Penicillium*-plant, and AMF-*Penicillium*-plant partnerships. In lettuce, we also tested *C. etunicatum* interaction with *Penicillium* under different soils (river sand, commercial soil, and river sand + commercial soil). *P. pinophilum* EU0013 showed phosphate solubilization, siderophore and organic acid production traits in in-vitro assays. In tomato and lettuce, mycorrhizal and *Penicillium* root colonization were increased during co-inoculation compared to single AMF or *Penicillium* treatment, indicating that a reciprocal fungal stimulation occurred during AMF-endophyte interaction in both hosts. In tomato, the inoculation with only *Penicillium* (from wheat medium) or in combination with AMF, significantly improved host weights. In lettuce, fresh weight was significantly improved when *Penicillium* from corn medium was used in combination with *C. etunicatum*. However, when the same AMF-*Penicillium* consortium was tested using *Penicillium* from wheat media, co-infection stress in the form of retarded growth, wilting of leaves and mortality occurred, depending on the soil. The pattern of negative

effects could be rapid and transient, where wilting, slow growth, mortality, and recovery occurred (in river sand), or gradual and persistent where there was slower growth but no mortality (as in commercial and mixed soil). Target host, soil, culture media of inoculum and fungal consortium composition are important considerations in the exploitation of AMF and *P. pinophilum* for improving vegetable growth.

2.2 Introduction

Plant growth-promoting fungi (PGPF) are non-pathogenic microbes that have been reported to be beneficial to several crops by promoting their growth and protecting them from disease (Shivanna et al., 1996). Broadly speaking, fungal symbionts that provide benefits to plants consists of diverse groups such as arbuscular mycorrhizal (AM) fungi (AMF) and other root-associated fungus, such as *Penicillium* sp. Many endophytes promote host growth via their ability to solubilize phosphate, produce organic acids and siderophores, stimulate phytohormones synthesis, aid trace element nutrition, etc. (Khan et al., 2009). AMF, on the other hand, are popular for enhancing phosphorus (P) nutrition, tolerance to drought, salinity, and excess heavy metals (Smith and Read, 2008). Due to the increasing cost of chemical fertilizers and its impact on the environment, there is considerable interest in harnessing these fungal bioresources as bioinoculants for improving growth and tolerance to sub-optimal conditions of economically important plants (Hossain et al., 2014; Berruti et al., 2015). Vegetables such as tomato and lettuce which are vital to the good health of humans (Wargovich, 2000), are widely consumed and cultivated, making them good targets for ecofriendly strategies like bio-inoculation. Many researchers have pointed to the issue of

consistency of beneficial effects of microbial inoculants, due to the context-dependent nature of the outcomes of plant-microbe interaction (Nadeem et al., 2014).

The use of dual inoculation or mixed inocula consisting of two (or more) different microbe species (consortia) is often reported as beneficial in this regard, due to synergistic tendencies (Meena et al., 2018), while negative effects are less often stated, due to a greater interest in positive outcomes. Ostensibly, a bio-inoculation strategy consisting of two groups of beneficial fungi might confer a wider range of benefits to host plants than single inoculation (Albrechtova et al., 2012), such as between biological control agents like *Penicillium* sp. and non-target beneficial fungi like mycorrhizas (Chandanie et al., 2006). But important questions such as the spectrum of interaction between fungal partners, effect of differences in inoculum substrate material, soil/growth conditions, target hosts, amongst others (Miransari, 2014), need to be considered; as fungal interactions with host, substrate, competing microbes, and other environmental factors, can range from synergistic to antagonistic, or neutral (Dix and Webster, 1995; Waing et al., 2015). *P. pinophilum* conferred benefits such as promoting plant growth and tolerance against Fusarium diseases (Teshima and Sakamoto, 2006; Alam et al., 2011a, 2011b) making it a potential candidate for commercial inoculum development. However, its partnership with AMF has not been evaluated, hence this study. Fungus-plant relationships are tightly dependent on genetic, physiological, and environmental factors (Kogel et al., 2006), and the host growth and colonization indices are valuable pointers to the interaction of these context-shaping conditions. I report a series of experiments evaluating the effect of inoculation with three species of AM fungi and *P. pinophilum* on the growth and root colonization of tomato and

lettuce hosts, using varied *Penicillium* inoculum substrate and/or soils. Single microbe inoculations generated AMF-plant and *Penicillium*-plant combinations for respective hosts, while AMF-*Penicillium*-plant partnerships were established using dual inoculation. Consistency of outcomes amidst modulating effects of AMF partnership, inoculum material, soil/growth condition, and host identity, were evaluated for the first time in *P. pinophilum*.

2.3 Materials and methods

2.3.1 Isolation and screening of *Penicillium pinophilum* EU0013

P. pinophilum EU0013 was previously isolated from eucalyptus roots and identified from morphological features of the conidiophores and sequence data on the internal transcribed spacer (ITS) region of rDNA (Teshima and Sakamoto, 2006) and it was screened for confirmation of some growth-promoting traits (Khan et al., 2009) at the outset.

2.3.1.1. Phosphate solubilization assay

Phosphate solubilization was determined using the Pikovskaya's broth medium (Pikovskaya, 1948). The powder (16.3 g) was suspended and dissolved in 1000 mL of distilled water, then sterilized by autoclaving for 15 min at 121°C and kept for at least one day before use. A tenfold serial dilution of homogenized spore suspension of *P. pinophilum* EU0013 was used. One mL (3.8×10^4 /μL) of the broth of *Penicillium* was added into a 100 mL of Pikovskaya broth media in an Erlenmeyer flask and incubated in a shaking incubator (Bioshaker, BR-23FP, Taitec, Japan) at 30 °C with a speed of 130 $rmin^{-1}$ for respective number of days. Samples (n = 3) from inoculated groups (EU0013+) and control (EU0013-) were collected at different intervals (2, 4, 6 and 8 days), centrifuged (Sakuma SS-1500X, Japan) at 12000×g for 5 min. Five mL of supernatant was filtered through a 0.2 μm membrane filter into plastic

tubes, and reacted with 3 mL of a mixture of sulfuric acid (10 mL), ammonium molybdate (3 mL), ascorbic acid (6 mL), and antimonyl potassium (1 mL), kept in the dark at 30 °C for 1 h before absorbance was measured (U-1800 Hitachi High Tech Corp, Tokyo, Japan) at 710 nm (Babu et al., 2014).

2.3.1.2. Organic acid assay

Organic acid production by *P. pinophilum* was determined using Rose-Bengal medium (Martin, 1950) with three replications. Fungal spore was inoculated (EU0013+) on petri dishes containing Rose-Bengal agar medium and incubated for two weeks before observation for color change around the halo zone. Control petri dishes (EU0013-) were maintained for the same period.

2.3.1.3. Siderophore assay

Siderophore assay was conducted by the method of Adriane *et al*. (1999), using Chrome-Azurol Sulphonate (CAS; 0.01452 mg) and hexadecyltrimethylammonium bromide (HDTMA) (0.01749 g dissolved in 9.6 mL of ultra-pure water). Both CAS and HDTMA were added together and poured into petri dishes (EU0013+ and EU0013-) containing half potato dextrose agar (PDA) and allowed to gel, giving a half PDA and a half CAS media. After 1hr, *P. pinophilum* was inoculated on the part containing the PDA and incubated for 3 weeks at 25 °C.

2.3.1.4. Phosphatase assay

Soil phosphatase activity was determined using p-nitrophenyl phosphate (PNP) according to the method of Tabatabai and Bremner (1969). For this, a sub-experiment was set-up with lettuce colonized by; *P. pinophilum* via inoculum from corn medium, AMF, *Penicillium* +

AMF, and control, grown for seven weeks. Rhizosphere soil attached to host roots (n = 4) was collected and 1 g (air-dried) of soil was put in a 50 mL Erlenmeyer flask, 4 mL of modified universal buffer (MUB), toluene (0.25 mL) and PNP (1 mL) was added and swirled for few seconds to mix properly. Flasks were corked with stoppers and placed in an incubator for 1 h at 37 °C. Afterwards, 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH was added and swirled to mix, filtered, and transferred into plastics tubes and measured at 420 nm in a spectrophotometer (U-1800 Hitachi High Tech Corp, Tokyo, Japan). P-nitrophenol content was calculated by reference to a calibration graph plotted from standards containing 0, 10, 20, 30, 40, and 50 µg of p-nitrophenol. To verify that increase in phosphatase activity around the roots contributed to P benefit to the plant (Morales et al., 2007), the phosphorus concentration in lettuce shoots were measured. Phosphorus concentration was determined using the vanadomolybdate method (Tandon et al., 1968). Ground samples were ignited in an electric furnace at 550 °C for 2 h, then digested in 0.6 mol L^{-1} HCl acid and reacted with vanadomolybdate acid solution. Samples were kept for 30 min after which absorbance was measured in a spectrophotometer at 420 nm.

2.3.1.5. Spore numbers in inoculum substrates

To determine spore number of *P. pinophilum* in media composed of different substrate materials, a spore count was done, using inoculum from corn and wheat substrate. One g of totally colonized, well-ground corn and wheat husks was dissolved in distilled water and filtered through a double fold cheese cloth into 50 mL tubes and centrifuged (Sakuma SS-1500×, Sakuma Co. Ltd, Japan) at 1300×g for 5 min. The supernatant was drained out and the harvested spores were diluted with sterile distilled water and mixed by shaking. To

determine spore number, 10 μL of the inoculum was loaded into Thoma cell counting chamber and viewed under a microscope (Nikon Co. Ltd, Japan. Controls were prepared in the same way but with no fungal inoculation.

2.4. Experiment on tomato

2.4.1. Soil

The soil utilized was river sand with pH of 7.29 and electrical conductivity of 4.8 mS m^{-1}. The soil was sieved with a 2 mm mesh, autoclaved at 121 °C for 1 h, then allowed to cool for at least 48 h before use.

2.4.2. Tomato cultivar

The dwarf tomato, *Solanum lycopersicum* L. cv. Micro-Tom was used as they are convenient to raise in growth chambers and yield fruits early (Meissner et al., 1997). Tomato seeds were sterilized in 70% ethanol for 5 min and 33.3% sodium hypochlorite solution for 15 min then rinsed in sterile distilled water before seeding. The seedlings were raised for 12 days on vermiculite moistened with half-strength Hoagland solution before being transplanted to soil in plastic pots (9 cm by 20 cm) in a growth chamber. Growth chamber conditions for plant cultivation were 140 μmol m^{-2} s^{-1} fluorescent light (25 °C, 14 h) and dark (18 °C, 10 h). All planted pots were equally fertilized with full strength Hoagland solution (50 mL pot^{-1}) applied every three days until harvest.

2.4.3. AM fungal species and inoculation

Three AM fungal species were used; *Claroideoglomus etunicatum* (Ce) (supplied by Kyowa Hakko Kirin Co. Ltd, Japan), *Rhizophagus irregularis* DAOM197198 (Rir) (Mycorise, Premier Tech, Rivi`ere-du Loup, Canada), and *Rhizophagus intraradices* 15S-1 (Rin)

(provided by Dr. M. Yamato, Faculty of Education, Chiba University). Ce consisted of soil bearing spores, while Rir and Rin consisted of soil bearing AM propagules containing spores, hyphae, and root fragments. AM fungal inoculation was performed on the day of seedling transplant from vermiculite to potted soil by applying the inoculum in the middle of soil, prior to transplant. The amount of inoculum applied for respective AMF are Ce, 2.2 g with an average of 400 spores pot^{-1}; Rir, 10 g pot^{-1}; Rin, 10 g pot^{-1}.

2.4.4. *Penicillium* inoculation

P. pinophilum was obtained from actively growing margins of 7–10 days old potato dextrose agar (PDA) culture medium. Two types of inoculums: wheat-substrate (WS) and corn-substrate (CS) based inoculum, were prepared for *Penicillium* inoculation. WS and CS inoculum were prepared as described by (Chandanie et al., 2006) but with incubation temperature of 25 °C. After 10 days of incubation, the completely colonized and dry corn and wheat were ground and sieved (1 mm mesh), and 0.5 g of the inoculum (CS and WS) was added to the middle of each pot before transplant. Sterile corn and wheat control inoculum were also prepared but without *Penicillium* inoculation.

2.4.5. Experimental set-up

The tomato experiment utilizing WS and CS inoculum of *Penicillium* was set up with the following treatment groups: non-inoculated control which received neither sterile nor active fungal inoculum (NI); wheat and corn controls which received respective sterile inoculum; *Penicillium* (WS); *Penicillium* (CS); AMF (Ce; Rir; Rin) and dual inoculations (Pen + Ce; Pen + Rir; Pen + Rin) for both WS and CS. Each treatment group consisted of five pots (n = 5) giving a total of 70 completely randomized experimental units maintained for 10 weeks.

2.5. Experiments on lettuce

2.5.1 Soil

The soil utilized were river sand with pH 6.75 and electrical conductivity of 7.9 mS m^{-1} and a commercial soil (Tsuchitaro, Sumitomo Forestry Co. Ltd., Japan) with pH of 6.70 and electrical conductivity of 0.9 mS m^{-1}. River sand was sieved with a 2 mm mesh, and both soils were mixed (1:1) where required and autoclaved at 121 °C for 1 h, then allowed to cool for at least 2 days before use.

2.5.2. Lettuce

The lettuce plant *Lactuca sativa* L. cv. Great lake 366 was used. Lettuce seeds were grown in cell trays and seedlings were raised for 21 days on commercial soil (Tsuchitaro) moistened with water, before been transplanted to soil in plastic pots (15 cm by 20 cm) in a growth chamber. Growth chamber conditions for plant cultivation were 140 μmol m^{-2} s^{-1} fluorescent light (25 °C, 14 h) and dark (18 °C, 10 h).

2.5.3. AM fungal and *Penicillium* inoculations

AM fungal inoculation was performed on the day of seedling transplant from nursery to potted soil by applying the inoculum in the middle of soil of each pot, prior to transplant. The amount of inoculum applied for respective AM fungi are Ce, 2.2 g with an average of 200 spores pot^{-1}; Rir, 10 g pot^{-1}; Rin, 10 g pot^{-1}. As in tomato experiments, 0.5 g of *Penicillium* inoculum was added to the middle of soil in each pot before transplant. Sterile control inoculum was prepared but without *Penicillium* inoculation.

2.5.4. Experimental set-up

Two experiments were conducted using lettuce. The first utilized river sand, three AMF species (Ce, Rir and Rin) and CS inoculum of *Penicillium* set up with the following treatment groups: non-inoculated control (NI) which received neither sterile nor active fungal inoculum; corn substrate control (CC) which received sterile inoculum; single *Penicillium* and AMF inoculations; and dual inoculations of *Penicillium* with respective AMF. Each treatment group consisted of five pots (n = 5) giving a total of 45 experimental units in a completely randomized design maintained for 8 weeks. All planted pots were equally fertilized at the time of transplant with $(NH_4)_2SO_4$ and K_2HPO_4 solution (50 mL pot^{-1}). The second experiment utilized only one AMF species (Ce) - chosen because it gave the best lettuce yield in dual inoculation with *Penicillium* in the previous experiment; WS inoculum of *Penicillium*; and three different soils for varied cultivation circumstance – river sand (RS), commercial soil (CMS), and river sand + commercial soil (RS + CMS). It was set up in a 4 × 3 factorial with "inoculation" as factor 1 with 4 levels (Control, *Penicillium*, Ce, and dual inoculation); and "soil" as factor 2 (RS, CMS, RS+CMS). Each treatment group consisted of four pots (n = 4) giving a total of 48 experimental units in a completely randomized factorial design, maintained for 8 weeks. All replications of dual inoculated plants in RS group died at 3 weeks after transplant but were replanted. No fertilization was administered in the second experiment due to the use of a commercial soil.

2.6. Plant harvests

Tomato and lettuce were harvested by carefully emptying the soil from the pots, breaking apart soil attached to the roots and washing in running tap water. Plant lengths and fresh weight were measured, and the plant was cut into root and shoot. A portion of the roots was subtracted for determination of fungal root colonization indices before plant tissues were put in an oven for drying at 80 °C for 48 h and the dry weights were recorded.

2.7. Root colonization by AM fungi

AM fungal colonization was assessed in the roots using the trypan blue staining technique as described by Rajapakse and Miller (1994). Root sections mounted on glass slides were observed in the light microscope for scoring according to Trouvelot et al. (1986) and mycorrhizal indices were then evaluated using "mycocalc" software.

2.8. Root colonization by *Penicillium*

Roots were washed thoroughly with running tap water and washed again with sterile water before use. Ten root segments of about 0.5 cm were cut and placed on PDA media in petri dishes amended with penicillin antibiotic (200 mg L^{-1}), to suppress the growth of other ubiquitous microorganisms. Petri dishes were placed in the incubator at 25 °C for 4–6 days, colonies of EU0013 growing from root segments were counted and colonization frequency was calculated as % CF = (Ncol/Nt) × 100, where Ncol = number of root segments colonized by the fungus and Nt = total number of segments of root studied, as described by Hata and Futai (1995).

2.9. Physiological parameters in lettuce

Due to the negative host responses observed in the second lettuce experiments, nutrient (Zn, Fe, Mn, and K) concentrations were determined in dry lettuce shoot tissues to ascertain if mineral nutrition was hampered (antagonism) in dual inoculation compared to single. Samples were ground in a laboratory electric miller and put (0.25 mg) in electric muffle furnace (ADVANTEC FUL220FA, Tokyo, Japan) at 550 °C for 6 h, then digested in 0.6 mol L^{-1} HCl acid, after which concentrations in solutions were measured using atomic absorption spectrophotometry (Shimadzu AA-6600F, Japan). Nitrogen content in shoot was measured with C-N coder (Yanaco Technical Science Co. Ltd., Japan), while chlorophyll content in shoot was measured using the SPAD meter (Minolta Co. Ltd., Japan), at five weeks of growth.

2.10. Statistical analysis

Data for the tomato and first lettuce experiments were processed statistically by one-way analysis of variance (ANOVA), while the second lettuce experiment which was a factorial was handled using two-way ANOVA. Significance (*P* values) was set at $P < 0.05$ and treatment group means were separated using Tukey-Kramer tests. For root colonization, control groups which were uncolonized were excluded from the ANOVA. In the pre-experiments ascertaining *Penicillium* attributes, the percentage difference between *Penicillium* (EU0013+) and control (EU0013-) samples was evaluated using the average of readings for invitro phosphate solubilization and pH, while rhizosphere soil acid phosphatase and corresponding shoot P concentrations were analyzed by one-way ANOVA.

2.11. Results

2.11.1. *Penicillium* characteristics

Phosphate solubilizing activity in EU0013+ showed 746, 807, and 1180% increases (respectively), at 4, 6, and 8 days, compared to EU0013-, indicating active phosphate solubilization by the endophyte (Fig. 2.1 (a)). The corresponding pH readings (Fig. 2.1 (b))

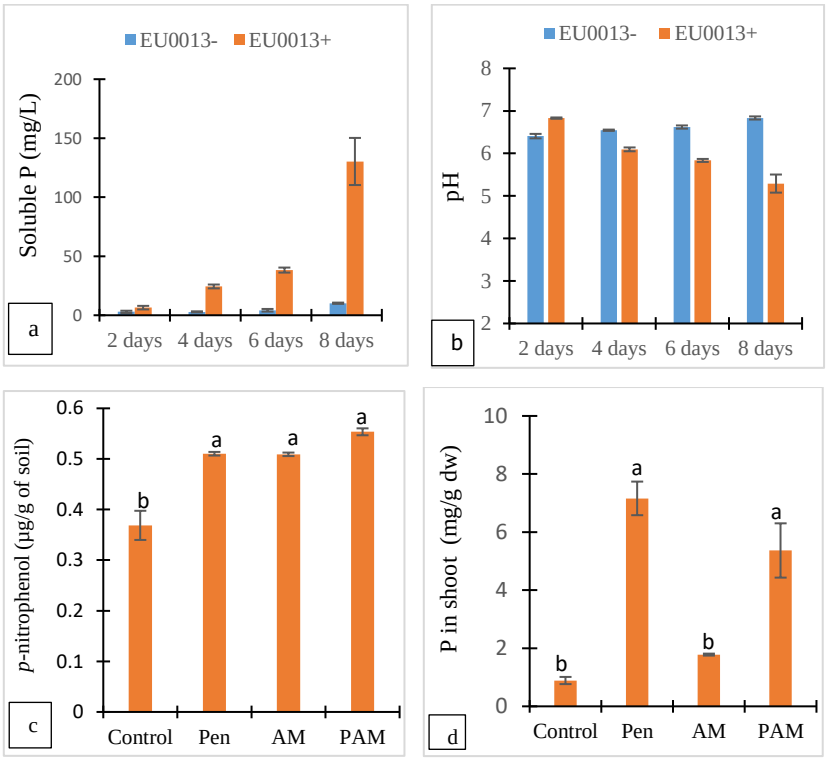

Fig. 2.1. Screening of *P. pinophilum* EU0013 for phosphate solubilization. Soluble P concentration (a) and corresponding pH readings (b) from day 2 to 8; Acid phosphatase (c) in rhizosphere soil and corresponding shoot P (d). Values are mean ± SEM. In (c) and (d), ^{ab}superscripts indicate differences based on Tukey-Kramer tests after one-way ANOVA. Pen= *Penicillium pinophilum* EU0013; AM= *C. etunicatum*; PAM= *C. etunicatum* + *Penicillium*.

in EU0013+ showed 6.8, 11.8, and 22.5% decreases (respectively), at 4, 6, and 8 days, compared to EU0013-. All inoculated soils showed higher acid phosphatase levels (p-nitrophenol released) (Fig. 2.1 (c)) indicating higher enzymatic activity in rhizosphere

compared to control. Corresponding phosphorus concentration in shoots (Fig. 2.1 (d)) was higher in *Penicillium* and dual inoculated groups. *P. pinophilum* exhibited organic acid production trait in Rose-Bengal media (Plate 2.1 a) as a clearer zone was observed around the colony. Siderophore production was indicated by the purple color change on the side containing the CAS-blue indicator, with positive confirmation of siderophore production in only the inoculated plates (Plate 2.1 b). Spore production in wheat and corn substrate by *Penicillium* (Plate 2.1 c & d) showed more spores in wheat than corn, indicating a variation in the endophyte proliferation due to the type of substrate used as inoculum media.

2.11.2. Root colonization indices

In tomato, AMF colonization indices were generally higher in dual inoculation than in single AM fungal inoculation. The frequency (Fig. 2.2 a) and intensity (Fig. 2.2 b) of mycorrhizal colonization showed significant differences between the treatment groups, with generally lower values in single AMF treatments compared to dual inoculation. Although not statistically significant, arbuscule abundance (Fig. 2.2 c) was generally higher in dual than in single AM inoculation. Compared to respective single AMF inoculations (Rin, Ce, and Rir), dual inoculated groups showed 30, 81, and 155 %, and 59, 140, and 121 % increases, for corn and wheat inoculum of *Penicillium*, respectively. The controls and single *Penicillium* groups

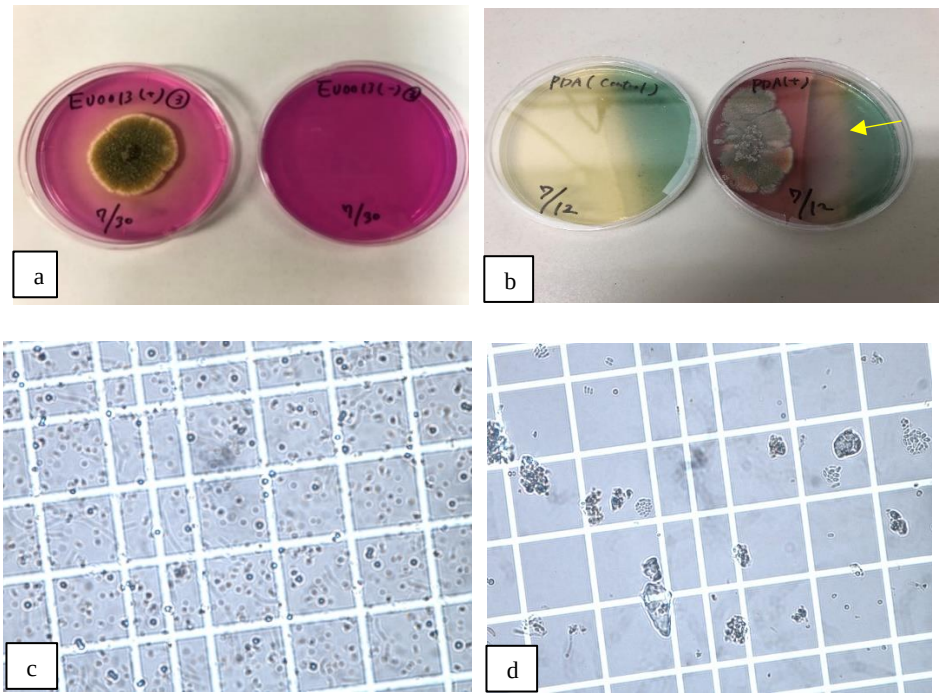

Plate 2.1. (a) Organic acid assay at 2 wpi; (b) Siderophore assay at 3 wpi; (c) Spore production by *Penicillium pinophilum* EU0013 in wheat (d) Spore production by *Penicillium pinophilum* EU0013 in corn inoculum

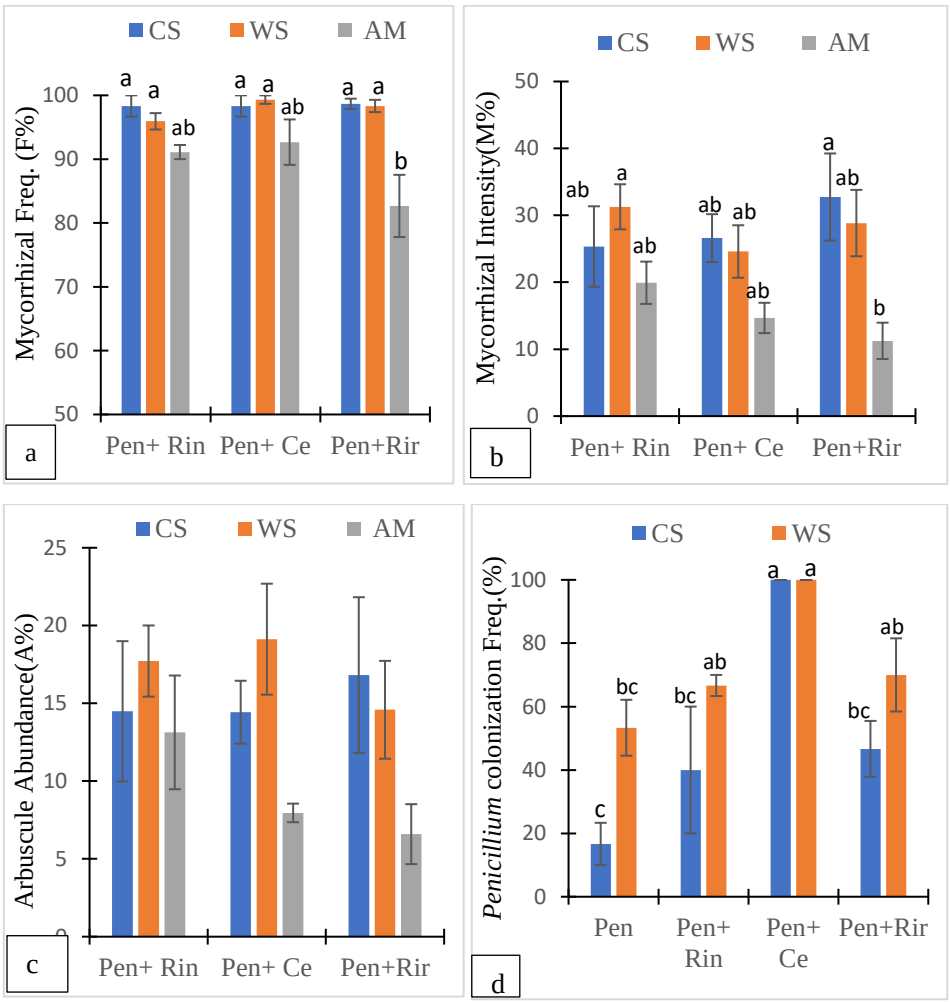

Fig. 2.2. Root colonization indices of tomato. (a) mycorrhizal frequency (b) mycorrhizal intensity (c) arbuscule abundance (d) Penicillium colonization. Values are Mean±SEM. [abc]Superscripts indicate significant differences. CS= corn substrate; WS= wheat substrate; Pen= *Penicillium pinophilum*. EU0013; AM= single AM inoculation.

were examined for AMF colonization but there was none. *Penicillium* colonization showed significant differences between the treatment groups (Fig. 2.2 d), with generally higher values in dual inoculation than in single inoculation of *Penicillium*. For corn substrate groups, *Penicillium* colonization during dual inoculation (with Rin, Ce, and Rir, respectively) increased by 140, 500, and 180 %, compared to single *Penicillium* treatment. For wheat substrate groups, dual inoculation (with Rin, Ce, and Rir, respectively) increased by 25, 87.5, and 31 %, compared to single *Penicillium* treatment, showing that the greatest increases occurred during partnership with *C. etunicatum*. The controls and single AMF groups were examined for *Penicillium* colonization but there was none. There was no indication of antagonistic effect of *P. pinophilum* EU0013 (neither corn nor wheat inoculum) on root colonization by the AMF species tested, and vice versa, but values tended to be generally higher in wheat than in corn inoculum, for *Penicillium* colonization.

Root colonization in lettuce indicate that while mycorrhizal frequency (Fig. 2.3 a) was not significant, mycorrhizal intensity (Fig. 2.3 b) and arbuscule abundance (Fig. 2.3 c) were significantly different between the treatment groups, with values in dual inoculation generally higher than in the single AMF group. Values of dual inoculation in commercial and mixed soils were significantly higher than in river sand. The control and single *Penicillium* groups were examined for AMF colonization but there was none. *Penicillium* colonization in lettuce (Fig. 2.3 d) showed significantly higher values in dual inoculation but effect of soil type was not significant. Compared to single *Penicillium* treatment, dual inoculation showed 100, 147, and 73.3% increases in river sand, commercial and mixed soils, respectively, indicating consistent increases in colonization during co-inoculation, under all soils.

