THE EFFECT OF RHIZOBIA ISOLATES AGAINST BLACK ROOT ROT DISEASE OF FABA BEAN (*Vicia faba* L) CAUSED BY *Fusarium solani*

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THE EFFECT OF RHIZOBIA ISOLATES AGAINST BLACK ROOT ROT DISEASE OF FABA BEAN (*Vicia faba* L) CAUSED BY *Fusarium solani*

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Gedyon Tamiru Mena

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APPROVAL SHEET

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As thesis adviser, we here by certify that we have read and evaluated the thesis prepared by Gedyon Tamiru Mena, entitled "**The Effects of Rhizobial Isolates on Black Root rot of Faba Bean (*Vicia faba* L) caused by *Fusarium solani***", we recommend that it be submitted as fulfilling thesis requirement.

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As members of the *Board of Examiner of the M.Sc. Thesis Open Defense Examination*, we certify that we have read, evaluated the thesis prepared by Gedyon Tamiru and examined the candidate. We recommended that it be accepted to fulfilling the thesis requirements for the Degree of Master of Science Plant Pathology.

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DEDICATION

To my beloved and affectionate mother **Brihanesh Chincho**, who taught me the first word to speak, the first alphabet to write and first step to take but passed away at early age during my study period.

DECLARATION/ STATEMENT OF THE AUTHOR

I, under signed, declare that this thesis is my original work and has not been presented for any institute or anywhere for the award of any academic degree or diploma. All the sources of materials used for this thesis have been dully acknowledged. This thesis is submitted in partial fulfillment of requirement for Msc degree at Jimma University college of Agriculture and Veterinary Medicine and is deposited at the University of Library to be made available to borrowers under rule of Jimma University Library.

Name: Gedyon Tamiru Mena

Place: Jimma University

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BIOGRAPHICAL SKETCH

Gedyon Tamiru Mena was born at, 'Bossa Waniche', Humbo Woreda, Wolaita Zone in October 1982. He attended his junior secondary school and high school at Humbo Tebela Junior secondary school and Wolaita Sodo comprehensive high school, respectively.

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LIST OF ACRONOMYS

AMF	Arbuscular mycorrhizal fungi
BNF	Biological nitrogen fixation
BRR	Black root rot
CFU	Colony forming unit
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DSI	Disease severity index
FRR	Fuasrium root rot
GP	Germination percent
HCN	Hydrogen cyanide
IAA	Indole-3-acetic acid
ISR	Induced Systemic Resistance
JUCAVM	Jimma University Collage of Agriculture and Veterinary Medicine
OD	Optical density
PA	Piskovskaya's Agar
PDA	Potato dextrose agar
PGPR	Plant Growth Promoting Rhizobacteria
PSB	Phosphate solubilasing bacteria
PSM	Phosphate solubilasing microorganism
SI	Solublisation index
SVI	Seedling vigor index
TSA	Trypticase soy agar
YEMA	Yeast extract mannitol agar
YEMB	Yeast extract mannitol broth

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The Effect of Rhizobia Isolates against Black Root rot disease of Faba Bean (*Vicia faba* L) caused by *Fusarium solani*

ABSTRACT

Ethiopia is the largest producer of Faba bean in world next to China. In spite of huge importance, the productivity of Faba bean remains far below the crops potential. Black root rot caused by Fusarium solani is the major treat to Faba bean production in Ethiopia and the World. The use of fungicides against the disease has been shown to induce negative effect on environment and results in the appearance of fungicide resistant pathogen biotypes. It is also unaffordable for farmers in countries like Ethiopia. Thus it is important to look for ecofriendly management strategies of the disease. The main purpose was to evaluate for potential biocontrol agent by assessing isolates for in vitro inhibitory efficacy, probable mechanisms to inhibit fungal pathogen and effect on growth of Faba bean infected with Fusarium solani. In PDA medium, the effect of 41 Rhizobium isolates on the radial mycelium development of F. solani were tested in vitro. The experiments were undertaken by applying the dual culture techniques. Then, 27 isolates that showed remarkable inhibitory effect against Fusarium solani in vitro were tested to assess hydrolytic enzymes and growth promoting traits. Subsequently, the three most inhibitors under in vitro Rhizobium isolates and their combination were tested in vitro to determine their effect on germination of Faba bean seeds against F. solani. These three isolates and combination were again tested in vivo against F. solani root rot on seedling by applying the cell suspension at three different time of application (at the time of inoculation, 7 days before and after) on the pathogen. In dual culture, 27 rhizobium isolates remarkably inhibited the mycelia radial growth of F. solani more than 25% and Rh26(1), Rh15(2) and fb-1WG, inhibited fungal radial growth by 70.58 %, 64.70% and 63.72%, respectively. Among the 27 Rhizobium isolates tested for hydrolytic enzymes 7(26.15%), 12(44.40%), 4(14.81%) were positive to chitinase, protease and lipase production, respectively. chitinase, protease and lipase positive isolates showed significant fungal mycelia inhibition (26.47-70.58%), (25.49-70.58%), (39.21-62.74%), respectively. Eight (29.63%) were positive to hydrogen cyanide production. Also, 24(88.88%) were positive for IAA production and Over 50% were formed visible dissolution haloes on PA. Rh21(4) isolate produced the largest (1.90cm) solublisation index. Concurrent production of protease, lipases, chitinase, IAA and phosphate solubilisation coupled with anti-fungal activity suggests the plant growth promotion and broad spectrum biocontrol potential of these isolates. The highest significant germination percent observed in combination and fb-1WG (79.69%) followed by Rh26(1) (60%) compared to control (45%). Maximum significant radicel length (5.92cm,) plumule length (5.25 cm), and vigor index (889.87), measured in combination, followed by (4.41), (3.77cm) (652.40) in fb-1WG, and minimum (1.28 cm) (0.49 cm) (79.5) was in the control, respectively. Furthermore, combination and fb-1WG consistently reduced disease incidence and severity; and increased growth parameters on seedling in greenhouse at all times of application compared to diseased (control). Maximum disease severity (73.31%) reduction percentage was observed with application of combination before the pathogen, followed by at same time. Combination resulted in the highest (48 cm/plant) shoot height when applied before. This suggested that beneficial traits strongly assist the efficiency of candidate antagonists for desired biocontrol, emphasizing the value of concerted mechanisms of action. The study indicated the possible use of Rhizobial isolates as an alternative means of BRR management but further study is needed to prove the result.

Key words: inhibition

1. INTRODUCTION

Ethiopia is the 2^{n} largest producer of Faba bean in the world next from to China (Hawitin and Hebblewaite, 1993). In Ethiopia, crops are grown annually on approximately 7.9 million hectares of land, of these, 1.2 million hectares is covered by pulses out of which 411,719 ha is dedicated to Faba bean with annual production of about 446,850 tone (CSA, 2006). Faba bean is a multi-purpose crop that plays an important role in the socio-economic life of farming communities (Agegnehu and Fessehaie, 2006). Faba bean makes a significant contribution to soil fertility restoration as a suitable rotation crop that fixes atmospheric nitrogen and reduce the dependence on external fertilizer inputs and also an important source of income for farmers and generates foreign currency for the country.

In spite of huge importance, the productivity of Faba bean in Ethiopia remains far below the crop's potential greater than 3 ton/ ha. Production of Faba bean has been constrained by several biotic and abiotic factors (Agegnehu *et al*., 2006). Surveys on diseases of Faba bean in Ethiopia showed that 17 pathogens infect Faba bean in different parts of the country **(**Agegnehu *et al*., 2006**).** Some diseases that are economically most important in the major Faba bean growing regions include black root rot (BRR), chocolate spot and rust caused by *Fusarium solani* (Mart.) Appel and Wr., *Botrytis fabae* Sard., and *Uromyces viciae-fabae* (Pers.) Schr. Important, repectively (Gorfu and Basher, 1994a).

Root rot is among the major production constraints limiting the yield of Faba bean in many countries of the world especially where poor nutrient supply and too wet soil condition prevails (Habtu and Gorfu, 1985). It is caused by soilborne fungi. A review by Ali (1996) indicated *Fusarium solani f.sp. fabae*, was associated with root rot of Faba bean in Sudan. In Egypt, the survey results showed that *Fusarium solani* was associated with Faba bean root rot (Doreiah *et al*., 1994). The fungus *Fusarium solani* has been encountered on a large number of hosts in Ethiopia including Faba bean (Tadesse *et al*., 2006). *Fusarium solani* produce Chlamydospores that can survive in the soil for more than a year. These structures can be spread by running water, wind blown soil and with plant debris mixed with seeds (Schipper and Old, 1973). Water logging is a key factor that predisposes Faba bean to this disease. Since the disease develops slowly, infected plants shown symptoms of BRR with black

discoloration and can is easily be observed. Death of the plants followed severe rotting (Gorfu and Beshir, 1994a; Tadesse *et al.*, 2006). Complete crop losses could occur in severe infection conditions and when favorable conditions prevail for the pathogen. In farmers' fields, a loss of about 45% was estimated due to this disease (Tadesse *et al*., 2006).

Modern agriculture, apart from improving the overall production and productivity, has also caused destruction of the environment. For instance, the use of agrochemicals has been necessitated to increase productivity. Nevertheless, the synthetic chemicals cause degradation of soil health (Cook, 1991). In addition, the price of these chemicals is alarmingly soaring and unaffordable to needy farmers particulary in developing countries. Hence the alternative methods are being envisaged in an ecofriendly approach for sustainable agriculture. While organic manures like compost, vermicompost, green manure etc. are satisfactory sources for the supply of plant nutrients, it is yet to find suitable alternatives to pesticides for the control of diseases of crop plants by microbes.

The search for biocontrol agents against black root rot has shown promising results in our country **(**Beshir, 1999a**).** Previous study by Beshir (1999a) indicated the role of *Trichoderma viride* in protecting plants from BRR infection. It has been tested on Faba bean under greenhouse condition. The results of this study suggest that the biological control agent *T. viride* can play a role in a strategy for the control of BRR in Faba bean. The radial growth of *Fusarium solani* on potato-dextrose-agar (PDA) was reduced when mixed with the cultural filtrate of *T. viride*.

Many soil microorganisms possess multiple beneficial traits such as nutrient mobilization, production of plant growth promoting substances (PGPS) and biocontrol ability **(**Scher and Baker, 1982; Boddey and Dobereiner, 1995**)**. Such organisms have a greater role in sustaining agricultural production **(**Boddey and Dobereiner, 1995**).** Plant growth promoting rhizobacteria (PGPR), a group of root associated bacteria, intimately interact with the plant roots and consequently influence plant health and fertility of soil. They offer an excellent combination of traits useful in disease control and plant growth promotion **(**Haggag *et al.,* 2007**)**. For instance, *in vitro* antagonism tests of Rhizobacteria isolates were carried out against emerging coffee pathogens as *Fusrium xylarioides, F. oxysporum* and *F. stilboides.* The isolates were very active against all the pathogens tested (Muleta *et al.,* 2007).

As compared to the other PGPR, rhizobia play an important role in legume plant nutrition through their ability of symbiotic nitrogen fixation (Peoples *et al*., 1995). Faba bean in association with rhizobia can fix up to 120 kg nitrogen/ha (Danso, 1992). The contribution of the fixed nitrogen is a key factor in low input agricultural systems to sustain long term soil fertility and plant growth promotion **(**Beshir, 1999**).** This is especially important in areas where there is high farming pressure and the fallow system is abandoned. Biologically fixed N (BNF) under such situations improves soil N content, which in turn increases the yield of crops (Habtegebrial and Singh, 2006).

Many species of *Rhizobium* are reported to inhibit significantly the growth of pathogenic fungi (Estevez de Jensen *et al*., 2002; Bardin *et al*., 2004). Among the *Rhizobium* group, *Rhizobium leguminosarum, Sinorhizobium meliloti,* and *Bradyrhizobium japonicum*, rhizobia are also reported to significantly inhibit the growth of pathogenic fungi, that is, *Macrophomina phaseolina* (Tassi) Gold, *Rhizoctonia solani* Kuhn and *Fusarium* sp., in both leguminous and non leguminous plants (Esteshamul-Haque and Ghaffar, 1993). *Rhizobium* spp. have a beneficial effect on plants including biological control of soil-borne pathogens (Seuk *et al*., 2000).

The rhizobia have several mechanisms of action to control pathogens that include competition for iron by production of siderophores (Arora *et al*., 2001), synthesizes Rhizobiotoxin (Deshwal *et al*., 2003), phosphate solubilization and promotion of plant growth in terms of better shoot height, root length, dry weight and root nodulation (Alikhani *et al*., 2006). Antoun *et al* (1998) working showed rhizobia able produce IAA.

An investigation of root rot disease is considered particularly important in light of its wide prevalence in Ethiopia; particularly in mid and high land area **(**Tadesse *et al.*, 2006**)**. Very few number of research activities using *Trichoderma* spp was done on the biocontrol aspects of black root rot **(**Beshir, 1999**)**. Even though, previous studies suggested the biocontrol agent against black root rot of Faba bean, still there is need to search other biocontrol agents against root rot (BRR) in the country.

Therefore, this study aimed at to bridge in the gap in search of alternative biocontrol against BRR using *Rhizobium* isolates with the potential of antifungal activities and growth

promotion. As a component of integrated disease management strategy, selecting potential *Rhizobium* isolates with multiple beneficial traits for production of Faba bean is very important.

1.1. Objectives

General objective

- The general objective of this study was to evaluate the potential of *Rhizobium* isolates as biocontrol agents against black root rot of Faba bean caused by soil-borne fungus, *Fusarium solani*

- *Specific objective*

The specific objectives of the present study were:

- To determine *in vitro* inhibitory effect of *Rhizobium* isolates against *Fusarium solani*
- To determine the probable mechanisms used by rhizobial isolates to inhibit fungal pathogen by studying their ability to produce: Protease, Chitinase, Lipase, Hydrogen cyanide (HCN), Indole acetic acid (IAA) and to mobilize phosphate
- To evaluate effect of *Rhizobium* isolates on *in vivo* growth of Faba bean (*Vicia faba* L) infected with *Fusarium solani*

2. LITERATURE REVIEW

2.1. Faba Bean

2.1.1. Taxonomy

Faba bean (*Vicia faba* .L) belongs to Kingdom: Plantae Class: Magnolipsida Order: Fabales Family: Fabaceae Subfamily: Faboideae; Tribe: Vicieae; Genus: *Vicia* and to the Species: *V. faba.* *Vicia faba* is an annual herb with coarse and upright stems, unbranched 0.3-2 m tall, with 1 or more hollow stems from the base (Bond *et al*., 1985). Several wild species *(V. narbonensis* L. and *V. galilaea* Plitmann and Zohary) are taxonomically closely related to the cultivated crop, but they contain 2*n* = 14 chromosomes, whereas cultivated faba bean has 2*n* = 12 chromosomes.

2.1.2. Origin and distribution

Broad bean or fava (faba) bean (also known as horse bean, field bean, tick bean or winds or bean) (*Vicia fava* L.) is cool- season legume popular in middle East, Europe, China and in the highlands of South America. Production is concentrated in nine major agroecological regions namely Meditrranean, the Nile valley, Ethiopia, Central Asia, East Asia, Oceania, Northern Europe, Latin America and North America. Ethiopia is considered as the secondary center of diversity and also one of the nine major agro-geographical production regions of Faba bean (Telaye *et al*., 1994). At present, Faba bean is the third most important cool-season food legume in the world (Torres *et al*., 2006).

Faba bean is assigned to the Central Asian, Mediterranean, and South American centers of Diversity. Cubero (I974) postulated a Near Eastern center of origin, with four radii: (1) to Europe (2) along the North African coast to Spain, (3) along the Nile to Ethiopia, and (4) from Mesopotamia to India. Secondary centers of diversity are postulated in Afghanistan and Ethiopia.

2.1.3. Uses

Cultivated Faba bean is used as human food in developing countries and as animal feed, mainly for pigs, horses, poultry and pigeons in industrialized countries. It can be used as a

vegetable, green or dried, fresh or canned. It is a common breakfast food in the Middle East, Mediterranean region, China and Ethiopia (Bond *et al*., 1985).

Faba bean is used as human food in developing countries and as animal feed in industrialized countries. Faba bean is widely used as a good sources of protein, starch, cellulose and minerals (Haciseferogullari *et al*., 2003) for human in developing countries. It provides essential amino acids (particularly lysine) that are not present in sufficient quantities in staple cereal corps (Giller, 2001). In Ethiopia, Faba bean is the leading protein source for the rural people and used to make various traditional dishes (Yetneberk and Wondimu, 1994). Moreover, it provides large cash for producers and foreign exchange for the country (Beyene, 1988).

The crop also plays an important role in improving the productivity of the cereal crops in the rainfed farming systems through improvement in physical, chemical and biological properties of the soil. Yields of rainfed cereal crops following Faba bean have been higher than those in a continuous cereal rotation and almost at par with the cereal crop following a fallow in Tel Hadya, Syria (Saxena, 1988).

2.1.4. Diseases

Diseases are among the important biotic factors that limit the production of Faba bean crop. Chocolate spot (*Botrytis fabae* Sard.) and rust (*Uromyces fabae* (Pers.) Schart) are one of the most economically important diseases that damage the foliage, limiting photosynthetic activity and reduce Faba bean production (Bouhassan *et al*., 2004; Torres *et al*., 2006). They may cause total crop failure under severe epidemic conditions (El-Bramawy & Abdul Wahid, 2005; Torres *et al*., 2006).

In Ethiopia, numerous pathogens that cause diseases of Faba bean (*Vicia faba L*.) include black spot (*Alternaria tenuis*), blight (*Ascochyta fabe*), leaf roll (Bean leaf roll virus), mosaic virus (Bean yellow mosaic virus), virus (Beet western yellows virus), chocolate spot (*Botrytis fabae*), stain virus (Broad bean stain virus), mosaic virus (Broad bean true mosaic virus), zonate leaf spot (Cercospora zonate), chlorotic virus (Chickpea chlorotic dwarf virus), Powdery mildew (Erysiphe polygoni), Necrotic yellow (Faba bean necrotic yellows virus), Foot rot (Fusarium avenceum), black root rot (*Fusarium solani*), dry root rot (Macrophomia

phaseolina), Root knot(*Meloidogyne incognita*), root lession (Pratylenchus sp.), root rot (*Rhizoctonia solani*), collar rot (Sclerotium rolfsii) and Rust (Uromyces viciae- fabae) (Gorfu and Beshir, 1994a; Tadesse *et al*.,2006; Gorfu & Yaynu, 2001). However, only a few of them have either major or intermediate economic significance. These include chocolate spot, rust, black root rot, foot rot and necrotic yellow (Gorfu and Beshir, 1994a).

