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ANTAGONISTIC EFFECTS OF *TRICHODERMA* SPP. AGAINST DAMPING OFF DISEASE OF HARICOT BEAN (*PHASEOLUS VULGARIS* L.), JIMMA ZONE, SOUTHWESTERN ETHIOPIA

BY

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A THESIS SUBMITTED TO DEPARTMENT OF BIOLOGY, COLLEGE OF NATURAL SCIENCES, JIMMA UNIVERSITY; IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTERS OF SCIENCE IN BIOLOGY (APPLIED MICROBIOLOGY)

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
BCA	Biological control agent
CIAT	International Center for Tropical Agriculture
IAR	Institute for Agricultural Research
masl	Meter above sea level
MEA	Malt Extract Agar
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PI	Percent Inhibition
SSF	Solid state fermentation
USDA	United State Department of Agriculture

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ABSTRACT

Rhizoctonia and Fusarium spp. are the common causative agents of the damping off disease of haricot bean (Phaseolus vulgaris L.). Trichoderma species are among the most important antagonists of plant pathogenic fungi. The antagonistic effects of two potential Trichoderma spp. (local and standard) were evaluated against damping off disease of haricot bean caused by Rhizoctonia and Fusarium spp. under in-vitro and greenhouse conditions. This study was carried out with the major objective of evaluating antagonistic effects of two Trichoderma isolates against damping off fungi isolated from diseased crops. Haricot bean fungal pathogens were isolated from 120 (30 each) apparently diseased faba bean, haricot bean, tomato and potato crops. Potato Dextrose Agar was used for fungal isolation following standard procedures. A total of 279 fungal pathogens were isolated from the four different types of diseased crops, out of which 215 (77.06%) isolates were identified to the genus level. Fifty three local Trichoderma isolates were investigated for their antagonistic properties against the test pathogens by dual culture techniques. The results showed that all of the isolates of Trichoderma inhibited the growth of the fungal pathogens with percentage of radial growth inhibition values ranging from 26.57 to 67.03%. In greenhouse experiment, Trichoderma isolates JUTr012+ standard fungus (T. harzianum) significantly ($p<0.05$) reduced the damping off disease incidence under greenhouse condition. Under greenhouse experiment, the highest number of leaves (25.30 leaves plant^{-1}), number of branch (9.67 plant^{-1}) and root fresh weight (17.52 g plant^{-1}) were recorded due to inoculation with isolates JUTr012+ T. harzianum compared to the control. Moreover, shoot height (58.59 cm plant^{-1}), root length (53 cm plant^{-1}), nodule number (206.89 plant^{-1}), nodule dry weight (0.59 g plant^{-1}), shoot fresh weight (89.53 g plant^{-1}), shoot dry weight (9.01 g plant^{-1}), number of pod (28.33 plant^{-1}), and seed weight (38.78 g plant^{-1}), were significantly($p<0.05$) increased in the presence of bio-control agents (JUTr012+ T. harzianum) compared to the control. The results of this study indicate that the possible use of Trichoderma spp. as a supplementary fungal disease (damping off) management option. Further studies under field conditions and other tests are also required to qualify the bio-control agents as potential inoculum in low inputs sustainable agriculture.

Keywords /phrases: Antagonistic effect, Bio-control agent, Damping off disease, Haricot bean, *Trichoderma* species

1. INTRODUCTION

Haricot bean (*Phaseolus vulgaris* L.) is a herbaceous annual plant domesticated independently in ancient Mesoamerica and the Andens (Ferris and Kaganzi, 2008). This crop is now cultivated worldwide as edible bean. It is an annual pulse crop with considerable variation in habit, vegetative characters, flower color and the size, shape and color of the pods and seeds (Onwueme and Sinha, 1991).

According to Ferris and Kaganzi (2008), haricot bean is rapidly evolving from a traditional subsistence to a market oriented crop with major impacts on household food, nutritional security and income with significant contribution to national economy. This crop has improved standard of living in some of the most marginalized communities in Africa. It continues to be the most valuable crop that widely grown and consumed grain legume in Eastern, Central and Southern Africa particularly in medium elevation and highland areas (Alemitu, 2011). The crop has been grown on about 4.5 million hectares annually by resource poor farmers particularly women (Belay, 2004). Haricot bean is the most preferred grain legume due to its short maturity period (≤ three months) and high nutritive value (Simane *et al.,* 1998; Ferris and Kaganzi, 2008).

Haricot bean is one of the most important food legumes of Ethiopia and is also considered as the main cash crop (IAR, 1995; EEPA, 2004). It is the least expensive source of protein for the farmers in many lowlands and mid altitude of the country. It is one of the most important food legumes for direct human consumption (Abd-El-Khair *et al*., 2010; Mahamune and Kakde, 2011). In addition, the crop helps to improve soil fertility through nitrogen fixation. Therefore, improving the productivity of this crop is one of the major objectives in agriculture in many countries of the globe (Boubekeur *et al*., 2012). Haricot beans are grown all over Ethiopia and are increasingly becoming important commodity in the cropping system of smallholder producers for their income and food security. The central part of the country, mainly the Rift Valley and lake areas rank first in hectare (48%) and production (55%) of low land pulses in Ethiopia (Alemitu, 2011).

Low productivity characterizes Ethiopian agriculture (Belay, 2004). The soil-borne pathogenic fungi have a broad host range and they also persist for longer periods in soil by means of

resistant resting structures. The fungal pathogens are the main causal organisms of many plant diseases and cause subsequent loss in the crop yields (Guleria *et al*., 2007; Haggag and El-Gamal, 2012; Subash *et al*., 2013) and productivity especially in subtropical and tropical regions (Gomathi and Ambikapathy, 2011). *Rhizoctonia* root-rot, caused by *R. solani,* is the commonest disease throughout the world (Vinale *et al.,* 2008). It is one of the most economically important root diseases of haricot beans. It has a broad host range that include most annual and many perennial plants. *Rhizoctonia* generally survives as sclerotia on crops or fungal mycelia in the soil. The young plants are more susceptible compared to older ones. Similarly, *Fusarium* root-rot to beans is caused by the fungus *F. solani* f. sp. *phaseoli.* The fungus can attack older seedlings, and causes severe disease on plants growing under stress conditions. The pathogen usually survives as thick-walled chlamydospores in soil (Abd-El-Khair *et al*., 2010).

According to Dev and Dawande (2010), soil-borne pathogens are complex not only in their behavioral pattern but also in their biochemical constituents. Hence, it is not very easy to control these pathogens. Understanding and dealing with soil-borne pathogens is a very difficult and challenging task. At present, effective management of plant diseases in several agricultural systems is generally achieved by the use of synthetic fungicides. However, the incessant and indiscriminate application of these chemical fungicides has caused health hazards in animals and humans due to residual toxicity necessitating for supplementary control agents (Rini and Sulochana, 2006). Various practices like use of resistant varieties, chemical control, cultural practices, plant volatile compounds, plant extracts and biological control have been adopted for the control of these fungal pathogens. Many researchers have demonstrated the potential of *Trichoderma* spp. in controlling damping off and wilt diseases of crop plants caused by *Rhizoctonia solani* and *Fusarium* spp. (Rojo *et al*., 2007; Abd-El-Khair *et al*., 2010).

Recently, the application of biological control agents (BCAs) in agriculture has gained popularity as a way to reduce or eliminate the use of synthetic pesticides (Vinale *et al*., 2008). The use of biological control against soil-borne pathogens such as *Rhizoctonia* and *Fusarium* spp.(Gohel *et al*., 2006; Dolatabadi *et al*.,2011) is more economical, self perpetuating and usually free from residual side effects (Pallavi *et al*., 2012). *Trichoderma* spp. is well recognized as effective biological control agents of plant diseases caused by soil-borne fungi (Abd-El-Khair *et al.,* 2010). *Trichoderma* spp. is common saprophytic fungi which are found in almost any soil

and plant rhizosphere. They have been investigated as potential biocontrol agents because of their ability to reduce the incidence of disease caused by plant pathogenic fungi, particularly many common soil-borne pathogens (Perveen and Bokhari, 2012).

Trichoderma species are among the most frequently isolated soil fungi and present in plant root ecosystems (Harman *et al*., 2004). The fungi are opportunistic, avirulent, plant symbionts and function as parasites and antagonists of many phytopathogenic fungi, thus protecting plants from diseases. *T. harizanum* reduced the mycelial growth of two (*F. solani* and *R. solani*) pathogenic fungi from 48.8 to 76.7%, while *T. viride* reduced the mycelial growth of the same fungi from 27.7 to 82.2 % (Haggag and El-Gamal, 2012). At present, *Trichoderma* spp. are among the most studied fungal biocontrol agents and commercially marketed as a potent biopesticides, biofertilizers and also used in soil amendments (Ramanujam *et al*., 2010; Sargin *et al*., 2013). For instance, Lo and Lin (2002) reported in cucumber a significant increase of plant growth treated with *T. harizianum* strain BR-61 and *T .virens* strain PR- 42 on the measured parameters (plant height, leaf area and plant root dry weight) when compared with untreated cucumber seedlings. Particularly, the seedling of cucumber treated with *T. harizianum* strain BR-61 and *T. virens* strain PR- 42 gave 1.4 fold heights compared to the non- treated seedlings 15 days after sowing.

A few number of research activities were done using *Trichoderma* spp. on the bio-control aspects of damping off disease in Ethiopia and still pertinent data are lacking on haricot bean crop. Currently, in developing countries including Ethiopia, there is an increasing interest and experience in the use of different types of bio-agents for the control of soil-borne fungal pathogens and storage insect pests because of drawbacks of conventional control measures. Therefore, this study was initiated to investigate the effects of bio-control agents against damping off disease of haricot bean under different conditions. As a component of integrated disease management strategy, selecting potential *Trichoderma* isolates with multiple beneficial traits for production of haricot bean has a paramount importance.

2. OBJECTIVES OF THE STUDY

2.1 General Objective

The general objective of this study was to evaluate antagonistic effects of *Trichoderma* spp. against soil-borne fungal pathogens.

2.2 Specific Objectives

- To isolate and characterize damping off fungi and *Trichoderma* spp. from different crops (faba bean, haricot bean, potato and tomato)
- To evaluate *in-vitro* and *in-vivo* antagonistic effects of *Trichoderma* spp. against haricot bean fungal pathogens, viz, *Fusarium solani* and *Rizoctonia solani*
- To evaluate damping off disease incidence and percentages of survival bean plants under greenhouse conditions
- To determine the effects of bio-control agents on growth parameters and yield of haricot bean plants in the presence of the fungal pathogens under *in-vivo* conditions

3. REVIEW OF LITERATURE

3.1 The haricot bean crop

Haricot bean (*Phaseolus vulgaris* L.) belongs to order Rosales, family Leguminosae subfamily Papilionideae (CIAT, 1986). The common bean was originated in Tropical America (Mexico, Guatemala, and Peru), but there are also evidences for its multiple domestication within Central America. The crop is now widely distributed throughout the world and consequently, it is grown in all continents except Antarctica (Singh, 1999).

According to Abd-El-Khair *et al.* (2010), haricot bean is a herbaceous annual plant domesticated independently in ancient Mesoamerica and the Andens, and now grown worldwide for its edible bean, popular both dry and as a green bean (Mahamune and Kakde, 2011). It is one of the most important food legumes for direct human consumption in the world. Haricot bean is considered as the main cash crop and protein source of the farmers in many low lands and mid altitude zones of Ethiopia (Negash, 2007).

3.2 Haricot bean production and its economic importance in Ethiopia

Haricot beans (*P. vulgaris*) are one of the most important grain legumes grown in most part of Ethiopia especially in the lowlands and in the Rift valley. They are produced primarily by small-scale farmers both for cash and subsistence. It has been cultivated for nutrition, food security and income source (Simane *et al.,* 1998; EARO, 2000). During, 2002, Ethiopia exported 42,127 tons of haricot beans and generated income of 13.2 million USD and it gradually increased up to 70,350-78,271 tons during 2007-2008 generated 32 to 49.7 million USD. The production of haricot bean increased significantly during 2003-2008 up to 24.5% (Ferris and Kaganzi, 2008).

According to Simane *et al.* (1998), haricot bean production is very varied in terms of yield, ecology and cropping system pattern. It predominantly grows from low land (300-1100 masl) to mid highland areas (1400-2000 masl) of the country. The national average yield of haricot bean is 0.5-0.8 tons per hectare. This common bean is one of the principal food and cash crop legume grown in the tropical world and it is widely grown in developing countries, particularly in Africa, India, Latin America and Mexico (Shimelis, 2005). Africa is considered to be a secondary center for bean genetic diversity. Beans are a major staple in Eastern, Southern and Great Lakes of

Africa, where they are the most important source of dietary protein and third most important source of calories after maize and cassava. The major haricot bean producing countries of Africa are Kenya, Tanzania, Malawi, Uganda, Zambia and Ethiopia (Shimelis, 2005; Hillocks *et al.*, 2006).

Haricot bean production has been practiced in all regions of the country. Oromia, Amhara, SNNPR, Benishangul-Gumuz and Tigray are the major producing regions that contribute more than 99 percent of the total haricot bean production. These are the major surplus producing areas of the crop. Almost all the export haricot beans are collected from these areas. Haricot bean production is increasingly becoming important commodities in the cropping system of smallholder producers for food security and income (Table 1).

Table 1. Production and forecast of haricot bean in Ethiopia (Source: Derese, 2012)

Production	**Amount**		
	2010	**2011**	**2012(forecasted)**
Area harvested(Ha)	244,012.88	237,366.39	346,662.16
Yield(Qtl/Ha)	14.87	14.34	13.77
Production(Qtls)	3,628,903	3,402,814	4,774,002

Around 30 species of grain legumes are grown both in the lowland and medium altitude areas of Ethiopia ranging from 700-2000 m above sea level. Among these, haricot bean is the most important food legume either as source of protein for local consumption or as an export crop for generating foreign currency (Tadele, 2006). Haricot bean is grown predominantly in low land area (300-1100m) and some mid highland areas (1400-2000m) of the country. Nationally, area under haricot bean production is estimated at 300-500 thousand hectares (IAR, 1995). Haricot bean has a wide range of adaptation and its production is very heterogeneous in terms of ecology, cropping system and agronomic performance. Under Ethiopian situation, the primary producers of haricot bean in Ethiopia lie between altitudinal ranges of 1400 to 2000 m for 9 rain fed agriculture and 700 m and beyond for irrigated agriculture. It also requires the minimum and maximum temperature of $10^{o}C$ and $32^{o}C$, respectively, and an annual rainfall distribution of 200 to 600 mm and relative humidity of 15% (Belay *et al.*, 1998; EARO, 2000).

There are three main types of haricot bean grown in Ethiopia. These can be distinguished on the basis of their color as red, speckled beans and white beans and further these can be sub classified according to their size. The red beans were grown by poor farmers for food security mainly in the area southern Rift Valley while, the white beans are produced almost exclusively for the export market in central eastern Rift Valley (Ferris and Kaganzi, 2008).

In Ethiopia, red beans are preferred by rural consumers, and there is a wide range of reds, red mottled varieties that are produced and sold in the rural markets. White beans are sold almost exclusively for the export markets; the leading white bean varieties include Awash 1, Awash Melka and Mexican 142, all of which are small white beans (Ferris and Kaganzi, 2008). The white beans are often referred to as white pea beans, due to their small size and round shape; they are otherwise known as navy beans (Figure 1).

Figure 1. Main commercial bean types grown in Ethiopia (Source: Ferris and Kaganzi, 2008).

3 .2.1 Nutritional status of haricot bean

Grain legumes are major sources of dietary proteins in the developing countries, as animal proteins are expensive. As source of food, it is extensively consumed in traditional dishes, and being part of the diet of the farming households, it serves as a source of protein to supplement the protein deficient main dishes like maize and enset in the southern parts of our country especially in Wolaita and Sidamo areas (Tenaw and Yeshi, 1990).They are considered as "poor man's meat" and are important inexpensive sources of protein, dietary fiber and starch (Perla *et al.*, 2003). In addition to their protein contributions, legumes are also rich in other nutrients such as starch, dietary fiber, oil, vitamins and mineral elements. Legumes contain about 60% carbohydrate (Fig.2) including starch, reducing and non reducing sugars, oligosaccharides of the raffinose family, etc. Among the commonly consumed food legumes, haricot bean is the most widely produced and consumed legume in the world and occupies an important place in human nutrition in the East and Great Lakes Regions of Africa by improving the nutritional status of many low income populations (Shimelis, 2005). They contain almost 2 or 3 times more protein than cereals. Because of their high protein and lysine content, they also represent good sources of supplementary protein when added to cereal grains and root crops, which are low in essential amino acids. In addition to their food value, pulses also play an important role in cropping systems because of their ability to fix nitrogen and thereby enrich the soil fertility (Deshpande *et al.*, 2000 and Perla *et al.*, 2003). Broughton *et al.* (2003) also suggested that bean is very important as a human food, animal feed and its beneficial effects in improving the soil fertility. The crop contains high protein content; is a good source of energy and provides folic acid, dietary fiber and complex carbohydrates. It is high in lysine, which is relatively deficient in maize, cassava and rice, making it a good complement to these staples in the diet (Gichangi *et al.,* 2012). Their role in reducing blood cholesterol level and combating chronic heart diseases, cancers and diabetics is also gaining recognition from human health point of view (Singh, 1999).

