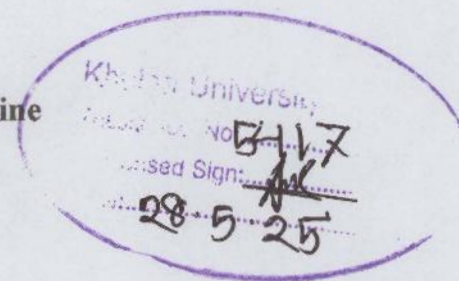


**Symbiosis of Soil Inhibiting Beneficial Microbes Boosts
Immunity to Root Rot and Foot Rot of Betelvine with
Enhanced Productivity**

A Thesis

**Submitted to
Agrotechnology Discipline
Khulna University**



By

STUDENT ID: MS-230824

**In Partial Fulfillment of the Requirements for the Degree of
MASTERS OF SCIENCE IN PLANT PATHOLOGY**



AGROTECHNOLOGY DISCIPLINE

LIFE SCIENCE SCHOOL

KHULNA UNIVERSITY

KHULNA – 9208

DECEMBER 2024

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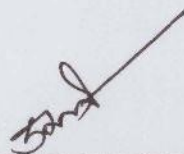
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the thesis)**

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**DEDICATED TO,
MY BELOVED PARENTS**

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The author

ABSTRACT

Betelvine (*Piper betle* L.) is an important cash crop grown primarily for its leaves, which are used in culinary, medicinal, and cultural practices. However, the yield of the culinary herb is greatly challenged by various pathogens, especially *Sclerotium rolfsii*. To reduce the target pathogen load, our study investigated the field efficiency of some selective soil-inhabiting beneficial microbes—*Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and Arbuscular Mycorrhizal Fungi (AMF)—on the growth and yield, metabolite profiling and immune response of the betelvine to *S. rolfsii*. The treatments were applied individually and in combination following RCBD design with triplicates. Results indicated that the combined application of Th, Pf, and AMF significantly enhanced nitrogen, phosphorus, and potassium accumulation which positively improved plant growth, yielding the highest values for vine length (142.77 ± 2.75 cm), number of laterals (22.00 ± 1.15), and leaf breadth (13.70 ± 0.75 cm). Yield parameters such as 100-leaf weight (277.23 ± 2.98 g), leaf number per plant (82.00 ± 1.57) as well as income (182.25 ± 1.72 taka per plant) were also statistically higher in the combined treatment compared to untreated controls and individual treatments. The disease incidence reduced by up to 74.32% in plants treated with Th + Pf + AMF. Biochemical analysis showed significant increases in flavonoids (Kaempferol $19.46 \mu\text{g/g} \pm 0.42$ mg/g), tannins (Catechuic acid 15.14 ± 0.33 mg/g), and phenolic acids (Gallic acid 12.50 ± 0.26 mg/g) in plants treated with the Th + Pf + AMF compared to check. In molecular docking analysis of eight bioactive ligands with the 1J58 (Oxalate Decarboxylase) and 1BHE (Polygalacturonase) proteins identified strong binding interactions, with Catechuic acid exhibiting the highest number of interactions with 1J58, and Quercetin demonstrating the broadest interaction profile with 1BHE, highlighting their potential in mitigating disease by effectively targeting these proteins through specific molecular binding. These findings suggest that the combination of these microbial treatments can effectively boost betelvine productivity by enhancing host immunity and concentration of bioactive compounds, offering a sustainable ecoefficient alternative to traditional chemical control of foot and root rot of betelvine.



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CHAPTER I

INTRODUCTION

The betel vine, scientifically known as *Piper betle* L., is a shade-loving plant that reproduces asexually and has separate male and female individuals. The plant is an evergreen and perennial creeper that belongs to the Piperaceae family. It has distinct heart-shaped leaves that are between 4 to 7 inches in length and 2 to 4 inches in width (Bhakta et al., 2016). Paan leaves are cultivated in the Philippines, Malaysia, India, Sri Lanka, Taiwan, Thailand, and other Southeast Asian nations for the purpose of being used as a mouth refresher after meals. The main consumers of this product are predominantly in South Asia and among specific Asian emigrants globally, who consume it as betel quid or paan. It is typically used in combination with areca nut or tobacco (Tutu et al., 2022). It is locally known as 'Pan' in Bangladesh and one of the vital cash crops of this country, where millions of people chew the item regularly. It is consumed mostly as betel quid which contains betel leaf, areca nut, and slaked lime (Flora et al., 2012). Bangladesh's betel leaf farming is estimated to be 11,230 hectares per year, with major districts like Rajshahi, Kushtia, Jashore, Khulna, and Satkhira producing around 6.5 metric tons per hectare, depending on leaf variety and cultivation practices (Mahfuza et al., 2020). The betel vine leaf, used as a mouth freshener, has medicinal, antiseptic, and antimicrobial properties due to its active phyto-chemicals, and is also used in cosmetics and ointments.

In betel vine cultivation farmers face a number of challenges, including root rot and foot rot diseases, which can have a significant negative impact on crop yield and overall productivity. The majority (55.3%) faced medium problems, while 23.5 percent faced low and 21.2% faced high problems. Factors such as age, education, experience, and knowledge had no significant impact on the problems faced. Leaf rot disease and lack of credit facilities were the most common problems faced by the farmers. (Imran, 2016; Afsana et al., 2014)

Disease damage to the crop is one of several known limiting factors. The betel vine is highly susceptible to diseases, pests some natural calamities (Sayeeduzzaman, 1988).

Humid and moist shaded conditions are favorable for betel vine growth and also favor a variety of root and foliage disease development (Goswami et al. 2002). Among the diseases of betel vine foot and root rot caused by *Sclerotium rolfsii* is the most overwhelming disease which decreases the production of betel leaf to a great extent. Farmers growing Piper betle in three upazilas of Rajshahi 3 incurred a huge loss as foot rot disease damaged about 60% of the cultivation in the year of 2004 (Jahana et al., 2014). *Sclerotium rolfsii* is a serious soil borne pathogenic fungus and harmful to many crops which are economically valuable in most of the tropical and subtropical region of the world. It has a wide host range and it has been referred as an almost omnipathogenic organism (Sadia et al., 2020). The fungus *Sclerotium rolfsii* is a facultative parasite and can maintain continuity of generation under adverse situation by the formation of sclerotia (Rahman et al., 2022).

Agriculture is a major economic activity in developing countries, with over 50% of the population engaged. However, low crop productivity due to factors like low soil moisture, nutrient capital, erosion risk, and pests has led to the green revolution. Microbial inoculants, such as rhizobium, plant growth promoting rhizobacteria, and arbuscular mycorrhizal fungi, can improve soil nitrogen, phosphorus, and potassium availability and uptake. They can also improve soil structure and alleviate plant stress from drought, soil contamination, and salinity. Microbial inoculants function through various mechanisms, such as producing plant hormones, expanding root systems, and eliciting resistance. The success of these strategies depends on the use of appropriate carrier material and the balance of soil microbial communities. (Ahirwar et al., 2020; Alori et al., 2017)

Microorganisms interact in ecosystems through synergistic, antagonistic, physical, and biochemical competition, influenced by biotic and abiotic factors. In the rhizosphere, microorganisms like arbuscular mycorrhizal fungi, *Trichoderma* fungi, and *Pseudomonas* bacteria play a crucial role in plant growth promotion and biocontrol. Their interactions can either synergistically or antagonistically affect plant growth and development (Cano, 2011).

Trichoderma spp., *Pseudomonas fluorescens*, and *Arbuscular Mycorrhizae* (AM) are beneficial microorganisms known for their ability to form symbiotic relationships with plants, promoting plant health and resilience. These microbes contribute significantly

to enhancing plant growth, defense, and nutrient acquisition, forming a crucial part of sustainable agriculture. The symbiotic relationship between these microbes and plants, particularly in the case of betel vines (*Piper betle*), has garnered significant interest due to its role in promoting plant immunity and improving soil health (Yang et al., 2022).

Trichoderma species (*T. harzianum*, *T. viride*, *T. aspergillum*), *Pseudomonas fluorescens*, and arbuscular mycorrhizal fungi (AMF) play crucial roles in promoting plant growth, enhancing disease resistance, and improving the overall health of plants, particularly betel vines (*Piper betle*). *Trichoderma* fungi act as biocontrol agents by colonizing plant roots, producing secondary metabolites that protect against pathogens such as *Fusarium spp.*, and stimulating the plant's immune system (Asad, 2022; Dutta et al., 2023). *Pseudomonas fluorescens* promotes plant growth by producing substances like auxins and siderophores, which improve nutrient uptake, while also suppressing pathogens through antibiotic production and competitive exclusion in the rhizosphere (Singh et al., 2003; Jetiyanon et al., 1998; Lugtenberg et al., 2009). AMF, in symbiotic relationships with plant roots, enhance nutrient uptake, especially phosphorus and nitrogen, and improve tolerance to environmental stressors like drought and salinity. In betel vines, the presence of AMF boosts growth, improves resistance to root diseases, and stimulates the production of defense-related compounds, promoting overall plant health (Smith & Read, 2010; Begum et al., 2019).

These microorganisms collectively contribute to the sustainable cultivation of betel vines by improving plant immunity, growth, and resilience to environmental challenges. The mutualistic interactions between *Trichoderma spp.*, *Pseudomonas fluorescens*, and AMF exemplify the power of microbial partnerships in enhancing plant health (Vyas et. al., 2024). These microbes work together to bolster the vine's natural defense mechanisms, making it more resistant to both biotic and abiotic stresses. *Trichoderma* species protect betel vines from pathogens, while *Pseudomonas fluorescens* promotes growth and suppresses harmful microbes in the rhizosphere. Meanwhile, AMF enhance nutrient uptake and water absorption, ensuring that the vine remains healthy and resilient. Together, these microbial allies create a synergistic effect that results in stronger, more disease-resistant betel vines, contributing to sustainable agricultural practices and improved crop yields.

Sustainable farming practices, including the use of beneficial microorganisms, offer numerous environmental and long-term benefits. These include reduced reliance on synthetic fertilizers and pesticides, improved soil health, enhanced biodiversity, increased productivity, reduced costs, and improved food security. Beneficial microorganisms also enhance the nutritional value of food by improving plant absorption and producing secondary metabolites. They also reduce food waste by extending fruit and vegetable shelf life and inhibiting spoilage-causing microorganism growth. Furthermore, beneficial microorganisms improve animal health and welfare by boosting immune systems, reducing disease susceptibility, and promoting weight gain. Thus, adopting sustainable farming practices is a win-win for both farmers and the environment, promoting a more sustainable and food-secure future (Oberč & Arroyo Schnell, 2020)

Considering the significance of these soil-inhabiting microbes, the current study is hypothesized to explore the symbiotic relationship between *Trichoderma* spp., *Pseudomonas fluorescens*, and AM fungi, which enhances plant immunity against Root Rot and Foot Rot, leading to increased productivity. It aims to quantify this impact on different betel vine varieties and provide practical recommendations for farmers. The study suggests developing biofertilizers and biopesticides containing these microbes to improve soil health, suppress diseases, and enhance productivity. It also suggests developing planting protocols that promote this relationship. The study also encourages farmers to educate about soil health and beneficial microbes for sustainable agriculture.

Objectives

- To investigate the mutualistic relationship among *Trichoderma harzianum*, *Pseudomonas fluorescens* and Arbuscular Mycorrhizae to enhance the plant's immunity to root rot and foot rot disease of betelvine.
- To assess their symbiotic impact on soil fertility and overall betel vine productivity.
- To offer insights into sustainable disease management practices for betel vine farmers for improved crop health, disease resistance, and productivity.

CHAPTER II

REVIEW OF LITERATURE

Root rot and foot rot are significant soil-borne diseases affecting a wide range of agricultural crops. These diseases, caused by various fungal pathogens, result in considerable yield losses and pose a threat to global food security. Traditional chemical treatments have been used to manage these diseases; however, growing concerns over environmental sustainability, soil health degradation, and the rise of pesticide resistance have prompted the search for more sustainable and eco-friendly alternatives. Biological control using beneficial microorganisms, such as *Trichoderma* species, *Pseudomonas fluorescens*, and arbuscular mycorrhizal fungi (AMF), offers a promising solution. These microorganisms exhibit various mechanisms that suppress pathogenic fungi, promote plant growth, and enhance plant immunity, thereby improving productivity and mitigating disease.

This literature review explores the roles of *Trichoderma* spp., *P. fluorescens*, and AMF in the biocontrol of root and foot rot diseases. The review delves into their individual and combined effects on plant immunity, disease suppression, and overall plant productivity.