2.2. *Fusarium solani*

2.2.1. Origin, taxonomy and distribution

Fusarium spp. may have one of the first fungi to become established on earth (Nelson *et al*., 1981). The genus *Fusarium* is among the heterogeneous fungal genera and classification of species within this genus is very difficult (Llorens *et al*., 2006). However, *Fusarium solani* is one of the few *Fusarium* species that is easily identifiable. The genus is sub divided into ten *formae specialis* (*f.sp*) based on host range test (Nelson *et al*., 1981).

The fungus was described and clearly illustrated by Martius in 1842, as *Fusisporum solani.* Forty year later, in 1881, Saccardo renamed it *Fusarium solani* (Booth, 1971). The genus *Fusarium* is imperfect fungi (Deutromycotina), belongs to the Kingdom fungi, order Hypocrealea, Family Hypocreaceae (Fry, 2004). The survival of *F. solani* in the soil depends on the production of Chlaymydospores, which are resistant structures capable of survival in the absence of the host plant (Schipper and Old, 1973).

Most *Fusarium* strains occure worldwide (Schollenberger *et al*., 2005). The distribution of *Fusarium* species is influenced by weather conditions such as temperature and humidity (Kosiak *et al*., 2004).

2.2.2. Morphology and cultural characteristic

The genus *Fusarium* is characterized by the production of septate, hyaline, delicately curved elongated macrocondia (Moss and Thrane, 2004). *F. solani* germinate in cultivated soils in the rhizospheres of host or non-host plants (Mondal *et al*., 1996). According to Song *et al*., (2004), the use of hydroponics cultivation systems in greenhouse also offers favorable

conditions for *F. solani*. *F. solani* f. sp. *Phaseoli* has specificity for nutrients of crop plants for germination (Mondal *et al*., 1996).

2.2.3. Economic significance

Fusarium solani that causes Fusarium root rot (FRR) is one of a complex of soil-borne pathogens causing root rots on beans, others being *Rhizoctonia solani* and *Macrophomina phaseoli* (Abawi & Pastor- Corrales, 1990; Rusuku *et al*., 1997). The pathogen has been reported to be particularly severe on large-seeded bean genotypes due to lack of genetic resistance in these seed types (Beebe *et al*., 1981; Burke & Miller, 1983; Schneider *et al*., 2001; Román-Avilès & Kelly, 2005). In addition, resistance to FRR has been associated with small seed size, black seed colour, and purple hypocotyls (Statler, 1970; Beebe, 1981), although these correlations have not been conclusive.

A review by Ali (1996) indicated *Fusarium solani f.sp. fabae,* was associated with root rot of Faba bean in Sudan. The fungi *Fusarium solani* has been encountered on a large number of hosts in Ethiopia, including Faba bean causing black root rot (Tadesse *et al*., 2006). Black root rot caused by *Fusarium solani* is the second most important disease of Faba bean.

2.2.4. Epidemiology, ecology and environmental conditions

According to Mondal *et al*., (1996), *Fusarium solani* germinates in cultivated soils in the rhizosphere of host or non-host plants. *F. solani* develops most rapidly at temperature ranging 24-29 ^{o}C. The effect of temperature on propagules density may influence the survival and the life cycle of *F. solani* (Seremi *et al*., 1999). The fungus is spread by infested plants and soil on farm machinery, drainage water and boots (Jones, 1997). When a susceptible crop is present, chlamydospores germinate and the fungus penetrates the plants through young roots. The mycelium enters the xylem and progresses up the root and stem into the leaves (Jones, 1997).

The majority of *Fusarium* species are normally found in or on soil, where they exist as colonizers of living plants or plants residues within the soil (Nelson *et al*., 1981). This pathogen can be found notably in the prairie soils, but is missing or rare in forest soils (Griffin, 1972). Furthermore, *Fusarium* can remain viable for up to 30 years (Thangavelus *et*

al., 2003). *Fusarium solani* levels in the field depend on the temperature and other factors such as rainfall. In our country, the *Fusarium solani* occurs mostly in clay soils where water logging is severe. Water logging is a key factor that predisposes Faba bean to this disease (Tadesse *et al*., 2006).

2.2.5. Symptoms

The disease develops slowly and infected plants develop chlorosis and dark black roots, which finally disintegrate. Pulling out of plants with symptoms of black root rot becomes easy and the black discoloration of the whole root is easily observed. Death of plant follows severe rotting (Gorfu and Beshir, 1994a; Tadesse *et al*., 2006).

2.2.6. Management of black root rot

2.2.6. 1. Cultural practice

Planting crops that are not hosts of *Fusarium solani*: noug (*Guizotia abyssinica*), rapessed (*Brassica napus*) and linseed (*Linum usitatissimum*) (Tadesse *et al*., 2006) in rotation with faba bean may reduce inoculum level in soil. However, it is still not known to what extent would this inoculums suppress BRR. The time interval occurring between the repeated cultivation of Faba bean (or other susceptible crop such as chickpea) is not determined, as well. As water logging is a key factor in predisposing plant to this disease, proper drainage of faba bean fields is essential to minimize the effect of this disease (Gorfu and Beshir, 1994a).

2.2.6. 2. Host plant resistance

The National Faba bean Improvement Program at Holeta Agricultural Research Centre and Ambo Plant Protection Reseasrch Centre, and Regional Research Centre, Sheno, made efforts to identify sources of resistance to black root rot (Beshir, 1995) and thereby develop BRR – resistant varieties possessing high yield. These efforts resulted in development and release of four BRR-resistant varieties of Faba bean: Wayu (Wayu 89-5), Salale (Salale Kasim 91-13), Lalo (Salale Kasim 89-4) and Dagm (Grarjarso 89-8) (NAIA, 2003). The first two varieties perform well under waterlogged conditions and have been released for general cultivation in the country in 2002, and the remaining have been released for North Shewa areas where BRR is a problem every year.

2.2.6. 3. Integrated disease management

An experiment integrating varieties, improved drainage system and seed rates were conducted to minimize root rot damage in Faba bean (Tadesse *et al*., 2006). The results obtained indicate that there was no significant variation in the seedling stage disease incidence for the combination of two or three of the treatments. But at flowering stage, disease incidence revealed significant difference for the separate effect of variety and seed rate treatments.

2.2.6.4. Chemical control

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. As agricultural production intensified over the past few decades, producers became more and more dependent on agrochemicals as a relatively reliable method of crop protection helping with economic stability of their operations.

Seed dressing with Agrosan and Furadan resulted in 38% reduction of *Fusarium* infecting okra (Dash and Narain, 1996). Combination of Benomyl and Captan was effective against all root rot fungi (Dash and Narain., 1996). Seed treatment with effective fungicides such as Thiram (Thiram 70s), benomyl (Benlate), and Captafol (Difolatan), are only partially effective in reducing damage, because damage occurs on fibrous roots at some distance from seed placement.

However, increasing use of chemical inputs causes several negative effects, i.e., development of pathogen resistance to the applied agents and their nontarget environmental impacts. Furthermore, the growing cost of pesticides, particularly in less-affluent regions of the world, and consumer demand for pesticide-free food has led to a search for substitutes for these products. Chemical pesticides contaminate groundwater, enter food chains, and pose hazards to animal health and to the spraying personnel of the chemical pesticides. Several members of the European Union (EU), such as Sweden, Denmark, and Netherlands have decided in the mid-late 1980s to decrease the chemical input in agriculture by 50% within a 10-year period (Butt *et al.,* 2001). There are also a number of fastidious diseases for which chemical

solutions are few, ineffective, or nonexistent. Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture.

2.3. Biological Control

Biocontrol organisms offer environmentally friendly alternatives to chemical control methods to manage plant diseases. Biological control agents could be used where chemical pesticides are banned (organochlorines) or being phased out (methyl bromide) or where pests or pathogens have developed resistance to conventional pesticides or to grow organic food to satisfy consumer perception (Butt *et al*., 2001). Biological control depends up on the establishment and maintenance of a threshold population and viability below that level may eliminate the possibility of biological control (Xu and Gross, 1986).

It has been reported that some bacterial species can serve as biological control agents against soil-borne pathogens (Arfaoui *et al*., 2007). Gupta *et al.* (1999) isolated *P. aeruginosa* from potato rhizosphere that displayed a strong antagonistic activity against important fungal pathogens, *viz. Macrophomina phaseolina* and *Fusarium oxysporum*. Muleta *et al*. (2007) reported that antagonistic rhizobacteria, more specifically *Fluorescent pseudomonads* and certain *Bacillus* species possessed the ability to inhibit emerging coffee fungal pathogens. Tripathi and Johri (2002) studied the biocontrol potential of *Fluorescent pseudomonas* isolated from rhizosphere of pea and wheat *in vitro* and *in vivo* against maize sheath blight caused by *Rhizoctonia solani*. They found some isolates to possess multiple disease control potential, while some others exhibited biocontrol potential against specific pathogens, which indicates that fluorescent pseudomonads are diverse with respect to their biocontrol potential.

Application of *P. fluorescens* or *B. subtilis* increased the growth and yield of chickpea and reduced the infection by *M. incognita* by minimizing the number of galls/root system, egg mass production and soil population (Khan *et al.*, 2009). Mortality of *M. incognita* juveniles was observed to be similar both in unheated and heated culture filtrates of *P. fluorescens* and the mortality increased with increase in concentration (Sirohi *et al.*, 2000).

Biological control method has potential to control crop diseases while causing no or minimal detrimental environmental impact (Haggag *et al.*, 2007). It has been used as alternative

control method of chemical due to harmful effects of some pesticides to human health and the environment (Cook, 1993). Controlling plant disease with biocontrol microorganisms reduce environmental pollution and resistance development as compared to chemical method. This is because they produce degradable chemical in low amounts at targeted locations. This approach fits well in the worldwide strategy to grow healthy plants in a sustainable way and, therefore produce high quality food (Haggag *et al.*, 2007).

2.4. Phytobeneficial Microorganisms

Growing plant roots influence the activities of soil microorganisms in the adjoining volumes of soil known as rhizosphere (Lynch, 1982). In other words, rhizosphere is a narrow zone of soil (soil–plant interface) subject to influence of living roots, where root exudates stimulate or inhibit microbial populations and their activities. The rhizoplane or root surface provides a highly favorable nutrient base for many species of microorganisms. In essence, the rhizosphere can be regarded as the interaction between soil, plants, and microorganisms (Brimecombe *et al.*, 2007).

The root rhizosphere is the place of an intense microbial life and a high microbial activity (Mohamed, 2009). The composition and number of microorganisms present in the rhizosphere of different plants may differ due to variations in the quantity and quality of compounds exuded by the different plants (Aldén *el al*., 2001). Root exudates selectively influence the growth of bacteria and fungi that colonize the rhizosphere by altering the chemistry of soil in the vicinity of the plant roots and by serving as selective growth substrates for soil microorganisms (Kremer *et al*., 1990).

The root colonizing ability is an essential prerequisite for the success of rhizobacteria. The introduced microorganisms in plant roots as biofertilizers, biocontrol agents and plant growth promoters have generally shown a progressive decline in population size leading to limit their effectiveness (Compeau *et al.*, 1988). So, the inoculants microbes must be able to establish themselves in the rhizosphere at population densities sufficient to produce beneficial effects. Therefore, efficient biocontrol agents should survive in the rhizosphere, make use of nutrients exuded by the plant roots, proliferate, be able to efficiently colonize the entire root system and

highly demonstrate rhizosphere competence with indigenous microorganisms (Mohamed, 2009).

Root exudates in the rhizosphere offer a carbon-rich diet to the rhizosphere microorganisms: organic acids (such as citrate, malate, succinate, pyruvate, fumarate, oxalate and acetate) and sugars (such as glucose, xylose, fructose, maltose, sucrose, galactose and ribose) constitute the major exudates, whereas variable amounts of α-amino acids, nucleobases and vitamins (such as thiamin and biotin) provide the minor exudates. The ability of rhizobacteria to use organic acids as carbon sources correlates with rhizosphere competence (Goddard *et al.,* 2001). In essence, rhizosphere is relatively rich in nutrients due to the loss of as much as 40% of plant photosynthesis from the roots (Lynch and Whipps, 1991). Consequently, rhizosphere microbes benefit because plant roots secrete metabolites that can be utilized as nutrients. Thus, the rhizosphere supports large and active microbial populations capable of exerting beneficial, neutral, or detrimental effects on plant growth.

2.4.1. Plant Growth Promoting Rhizobacteria (PGPR)

Plant growth promoting microorganisms (PGPM) are a heterogeneous group of microbes that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth. They exert beneficial effects on plant development via direct or indirect mechanisms. The exact mechanisms by which PGPR (Plant growth promoting Rhizobacteria) promote plant growth are not fully understood but are thought to include: the ability to produce or change the concentration of plant growth regulators like indole acetic acid, gibberellic acid, cytokinins and ethylene; biofertilization (Boddey and Dobereiner, 1995); antagonism against phytopathogenic microorganisms by production of siderophores (Scher and Baker, 1982); antibiotics (Shanahan *et al*., 1996) and cyanide (Flaishman *et al*., 1996); solubilization of mineral phosphates and other nutrients (Gaur, 1990); rhizoremediation (Kuiper *et al.,* 2004), induction of systemic resistance and competition for nutrients and niches (Lugtenberg and Kamilova, 2009).

2.5. Mechanisms of Biological Control of Plant Pathogens

2.5.1. Antibiosis

Antibiosis is the inhibition or destruction of one organism by a metabolite produced by another organism. Antagonists may produce powerful growth inhibitory compounds that are effective against a wide variety of microorganisms (Ownley and Windham, 2006). These compounds are low molecular weight organic substances that are produced as secondary metabolites by certain groups of microorganisms at low concentration (George, 2002). These metabolites may have acidic (killing) effect or a static (inhibiting) effect on the growth of the target pathogen, and this is the best-known mechanism by which microbes can control plant diseases (Lecrere *et al*., 2005). The best-known antibiotics produced by gram-negative bacteria are phenazines, 2, 4- diacetylphloroglucinol (Bangera and Thomashow, 1999), pyrrolnitrin (Pfender *et al.,* 1993), pyoluteorin (Kraus and Loper, 1995) and oomycin A (Gutterson, 1990). Some biocontrol Bacilli produce the antibiotics zwittermycin A and kanosamine (Silo-suh, 1994). It was also reported that *Trichoderma* and *Gliocladium* produce antimicrobial compounds such as gliovirin and gliotoxin (Howell *et al*., 1993). Different antibiotics have different mode of action on bacterial pathogens. These include: prevent proper cell wall formation; inhibition of protein synthesis and damage to memberane integrity; disruption of plasma and outer membrane function and inhibition of DNA synthesis (Walker *et al.,* 2001).

Volatiles other than hydrogen cyanide, such as 2,3-butanediol, or blends of volatiles produced by *Bacillus* spp. (Ruy *et al*., 2003) can be involved in plant protection. Finally, lipopeptide biosurfactants produced by *B. subtilis* (Ongena *et al*., 2007) and by pseudomonads (de Bruijn *et al*., 2007) have been implied in biocontrol. Rhamnolipid and phenazine act synergistically against soilborne diseases caused by *Pythium* spp. (Perneel *et al*., 2008).

2.5.2. Enzyme production

Biological control microorganisms attack pathogens by excreting lytic enzymes. Muleta *et al*. (2007) revealed that rhizobacterial isolates from coffee rhizosphere produced Chitinase, β - 1,3-glucanase, protease and lipase enzymes to control coffee pathogens such as *Fusarium xylarioides, Fusarium oxysporum* and *Fusarium stilboides.* It has reported that chitinase

produced by *Serratia plymuthica* inhibited spore germination and germ-tube elongation in *Botrytis cinerea* (Frankowski *et al.,* 2001); β -1,3-glucanase synthesized by *Paenibacillus* and *Streptomyces* sp lyse fungal cell walls of *F. oxysporum* f. sp. *cucumerinum* (Singh *et al.,*1999). Lysis of pathogen by hydrolytic enzymes is often characteristic of mycoparasitism. This has been demonstrated for several *Trichoderma* species that control fungal pathogens (Harman *et al.,* 2004). Chitinase and β-1,3 glucanase (laminarase) are particularly important enzymes secreted by fungal mycoparasites capable of degrading the fungal cell wall components, chitin, and β-1,3 glucan (Schroth and Hancock, 1981).

2.5.3. Competition for nutrients and ecological niche

According to Elad and Chet (1987), the competition for available carbon and nitrogen sources may account for observed disease reduction. These authors found that competition for nutrients between germinating oospores of *Pythium aphanidermatum* and bacterial biocontrol strains significantly correlated with suppression of the disease. It appeared that bacteria were competing with germinating oospores for available carbon and nitrogen and by eliminating these resources, the bacteria effectively reduced oospores germination. Wilson and Lindow (1993) demonstrated that *P. fluorescens A* 506 colonized by competing for a limiting nutrient, thus making this nutrient unavailable to *Erwinia amylovora.* Niche exclusion is an alternative mechanism involved in biocontrol agents. *Pseudomonas* strains, for example, are able to establish on inoculated seeds easily (Brimecombe *et al.,* 2007).

2.5.4. Hydrogen cyanide (HCN) production

The cyanide ion is exhaled as HCN and metabolized to a lesser degree into other compounds. HCN first inhibits the electron transport and energy supply to the cell leading to the death of the organisms. It inhibits proper functioning of enzymes and natural receptors (Corbett, 1974) and it also known to inhibit the action of cytochrome oxidase (Gehring *et al.,* 1993).

Hydrogen cyanide is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens (Defago *et al.,* 1990). Muleta *et al*. (2007) have demonstrated that all the *Pseudomonas* spp. that showed remarkable inhibitory effects against the test coffee fungal pathogens produced HCN. Production of HCN by certain strains of fluorescent pseudomonads has been involved in the suppression of soil borne pathogens (Voisard *et al.,*

1989). HCN producing *Pseudomonas fluorescens* inhibited the mycelial growth of *Pythium* under *in vitro* condition (Westsleijn, 1990).

The cyanide producing *Pseudomonas* strain CHAO stimulated root hair formation, indicating that the strain induced and altered plant physiological activities (Voisard *et al.,* 1989). Four of the six PGPR strains that induced systemic resistance in cucumber against *Colletotrichum orbiculare* produced HCN (Wei *et al.,* 1991). Fluorescent *pseudomonas* strain RRS1 isolated from Rajanigandha (tuberose) produced HCN and the strain improved seed germination and root length (Saxena *et al.,* 1996).