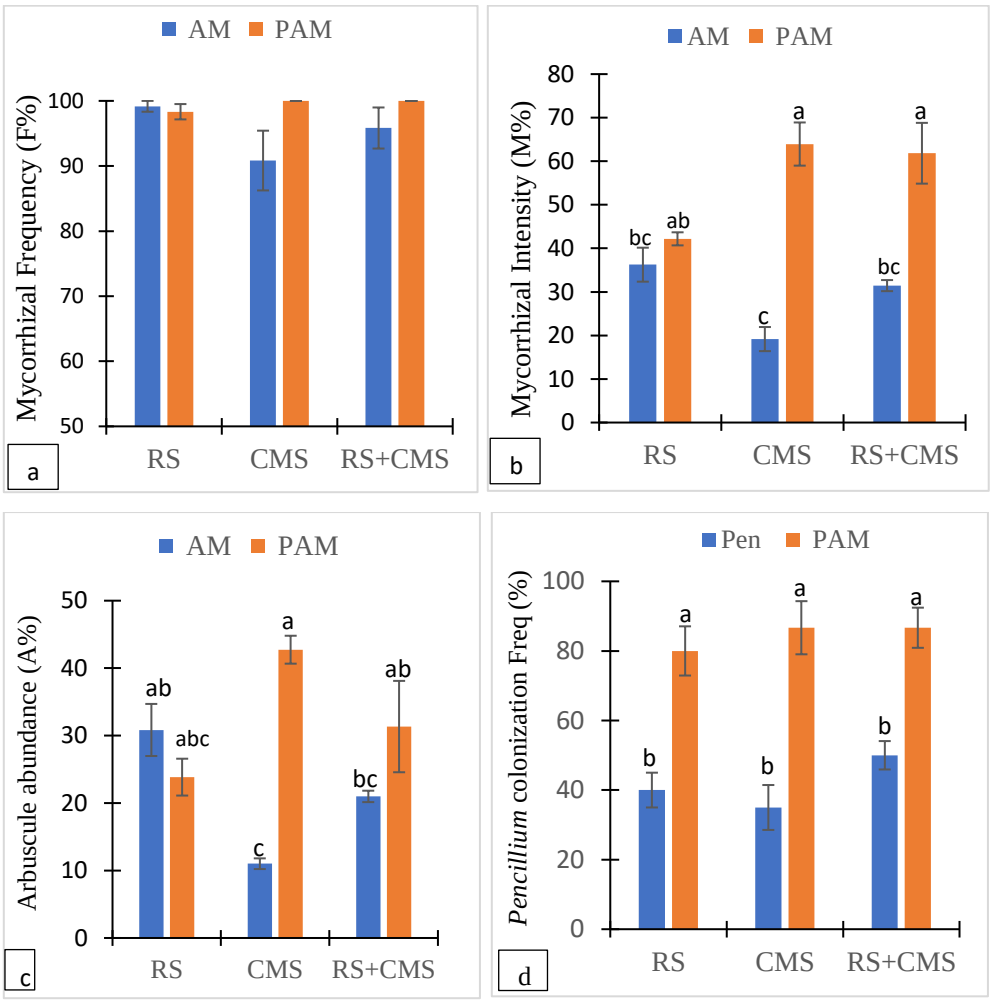

Fig. 2.3. Root colonization indices of lettuce. (a) mycorrhizal frequency (b) mycorrhizal intensity (c) arbuscule abundance (d) *Penicillium* colonization. RS= river sand; CMS= commercial soil; AM= single AM inoculation; Pen= *Penicillium sp*. EU0013; PAM= dual inoculation of AM and *Penicillium* sp. EU0013.

The control and single AMF groups were examined for *Penicillium* colonization but there was none. There was no indication of AM fungal antagonism to *Penicillium* colonization.

2.11.3. Plant growth indices

In tomato, plant length (Fig. 2.4 a) showed significant differences between the treatment groups, with the highest value observed in the *R. irregularis* + *Penicillium* (WS) dual inoculated group. Controls which received sterile inoculum (CS and WS) had higher values than non-inoculated control which did not (NI). Total fresh weights (Fig. 2.4 b) were highest in the *Penicillium* + *C. etunicatum* treatment (Pen (WS)+ Ce), and generally lower in corn substrate groups. Total dry weight (Fig. 2.4 c) was also highest in Pen (WS)+Ce and this was significantly higher than in Pen (CS)+Ce. Fruit dry weights (Fig. 2.4 d) were highest in the wheat substrate groups (Pen (WS), Pen (WS)+Ce, and Pen (WS)+ Rin) and single AM inoculation (Rin and Rir).

In the first lettuce experiment, when corn inoculum of *Penicillium* was used, shoot length (Fig. 2.5 a) showed significant differences between the treatment groups, with values in dual inoculation and corn control (CC) higher than single AM and *Penicillium* inoculation. Total fresh weight (Fig. 2.5 b) was highest in *Penicillium* + *C. etunicatum* inoculation and was significantly higher than control and all single inoculations. Total dry weights (Fig. 2.5 c) were highest in *Penicillium* + *C. etunicatum* and single *R. intraradices* inoculations. In the second lettuce experiment when wheat inoculum of *Penicillium* was used with only *C. etunicatum* under three different soils, shoot length (Fig. 2.6 a) was significantly affected by

soil and inoculation (see Table 2.1), with the highest values in the commercial soil (CMS), while river sand (RS) had the lowest. The AM groups had the lowest shoot lengths under all

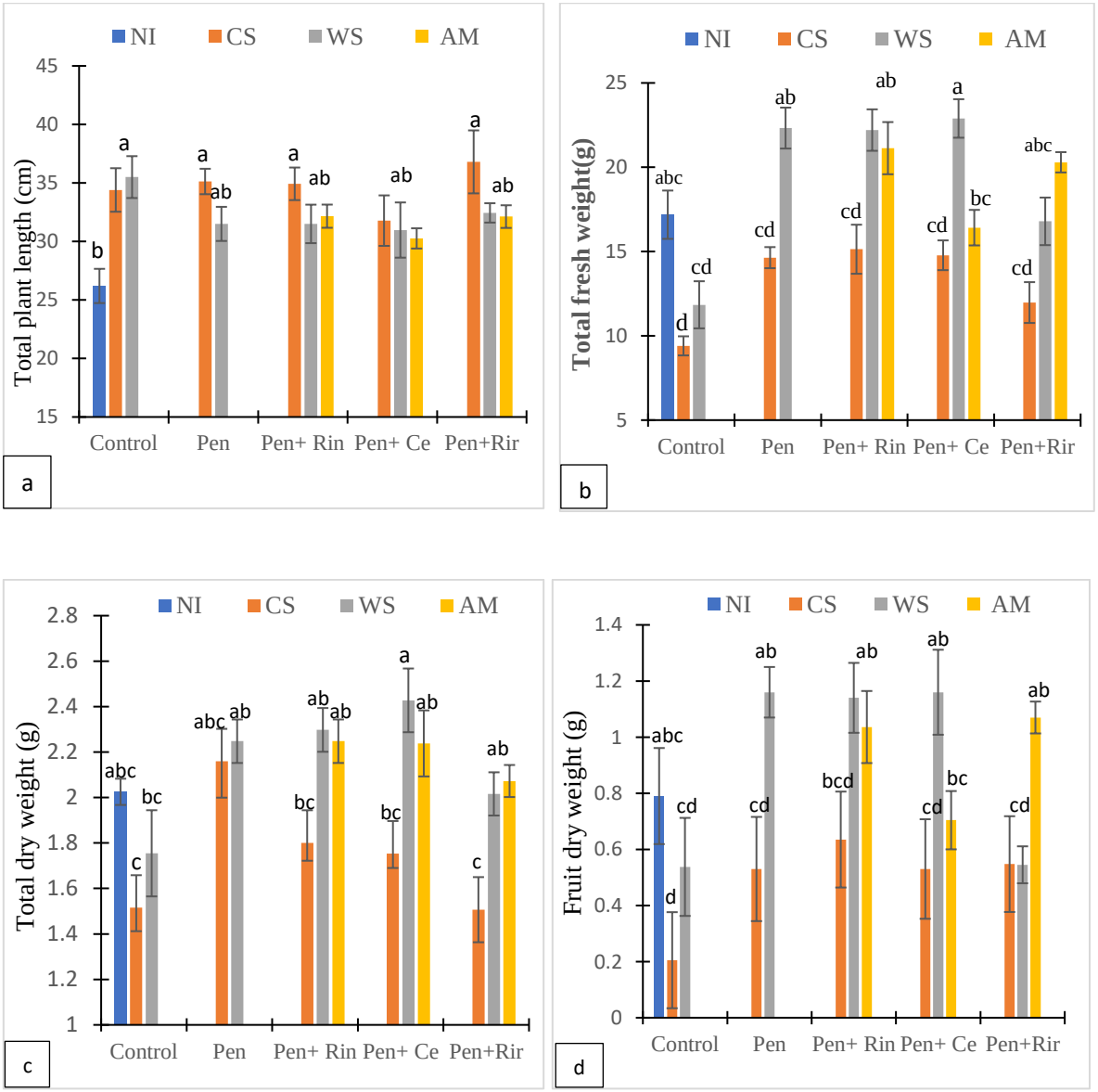

Fig. 2.4. Growth indices of tomato. Values are Mean±SEM. [abc]Superscripts indicate significant differences. NI= no inoculation; CS= corn substrate; WS= wheat substrate; Pen= *Penicillium* sp. EU0013; AM= single AM inoculation.

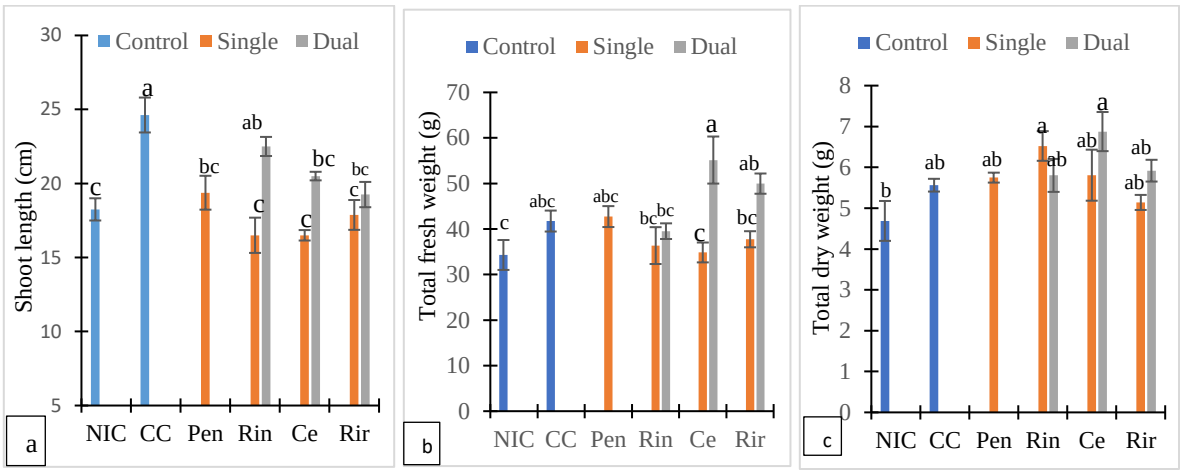

Fig. 2.5. Growth indices of lettuce (first experiment). NIC=non-inoculated control; CC=corn substrate control; Pen= *Penicillium* sp. EU0013; Rin, Ce, and Rir = single AM inoculation.

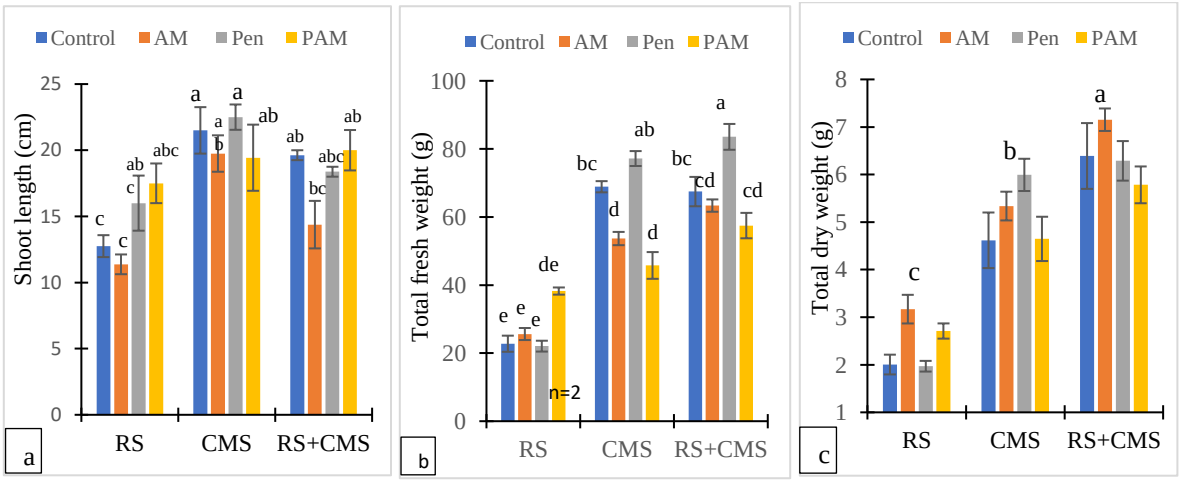

Fig. 2.6. Growth indices of lettuce (second experiment). RS= river sand; CMS= commercial soil; AM= single AM inoculation; Pen= *Penicillium* sp. EU0013; PAM= dual inoculation of AM and *Penicillium* sp. EU0013.

Table 2.1. Significance (*P*-values) of experimental factors and their interaction in the second lettuce experiment

	Parameter	Inoculation	Soil	Interaction
1	Shoot length	0.002535**	4.3E-07 ***	0.158439 ns
2	Fresh weight	4.25E-08 ***	5.4E-20 ***	2.65E-07 ***
3	Dry weight	0.069508 ns	3.77E-13 ***	0.126177 ns
4	Chlorophyll content	5.8472E-05 ***	0.0584790 ns	0.45533919 ns
5	Nitrogen in shoots	1.55E-06 ***	7.1E-15***	0.004411 **
6	K concentration	0.01490 *	0.28888 ns	0.135762 ns
7	Zn concentration	0.024877 *	8.08E-09 ***	0.049475 *
8	Mn concentration	0.112195 ns	1.76E-08 ***	0.252251 ns
9	Fe concentration	0.708907 ns	0.016519 *	0.000102 ***

* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; ns= not significant. Two-way ANOVA

soils. Total fresh weight (Fig. 2.6 b) was highest in mixed soil (RS + CMS) and lowest in RS. Inoculation, soil, and interaction were significant (Table 2.1) as single *Penicillium* inoculation gave better yield in commercial and mixed soils. In river sand, the dual inoculated group (which was replanted after all replicates initially died) showed initial retarded growth, wilting and 50% mortality (Plate. 2.2 (a & b)), but the surviving plants (n = 2) recovered and grew larger shoots. In commercial and mixed soils, dual inoculation also showed retarded growth and had lower fresh weights but there was no mortality. For total dry weights (Fig. 2.6 c), the effect of soil was significant as mixed soil had the highest values.

2.11.4. Lettuce physiological parameters

As dual inoculated plants suffered negative effects when *Penicillium* inoculum from wheat substrate was used, some physiological parameters were measured to assess host nutrient status. Chlorophyll levels in leaf (Fig. 2.7 a) was significantly affected by inoculation and

soil, with values in control generally lower than in inoculated groups. The highest values were in mixed soil and the lowest in river sand. Nitrogen in shoots (Fig. 2.7 b) showed significant differences with the highest values in commercial soil and the lowest in river sand, with generally higher values in inoculated plants. K concentration (Fig. 2.7 c) showed significant differences, with *Penicillium* group having the lowest values in commercial soil. Zn concentration in shoot (Fig. 2.7 d) showed significant differences with generally higher values in commercial soil. There was significant interaction between inoculation and soil (Table 2.1), as inoculated plants showed increases depending on soil. Mn concentration in shoot (Fig. 2.7 e) showed significant differences due to soil with the highest values observed

Plate 2.2. (a) Lettuce plants grown in river sand showing wilting of leaves in dual inoculated pots (L-R; control, *C. etunicatum*, *Penicillium* (WS), *C. etunicatum* + *Penicillium* (WS); (b) pots of dead plants in *C. etunicatum* + *Penicillium* (WS) treatment at time of harvest.

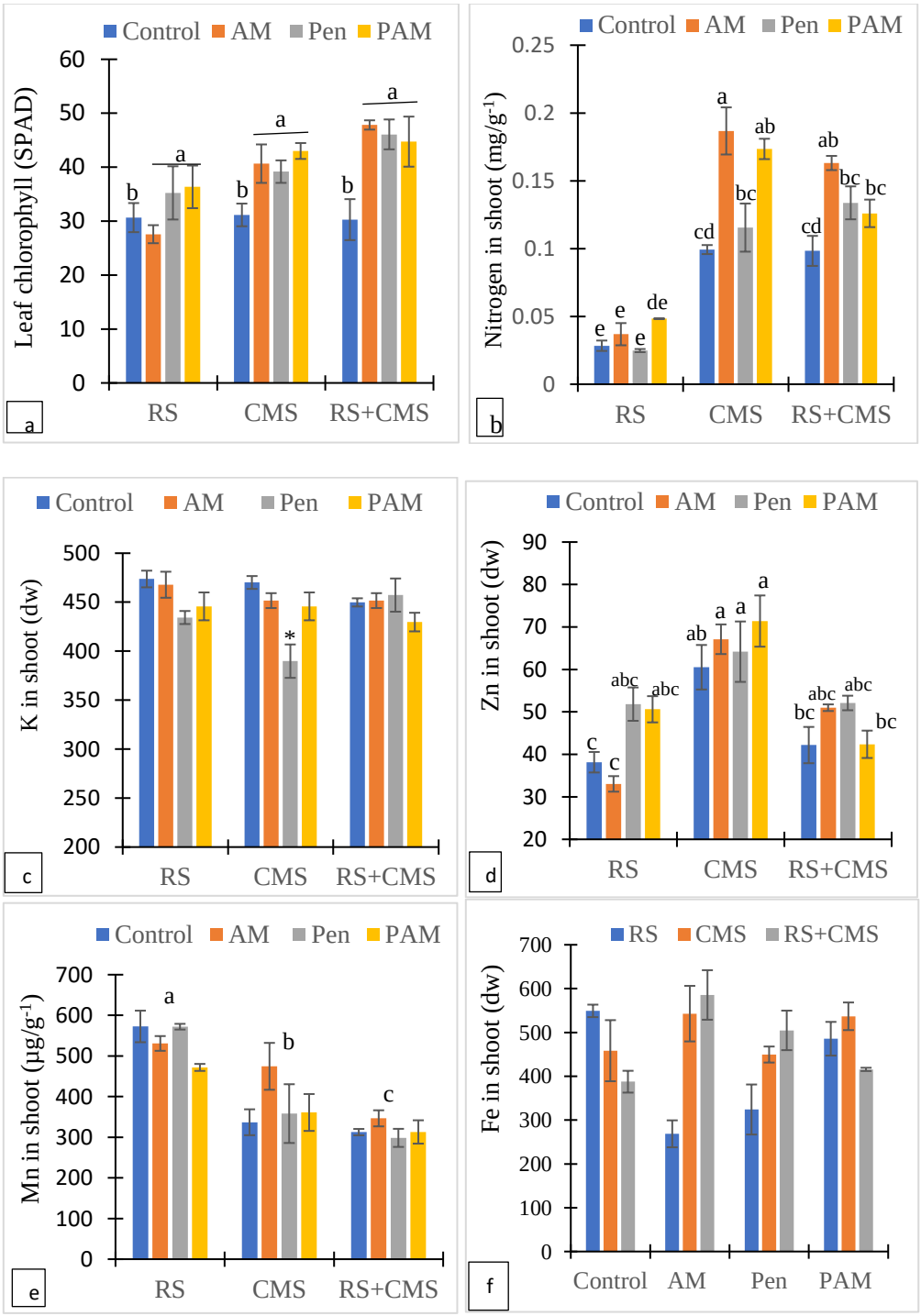

Fig. 2.7. Physiological parameters of lettuce (second experiment). RS= river sand; CMS= commercial soil; AM= single AM inoculation; Pen= *Penicillium* sp. EU0013; PAM= dual inoculation of AM and *Penicillium*. In (c), *=significantly different from control.

in river sand and the lowest in mixed soil. Fe concentration in shoot (Fig. 2.7 f) was significantly affected by soil and interaction (Table 2.1). In control plants, shoot Fe was highest in river sand and lowest in mixed soil, but the opposite trend was observed in single AM and *Penicillium* treatment. In general, the dual inoculated plants which showed negative growth indices did not exhibit a trend of lower chlorophyll, nitrogen, K, Zn, Fe, and Mn, compared to single inoculations or control.

2.12. Discussion

In both tomato and lettuce, AM fungal and *Penicillium* root colonization indices were generally higher in dual inoculation, compared to single. This suggests no indication of antagonism between both groups of fungi, for the species tested, but rather a stimulatory effect of each fungus on the other. The consistency of this feature in both tomato and lettuce makes it more likely an outcome of fungus-fungus interaction. A possible reason for this bio-stimulation could be competition for rhizosphere resources such as space and nutrients in host roots (Pearson et al., 1993). Compared to single inoculation, an observation of higher root colonization levels in dual inoculation of AMF (*Funneliformis mosseae*) and a phosphate-solubilizing fungus (*Mortierella* sp.), was reported by Xueming et al. (2014). However, Chandanie et al. (2009) reported similar levels of colonization between single and dual inoculations of *Glomus mosseae* and *Penicillium simplicissimum*. Such contrasts point to the significance of fungal identity in these partnerships. In our study, a potential significance of the fungus-fungus interaction (in *C. etunicatum* - *P. pinophilum* partnership) was revealed in lettuce in the form of retarded growth, wilting of leaves, and mortality in river sand, when the *Penicillium* inoculum was based on wheat medium. Although *P.*

pinophilum showed typical PGPF traits such as phosphate solubilization and increase in P concentration in shoots, and production of organic acid and siderophore (Khan et al., 2008, 2009), the susceptibility of host to a possible spectrum of co-infection stress, including mortality, was observed. The importance of inoculum substrate material is also seen where lettuce dually inoculated with *Penicillium* (CS) + *C. etunicatum* and *Penicillium* (CS) + *R. irregularis*, had higher biomass than control, with zero mortality. It is likely that the different outcomes between corn and wheat-based inoculum could be due to the preponderance of *Penicillium* propagules in the latter, as fewer number of spores were observed in corn than in wheat inoculum. While fewer studies have examined the impacts of variation in formulation and application methods, as well as substrate-induced effects (Bashan et al., 2014), growth-promoting effect can depend on rate and type of inoculum and substrates used (Marin-Guirao et al., 2016). Substrates such as wheat, barley and sorghum with high organic matter could favor development, reproduction, and maintenance of fungi, and promote their retention in substrate with high levels of propagules (Martinez-Medina et al., 2009; Rajput et al., 2014). Given that *C. etunicatum* is a spore producing AM fungi, the higher *Penicillium* spore numbers in wheat inoculum apparently caused higher infection stress during dual inoculation. In terms of inoculation strategy (single vs dual) in lettuce, dual inoculation (Pen + Ce) was better for improving lettuce biomass using corn-based inoculum, while single *Penicillium* inoculation using wheat-based inoculum gave better fresh weights in commercial and mixed soils. In addition to higher *Penicillium* spore numbers in wheat inoculum than in corn, differences in endophyte growth patterns as it colonizes roots may stem from variation in nutrient composition of the two substrates, and potentially compound the co-infection

outcomes in lettuce. Differences in substrate effect on fungal composition and promotion of growth and yield of host has been reported by Nieto and Chegwin (2013) and Ha et al. (2015). Other substrates used for *Penicillium* inoculum preparation includes barley grain, which showed growth promoting effect and high colonization of host roots (Hossain et al., 2014). Comparing the host fresh weights (yield) in both lettuce experiments (with respect to river sand), shows that the absence of nitrogen and phosphorus fertilizations ($(NH_4)_2SO_4$ and K_2HPO_4) in the second experiment, led to lower yields, compared to the first. Ostensibly, the absence of fertilization affected hosts in river sand more than those in commercial and mixed soils, and likely exacerbated the dual infection stress on lettuce seedling in river sand. The repeated occurrence of plant mortality in river sand only, shows that in commercial and mixed soils where mortality was not seen, greater seedling stamina was displayed during co-infection, although fresh weight values were lower at harvest. Higher shoot nitrogen content and chlorophyll levels in commercial and mixed soils than in river sand, clearly indicate a lower fertility of the latter for the host. Nitrogen is essential for building tissues, hence the mycobionts-induced wilting of leaves in co-inoculated plants under river sand. The recovery of the surviving dual inoculated plants in river sand, might be linked to subsequently improved nitrogen status in hosts since the survivors had the highest nitrogen content in shoots within river sand treatment. Considering that mortality of dually inoculated host plant occurred twice when wheat inoculum of *Penicillium* was used with *C. etunicatum*, it appears the co-infection stress build-up in the lettuce host could be rapid and transient (as in river sand where wilting, slow growth, mortality, and recovery occurred), or gradual and persistent (as in commercial and mixed soil where slower growth occurred but no mortality). Since the

groups which showed negative growth indices did not exhibit lower chlorophyll, nitrogen, K, Zn, Fe, and Mn compared with other treatment groups, the phytohormones, soluble sugars, and enzymes in the *C. etunicatum – P. pinophilum* interaction, are targets for further exploration. Plant growth at root and shoot apices are under the control of hormones such as gibberellins and auxins. While AMF influences plant hormones, *Penicillium* sp. are known to stimulate gibberellins and indole-3-acetic acid (IAA) production to enhance host growth (Khan et al., 2008; Radhakrishnan et al., 2013; Smith and Read, 2008). Overall, this indicates a complex interaction between the environmental conditions (soil and nutrients), inoculum substrate material vis-a-vis inoculation strategy (single vs dual), fungal consortium composition and target host. While Wakelin et al. (2007) reported that *Penicillium* growth-promoting effect may be related to soil condition, nutrient limitation has been known to influence endophyte-host interaction negatively (Cheplick et al., 1989). Depending on soil, AMF, and *P. pinophilum* showed ability to improve Zn in shoots, as well as Fe, but not K. Fungal mobilization of trace elements is one of their mechanisms for affecting host growth (Smith and Read, 2008; Khan et al., 2009), but it appears that the soil used for cultivation had controlling effect on plant nutrient concentrations and mycobionts effects, as evident in Mn and Fe. In tomato and lettuce, uninfected controls that received sterile inoculum had higher total plant lengths than those that did not. It is known that nutrients in inoculum material may, on their own, influence plant growth. For plant dry weights in tomato, increases in dual inoculation was indicated when wheat substrate was used for *Penicillium* inoculum, while single *C. etunicatum* improved dry weight showing a consistent effect in-line with a previous report using this AMF in soybean (Ibiang and Sakamoto, 2018).

2.13. Conclusion

The fungus-fungus interaction between *P. pinophilum* and AM fungi stimulates higher levels of host root colonization during dual inoculation in both tomato and lettuce. Depending on the soil, host, substrate used for inoculum formulation and fungal consortium composition, the growth response of tomato and lettuce to colonization by arbuscular mycorrhizal fungi and *P. pinophilum* show potential for a spectrum of negative effects during AMF-*Penicillium*-plant interaction. While no negative effects on host growth was observed in dually inoculated tomato, lettuce plant co-inoculated with *P. pinophilum* and *C. etunicatum* suffered retarded growth, wilting of leaves, and mortality when wheat rather than corn-based inoculum of *Penicillium* was used, in river sand. Mortality of lettuce plants did not occur in commercial soil and mixed soil, but growth depression and reduced fresh weights were observed, indicating an influence of the soil on the host response. The pattern of negative effects could be rapid and transient, where wilting, slow growth, mortality, and recovery occurred (in river sand); or gradual and persistent, where there was slower growth but no mortality (as in commercial and mixed soils). Tomato responded better to dual inoculation of wheat substrate than corn and single fungal treatments, while lettuce responded better to *Penicillium* inoculation using wheat inoculum, or dual inoculation with *C. etunicatum* using corn inoculum of *Penicillium*. Although *P. pinophilum* improved Zn and P uptake and showed typical PGPF characteristics such as phosphate solubilization, organic acid and siderophore production *in vitro*, a susceptibility of host to potential co-infection stress (during partnership with a spore producing AM fungi) has been demonstrated. The target plant, soil, inoculum substrate material, and inoculation strategy (single or dual) vis-a-vis´fungal

consortium composition, needs to be considered carefully in the deployment of AMF and *P. pinophilum* for use in boosting vegetable production.

CHAPTER THREE

Modulation of phytochemicals and essential trace elements in fruits of different tomato cultivars by the endophytic fungus *Penicillium pinophilum* EU0013

3.1. Abstract

The present study investigated the effects of the endophytic fungus, *Penicillium pinophilum* EU0013 on fruit phytochemical indices and essential trace elements in five tomato cultivars. In a completely randomized design, inoculated and uninoculated seedlings of tomato cultivars (Momotaro, Rodeo, Anaya, Reika, and Cherry) were raised for sixteen weeks in a greenhouse. Fruit fresh weights and root colonization by *P. pinophilum* were significantly higher in the Rodeo cultivar than in the other cultivars tested. Significant effects of the cultivar, inoculation, and interaction on fruit dry weights were observed with higher values in Anaya inoculated with *P. pinophilum*. Cultivar and inoculation effects were significant for ascorbic acid and soluble sugars in four cultivars, with increases being observed due to the *P. pinophilum* inoculation. Lycopene levels increased in Rodeo and decreased in Anaya, while β-carotene levels increased in four cultivars due to the inoculation. Manganese concentrations were significantly increased in Cherry, while iron concentrations were increased in Reika and Cherry. Increases due to the inoculation were observed for gibberellic acids (GA_1 and GA_4) in Reika and Anaya, whereas decreases were detected in Cherry and Rodeo. Similar results were obtained for abscisic acids (ABA) with increases in Reika and Anaya due to the inoculation. *P. pinophilum* demonstrated the ability to improve the nutritive value of tomato fruits via modulations of phytochemicals in addition to increases in Mn and Fe concentrations, particularly in Cherry and Rodeo. Cultivar responses to *P. pinophilum*

inoculation are a factor that need to be considered for its use in increasing fruit quality indices in tomato.