2.1. Historical context

Foot and root rot of betel vine was first recognized as a serious problem in the early 20th century. In India, where betel vine cultivation is widespread, farmers observed severe wilting and decay of vines, leading to significant yield losses. The disease was attributed to poor soil conditions and high humidity, but its exact cause remained uncertain until systematic studies were undertaken (Kharwar et al., 2021). Initial research identified fungal pathogens as the primary culprits behind foot and root rot. *Phytophthora parasitica* and *Sclerotium rolfsii* were among the first pathogens isolated and reported as causal agents (Parvin et al., 2020). Subsequent studies revealed that other fungi, including *Rhizoctonia solani*, also contributed to disease severity (Mehrotra & Aggarwal, 1974). The disease thrives in warm and humid environments, which are typical of betel vine-growing regions. It spreads through contaminated soil, water, and infected planting materials. By the mid-20th century, researchers emphasized the role of improper drainage and monocropping in exacerbating the disease (Ramakrishnan,

1949). In the latter half of the 20th century, research shifted toward developing effective control measures. Cultural practices such as crop rotation, soil solarization, and the use of disease-resistant cultivars were recommended. Chemical fungicides like copper oxychloride and carbendazim showed promise in managing the disease but raised concerns about environmental impact and pathogen resistance (Kumar et al., 2016). In recent decades, integrated disease management (IDM) approaches have gained traction. These combine biocontrol agents like *Trichoderma harzianum*, organic amendments, and precision irrigation to reduce the disease burden. The role of microbial diversity in suppressing pathogens has also been explored, offering sustainable alternatives to chemical control (Parvin, 2013).

2.2. Root Rot and Foot Rot Pathogens

Root rot and foot rot are major diseases caused by a variety of fungal pathogens that infect plant roots and lower stems. Common pathogens responsible for these diseases include species from the genera *Fusarium*, *Rhizoctonia*, *Pythium*, *Phytophthora*, and *Sclerotinia* (Agrios, 2005). The pathogens thrive in poorly-drained soils and typically cause severe damage during conditions of high humidity, temperature, and moisture stress.

Fusarium spp., for example, are responsible for root rot in many crops, including tomatoes, potatoes, and cereals. These fungi infect plant roots, causing vascular wilt, nutrient deficiency, and ultimately plant death (Leslie & Summerell, 2006). Similarly, *Pythium* and *Phytophthora* species cause root rot, often leading to the decay of the root system and significant yield losses (Erwin, 1996).). Foot rot, caused by pathogens like *Rhizoctonia solani* and *Phytophthora* spp., affects the lower stem area and can lead to severe root damage and stunting, especially in plants grown in heavy, poorly aerated soils (Akber et al., 2024).

These diseases often result in severe economic losses, especially in crops that are susceptible to soil-borne pathogens. Chemical fungicides have been traditionally used to control these diseases; however, the increasing environmental and regulatory concerns related to their use have made biological control an attractive alternative (Lahlali et al., 2002).



2.3. Biological Control Agents

Biological control involves the use of beneficial organisms to suppress or control plant pathogens, promoting plant health. The primary mechanisms of biological control include competition for resources, production of antifungal metabolites, pathogen inhibition via direct antagonism, and induction of plant immune responses (Tyagi et al., 2024). Among the most effective biological control agents are *Trichoderma* species, *Pseudomonas fluorescens*, and arbuscular mycorrhizal fungi (AMF). These microorganisms not only suppress plant pathogens but also promote plant growth and enhance resistance to environmental stress.

Betelvine, an important medicinal and aromatic plant, is susceptible to various diseases, including root rot and leaf spot. *Trichoderma harzianum* and *Pseudomonas fluorescens* have been identified as effective BCAs for controlling these diseases. For instance, research by Dasgupta and Sen (1997) demonstrated that *Trichoderma harzianum* significantly reduced the incidence of root rot in betelvine. Similarly, Rahman et al., 2008 reported that *Pseudomonas fluorescens* effectively suppressed leaf spot disease in betelvine.

Tomato is a widely cultivated vegetable crop affected by various diseases, such as late blight and root-knot nematode. *Trichoderma harzianum* and *Pseudomonas fluorescens* have been used to control these diseases. Research by Anjum et al. (2019) demonstrated that *Trichoderma harzianum* effectively suppressed late blight in tomato. Similarly, studies by Norabadi et al. (2014) showed that *Pseudomonas fluorescens* reduced the incidence of root-knot nematode in tomato.

Melon cultivation is often hampered by powdery mildew and Fusarium wilt. Several BCAs, including *Trichoderma* spp. and *Bacillus subtilis*, have shown promise in managing these diseases.² Studies by Ahmed (2015) revealed that *Trichoderma viride* and *Bacillus subtilis* significantly reduced the severity of powdery mildew in melon.

2.4. *Trichoderma harzianum* and Their Role in Disease Suppression

Trichoderma spp. are well-known for their antagonistic activity against a wide range of fungal pathogens. Species such as *Trichoderma harzianum*, *Trichoderma viride*, and *Trichoderma koningii* have been widely studied for their biocontrol properties. The mechanisms through which *Trichoderma* suppresses pathogens include:

Antagonistic Activity: *Trichoderma* spp. suppress fungal pathogens by producing hydrolytic enzymes such as chitinases, glucanases, and proteases, which degrade the cell walls of pathogenic fungi (Harman et al., 2004). For instance, *T. harzianum* produces gliotoxins and peptaibols, secondary metabolites that inhibit pathogens like *Fusarium oxysporum* and *Phytophthora capsici* (Vinale et al., 2008). These metabolites disrupt cellular processes, thereby reducing pathogen viability.

Mycoparasitism: *Trichoderma* spp. exhibit mycoparasitic behavior, where they parasitize and kill other fungi by growing over their hyphae and secreting lytic enzymes (Alfiky et al., 2021). This is particularly effective against pathogens like *Fusarium* and *Rhizoctonia*, which are responsible for root and foot rot.

Induced Systemic Resistance (ISR): *Trichoderma* spp. enhance plant defense by inducing systemic resistance. This involves activating the plant's immune system and boosting the production of defensive enzymes such as phenylalanine ammonia lyase (PAL), peroxidases, and β -1,3-glucanase (Shoresh et al., 2010). In betel vine, ISR mediated by *Trichoderma* spp. has been shown to reduce disease severity and improve plant health (Behiry et al., 2023).

Competition for Resources

In the rhizosphere, *Trichoderma* spp. compete with pathogens for nutrients and space. Their rapid colonization of root zones limits the availability of resources for pathogens, thereby preventing their establishment (Howell, 2003).

2.5. The Role of *Pseudomonas fluorescens* in Plant Health

Pseudomonas fluorescens is another well-studied biological control agent, known for its ability to suppress soil-borne pathogens through multiple mechanisms:

Production of Antibacterial and Antifungal Compounds: *P. fluorescens* produces a range of antimicrobial compounds, including 2,4-diacetylphloroglucinol (DAPG), which has been shown to inhibit the growth of pathogens like *Fusarium*, *Rhizoctonia*, and *Pythium* (Mavrodi et al., 2007). These compounds disrupt pathogen growth by interfering with cell membrane integrity or inhibiting pathogen-specific enzymes.

Siderophore Production: *P. fluorescens* secretes siderophores that bind to iron in the soil, making it unavailable to pathogens. By outcompeting pathogenic fungi for this

essential nutrient, *P. fluorescens* limits the growth of harmful microbes in the rhizosphere (Duffy et al., 1999).

Induction of Systemic Resistance: Similar to *Trichoderma*, *P. fluorescens* can induce systemic resistance in plants, activating the plant's defense mechanisms against a wide range of pathogens. This is particularly important in preventing root rot and foot rot diseases caused by soil-borne fungi (Kloepper et al., 2004).

2.6. Arbuscular Mycorrhizal Fungi (AMF) and Their Role in Disease Resistance

Arbuscular mycorrhizal fungi (AMF) are a group of fungi that form mutualistic relationships with plant roots. The primary benefits provided by AMF include enhanced nutrient uptake, particularly phosphorus, improved drought resistance, and increased plant resistance to pathogens. AMF play a critical role in enhancing plant resistance to root rot and foot rot diseases:

Physical Barrier Against Pathogens: AMF form a dense network of hyphae around the root system, preventing pathogen invasion. This physical barrier makes it harder for pathogens such as *Fusarium* and *Phytophthora* to penetrate the root tissues (Vyas et al., 2024).

Induced Resistance: AMF can stimulate the production of defensive enzymes, such as chitinases and peroxidases, which help protect plants from pathogen attack (Ruiz-Lozano et al., 2012). Additionally, AMF activate plant immune responses, enhancing the plant's ability to resist root and foot rot pathogens.

Improved Root Health and Nutrient Uptake: AMF improve the health of the root system by increasing the root surface area for nutrient absorption. This is particularly beneficial in combating diseases that weaken the root system, such as root rot caused by *Fusarium* and *Pythium* (Smith & Read, 2008).

2.7. Synergistic Effects of *Trichoderma harzianum*, *P. fluorescens*, and AMF in Disease Suppression

The use of synergistic microbial consortia involving *Trichoderma* spp., *Pseudomonas fluorescens*, and arbuscular mycorrhizal fungi (AMF) has gained prominence as an eco-friendly approach to disease suppression in betel vine (*Piper betle* L.) and various other crops (Mishra et al., 2021; Pandey et al., 2023). These microbes, through

complementary and interactive mechanisms, enhance plant resilience against pathogens while promoting growth and productivity. *Trichoderma* spp., particularly *T. harzianum* and *T. viride*, are widely recognized for their mycoparasitic activity, where they degrade the cell walls of fungal pathogens using hydrolytic enzymes like chitinases and glucanases. This action is particularly effective against soil-borne pathogens such as *Fusarium* spp. and *Phytophthora* spp., which cause root and foot rot in betel vine and other plants (Harman et al., 2004). The ability of *Trichoderma* to produce secondary metabolites, such as gliotoxin and 6-pentyl- α -pyrone, further enhances its antifungal properties and inhibits the growth of plant pathogens.

The inclusion of *P. fluorescens* in microbial consortia adds a bacterial dimension to disease suppression. This bacterium produces a range of antibiotics, including phenazines, pyrrolnitrin, and pyoluteorin, which are effective against bacterial and fungal pathogens. It also induces systemic resistance (ISR) in plants by stimulating the production of pathogenesis-related proteins, such as peroxidases and chitinases, and phenolic compounds, which collectively enhance plant defenses (Van Loon et al., 1998). Moreover, *P. fluorescens* contributes to nutrient mobilization by solubilizing phosphorus and producing siderophores that chelate iron, thereby limiting its availability to pathogens while enhancing plant nutrient uptake (Kloepper et al., 2004). Studies in crops like tomato and cucumber have shown that *P. fluorescens* significantly reduces the incidence of wilt and blight when used in combination with *Trichoderma* spp., underscoring the effectiveness of such microbial partnerships (Rajeswari, 2019).

AMF complement the activities of *Trichoderma* and *P. fluorescens* by forming symbiotic associations with plant roots. These fungi extend hyphal networks into the soil, facilitating the uptake of immobile nutrients like phosphorus and micronutrients such as zinc and copper (Smith & Read, 2008). The improved nutrient status not only enhances plant growth but also strengthens the plant's ability to resist infections. Additionally, AMF contribute to better soil aggregation and water retention, which indirectly benefit microbial consortia by providing a stable rhizosphere environment. In crops like maize and wheat, AMF have been shown to enhance the colonization efficiency of *Trichoderma* and *P. fluorescens*, resulting in improved disease suppression and higher yields (Goswami et al., 2016). Similar benefits have been observed in betel vine, where the combination of these microbes has reduced disease severity caused by *Sclerotium rolfsii* and *Rhizoctonia solani* (Pandey et al., 2023).

The synergistic effects of these microbes stem from their ability to occupy distinct ecological niches in the rhizosphere. While *Trichoderma* targets fungal pathogens through direct antagonism and enzymatic degradation, *P. fluorescens* provides bacterial pathogen suppression and nutrient mobilization. AMF, on the other hand, enhance nutrient acquisition and plant resilience against abiotic stresses such as drought and salinity (Miransari, 2010). This division of labor allows the microbes to function together without competition, maximizing their collective benefits. Field studies have confirmed that the combination of *Trichoderma*, *P. fluorescens*, and AMF is more effective in disease suppression than individual applications. For instance, Singh et al. (2014) reported a 40–60% reduction in the incidence of root rot and an increase in yield in betel vine when these microbes were applied together compared to single treatments.

The application of these microbial consortia extends beyond betel vine to other economically important crops. In rice, the combined use of *Trichoderma*, *P. fluorescens*, and AMF has been shown to suppress sheath blight caused by *Rhizoctonia solani*, while also enhancing grain yield and nutrient uptake (Dubey et al., 2017). Similarly, in chickpea, this microbial trio effectively reduced the incidence of wilt caused by *Fusarium oxysporum* while improving plant biomass and pod production (Chohan et al., 2024). These outcomes highlight the broad-spectrum applicability of such microbial combinations in sustainable agriculture. The mechanisms driving these benefits include improved root colonization, increased production of antifungal compounds, and enhanced plant metabolic activities that collectively improve plant health and productivity.

Organic amendments and soil management practices further enhance the effectiveness of these microbial consortia. The addition of compost or neem cake, for example, has been shown to improve the survival and activity of *Trichoderma*, *P. fluorescens*, and AMF in the rhizosphere, leading to more consistent disease suppression and plant growth promotion (Pandey et al., 2016). This approach not only reduces reliance on chemical pesticides and fertilizers but also aligns with sustainable agricultural practices that emphasize soil health and environmental conservation.