2.5.5. Induced systemic resistance (ISR)

It is defined as the process of active resistance dependent on the host plant's physical and chemical barriers, activated by biotic and abiotic agents (inducing agent) (Leeman *et al*., 1996). Induced systemic resistance (ISR) is a mechanism by which biocontrol agents induce a certain change in the plant and increase its tolerance to infection of plant diseases. ISR protects the plant systemically following induction with an inducing agent to a single part of the plant (Brimecombe *et al.,* 2007). The action of ISR is based on plant defense mechanisms that are activated by inducing agents (Kloepper *et al.,* 1992). Induced systemic resistance makes susceptible plant resistant to a wide array of subsequent pathogen attack (van Loon and Glick, 2004) by activation of host responses that directly attack the pathogen through enzymatic action, production of plant produced antibiotic substances or by lignifying cell walls so that pathogen movement is slowed. ISR activates multiple potential defense mechanisms, which include increases in activity of chitinases, ß-1,3- glucanases, peroxidases, and other pathogenesis-related proteins which mainly include (a) phenol oxidases, peroxidases and polyphenoloxidases (Lawton and Lamb,1987); accumulation of antimicrobial low molecular-weight substances such as phytoalexins (Kuc and Rush,1985); and the formation of protective biopolymers such as lignin, cellulose, and hydroxyproline-rich glycoproteins (Hammerschmidt *et al.,*1984). Phytoalexins produced in plants act as toxins to the attacking organism. They may puncture the cell wall, delay maturation, disrupt metabolism or prevent reproduction of the pathogen in question. As part of the induced resistance, the short-term response, the plant deploys reactive oxygen species such as superoxide and hydrogen peroxide to kill invading cells. In pathogen interactions, the

common short-term response is the hypersensitive response, in which cells surrounding the site of infection are signaled to undergo apoptosis, or programmed cell death, in order to prevent the spread of the pathogen to the rest of the plant (Glazebrook and Ausubel., 1994). A single inducing agent can control a wide spectrum of pathogens. In cucumber, it has indicated that treatment of the first leaf with a necrosis-forming organism protects the plant against at least 13 pathogens, including fungi, bacteria, and viruses (Dean and Kuc, 1985).

2.5.6. Competition for Iron and role of siderophores

Rhizobacteria produce siderophores that chelates the available iron and prevent the iron nutrition of respective phytopathogens, thereby restricting the proliferation and colonization by phytopathogens (Lemanceau and Albouvette, 1993). Siderophores are iron chelating ligands which can be beneficial also to plants by increasing the solubility of ferric iron (Fe III), which otherwise is unavailable for plant nutrition (Renshaw *et al*., 2002).

Muleta and co-workers (2007) revealed that all the potent *in vitro* antagonistic *Pseudomonas* spp. produced siderophores, mainly of the hydroxamate type. Siderophore-producing microorganisms are also known to impart induced systemic resistance to plants (Pieterse *et al.*, 2001) and suppressive to soil-borne pathogens (Mazzola, 2002) and have been implicated in the biocontrol of several plant diseases (Sayyed *et al*., 2005). Siderophore-based biological control agents provide iron nutrition; thereby promote the plant growth (Sayyed *et al*., 2007).

2.5.7. Indole-3-acetic acid (IAA) production

IAA is phytohormone which is known to be involved in root initiation, cell division and cell enlargement (Salisbury, 1994). This hormone is very commonly produced by PGPR (Barazani and Friedman, 1999; Muleta *et al*., 2009). Vessey (2003) has reviewed the production of this hormone and implicated it in the growth promotion by PGPR. However, the effect of IAA on plants depends on the plant sensitivity to IAA and the amount of IAA produced from plant associated bacteria and induction of other phytohormones (Peck and Kende, 1995). Patten and Glick (2002) demonstrated that bacterial IAA from *Pseudomonas putida* played a major role in the development of host plant root system. Similarly, IAA production in *P. fluorescens* HP 72 correlated with suppressing of creeping bent grass brown patch (Suzuki *et al.*, 2003).

2.5.8. Phosphate solubilization

Several soil microorganisms, including bacteria, improve the supply of phosphorus to plants as a consequence of their capability for inorganic or organic phosphate solubilization (Lifshitz *et al*., 1987).

Chabot *et al*. (1993) demonstrated growth stimulation of maize and lettuce by several microorganisms capable of mineral phosphate solubilization. A strain of *Burkholderia cepacia* showing no indoleacetic acid production, but displaying significant mineral phosphate solubilization and moderate phosphatase activity (Rodríguez *et al*., 1996) has improved the yield of tomato, onion, potato, banana, and coffee, among other cultivars, in field tests.

Furthermore, several examples of simultaneous growth promotion and increase in phosphorus uptake by plants as the result of phosphate-solubilizing bacteria inoculations have been reported. Inoculation with two strains of *Rhizobium leguminosarum* selected for their Phoosphate solubilization ability has been shown to improve root colonization and growth promotion and to increase significantly the P concentration in lettuce and maize (Chabot *et al*., 1996b). Chabot *et al*. (1996b) concluded that the phosphate-solubilization effect of *Rhizobia* and other mineral phosphate-solubilizing microorganisms seems to be the most important mechanism of plant growth promotion in moderately fertile and very fertile soils.

PGPR can affect plant growth by a wide range of mechanisms such as solubilisation of inorganic phosphate, production of phytohormones, siderophores and organic acids, lowering of plant ethylene levels, N2 fixation and biocontrol of plant diseases (Muleta *et al*., 2007b; Datta *et al*., 2011). The use of such beneficial bacteria as biofertilisers and biocontrol agents has currently attracted increased interest world-wide in attempts to achieve sustainability, particularly in agriculture, forestry and horticulture (Datta *et al*., 2011).

The list of important genera of mineral P solubilisers is increasing over time as more research groups are engaged in screening potential rhizobacteria. The commonly reported genera include Achromobacter, Aereobacter, Agrobacterium, Bacillus, Burkholderia, Erwinia, Flavobacterium Micrococcus, Rhizobium and Pseudomonas (Rodriguez and Fraga, 1999).

Accordingly, Tilak *et al*. (2005) emphasise that the most efficient phosphate-solubilising microorganisms (PSM) belong to the bacterial genera Bacillus and Pseudomonas and the fungal genera Aspergillus and Penicillium. These authors further note that the use of PSB as inoculants simultaneously increases P uptake by the plant and crop yield. Many PSB also exhibit other beneficial traits such as production of siderophores, phytohormones and 1-amino-cyclopropane-1-carboxylate deaminase activity (Poonguzhali *et al*., 2006; Muleta *et al.*, 2007b).

2.6. Rhizobia

2.6.1. Characteristics of rhizobia

Rhizobia (the fast-growing *Rhizobium* spp. and the slow-growing *Bradyrhizobium* spp.) or root nodule bacteria are medium-sized, rod-shaped cells, 0.5-0.9 μm in width and 1.2-3.0 μm in length. They do not form endospores, are Gram-negative, and are mobile by a single polar flagellum or two six peritrichous flagella. Rhizobia are predominantly aerobic chemoorganotrophs and are relatively easy to culture. They grow well in the presence of oxygen and utilize relatively simple carbohydrates and amino compounds. Some strains of rhizobia require vitamins for growth. Rhizobia are likely to lose viability rapidly in water. Optimal growth of most strains occurs at a temperature range of 25-30 ^{o}C and a pH of 6.0- 7.0 but despite their usual aerobic metabolism, many strains are able to grow well under microaerophilic conditions at oxygen tensions of less than 0.01 atm. Generally, most rhizobia produce white colonies. Fast-growing rhizobia produce an acid reaction in yeast mannitol medium containing bromthymol blue (pH 6.8) while slow growers produce an alkaline reaction.

2.6.2. Rhizobia as natural endophytes of the leguminosae

Rhizobia (genera of *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, and *Sinorhizobium*) form intimate symbiotic relationships with legumes by responding chemotactically to flavonoid molecules released as signals by the legume host. These plant compounds induce the expression of nodulation (*nod*) genes in rhizobia, which in turn produce lipo-chito-oligosaccharide (LCO) signals that trigger mitotic cell division in roots, leading to nodule formation (Dakora 1995; Lhuissier *et al*., 2001).

Nitrogen is required for cellular synthesis of enzymes, proteins, chlorophyll, DNA and RNA, and is therefore important in plant growth and the production of food and feed. For nodulating legumes, nitrogen is provided through symbiotic fixation of atmospheric N_2 by nitrogenase in rhizobial bacteroids. This process of biological nitrogen fixation (BNF) accounts for 65% of the nitrogen currently utilized in agriculture, and will continue to be important in future crop productivity, especially in sustainable systems. In Africa, grain legumes fix about 15-210 kg N ha^{-1} seasonally, while tree legumes fix about 43-581 kg N ha^{-1} y^{-1} (Dakora and Keya, 1997).

2.6.3. Rhizobia as biocontrol agent of plant pathogens

Plant growth-promoting bacteria can also stimulate growth by producing and/or inducing the plant to release secondary metabolites, facilitating the uptake of nutrients, and/or inhibiting plant pathogenic organisms in the rhizosphere. The control of plant pathogens by PGPR can be achieved by involving different mechanisms, acting either alone or in combination with other compatible microbes.

2.6.3.1. Inhibiting plant pathogenic organisms

Many rhizobia can produce antibiotics, especially bacteriocins, proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strains, conferring competitive advantage to bacteriocin - producer strains (Hafeez *et al*. 2005). Robleto *et al*. (1998) described the effects of the antibiotics Trifolitoxin, Abacteriocin produced by a *R. etli*, on the microbial composition in the rhizosphere of common bean. They observed a significant reduction in the genetic diversity of Alpha proteobacteria, with little apparent effect on most microbes. Though bacteriocins are a narrow – spectrum antimicrobial compound, yet it is an effective metabolite that inhibits bacterial plant pathogens (Hafeez *et al*. 2005). *Rhizobium* sp. Strains ORN 24 and ORN 83, isolated from Algerian soil, were found to produce bacteriocins with antimicrobial activists against *Pseudomonas savastanoi*, the agent responsible for olive knot disease (Mourad *et al*. 2009).

Additionally, it has been shown that rhizobia are able to elicit reactions of plant defense against pathogens, as demonstrated by Elbadry *et al*. (2006). The authors verified the occurrence of induced systemic resistance (ISR) against bean yellow mosaic potyvirus

(BYMV) in Faba bean (*Vicia faba* L.) inoculated with *P. fluorescence* FBII and *R. leguminosarum* bv. *viceae* FBG05. Plants inoculated showed a pronounced and significant reduction in percent disease incidence and a significant reduction in virus concentration. Since the PGPR inoculants and the pathogen remained spatially separated, it could be concluded that the tested *Pseudomonas* or *Rhizobium* strains induced systemic resistance in Faba bean against BYMV. The activation of ISR by PGPR can be optimized when more than one microorganisms are used as elicitors as reported by Dutta *et al.* (2008), who evaluated the occurrence of the process in pigeon pea (*Cajanus cajan*). They exposed separately plant root system to the pathogenic fungus *Fusarium udum* and part to PGPR *B. cereus* or *P. aeruginosa*, and also evaluated the interaction of these PGPR with *Rhizobium* sp. It was evidenced by an enhancement of resistance in treated plants, mainly when PGPR strains were used with *Rhizobium*.

Plants with mixture of PGPR and *Rhizobium* survived longer and showed higher level of defense – related enzymes than individual organism and nonbacterized control. Plant resistance to pathogens is, however, based on the deployment of a multicomponent defense response, which includes the hypersensitive response, chemical weapons, and structural defensive barriers. Arfaoui *et al*. (2007) in an experiment, analyzed the effect of rhizobial inoculation on chickpea, and noticed the activation of plant defense response against wilt caused by *F. oxysporum*. As a result, there was an accumulation of phytoalexins and a consequent activation of the defense enzymes such as ammonia–lyase, chalcone synthase, and isoflavone reductase. These findings complemented previous work of the same research group, who reported that the inoculation of phytoalexins with rhizobial strains induced defense responses, reduced disease severity in chickpea plants infected with *F. oxysporum* (Arfaoui *et al*., 2006), and increased in activity of other defense – related enzymes, such as peroxidases and polyphenoloxidases, as well as led to the accumulation of phenolic compounds (Arfaoui *et al*., 2005). Similarly, Mishra *et al*., (2006) observed that the inoculation of rice plants with *R. leguminosarum* bv. *Phaseoli* caused an increase in the production of phenolic compounds, which are indicative of plant defense response, was more remarkable in the presence of *Rhizoctonia solani*. Moreover, Rhizobia are reported to reduce infections by the parasitic weed *Orobanche crenata* (Mabrouk *et al*., 2007). As an example, *R. leguminosarum* strains were able to promote pea development and simultaneously

controlled *0. crenata,* notably by inducing necrosis in the parasite. Besides, *R. leguminosarum* can also elicit IRS of pea plants, as indicated by the accumulation of toxins and phenolic compounds in plant tissues.

2.6.3.2. Plant Growth Promotion

Plant growth regulators are organic molecules analogous to plant hormones, which, at low concentrations, cause a physiological response and influence plant development. They are divided into five general groups of compounds based on their chemical structures and effects on plant: auxins, gibberellins, cytokinins. All these compounds are produced by soil bacteria, but vary in concentration. The production of auxins and ethylene by bacteria is considered a common trait, while the synthesis of cytokinins is less common and the gibberellins secretion at high concentrations is very rare (Solano *et al*., 2008). It been estimated that more than 80% of the soil bacteria are able to produce auxins especially Indoleacetic acid (IAA). Indolebutyric acid, or similar compounds derived from tryptophan metabolism (Solano *et al*., 2008). Auxins are plant growth regulators they stimulate cell division and elongation and its production by PGPR is one of the most studied and perhaps the most effective mechanism of plant growth promotion by rhizobia (Schlindwein *et al*., 2008).

Many rhizobial strains are reported to produce auxins in variable amounts. For example, Antoun *et al* (1998) working with 266 rhizobial strains, from different species and genera, found that 58% of the strains produced IAA, while Vargas *et al*. (2009) in a similar study found a considerably lower frequency of Auxin producers (23%) among populations of clover modulating *R. leguminosarum* by *trifolii*. However, they noticed a very distinct behavior between strains isolated from arrow leaf clover and those isolated from white clover nodules. In the first group, IAA production was much more frequent accounting for more than 90% of the isolates. On the contrary, IAA production was considerably less frequent (only 15%) in rhizobia isolated form white clover nodules (Vargas *et al*., 2009). In the first group, IAA production was much more frequent accounting for more than 90% of the isolates. On the country, IAA production was considerably less frequent (only 15%) in rhizobia isolated form white clover nodules (Vargas *et al*., 2009). Auxins produced by rhizobia may be related to nodulation, and hence, IAA synthesizing rhizobia have been found to nodulate more intensely than IAA negative mutants (Boiero *et al*., 2007). In nonlegumes, IAA produced by rhizobia

may stimulate plant root system, increasing its size and weight, benching number and the surface area in contact with soil, resulting in the development of more expansive root architecture (Dazzo and Yanni, 2006). Inoculation with Auxin producing bacteria may also result in the formation of adventurous roots (Solano *et al*., 2008). All these changes in root system increase its ability to prospect the soil for nutrient exchange, therefore improving plant nutrition and growth capacity (Gutierez *et al*., 2001). Noel *et al.,* (1996) verified that the inoculation with IAA producing strains of *R. leguminosarum* accelerated there germination of canola and lettuce. Similarly, Biswas *et al.,* (2000a) observed that the inoculation of rice with *R. leguminosaum* bv. *trifolii* increased dry matter and grain production, besides an increment in N, P, K, and Fe content in plant tissue. All these effects were credited to IAA accumulation in photosphere by rhizobia inoculation, resulting in physiological changes in root system with consequent improvement in nutrient uptake.

Phosphorus (P) is one of the major mineral nutrients required by plants whose deficiency is extremely limiting crop production. In nature, phosphorus is found in a variety of organic and inorganic forms that are very poorly soluble. It is considered as one of the less soluble elements in the natural environment, with less than 5% of the total soil phosphorus content being available to the plants (Dobbelaere *et al*., 2003). So phosphatic fertilization is needed to obtain optimum crop production. However, a large portion of the soluble inorganic phosphorus applied to soil as fertilizer is rapidly immobilized by the iron and aluminum oxides in acid soils and by calcium in calcareous soils soon after application, thus becoming unavailable to plants (Khan *et al*., 2007).

Many soil microorganisms can solubilze mineral phosporus generally via the production of organic acids (Khan *et al*., 2010). A large number of P solubizing bacteitra have been isolated from the Rhizosphere of several crops (Chabot *et a.*, 1993). There have been a number of reports on plant growth promotion by bacteria that have the ability to solubilze phosphorus. However, the production of other metabolites beneficial to the plant are produced by these microorganisms such as phytohormones, antibiotics, or siderophores. Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphrous compounds such as Tricalcium phosphate (TCP), dicalcium phosphate (DCP), hydroxyapatite, and rock phosphate (RP). There are considerable populations of phosphrous

mobilizing bacteria in soil and in plant rhizospheres, including many genera of both aerobic and anaerobic strains.

According to Rodriguez and Fraga (1999), the genus *Rhizobium* is one of the major P solubilizers, along with bacteria belonging to the genera *Pseudomonas* and *Bacillus.* Among the PGPR traits evaluated for 252 isolates of *R. leguminosarum* bv. *Trifolii*, solubiliztion of P was the most usual characteristics. Like *Rhizobium* species, other rhizobia also possess this PGPR trait. For example, Alikhani *et al.*, (2006) while working with 446 bacteria belonging to the genera *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Rhizobium* evaluated the solubilizaiton of inorganic and organic P under in vitro conditions. They observed that 44% of the isolates solubilized TCP while 76% solubilized inositiol hexa phosphate. However, the rhizobial isolates differed in their phosphrous solubilization ability.

2.6.4. Rhizobia and AMF

A great deal of work has been carried out on the triplicate symbiosis legume- mycorrhiza-Rhizobium in the control of soil-borne pathogens. Evidence reveals that mycorrhi- zal symbionts offer increased resistance to certain wilt and root rot pathogens such as *Olphidium brassicae*, *Macrophomina phaseolina, Fusarium oxysporum, Pythium splendens*, and *Rhizoctonia solani* (Jalali *et al*., 1990; Ramaraj *et al*., 1988; Giovannetti *et al*., 1991). However, the effect of VA mycorrhizas and Rhizobium on plant disease, nutrient uptake, and rhizophere microbial biomass and their activities are very difficult to generalize because the interactions involving VA mycorrhiza, root rot fungi, and Rhizobium vary with the microbial species and plant cultivars.