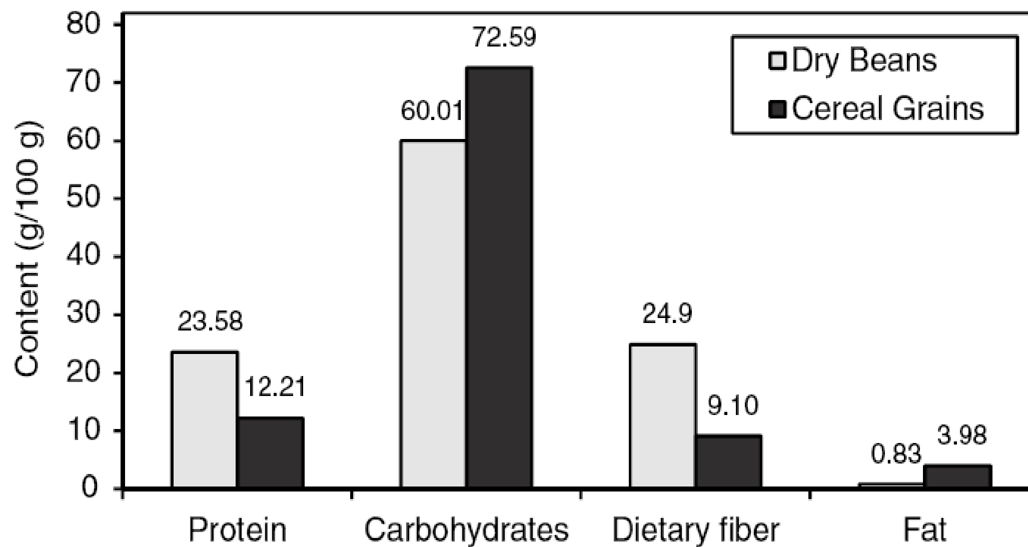


Figure 2. Nutritional benefits of dry beans versus cereal grains (Adapted from USDA, 2012)

3.3 Major constraints to haricot bean production

Bean is primarily grown by small-scale farmers who have limited resources and usually produce the crop under adverse conditions such as poor agronomic practices, low input use, marginal land, intercropping with competitive crops, low soil fertility, periodic water stress, weed competition and damage caused by diseases and insect pests (Allen *et al*., 1989; Allen and Edje, 1990; Wachenje, 2002). Major abiotic constraints include nitrogen and phosphorus deficiency, low pH complex and drought. Severity of root rots and bean stem maggot is aggravated by certain abiotic stresses. Bad weather, lack of improved cultivars and clean planting seeds are also major contributing factors to low yields (Fininsa and Yuen, 2002).Continuous cropping characterized by minimal or no rotation has led to a decline in soil fertility concomitant with an increase in pest and disease pressure.

Fungal and bacterial diseases are among the main production constraints in the major bean growing areas of the country (Fininsa and Yuen, 2002). The effect of diseases may be restricted to certain production systems, locations and cropping seasons (Opio *et al*., 2001). Among the listed disease of beans in Ethiopia, angular leaf spot (caused by *Phaeoisariopsis griseola*), common bacterial blight *(Xanthomonas campestris* pv. *Phaseoli*), anthracnose (*Collectotrichum*

lindemuthianum) and damping off (*Fusarium and Rhizoctonia solani*) are economically important (Allen *et al*., 1996; Fininsa and Yuen, 2002; Lemessa *et al*., 2011).

3.4 Diseases of haricot bean

Soil-borne pathogens cause important yield losses, but fungi are the most aggressive soil microbes (Haggag, 2011). Some of the important soil borne plant pathogens such as *Rhizoctonia* spp., *Fusarium* spp., *Pythium*, *Phytopthora*, and *Botrytis* have spread very fast and have detrimental effects on crops like haricot bean having high economic importance (Mohiddin *et al.,* 2010). Plant disease continues to threaten crop production in modern agriculture and plays a direct role in the destruction of natural resources in agriculture.

3.5 Extent of haricot bean yield loss in Ethiopia

About 90 percent of the country's agricultural output is generated by subsistence farmers who use traditional tools and farming practices (Belay, 2004). Low productivity characterizes Ethiopian agriculture. The average grain yield for various crops is only about one metric ton per hectare (Byerlee *et al*., 2007).

Disease has been one of the major contributors to low productivity and hence production of dry beans in many sub-Saharan countries (Allen *et al.,* 1989). Common beans are susceptible to a number of pests and disease constraints, but farmers and traders were of the view that beans are fairly robust. In Ethiopia, the crop suffers from few field diseases, unless there are exceptionally high rains, which leads to root rots. Farmers do not spray beans, and protection costs are mainly to avoid losses caused by bird damage.

Among the listed disease of beans in Ethiopia, common bacterial blight, rust, anthracnose, damping off and angular leaf spot are economically important (Fininsa and Yuen, 2002; Lemessa *et al*., 2011). According to Fininsa and Yuen (2002), bean root rots adversely affected bean production in Western Kenya. This is particularly in areas where soil fertility is low and bean production is intensive with two plantings in a year. Root rot has been observed to be serious under similar conditions in parts of Burundi, Rwanda, Central Kenya, Uganda and Zaire (CIAT, 1992). Root rots attack bean plant at all stages of growth causing damping off at seedling stage, yellowing of leaves, stunted growth and death of plant. The small land parcels on which beans

are grown do not allow for crop rotation. Small-scale farmers are also reluctant to reduce the frequency of growing beans on a piece of land and this may lead to a buildup of the root rot pathogens.

3.6 Disease management

Different management options have been developed to reduce the yield losses by fungal diseases. These include the use of chemical fungicides, introducing resistant/ tolerant varieties, use of certain cultural practices such as crop residue management and altering planting date (Dereje, 1999; Yonas, 2010).

Plant diseases may be managed by growing resistant plant varieties, planting pathogen-free seed or stock, applying a biological control agent, modifying environmental conditions to decrease disease, and using plant medicines that inhibit or kill the pathogen without harming the plant or the environment. Using disease resistance varieties, clean seed and intercropping are some of the control measure for haricot bean diseases (Flood and Brayford, 1997).

3.6.1 Cultural practices

Systematic elimination of affected plants over vast areas combined with the development of breeding programmes effectively reduced its impact (Sahile *et al*., 2011). Affected beans and tomatoes adjacent to affected plants should also be uprooted and burnt although appear healthy because while symptoms of the disease may not be visible, the fungus may be inside the plant (Rutherford, 2006). When symptoms are recognized quickly and uprooting and burning done efficiently, the farmers may save some of the crops. If the farmers delay, the infected trees act as source of inoculums to other trees and leads to whole crop losses (Lepoint *et al.,* 2005; Leslie *et al.,* 2005).

3.6.2 Chemical Control

Synthetic chemical disease control products, with a few exceptions, are based on toxicity of the chemicals to target organisms. As chemical methods and products have evolved, they have become much more specific in their modes of action. This specificity has created an increased probability that target microbes will develop resistance to them. Their ability to directly inhibit a high proportion of target organisms means that they can be highly effective; however, their

period of efficacy is limited because the amount of toxic substance applied is finite and effects rarely last more than a few weeks. Furthermore, the active chemical control ingredient must be applied to site of infection, either through topical application or via systemic activity of the pesticide (Harman *et al.,* 2004).

Chemical control of soil-borne pathogens provides certain degree of control but at the same time have adverse effects on environment affecting the beneficial soil microorganisms (Radwan *et al*., 2007; Yonas, 2010). On the other hand, biological control of plant pathogens has been considered as a potential control strategy in recent years and search for these biological agents is increasing (Muleta, 2007).

3.6.3 Biological control

Biological control is, which has not been fully exploited is economical, self-perpetuating and usually free from residual effects and can be an important component of integrated disease management (Sahile *et al*., 2011). Biological control of plant pests and pathogens continues to inspire research and its application in many fields. Plant pathogens are just one class of targets of biological control, which also is designed to limit other pests such as insects, parasitic nematodes, and weeds. Several plant diseases caused by fungi can be potentially controlled by *Trichoderma* species (Table 2). In the narrowest sense, bio-control involves suppressing pest organisms with other organisms. However, the interrelationships of many environmental variables can result in multiple interactions among organisms and their environment, several of which might contribute to effective biological control (Amin *et al.,* 2010).

Biological control means control of disease through some biological agency that is any living microorganism. Bio-control may be defined as any condition or practice where by survival or activity of pathogen is reduced through the effect of any other living organism with the result that there is reduction in the incidence of disease caused by the pathogen (Dev and Dawande, 2010).

According to Jegathambigai *et al*. (2009), *Trichoderma* spp. is one efficient biocontrol which is commercially produced to prevent development of several soil pathogenic fungi. Indigenous *Trichoderma* spp. has shown more effective, enhanced adaptability and antagonist activity in controlling the phytopathogen.

Table 2. Plant diseases controlled by *Trichoderma* species (Source: Ha, 2010).

Name of disease	**Crop**	**Fungal pathogens**	***Trichoderma* species**
Wilt	Tomato,Chili, Peanut, Potato, Coffee, Black pepper,	*Fusarium* spp.	*T. hamatum, T. harzianum, T. viride,*
Root rot	Citrus,Tobacco, Pineapple,Rubber,Black pepper	*Phytophthora* spp.	*T. harzianum, T. viride*
Damping off	Tomato,Chili, Peanut, Potato, Bean, Maize, Cabbage,Chinese cabbage	*Pythium* spp. *R. solani*	*T. hamatum, T. harzianum, T. viride.*
Southern stem rot	Tomato,Chili, Peanut, Potato, Soybean,	*Sclerotium rolfsii*	*T. hamatum, T. harzianum, T. viride.*
Cottony rot	Cabbage, Soybean	*S. sclerotiorum*	*T. harzianum, T. viride*
Sheath blight	Rice, Maize	*R. solani*	*T. harzianum, T. viride*

Trichoderma spp.

One of the most important biocontrol agents is *Trichoderma* spp. which is the most frequently isolated soil fungi and present in plant root ecosystems. *Trichoderma* is the most commonly used fungal biological control agent and have long been known as effective antagonists against plant pathogenic fungi (Amin *et al*., 2010). The use of *Trichoderma* fungi in agriculture can provide numerous advantages ; 1) Colonization of the root and rhizosphere of plant, 2) Control of plant pathogens by different mechanisms such as parasitism, antibiotic production and induce systemic resistance , 3) Improvement of the plant health by promoting plant growth , and 4) Stimulation of root growth (Harman *et al.,*2004). *Trichoderma* species are used widely as bio-control agents because they have more benefits on plant growth such as promoting plant growth, increasing the

nutrient uptake from the soil, and decreasing the activity of the soil borne pathogens that ultimately affect the growth of the plant (Cumagun, 2012).

Members of the *Trichoderma* genus are known as imperfect fungi, fast growing in culture and produce numerous green spores. These occur worldwide and are commonly associated with root, soil and plant debris (Howell, 2003). *Trichoderma* spp. is the common soil inhabitants, especially in organic soils. These fungi can live either saprophytically or parasitically on other fungi. One of the most salient characteristics of the group is their ability to parasitize other fungi. *Trichoderma* has been an exceptionally good model to study bio-control mechanisms, as it is ubiquitous, easy to isolate and culture, grows rapidly on many substrates, affects a wide range of plant pathogens, is rarely pathogenic on higher plants, acts as mycoparasite, produces antibiotics, and has an enzyme system capable of attacking a wide range of plant pathogens (Shalini and Kotasthane, 2007).

The genus *Trichoderma* was first reported from Germany and four species were originally described on the basis of color and shape of their conidia and on the colony appearance. Most species were identified as *T. lignorum* (= *T. viride*) because of its globose conidia or as *T. koningii* because of its oblong conidia. The potential for use of *Trichoderma* sp. as biocontrol agents was suggested more than 75 years ago first to demonstrate the parasitic activity of members of this genus to pathogens such as *R. solani*. *Trichoderma* is perhaps the best known mycoparasite suggested as a biocontrol agent against many soil-borne plant pathogens (Harman *et al.,* 2004). According to Haggag and El-Gamal (2012) report, *T. harizanum* reduced the mycelial growth of two pathogenic fungi by 76.7%, while *T. viride* reduced the mycelial growth of the same fungi from by 82.2 % under *in-vitro* condition (Table 3). A research conducted by Abd-El-Khair *et al.*(2010) revealed that the average of bean plant height with *Trichoderma* application are in the range of 46.0-49.8 cm compared to 37.3 cm in the control plants under greenhouse conditions. According to their report, *T. hamatum* resulted highest increase of plant height (34%), followed by *T. harzianum* (26%), *T. viride* (26%) and *T. album* (23%).

Table 3. Antagonistic effect of biocontrol agents against mycelial growth of *F. solani* and *R.solani* isolates *in-vitro* tests (Source: Haggag and El-Gamal, 2012)

Fungal isolates	Mycelial Growth Reduction (%)			
	Trichoderma harzianum	*Trichoderma viride*	*Bacillus subtilis*	*Pseudomonas fluorescens*
F.solani				
Fs_1	75.5a	56.6c	20.8 a	20.7 a
Fs_2	66.6b	67.8b	19.6 bc	20.9 a
Fs_3	76.7a	81.1a	20.4 ab	20.4 a
Fs_4	63.3c	82.2a	21.0 a	20.6 a
Mean	70.5A	71.9A	20.5 A	20.7 A
R.solani				
Rs_1	53.3d	27.7d	19.1 c	19.1 b
Rs_2	48.8e	50.0e	19.9 bc	19.2 b
Mean	51.1B	38.9B	19.5 A	19.2 B

Means in each column followed by the same capital and/or small letter are not significantly different according to LSD test (P = 0.05)

Factors influencing bio-control agent

From the microbial perspective, soils and living plant surfaces are frequently nutrient limited environment. So to colonize the phytosphere, a microbe must effectively compete for the available nutrients (Pal and Gardener, 2006). Both the bio control agents and the pathogens compete with one another for the nutrients and space to get established in the environment. This process of competition is considered to be an indirect interaction between the pathogen and the

bio control agent whereby the pathogens are excluded by the depletion of food base and by physical occupation of site (Lorito *et al.,* 1994).

Abiotic and biotic environmental parameters may have negative influence on the bio-control efficacy of *Trichoderma* strains, therefore it is very important to collect information about the effects of environmental factors on the different activities of *Trichoderma* strains with bio-control potential (Kredics *et al*., 2003).

Rhizosphere competence

Rhizosphere competence is the ability to colonize and grow in association with plant roots. This is possibly the most important factor in considering the potential of any given isolate for biological control because it is a measure of the ability of an isolate to survive in the soil (Pal and Gardener, 2006). *Trichoderma* strains grow rapidly when inoculated in the soil because they are naturally resistant to many toxic compounds including herbicides, fungicides and pesticides such as DDT and phenolic compounds. *Trichoderma* strains are very efficient in controlling several phytopathogens such as *R. solani*, *F. solani, P. ultimum* and *S. rolfsii* when alternated with methyl bromide, benomyl, captan or other chemicals due to the presence of ABC-transport system (Vinale *et al*., 2008). Sivan and Chet (1989) demonstrated that competition for nutrients is the major mechanism used by *T. harzianum* to control *F. oxysporum*. Moreover, *Trichoderma* has a strong capacity to mobilize and take up soil nutrients, thus making it more efficient and competitive than many other soil microbes (Benitez *et al*., 2004).

Temperature

Studies are available on the effects of temperature on the spore germination, mycelial growth, and competitive saprophytic abilities and on volatile and non-volatile metabolite production of *Trichoderma* strains (Samuels, 1996). Most *Trichoderma* strains are mesophilic, and cannot protect germinating seeds from soil-borne diseases caused by cold-tolerant strains of plant pathogenic fungi during cold autumn and spring conditions (Kredics *et al*., 2003).

