Single and Co-Inoculation of Native Arbuscular Mycorrhizal Fungi (AMF) and a Plant Growth Promoting *Bacillus subtilus* (ALCR46), on the growth of three Vegetable Species in a Low Phosphorus Soil

Sara Gebreslassie

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Applied Biology (Applied Microbiology)

Office of Graduate Studies

Adama Science and Technology University

March, 2022

Adama, Ethiopia

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DECLARATION

I hereby declare that this Master Thesis entitled "**Single and Co-Inoculation of Native Arbuscular Mycorrhizal Fungi (AMF) and a Plant Growth Promoting *Bacillus subtilus* (ALCR46), on the growth of three Vegetable Species in a Low Phosphorus Soil**" is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation

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RECOMMENDATION

We, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled "**Single and Co-Inoculation of Native Arbuscular Mycorrhizal Fungi (AMF) and a Plant Growth Promoting *Bacillus subtilus* (ALCR46), on the growth of three Vegetable Species in a Low Phosphorus Soil**" prepared under our guidance by Sara Gebreslassie submitted in partial fulfillment of the requirements for the degree of Mater's of Science in Applied Microbiology. Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

Major Advisor	Signature	Date
Co-Advisor	Signature	Date

APPROVAL PAGE

We, the advisors of the thesis entitled "**Single and Co-Inoculation of Native Arbuscular Mycorrhizal Fungi (AMF) and a Plant Growth Promoting *Bacillus subtilus* (ALCR46), on the growth of three Vegetable Species in a Low Phosphorus Soil**" and developed by **Sara Gebreslassie**, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	Signature	Date
Co-Advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by **Sara Gebreslssie** have read and evaluated the thesis entitled "**Single and Co-Inoculation of Native Arbuscular Mycorrhizal Fungi (AMF) and a Plant Growth Promoting *Bacillus subtilus* (ALCR46), on the growth of three Vegetable Species in a Low Phosphorus Soil**" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Microbiology.

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Internal Examiner	Signature	Date
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Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

Department Head	Signature	Date
School Dean	Signature	Date
Office of Postgraduate	Signature	Date

ACKNOWLEDGMENTS

First of all, I would like to express my heartfelt gratitude to My Major advisor, Dr. Zerihun Belay, for his continuous encouragement and constructive comments starting from the beginning to the end of this research work. My advisor, Dr. Zerihun, has been an enormous resource to me in all facets of my academic life during my Master's studies. He made every effort possible to help me in every way, including financially, and he teach me kindness practically, for which I'm sincerely indebted. I'm also thankful to my co-advisor, Dr. Mulissa Jida, for allowing me to work on my thesis with their research project and for his guidance throughout the research work and critical comments.

I extend my deep gratitude to Mr. Solomon Girmay for his continuous encouragement, brotherly approach, inspiring advice, and supporting me financially during my study period. I would also like to express my appreciation to Mr. Mohammod Fili and Elsa Gebreslassie for their supporting me financially. My thanks also go to my friends, Tihitna Atle, Tseday Temesgen, Rediet Solomon, Yonatan Legesse, and Urgessa Enssermu for their advice, help, and encouragement. I would also like to express my special thanks to my family for nurturing me with love and inspiration throughout my life.

I acknowledge the Department of Applied Biology, School of Applied Natural Science, Adama Science and Technology University for giving me an M.Sc. scholar by covering all the tuition expenses, including thesis work. I extend my deep gratitude to Ethiopian Biotechnology Institution for their willingness and support me financially. Last but not least, I am also highly indebted to Melkassa agricultural research center for their supply of selected seed varieties for my research work.

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LISTS OF ACRONYMS

ACC	1-Aminocyclopropane-1-Carboxylate
AFMs	Antifungal Metabolites
ALP	Alkaline Phosphatase
AMF	Arbuscular Mycorrhiza Fungi
ANOVA	Analysis of Variance
DAPG	Phenazines, Pyrrolnitrin, 2, 4-Diacetylphloroglucinol
ePGPR	Extracellular Plant Growth-Promoting Rhizobacteria
EPS	Exopolysaccharides
EPS	Extracellular Polymeric Substance
INVAM	International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi
iPGPR	Intracellular Plant Growth-Promoting Rhizobacteria
MD	Mycorrhizal Dependency
MHB	Mycorrhiza Helper Bacteria
MRC	Melkasa Agricultural Research Center
PEG	Polyethylene glycol
PGPB	Plant Growth Promoting Bacteria
PGPR	Plant Growth-Promoting Rhizobacteria
PPA	Prepenetration Apparatus
PVLG	Polyvinyl-Lactic Acid-Glycerol
SDH	Succinate Dehydrogenase

ABSTRACT

Arbuscular mycorrhizal fungi (AMF) and Plant Growth Promoting Rhizobacteria (PGPR) are the major component of the soil microbial populations, which play significance roles in improving soil fertility, plant growth and health; Hence, this study was aimed to investigate the effect of single and co-inoculation of AMF and a PGPR, Bacillus subtilus (ALCR46) on three selected vegetables, Lycopersicum esculentum (Tomato); Allium cepa (Onion) and Cucurbita pepo (Squash). The experimental design was a randomized complete block design with three replications in a greenhouse. Seven treatments were set up as follows: plants without inoculation (negative and positive controls), with the consortium and single AMF inoculation, with Bacillus subtilus inoculation, and with co-inoculation of PGPR and a single and consortium of AMF. In this work, the growth parameters like root length and shoot height, fresh and dry weight of root and shoot, and the number of branches; percentage of root colonization, spore number were measured and all data were analyzed by ANOVA. The result shows that the growth of the vegetables significantly increased by co-inoculation of the consortium species of AMF and Bacillus subtilus (ALCR46). AMF spore density and colonization rate were also increased in co-inoculation. Moreover, results indicated that the highest root colonization, spore number, and mycorrhizal dependency were observed in Allium cepa among the selected vegetables. Furthermore, Pearson's correlation analysis showed that positively correlated between the parameters. All of these results suggested that co-inoculation of native AMF and PGPR could replace the inorganic fertilizer, and support the conclusion that there is a synergistic effect between the AMF and a PGPR (Bacillus subtilus (ALCR46); and within AMF species. Moreover, concluded that among the vegetable crops, Allium cepa was particularly found reactive to soil mycorrhiza with high growth dependency. However, the application of present findings under field conditions required to be confirmed by further studies.

Keywords: Arbuscular Mycorrhizal Fungi (AMF), Co-inoculation, Consortium of AMF sp., Plant Growth Promoting Rhizobacteria (PGPR), *Lycopersicum esculentum, Allium cepa, Cucurbita sp.*

CHAPTER ONE

1. INTRODUCTION

1.1. Background of the Study

Vegetables are essential for food and nutrition security. They are key sources of micronutrients including vitamins; trace minerals, and dietary fiber, phytochemicals, and many other classes of biologically active compounds (Schreinemachers *et al*., 2018). They are involved in different biological activities such as stimulation of the immune system, reduction of platelet aggregation, modulation of cholesterol synthesis and hormone metabolism, reduction of blood pressure, and antioxidant, antibacterial, and antiviral effects (Lenka *et al*., 2021).

In 2018, the world produced a total of 1089 million tonnes of vegetables most of which was produced in Asia, mainly in China (FAO, 2020). In contrast, the production of vegetables in Africa as well as in Ethiopia is lagging far behind the worldwide average, and is not well-developed due to lack of advanced agricultural technologies and yield losses caused due to their extremes sensitivity to environmental factors (biotic and abiotic) (Habtamu Mokennen and Mulugeta Kibret, 2021).

Most vegetables are produced using synthetic fertilizers and pesticides which have adverse effects on humans, animals, and the environment (Hakim *et al*., 2021).This requires for urgent need of developing other strategies to solve these problems (Nagargade *et al*., 2018). In recent years, several technical and technological innovations proposed in order to improve the sustainability of production systems through a significant reduction in agrochemicals. A promising practice would be the use of soil microorganisms (bacteria, fungi, algae, etc.) that enhance plant growth, increase tolerance to disease and extreme environmental conditions (Nanjundappa *et al*., 2019).

Among soil microorganisms, Arbuscular Mycorrhizal Fungi (AMF), and Plant Growth Promoting Rhizobacteria (PGPR) are the most promising to improve the sustainability of production systems through a significant reduction in agrochemicals. AMF are soil microorganisms that form a symbiotic relationship with 80–90% of vascular plant species including cereals, vegetables, and horticultural plants (Smith and Read, 2008). AMF benefits the host by increasing nutrient uptake mainly, N, P, K, Ca, S, Cu, and Zn. Moreover, they

improve drought, salinity, heavy metals, and pathogen resistance of the host (Carvalho *et al*., 2010).

Recent studies confirmed that, a number of bacterial species mostly associated with the plant rhizosphere, are found to be beneficial for plant growth, yield and crop quality. Some of these organisms are known as Plant growth promoting rhizobacteria (PGPR) that are beneficial microorganisms capable of improving plant growth through supply of nutrients and sustaining environmental health and soil productivity (Pratap *et al*., 2016). *Bacillus* is one of the important genera of PGPR that exists in soil or as an endophyte and a spore former has better saprophytic ability and competitiveness and can survive in soil for long period of time under harsh environmental conditions. *Bacillus* spp. assist plants in their defense against pathogen attack and also enhance stress tolerance by inducing the expression of stress-response genes, and producing phytohormones and stress related metabolites (Nanjundappa *et al*., 2019).

Studies show that combined inoculation of AMF with other PGPR exerted positive effects on the growth of several crop plants, enhances nutrient uptake and productivity compared to a single inoculation under both normal and stressed environments (Sagar *et al*., 2021). Kavatagi& Lakshman, (2014) showed that co-inoculation of AMF and PGPR increased root length, fresh weight of root, dry weight of root, fresh weight of shoot, dry weight of shoot, percentage of root colonization, spore number and number of leaves in the two variety of *Lycopersicum esculentum*. Lee *et al*. (2015), evaluated the efficacy of co-inoculation of AMF (*Glomus etunicatum*) and *Methylobacterium oryzae* to alleviate salt stress and improve maize growth; and concluded that co-inoculation was more effective to ameliorate salt stress and improve plant growth, and showed higher AMF density and AMF colonization compared to single AMF treatment.

Although the use of commercial inoculants consisting of native AMF and PGPR is highly recommended as supplementary method to reduce the use of excessive chemicals in vegetable production (Séry *et al*., 2016), they may not necessarily effective as indigenous AMF and PGPR to use them under different local environments Williams *etal*., (2012) made a comparative study on the effect of indigenous AMF inoculums with commercially available AMF on growth and survival of *Podocarpus cunninghamii* (*mountain tōtara*). They reported that plants treated with indigenous and forest AMF significantly showed a greater survival than those treated with commercial AMF. Therefore, the aim of the study

was to evaluate the potential of native inoculants (*Bacillus* sp. and AMF) isolated from potential regions of Ethiopia in improving growth of selected vegetables.

1.2. Statement of the Problem

In Ethiopia, vegetable crops are produced in different agro-ecological zones through commercial as well as smallholder farmers both as a source of income and food. Onion (*Allium cepa*), tomato (*Lycopersicum esculentum*), and squash (*Cucurbita pepo*) are widely cultivated in Ethiopia in most parts of the country throughout the year. However, it is well recognized fact that vegetable production is extremely sensitive to environmental factors (biotic and abiotic stresses), that resulted into low yield. In Ethiopia, farmers get lower yield mainly due to diseases and pests as well as due to sub-optimal fertilization.

In Ethiopia, a lot of works have been conducted related to abundance and diversity of the AMF in different land use system in Ethiopia (Zerihun Belay *et al*., 2013;205; 2020). In addition, several other studies have been in Ethiopia related to the identification and characterization of symbiotically effective indigenous rhizobia strains isolated from faba bean, field pea, chickpea and lentil and etc.(Ayneabeba Adamu *et al*., 2001;Zerihun Belay &Fassil Assefa, 2011; Anteneh Argaw, 2013; Mulissa Jida *et al*., 2016; Nigusie Girma *et al*., 2019). Attempts were made in mass-production and distributing selected strains of rhizobia to farmers in various parts of the country (Nigussie Alemayehu, 2020). Moreover, the most research activities on biofertilizer have also focused mainly on symbiotic nitrogen fixation of major legume crops (Mulissa Jida *et al*., 2016).

Nevertheless, Limited researches have been conducted on the evaluation of potential of co-inoculation of native PGPR and AMF to improve the growth and yield of crops particularly, vegetables. Therefore, this study was initiated to evaluate a potential of single and co-inoculation of native AMF and *Bacillus* species to the alleviate the problems related to growth and yield of different vegetables with low-input cropping system.

1.3. Research Questions

This research project is designed to answer the following questions:

- What is the potential of the native co-inoculants of AMF and a PGPR, (*Bacillus subtilus*) in the growth of the selective vegetables?
- What is the effect of the *Bacillus* on AM establishment?
- Which selected vegetable is more benefited by inoculation?
- What is the difference in the potential of inoculation of consortium and single species of AMF on vegetable growth?

1.4. Objective of the Study

1.4.1. General Objective

❖ To evaluate growth response of selected vegetables (*Lycopersicum esculentum*, *Allium cepa*, and *Cucurbita pepo*) to single and co-inoculation of native Arbuscular Mycorrhizal Fungi (AMF) and a Plant Growth Promoting Rhizobacterium (PGPR), (*Bacillus subtilus*) isolated from some parts of Ethiopia.

1.4.2. Specific Objectives

- To determine the AMF spore abundance, and physical and chemical parameters of the different AMF inoculants.
- To determine the effect of single and consortium species of AMF inoculations on the growth of the selected vegetables.
- To compare the effects of inorganic fertilizer and microbial inoculants on the growth of vegetables.
- To assess the effect of a PGPR *Bacillus subtilus* on AM establishment.
- To compare the level of mycorrhizal dependency, AMF root colonization, and spore density among the selected vegetables.

1.5. Significance of the Study

This study provided sufficient information on the potential of native AMF and PGPR inoculum to be readily generated using local soil as a source of native inoculum. In addition, provided evidence that the potential of using dual inoculums (AMF and PGPR) rather than a single inoculation of either of them, and the use of consortiums rather than a single species of AMF to simultaneously increase vegetable growth. Moreover, data obtained from this

study have implications for management strategy with regard to the use of bio-fertilizer in agricultural systems, especially in the production of vegetables. As a result, professional horticulturists, including greenhouse and nursery growers, as well as large-scale and small-scale vegetable farmers, will benefit from the study.

Furthermore, this study contributes on the restoring and preserving soil nutrients, reduce the growing cost of fertilizers, and protection environmental problems due to agro-pollutions such as surface water and groundwater, as well as air pollution, and reduced biodiversity. This research may also help to make this topic more noticeable and help to share the latest information to more studies should focus on microbial technologies and emerging microbiome approaches potentially can significantly increase agriculture productivity & human healthcare and henceforth can contribute to meet several sustainable development goals.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Vegetable crops

Vegetables are a diverse group of plants and herbaceous species grown for human consumption in which the edible portion consists of leaves (e.g. lettuce, cabbage), swollen tap roots (e.g. carrots), lateral roots (e.g. sweet potato), hypocotyls (e.g. radish, turnip), aboveground stems (e.g. asparagus), below ground stems (e.g. Irish potato), petioles (e.g. celery), and flower buds (e.g. cauliflower) (Baum *et al*., 2015). Some species grow from year to year; others grow and die within one or two years. They have diverse forms of propagation: by seeds or vegetative parts. They may be herbaceous, viny, shrubby, or tree-like in growth habit. They differ in growth requirements (AVRDC, 1990; Keatinge *et al*., 2010).

Vegetables are grown worldwide in almost 200 countries and make up a major portion of the diet of humans in many parts of the world. They play a significant role in human nutrition, especially as sources of vitamins (C, A, B1, B6, B9, E), minerals, dietary fiber and phytochemicals (Varadaraju, 2019). A range of vegetable crops are grown in Ethiopia under rain-fed and/or irrigation systems. The major economically significant vegetables include hot and sweet peppers (*Capsicum spp*.), Ethiopian mustard/ kale (Brassica carinata), onion (*Allium cepa*), tomato (*Lycopersicum esculentum*), chili (*C. chinense*), carrot (*Daucus carota*), garlic (*A. sativum*) and cabbage (*B. oleracea var. capitata*). According to the Ethiopian Investment Agency, green beans (*Phaseolus spp*.) and peas (*Pisum sativum*), okra (*Abelmoschus spp*.), asparagus (Asparagus officinalis), cauliflower (*B. oleracea var. botrytis*), broccoli (*B. oleracea var. italica*), celery (*Apium graveolens L.*), eggplant (*S. melongena*) and cucumbers (*Cucumis sativus*) have also recently emerged as important export vegetables (Bezabih Emana *et al*., 2015).

2.1.1. *Allium cepa L.* (Onion)

Onion (*Allium cepa L.*) belongs to the family *Alliaceae* or *Amaryllidaceae*, belongs to the genus *Allium*, which is one of the most important monocotyledonous crops commercially grown in the world (Guesh Tekle, 2015). It probably originated from Central Asia between Turkmenistan and Afghanistan where some of its relatives still grow in the wild. Onion from Central Asia, the supposed onion ancestor had probably migrated to the Near East and areas

around the Mediterranean Sea are secondary centers of development. It contributes significant nutritional values to the human diet and has medicinal properties and is primary consumed for its unique flavors or for its ability to enhance the flavors of other foods (Demis Fikre, 2021).

Onion is a shallow rooted, biennial crop which is grown as annual. The leaves are long, hollow with widening, overlapping bases. The tubular leaf blades are flattened on the upper surface, and the stem of the plant also is flattened. Roots arise from the bottom of the growing bulb (Demis Fikre, 2021). Leaf initiation stops when the plant begins to bulb. The base of each leaf becomes one of the "scales" of the onion bulb, so the final bulb size depends in part on the number of leaves present at bulb initiation. The leaf base begins to function as a storage organ at bulb initiation, so the size of the leafy part of the plant also influences bulb size. Thus, the more leaves present and the larger the size of the plant at the onset of bulb initiation, the larger will be the bulbs and the greater will be the crop yield (Aklilu Nigussie *et al*., 2015).

Onion is more widely grown in Ethiopia for local consumption and for flower export (Demis Fikre, 2021), and one of the most important vegetable crops in Ethiopia which is used almost daily as a spice and vegetable in the local dish regardless of religion, ethnicity, and culture. The diverse agro-climatic conditions that prevail in the country provide the opportunity of producing onion bulb, seeds and cut flower for local use and export market (Gebretsadkan Gebremicael *et al*., 2019). As cited by Agidew Abebe, (2018), recent statically data indicated the total hectare under onion was about 20,444 hectare with total production of 2,572,053 quintals dry bulbs per annum. Globally onion is produced to, at nearly 35 million meters per annum. However, there are a number of constraints that cause low productivity of onion in Ethiopia. The low yield of onion in the country is reported to be due to low fertility of soil, inappropriate fertilizer rate, lack of improved varieties, and poor management practices (Gebretsadkan Gebremicael *et al*., 2019).

Onion grows between 500-2400 m.a.s.l. The best growing altitude so far known in Ethiopia is between 700-1800 m.a.s.l. (Demis Fikre, 2021). Besides altitude which has an indirect bearing on climate, onion production is affected by temp, rainfall and soil. It is grown under mild seasons without extremes of heat, cold or moderate rainfall. Optimum temperature of 18.3-23.90 day and 10-120°C of night temp is ideal for onion bulb production. But lower temperature is referred for seed stalk development. Onion requires deep alluvial and friable

or sandy loam soil with a pH of 5-6.8. Onion does not tolerate badly drained soil and also it is moderately sensitive to soil salinity (Aklilu Nigussie *et al*., 2015).