2.6.5. Effect of Rhizobia on yields at greenhouse and field

Yield increases caused by inoculation of nonlegumes with PGPR rhizobia have been reported in pot and field experiments. *R. leguminosarum* bv.*trifolii* R39 promoted the growth of maize, spring wheat, and spring barley in field trials performed between 1985 and 1993 in a loamy sand soil in Germany (Höflich *et al*., 1994). Inoculation of these nonleguminous plants with R39 resulted in yields that were significantly ($P<0.05$) increased by 6 to 8%. The two rice endophytes *R. leguminosarum* bv. *trifolii* E11 and E12 enhanced rice grain yield by 46 and 42%, respectively, under field conditions (Yanni *et al*., 1997). A 20% increase in shoot

growth and grain yield of the wild rice *Oryza breviligulata* was obtained under greenhouse conditions by inoculation with photosynthetic endophytic bradyrhizobia (Chaintreuil *et al*., 2000).

Greenhouse wheat inoculation assays performed in two different soils showed that the endophytic strain IAT168 behaved like a PGPR (the 24% increases in shoot dry matter and grain yields were significant at $P<0.1$) in the loamy sand Rabat soil. However, in the silty clay Merchouch soil, six strains had significant ($P<0.05$) deleterious effects, stressing the importance of choosing rhizobial strains that are PGPR for all the plants involved in crop rotation systems. Maize and lettuce growth promotion under field conditions were obtained by inoculation with two mineral phosphate-solubilizing PGPR strains R1 and P31, of *R. leguminosarum* bv. *phaseoli* (Chabot *et al*., 1996b). Interestingly, in a P-depleted loam soil in Quebec, strain P31, significantly ($P<0.05$) increased lettuce shoot dry matter yield when the soil was fertilized with half of the recommended amount of P (35 Kg ha^{-1} superphosphate). The yield was equivalent to that obtained with the uninoculated control fertilized with the recommended 70 kg ha–1 superphosphate Greenhouse radish inoculation trials with 266 strains of *Rhizobium* and *Bradyrhizobium* revealed the existence of potential PGPR strains among all the genera and species tested (Antoun *et al*., 1998). No significant correlation was found between the *in vitro* bacterial characteristics generally associated with PGPR activity (i.e., production of indole 3-acetic acid, siderophores, or HCN and Psolubilization) and radish yield. In fact, strain Soy 213 of *B. japonicum*, which produced the highest increase of radish cultivar Pocker dry matter yield (60% compared with uninoculated control), did not exhibit any of the *in vitro* characteristics tested. Other mechanisms of action such as induced systemic resistance against pathogens (Ramamoorthy *et al*., 2001) or competition and antagonism toward other deleterious microorganisms might be responsible for the beneficial effect of strain Soy 213. Beneficial genes expressed solely *in planta* might also be present in this strain. However, many strains harboring all these *in vitro* characteristics did not produce plant growth promotion. These observations indicate that plant yield is the outcome of very complex interactions taking place in the rhizosphere between plant roots, soil, and rhizobacteria, and they underline the importance of developing model systems to study the PGPR mechanisms of action *in planta*.

2.7. Characteristics of a Successful PGPR for Formulation Development

High rhizosphere competence, high competitive saprophytic ability, enhanced plant growth, ease for mass multiplication, broad spectrum of action, excellent and reliable control, safe to environment, compatible with other rhizobacteria, should tolerate desiccation, heat, oxidizing agents and UV radiations (Jeyarajan and Nakkeeran, 2000).

2.8. Bioformulation of biocontrol agents

2.8.1. Individual strain based formulations

Plant growth promoting rhizobacteria has diverse applications for the management of plant diseases in agriculture, horticulture and forestry. In addition it also plays a vital role in environmental remediation (Lucy *et al*., 2004). Seed treatment of groundnut and pigeon pea with peat based formulation of *B. subtilis* supplemented with 0.5% chitin or with 0.5% of sterilized *Aspergillus* mycelium controlled crown rot and wilt of groundnut and pigeon pea, respectively. It also increased growth promotion even in the presence of inoculum pressure (Manjula and Podile, 2001). Chitin supplementation enhances the biocontrol efficacy of formulations. But incorporation of chitin will increase the production cost of biopesticides. Hence, identification of cheap and easy available source of chitin is essential. Seed treatment and soil application of *P. aeruginosa* strain 78 reduced root knot incidence of mungbean besides the reduction in the population density of *Meloidogyne javanica* under field conditions (Ali *et al*., 2002). Seed treatment with wettable powder formulation of *P. putida* strain 30 and 180 suppressed wilt of musk melon to the extent of 63 and 50% after 90 days of transplanting muskmelon in the field. However, seed treatments with strain mixtures were not as effective as that of individual strains (Bora, 2004). The decrease in efficacy might be due to the incompatibility of the isolates, which might suppress the genetic expression of defense genes in either bacterial strain.

2.8.2. Strain combination based formulations

Several research outcomes on formulations explain that a single biocontrol agent has the ability to combat a plant pathogen. Nevertheless, usage of single biocontrol agent in disease management might be also responsible for its inconsistent performance under field conditions.

A single biocontrol agent may not perform well at all times in all kinds of soil environment to suppress plant pathogens (Raupach and Kloepper, 1998). In addition, application of single biocontrol agent based formulation might have resulted in inadequate colonization, inability to tolerate the extremes of soil pH, moisture and temperature and fluctuations in the production of antimicrobial substances (Weller and Thomashow, 1994). Inconsistent performance of biocontrol agents was overcome by the combined application of several biocontrol strains that mimic the natural environment (Raupach and Kloepper, 1998). Development of cocktail formulation with compatible isolates will improve disease control through synergy in cross talk between the isolates that lead to increased production of antibiotics at the site of colonization and thereby could suppress the establishment of pathogenic microbes. Advantages of strain mixtures include, broad spectrum of action, enhanced efficacy, reliability and also allow combination of various traits without genetic engineering (Janisiewicz, 1996). Application of mixed PGPR strains based formulations to field might ensure at least one of the mechanism to operate under variable environment that exist under field conditions (Duffy *et al*., 1996).

Application of talc based strain mixture formulation of fluorescent pseudomonads through seed, root, soil and foliage to rice crop suppressed sheath blight under field conditions better than individual strains based formulations. The average disease reduction for mixtures was 45.1% compared to 29.2% for individual strains. In addition to disease reduction strain mixtures increased biomatter production and yield compared to individual strains (Nandakumar *et al*., 2001). Combined application of *Pichia guilermondii* and *Bacillus mycoides* (B16) reduced the infection of *Botrytis cinerea* by 75% on fruits in strawberry plants grown commercially under greenhouse conditions. However, the individual application of either antagonist resulted in 50% reduction of strawberry fruit infection. Population of yeast increased when applied as mixture rather than single application (Guetsky *et al*., 2002). Delivering of talc based strain mixtures of *P. fluorescens* strains (Pf1 and FP7) through seed, soil and foliar reduced sheath blight and leaf folder incidence in rice under greenhouse and field conditions. It also reduced the feeding behavior of leaffolder, reduced larval and pupal weight, and increased larval mortality. Besides, population of parasitoids and spiders also increased in PGPR treated plots (Radja *et al*., 2002).

2.9. Limitation of Biological Control Agents

Although biological control agents have shown to protect crop plants from disease under experimental conditions, inconsistent performance between under experiment conditions and field locations has been reported. Biocontrol is less consistent than chemical control in field condition. Variation in consistence and performance of biological control agents has been attributed to many factors like biotic and ecological factors (Kamilova *et al*., 2005). Moreover, the survival and efficacy of biocontrol agents affected by host plant genotype, agricultural practices, mutation of biocontrol organism and resistance of pathogen to biocontrol mechanisms (Ownley and Windham, 2006). Biological control may also competitively displace non-target organisms (Cook *et al.*, 1996).

3. MATERIALS AND METHODS

3.1. Descriptions of the Experimental Site

Jimma is located in southwest Ethiopia in Oromia National Regional State and it is situated at latitude of 070 41' 5.41''N and longitude 0360 49' 48.58''E Ethiopia. The average annual rainfall is 1510 mm with binomial distribution per year and the maximum and minimum temperatures about 28^0C and 11.4^0C, respectively (Gemechu, 1977). The maximum and minimum relative humidity of the site is 91.4% and 37.92%, respectively. Dedo is located in southwest Ethiopia in Oromia National Regional State and situated at latitude of 070 41' 5.41''N and longitude 0360 49' 48.58''E Ethiopia. Annual rainfall is 2003 mm with binomial distribution per year and the maximum and minimum temperatures about 23.18^0C and 17.4^0C, respectively (Gemechu, 1977).

3.2. Preparation of Fungal Pathogen

3.2.1. Culture media

Potato dextrose agar (PDA) was prepared by dissolution of commercially formulated dehydrated (powder) PDA (Difco). The PDA powder was mixed with sterilized distilled water in a flask at rate of 39 g/ml, and heated until melting. The solution was autoclaved at 121 ^{0}c at atmospheric pressure for 15 minutes to sterilize the media. The liquid media maintained under aseptic condition and allowed to cool to about 50 ^{0}c. Streptomycin sulphate powder was added to the nutrient media at the rate of 1 g/lit to avoid bacterial contamination (Christensen, 1957). The media was poured into sterilized Petri dishes at the rate of about 15 ml/Petri dish and allowed to solidify.

3.2.2. Isolation and identification of isolates

Faba bean plants infected with root rot were collected from Dedo, Jimma, Ethiopia. Infected roots were washed with running tap water to remove any soil remains, and then cut into small pieces before being dipped in sodium hypochlorite solution (2.5%) for two minutes for surface sterilization. These roots were then passed through changes of distilled water, dried

between sterilized filter paper. The treated root pieces placed on PDA medium. The plates were incubated at 25°C±1 and checked daily for fungal development. Preliminary microscopic examination of the isolated fungi was done and classified to genus level (Christensen, 1957). The isolates were identified according to their morphological features as described by Booth, (1985) and Barnett and Hunter, (1986). Identification was confirmed by the Department of Mycology, Ambo Plant Protection Research Centre, Ambo, Ethiopia. Fungal pure cultures were maintained on PDA slants for further studies at 4 ^{o}C in refrigerator.

3.2.3. Characterization of fungal isolates

3.2.3.1 Slide culture

To identify *Fusarium solani*, the technique of slide culture was used (Stevens, 1974) which allows the direct microscopic observation of morphological structures of taxonomic value. A bent glass rod was placed inside the Petri dish layered with filter paper. A slide was put on the glass rod with soaked cotton pad on the corner of the Petri dish. The set up was covered with the lid and autoclaved at 121 Petri dish was cut into 1cm^{2} ^{0}C and 15 lb. Sterilized PDA from a different blocks and put at the center of the slide on the beat glass rod. Fungal isolates were inoculated at the center of four sides of agar block and covered with sterile cover slip under aseptic condition and incubated at 25 ^{0}C for 5 days. After 5 days the cover slip was lifted up carefully and placed on another clean slide. Likewise, a clean cover slip was placed on the slide after agar block was discarded. The slides were then stained with Lactophenol cotton blue and Mounted on a microscopic and observed under high power objection (400X). The Spore type, mycelia morphology were recorded.

3.3. Origin of Rhizobium Isolates and Culture Conditions

A total of 41 rhizobium isolates (Table 1) collected from different parts of the country, were tested for their biocontrol potential under *in vitro* conditions. From these, fifteen (15) rhizobium isolates were obtained from the culture collection of Holeta Agricultural Research Centre, Microbial Biotechnology Laboratory. The other Twenty six (26) isolates were obtained from Jimma University, Department of Biology, Applied Microbiology Laboratory. Isolates from Jimma, were originally isolated from rhizospheres of Faba bean *(Vicia faba* L.), Haricot bean (*Phaseolus vulgaris L.)* and Pea (*Pisum sativum L*) grown at different locations

in Jimma zone, Oromia Regional State, Southwestern Ethiopia. Jimma isolates have been morphologically, and biochemically tested in Applied Microbiology Laboratory at Department of Biology (Demissie, 2012).

Table1. Types, origin and source of *Rhizobium* isolates

S no	Isolates code	Host	Geographic origin	source	S no	Isolates code	Host plant	Geographic origin	source
1	Rh2(4)	Faba bean	Mena	Jimma	23	Rh3(1)	Faba bean	Mena	Jimma
2	RhE(1)	Haricot bean	-	Jimma	24	fb-4-1SG	Faba bean	S. Gonder	Jimma
3	Rh1(2)	Faba bean	Mena	Jimma	25	Rh47(2)	Faba bean	Mena	Jimma
4	Rh1(3)	Faba bean	Mena	Jimma	26	Rh2(2)	Faba bean	Mena	Jimma
5	Rh8(1)	Pea	Mena	Jimma	27	Rh2(3)	Faba bean	Mena	Jimma
6	Rh1(5)	Faba bean	Mena	Jimma	28	Rh Y(1)	Haricot b	Yebu	Jimma
7	fb-1-1NG	Faba bean	N. Gonder	Holeta	29	Rh3(2)	Faba bean	Mena	Jimma
8	fb-1018	Faba bean	N. Gonder	Holeta	30	Rh8(2)	Faba bean	Mena	Jimma
9	fb-3WG	Faba bean	W. Gonder	Holeta	31	Rh8(3)	Faba bean	Mena	Jimma
10	Rh26(1)	Faba bean	Mena	Jimma	32	Rh D(1)	Haricot b	Dedo	Jimma
11	Rh3(1)	Faba bean	Mena	Jimma	33	fb-3-1SG	Faba bean	S. Gonder	Jimma
12	Rh15(2)	Faba bean	Mena	Jimma	34	Rh5(2)	Faba bean	Mena	Jimma
13	Rh S(1)	Haricot b	Seribo	Jimma	35	Rh21(4)	Faba bean	Mena	Jimma
14	Rh11(1)	Faba bean	Mena	Jimma	36	Rh Sr(1)	Haricot b	Seribo	Jimma
15	Rh G(2)	Haricot b	Ginibbe	Jimma	37	Rh26(2)	Faba bean	Mena	Jimma
16	fb-2ST	Faba bean	S. Tigria	Holeta	38	fb-7EG	Faba bean	E. Gonder	Holeta
17	fb-1WG	Faba bean	W. Gonder	Jimma	39	fb-1035	Faba bean	N. Gonder	Holeta
18	Rh3(3)	Faba bean	Mena	Jimma	40	fb-2-1ST	Faba bean	S. Tigria	Holeta
19	fb-5EG	Faba bean	E. Gonder	Holeta	41	fb-2-1WG	Faba bean	W. Gonder	Holeta
20	fb-2EG	Faba bean	E. Gonder	Holeta					
21	fb-1EG	Faba bean	E. Gonder	Holeta					
22	Rh1(4)	Faba bean	Mena						

All pure culture rhizobium isolates were routinely maintained on yeast extract mannitol (YEMA) agar plates (Vincent, 1970) at 4°C and in YEM broth containing 20% (v/v) glycerol at -80^{o}c.

3.4. *In vitro* Screening for Antagonism

The inhibitory effect of the *Rhizobium* isolates against the radial mycelial growth of the pathogenic fungus was evaluated using the dual culture technique (Gupta *et al*., 2006). In order to quantify the radial growth of the pathogen and percent reduction, a small fungal agar block (5 mm) in diameter of grow on potato dextrose agar for seven days at 25°C was centrally placed on pre-solidified PDA medium. Exponentially grown one loopful of 24 h old culture (10^8 cfu/ml) bacterial cultures were streaked on PDA as a broad band (making a straight short bar) approx. 2 cm away from the fungal agar block at one opposite edge of triplicate Petri dishes (90 mm diameter). In all antagonistic studies, a medium inoculated only with a test fungus at centre served as a control. Both experimental and control Petri plates were arranged in a completely randomized design with three replicates per treatment. Petri plates were incubated at 28 ± 2 °C for 7 days. The percentage fungal radial growth inhibition was calculated by using the following formula of Lokesha & Benagi (2007).

$$Percent\ inhibition\ over\ control = \frac{C - T}{C}\ X\ 100$$

Where, C is the radial growth of fungus in control, and T is the radial growth of fungus in dual culture after 7 days of incubation

3.5. Determination of Possible Mechanisms of Inhibition

3.5.1. Hydrolytic enzymes production

3.5.1.1. Chitinolytic test

To test for chitinolytic activity of isolates were spot inoculated individually on medium containing fine powdered chitin (Renwick and Campbell., 1991) (g/L): chitin powder, 5.0; yeast extract, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; agar, 20.0. The pH of the medium was adjusted to 7.0 and the medium was sterilized by autoclaving at 121^{o}C for 15 min. Single bacterial isolate was inoculated onto solidified plates of the medium. The uninoculated plates were used as control in the experiment. The plates were incubated at 28^{o}C for 72 to 96 h until zones of chitin clearing could be seen around the colonies.

3.5.1.2. Proteolytic test

Proteolytic activity was assessed on skimmed milk agar (Smibert and Krieg, 1994). The medium contains g/liter: skim milk 100.0, trypitcase soy 25.0, and agar 15.0. 24 h old culture single bacterial isolate was spot inoculated onto solidified plates of Skim-milk agar. Uninoculated plates were used as control. The plates were incubated at 28^{o}C for 2 to 3 days until zones of clearing could be seen around the colonies.

3.5.1.3. Lipolytic test

Lipolytic activity was assessed on Yeast Extract Mannitol Agar medium containing 1 % Tween 80^{R}. The medium had the following composition (g/L): mannitol 10, KH2PO4 0.5, MgSO4.7H20 0.2, NaCl 0.1, yeast extract 0.5, Agar 10, 1% Tween. Single bacterial isolate was spot inoculated onto solidified plates of medium. The uninoculated plates served as control. The presence of lipase activity was indicated by insoluble olic acid forming halos around the bacterial colonies (Kumari *et al*., 2010).

3.5.2. Hydrogen cyanide test

Hydrogen cyanide production was detected as described by Bakker and Schippers (1987). Single bacterial isolates were streaked onto Trypticase soy agar (TSA, Difco, Franklin Lakes, NJ USA) supplemented with 4.4 g glycine per liter to screen for cyanide production. A piece of filter paper impregnated with 0.5% picric acid (yellow) and 2.0% sodium carbonate was placed in the lid of each Petri dish. The uninoculated and inoculated plates without picric acid impregnated used for control. The Petri dishes were sealed with parafilm and held at 28^{o}C for 3 to 5 days. Discoloration of the filter paper to orange brown after incubation indicates microbial production of cyanide.

3.5.3. Plant growth promoting mechanisms

3.5.3.1. Production of indole acetic Acid (IAA)

Indole Acetic Acid (IAA) production was detected as described by Gordon and Weber (1951). *Rhizobium* isolates were grown separately in YEM broth medium supplemented with L-tryptophan (100 µg/ml). The flasks were incubated at room temperature on rotatory shaker at 150 rpm for 72 h. After incubation, the medium was centrifuged at 6000 rpm for 15 min and

the cell-free supernatant was used for IAA test. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Uninoculated liquid media were used as control. Development of pink colour indicates IAA production.