3.2. Introduction

Tomato (Solanum lycopersicum L.) is a popular and highly consumed fruit/vegetable worldwide. It is considered to be one of the most economically important crops worldwide and is a rich source of minerals, vitamins, organic acids, essential amino acids, and dietary fibers, which are vital for the general health of humans by minimizing the risk of diseases and other medical conditions (Wargovich, 2000; Oguntibeju, 2013). The nutritive value of tomato fruits, such as color, flavor, and taste, mainly depends on lycopene, β-carotene, ascorbic acid, gibberellic acids (GA), sugars, indole acetic acids (IAA), abscisic acid (ABA), and essential trace elements (Ilahy et al., 2009). Lycopene and β-carotene are among the most important carotenoids in tomato and function as antioxidants. However, their accumulation in fruits depends on the cultivar/genotype, ripening stage, and environmental factors (Tilahun et al., 2017). Environmental factors include the prevailing abiotic and biotic conditions impacting plants, which interact with genetic factors, such as the cultivar type, to shape the plant nutrient physiology. Food quality and human health are intertwined, from essential trace elements, such as iron (Fe), zinc (Zn), and manganese (Mn), to antioxidative phytochemicals, including polyphenols, β-carotene, and ascorbic acids. The nutrient quality of foods needs to be assessed and improved for many individuals who may be affected by insufficient intake via the diet (FAO, 2020). There is also an increased recognition of the value of natural over synthetic antioxidants in our diets for the modulation of oxidative stress stemming from injury and metabolic disorders (Mahmood et al., 2021). Microbial

biofortification is a potentially attractive plant-microbe interaction strategy for improving the nutrient quality of foods because the bacterial and fungal modulation of phytochemicals in host plants and the rhizosphere may lead to higher levels of polyphenols, organic acids, sugars, and micronutrients (Khan et al., 2009; Ibiang et al., 2018). Endophytes are microorganisms that inhabit the internal parts of plants for at least part of their life cycle without causing disease and include *Penicillium* sp., *Colletotrichum tofieldiae* (Hiruma et al., 2016), and *Acremonium* sp. (Khan et al., 2021). *Penicillium* sp. are popular endophytes for their secretion of bioactive compounds and roles as biocontrol agents against plant diseases (Guijarro et al., 2017). The endophytic fungus *Penicillium pinophilum* EU0013, isolated from eucalyptus roots (Teshima and Sakamoto, 2006), has been shown to improve seed germination, plant growth, and tolerance to Fusarium wilt in tomato (Alam et al., 2011). Its abilities to solubilize phosphate, produce organic acids and siderophores *in vitro*, and improve tolerance to *Verticillium* wilt in tomato have been demonstrated (Ibiang et al., 2020, 2021). It is now being considered as a candidate for more widespread deployment; however, its impact on fruit quality indices in tomato remain unclear. Therefore, the present study investigated the effects of *P. pinophilum* EU0013 inoculation on fruit phytochemical indices and essential trace elements, namely Zn, Fe, and Mn, in five tomato cultivars obtained from Nigeria and Japan. Since *P. pinophilum* was originally isolated in Japan, we broadened the cultivar domicile by utilizing two from Nigeria (tropical region) and three from Japan (temperate region).

3.3. Materials and Methods

3.3.1. Soil

The soil utilized was a mixture of river sand and commercial soil (Premium soil, Setoharakaen) with pH 7.4, EC of 75.8 mS m^{-1}, available Zn of 25.4 μg g^{-1} soil, Fe of 16.1 μg g^{-1} soil, Mn of 11.9 μg g^{-1} soil, and Cu of 0.3 μg g^{-1} soil. River sand was sieved with a 2-mm mesh and both soils were mixed (1:1 v/v), autoclaved at 121 °C for 2 h, and then allowed to cool for 2 days before use.

3.3.2. Plant

Five tomato (*Solanum lycopersicum* L.) cultivars (Momotaro, Rodeo, Anaya, Reika, and Cherry) were examined in the present study. Anaya and Rodeo (supplied by AGRITROPIC, Nigeria) are planted by farmers in Nigeria, while Reika, Cherry, and Momotaro (supplied by Takii Seed) are cultivated in Japan. Tomato seeds were sterilized in 70 % ethanol for 5 min and 33.3 % sodium hypochlorite solution for 15 min and then rinsed repeatedly in sterile distilled water before seeding in a growth chamber to obtain seedlings. The conditions in the growth chamber were as follows: 140 μmol m^{-2} s^{-1} fluorescent light (25 °C, 14 h) and dark (18 °C, 10 h). Seedlings were raised on vermiculite moistened with half-strength Hoagland solution for four weeks before being transplanted to potted soils.

3.3.3. *Penicillium* inoculation

P. pinophilum EU0013 (available from the National Institute of Technology and Evaluation, NBC accession number 100411) was obtained from actively growing margins of 7-10 days old potato dextrose agar (PDA) culture medium. A wheat bran substrate-based inoculum was prepared for the *Penicillium* inoculation. The inoculum was prepared as described by

Chandanie et al. (2006), but with an incubation temperature of 25 °C. After a 10 days incubation, completely colonized wheat was blended, and 0.5 g (3.6×103 spores) was added to the middle of the designated pots before seedling transplant, while non-penicillium pots received no inoculum (Ibiang et al., 2020).

3.3.4. Experimental design and plant cultivation

The experiment was laid out in a randomized 5×2 factorial representing cultivars and inoculation treatments. Regarding the inoculation, uninoculated control (EU0013-) and inoculated (EU0013+) groups were set up for each cultivar, with three replicates (n=3), giving 30 pots. Plant cultivation was performed between May and August 2020 in a greenhouse for 16 weeks. All planted pots were equally fertilized with full-strength Hoagland solution applied (100 mL pot^{-1}) once every two weeks until harvest, while regular watering was conducted with tap water.

3.3.5. Data collection

All fruits produced by the respective plants were harvested after 16 weeks, weighed, and sorted into green, breaker, turning, pink, light red, and red to cover all of the ripening stages of tomato (de Jong et al., 2009). Light red and red fruits (stages 5 and 6) were more abundant than the others and were utilized in fruit phytochemical and essential element analyses. Selected fruits were longitudinally sectioned into four equal parts, and one randomly selected portion for the phytochemical analysis was wrapped with aluminum foil and submerged in liquid nitrogen before being stored at -80 °C. Leftover fruit tissues were placed in an oven for drying at 105 °C until a constant weight, and dry fruit weights were recorded for each

plant. Roots were obtained and washed under running tap water, and a portion was subtracted to assess endophyte root colonization.

3.3.6. Root colonization by *Penicillium*

Root samples were washed thoroughly with running tap water and washed again with sterile water before use. Ten root segments of 0.5 cm were cut and placed on PDA media in Petri dishes amended with a penicillin antibiotic (200 mg L^{-1}) to suppress the growth of oth.er ubiquitous microorganisms. Petri dishes were placed in an incubator at 25 °C for 6 days, colonies of *P. pinophilum* growing from root segments were counted, and the colonization frequency was calculated as %CF=(Ncol/Nt)×100, where Ncol=the number of root segments colonized by the fungus and Nt=the total number of segments of roots studied, as described by Hata and Futai (1995).

3.3.7. Total soluble sugars

Total soluble sugars were assessed using a brix refractometer (PAL-BX/ACID F5 Master Kit; ATAGO) according to the method of Tefera et al. (2007). Freshly harvested tomato fruits were diced into smaller pieces. Approximately 2 g of each diced fruit was placed into tea bags and the juice was extracted by squeezing the tea bag. Two drops of the juice were placed on the prism and the measurement time was within 3 s. Distilled water was used to clean between readings and values were shown as percentages.

3.3.8. Measurement of Zn, Fe, and Mn

Dried fruit samples were ignited in an electric furnace (ADVANTEC FUL220FA) at 550 °C for 6 h (heat-up time of 20 min) and then digested in 0.6 mol L^{-1} HCl. Element concentrations in solutions were assessed by atomic absorption spectrophotometry (Shimadzu AA-6600F).

3.3.9. Polyphenol content in fruits

Total polyphenols in fruit samples were measured via the Folin-Ciocalteu method (Amerine and Ough, 1980). Dried samples (0.3 g) were extracted in 70% acetone and 2.5 mL of 10-fold diluted Folin-Ciocalteu solution was added, followed by 2.0 mL of Na_2CO_3 solution (75 g L^{-1}) after 2 min. Chlorogenic acid was used as the standard and absorbance was measured in a spectrophotometer (U-1800 Hitachi High Tech) at 760 nm.

3.3.10. Ascorbic acid assessment

Ascorbic acid measurements were performed using the Shimadzu Prominence HPLC system (Shimadzu) according to Brause et al. (2003). The extraction solution was freshly prepared by dissolving 56 g of metaphosphoric acid in 1 L of water on the day of extraction. Plastic tubes (50 mL) were filled into racks and 35 mL of extraction solution was added to each tube. Frozen tomato samples (5 g) were placed into tubes and an additional 10 mL of extraction solution was added. Samples were homogenized at 200×g for 30 s and then filtered through a 110-mm filter paper. The filtrate was further filtered with a 0.45-μm sterile filter connected to a syringe and collected into a 1.5-mL tube. The HPLC syringe was used to collect 0.05 mL of the filtrate and injected into the machine to measure concentrations. During the analysis, the fitted column was a Unison UK-C18 (3 μm, 4.6×150 mm) ODS column (Imtakt); the mobile phase was 2 mM $HClO_4$ and 100 mM $NaBH_4$, which were pumped at flow rates of 1.0 mL min^{-1} and 0.5 mL min^{-1}, respectively; the injection volume was 20 μL; the column temperature was maintained at 40 °C; and UV detection was performed at 300 nm.

3.3.11 Measurement of lycopene and β-carotene

Pigment concentrations were measured according to the method of Nagata and Yamashita (1992). Sample pigments were extracted once with acetone-hexane (4:6) solvent. Twenty milliliters of the solvent were measured into glass tubes, and 1 g of the frozen tomato sample was added. Samples were then homogenized for 2 min and kept until the solvent was separated from the solute. Three milliliters of the clear solvent/supernatant were placed in a glass cuvette and absorbance (A) was measured at 663, 645, 505, and 453 nm in a spectrophotometer (U-1800 Hitachi High Tech). Pigment concentrations (mg 100 mL^{-1}) were calculated as stated below:

Chlorophyll a = (0.999×A663)–(0.0989×A645)

Chlorophyll b = (–0.328×A663) + (1.77×A645)

Lycopene = (–0.0458×A663) +(0.204×A645) +(0.372×A505)–(0.0806×A453)

β-Carotene = (0.216×A663) -(1.22×A645) -(0.304×A505) +(0.452×A453)

3.3.12 Measurement of GA_1, GA_4, IAA, and ABA

The extraction and quantification of GAs, IAA, and ABA were performed as described by Manzi et al. (2015) with some modifications (Opio et al., 2020). Approximately 0.5 g of diced and frozen tomato fruit was crushed in 20 mL methanol (80 % [v/v]) containing butylhydroxytoluene (0.1 g L^{-1}) and ascorbic acid (0.1 g L^{-1}). Samples were homogenized and all homogenates were immediately fortified with 100 ng of the deuterated isotopes of [$2H_2$]-GA_1 and [$2H_2$]-GA_4, with 200 ng each of the stable isotopes of phenyl-13C6 IAA (Cambridge Isotope Laboratories) and 3′,5′,5′,7′,7′,7′-hexadeuterated ABA (ABA-d6) being

used as internal standards. Samples were vortexed and stored at 4 °C overnight before use. Homogenates were centrifuged the following day at 19,000×g at 4 °C for 15 min before filtering through a membrane filter (pore size 0.22–0.4 μm) and then through a Sep-Pak cartridge. Filtrates were rotary-evaporated at 40 °C to dryness. The aqueous layer was recovered, dissolved in 2 mL of 1% (v/v) acetic acid, and the solution was purified using Sep-Pak C18 cartridges (Waters). Cartridges were initially conditioned using 5 mL of methanol in 1% acetic acid, and 5 mL of 1% acetic acid was eluted through and discarded before samples were introduced. Once more, 5 mL of 1% acetic acid was added with all the eluates discarded, and the solution containing phytohormones was eluted using 5 mL of 80 % methanol containing 1 % acetic acid, with the eluates being collected and evaporated to dryness. The residue was then dissolved with 1 mL of 1 M formic acid. Samples were loaded into an Oasis MCX column (60 mg sorbent; Waters), which had been pre-conditioned with 5 mL methanol and equilibrated with 5 mL formic acid. The column was washed with 5 mL formic acid with the eluent discarded. Phytohormones were subsequently eluted through the column with 5 mL methanol (100 %) and collected for purification and quantification. The quantification of GAs was performed using a LC/MS-2010EV (Shimadzu) with a cooled autosampler and LC-10ADvp pump (Shimadzu) at a voltage of 1.5 kV and column temperature of 40 °C connected to a mass spectrometer equipped with an electron spray ionization (ESI) source operated in the positive analytical mode. Separation was achieved by an ODS Mightysil RP-18 column (150 × 2.0 mm i.d., 5 μm), and data acquisition software was Labsolutions Ver. 3. Using an injection volume of 1 μL, compounds were isocratically eluted with methanol containing 20 mM formic acid (80:20 [v/v]) at a flow rate of 0.3 mL

min^{-1}. LC-MS conditions were as follows: 4.5 kV for the ESI spray voltage, 250 °C for the curved desolvation line (CDL) and block heater temperatures, and 1.5 mL min^{-1} for the nebulizer gas (N_2) flow. The quantification of phytohormones was performed using selected ion monitoring. The parent and fragment ions used for quantification were as follows: m/z=347 and 349 for GA_1 and 331 and 333 for GA_4, respectively (Opio et al., 2020). The column temperature was a step gradient of 60 °C for 2 min, 60–270 °C at 10 °C min–1, and 270 °C for 35 min. The mass-to-charge ratios (m/z) were 130 and 189 for methyl-IAA, 135 and 195 for methyl- [13C6] IAA, 162 and 190 for methyl-ABA-d0, and 169 and 194 for methyl-[ABA-d6]. Endogenous IAA and ABA concentrations were calculated from the peak ratios of m/z 130/135 and 190/194, respectively (Opio et al., 2020).

3.3.13 Statistical analysis

Data were subjected to a two-way analysis of variance (ANOVA) using STATCEL version 4 (OMS) with significance set at $P<0.05$ followed by the Tukey-Kramer test for mean separation. Regarding root colonization, EU0013– groups were devoid of *Penicillium* colonies and were excluded from the statistical analysis; therefore, a one-way ANOVA was performed for the five cultivars in the EU0013+ series.

3.4. Results

3.4.1. Fruit biomass, leaf chlorophyll content, and root colonization

Fruit fresh weight (Fig. 3.1a) showed significant differences (Table 3.1) due to the cultivar, with the highest values being observed in Rodeo and the lowest in Cherry. Fruit dry weight (Fig.3.1b) showed significant differences due to the cultivar, inoculation, and

cultivar×inoculation interaction. In Anaya, the *P. pinophilum* inoculation significantly increased the fruit dry weight over that of the uninoculated control. The leaf chlorophyll

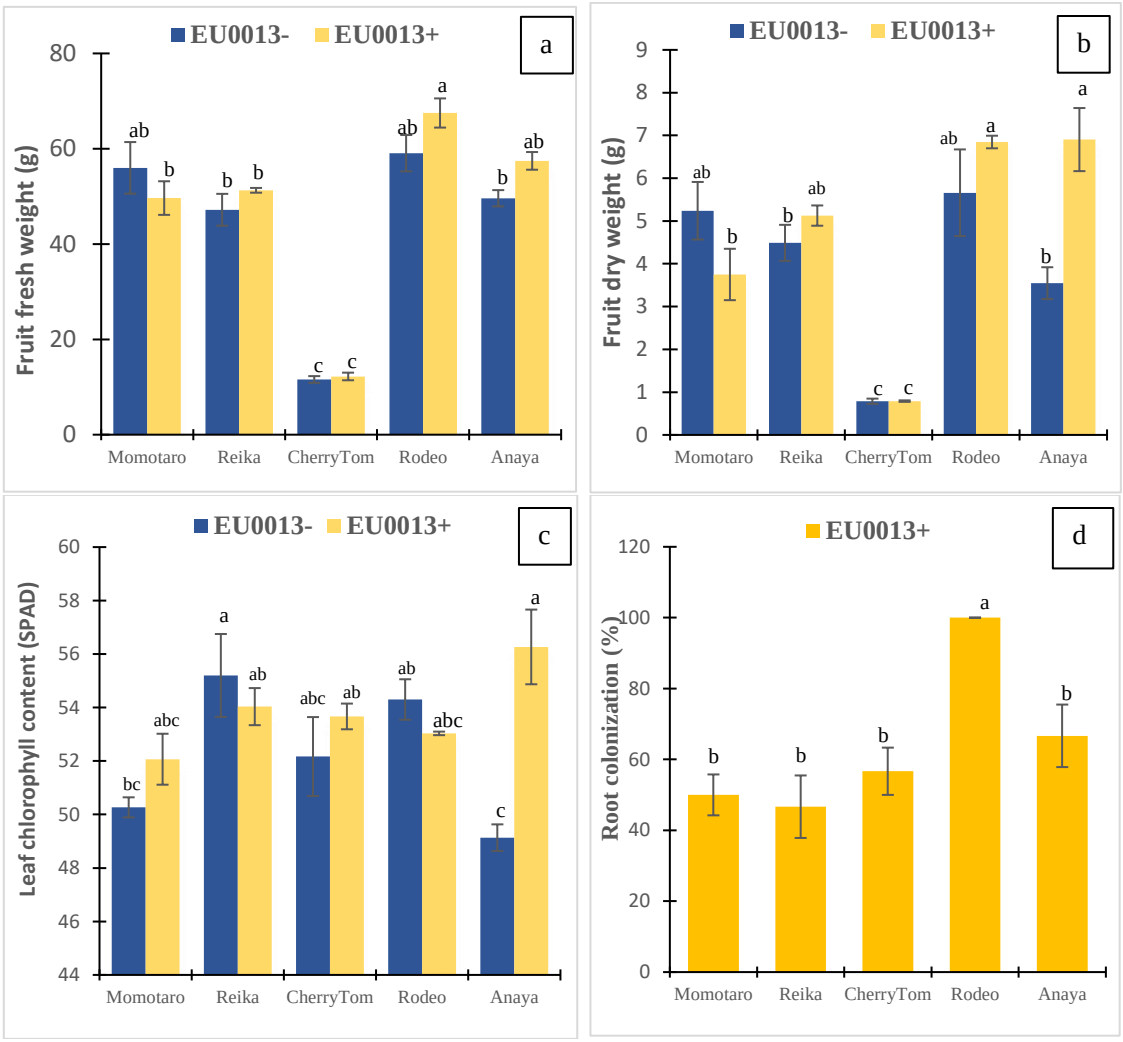

Fig. 3.1. Effects of the *Penicillium pinophilum* EU0013 inoculation on growth and root colonization of five tomato cultivars (a) fruit fresh weights, (b) fruit dry weights, (c) leaf chlorophyll contents, and (d) root colonization in tomato cultivars. Values are means±SEM (n=3). ^{abc} indicate differences based on the Tukey-Kramer test.

Table 3.1. *P* values showing significance of treatment effects and their interaction based on ANOVA

	Parameter	Cultivar	Inoculation	Cultivar × Inoculation
1	Lycopene	0.008**	0.794 ns	0.064 ns
2	β-carotene	1.96E-05***	0.00049***	0.0204*
3	GA_1	0.259 ns	0.705 ns	0.002**
4	GA_4	0.0065**	0.0052**	1.05E-08***
5	IAA	5.69E-06***	0.001**	1.68E-06***
6	ABA	0.259 ns	0.38 ns	0.047*
7	Soluble sugars	0.020*	0.007**	0.514 ns
8	Ascorbic acids	1.69E-06***	0.0013**	0.185 ns
9	Polyphenols	0.0015**	0.6614 ns	0.6913 ns
10	*Penicillium* root colonization	0.0016 **		
11	Leaf chlorophyll	0.023*	0.015*	0.001**
12	Fruit fresh wt.	1.23E-12***	0.126 ns	0.109 ns
13	Fruit dry wt.	2.49E-08***	0.038*	0.003**
14	Mn	0.001**	0.779	1.27E-05***
15	Zn	0.03*	0.797 ns	0.625 ns
16	Fe	0.022*	0.0259*	0.048*

*=P<0.05; **=P<0.01; ***=P<0.001; ns=not significant. One-way ANOVA (*Penicillium* root colonization), Two-way ANOVA (other parameters).

content (Fig. 3.1c) showed significant differences due to the cultivar, inoculation, and cultivar×inoculation interaction. In Anaya, the *P. pinophilum* inoculation significantly increased the leaf chlorophyll content over that of the uninoculated control. Root colonization by *P. pinophilum* (Fig. 3.1d) was significantly higher in Rodeo than in the other cultivars. Uninoculated plants were devoid of *P. pinophilum* colonies.

3.4.2. Pigmentation indices

Lycopene (Fig. 3.2a) showed significant differences due to the cultivar, with the highest values being observed in Reika and Rodeo inoculated with *P. pinophilum;* however, the effect of inoculation was not significant. β-carotene (Fig. 3.2b) showed significant effects of the cultivar, inoculation, and cultivar×inoculation interaction. The highest values were observed in Cherry inoculated with *P. pinophilum*, while the lowest were in Momotaro inoculated with *P. pinophilum*. In four out of five cultivars, the inoculation with *P. pinophilum* increased the β-carotene content over that of the uninoculated control.

3.4.3. Total soluble sugars, ascorbic acid, and polyphenol

Total soluble sugars (Fig. 3.3a) showed significant differences due to the cultivar and inoculation. Except for Reika, the *P. pinophilum* inoculation increased soluble sugars in four cultivars over those in the uninoculated control. Ascorbic acid (Fig. 3.3b) also showed significant differences due to the cultivar and inoculation, with the highest values being observed in Cherry. Total polyphenols (Fig. 3.3c) showed significant differences due to the cultivar and cultivar×inoculation interaction, with the highest values being detected in Cherry and Rodeo and the lowest in Reika.

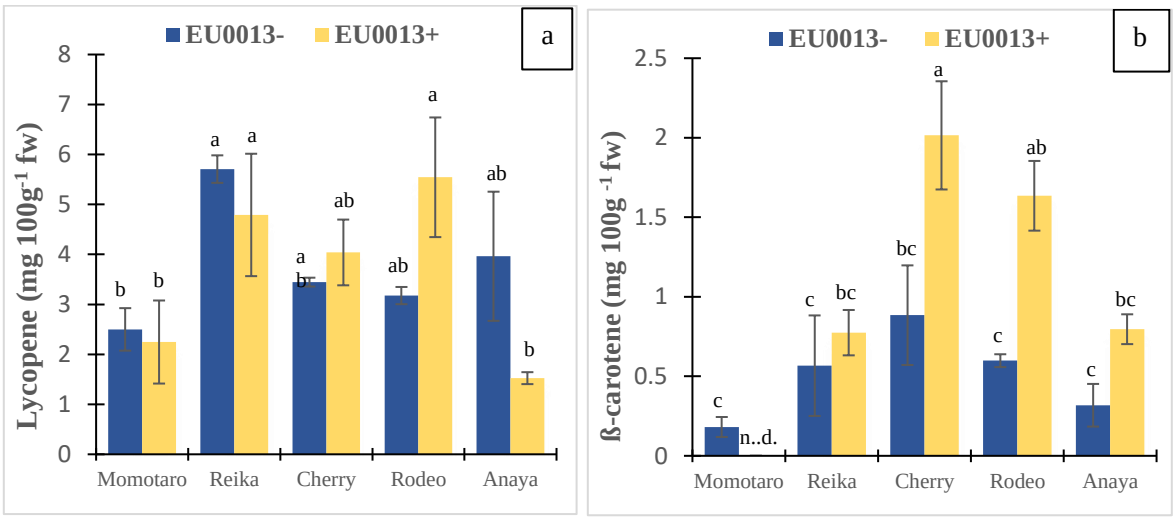

Fig 3.2. Effects of the *P. pinophilum* EU0013 inoculation on fruit pigmentation indices in tomato cultivars. (a) Lycopene, (b) β-carotene.Values are means±SEM (n=3). [abc] superscripts indicate differences based on the Tukey-Kramer test. n.d.=not determined.

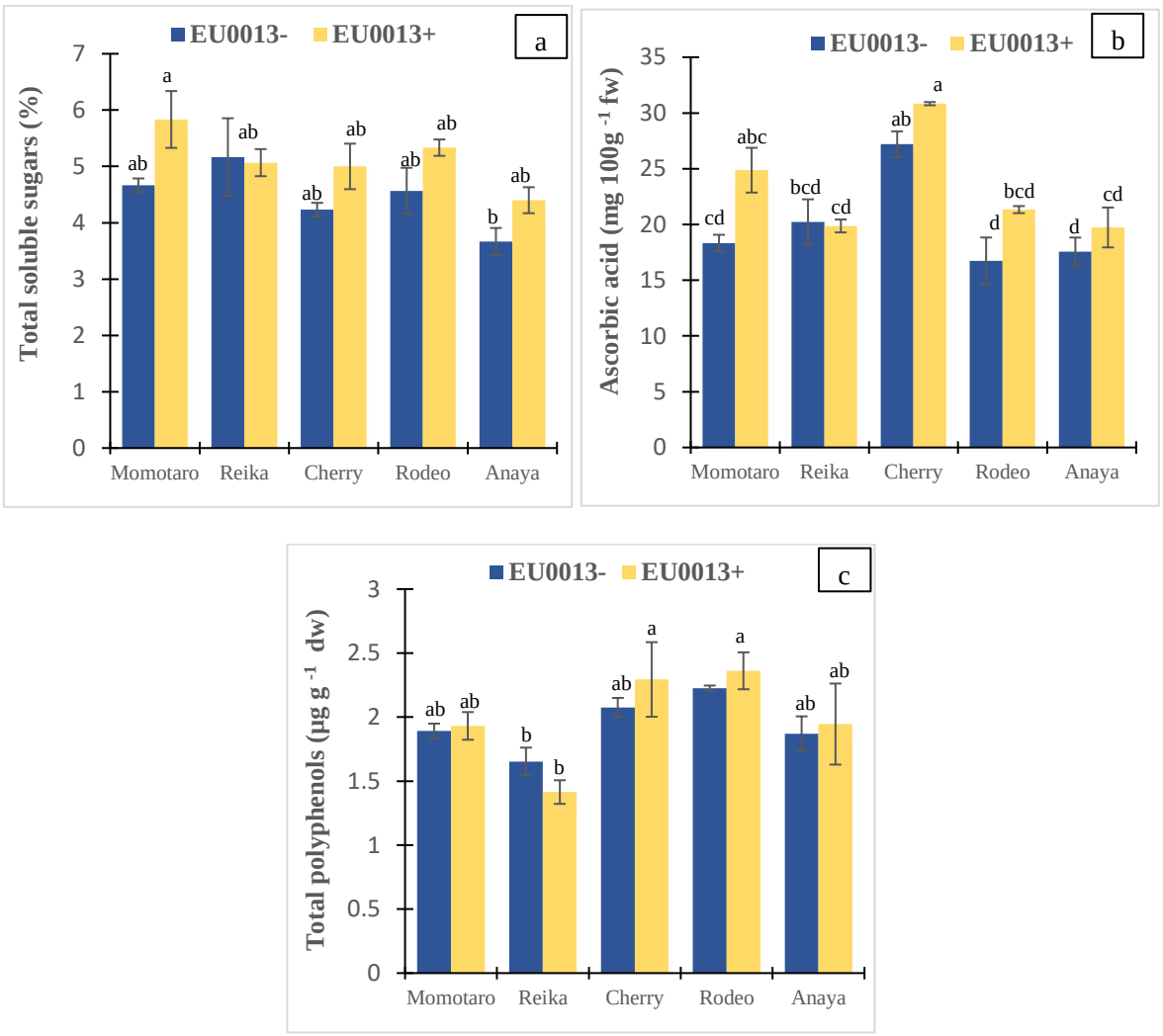

Fig 3.3. Effects of the *P. pinophilum* EU0013 inoculation on fruit biochemical indices in tomato cultivars. (a) total soluble sugars, (b) ascorbic acid, and (c) total polyphenols. Values are means±SEM (n=3). ^{abc} indicate differences based on the Tukey-Kramer test. n.d.=not determined.

3.4.4. GAs, IAA, and ABA

GA_1 (Table 3.2) showed significant differences due to the cultivar×inoculation interaction. The *P. pinophilum* inoculation increased GA_1 levels in Reika and Anaya, but decreased those in Momotaro, Cherry, and Rodeo. GA_4 (Table 3.2) showed differences due to the cultivar, inoculation, and cultivar×inoculation interaction. Reika and Anaya also showed increases in GA4 levels due to the *P. pinophilum* inoculation, while the reverse was observed in Momotaro, Cherry, and Rodeo. IAA (Table 3.2) showed significant differences due to the cultivar, inoculation, and cultivar×inoculation interaction. The highest values were observed in Momotaro inoculated with *P. pinophilum*; however, values in Cherry, Rodeo, and Anaya with the *P. pinophilum* inoculation were lower than that in the uninoculated control. ABA (Table 3.2) showed significant differences due to the cultivar and cultivar×inoculation interaction. The *P. pinophilum* inoculation increased ABA levels in Reika and Anaya but reduced them in Momotaro. In Reika and Anaya, the *P. pinophilum* inoculation consistently increased ABA, GA_1, and GA_4 levels over those in the uninoculated control.

3.4.5. Zn, Fe, and Mn concentrations

Mn concentrations (Table 3.3) showed significant differences due to the cultivar and cultivar×inoculation interaction. The *P. pinophilum* inoculation increased Mn concentrations in Cherry, but decreased them in Momotaro, Rodeo, and Anaya. Zn concentrations (Table 3.3) showed significant differences due to the cultivar, with higher values being observed in Cherry. Fe concentrations (Table 3.3) showed significant differences due to the cultivar, inoculation, and cultivar×inoculation interaction. The *P. pinophilum* inoculation increased Fe concentrations in Reika and Cherry over that in the uninoculated control.

Table 3.2: Effect of *P. pinophilum* EU0013 inoculation on Gibberellic acids, IAA, and ABA contents in fruits of tomato cultivars

		Momotaro		**Reika**		**Cherry**		**Rodeo**		**Anaya**	
		EU0013-	EU0013+	EU0013-	EU0013+	EU0013-	EU0013+	EU0013-	EU0013+	EU0013-	EU0013+
1	GA_1	87.06^{ab}	66.98^{ab}	69.91^{ab}	126.87^{ab}	177.05^{a}	100.73^{ab}	138.17^{ab}	67.17^{ab}	42.16^{b}	184.97^{a}
	(**µmol kg^{-1}fw**)	±4.55	±2.65	±17.55	±34.13	±34.02	±37.03	±46.51	±9.63	±11.52	±26.57
2	GA_4	221.02^{bc}	127.02^{c}	52.02^{c}	697.15^{a}	368.70^{bc}	125.07^{c}	520.55^{ab}	103.27^{c}	54.03^{c}	799.05^{a}
	(**µmol kg^{-1} fw**)	±46.02	±32.46	±10.99	±142.36	±71.36	±14.64	±25.73	±42.51	±9.11	±98.62
3	IAA	3.51^{b}	130.52^{a}	2.23^{b}	2.80^{b}	2.90^{b}	0.91^{b}	7.54^{b}	1.43^{b}	13.90^{b}	3.98^{b}
	(**µmol kg^{-1} fw**)	±1.31	±30.02	±0.81	±0.70	±1.34	±0.24	±1.89	±0.33	±4.89	±1.67
4	ABA	2.03^{ab}	0.81^{ab}	1.49^{a}	3.96^{a}	1.93^{ab}	0.57^{ab}	0.29^{b}	0.36^{b}	1.30^{ab}	3.98^{a}
	(**µmol kg^{-1} fw**)	±0.79	±0.40	±0.63	±1.10	±1.60	±0.05	±0.12	±0.14	±0.76	±0.13

Values are Mean ±SEM (n=3). ^{abc}Superscripts indicates differences based on Tukey-Kramer test.