2.1.2. *Solanum lycopersicum* (Tomato)

Tomato (*Lycopersicum esculentum*) belongs to the family *solanaceae*, which contains more than 3,000 species, including plants of economic importance such as potatoes, eggplants, tobacco, petunias and peppers (Gerszberg *et al*., 2015). Tomato is one of the most widely grown vegetable crops in the world, second to potato (Dam *et al*., 2005). It originally came from tropical area from Mexico to Peru. Its use as a food originated in Mexico, and spread throughout the world following the Spanish colonization of the Americas (Kassa Melese, 2018). It is a fruit of good nutritive value as it is fairly rich in vitamins (vitamin C), and other minerals like calcium, phosphorus and iron. Tomato juice is used alone or in combination with lime, honey, buttermilk, yoghurt and avocado in several homemade remedies to keep skin healthy (Harminder *et al*., 2011).

The species have been used in traditional medicine to cure human diseases including cancer diseases such as lung, prostate, stomach, cervical, breast, oral, colorectal, oesophageal, pancreatic, and many other types of cancer, high blood pressure, treat oedema, kidney and liver problems, antioxidant cathartic. Tomato is also good for liver health (Bhowmik *et al*., 2012). Tomato has detoxification effect in the body. Probably it is due to the presence of chlorine and sulfur in tomatoes. Sulfur in tomatoes protects the liver from cirrhosis (Harminder *et al*., 2011).

Tomato is mainly propagated from seeds, which are started in the nursery and later transplanted. Intensive agronomic practices such as watering, weeding, pruning, training, pest and disease control are carried out to ensure optimum production. The plant typically grows to 1–3 meters in height and has a weak stem that often sprawls over the ground and vines over other plants, if left untrained, it exhibits decumbence. It is perennial in its native habitat, although often grown outdoors in temperate climates as an annual. An average common tomato weighs approximately 100 grams (Gatahi, 2020).

In Ethiopia tomato is one of the most important and widely grown vegetable crops, both during the rainy and dry seasons for its fruit by smallholder farmers, commercial state and private farms. In Ethiopia, the crop is grown between 700 and 2000 m above sea level with about 700 to over 1400 mm annual rain fall, in different areas and seasons, in different soils, under different weather conditions (Getahun Lendabo, 2021). Tomato is cultivated mainly

in the central Rift Valley part of the country under irrigation. Tomato served as an ingredient in many dishes (local sauce), fresh produce is sliced and used as a salad; and processed products such as tomato paste, tomato juice, soups, stews, and tomato catch-up are consumed in large quantities when compared to other vegetables (Francis Abuye & Berhanu Achamo, 2016).

In Ethiopia, several tomato varieties (such as Melkashola) had been released nationally and recommended by the Melkassa Agricultural Research Center for commercial production and small-scale farming systems. The total production of this crop in the country has shown a marked increase since it became the most profitable crop providing a higher income to small scale farmers compared to other vegetable crops. However, The average yield of tomato in Ethiopia is low (8 $tonha^{-1}$) compared with world average yields of 34 $tonha^{-1}$ (Kassa Melese, 2018).

Tomato production is faced with a number of constraints which are biotic and abiotic that resulted into low yield, and requires large quantities of fertilizer (Francis Abuye & Berhanu Achamo, 2016). Biotic factors contributing for lower yield of tomato in Ethiopia include insect pests. Plant parasitic weeds are also one of the factors affecting tomato yield. Drought, heat, and poor cultural practices constitute abiotic factors for lower productivity of tomato. The shortage of varieties that are adaptable to different agro-ecologies, poor quality seeds, disease and insect pests, high post-harvest loss, lack of awareness of existing improved technology and poor marketing systems are some of the major constraints associated with tomato production in Ethiopia (Sora SA, 2018).

2.1.3. *Cucurbita* Species (Squash)

Squash is a member of the *Cucurbitaceae* family, which comprises about 118 genera and 825 species which are of consumed by humans. including a number of warm-season vegetables including watermelon, cantaloupe, cucumber, and pumpkins (Jyoti *et al*., 2019). The fruit is large and variable in shape, size, colour and markings with a peduncle that is large, soft and corky on the surface at maturity. Numerous *cucurbit* crops are economically important worldwide. *Cucurbits* are consumed in different ways as fruits or vegetables, providing essential nutrients and dietary fiber (Jahan *et al*., 2013).

Cucurbita species is mostly grown in North America, Central America, Mexico and the United Kingdom. However, it is becoming widely available in export stores across the world due to its numerous culinary applications and nutrient dense competition (Khoury *et al*.,

2020). Squash fruit is rich with some essential nutrients (e.g., manganese, potassium, phosphorus, copper, and magnesium). Furthermore, it is rich in protein and bioactive compounds such as antioxidants, flavonoids, vitamins, and medicinal prospects. Squash plants are widely cultivated in all soils, and can be planted in many temperature conditions (e.g., Mediterranean, tropical, and sub-tropical regions) (Youssef *et al*., 2021).

This vegetable is used not only as a food source in soups, stews and stand-alone side dishes, but also as an element in poultices for scratches, salves for rheumatism, and as a number of other traditional medicine applications (Mkhabela *et al*., 2021). It has various health benefits to humans as well as medicinal potentials such as anti‑fungal infection of the skin in the management of HIV/AIDS-related diseases, anxiolytic, antipyretic, anti-diarrhoeal, carminative, antioxidant, antidiabetic, antibacterial, laxative, anthelmintic, antitubercular, purgative and hepatoprotective. It is an important vegetable as its fruit, flowers and leaves are edible, and the peel is where most of the nutrients are hence peeling of the skin is not recommended (Jyoti *et al*., 2019). As cited by Olarewaju *et al*, (2021) Ethiopia is an old country with the ancient use of *Cucurbita pepo* leaves for the treatment of dandruff (a skin condition that mainly affects the scalp) in the Ghimbi district, southwestern Ethiopia. *Coccinia abyssinica* is used to treat gonorrhoea, tuberculosis, and cancer in Wollega, Ethiopia. It is used during traditional ceremonies and celebrations. Oral administration of cultivated seed of *C. pepo* is used for the treatment of gonorrhoea in the Ghimbi district of southwestern Ethiopia (Salehi *et al*., 2019).

2.2. Microbial Inocula Technologies as Biofertilizers in Agricultural System

Growing crops in sustainable systems with reduced chemical inputs has increasing interest. In low-input systems, the main requirements for crops to achieve high and profitable yields are sufficient uptake of nutrients and coping with unpredictable and varying levels of soil nutrients (Okur, 2018). Biological fertilizer (Microbial Inocula) is a substance which contains living microorganisms which when applied to plants either on the surfaces or soil has the ability to colonize the rhizosphere or the interior of the plant (Malusá *et al*., 2012). They promote growth by increasing the supply or availability of primary nutrients to the host plant (Saritha & Tollamadugu, 2019). Biological fertilizer boosts the nutrient composition of soil through the processes of nitrogen fixation and solubilizing mineral ions and thereby stimulates plant growth through the synthesis of growth-promoting substances. The use

of microbial inoculant is expected to reduce the use of chemical fertilizer and synthetic pesticides (Habtamu Deribe, 2020).

The global biofertilizer market is currently growing, and countries such as Argentina, Canada, China, Europe, India and the United States are the drivers. These countries have realized the enormous benefits of biofertilizers and are making substantial efforts to promote its adoption, as evidenced by their well-developed biofertilizer market (Odoh, 2017). Moreover, these countries have strategies in place to support biofertilizer development. Such strategies include industry privatization, product commercialization, tax incentives, input subsidies, as well as market development and financial support. Unfortunately, the potential benefits of biofertilizer have been largely untapped in Africa due to lack of market development, inadequate regulatory framework and ineffective quality control systems (Saritha & Tollamadugu, 2019). These challenges have contributed to the increase in unregulated production and influx of commercial biofertilizers whose real quality is questionable. The poor-quality products supplied to the agro-market has contributed to the low acceptability and adoption of biofertilizer technology amongst farmers in Africa (Raimi *et al.*, 2021).

The groups of microbes mostly used for inoculant production are the AMF, PGPB and nitrogen-fixing rhizobia. The genera *Gigaspora*, *Glomus*, *Bacillus*, *Pseudomonas*, *Azotobacter*, *Azospirillum*, *Bradyrhizobium* and *Rhizobium*, are commonly used for inoculant formulation (Raimi *et al.*, 2021). These microbes play essential roles in soil ecosystem functions such as restoring and preserving soil nutrient richness. Several studies have been conducted on biofertilizers and their various benefits in sustainable agriculture, especially in promoting plant nutrition. Some of these benefits include nitrogen fixation, nutrient solubilization and mobilization, phytohormone production, microbial community diversification, and soil physicochemical property improvement (Nigussie Alemayehu, 2020).

2.3. Arbuscular Mycorrhizal Fungi (AMF)

Most plants in their natural habitats function under the influence of a special group of soil fungi known as arbuscular mycorrhizal fungi (“AM fungi” or AMF). The term “mycorrhiza” was coined by A. B. Frank in 1885, a scientist in Germany. It literally means fungus-root, and describes the mutualistic association existing between a group of soil fungi and higher plants (Ajit, 2008). The association is based on the plant component providing carbohydrates

and other essential organic compounds to the fungi. In return, the fungal component, which colonizes both the root and the adjacent soil, helps the plant take up nutrients and water by extending the reach of its root system. Mycorrhizal fungi are symbionts in roots of the majority of higher plants. These associations vary widely in structure and functions, but the vesicular arbuscular (VA) type endomycorrhizal connection is the most common both geographically and within the plant kingdom (Lies *et al*., 2018; Nanjundappa *et al*., 2019).

Arbuscular mycorrhizas (AM) are formed in More than 80% of all terrestrial plants by obligatory symbiotic fungi which have recently been reclassified on the basis of DNA sequences as a member of subkingdom *Mucoromyceta* and the phylum *Glomeromycota* including three classes (*Glomeromycetes*, *Archaeosporomycetes*, and *Paraglomeromycetes*. The plants include angiosperms, gymnosperms and the sporophytes of pteridophytes, all of which have roots, as well as the gametophytes of some hepatics and pteridophytes which do not (Smith & Read, 2008; Bartha, 1984).

The association between plants and AMF is one of the most important symbioses on earth, linking the root and the soil system, and represents an ancient symbiosis(Smith and Ready, 2008). Hyphae and arbuscules have been reported in fossils of *Aglaophyton* isolated from the Rhynie chert, and this evidence has established the existence of AM symbiosis in the early Devonian (Giovannini *et al*., 2020). Furthermore, molecular works based on the nucleotide sequence divergence of 18s rDNA suggests that the *Glomales* arose 350–460 million years ago and that the symbiosis was instrumental in the successful colonization of land by plants. In early October 2019, the taxonomy of AMF from the total number of described AMF species was 318. (www.amf-phylogeny.com). There are 34 genera and 12 families among these species (Belay *et al*., 2020). Some of the important genera are *Glomus*, *Acaulospora*, *Gigaspora*, *Scutellospora*, *Enterophospora* and *Sclerocystis* (Aggarwal *et al*., 2011).

The symbiosis between AMF and autotrophic plants is regarded as mutualistic. With the exception of a few achlorophyllous species, AM host plants are autotrophic and, although normally colonized by mycorrhizal fungi in the field, they are usually capable of satisfactory growth in the absence of mycorrhizal colonization, provided that mineral nutrient supplies are adequate (Smith and Ready, 2008). In contrast, the *glomeromycotan* fungi are obligate symbionts, as their extraradical hyphae are unable to take up carbohydrates. Thus, they

depend on photosynthates supplied by the plant and utilize a considerable proportion of its assimilated carbon probably 20 % of net photosynthate (Sýkorová, 2014).

In return, the fungi improve the supply of water and nutrients, such as phosphorus (P), and nitrogen (N) to the host plant through extraradical and intraradical hyphae, arbuscules, and the root apoplast interface. And AMF benefit the plants by alleviate biotic and abiotic stress from their host by inhibiting the growth of pathogenic organisms and increasing plant resistance to drought and other unfavorable conditions such as tolerance to drought, high soil temperatures, toxic heavy metals, extremes in pH and transplant shock (Millar & Bennett, 2016). As a result of mycorrhizal colonization, the host plant is increase photosynthetic activity, to compensate for the funguses "lost" carbon (Johansson *et al*., 2004).

The movement of carbon to a fungal partner in an ecosystem performs many important functions. For instance, it is used in intra- and extra-radical mycelium growth and maintenance, thus promoting nutrient uptake in turn. Consequently, a greatly increased growth of extraradical mycelium and production of spores are often detected once the AM fungal root colonization is established (Lies *et al*., 2018). In addition, AMF and their products such as glomalin, directly contribute to soil structural formation. By virtue of their roles in soil aggregate formation and stabilization, they increase C and N storage, implicating them in the reduction of greenhouse gas emissions (Pal, 2014). With these beneficial roles of AMF, it is conceivable that among the numerous soil organisms, AMF play a key role in ecosystem processes (Mardhiah *et al*., 2016).

2.3.1. The Main Structure of AMF

AMF are unique endomycorrhizae (i.e., fungi found within plant roots) distinguished from other mycorrhizas, such as ectomycorrhiza and ericoid by structures such as arbuscules and vesicles. The name ‘arbuscular’ is derived from characteristic structures, named by Gallaud (1905) because they look like trees (Aggarwal *et al*., 2011). It is highly branched structure produced by arbuscular mycorrhizal fungi inside the cell lumen of their host, and are considered the most important site of key element of the symbiotic nutrient exchanges between the fungus and host plan (Lies *et al*., 2018).

Vesicles are thin-walled or thick-walled globose to subglobose, irregular shaped structures (Aggarwal *et al*., 2011). And vesicles do not occur in some genus from the Order *Diversisporales*, such as *Gigaspora* and *Scutellospora* (Souza, 2015). They play an important role in nutrient storage, since their cells contain high levels of lipids and glycogen;

but in some cases they might assume a reproductive function, because vesicles may form spores that act as propagules (Bagyaraj, 2014; Johansson *et al*., 2004).

Also, AMF possess intraradical hyphae located within the host and extraradical hyphae found outside the root, in the soil environment (Souza, 2015). Collectively, the, arbuscules, vesicles, and intraradical hyphae are regarded as the intraradical mycelium, and the collection of extraradical hyphae is known as extraradical mycelium (Ajit, 2008). The mycelium in soil is involved in searching for new plants and in acquisition of mineral nutrients that are used by both fungus and plant. Apart from nutrient uptake, the extraradical mycelium also is involved in spore formation and initiation of root colonization, whereas the intraradical interfaces are concerned with nutrient transfers between the symbionts (Smith and Ready, 2008).

AM colonization patterns within host roots are divided into three types: Arum- , Paris- and intermediate-types (Mohin, 2015). In the Arum -type, the hyphae grow intercellularly in the root cortex and penetrate to form ‘arbuscules’ intracellularly, whereas in Paris - type association, intracellular hyphal coils frequently having intercalary arbuscules spread cell to cell in the cortex. Intermediate-type AM exhibits characteristics of both Arum- and Paris types. Most of the cultivated crops form Arum type, while Paris-type is common in plants of natural ecosystem (Aggarwal *et al*., 2011).

2.3.2. Development of Arbuscular Mycorrhizal (AM) Association

In the absence of host plants, the life cycle of the AMF cannot be completed and unlike certain classes of fungi, they do not reproduce sexually. Their life cycle begins with an asexual spore being germinated and forming hyphae with a limited life span in the soil; and it begins with an asymbiotic process in the response to physical influences, such as humidity, temperature and pH (Giovannini *et al*., 2020).

But the spores respond with an increase in hyphal branching and metabolic activity to root exudates. This constitutes the Pre-symbiotic phase and both AMF species and host plants start to contact and exchange chemical and molecular signals (Lies *et al*., 2018). Its success is very dependent on two conditions: soil properties (e.g., pH, moisture, and temperature); and the host plant (e.g., root exudates, like flavonoids, CO_2, and unknown ramification factors) (Souza, 2015). Plant roots release for example strigolactones, which results in the branching of the hyphae. In response, the branched hyphae secrete a diffusible signal to the host roots, which initiates the expression of symbiotic-related genes (Ajit, 2008).

Communication between the plant and the fungus is followed by the hypopodium's adhesion to the surface of the root. This stimulates the assembly in the contact epidermal cell and the underlying cortical cell of a large aggregation of cytoplasm called the prepenetration apparatus (PPA)(Figure 2.1) (Lies *et al*., 2018). After to the establishment of physical contact with the root surface, extraradical hyphae cells differentiate in appressorium cells, and the symbiotic phase starts. Appressorium are specialized structures that play an extremely important role in the penetration of root epidermis by the intraradical hyphae. Once inside the root, AMF are able to develop both extra- and intraradical hyphae (Souza, 2015). Hyphopodium is specialized hypha of an arbuscular mycorrhizal fungus, often branched and swollen, which adheres to the root epidermis before intracellular fungal penetration (Figure 2.1).

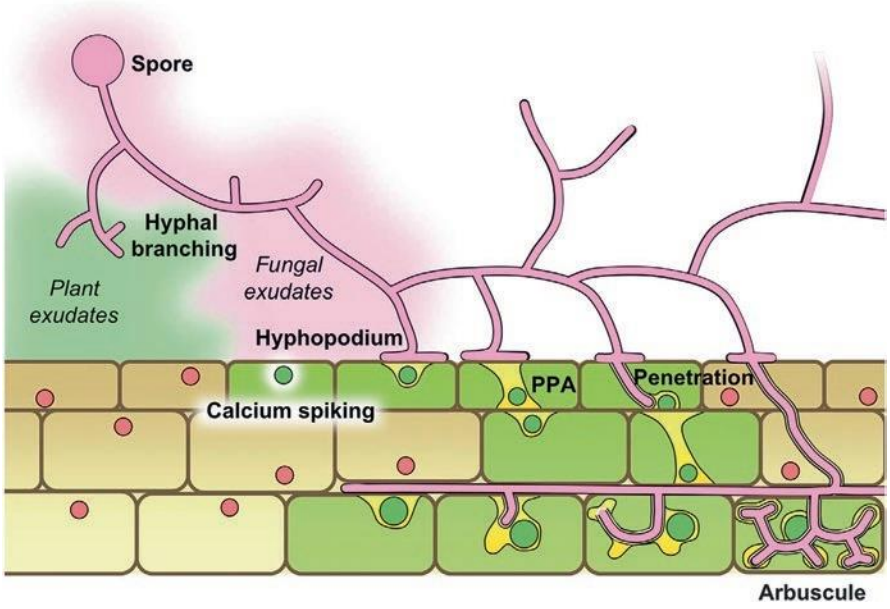

Figure 2. 1 Development of Arbuscular Mycorrhizal (AM) Association under soil-plant system (Lies *et al*., 2018).

2.3.3. Role of Arbuscular Mycorrhizal Fungi on Sustainable Crop Production

Climate change and food security have now become major problems mainly in developing countries. Therefore, there is need to fulfill demand of growing population of developing countries by increasing food production through high-input agricultural practices and at the same time by minimizing negative environmental impact (Gupta & Tuohy, 2019). Different research studies conducted on AMF during the past two decades have highlighted their countless benefits on soil health and crop productivity. And it is widely believed that AMF could be considered as a replacement or lower down the use of chemical fertilizers up to

50% for best agricultural production in the near future, because mycorrhizal application can effectively reduce the quantitative use of chemical fertilizer input especially of phosphorus (Begum *et al*., 2019).

Arbuscular mycorrhizal fungi, often referred to as agroecosystem engineers, represent a key functional group of soil microbiota that are fundamental for soil fertility, crop productivity, yield quality and ecosystem resilience. They form a critical symbiotic relationship with most agricultural crops improving the nutritional status of their hosts, besides protecting them against several soil-borne plant pathogens and environmental stresses. In addition, they improve soil structure and quality mainly through the external hyphal network which creates a skeletal structure that enmeshes the soil particles, and by production of glomalin related soil protein (GRSP) which binds soil particles together (Njeru, 2018).