3.5.3.2. Phosphate Solubilization

Isolates were tested for phosphate solubilizing ability on Pikovskaya medium (Pikovskaya, 1948) Consisting of (g/L) glucose 10, $(NH_4)_2SO_4$ 0.5, $MgSO_4$.7H2O 0.1, Yeast extract 0.5, KCL0.2, NaCl 0.2, $FeSO_4$.7H2O 0.002, $MnSO_4$. H_2O 0.002, $Ca_3(PO_4)$ 5 and agar 15 . The pH of the medium was adjusted to 7 before autoclaving. The medium was distributed in 9 cm diameter Petri plates and inoculated with a loopful (10^8cfu/ml) of single isolate at the centre. Uninoculated plates were used as control. Both inoculated and uninoculated plates were incubated at 28±2 ^oC for 7 days and clear zone (halo) diameter surrounding the bacterial colony was measured. The ability of the rhizobium isolates to solubilise insoluble phosphate was primarily described by Edi-Premono *et al*. (1996).

$$SI = \frac{colony\ diameter + halozone\ diameter}{colony\ diameter}$$

3.6. *In vitro* Effect of single and mixed Rhizobium isolates together with *Fusarium solani* on Faba Bean Seed Germination

3.6.1. Faba bean seeds and disinfection

In this experiment, the seed 'Kass', the highest susceptible seed to infection by *Fusarium solani* was used to study the effects of rhizobial isolates on root rot disease in Faba bean. The seeds were obtained from Holeta Agricultural Research Centre. Before use, seeds were surface disinfested by 2.5% NaOCl for 3 min, washed three times in sterilized distilled water, and dried between sterilized filter paper layers.

3.6.2. *Fusarium solani* and Rhizobium isolates

One our *Fusarium* isolate was obtained from the purified culture slant in test tube and maintained on PDA medium to produce conidia. Isolates of *Rhizobium* (Rh15(2), Rh26(1) and fb-1WG) which showed high potential of *in vitro* inhibition were used for experiment.

3.6.3 *Fusarium solani* inoculum preparation

The culture of fungal pathogen was multiplied by cultivating on potato dextrose agar (PDA) medium in Petri plates (9cm) at 25±2 ^{o}C for 15 days in an incubator. The colonies were harvested by scraping the surface with spatula and homogenized with 200 ml of sterile distilled water and filtered through a cheese cloth to make a fungal suspension for inoculation. The spores 1 x 10^{6} / mL were counted by haemocytometr (Estevez de *et al*., 2002).

3.6.4. Rhizobium isolates inoculum preparation

For single isolate inoculum production, a loopful of isolate (Rh26(1), Rh15(2) and fb-1WG) was separately inoculated into the 250 ml Erlenmeyer flasks contain sterilized yeast extract mannitol broth (Vincent, 1970). The medium had the following composition (g/l): mannitol 10, KH2PO4 0.5, MgSO4.7H20 0.2, NaCI 0.1, yeast extract 0.5. The pH of the medium was adjusted to 6.8 before autoclaving at 121 ^{O}C for 15 minutes. The flasks were incubated on a rotary shaker at 120 rpm for 72 h at room temperature (28 ± 2 ^{o}C). After 48 hr of incubation, bacterial concentration was adjusted to 10^{8}CFU/ ml (OD620 0.8-0.9) using spectrophotometer (Arfaoui *et al.*, 2006). Bacterial concentrations were confirmed by dilution plating. For preparation of combination of inoculums, the bacterial isolates (Rh26(1), Rh15(2) and fb-1WG) were grown separately and mixed with equal volume (1:1:1 v/v ratio) (Nandakumar *et al.*, 2001a).

3.6.6. Blotter method

The effects of *Rhizobium* isolates (Rh26(1), Rh15(2), fb-1WG and combination) on germination seeds of Faba been inoculated with pathogen was examined in Petri dishes by blotting paper method (ISTA, 1993).

A total of 15 seeds were inoculated with the conidial suspension of (5 ml) of *Fusarium isolate* (10^{6} condia/ml) in 9 cm diameter Petri plates and allowed to dry overnight and the next day treated per suspension of (5 ml) of *Rhizobium* isolate containing cells (10^{8} CFU/ ml) for 5 hrs. Inoculation of the disinfected seeds was carried out following the standard methods of Bhutta *et al*., (2004). Treated and inoculated seeds were rolled in moist blotter and placed on Petri plates (Ten seeds per plate). The seeds inoculated with *F. solani* but not treated with

antagonists served as diseased (control). The seeds untreated and uninoculated were served as health. Petri plates were arranged in a completely randomized design with three replicates per treatment. The seeds were allowed to germinate at 28 ± 2^0C for 10 days. Seeds were considered to be germinated with the emergence of both radicle and plumule. After 10 days, germination percentage as well as radicle length and plumule lengths were recorded. Vigour index was calculated following the procedure of Abdul Baki and Anderson (1973).

$$Germination\ percent\ (GP) = \frac{Number\ of\ germinated\ seeds}{Number\ of\ total\ seeds\ tested}\ x\ 100$$

$$Seedling\ vigor\ index(SVI) = (mean\ radicle\ length + mean\ plumule\ length)x\ \%\ germination$$

3.7. Soil analysis

Soil samples were collected from the field area of Jimma University Collage of Agriculture and Veterinary Medicine, Jimma. Some soil physico-chemical properties (Organic matter, Walkey Black (1934); Total nitrogen, Kjeldahl (1883); Phosphorous, Bray II (1945); Soil texture, Hydrometer method (Day, 1965); pH and EC were determined following standard procedures. A soil suspension was prepared with 1:2.5 (soil: water ratio).

3.8. *In Vivo* Seedling Test

The soil was placed evenly but not more than 40 cm deep in non-plastic containers such as metal baking pans. Each container was tightly covered with aluminum foil. Thermometer was inserted through the foil into the center of the soil. The oven was set between 82.2-93.3^{o}C. The soil was heated to at least 82.2^{o}c and allowed to remain at this temperature for 30 min (*http://www.momsbudget.com/containergardening/sterilizeoven.html*).

Table 2. Some physico-chemical properties of soil samples

Properties	Units	amount
Sand	%	8.00
Silt	%	18.00
Clay	%	74.00
Texture class	-	Clay
pH (1:2.5 soil : water)	-	5.29
E. C. (1:2.5 soil: water)	ds/m	0.07
Organic matter	%	3.83
Organic carbon	%	2.22
Total nitrogen	%	0.19
Available phosphorus	ppm	3.41

Antagonistic effect of *Rhizobium* isolates against *Fusarium* isolate was studied under greenhouse conditions (25-28^{o}C) by pot culture method (Yaqub *et al.*, 2012). The pots were surface sterilized for 3 min with 5% sodium hypochlorite and washed five times with sterile water and left to dry before use. Surface sterilized healthy looking seeds of Faba bean were sown (4 seeds/pot) in 18-cm diameter plastic pots containing sterilized clay soil. After 10 days, 20 ml of pathogen (1×10^6 condia/ml) and 4 ml (10^8 CFU/ ml) of *Rhizobium* were added per seedling to the base of stem at 2-cm depth of soil. The antagonists (Rh26(1), Rh15(2), fb-1WG and combination) were inoculated to seedling in pot before or at the same time or after the pathogen inoculation with an interval of 7 days. The following treatments were carried out in triplicate: T1= health (uninoculated); T2=Diseased (control, only *Fusarium solani*); T3= Rh26(1)+*F. isolate*; T4=Rh15(2)+*F. isolate*; T5=fb-1WG+*F. isolate;* T6=(Rh26(1)+ Rh15(2) + fb-1WG)+ *F. isolate* (combination). Pots were arranged in a randomized complete block design.

All plants were harvested from each pot at full bloom flowering stage (45 days). Excess soil was removed from the roots by placing the root mass on a sieve and washing with running tap water. Cleaned plants were separated into nodules, root (everything below the first node except nodules) and shoots and the following parameter were assessed.

Disease incidence was evaluated after 45 days of planting. The number of plants exhibiting root rot symptoms and percent disease incidence were estimated according the equation by Reddy *et al* (1983).

$$Percent\ disease\ incidence = \frac{Number\ of\ symptomatic\ plants}{Total\ number\ of\ plants}\ x\ 100$$

To determine disease severity, individual plant root tissues assessed according to a 1-9 scale proposed by Abawi and Pastor-Corrales (1990).

Table 3. The disease severity score scale

score	Signs and symptoms of infection
1	No visible symptoms
3	Light discoloration either without necrotic lesions or with approximately 10% of the hypocotyl and root tissues covered with lesions.
5	Approximately 25% of the hypocotyls and root tissues covered with lesions but tissues remain firm, with some deterioration of the root system. Heavy discoloration symptoms may be evident.
7	Approximately 50% of the hypocotyls and root tissues covered with lesions combined with considerable softening, rotting, and reduction of root system.
9	Approximately 75% or more of the hypocotyl and root tissues affected with advanced stages of rotting combined with severe reduction in the root system.

With these data, for each replicates a disease severity index (DSI) was calculated as follows

$$Disease\ severity\ index(DSI) = \frac{\sum(score\ x\ number\ of\ plants\ with\ this\ score)}{Total\ number\ of\ plants\ x\ greater\ score}\ x\ 100$$

The percentage of disease reduction (DR%) was calculated according to Edginton *et al* (1971):

$$DR\ (\%) = \frac{Dc - Dt}{DC}\ X\ 100,$$

where Dc is disease on the control plants that treated with only pathogen and Dt is disease on the treated with antagonist and pathogen

Shoots (in terms of height (cm), fresh and dry weight (g)), roots in terms of length (cm), fresh and dry weight (g), root nodulation (in terms of number, fresh and dry weight (g)), and also flower and leaf number were recorded. In order to measure dry weight of nodules, roots and shoots from each plant was placed in paper bags, dried at 70^{o}C for 72 h in a hot oven, and weighed (Mazen *et al*., 2008).

2.9. Statistical analysis

All data were subjected to analysis of variance using SAS version 9.2 (SAS institute, 2008). The data on germination percent, disease incidence and severity was arcsine transformed before undergoing statically analysis (Gomez and Gomez, 1984). Single and interaction effect of factors were determined using the ANOVA procedure of SAS. Whenever significant interactions were observed between factors, the level of one factor was compared at each level of other factors. Mean values among treatments were compared by the Tukey's test at α =0.05% level of significance.

4. RESULTS AND DISCUSSION

4.1. Isolation and Identification

The *Fusarium* isolates from rotten roots of Faba bean plants were further identified as *Fusarium solani* by analyzing morphological characters using the slide culture technique (Fig. 1). The Colony was grown, 3.5cm, 5.7cm and 6.9 cm diameter after 3 days, 5 days and 7 days, respectively at 25 ^{o}c on PDA plates. The colony had white felty mycelia with pinkish areas that later changed to purplish which is one of known characteristics of *Fusarium solani* (Fig.1A, 1B). The reverses are pale pink, but darken purplish within 2 weeks (Fig. 1C).

When the fungus was slide cultured on a block of PDA for 5-7 days at 25^{o}C, its macroconidia were produced from short multi-branched conidophores (Fig. 1D). They were fusiform and moderately curved in shape with 3-5 septa (usually 3-septate). Chlamydospores were globose and formed either paired from the lateral hyphal branches after 15 days (Fig. 1E). The observed cultural and morphological characteristics of the identified isolates were compared with the reports of Booth (1971) and Barnett and Hunter (1986). Most of the mycological features of the *Fusarium solani* isolate from this study agreed with the known features of *Fusarium solani*.

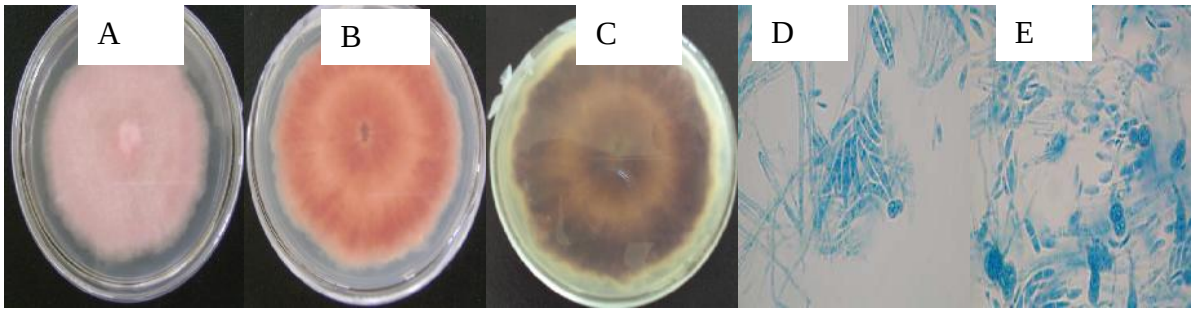

Fig.1. Morphological features of *Fusarium solani* isolated form rotten roots of Faba bean. The fungal isolate was grown on PDA for microscopic observation. Colony shape: front (A) and back (B) side of a PDA plate. Back (C) side of a PDA plate after 15 days, and (D) Macroconidia and (E) chlamydospore

4.2. *In Vitro* Screening for Antagonism

A total of 41 *Rhizobium* isolates were screened for their potential to inhibit the *Fusarium solani* under *in vitro* assays (Table 4). The antagonistic isolates tested showed varying levels of effects against the *Fusarium solani*. Most of the *Rhizobium* isolates showed potent antifungal activity by restricting mycelial expansion on dual culture medium (Figs. 2B, C and D). Some of the *Rhizobium* isolates did not show any antagonistic effects (Fig. 2E).

There were significant differences ($p < 0.0001$) between rhizobial antagonists in inhibiting the mycelial expansion of *F. solani*, ranging from 0.02-70.58% radial growth inhibition, respectively (Table 4). Over 65.85 % of the rhizobial antagonists tested ($n = 27$) exhibited remarkable fungal radial growth inhibition, against *F. solani* as compared to the control. The isolates fb-1WG, Rh15(2) and Rh26(1), exerted their maximum inhibitory effect 63.72%, 64.70% and 70.58% against *F. solani*, respectively (Table 4). However, the isolates fb-5EG, fb-2-1ST and Rh 1(5) showed the least inhibition percent 11.76 %, 11.76% and 14.7% against *F.solani*, respectively. It was pointed out by Chao (2002) that the *Rhizobium leguminosarum biovar phaseoli* had an effect on the inhibition of the *Pythium*, *Fusarium* and *Rhizoctonia* species. Also in a study Omar and Abd-Alla (1998) determined that rhizobia significantly inhibited the mycelial growth of *Fusarium solani* and *Rhizoctonia solani*. In general, Lalande and Bissonette (1989) showed that the inhibitory effect of the *Rhizobium* isolates vary from one isolates to another.

Table 4. Effect of *Rhizobium* isolates on the radial growth of *Fusarium solani*

S No	Isolates code	host	Percent inhibition (%)	S No	Isolates code	host	Percent inhibition (%)
1	Rh2(4)	Faba bean	50.00^{f}	22	Rh1(4)	Faba bean	36.27^{ji}
2	Rh E(1)	Haricot bean	29.41^{lmn}	23	Rh13(1)	Faba bean	24.50^{op}
3	Rh1(2)	Faba bean	56.94^{e}	24	fb-4-1SG	Faba bean	35.29^{jk}
4	Rh1(3)	Faba bean	44.11^{g}	25	Rh47(2)	Faba bean	26.47^{opn}
5	Rh8(1)	Pia	25.49^{op}	26	Rh2(2)	Faba bean	23.52^{qp}
6	Rh1(5)	Faba bean	14.70^{r}	27	Rh2(3)	Faba bean	30.39^{lm}
7	fb-1-1NG	Faba bean	0.02^{s}	28	Rh Y(1)	Haricot bean	61.76^{cbd}
8	fb-1018	Faba bean	27.45^{omn}	29	Rh3(2)	Faba bean	32.32^{lk}
9	fb-3WG	Faba bean	62.74^{cbd}	30	Rh8(2)	Faba bean	24.50^{op}
10	Rh15(2)	Faba bean	64.70^{b}	31	Rh8(3)	Faba bean	59.80^{ed}
11	Rh3(1)	Faba bean	41.15^{hg}	32	Rh D(1)	Haricot bean	60.78^{cd}
12	Rh26(1)	Faba bean	70.58^{a}	33	fb-3-1SG	Faba bean	41.17^{hg}
13	Rh S(1)	Haricot bean	29.41^{lmn}	34	Rh5(2)	Faba bean	29.41^{lmn}
14	Rh11(1)	Faba bean	39.21^{hi}	35	Rh21(4)	Faba bean	39.21^{hi}
15	Rh G(2)	Haricot bean	59.80^{ed}	36	RhSr(1)	Haricot bean	20.58^{q}
16	fb-2ST	Faba bean	0.02^{s}	37	Rh26(2)	Faba bean	20.58^{q}
17	fb-1WG	Faba bean	63.72^{cb}	38	fb-7EG	Faba bean	35.29^{jk}
18	Rh3(3)	Faba bean	24.50^{op}	39	fb-1035	Faba bean	20.58^{q}
19	fb-5EG	Faba bean	11.76^{r}	40	fb-2-1ST	Faba bean	11.76^{r}
20	fb-2EG	Faba bean	20.58^{q}	41	fb-2-1WG	Faba bean	26.47^{opn}
21	fb-1EG	Faba bean	32.35^{lk}	42	Control		0.00^{s}

Values are mean of three replications. In a column, mean followed by a similar letter (s) are not significantly different at the 5% level by Tukey.

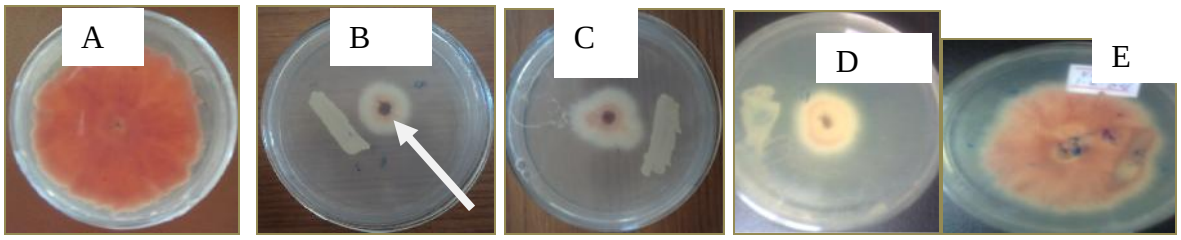

Fig.2. Control plate and dual culture media showing some *Rhizobium* isolate and *Fusarium solani* interactions: (A) *Fusarium solani* alone, (B) Rh26(1) vs *F. solani*, (C) Rh15(2) vs *F. solani*, (D) fb-1WG vs *F. solani* and (E) fb-2ST vs *F. solani.* Arrow indicate the spots of inhibition

4.3. *In Vitro* Assay of Possible Biocontrol Mechanisms of Rhizobium Isolates

In this study, seven (25.94%) isolates that had showed remarkable inhibitory effect against *Fusarium solani* were tested positive for chitinase (Table 5). Variation was observed among the isolates for the chitinase production. Mazen *et al*. (2008) reported that chitinase enzyme was produced by rhizobial isolates but with different degrees. The *Rhizobium* isolates that were positive for chitinase activity in present study had significant mycelia radial growth inhibition (26.47-70.58%) against *Fusarium solani* (Table 4). In recent study, the rhizobacterial isolates that were positive for chitinase activity had significant radial growth inhibition (> 40 %) against *F. oxysporum* and *F. stilboides* and caused large inhibition zones (> 3 cm) against *F. xylarioides* (Muleta *et al*., 2007).