Table 3.3: Effect of *P. pinophilum* EU0013 inoculation on Zn, Fe and Mn concentrations in fruits of tomato cultivars.

		Momotaro		**Reika**		**Cherry**		**Rodeo**		**Anaya**	
		EU0013-	EU0013+	EU0013-	EU0013+	EU0013-	EU0013+	EU0013-	EU0013+	EU0013-	EU0013+
1	Zn (µg g^{-1})	27.99^{ab} ± 2.34	27.19^{ab} ± 2.37	25.92^{ab} ± 1.41	28.72^{ab} ± 1.74	37.28^{a} ± 6.90	32.03^{a} ± 1.84	25.86^{b} ± 1.15	24.89^{b} ± 0.72	28.65^{ab} ± 0.78	30.62^{ab} ± 2.35
2	Fe (µg g^{-1})	53.87^{b} ± 7.24	45.08^{b} ± 6.96	62.47^{ab} ± 7.29	107.78^{a} ± 14.23	52.85^{b} ± 11.25	90.45^{ab} ± 10.88	77.96^{ab} ± 6.78	76.84^{ab} ± 11.20	62.21^{ab} ± 10.67	66.24^{ab} ± 11.78
3	Mn (µg g^{-1})	20.93^{b} ±5.27	11.45^{bc} ± 1.55	11.60^{bc} ± 3.26	14.72^{bc} ± 1.72	12.38^{bc} ± 1.95	36.33^{a} ± 2.71	16.11^{bc} ± 1.90	9.16^{c} ± 1.08	23.77^{ab} ± 0.94	15.51^{bc} ± 3.02

Values are Mean ±SEM (n=3). ^{abc}Superscripts indicates differences based on Tukey-Kramer test.

3.5. Discussion

Tomato fruit development is very sensitive to environmental conditions and is strongly influenced by hormones, such as IAA and GAs (de Jong et al., 2009). IAA and GAs stimulate and regulate each other during the early stages of fruit development (Koshioka et al., 1994; Pattison and Catala, 2012) and are modulated by ethylene and ABA responses (Nitsch et al., 2009). Furthermore, the accumulation of soluble sugars and acids in tomato fruits influence their sweetness or sourness and often depend on the ripening stage (Viskelis et al., 2015). Therefore, an evaluation of endophyte effects on these fruits requires the assessment of some of these phytochemicals because of their impact on color, taste, and flavor. The *P. pinophilum* inoculation increased GA_1, GA_4, and ABA levels in Reika and Anaya, but reduced them in Cherry and Rodeo. This result indicates cultivar-based differences in the modulation of these phytochemicals by the endophyte against the backdrop of their physiological differences in development and responses to environmental conditions. Endophyte effects may be stimulated from the modulated expression of associated genes and their regulators (Pattison and Catala, 2012). Except for Momotaro, the *P. pinophilum* inoculation did not enhance IAA levels in the fruits. Crosstalk signaling between GAs and IAA may have minimized concurrent fluctuations in IAA levels due to the endophyte, but this was not observed in ABA (Quinet et al., 2019). Lycopene and β-carotene are important pigments in tomato and play crucial roles as dietary antioxidants (Ilahy et al., 2009). Their amounts are influenced by the environmental conditions of the growth, cultivar, and ripening stage of tomato (Viskelis et al., 2015). Therefore, increases in β-carotene due to the *P. pinophilum* inoculation in three

(Cherry,Rodeo and Anaya) out of five cultivars are remarkable. Increases were observed in ascorbic acids and total soluble sugars in four out of five cultivars, indicating the significant effects of the inoculation. These increases were not associated with the countries from which the cultivars were obtained, but rather to individual cultivar identities. Ascorbic acid is a major vitamin that enriches the human diet, scavenges free radicals, and fights against oxidative stress. Factors such as growth conditions, the cultivar, and ripening stages affect their accumulation in tomato (Viskelis et al., 2015). The ascorbic acid biosynthesis pathway in plants involves D-glucose-6-P, fructose-6-P, and galactose (Hemavathi et al., 2010), and these are among the main soluble sugars found in tomato (Zhao et al., 2016). Therefore, similarities in the results obtained on total soluble sugars and ascorbic acid concentrations (Fig. 3.3 a & b) are indicative of this relationship. Cherry had significantly smaller fruit weights due to its genotype/physiology. The significant effect of the *P. pinophilum* inoculation on fruit dry weights in Anaya indicates the influence of the endophyte on the accumulation of the dry biomass. This corresponds to higher leaf chlorophyll levels in Anaya inoculated with *P. pinophilum* and ties into the effects on sugars and ripening because the levels of total soluble sugars and dry matter in fruits slightly increase with the advancement of ripening (Viskelis et al., 2015). Phytochemicals are largely linked to the phenylpropanoid pathway/polyphenol metabolism in plants (Dong and Lin, 2020). Since many plant antioxidants are phenol derivatives, the minimal impact of the inoculation on total polyphenols indicates a more targeted physiological effect of microbe-host symbiosis rather than a broad-based increase in the biosynthesis of total polyphenols given the absence of a pathogen or abiotic stress conditions (Dong and Lin, 2020; Ibiang et al., 2021). Zn, Fe, and

Mn are essential trace elements with important roles in the activities of many enzymes (Pilon et al., 2009). Fe is essential for oxygen transport in red blood cells, Mn is involved in bone formation and some antioxidative activity, such as the healing of wounds, and Zn boosts the immune system and aids taste and smell. Since they may be deficient (e.g., Fe) in the staples consumed in some communities (Okwuonu et al., 2021), their natural enrichment in vegetables is obviously beneficial. Fruit Mn was increased by the *P. pinophilum* inoculation in Cherry only, while fruit Fe increased in Cherry and Reika. Enhancements in the accumulation of trace elements in tomato fruits may be achieved by the fungal modulation of root-to-shoot translocation and/or siderophore and organic acid production in the rhizosphere for improved element availability in soil, as previously reported (Khan et al., 2009; Ibiang et al., 2020). Significantly higher endophyte root colonization was observed in Rodeo than in the other cultivars; however, this did not clearly appear to be linked to fruit biomass. Higher root colonization by *Penicillium* may be due to signals that occur in the rhizosphere between the host and endophyte under different cultivation conditions (Ibiang et al., 2021) and indicates greater compatibility between the host and endophyte.

3.6. Conclusion

In conclusion, *P. pinophilum* EU0013 modulated phytohormones and increased total soluble sugars, ascorbic acids, β-carotene, Mn, and Fe concentrations in tomato fruits. Its potential to enhance the nutraceutical value of tomato is indicated, particularly in Cherry and Rodeo. Cultivar responses to the *P. pinophilum* inoculation are important factor to consider in its deployment in tomato, and responses were related to individual cultivar identities rather than the countries from which they were obtained.

CHAPTER FOUR

Reduction of *Verticillium* wilt in tomato by an arbuscular mycorrhizal fungus-*Rhizophagus intraradices* and an endophytic fungus-*Penicillium pinophilum* is cultivar dependent

4.1. Abstract

Verticillium wilt is a major concern in vegetable production due to its broad host range, world-wide distribution, vascular colonization, and resilient microsclerotia. This study was conducted to evaluate the growth and tolerance to *Verticillium dahliae* in two tomato cultivars inoculated with arbuscular mycorrhizal fungi (AMF)-*Rhizophagus intraradices* 15S-1 and an endophytic fungus- *Penicillium pinophilum* EU0013. In a randomized factorial layout, seedlings of tomato cultivars (Momotaro, Rodeo) were inoculated with beneficial microbes and *V. dahliae* pathogen and raised until maturity under greenhouse condition. *In vitro* dual culture indicated the growth inhibition of *V. dahliae* by *P. pinophilum* after one month culture on PDA media. Root colonization by *R. intraradices* and *P. pinophilum* were modulated in response to *V. dahliae* in a cultivar-dependent manner. While Momotaro showed greater foliar and internal disease symptoms than Rodeo, *P. pinophilum* and *R. intraradices* significantly reduced disease incidence, compared to control. *Verticillium*-induced reductions in root and fruit dry weights in Rodeo and Momotaro were reduced by *P. pinophilum*, while AMF generally boosted fruit weights. Co-inoculation of *R. intraradices* and *P. pinophilum* did not consistently improve host growth indices compared to single inoculation, and modulations in mineral element nutrition by both beneficial microbes in

response to *V. dahliae* was cultivar dependent. In Momotaro, *R. intraradices* and *P. pinophilum* increased SlSOD2, SlSOD5 and SlSOD9 gene expression, but this was not generally the case in Rodeo, where lower expression levels were observed. Cultivar-dependent modulation of root colonization, element nutrition, and SlSODs gene expression, by *R. intraradices* and *P. pinophilum* underpin their promotion of host growth and tolerance to *Verticillium* wilt.

4.2. Introduction

Plant diseases are a major contributor to economic losses in agricultural industries. They affect plant development, quality, and yield and are a threat to food security. *Verticillium dahliae* is a pathogenic soilborne fungus that causes foliar yellowing and wilting, vascular discoloration, growth reduction, yield losses, poor fruit quality and plant mortality in many vegetables (Witzel et al., 2017). The fungus infects the root tips and invades the vascular system of the host, gradually spreading to the stem and other parts of the shoots, inducing characteristic symptoms (Tao et al., 2020). Over 200 plant species are susceptible to *V. dahliae* including cotton, olive, potato, eggplant, peppers, cabbage, tomatoes, etc. (Sun et al., 2014). Considered to be one of the most economically important crops worldwide, tomato is a rich source of minerals, vitamins, organic acids, essential amino acids, and dietary fibers. Its successful production in field and greenhouses depend substantially on pathogen control and adequate mineral nutrition. *Verticillium* wilt can be a major constraint in tomato production due to several reasons such as its world-wide distribution, vascular colonization, infectivity rate, spore resistance and the existence of different pathogen races (Acharya et al.,

2020). *Verticillium* wilt resistance in tomato is controlled by the Ve1 gene (Kawchuk et al., 2001; Fradin et al., 2009) which is operative against race 1 of the pathogen that carries the avirulence gene, VdAve1, which codes for a peptide elicitor (de Jonge et al., 2012). However, Ve1 is overcome by race 2 which lacks VdAve1. Thus, in areas where race 1 resistant tomato cultivars and rootstocks were cultivated, it was presumed that the causal agent was race 2 in farms reporting infection in Japan (Usami et al., 2017). Although chemical fumigation for control of *Verticillium* can be done, the potential human and environmental health concerns regarding chemical control subsists (Aktar et al., 2009; Hassaan and El Nemr, 2020). Particularly, the eradication of *V. dahliae* may prove difficult due to its long-lasting microsclerotia in the soil. Many studies have reported the use of plant growth-promoting fungi (PGPF) to control pathogens/reduce disease in plants, including *Verticillium* wilt (Deketelaere et al., 2017). Such integrated pest management tools, which are more ecofriendly, are gaining traction (Alam et al., 2011; Acharya et al. 2020). The mechanisms involved in fungal biocontrol include competition for space and nutrients, antibiosis, induced systemic resistance involving an upregulation of antioxidant enzymes biosynthesis, etc. (Ghorbanpour et al., 2018). Arbuscular mycorrhizal fungi (AMF) which establishes a symbiotic association with plants and colonizes the root system, improves plant absorption of water and mineral nutrients, especially phosphorus, thereby improving plant growth and fitness (Smith and Read, 2008). Due to their protective effects against biotic and abiotic environmental stresses, they are deployed as bioinoculants in commercial formulations. Examples of AMF species used in commercial formulations include *Rhizophagus intraradices, Funneliformis mosseae, Gigaspora margarita,* amongst others (Chenchouni et

al., 2020). To minimize the adverse effects of environmental factors on bioinoculants, the use of dual inoculation or mixed inoculants consisting of two or more species (consortia) might engender synergistic effects that optimize inoculation outcomes (Zhang et al., 2012; Meena et al., 2018). The endophytic fungus *Penicillium pinophilum* EU0013 isolated from eucalyptus roots (Teshima and Sakamoto, 2006) was reported to improve seed germination, plant growth, and tolerance to *Fusarium* wilt disease of tomato (Alam et al., 2011). It is a potential active ingredient in the formulation of commercial inoculum consisting of a consortium of non-target beneficial fungi, but knowledge on its partnership with AMF is limited, neither its action against verticillium disease (and associated mechanisms). According to Deketelaere et al. (2017), the ability to mitigate *Verticillium* in host plants is a valuable attribute of a biological control agent in terms of its global market potential. The aim of this study was to investigate the growth and tolerance to *V. dahliae* of two tomato cultivars inoculated with *P. pinophilum* EU0013, *Rhizophagus intraradices* 15S-1, and their co-inoculation.

4.3. Materials and methods

4.3.1. Soil

The soil utilized was a mix of river sand and commercial soil (Premium soil, Setoharakaen Co. Ltd, Japan) with pH (7.4), EC (75.8 mS m^{-1}), available Zn (25.4 μg^{-1} g of soil), Fe (16.1 μg^{-1} g of soil), Mn (11.9 μg^{-1} g of soil), and Cu (0.3 μg^{-1} g of soil). River sand was sieved with a 2 mm mesh, and both soils were mixed (1:1 v/v), autoclaved at 121 °C for 2 h then allowed to cool for at least 2 days before use.

4.3.2. Plant

Two tomato (*Solanum lycopersicum* L.) cultivars (Momotaro and Rodeo) were used for this experiment. Rodeo (supplied by AGRITROPIC, Nigeria Ltd) is planted by farmers in Nigeria, while Momotaro was supplied by (Takii Seed Co. Ltd, Japan). Tomato seeds were sterilized in 70 % ethanol for 5 min and 33.3 % sodium hypochlorite solution for 15 min then rinsed repeatedly in sterile distilled water before seeding in a growth chamber to obtain seedlings. Growth chamber conditions were: 140 μmol m^{-2} s^{-1} fluorescent light (25 °C, 14 h) and dark (18 °C, 10 h).

4.3.3. Microbe inoculations

4.3.3.1. AM fungi

The AM fungus used was *Rhizophagus intraradices* 15S-1 (provided by Dr. M. Yamato, Faculty of Education, Chiba University). AM fungal inoculum consisted of river sand bearing AMF propagules such as hyphae and root fragments. The amount of inoculum applied was 10 g per pot, at the time of seedling transplant to potted soil. My previous study indicated that this quantity of inoculum per pot is adequate for tomato root colonization (Ibiang et al., 2020). Since *R. intraradices* is not a readily sporulating AMF, any number of spores per pot would be largely incidental. The non-AM pots received no inoculum.

4.3.3.2. *Penicillium pinophilum*

Penicillium pinophilum EU0013 (available from the National Institute of Technology and Evaluation (NITE) NBRC accession number 100411, http://www.nite.go.jp/en/nbrc/cultures/index.html) was obtained from actively growing margins of 7–10 days old potato dextrose agar (PDA) culture medium. Wheat bran substrate-

based inoculum was prepared for *Penicillium* inoculation. The inoculum was prepared as described by Chandanie et al. (2006) but with incubation temperature of 25 °C. After 10 days of incubation, the completely colonized wheat was ground and sieved through a mesh (1 mm), and 0.5 g (3.6×10^3 spores) was added to the middle of each pot before transplant (Ibiang et al., 2020). The non-*Penicillium* pots received no inoculum.

4.3.3.3. *Verticillium dahliae*

Verticillium dahliae race 2 GF1207 was used as pathogen inoculant and is available from NITE (Usami et al., 2017). *V. dahliae* inoculum was prepared using fresh Irish potato as described by Usami et al. (2017). The media was allowed to cool on a clean bench, then 5 fungal plugs of *V. dahliae* spores and mycelium from 7 days old active margins agar media was added and incubated in a bio-shaker (Eyela Co. Ltd, Japan) for 7 days at 25 °C. After 7 days, culture media was sieved with tissue mesh into 50 mL tubes and centrifuged (Sakuma SS-1500×, Sakuma Co. Ltd, Japan) at 1300×g for 5 min. The supernatant was drained out and the harvested spores were diluted with sterile distilled water and mixed by shaking. To determine spore concentration, 10 µL of the inoculum was loaded into Thoma cell counting chamber and viewed under a microscope (Nikon Co. Ltd, Japan) and determined to be 7.3×10^8 spores. To dilute *V. dahliae* inoculum density, a 1:3 dilution was done with sterile distilled water, then seedling roots were dipped, and transferred into designated potted soil for planting. To ensure similar root moistening prior to transplant, the non-verticillium plants were dipped in sterile distilled water.

4.3. 4. Experimental design and plant cultivation

The experiment was laid out in a randomized 2 × 4 × 2 factorial layout, with cultivar (Momotaro, Rodeo); beneficial fungus inoculation – control, *R. intraradices* (AMF), *P. pinophilum*, (Pen) and co-inoculation (PAM); and *Verticillium* disease; V- (control) and V+ (*V. dahliae*), as the treatments (n = 3). Plant cultivation was from May to August 2020 in the Faculty of Horticulture greenhouse for a total of 13 weeks. The seedlings were initially raised for 4 weeks on a commercial soil in a growth chamber before being transplanted to potted (15 cm by 20 cm) soil in the greenhouse. All 48 pots were equally fertilized with full strength Hoagland solution applied (50 mL pot^{-1}) on the day of transplant and once a week from flowering until harvest, while regular watering was done with borehole water.

4.3.5. *In vitro* dual culture of *P. pinophilum* EU0013 and *V. dahliae* race 2 GF1207

A dual culture of *P. pinophilum* and *V. dahliae* was done *invitro* on PDA media. The culture media was prepared according to manufacturer's instruction, and fungal plugs of both microbes were placed at 2 cm and 4 cm apart. The culture was incubated for 21–30 days at 25 °C.

4.3.6. Disease symptoms evaluation

Disease symptoms of *V. dahliae* were observed at 3, 5, and 7 weeks, post inoculation (wpi). The symptoms were quantified as incidence, using external foliage symptoms visually rated on a scale of 0 –3 (Usami et al., 2017) as follows; 0 (none), 1 (yellowing/wilting of one or a few leaves), 2 ($^1/_3$ to ½ of the leaves are wilted or yellowed) and 3 (Most of the leaves are wilted or yellowed), with the severity calculated as follows:

$$Foliar\ disease\ severity = \frac{Total\ leaf symptoms}{Total\ number\ of\ leaves\ per\ plant * 3} * 100$$

Vascular browning was determined by dividing stems diagonally and visually rated as follows; 0 (no color change), 1 (a part of the vascular tissue is changed), 2 ($^1/_3$ to ½ of the vascular tissue is changed) and 3 (More than half or all the conduit is changed). The vascular disease incidence was calculated as follows:

$$Vascular\ disease\ severity = \frac{Total\ internal\ symptoms}{Total\ number\ of\ stem\ shares * 3} * 100$$

4.3.7. Plant harvest

Plants were harvested by carefully emptying the soil from the pots, breaking apart soil attached to the roots and washing in running tap water. Plant lengths and fresh weight were measured, and the plant was cut into root and shoot. A portion of the roots was subtracted for determination of fungal root colonization and gene expression analysis. Left-over plant tissues were put in an oven for drying at 105 °C for 48 h and the dry weights were recorded.

4.3.8. Root colonization by AM fungi

AM fungal colonization was assessed in the roots using the trypan blue staining technique as described by Rajapakse and Miller (1994). Root sections mounted on glass slides were observed in the light microscope for scoring according to Trouvelot et al. (1986) and mycorrhizal indices were then evaluated using "mycocalc" software.

4.3.9. Root colonization by *Penicillium*

Roots were washed thoroughly with running tap water and washed again with sterile water before use. Ten root segments of 0.5 cm were cut and placed on PDA media in petri dishes

amended with penicillin antibiotic (200 mg L^{-1}), to suppress the growth of other ubiquitous microorganisms. Petri dishes were placed in the incubator at 25 °C for 6 days, colonies of *P. pinophilum* growing from root segments were counted and colonization frequency (CF) was calculated as % CF = (Ncol/Nt) × 100, where Ncol = number of root segments colonized by the fungus and Nt = total number of segments of root studied as described by Hata and Futai (1995).

4.3.10. Determination of chlorophyll, macro, and micronutrients

Chlorophyll content in leaves was measured using a SPAD meter (Minolta Co Ltd, Japan) at 6 weeks, while carbon and nitrogen content in dried shoot samples was measured using a C–N coder (Yanaco Technical Science Co Ltd, Japan). Phosphorus concentration was determined using the vanadomolybdate method (Tandon et al., 1968). Dried ground plant samples were ignited in an electric furnace (ADVANTEC FUL220FA, Tokyo, Japan) at 550 °C for 3 h, then digested in 0.6 mol L^{-1} HCl acid and reacted with vanadomolybdate acid solution. The samples were kept for 30 min after which the absorbance was measured in a spectrophotometer (U-1800 Hitachi High Tech Corp, Tokyo, Japan) at 420 nm. Zn, Fe, and Mn concentrations were determined by atomic absorption spectrophotometry (Shimadzu AA-6600F, Japan). Three hundred and fifty mg of dried plant samples were ignited in electric furnace at 550 °C for 6 h, digested in 0.6 mol L^{-1} HCl acid, then element concentrations in solutions were measured.

4.3.11. Determination of polyphenol content

Total polyphenols in shoot samples were determined via the Folin Ciocalteu method (Amerine and Ough 1980). Dried shoot samples (0.3 g) were extracted in 70 % acetone. 2.5

mL of 10-fold diluted Folin-Ciocalteu solution was added to the solutions, and after 2 min, 2.0 mL of Na_2CO_3 solution (75 gL^{-1}) was added. Chlorogenic acid was used as the standard and the absorbance was measured in a spectrophotometer at 760 nm.

4.3.12. SOD gene expression

Using fresh root samples, total RNA extraction, cDNA synthesis and Real-Time RT-PCR was performed for members of the superoxide dismutase (SOD) gene family (SOD2, SOD7 and SOD9). Total RNA extraction of root sample was carried out using FavorPrep™ kits (FAVORGEN BIOTECH CORP) according to the manufacturer recommendations, including DNA elimination step using Dnase I. The concentration of extracted RNA was confirmed using NanoDrop Lite Spectrophotometer (Thermo Scientific, USA) to be > 20 ng μL^{-1} RNA then kept at – 80 °C. cDNA synthesis (BIORAD T100™ Thermal Cycler) was then performed using total RNA volume equivalent to 200 ng in 20 µL total volume mix containing 10 mM dNTPs (Takara, Japan), 5× RT buffer (Toyobo, Japan), oligo (dT)15 primer (Promega, USA) and ReverTraAce® (Toyobo, Japan) as follows; 30 °C (10 min) → 42 °C (60 min) → 99 °C (5 min) → 12 °C (∞). The cDNA samples were kept in – 20 °C refrigerator until use. Real-time RT-PCR (40 cycles, 25 µL final volume) was performed (StepOnePlus™ Real-time PCR System, Applied Biosystems, Singapore) on the cDNA fractions and primers using THUNDERBIRD® SYBR qPCR Mix (Toyobo, Japan). The sequences for the primers were designed using GenScript server10 as obtained from Feng et al. (2016), while Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control (Exposito-Rodríguez ´ et al., 2008). The primer sequences are as follows: SOD2; 5′ -AATCTCCGGGAACGATAGTG-3’ (forward) and 5′ -

AAGGCATGGATATGGAAAGC-3’ (reverse), SOD5; 5′ - AGCATTCAACAATGCTGCTC-3’ (forward) and 5′ -CTCCGTTGGGCTTCATAGAT-3′ (reverse), SOD9′ -5′ - GCAGAAGGTGCTGCTTTACA-3’ (forward) and 5′ - AGGCGCTTAAGCTCTTTGTC -3’ (reverse), and GAPDH; 5′ - GGCTGCAATCAAGGAGGAA- 3’ (forward) and 5′ -AAATCAATCACACGGGAACTG -3’ (reverse). Real-time RT-PCR conditions were as follows; 95 °C (15 min) → 95 °C (10 s) → 55 °C (20 s) → 72 °C (30 s) (Feng et al., 2016). The relative expression of SODs was calculated after normalization of gene expression using GAPDH as the internal reference gene (Livak and Schmittgen 2001).

4.3.13. Statistical analysis

Data were subjected to a three-way analysis of variance (ANOVA), using the SPSS 26 (software package) with significance level set at $P < 0.05$. For foliar and vascular disease incidence (estimated in only V+ plants), a two-way ANOVA was performed. Differences between the means of treatment groups were determined using Tukey-Kramer test.

4.4. Results

4.4.1. *In vitro* fungal bioassay

P. pinophilum showed antagonism towards *V. dahliae* in an *in vitro* culture, by growth impediment and a clearly defined inhibition zone, after one month of culture (Plate 4.1 a–d).

4.4.2. Disease incidence and root colonization

Foliar disease symptoms of *V. dahliae* were observed 3, 5 and 7 wpi to include chlorosis and wilting of mostly the lower parts of leaves (see Plates. 4.2, 4.3, 4.4, and 4.5). At 3, 5, and 7

weeks, cultivar, and beneficial microbe inoculation had significant effects (Table 4.1) on foliar disease severity (Fig. 4.1 a, b, & c). Momotaro showed higher chlorosis than Rodeo, with greater symptoms in controls than AMF and/or *P. pinophilum* inoculated groups. Vascular disease severity at harvest (Fig. 4.1 d) showed significant differences between the cultivars.

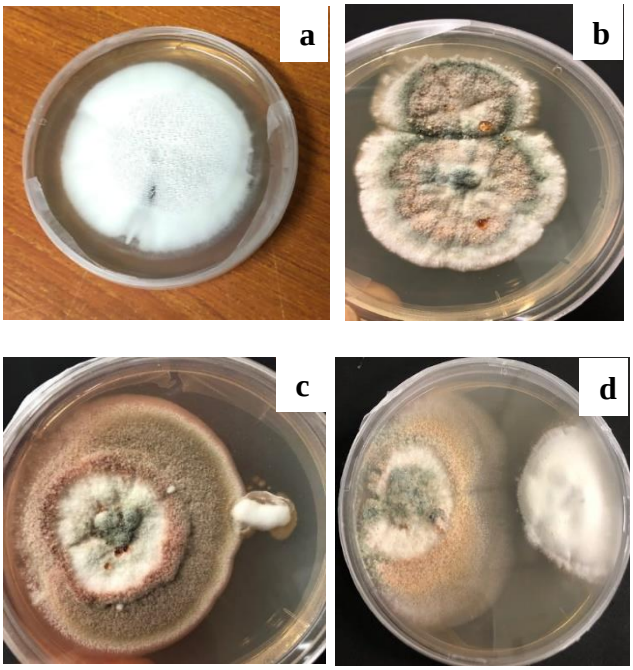

Plate 4.1. *In vitro* culture of (a) *Verticillium dahliae* at 1 month old in single culture (b) *Penicillium pinophilum* EU0013 at 1 month old in single culture (c) Dual culture of *P. pinophilum* EU0013 and *V. dahliae* (2cm apart) indicating growth suppression of *V. dahliae* by *P. pinophilum* EU0013 after 1 month, with a dense inhibition zone (d) Dual culture of *P. pinophilum* EU0013 and *V. dahliae* (4cm apart) indicating growth suppression of *V. dahliae* by *P. pinophilum* EU0013 after 1 month, with a clearer inhibition zone.

Plate 4.2. Momotaro cultivar subjected to *Verticillium* treatment at 5wpi. From left to right (control, AMF, Penicillium, AMF+*Penicillium*).

Plate 4.3. Rodeo cultivar subjected to *Verticillium* treatment at 5wpi. From left to right (Control, AMF, *Penicillium*, AMF+*Penicillium*).

Plate 4.4. Momotaro cultivar subjected to *Verticillium* treatment at 7wpi. From left to right (Control, AMF, *Penicillium*, AMF+*Penicillium*).

Plate 4.5. Rodeo cultivar subjected to *Verticillium* treatment at 7wpi. From left to right (Control, AMF, *Penicillium*, AMF+*Penicillium*).

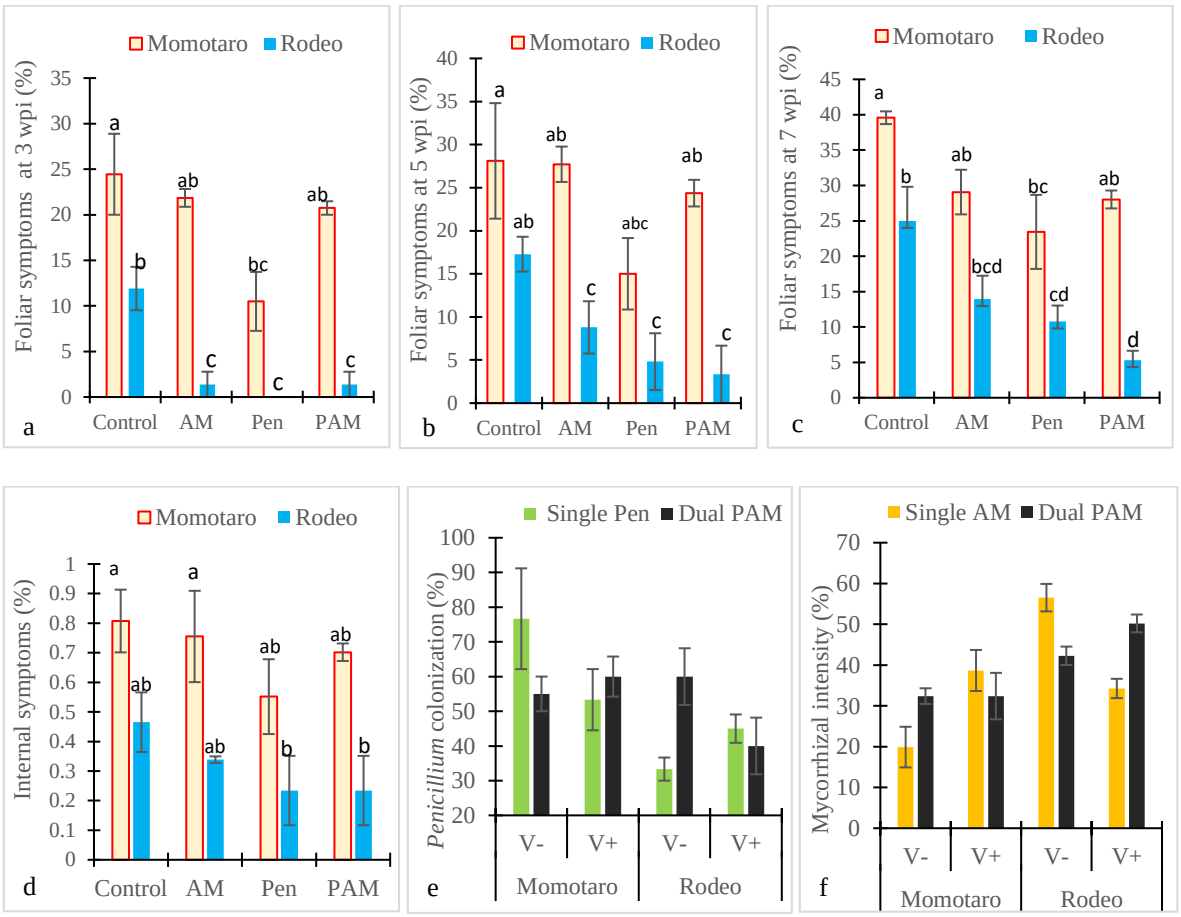

Fig 4.1. Disease symptoms and root colonization of tomato cultivars (Mean±SEM). Foliar symptoms at (a) 3wpi, (b) 5wpi, (c) 7wpi, (d) Internal symptoms in stems, (e) *Penicillium* root colonization, (f) Intensity of arbuscular mycorrhizal root colonization. [abc]Superscripts denote differences based on Tukey-Kramer tests.