It is important to understand the cycle of the pathogen in order to determine the best time for application of a biocontrol agent. Potential bio-control strains need to cover the thermal spectrum of the target organism, e.g., *Botrytis* has a very wide spectrum and to grows at a higher optimum

temperature than *T. stromaticum* thus limiting the ability of the *Trichoderma* to control the plant parasite (Dominguesa *et al*., 2000).

According Al-Mahareeq (2005) report, most *Trichoderma* isolates reached a peak in mycelial growth rate at 30 °C and was completely inhibited at 40 °C (Fig. 3).

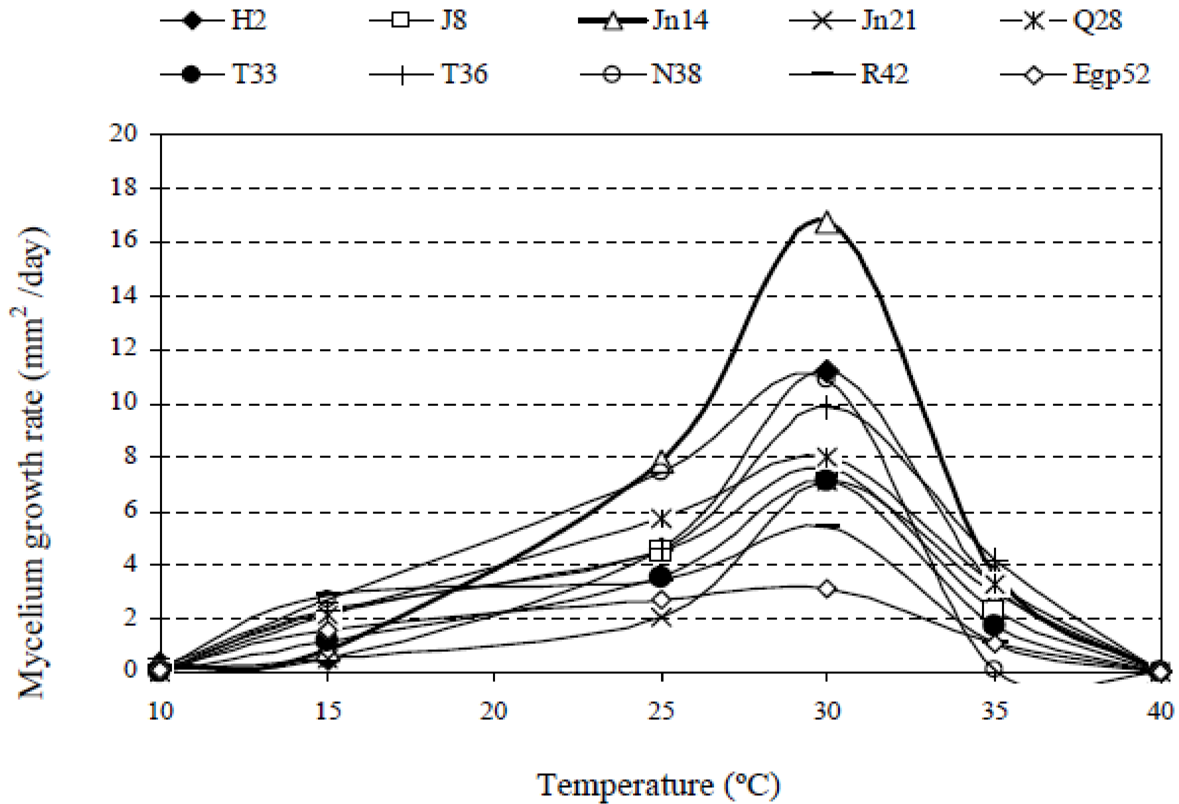


Figure 3. Mycelium growth rate (mm^2/day) of *Trichoderma* isolates (H2, J8, Jn14, Jn21, Q28, T33, T36, N38, R42, and Egy52) growing on PDA medium and incubated at different temperatures (Source: Al-Mahareeq, 2005)

Optimum temperature for linear growth on PDA is 30 °C. After 96 h at 30 °C in darkness on PDA conidia filling most Petri dishes; conidia formed densely over the center and in undulating concentric rings toward the edge; no pustules formed; in many colonies conidia first yellow, becoming yellow-green; often with yellow pigment diffusing in medium(Samuels *et al*., 2002).

Moisture

Soil moisture greatly affected the population of *Trichoderma* species. Populations of *Trichoderma* spp. have been reported to be greater in moist soils or even in soils with excessive moisture as compared to dry soils (Rahman *et al*., 2011). Moisture content and water activity are the key factors (Benitez *et al*., 2004). The availability of water strongly affects microbial growth. Therefore, the moisture content of the substrate or inert material should be within a suitable range.

Moisture can limit the ability of a bio-control agent to colonize the habitat. Lack of moisture can limit the ability of the bio-control spores to germinate. Moisture controls availability of nutrients essential to growth of the bio-control agent. Applications can be timed to periods when there will be enough moisture to stimulate spore germination (Sivan and Chet, 1989).

Nutrients

Starvation is the most common cause of death for microorganisms, so that competition for limiting nutrients results in biological control of fungal phytopathogens (Benitez *et al*., 2004). *Trichoderma* conidia are very small. They must take up water and swell before germination. That process requires the presence of nutrients (Sharma, 2011). On the other hand, *Trichoderma* species are good in mobilizing and up taking of nutrients compared to other organisms. They compete for nutrients, growth factors and space with plant pathogens (Vinale *et al*., 2008). Lack of easily accessible nutrients in the soil starved the pathogens and thus controls the growth of pathogens. For example, biological control strains of *Trichoderma* are able to make highly efficient siderophores that chelate iron from other filamentous fungi (Benitez *et al*., 2004).

Mechanisms of disease suppression

The activities of bio-control agents mainly depend on different physicochemical environmental conditions to which they are subjected. Bio-control results either from competition for nutrients and space or as a result of the ability of *Trichoderma* bio-control agents to produce and/or resist metabolites that either impede spore germination (fungistatis), kill the cells (antibiosis) or modify the rhizosphere, e.g., by acidifying the soil, so that pathogens cannot grow. Biocontrol may also result from a direct interaction between the pathogen itself and the bio-control agent, as

in mycoparasitism, which involves physical contact and synthesis of hydrolytic enzymes, toxic compounds and/or antibiotics that act synergistically with the enzymes (Ghildiyal and Paney, 2008). *Trichoderma* spp. can even exert positive effects on plants with an increase in plant growth (mineralization) and the stimulation of plant defense mechanisms (Dev and Dawande, 2010).

Different mechanisms have been suggested as being responsible for their bio-control activity, which include competition for space and nutrients, secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds (Ghildiyal and Paney, 2008; Hassan, 2011).

According to Kamala and Indira (2011), the antagonistic action of *Trichoderma* species against phytopathogenic fungi might be due to either by secretion of extracellular hydrolytic enzymes or by the production of antibiotics. It also produces a large variety of volatile secondary metabolites such as ethylene, hydrogen cyanide, aldehydes and ketones which play an important role in controlling the plant pathogens (Haran *et al.,* 1996).

Mycoparasitism

Mycoparasitism is a complex process in which a *Trichoderma* species grows chemotropically toward its host and attaches to and coils around the host hyphae, sometimes penetrating them (Elad *et al*., 1983)**.** The mycoparasitic activity of *Trichoderma* spp. may be due to production of cell wall-degrading enzymes (Boubekeur *et al*., 2012). Partial degradation of the host cell wall is normally observed in later stages of the parasitic process. The degradation and further assimilation of phytopathogenic fungi, namely, mycoparasitism, has been proposed as the major mechanism accounting for the antagonistic activity of *Trichoderma* species against fungal pathogens (Cherif and Benhamou, 1990). *Trichoderma* spp. produce extracellular β-(1, 3)-glucanases, chitinases, lipases, and proteases when they are grown on cell walls of pathogenic fungi. In addition, several lines of evidence have shown that the production of some lytic enzymes is induced during the parasitic interaction between *Trichoderma* spp. and some pathogenic fungi (Haran *et al.* 1996).

Mycoparasitic *Trichoderma* species are used commercially as biological control agents against plant-pathogenic fungi such as *R. solani, B. cinerea, S. rolfsii, S. sclerotiorum*, *Pythium* spp., and *Fusarium* spp. in, among others, the United States, India, Israel, New Zealand, and Sweden as a promising alternative to chemical pesticides (Howell, 2003). The antagonistic activity of the genus *Trichoderma* to *F. solani* and *R. solani* has been widely demonstrated (Lewis *et al*., 1998).

Antibiosis

Most microbes produce and secrete one or more compounds with antibiotic activity. Antibiotics produced by microorganisms have been shown to be particularly effective at suppressing plant pathogens (Yonas, 2010). Both volatile and non-volatile antibiotics are known to be produced from *Trichoderma* species (Okigbo and Ikediugwu, 2000). Some antibiotics produced include trichorizianines, trichokindins, trichorozins and harzianins, a class of antibiotics, are produced by most species and strains of *Trichoderma* species (Barbaso *et al*., 2001). These antibiotics are thought to act on the membrane of the target fungus to inhibit membrane-associated enzymes involved in cell wall synthesis (Okigbo and Ikediugwu, 2000).

Production of lytic enzymes

Trichoderma known for its mycoparasitic activity against several fungal plant pathogens is aided by the production of different chitinases, ß-1, 3-glucanases and proteases and cellulase (Cortes *et al*., 1998; Cumagun, 2012). These extra cellular enzymes such as ß-1, 3-glucanase, chitinase and cellulase are effective in disrupting the mycelium of plant pathogenic fungi (Elad *et al.*, 1982; Samuels, 1996). These lytic enzymes are probably responsible for hyphal lysis through the digestion of major cell wall components (Cortes *et al*., 1998). It is believed that these enzymes act synergistically with the antibiotics to inhibit the growth of fungal pathogens (Mora and Earle, 2001). It appears that the weakening of the host cell wall by the enzymes increases the rate of diffusion of the antibiotics through the cell wall. For instance, cucumber roots treated with *T. harzianum* showed higher activities of chitinase, ß-1, 3-glucanases and peroxidase (Yedidia *et al.*, 2000).

Competition and Rhizosphere Competence

Competition for substrates (‘‘rhizosphere competence’’), is the most important factor for space and nutrients with plant pathogenic fungi (Barbaso *et al*., 2001; Cumagun, 2012). The pathogen carryover capacity of the cropping systems depends upon the survival ability of the pathogen in stubbles after rice in the soil (Mew and Rosales, 1985). In rice, *Trichoderma* is responsible for the reduction of inoculum potential of *R. solani* by decomposing rice straw and stubbles after rice harvest. Since *R. solani* also infects crops after rice, such as corn (maize), *Trichoderma* could also be efficient in controlling diseases inflicted on these crops.

Trichoderma species are able to survive under extreme competitive conditions. They are able to overcome fungistatic effects (Benitez *et al*., 2004). Moreover, they are resistant against many toxic compounds, including metabolites produced by soil microflora and plants, fungicides, herbicides and antibiotics. These abilities might be due to the presence of ATP-binding cassette (ABC) transporter. The increased expression of these ABC- transporter genes reduces toxicant accumulation in the cells. Thus, allowing them to survive under extreme conditions and become more competitive compared to other soil-borne fungi (Harman *et al.,* 2004).

Induced Resistance

penetration stimulate the activation of plant defense system, causing an increase in the production of defense-related plant enzymes, such as chitinase, glucanase, and enzymes associated with the Induced systemic resistance is believed to be one of the most important mechanisms of bio-control effects of *Trichoderma*. The mechanism for increased plant growth could be due to inhibition of minor pathogens and the production of a growth stimulating factor (Harman, 2006).

Trichoderma species are usually found colonizing plant root ecosystems, establishing symbiotic relationship with plants. However, colonization of the root tissues is only limited to cortex due to the deposition of callose which restricts the penetration of hyphae. The callose barriers made *Trichoderma* harmless to the plants (Vinale *et al*., 2008). However, elicitors produced by *Trichoderma* species during biosynthesis of phytoalexins. This has been shown in the plants treated with *Trichoderma (*Benitez *et al*., 2004; Vinale *et al*., 2008).

Furthermore, presence of *Trichoderma* species at the root ecosystems had shown to enhance plant root development (Harman *et al.,* 2004; Benitez *et al*., 2004; Vinale *et al*., 2008). In

addition, *Trichoderma* species are capable of controlling deleterious microbes that reduce root development. *Trichoderma* species are resistant to cyanide produced by these deleterious microbes and even able to remove the microbes from the root zone through mycoparasitic effects. Therefore, *Trichoderma*- plant interactions are always associated with improvements in plant biomass and yield. For example, maize treated with *Trichoderma* strain T-22 had shown to increase about 5% in average yield (Harman *et al.,* 2004).

3.7 Greenhouse and field success of *Trichoderma* species: potentials and challenges

Trichoderma spp. is active mycoparasites against a range of economically important aerial and soil-borne plant pathogens, and is successfully used as a biocide in greenhouse and field applications (Chet, 1987; Papavizas, 1985). The method of application of BCAs is simple, and it is easy to see the effects of treatments because the disease is visible. Once applied and established, BCAs can move onto different parts of the foliage, and may provide long-term control without the risk of resistance (Verma *et al*., 2007).

Numerous opportunities are open for the development of biological control procedures for postharvest diseases of fruits and vegetables as an alternative to fungicides. Exploration of natural plant products as fungicides should be expanded. Also, a basic understanding of resistance and defense strategies in harvested commodities should reveal a multitude of new approaches for control of damping off diseases. Promising research on the use of antagonistic microorganisms to control postharvest diseases indicates that we should be able to develop "biologicals" that will be as effective as fungicides and presumably safer for man and the environment (Junaid *et al.,* 2013).

Presently, *Trichoderma* spp. based products are considered as relatively novel type of BCAs. In addition, the actual/true market size is vague and only scattered information could be obtained based on registered as well as non-registered bio-fungicides. However, a general consents that *Trichoderma* spp. based BCAs share about 60% of all fungal based BCAs and an increasing number of *Trichoderma* spp. based BCAs products are registered regularly. Moreover, field

application/trials throughout the world are being accepted and many bio-pesticide companies are endorsing this production on regular basis (Verma *et al*., 2007).

With regarding to the challenges using bio-control agent is mainly because the research in this area is still confined to the laboratory and very little attention has been paid to produce the commercial formulations of bio agents. Moreover, whatever has been commercially produced has not been used efficiently by the farmers owing to the lack of information regarding its use. So to popularize the concept of biological control extension at University level in this direction needs to be improved (Junaid *et al.,* 2013).

Most of the bio agents perform well in the laboratory conditions but fail to perform to their fullest once applied to the soil. This is probably attributed to the physiological and ecological constraints that limit the efficacy of bio agents. To overcome this problem, genetic engineering and other molecular tools offer a new possibility for improving the selection and characterization of bio-control agents. Various methods that can contribute to increase the efficacy of bio agent include mutation or protoplasm fusion utilizing poly ethylene glycol. There is also an urgent need to mass produce the bio agents, understand their mechanism of action and to evaluate the environmental factors that favor the rapid growth of bio-control agents (Lorito *et al.,* 1994; Junaid *et al.,* 2013).

3.7.1 Inoculum development of bio-control agents

Several growth media and protocols for *Trichoderma* spp. spore production were reported as mass scale production would have great potential for commercial use (Ha, 2010; Sargin *et al*., 2013). At the beginning, *Trichoderma* propagules, in the form of conidia, mycelium and chlamydospores, were mass produced on conventional synthetic media. However the cost of these materials is too high. To overcome cost limitation, alternative substrates such as coffee husks, sugarcane waste, rice bran, corn meal are used. *Trichoderma* products can be applied to the soil, used as seed treatment, seedling root dip or added to organic fertilizers/or compost (Ramanujam *et al*., 2010). *Trichoderma* spp. is commercially marketed as bio-pesticides, bio-fertilizers and soil amendments.

Aziz *et al*. (1997) found that application of *Trichoderma lignorum* as a seed coating (8×10^6 conidia/seed) or wheat bran preparation (1×10^6 cfu/g) at a rate of 20 g/kg soil greatly reduced the number of bean seeds infested by *R. solani* and the percentage of healthy seeds reached 92%.