In this investigation, twelve (44.4%) out of those that showed remarkable inhibitory effect against *Fusarium solani* were tested positive for protease (Table 5). Variation was observed among the isolates for the protease activity. The variation in enzymatic activities of rhizobial isolates was reported by various workers. Kumari *et al*. (2010) reported that out of 5 strains of rhizobia studied for protease production, 4 were positive.

Table 5. PGPR properties of Rhizobium isolates

No	Isolates code	Hydrolytic enzymes			HCN	IAA	Growth promoting traits Phosphate solubilisation	
		Chitinase	Protease	Lipase			(halo zone diameter in cm)	SI
1	Rh D(1)	+	+	-	-	+	0.15	1.08^{h}
2	fb-1 WG	+	+	-	-	+	1.30	1.90^{a}
3	RhY(1)	-	+	+	-	-	0.20	1.12^{fhg}
4	Rh 47(2)	+	+	-	-	+	0.20	1.11^{hg}
5	Rh8(3)	-	+	-	-	+	-	-
6	Rh1(2)	-	+	-	-	+	0.30	1.08^{h}
7	RhE(1)	-	-	-	-	-	-	-
8	RhS(1)	+	-	-	-	+	-	-
9	fb-2-1WG	-	-	-	-	+	0.50	1.37^{c}
10	Rh11(1)	-	-	+	-	+	0.30	1.18^{feg}
11	Rh4-1SG	-	-	-	+	+	-	-
12	RhG(2)	-	+	-	-	+	0.20	1.20^{fe}
13	Rh1(3)	-	-	-	+	+	-	-
14	Rh3(2)	+	+	-	-	+	-	-
15	Rh3(1)	-	-	-	-	+	0.40	1.67^{b}
16	fb-3-1SG	-	-	-	+	+	0.20	1.09^{h}
17	Rh21(4)	-	+	-	-	-	0.75	1.23^{de}
18	fb-3WG	-	-	+	-	+	-	-
19	Rh5(2)	-	-	-	+	+	-	-
20	Rh26(1)	+	+	-	-	+	0.30	1.32^{dc}
21	Rh1(4)	-	-	-	+	+	-	–
22	Rh2(3)	-	-	-	+	+	0.50	1.38^{c}
23	Rh8(1)	-	+	-	-	+	0.70	1.88^{a}
24	fb-1EG	-	-	-	+	+	-	-
25	Rh2(4)	-	-	+	-	+	-	-
26	Rh15(2)	+	+	-	-	+	0.20	1.17^{fheg}
27	fb-1018	-	-	-	+	+	-	-
	Control	-	-	-	-	-	0.00	0.00^{i}

Mean from two replications and values followed by the same letter(s) indicate no significant difference ($p>0.05$) at 95% confidence interval. SI = solubilization index

The *Rhizobium* isolates that were positive for protease activity in this study had showed significant mycelia growth inhibition (25.49-70.58%) against *Fusarium solani* (Table 5). Kishore *et al.* (2005) demonstrated that *Pseudomonas aeruginosa* which produced protease had significant inhibition (> 32%) against *Sclerotium rolfsii.* This is an indication that the enzyme protease has responsible effect on the phytophatogens. Earlier, Dunne *et al.* (1997) confirmed that biocontrol of *Pythium ultimum* in the rhizosphere of sugar beet with *Stenotrophomonas maltophila* (Hugh) Palleroni and Bradbury W81 was due to the production of extracellular protease. Although, chitinolytic activity appears less essential for Plant growth promoting bacteria such as *S. plymutica* IC14 when used to suppress *S. sclerotiorum* and *Botrytis cinerea*, synthesis of proteases are involved (Kamensky *et al.*, 2003). Our result seems to confirm the previous study on the responsibility of protease on biocontrol activity.

In this study, 4(14.81%) out of those that showed remarkable inhibitory effect against *Fusarium solnai* were positive for lipase (Table 5). Variation was observed among the isolates for the lipase activity as reported elsewhere (Kumari *et al*., 2010).

The *Rhizobium* isolates that were positive for lipase activity in this study had showed significant ($p < 0.05$) mycelial growth inhibition (39.21-62.74%) against *Fusarium solani* (Table 4). Muleta *et al*. (2007) indicated that all rhizobacterial isolates which were able to produce lipase had significant radial growth inhibition (> 37%) against *Fusarium stilboides.* This indicates that lipase enzyme is responsible for antifungal activity exhibited by the rhizobial isolates. Srividya *et al*. (2012) verified that *Streptomyces* sp. 9p which produced lipase enzyme showed a significant inhibitory effect against fungal soilborne phytopathogens such as *Alternaria brassisicola* (OCA1), *Alternaria brassiceae* (OCA3) *Alternaria alternate* (OTA36), *Colletotrichum gloeosporioides* (OGC1), *Phytophthora capsici* (98-01) and *Rhizoctonia solani* (MTCC 4633).

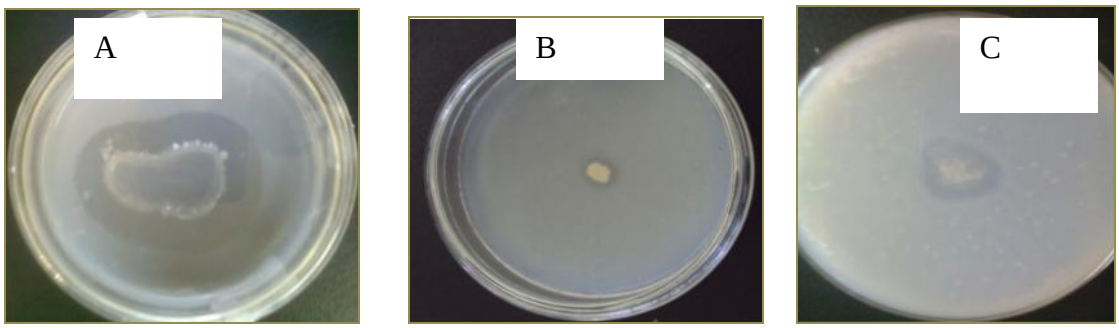

Fig.3. Qualitative assay of lytic enzymes production: (A) Protease positive (Rh15(2)), (B) Lipase positive (fb-3WG) and (C) Chitinase positive (Rh26(1)).

Color of the picrate/ Na2CO3-impregnated paper strips changed from yellow (control) to light brown, brown or reddish brown as an indication of the strength of the cyanide produced (Fig 4**).** In the current study, eight (29.63%) of the antagonists produced HCN (Table 5). This proportion is by far higher than that reported by Beauchamp *et al*. (1981). These authors further remarked that among the rhizobial isolates tested, 12.5% of them were found to be HCN producers.

A growing body of evidence showed that the inhibitory effect of *Bradyrhizobium* (Arachis) on *Macrophomina phaseolina* was due to HCN production (Deshwal *et al*. 2003). Moreover, some works explained the antagonistic properties of *Rhizobium leguminosarum* against *Fusarium oxysporum f.sp. lentis* due to excretion of antibiotics that have fungicidal action on condia of *F. oxysporum* (Essalmani and Lahlou., 2002). *Rhizobium* was reported to produce toxic metabolites which have inhibitory effect against soilborne plant pathogens (Chakraborth and Purkayastha, 1984). Defago *et al.* (1990) have also demonstrated by mutational analysis and complementation that production of HCN by *Pseudomonas fluorescens* strain, CHAO accounted for about 60 % of the biocontrol activity. Hydrogen cyanide from *P. fluorescens* strain CHAO not repressed by fusaric acid and played a significant role in disease suppression of *F. oxysporum* f.sp. *radicis- lycopersici* in tomato (Duffy *et al.*, 2003).

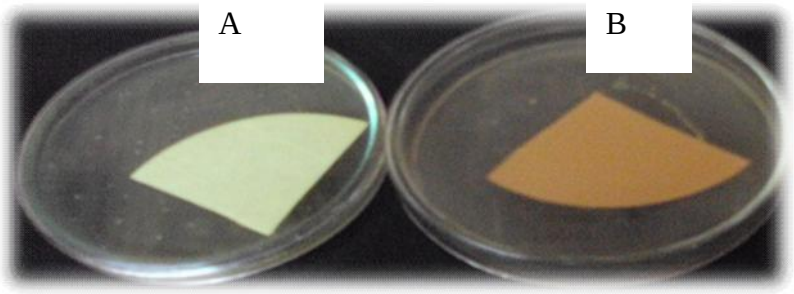

Fig.4. HCN production by Rhizobium isolates: (A) Control and (B) HCN production positive (RhG(2)

Most of the *Rhizobium* isolates tested in the present work were able to produce IAA in the presence of tryptophan (Table 5). Among the 27 rhizobial isolates tested for IAA, 24(88.88%) of them produced IAA. Wang *et al*. (1982) observed nodulating and non-nodulating strains of

Rhizobium leguminosarum were able to produce indole-3-acetic acid. Indole-3-acetic acid which was produced by rhizobacteria increased root hair density, length and enhanced rice seed germination (Vessey, 2003; Ashrafuzzaman *et al*., 2009). Under gnotobiotic conditions, Noel *et al*. (1996) observeda direct growth promotion of the early seedling root of canola (*Brassica campestris*) and lettuce (*Lactuca sativa*) by *R. leguminosarum*. This direct growth-promotion effect appears to involve the plant growth regulators such as indole-3-acetic acid.

Over 50% of the total *Rhizobium* isolates showed clearly visible haloes (>0.15 cm) around their colonies (Fig. 5B) on PA after 7 days of incubation. The phosphate solubilizing frequency of isolates of this study is comparable to other studies (Hani *et al*., (1998). Among the 27 isolates tested for phosphate solubilisation, isolate Rh21(4) produced the largest halo zone (1.3 cm), on Piskovskaya's Agar plates(Table 5). Solubilisation indices (SI) in PA are presented in Table 5.

The SI of the potential P solubilising rhizobial isolates differed significantly ($p < 0.0001$) and ranged from 1.08 to 1.90. The bacterial strain fb-1WG showed the largest SI (1.90 cm) of solubilisation, followed by Rh8(1) and Rh3(1). Phosphate solubilizing rhizobacteria convert insoluble phosphates into soluble monobasic form for plant uptake (Gyaneshwar *et al*., 2002). In a field study, Chabot *et al* (1996a) have observed that phosphate solubilization by strains of *R. leguminosarum* bv. *phaseoli* was the most important mechanism of maize and lettuce growth promotion, in moderately fertile and very fertile soils. Barley yield and uptake of P, N, K, Ca, and Mg were significantly increased by inoculation with a P-solubilizing strain of *Mesorhizobium mediterraneum* (Peix *et al*., 2001). A recent extensive review (Muleta, 2009) from both greenhouse and field trials has demonstrated a remarkable improvement in growth responses of various crops to phosphate solubilising microorganisms' inoculations.

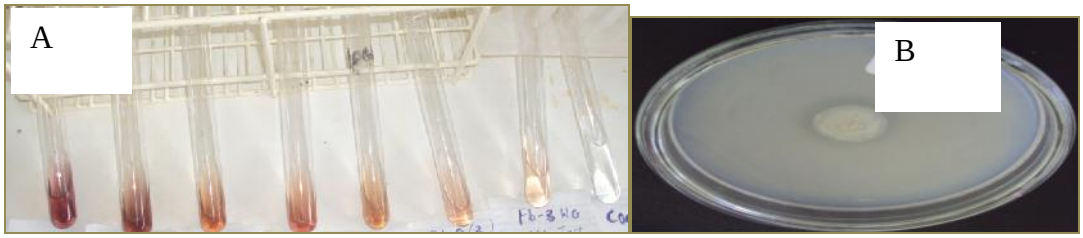

Fig.5. Possible Plant Growth promoting mechanisms. (A) IAA production by *Rhizobium* isolates: RhD(1), fb-1018, Rh5(2), Rh1(4), Rh26(1), Rh15(2), fb-1WG and Control, left to right respectively. (B) Phosphate Solubilization: fb-1WG

4.5. Effects of Rhizobium Isolates on Seed Germination

In blotter experiment, the *Rhizobium* isolates affected germination of seeds in the presence of *Fusarium solani*. The germination test showed that all *Rhizobium* isolates and their combination significantly ($p<.0001$) increased germination percent of Faba bean seeds compared to diseased (control) (Table 6). The mean germination percent in diseased (control) seeds was 45%. The fb-1WG and combination of all isolates showed 79.69% germination. Isolates Rh15(2) and Rh26(1) showed 52.33% and 60.00% germination, respectively. The health seeds showed 52.24% germination of seeds after 10 days of experiment.

The data of radicle length of germinated seeds showed significant ($p<.0001$) differences between treatments (Table 6). The radical length in diseased (control) seeds significantly retarded compared to health. The mean radicle length in control seeds was 1.28 cm/ seed. The radicle length of seeds was significantly increased when treated with all *Rhizobium* isolates and their combination compared to diseased (control). The combination, fb-1WG, Rh15(2) and Rh26(1) treated seeds showed 5.92 cm/seed radicle length, 4.41 cm/seed, 2.60 cm/seed and 1.85 cm/seed, respectively. The health seeds showed 2.15 cm/seed radicle length.

Table 6. Effects of isolates on seed germination

Parameters	Uninoculated	Control	Rh26(1)	Rh15(2)	fb-1WG	Combination
Germination%	52.24^{c}	45.000^{d}	60.000^{b}	52.327^{c}	79.690^{a}	79.690^{a}
Radical length	2.15333^{d}	1.2800^{f}	1.85333^{e}	2.60333^{c}	4.41333^{b}	5.91667^{a}
Plumul length	1.00000^{c}	0.48667^{e}	0.93333$_{c}$	0.85000^{d}	3.77333^{b}	5.25000^{a}
Vigor Index	164.713^{d}	79.500^{e}	167.200^{dc}	180.6970^{c}	652.397^{b}	889.870^{a}

Values are mean of three replications. In a column, mean followed by a similar letter (s) are not significantly different at the 5% level by Tukey.

The result regarding plumule length showed significant ($p<.0001$) difference between *Rhizobium* isolates (Table 6). The plumule length in diseased (control) seeds significantly reduced compared to health. The mean plumule length in diseased (control) seeds was 0.49 cm/seed. The plumule length of seeds was significantly increased by treatment of all *Rhizobium* isolates and combination compared to diseased (control). The combination, fb-1WG, Rh26(1) and Rh15(2) treated seeds showed 5.25 cm/seed, 3.77 cm/seed, 0.93 cm/seed

and 0.85 cm/seed plumule length, respectively. The health seeds showed 1.00 cm/seed plumule length.

All the *Rhizobium* isolates and their combination were significantly ($p<.0001$) effective in control of *F. solani* and thus vigor index was increased in treated seeds (Table 6). In diseased (control), *F. solani* inoculation significantly affected the vigor index compares to health. The mean vigor index in diseased (control) seeds was 79.50/seed. The combination, fb-1WG, Rh15(2) and Rh26(1) treated seeds showed 889.87/seed, 652.40/seed, 180.70/seed and 167.20/seed vigor index, respectively. The health seeds showed 164.71/seed vigor index.

Rhizobium isolates were able to improve the germination parameters such as germination percent, radicle length, plumule length and vigor index of *Fusarium solani* infected Faba bean seeds compared to control. This indicates that *Rhizobium* isolates were beneficial for germination of Faba bean seeds. Studies (e.g Duffy *et al*., 1996) demonstrated that microorganisms such as *Azospirillum* and *Pseudomonas* from natural sources enhanced germination. The results clearly present a protecting and growth-promoting role of these species in the germination process of many cereals (Arsac *et al*., 1990; Hofte *et al*., 1991). Seed treatment of biocontrol agents can suppress pathogenic fungi residing on the surface of seed or inside the seed (Martha *et al.*, 2003). Study (Mohamed, 2009) demonstrated that *Rhizobium* showed significant effect on increase of germination.

Application of combination of *Rhizobium* isolates has resulted in much more intensive germination percent, vigor index and radicle and plumule length compared to single isolates. Kumari *et al* (2010) proved that seed treatment with a rhizobial isolate alone and together with *P. fluorescens* isolates reduced the number of infected peas grown in *Fusarium oxysporum* infected soils. Esteve de Jensen *et al* (2002) demonstrated that the application of *Rhizobium* with *Bacillus subtilis* is a promising approach for the improvement of bean root rot control. Application of mixed PGPR strains based formulations might ensure at least one of the mechanisms to operate under variable environment conditions (Duffy *et al*., 1996).

The present results suggest that, *Rhizobium* isolates effect on germination might be antagonism to *Fusarium solani* and/or growth promotion due to the production of some metabolites (protease, chitinase, lipase, HCN, IAA). Ryu *et al*. (2003) also observed that

PGPR treatment increased germination rate and radicle/ plumule growth in way similar to IAA, cytokinin and gibberellins treatments. Moreover, Dal-Bello *et al*. (2002) observed that seed bacterization proved a successful method for enhancing biological control of plant disease.

4.6. Effects of the Rhizobium Isolates on Seedlings

4.6.1. Disease

The results showed that there was significant ($p < 0.0001$) interaction effect among types of treatment used and time of application of the isolates in reduction of disease incidence (Table 7). Under greenhouse conditions, all of the treatment with the *Rhizobium* isolates applied at time of pathogen inoculation and 7 days before and after have significantly reduced incidence of disease compared to the diseased (control) (Table 7).

Application of combination of *Rhizobium* isolates to seedling before the time of pathogen inoculation gave the highest (88.60%) reduction in disease incidence of root rot on Faba bean plants. The lowest (50.00%) percentage of disease incidence reduction was obtained by application of Rh26(1) to seedling. There was no significant ($p>0.05$) difference obtained in the disease incidence when the rhizobial treatments were added 7 days before pathogen inoculation and at the same time of pathogen inoculation (Table 7). Among different treatments applied to seedlings, disease (black root rot) count was maximum (100%) in diseased (control).