2.3.3.1. Increase Soil Fertility (Nutrient Uptake)

Many thousands of experiments have shown that AMF can overcome nutrient limitation to plant growth by enhancing nutrient acquisition (Aggarwal *et al*., 2011). Results of experiments suggest that AMF absorb N, P, K, Ca, S, Cu, and Zn from the soil and translocate them to associated plants(Smith & Ready, 2008). However, the most prominent and consistent nutritional effect of AM fungi is in the improved uptake of immobile nutrients, particularly P, Cu, and Zn (Nogueira *et al*., 2006).

The supply of immobile nutrients to roots is largely determined by the rate of diffusion. In soils not adequately supplied with nutrients, uptake of nutrients by plants far exceeds the rate at which the nutrients diffuse into the root zone, resulting in a zone around the roots depleted of the nutrients (Table 2.3). Mycorrhizal fungi help overcome this problem by extending their external hyphae to areas of soil beyond the depletion zone, thereby exploring a greater volume of the soil than is accessible to the unaided root (Habte, 2000). As the fungal mycelium grows through soil, it scavenges for mineral nutrients and is able to make contact with uninfected roots, sometimes of different host species. The small extra-radical mycelium compared to roots which allows penetration into some crystalline minerals, aggregates and organic matter with smaller pores than could be exploited by root alone (Aggarwal *et al*., 2011).

Moreover, phosphorus acquisition is a major contribution of AMF to plant growth and development (Smith & Ready, 2008). Phosphorus is an essential nutrient, but may be limiting; a considerable fraction of soil P is in organic forms, and a percentage of the

inorganic P is insoluble. Both the organic P and the insoluble inorganic P are unavailable for plant uptake. Only a few plants on their own are capable of producing organic acids and phosphatases to hydrolyze and release P (Hakim *et al*., 2021).

The various mechanisms proposed to account for enhanced P uptake include, increased exploration of soil; increased translocation of phosphorus into plants through arbuscules; modification of root environment; efficient utilization of P within plants; efficient transfer of P to plant roots; and increased storage of absorbed P (Aggarwal *et al*., 2011). AM fungi may have biochemical capabilities for increasing the supply of available P and other immobile nutrients. AMF phosphatases are able to mineralize organic P sources (Recorbet & Courty, 2019). Alkaline phosphatase activity is related to phosphate metabolism of fungus as it is present within the fungal vacuoles where polyphosphate granules were observed. The polyphosphate granules in fine branches of arbuscules are broken down by enzymatic activities releasing inorganic phosphorus in the cytoplasm (Khade & Rodrigues, 2009).

Although AMF influence on plant N nutrition is not as high as in the case of P, they give their host access to different forms of N, thereby increasing plant N uptake (Khade & Rodrigues, 2009). Hodge *et al*. (2001) demonstrated the ability of an AMF to decompose organic matter and acquire N from the organic source. They also showed that AMF increased the diffusion rate of N to its host. Hence, mycorrhizal plants have additional access to N sources compared to non-mycorrhizal plants. The most exciting part of their findings is that AMF could be saprophytic, especially when decomposition is required for nutrient uptake (Mohin, 2015).

While exploring N transfer by AMF, Govindarajulu *et al*. (2005) showed that AMF transfer a substantial amount of N from the soil to the root system. Using a proposed model, the authors explained that extraradical hyphae of AMF take up inorganic N, and transfer it to intraradical hyphae as amino acids (mainly arginine). The intraradical hyphae decompose the amino acids to access the C, and then transfer the remaining N as ammonium to the host plant. These authors are among several that have demonstrated the remarkable contributions of AMF to N uptake by plants (Adeleke, 2010).

Nitrogen and P are not the only nutrients transferred by AMF; the ability of AMF to supply micronutrients to the host plant has been documented (Smith & Ready, 2008). These elements are copper, zinc, magnesium, manganese and cobalt. AM fungi help the plant in two mechanisms: firstly, they help in the uptake of these elements which are considered to

be relatively immobile, and secondly, they take up these elements and store them so as to prevent their concentrations to reach toxic levels. AM fungi could act as a sink for copper, cobalt and zinc (Lies *et al*., 2018).

2.3.3.2. Increase Resistance to Phytopathogen

There is also sufficient evidence that AMF protect plants against phytopathogens by reducing the numbers and activities of pathogens (Diagne *et al*., 2020); competing with pathogens for colonization sites, nutrients, and photosynthates; and by improving plant resistance to disease (Menge, 1985). The major ways AMF perform the latter role is by inducing the release of pathogenesis-related (PR) proteins by the host plant, and increasing nutrient acquisition of the affected plant to compensate for the damage caused by the pathogen.

However, biocontrol of AMF is dependent on the degree of mycorrhization (Slezack et al., 1999). At a high percent colonization, Slezack *et al*.(1999) found that *G. mosseae* significantly decreased the disease index of Aphanomyces euteiches, regardless of the density of the pathogenic inoculum, whereas when AMF colonization was low, the AMF exhibited no protection against the pathogen. Despite the numerous reports of AMF on plants, studies have shown that the known beneficial effects of mycorrhizal symbioses on plant productivity are a mere estimate and other functional roles are yet to be determined (Diagne *et al*., 2020).

2.3.3.3. Increase Tolerance to Drought Stress

Drought stress affects plant life in many ways; for example, shortage of water to roots reduces rate of transpiration as well as induces oxidative stress. Drought stress imparts deleterious effects on plant growth by affecting enzyme activity, ion uptake, and nutrient assimilation. The AMF association improves the hydraulic conductivity of the roots and improves water uptake by the plants or otherwise alters the plant physiology to reduce the stress response to soil drought (Begum *et al*., 2019).

Mycorrhizal plants show better survival than nonmycorrhizal plants in extreme dry conditions. It reveals that mycelial network extends deeper and wider in the soil in search of water and nutrients. The permeability of cell membrane to water may also be altered by mycorrhizal colonization though the improved phosphorus nutrition and colonization by AMF can improve the drought resistance of plants (Lies *et al*., 2018).

Under conditions of drought stress, AMF exert their influence by increasing the transpiration rate and lowering stomatal resistance or by altering the balance of plant hormones. The change in leaf elasticity due to AMF inoculation improves water and turgor potential of leaf and also increase root length and depth and may also influence water relations and therefore, the drought resistance of the plants (Aggarwal *et al*., 2011).

2.3.3.4. Tolerance to Heavy Metals

AMF are widely believed to support plant establishment in soils contaminated with heavy metals, because of their potential to strengthen defense system of the AMF mediated plants to promote growth and development. Heavy metals may accumulate in food crops, fruits, vegetables, and soils, causing various health hazards. And there are many reports in the literature on uncovering the AMF-induced effects on the buildup of metals in plants (Begum *et al*., 2019). Heavy metal remediation by AMF can happen through hyphal "metal binding", which reduces the bioavailability of elements, such as Cu, Pb, Co, Cd, and Zn (Smith and Ready, 2008).

As cited by Diagne *et al*. (2020) inoculation with AMF showed the best results in terms of percentage of seed germination, the sustainability of seedlings, fresh weight, and dry weight of plants. In two different heavy metal-polluted soils, root colonization of maize plants with Glomus isolates reduced heavy metal concentrations in shoots and roots, and increased the contents of essential elements like K, P, and Mg in roots. When maize was grown in soil contaminated with Cd, AMF inoculation significantly increased growth and reduced Cd uptake, suggesting that AMF can be used in association with plants for the mitigation of heavy metal such as Cd in soils (Kaldorf *et al*., 1999).

Heavy metals can be immobilized in the fungal hyphae of internal and external origin that have the ability to fix heavy metals in the cell wall and store them in the vacuole or may chelate with some other substances in the cytoplasm and hence reduce metal toxicity in the plants. The strong effects of AMF on plant development and growth under severe stressful conditions are most often due to the ability of these fungi in increasing morphological and physiological processes that increase plant biomass and consequently uptake of important immovable nutrients like Cu, Zn, and P and thus reduced metal toxicity in the host plants (Begum *et al*., 2019).

2.3.3.5. Increase Tolerance to Salinity

It is widely known that the soil salinization is an increasing environmental problem posing a severe threat to global food security. Salinity stress is known to suppress growth of plants by affecting the vegetative development and net assimilation rate resulting in reduced yield productivity (Begum *et al*., 2019). AMF have been known to occur naturally in saline environments. Their contribution to the improvement of the growth of several plant species under saline conditions is well known. This is mainly related to a combination of biochemical, physiological, and nutritional effects. Among the mechanisms involved in salinity tolerance in AMF inoculated plants, we have the enhancement of water absorption capacity and nutrient uptake, the accumulation of osmoregulatory like proline and sugars, the ionic homeostasis, and the reduction in Na+ and Cl− uptake (Aggarwal *et al*., 2011).

In addition, it has been demonstrated that AMF colonization improves stomatal conductance and reduces the oxidative damage in plants exposed to salinity. For example, inoculation with *F. mosseae* of tomato plants irrigated with saline water significantly increased plant biomass, fruit fresh yield, and shoot contents of P, K, Cu, Fe, and Zn (Diagne *et al*., 2020). Santander *et al*. (2019) have shown with lettuce that the mycorrhizal plants had higher biomass production, increased synthesis of proline, increased N uptake, and noticeable changes in ionic relations, particularly reduced accumulation of Na+, than those in non-mycorrhizal plants under stress conditions.

2.3.4. Methods in Arbuscular Mycorrhizal Fungi on Inoculum Production

Mukhongo *et al*. (2016) reported that there are about 12 mycorrhizal inocula producers in the European Union, with the producers in the United Kingdom, Czech Republic, Germany, Switzerland, Spain, and France and more than 20 others worldwide. The un-culturability and obligate biotrophy of arbuscular mycorrhizal (AM) fungi have not allowed the development of large-scale inoculation programs (Saritha & Tollamadugu, 2019). The only feasible means for production of infective propagules is growing the inoculum in symbiosis with living host plants or in root organ cultures (Varma & Kharkwal 2009). Although such production systems provide an advantage by allowing a continuous monitoring of the infective capability of the inoculum, their major drawbacks include extensive production costs, slow turnover time, and difficulty excluding secondary root colonizers such as root pathogens (Ajit, 2008).

Large-scale multiplication of AMF aiming to produce mycorrhizal inoculant for field applications is generally carried out in substrate-based (nursery beds, pots, concrete tanks) (Habte & Osorio, 2001), substrate-free (i.e., hydroponics and aeroponics boxes) (Saritha & Tollamadugu, 2019), and in vitro systems (Karti *et al*., 2021). Commercial inocula produced using these systems are available in several countries, especially in Asia and Europe (Mukhongo *et al*., 2016).

AMF inoculum can be applied as spores, fragments of roots colonized by AMF, or a combination of the two and incorporated soil mycelium. AMF spores and hyphae can be isolated from the soil substrate and mixed with carrier substrate (Smith and Ready, 2008). Commonly used carriers include pumice or clay, sand, perlite, vermiculite, soilrite, and soil or glass pellets. AMF taxa and strains may vary in their ability to colonize host plants depending on the source of inoculum (Habte, 2000). Spores may be the most reliable source of inoculum across various AM taxa, whereas fragments of colonized roots are effective for some taxa but not others. The entire substrate can also be used and homogenized into a crude soil carrier that includes plant roots and fungal spores as well as the soil mycelium (Rai, 2006).

2.3.4.1. Trap Culturing

Field-collected AM spores are found to be low in numbers, parasitized with microbes and fungi, lacking suitable information for taxonomic characters, and hindering a more accurate identification. Trapping is necessary to obtain healthy AM spores for identification as well for using as inoculum to establish monospecific culture (Gupta & Tuohy, 2019). Spores collected directly from a field soil suffer from many problems: they appear healthy but are not viable (some persisting as dead husks for years or possibly decades); they lose or change appearance of their structural characters in response to root pigments, soil chemistry, temperature, moisture, and microbial activity; and they represent only those colonizing arbuscular fungi with enough activity and biomass to trigger sporulation. Trap cultures yield very different results, depending on all of the biotic and abiotic factors that go into growth, reproduction, and the partitioning of colonization and sporulation (INVAM, 2020).

2.4. Plant Growth-Promoting Rhizobacteria (PGPR)

Plant Growth-Promoting Rhizobacteria (PGPR) is a heterogeneous group of bacteria that can be found in the rhizosphere, at root surface and in association with roots, which can improve plant growth. The word PGPR was proposed by Kloepper *et al*. (1980), And, was

coined for fluorescent *Pseudomonas*, a plant growth enhancer that fought against pathogens (Parewa *et al*., 2014). Since then, the term has metamorphosed and extended to include all rhizobacteria capable of directly enhancing plants growth. Recently, it was used to include wide range of rhizobacteria that improve plant growth through different mechanisms (Odoh, 2017). Plant growth-promoting rhizobacteria can be classified into extracellular plant growth-promoting rhizobacteria (ePGPR) and intracellular plant growth-promoting rhizobacteria (iPGPR) or endophytes. ePGPR may exist in the rhizosphere, on the rhizoplane, or in the spaces between the cells of root cortex, while iPGPR locate generally inside the specialized nodular structures of root cells (I. Singh, 2018b).

The rhizobacteria genera included under ePGPR are: *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcus, Pseudomonas,* and *Serratia* belong to ePGPR. whereas the rhizobacteria genera under iPGPR belongs to the family of *Rhizobiaceae* which includes *Allorhizobium, Bradyrhizobium, Mesorhizobium and Rhizobium,* and *Frankia* species, both of which can symbiotically fix atmospheric nitrogen with the higher plants (Lies *et al*., 2018; Nagargade *et al*., 2018).

2.4.1. Role of PGPR on Enhancing of Plant Growth

PGPR enhance plant growth and health by the beneficial mechanisms which are direct or indirect (Figueiredo *et al*., 2016). Any mechanism that protects the plant against infections (biological stress) or helps the plant grow healthy under abiotic stress is considered as indirect mechanics, whereas any mechanism that directly increases plant growth through the provision of nutrients or the production of growth regulators is considered as a direct mechanism. This section focuses on plant growth promotion by PGPR directly (Odoh, 2017).

2.4.1.1. Direct Mechanisms of Action

2.4.1.1.1. Increasing Nutrient Availability to Plants

PGPR directly affect plant metabolism by increasing nutrient supply of plants or providing nutrients that are usually scarce in the rhizosphere, such as nitrogen (N) by fixing atmospheric nitrogen (Figueiredo *et al*., 2016). The capture and subsequent release of nitrogen to plants is carried out by bacteria present in the rhizo- and endosphere through a diverse set of processes. PGPR may convert nitrogen trapped in the molecular or atmospheric form N_2 into biologically useful forms in a process known as biological nitrogen fixation (BNF) (Odoh, 2017).

The biological fixation of atmospheric nitrogen is an important microbial activity for the maintenance of life on earth through photosynthesis performed by photosynthetic organisms (Singh, 2018b). This process take place when atmospheric nitrogen is converted to ammonia by an enzyme called nitrogenase; a highly complex oxygen labile enzyme conserved in free-living symbiotic diazotrophs. The process is coupled with the hydrolysis of sixteen equivalents of ATP and is accompanied by the co-formation of one molecule of H_2 (Parewa *et al*., 2014).

Only diazotrophic bacteria execute BNF, as the nitrogenase enzyme is present only in these organisms. Members of the genera *Anabaena*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Clostridium*, *Klebsiella*, *Nostoc*, *Paenibacillus*, and *Rhodobacter* are examples of free-living diazotrophic bacteria that provide available N to several plants (Figueiredo *et al*., 2016). The role of bacteria in biological N_2 fixation has been reported as early as 1800s, and there are reports that certain PGPR promote growth, mainly because of their role in N_2 fixation. For example, *Bacillus polymyxa*, found in wheat rhizosphere, was regarded as a PGPR due to its ability to fix N_2 as sited by Adeleke, (2010). Furthermore, some bacteria (e.g., ammonifiers and nitrifiers) convert organic N compounds into inorganic forms (i.e., NH_4^+ and NO_3^-) that are available for root uptake (Etesami & Adl, 2020).

After N, the essential mineral element that most frequently limits the growth of plants is phosphorus (P), which is taken up only in monobasic ($H_2PO_4^-$) or dibasic (HPO_4^{2-}) soluble forms. P is found mainly in inorganic fractions, which are either adsorbed into the soil's inorganic surfaces or found as sparingly available precipitates, and in organic forms that are either adsorbed, incorporated within biomass, or associated with soil organic matter (Odoh, 2017). Even in soils with abundant P ranging from 400 to 1200 mg kg^{-1} of soil, usually only about 1% of the soil P is actually in a readily available, soluble form, and over 90% is generally bound tightly to soil particles and inorganic minerals such as apatite, hydroxyapatite, and oxyapatite or appear as one of several organic forms including inositol phosphate (soil phytate), phosphomonoesters, and phosphotriesters, which require mineralization before they become plant available (Etesami & Adl, 2020; Nanjundappa *et al*., 2019).

PGPRs have been identified to play an important role in mediating phosphorus available to plants through their participation in the soil phosphorus cycle. PGPRs either directly solubilize and mineralize inorganic phosphorus or facilitate the mobility of the organic form

through biogeochemical cycle for more efficient root uptake (Nagargade et al., 2018). Specifically, Phosphate Solubilizing Bacteria (PSB) is *Arthrobacter*, *Pseudomonas*, *Alcaligenes*, *Bacillus*, *Burkholderia*, *Serratia*, *Enterobacter*, *Acinetobacte*r, *Azospirillum*, *Azotobacter*, *Flavobacterium*, *Rhizobium*, and *Erwinia*. Explicitly, each genus act independently to facilitate the dissolution and uptake of phosphate via in vitro condition or other mechanisms (Odoh, 2017).

The PGPR secrete different types of organic acids e.g., carboxylic acid, formic acid, propionic acid, lactic acid, glycolytic acid, fumaric and succinic acid. Kaur *et al*., (2016) in their discovery established that these organic acids lower the pH in the rhizosphere, thus causing release of the bound forms of phosphate like Ca_3 $(PO_4)_2$ in the calcareous soils (Figueiredo *et al*., 2016). Apart from creating the availability of accumulated phosphate, phosphorus biofertilization also help in increasing the efficiency of biological nitrogen fixation and render availability of Fe, Zn, etc., through production of plant growth promoting substances. PSB are also able to mineralize the insoluble organic phosphate through the excretion of extracellular enzymes such as phytases and C-P lyases phosphatases (Odoh, 2017).

Potassium (K), iron (Fe), calcium (Ca), and magnesium (Mg), are among other essential nutrients supplied by PGPR to their host. Some PGPR enhance nutrient availability via production of siderophore (Parewa *et al*.,2014). For example, iron though abundant in soil, cannot be assimilated directly by plants, certain organisms, such as the fluorescent *pseudomonads*, produce an iron chelator (siderophore) which increases Fe availability for plant uptake. Siderophores are yellow-green fluorescent pigments with high affinity for Iron (III) ($Fe^{3+)}$ and are capable of reducing this Fe form to Iron (II) Fe^{2+} which can be absorbed by plant cells. This mechanism is especially important under Fe limiting conditions (Adeleke, 2010).

2.4.1.1.2. Production of Phytohormones

In addition to improved plant nutrition, the biosynthesis of phytohormones is also considered to directly stimulate plant growth. In this context, there are nutrients in the soil and their solubility is also high, but plants do not have any access to them (Parewa *et al*., 2014). Therefore, PGPR enhances the access of plants to nutrients and increases the uptake of them by increasing the root growth of plants by different mechanisms (Etesami & Adl, 2020). Auxin, gibberellin, cytokinin, ethylene, and abscisic acid are examples of phytohormones

produced and released by numerous members of the genera *Alcaligenes, Azospirillum, Azotobacter, Bacillus, Bradyrhizobium, Brevibacillus, Burkholderia, Enterobacter, Klebsiella, Mycobacterium, Pseudomonas, Rhizobium*, and *Serratia* (Figueiredo *et al*., 2016).