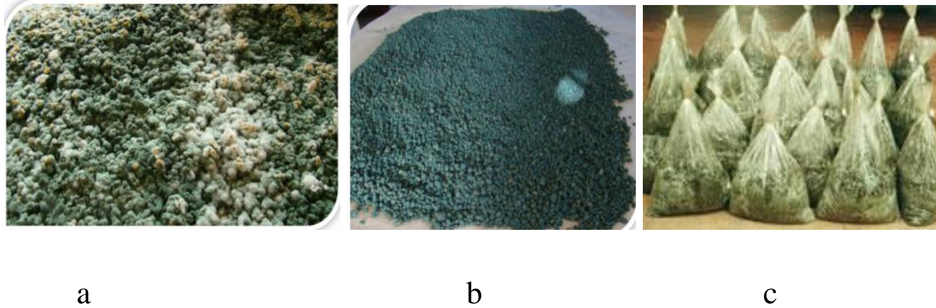
The major aspects of successful biological control technologies include the establishment of product, formulation and delivery system for microorganism that enable them for efficient disease control (Ramanujam *et al*., 2010). The mass production system should be compatible with industrial and commercial development methods and field application. There are two major methods of inoculum production as (a) Solid state fermentation and (b) Liquid state fermentation.

Solid state fermentation

Solid media for the experimental production of *Trichoderma* has frequently been used in laboratory and greenhouse studies (Elad *et al*., 1980a). Agro-industrial residues are generally considered to be the best substrates for the SSF processes. Some of the substrates that are commonly used for SSF include; sugar cane bagasse, wheat bran, rice bran, maize bran, wheat straw, saw dust, corn flour, wheat flour and pre-treated willow (Joshi *et al*.,2010; Ramanujam *et al*., 2010).

Solid fermentation is a very common method for mass production of *Trichoderma*. The most widely used fungal antagonist *Trichoderma* spp. have been grown on solid substrates like sorghum grain, wheat straw, wheat bran, spent tea leaf waste, coffee husk, saw dust (Jeyarajan, 2006; Zaidi and Singh ,2004) etc.

Investigation on mass production of *Trichoderma* spp. by solid substrate fermentation revealed that sorghum seed is a suitable material for production of spores. Appropriate procedure consisted of soaking sorghum seeds in running water for 24 hr, filling in autoclavable bags, sterilizing for 30 min. *Trichoderma* suspension is inoculated on sterilized sorghum seeds and then all bags were incubated at 25-30 ^{o}C for 10 days (Fig. 4). Finally, *Trichoderma* culture is prepared as powder formulation. Culturing of *Trichoderma* on sorghum seeds contained in autoclavable bags is suitable for mass production of culture used for greenhouse and field testings (Ramanujam *et al*., 2010).



a b c

Figure 4. *Trichoderma* inoculum on sorghum (a) 3 days after inoculation, (b) ten days after inoculation and inoculum in polyeten bag (Source: Ramanujam *et al*., 2010)

Solid fermentation results in a product that is generally used as it is for soil application or for enriching organic manures. This technique is suitable for small-scale production in cottage industries or at individual farmer level. The disadvantage of technique is laborious and results in a product which is bulky and prone for contamination. Solid state fermentation technique for commercial/industrial scale production of bio-control fungi has to work out in collaboration with industry (Zaidi and Singh, 2004).

Liquid fermentation

In liquid fermentation system, *Trichoderma* is grown in liquid media in stationary/shaker/ fermentor cultures and formulated and used for field application (Ramanujam *et al*., 2010).

A key requirement for successful deployment of inoculants is the development of appropriate formulation and delivery systems to ensure survival and effective establishment of target microorganisms within the rhizosphere. Poor competitive ability and lack of persistence of inoculants in soils is commonly considered to be an important factor that may restrict their effectiveness (Richardson, 2001).

3.7.2 Formulation methods

Formulation is blending of active ingredients such as fungal spores with the inert material such as diluents and surfactants in order to alter the physical characteristics of to a more desirable form (Junaid *et al.,* 2013).

Different formulations have been used in control soil borne pathogens, these are, fungal spores (Harman *et al*., 1980), and powdery preparations of fungal mycelium (Latunde -Dada, 1993). A bio-control formulation with agricultural potential should possess several desirable characteristics such as: easy preparation and application be easy to handle, stable over a range of -5 to 35ºC, adequate shelf life, abundant viable propagules, and low cost (Zaidi and Singh, 2004; Junaid *et al.,* 2013).

Shelf life of the formulated product of a bio-control agent plays a significant role in successful marketing. In general the antagonists multiplied in an organic food base have longer shelf life than the inert or inorganic food bases. Shelf life of *Trichoderma* in coffee husk was more than 18 months. Talc, peat, lignite and kaolin based formulation of *Trichoderma*, have kaolin based formulation of *Trichoderma*, have a shelf life of 3-4 months (Sankar and Jeyarajan, 1996).

Shelf life of the bio control product is dependent on the storage temperature and the carriers used in the formulation of bio-control product. A talc based preparation of the *T. virens* conidia retain 82% viability at 5 ^{o}C in refrigerator after 6 months while at room temperature same level of viability was observed for a period of 3 months (Chaube *et al.,* 2003).

Delivery of *Trichoderma* for disease Management

The ease of formulation and delivery system is an advantage as *Trichoderma* can be grown on a wide range of carbon and nitrogen source (Harman and Kubicek, 1998). For successful diseases control, delivery and establishment of *Trichoderma* to the site of action is very important. The most common methods of application of *Trichoderma* are by seed treatment, seedling dip, soil application and wound dressing (Zaidi and Singh, 2004; Ha, 2010).

Seed Treatment

Seed coating with *Trichoderma* is one of the easy and effective methods of delivering the antagonist for the management of seed/soil-borne diseases. Seed is coated with dry powder/dusts of *Trichoderma* just before sowing. Propagules of biocontrol agents germinate on the seed surface and colonize roots of germinated seedlings and rhizosphere (Tiwari, 1996). *T. harzianum, T. virens* and *T. viride* are found to be effective seed protectants against *Pythium* spp. and *R. solani* (Joshi *et al*., 2010).

Root treatment

Seedling roots can be treated with spore or cell suspension of antagonists either by drenching the bio-agent in nursery beds or by dipping roots in bio-agent suspension before transplanting. This method is generally used for the vegetable crops, rice and tomato where transplanting is practiced (Singh and Zaidi, 2002). Root dipping of tomato seedlings reduces the severity of root knot caused by *Meloidogyne incognita*. Root dipping in antagonist's suspension not only reduces disease severity but also enhances seedling growth in rice, tomato, chili and capsicum (Singh and Zaidi, 2002).

Soil treatment

There are several reports on the application of bio-control agents to the soil and other growing media either before or at the time of planting for control of a wide range of soil-borne fungal pathogens (Baby and Manibhushanrao, 1996). Such applications are ideally suited for green house and nursery. *Trichoderma* is capable of colonizing farm yard manure (FYM) and therefore application of colonized FYM to the soil is more appropriate and beneficial. This is the most effective method of application of *Trichoderma* particularly for the management of soil-borne diseases.

Aerial spraying/Wound dressing

Trichoderma has been successfully applied to the aerial plant parts for the bio-control of decay fungi in wounds on shrubs and trees (Papavizas, 1985).

3.7.3 Challenges of using bio-control agents

One of the greatest obstacles to the biological control by introducing microorganisms is the lack of the methods for the mass production (the development of appropriately formulated products) of the bio agent (Subash *et al*., 2013). The most widely used fungal antagonist *Trichoderma* spp. have been grown on solid substrates like sorghum grain, wheat straw, wheat bran, spent tea leaf waste, coffee husk, saw dust (Zaidi and Singh ,2004) etc. The *in-vitro* ability of the antagonists and their ability to suppress diseases in the field; accordingly, the strains producing largest inhibition zones or overgrowth over pathogens on media do not always make the best biocontrol

agents (Cook, 1993). It is, therefore, necessary to develop, suitable *in-vitro* assay procedures which closely simulate natural conditions. Biological control essentially depends upon maintaining a threshold population of the antagonist on planting material or in soil; any drop in the viable count below this critical level may render biological control ineffective. A large number of edaphic and environmental factors affect the viability of antagonists (Weller, 1988).

4. MATERIALS AND METHODS

4.1 Description of the study area

The study was carried out in Postgraduate research Laboratory, Department Biology, and the greenhouse experiments were conducted at College of Agriculture and Veterinary Medicine, Jimma University. Jimma town is located 353 km to the south-west of Addis Ababa. The town's geographical coordinates are 7°41' N latitude and 36° 50' E longitude. The town is found at an average altitude of 1,780 m above sea level. It lies in the climatic zone locally known as "Woyna Daga" (1,500-2,400 m above sea level). The town is generally characterized by warm weather with a mean annual maximum temperature of 30°C and a mean annual minimum temperature of 14°C. The annual rainfall ranges from 1138-1690 mm (Alemu *et al*, 2011). The soils are mainly acidic pH that ranges from 5.5-6.5 due to the presence of high rainfalls and deforestation. The soil type is mostly low fertile, black in color, and has a good retention capacity (Kifle, 1997).

4.2 Study sites and Samples collection

A total of 120 infected plant samples were collected, which comprised of 30 plant samples of each faba bean (*Vicia faba* L.), haricot bean (*Phaseolus vulgaris* L.), tomato (*Lycopersicon esculentum* Mill.) and potato *(Solanum tubersom)* from Jimma Zone, Oromia Regional State. Root samples of faba bean, haricot bean, tomato, and potato plants showing damping-off disease symptoms were selected from four districts (Dedo, Kersa , Mana , and Seka Chekorsa) in April 25-June 25, 2012. The districts were purposely selected based on the preliminary survey made to identify potential growing areas for experimental crop. The samples were randomly collected from farm lands within 1 to 2 km interval between the same samples.The four areas are located at elevation of 1600-2400 m above sea level in Jimma Zone, south-western Ethiopia. Roots with adhering soils of infected plants were collected and transferred to sterile plastic bags and transported to the Postgraduate and Research Laboratory, Department of Biology, Jimma University and stored at 4°C for further analysis.

4.3 Isolation and identification of fungal pathogens

Samples of infected plants (faba bean, haricot bean, tomato, and potato seedlings) were collected and used for isolation of the fungal pathogens. Isolation was done from small root pieces (3-5

mm), cut from diseased areas of the plants. Root pieces were washed under tap water for about 30 min to remove adhering soil particles (Jiskani *et al.*, 2007). The washed diseased root fragments were surface sterilized with 0.5% sodium hypochlorite (NaOCl) solution, for 5 min and washed three times thoroughly with the sterile distilled water and placed in Petri plates containing pre-solidified Potato Dextrose Agar (PDA) medium. These Petri plates were incubated at $25\pm1^{o}C$ for 7 days.

4.4 Isolation and identification of *Trichoderma* species

Trichoderma spp. were isolated from the roots of healthy faba bean, haricot bean, tomato and potato plants grown in four districts (Dedo, Seka Chekorsa, Mana and Kersa) of Jimma Zone, southwestern Ethiopia. A total of 120 soil samples were collected from rhizosphere soils of faba bean, haricot bean, tomato, and potato crops. For rhizospheric soil, the plant was gently and carefully uprooted. Tightly adhered soil particles were collected, mixed and used as composite rhizosphere soil samples (Al-Mahareeq, 2005). The soil samples were taken from a depth of 10 to 15 cm around the rhizosphere area of healthy plants. The soil samples were collected in plastic bags, sealed and labeled with information of collection sites and origin of samples. Then, the samples were transported to the laboratory and processed within 24 hours.

Collected soil samples were air dried for four hours and isolation was done by serial dilution (soil washing) technique according to Aneja (2005). Samples were shaken for 20-30 min on a rotary shaker at 250 rpm. Malt Extract Agar (MEA, Difco, USA) was used for isolation of the bio-agents. To the autoclaved medium, chloramphenicol 0.25 g/l was added to prevent bacterial contamination. Serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} were made for each soil sample. A 0.1 ml aliquot of soil suspension was dispensed onto pre-solidified MEA surface and spread with a glass rod. The plates were incubated at $28\pm1^{o}C$ for 5 days. The plates were observed daily Appearances of colonies were recorded from 3rd to 5th days. Individual colonies were picked and purified using PDA for further study (Al-Mahareeq, 2005; Joshi *et al.,* 2010).

The isolated fungi (antagonists and pathogens) were identified with the aid of keys using taxonomic and morphological references (Cheesbrough, 2000). The identification of the fungal isolates was based on morphological characterization that included colonial characteristics, conidial morphology and other pertinent features of the isolates. The identified fungi were

maintained on Potato Dextrose Agar (PDA) slant and stored in a refrigerator (+4°C) with periodic transfer to keep the microorganisms viable.

The *Trichoderma* spp. and pathogenic fungi were identified on the basis of their cultural and morphological characters. After incubation, *Trichoderma* spp. was identified from other fungi based on color, size, shape, and appearance of colony on surface of PDA. Thereafter, the isolates were transferred to a Potato Dextrose Agar (PDA) medium for purification and further identification.

4.5 Characterization of the fungal isolates

4.5.1 Slide culture preparation

The morphology of spores and mycelia of fungal isolates were examined by growing them on slide according to Stevens (1974). Fungal isolates were grown on PDA in Petri dishes, and plates incubated at 25°C for 7 days. Sterile glass cover slips, 50 × 25 mm, were held with forceps and immersed in autoclaved melted water agar (20 g Difco agar / L distilled water) at about 45°C for 1-2 seconds, and allowed to drain. The cover slip was then laid singly on the surface of 2% solidified water agar in center of Petri dishes, so that a thin film of agar was set on the upper surface (Laing and Deacon, 1991). Each plate was inoculated with a 5 mm plug cut from the edge of 7 days old growing colonies of each fungal isolates on PDA. The plug was placed 2cm apart of the coverslip placed on the agar surface, so that fungal colony grew across the coated cover slips. Plates containing coated cover slips were incubated at 25 ± 2 °C and inspected daily for four days for fungal mycelium growth. Each cover slip were removed carefully without damaging the fungus mycelium, and then it was inverted on sterile microscopic slide (24.4 × 76.2) and sealed by nail varnish to prevent drying. The growing isolates were studied using fresh direct mounts in Lactephenol cotton blue under low and high magnifications, ×10, and ×40, respectively. The top of the cover slip was cleaned, and microscopic observations were made throughout the coated cover slip and thin film of agar.

For *R. solani* and *F. solani-Trichoderma* interaction (mycoparasitism test), a clean glass sterile slide was placed on an L-shaped glass rod in a 9 cm diameter Petri dish and autoclaved. Then, a small amount of autoclaved PDA was spread over the slide to make a thin film on the slide. One end of the slide was kept free of the medium to facilitate handling. Five mm discs of one week

old growing colonies cut from the margin of each pathogen and *Trichoderma* isolates were placed on the opposite sides of the slide 3 cm apart on the PDA surface and incubated at 25 ± 1 ^{o}C for a week. At the end of incubation period, meeting area was observed microscopically by staining with Lactophenol and Cotton blue (Himedia, India) for presence of coiling of *Trichoderma* hyphae on *R. solani* and *F. solani* hyphae, mycelia penetration and cell wall disintegration in the area of interaction (Stevens, 1974; Laing and Deacon, 1991).

4.5.2 *Trichoderma* isolates' mycelia growth rate

Mycelial growth rate of *Trichoderma* strains were observed on plates containing PDA as follows: Four Petri dishes (90 mm diameter) containing PDA were centrally inoculated with a 5-mm of agar plugs from 7-day-old PDA cultures of each *Trichoderma* isolate to determine the average linear growth of each isolate. Plates were incubated at 25 ±2°C under continuous light and inspected daily for three consecutive days. Radial mycelial growth was recorded every 24 hours during this period. The colony diameter was measured as the mean of two perpendicular diameters measured at the third day minus the diameter at first day. Average linear growth rates (ALG) was calculated by using formula (Elad *et al*., 1981):

$$\mathbf{ALG}\ (\text{mm /day}) = \mathbf{C_3 - C_1/T}$$

Where, $\mathbf{C_3}$: colony diameter in mm after three days,

$\mathbf{C_1}$: colony diameter in mm after one day of incubation and

T: the difference in time (day).

The reference strains of plant pathogens (*R. solani* and *F. solani)* and the biocontrol agent (*Trichoderma* strain) were obtained from Ambo Crop Protection Center were maintained on Potato Dextrose Agar (PDA) slant and stored in a refrigerator (+4°C) with periodic transfer to keep the microorganisms viable.