Table 4.1. *P* values showing significance of treatment effects and their interaction based on two-way ANOVA.

		Cultivar	PGPF Inoculation	Interaction
1	Foliar symptoms at 3wpi	3.7E-08***	0.00039***	0.1095 ns
2	Foliar symptoms at 5wpi	1.9E-05***	0.0225*	0.217 ns
3	Foliar symptoms at 7wpi	1.435E-06***	0.000302***	0.3764 ns
4	Internal symptoms at harvest	9.64E-05***	0.1626 ns	0.886 ns

*=*P*<0.05; **=*P*<0.01; ***=*P*<0.001; ns=not significant.

Table 4.2. *P* values showing significance of treatment effects and their interaction based on three-way ANOVA.

		Cultivar	Disease	PGPF	Cultivar×Disease	Cultivar×PGPF	Disease×PGPF	Cultivar×Disease×PGPF
1	Penicillium colonization	0.003**	0.129 ns	0.753 ns	0.753 ns	0.129 ns	0.753 ns	0.011*
2	Mycorrhizal Intensity	0.0007***	0.2525 ns	0.668 ns	0.087 ns	0.24 ns	0.843 ns	0.0013**
3	Root Length	2.38E-10***	0.001**	0.000019***	1.47E-06***	0.095 ns	0.013*	0.028*
4	Shoot Length	0.003**	0.002**	0.143 ns	0.071 ns	0.579 ns	0.223 ns	0.561 ns
5	Root dry weight	0.001**	0.000023***	4.07E-06***	0.8387 ns	0.399 ns	0.182 ns	0.233 ns
6	Shoot dry weight	0.551 ns	0.000029***	0.068 ns	0.0375*	0.109 ns	0.008**	0.562 ns
7	Fruit dry weight	5.65E-11***	0.890 ns	2.74E-10***	0.250 ns	0.014*	0.00023***	0.0000024***
8	Chlorophyll	0.207 ns	0.013*	0.507 ns	0.046*	0.507 ns	0.458 ns	0.043*
9	Polyphenols	0.585 ns	0.443 ns	0.272 ns	0.024*	0.451 ns	0.012*	0.003**
10	Carbon	0.234 ns	0.00006***	0.000013***	0.24 ns	0.269 ns	0.124 ns	0.124 ns
11	Nitrogen	0.488 ns	0.139 ns	0.018*	0.823 ns	0.457 ns	0.009**	0.628 ns
12	Phosphorus	0.881 ns	0.007**	0.017*	0.441 ns	0.374 ns	0.514 ns	0.229 ns
13	Zn	0.014*	0.047*	0.405 ns	0.09 ns	0.088 ns	0.553 ns	0.745 ns
14	Mn	0.509 ns	0.011*	2.76E-10***	0.2693 ns	0.00509**	5.85E-09***	0.169 ns
15	Fe	0.0721 ns	0.8701 ns	1.80E-06***	0.1663 ns	1.11E-06***	0.043*	0.000022***
16	*SOD2* expression	4.98E-24***	5.73E-13***	1.71E-26***	9.03E-13***	1.50E-26***	3.32E-22***	3.01E-22***
17	*SOD7* expression	4.73E-15***	0.244 ns	3.15E-15***	0.205 ns	1.79E-15***	5.44E-11***	3.21E-11***
18	*SOD9* expression	0.09 ns	0.133 ns	0.0016**	0.000044***	0.000281***	0.691 ns	0.00001***

*=*P*<0.05; **=*P*<0.01; ***=*P*<0.001; ns=not significant.

Momotaro showed higher vascular discoloration than Rodeo, and in both cultivars, *P. pinophilum* inoculated groups showed less discoloration compared to controls and AMF groups. Frequency of *Penicillium* root colonization (Fig. 4.1 e) was significantly affected by cultivar, with significant cultivar × disease × beneficial microbe interaction (Table 4.2). In Momotaro, root colonization in *Penicillium* groups was lower in V+ compared to V- treatment, while in co-inoculated groups, the reverse was the case. In Rodeo, *Penicillium* root colonization was higher in V+ compared to V- groups, while in co- inoculation, it was higher in V- groups compared to V+. Mycorrhizal intensity (Fig 4.1f) was significantly affected by cultivar, with significant cultivar × disease × beneficial microbe interaction (Table 4.2). For AMF treatment, mycorrhizal intensity was higher in V+ compared to V- in Momotaro, while in Rodeo, it was lower in V+ treatment compared to V-. In co-inoculation, under V+ treatments, mycorrhizal intensity was 50 % in Rodeo and 32 % in Momotaro.

4.4.3. Plant growth indices

Root lengths (Fig. 4.2a) showed significant differences due to cultivar, disease, and beneficial microbe inoculation (Table 4.2). In Momotaro, V+ groups had higher values than V-, with AMF and *P. pinophilum* groups higher than control. In Rodeo, inoculated groups were higher than control, especially in V+ treatment. Shoot lengths (Fig. 4.2 b) showed significant differences due to cultivar and disease. In Momotaro, V+ had higher values than V- groups, but this was not observed in Rodeo. Root dry weights (Fig. 4.2 c) was significantly affected by cultivar, disease, and beneficial microbe inoculation (Table 4.2).

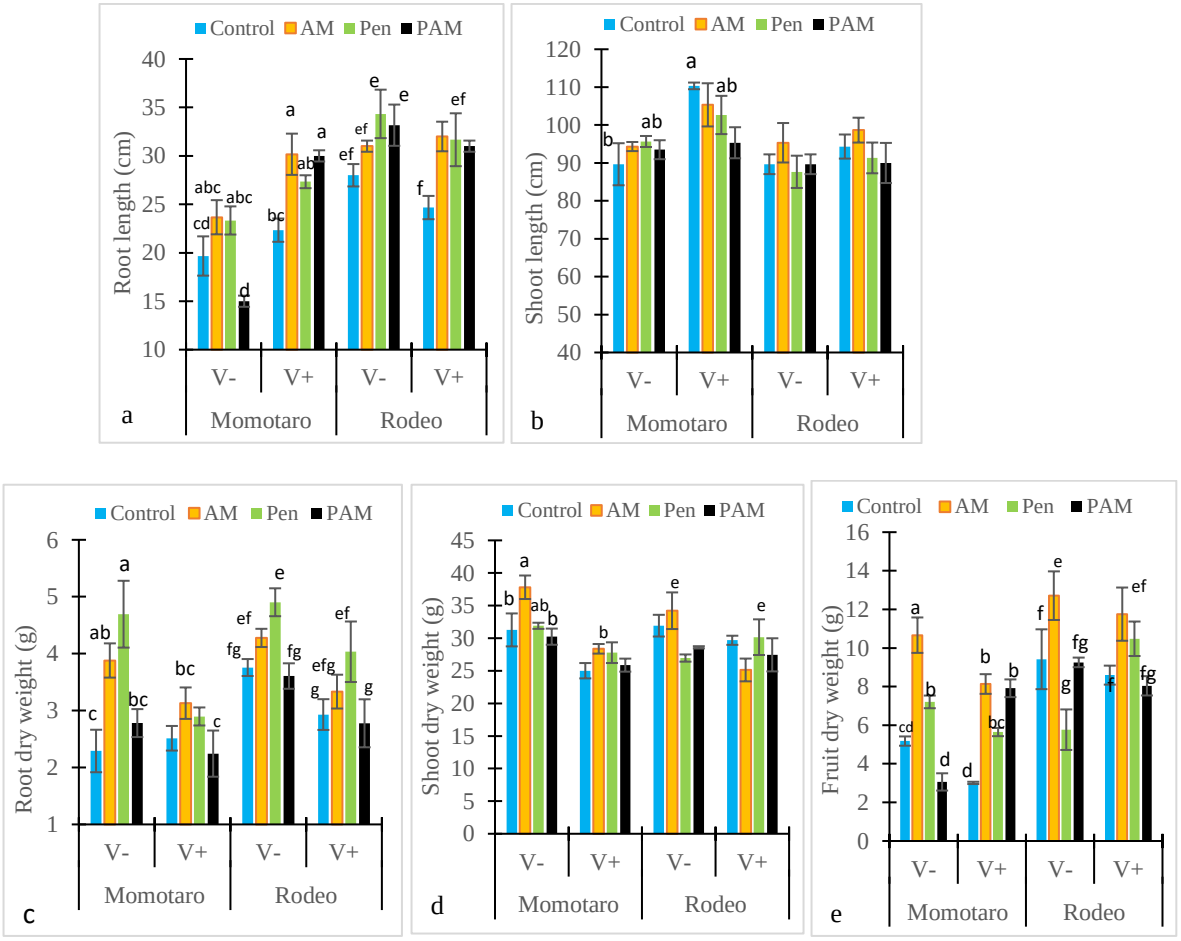

Fig 4.2. Plant growth indices of tomato cultivars (Mean±SEM). (a) Root length, (b) Shoot length, (c) Root dry weight, (d) Shoot dry weight, (e) Fruit dry weight. [abcefg]Superscripts denote differences within each cultivar based on Tukey-Kramer tests.

Rodeo showed higher root biomass than Momotaro; groups with no disease (V-) had higher values than diseased groups (V+); while *P. pinophilum* and AMF inoculations gave higher values than control and co-inoculation in both cultivars. In V- groups, *P. pinophilum* increased root weights by 104% and 30.6 % in Momotaro and Rodeo, respectively, compared to control. In V+ groups, it was 15 % (Momotaro) and 37.5 % (Rodeo). Shoot dry weights (Fig. 4.2 d) was significantly affected by disease, with significant cultivar × disease, and

disease × beneficial microbe interactions (Table 4.2). In Momotaro, V+ generally had lower shoot weights compared to V-. In Rodeo, a reduced shoot weight was seen in V+ groups, except in *P. pinophilum* inoculation. Fruit dry weight (Fig. 4.2 e) was significantly affected by cultivar and beneficial microbe inoculation, with significant cultivar × disease × beneficial microbe interaction (Table 4.2). Fruit weight was higher in Rodeo than in Momotaro, and in both cultivars, AMF inoculation gave the highest values. Compared to control, fruit dry weights were generally higher in AMF and *P. pinophilum* inoculated plants, in both V- and V+ groups. In Momotaro, *V. dahliae* treatment reduced fruit weight in control plants by 41.9 %, compared with V- group. Within V+ groups, AMF, *P. pinophilum* and co-inoculation countered *V. dahliae* effect with 62.9 %, 46.7 %, and 62 % increases, respectively, in fruit weight compared to control. In Rodeo, a much smaller reduction in fruit weight by *V. dahliae* (8.7 %) was observed in control plants, but AMF and *P. pinophilum* inoculated groups had higher fruit dry biomass. AMF inoculated plants in both V- and V+ groups, flowered the earliest but the reverse was observed in *P. pinophilum* and co-inoculated plants.

4.4.4. Chlorophyll and polyphenol concentration

Chlorophyll content in leaf (Fig. 4.3 a) was significantly affected by disease, with significant cultivar × disease and cultivar × disease × beneficial microbe interaction (Table 4.2). In Momotaro, chlorophyll content in control plants was decreased in V+ treatment, but

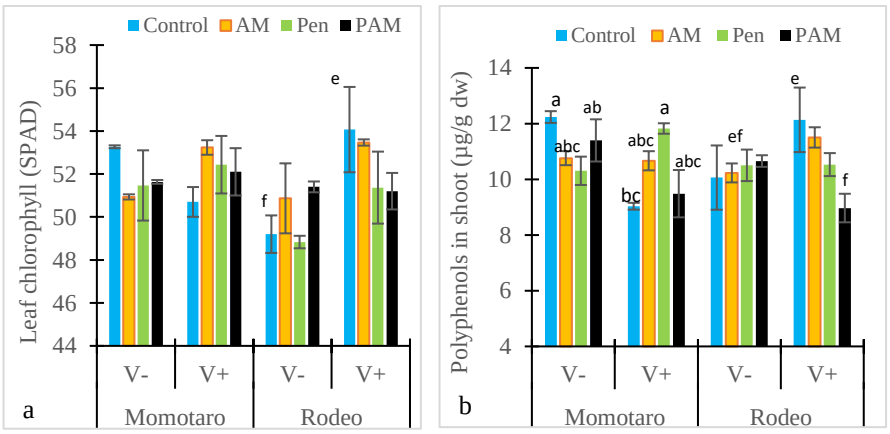

Fig 4.3. Physiological indices of tomato cultivars (Mean±SEM). (a) Leaf chlorophyll, (b) Polyphenols in shoot. [abcefg]Superscripts denote differences within each cultivar based on Tukey-Kramer tests.

beneficial microbe inoculations countered the effect of *V. dahliae*. In Rodeo, V+ groups generally had higher values than V-. Polyphenols in shoots (Fig. 4.3 b) was significantly affected by cultivar × disease, disease × beneficial microbe, and cultivar × disease × beneficial microbe interactions (Table 4.2). In control Momotaro plants, polyphenol content was decreased in V+ compared to V- groups and was lower than AM and *P. pinophilum* inoculated groups. In Rodeo, however, polyphenol content in control plants of V+ group was increased compared to V-, while co-inoculated groups were the lowest within V+ series.

4.4.5. Carbon, nitrogen, phosphorus, and trace element concentrations

Carbon content in shoot (Fig. 4.4 a) was significantly affected by disease and beneficial microbe inoculation. In both cultivars, inoculated plants generally showed lower carbon content than control. Decreases due to *V. dahliae* treatment was observed in AMF, *P. pinophilum* and co-inoculated plants in Momotaro, while in Rodeo, decreases were observed in control and AMF groups. Nitrogen content in shoots (Fig. 4.4 b) was significantly affected

by beneficial microbe inoculation, with significant disease × beneficial microbe interaction (Table 4.2). In Momotaro, co-inoculation increased shoot nitrogen content in V-, while *Penicillium* inoculation increased it in V+ groups, compared to control. In Rodeo, *P. pinophilum* and co-inoculation increased shoot nitrogen in V+ treatment groups. Phosphorus concentration in shoot (Fig. 4.4 c) was significantly affected by disease and beneficial microbe inoculation. In both cultivars, inoculated groups generally had higher phosphorous than controls, with V+ groups higher than V-. Zn concentration in shoots (Fig. 4.4 d) was significantly affected by cultivar and disease. In Rodeo, shoot Zn concentration was increased in control and inoculated plants in V+ groups compared to V-, with the highest value observed in *Penicillium* treatment. In Momotaro, *R. intraradices* inoculation insignificantly increased shoot Zn in response to *V. dahliae.* The AMF effect on shoot Zn in response to *V. dahliae* was consistent in both cultivars. Mn concentration in shoots (Fig. 4.4e) was significantly affected by disease and beneficial microbe inoculation, with significant cultivar × beneficial microbe and disease × beneficial microbe interactions (Table 4.2). *R. intraradices* inoculation inhibited shoot Mn in both cultivars, compared to control. In V+ group of Rodeo, control plants showed decreased shoot Mn concentrations while *P. pinophilum* and co-inoculation countered *Verticillium* effect by an increased Mn concentration. Increased Mn concentration in shoot by *P. pinophilum* inoculation was consistent in both cultivars and a higher modulation was observed in response to *V. dahliae* treatment. Fe concentration in shoots (Fig. 4.4 f) was significantly affected by beneficial microbe inoculation and cultivar × disease × beneficial microbe interaction (Table 4.2). In Rodeo, *P. pinophilum* inoculation increased shoot Fe concentration in both V+ and V- groups,

compared to control. In Momotaro, *P. pinophilum* and *R. intraradices* increased shoot Fe, in response to *V. dahliae* treatment, while co-inoculation decreased it.

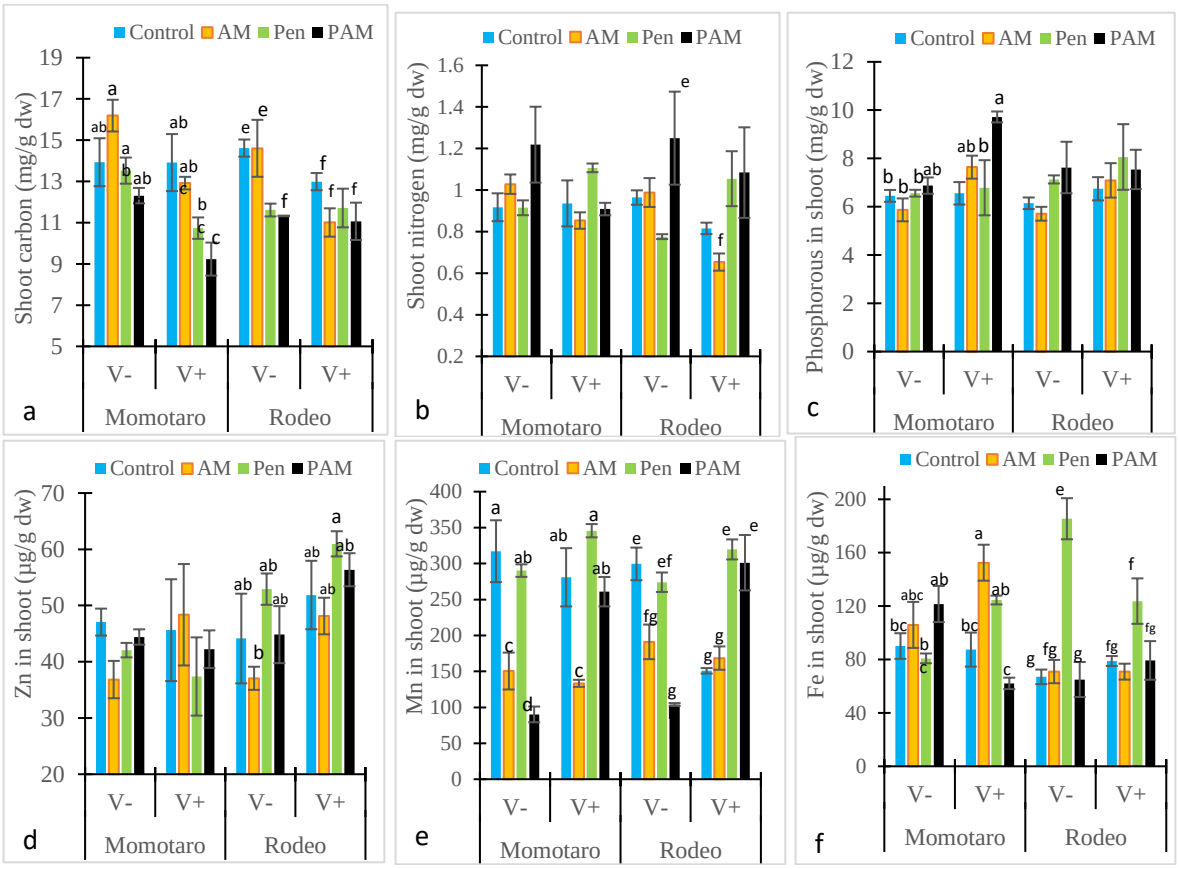

Fig 4.4. Nutritional indices of tomato cultivars (Mean±SEM). (a) Shoot carbon, (b) Shoot nitrogen, (c) Shoot phosphorus, (d) Shoot Zinc, (e) Shoot Manganese, (f) Shoot Iron. [abcefg]Superscripts denote differences within each cultivar based on Tukey-Kramer tests.

4.4.6. SOD gene expression in roots

The trend of SOD expression observed was similar in all three SOD family genes evaluated and variation was seen between cultivars. Generally, higher expression was observed in Momotaro than in Rodeo, and in *R. intraradices* inoculated groups. SlSOD2 (Cu/ZnSOD) relative expression (Fig. 4.5 a) showed significant effects of the experimental treatments and their interactions (Table 4.1). In Momotaro, the highest value was observed in AMF inoculated groups, and the beneficial microbe inoculated groups were higher than control, under V+ treatment. Values in Rodeo were lower than in Momotaro. SlSOD5 (FeSOD) relative expression (Fig. 4.5 b) showed significant effect of cultivar, disease, beneficial microbe inoculation, and their interaction (Table 4.2). In Momotaro, *R. intraradices* and *P. pinophilum* inoculated groups had higher values than control, with or without *V. dahliae* treatment.

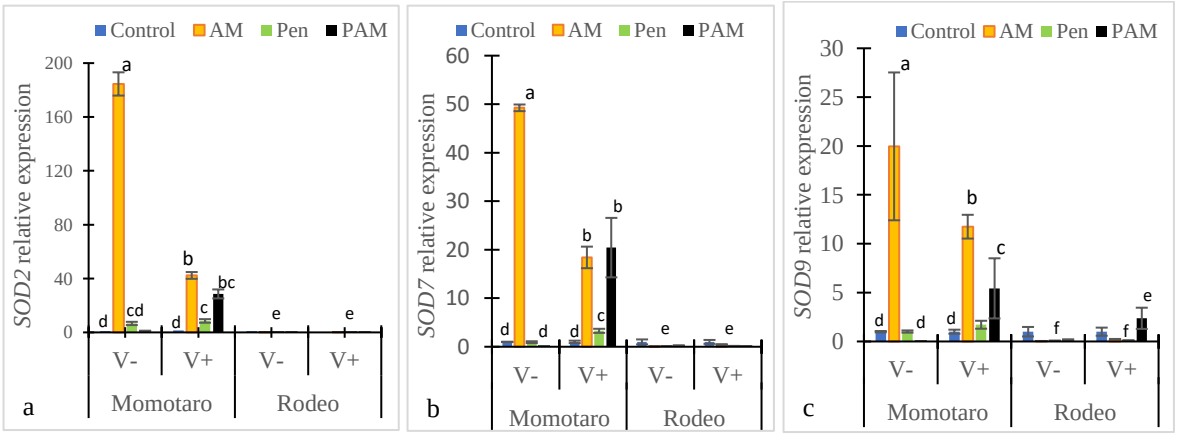

Fig 4.5. SOD relative gene expression of tomato cultivars. (a) *SlSOD2*, (b) *SlSOD7*, (c) *SlSOD9*, relative expression. [abcefg]Superscripts denote differences within each cultivar based on Tukey-Kramer tests.

Values in Rodeo were generally lower than in Momotaro, in both V- and V+ groups. SlSOD9 (MnSOD) relative expression (Fig. 4.5 c) showed significant differences due to beneficial microbe inoculation, and three-factor interaction. In Momotaro, AMF groups had higher values than control in both V- and V+ treatments. In Rodeo, *P. pinophilum* and co-inoculation had higher values than control, in V- treatment.

4.5. Discussion

4.5.1. *In vitro* assay, root colonization and disease incidence

An efficient root colonization is not only important for the proper establishment and functioning of symbiosis, but it also serves as a mechanism for competition with *Verticillium* for space and nutrients in host tissue, thereby giving protection to the host (Qiang et al., 2018; Ghorbanpour et al., 2018). This is indicated for *P. pinophilum*, as its colonization was higher in Momotaro (the cultivar with higher disease incidence) than in Rodeo, while the reverse was the case for mycorrhizal intensity. Significant three-factor interaction in root colonization indices indicate that these features were modulated to fit the unique (treatment) scenario. Since the growth and development of fungal symbionts like AMF is basically dependent on host root colonization to obtain sugars and lipids (Jiang et al., 2017; Luginbuehl et al., 2017), a possible explanation for this regulation of colonization could be changes in photosynthate availability in the roots, and/or in the root exudation (Zhang et al., 2012), due to the presence of *V. dahliae*. A lower shoot carbon content in dual inoculation treatments also points to rhizosphere microbe drain on the host photosynthates. While Rodeo showed lower foliar symptoms of *Verticillium* than Momotaro, foliar symptoms in Rodeo was lowest

in dual inoculation, and this group had the highest mycorrhizal intensity among the V+ treated groups. But in Momotaro, *P. pinophilum* inoculation had the lowest foliar symptoms in V+ groups. These indicate possible cultivar-inoculant preferences, in terms of *R. intraradices* or *P. pinophilum* or co-inoculation. There are reports pointing to varied responses due to plant genotype/cultivar compatibility in plant-microbe interaction (Vidotti et al., 2019; Ibiang 2021). The protective effect of biocontrol agents against *Verticillium* could involve the inhibition of the germination/spread of pathogen structures in the host rhizosphere and cortex (Antonopoulos et al., 2008; Papasotiriou et al., 2013). In this regard, the *in vitro* inhibition of *V. dahliae* may coincide with *in vivo* protective effect of a beneficial fungus (Sun et al., 2014). The biocontrol strategies of *Penicillium* sp. include plant growth promotion, induced resistance, antibiosis and mycoparasitism (Deketelaere et al., 2017). Here, the *in vitro* suppression of *V. dahliae* growth by *P. pinophilum* may be categorized as falling within the sphere of antibiosis (Ghorbanpour et al., 2018) and competition. These *in vitro* observations align with the reduced foliar disease incidence observed in the *P. pinophilum* treated plants.

4.5.2. Plant biomass

In both cultivars, plant dry weights indicate increases due to *R. intraradices* and *P. pinophilum*. Fruit dry weights were mostly boosted by *R. intraradices* inoculation across the board, while *P. pinophilum* boosted root weights. These indicate their competency for growth promotion, but synergic effect was not generally indicated in dual inoculation. Synergic effect between AMF and *P. pinophilum* EU0013 on host growth is influenced by the AM

fungal identity and soil/growth conditions, amongst others (Ibiang et al., 2020). Among the cultivars, Rodeo showed more tolerance to *V. dahliae* than Momotaro, in terms of shoot biomass indices. This is in line with the foliar disease incidence between both cultivars. Such differences in cultivar response to pathogen are normal, as genetic factors influence plant-microbe interaction outcomes (Vannier et al., 2015; Ibiang 2021). While *V. dahliae* treatment decreased root dry weights in both tomato cultivars, *P. pinophilum* treatment countered this in Rodeo (Fig. 4.2 c). *Verticillium*-induced reduction in fruit dry weights in Momotaro, was countered by single and dual inoculations of *R. intraradices* and *P. pinophilum*. These indicate that protective effect of *R. intraradices* and *P. pinophilum* occurred in both cultivars, and that in AMF groups, the mechanisms are largely connected to growth-promoting effects, as reflected in increased fruit weights across the board. Growth-promoting ability compensates for any deleterious effects of *Verticillium* and dilutes the adverse effects on hosts (Deketelaere et al., 2017). Beneficial microbe effects on root lengths and biomass are often linked to stimulation of host phytochemicals (phenols, organic acids, auxins, gibberellins, etc.) and nutrient uptake (Khan et al., 2009). Disparate effects on plant parts are based on intricacies of host-microbe interaction, and potentially influenced by the environmental factors stemming from the cultivation conditions (Ibiang 2021).

4.5.3. Physiological indices and SOD relative expression

Dual inoculation increased shoot phosphorus concentrations in both cultivars, compared to control. This is a previously reported outcome of AM fungi and *Penicillium* co-inoculation (Smith and Read, 2008; Ibiang et al., 2020). In addition, my findings show that the

improvements in host phosphorus concentrations was enhanced in response to *Verticillium*. Symbiont's improvement in phosphorus nutrition for enhanced tolerance to stress is known, as phosphorus is an essential element for leaf and stem production and overall plant growth (Mehta et al., 2019). Since *Verticillium* diminishes stem and leaf integrity, improvements in host phosphorus and leaf chlorophyll are likely to counter *Verticillium* stress. This is in line with the lower foliar symptoms recorded in the plants inoculated with *P. pinophilum* and its co-inoculation with *R. intraradices*. Modulation of phosphorus nutrition in shoots fits into the systemic protective effects of beneficial microbes against stress. With respect to cultivar, Rodeo displayed greater chlorophyll and polyphenols levels than Momotaro, and this was manifested in the generally lower disease symptoms and higher dry weights it showed. Decreases in shoot carbon content in microbe inoculated plants, including *Verticillium*-inoculated groups, indicate carbon/photosynthate drain due to inoculants. Increases in shoot nitrogen was observed in *P. pinophilum* and co-inoculated groups. It appears that while *Penicillium* increased shoot nitrogen in both cultivars in response to *Verticillium*, decreases were observed in AMF plants. This indicates the existence of unique microbe-induced effects for achieving host protection and may partly account for some of the disparities in host biomass improvements stated earlier. Since *Verticillium* colonizes and weakens the host stem, modulation of one or more nutrient translocation from roots to shoots could occur as a tolerance mechanism. In Rodeo, increase in shoot Zn concentration in both control and inoculated plants in response to *V. dahliae* indicate a cultivar response. Many enzymes require Zn, Fe, Mn, and Cu ions for their activity (Pilon et al., 2009). Thus, a boost in shoot Zn levels appeared to be involved in the protective effect of *P. pinophilum* in Rodeo. *R.*

intraradices decreased Mn generally in tomato shoots, as previously reported (Ibiang et al., 2018). While Mn concentration in the presence of *V. dahliae* varied between the cultivars, *P. pinophilum* and co-inoculation increased shoot Mn in both cultivars. Fe is essential for the formation of chlorophyll in the leaves and is a component of vital enzymes involved in electron transfer. Shoot Fe concentrations showed increases due to *P. pinophilum* (in both cultivars) and *R. intraradices* (in Momotaro) in response to *V. dahliae*. Since *Verticillium* causes yellowing of leaves, boosting of the shoot Fe by *P. pinophilum* and *R. intraradices* likely supported leaf integrity and minimization of foliar disease symptoms. Taken together, unique cultivar-based differences are indicated in the modulation of trace element nutrition by beneficial microorganism, in response to *Verticillium*. Beneficial fungi such as *Penicillium* sp. Can influence the trace element nutrition of hosts via siderophore and organic acid production in the rhizosphere (Khan et al., 2009). A previous study confirmed the siderophore and organic acid production ability of *P. pinophilum* EU0013 (Ibiang et al., 2020). Increase in tomato defense gene expression by AMF in response to pathogens has been previously reported (Nair et al. 2014). SODs represent a class of antioxidant enzymes that catalyze the dismutation of superoxide anion (O_2^-) to H_2O_2 and are produced in response to oxidative stress, including microbe infection. They are among the first line of defense and are encoded by nuclear genes and present in mitochondria, peroxisomes, chloroplasts, and cytosol (Wang et al., 2017). In addition to the above, the SOD family consists of different members that might show varied expression levels in the cultivars, and in the roots where the target microbes associate with the host. Generally, SOD activity is higher in mycorrhizal plants than in non-mycorrhizal (Wu et al., 2014). This is in line with our results, as *R.*

intraradices inoculated plants generally exhibited higher SOD gene expression. However, the members of the SOD gene family in tomato respond differently to stress as expression could be upregulated or downregulated (Feng et al., 2016). We observed cultivar-based differences in the modulation of SlSOD gene expression by *R. intraradices* and *P. pinophilum*. In Momotaro, *R. intraradices* increased SlSOD2, SlSOD5 and SlSOD9 expression compared to control, in the presence or absence of *Verticillium*. In Rodeo, however, SlSlSOD2 and SlSOD5 expression was not elevated by *R. intraradices*, while elevation of SlSOD9 was observed in co-inoculation or *P. pinophilum* treatment. Regulation of SlSOD gene expression is believed to be controlled by cis elements acting at the promoter regions (Feng et al., 2016). Thus, especially for Rodeo, minimal impact of *R. intraradices* and *P. pinophilum* on gene expression might be due to cultivar-based differences in the functioning of the regulatory sections or may stem from plant part or growth stage. Cis-elements in promoters of SlSODs could be stress-responsive, hormone-responsive, light-responsive, while others are linked to fungal elicitors (Feng et al., 2016), thus there is ample latitude through which cultivar-unique scenarios could take effect. Further studies examining the SODs gene expression between tomato cultivars in different parts of plants such as the leaves, could reveal other effects of beneficial microbes and *V. dahliae*.