Among plant growth regulators, Indole acetic acid (IAA) is the most common natural auxin found in plants and its positive effect on root growth (Maharana, 2019). Rhizobacterial strains synthesize ~80% of IAA colonized the seed or root surfaces is proposed to act in conjunction with endogenous IAA in plant to stimulate cell proliferation and enhance the host's uptake of minerals and nutrients from the soil (Adeleke, 2010). The IAA affects plant cell division, extension, and differentiation; stimulates seed and tuber germination; increases the rate of xylem and root development; controls processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light, gravity, and florescence; and affects photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stressful conditions (Etesami & Adl, 2020).

Tryptophan is an amino acid commonly found in root exudates and has been identified as main precursor molecule for biosynthesis of IAA in bacteria (Rehman *et al*., 2020). The biosynthesis of indoleacetic acid by plant growth-promoting rhizobacteria involves formation via indole-3-pyruvic acid and indole-3-acetic aldehyde, which is the most common mechanism in bacteria like *Pseudomonas*, *Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, *Enterobacter*, and *Klebsiella*. Furthermore, beneficial effects of IAA-producing PGPR on root growth increases colonization sites for other beneficial microorganisms, such as AMF and symbiotic N_2 fixers (Etesami & Adl, 2020)

Abscisic acid (ABA) is a plant growth hormone that is produced by plants in response to abiotic stresses such as drought, salt stress, cold, or soil pollution, among others. It stimulates the expression of stress-resistance genes. Abscisic acid is produced by several strains of PGPR. The internal content of ABA is raised when plants are inoculated with Abscisic acid-producing bacteria such as *Bacillus licheniformis Rt4M10*, *Azospirillum Brasiliense* Species, and *Pseudomonas fluorescens Rt6M10*. As a result, the plant becomes more drought resistant (Rehman *et al*., 2020).

Moreover, certain PGPR generate enzymes that reduce phytohormone levels, allowing for the maintenance of appropriate Phytostimulator concentrations for plant growth (Martínez-Viveros *et al*., 2010). Some PGPR produce the enzyme 1- aminocyclopropane-1-carboxylate

(ACC) deaminase, which promotes plant growth by inhibiting ethylene synthesis (Singh, 2018a). Ethylene inhibits seedling emergence and root elongation at high concentrations, resulting in reduced plant growth. Hormone production by PGPR is a crucial part of their plant growth-promoting abilities; however, additional research is needed to fully comprehend the growth regulators' functional responsibilities (Adeleke, 2010).

2.4.1.2. Indirect Mechanisms of Action

The indirect mechanism involves the ability of PGPR to influence plant health by suppressing phytopathogens and other deleterious microorganisms through different mechanisms (Singh, 2018). The important mechanism used by PGPR in the control of pathogens is the parasitism of the phytopathogens, mostly fungi, and antagonism related to producing antibiotics and antifungals (phenazine-1-carboxylic acid, pyocyanine, pyrrolnitrin), and lytic enzymes (chitinases, glucanases, and proteases) (Figueiredo *et al*., 2016). These compounds lyse the cells of pathogenic fungi and degrade toxic compounds synthesized by the detrimental organisms (Rehman *et al*., 2020). Other important mechanisms used by PGPR to control phytopathogens include inducing systemic resistance in plants against the pathogens, the production of siderophores, such as *pseudobactin*, reducing the numbers and activities of pathogenic bacteria and fungi by depriving them of Fe3+, and competition for niche and nutrients with phytopathogens and other harmful microorganisms in the rhizosphere (Basu *et al*., 2021).

Pseudomonas, *Enterobacter*, *Bacillus*, and *Rhodococcus* genera are known for the production of siderophore. The most researched PGPR for the production of siderophore has been *P. fluorescens* and *Pseudomonas aeruginosa*, which produce pyroverdine and pyochelin kinds of siderophores (Singh, 2018a). They have been known for their influential role in improving iron uptake, inhibiting the growth of pathogens through antibiotic production, and inhibiting fungal growth in the locality (Martínez-Viveros *et al*., 2010).

2.4.2. PGPR Inoculant Biomass Production

Biomass production, formulation, shelf life determination, determination of a suitable carrier, and the choice of an appropriate culture medium for the development of high amounts of microbial biomass are crucial steps during the development of bacterial inoculants , which should ensure the application of the required number of viable and active microbial cells (Lobo *et al*., 2019). The cost of biological fertilizers, which comprises mainly

raw material costs, equipment, and staff, must be competitive in relation to the production cost of chemical fertilizers (Singh *et al*., 2016).

In several studies, different culture media have been assayed or optimized for the growth of PGPR in submerged and solid-state fermentation processes. Standard culture media such as ammonium mineral salt (AMS) broth, nitrogen-free broth (NFb) and nutrient broth are suitable for laboratory assays, but they are expensive for PGPR biomass production at larger scales. Several new culture media were optimized based only on conventional ingredients (Malusá *et al*., 2012). However, the inclusion of some components such as peptones, yeast extract and tryptone continues to generate high inoculant production prices. In this sense, argued that the decrease in nutrient concentrations or incubation time needed to reach maximum PGPR biomass can represent a reduction in production costs (Lobo *et al*., 2019).

On the other hand, traditional culture medium components have been successfully combined with low-cost substrates for biomass production during inoculant development. Vyas *et al*. (2014) and Posada-Uribe *et al*. (2015) were used industrial wastes or by-products such as crude glycerol, corn flour, soybean meal, dairy sludge and maize bran residue as low-cost carbon or nitrogen sources for microbial growth. Interestingly, glycerol and maize bran residue can be employed in both biomass production and formulation processes (glycerol as a protective agent of microbial cells and maize bran residue as a carrier). The use of culture media formulated with industrial wastes or by-products to produce bacteria of agronomic interest is a viable way to contribute to a sustainable agro industry, handling low-cost substrates of high nutritional value (Vyas *et al*., 2014).

Product quality is major cause of concern to both producers and users of biocontrol inoculants and it is essential that producers have an adequate quality control system. In the case of PGPR inoculants, product cfu/g can be determined by plate counts following serial dilutions, and purity may be determined by examination of colony morphology (Uribe *et al*., 2015). However, confirming the identity of the microorganisms in a formulation and inoculant persistence in the growth substrate may be more problematic. This can be done using molecular methods but at considerable cost. Emerging techniques such as MALDI-TOF mass spectrometry of intact bacteria may offer rapid identification with limited preparation and consumable costs (Varma, A., & Kharkwal, 2009).

In addition to that, a suitable carrier is required for field application of biofertilizer because of the short shelf life of the bioinoculant agent. Thus, the unavailability of an appropriate

carrier proves to be one of the major constraints for its large-scale use in fields. Ideal carriers used in biofertilizer production are peat, charcoal, lignite, etc. These carriers again pose technical constraints because most of them are unavailable in developing countries (Kumar Arora, 2014).

2.5. Synergistic Interaction of AMF and PGPR

Alteration in the composition of root exudates is the most important physiological change that occurs during AM root colonization of plants. This chemical alteration in the rhizosphere of mycorrhizal plants is responsible for the bacterial community change, resulting in the mycorrhizosphere effect. Bacteria adhering to the AM mycelium get benefits by feeding on hyphal exudates and/or using the mycelium as a vehicle for colonization of the rhizosphere (Ramasamy *et al*., 2011).

PGPR are reported to play a significant role in the establishment of AM symbiosis with host plant growth. Bacteria may be found adhering to the AM hyphae (Bianciotto *et al*., 1996a)as well as embedded within the AM spore walls (Figure 2. 2). PGPR produces biologically active compounds (e.g. amino acids, plant hormones, vitamins, other organic compounds, and volatile substances) that increase root cell permeability, thereby increasing the rates of root exudation and stimulating the growth of arbuscular mycorrhizal fungi (AMF) hyphae in the rhizosphere (Parewa *et al*., 2014).

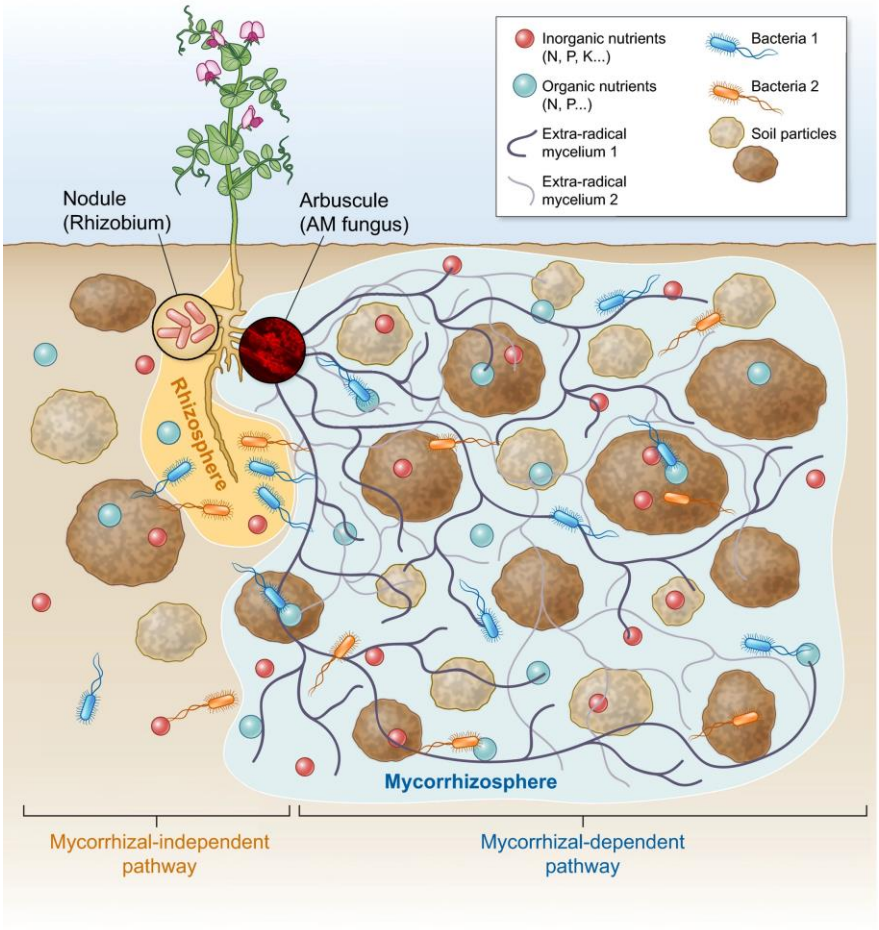

Figure 2. 2 Schematic view of PGPR and AMF association, soil nutrients, bacteria and mycorrhizal vs nonmycorrhizal pathways (Recorbet & Courty, 2019).

The extraradical mycelium 1 and 2 correspond either to two different arbuscular mycorrhizal fungal (AMF) species or two isolates from the same species. The extraradical mycelium is an extension of the root system, foraging soil that is not accessible to the root system. Some bacteria interact with the extraradical mycelium to mobilize nutrients. Leguminous plants have two different root symbioses: arbuscular mycorrhizal (AM) symbiosis and rhizobia.

Paenibacillus, known for its antagonistic activity over a broad range of root pathogens, is capable of stimulating mycorrhizal colonization in sorghum (Budi *et al*., 1999). Furthermore, Budi *et al*. (1999) reported an increase in total bacterial population, including nitrogen fixers and beneficial gram-negative bacteria in the rhizosphere of mycorrhizal plants. PGPR are known to enhance AM fungal growth, by supporting the spore germination and mycelia extension of AMF (Ramasamy *et al*., 2011). Hildebrandt *et al*. (2002) reported that *Paenibacillus validus* under in vitro conditions supported the mycelial growth of *G.*

intraradices in the absence of a host root, through the production of sugars such as raffinose. Nutrient exchange occurs between PGPR and AM fungi by close contact (Artursson *et al*., 2006), and these bacteria are considered to be mycorrhiza helper bacteria (MHB). MHB stimulates AM propagule germination, hyphal growth and root colonization (Ramasamy *et al*., 2011). Further studies by Hildebrandt *et al*. (2002) showed an increased auxin level in mycorrhizal plants suggested that these hormones could be signals for the AM colonization process.

Bianciotto *et al.* (2001) reported the role of surface components in the physical interactions between beneficial rhizosphere bacteria and the AMF. Bacterial extracellular polymeric substance (EPS) plays a significant role in the attachment of PGPRs to AM fungal structures and roots. Mutants of *A. brasilense* and *R. leguminosarum* strains impaired in EPS failed to colonize AM fungal structures and spores when compared with *Pseudomonas fluorescens* strains with an increased production of EPS. In vitro assay confirmed the attachment of EPS bacteria to transformed mycorrhizal carrot roots and AM hyphae. EPS also play a general role in the protection of bacteria against desiccation. These studies demonstrate that EPS is involved in the attachment of bacteria to both the surfaces (roots and fungal hyphae) and biofilm formation to enhance their survivability under limiting environments (Ramasamy *et al*., 2011).

The PGPR activity of some of these strains has been known, resulting in a broad knowledge of the mechanisms involved. There are a number of metabolites that are released by these strains, which strongly affect the environment by increasing nutrient availability of the plants. Multiple species of *Bacillus* and *Paenibacillus* are known to promote plant growth (Desai *et al*., 2016). Moreover, among the PGPR, *Bacillus* is one of the important genera that exists in soil or as an endophyte and being a spore former with better saprophytic ability and competitiveness, it can survive in soil for long period of time under harsh environmental conditions. *Bacillus* spp. assist plants in its defense against pathogen attack and also enhance stress tolerance by inducing the expression of stress-response genes, phytohormones and stress related metabolites (Nanjundappa *et al*., 2019).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Study Area Description

The study was performed in selected areas of central Rift Valley and highland, regions of Ethiopia. The Central Rift Valley region is involving Batu and Bishoftu woody grassland where naturally different acacia tree species are dominating and the high land region is Sululta, the tertiary of Addis Ababa. Bishoftu and Batu are located in the East Showa zone, Oromia Regional State at the altitude of 1600 to 1960 m a.s.l. The Zone extends between 7 0 33’ N – 9 0 08’ N and from 38^0 24’ E – 40^0 05’ E, with a total area coverage of approximately 8,370.90 square kilometers. Since a large part of the Zone is situated along with the Rift Valley system, rainfall ranges from 600 mm to 1000 mm with an average annual rainfall of 816 mm, with the temperature range from 10ºC in the uplands to over 30ºC in the depressions of the Rift Valley with a mean temperature of 20ºC (CSA, 2020).

Sululta is located in the central part of Ethiopia, Oromia Special Zone surrounding Addis Ababa. It is found on the road to Fiche (north Showa Zone, Oromia) 23 km from Addis Ababa to north. Geographically, the study area extends from $9^0$30'00"N to $9^0$12'15"N latitude and $38^0$42'0"E to $38^0$46'45" E longitude. The administrative area of the town is about 4471 hectares. Sululta has the similar climatological characteristics to Addis Ababa. Globally it is a part of a tropical humid climatic region, which is characterized by warm temperature and high rainfall (The high annual rain fall is 1447 mm with mean of 1140 mm and minimum of 834 mm). The soils of the area are derived from Mesozoic sedimentary and volcanic rocks. The major soil types of the Sululta area are Chromic Luvisols (Eshetu Gelan, 2021).

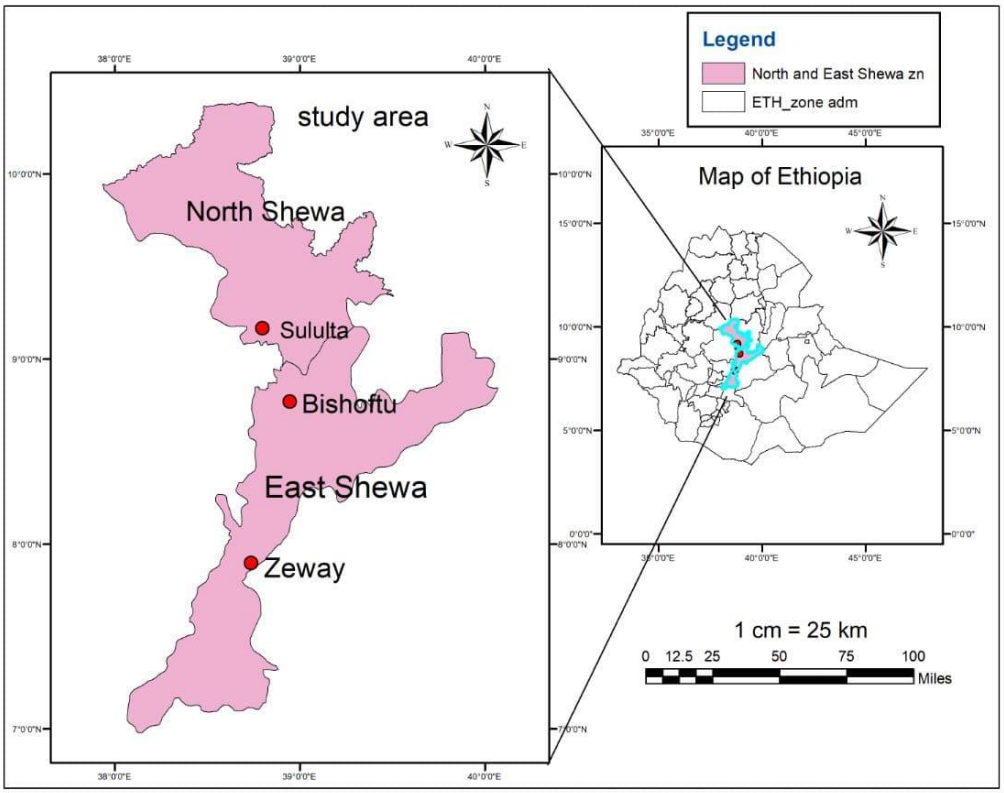

Figure 3. 1 Map of study area, Oromia special zone and East Showa in Central Rift Valley of Oromia Regional State, Ethiopia.

3.2. Soil Sampling

Rhizosphere soil samples from acacia trees were collected during January 2021 from three locations. Four species of acacia tree including *A. seyal*, *A. tortilis*, and *A. saligna* each from the lowland area of Batu and Bishoftu, respectively and *A. abyssinica* from the highland area (Sululta town) were selected for sampling. As result, seven samples (six from lowland and one from highland) were collected. The acacia tree species were characterized by relatively high AMF colonization and very high AMF abundance and diversity according the report of Zerihun Belay *et al.* (2013). Within each of these areas (approximately 100 m^2), three replicates of each acacia tree species were randomly selected and 3kg of rhizosphere soil were taken into a depth of 30 cm and that were pooled into a composite sample (9kg from each sample location for each species). The samples were collected in alcohol sterilized plastic bags and stored at room temperature until further analysis is conducted.

3.3 Analysis of Soil Physicochemical Characteristics

The physical and chemical properties of sample soils were analyzed at Melkasa Agricultural Research Center (MRC). Soil pH was measured potentiometrically using a digital pH-meter in the supernatant suspension of (1:2.5 soil: water), and soil texture was measured with the Boyacus hydrometer method (g/cm) using USDA textural triangle (Gee & Bauder, 1986). The total organic carbon was determined by using the method of Walkley & Black (1934), and available phosphorus was determined by Olsen methods (Olsen, 1954). In the Olsen procedure, NaHCO3 at nearly constant pH of 8.5 was used as extracting solution. The total N content was determined using the Kjeldahl method by oxidizing soil samples with sulfuric acid and converting the N compounds into NH^{4+} as ammonium sulfate (Hinds & Lowe, 1980) (Table 4.1).