4.5.3 *In-vitro* evaluation of antagonism of *Trichoderma* spp. against *R.solani* and *F.solani*

The antagonistic effect of tested biocontrol agents against mycelial growth of both *F. solani* and *R. solani* isolates were tested under *in vitro* condition using dual culture technique (Coskuntuna and Ozer, 2008). The *Trichoderma* isolates, *F. solani* and *R. solani* were cultured on PDA medium for 7 days at 25ºC. Disc (5 mm diameter) from bio-control fungi was inoculated on

surface of PDA medium. A disc (5 mm - diameter) of *F. solani* and *R. solani* was separately inoculated at equal distance of the opposite side of Petri dish (Radwan *et al*., 2007). Plates which were inoculated with each pathogenic fungus only were used as control. The inoculated Petri dishes were incubated at 25° C for 7 days. At the end of the incubation period, radial growth (linear growth of *R. solani* and *F. solani*) was measured using transparent ruler. The colony interaction was assayed as percentage inhibition of the radial growth of the pathogens by *Trichoderma* spp. using the following formula (Gomathi and Ambikapathy, 2011):

$$PI = (C-T)/C \times 100$$

Where, PI = Percent inhibition of mycelia growth

C = radial growth measurement of the pathogen in the control and

T= radial growth of the pathogen in the presence of *Trichoderma* spp.

4.6 Greenhouse experiment

4.6.1 Preparation of *R. solani* and *F. solani* inoculums

Fifty milliliters of Potato Dextrose Broth was added to 250 ml capacity Erlenmeyer flasks and inoculated with two 7 mm-diameter mycelial disks from 7 days old PDA cultures of *R. solani* and *F. solani* and incubated at 27°C for 10 days separately. After incubation, the upper solid layers with growth was washed and air-dried on tissue paper layers. Then, the amount needed from this preparation (30mlwith $5x10^6$ cfu/ml per pot) was calculated for the whole experiment. The air-dried mycelium in sterile 250 ml Erlenmeyer flasks was shredded by Ultra thorax in distilled water and then passed through a 2-mm sieve to obtain inoculums pieces of 1-2 mm in diameter (Al-Mahareeq, 2005).

4.6.2 Preparation of *Trichoderma* spp. inoculums

One isolate of *Trichoderma* sp. (JUTr012) was selected for the production of inoculums to test its ability to control haricot bean damping off disease. One of the most important selection criteria for JUTr012 isolate was its highest mycelial growth rate (32.07±0.38) and rapid colonization of medium than other *Trichoderma* isolates (Table 10). The other selection criterion for JUTr012 isolate was its percent inhibition against *F. solani* and *R. solani*. JUTr012 showed highest percent inhibition over the other *Trichoderma* isolates (Table 9).

The colony forming unit (cfu) from suspension of *Trichoderma* fungus was prepared on steam sterilized sorghum grain from 7 days old culture on potato dextrose agar (Rojo *et al*., 2007). Mass cultures of *Trichoderma* isolates were made on sorghum grains. Grains (250 g) were soaked in tap water in 750 ml flask for twelve hours. The soaked grains were autoclaved at 121°C at 15 psi pressure for half an hour. After cooling, the grains were inoculated with three days old cultures of efficient *Trichoderma* isolates and incubated at 28±1°C for 12 days. Colonized grains were allowed to dry in open shade and grounded with the help of wily mill to get fine powder which passed through 50 and 80 mesh size sieves and used for glass house experiments. For soil infestation, fungal suspensions (pathogens) containing 5x10^{6} cfu/ml was added to plastic pots, filled with steam sterilized sandy loam soil 7 days before planting (Sallam *et al*., 2008) and for bio-control agents (both reference and local isolate), fungal suspensions (30ml of each) was determined by counting using a haemocytometer slide (adjusted at 1x10^{8} cfu / ml).

In greenhouse experiment, the efficient antagonistic strain was evaluated for its ability to protect haricot bean plant against artificially inoculated soil-borne pathogens of damping off disease (Sallam *et al.,* 2008).

4.6.3 Treatments and the Experimental Design

The soil sample was thoroughly mixed and prepared for potting after clearing the debris and sieved through 2-mm mesh size. A total of 27 sterile (with 95 % ethanol by swabbing) plastic pots were filled with 4 kg homogenized and sieved soil sample in 5 kg capacity plastic pots size (w=10.5cmxh=8.5cm). For inoculation, a haricot bean seed (Awash Melka variety) was kindly obtained from Agricultural Research Center, Jimma, Ethiopia. The healthy seeds were briefly surface sterilized in 0.1% sodium hypochlorite for 2 minutes and washed repeatedly three times with sterile distilled water (Siddiqui and Akhtar, 2007).

Antifungal activity of *Trichoderma* spp. against *R. solani* and *F. solani* was evaluated in pots under artificially infestation conditions. The experiments were conducted under greenhouse conditions at College of Agriculture and Veterinary Medicine, Jimma University. For soil infestation, 30 ml of fungal suspensions (pathogens) containing 5x10^{6} cfu/ml was added to plastic pots, filled with steam sterilized sandy loamy soil 7 days before planting (Sallam *et al*.,

2008). The pots were irrigated for 7 days before bio-control agents' inoculation. Soil in each pot was inoculated with 30 ml of *Trichoderma* fungi (adjusted at $1x10^8$ cfu/ml) using a haemocytometer slide. The inoculated pots were watered for 7 days before sowing. Ten white colored haricot bean seeds were sown in each pot. Three replicate pots were used for each treatment as well as the control. The experiment was included the following treatments (Table 4): 1) Non-infested soil (control), 2) Soil treated with *F. solani* only, 3) Soil treated with *F. solani* + *Trichoderma* fungus (standard strain), 4) Soil treated with *F. solani* + *Trichoderma* fungus (local isolate), 5) Soil treated with *R. solani* only,6) Soil treated with *R. solani* + *Trichoderma* fungus (standard strain), 7) Soil treated with *R. solani* + *Trichoderma* fungus (local isolate), 8) Soil treated with *R. solani*+ both *Trichoderma* spp. and 9) Soil + *F. solani* + both *Trichoderma* spp. Disease incidence of pre- and post-emergence of damping off disease and survival (%) of bean plants were recorded after 15, 30 and 45 days, respectively (Abd-El-Khair *et al*., 2010). The design of greenhouse condition was completely randomized block design (CRBD) which was used for statistical analysis.

Table 4. Treatment combinations for greenhouse experiments

S. No.	**Treatment**	**Description**
1.	Soil only	Non-infested soil + 10 bean seeds
2	Soil +R	Soil + *R. solani*+ 10 haricot bean seeds
3	Soil +R +T_{stan}	Soil+ *R. solani* +*Trichoderma*(standard) + 10 bean seeds
4	Soil +R +JUTr012	Soil+ *R. solani* +*Trichoderma* (local isolate) + 10 bean seeds
5	Soil +F	Soil + *F. solani* + 10 bean seeds
6	Soil+F +T_{stan}	Soil + *F.solani* + *Trichoderma* (standard) + 10 bean seeds
7	Soil+F + JUTr012	Soil + *F. solani* + *Trichoderma* (local isolate) + 10 bean seeds
8	Soil +R + JUTr012+T_{stan}	Soil+ *R. solani* + both *Trichoderma* spp. + 10 bean seeds
9	Soil+F + JUTr012 +T_{stan}	Soil + *F. solani* + both *Trichoderma* spp. + 10 bean seeds

Key: F= *F. solani,* R= *R. solani,* JUTr012 = *Trichoderma* sp. (local isolate) and T_{stan}= *Trichoderma* sp. (standard).

Effect of the *Trichoderma* spp. in reducing the damping off disease incidence at pre-and post-emergence stages as well as the percentages of the survival of healthy plants were recorded after 15 , 45 and 60 days (Abd-El-Khair *et al.*, 2010). Pre-emergence damping off (%) was based on the number of non-emerged seeds in relation to the number of sown seeds, while post-emergence (%) was based on the number of plants showing disease symptoms in relation to the number of emerged seedlings.

Forty five days after planting, a random sample was taken from each pot to measure some morphological characteristics for each treatment and the control plants. The plant growth parameters (number of branches per plant, shoot height, number of leaves per plant, shoot fresh weight, number of pod per plant, pod weigh(g), number of nodule per plant, shoot and root length (cm) and dry weight per plant (g)) were determined. At 60 days of harvest, the whole plants (three plants per pot) were carefully uprooted from the pots, washed gently under running tap water to remove the adhering soil particles. Plants (in the same treatment) were pulled carefully without damaging the shoot and roots. Shoot height of the plant from ground level of growing point of the stem was measured and the mean was calculated and expressed in centimeter. The length of the roots from the point of attachment to the stem and the tip of the roots were measured and expressed in centimeter. Shoot and root tissues were separated for the determination of fresh and dry weights, number of pods/plant, number of nodules and 100–seed weight (g). After recording the plant height, the dry weight of shoot and root were measured and recorded after drying in oven at 70° C for 3 days to get a constant weight but the fresh weights of samples were recorded before being dried. The yield parameters were also recorded as number of pods per plant and the average pod weight.

4.7 Data Analysis

All data were subjected to one-way analysis of variance (ANOVA) using the Statistical Analysis System software (SAS soft ware) Program (Version 9.1). Treatment means were compared using Tukey's test and differences were accepted as significant when $p<0.05$.

5. RESULTS

5.1 Isolation and identification of plant fungal pathogens

In this study, a total of 279 fungal isolates were isolated from 120 infected plant samples of faba bean (*Vicia faba* L.), haricot bean (*Phaseolus vulgaris,* L.*),* tomato *(Lycopersom esculentum* Mill*)* and potato *(Solanum tubersom)* collected from four different (Dedo, Kersa, Mana and Seka Chekorsa) districts in Jimma Zone (Table 5). Of the total isolates, the highest numbers of damping off fungal pathogens were isolated from *L. esculentum* Mill. (29.76 %) followed by *P. vulgaris* L. (24.19 %), *V. faba* L. (24.19%) and *S. tubersom* (21.86 %).

Table 5. Total number of damping off fungi isolated from crops growing in four (Dedo, Kersa, Mana & Seka Chekorsa) districts of Jimma Zone farmlands

Crops	**No. of samples**	**No. of damping-off isolates**	**No. of other Moulds**	**Proportion of damping-off isolates (%)**
***L. esculentum* Mill.**	30	64	10	29.76
***P. vulgaris* L.**	30	52	25	24.19
***V. faba* L.**	30	52	15	24.19
S. tubersom	30	47	14	21.86
Total	120	215	64	100

Of the total 279 damping off fungi isolated from the four different types of crops (*V. faba* L., *P. vulgaris, L .esculentum* and *S. tuberosum),* 215 (77.06%) isolates were characterized and identified as *Fusarium, Rhizoctonia, Aspergillus* and *Penicillium* spp. The remaining 64 (22.93%) were uncharacterized since they did not belong to the four important damping-off fungi (Table 6). Predominant fungi species observed on faba bean, haricot bean, potato and tomato plants were *Penicillium, Aspergillus, Fusarium* and *Rhizoctonia* spp. Generally, of all isolated fungi, *Penicillium* spp. was the dominant isolate (21.14 %) followed by *Fusarium* spp. [20.07 %(Table 6)].

Table 6. Occurrence of damping-off fungi isolated from crops collected from Dedo, Kersa, Mana & Seka Chekorsa districts

Crops	No. samples	No. isolates	*Fusarium* spp.	*Rhizoctonia* spp.	*Asperigillus* spp.	*Penicillium* spp.	Unidentified
P. vulgaris L.	30	77(27.59)	11(3.94)	10(3.58)	14(5.01)	17(6.09)	25(8.96)
S. tubersom	30	61(23.65)	15(3.37)	9(3.22)	11(3.94)	12(4.30)	14(5.01)
L. esculentum Mill	30	74(26.52)	17(6.09)	13(4.65)	18(6.45)	16(5.73)	10(3.58)
V. faba L.	30	67(24.01)	13(4.65)	15(5.37)	10(3.58)	14(5.01)	15(3.37)
Total	120	279(100)	56(20.07)	47(16.84)	53(18.99)	59(21.14)	64(22.93)

Note: Values in parenthesis are percentages of the isolated fungi.

5.2 Isolation and identification of *Trichoderma* spp.

A total of 317 fungal isolates were isolated from 120 rhizosphere soil samples collected from four different locations in Jimma Zone (Table 7). Of the total 317 fungi isolated from four different types of crops *V. faba*, *P. vulgaris*, *L. esculentum,* and *S. tuberosum,* 223 (70.35%) isolates were characterized and identified as *Trichoderma, Fusarium, Aspergillus*, and *Penicillium* spp. The remaining 94(29.65%) were uncharacterized. Out of 223 isolates, 53 (16.71%) were *Trichoderma* isolates. *Trichoderma* isolates were found to occur in faba bean, haricot bean, tomato, and potato plants rhizosphere soil samples in all districts. The highest number 15 (4.73%) of *Trichoderma* isolates were obtained from *L. esculentum* followed by *P. vulgaris* 14 (4.41%), *S. tubersom* 13 (4.10) and *V. faba* 11 (3.47). Highest number (15) of *Trichoderma* isolates were obtained from Mana district (Table 8). Other predominant fungi species isolated from rhizosphere soil samples were *Penicillium* (19.87%), *Aspergillus* (18.29%) and *Fusarium* [15.45% (Table 7)].

Table 7. Total number of molds isolated from rhizosphere soil collected from four different districts of Jimma Zone

Crops	No. Samples	No. Isolates	*Trichoderma* spp.	*Fusarium* spp.	*Aspergillus* spp.	*Penicillium* spp.	Unidentified
P. vulgaris L.	30	85(26.81)	14(4.41)	17(5.36)	12(3.78)	19(5.99)	23(7.25)
S. tubersom	30	72(22.71)	13(4.10)	10(3.15)	16(5.04)	14(4.41)	19(5.99)
L. esculentum Mill	30	83(26.18)	15(4.73)	13(4.10)	17(5.36)	10(3.15)	28(8.83)
V. faba L.	30	77(24.29)	11(3.47)	9(2.83)	13(4.10)	20(6.30)	24(7.57)
Total	120	317(100)	53(16.71)	49(15.45)	58(18.29)	63(19.87)	94(29.65)

Note: Values in parenthesis are percentages of the isolated fungi

Characterization of the isolates

Characterization pathogenic isolates using slide culture

The fungal isolates were characterized and identified as *Fusarium, Aspergillus, Penicillium* and *Rhizoctonia* spp. (Table 9). The results of colony morphology and microscopic, characteristics of each isolate obtained are presented in Table 9 & Fig. 5-8.

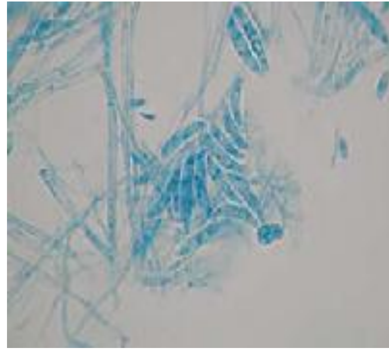
Table 8. Number of *Trichoderma* isolates isolated from soil samples collected from four different districts of Jimma Zone

District	Sites	No. of soil samples collected	No. of *Trichoderma* isolated	Local *Trichoderma* isolates
Dedo	1. Affele Dawe 2. Waro Kolobo 3. Bilo	30	14	JUTr001- JUTr 014
Kersa	1. Babo 2. Tikur Balto 3. Away Sebo	30	13	JUTr 015- JUTr 027
Mana	1. Doyo Bikila 2. Ittisa Guda 3. Haro Mana	30	15	JUTr 028- JUTr 042
Seka Chekorsa	1. Debo Yaya 2. Komo Hare 3. Gibe Beso		11	JUTr 043- JUTr 053

Table 9. Colony morphology and microscopic characteristics of fungal pathogens associated with the study crops.