4.6. Conclusion

The growth of *V. dahliae* race 2 GF1207 was inhibited *in vitro* by *P. pinophilum*. Inoculation with *P. pinophilum* and/or *R. intraradices*, reduced the impacts of *Verticillium* wilt and improved the growth of tomato-hosts. The mechanisms induced by *R. intraradices* include

improved shoot Fe and Zn concentrations, modulation of root colonization, host growth-promotion with increases in root lengths and upregulation of SOD gene expression. On the other hand, *P. pinophilum* increased trace elements concentration in shoots, biomass indices of roots, modulated root colonization, and ameliorated foliar disease symptoms. How these mechanisms were deployed was determined by the cultivar of the host. Cultivar-dependent modulation of root colonization, mineral element (Zn, Fe, Mn, P) nutrition, and SlSOD expression, by *R. intraradices* and *P. pinophilum* underpin their promotion of host growth and tolerance to *V. dahliae*.

CHAPTER FIVE

Lettuce tolerance to *Verticillium* wilt after inoculation with *Penicillium pinophilum* and *Rhizophagus intraradices*

5.1. Abstract

This study evaluated lettuce growth and tolerance to *Verticillium* wilt after inoculation with an arbuscular mycorrhizal (AM) fungus - *Rhizophagus intraradices* and an endophytic fungus - *Penicillium pinophilum.* In a randomized layout with four treatment groups (Control, AM, *Penicillium*, AM+*Penicillium*), seedlings of lettuce (cv. Great Lake 366) inoculated with the plant growth-promoting microbes in the presence (V+) and absence (V-) of the pathogen (*Verticillium dahliae*), were raised for six weeks in a growth chamber. Leaf chlorophyll content was significantly lower due to disease across board. Within V+ groups, leaf number, leaf width, shoot and root lengths, shoot and root fresh weights were significantly higher in *Penicillium* group when compared to other groups. Foliar disease symptoms were lowest in *Penicillium* inoculation and highest in Control. Relative DNA quantitation of *V. dahliae* in lettuce stem, was significantly higher in Control, when compared to inoculated groups, indicating a reduction of *V. dahliae* proliferation in host. Disease significantly increased hydrogen peroxide (H_2O_2) and polyphenol contents in Control and dual inoculated groups, while superoxide dismutase (SOD) activity was significantly higher in the dual inoculated groups. Significant decreases were observed in trace elements concentration, total carbon and nitrogen of V+ groups when compared to V-, with some mitigation by *P. pinophilum*. *P. pinophilum* inoculation inhibits the systemic spread of *V. dahliae* from roots to shoots, suppressing foliar wilting, modulates reactive oxygen species (H_2O_2) and SOD activity in

shoots to enhance host tolerance. Its partnership with AM showed no benefit to lettuce tolerance against *V. dahliae*.

5.2. Introduction

Lettuce (*Lactuca sativa* L) is a popular leafy vegetable that is highly consumed worldwide due to its nutritional benefits in human diet. They are a source of vitamins, beta-carotene, folate, and iron. Their nutritional content depends on the type of lettuce cultivated and the growth conditions (Murray *et al*. 2021). Also, lettuce is a fast-growing vegetable that can be cultivated within a short period of time or sometimes for longer periods, which make it less demanding for farmers. Lettuce has been ranked as the third most consumed vegetable crop in the United States (USDA,2015). Challenges of lettuce growth and production include susceptibility to phytopathogens such as *Rhizoctonia solani* and *Pythium* sp. which causes bottom rot and damping-off, *Bremia lactucae* that causes downy mildew, *Fusarium oxysporum* and *Verticillium dahliae* which causes foliar and vascular discoloration, amongst others (ADAS, 2019). *Verticillium* wilt is a widespread plant disease caused by the soil borne pathogen *V. dahliae* and is a major threat to vegetable production. In lettuce, *V. dahliae* colonization begins with the penetration of secondary root through the tap roots into the vascular system (Vallad and Subbarao, 2008). The fungus continues spreading to other parts of the plant causing symptoms such as chlorosis, wilting, necrosis, and under severe conditions may lead to host death (Fradin and Thomma, 2006). Control of this disease is difficult once established due to its resting structure – microsclerotia. Host resistance is one of the long-term control methods used in lettuce, due to its sustainability and eco-friendly nature (Lebeda *et al*., 2009). Another effective method is chemical fumigation of the soil.

However, this is costly, difficult to sustain and potentially dangerous to the environment and human health upon consumption of vegetable with high amounts of pesticide residues. The negative impacts of pesticides on non-target organisms in the environment and food safety are well documented (Ibiang *et al*. 2014) and have led to sustained interest in alternative approaches for managing plant diseases, such as the use of biocontrol agents.

Many biocontrol agents are bacteria or fungi, that could be referred to as plant growth-promoting microorganisms (PGPM). These PGPM colonizes their host roots, confer benefits such as enhancement and facilitation of mineral nutrition, promote host growth and increase resistance against phytopathogens. The effective use of PGPM minimizes the need for chemical fertilizers. Besides this benefit, they are also very easy to handle, cost and environmentally friendly, which is of major interest in agricultural production. PGPM are classified into different groups based on the sphere of their association with the host such as rhizosphere, phyllosphere, and endosphere organisms (Orozco-Mosqueda *et al*., 2018). The endophytic microorganisms are those that colonizes the inner parts of plants without causing disease. Their major defense mechanisms against phytopathogens include parasitism, competition for nutrients and space, antibiosis, induced systemic resistance (ISR), etc. (Olarewaju *et al*., 2017, Nishad *et al*., 2020) An example of an endophytic fungus is *Penicillium pinophilum* EU0013 which was previously isolated from eucalyptus roots, identified from morphological features of the conidiophores and sequence data on the internal transcribed spacer (ITS) region of rDNA (Teshima and Sakamoto, 2006). It was screened to confirm its growth-promoting (Ibiang *et al*., 2020) and disease resistance ability in cabbage and tomato (Alam *et al*., 2011; Ibiang *et al*., 2021), but it has not been tested on lettuce.

Arbuscular mycorrhizal (AM) fungi form symbiotic association with over 80% of plants (Smith and Read, 2008). AM fungi consist of an intra and extraradical mycelium which forms external network in the soil. They confer multiple benefits to plants such as enhance nutrient availability, increase drought resistance, reduce soil pathogen, and improve tolerance to salinity and heavy metals. There are also reports of negative impact on their host such as decrease in growth (Lerat *et al.*, 2003). The use of mixed inoculum has been reported by many researchers to promote synergistic benefit to host (Tanwar *et al*., 2013; Hashem *et al.*, 2016; Ujvari *et al*., 2021), but this is not always the case (Ibiang *et al*., 2020). This research was conducted to evaluate lettuce growth and tolerance to Verticillium wilt via the use of single and dual inoculation of an arbuscular mycorrhizal (AM) fungus - *Rhizophagus intraradices*, and an endophytic fungus - *Penicillium pinophilium.*

5.3. Materials and methods

5.3.1. Plant and cultivation conditions

Lettuce, *Lactuca sativa* L. cv. Great lake 366 was used for this experiment. Seeds were supplied by Takii Seed Co. Ltd, Japan. The seedlings were initially raised for 3 weeks on a commercial soil (Premium soil, Setoharakaen) in a growth chamber before being transplanted to pots (15 cm by 20 cm) in same growth chamber for an additional 3 weeks. The soil for transplant was a mixture of the commercial soil and river sand (1:1 v/v) with pH (5.63 ±0.06), and plant-available (mg kg^{-1}) Zn (3.93 ±0.06), Fe (12.67 ±0.12), Mn (19.04 ±1.03), Cu (0.52 ±0.01). All 32 pots were equally fertilized with $(NH_4)_2SO_4$ and K_2HPO_4 solution (50 mL pot^{-1}) on the day of transplant until harvest, while regular watering was done with tap water.

Growth chamber conditions were: 140 μmol m^{-2} s^{-1} fluorescent light (25 °C, 14 h) and dark (18 °C, 10 h).

5.3.2. Microbe inoculations

The AM fungi used was *Rhizophagus intraradices* 15S-1 (provided by Dr. M. Yamato, Faculty of Education, Chiba University). The AM fungal inoculum consisted of river sand bearing AM fungal propagules such as hyphae and root fragments. The amount of inoculum applied was 10 g per pot, done on the day of seedling transplant to potted soil, in line with previous report indicating its adequacy for lettuce root colonization (Ibiang et al. 2020). *R. intraradices* is not a readily sporulating AM fungi, so spore number was not determined. The non-AM pots received no inoculum.

Penicillium pinophilum EU0013 (available from the National Institute of Technology and Evaluation (NITE) NBRC accession number 100411, http://www.nite.go.jp/en/nbrc/cultures/index.html) was obtained from actively growing margins of 7 days old potato dextrose agar (PDA) culture medium. Corn substrate-based inoculum was prepared for *Penicillium* inoculation. The inoculum was prepared as described by Chandanie *et al*. (2006) with incubation temperature of 25 °C. After 10 days of incubation, the completely colonized corn was blended into fine powder, and 0.5 g (0.6×10^3 spores) was added to the middle of each pot before transplant (Ibiang *et al.,* 2020). The non-*Penicillium* pots received no inoculum.

Verticillium dahliae race 2 GF1207 was used as the pathogen and is available from NITE (Usami *et al.,* 2017). *V. dahliae* inoculum was prepared by liquid culture using fresh Irish potato as described by Usami *et al*. (2017). Ten fungal plugs of *V. dahliae* from 7 days old

culture on potato dextrose agar were added and incubated in a bio-shaker (Eyela Co. Ltd, Japan) for 7 days at 25 °C. At the end of the incubation days, the culture media was sieved with tissue mesh into 50 mL tubes and centrifuged (Sakuma SS-1500×, Sakuma Co. Ltd, Japan) at 1300×g for 5 min. The supernatant was drained out and spores were harvested. Harvested spores were diluted with sterile distilled water and mixed by shaking until a clear appearance. To determine spore concentration, 10 μL of the inoculum was loaded into Thoma cell counting chamber and viewed under a microscope (Nikon Co. Ltd, Japan) and determined to be 6.8×10^8 per milliliter.

5.3.3. Experimental design and statistical analysis

The experimental layout was a completely randomized 4 × 2 factorial, with beneficial microbe inoculation - Control, *R. intraradices* (AM), *P. pinophilum*, (Pen) and dual inoculation (Pen+AM); and pathogen (*V. dahliae*) inoculation, V+ (disease) and V- (no disease). Treatments were replicated four times (n=4), giving a total of 32 pots. Data collected was subjected to two-way analysis of variance (ANOVA) (STATCEL ver4) and conclusion drawn at 5% probability level. The separations of treatment group means were done using Tukey-Kramer post hoc tests. For disease incidence (in only V+ groups) and *Penicillium* colonization, a one-way ANOVA was used, while a t-test was used for AM colonization between AM and dual in V- series.

5.3.4. Disease symptoms evaluation

Disease symptoms of *V. dahliae* were observed at 2 and 3 weeks, post inoculation (wpi). The symptoms were quantified as disease incidence, using external foliage symptoms visually rated on a scale of 0 - 3 (Usami *et al*., 2017) as follows; 0 (none), 1 (yellowing/wilting of one

or a few leaves), 2 ($^1/_3$ to ½ of the leaves are wilted or yellowed) and 3 (most of the leaves are wilted or yellowed), with the incidence calculated as follows:

Foliar disease severity = $\frac{Total\ leaf\ symptoms}{Total\ number\ of\ leaves\ \ per\ plant \times 3} \times 100$

5.3.5. Plant harvest

Plants were harvested after six weeks by carefully emptying the soil from the pots, breaking apart soil attached to the roots and washing in running tap water. Plant lengths and fresh weight were measured, and the plant was cut into root and shoot, and photos taken. Portions of shoots was subtracted for further analysis.

5.3.6. Determination of leaf chlorophyll, C, N, and trace element concentrations

Chlorophyll content in leaves was measured using a SPAD meter (Minolta Co Ltd, Japan) on the day of sampling. Total carbon and nitrogen content in dried shoot samples was measured using a C–N coder (Yanaco Technical Science Co. Ltd, Japan). Trace elements (Zn, Fe, Cu, and Mn) concentration were determined by atomic absorption spectrophotometry (Shimadzu AA-6600F, Japan). Dried plant samples (3.5 g) were ignited in electric furnace at 550 °C for 6 h, digested in 0.6 mol L^{-1} HCl acid, then element concentrations in solutions were measured.

5.3.7. Total polyphenol in lettuce leaves

Polyphenol content in leaf samples were determined using the Folin-Ciocalteu method (Amerine and Ough, 1980). Fresh samples (0.3 g) were extracted in 70 % acetone, 2.5 mL of 10-fold diluted Folin-Ciocalteu solution was added, and kept for 2 min, 2.0 mL of Na_2CO_3 solution (75 gL^{-1}) was added. Chlorogenic acid was used as the standard and absorbance was measured in a spectrophotometer (U-1800 Hitachi High Tech Corp, Tokyo, Japan) at 760 nm.

5.3.8. Hydrogen peroxide determination

Hydrogen peroxide (H_2O_2) concentration was estimated using the method of Patterson *et. al* (1984) as an indicator of reactive oxygen species (ROS). Plants were harvested and a portion was cut, digested, and stored at -80 °C until day of experiment. Plant samples (1 g) were crushed and homogenized in 3 mL acetone. Samples were centrifuged at 1000×g for 3 mins, then 200 µL of supernatant was added to 2 mL of 20 % TiCl in conc HCl. Blank samples were prepared in same way but with no plant tissue. A precipitate was formed after the addition of 0.2 mL of ammonia solution to 1 mL of above mixture. The precipitate formed was washed repeatedly, drained out carefully, and dissolved in 3 mL sulfuric acid (2 N) for all samples. Absorbance was measured at 410 nm with a spectrophotometer (U-1800 Hitachi High Tech Corp, Tokyo, Japan). Standard curve was obtained by serial dilution of 30 % H_2O_2 at different concentrations, and H_2O_2 concentration in the samples were determined from values obtained from curve.

5.3.9. Superoxide dismutase (SOD) activity

Total SOD activity was determined using the method of Elavarthi and Martin (2010) with slight modifications. Plant samples submerged in liquid nitrogen were crushed with a mortar and pestle, then 0.5 g was added to tubes already containing 2 mL of 50 mM phosphate buffer. Samples were vortexed for 5 sec before centrifuging at 14000×g for 30 mins. Supernatant was transferred into clean tubes in racks. The reaction mixture contained 50 mM phosphate buffer (pH 7.6), 2 mM EDTA, 9.9 mM L-methionine, 0.01 M NBT, 0.2 mM Riboflavin and 0.025 % Triton -X100. Tubes were prepared for both light and dark reaction for the number of treatment groups, and 3mL of the reaction mixture was added to all tubes, thereafter, 50

µL of samples were added. A box wrapped with an aluminum foil was placed on an oscillating platform under a 15 W fluorescent tube 15 cm away. Samples for the light reaction were transferred into the box and kept for 10 mins, while those for the dark reaction were in the dark for same time and used as blanks. Absorbance was measured at 560 nm at end of the reaction with a spectrophotometer (U-1800 Hitachi High Tech Corp, Tokyo, Japan). SOD activity in samples were determined from standard curve of pure SOD from Sigma Aldrich.

5.3.10. Quantitation of *V. dahliae* DNA in lettuce stem

As an index of pathogen biomass in host stem, DNA quantitation of *V. dahliae* was done according to the method of de Jonge *et al*. (2012). The lower part (close to the root) of fresh shoot were used. Labelled 7 mL Precellys homogenizing kit (CK28) were filled into racks for the number of samples. Stems (5 cm from the end of root upward) were diced with a knife and about 1.7 g was weighed and put into tubes. Two milliliter of DNA extraction buffer (0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1 % SDS), 1 mL of chloroform, and 1 mL of TE saturated phenol was added to tubes. Each tube was homogenized for 1 min using the Minilys homogenizer. Samples were then transferred to the centrifuge (HIMAC SCT5B) for 5 mins at 5000×g. Supernatant was taken out into new tubes, 2 mL of isopropanol was added and mixed by swirling. Samples were centrifuged again for 10 mins at 12000×g using a high speed refrigerated centrifuge (HIMAC CR-209). Supernatant was completely drained, 1 mL of 70 % ethanol was added to rinse pellets, then drained again for another 5 mins. Pellets were dissolved with 300 µL of TE buffer containing RNase. DNA concentration in extracted samples were determined using a Qubit fluorometer (Invitrogen) according to manufacturer recommendations, then samples concentration was diluted to 50 µg μL^{-1} and

stored at -20 °C. Real-Time PCR (40 cycles, 25 μL final volume) was performed (Thermal Cycler Dice® Real Time System ιιι, Japan) on the DNA fractions and primers using TB Green® premix Ex Taq^{TM} (Tli RNaseH Plus) (Takara, Japan). The sequences for the primers were obtained from de Jonge *et al*. (2012). The primer sequence for *V. dahliae* elongation factor 1-alpha; VdELFE-1a-F, 5′ -CCATTGATATCGCACT- 3', and VdELFE-1a-R, 5′ -TGGAGATACCAGCCTCGAAC- 3'were used, with the PCR conditions as follows; 95 °C (30 s) → 95 °C (5 s) → 60 °C (30 s) → 95 °C (15 s) → 60 °C (30 s) → 95°C (15 s).

5.3.11 Determination of root colonization by *Penicillium* and AM fungus

For *Penicillium* colonization, roots were washed thoroughly with running tap water and washed again with sterile water before use. Ten root segments of about 0.5 cm were cut and placed on PDA media in petri dishes amended with penicillin antibiotic (200 mg L^{-1}), to suppress the growth of other ubiquitous microorganisms. Petri dishes were placed in the incubator at 25 °C for 4 – 6 days, the colonies of *P. pinophilum* EU0013 growing from root segments were counted and colonization frequency was calculated as %CF = (Ncol/Nt)×100, where Ncol = number of root segments colonized by the fungus and Nt = total number of segments of root studied as described by Hata and Futai (1995). AM fungal colonization was assessed in the roots using the trypan blue staining technique as previously described by Rajapakse and Miller (1994). The root sections (approx. 1 cm each, n=30) mounted on glass slides were observed in the light microscope (Nikon Eclipse 50*i*, Japan) for scoring according to (Trouvelot et al.1986) and intensity of mycorrhizal colonization was determined using "mycocalc" software.

5.4. Results

5.4.1. Plant growth indices

Leaf number (Fig. 5.1a) and leaf width (Fig. 5.1b) showed significant differences due to disease, inoculation, and their interaction (Table 5.1). The values in Control, AM, and dual inoculation of V+ group were significantly lower than V-. This was not the case in *Penicillium* groups, as it ameliorated the effect of verticillium disease on both leaf number and leaf width. In shoot length (Fig. 5.1c) and root length (Fig. 5.1d), highly significant differences were seen due to disease, inoculation, and their interaction. *V. dahliae* inhibited shoot and root length in V+ groups except in *Penicillium* inoculation (see Plates 5.1 & 5.2). Fresh weights of shoot (Fig. 5.1e) and root (Fig. 5.1f) showed significant differences due to disease, inoculation, and their interaction, with decreases due to *Verticillium*. *Penicillium* inoculated groups showed a consistent pattern of shoot and root weight in both V- and V+ groups, indicating its whole-plant amelioration of *V. dahliae's* effect.

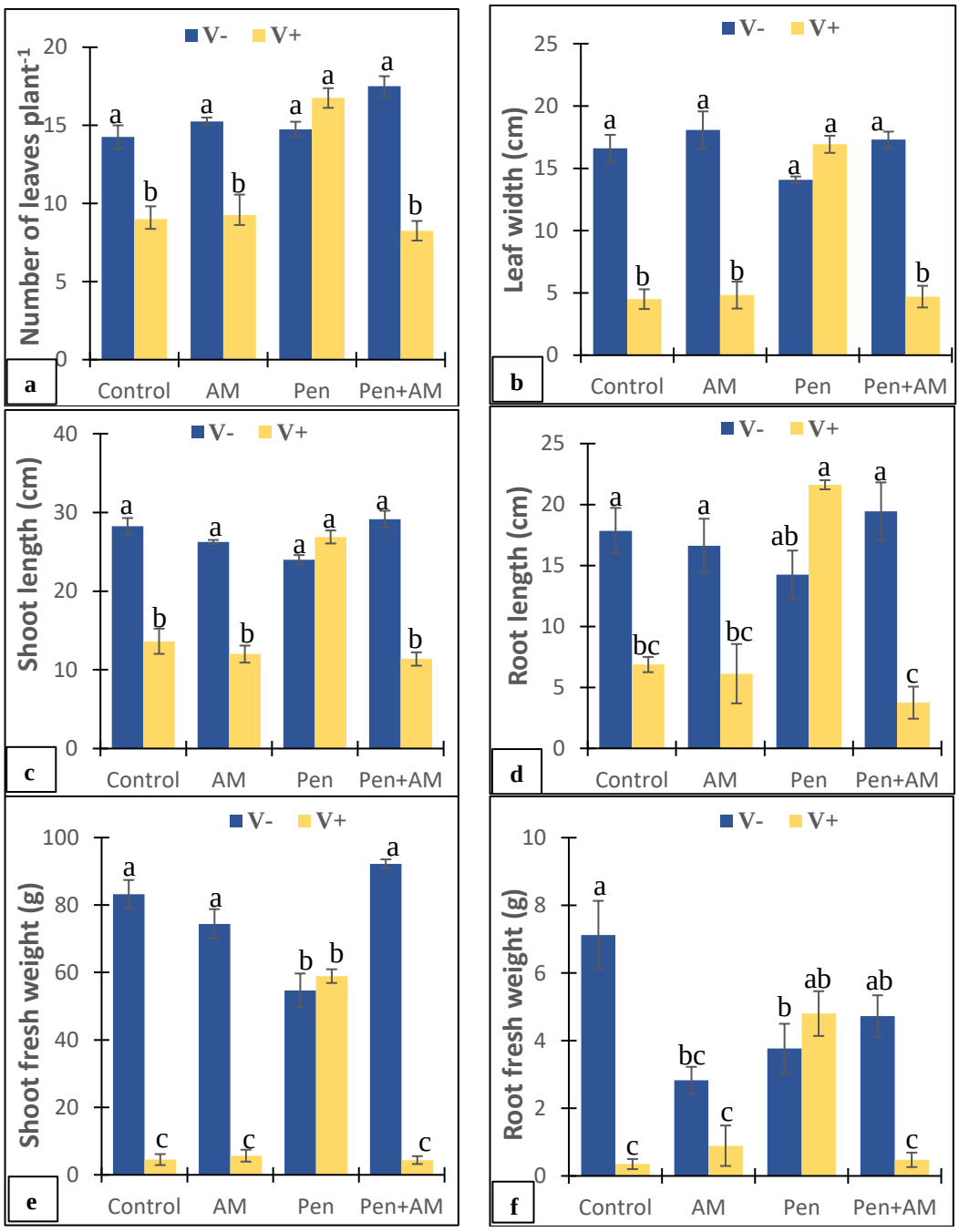

Figure 5.1. Growth indices of lettuce inoculated with *P. pinophilum* and *R. intraradices* under *Verticillium* stress. Values are Mean ±SEM (n=4). [abc]Superscripts indicates differences based on Tukey-Kramer Post hoc tests.

Table 5.1: *P* values showing significance of treatment effects and their interaction based on ANOVA

	Parameter	Inoculation	Disease	Disease× Inoculation
1	Leaf number	5.60841E-05***	6.08201E-09***	9.38317E-07***
2	Leaf width	5.26437E-05***	1.32988E-12***	6.4969E-09***
3	Shoot length	6.99263E-06***	4.03651E-14***	5.33109E-10***
4	Root length	0.003573787**	5.13799E-06***	6.90237E-06***
5	Shoot fresh weight	9.63551E-05***	2.54354E-19***	2.14125E-13***
6	Root fresh weight	0.002223252**	3.49329E-07***	1.03521E-05***
7	Leaf chlorophyll	0.162718 ns	0.000104***	0.666651ns
8	Polyphenols	0.032368226*	0.000622852***	0.003971464**
9	H_2O_2	0.00165564**	3.07898E-05***	2.22301E-06***
10	SOD activity	0.002309075**	0.93274228 ns	0.092136355ns
11	DNA quantity	3.50405E-12***	4.1201E-12***	3.25669E-11***
12	Foliar symptoms	0.013374286*	-	-
13	Zn	0.01157564*	1.4503E-09***	0.898555539 ns
14	Fe	0.287607383 ns	0.575354729 ns	0.232294228 ns
15	Mn	0.112180939 ns	2.65664E-07***	0.398451262 ns
16	Cu	0.960246333 ns	0.000433314***	0.952961857 ns
17	Carbon	0.019382107*	3.16207E-16***	8.68755E-10***
18	Nitrogen	0.000112876***	3.35247E-17***	3.84111E-12***

*=P<0.05; **=P<0.01; ***=P<0.001; ns=not significant.

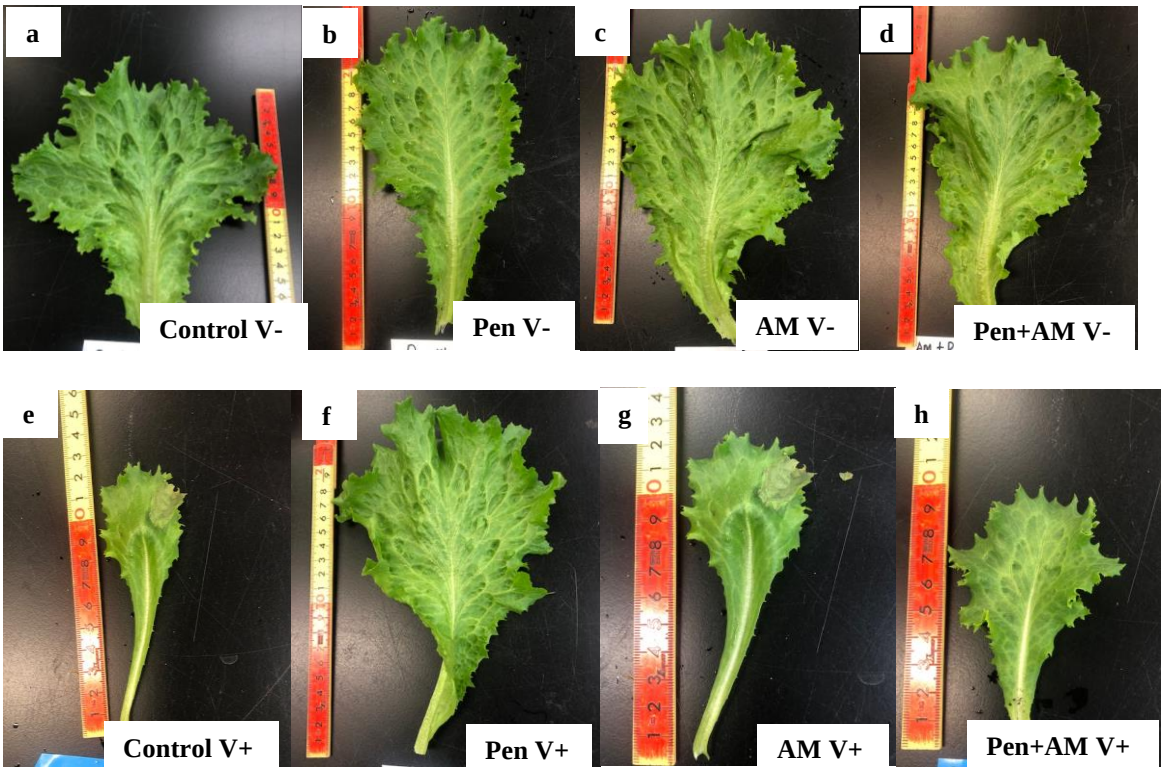

Plate 5.1. Effect of *P. pinophilum* and *R. intraradices* on lettuce tolerance to *V. dahliae* inoculation (leaf width at 3 wpi)

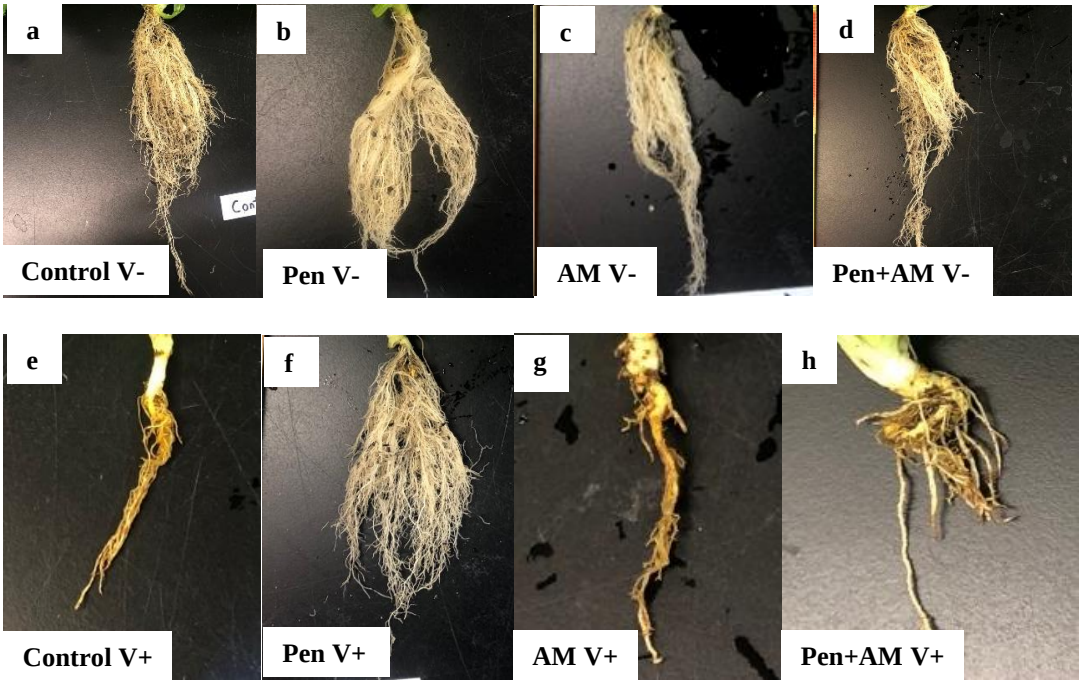

Plate 5.2. Effect of *P. pinophilum* and *R. intraradices* on lettuce tolerance to *V. dahliae* inoculation (roots at 3 wpi)

5.4.2. Physiological indices, disease incidence, and pathogen DNA quantitation

Leaf chlorophyll (Fig. 5.2a) was significantly affected by disease as values in V+ groups were generally lower than V- across the board. H_2O_2 levels (Fig. 5.2b) showed significant differences due to disease, inoculation, and their interaction with highest values in Control plants of V+ groups. In dual inoculated groups, values were significantly higher in V+ than V-groups, while in AM and *Penicillium* inoculations, insignificant differences were seen between V- and V+ groups. SOD activity (Fig. 5.2c) showed significance due to inoculation. Generally, the lowest values were in *Penicillium* groups and highest were in dual inoculation. In Control, AM, and *Penicillium*, values in V+ were higher than V- but were not significant. Polyphenol content (Fig. 5.2d) was significantly different due to disease, inoculation, and their interactions. Except for *Penicillium* inoculated groups, other V+ groups showed increases in polyphenol content. This result mirrored H_2O_2 especially in Control and dual inoculated groups, indicating increase of both indices due to *Verticillium* pathogen. The relative quantity of *V. dahliae* DNA in lettuce stem (Fig. 5.2e) showed significant differences due to disease, inoculation, and their interactions. Highest values were seen in Control which was significantly higher than all inoculated groups. This indicates that both *P. pinophilum* and *R. intraradices* reduced *V. dahliae* biomass penetration into the stem of lettuce. In addition, AM and dual inoculated group were significantly lower than *Penicillium* group.