2.8. Impact on Plant Productivity

The combination of *Trichoderma*, *Pseudomonas fluorescens*, and arbuscular mycorrhizal fungi (AMF) has demonstrated significant potential in enhancing plant

productivity in betel vine a crop of immense cultural and economic importance in tropical and subtropical regions (Pandey et al., 2016; Raut et al., 2012; Goswami et al., 2016). These beneficial microbes, through their synergistic interactions, offer a sustainable alternative to chemical inputs for improving plant growth, yield, and resistance to pathogens (Smith & Read, 2008; Goswami et al., 2016). Their multifaceted roles include nutrient mobilization, disease suppression, and stress tolerance, making them indispensable components in sustainable agricultural systems for betel vine cultivation (Harman et al., 2004; Pandey et al., 2016).

Trichoderma spp. are widely recognized for their ability to suppress plant pathogens and promote plant growth. These fungi produce an array of hydrolytic enzymes such as chitinases, glucanases, and proteases that degrade the cell walls of pathogenic fungi, thereby curbing diseases like root and foot rot caused by *Phytophthora parasitica* and *Sclerotium rolfsii* (Harman et al., 2004). In addition to their biocontrol properties, *Trichoderma* spp. contribute to nutrient cycling by solubilizing phosphates and producing phytohormones like indole-3-acetic acid (IAA), which enhance root development and nutrient absorption (Vinale et al., 2008). Their role in stimulating plant defense mechanisms through induced systemic resistance (ISR) further strengthens plant immunity, making them highly effective in disease-prone crops like betel vine (Shores et al., 2010). Moreover, *Trichoderma* spp. have been shown to improve the colonization efficiency of AMF, resulting in enhanced plant-microbe interactions and greater nutrient uptake (Poveda et al., 2019).

Similarly, *Pseudomonas fluorescens*, a rhizosphere-associated bacterium, plays a pivotal role in enhancing plant health through its biocontrol and plant growth-promoting properties. This bacterium produces a wide range of secondary metabolites, including antibiotics like pyoluteorin and phenazines, which suppress soil-borne pathogens (Weller, 2007). Additionally, *P. fluorescens* induces systemic resistance in plants by activating defense-related enzymes such as peroxidases, polyphenol oxidases, and phenylalanine ammonia-lyase (Van Loon et al., 1998). Its ability to solubilize phosphorus and fix atmospheric nitrogen further supports nutrient availability, leading to improved plant growth and yield (Kloepper et al., 2004). The synergistic interaction between *P. fluorescens* and *Trichoderma* has been observed to enhance both pathogen suppression and nutrient acquisition, resulting in better crop productivity (Sandheep et al., 2013).

Arbuscular mycorrhizal fungi (AMF) are another crucial component in this microbial consortium. AMF form mutualistic associations with plant roots, extending their hyphal networks to access immobile nutrients such as phosphorus, zinc, and copper, which are otherwise unavailable to plants (Smith & Read, 2008). In addition to their role in nutrient acquisition, AMF improve plant tolerance to abiotic stresses like drought and salinity by enhancing water uptake and regulating stress-responsive pathways (Miransari, 2010). AMF also interact positively with both *Trichoderma* and *P. fluorescens*, creating a conducive environment for their colonization and activity in the rhizosphere (Goswami et al., 2016). This mutual reinforcement among the microbial partners maximizes their individual and collective benefits, significantly boosting plant productivity in betel vine.

Field studies and greenhouse experiments have confirmed the effectiveness of this microbial consortium in betel vine cultivation. Pandey et al. (2016) reported a marked increase in betel vine yield and biomass when plants were inoculated with a combination of *Trichoderma*, *P. fluorescens*, and AMF compared to individual applications. The consortium not only enhanced nutrient uptake, particularly phosphorus and nitrogen, but also reduced the incidence of root and foot rot diseases by up to 40%. Another study by Singh et al. (2014) demonstrated that integrating microbial inoculants with organic amendments such as compost or neem cake further improved the efficacy of these microbes, resulting in healthier plants and higher quality leaves. These findings underscore the potential of microbial consortia as a sustainable alternative to chemical fertilizers and fungicides in betel vine cultivation.

Despite the proven benefits, challenges remain in the practical application of these microbial consortia. Variability in soil conditions, environmental factors, and strain compatibility can influence their effectiveness, necessitating localized research to optimize their use. Additionally, the development of stable, cost-effective formulations that maintain the viability of *Trichoderma*, *P. fluorescens*, and AMF during storage and field application is critical for their widespread adoption (Santoyo et al., 2021). Future research should also explore the integration of these consortia into holistic disease and nutrient management systems tailored to the specific needs of betel vine cultivation.

CHAPTER III

MATERIALS AND METHODS

3.1 Location Soil and Climate

The study was carried out in the Khulna region, located in the southwestern part of Bangladesh, a region known for the cultivation of betel vine. The experiment was set up at the experimental farm of Chuknagar, Dumuria upazila in the Khulna district. The experimental site, located 16 feet above sea level, experienced extreme heat and humidity during the monsoon season. It has a tropical monsoon climate with an annual rainfall of 1,400–1,800 mm, making it suitable for betel vine cultivation. The average temperature during the experimental period ranged from 24°C to 30°C, with humidity levels between 70–80%, providing ideal conditions for pathogen development and biological control. The site's soil is in the Ganges Tidal Floodplain, an Agro-Ecological Zone (AEZ No. 13), with a flat topography and high salt content. The soil type is non-calcareous grey flood plain, with acid sulphate soil present in a significant portion of the region. The upazillas in Khulna have a range of terrain types, characterized by silty clay and loam texture, a pH level of 6.5 to 7, and a gray hue. Fertility levels are typically high, with medium to high organic matter concentration.

3.2 Study Duration

The experiment was conducted over a period of six months from November 2023 to April 2024, covering the full growing cycle of betel vine, which typically spans six to eight months.

3.3 Experimental Design

3.3.1 Design

The experiment was arranged using a Randomized Complete Block Design (RCBD) with three replications to minimize variability in the field. Each treatment was applied to a plot sizing one decimal, with each replication containing eight plots.

3.3.2 Treatments

1. **T₁**: Control (no treatment).
2. **T₂**: Two applications of *T. harzianum* inoculated in 500 kg oil cake ha⁻¹ at pre and post monsoon
3. **T₃**: Two applications of *Pseudomonas fluorescens* inoculated in 500 kg oil cake ha⁻¹ at pre and post monsoon
4. **T₄**: Two applications of Arbuscular Mycorrhizae inoculated in 500 kg oil cake ha⁻¹ at pre and post monsoon
5. **T₅**: Two applications of *T. harzianum* + *P. fluorescens* inoculated in 500 kg oil cake ha⁻¹ at pre and post monsoon
6. **T₆**: Two applications of *T. harzianum* + AM inoculated in 500 kg oil cake ha⁻¹ at pre and post monsoon
7. **T₇**: Two applications of *P. fluorescens* + AM inoculated in 500 kg oil cake ha⁻¹ at pre and post monsoon
8. **T₈**: Two applications of *T. harzianum* + *P. fluorescens* + AM. inoculated in 500 kg oil cake ha⁻¹ at pre and post monsoon

3.4 Soil and Field Preparation

Soil Sterilization: Soil samples were collected from the experimental field and sterilized using formalin (1:5 v/v). The soil was then covered with polythene sheets for 15 days to eliminate any soil-borne pathogens, ensuring that the treatment effects would not be confounded by pre-existing pathogens.

3.5 Application of Treatments

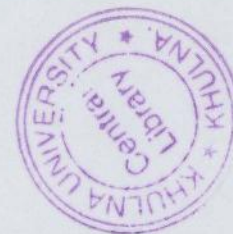
3.5.1 Seedling Treatment

Healthy betel vine cuttings were selected for the experiment. Each cutting was dipped into a spore/bacterial suspension of the respective biological agent for 30 minutes before planting. This was done to promote early colonization of the roots by the biological agents.

3.5.2 Soil Application of experimental micobes

Trichoderma harzianum

Application Rate: Apply 2.5 kg per hectare



3.8. Analyze the biochemical activities and phenolic content of plant root and leaf extracts

Fresh root and leaf samples of betel vine were collected from the field. The plant material was washed thoroughly with distilled water to remove any adhering soil particles. The washed samples were dried in an oven at 40°C for 48 hours and then ground into a fine powder using a mortar and pestle.

3.8.1. Metabolite Profiling with HPLC

High-Performance Liquid Chromatography (HPLC) is widely utilized for the detection and quantification of phytochemical compounds, including caffeic acid, catechuic acid, chlorogenic acid, ferulic acid, gallic acid, kaempferol, protocatechic acid, and quercetin as per recommendation of Ingle et al., (2017). The process begins with the preparation of samples, where phytochemicals are extracted from plant material using solvents such as methanol, ethanol, or water. The extracts are then filtered through a 0.45 μm membrane filter to remove particulate matter. The HPLC conditions involve the use of a C18 reverse-phase column, with a mobile phase consisting of acetonitrile and water, both containing 0.1% formic acid. The flow rate is set to 1.0 mL/min, and an injection volume of 10-20 μL of the sample is used. Detection is carried out using a UV-Vis detector set at appropriate wavelengths for each compound. A gradient elution program is employed, starting with a higher proportion of water and gradually increasing the acetonitrile concentration over time. Calibration and quantification are achieved by preparing standard solutions of known concentrations for each phytochemical compound, creating a calibration curve by plotting the peak area against the concentration, and quantifying the compounds in the sample by comparing the peak areas to the calibration curve. This method ensures accurate detection and quantification of the specified phytochemical compounds using HPLC.

3.9. Molecular docking

3.9.1. Protein preparations

To perform molecular docking, protein models PDB ID: 1J58 (Oxalate Decarboxylase) and PDB ID: 1BHE (Polygalacturonase) were chosen and obtained from the protein

data bank (<https://www.rcsb.org/>). In order to provide the ideal conditions for carrying out the intended interaction with the chosen ligand molecules later, Discovery Studio 2020 clients then altered protein models by eliminating all water molecules and related ligands (Kundu et al., 2023).

3.9.2. Ligand preparation

The 3D structures of standard drugs used in pharmacological tests, including caffeic acid (CID: 689043), catechuic acid (CID: 9064), chlorogenic acid (CID: 1794427), ferulic acid (CID: 445858), gallic acid (CID: 370), kaempferol (CID: 5280863), protocatechuic acid (CID: 72), and quercetin (CID: 5280343), were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). Based on compounds previously reported in the literature for *Piper betle* L. leaves and our HPLC analysis, selected compounds were used as ligands for conducting in silico analysis (Waikedre et al., 2010).

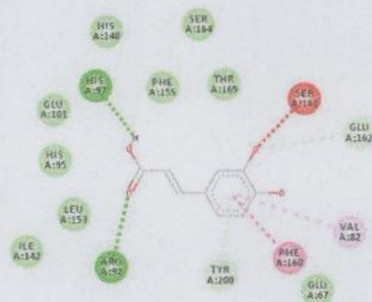
3.9.3 Site-specific binding and visualization

In order to make every region of the selected proteins available for binding, we decided to do blind docking. These processes were done out by utilizing PyRx and auto dock vina 4.2, where the grid dimensions were x:y:z =44.0384:91.9102:58.0255 and 62.9873:61.0013:67.1651 for 1J58 and 1BHE respectively. Site-specific molecular docking analysis was performed in PyRx loaded with auto dock vina 4.2, and ultimately, the 3D interactions of the protein-ligand complex were produced in PyMOL (Rahman et al., 2023).