Table 7. Interaction effects of types of isolates and time of application on disease incidence and severity on Faba bean

Treatments	Parameters	Time of application		
		Before	At same time	After
Rh26(1)	Incidence	(50)45.00^{b}±0.00	(50)45.00^{b}±0.00	(50)45.00^{b}±0.00
	DR%	50.00	50.00	50.00
	Severity	(55.5)48.16^{cd}±00	(61.1)51.41^{bc}±0.0	(64.8)53.61^{b}±1.90
	DR%	37.50	27.70	27.03
Rh15(2)	Incidence	(25)30.00^{c}±0.00	(25)30.00^{c}±0.00	(41)40.00^{b}±8.66
	DR%	75.00	75.00	59.00
	Severity	(38.8)38.53^{e}±0.00	(51.8)46.03^{d} ±1.8	(55.5)48.16^{cd}±0.00
	DR%	56.31	41.66	37.50
fb-1WG	Incidence	(25)30.00^{c}±0.00	(25)30.00^{c}±0.00	(50)45.00^{b}±0.00
	DR%	75.00	75.00	50.00
	Severity	(31.4)34.08^{f}±2.00	(38.5)38.53^{e} ±0.0	(57.3)49.20^{cd}±1.80
	DR%	64.64	56.64	35.47
Combination	Incidence	(0)1.40^{d} ±0.00	(0)1.40^{d} ±0.00	(25)30.00^{c}±0.00
	DR%	100.00	100.0	75.00
	Severity	(23.7)29.13^{g}±1.80	(31.2)33.96^{f} ±2.3	(35.1)36.33^{ef}±1.90
	DR%	73.31	64.86	60.47
Control (diseased)	Incidence	(100)88.60^{a} ±0.00	(100)88.60^{a}±0.00	(100)88.60^{a} ±0.00
	DR%	-	-	-
	Severity	(88.8)70.45^{a}±0.00	(88.8)70.45^{a}±0.00	(88.8)70.45^{a} ±0.00
	DR%	-	-	-

Means with the same letter is not significantly different at p = 0.05 according to Tukey's honest significant difference (HSD). There were 3 replicates in each treatment (mean ± SD). Values in bracet are actual data before transformation. Disease reduction, Dc is disease on the control plants; Dt is disease on the treated

The results also showed that there was significant ($p < .0001$) interaction effect among types of treatments used and time of application in reduction of disease severity (Table 7). All of the *Rhizobium* treatments applied at the same time with pathogen and 7 days before and after significantly reduced disease severity compared to the control (Table 7). However, the highest reduction in black root rot (BRR) severity (73.31%) was observed with application of combination of isolates before the pathogen inoculation followed by at the same time with pathogen inoculation with mean value of 64.86%. The lowest (27.03%) percentage of disease severity reduction was obtained by application of Rh26(1) to seedling after 7 days of pathogen inoculation followed by at the same time of pathogen inoculation with mean value of 27.70%.

The present study showed that *Rhizobium* isolates proved to be effective in controlling *Fusarium solani* the causative agent of Faba bean root rot disease. Combination of the isolates proved to be more effective in controlling the disease than all the tested individual isolates. This indicates *Rhizobium* isolates were beneficial for suppressing root rot. Rhizobia have been reported as best control of root infecting fungi on both leguminous and non-leguminous plants (Ehteshamul-Haque and Ghaffar, 1993). The potentiality of *Rhizobium* as biocontrol agents of phytopathogenic fungi in soybean is well known especially to *Macrophomina phaseolina*, causative agent of charcoal rot disease (Chakraborty and Purkayastha, 1984).

Combined application of plant growth promoting rhizobacteria has significantly lowered *Fusarium* wilt disease of *Capsicum annum* L. caused by *Fusarium solani* compared to individual isolate (Sundaramoorthy *et al*., 2012). Combined application of *Pichia guilermondii* and *Bacillus mycoides* (B16) reduced the infection of *Botrytis cinerea* by 75% on fruits in strawberry plants grown commercially under greenhouse conditions. However, the individual application of either antagonist resulted in 50% reduction of strawberry fruit infection. Advantages of strain mixtures include, broad spectrum of action, enhanced efficacy, reliability and also allow combination of various traits without genetic engineering (Janisiewicz, 1996).

The reduction of root rot incidence and severity obtained in *Rhizobium* isolates inoculated plants in this study could be related to antifungal activity of *Rhizobium* isolates. There are many mechanisms suggested to clarify the role of antagonistic organisms in suppression of pathogens growth and thus to control diseases. *Rhizobium* spp. isolated from Algerian soil, were found to produce bacteriocins with antimicrobial activities against *Pseudomonas savastanoi*, the agent responsible for olive knot disease (Mourad *et al*., 2009). The Rhizobia present in the rhizosphere of plants presumably prevent the contact of pathogenic fungi on roots by covering the hyphal tip of *Rhizocotonia solani* and by parasitizing it (Tu, 1983). *Serratia plymuthica* was found to produce chitinase that inhibited spore germination and germ-tube elongation in *Botrytis cinerea* (Frankowski *et al.,* 2001). The isolates used for inoculation in the present study exhibited the capacity to produce protease, lipase, chitinase and hydrogen cyanide (Table 5) and therefore might have significantly contributed to the reduction of disease incidence and severity of root rot.

Timing of *Rhizobium* inoculation was also a factor in determining the level of root rot reduction. The incidence and severity of root rot incited by the *Fusarium solani* were highly reduced when Faba bean plants were inoculated 7 days before the pathogen whereas, the damage was of intermediate order in simultaneous application. These findings are in agreement with Kokalis - Burelle *et al* (2002). Similarly, Tu (1980) found less root rot development when alfaalfa plants were inoculated simultaneously with *Rhizobium* and *Fusarium oxysporum* or *Phytophthora megasperma* rather than sequentially with Rhizobium being added several weeks after pathogen. It seems that earlier establishment of *Rhizobium* protected the plant. It might have due to induced systemic resistance.

4.6.2. Growth parameters

The result showed that there was significant ($p<.0001$) interaction effect among types of treatments used and time of application on number of nodules. The roots of uninoculated and those infected with *Fusarium solani* only (control) were devoid of any nodules (Table 8). Root nodule number of control plant infected by *Fusarium solani* was not significantly ($p>0.05$) different from that of uninoculated (Table 8). Under greenhouse conditions, all treatments applied before pathogen inoculation significantly increased root nodule number compared to the control except Rh26(1) inoculation. Application of fb-1WG isolates to seedling before pathogen inoculation gave the highest (26.42) root nodule number followed by at the same time of pathogen inoculation and after inoculation. The lowest (0.00) root nodule number was obtained by application of Rh26(1) after and at the same time of pathogen inoculation and followed by before inoculation.

The result showed that there was significant interaction ($p<.0001$) effect among types of treatment used and time of application in increasing of nodule fresh weight on the plants. Results also, indicate that all tested rhizobial treatments when applied before have significantly ($p<.0001$) increased nodule fresh weight relative to control except Rh26(1) inoculation (Table 8).

Inoculation of fb-1WG isolates to seedling before resulted in highest (0.19 g/plant) nodule fresh weight followed by at time of pathogen inoculation. Although, application of fb-1WG caused highest nodule fresh weight, no significant differences were found between fb-1WG

and combination. Lowest (0.00 g/plant) nodule fresh weight was obtained by application of Rh26(1) to seedling after, and at same time followed by before.

Table 8. Nodulation of Faba bean as influenced by the intraction effects of types of isolates and time of application

Treatments	Parameters	Time of application		
		Before	At same time	After
Rh26(1)	Number	$0.97^{g} \pm 0.06$	$0.00^{g} \pm 0.00$	$0.00^{g} \pm 0.00$
	FW	$0.01^{f} \pm 0.00$	$0.00^{f} \pm 0.00$	$0.00^{f} \pm 0.00$
	DW	$0.004^{g} \pm 0.00$	$0.00^{g} \pm 0.00$	$0.00^{g} \pm 0.00$
Rh15(2)	Number	$4.43^{f} \pm 0.06$	$1.00^{g} \pm 0.00$	$0.00^{g} \pm 0.00$
	FW	$0.04^{e} \pm 0.00$	$0.010^{f} \pm 0.00$	$0.00^{f} \pm 0.00$
	DW	$0.02^{ef} \pm 0.00$	$0.01^{fg} \pm 0.00$	$0.00^{g} \pm 0.00$
fb-1WG	Number	$26.42^{a} \pm 0.95$	$24.33^{b} \pm 0.29$	$16.33^{d} \pm 0.14$
	FW	$0.19^{a} \pm 0.01$	$0.14^{b} \pm 0.01$	$0.07^{d} \pm 0.01$
	DW	$0.15^{b} \pm 0.01$	$0.02^{de} \pm 0.01$	$0.01^{fg} \pm 0.00$
Combination	Number	$25.33^{b} \pm 0.58$	$22.33^{c} \pm 0.58$	$14.42^{e} \pm 0.38$
	FW	$0.19^{a} \pm 0.01$	$0.08^{c} \pm 0.01$	$0.06^{d} \pm 0.01$
	DW	$0.17^{a} \pm 0.01$	$0.10^{c} \pm 0.00$	$0.03^{d} \pm 0.01$
Health (uninoculated)	Number	$0.00^{g} \pm 0.00$	$0.00^{g} \pm 0.00$	$0.00^{g} \pm 0.00$
	FW	$0.00^{f} \pm 0.00$	$0.00^{f} \pm 0.00$	$0.00^{f} \pm 0.00$
	DW	$0.00^{g} \pm 0.00$	$0.00^{g} \pm 0.00$	$0.00^{g} \pm 0.00$
Control (diseased)	Number	$0.00^{g} \pm 0.00$	$0.00^{g} \pm 0.00$	$0.00^{g} \pm 0.00$
	FW	$0.00^{f} \pm 0.00$	$0.00^{f} \pm 0.00$	$0.00^{f} \pm 0.00$
	DW	$0.00^{g} \pm 0.00$	$0.00^{g} \pm 0.00$	$0.00^{g} \pm 0.00$

Means with the same letter is not significantly different at $p = 0.05$ according to Tukey's honest significant difference (HSD). There were 3 replicates in each treatment (mean ± SD).FW=fresh weight; DW= dry weigth

The result also showed that there was significant interaction ($p<.0001$) effect among types of treatment used and time of application on nodule dry weight (Table 8). Before and at time of pathogen inoculation of fb-1WG, combination and Rh15(2) were significantly($p<0.05$) increased nodule dry weight compared to the control (diseased) and health (uninoculated). The of suspension isolate Rh26(1) was not significantly increased nodule dry weight in either of time of application. Application of isolate combination resulted in the highest (0.17g/plant) nodule dry weight when applied before followed by at the same time of pathogen inoculation.

Results reported herein indicated that *Rhizobium* isolates treatments not only suppressed both disease incidence and severity but also enhanced nodule number, nodule fresh weight and nodule dry weight of Faba bean plants compared to infected control. The findings suggest that

incorporation of *Rhizobium* is important for plant growth promotion by increasing mineral uptake of plants and producing IAA. Similar studies (Pal, 1997; Akhtar and Siddiqui, 2008a) have shown that the addition of specific *Rhizobium and Bacillus* isolates to the rhizosphere can result in increased nodulation of chickpea and soybean plants.

IAA synthesizing rhizobia have been found to nodulate more intensely than IAA negative mutants (Boiero *et al*., 2007). Sheng (1993) reported that besides fixation of atmospheric nitrogen, the nodulation effect of rhizobial isolates is due to the production of plant growth regulators such as Auxins and cytokinins like substance. Inoculation of peanut seeds with plant growth promoting and phosphorus solubilizing *Fluorescent pseudomonad* isolates, PGPR1, PGPR2 and PGPR4 significantly enhanced the nodule number and dry weight over the control in a three-year study (Dey *et al*., 2004). The isolates used for inoculation in the present study exhibited the capacity to solubilize phosphorus and produced IAA (Table 5) and therefore might have contributed to the enhanced nodulation.

Over all, significant results have been shown in before application followed by at time of pathogen inoculation. The result of this study corresponds with work done by Yaqub *et al.* (2012) who found that increased nodulation in treatments with prior application of *Bradyrhizobium* and decreased nodule formation in simultaneous or delayed applications on *Meloidogyne incognita* inoculated black gram, *Vigna mungo* plants.

The result showed that there was significant (<.0001) interaction effect among types of isolates used and time of application isolates in increase of shoot height on the Faba bean plants (Table 9). Shoot height of control plant (infected with *Fusarium solani*) was significantly lower than that of uninfected (health) (Table 9). Generally, all treatments applied at time of pathogen inoculation and 7 days before and after pathogen inoculation significantly increased shoot height compared to the control (diseased).The combination of isolates produced the maximum (48.00 cm/plant) shoots height in response to before inoculation followed by at the same time of pathogen inoculation. Isolate Rh26(1) produced the lowest (34.36 cm/plant) shoot height when applied after pathogen inoculation followed by before inoculation and at the same time of pathogen inoculation. There was no significant ($p>0.05$) difference between isolates Rh15(2) and Rh26(1) with regard to shoot height in three time of applications**.** The significant shoot height variation was observed between treatments.

Data regarding shoot fresh weight per plant showed significant ($p<.0001$) interaction between treatments and the time of inoculation (Table 9). Shoot fresh weight of control plant infected with *Fusarium solani* was significantly lower than that of uninfected (health). Before inoculation treatments produced the highest shoot fresh weight in all the treatments of *Rhizobium* followed by at the same time of pathogen inoculation treatment.

The combination of isolates produced the highest (18.86 g/plant) shoot fresh weigh in case of before inoculation, which was at par with inoculation after. Fresh weight of shoot per plant in fb-1WG significantly increased by before, after inoculation and at the same time of pathogen inoculation compared to diseased (control). Both Rh26(1) and Rh15(2) showed the same fresh weight of shoot plant during all application times. Isolate Rh15(2) produced the lowest (9.51 g/plant) shoot fresh weight when inoculated after the pathogen followed by at the same time and before inoculations.

The result showed that there was significant ($p<.0001$) interaction effect among types of isolates used and times of application of isolates in increase of shoot dry weight of the Faba bean plants (Table 9). Shoot dry weight of control plant infected with *Fusarium solani* was significantly lower than that of uninfected (health). Combination of isolates produced the highest (3.66 g/plant) shoot dry weigh per plant in case of before inoculation, which was not significantly different than at time of inoculation. After inoculation treatment in case of Rh26(1) produced lowest (1.01 g/plant) shoot fresh weight followed by at time inoculation, which not significantly different with diseased control.

Table 9. Combined effect of types of isolates and time of application on shoot, root, flower and leaf on Faba bean

Treatments	Parameters		Time of application		
			Before	At same time	After
Rh26(1)	Shoot	Height	$38.25^{cd} \pm 0.66$	$35.83^{de} \pm 1.53$	$34.36^{de} \pm 1.80$
		FW	$11.46^{c} \pm 0.01$	$10.13^{cd} \pm 0.02$	$10.18^{cd} \pm 0.01$
		DW	$1.88^{ef} \pm 0.00$	$1.40^{gh} \pm 0.03$	$1.01^{h} \pm 0.01$
	Root	Length	$28.67^{bcd} \pm 1.15$	$25.33^{e} \pm 0.58$	$22.33^{fg} \pm 0.58$
		FW	$12.10^{cd} \pm 0.09$	$12.60^{c} \pm 0.00$	$8.15^{efg} \pm 0.69$
		DW	$1.04^{e} \pm 0.03$	$0.87^{f} \pm 0.06$	$0.60^{gh} \pm 0.045$
	Flower	Number	$14.67^{cd} \pm 0.58$	$9.67^{e} \pm 0.58$	$6.67^{f} \pm 0.58$
	Leaf	Number	$30.67^{cd} \pm 0.29$	$28.92^{def} \pm 0.72$	$27.33^{efg} \pm 0.29$
Rh15(2)	Shoot	Height	$37.00^{cde} \pm 0.66$	$35.83^{de} \pm 1.53$	$35.00^{de} \pm 1.30$
		FW	$11.20^{cd} \pm 0.01$	$10.13^{cd} \pm 0.02$	$9.51^{de} \pm 0.01$
		DW	$2.03^{e} \pm 0.01$	$1.38^{gh} \pm 0.01$	$1.17^{gh} \pm 0.03$
	Root	Length	$29.67^{bc} \pm 0.58$	$29.33^{bc} \pm 1.53$	$21.00^{g} \pm 1.00$
		FW	$13.95^{bc} \pm 1.72$	$10.19^{de} \pm 0.16$	$9.50^{ef} \pm 1.28$
		DW	$1.05^{e} \pm 0.05$	$1.04^{e} \pm 0.01$	$0.71^{g} \pm 0.02$
	Flower	Number	$17.00^{b} \pm 1.00$	$13.67^{d} \pm 0.58$	$13.00^{d} \pm 1.00$
	Leaf	Number	$29.42^{de} \pm 0.14$	$28.00^{ef} \pm 0.66$	$25.25^{gh} \pm 0.43$
fb-1WG	Shoot	Height	$40.67^{bc} \pm 1.80$	$36.42^{de} \pm 0.38$	$36.83^{cde} \pm 2.52$
		FW	$15.51^{b} \pm 1.18$	$13.46^{b} \pm 0.96$	$10.19^{cd} \pm 0.73$
		DW	$2.90^{cd} \pm 0.05$	$2.66^{d} \pm 0.01$	$1.56^{fg} \pm 0.51$
	Root	Length	$31.00^{b} \pm 1.00$	$29.33^{bc} \pm 1.15$	$26.00^{de} \pm 1.00$
		FW	$14.83^{b} \pm 0.29$	$12.20^{cd} \pm 0.20$	$7.53^{fg} \pm 0.06$
		DW	$1.53^{c} \pm 0.06$	$1.28^{d} \pm 0.02$	$0.93^{ef} \pm 0.03$
	Flower	Number	$20.33^{a} \pm 1.15$	$14.67^{cd} \pm 0.58$	$14.33^{cd} \pm 0.58$
	Leaf	Number	$33.92^{ab} \pm 0.95$	$32.33^{bc} \pm 1.15$	$29.08^{def} \pm 0.14$
Combination	Shoot	Height	$48.00^{a} \pm 0.75$	$42.8^{b} \pm 1.04$	$37.50^{cde} \pm 1.09$
		FW	$18.86^{a} \pm 0.12$	$15.04^{b} \pm 0.80$	$11.45^{c} \pm 1.86$
		DW	$3.66^{a} \pm 0.15$	$3.39^{ab} \pm 0.03$	$3.13^{bc} \pm 0.00$
	Root	Length	$35.67^{a} \pm 1.15$	$27.33^{cde} \pm 1.15$	$24.67^{ef} \pm 0.58$
		FW	$17.78^{a} \pm 0.07$	$15.81^{ab} \pm 0.07$	$12.34^{cd} \pm 0.13$
		DW	$2.73^{a} \pm 0.02$	$2.50^{b} \pm 0.01$	$0.70^{gh} \pm 0.00$
	Flower	Number	$20.67^{a} \pm 0.58$	$15.67^{bc} \pm 1.15$	$14.33^{cd} \pm 0.58$
	Leaf	Number	$36.08^{a} \pm 0.76$	$32.00^{bc} \pm 1.09$	$27.08^{fg} \pm 0.95$
Health (uninoculated)	Shoot	Height	$34.00^{e} \pm 0.00$	$34.00^{e} \pm 0.00$	$34.00^{e} \pm 0.00$
		FW	$9.50^{d} \pm 0.00$	$9.50^{d} \pm 0.00$	$9.50^{d} \pm 0.00$
		DW	$1.55^{fg} \pm 0.00$	$1.55^{fg} \pm 0.00$	$1.55^{fg} \pm 0.00$
	Root	Length	$27.00^{cde} \pm 1.00$	$27.00^{cde} \pm 1.00$	$27.00^{cde} \pm 1.00$
		FW	$9.77^{e} \pm 0.38$	$9.77^{e} \pm 0.38$	$9.77^{e} \pm 0.38$
		DW	$1.23^{d} \pm 0.06$	$1.23^{d} \pm 0.06$	$1.23^{d} \pm 0.06$
	Flower	Number	$7.33^{f} \pm 0.29$	$7.33^{f} \pm 0.29$	$7.33^{f} \pm 0.29$
	Leaf	Number	$24.67^{h} \pm 0.76$	$24.67^{h} \pm 0.76$	$24.67^{h} \pm 0.76$
Control (diseased)	Shoot	Height	$25.25^{f} \pm 1.25$	$25.25^{f} \pm 1.25$	$25.25^{f} \pm 1.25$
		FW	$6.78^{e} \pm 0.16$	$6.78^{e} \pm 0.16$	$6.78^{e} \pm 0.16$
		DW	$1.00^{h} \pm 0.01$	$1.00^{h} \pm 0.01$	$1.00^{h} \pm 0.01$
	Root	Length	$17.33^{h} \pm 0.58$	$17.33^{h} \pm 0.58$	$17.33^{h} \pm 0.58$
		FW	$6.13^{g} \pm 0.95$	$6.13^{g} \pm 0.95$	$6.13^{g} \pm 0.95$
		DW	$0.58^{h} \pm 0.04$	$0.58^{h} \pm 0.04$	$0.58^{h} \pm 0.04$
	Flower	Number	$4.33^{g} \pm 0.29$	$4.33^{g} \pm 0.29$	$4.33^{g} \pm 0.29$
	Leaf	Number	$20.67^{i} \pm 0.76$	$20.67^{i} \pm 0.76$	$20.67^{i} \pm 0.76$