3.4. Determination of AMF Spore Abundance of the Soil Samples

Before establishing trap culture, the abundance of AMF in the soil samples collected from sample locations was examined to determine the initial mycorrhizal status of the soil sample (Table 4.1). Soil samples from the field were air-dried and sieved through a 2-mm sieve to remove coarse debris. AMF spores occurring in the soil samples were extracted by the wet sieving and decanting method (Gerdemann & Nicolson, 1963) followed by centrifugation in water and in a 60% sucrose solution (Walker *et al*., 1982). One hundred grams of soil was suspended in one liter of tap water in each batch and left to settle the heavier fractions to settle for two to three minutes. Then, the liquid fraction was decanted through four stacked sieves of decreasing pore size: 750,500, 250, and 63 µm.

The first sieve was used to catch large pieces of floating organic debris, and the second sieve was used to capture some AMF sporocarps, and the third and fourth also to capture AMF spores and fine particles. The contents of the 500, 250, and 63 µm sieves were then, put into a centrifuge tube containing water and centrifuged at 2000 rpm for five minutes. After pouring off the supernatant, 60% sucrose was added to the pellet and mixed it carefully, then centrifuged for one min at 2000 rpm. The supernatant was carefully poured through a 63 µm sieve and carefully washed with tap water to remove the sucrose. Finally, spores, spore clusters, and sporocarps were carefully washed and transferred to a plastic Petri dish for examination under the stereomicroscope. Enumeration of spore numbers per gram of dry soil was undertaken according to INVAM, http://invam.caf.wvu.edu.

3.5. Establishment of Trap Cultures (AMF Inoculum Production)

In order to obtain many healthy and infective AMF spores and propagules for inoculum production, a trap culture was established in the greenhouse at ASTU for four months (March to June 2021), according to INVAM (2020). The trap cultures in pots were set up in triplicates for all seven soil samples (*A. seyal, A. tortilis,* and *A. saligna* each from Batu and Bishoftu, and *A, abyssinica* from Sululta), as result a total of 21 pots was established. Each soil sample was thoroughly mixed with autoclaved sand (sterilized twice in autoclave at 121 ^{o}C, 1 hour with intervals of 24 hrs.) (1:1; v/v), and about 3kg of mixed soil: sand sample was transferred to a plastic pot, and were irrigated for three days before sowing.

Seeds of "*Melkam"* a sorghum cultivar and a mycotrophic crop from Melkasa Agricultural Research Center (MRC) were selected as an appropriate trap plant for its ability to induce high spore density, diversity, and species richness according to INVAM (2020). Surface sterilization of sorghum seeds was performed by soaking them for 15 minutes in a 0.5% sodium hypochlorite solution. Then, after washing the seeds with sterile water, they were sown at a two cm depth in each plastic pot and covered with sterilized sand. Pots were irrigated daily as needed. During the growing phase, no fertilizer was used. All seedlings were grown in the greenhouse under natural light and temperature conditions for four months.

Finally, watering was reduced during the final weeks of the four months to maximize spore production. At the end of 4th month, the plants were cut near the base, and the cultures was air-dried and stored in zipped plastic bags at room temperature for 30 days before inoculation (INVAM, 2021).

3.6. Assessment of root Colonization and Spore Density of Trap Culture

The root samples were washed several times in tap water cut into segments about 1–2 cm long. About 0.5 g of root segments was cleared in 10 % (w/v) KOH at 90 ^{0}C for 1 hour in water bath. And roots were further bleached with alkaline hydrogen peroxide (10% H_2O_2) for three minutes at room temperature. Thereafter, the roots were treated with 2% HCl (v/v) for 15-20 minutes at room temperature and finally stained in 0.05% w/v trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water) at 90 ^{0}C for one hour in water bath. Samples were drained and washed thoroughly with distilled water at the end of every step except HCl treatment. The samples were left in distaining solution (lactoglycerol) for more than two days in a dark room. Finally, roots were mounted in PVLG mountant on

microscopic slides and covered with 24×24 mm coverslips (Brundrett *et al*., 1996). AMF colonization was assessed from cleared and stained roots according to McGonigle *et al*. (1990). A total of 150 intersections were taken for each subsample to estimate percent AM root colonization under a compound microscope at a magnification of 100X. The presence of arbuscular colonization (AC) and vesicular colonization (VC) were calculated by dividing the count for the 'arbuscules' and 'vesicles' categories, respectively by the total number of intersections. Hyphal colonization (HC) was calculated by dividing the proportion of non-negative intersections to total number of intersections. And the assessment of spore density also performed by wet sieving and decanting method (Gerdemann & Nicolson, 1963). followed by centrifugation in water and in a 60% sucrose solution (Walker *et al*., 1982) as described above.

3.7. Experimental Design and Inoculation of AMF and PGPR Inocula for Vegetables

The experiment set up in a randomized complete block design with three replications in a greenhouse. The experiment consisted of seven treatments for each crop. T1 [consortium of AMF inoculum, (32.4 spores g-1 of soil)]; T2 (dual inoculation of consortium spp. of AMF and *Bacillus subtilus*); T3 [*Rhizophagus clarus* (29 g-1 of soil)]; T4 (dual inoculation with *Rhizophagus clarus* and *Bacillus subtilus*); T5 [*Bacillus subtilus (*~10^8 CFU/ml)]; T6 [negative control (non-inoculated or not fertilized)]; T7 [positive control (treated with chemical fertilizer)]. The PGPR strain of *Bacillus subtilus* (ALCR46) was obtained from the Addis Ababa University/ Ethiopian Biotechnology Institute, which was isolated from rhizosphere soil of chickpea and reported as plant growth-promoting bacteria and phosphate solubilizer (Mulissa Jida *et al*., 2016). The commercial chemical fertilizer, Urea and Diammonium Phosphate (DAP), and foliar fertilization (N.P.K 12.10.8 plus chelated trace elements: Iron, Copper, Manganese Molybdenum, Boron and Zinc. plus 2% Magnesium, 4% Sulphur, and 2% Calcium) also used in the study was procured from the market.

The vegetable seed varieties used in the test were tomato (*Lycopersicum esculentum*) Melkashola variety, onion (*Allium cepa*), and squash (*Cucurbita pepo*) Jp-10 variety, obtained from the vegetables and flowers division, Melkasa Agricultural Research Institute (MRC), Ethiopia's. The single strain of AMF (*Rhizophagus clarus*) used in the experiment was obtained from Hawassa University, Department of Biology.

In order to prepare PGPR inoculum, a loopful of bacterial biomass of *Bacillus* ALCR46 was transferred from slant culture into 250 mL of nutrient broth (NB) and incubated with shaking at 30 °C, for 24 h to adjust the final concentration of the cells to ~10^8 CFU/ml (viable plate count method and optical density measurement using spectrophotometer (SM-1600 Double Beam UV/VIS Spectrophotometer) at 600 nm.

Autoclaved soil with low pH and low phosphorus level was collected from Holeta for the experiment to evaluate the efficiency of the inoculants. The physical and chemical characteristics of the test soil was as follows: P (6.44 Ppm), N (1.66%), organic matter content (1.549 %), pH (4.75) and EC (0.059 ds/m) (Zerihun Belay & Fassil Assefa, 2011). Healthy selected seeds of all three vegetables were surface sterilized with 0.5% sodium hypochlorite solution for 15 minutes and allowed to germinate on a 0.75% (w/v) water agar for three days at 25 ^{0}C before planting or sowing.

One hundred gram of inoculum of cAMF (with spore number of 32 g^{-1} of soil), and Rc (29 g^{-1} of soil) was applied into the designed plastic pot (18.5 cm upper mouth diameter × 18 cm depth × 16 cm bottom mouth diameter) containing 4 kg of autoclaved growth substrates (experimental soil and sand soil, 1:1 v/v), Placing the inoculum as one layer at 8 cm depth below the growth substrates (Wu & Zou, 2012). The inoculum contained the infected root segments of host plant, spores, and extraradical hyphae (INVAM, 2020). Equal volume of sterilized inoculums was applied in to the negative control, to ensure that trace elements were consistent. Five germinated seeds of each vegetable were moved into each plastic pot, which were later thinned down to three.

For the PGPR treatment, one ml of culture suspension adjusted their number approximately to be 10^8 CFU/ml was flooded on each seedling for one hour. Then the inoculated seedlings were planted on each pot. During the trial, bacterial inoculation was performed three times. A first dose of 1 mL $plant^{-1}$ was applied during seedling planting, followed by 5 mL amount of the cell suspension in sterile water was added to pots immediately after planting and one week after planting (Samayoa *et al*., 2020). In the positive control two fertilization schemes were applied, one directly to the soil with 1.32 g $plant^{-1}$ of urea and DAP ($(NH_4)2HPO_4$ contains 18% Nitrogen and 46% Phosphorus (P_2O_5)), and the other a foliar fertilization with the formulation 12.10.8 (N-P-K) by dissolving 7.5 ml in 3 L of distilled water. Every 15 days, the solution (50 mL) was applied per plant during the crop cycle (two

months) (AVRDC, 1990). All pots were irrigated daily as needed. All seedlings were grown in the greenhouse under natural light and temperature conditions for 60 days.

3.8. Mycorrhization and Growth Parameters

During the growth process, root colonization of the seedlings was measured two times, after 35 days, and after 60 days, harvested fine roots from each plant were washed and cut into 1 cm long segments, cleared by soaking in 10% (w/v) KOH and stained in 0.05% (w/v) trypan blue solution. AMF colonization rate was estimated based on the intercept method and calculated using the following formula: root colonization rate (%) = number of infected root segments/total number of segments × 100% (Brundrett *et al*., 1996).

Plant height (height from cotyledon to growing point), and number of branches per plant were directly determined after 60 days. At the end of 60 days, shoots and roots were harvested and rinsed with tap water to remove surface debris, then rinsed with deionized water; after removed moisture, the fresh weight was measured, and the average fresh weight per plant was obtained; afterwards, the samples were oven-dried (75°C, 48 h) to constant weight; the samples were weighted, to get average dry weight per plant.

AMF spores occurring in the soil rhizosphere after completing the growth of all the seedlings were examined by the wet sieving and decanting method (Gerdemann & Nicolson, 1963) as described previously. Moreover, for each plant that formed mycorrhizae a Mycorrhizal Dependency (MD) value was calculated. It was determined by expressing the difference between the total dry mass of mycorrhizal plant and the total dry mass of the non-mycorrhizal plant as a percentage of the dry mass of the mycorrhizal plant. The MD indexes calculated from these values, %MD = [(dry mass mycorrhizal plant - dry mass nonmycorrhizal plant)/dry mass mycorrhizal plant] * 100 (Plenchette *et al*., 2005).

3.9. Data Analysis

The analysis was performed using the SPSS software package (version 26.0). the AMF spore abundance and root colonization of the field soil and trap culture of the inoculum data; and the growth response (plant shoot height, root height, number of branches, stem diameter, dry and fresh weight of root and shoot) data were subjected to one-way analysis of variance (ANOVA) using the treatments as factor. In the same pattern, data on root colonization, mycorrhizal dependency, and spore number of the vegetables were analyzed by two-way ANOVA using the crops and treatments as factor. A two-way ANOVA was used to

determine the significance of the vegetables and inoculation treatments, and their interaction with the experimental parameters (Chen *et al*., 2020). Statistically significant differences based on two-way ANOVA are reported as crop effect, treatment (inoculum) effect and (interaction between crop and treatment). Tukey's honestly significant difference (HSD) post hoc test was used for pair-wise multiple mean comparisons tests. Pearson's correlation coefficients among parameters were analyzed. All the tests of statistical significance were decided at $p < 0.05$. And data are reported as means ± SE, based on three replicates.

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CHAPTER FOUR

4. RESULT

4.1. AMF spore abundance and Physicochemical Analysis of Soil Sample

The soil sample used for the production of AMF inoculum in this experiment was dominated by the soil texture of the sandy loam in all the acacia species, while the *A. abyssinica* showed a clay loam fraction. The physico-chemical properties and AMF status of the field soil were shown in Table 4.1. The soil samples were close to neutral, which ranged between pH values of 6.54 in *A. seyal* and 7.33 in *A. tortilis* from Batu. The soil organic carbon concentration varied between 0.87 in *A. saligna* and 2.1% in *A. abyssinica* from Bishoftu and Sululta. Similarly, the soil total nitrogen concentration varied between 0.09 and 0.21% for Batu (*A. tortilis*) and Sululta, respectively. Additionally, the P content was observed to be the highest in Sululta (13.5 ppm) and the lowest in Batu with *A. seyal* (6.32 ppm).

Regarding AMF analysis, it shows that the spore density of the rhizosphere soils sampled from all acacia species in highland and lowland showed numbers of AMF spores ranging from 10.47 to 18.89 spores g-1 soil. The highest average spore density of 18.89 spores g-1 soil was observed under *A. seyal* from Batu, and the lowest of 10.47 spores g-1 soil under *A. tortilis* from Bishoftu.

Table 4. 1 AMF spore abundance and Physico-chemical parameters of soil samples from rhizosphere of acacia trees.

Sampling area	Acacia sp.	pH	T.N (%)	Ave.P (ppm)	OC (%)	%Sand	%Clay	%Silt	Class of texture	SD (gm^{-1})
Sululta	*A. abyssinica*	6.84	0.21	13.5	1.08	32	36	32	clay loam (CL)	10.55±0.3d
Batu	*A. saligna*	6.9	0.15	12.8	1.71	56	4	40	sandy loam (SL)	18.19±0.1a
	A. seyal	6.58	0.15	6.32	2.1	62	4	34	sandy loam (SL)	18.89±0.07a
	A. tortilis	7.33	0.09	11.1	1.61	58	6	36	sandy loam (SL)	13.73±0.06c
Bishoftu	*A. saligna*	7.04	0.12	12.7	0.87	50	17	33	loam (L)	14.48±0.09c
	A. seyal	7.08	0.13	10.1	0.99	66	6	28	sandy loam (SL)	16.61±0.19b
	A. tortilis	7.13	0.14	9.58	1.66	50	14	36	sandy loam (L)	10.47±0.17d

Note- TN - total nitrogen, Ave.P - available phosphorus, OC - organic carbon, and SD – spore density.

4.2. AMF Root Colonization and Spore Density of the Trap Culture

The average number of AMF spore density enumerated in the trap culture ranged from 18.74 to 32.41 g^{-1} (Table 2). There was a significant difference in AMF spore density among the areas of sampling and the acacia species. The highest spore densities were found associated with A. *seyal* from Batu. Sorghum that treated with highland *A, abyssinica* rhizosphere, recorded the lowest *(*10.55 g^{-1} soil*)* AMF spore density. All seedlings of sorghum in trap culture were colonized by AMF. The percentage of AMF root colonization significantly differs between tree species in the lowlands and highland ($p < 0.05$) (Table 2). Average hyphal colonization ranged between 56 and 94%. The highest colonization was found in *A. seyal* (94%) species from lowland Batu. The lowest colonization was in *A. abyssinica* species (56%).

Table 4. 2 AMF spore abundance & percentage of root colonization in trap cultures of the inoculum.

sampling area	*Source of the soil inoculum*	SD (gm^{-1})	RC%		
			AC%	VC%	HC%
Sululta	*A. abyssinica*	18.74±0.47e	25.26±0.08e	14.6±0.15d	56.96±0.23e
Batu	*A. saligna*	27.34±0.06b	28.7±0.23d	21.03±0.61bc	76±0.78b
	A. seyal	32.41±0.21a	45.7±0.86a	25.6±0.45a	94.9±0.4a
	A. tortilis	22.43±0.06d	34.4±0.4c	19.9±0.45c	62.93±1d
Bishoftu	*A. saligna*	25.64±0.16c	27.26±0.28d	15.15±0.2d	67.03±0.65c
	A. seyal	28.44±0.16b	41.76±0.48b	27.33±0.49a	79.1±1.17b
	A. tortilis	21.85±0.11d	28.46±0.4d	22.53±0.44b	61±0.56d

Note- SD- spore density, RC- root colonization, AC- arbuscular colonization, VC – vascular colonization, and HC- hyphae colonization. Different lowercase letters in the same column represent significant difference at 0.05 level; Mean values followed by the same letter are not significantly different at P=0.05. Mean ± Standard error.

4.3. Growth Response of the Vegetables to AMF and PGPR Inocula

4.3.1. *Lycopersicum esculentum* (Tomato)

Data on the growth parameters of *Lycopersicum esculentum* is presented in Table 4.3. It was observed that the seedlings treated with chemical fertilizer (T7) and inoculated with dual inoculation of consortium of AMF and PGPR application (T2) showed significantly greater response at all growth parameters over the uninoculated control (T6) and other treatments. Interestingly, no significant differences were observed between the positive control and dual

inoculation in all parameters. The growth parameters recorded by the T7 and T2 were followed by the seedlings treated with dual inoculation of a single strain of AMF and the PGPR (T4) and the single inoculation of a consortium of AMF inoculum (T1), respectively. Except for the T2, all seedlings treated with microbial inoculations showed significantly lower average values of growth in all parameters when compared to the T7 (chemical fertilizer). For example, the average root and shoot height of seedlings inoculated with T4 and T1 was lowered by 18% and 13%, 39% and 24%, compared to seedlings treated with T7 (chemical fertilizer), respectively.

The T2 inoculation received the best effort, which had a significant difference with the other single inoculation treatments. The average root and shoot height and the number of branches of *Lycopersicum esculentum* inoculated with T2 inoculum were increased by 33%, 18%, and 10% compared with single inoculation with a consortium of AMF (T1). As shown in Table 4.3, there was also a significant increase with the T2 inoculum in the average of fresh weight of shoot (36%) and root (62%), dry weight of shoot (112%) and root (156%) when compared to the T1 inoculation.

Moreover, the result showed that the growth potential of *Lycopersicum esculentum* seedlings inoculated with single ether T1, T3, or T5 was better than in the negative control (T6). The T1 inoculation treatment treated seedlings showed the best growth response compared to other single treatments, with a significant difference in most growth parameters. It is followed by the T3 and T5 treatments, respectively. The average root height, fresh weight of shoot and root, and dry weight of shoot and root in the T1 inoculation treatment were increased by 15%, 19%, 47%, 49%, and 39%, respectively, compared to T3, and the difference reached a significant level. However, no significant difference was found in shoot height or the number of branches between T3 and T1.