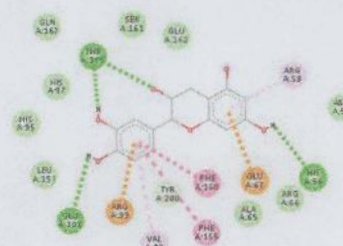
Table 1: Docking score (kcal/mole) of different ligands with 1J58 and 1BHE proteins

Name of ligand	Pubchem ID	1J58	1BHE
Caffeic acid	689043	-7.1	-6.0
Catechuic acid	9064	-8.6	-7.3
Chlorogenic acid	1794427	-7.8	-7.4
Ferulic acid	445858	-6.2	-6.1
Gallic acid	370	-6.2	-5.8
Kaempferol	5280863	-9.3	-7.4
Protocatechic acid	72	-6.0	-5.7
Quercetin	5280343	-9.3	-8.1

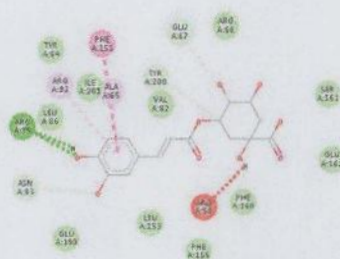
1J58 (Oxalate Decarboxylase)



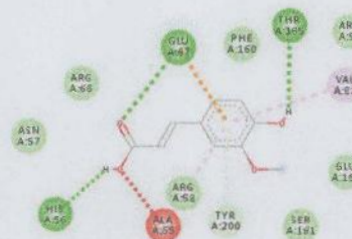
A. Caffeic acid



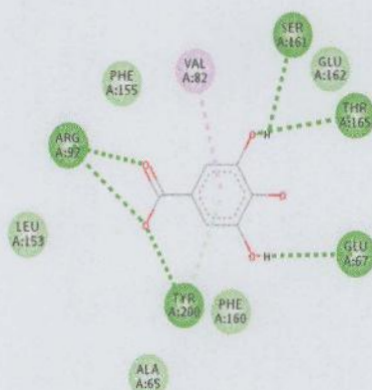
B. Catechuic acid



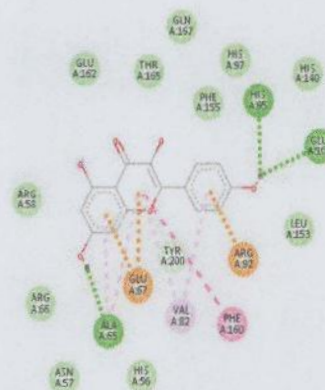
C. Chlorogenic acid



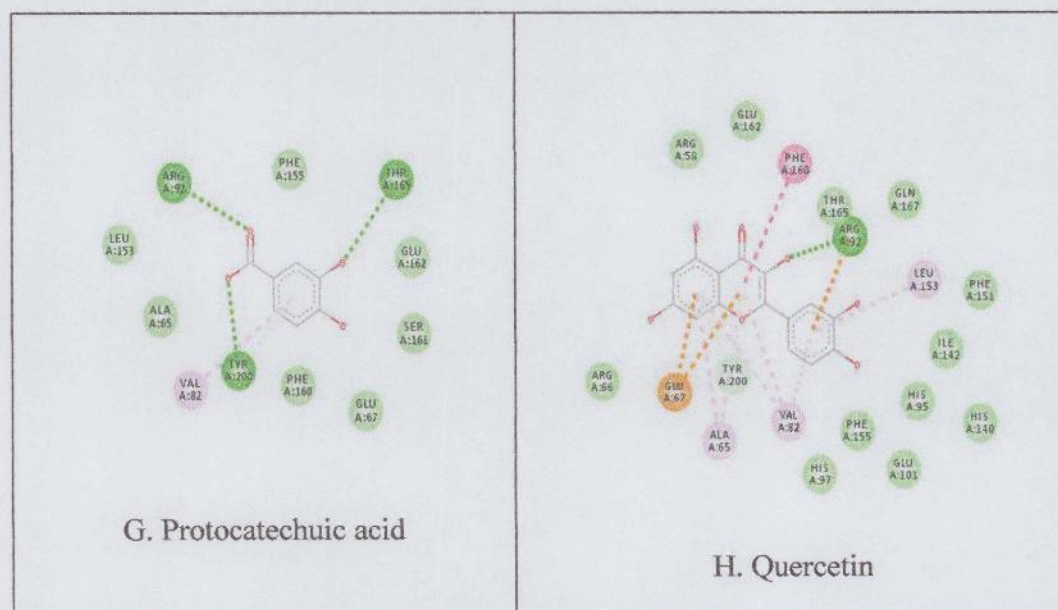
D. Ferulic acid



E. Gallic acid



F. Kaempferol

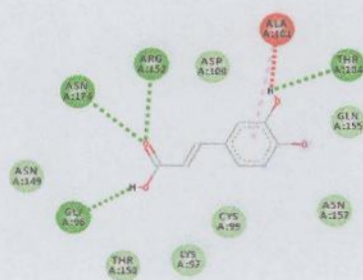


Interactions

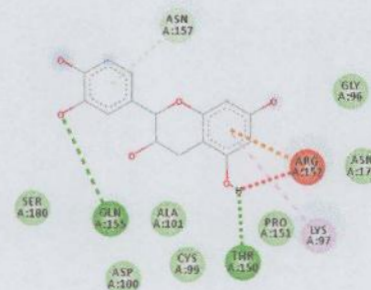
 Van der Waals	 Pi-Donor Hydrogen bond
 Conventional Hydrogen bond	 Pi-pi T-shaped
 Pi-Cation/ Pi-Anion	 Pi-Alkyl
 Unfavorable donor-donor/ Unfavorable acceptor-acceptor	

Figure 2. 2D interactions of amino acids of 1J58 (Oxalate Decarboxylase) proteins with A. Caffeic acid, B. Catechuic acid, C. Chlorogenic acid, D. Ferulic acid, E. Gallic acid, F. Kaempferol, G. Protocatechuic acid and H. Quercetin

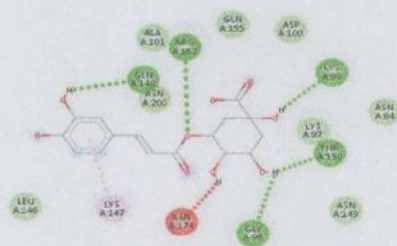
1BHE (Polygalacturonase)



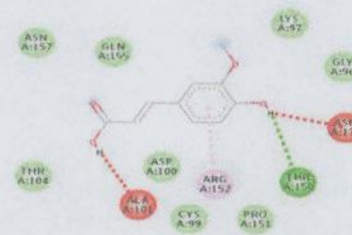
A. Caffeic acid



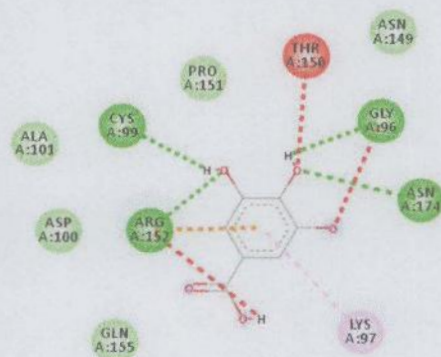
B. Catechuic acid



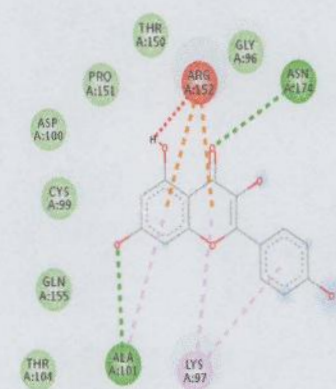
C. Chlorogenic acid



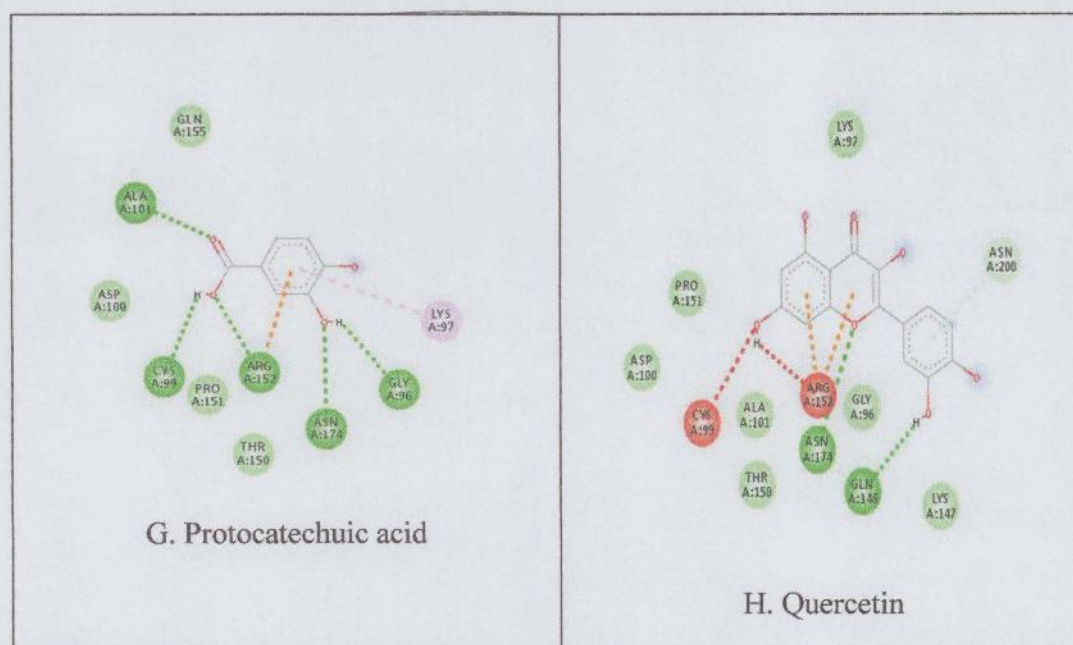
D. Ferulic acid



E. Gallic acid



F. Kaempferol



Interactions

Van der Waals	Pi-Donor Hydrogen bond
Conventional Hydrogen bond	Pi-pi T-shaped
Pi-Cation/ Pi-Anion	Pi-Alkyl
Unfavorable donor-donor/ Unfavorable acceptor-acceptor	

Figure 3. 2D interactions of amino acids of 1BHE (Polygalacturonase) proteins with A. Caffeic acid, B. Catechuic acid, C. Chlorogenic acid, D. Ferulic acid, E. Gallic acid, F. Kaempferol, G. Protocatechuic acid and H. Quercetin

3.10. Collection and Recording Data on Growth, Yield and Disease Parameters

Plant Growth: Parameters such as vine length, laterals vine⁻¹, Internodal length (cm), leaf petiole length (cm), leaf length (cm), leaf breadth (cm) and number of leaves were recorded. These measurements provide insight into how well the plants are developing in response to the treatments.

Leaf Yield: At harvest, the 100leaf weight (g) and the total number of leaves per plant were recorded. These are key productivity indicators.

Data were also collected on days to appear symptom, % disease incidence at different days after inoculation (DAI), % disease reduction at 120 DAI over control, accumulation of phenolic acids in leaf and roots, biochemical compounds, and N P K

concentration in soil before and after colonization. Disease incidence was calculated by the following formula:

$$\% \text{ Disease incidence} = \frac{\text{Number of diseased plants}}{\text{Number of total plants inspected}} \times 100$$

3.11. Statistical Analysis

Recorded data were subjected to one-way ANOVA wherein means with significant treatment effects were identified by F test and separated by Tukey's HSD test at $p \leq 0.05$ (Minitab 19.0, NY, USA). To determine the relations among the studied variables, the correlation matrix was performed on the organized raw data.

CHAPTER IV

RESULT AND DISCUSSION

Results

4.1 Growth parameters

The growth parameters of betelvine were statistically stimulated by different treatments involving *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and Arbuscular Mycorrhizal Fungi (AMF), applied individually and in combination (Table 1). The results underscore the combined efficacy of these biological agents in promoting various growth attributes, such as vine length, number of laterals per vine, internodal length, and leaf characteristics, compared to untreated control plants (Table 1). These microbial treatments demonstrated significant improvements in all measured parameters compared to the untreated control, with the synergistic combination of Th + Pf + AMF showing the most pronounced effects.

In terms of vine length, the Th + Pf + AMF treatment recorded the highest value (142.77 ± 2.75 cm), which was significantly greater than all other treatments. The second-best result was observed in the Th + Pf combination (132.63 ± 1.66 cm), followed by Th + AMF (122.97 ± 1.35 cm) and Pf + AMF (122.67 ± 1.44 cm). In contrast, the control group exhibited the shortest vine length (96.90 ± 1.37 cm), highlighting the considerable role of microbial symbiosis in enhancing vine growth (Table 2).

The number of laterals per vine was also significantly influenced by the treatments. The Th + Pf + AMF combination yielded the highest number of laterals (22.00 ± 0.15), followed by the Th + Pf treatment (19.10 ± 0.11) and Th + AMF (17.23 ± 0.06). The control group recorded the lowest number of laterals (14.00 ± 0.11), underscoring the effectiveness of the treatments in promoting lateral branching (Table 2).

For internodal length, plants treated with Th + Pf + AMF exhibited the longest internodes (6.50 ± 0.05 cm), followed closely by the Th + Pf combination (6.23 ± 0.06 cm) and Th + AMF (6.00 ± 0.05 cm). The control plants displayed the shortest internodal length (4.20 ± 0.06 cm), indicating that the combined microbial treatments significantly improved plant structure by elongating internodes (Table 2).

Leaf petiole length was similarly enhanced by the treatments. The Th + Pf + AMF combination produced the longest petioles (7.70 ± 0.05 cm), followed by Th + Pf (7.40 ± 0.05 cm) and Th + AMF (6.96 ± 0.09 cm). The control group, in comparison, had the shortest petioles (5.30 ± 0.05 cm), showing that the treatments positively influenced leaf attachment and support structure (Table 2).

In terms of leaf size, the Th + Pf + AMF treatment demonstrated the most significant improvements, with leaf lengths of 15.76 ± 1.08 cm and leaf breadths of 13.70 ± 0.05 cm. This was followed by Th + Pf (14.80 ± 0.05 cm in length and 13.40 ± 0.05 cm in breadth) and Th + AMF (14.30 ± 0.11 cm in length and 13.10 ± 0.05 cm in breadth). The control plants had the smallest leaves, with lengths of 9.43 ± 0.08 cm and breadths of 10.30 ± 0.05 cm, demonstrating the impact of microbial symbiosis on improving leaf size and surface area (Table 2).