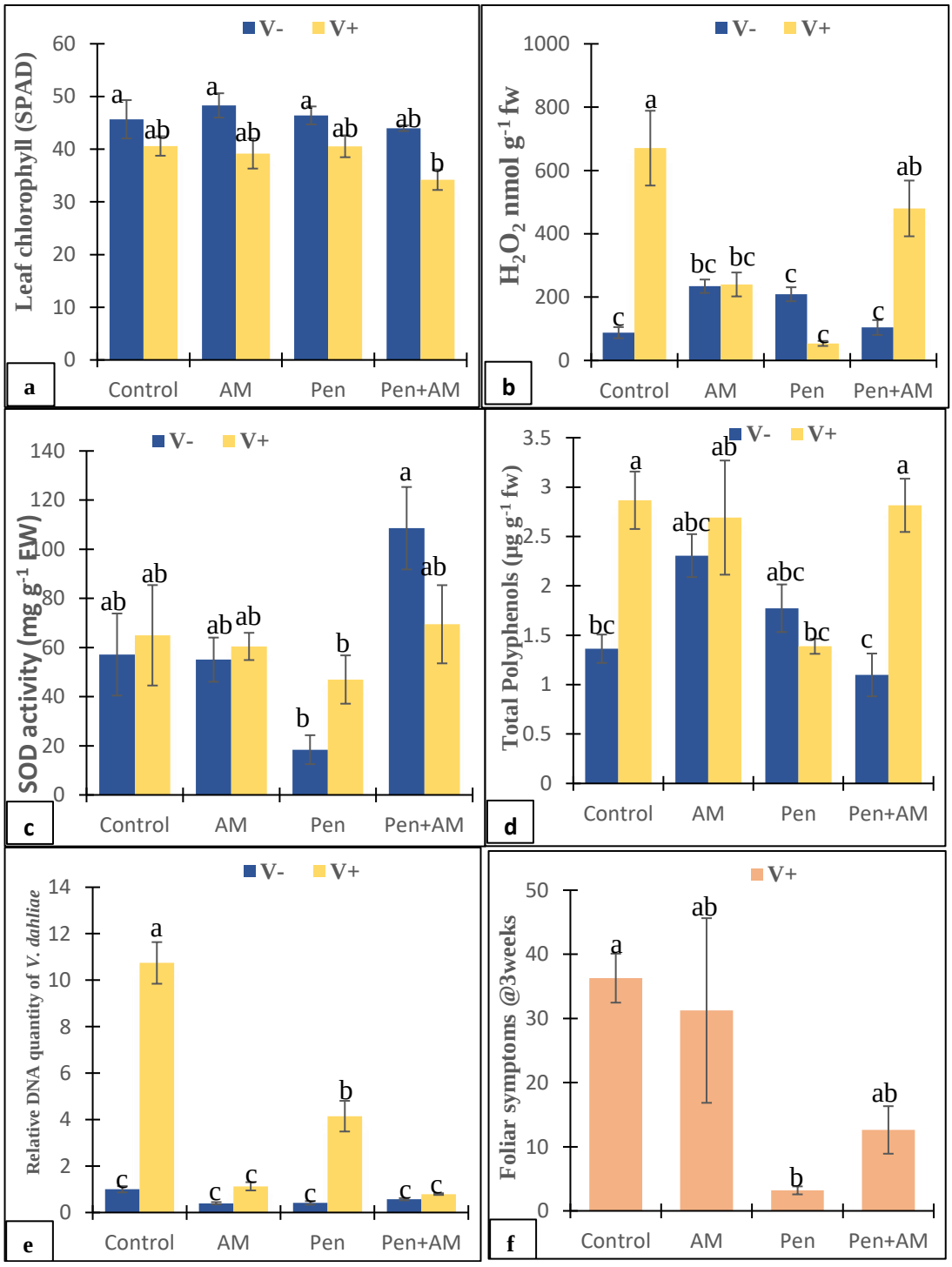

Figure 5.2. Physiological indices, pathogen DNA quantitation, and foliar disease symptoms of lettuce inoculated with *P. pinophilum* and *R. intraradices* under *Verticillium* stress. Values are Mean ±SEM (n=4). [abc]Superscripts indicates differences based on Tukey-Kramer Post hoc tests.

Foliar disease symptoms (Fig. 5.2f) were lower in *Penicillium* inoculated group compared to Control which had the highest values. This was consistent at two- and three-weeks post inoculation (see Plate 5.3).

5.4.3. Carbon, nitrogen, and trace elements concentration

Nitrogen content (Fig. 5.3 a) in shoot showed significant differences due to disease, inoculation, and their interactions. *Verticillium* inoculation reduced nitrogen content in shoots except in *Penicillium* groups. In V- groups, *Penicillium* inoculation was lower than the others. Carbon content (Fig. 5 b) was also significantly different due to disease, inoculation, and their interactions, with a similar trend as nitrogen, where decreases due to *Verticillium* were ameliorated by *Penicillium*. Zn concentration in shoot (Fig. 5.3 c) of lettuce was significantly reduced by *Verticillium* disease, as seen in V+ groups compared to V-. *Penicillium* moderately increased the concentration of Zn in lettuce shoots of V+ plants. Fe concentration in shoot (Fig. 5.3 d) showed no significant difference, but values in AM and dual inoculation were higher in V+ group. Mn (Fig. 5.3 e) and Cu (Fig. 5.3 f) concentration showed significant differences due to disease, as concentration of these elements were reduced by the presence of *Verticillium*. *Penicillium* inoculation moderately increased the concentration of Mn, but this was not the case in Cu.

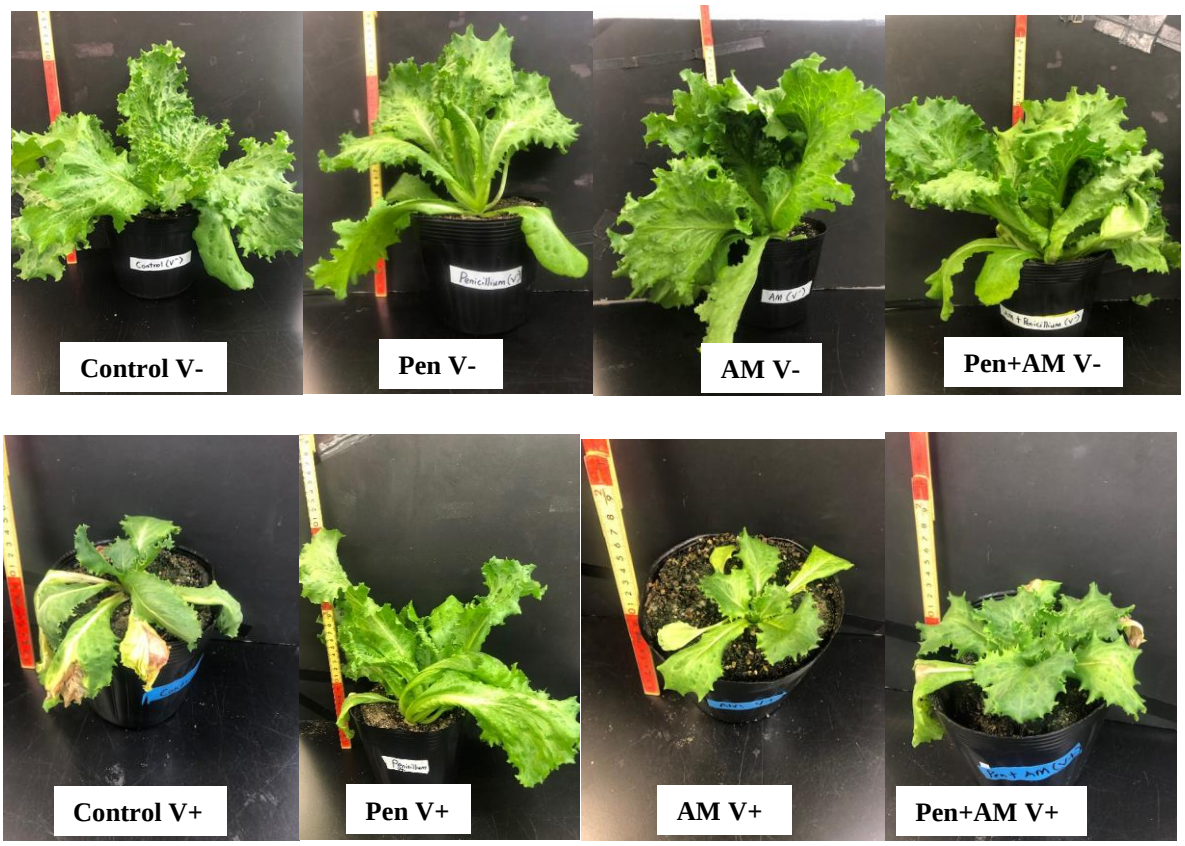

Plate 5.3. Effect of *P. pinophilum* and *R. intraradices* on lettuce tolerance to *V. dahliae* inoculation (foliar disease symptoms at 3 wpi)

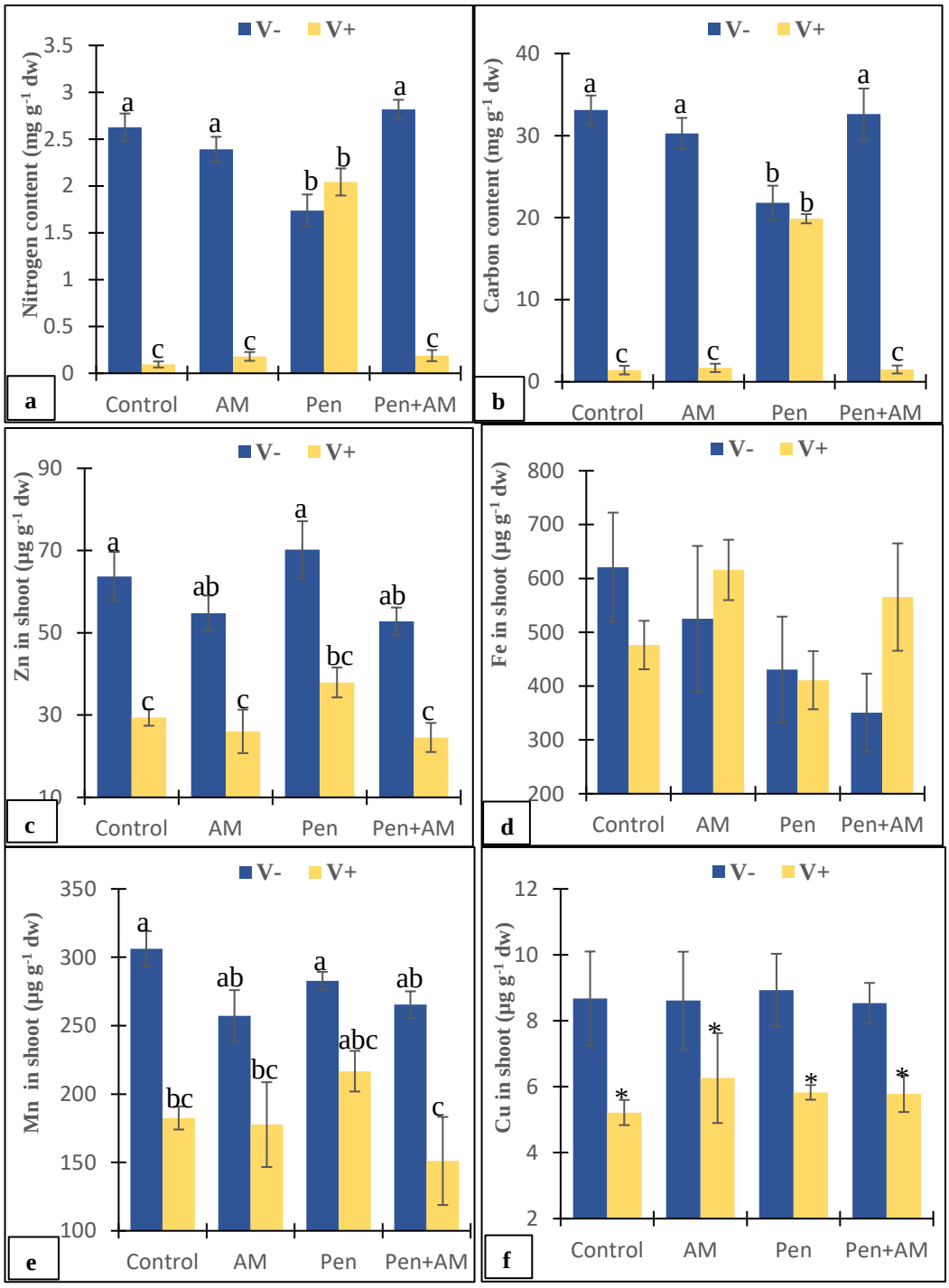

Figure 5.3. Shoot carbon, nitrogen and trace element concentrations of lettuce inoculated with *P. pinophilum* and *R. intraradices* under *Verticillium* stress. Values are Mean ±SEM (n=4). [abc]Superscripts indicates differences based on Tukey-Kramer Post hoc tests. *Significantly different based on ANOVA.

5.4.4 Root colonization

Due to the negative impact of *V. dahliae* on the roots leading to insufficient material, root colonization was not determined in V+ groups, except in *Penicillium* treatment. In V- groups, the control plants were devoid of root colonization while *Penicillium*, AM fungus, and dual inoculation were colonized accordingly. The intensity of AM colonization (Fig. 5.4 a) was higher in dual inoculation compared to single AM in V- groups, but not significant (based on t-test). *Penicillium* colonization (Fig. 5.4 b) was significantly different (P=0.036) between the groups, with higher values occasioned by the presence of AM fungus, and to a lesser extent, *Verticillium*.

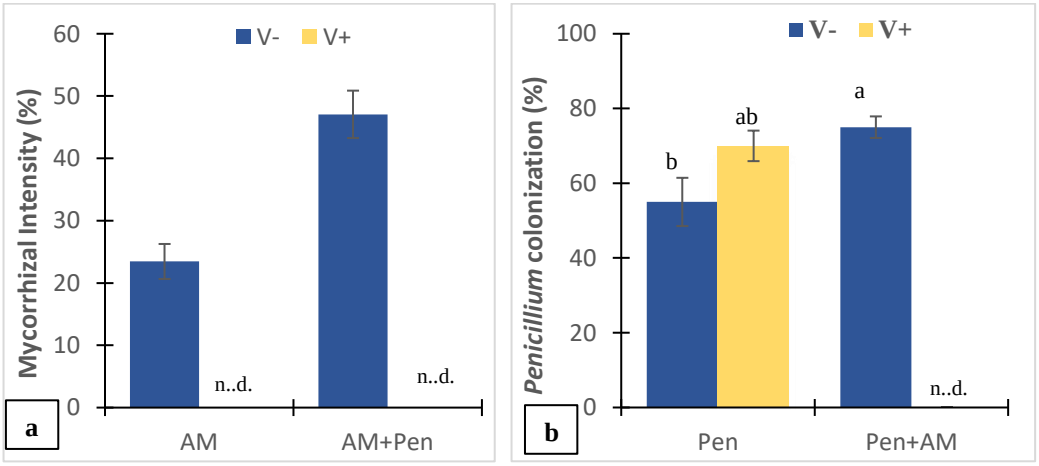

Fig 5.4. Root colonization indices of lettuce inoculated with *P. pinophilum* (Pen) and *R. intraradices* (AM) under *Verticillium* stress. Values are Mean ±SEM (n=4); [ab]Significance based on one-way ANOVA and Tukey-Kramer Post hoc test; n.d. (not determined).

5.5. Discussion

This experiment demonstrated the suppression of *Verticillium* wilt disease by *P. pinophilum* inoculation in lettuce. Chlorophyll content in shoot, which is essential for the general wellbeing of the plant due to its role in photosynthesis, was significantly affected by disease, in line with previous report on race 2 of *V. dahliae* (Ibiang et al. 2021), and this played into the *Verticillium*-induced reductions in carbon content and growth indices observed in V+ plants. This indicate an overall negative impact of *V. dahliae* on the health of the plant. The negative plant growth indices induced by *V. dahliae*, were ameliorated by *P. pinohilum* inoculation, with elongation of the roots and shoots, and increases in leaf number and width. The physiological basis of this may be an increased production of plant growth regulators such as auxin, gibberellins, and ethylene by the endophyte. Endophytes have been reported by many researchers as major source of bioactive compounds, including their stimulation of the production of plant growth hormones (Atanasov *et al*. 2015; Liarzi *et al.* 2016; Guijarro *et al*. 2017). These compounds influence plant growth, development, and their response to biotic and abiotic stress (Khan and Dotyl, 2009; Ali *et al.* 2012; Ullah *et al.* 2019; Gao *et al.* 2021). Indole acetic acids (IAA) are usually produced at the apical meristem of different parts of plants (stem, roots, and buds) where they function in the elongation of plant cells and coordinate major signaling in plant growth and development (Taiz *et al*. 2010). Our previous result showed that *P. pinophilum* significantly modulated IAA, abscisic acids (ABA), and gibberellic acids (GA) in tomato (Ibiang and Sakamoto 2022). Thus, it is possible that similar modulation of phytohormones occurred in lettuce in response to the presence of *V. dahliae*, leading to the increases in shoot and root length of *Penicillium* inoculated plants in V+ groups.

R. intraradices inoculation, including its partnership with *P. pinophilum* in the dual inoculated groups, did not give a substantial protective effect to lettuce plant against *V. dahliae*. Plant-microbe interaction regulates the production of different bioactive compounds, including the stimulation of a range of antimicrobial compounds in plants such as phytoalexins, pathogenesis related proteins, phenolics, etc., in response to pathogen presence (Ahuja *et al*. 2012). Since pathogen DNA quantitation indicated that the inhibition of *V. dahliae* colonization in host stem was greater in AM and dual inoculated group, than in *Penicillium* group, a mycorrhizal effect in the production of inhibitory compounds likely occurred (Dowarah et al. 2021). Phytoalexins, for example, are generally considered as antimicrobials against pathogens, but higher plants are potentially susceptible to their biocidal and biostatic effects (Guest, 2017). Thus, in the presence of *V. dahliae*, the production of such antimicrobial compounds by the AM fungus when it becomes antagonistic to the host, would appear to subsist rather than mitigate the impact of the disease on host growth. Aside potential inhibitory compounds stimulated by the AM fungus, a more obvious reason why AM and dual inoculated plants didn't show tolerance to the pathogen is because their roots were harmed by *V. dahliae* (see Fig S2), and plant nutrition (e.g., nitrogen) was impacted. Apparently, the *in-vivo* inhibition of *V. dahliae* by AM was not on its own sufficient in suppressing *Verticillium* disease. Unlike in single *Penicillium* inoculation, it appears that the additional microbial load of *R. intraradices*, in the dual inoculated treatments minimized the efficacy of *P. pinophilum*. A previous report indicates that upon co-inoculation of *P. pinophilum* and AM fungi, each symbiont may increase its colonization of host roots (Ibiang *et al*. 2020), in line with the observations for mycorrhizal intensity and

Penicillium colonization reported here. Since the growth and development of fungal symbionts is basically dependent on host root colonization to obtain sugars and lipids (Jiang *et al*. 2017; Luginbuehl 2017), the interaction between both symbionts in dual inoculated plants may result in a higher drain on host photosynthates which would detract from the plant under sub-optimal (e.g., disease) conditions (See plates 5.1 – 5.3).

Plant stress attributes from the increased production of ROS such as H_2O_2, and O_2^-. High ROS level can cause lipids, proteins and DNA damage and may finally lead to plant death (Su et al., 2019). Under *Verticillium* disease stress, H_2O_2 production was significantly increased in control and dual inoculated groups. Increased ROS in dual inoculated groups may be as a result of fungal load, while in control plants, it may be due to lack of protection. This result mirrored total polyphenol content as accumulation of phenols was elevated in all V+ groups except *Penicillium* inoculation. Under varying stress conditions, plants respond through a vast range of defense mechanisms which helps plants mitigate or scavenge the stress. Increased production of phenolic compounds in plants is an indication of its response to stress, as phenolics constitute a large portion of the antioxidants which scavenges ROS and offers protection/stress tolerance in plants (Meena *et al*., 2000). SOD activity showed no significant increase due to disease. However, increase was observed in V- group of dual inoculated plants. SOD is one of the first lines of defense plants poses against the invasion of a foreign body. Thus, it may increase due to infection and/or early colonization by both beneficial microbes (Duc et al., 2017). It appears that a high SOD activity was required to modulate dual infection stress in V- groups as this corresponded to a low H_2O_2 levels in same group. Between V- and V+ groups of *Penicillium* inoculation, the H_2O_2 and SOD levels

indicate possible attenuation by reciprocity. Overall, the presence of beneficial microbes modulated the production of SOD as seen in *Penicillium* and dual inoculation.

The concentration of trace elements was significantly reduced by *Verticillium* disease, indicating an adverse impact on host nutrition. Across board, Zn, Mn, and Cu nutrition were lower in V+ groups when compared to V- groups, while Fe nutrition varied but showed no significance. Reduction of root uptake and root-to-shoot translocation may have been the reason V+ groups showed less nutrients, as pathogen colonization of host affects nutrients physiology, including translocation and utilization (Marschner, 1995). Nitrogen content in shoot were also significantly affected by disease in all V+ groups, except in *Penicillium* inoculated group. Plate 5.2 shows a degradation of root tissues/biomass due to *V. dahliae*, which likely contributed to reduced nutrients uptake. N is a macro nutrient required for many biological processes in plants such as respiration, photosynthesis, amino acid synthesis, etc. Some researchers have reported that sufficient plant N levels, and even its forms (nitrate and ammonia) can inhibit pathogen proliferation and support plant defense (Bolton *et al*., 2008; Huang *et al*., 2017). Increased N in shoot by *Penicillium* may have been another strategy to suppress *Verticillium* growth in lettuce shoot, and this likely increased the host biomass and enhanced tolerance observed. With higher nitrogen status of shoots, bioproduction of wider leaves that optimize photosynthates production (inferred from higher shoot carbon contents) was obtained in *P. pinophilum*-inoculated plants, to counter *V. dahliae*.

5.6. Conclusion

P. pinophilum EU0013 inoculation improved lettuce tolerance to *Verticillium* wilt by elongating the roots and shoots, enhancing leaf width and number, increasing plant biomass

and N nutrition, inhibiting systemic proliferation of *V. dahliae* and foliar necrosis, reducing ROS production, and modulating SOD activity in shoots. This study confirms that *in vivo* inhibition of *V. dahliae* by *P. pinophilum* previously reported in tomato in chapter four of this thesis (Ibiang *et al*., 2021), also occur in lettuce. While this is, perhaps the first report utilizing pathogen DNA quantification for verification of the *in vivo* antibiosis of *P. pinophilum* EU0013 and *R. intraradices* against *V. dahlia*e in lettuce, the co-inoculation of *R. intraradices*, despite its *in vivo* antibiosis activity against the pathogen, appeared to interfere with the efficacy of *P. pinophilum*.

CHAPTER SIX

Metabolome profiling of the wheat inoculum of *Penicillium pinophilum* - an endophytic fungus with biocontrol abilities – by LC-TOFMS

6.1 Abstract

Metabolome analysis was performed on the wheat inoculum of *P. pinophilum* using Liquid Chromatography Time-of-Flight Mass Spectrometry (LC-TOFMS) in two modes (for cationic and anionic metabolites), to determine the metabolic contents of the fungal inoculum, and extrapolate the potential implications for its efficacy, food safety, and ecological impact. Two samples were tested, one of which was colonized by the fungus while the other was the uncolonized control. A hundred and twenty-nine metabolites (82 metabolites in positive mode and 47 metabolites in negative mode) were detected on the basis of HMT's standard library.

6.2 Introduction

Secondary metabolites are bioactive compounds that are not directly essential for growth, development, and survival of the organism, but are important biological signal molecules involved in ecological and adaptive interactions of plants/organisms with the environment (Yang et al., 2018). Most of these secondary metabolites are biosynthesized from the primary metabolites and are stored/accumulated in tissues or cell. They are mainly produced by plants, fungi, bacteria, etc. Secondary metabolites can function in diverse ways such as stimulation of symbiosis between plants and beneficial microbes (Guerrieri et al., 2019), trigger defense response (Isah, 2019), induce resistance to biotic and abiotic stress (Yang et al., 2018;

Guerrieri et.al., 2019). These roles have been employed in medicine, chemical and agricultural industries, and are on the increase as sustainable methods of pest/pathogen control, production of drugs, source of flavors and fragrance (Maffei, 2010). Bioactives are a heterogenous group and have been classified into the following groups: alkaloids, phenolics, terpenes (Kabera et al., 2014), non-ribosomal peptides, polyketides (Boruta, 2018), saccharides (Mishra et al. 2022). In plants, the production of secondary metabolites depends on the growth stage, and response to environmental condition (Korenblum et al., 2020. Plant roots secrete these metabolites into the rhizosphere which are put to use by soil microbes and may stimulate the interaction between surrounding microbes (Sugiyama and Yazaki, 2014; Pascale et al., 2020).Microbes on the other hand, produce secondary metabolites based on their phase of growth, and response to environmental conditions. Generally, it is widely accepted that secondary metabolites are important in the establishment and maintenance of plant-microbe interactions (Mishra et al., 2022). Plant endophytes are considered to be a source of metabolites (Rai et al., 2001). The production of bioactive compounds has received increased interest, with metabolomics facilitating the identification of novel compound and endophytes markers. It has been reported that the choice of substrate used for propagation of fungi inoculum is important (Bashan et al. 2014; Marin-Guirao et al., 2016). Substrate with high organic matter such as wheat and barley favor high proliferation of fungi (Rajput et al., 2014). In *Aspergillus aculectus,* an endophyte isolated from *Terminalia laxiflora* was observed to produce higher metabolites in rice substrate inoculum than in liquid media (Tawfike et al., 2017).

Recently, Nischitha and Shivana (2021) reported that the extract of the endophytic fungus, *P. pinophilum* contained 21 antimicrobial and 13 antioxidative compounds. My previous study on substrate inoculum of *P. pinophilum* indicates its higher proliferation in wheat inoculum than in corn and this inoculum was observed to be more effective in its growth promotion and disease suppression in tomato. The aim of this study was to determine the metabolic contents of the wheat inoculum of *P. pinophilum* EU0013 using Liquid Chromatography Time-of-Flight Mass Spectrometry (LC-TOFMS) and extrapolate the potential implications for its efficacy, food safety, and ecological impact.

6.3 Material and Methods

6.3.1 Inoculum preparation

Wheat bran (20 g) was autoclaved for 1 hr at 121 °C and allowed to cool down before inoculating *P. pinophilum*. Samples were incubated for 3weeks at 25 °C. Control sample was prepared in same way but without fungal inoculation. At the end of the incubation period, 20mg of both control and inoculated samples were added into small plastic tubes and sent to Human Metabolome Technologies, Inc. Japan for further study.

6.3.2. Sample preparation for analysis

The sample was placed in a homogenization tube, along with zirconia beads (5 mmφ and 3 mmφ). Next, 1% formic acid in acetonitrile (v/v) containing internal standards (10 μM) was added to the tube, and the sample was completely homogenized at 3,500 rpm at 4 °C for 60 sec × 2 times using a beads shaker. Following this, 167 μL of Milli-Q water was added to the mixture, and further homogenization was performed for another 60 sec. The homogenate was then centrifuged at 2,300 × g at 4 °C for 5 min, after which the supernatant was transferred

to a fresh 1.5 mL microtube. 500 µL of 1% formic acid in acetonitrile (v/v) and 167 µL of Milli-Q water were then added to the homogenization tube. The homogenization and centrifugation steps were repeated once more, and the supernatant was mixed with the previously collected one. The mixed supernatant was filtered through a 3-kDa cut-off filter (NANOCEP 3K OMEGA, PALL Corporation, Michigan, USA) at 9,100 × g at 4 ℃ for 30 min to remove macromolecules, and further filtered through a column (Hybrid SPE Phospholipid 55261-U, Supelco, Bellefonte, PA, USA) to remove phospholipids. The filtrate was evaporated to dryness under nitrogen and reconstituted in 50 % isopropanol in Milli-Q water (v/v) for metabolome analysis.

6.3.3. Measurement

The compounds were measured in the Positive and Negative modes of LC-TOFMS based metabolome analysis in the following conditions. The samples were diluted as shown in Table 6.1 for the measurement, to improve analysis qualities of the CE-MS analysis.

Cationic Metabolites (Positive Mode)

Device:

LC system: Agilent 1200 series RRLC system SL (Agilent Technologies Inc.)