Table 4. 3 Effect of microbial inoculums and chemical fertilizer on growth characteristics of *Lycopersicum esculentum*, *Allium cepa,* and *Cucurbita pepo*

Treatments	Parameters						
	SH	RH	NB	SFW	RFW	SDW	RDW
Lycopersicum esculentum							
T1	52.3±0.15c	19.43±0.35c	13±0.00bc	28.63±0.18c	4.45±0.09c	6±0.28c	1.06±0.03c
T2	61.46±0.31ab	25.93±0.18a	14.33±0.33ab	38.9±0.11a	7.22±0.35a	12.73±0.09a	2.71±0.01a
T3	50.33±0.44c	16.83±0.37d	13±0.00bc	24±0.17d	3.03±0.01d	4.03±0.01d	0.76±0.03d
T4	57.86±0.14b	22.8±0.4b	13.66±0.33ab	31.4±0.45b	5.48±0.12b	7.79±0.16b	1.93±0.08b
T5	44.2±2.05d	14.6±0.26e	11.66±0.33cd	21.04±0.63e	1.98±0.06e	2.02±0.01e	0.14±0.00e
T6	29.2±1.34e	10.76±0.35f	10.33±0.33d	15.43±0.68f	1.05±0.09f	0.75±0.01f	0.013±0.00f
T7	65.1±0.11a	26.96±0.54a	14.66±0.33a	39.76±0.27a	7.54±0.26a	12.15±0.16a	2.64±0.12a
Allium cepa							
T1	50.43±0.66c	12.2±0.2bc	3.66±0.33ab	11.94±0.25b	0.86±0.04b	0.79±0.03b	0.04±0.00b
T2	57.9±0.1b	15.2±0.35a	5±0.00a	15.11±0.37a	1.68±0.08a	1.46±0.02a	0.06±0.00a
T3	45.56±0.21d	11.7±0.2c	3.66±0.33ab	9.27±0.28c	0.43±0.05c	0.61±0.04c	0.02±0.01c
T4	52.43±0.74c	13.3±0.3b	4.66±0.33a	13.05±0.41b	1.09±0.11b	0.86±0.04b	0.04±0.00b
T5	20.66±0.95e	4.66±0.44d	2.66±0.33b	1.26±0.02d	0.05±0.02d	0.04±0.00d	0.007±0.00d
T6	18.56±0.34e	4.36±0.4d	2.33±0.33b	1.18±0.02d	0.03±0.01d	0.03±0.00d	0.004±0.00d
T7	59.43±0.78a	15.23±0.29a	5±0.00a	16.59±0.6a	1.92±0.06a	1.51±0.01a	0.06±0.00a
Cucurbita pepo							
T1	66.86±0.56c	8±36bc	8.33±0.33ab	48.26±0.18bc	3.49±0.26b	7.99±0.11c	1.02±0.00c
T2	71.9±0.8ab	10.86±0.36a	9±0.00a	54±0.3a	5.08±0.16a	14±0.06a	2.21±0.06a
T3	64.8±0.56c	6.23±0.21cd	7.33±0.33b	45.56±0.93c	2.15±0.09c	6.76±0.03d	0.89±0.03c
T4	68.76±0.24bc	9.53±0.31ab	8.66±0.33ab	52.6±0.26ab	3.82±0.25b	9.81±0.03b	1.44±0.05b
T5	58.76±1.14d	4.9±0.15de	5.66±0.33c	38.93±0.78d	1.45±0.08cd	5.02±0.01e	0.54±0.00e
T6	46.13±1.53e	3.7±0.11e	5.33±0.33c	28.43±0.17e	0.97±0.03d	2.75±0.01f	0.34±0.03e
T7	73±0.5a	11.16±0.78a	9.33±0.33a	57.33±1.18a	5.47±0.22a	14.25±0.2a	2.24±0.02a

Note: SH-Shoot Height, RH-Root Height, NB - Number of Branches, SFW-Fresh Weight of Shoot, RFW-Fresh Weight of Root, SDW-Dry Weight of Shoot, RDW-Dry Weight of Root. +cAMF- Consortium AMF species, +Rc- *Rhizophagus clarus*, +Bs- *Bacillus subtilus* (ALCR46), -Ve C- Negative Control, and +Ve C- Positive Control. The same letter in the row indicates not significantly different values based on one-way ANOVA and Tukey's honestly significant difference (HSD) post hoc test ($P < 0.05$).

4.3.2. *Allium cepa* (Onion)

In this study, *Allium cepa* seedlings treated with +cAMF+Bs resulted in higher growth and biomass, and significantly superior in most growth parameters compared to the dual inoculation of T4 or single inoculations of T1 or T3 (Table 4.3). Additionally, the experiment results in the analysis showed that +cAMF+Bs and chemical fertilizer did not show a significant difference in all growth parameters except in shoot height. As shown in Table 4.3, the growth potential of *Allium cape* seedlings inoculated with T2 and T7 (chemical fertilizer) was better than that in other treatments; the average shoot height, root height, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight in the T2 inoculation

treatment were increased by 15%, 25%, 27%, 95%, 85%, and 50%, respectively compared with +cAMF, and the difference reached a significant level.

Likewise, it was found that T2 inoculated seedlings increased significantly more than other single inoculums in most growth parameters. The average weight of fresh shoot (29%), root (100%), dry weight of the shoot (30%), and root (100%) increased significantly more in T1 treated seedlings than in T3 treated seedlings (Table 4.3). In the same way, in the T3 treatment, all average growth responses had reached the significance difference level when compared with the T5 inoculum and the negative control, except in the number of branches. However, there was no significant difference in growth response between the T5 and T6 (negative controls) (Table 4.3).

4.3.3. *Cucurbita pepo* (Squash)

In the same trend, the results showed that all single and co-inoculation of AMF and a PGPR significantly increased the growth of *Cucurbita pepo* including fresh and dry biomass and other growth parameters of the seedlings when compared with non-inoculated plant growth (Table 4.3). The highest average growth parameters were recorded in the T7 (chemical fertilizer treated), followed by dual inoculations of T2, and no significant differences were observed between T2 and T7 treated seedlings ($p > 0.05$). Moreover, the average of the fresh and dry weight of the shoot and root of *Cucurbita* seedlings in the T2 treatment was higher than the single inoculation treatment of T1 by 12%, 46%, 75%, and 117%, respectively. It was followed by T4, which significantly increased the dry weight of shoot and root by 33% and 41%, compared to the single inoculation of T1.

Among the single inoculations, the effect of T1 treatment was superior to T3 and T5, and showed increased growth of root fresh weight by 62% and shoot dry weight by 18%, but no significant differences were observed in the other parameters. Correspondingly, seedlings treated with T1 showed an increase in all growth parameters compared to T5 and T6 (negative control). Among them, the dry weight of shoot and root increased by 59%, 89%, 191%, and 200% in T1 than in T5 inoculum and T6, respectively. There was also a significant difference between the single inoculums (T3 and T5) and the negative control (T6), and the result showed that the effect of the T3 inoculum treatment on the seedling was higher than the T5 inoculum in most growth responses of the seedling, with a statistically different apart from the root height and fresh weight of the shoot. However, no differences were found in most growth parameters between T5 treated seedlings and negative control

(T6) except in shoot height and fresh and dry weight of shoot by 27%, 37%, and 83%, respectively.

4.4. Arbuscular Mycorrhizal Fungi Parameters

4.4.1. AMF Root Colonization and Spore Density

Data on the root colonization parameters was measured twice, after 35 and 60 days. Apart from the single inoculation treatment of the T5 and the controls (T6 and T7), AMF root colonization was found in all vegetable seedlings in all inoculation treatments. The AMF hyphal root colonization recorded after 35 days was found highest in roots inoculated with dual inoculation, T2 (28.33%, 36.66%, and 23.66%), and the lowest in single inoculation of single species of mycorrhiza inoculation T3 (11.3%, 7.63%, and 2.96%) in *Allium cape*, *Lycopersicum esculentum*, and *Cucurbita pepo*, respectively (Figure 4.1).

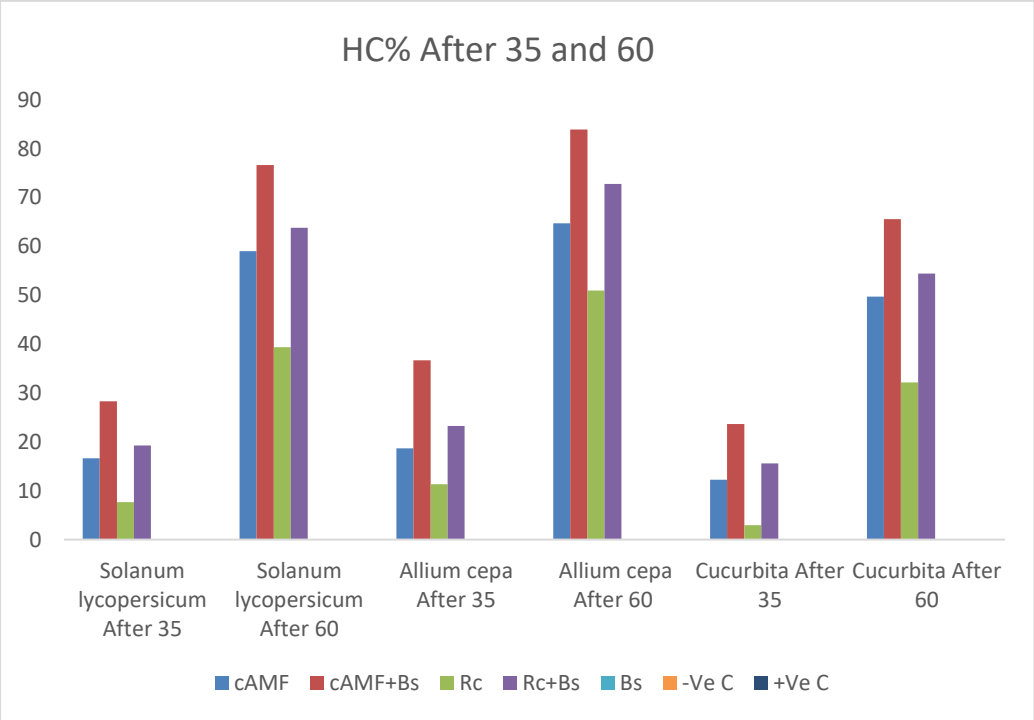


Figure 4. 1 Showing an increasing and compering of percent of hyphal root colonization after 35 and 60 days of growth in *Lycopersicum esculentum*, *Allium cape*, and *Cucurbita pepo*. with the single and co inoculation of AMF and PGPR (*Bacillus subtilus*). cAMF (T1), cAMF+Bs (T2), Rc (T3), Rc+Bs (T4), Bs (T5), -Ve C (T6), and +Ve C (T7).

As expected, after 60 days, the rate of AMF colonization increased significantly (Figure 4.1). Similarly, the highest HC% was recorded in T2 and increased by 171%, 129%, and 177% in *Lycopersicum esculentum*, *Allium cape*, and *Cucurbita pepo* seedlings, compared to a 35-

day rate of colonization, respectively. Moreover, the spore abundance after 60 days was high in the dual inoculation treatment, T2 (27.18 g-1, 22.47 g^{-1}, and 10.95 g^{-1}), and followed by T4 (17.2 g^{-1}, 14.26 g^{-1}, and 8.33 g-1), T1 (15.31 g^{-1}, 12.28 g^{-1}, and 8.5 g^{-1}), and T3 (11.22 g^{-1}, 9.65 g^{-1}, and 6.4 g^{-1}) in *Allium cape*, *Lycopersicum esculentum*, and *Cucurbita pepo*, respectively (Table 4.4).

Table 4. 4 Effect of single and co-inoculation of AMF and PGPR inoculums on rate of AMF root colonization (HC) and spore density (SD) of *Lycopersicum esculentum*, *Allium cape*, and *Cucurbita pepo* seedlings after 60 days.

Treatments	***Solanum lycopersicum***		***Allium cepa***		***Cucurbita SP.***	
	RC%	SD g^{-1}	RC%	SD g^{-1}	RC%	SD g^{-1}
T1	59.03±0.16c	12.28±0.04c	64.74±0.58c	15.31±0.02c	49.73±0.17c	8.5±0.16b
T2	76.66±0.68a	22.47±0.04a	83.9±0.87a	27.18±0.02a	65.6±0.29a	10.95±0.42a
T3	39.38±0.36d	9.65±0.03d	50.96±0.24d	11.22±0.02d	32.18±0.71d	6.4±0.31c
T4	63.79±0.58b	14.26±0.04b	72.8±0.37b	17.2±0.01b	54.45±0.32b	8.33±0.12b
T5	-	-	-	-	-	-
T6	-	-	-	-	-	-
T7	-	-	-	-	-	-

Note: +cAMF- consortium AMF species, +Rc- *Rhizophagus clarus*, +Bs- *Bacillus subtilus* (ALCR46), -Ve C- negative control, and +Ve C- positive control. Different lowercase letters in the same column represent significant difference at 0.05 level; Mean values followed by the same letter are not significantly different at P=0.05. Mean ± Standard error.

Furthermore, the additional comparing of root colonization, spore density, and mycorrhizal dependency among the vegetables and the treatments were subjected to two-way ANOVA. The root colonization and spore number were increased in *Allium cape* by 35% & 14% and 106% & 21%, compared to *Cucurbita pepo* and *Lycopersicum esculentum*, respectively (Figure 4.1). In the same pattern, the statistical analyzing and comparing of mean between treatments in all vegetables shows that the dual inoculation of T2 was the highest than single inoculations (T1 and T3) and dual inoculation of single species of AMF and PGPR (T4) by 30%, 85%, and 19% in root colonization and by 67%, 122%, and 52% in spore density with a statistical difference, respectively.

In addition to that, the difference between the total dry mass of the mycorrhizal plant and the total dry mass of the non-mycorrhizal plant as a percentage of the dry mass of the mycorrhizal plant (mycorrhizal dependency) was calculated (Figure 4.3). All selected vegetables were showed a high degree of dependency on AM fungi. Among the selected

vegetables, *Allium cape* (97.49%) was shown to have higher mycorrhizal dependence than *Lycopersicum esculentum* (90.11%) and *Cucurbita pepo* (72.33%) (Figure 4. 2). In terms of treatments, the vegetables were more dependent on both dual inoculations T2 (92.13%) and T4 (88.29%) than on a single inoculation of T1 (85.2%) or T3 (80.62%) (Figure 4.3).

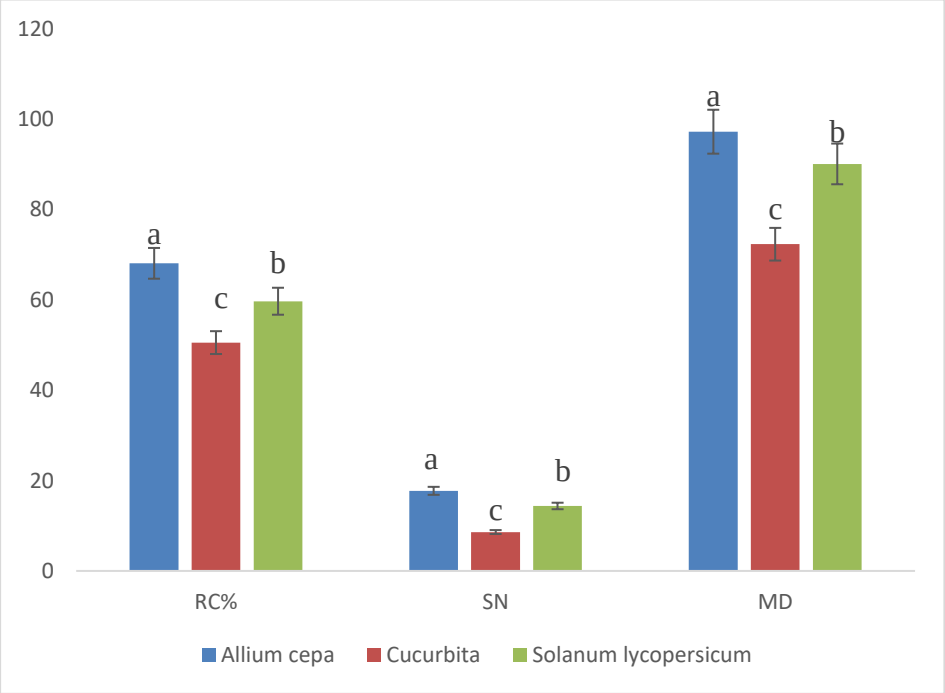


Figure 4. 2 Comparison among the vegetables in average value of all treatments in percent of hyphal root colonization, mycorrhizal dependency, and spore density (number).

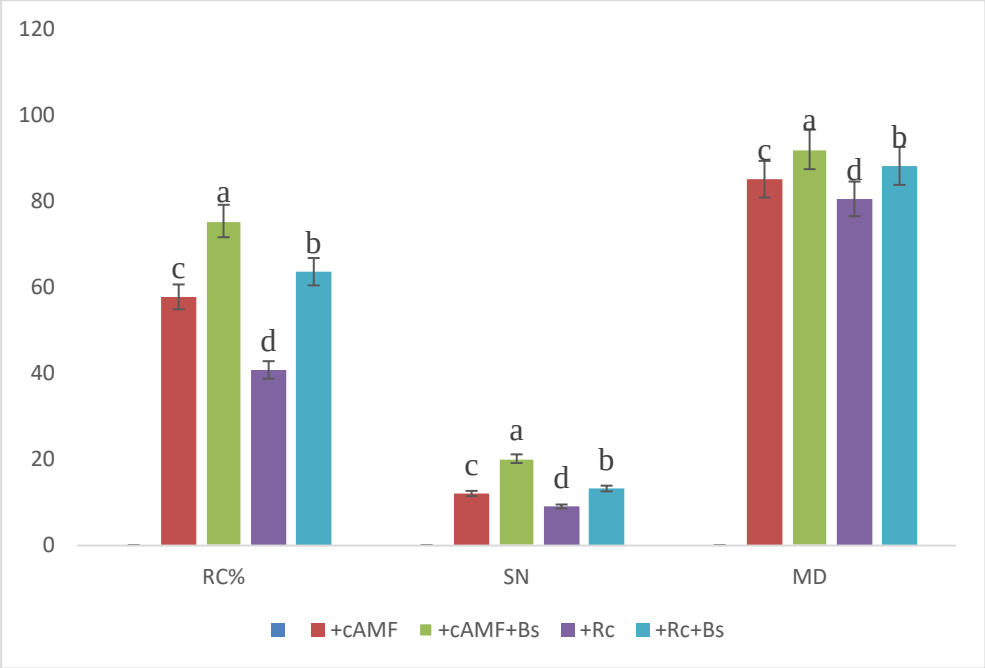


Figure 4. 3 Comparing the mean of hyphal root colonization, spore density (number), and mycorrhizal dependency regarding the treatments. cAMF (T1), cAMF+Bs (T2), Rc (T3), Rc+Bs (T4), Bs (T5), -Ve C (T6), and +Ve C (T7).

4.5. Correlation Between the Parameters

Pearson correlation analysis between the parameters in each vegetable was carried out to clarify the potential of inoculums and growth response of the seedlings ($p<0.05$). Therefore, Pearson's correlation results indicated that strong positive and significant correlations were observed between the plant growth parameters in each vegetable (Table 4.5). Similarly, the analysis shows that, there was positive correlation between the arbuscular mycorrhizal parameters and plant growth parameters (Table 4.5). For instance, root colonization and total dry weight in *Lycopersicum esculentum* ($r = 0.461$, $p = 0.03$), *Cucurbita pepo* ($r = 0.433$, $p = 0.05$), and *Allium cepa* ($r = 0.458$, $p = 0.03$) was correlated positively. Moreover, Pearson's correlation results indicated also strong positive correlation between root colonization and plant height (root height $r = 0.618$, 0.508, and 0.465, and shoot height $r = 0.574$, 0.543, and 0.475) in *Allium cepa*, *Cucurbita pepo*, and *Lycopersicum esculentum*, respectively (Table. 4.5). In the same trend, very strong positive correlations were found between the percent of AMF colonization and spore density ($r_s = 0.882$, $p = 0.000$) and between percent of AMF root colonization and mycorrhizal dependency ($r_s = 0.744$, $p = 0.000$), when all the vegetables species were considered (Figure 4.4).

Table 4. 5 Pearson correlation coefficients between the parameters in each selected vegetable.