Table 2. Interaction of *Trichoderma harzianum*, *P. fluorescens* and AMF, on different growth parameters of Betelvine

No	Treatments	Vine length(cm)	Laterals vine ⁻¹ (cm)	Internodal length (cm)	Leaf petiole length(cm)	Leaf length(cm)	Leaf breadth (cm)
1	Control	96.90± 0.37f	14.00± 0.11e	4.20±0.06f	5.30±0.05f	9.43±0.08f	10.30±0.05f
2	Th	114.73± 0.34d	15.70±0.15d	5.70±0.06d	6.70±0.05d	12.50±0.11d	12.40±0.05d
3	Pf	103.30±0.11e	15.43±0.12d	5.20±0.06e	5.96±0.18e	11.24±0.12e	11.10±0.05e
4	AMF	102.93±0.08e	14.83± 0.41de	5.03±0.08e	5.86±0.03e	11.20±0.05e	11.12±0.07e
5	Th + Pf	132.63± 1.66b	19.10±0.11b	6.23±0.06ab	7.40±0.05b	14.80±0.05b	13.40±0.05b
6	Th+AMF	122.97± 1.35c	17.23± 0.06c	6.00±0.05bc	6.96±0.09c	14.30±0.11c	13.10±0.05c
7	Pf+AMF	122.67±1.44c	16.96± 0.06c	5.86±0.08cd	6.90±0.05cd	14.30±0.15c	13.06±0.03c
8	Th+Pf+AMF	142.77±2.75a	22.00±1.15a	6.50±0.05a	7.70±0.05a	15.76±1.08a	13.70±0.75a
	LS (P<0.05)	*	*	*	*	*	*

(Means followed by same small case letters are statistically similar within a column based on Tukey's honest significant difference test at $p < 0.05$; data are means of three replications $\pm$ standard errors; LS = Level of Significance, * = Significant at 5% level of significance; Th = *Trichoderma harzianum*, Pf = *Pseudomonas fluorescens*, AMF = Arbuscular mycorrhizal fungi)

4.2 Yield Attributes

Significant variations were observed in the parameters analyzed, including 100-leaf weight, leaf number per plant, and leaf number per decimal, with the treatments showing a considerable impact compared to the control (Table 3).

In terms of 100-leaf weight, the Th + Pf + AMF treatment resulted in the highest weight (277.23 ± 2.98 g), significantly outperforming all other treatments. This was followed by the Th + Pf treatment (267.07 ± 2.81 g) and Th + AMF (260.22 ± 2.51 g). The control group recorded the lowest 100-leaf weight (237.67 ± 2.51 g), highlighting the contribution of microbial treatments to enhancing leaf size and quality (Table 3).

The plant count per decimal remained constant at 50 across all treatments, ensuring that the observed variations in other parameters were solely attributable to the effects of microbial treatments rather than differences in plant density. This consistency allows for a more accurate comparison of yield parameters (Table 3).

The leaf number per plant significantly increased with the microbial treatments. The combination of Th + Pf + AMF produced the highest leaf number per plant (82.00 ± 0.57), followed by Th + Pf (79.00 ± 0.57) and Pf + AMF (75.00 ± 0.57). In contrast, the control group showed the lowest leaf number per plant (64.00 ± 0.57), emphasizing the role of microbial combinations in promoting leaf production (Table 3).

Similarly, the leaf number per decimal followed the same trend as the leaf number per plant. The Th + Pf + AMF treatment achieved the highest leaf number per decimal (4060.0 ± 5.77), indicating a marked improvement in yield. The Th + Pf and Pf + AMF treatments also recorded high values (3963.3 ± 8.81 and 3750.0 ± 5.77 , respectively), while the control group produced the least leaves per decimal (3210.0 ± 5.77). This demonstrates the positive effect of microbial treatments on overall leaf yield. The increase in leaf number per plant and per decimal over the control was most prominent in the Th + Pf + AMF treatment. This combination resulted in an increase of 26.82 leaves per plant and per decimal compared to the control. The second-highest increase was observed in the Th + Pf treatment (23.41), followed by Pf + AMF (16.19). The control group, as expected, exhibited no increase, further validating the impact of microbial symbiosis on yield enhancement. (Table 3).

Table 3. Influence of *Trichoderma harzianum*, *P. fluorescens* and AMF on Yield Attributes of Betelvine

No	Treatments	100 leaf weight(g)	Plant no/ decimal	Leaf no /plant	Leaf no/ decimal	Leaf no increase/plant over control	Leaf no increase/decimal over control
1	Control	237.67 ±1.51f	50	64.00 ±0.57f	3210.0±5.77f	0.00±0.00f	0.00±0.00f
2	Th	252.53 ±2.37d	50	71.00±0.57d	3553.3±8.81d	10.97± 0.11d	10.97± 0.11d
3	Pf	247.01 ±1.55e	50	67.66±0.88e	3323.3±8.81e	6.75± 0.25e	6.75± 0.25e
4	AMF	246.22 ±2.85e	50	66.66±0.88e	3311.7±6.00e	6.14±0.51e	6.14±0.51e
5	Th + Pf	267.07 ±2.81b	50	79.00±0.57b	3963.3± 8.81b	23.41±0.34b	23.41±0.34b
6	Th+AMF	260.22 ±2.51c	50	74.00±0.57c	3740.0±2.88c	15.99±0.05c	15.99±0.05c
7	Pf+AMF	259.63 ±2.37c	50	75.00±0.57c	3750.0±5.77c	16.19±0.05c	16.19±0.05c
8	Th+Pf+AMF	277.23 ±2.98a	50	82.00±1.57a	4060.0±5.77a	26.82± 0.15a	26.82± 0.15a
LS (P≤0.05)		*	NS	*	*	*	*

(Means followed by same small case letters are statistically similar within a column based on Tukey's honest significant difference test at $p < 0.05$; data are means of three replications ± standard errors; LS = Level of Significance, * = Significant at 5% level of significance, NS = not significant at 5% level of significance; Th = *Trichoderma harzianum*, Pf = *Pseudomonas fluorescens*, AMF = Arbuscular mycorrhizal fungi)

4.3 Evaluation of Income Metrics

The control group serves as the baseline with an income of 48.43 ± 0.53 per plant and 2410.0 ± 5.77 per decimal. This group shows no income increase compared to itself, as reflected in the values of 0.00 ± 0.00 for both income increase per plant and per decimal (Table 4).

In contrast, the application of individual treatments such as Th, Pf, and AMF all led to increases in both income per plant and per decimal when compared to the control. Specifically, Th resulted in an income of 71.33 ± 0.60 per plant and 3563.3 ± 8.81 per decimal, yielding increases of 23.16 ± 0.44 per plant and 47.73 ± 0.49 per decimal. The Pf treatment showed slightly lower results, with income per plant at 68.53 ± 0.31 and income per decimal at 3410.0 ± 5.77 , reflecting increases of 20.16 ± 0.44 per plant and 41.42 ± 0.45 per decimal. Similarly, the AMF treatment resulted in 67.36 ± 0.41 per plant and 3398.3 ± 10.13 per decimal, with increases of 19.26 ± 0.37 per plant and 40.23 ± 0.48 per decimal (Table 4).

When treatments were combined, a substantial increase in income was observed. The Th + Pf combination yielded the highest increase among the treatments, with 138.25 ± 0.43 income per plant and 6920.8 ± 15.02 income per decimal, resulting in increases of 90.50 ± 0.52 per plant and 188.17 ± 1.01 per decimal. The Th + AMF combination followed closely with 130.33 ± 0.60 income per plant and 6518.3 ± 31.66 per decimal, showing increases of 81.66 ± 1.01 per plant and 171.36 ± 1.57 per decimal. The Pf + AMF combination produced similar results, with income per plant at 130.68 ± 0.22 and per decimal at 6507.5 ± 25.29 , and income increases of 82.11 ± 0.94 per plant and 172.98 ± 1.05 per decimal (Table 4).

The highest income values were recorded for the Th + Pf + AMF combination, with 182.25 ± 0.72 income per plant and 9120.8 ± 15.02 income per decimal. This combination showed the greatest increase in income, with 134.42 ± 0.58 per plant and 279.40 ± 0.73 per decimal (Table 4).

Table 4. Influence of *Trichoderma harzianum*, *Pseudomonas fluorescens*, and AMF on Economic Performance Indicators of Betelvine

No	Treatments	Income/plant	Income/decimal	Income increase/Plant over control	Income Increase/Decimal over control
1	Control	48.43 ±0.53f	2410.0 ±5.77f	0.00 ±0.00f	0.00 ±0.00f
2	Th	71.33 ±0.60d	3563.3 ±8.81d	23.16 ±0.44d	47.73 ±0.49d
3	Pf	68.53 ±0.31e	3410.0 ± 5.77e	20.16 ±0.44e	41.42 ±0.45e
4	AMF	67.36 ±0.41e	3398.3 ±10.13e	19.26 ±0.37e	40.23 ±0.48e
5	Th + Pf	138.25 ±0.43b	6920.8 ±15.02b	90.50 ±0.52b	188.17 ±1.01b
6	Th+AMF	130.33 ±0.60c	6518.3 ±31.66c	81.66 ±1.01c	171.36 ±1.57c
7	Pf+AMF	130.68 ±0.22c	6507.5 ±25.29c	82.11 ±0.94c	172.98 ±1.05c
8	Th+Pf+AMF	182.25 ±0.72a	9120.8 ±15.02a	134.42 ±0.58a	279.40 ±0.73a
	LS	*	*	*	*

(Means followed by same small case letters are statistically similar within a column based on Tukey's honest significant difference test at $p < 0.05$; data are means of three replications ± standard errors; LS = Level of Significance, * = Significant at 5% level of significance; Th = *Trichoderma harzianum*, Pf = *Pseudomonas fluorescens*, AMF = Arbuscular mycorrhizal fungi)



4.4 Disease Management

The results from the experiment, as presented in the table, examine the effects of various treatments on disease incidence and disease reduction at different time points following inoculation. The data includes the number of days to symptom appearance, disease incidence at 60, 80, 100, and 120 days after transplant (DAT), as well as the average disease reduction at 120 days post-treatment compared to the control group.

4.4.1 Days required for the appearance of disease symptom

This study examined the influence of *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and arbuscular mycorrhizal fungi (AMF) on the time taken for symptoms to appear in betelvine plants. The number of days to symptom appearance was significantly affected by the treatments, with the combination of Th, Pf, and AMF (Th+Pf+AMF) demonstrating the most substantial delay (Table 5).

The control group exhibited symptoms the earliest, with an average of 28.00 ± 0.57 days, reflecting the baseline susceptibility of untreated plants. In contrast, plants treated with individual microbial agents displayed a moderate delay in symptom onset. Treatment with Th resulted in symptoms appearing at 33.33 ± 0.88 days, while Pf and AMF extended this period to 34.33 ± 0.88 and 32.33 ± 0.88 days, respectively (Table 5).

The application of combined treatments further delayed symptom appearance, suggesting a synergistic effect. The Th+Pf combination delayed symptoms to 40.66 ± 0.33 days, followed closely by Th+AMF (40.33 ± 0.88 days) and Pf+AMF (40.00 ± 0.00 days). The most significant delay was observed with the Th+Pf+AMF treatment, which extended symptom appearance to 48.00 ± 1.15 days, demonstrating the highest level of effectiveness among all treatments (Table 5).

4.4.2 Disease incidence

This study investigated the impact of microbial treatments, including *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and arbuscular mycorrhizal fungi (AMF), on disease incidence in betelvine at different days after transplanting (DAT) and their overall effectiveness in reducing disease incidence at 120 DAT compared to the control.

The control group exhibited consistently high levels of disease incidence, reaching $42.00 \pm 0.57\%$ at 120 DAT. In contrast, all treatments significantly reduced disease incidence across all time points (60, 80, 100, and 120 DAT). Among individual treatments, Th and Pf showed moderate reductions, with Th-treated plants recording $31.39 \pm 0.20\%$ and Pf-treated plants recording $31.50 \pm 0.76\%$ disease incidence at 120 DAT. AMF-treated plants showed slightly higher incidence at $32.05 \pm 0.27\%$ (Table 5).

Combined treatments provided greater disease control than individual treatments. The Th+Pf treatment reduced disease incidence to $20.00 \pm 0.57\%$, while Th+AMF and Pf+AMF further lowered it to $22.07 \pm 0.79\%$ and $22.00 \pm 0.76\%$, respectively. The most effective treatment was the combination of Th, Pf, and AMF (Th+Pf+AMF), which minimized disease incidence to $10.77 \pm 0.39\%$ at 120 DAT (Table 5).

4.4.3 Average Disease Reduction

The percentage of disease reduction at 120 DAT compared to the control was calculated to assess the effectiveness of the treatments. The control group had no reduction ($0.00 \pm 0.00\%$), whereas the Th+Pf+AMF treatment achieved the highest reduction of $74.32 \pm 1.27\%$. Other combined treatments, including Th+Pf ($52.34 \pm 1.77\%$), Th+AMF ($47.42 \pm 1.98\%$), and Pf+AMF ($47.60 \pm 1.81\%$), also showed substantial reductions. Individual treatments (Th, Pf, and AMF) displayed moderate reductions ranging from $23.63 \pm 1.65\%$ to $25.23 \pm 1.03\%$ (Table 5).