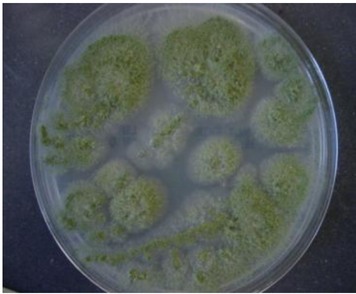
Isolates	Colonial characteristics	Microscopic morphology	Suggested genus
JUFb001-013 JUHb 001-011 JUPo 001-015 JUTo 001-017	Light yellow, moist appearance, red with cottony and orange brown mycelium on PDA (Fig. 5a).	Abundant, cylindrical, dorsal and ventral surface paralleel,3-5 septa (Fig. 5b)	*Fusarium* spp.
JUFb014-027 JUHb 012-028 JUPo 016-027 JUTo 018-033	Greenish or blue green colonies (Fig.7a).	Conidia in long chains on repeatedly branched conidiophores resembling a brush like head (Fig.7b) (penicllus). Conidiophores smooth, relatively short. Penicillia mycelia arranged very irregular and asymmetrical with branches of various lengths.	*Penicillium* spp.
JUFb028-037 JUHb 029-042 JUPo 028-045 JUTo 034-051	Colonies with loose white to yellow mycelium rapidly becoming dark brown to black on the development of conidia. Colonies light green-yellow (Fig.6a).	Black, brownish black, purple brown Conidiophores and yellow to green conidia with dark sclerotia. Microscopically (Fig.6b) Conidiophores arising from a foot-cell, catenate (basipetal) conidia on phialides (1or 2 series) on vesicle. At maturity conidia are straw-like and yellow-green.	*Aspergillus* spp.
JUFb038-052 JUHb 043-052 JUPo 046-054 JUTo 052-064	White to dark brown with irregularly shaped colonies, fast growing and filling the Petri dish with dense cottony mycelium, producing light brown to dark brown mass of sclerotia(Fig.8c).	Non-septate mycelium with hyphal branches occurs at 90° angles; (Fig.8b&c).	*Rhizoctonia* spp.

Legend: Fb = Faba bean; Hb= Haricot bean; Po= Potato; To= Tomato



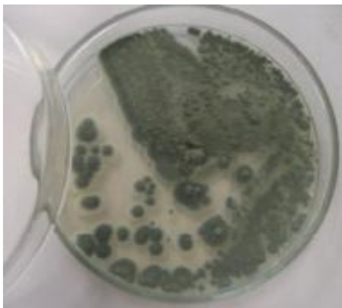
a b

Figure 5. *Fusarium* spp. isolated from haricot bean. Colony features *Fusarium* spp. grown on PDA (a) and Microscopic features of *Fusarium* spp. (b&c)



a b

Figure 6. *Aspergillus* spp. isolated from crops haricot bean. *Aspergillus* spp. grown on PDA (a) and microscopic structure of *Aspergillus* spp. (b)



a b

Figure 7. *Penicillium* spp. isolated from tomato. *Penicillium* spp. grown on PDA (a) and microscopic structure of *Penicillium* spp. (b)

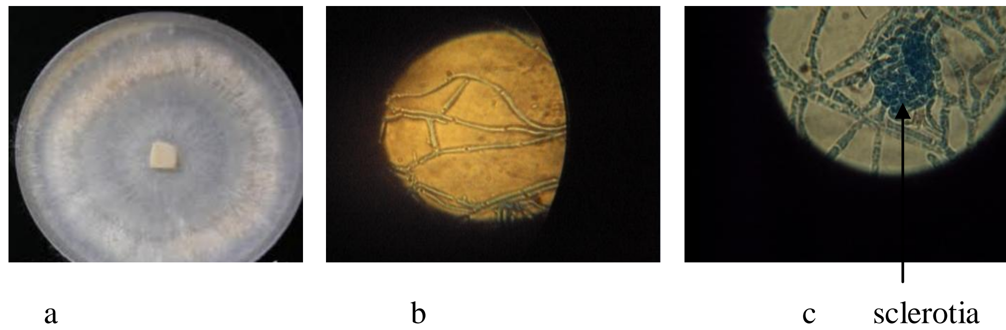


Figure 8. *Rhizoctonia solani* isolated from haricot bean. *R. solani* grown on PDA (a), Microscopic structure of *R. solani* (b) & sclerotia of *R. solani*(c)

5.3 Characterization of the *Trichoderma* isolates using slide culture

Most *Trichoderma* species grow rapidly in artificial culture and produce large numbers of small green or white conidia from conidiogenous cells situated at the ends of widely branched conidiophores (Fig. 9c & d). The colony color was initially watery white and turned bright green to dark green and dull green with compact conidiophores throughout the Petri plates (Fig. 9).

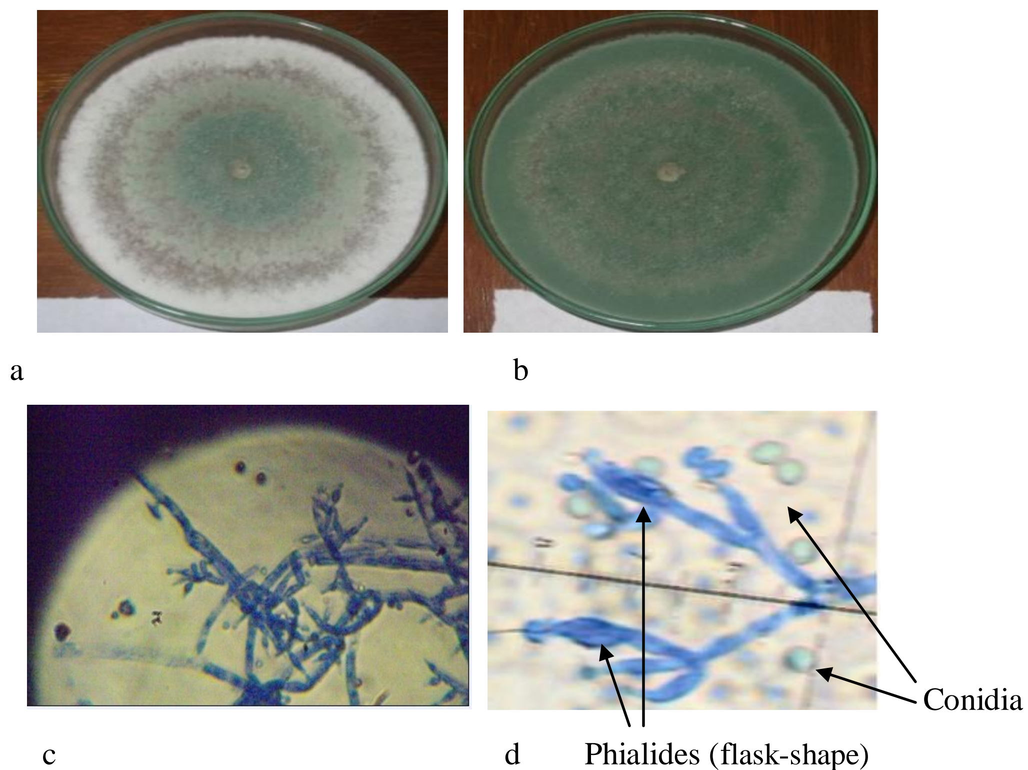


Figure 9. *Trichoderma* spp. isolated from haricot bean crop. Colony morphology on PDA five days (a), seven days (b) & microscopic structure of *Trichoderma* (c & d)

Mycoparasitism test using slide culture technique

In the slide culture examination, the pathogenic fungal hyphae came into contact, JUTr012, growth alongside, then the JUTr012 growth attached to the pathogenic hyphae and able to coil around it (Fig.10).

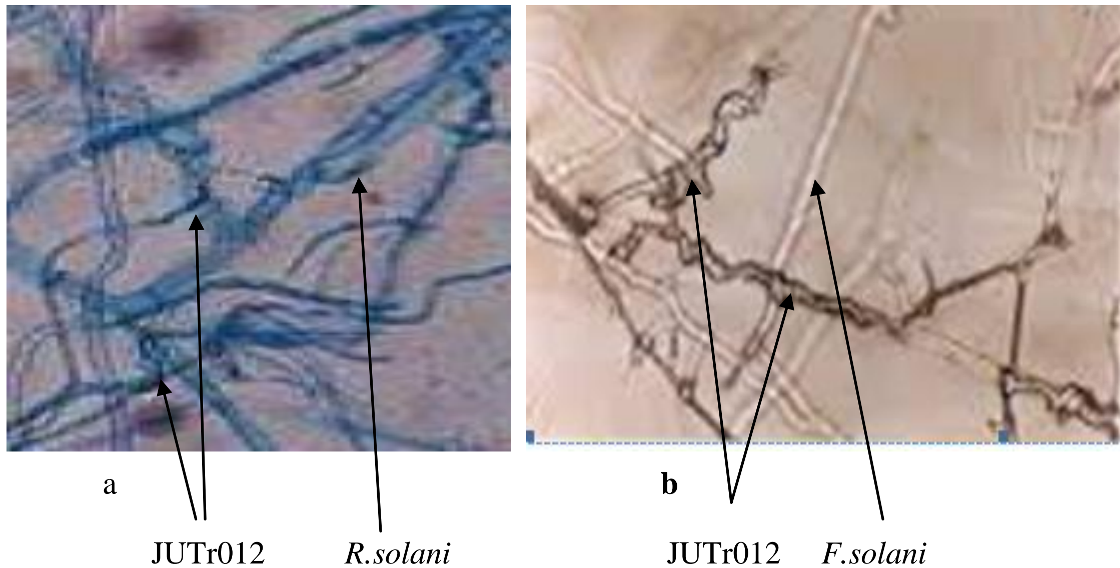


Figure 10. Coiling of JUTr012 hyphae on *R. solani* (a) & *F.solani* (b) mycelium

5.4. Mycelial growth rate of *Trichoderma* isolates

Further studies on PDA colony diameters of 53 *Trichoderma* isolates varied from 13.60 to 32.07 mm/day (Table 10).The mean of average linear growth rate for isolates ranged from 13.60 mm/day for isolate JUTr015 and 32.07mm/day for isolate JUTr012. Isolates JUTr027, JUTr047, JUTr033, JUTr045, JUTr040 and JUTr002 had fairly fast growth rates (Table 10).

Table 10. *Trichoderma* isolates average linear mycelial growth rate (mm/day) growing on PDA after 4-days of incubation at 25°C

Isolate code	Mycelium Growth Rate (mm/day)	Isolate code	Mycelium Growth Rate (mm/day)
JUTr001	26.53 ± 0.75^{fg}	JUTr028	22.83 ± 0.49^{kl}
JUTr 002	28.93 ± 0.50^{cd}	JUTr029	27.50 ± 0.66^{ef}
JUTr003	27.17 ± 0.32^{efg}	JUTr030	19.03 ± 0.42^{q}
JUTr004	25.07 ± 0.40^{hi}	JUTr031	14.93 ± 0.25^{w}
JUTr005	25.00 ± 0.36^{hi}	JUTr032	17.10 ± 0.36^{st}
JUTr006	24.10 ± 0.30^{ij}	JUTr033	30.17 ± 0.40^{c}
JUTr007	23.20 ± 0.46^{jk}	JUTr034	16.27 ± 0.35^{uv}
JUTr008	22.87 ± 0.57^{jkl}	JUTr035	16.50 ± 0.66^{tu}
JUTr009	22.33 ± 0.47^{kl}	JUTr036	20.53 ± 0.87^{o}
JUTr010	22.00 ± 0.62^{klm}	JUTr037	19.10 ± 0.30^{q}
JUTr011	22.03 ± 0.35^{klm}	JUTr038	15.80 ± 0.56^{v}
JUTr012	32.07 ± 0.38^{a}	JUTr039	19.17 ± 0.31^{q}
JUTr013	16.90 ± 0.62^{stuv}	JUTr040	29.20 ± 0.46^{d}
JUTr014	19.13 ± 0.31^{pq}	JUTr041	21.77 ± 0.25^{mn}
JUTr015	13.60 ± 0.66^{y}	JUTr042	14.03 ± 0.38^{x}
JUTr016	27.73 ± 061^{ef}	JUTr043	22.13 ± 0.42^{mn}
JUTr017	18.03 ± 025^{r}	JUTr044	19.17 ± 0.25^{q}
JUTr018	18.17 ± 0.45^{r}	JUTr045	30.00 ± 0.53^{c}
JUTr019	18.60 ± 0.60^{qr}	JUTr046	18.20 ± 0.46^{r}
JUTr020	16.27 ± 0.59^{uv}	JUTr047	30.30 ± 0.44^{c}
JUTr021	19.10 ± 0.36^{q}	JUTr048	18.19 ± 0.40^{r}
JUTr022	16.87 ± 0.31^{stu}	JUTr049	17.13 ± 0.31^{s}
JUTr023	23.93 ± 0.32^{i}	JUTr050	19.80 ± 0.62^{p}
JUTr024	23.80 ± 0.46^{ij}	JUTr051	21.70 ± 0.26^{n}
JUTr025	28.07 ± 0.21^{e}	JUTr052	20.83 ± 0.32^{o}
JUT026	25.03 ± 0.25^{h}	JUTr053	15.80 ± 0.79^{v}
JUTr027	30.97 ± 0.25^{b}	Standard(*T. harzianum*)	25.93 ± 0.32^{g}

Mean of three replicates ± standard deviation followed by the same letters within columns are not statistically significant according to Fisher LSD method (p=0.05)

5.5 *In-vitro* evaluation of antagonism of *Trichoderma* spp. against *R.solani* and *F.solani*

Bio efficacy of all fifty three *Trichoderma* isolates was studied against *F. solani* and *R. solani* causing damping off disease presented in Table 11. The results revealed that the antagonists significantly ($p<0.05$) reduced the growth of *F. solani* and *R. solani* either by over growing or by exhibiting inhibition zones (Fig. 11&12). All *Trichoderma* isolates tested under *in vitro* condition, were effective in suppressing *R. solani and F. solani*. Two isolates (JUTr012 and JUTr027) were the most effective against *R. solani and F. solani*. These isolates completely overgrew the pathogens and suppressed within 7 days of inoculation (Fig. 11b & 12b).

The antagonistic effects of *Trichoderma* spp. against *F. solani* were in the range of 26.57-59.83% (Table 11). Isolate JUTr027 gave the highest suppression effect (59.83%) followed by JUTr047 (57.93%), JUTr040 (55.60%) and JUT012 (55.37%). The growth inhibition of *R. solani* by *Trichoderma* spp. was in the range of 31.20 - 67.03 %. Isolate JUTr012 showed the highest mycelial growth inhibition (67.03%) of *R. solani* followed by JUTr027 (62.07%) and JUT033 (61.83%). The maximum growth inhibition (%) of the two pathogenic fungi was obtained by JUTr012 (Table 11), while the minimum was exhibited by JUT002 (26.57±0.75%). The remaining isolates, although inhibited the growth of *R. solani* and *F. solani*, were inferior to the ones listed above in performance.

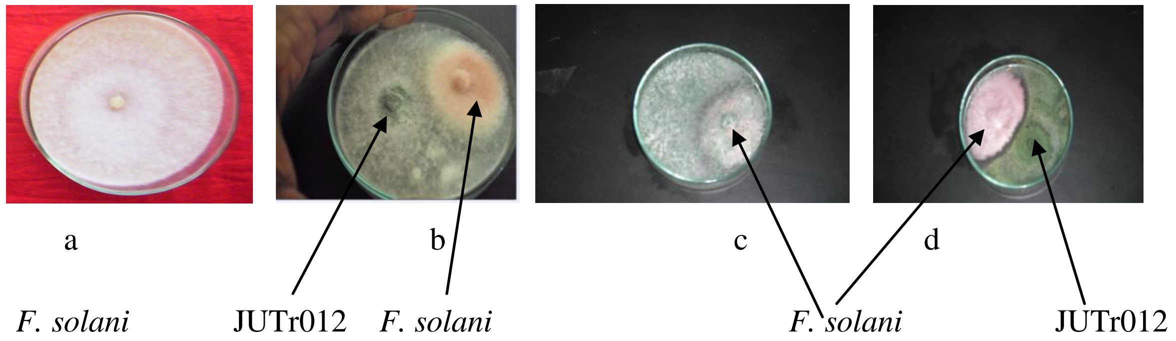


Figure 11. Antagonistic activity of the *Trichoderma* isolate against *F. solani*, (a) *F. solani* (control),(b) JUTr012 + *F. solani*, (c) JUTr012 completely over grow on *F .solani* and clear zone formed by *Trichoderma against F. solani* (d)

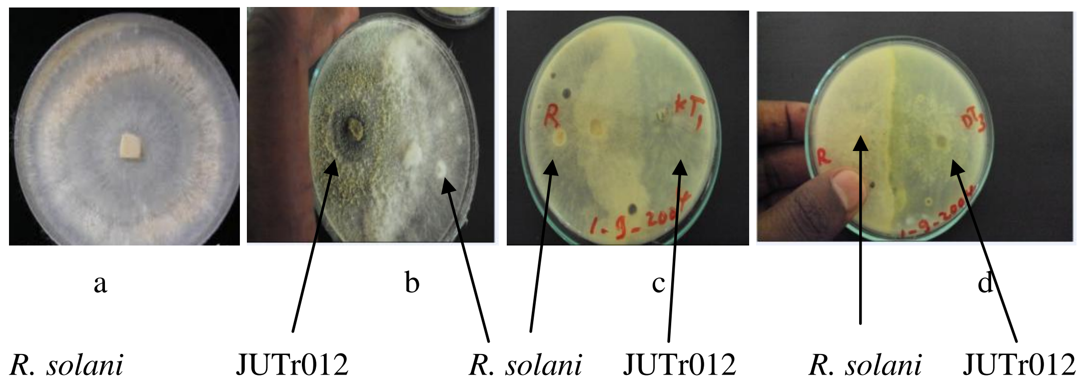


Figure 12. Antagonistic effect of the *Trichoderma* isolate against *R.solani,* (a) *R. solani* only(control),(b) JUTr012 + *R. solani*, (c) JUTr012 completely over grow on *R. solani* and clear zone formed by *Trichoderma against R. solani* (d)