Lycopersicum esculentum

	SH	RH	NB	SDW	RDW	TDW	RC	SD
SD	1	.959**	.958**	.905**	.899**	.906**	.475*	.489*
RH	.959**	1	.930**	.975**	.972**	.976**	.465*	.500*
NB	.958**	.930**	1	.893**	.884**	.893**	.487*	.509*
SDW	.905**	.975**	.893**	1	.988**	1.000**	.457*	.522*
RDW	.899**	.972**	.884**	.988**	1	.992**	.473*	.528*
TDW	.906**	.976**	.893**	1.000**	.992**	1	.461*	.524*
RC	.475*	.465*	.487*	.457*	.473*	.461*	1	.978**
SD	.489*	.500*	.509*	.522*	.528*	.524*	.978**	1

Allium cepa

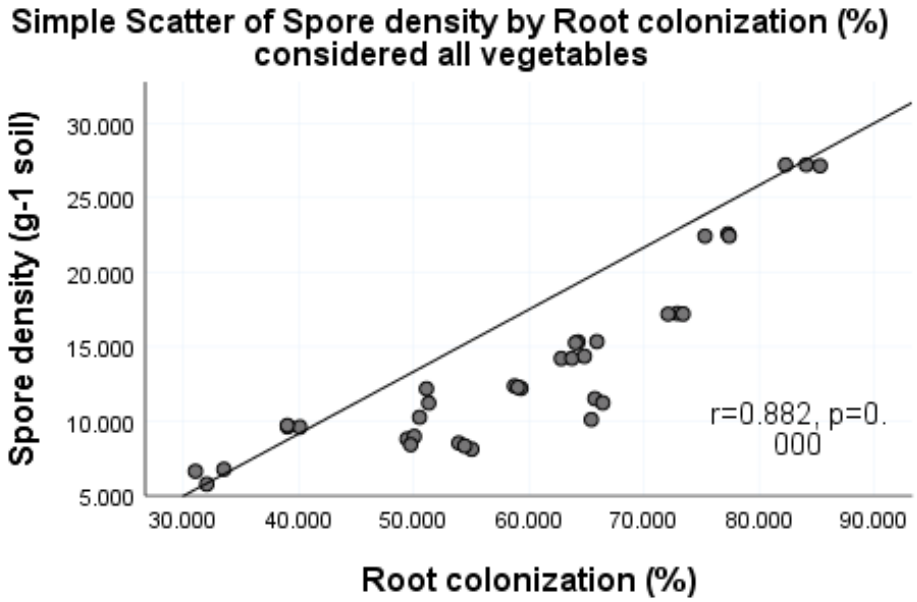
	SH	RH	NB	SDW	RDW	TDW	RC	SD
SD	1	.991**	.877**	.952**	.949**	.953**	.574**	.556**
RH	.991**	1	.876**	.947**	.944**	.947**	.618**	.599**
NB	.877**	.876**	1	.890**	.878**	.890**	.501*	.544*
SDW	.952**	.947**	.890**	1	.981**	1.000**	.456*	.536*
RDW	.949**	.944**	.878**	.981**	1	.982**	.506*	.593**
TDW	.953**	.947**	.890**	1.000**	.982**	1	.458*	.538*
RC	.574**	.618**	.501*	.456*	.506*	.458*	1	.930**
SD	.556**	.599**	.544*	.536*	.593**	.538*	.930**	1

Cucurbita pepo

	SH	RH	NB	SDW	RDW	TDW	RC	SD
SD	1	.900**	.871**	.892**	.840**	.885**	.543*	.539*
RH	.900**	1	.915**	.955**	.960**	.959**	.508*	.473*
NB	.871**	.915**	1	.886**	.892**	.890**	.568**	.553**
SDW	.892**	.955**	.886**	1	.981**	.999**	0.427	0.401
RDW	.840**	.960**	.892**	.981**	1	.987**	.453*	0.418
TDW	.885**	.959**	.890**	.999**	.987**	1	.433*	0.405
RC	.543*	.508*	.568**	0.427	.453*	.433*	1	.991**
SD	.539*	.473*	.553**	0.401	0.418	0.405	.991**	1

Note: SH-shoot height, RH-Root height, NB - Number of Branches, SDW- Shoot Dry Weight, RDW- Root Dry Weight, TDW- Total Dry Weight, RC- Hyphal Root Colonization, and SD – Spore Density. **. Correlation is significant at the 0.01 level (2-tailed) and *. Correlation is significant at the 0.05 level (2-tailed).

A



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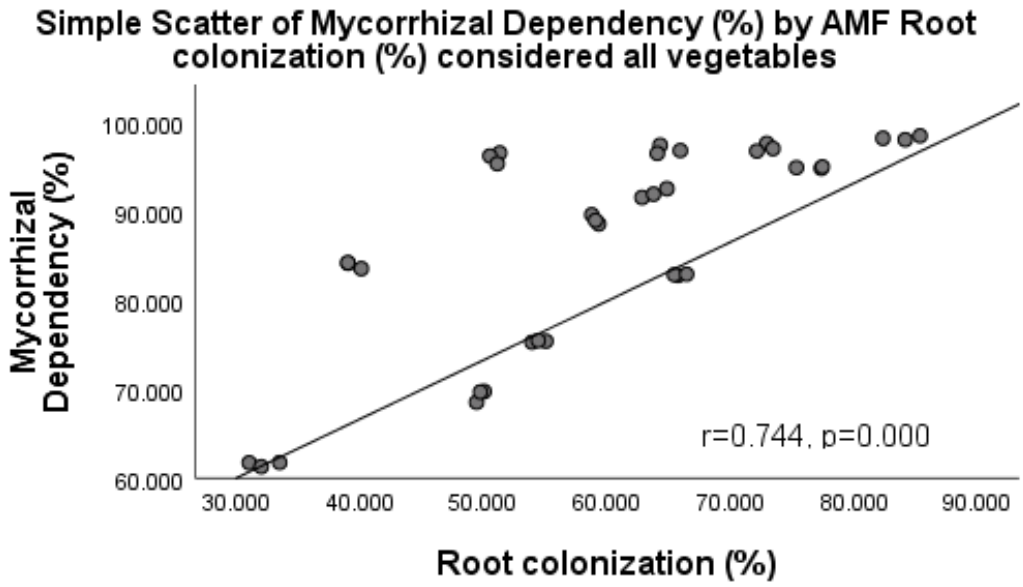

Figure 4. 4 Correlation between AMF root colonization with spore density and mycorrhizal dependency considering all selected vegetables. A) shows the correlation between %RC and SD, B) shows the correlation between %RC and %MD.

CHAPTER FIVE

5. DISCUSSION

The data showed that the highest average spore density of AMF was recorded in the rhizosphere soil of *A. seyal* (18.89 spores g-1) and followed by *A. saligna* (18.19 spores g^{-1}) from Batu. Thus, this result agrees with Zerihun Belay *et al.* (2013), where the highest average spore density and root colonization were observed under *A. seyal* from Batu. Similarly, the significantly highest average value of AMF spore density and root colonization after the trap culture was recorded from *A. seyal* (32.41 spores g^{-1} and 94%), respectively. Therefore, the propagules obtained from the rhizosphere soil of *A. seyal* from Batu were found to be the best source of AMF inoculums.

The current study was focused on evaluating the effects of single and co-inoculation of AMF (single and consortium) and a PGPR, *Bacillus subtilus* (ALCR46) strain, on the growth of selected vegetables (*Lycopersicum esculentum, Allium cepa*, and *Cucurbita pepo*) under low fertility of soil. Generally, the results showed that, except from the single inoculation of *Bacillus* (T5) in *Allium cepa* that did not show a significant difference, all the inoculation treatments of single and dual inoculation notably and significantly increased all growth parameters compared to non-inoculated vegetables (T6). Moreover, it was observed by Gupta *et al*. (2021) that the single application of *Bacillus licheniformis* and *Pseudomonas fluorescens* (PF) in *Allium cepa* had no significant effect on most of the leaf and root growth parameters and was inhibitory on bulb growth. The rhizospheric bacteria *Azotobacter chroococcum, Pseudomonas fluorescens,* and *Bacillus subtilis* enhanced the bulb diameter of onion, although not statistically significant (Čolo *et al*., 2014). Gupta *et al*. (2021), pointed out that the variable responses reported in different studies possibly due to the interactions between PGPR and plants not being stable in nature or to plant-PGPR specificity. The antimicrobial properties of *Allium cepa* in its root exudates, which may exist in the rhizosphere could probably suppress the strain's growth and colonization.

Moreover, this study shows that co-inoculation of consortium AMF and *Bacillus* (T2) significantly increased the growth of the seedlings with all growth parameters, which is statistically equivalent to the growth treated with chemical fertilizer (T7) in all vegetables (Table 4.3). This suggests the potential use of both AMF and PGPR as biofertilizers to enhance the growth of different vegetables instead of the application of chemical fertilizers. This finding is quite in agreement with the results reported by Sreedhar & Mohan, (2016),

who showed that co-inoculation of AMF and PGPR was not significantly different from those found in commercial chemical fertilizer. In addition, Khaekhum *et al*. (2021) also reported that the application of biofertilizer was more efficient than chemical fertilizers in improving plant growth. They evaluated the co-inoculation of an EPF, *Exserohilum rostratum* NMS1.5, and an AMF, *Glomus etunicatum* UDCN52867, on the growth and yields of sunchoke (*Helianthus tuberosus* L.) compared to the effects of full-dose and half-dose chemical fertilizer under field conditions and, concluded that the growth parameters of the co-inoculated plants were relatively equal to or even better than the chemical fertilizers.

Furthermore, in this study, all vegetable seedling growth showed significantly higher growth in the treatments of co-inoculation of consortium T2 compared to the single inoculations (T1, T2, and T3). This indicated the compatibility and synergy between them in enhancing the growth and biomass of the seedlings. Synergistic interaction between AMF and *Bacillus* spp. promoting plant growth compared to single inoculation with either of them has been reported elsewhere (Medina *et al*., 2003; Alam *et al*., 2011; Awasthi *et al*., 2011; Nacoon *et al*., 2021). A similar synergistic effect has been reported in a study done by Timonen *et al*. (2018) who evaluated the plant growth promoting effects of inoculating *Rhizophgus irregularis* and/or *Bacillus amyloliquefaciens* in autoclaved substrates. They found that dual inoculation with *B. amyloliquefaciens* and *R. irregularis* resulted in the greatest increase in shoot weight and photosynthetic efficiency compared to a single inoculation. Another study was undertaken by Awasthi *et al*. (2011) in India to determine the effect of arbuscular mycorrhizal (AM) fungi species and two free-living nitrogen-fixing bacteria (NFB) inoculated alone or in combinations. The plants inoculated with *G. mosseae* and *B. subtilis* performed better than any other treatment or uninoculated control plants. The results of the experiment clearly indicated the compatibility and synergy between the two strains in enhancing growth and biomass yield.

Roots of the seedlings treated with co-inoculation of AMF and PGPR were not only increased in height and weight but also showed significantly higher colonization by AMF and spore density than treated with only AMF, indicating that AMF infection in the soils was improved directly by the support of the PGPR. Singh *et al*. (2013) conducted a study on co-inoculation of a native *Pseudomonas monteilii* strain and the exotic AMF *Glomus fasciculatum* under organic field conditions in order to evaluate their potential for controlling root rot and wilt, and they found that co-inoculation of *P. monteilii* with *G. fasciculatum* significantly improved the percent of AM root colonization and spore density retrieved from

soil. Similar results obtained by Marulanda-Aguirre *et al*. (2008) showed *Bacillus megaterium* inoculated with *G. intraradices* increased the percentage of mycorrhizal root length of *Lactuca sativa* plants compared to the single inoculation of *G. intraradices*. Therefore, this finding indicates that *B. subtilus* (ALCR46 strain) not only acts as a specific combination (*B. subtilus* strain plus AMF*)* through increasing the growth of the crops, but also acts as "mycorrhiza helper bacteria" (MHB) through increased spore germination and root colonization. As cited by Ramasamy *et al*. (2011), MHB stimulates AM propagule germination, hyphal growth, and root colonization.

Likewise, the effect of a single inoculation of T1, T3, and T5 was found to have a significant increase in height, fresh and dry mass, compared to T6 (non-inoculated) in all vegetables. This agrees with the finding of Carrillo *et al*. (2014) who worked on the tomato hybrid 'El Cid' (*Lycopersicum esculentum*; Saladette) that *Rhizophagus intraradices* significantly increased the height of the plant, the content of chlorophyll, and the radicular mycorrhizal colonization, making an emphasis on the greater chlorophyll index (12.0%, $p \leq 0.01$) in comparison to the non-inoculated plants. Another aspect of the present study was to test if the mixtures of different AMF species improved the growth of the selected vegetables compared to one AMF species alone. In this case, in all selected vegetables, a superior response was obtained from consortium species of AMF (T1). Interestingly, although there was a significant difference between the co-inoculation treatments of dual inoculations of consortium of AMF and PGPR (T2) and a single species of AMF and a PGPR (T4), the growth response treated with T2 in most parameters was significantly greater than T4 in all vegetables. This phenomenon implies the existence of synergism within the AMF community colonizing a single root system. Similarly, it was shown that the inoculation of a woody shrub and herbaceous plant with an AM fungus, either *Funneliformis mosseae*, *Diversispora versiformis,* or *Glomus diaphanum,* or with an inoculum mixture of all three AM fungi, enhanced significantly the biomass, nitrogen, and phosphorus acquisition better than inoculation with only one (He *et al*., 2017). Additionally, Jansa *et al*. (2008) reported that, *Allium porrum* colonized by a mixture of AMF species acquired more P than with either of the two AMF species separately. Direct evidence is provided for functional complementarity among species within the AMF community colonizing a single root system. Moreover, inoculation with AMF consortia, which co-evolve in local ecological niches, is more robust and sustaining than inoculation with a single species (Szczałba *et al*., 2019).

In regards to evaluating the differences in AMF response (mycorrhizal dependency), root colonization, and spore density among the selected vegetables, the vegetables showed high growth response, root colonization, and spore density. The highest values were recorded in *Allium cepa* growing under T2 and followed by T4, T1, and T3 inoculations, respectively (Figure 4.2). Then, *Allium cepa,* followed by *Lycopersicum esculentum* and *Cucurbita pepo,* respectively. This phenomenon was also previously recorded by Hernandez *et al*. (2019), who showed a greater response on onion, melon, and tomato. The plants from the *Amaryllidaceae* (onion and leek), *Cucurbitaceae* (cucumber), and *Solanaceae* (tomato and bell pepper) families possess a high dependence on mycorrhizal colonization (Baum *et al*., 2015).

Mycorrhizal dependency is related in part to the morphology of the plant root system. Plants having extensive fibrous roots are often less dependent on mycorrhiza than those with less extensive root systems (Kahiluoto *et al*., 2001). Such plants are often obligate mycorrhizal crops that are unable to complete their life cycle in the absence of AMF because of insufficient P uptake and hence insufficient growth (Charron *et al*., 2001; Galván *et al*., 2011). *Allium* species, in particular *Allium cepa,* are regarded as highly AMF responsive plants because they have a coarse root system without root hairs (Portas, 1973; Plenchette *et al*., 1983). In this study, *Allium cepa* has been found to depend more on AMF than other plants due to their scarce radicular branching and lack of root hairs. There is also evidence that onion genetics influence the response to mycorrhizal symbiosis (Taylor *et al*., 2015). Mycorrhiza helps plants with such a shallow and sparse root system to increase phosphorus uptake.

Moreover, in this study all parameters were positively correlated with each other. And colonization by AMF had positive effects on the growth parameters of the selected vegetable seedlings and on spore density and mycorrhizal dependency. Similar effects were previously reported in other studies (Khakpour & Khara, 2012; Bhale, 2016; Chen *et al*., 2020; Pei *et al*., 2020). In addition, very strong positive correlations ($p<0.05$) in AMF root colonization with AMF spore density and mycorrhizal dependency of the vegetables were found. , This was also in agreement with previous report of Bhale, (2016) Where a Pearson correlation was studied between root colonization percentage and spore density in the rhizosphere soil, and it showed strong positive correlation in Chilli ($r = 0.799$) and Brinjal ($r = 0.899$). Khakpour & Khara, (2012) also reported that, there was positive correlation between percentage root colonization and spore number with correlation coefficient 0.73 in

Hyoscyamus niger L. of family *Solanaceae.* Comparable to other studies, this study also found that AMF colonization with total dry biomass, root height and shoot height were positively correlated (Figure. 4.4), indicating that likely this growth benefit is due to the large extracellular hyphae AMF network, which transports water and nutrients to roots, increasing their absorption range and helping plants in the low nutrient soil (Chen *et al*., 2020). This findings were quite in agreement with the results reported by Pei *et al*. (2020) who showed positive correlation of AMF colonization with Flavonoids and plant biomass in Chinese tallow tree (*Triadica sebifera*). They reported that the plant biomass and AMF colonization rate were positively correlated ($r = +0.15$, $P = 0.0346$).

6. CONCLUSION

In the present study, application of microbial inoculation containing both indigenous AMF and a PGPR, which were isolated from legumes, *Acacia* trees and check pea rhizosphere, respectively, showed better growth in all vegetables including *Allium cepa*, *Lycopersicum esculentum* and *Cucurbita pepo* tested on infertile soil. It is evident from the data that the combined application of AMF and PGPR has greatly improved seedling growth and biomass of the seedlings almost as equal to as the chemical fertilizer. This could be important to development of such a sustainable biofertilizer technology for maximum and environmentally friendly vegetable production. Therefore, from the present research, it could be concluded that application of both the AMF and PGPR inocula used in this experiment demonstrate the possibility of substituting chemical fertilizers with bio-fertilizers.

Moreover, the results showed that the co-inoculation of AMF and PGPR (AMF + *Bacillus* sp.) could significantly improve the growth of vegetables better than inoculation of AMF or a PGPR alone. In this regard, plants with co-inoculation had a significant increase in many parameters including height, total fresh and dry plant biomass. In addition, plants with co-inoculation also had a significant increase in root colonization and spore density. Therefore, this was evidence that a PGPR had synergistic effects on AMF development. Additionally, this study determined the influence of consortium species of AMF and single (*R. clarus*) inoculation on plant growth parameters, root colonization, and spore density of AMF. Therefore, the result of the study supports the conclusion that there is a synergistic effect within AMF species. Thus, inoculation of AMF species consortia usually provides higher beneficial effects than the single AMF species application. Moreover, it can be concluded that among the vegetable crops, *Allium cepa* was particularly reactive to soil mycorrhiza with high growth dependency, and AMF spore density and root colonization than *Lycopersicum esculentum* and *Cucurbita pepo*.

7. RECOMMENDATIONS

During this study, the potential for tolerance to environmental stress and pathogen resistance of these microbial inoculums was not evaluated. Therefore, it is necessary in the future to evaluate their effectiveness under different environmental conditions and against different plant pathogens, both in the glass house and in field trials. Moreover, the application of the inoculums showed increases in all growth parameters, however during this experiment no investigation was carried out regarding the effect of these inoculums in nutrient acquisition. Therefore, it should be determined what the potential of these inoculum on these parameters. Moreover, further study needs to be conducted to compare the effectiveness of these native inoculums with commercial microbial inoculants.

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9. APPENDIXCES

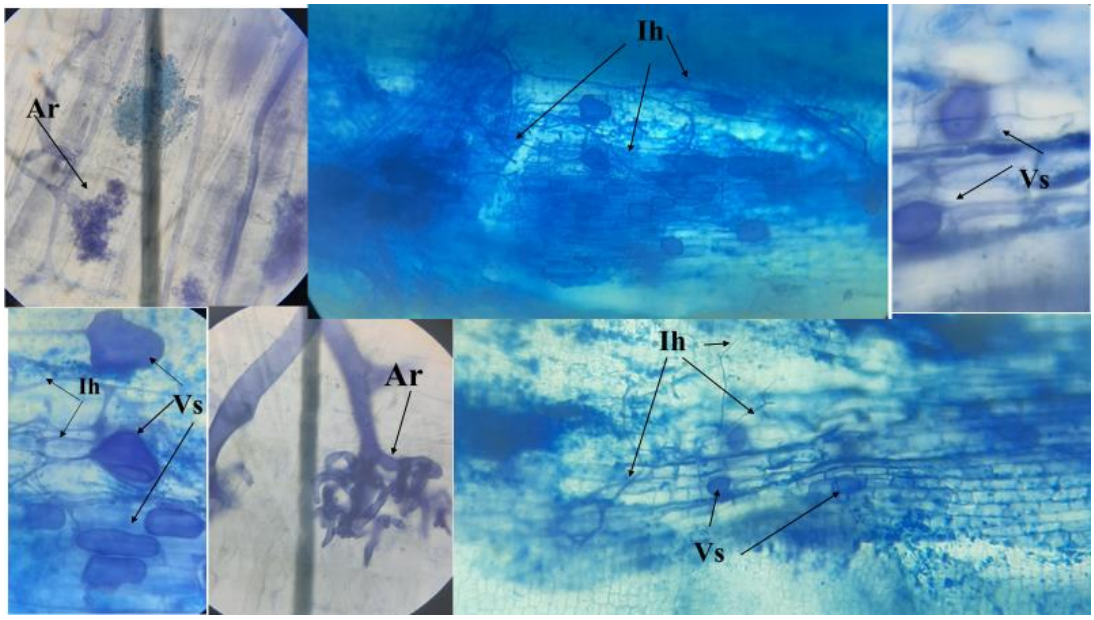
Appendix 1. Representative pictures to showing the growth response of *Solanum lycopersicum* to the different inoculums. 1 (Negative control), 2 (Positive control), 3 (+Rc), 4 (+Rc+Bs), 5 (+cAMF), 6 (+cAMF+Bs), 7 (+Bs and).