Table 5. Influence of *Trichoderma harzianum*, *Pseudomonas fluorescens*, and AMF on Disease Incidence and Reduction in Betelvine

No	Treatments	Days to appear symptom	% Disease incidence at different days after Transplant (DAT)				% Avg Disease reduction at 120 DAT over control
			60 DAT	80 DAT	100 DAT	120 DAT	
1	Control	28.00±0.57e	26.00±0.57a	31.00±0.57a	41.00±0.57a	42.00±0.57a	0.00±0.00d
2	Th	33.33±0.88cd	16.00±0.57bcd	25.00±0.57b	31.00±0.57b	31.39±0.20b	25.23±1.03c
3	Pf	34.33±0.88c	18.00±0.57bc	24.00±0.57b	34.00±0.57b	31.50±0.76b	24.93±2.65c
4	AMF	32.33±0.88d	20.00±0.57b	30.00±0.57a	35.00±0.57b	32.05±0.27b	23.63±1.65c
5	Th + Pf	40.66±0.33b	15.00±0.57cd	17.16±0.44cd	20.16±0.85c	20.00±0.57c	52.34±1.77b
6	Th+AMF	40.33±0.88b	14.50±2.02cd	20.00±0.57c	22.50±0.57c	22.07±0.79c	47.42±1.98b
7	Pf+AMF	40.00±0.00b	13.66±2.18cd	16.33±1.85d	20.00±0.57c	22.00±0.76c	47.60±1.81b
8	Th+Pf+AMF	48.00±1.15a	11.83±2.35d	12.66±1.20e	13.50±0.57d	10.77±0.39d	74.32±1.27a
LS (P≤0.05)		*	*	*	*	*	*

(Means followed by same small case letters are statistically similar within a column based on Tukey's honest significant difference test at $p < 0.05$; data are means of three replications ± standard errors; LS = Level of Significance, * = Significant at 5% level of significance; DAT=Days after Transplant; Th =*Trichoderma harzianum*, Pf =*Pseudomonas fluorescens*, AMF =Arbuscular mycorrhizal fungi)

4.5 Biochemical Analysis

4.5.1 Flavonoid Content

The impact of various treatments on the flavonoid content, specifically Kaempferol and Quercetin, in the leaf and root of Betelvine (Table-6). The control group showed the lowest flavonoid levels for both Kaempferol and Quercetin, with values of 12.93 mg/g and 10.50 mg/g in the leaf, and 10.10 mg/g and 8.30 mg/g in the root, respectively.

Among the treatments, the combined application of *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and AMF (Th+Pf+AMF) resulted in the highest flavonoid content. The leaf samples under this treatment exhibited the maximum accumulation of both Kaempferol (16.60 mg/g) and Quercetin (13.50 mg/g), significantly higher than those observed in the control. Root flavonoid levels were similarly enhanced in this treatment, with Kaempferol reaching 13.10 mg/g and Quercetin reaching 11.00 mg/g (Table-6).

Other treatments, including Th, Pf, and AMF applied individually or in combinations (Th+Pf, Th+AMF, and Pf+AMF), also led to an increase in flavonoid content compared to the control, with values ranging from 12.80 mg/g to 14.93 mg/g for Kaempferol and 9.60 mg/g to 12.35 mg/g for Quercetin in the leaves. The root flavonoid content was generally lower in all treatments compared to the leaves, but still showed a marked increase over the control (Table-6).

Table 6. Effect of *Trichoderma harzianum*, *Pseudomonas fluorescens*, and AMF on the Accumulation of Flavonoid Content (Kaempferol and Quercetin) in Leaf and Root of Betelvine

No	Treatments	Flavonoids(mg/g)			
		Kaempferol		Quercetin	
		Leaf	Root	Leaf	Root
1	Control	12.93±0.37c	10.10±0.05c	10.50±0.30c	8.30±0.10c
2	Th	13.03±0.12c	8.50±0.05d	10.60±0.20c	7.00±0.08d
3	Pf	13.00±0.05c	8.60±0.05d	10.55±0.25c	7.10±0.05d
4	AMF	12.80±0.05c	8.30±0.05d	10.40±0.18c	6.95±0.05d
5	Th + Pf	14.83±0.38b	11.60±0.25b	12.20±0.35b	9.60±0.20b
6	Th+AMF	14.90± 0.25b	11.90±0.05b	12.30±0.25b	9.80±0.10b
7	Pf+AMF	14.93±0.38b	11.66±0.12b	12.35±0.30b	9.70±0.15b
8	Th+Pf+AMF	19.46± 0.42a	13.10±0.05a	13.50±0.10a	11.00±0.05a
	LS (P≤0.05)	*	*	*	*

(Means followed by same small case letters are statistically similar within a column based on Tukey's honest significant difference test at $p < 0.05$; data are means of three replications $\pm$ standard errors; LS = Level of Significance, * = Significant at 5% level of significance; Th = *Trichoderma harzianum*, Pf = *Pseudomonas fluorescens*, AMF = Arbuscular mycorrhizal fungi)



4.5.2 Tannin Content

The effects of *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and AMF on tannin content, specifically Catechuic acid and Gallic acid, in the leaf and root tissues of Betelvine (Table-6). The data reveals that all treatments resulted in a significant increase in tannin content compared to the control, with the highest concentrations observed in the combined application of Th, Pf, and AMF.

In the control treatment, the tannin content for Catechuic acid was 7.71 mg/g in the leaf and 7.56 mg/g in the root, while for Gallic acid, it was 6.50 mg/g in the leaf and 6.40 mg/g in the root. This baseline data reflects the natural levels of tannins in Betelvine under untreated conditions (Table 7).

When individual treatments were applied, the tannin content increased notably. For example, the Th treatment resulted in a Catechuic acid content of 9.53 mg/g in the leaf and 7.96 mg/g in the root, while the Gallic acid content was 8.00 mg/g in the leaf and 6.80 mg/g in the root. Similarly, Pf and AMF treatments also caused increases in tannin content, with no significant difference between these treatments in either leaf or root tissues. However, the combined treatments produced more substantial increases in tannin content (Table 7).

The combination of Th and Pf led to an increase in Catechuic acid content to 11.93 mg/g in the leaf and 10.46 mg/g in the root, and the Gallic acid content was 10.00 mg/g in the leaf and 8.80 mg/g in the root. Th+AMF and Pf+AMF treatments resulted in similar increases, with Catechuic acid values ranging from 11.86 to 12.06 mg/g in the leaf and 10.20 to 10.23 mg/g in the root, and Gallic acid values ranging from 9.95 to 10.10 mg/g in the leaf and 8.55 to 8.60 mg/g in the root (Table 7).

The highest tannin content was observed in the Th+Pf+AMF treatment, where Catechuic acid reached 15.14 mg/g in the leaf and 13.40 mg/g in the root, while Gallic acid content was 12.50 mg/g in the leaf and 11.00 mg/g in the root. This treatment exhibited the most significant enhancement in tannin accumulation, both in the leaf and root tissues (Table 7).

Table 7: Effect of *Trichoderma harzianum*, *Pseudomonas fluorescens*, and AMF on the Accumulation of Tannin Content (Catechuic Acid and Gallic Acid) in Leaf and Root of Betelvine

No	Treatments	Tanins(mg/g)					
		Catechuic acid			Gallic acid		
		Leaf	Root		Leaf	Root	
1	Control	7.71±0.12d	7.56±0.08c		6.50±0.10d	6.40±0.07c	
2	Th	9.53±0.03c	7.96±0.09c		8.00±0.08c	6.80±0.10c	
3	Pf	9.46±0.08c	8.06±0.08c		7.95±0.10c	6.85±0.08c	
4	AMF	9.36±0.03c	7.83±0.06c		7.85±0.05c	6.70±0.07c	
5	Th + Pf	11.93±0.44b	10.46±0.23b		10.00±0.40b	8.80±0.18b	
6	Th+AMF	12.06±0.32b	10.23±0.24b		10.10±0.30b	8.60±0.20b	
7	Pf+AMF	11.86± 0.32b	10.20±0.20b		9.95±0.25b	8.55±0.15b	
8	Th+Pf+AMF	15.14±0.33a	13.40±0.11a		12.50±0.26a	11.00±0.10a	
	LS (P≤0.05)	*	*		*	*	

(Means followed by same small case letters are statistically similar within a column based on Tukey's honest significant difference test at $p < 0.05$; data are means of three replications $\pm$ standard errors; LS = Level of Significance, * = Significant at 5% level of significance; Th = *Trichoderma harzianum*, Pf = *Pseudomonas fluorescens*, AMF = Arbuscular mycorrhizal fungi)

4.5.3 Phenolic Content

The study evaluated the influence of different treatments, including *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and arbuscular mycorrhizal fungi (AMF), on the accumulation of phenolic acids in the leaves and roots of betelvine (*Piper betle* L.). The treatments significantly affected the accumulation of four phenolic acids—protocatechuic acid, chlorogenic acid, caffeic acid, and ferulic acid—in both the leaf and root tissues, as shown by the statistical analysis (LS at $P \leq 0.05$).

In the control group, the accumulation of phenolic acids was relatively low compared to the treated groups. Specifically, the levels of protocatechuic acid, chlorogenic acid, caffeic acid, and ferulic acid in both leaves and roots of the control plants ranged from 5.3–17.5 $\mu\text{g/g}$ in the leaves and 2.2–11.0 $\mu\text{g/g}$ in the roots (Table 8).

The treatment with *Trichoderma harzianum* (Th) significantly increased the accumulation of phenolic acids in both the leaf and root tissues. Notably, the accumulation of protocatechuic acid in leaves (17.3 $\mu\text{g/g}$) and roots (13.1 $\mu\text{g/g}$) was substantially higher than the control. Similarly, the levels of chlorogenic acid, caffeic acid, and ferulic acid were also elevated compared to the control (Table 8).

Treatment with *Pseudomonas fluorescens* (Pf) resulted in a significant increase in the accumulation of protocatechuic acid (26.3 $\mu\text{g/g}$ in leaves) but had a lesser effect in the roots (10.7 $\mu\text{g/g}$). Chlorogenic acid levels in the leaves (6.0 $\mu\text{g/g}$) were also increased compared to the control, though the root concentrations were lower than in Th-treated plants (Table 8).

The application of AMF showed a moderate effect, with protocatechuic acid accumulation reaching 19.2 $\mu\text{g/g}$ in the leaves and 12.5 $\mu\text{g/g}$ in the roots. While the levels of chlorogenic acid, caffeic acid, and ferulic acid were also elevated, the increase was less pronounced compared to the Th and Pf treatments (Table 8).

Combining Th and Pf (Th + Pf) or Th and AMF (Th + AMF) resulted in further improvements in phenolic acid accumulation, particularly in the leaves. The combination of Th and Pf (26.3 $\mu\text{g/g}$ of protocatechuic acid in leaves) or Th and AMF (27.3 $\mu\text{g/g}$ of protocatechuic acid in leaves) showed considerable enhancement in phenolic acid content in both plant parts (Table 8).

The highest accumulation of phenolic acids was observed in the triple combination of Th, Pf, and AMF (Th+Pf+AMF). This treatment resulted in the highest concentrations of protocatechuic acid, chlorogenic acid, caffeic acid, and ferulic acid in both leaves and roots, with values of 29.9 $\mu\text{g/g}$, 16.3 $\mu\text{g/g}$, 23.2 $\mu\text{g/g}$, and 11.2 $\mu\text{g/g}$, respectively, in the leaves. This indicates a synergistic effect of the three treatments in enhancing phenolic acid production in betelvine (Table 8).

Table 8: Influence of *Trichoderma harzianum*, *Pseudomonas fluorescens*, and AMF on the Accumulation of Phenolic Acids in Leaf and Root of Betelvine

No	Treatments	Accumulation of phenolic acids in leaf and roots							
		Protocatechuic acid (µg/g)		Chlorogenic acid (µg/g)		Caffeic acid (µg/g)		Ferulic acid (µg/g)	
		Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
1	Control	5.3±0.13e	8.2±3.05c	3.2±0.22f	2.2±0.12e	17.5±0.51de	11.0±0.57f	6.7±0.14f	4.7±0.24f
2	Th	17.3±0.12d	13.1±0.06abc	7.6±0.08de	6.3±0.63bc	20.7±0.37bc	14.0±0.57cd	8.1±0.20cde	6.2±0.16cd
3	Pf	26.3±0.54b	10.7±0.43bc	6.0±0.57e	4.2±0.64d	18.5±1.19cd	12.7±0.61e	7.5±0.23de	5.5±0.17de
4	AMF	19.2±0.60d	12.5±0.31abc	13.3±0.88b	7.1±0.58b	16.2±0.43e	13.2±0.53de	7.4±0.23ef	5.3±0.17ef
5	Th + Pf	26.3±0.67b	14.6±0.30ab	9.8±0.14c	5.5±0.23bcd	22.3±0.35ab	14.9±0.19b	9.2±0.14b	7.1±0.05b
6	Th+AMF	27.3±0.13b	16.4±0.30a	9.7±0.27cd	5.5±0.51cd	20.4±0.23bc	14.7±0.32bc	8.5±0.12bc	6.5±0.11bc
7	Pf+AMF	23.2±0.58c	13.9±0.30ab	10.2±0.31c	6.1±0.30bc	19.9±0.05c	14.8±0.40bc	8.2±0.17cd	6.2±0.08cd
8	Th+Pf+AMF	29.9±0.98a	17.5±0.28a	16.3±0.88a	10.8±0.60a	23.2±0.60a	17.3±0.44a	11.2±0.44a	9.1±0.20a
	LS (P<0.05)	*	*	*	*	*	*	*	*

(Means followed by same small case letters are statistically similar within a column based on Tukey's honest significant difference test at $p < 0.05$; data are means of three replications $\pm$ standard errors; LS = Level of Significance, * = Significant at 5% level of significance; Th = *Trichoderma harzianum*, Pf = *Pseudomonas fluorescens*, AMF = Arbuscular mycorrhizal fungi)

4.5.4 HPLC analysis

HPLC-DAD analysis of Betelvine extract provided identification and quantification of eight phenolic compounds. Among these compounds, Protocatechuic acid ($29.9 \pm 0.98a$) was found to be present in the highest quantity. (–)-Caffeic acid, Kaempferol, Quercetin, Catechuic acid and Gallic acid were found in quite enough while chlorogenic acid and Ferulic acid ($\mu\text{g/g}$) were found in very small amounts. (Table 6,7,8).