Table 11. Inhibition effects of *Trichoderma* spp. against *R.solani* & *F.solani*

***Trichoderma* spp.**	**% Inhibition**	
	R.solani	***F.solani***
JUTr001	40.10±0.79^{tuvw}	29.50±0.75^{wxyz}
JUTr0 02	31.97 ±1.46^{z}	26.57±0.75^{wzy}
JUTr003	35.30±1.75^{xyz}	27.60±0.75^{yz}
JUTr004	37.10±1.87^{xyw}	35.93±0.42^{pqrs}
JUTr005	42.50±0.62^{stuv}	30.57±0.91^{vwx}
JUTr006	40.83±1.27^{tuvw}	39.43±0.85^{mno}
JUTr007	54.50±0.66^{hij}	50.57±1.00^{e}
JUTr008	43.43±2.17^{rstu}	44.57±0.71^{hij}
JUTr009	39.53±0.65^{vw}	33.57±0.90^{stu}
JUTr010	35.40±0.66^{xyz}	30.50±0.95^{vwx}
JUTr011	43.93 ±1.40^{pqrst}	34.60±0.80^{rst}
JUTr012	67.03±1.76^{b}	55.37±0.59^{cd}
JUTr013	49.67±0.70^{klm}	46.53±0.87^{fgh}
JUTr014	47.53±0.85^{lmnopq}	42.23±0.40^{jklm}
JUTr015	30.37±0.96^{yz}	27.50±0.80^{z}
JUTr016	48.63±0.91^{klmn}	43.43±0.75^{ijkl}
JUTr017	47.67±0.86^{lmnop}	44.23±0.40^{hijk}
JUTr018	50.70±1.21^{jkl}	48.47±0.71^{efg}
JUTr019	38.40±0.9^{wx}	30.47±0.85^{vwxy}
JUTr020	37.53±0.75^{wx}	34.00±0.36^{stu}
JUTr021	33.00±1.35^{z}	28.60±0.80^{xyz}
JUTr022	39.47±0.85^{vw}	33.47±0.80^{stu}
JUTr023	59.60±0.96^{def}	35.60±0.80^{qrst}
JUTr024	53.77±1.44^{hij}	45.57±0.65^{hi}
JUTr025	48.43±0.78^{klmno}	34.63±0.75^{rst}
JUTr026	59.70±0.80^{def}	41.50±0.80^{klmn}
JUTr027	62.07±3.00^{cd}	59.83±0.67^{b}
JUTr028	39.80±1.08^{uvw}	36.90±0.36^{opqr}

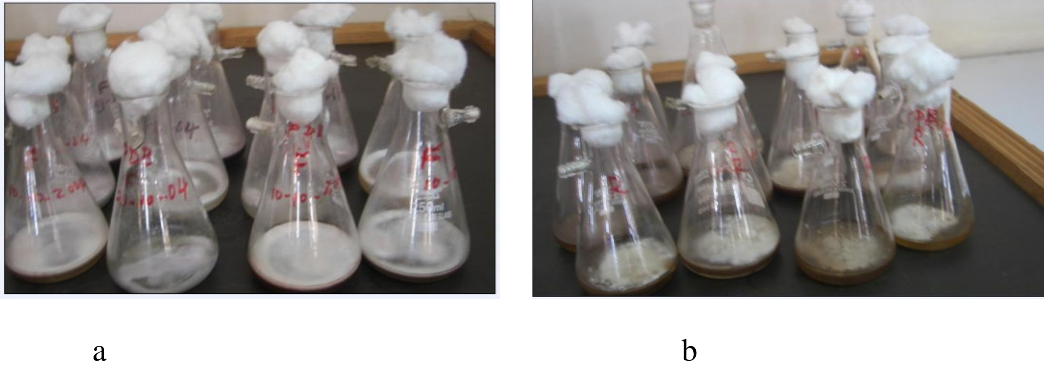
JUTr029	37.47±1.65^{wx}	29.97±0.25^{wxyz}
JUTr030	40.53±0.86^{tuvw}	31.63±0.80^{uvw}
JUTr031	56.43±0.70^{fgh}	50.47±0.70^{e}
JUTr032	45.50±0.89^{nopqrs}	37.60±0.75^{opq}
JUTr033	61.83 ±1.38^{cd}	54.27±4.03^{d}
JUTr34	40.57±0.70^{tuvw}	35.00±0.40^{qrst}
JUTr035	52.13±1.22^{ijk}	49.63±0.42^{e}
JUTr036	43.70 ±0.79^{qrst}	41.27±0.45^{lmn}
JUTr037	45.33±1.93^{nopqrs}	34.30±0.36^{rstu}
JUTr038	31.20±2.02^{z}	28.87±0.47^{wzy}
JUTr039	37.83±1.23^{wx}	33.23±0.45^{stuv}
JUTr040	59.60±0.70^{def}	55.60±0.75^{cd}
JUTr041	58.57±0.70^{defg}	45.60±0.66^{ghi}
JUTr042	55.07±0.40^{ghi}	35.00±0.44^{rst}
JUTr043	33.50±0.70^{yz}	28.47±0.87^{bc}
JUTr044	54.60±0.70^{hi}	49.00±0.40^{fg}
JUTr045	61.03±0.42^{cde}	41.23±0.40^{m}
JUTr046	55.10±0.36^{ghi}	41.27±0.42^{m}
JUTr047	63.80±0.44^{bc}	57.93±0.42^{c}
JUTr048	46.70±0.80^{mnopr}	32.90±0.53^{wx}
JUTr049	54.37±0.60^{hij}	38.70±0.30^{no}
JUTr050	55 .73±0.80^{ghi}	45.53±0.70^{hi}
JUTr051	58.77±0.47^{defg}	43.17±0.42^{kl}
JUTr052	60.63±0.80^{cde}	46.70±0.44^{h}
JUTr053	44.70±0.44^{opqrs}	35.50±0.66^{rs}
Standard(*T. harzianum*)	57.53±0.80^{efgh}	50.07±0.35^{ef}
Control	90.00±0.00^{a}	85.23±0.47^{a}

Mean of three replicates ± standard deviation followed by the same letters within columns are not statistically significant according to Fisher LSD method (P=0.05)

5.6 Greenhouse Experiment

5.6.1 Preparation of *R. solani* and *F. solani* inoculums

Fifty milliliters of PDB was inoculated with two 7mm-diameter mycelial disks from 7 days old PDA cultures of *R. solani* and *F. solani* and incubated at 27°C for 10 days separately. The inoculums grew over PDB and become white layer (Figure 13 a & b).



a b

Figure 13. Preparation of *F. solani* (a) and *R. solani* (b) inoculums on PDB

5. 6.2 Preparation of *Trichoderma* spp. inoculums

The fungal mycelia of JUTr012 and standard strain formed on the PDA were used to inoculate sorghum seeds and spore biomass was prepared in boiled sorghum seeds. The fungal mycelium slowly colonized the sorghum seeds and after 7 days the entire grain was full of green colored mycelial growth and spores on the seeds (Figure 14).

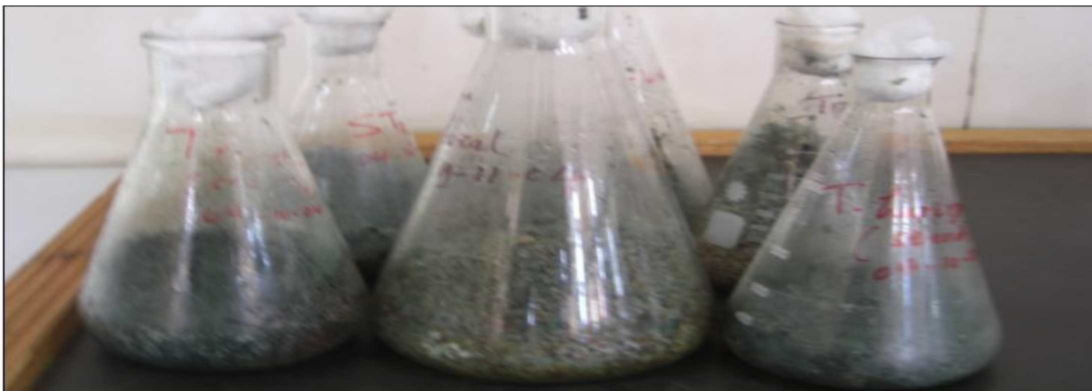


Figure 14. Preparation of *Trichoderma* spp. inoculums on sorghum grain

5.6.3 Damping off disease incidence

Soil treatment with bio-control agents significantly ($p<0.05$) reduced damping off disease incidence with *F. solani* and *R. solan*i in greenhouse conditions (Table 12). The damping off disease incidence caused by *F. solani* under application of *Trichoderma* spp. were in the range of 42.80– 72.36% and 19.22-5.70%, compared to 73.33% and 56.43% in the control plants, at pre- and post-emergence stages, respectively**.** At pre-emergence**,** R+ JUTr012+ standard gave the highest reduction of disease incidence (76.40%) followed by F+JUTr012 +standard (72.36 %), R+ JUTr012 (66.23%), F + JUTr012 (61.40%), R+standard (50.86%) and F + standard (42.80%). At post-emergence stage, again the combination of R+JUTr012+standard gave the best reduction to disease incidence (81.59%) followed by F+JUTr012+ standard (77.05%), R+ JUTr012 (71.35%), R+standard (55.24%) and F +standard [55.12 % (Table 12)].

The percentages of survival bean plants were in the range of 56.67– 80.00% compared to 45.00% in the control treatment. F + Standard+ JUTrO12 gave 80 % healthy plants followed by R + Standard + JUTr012 (76.67%), F+ JUTr012 (70%), R+ JUTr012 (66.67 %), F+ Standard (66.67%) and R+standard [56.67% (Table 12)].

Combination of JUTr012+standard isolates had strong bio-control activity against damping off disease caused by *R. solani and F. solani* under pot experiment (Table 12/ Fig. 15c). Data presented in Table (12) revealed that soil infested with *F. solani* has significantly increased damping off of haricot bean seedlings and severely reduced survival rate (20%) compared to the combined treatment[80%($p< 0.05$)].

Table 12. Effect of *Trichoderma* species treatments on the percentage of damping off disease of bean plants under greenhouse condition

Treatments	Damping off (%)				Survival plants (%)
	Pre-emergence		Post-emergence (%)		
	Incidence %	Reduction %	Incidence %	Reduction %	
Soil only	44.53^{c}	-	42.83^{b}	-	45.00^{c}
Soil +R	55.57^{b}	24.80	46.33^{b}	8.17	10.00^{d}
Soil +R+ JUTr012	15.04^{g}	66.23	12.27^{de}	71.35	66.67^{ab}
Soil +R+ Standard	21.88^{e}	50.86	19.17^{c}	55.24	56.67^{bc}
Soil + F	64.99^{a}	45.95	57.11^{a}	33.34	20.00^{d}
Soil + F+ JUTr012	17.19^{f}	61.40	14.28^{d}	66.66	70.00^{ab}
Soil +F+ Standard	25.47^{d}	42.80	19.22^{c}	55.12	66.67^{ab}
Soil+R+ Standard+ JUTr012	10.51^{i}	76.40	7.89^{f}	81.59	76.67^{a}
Soil+F+ Standard+ JUTr012	12.31^{h}	72.36	9.83^{ef}	77.05	80.00^{a}
LSD	0.98		2.50		8.69
CV	1.9		5.67		9.19
P-value	*		*		*

a

b **c**

Figure 15. Effect of *Trichoderma* species treatments on the damping-off disease of bean plants under greenhouse condition (a) experimental design, (b) control and (c) treated with JUT012+ standard

5.6.4 Growth and Yield parameters

Results revealed that the averages of bean plant height with *Trichoderma* application were in the range of 42.81 - 58.59 cm compared to 35.15 cm in the control (Table13). Soil +F + Standard+ JUTrO12 gave the highest plant height (58.59 cm) followed by Soil +R + Standard+ JUTrO12 (56.37 cm), Soil + F+ JUTO12 (53.02cm), Soil + R+ JUTrO12 (43.69 cm), Soil +R + Standard (42.81cm) and Soil +F + Standard (42.79cm). Significant ($p<0.05$) variations were recorded between *Trichoderma* treatments and the control plants.

The average branches number per plant as a result of *Trichoderma* spp. application ranged 5.78 – 9.67 branch/plant compared with 5.44 branch/plant for control. Soil +F + JUTO12 + Standard

significantly increased the branches number (9.67/plant) followed by Soil +R + JUTrO12 + Standard (9.22/plant), Soil +F + JUTrO12 (7.22/plant), Soil +R + JUTrO12 (6.78/plant), Soil +F + Standard (6.55/plant) and Soil +R+ Standard (5.78/plant). The average leaves number in treated bean plants ranged from 18.00 – 25.30 leaves/plant (Table 13). Soil +F + JUTrO12 + Standard significantly increased number of leaves (25.30/plant) followed by Soil +R + JUTrO12 + Standard (24.78 /plant), Soil +F + JUTO12 (22.00/plant), Soil +R + JUTrO12 (20.44/plant), Soil +F + Standard (20.33/plant) and Soil +R Standard (18.00/plant). Average root/shoot fresh weight was in the range of 79.33g/plant - 89.53/plant compared to 53.72 /plant in the control (Table, 11). Soil +F + JUTO12 + Standard significantly increased the shoot fresh weight of bean plant (89.53/plant) followed by Soil +R + JUTrO12+ Standard (89.30/plant), Soil + F + JUTrO12 (86.22 /plant), Soil +R + JUTrO12 (85.50/plant), Soil +R Standard (81.77/plant) and Soil +F + Standard (79.33/plant).

Application of *Trichoderma* spp. highly increased the average pods number that ranged from 22.00 – 28.33 pods/plant compared to 15.45 pods per plant in the control treatment (Table 13). Soil +F + JUTrO12 + standard considerably increased the mean pods number (28.33/plant) followed by Soil +R + JUTrO12+ Standard (27.22/plant), Soil + F + JUTrO12 (25.56 /plant), Soil +R + JUTrO12 (25.33/plant), Soil +F + Standard (22.67/plant) and Soil +R Standard (22.00/plant).