Appendix 2. Representative pictures to showing the growth response of *Allium cepa* to the different inoculums.

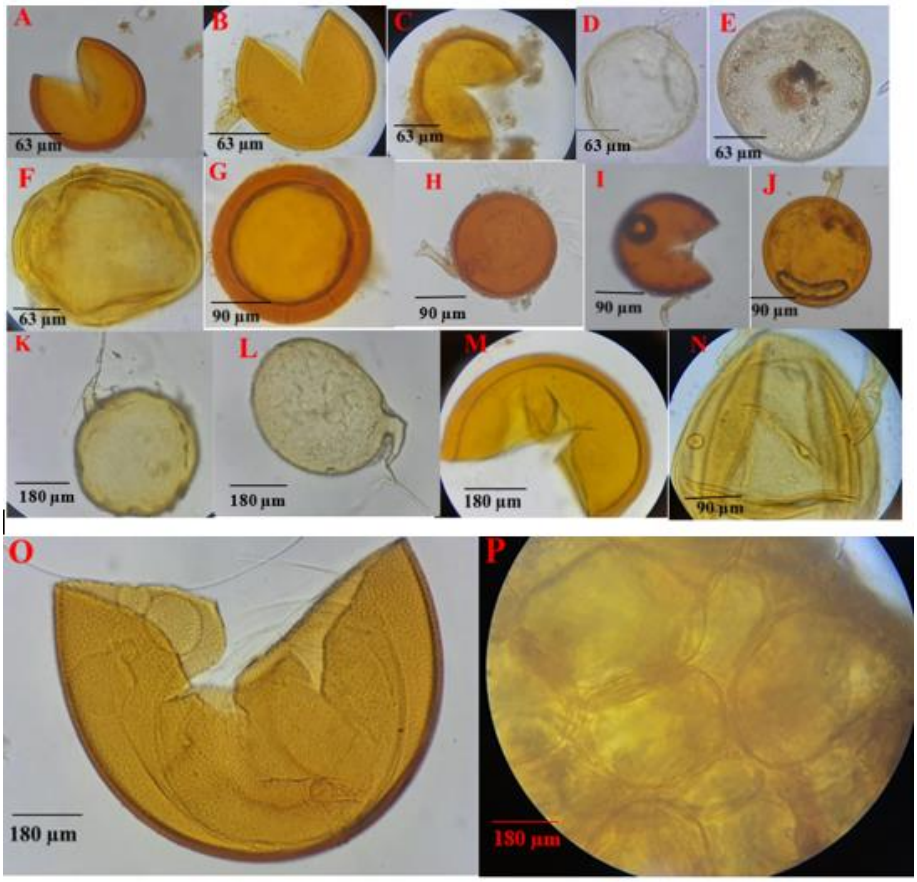
Appendix 3. Representative pictures to showing growth response of *Cucurbita sp.* to the different inoculums.

1 (Negative Control), **2** (+Bs), **3** (Positive Control), **4** (+cAMF+Bs), **5** (+cAMF), **6** (+Rc+Bs), and **7** (+Rc).

Appendix 4. Microscopic picture of root colonization. Intraradical hyphae (Ih), Arbuscule (Ar), and vesicles (Vs).



Appendix 5. Microscopic photos of some morphotypes extracted and identified spores from the consortium of AMF inoculum and the single AMF inoculum (*Rhizophagus clarus*). All photos are from slides made in PVLG.



A (*Claroideoglomus etunicatum*), **B** and **C** (*Claroideoglomus claroideum*), **D** (*paraglomus occultum*), **E** (*Acaulospora sp.*), **F** (*Rhizophagus aggregatus*), **G** (*Glomus sp.* (Sporcarpic

thick wall)), **H** and **I** (*Septoglomus constrictum*), **J** (*Funneliformis geosporum*), **K** and **L** *(Funneliformis mosseae*), **M** (*Gigaspora sp.*,) **N** (*Rhizophagus clarus*), **O** (*Acaulospora scrobiculata*/ *tuberculate*), **P** (*Sclerocystis sinuosum* (sporocarps, A dense layer of tightly interwoven hyphae, thick, covering all spores to keep them tightly packed)).

Appendix 6. Representative pictures to give further illustration of all practical works beginning from trap culturing to the end of laboratory session.

A (Trap culturing), **B** (Intation of sporylation), **C** (AMF inoculum storing), **D** (Pot setup before planting the vegetables), **E** (Growing of the vegetables), **F** (OD mesurment of the PGPR inoculum), **G** (Inoculation of PGPR), **H** and **I** (Root stainig), **J** (Estimation percent AM root colonization under a compound microscope), **K** (Centrifugation in

water and in a 60% sucrose solution), and **L** (Enumeration and extraction of AMF spore under stereomicroscope).

Appendix 7. One-way ANOVA result analysis output of the plant growth parameters in each vegetable from SPSS software. *Allium cepa*, *Cucurbita sp*., and *Solanum lycopersicum*, respectively.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Shoot height of Allium cepa after 2 monthsof growth time		5517.780	6	919.630	959.376	.000
		13.420	14	.959		
		5531.200	20			
Root height of Allium cepa after 2 months of growth time		380.632	6	63.439	199.135	.000
		4.460	14	.319		
		385.092	20			
Number of branch of Allium cepa after 2 months of growth time		21.238	6	3.540	14.867	.000
		3.333	14	.238		
		24.571	20			
Shoot fresh mass of Allium cepa after 2 monthsof growth time		710.627	6	118.438	336.603	.000
		4.926	14	.352		
		715.553	20			
Root fresh mass of Allium cepa after 2 months of growth time		10.132	6	1.689	135.655	.000
		.174	14	.012		
		10.306	20			
Total fresh mass of Allium cepa after 2 monthsof growth time		880.323	6	146.721	365.643	.000
		5.618	14	.401		
		885.941	20			
Shoot dry mass of Allium cepa after 2 monthsof growth time		6.548	6	1.091	1455.110	.000
		.010	14	.001		
		6.558	20			
Root dry mass of Allium cepa after 2 months of growth time		.012	6	.002	147.063	.000
		.000	14	.000		
		.013	20			
Total dry mass of Allium cepa after 2 months of growth time		7.124	6	1.187	1488.458	.000
		.011	14	.001		
		7.135	20			
Mycorrhizal dependency of Allium cepa after 2 months of growth time		48643.448	6	8107.241	65417.824	.000
		1.735	14	.124		
		48645.183	20			
Root colonization of Allium cepa after 2 months of growth time		25582.478	6	4263.746	7630.248	.000
		7.823	14	.559		
		25590.301	20			
Spore number of Allium cepa after 2 months of growth time		2084.525	6	347.421	425250.569	.000
		.011	14	.001		
		2084.537	20			

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Shoot height of Cucurbita after 2 monthsof growth time		1562.646	6	260.441	115.948	.000
		31.447	14	2.246		
		1594.092	20			
Root height of Cucurbita after 2 months of growth time		154.356	6	25.726	56.217	.000
		6.407	14	.458		
		160.763	20			
Number of branch of Cucurbita after 2 months of growth time		46.667	6	7.778	27.222	.000
		4.000	14	.286		
		50.667	20			
Shoot fresh mass of Cucurbita after 2 monthsof growth time		1795.359	6	299.227	71.398	.000
		58.673	14	4.191		
		1854.032	20			
Root fresh mass of Cucurbita after 2 months of growth time		54.918	6	9.153	94.120	.000
		1.361	14	.097		
		56.279	20			
Total fresh mass of Cucurbita after 2 monthsof growth time		2435.100	6	405.850	99.568	.000
		57.065	14	4.076		
		2492.165	20			
Shoot dry mass of Cucurbita after 2 monthsof growth time		340.126	6	56.688	2037.727	.000
		.389	14	.028		
		340.515	20			
Root dry mass of Cucurbita after 2 months of growth time		15.971	6	2.662	657.882	.000
		.057	14	.004		
		16.028	20			
Total dry mass of Cucurbita after 2 months of growth time		501.146	6	83.524	2907.763	.000
		.402	14	.029		
		501.548	20			
Mycorrhizal dependency of Cucurbita after 2 months of growth time		27651.124	6	4608.521	57588.248	.000
		1.120	14	.080		
		27652.244	20			
Root colonization of Cucurbita after 2 months of growth time		14908.801	6	2484.800	7842.492	.000
		4.436	14	.317		
		14913.237	20			
Spore number of Cucurbita after 2 months of growth time		412.671	6	68.779	496.678	.000
		1.939	14	.138		
		414.610	20			

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Shoot height of Solanum lycopersicum after 2 monthsof growth time		2632.230	6	438.705	137.034	.000
		44.820	14	3.201		
		2677.050	20			
Root height of Solanum lycopersicum after 2 months of growth time		645.979	6	107.663	263.819	.000
		5.713	14	.408		
		651.692	20			
Number of branch of Solanum lycopersicum after 2 months of growth time		41.619	6	6.937	29.133	.000
		3.333	14	.238		
		44.952	20			
Shoot fresh mass of Solanum lycopersicum after 2 monthsof growth time		1470.449	6	245.075	459.347	.000
		7.469	14	.534		
		1477.918	20			
Root fresh mass of Solanum lycopersicum after 2 months of growth time		113.664	6	18.944	189.539	.000
		1.399	14	.100		
		115.063	20			
Total fresh mass of Solanum lycopersicum after 2 monthsof growth time		2398.580	6	399.763	579.727	.000
		9.654	14	.690		
		2408.234	20			
Shoot dry mass of Solanum lycopersicum after 2 monthsof growth time		395.818	6	65.970	1042.803	.000
		.886	14	.063		
		396.704	20			
Root dry mass of Solanum lycopersicum after 2 months of growth time		22.586	6	3.764	346.567	.000
		.152	14	.011		
		22.738	20			
Total dry mass of Solanum lycopersicum after 2 months of growth time		605.728	6	100.955	1064.869	.000
		1.327	14	.095		
		607.055	20			
Mycorrhizal dependency of Solanum lycopersicum after 2 months of growth time		41958.488	6	6993.081	72937.354	.000
		1.342	14	.096		
		41959.830	20			
Root colonization of Solanum lycopersicum after 2 months of growth time		20494.178	6	3415.696	8286.024	.000
		5.771	14	.412		
		20499.949	20			
Spore number of Solanum lycopersicum after 2 months of growth time		1382.846	6	230.474	65444.528	.000
		.049	14	.004		
		1382.896	20			

c

Appendix 8. Two-way ANOVA result analysis output of the mycorrhizal dependency, hyphal root colonization, and spore density using crop and treatment as factors in order to test for interactions.

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Mycorrhizal Dependency	4893.824[a]	11	444.893	2543.659	.000
	Root colonization	7495.084[b]	11	681.371	906.983	.000
	Spore density	1238.471[c]	11	112.588	705.510	.000
Intercept	Mycorrhizal Dependency	269770.366	1	269770.366	1542401.292	.000
	Root colonization	127275.319	1	127275.319	169418.062	.000
	Spore density	6729.536	1	6729.536	42169.178	.000
crop	Mycorrhizal Dependency	3949.112	2	1974.556	11289.446	.000
	Root colonization	1848.336	2	924.168	1230.174	.000
	Spore density	517.587	2	258.794	1621.675	.000
Treatment	Mycorrhizal Dependency	641.123	3	213.708	1221.864	.000
	Root colonization	5612.206	3	1870.735	2490.163	.000
	Spore density	596.315	3	198.772	1245.560	.000
crop * Treatment	Mycorrhizal Dependency	303.590	6	50.598	289.293	.000
	Root colonization	34.542	6	5.757	7.663	.000
	Spore density	124.569	6	20.761	130.097	.000
Error	Mycorrhizal Dependency	4.198	24	.175		
	Root colonization	18.030	24	.751		
	Spore density	3.830	24	.160		
Total	Mycorrhizal Dependency	274668.388	36			
	Root colonization	134788.433	36			
	Spore density	7971.837	36			
Corrected Total	Mycorrhizal Dependency	4898.022	35			
	Root colonization	7513.114	35			
	Spore density	1242.301	35			

a. R Squared = .999 (Adjusted R Squared = .999)

b. R Squared = .998 (Adjusted R Squared = .997)

c. R Squared = .997 (Adjusted R Squared = .996)

Appendix 9. Output SPSS data analysis with Pearson's correlation coefficients between the parameters. a) *Solanum lycopersicum*, b) *Cucurbita sp.*, c) *Allium cepa*, and d) considering all the vegetables.

Correlations

		SH	RH	NB	SDW	RDW	TDW	RC	SD
SH	Pearson Correlation	1	.959**	.958**	.905**	.899**	.906**	.475*	.489*
	Sig. (2-tailed)		.000	.000	.000	.000	.000	.030	.024
	N	21	21	21	21	21	21	21	21
RH	Pearson Correlation	.959**	1	.930**	.975**	.972**	.976**	.465*	.500*
	Sig. (2-tailed)	.000		.000	.000	.000	.000	.034	.021
	N	21	21	21	21	21	21	21	21
NB	Pearson Correlation	.958**	.930**	1	.893**	.884**	.893**	.487*	.509*
	Sig. (2-tailed)	.000	.000		.000	.000	.000	.025	.019
	N	21	21	21	21	21	21	21	21
SDW	Pearson Correlation	.905**	.975**	.893**	1	.988**	1.000**	.457*	.522*
	Sig. (2-tailed)	.000	.000	.000		.000	.000	.037	.015
	N	21	21	21	21	21	21	21	21
RDW	Pearson Correlation	.899**	.972**	.884**	.988**	1	.992**	.473*	.528*
	Sig. (2-tailed)	.000	.000	.000	.000		.000	.030	.014
	N	21	21	21	21	21	21	21	21
TDW	Pearson Correlation	.906**	.976**	.893**	1.000**	.992**	1	.461*	.524*
	Sig. (2-tailed)	.000	.000	.000	.000	.000		.035	.015
	N	21	21	21	21	21	21	21	21
RC	Pearson Correlation	.475*	.465*	.487*	.457*	.473*	.461*	1	.978**
	Sig. (2-tailed)	.030	.034	.025	.037	.030	.035		.000
	N	21	21	21	21	21	21	21	21
SD	Pearson Correlation	.489*	.500*	.509*	.522*	.528*	.524*	.978**	1
	Sig. (2-tailed)	.024	.021	.019	.015	.014	.015	.000	
	N	21	21	21	21	21	21	21	21

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

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

Correlations

		SH	RH	NB	SDW	RDW	TDW	RC	SD
SH	Pearson Correlation	1	.991**	.877**	.952**	.949**	.953**	.574**	.556**
	Sig. (2-tailed)		.000	.000	.000	.000	.000	.006	.009
	N	21	21	21	21	21	21	21	21
RH	Pearson Correlation	.991**	1	.876**	.947**	.944**	.947**	.618**	.599**
	Sig. (2-tailed)	.000		.000	.000	.000	.000	.003	.004
	N	21	21	21	21	21	21	21	21
NB	Pearson Correlation	.877**	.876**	1	.890**	.878**	.890**	.501*	.544*
	Sig. (2-tailed)	.000	.000		.000	.000	.000	.021	.011
	N	21	21	21	21	21	21	21	21
SDW	Pearson Correlation	.952**	.947**	.890**	1	.981**	1.000**	.456*	.536*
	Sig. (2-tailed)	.000	.000	.000		.000	.000	.038	.012
	N	21	21	21	21	21	21	21	21
RDW	Pearson Correlation	.949**	.944**	.878**	.981**	1	.982**	.506*	.593**
	Sig. (2-tailed)	.000	.000	.000	.000		.000	.019	.005
	N	21	21	21	21	21	21	21	21
TDW	Pearson Correlation	.953**	.947**	.890**	1.000**	.982**	1	.458*	.538*
	Sig. (2-tailed)	.000	.000	.000	.000	.000		.037	.012
	N	21	21	21	21	21	21	21	21
RC	Pearson Correlation	.574**	.618**	.501*	.456*	.506*	.458*	1	.930**
	Sig. (2-tailed)	.006	.003	.021	.038	.019	.037		.000
	N	21	21	21	21	21	21	21	21
SD	Pearson Correlation	.556**	.599**	.544*	.536*	.593**	.538*	.930**	1
	Sig. (2-tailed)	.009	.004	.011	.012	.005	.012	.000	
	N	21	21	21	21	21	21	21	21

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

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

b

Correlations

		SH	RH	NB	SDW	RDW	TDW	RC	SD
SH	Pearson Correlation	1	.991**	.877**	.952**	.949**	.953**	.574**	.556**
	Sig. (2-tailed)		.000	.000	.000	.000	.000	.006	.009
	N	21	21	21	21	21	21	21	21
RH	Pearson Correlation	.991**	1	.876**	.947**	.944**	.947**	.618**	.599**
	Sig. (2-tailed)	.000		.000	.000	.000	.000	.003	.004
	N	21	21	21	21	21	21	21	21
NB	Pearson Correlation	.877**	.876**	1	.890**	.878**	.890**	.501*	.544*
	Sig. (2-tailed)	.000	.000		.000	.000	.000	.021	.011
	N	21	21	21	21	21	21	21	21
SDW	Pearson Correlation	.952**	.947**	.890**	1	.981**	1.000**	.456*	.536*
	Sig. (2-tailed)	.000	.000	.000		.000	.000	.038	.012
	N	21	21	21	21	21	21	21	21
RDW	Pearson Correlation	.949**	.944**	.878**	.981**	1	.982**	.506*	.593**
	Sig. (2-tailed)	.000	.000	.000	.000		.000	.019	.005
	N	21	21	21	21	21	21	21	21
TDW	Pearson Correlation	.953**	.947**	.890**	1.000**	.982**	1	.458*	.538*
	Sig. (2-tailed)	.000	.000	.000	.000	.000		.037	.012
	N	21	21	21	21	21	21	21	21
RC	Pearson Correlation	.574**	.618**	.501*	.456*	.506*	.458*	1	.930**
	Sig. (2-tailed)	.006	.003	.021	.038	.019	.037		.000
	N	21	21	21	21	21	21	21	21
SD	Pearson Correlation	.556**	.599**	.544*	.536*	.593**	.538*	.930**	1
	Sig. (2-tailed)	.009	.004	.011	.012	.005	.012	.000	
	N	21	21	21	21	21	21	21	21

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

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

c

Correlations

		Root colonization	Spore density	Mycorrhizal Dependency
Root colonization	Pearson Correlation	1	.882**	.745**
	Sig. (2-tailed)		.000	.000
	N	36	36	36
Spore density	Pearson Correlation	.882**	1	.744**
	Sig. (2-tailed)	.000		.000
	N	36	36	36
Mycorrhizal Dependency	Pearson Correlation	.745**	.744**	1
	Sig. (2-tailed)	.000	.000	
	N	36	36	36

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

d