Table 9. Interacting amino acids with 1J58 protein

Name of the ligand	Interacting amino acids
Caffeic acid	Ser161, Ser164, Glu67, Glu101, Glu162, Phe155, Phe160, Val82, Tyr200, Arg92, Leu153, Ile142, His95, His97, His140, Thr165
Catechuic acid	Ala65, Arg58, Arg66, Arg92, Asn57, Glu67, Glu101, Glu162, Gln167, Ser161, His56, His97, His95, Leu153, Phe155, Phe160, Thr165, Tyr200, Val82,
Chlorogenic acid	Arg66, Glu67, Tyr200, Val82, Ala65, Phe151, Ile201, Arg92, Tyr64, Leu86, Arg85, Asn83, Glu150, Leu153, Phe155, Arg58, Phe160, Glu162, Ser161
Ferulic acid	Arg66, Asn57, His56, Ala65, Arg58, Tyr200, Ser161, Glu162, Val82, Arg92, Thr165, Phe160, Glu67
Gallic acid	Ala65, Tyr200, Phe160, Glu67, Thr165, Glu162, Ser161, Val82, Phe155, Arg92, Leu153
Kaempferol	Arg58, Arg66, Ala65, Asn57, His56, Glu67, Val82, Phe160, Tyr200, Arg153, Leu153 Glu101, His140, His95, His97, Phe155, Gln167, Thr165, Glu162
Protocatechuic acid	Arg92, Leu153, Ala65, Val82, Tyr200, Phe160, Glu67, Ser161, Glu162, Thr165, Phe155
Quercetin	Arg66, Glu67, Ala65, Tyr200, Val82, His97, Phe155, Glu101, His95, His140, Ile142, Phe151, Leu153, Arg92, Gln167, Thr165, Phe160, Glu162, Arg58



Fig. 4. Binding pocket of caffeic acid (red), catechuic acid (salmon), chlorogenic acid (green), ferulic acid (cyan), gallic acid (blue), kaempferol (yellow), protocatechuic acid (magenta), quercetin (orange) with 1J58 protein

Table 10. Interacting amino acids with 1BHE receptor

Name of the ligand	Interacting amino acids
Caffeic acid	Ala101, Asp100, Arg152, Asn149, Asn157, Asn174, Cys99, Gly96, Gln155, Lys97, Thr104, Thr150
Catechuic acid	Ala101, Arg152, Asp100, Asn157, Asn174, Cys99, Gln155, Gly96, Lys97, Pro151, Ser180, Thr150
Chlorogenic acid	Ala101, Asn84, Asn149, Asn174, Asn200, Asp100, Arg152, Cys99, Gly96, Gln148, Gln155, Lys97, Leu146, Lys147, Thr150
Ferulic acid	Ala101, Asn157, Asn174, Asp100, Arg152, Cys99, Gly96, Lys97, Gln155, Pro151, Thr150, Thr104,
Gallic acid	Ala101, Asn149, Asn174, Asp100, Arg152, Cys99, Gln155, Gly96, Lys97, Thr150, Pro151
Kaempferol	Ala101, Arg152, Asn174, Asp100, Cys99, Gln155, Gly96, Lys97, Pro151, Thr104, Thr150,
Protocatechuic acid	Ala101, Arg152, Asn174, Asp100, Cys99, Gln155, Gly96, Lys97, Pro151, Thr150
Quercetin	Ala101, Arg152, Asn174, Asn200, Asp100, Cys99, Gly96, Gln148, Lys147, Lys97, Pro151, Thr150,

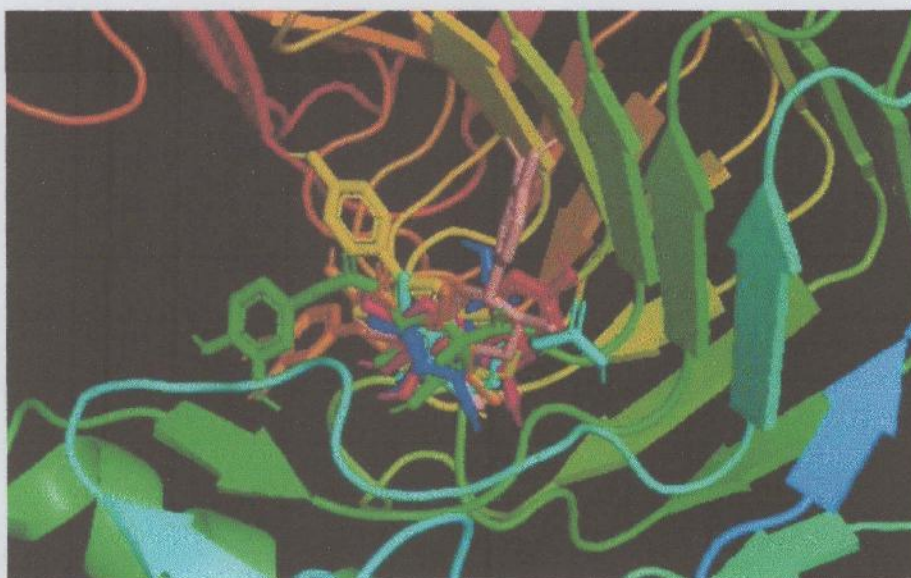


Fig. 5. Binding pocket of caffeic acid (red), catechuic acid (salmon), chlorogenic acid (green), ferulic acid (cyan), gallic acid (blue), kaempferol (yellow), protocatechuic acid (magenta), quercetin (orange) with 1BHE protein

4.5.5 Molecular Docking Analysis

The molecular docking analysis revealed significant interactions between eight bioactive ligands and two target proteins, 1J58 and 1BHE, offering insights into the molecular basis of ligand-protein binding. Each ligand exhibited unique interaction profiles, characterized by specific amino acid residues involved in the binding.

4.5.5.1 Interactions with 1J58 Protein

The docking analysis with the 1J58 protein identified a range of interacting amino acids within its binding pocket. Among the ligands, Catechuic acid demonstrated the most extensive interaction, forming bonds with 20 amino acids, including Ala65, Arg58, Arg66, Glu101, His56, His95, and Thr165. Similarly, Kaempferol and Quercetin showed broad interaction profiles, each interacting with 18 amino acids, including Arg66, Glu67, His140, and Gln167. These ligands displayed high binding specificity and affinity, indicating their strong potential for protein interaction.

Other ligands, such as Caffeic acid and Chlorogenic acid, exhibited moderate binding activity, forming interactions with 16 and 18 amino acids, respectively. Notable interacting residues included Ser161, Glu162, Phe155, and Tyr200. In contrast, Ferulic acid, Gallic acid, and Protocatechuic acid showed fewer interactions, engaging with 11 to 13 amino acids. Key residues for these ligands included Arg66, Glu67, Ala65, Val82, and Thr165. The distinct spatial orientation of these ligands within the protein's binding pocket was visualized with color coding, highlighting the specific positioning of each ligand. (Table 9)

4.5.5.2 Interactions with 1BHE Protein

The docking results for the 1BHE protein revealed a slightly different interaction profile. Among the ligands, Quercetin exhibited the broadest interaction network, binding to 13 amino acids, including Ala101, Arg152, Lys97, and Thr150. Other ligands, such as Caffeic acid, Catechuic acid, and Chlorogenic acid, displayed substantial binding activity, interacting with 12 to 15 amino acids. Key residues involved included Asp100, Gly96, Asn174, and Gln155 (Table 10).

In comparison, ligands like Ferulic acid, Gallic acid, Kaempferol, and Protocatechuic acid displayed selective interactions, engaging with 11 to 12 amino acids, such as Ala101, Cys99, Pro151, and Thr104. These ligands demonstrated a consistent binding pattern involving residues like Arg152, Lys97, and Thr150. The binding pocket visualization depicted the spatial orientation of the ligands with distinct colors, providing a clear representation of their interaction patterns (Table 10).

The analysis highlighted that Catechuic acid exhibited the highest number of interactions with the 1J58 protein, while Quercetin demonstrated the broadest interaction profile with the 1BHE protein. Conversely, Gallic acid consistently showed fewer interactions across both proteins. These findings underscore the structural specificity and binding potential of the ligands, providing valuable insights into their potential efficacy in targeting these proteins. The diversity and specificity of the interactions suggest a strong foundation for further experimental validation and exploration of these ligands as potential bioactive compounds (Table 10).

4.6 Nutrient Accumulation

This study evaluated the impact of different microbial treatments—*Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and arbuscular mycorrhizal fungi (AMF)—on nitrogen (N), phosphorus (P), and potassium (K) accumulation in betelvine. The results demonstrated that all treatments significantly influenced nutrient accumulation compared to the control, with the combination of Th, Pf, and AMF yielding the greatest enhancement in nutrient uptake.

4.6.1 Before the Experiment

The initial nutrient levels were consistent across treatments, with nitrogen (N) ranging from 6.2–6.7 mg/g, phosphorus (P) from 28.3–31.6 mg/g, and potassium (K) from 108.5–119.9 mg/g. The highest baseline levels of N, P, and K were observed in plants assigned to the Th treatment, while the lowest values were recorded in the control.

4.6.2 After the Experiment

Post-treatment analysis revealed significant variations in nutrient levels. Nitrogen content increased across all treatments, with the highest accumulation observed in the Th+Pf+AMF group (68.6 ± 0.77 mg/g), followed closely by Pf (66.6 ± 0.78 mg/g), Th+AMF (66.9 ± 0.67 mg/g), and AMF alone (66.0 mg/g). The control group exhibited the lowest nitrogen accumulation (49.5 mg/g). Phosphorus content also showed notable increases, with the Th+Pf+AMF treatment leading (37.1 ± 0.47 mg/g) and significantly surpassing the control (31.5 ± 0.25 mg/g). Potassium accumulation followed a similar trend, with AMF-treated plants achieving the highest levels (155.7 ± 1.15 mg/g), while the control group recorded the lowest (127.3 ± 1.22 mg/g) (Table 11).

4.6.3 Difference in Nutrient Accumulation

The net difference in nutrient accumulation highlighted the effectiveness of the microbial treatments. Nitrogen accumulation ranged from 43.37 mg/g in the control to 62.17 mg/g in the Th+Pf+AMF group, demonstrating the highest nitrogen uptake efficiency. Phosphorus showed a marked improvement, with differences ranging from 3.30 mg/g in the control to 6.80 mg/g in the Th+Pf+AMF group. Similarly, potassium accumulation varied between 18.78 mg/g in the control and 37.21 mg/g in AMF-treated plants (Table 11).

Table 11. Influence of *Trichoderma harzianum*, *Pseudomonas fluorescens*, and AMF on Nutrient Accumulation in Betelvine

No	Treatments	Before Experiment			After Experiment			Difference		
		N	P	K	N	P	K	N	P	K
1	Control	6.2±0.03e	28.3±0.14c	108.5±1.26b	49.5±0.74g	31.5±0.25d	127.3±1.22d	43.37g	3.30d	18.78d
2	Th	6.7±0.05a	31.6±0.47a	119.9±1.16a	64.7±0.86e	36.6±0.27b	155.7±1.15c	58.05e	4.94c	35.87c
3	Pf	6.5±0.03abc	30.6±0.08b	119.5±1.02a	66.6±0.78bc	36.5±0.37bc	156.4±1.34abc	60.08bc	5.88abc	36.86ab
4	AMF	6.6±0.03ab	30.8±0.05ab	119.8±1.04a	66.0±0.77cd	36.7±0.33b	157.0±1.8a	59.46cd	5.86abc	37.21a
5	Th + Pf	6.4±0.03cd	30.6±0.03b	119.7±1.02a	65.6±0.72d	36.2±0.45c	155.8±1.10c	59.32d	5.60bc	36.13bc
6	Th+AMF	6.6±0.06ab	30.7±0.07ab	119.7±1.05a	66.9±0.67b	36.4±0.42bc	156.6±1.10ab	60.33b	5.71bc	36.89ab
7	Pf+AMF	6.2±0.03de	30.4±0.07b	119.6±1.10a	59.1±0.61f	36.8±0.29ab	155.8±1.03c	52.89f	6.37ab	36.28bc
8	Th+Pf+AMF	6.5±0.03bc	30.3±0.12b	119.4±2.09a	68.6±0.77a	37.1±0.47a	156.0±2.15bc	62.17a	6.80a	36.56abc
	LS (P≤0.05)	*	*	*	*	*	*	*	*	*

(Means followed by same small case letters are statistically similar within a column based on Tukey's honest significant difference test at $p < 0.05$; data are means of three replications ± standard errors; LS = Level of Significance, * = Significant at 5% level of significance; Th = *Trichoderma harzianum*, Pf = *Pseudomonas fluorescens*, AMF = Arbuscular mycorrhizal fungi)



4.7. Correlation with Growth Parameters, Biochemical Compounds, and Nutrient Concentrations in Plants

The significant relationships between various growth parameters, biochemical compounds, and nutrient concentrations in the studied plants. High positive correlations are observed between key growth traits such as vine length, leaf length, leaf breadth, and leaf number per plant, indicating their interdependence in influencing overall plant development. For instance, vine length shows strong positive correlations with leaf length ($r = 0.972^{**}$) and leaf breadth ($r = 0.957^{**}$), suggesting that longer vines are associated with larger, more abundant leaves (Table 12).