Table 13. Effect of *Trichoderm*a inoculation on shoot growth and yield of haricot bean under greenhouse condition

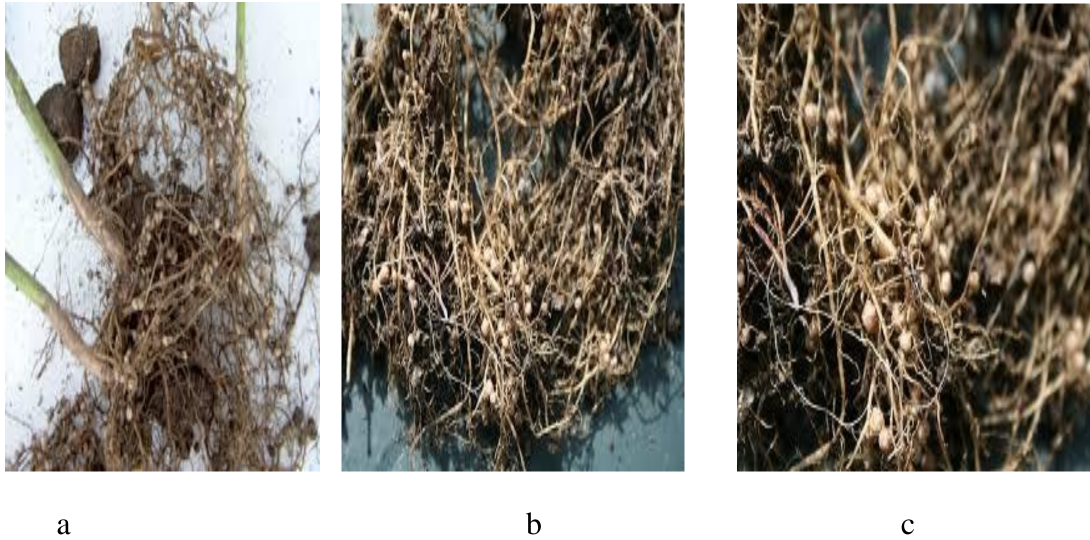
Treatments	SH (cm)	SFW (g/plant)	SDW (g/plant)	BN /plant	LN (g/plant)	PN /plant	100-seed weight(g)
Soil only	35.15^{e}	53.72^{d}	5.53^{d}	5.44^{d}	15.67^{d}	15.45^{d}	33.73^{bc}
Soil +R	15.71^{f}	29.67^{e}	3.02^{e}	2.78^{e}	7.89^{e}	7.89^{f}	28.27^{e}
Soil +R+ JUTrO12	43.69^{d}	85.50^{b}	6.56^{c}	6.78^{bc}	20.44^{bc}	25.33^{b}	34.69^{b}
Soil +R+ Standard	42.81^{d}	81.77^{c}	6.14^{cd}	5.78^{cd}	18.00^{cd}	22.00^{c}	32.69^{cd}
Soil + F	13.96^{f}	28.34^{e}	2.90^{e}	3.22^{e}	8.00^{e}	10.33^{e}	27.60^{e}
Soil + F+ JUTrO12	53.02^{c}	86.22^{ab}	7.78^{b}	7.22^{b}	22.00^{b}	25.56^{b}	33.96^{bc}
Soil +F+ Standard	42.79^{d}	79.33^{c}	6.78^{c}	6.55^{bcd}	20.33^{bc}	22.67^{c}	31.48^{d}
Soil + R + Standard + JUTrO12	56.37^{b}	89.30^{a}	8.48^{ab}	9.22^{a}	24.78^{a}	27.22^{ab}	38.23^{a}
Soil +F +Standard+ JUTrO12	58.59^{a}	89.53^{a}	9.01^{a}	9.67^{a}	25.30^{a}	28.33^{a}	38.78^{a}
LSD	1.23	2.08	0.45	0.75	1.47	1.45	0.98
CV	1.77	1.73	4.20	6.92	4.70	4.08	1.71
P-value	*	*	*	*	*	*	*

SH= shoot height, SFW= shoot fresh weight, SDW= shoot dry weight, BN=branch number, LN= leaves number, PN = pods number. Mean values followed by the same superscripts within a column are not significantly different at $p<0.05$. LSD= Least significant significance difference, CV=Coefficient of variance

Nodules were scattered along the length of the root system. Nodules showed variations in sizes (from small individual nodules to large globular clusters). Root length varied with treatment used and ranged from 43.44 to 53.00 cm long (Table14 /Fig.16).

The number of nodules per plant was variable. Soil+ F+JUT012 + standard gave the highest number of nodules per plant (206.89/plant) followed by Soil+R+ JUT012+ standard

(204.22/plant), Soil+ F+JUT012 (173/plant), Soil+ R+JUT012 (158.22/plant), Soil+ R+ standard (155.22/plant) and Soil+ F+ standard (152.2/plant). There were significant ($p < 0.05$) differences in nodule dry weight (Table14).



a b c

Figure 16. Effect of *Trichoderma* isolates on root growth of haricot bean. Soil only (control) (a), Soil+R+JUT0r12+ standard (b) and Soil+F+JUT0r12+ standard (c)

Table 14. Effect of *Trichoderm*a inoculation on root growth and nodulation of haricot bean under greenhouse condition

Treatments	**RL (cm/plant)**	**RFW (g/plant)**	**RDW (g/ plant)**	**NN /plant**	**NDW (g/plant)**
Soil only	35.44^{c}	13.14^{c}	2.68^{c}	133.11^{d}	0.26^{d}
Soil +R	22.23^{d}	5.85^{e}	2.58^{d}	95.66^{e}	0.21^{e}
Soil +R+ JUTO12	44.89^{b}	12.18^{c}	2.71^{bc}	158.22^{c}	0.45^{b}
Soil +R+ Standard	43.44^{b}	8.51^{d}	2.69^{bc}	155.33c	0.41^{c}
Soil + F	22.33^{d}	6.51^{e}	2.60^{d}	85.78^{e}	0.21^{e}
Soil + F+ JUTO12	45.22^{b}	8.84^{d}	2.72^{b}	173.00^{b}	0.43^{bc}
Soil +F+ Standard	43.44^{b}	12.28^{c}	2.68^{c}	152.22^{c}	0.40^{c}
Soil + R + Standard + JUTO12	53.00^{a}	16.28^{b}	2.97^{a}	204.22^{a}	0.57^{a}
Soil +F + Standard+ JUTO12	51.89^{a}	17.52^{a}	2.97^{a}	206.89^{a}	0.59^{a}
LSD	2.94	0.64	0.02	7.22	0.02
CV	4.22	3.29	0.41	2.75	2.96
P-value	*	*	*	*	*

RL= root length, RFW= root fresh weight, RDW= root dry weight, NN = nodule number, NDW = nodule dry weight. Mean values followed by the same superscripts within a column are not significantly different at (Tukey, $p<0.05$). LSD= Least significant Significance difference, CV= Coefficient of variation

6. DISCUSSION

Damping off fungi were retrieved with higher proportion (29.76%) from tomato plants. The present study showed that *F. solani* and *R. solani* are considered as important soil-borne pathogenic fungi capable of causing severe damage to agricultural crops such as bean, potato, and tomato (Lartey *et al.,* 1991). They are capable of attacking a tremendous range of host plants causing seed decay, damping off, stem cankers, root rot, fruit decay and foliage disease (Elad *et al*., 1980b). It might be due to favorable weather conditions prevailing in the study areas and hence caused severe yield losses every year. Haggag and El-Gamal (2012) also reported that damping off diseases caused by *R. solani* and *F. solani* fungi spread worldwide in crop growing areas and cause significant economic losses.

The overall weather conditions of Jimma zone, especially the high relative humidity favors the growth of fungi on crops and vegetables in these areas (Dedo, Kersa, Mana and Seka Chekorsa). Jimma Zone is characterized by the climatic zone locally known as "Woyna Daga" (1,500-2,400 masl) which is considered ideal for agriculture as well as human settlement (Gemechu, 1977). The zone is generally characterized by warm climate with a mean annual maximum temperature of 30°C and a mean annual minimum temperature of 14°C (Kifle, 1997). The annual rainfall ranges from 1138-1690 mm (Alemu et *al*., 2011). The authors further remarked that maximum precipitation occurs during the three months period from June through August, with minimum rainfall occurring in December and January.

The development of a proper isolation and *in-vitro* screening protocol that provides rapid, repeatable and reliable results is an important initial step in screening efficient antagonists for bio-control of plant diseases. This is because the success of all subsequent stages depends on the ability of the initial screening procedure to identify appropriate candidates (Whipps *et al*., 1988). One of the limitations of this research was that all of the isolates were characterized by conventional (traditional) methods. In the presence of molecular (PCR-based) methods, more *Trichoderma* isolates can be recovered and characterized.

The results of this study revealed that the JUT012 isolate, which was obtained from the rhizosphere soil of healthy plants had strong bio-control activity against *R. solani* and *F. solani* under *in-vitro* experiment thereby reducing the mycelial growth of the pathogenic fungi. Under

in-vitro condition, antagonistic capability of JUT012 isolate showed the highest inhibitory effect against growth of both *R.solani* and *F. solani.*

Trichoderma, *Penicillium, Aspergillus*, and *Fusarium* were also found to be prevalent in haricot bean, potato and tomato rhizosphere soil samples. This clearly indicates their wide adaptability to different environments. The current study assures the efficiency of *Trichoderma* as bio-control agents against soil-borne fungal pathogens and indicates the need for production and development of *Trichoderma* based bio-control agents to serve as a model eco-friendly bio-control agent. In this work, the results of dual culture revealed rapid colonization of the medium by *Trichoderma* isolates. All *Trichoderma* isolates evaluated were effective in suppressing colony growth of the soil-borne plant pathogens. For instance, JUTr012 was found to be an effective antagonistic agent against both *R. solani* and *F. solani.* It was capable of directly attacking and lysing both pathogens. The present results are in line with the findings of Padgan and Gade (2006) who reported that *T. harzianum* inhibited the mycelial growth of *F. oxysporum* to the extent of 77.10 %. Species of *Trichoderma viz*., *T. harzianum*, *T. viride*, *T. koningii* and *T. virens* showed more mycelial inhibition of pathogenic organisms compared to bacterial antagonists (Haggag and El-Gamal, 2012). This can be attributed to higher competitive ability of *Trichoderma* spp. by different possible mechanisms *viz.,* mycoparasitism, antibiosis and siderophore production (Kishore, 2007). Cumagun (2012) also stated that *Trichoderma* spp. are known to have strong antifungal effect partly as a result of their production of extracellular protease and chitinase enzymes which hydrolyse the main constituent of the pathogenic fungal cell wall.

The findings of this study showed that *Trichoderma* spp. proved to be effective in controlling *R. solani and F. solani*, the causative agents of haricot bean damping off disease under greenhouse conditions. The selected *Trichoderma* isolate (JUTr012) can be a potential bio-control agent to control damping off disease in haricot bean grown in the study areas which might be related to location specific and good adaptation with the existing environmental conditions of the region added to pathogen specificity.

The present finding indicated that all the *Trichoderma* spp. significantly ($p<0.05$) reduced disease incidence at pre- and post-emergence stages in greenhouse experiments. These results are

in agreement with the results of Snoeijers *et al.* (2000) who reported that *T. harzianum*, *T. koningii* and *T. viride* protected the germinating bean seedlings against *Fusarium* spp. and *R. solani* infection. Similarly, the tested *Trichoderma* spp. demonstrated a highly significant reduction to incidence of damping off disease in haricot bean plants under greenhouse infection as reported by other investigators (El-Kafrawy, 2002, Gonzalez *et al*., 2005; Malik *et al*., 2005). The ability of plant growth promotion and plant pathogens suppression by *Trichoderma* sp. has also been reported (Harman, 2006). Rapid colonization and rhizosphere competence may be the main mechanisms in its bio-control effects. Evidently, rhizosphere competent strains of *Trichoderma* species completely colonize roots surface for a few weeks or months and protect them from invading pathogenic fungi (Harman, 2000).

Application of *Trichoderma* spp. significantly increased average branch number per plant, when compared to the control treatment. The highest number of branches per plant was observed in JUTr012 + *T. harzianum* (standard strain).This result is in agreement with previous reports (Sallam *et al.,* 2008, Abd-El-Khair *et al*., 2010 Sahile *et al*., 2011).

The *Trichoderma* isolates JUTr012 + *T. harzianum* (standard) significantly ($p<0.05$) increased the shoot length, root length, of haricot bean plant compared to control plant. The highest shoot height was observed in case of soil inoculated with combination of JUTr012 + *T. harzianum* (standard) isolates. The increase in plant shoot height due to this bio-control application could be the result of modifying the environmental conditions, or promoting plant growth and plant defensive mechanisms (Benitez *et al*., 2004). Similar results of increase in shoot height due to bio-control inoculation have been reported on cucumber plant (Kleifeld and Chet, 1992). *Trichoderma* isolates significantly promoted root growth. These findings are similar to the results reported by Yedidia *et al*. (2001) who showed that treatment of cucumber plants with *T. harzianum* resulted in increase in cumulative root lengths and significant increase in dry weight, shoot length and leaf area over that of the untreated control. Harman and Kubicek (1998) also demonstrated that the colonization of the root system by rhizosphere competent strains of *Trichoderma* resulted in increased development of root and/or aerial systems and crop yields. In addition, Harman (2000) verified that *Trichoderma* spp. are opportunistic plant colonizers that affect plant growth by promoting abundant and healthy plant roots, possibly via the production of plant hormones.

The *Trichoderma* isolates JUT012 + standard (*T. harzianum*) significantly ($p<0.05$) increased the shoot dry weight (g/plant) of haricot bean plant compared to untreated plant. The highest shoot weight was observed in case of soils with the combined treatments causing the greatest effect as reported elsewhere (Siddiqui and Akhtar, 2009). Bean seedlings grown in *Trichoderma* treated soils recorded higher values of plant heights and weights as reported by Kleifeld and Chet (1992) who found that dry weights of cucumber plants grown in autoclaved sandy loam soils treated with a conidial suspension (10^6 CFU/g soil) of *Trichoderma* isolate. This variation could be related to increased growth response of plants that depends on the ability of the fungus (*Trichoderma*) to survive and develop in the rhizosphere.

The growth and yield parameters of bean plants were significantly ($p<0.05$) increased with the combined inoculation of JUTr012+*T. harzianum* (standard strain) compared to the individual bio-control agent. Inoculation of combination of *Trichoderma* isolates has resulted in much more increased shoot height, shoot fresh weight, shoot dry weight, root length, root fresh weight, root dry weight, leaf number per plant , branch number per plant and pod number per plant,100-seed weight compared to single inoculation**.** This indicates *Trichoderma* isolates had synergic interaction (Ghildiyal and Paney, 2008). The combination of bio-control agents may better adapt to the environmental changes, protect against a broader range of pathogens, increase the genetic diversity of bio-control systems that persist longer time in the rhizosphere, utilize a wider array of bio-control mechanisms (Pierson and Weller, 1994), enhance the efficacy and reliability of control (Duffy and Weller,1995; Duffy *et al*., 1996), allow the combination of various mechanisms of bio-control without the need for genetic engineering (Janisiewicz,1988). In addition, combination of bio-control agents with different plant colonization patterns may be useful for the bio-control of several plant pathogens via multiple mechanisms of disease suppression (Siddiqui and Akhtar, 2009). Similar studies by Nandakumar *et al*. (2001) showed that inoculation of combined *Trichoderma* isolates through seed, root, soil and foliage to rice crop suppressed sheath blight under field conditions better than individual strains. The earlier studies also revealed that antimicrobial metabolites produced by *Trichoderma* are effective against a wide range of fungal phytopathogens for example, *Fusarium* spp., *R. solani, Pythium aphanidermatum, Curvularia lunata, Bipolarizes sorokiniana* and *Colletotrichum gloeosporiodes* (Xiao-Yan *et al*., 2006; Zivkovic *et al.,* 2010).

It was suggested that these multiples of beneficial traits strongly assist the efficiency of candidate antagonists for desired biocontrol methods emphasizing the great value of concerted mechanisms of action. It was strongly suggested that the main success of biocontrol agents is largely attributed to their multifunctional characteristics and synergic interactions (Vassilev *et al*., 2006).

The present study clearly indicates the high potential of biocontrol agent (*Trichoderma* isolates) for different fungal pathogens. Thus, these biocontrol agents are important in reduction of damping off pathogens, improve soil fertility to reduce or supplement chemical fertilizers, fungicides, and enhance production system of *P. vulgaris*.

7. CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

During co-culturing, the most effective isolate (JUTr012) overgrew the pathogens *F. solani* and *R. solani*. This study showed that some *Trichoderma* isolates were efficient in controlling the damping off of haricot bean under both *in-vitro* and greenhouse conditions. Thus, soil inoculation with efficient *Trichoderma* isolates showed strong antagonistic activities against damping off disease that can be an acceptable option to minimize chemical fungicides application after fulfilling the necessary requirements for inoculum development.

7.2 Recommendations

The present study contributes to the understanding and utilization of *Trichoderma* isolates as bio-control agent. Therefore, further investigations are needed to explore the possibilities of using potent *Trichoderma* isolates as one of the most suitable candidates in biocontrol strategies to suppress phytopathogenic fungi. Thus, based up on the results of this work, the following points are recommended

- ❖ The most effective *Trichoderma* isolates that have been tested with *P. vulgaris* in greenhouse of sandy loam soil need to be re-tested in the field conditions to check their performance. `
- ❖ Evaluation of some locally available (like coffee husk) and cheap organic substrates for mass production of *Trichoderma* isolates could be worth to try.
- ❖ The potential of these biocontrol agents can be improved by continual improvement in isolation, inoculum preparation, and formulation methods, particularly in the field.
- ❖ Further studies are recommended for the expansion and commercialization of these potential biocontrol agents.

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9. DECLARATION

I, the undersigned, declare that the thesis here by submitted to Jimma University for the degree of Master of Science in Applied Microbiology has not previously been submitted by any other person for a degree at this or any other university, that it is my own work in design and in completing and that all material contained herein has been duly acknowledged.

Name: Solomon Bogale

Signature: ___________ Date ___________

The work has been done under the supervision and approved for final submission by:
Diriba Muleta (PhD)

Signature: ___________ Date ___________