Column: ODS column, 2×50 mm, 2 µm

MS system: Agilent LC/MSD TOF (Agilent Technologies Inc.) Machine No. 9

Analytical Condition:

Column temp.: 40 ℃

Mobile phase A: H_2O / 0.1 % HCOOH Mobile phase

B: Isopropanol: Acetonitrile: H_2O (65:30:5) / 0.1 % HCOOH, 2 mM $HCOONH_4$

Flow rate: 0.3 mL / min

Run time: 20 min

Post time: 7.5 min

Gradient condition: 0-0.5 min: B 1%, 0.5-13.5 min: B 1-100 %, 13.5-20 min: B 100%

MS ionization mode: ESI Positive

MS Nebulizer pressure: 40 psi

MS dry gas flow: 10 L / min

MS dry gas temp: 350 ℃

MS capillary voltage: 4,000 V

MS scan range: m/z100-1,700

Sample injection: 1 μL

Anionic Metabolites (Negative Mode)

LC system: Agilent 1200 series RRLC system SL (Agilent Technologies Inc.)

Column: ODS column, 2×50 mm, 2 μm

MS system: Agilent LC/MSD TOF (Agilent Technologies Inc.) Machine No. 9

Analytical Condition

Column temp.: 40 ℃

Mobile phase A: H_2O / 0.1 % HCOOH

B: Isopropanol: Acetonitrile: H_2O (65:30:5) / 0.1 % HCOOH, 2 mM $HCOONH_4$

Flow rate: 0.3 mL / min

Run time: 20 min

Post time: 7.5 min

Gradient condition: 0-0.5 min: B 1%, 0.5-13.5 min: B 1-100 %, 13.5-20 min: B 100 %

MS ionization mode: ESI Negative

MS Nebulizer pressure: 40 psi

MS dry gas flow: 10 L / min

MS dry gas temp: 350 ℃

MS capillary voltage: 3,500 V

MS scan range: m/z 100-1,700

Sample injection: 1 μL

Table 6.1. **Sample and dilution factor for the LC-TOFMS analysis**

Sample name	**Quantity**	**Dilution(positive)**	**Dilution (negative)**
Control (*P. pinophilum* -)	13.7	1	1
WP (*P. pinophilum +)*	11.8	1	1

6.3.4 Data processing and analysis

6.3.4.1 Data Processing

Peaks detected in the LC-TOFMS analysis were extracted using automatic integration software (MasterHands ver. 2.19.0.2 developed at Keio University) in order to obtain peak information, which includes *m/z*, retention time (RT) and peak area. The peak area was then converted to relative peak area by the following equation. The peak detection limit was determined based on signal-noise ratio; S/N = 3.

$$Relative\ Peak\ Area = \frac{Metabolite\ Peak\ Area}{Internal\ Standard\ peak\ Area * Sample\ Amount}$$

6.3.4.2 Annotation of peaks

Putative metabolites were then assigned from HMT's standard library on the basis of *m/z* and RT. The tolerance was ±0.3 min in RT and ±25 ppm. If several peaks were assigned the same candidate, the candidate was given the branch number.

$$Mass\ error\ (ppm) = \frac{Measured\ value - Theoritical\ value}{Measured\ value} * 10^6$$

6.3.4.3. Plotting on metabolite cluster

The profile of peaks with putative metabolites were represented on metabolite cluster using VANTED (Visualization and Analysis of Networks containing Experimental Data) 2) software.

6.4 Results and discussion

6.4.1 Putative metabolites

From the measurement, 129 peaks (82, 47 peaks in Positive and Negative Mode, respectively) were detected and annotated according to HMT's standard library. We selected and discussed some of the metabolites produced by *P. pinophilum* according to their groups.

a. Phenolics

Caffeic acids are phenolic compounds and are classified as phenylpropanoid hydroxycinnamic acids (HCA) (Raiz et al., 2019). They are secondary metabolites present in the biosynthesis of lignin and occurs in many plants. They are also involved in cell expansion, phototropism, growth, etc. (Lattanzio et al., 2006). Caffeic acids are widely studied due to their pharmaceutical relevance and act as antioxidants, antitumor and antiplatelet. They have also been reported to be involved in plant biotic stress tolerance, where their increased accumulation was observed to inhibit the proliferation of brown rot disease (Martinez, 2012). They are the most occurring phenolic acids in plants, responsible for scavenging reactive oxygen species. They increase plant tolerance to stress based on their high antioxidant activity (Siquet et al., 2006; Raiz et al., 2019). *P. pinophilum* was observed to produce caffeic acid in wheat inoculum which explain some of its antioxidative characteristics (Fig 6.1).

Formononetin is an iso-flavonoid responsible for the biosynthesis of phytoalexins that function to defend plants from biotic and abiotic stress (Tay et al., 2019). They are common in leguminous plants and have gained interest in pharmaceutical industry due to their antitumor and neuroprotective characteristics (El-Bakoush and Olajide, 2018; Li et al., 2018).

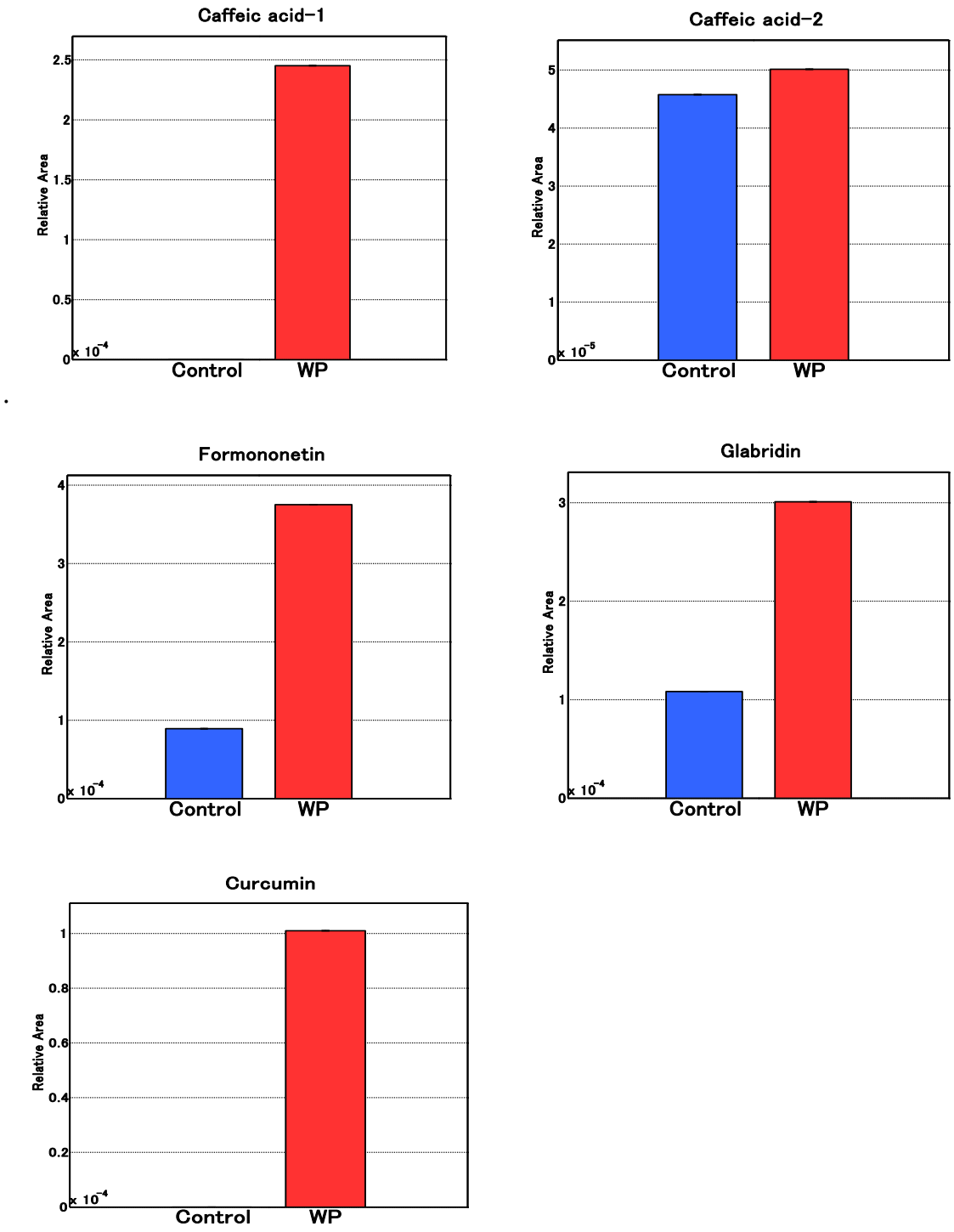

Figure 6.1. Phenolic compounds present in inoculated wheat substrate of *P. pinophlium.*

There are reports of formononetin inhibiting cell proliferation, cytotoxicity against cancer cells (Zhang et al., 2018; Kim et al., 2018a; Qi et al., 2016) indicating a potential drug against cancer. *P. pinophilum* production of formononetin is a potential candidate that can be used for an anticancer drug.

Glabridin is an isoflavonoid with growing impact in the cosmetic and dietary industry. It is known as a phytoestrogen and possess characteristics such as regulating energy metabolism, anti-inflammatory, neuroprotective, antioxidative and anti-atherogenic (Tay et al., 2019). It is also used as a skin lightening agent in the cosmetic industry. In the dietary industry, Glavanoid® an extract rich in glabridin are a relatively novel dietary supplement (Simmeler et al., 2013).

b. Alkaloids and steroids

Ergosterol is the main sterol found in fungi membrane, responsible for membrane signaling and stabilization (Lochman and Mikes, 2006). Ergosterol are not produced by plants and can be perceived as a non-plant metabolite (Sanabria et al., 2009). They function as microbe-associated molecular pattern (MAMP) molecules, which results in microbe-triggered immunity response (MTI). This defense response is associated with the production of ROS and defense related metabolites, activation of ion fluxes, and defense genes (Tugizimana et al., 2012). The identification of ergosterol in inoculated sample is an indication of fungal presence and a confirmation of *P. pinophilum* propagation in the inoculum (Fig 6.2) Other sterols confirmed are cycloartenol-lanosterol and androstanediol. Androstanediol is a

steroidal hormone produced in the male and female gonad and adrenal glands (Badawy et al. 2021). Its main function is the production of testosterone and estrogen.

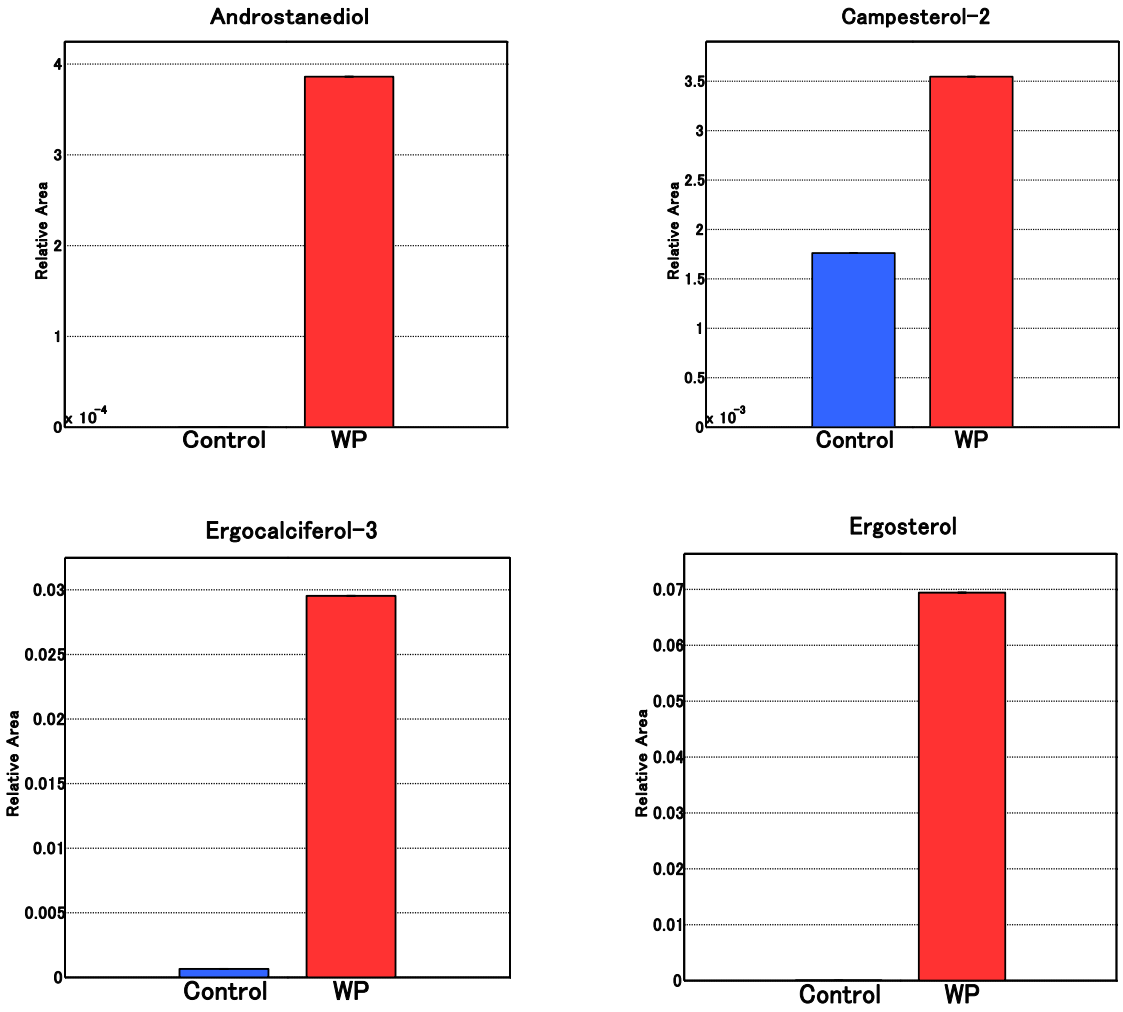

Fig. 6.2. Alkaloids and steroids present in inoculated wheat substrate of *P. pinophilum*.

It is sold as a drug supplement to boost energy, support athletic performance, enhance testosterone quantities, promote healthy red blood cells, develop muscles, etc.

Glycoalkaloids are secondary metabolites produced mainly by plants in the Solanaceae family. They produce these compounds during growth and post-harvest process, with higher concentrations found in young leaves, flowers, sprouts, etc. It has been reported to be abundant in potato. They are allelopathic and their accumulation poses toxicity to certain phytopathogens, predators, and even humans (Al Sinani and Eltayeb, 2017; Friedman, 2006). The highly produced glycoalkaloids are the α-chaconine (solanidine-glucose-rhamnose) and the α-solanine (solanidine galactose-glucose-rhamnose). Solanidine production by *P.pinophilum* was confirmed, indicating another toxic property it possess, perhaps against *Verticillium*. The production of solanidine by *P. pinophilum* also indicates that food safety analysis in animals needs to be evaluated before it can be deployed in a more widespread way.

c. Fatty acids and lipids

Sphingolipids are vital groups of lipids abundant in eukaryotic organisms. Some examples are sphingosine, N-acetylsphingosine, ceramide, etc. They play vital roles such as regulation of cell growth and differentiation, moderation of cell-to-cell interactions (Bartke and Hannun 2009), receptors for antibiotics, bacteria, and viruses. Sphingosine can be phosphorylated *in vivo* by sphingosine kinase, to form sphingosine-1-phosphate which is a signaling lipid. Its amino group is mainly neutral, giving it the ability to transfer and translocate freely among membranes. This enables it to play an important role in cell death (apoptosis), differentiation,

and growth (Li et al., 2021; Chen et al. 2021). N-acetylsphingosine and sphingosine were identified as lipids produced by *P. pinophilum* (Fig 6.3).

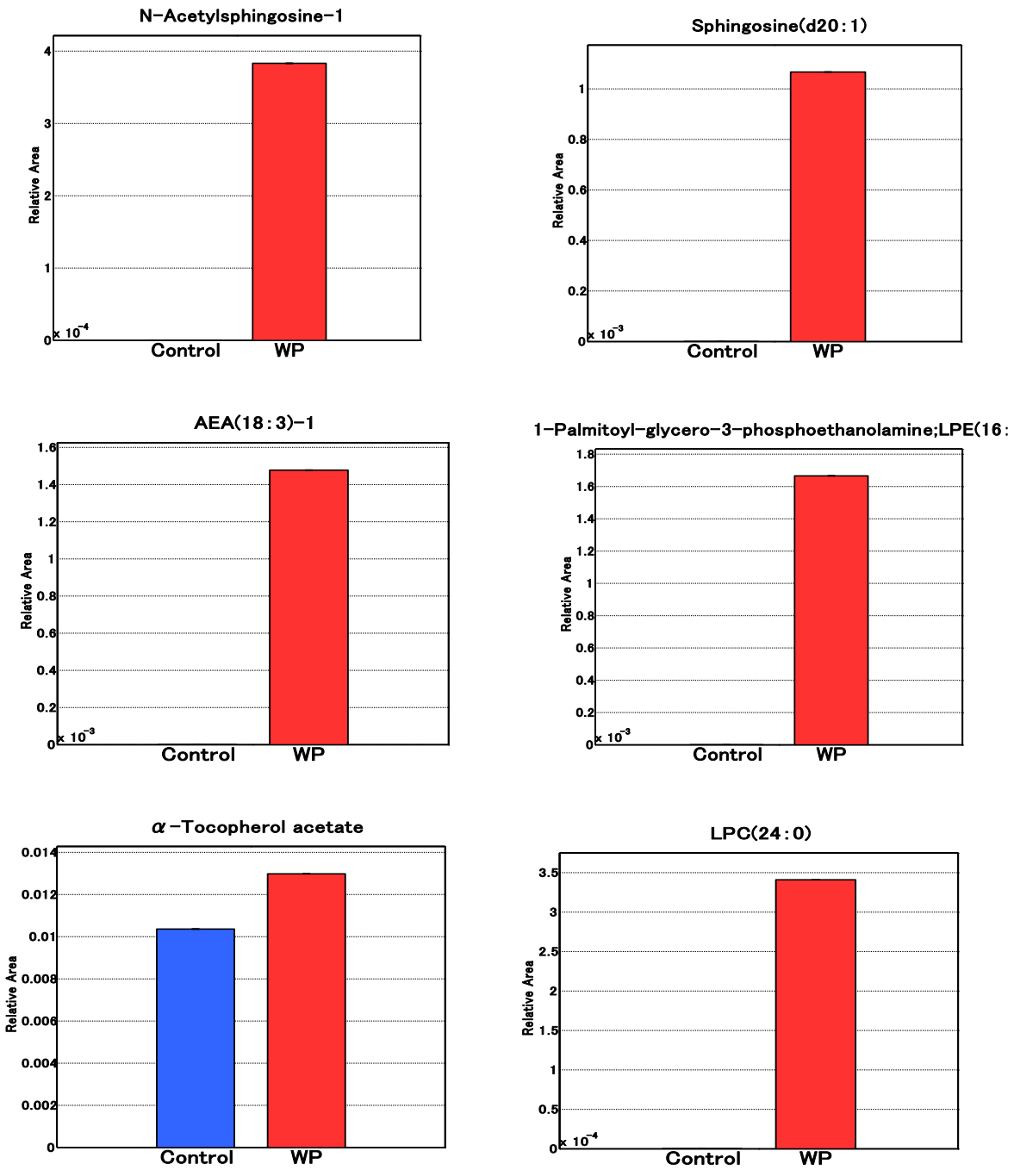

Fig.6.3. Fatty acids and lipids present in inoculated wheat substrate of *P. pinophlium*

d. **Coenzymes**

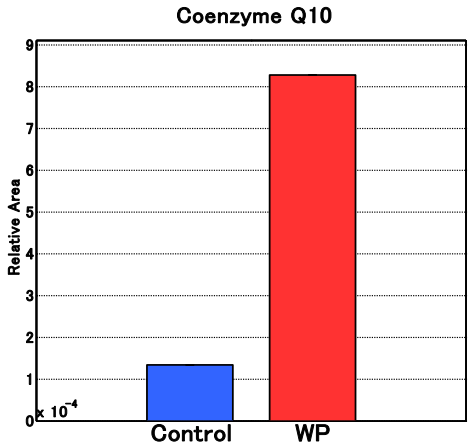

Fig.6.4. Coenzyme present in inoculated wheat substrate of *P. pinophlium.*

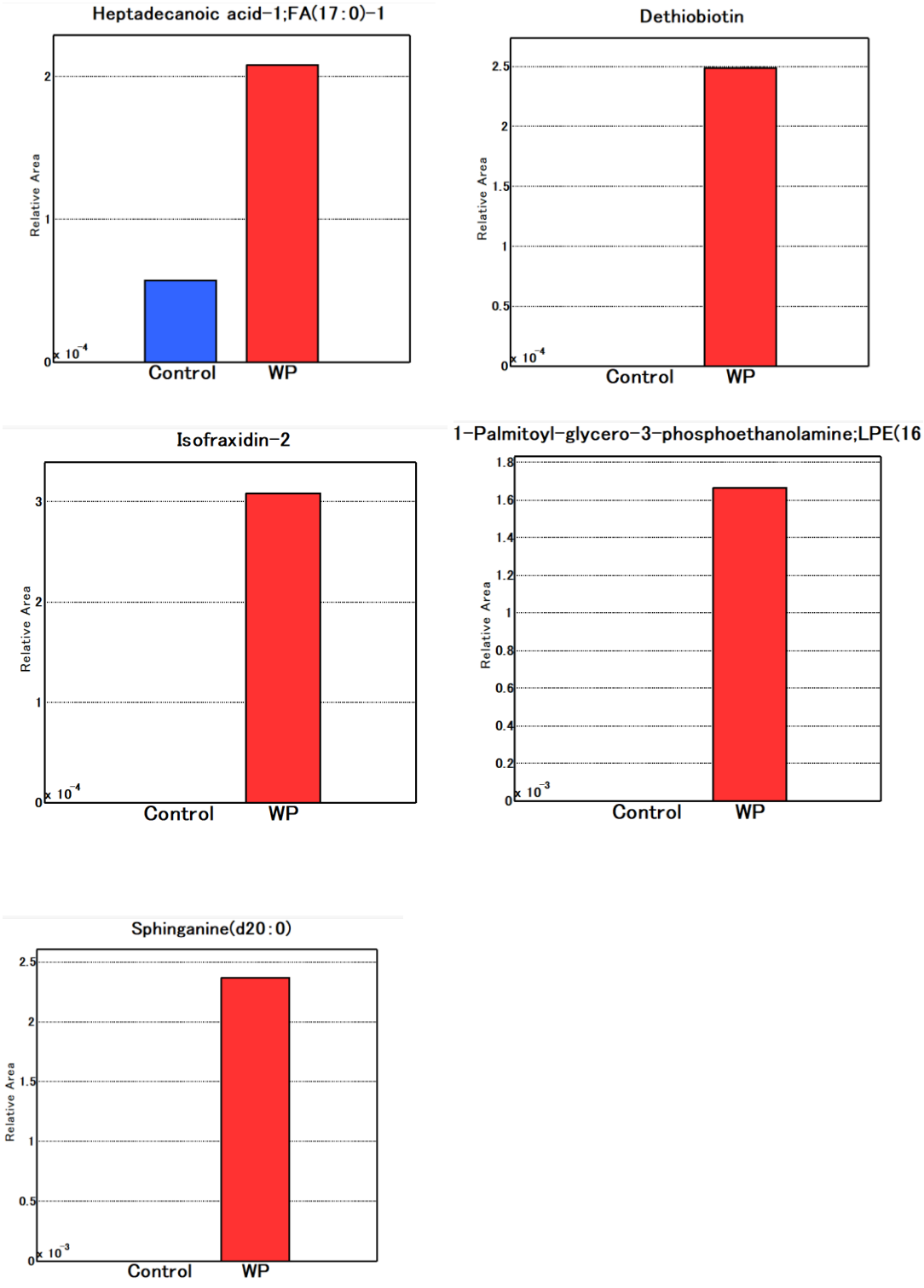

Fig 6.5. Other metabolites present in inoculated wheat substrate of *P. pinophlium.*

6.5. Conclusion

P. pinophilum ability to produce varying compounds in wheat inoculum is an indication that it can produce useful chemicals of economic importance that can be applied in agriculture, pharmaceutical and cosmetics industries. More experiments are required to determine possible compounds that could be produced in other inoculum types, and their safety during application.

CHAPTER SEVEN
GENERAL DISCUSSION AND CONCLUSION

7.1 General discussion

Series of experiments were conducted to understand the efficacy and mechanisms deployed by *P. pinophilum* and AM fungi as plant growth promoters and as biocontrol agents against *Verticillium* disease, in tomato and lettuce. This study established that a bio-stimulatory interaction exists between the AM fungi species tested and *P. pinophilum*, which resulted in higher root colonization and regulation of host physiological parameters. In tomato, negative outcomes were not observed but this occurred in lettuce, where wilting and retarded growth was observed. The results implied that the outcome of their interactions is dependent on the type of inoculum substrate used for *P. pinophilum*, the target host and soil type, such that single inoculation was better for lettuce. Differences in host response due to plant and soil type has been reported (Miransari 2014; Ibiang et al. 2020). *P. pinophilum* demonstrated the potential of improving nutraceutical value in tomato but this was cultivar dependent. However, four out of five cultivars showed a positive impact of the fungus presence by promotion of biochemical and physiological properties. Furthermore, during the interaction between beneficial microbes and phytopathogen in different hosts, *P. pinophilum* exhibited higher resistance against *V. dahliae* and this was consistent in both lettuce and tomato confirming a biocontrol property of the fungus. AM fungi (*R. intraradices*) inoculation conferred some protection in tomato, but not in lettuce, and its co-inoculation with *P. pinophilum* did not improve lettuce protection against Verticillium wilt disease. Further studies on the interaction of *P. pinophilum* and the AM fungi under disease stress is required to determine the reason for this, but it can be inferred that *R. intraradices* has some

antagonism to *V. dahliae,* but the compounds it produced were tolerated by tomato than by lettuce. This study established that *V. dahliae* biomass in shoot of lettuce was reduced by AM fungi, but this had no positive impact on the host. *P. pinophilum* production of varying metabolites with antimicrobial, antiviral, and anti-inflammatory characteristics are the possible reasons for its growth promoting and biocontrol traits. Further experiments are required to test its consistency on other vegetables.

7.2. General conclusion

All AM fungi inoculants used in these studies provided diverse support to hosts as growth-promoters. *P. pinophilum* co-inoculation with AM fungi showed varying interactions, ranging from synergism to antagonism in both lettuce and tomato (see Fig. 7.1). These results implied that both partners can coexist together in the rhizosphere but their synergistic impact on the host is dependent on the inoculum substrate of *P. pinophilum*, inoculation strategy used, soil and host type. Also, co-inoculation of both partners under stress conditions (soil and disease) are not required as this led to negative outcomes in tomato and lettuce. *P. pinophilum* was consistent as a biocontrol agent against *V. dahliae* by suppressing its proliferation *in vitro* and *in vivo* (in both tomato and lettuce). *P. pinophilum* also acted as a growth-promoter via modulation of phytohormones and mineral nutrition. To ascertain uniformity in the outcome of different hosts, a more suitable mode of its application to fields and plants is necessary. Some of its mechanisms utilized during the course of these studies include root elongation, modulation of phytohormones and mineral nutrition, antagonism (*in*

vitro and in vivo), and antioxidants production. More experiments are required to test its efficacy against other phytopathogens before its widespread use as a bioinoculant.

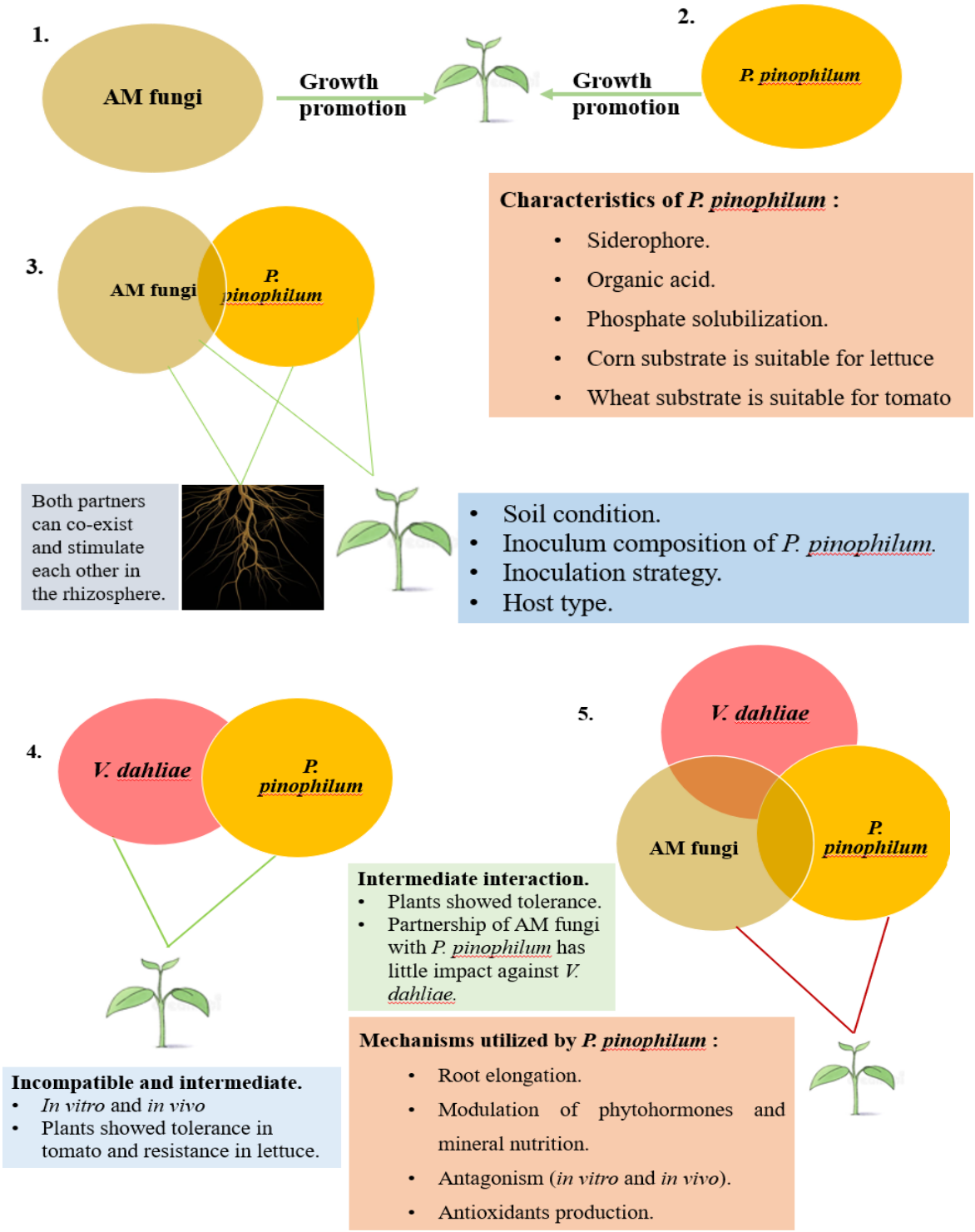

Fig.7.1. Diagrammatic conclusion

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