Several biochemical compounds, including Kaempferol, Quercetin, and Catechuic acid, demonstrate significant negative correlations with disease incidence, highlighting their potential in disease resistance. Leaf Kaempferol ($r = -0.964^{**}$) and Root Quercetin ($r = -0.818^{*}$) are strongly negatively correlated with disease incidence, suggesting their role in mitigating disease effects leaves (Table 12).

Nutrient concentrations, specifically Nitrogen (N), Phosphorus (P), and Potassium (K), exhibit moderate to strong positive correlations with growth parameters like leaf size and income per plant, indicating that these nutrients contribute to enhanced growth and productivity. N (kg/ha correlates positively with leaf length ($r = 0.626$) and leaf number per plant ($r = 0.555$), suggesting that adequate nutrient availability supports better growth and yield. Strong intercorrelations among the biochemical compounds themselves, with significant positive relationships between Leaf and Root concentrations of various phenolic acids such as Gallic acid ($r = 0.998^{**}$) and Chlorogenic acid ($r = 0.941^{**}$), further supporting their role in plant health and stress tolerance leaves (Table 12).

Table 12. Correlation of Growth Parameters, Biochemical Compounds, and Nutrient Concentrations in Plant

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1 Vine length(cm)	1																													
2 Lateral vine-1 (cm)	.954**	1																												
3 Internodal length (cm)	.911**	.853**	1																											
4 Leaf petiole length(cm)	.973**	.872**	.932**	1																										
5 Leaf length(cm)	.972**	.898**	.958**	.973**	1																									
6 Leaf breadth (cm)	.957**	.833**	.926**	.993**	.978**	1																								
7 100 leaf weight (g)	.988**	.970**	.925**	.951**	.975**	.936**	1																							
8 Leaf No / plant	.992**	.940**	.955**	.966**	.981**	.957**	.981**	1																						
9 Incidence/plant	.967**	.943**	.854**	.914**	.957**	.913**	.977**	.980**	1																					
10 Days to appear symptom	.953**	.931**	.888**	.905**	.967**	.911**	.977**	.959**	.983**	1																				
11 Average Disease Incidence	-.959**	-.917**	-.905**	-.931**	-.975**	-.938**	-.979**	-.963**	-.973**	-.992**	1																			
12 N (kg/ha) After	0.523	0.568	.787*	0.544	0.626	0.532	0.606	0.555	0.492	0.568	-0.569	1																		
13 P(kg/ha) After	0.561	0.532	.817*	0.62	0.7	0.636	0.64	0.609	0.546	0.644	-0.678	.892**	1																	
14 K(kg/ha) After	0.481	0.437	.777*	0.554	0.64	0.577	0.556	0.541	0.463	0.565	-0.595	.892**	0.507	0.546	1															
15 Leaf Kaempferol	.938**	.947**	.817*	.871**	.922**	.866**	.966**	.920**	.987**	.979**	-.964**	0.507	0.546	0.449	1															
16 Root Kaempferol	.826*	.784*	0.568	.752*	.770*	.761*	.803*	.791*	.895**	.837**	-.806*	0.107	0.147	0.059	.884**	1														
17 Leaf Quercetin	.935**	.903**	.768*	.875**	.912**	.880**	.938**	.917**	.986**	.962**	-.946**	0.363	0.431	0.34	.979**	.952**	1													
18 Root Quercetin	.837**	.802*	0.584	.758*	.782*	.765*	.816*	.806*	.908**	.851**	-.818*	0.125	0.168	0.08	.885**	.998**	.939**	1												
19 Leaf Catechin acid	.946**	.959**	.861**	.888**	.956**	.878**	.980**	.931**	.977**	.980**	-.973**	0.588	0.622	0.526	.993**	.827*	.933**	.839**	1											
20 Root Catechin acid	.950**	.953**	.802*	.876**	.918**	.868**	.963**	.932**	.992**	.971**	-.950**	0.45	0.473	0.38	.993**	.918**	.989**	.929**	.998**	1										
21 Leaf Gallic acid	.953**	.952**	.874**	.902**	.953**	.897**	.984**	.943**	.987**	.989**	-.981**	0.586	0.63	0.541	.993**	.833**	.962**	.851**	.994**	.997**	.983**	1								
22 Root Gallic acid	.947**	.963**	.800*	.867**	.908**	.853**	.962**	.926**	.985**	.967**	-.948**	0.463	0.482	0.383	.994**	.899**	.979**	.911**	.994**	.997**	.983**	.983**	1							
23 Leaf Protocatechuic acid (µg/g)	.726*	0.704	.830**	.718*	.843**	.750*	.789*	.779*	.765*	.841**	-.830*	.826*	.866**	.851**	.751*	.984	0.687	0.501	.785*	.714*	.803*	.706	0.706	1						
24 Root Protocatechuic acid (µg/g)	.794*	.748*	.940**	.837**	.890**	.845**	.849**	.811*	.781*	.835**	-.857**	.870**	.924**	.886**	.777*	.966	0.694	0.478	.830*	.727*	.839**	.728*	.908**	.728*	1					
25 Leaf Chlorogenic acid (µg/g)	.726*	.775*	.717*	0.705	.734*	0.667	.781*	.708*	.731*	.741*	-.739*	0.62	0.663	0.588	.772*	.935	0.505	0.681	.830*	.735*	.796*	.753*	.587	.769*	.587	1				
26 Root Chlorogenic acid (µg/g)	.760*	.842**	0.69	.710*	.713*	0.655	.811*	.724*	.728*	.749*	-.763*	0.546	0.583	0.47	.805*	.939	0.706	0.562	.838**	.768*	.816*	.801*	.531	.707*	.941**	.531	1			
27 Leaf Caffeic acid (µg/g)	.935**	.823*	.810*	.947**	.876**	.929**	.871**	.916**	.841**	.804*	-.830*	0.32	0.365	0.295	.784*	.777*	.829*	.777*	.785*	.820*	.796*	.808*	.808*	.52	.629	.525	.835	.52	1	
28 Root Caffeic acid	.910**	.917**	.900**	.892*	.912**	.871**	.952**	.892**	.892**	.916**	-.940**	0.692	.730*	0.652	.915**	.652	.840**	.664	.954**	.878**	.944**	.895**	.774*	.897**	.829*	.891**	.829*	.891**	1	
29 Leaf Ferulic acid	.943**	.984**	.848**	.873**	.884**	.836**	.960**	.918**	.917**	.909**	-.906**	0.582	0.529	0.427	.933**	.767*	.884**	.776*	.953**	.933**	.948**	.948**	.689	.756*	.720*	.827*	.827*	.834*	.932**	1
30 Root Ferulic acid	.933**	.981**	.865**	.867**	.887**	.831*	.961**	.912**	.910**	.913**	-.912**	0.634	0.591	0.49	.932**	.729*	.869**	.740*	.959**	.922**	.944**	.940**	.722*	.794*	.755*	.849**	.802*	.955**	.996**	1

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Discussion

Betelvine is a significant economic crop widely cultivated for its leaves, valued for medicinal and cultural uses. The cultivation of betelvine often encounters challenges such as root rot and foot rot, which significantly diminish productivity and income for farmers. This discussion examines the role of beneficial soil microbes in enhancing plant immune responses to combat these diseases alongside accelerating growth and yield attributes and overall productivity. The analysis also includes biochemical changes, nutrient accumulation, and evaluation of income metrics.

Incorporating soil-inhabiting beneficial microbes like *Trichoderma harzianum*, *Pseudomonas fluorescens*, and AMF has shown marked improvement in the growth parameters of betelvine in our study. The enhanced nutrient accumulation, particularly nitrogen, phosphorus, and potassium uptake due to microbial treatments (Th, Pf, and AMF), also showed a strong correlation with improved growth parameters such as increased plant height, leaf number, and biomass production in betelvine. Alike results were revealed by Sandheep et al., (2021) wherein they reported a significant growth increase in terms of vine length, shoot weight and leaf number of vanilla spice in plot treated with *T. harzianum* and *P. fluresencs* with reference to untreated control. Enhanced microbial symbiosis promotes hormonal balance, nutrient uptake, and stress resistance, leading to robust vegetative growth. Our results also the application of beneficial microbes not only boosts growth but also improves yield attributes. The Th + Pf + AMF treatment significantly improved yield attributes of betelvine, including 100-leaf weight and leaf numbers per plant and decimal, compared to the control. This highlights the potential of microbial symbiosis for sustainable yield enhancement. A corresponding piece of research works done by Ramesh et al. (2020) who demonstrated a 35% increase in the number of leaves per vine and a 40% improvement in leaf quality parameters, such as texture, color, and shelf life. The enhanced nutrient availability and systemic resistance induced by the microbial inoculants contributed to these improvements.

Root rot and foot rot, caused by pathogens like *Sclerotium rolfsii* pose significant threats to betelvine cultivation. Beneficial microbes act as biocontrol agents by competing with pathogens for resources and space, producing antagonistic metabolites, and inducing systemic resistance in plants. Our study demonstrates the effectiveness of

microbial treatments in delaying disease symptom onset, reducing disease incidence, and enhancing disease control in betelvine plants. The combination of Th, Pf, and AMF exhibited the most significant delay in symptom appearance and achieved the lowest disease incidence at 120 DAT, with a remarkable disease reduction compared to the control. Field trials have reported a 60-70% reduction in disease incidence and severity with microbial treatments (Rahman et al., 2021).

The biochemical analysis reveals that microbial treatments significantly enhanced the flavonoid, tannin, and phenolic acid content in both the leaves and roots of Betelvine, with the combination of Th, Pf, and AMF showing the highest accumulation of these compounds. This synergy suggests that combined microbial treatments can be highly effective in improving the plant's biochemical profile, potentially contributing to enhanced disease resistance and overall plant health (Das et al., 2016). Our study also revealed that molecular docking analysis confirmed the increased binding interactions of phenolic compounds with target proteins, such as 1J58 and 1BHE, correlate with their enhanced disease resistance potential, indicating that stronger binding capability translates to better disease control efficacy.

CHAPTER V

SUMMARY AND CONCLUSION

This study evaluated the effects of microbial treatments, including *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and Arbuscular Mycorrhizal Fungi (AMF), applied individually and in combination, on the growth, yield, disease resistance, and biochemical composition of betelvine. The treatments were aimed at improving plant growth, enhancing yield attributes, and controlling root rot and foot rot diseases, while also boosting the plant's biochemical content, including flavonoids, tannins, and phenolic acids. The results demonstrated that the combination of Th, Pf, and AMF provided the most significant improvements in growth parameters, such as vine length, number of laterals, internodal length, leaf size, and petiole length, compared to untreated control plants. Similarly, yield attributes including 100-leaf weight, leaf number per plant, and leaf number per decimal were also significantly higher in the combined treatment. The highest leaf yield and income were recorded for the Th + Pf + AMF combination, showing substantial increases over the control group. Disease management was another area where microbial treatments showed considerable effectiveness. The combined treatment of Th + Pf + AMF delayed disease symptom appearance and reduced disease incidence, achieving the highest disease reduction of 74.32% at 120 days post-treatment. Other combinations, such as Th + Pf and Th + AMF, also showed effective disease control, but with less pronounced reductions. Biochemical analysis revealed significant increases in flavonoid, tannin, and phenolic acid content in the leaves and roots of plants treated with the combined microbial treatments. The Th + Pf + AMF combination resulted in the highest accumulation of bioactive compounds, particularly flavonoids like Kaempferol and Quercetin, and tannins such as Catechuic acid and Gallic acid. The molecular docking analysis further supported these findings, showing strong interactions between bioactive compounds and target proteins, suggesting potential bioactivity. In conclusion, the study highlights the synergistic effects of *Trichoderma harzianum*, *Pseudomonas fluorescens*, and Arbuscular Mycorrhizal Fungi in enhancing the growth, yield, disease resistance, and biochemical content of betelvine. The combination of these microbial treatments offers a sustainable and effective biological approach to

improving betelvine productivity by enhancing the plant's bioactive compounds leading to resilient disease management of betelvine.

CHAPTER VI

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