Means with the same letter is not significantly different at $p = 0.05$ according to Tukey's honest significant difference (HSD). There were 3 replicates in each treatment (mean ± SD)

The result showed that there was significant ($p<.0001$) interaction effect among types of isolates used and times of application in increase of root length on the Faba bean plants (Table 9). Root length was significantly lower in plants infected with *Fusarium solani* than in uninfected plants, or those inoculated with either treatment (Table 9). The pathogenic influence on root length in *Fusarium solani* -inoculated plants was significantly less when applied at the same time of pathogen inoculation and 7 days before and after inoculated with each biocontrol agent compared to the infected control (diseased control). The combination of isolates produced the maximum (35.67 cm/plant) root length in response to before inoculation followed by at the same time of pathogen inoculation. After inoculation treatment with isolate Rh15(2) produced the lowest (21.00 cm/plant) root length followed by other time of applications. The significant root length variation was observed in each combination and Rh26(1) treatment.

Data concerning root fresh weight per plant showed significant ($p<.0001$) interaction between treatments and the time of inoculation (Table 9). Root fresh weight was significantly lower in plants infected with *Fusarium solani* than in uninoculated plants (Table 9). The pathogenic influence on fresh weight in *Fusarium solani* -inoculated plants was significantly less when at time of pathogen inoculation and 7 days before inoculation with each of treatments compared to the infected control (diseased control). The combination of isolates produced the highest (17.78 g/plant) root fresh weight in response to before inoculation followed by at the same time of pathogen inoculation. After inoculation of isolate Rh26(1) produced the lowest(8.15 g/pant) root fresh weight followed by before inoculation and at the same time of inoculation. There was significant ($p<0.05$) difference in root length with at the same time of pathogen inoculation, 7 days before and after inoculation for treatments of Rh15(2) and fb-1WG .

Data regarding root dry weight per plant showed significant ($p<.0001$) interaction between treatments and the time of inoculation (Table 9). Root dry weight was significantly lower in plants infected with *Fusarium solani* than in uninfected plants (Table 9). Root dry weight was significantly ($p<0.05$) less when applied at the same time of pathogen inoculation and 7 days before inoculated with each of treatments compared to the infected control. The highest (2.73 g/plant) root dry weight was measured in response to before inoculation followed by at the same time of pathogen inoculation with combination of isolates. After inoculation treatment

with Rh26(1) produced the lowest (0.60 g/plant) root dry weight followed by at the same time and before inoculations.

There was significant ($p<.0001$) interaction effect among isolates used and time of applications with regard to flower number (Table 9). The highest (20.67 /plant) flower number was recorded in response to before inoculation followed by after inoculation. No significant ($p>0.05$) difference was observed between combination and fb-1WG application on flower number of Faba bean plants.

There was significant ($p<.0001$) interaction effect between treatments and the time of inoculation with regard to leaf number (Table 9). The combination of isolates produced the highest (36.08/plant) leaf number in response to before inoculation followed by at time of pathogen inoculation. After inoculation of Rh15(2) produced the lowest (25.25/plant) leaf number followed by at the same time.

The Faba bean plants remained stunted and showed poor growth response in the absence of *Rhizobium* isolates. However, the biocontrol agents posed increased shoot height, shoot weight, root length, and lesser damage of flower number to *Fusarium solani* inoculated plants. Multiple lines of evidence (Lin *et al.*, 1983; Antoun *et al.*, 1998; Alikhani *et al*., 2006; Arfaoui *et al*., 2007) demonstrates that rhizobial isolates enhance plant health and growth through manifold mechanisms. For instance, Elsiddig *et al*. (1999) studied the inoculation effect of *Bradyhizobium* strains on five guar (*Cyamopsis tetragonoloba*) cultivars in a field experiment. Most of the *Bradyhizobium* strains significantly increased yield, protein, crude fiber and mineral content. Increase in shoot and root length of several crop plants due to inoculation of phosphate solublizing *Rhizobium* isolates have been reported (Estevez de Jensen *et al*., 2002). Increased cell elongation and multiplication due to enhanced nutrient uptake by plants following inoculation of Phosphate solubilizing microorganisms may have caused the increased plant height (Peix *et al*., 2001). In nonlegumes, IAA produced by rhizobia may stimulate plant shoot and root systems, (Dazzo and Yanni, 2006). The role of these plant growth promoting substances in shoot and root elongation as well as plant growth is well established (Dazzo and Yanni, 2006). The isolates used for inoculation in the present study exhibited the capacity to solubilize phosphorus and produce IAA which could contribute to improved growth parameters.

The findings suggest that incorporation of *Rhizobium* is important for plant growth promotion by increasing mineral uptake of plants and producing IAA. Similar studies Noel *et al*. (1996) observed that several strains of *R. leguminosarum* bv. *viciae* promoted the early seedling root growth of canola and lettuce. The observed growth stimulation was associated with the production of the plant growth regulators indole- 3-acetic acid (IAA) and cytokinin. The growth promotion of wheat and maize inoculated with *R. leguminosarum* bv. *trifolii* R39 in greenhouse and field experiments was mediated by auxine and cytokinine production (Höflich *et al*., 1994). The role of inorganic P-solubilization as a mechanism in maize growth promotion was analyzed by using two Lux+ mutants of *R. leguminosarum* bv. *phaseoli* R1 with reduced solubilization activity (Chabot *et al.,* 1998).

Inoculation of combination of *Rhizobium* isolates has resulted in much more demanding height, shoot fresh weight, shoot dry weight, root length, root fresh weight, root dry weight, flower and leaf number of compared to single isolates. This indicates *Rhizobium* isolates had synrigetic intraction. The combination of biocontrol agents may better adapt to the environmental changes, protect against a broader range of pathogens, increase the genetic diversity of biocontrol systems that persist longer in the rhizosphere, utilize a wider array of biocontrol mechanisms (Pierson & Weller, 1994), enhance the efficacy and reliability of control (Duffy & Weller, 1995), and allow the combination of various mechanisms of biocontrol without the need for genetic engineering (Janisiewicz, 1988). Similar studies Nandakumar *et al*. (2001) showed that inoculation of combine formulation of *Fluorescent pseudomonads* through seed, root, soil and foliage to rice crop suppressed sheath blight under field conditions better than individual strains based formulations.

The average disease reduction for mixtures was 45.1% compared to 29.2% for individual strains. In addition to disease reduction strain mixtures increased biomatter production and yield compared to individual strains (Nandakumar *et al*., 2001). Under gnotobiotic conditions, in dual inoculation trials of lettuce, a very significant interaction was observed between *Sinorhizobium meliloti* and the AM fungus *Glomus mosseae.* This translated into a 476% increase in shoot dry matter yield of 40-dayold plants (Galleguillos *et al*., 2000), and the growth promoting effect reported was not accompanied by an increase in root colonization by the AM fungus. This observation suggests that the PGPR rhizobial strain did not act as a

mycorrhizal helper bacterium (Budi *et al*., 1999), but that rather that the fungus stimulated the PGPR activity.

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 has been strongly suggested that the main success of biocontrol agents is largely attributable to their multifunctional characteristics (Vassilev *et al*., 2006) and synergistic interactions (Hassan Dar *et al*., 1997).

5. SUMMERY AND CONCLUSIONS

Fourty one (41) rhizobial isolates were screened for antagonism, of which twenty seven (27) 66% rhizobial isolates showed potential to inhibit the test pathogen in *in vitro* evaluation. Rhizobial isolates tested showed varying levels of effects against the *Fusarium solani*. Isolates of fb-1WG, Rh15(2) and Rh26(1), exerted their highest inhibitory effect 63.72%, 64.70% and 70.58% against *F. solani*, respectively.

Regarding to hydrolytic enzymes production and growth promoting properties of antagonistic isolates, seven rhizobium isolates produced chitinase, 12 isolates were protease producer, four isolates produced lipase, eight were produced hydrogen cyanide, twenty four produced indole acetic acid, and fifteen were solublized inorganic phosphate. The antagonistic isolates like RhD(1), fb-1WG, Rh47(2), Rh15(2) and Rh26(1) found positive for protease, chitinase, indole acetic acid and phosphate solubilization but negative for Hydrogen cyanide.

In blotter experiment, Rh26(1), Rh15(2), fb-1WG and combination showed significant increase on germination percent, radical and plumule length, and vigor index relative to the positive control. It was observed that the isolates Rh26(1), Rh15(2), fb-1WG and combination showed germination percent 52.33%, 60.00% 79.69% and 79.69%, respectively. *Rhizobium* isolates were valuable for germination of Faba bean seeds.

In pot experiment, Rh26(1), Rh15(2), fb-1WG and combination were tested to evaluate their effect against Black root rot of Faba bean at three different time of application. It was observed that the isolates Rh26(1), Rh15(2), fb-1WG and combination showed maximum disease incidence reduction percent 50.00%, 75.00%, 75.00% and 100% due to before application, respectively. Maximum disease severity reduction percent 37.50%, 56.31%, 64.64%, and 73.31% was due to before application followed by at same time of pathogen inoculation with value 27.70%, 41.66%, 56.64%, and 64.86%, respectively. The isolates showed significanct diseases reduction relative to the positive control.

The result showed that there was significant interaction effect among types of isolates used and time of application on growth of Faba bean plant. Application of fb-1WG isolates to seedling before pathogen inoculation gave the highest (26.42) root nodule number followed

by at the same time of pathogen inoculation. Combination of isolates resulted in the highest (0.17g/plant) nodule dry weight when applied before followed by at the same time of pathogen inoculation. Combination of isolates produced the maximum (48.00 cm/plant) shoots height in response to before inoculation followed at the same time of pathogen inoculation. *Rhizobium* isolates enhanced the growth of Faba bean plants compared to infected control and uninoculated. Generally, isolates showed significant disease reduction and enhanced growth relative to control. Finally, controlling *Fusarium solani* root rot with combination of the isolates applied 7 days before pathogen inoculation under greenhouse condition was found to be the most promising.

Thus, it is evident from the present study that *Rhizobium* isolates Rh26(1), Rh15(2), fb-1WG and combination under investigation are capable of producing plant growth promoting substances and mediate antagonism against *Fusarium solani* through mixed type of mechanism of hydrolytic enzymes. Hence, Rh26(1), Rh15(2), fb-1WG and their combination were a potential candidate for the development of bioinoculants for *Fusarium solani* root rot of Faba bean plant.

The effective isolates like Rh26(1), Rh15(2), fb-1WG and combination against *Fusarium solani in vitro* test and *in vivo* under green house conditions should be checked under field experiment to evaluate and confirm their efficacy, potentiality and survival ability. In addition to these their antibiotic production and efficacy determination will be done further study.

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7. APPENDIX

Appendix Table 1. Variance analysis of effect of Rhizobium isolates on radial growth of *Fusarium solani in vitro* test

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	41	42290.97199	1031.48712	1000.97	<.0001
Rep.	2	1.64121	0.82061	0.80	0.4544
Error	82	84.50012	1.03049		
Corrected Total	125	42377.11332			
R^2 = 0.998006	MSD= 3.37	CV= 2.982975	Root MSE= 1.015130		

Appendix Table 2. Variance analysis of phosphate solublization index

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	27	121.2757143	4.4916931	6174.04	<.0001
Rep.	2	0.0007143	0.0003571	0.49	0.6148
Error	54	0.0392857	0.0007275		
Corrected Total	83	121.3157143			
R^2= 0.999676	MSD= 0.0863	CV=2.982975	Root MSE=2.468068		

Appendix Table 3. Variance analysis of effect of Rhizobium isolates on germination percent in Faba bean seeds under *in vitro* conditions

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	3318.448111	663.689622	176722	<.0001
Rep.	2	0.007511	0.003756	1.00	0.4019
Error	10	0.037556	0.003756		
Corrected Total	17	3318.493178			
R^2 =0.999989	MSD= 0.17	CV= 0.099661	Root MSE=0.061283		

Appendix Table 4. Variance analysis of effect of Rhizobium isolates on radicle lenght in Faba bean seeds under *in vitro* conditions

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	46.93146667	9.38629333	1163.11	<.0001
Rep.	2	0.00463333	0.00231667	0.29	0.7564
Error	10	0.08070000	0.00807000		
Corrected Total	17	47.01680000			
R^2= 0.998284	MSD= 0.25	CV= 2.958283	Root MSE= 0.089833		

Appendix Table 5. Variance analysis of effect of Rhizobium isolates on plumule lenght in Faba bean seeds under *in vitro* conditions

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	58.32997778	11.66599556	17016.8	<.0001
Rep.	2	0.00114444	0.00057222	0.83	0.4621
Error	10	0.00685556	0.00068556		
Corrected Total	17	58.33797778			
R^2 = 0.999882	MSD= 0.07	CV= 1.277918	Root MSE= 0.026183		

Appendix Table 6. Variance analysis of effect of Rhizobium isolates on vigor index in Faba bean seeds under *in vitro* conditions

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	1656861.778	331372.356	12504.9	<.0001
Rep.	2	14.777	7.388	0.28	0.7624
Error	10	264.993	26.499		
Corrected Total	17	1657141.548			
R^2 = 0.999840	MSD=14.56	CV= 1.447097	Root MSE= 5.147751		

Appendix Table 7. Variance analysis of effects of types of isolates and time of application on disease incidence (%) on Faba bean *in vivo* condition

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	4	29413.48800	7353.37200	1470.67	<.0001
Time	2	1149.18400	574.59200	114.92	<.0001
Rep.	2	10.00000	5.00000	1.0	0.3806
Treatment* Time	8	1136.73600	142.09200	28.42	<.0001
Error	28	140.00000	5.00000		
Corrected Total	44	31849.40800			
R^2 = 0.995604	MSD= 1.71	CV= 5.252274	Root MSE= 2.236068		

Appendix Table 8. Variance analysis of effects of types of isolates and time of application on disease severity (%) on Faba bean *in vivo* condition

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	4	7227.251542	1806.812886	992.63	<.0001
Time	2	420.279951	210.139976	115.45	<.0001
Rep.	2	2.178258	1.089129	0.60	0.5566
Treatment* Time	8	222.068404	27.758551	15.25	<.0001
Error	28	50.966542	1.820234		
Corrected Total	44	7922.744698			
R^2= 0.993567	MSD=0.78		CV=2.816632	Root MSE= 1.349160	

Appendix Table 9. Variance analysis of effects of types of isolates and time of application on nodule number on Faba bean *in vivo* condition

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	5321.915926	1064.383185	10102.7	<.0001
Time	2	178.844537	89.422269	848.76	<.0001
Rep.	2	0.056204	0.028102	0.27	0.7675
Treatment* Time	10	216.327685	21.632769	205.33	<.0001
Error	34	3.582130	0.105357		
Corrected Total	53	5720.726481			
R^2 = 0.999374	MSD=0.19		CV= 4.309737	Root MSE=0.324587	

Appendix Table 10. Variance analysis of effects of types of isolates and time of application on nodule fresh weight on Faba bean *in vivo* condition

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	0.16888481	0.03377696	2168.34	<.0001
Time	2	0.02355615	0.01177807	756.10	<.0001
Rep.	2	0.00000370	0.00000185	0.12	0.8883
Treatment* Time	10	0.03114963	0.00311496	199.97	<.0001
Error	34	0.00052963	0.00001558		
Corrected Total	53	0.22412393			
R^2 = 0.997637	MSD=0.0023		CV= 8.977589	Root MSE= 0.003947	

Appendix Table 11. Variance analysis of effects of types of isolates and time of application on nodule dry weight on Faba bean *in vivo* condition

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	0.08072148	0.01614430	1385.09	<.0001
Time	2	0.02324904	0.01162452	997.32	<.0001
Rep.	2	0.00000370	0.00000185	0.16	0.8537
Treatment* Time	10	0.03947185	0.00394719	338.65	<.0001
Error	34	0.00039630	0.00001166		
Corrected Total	53	0.14384237			
R^2 = 0.997245	MSD=0.002		CV= 11.87880	Root MSE =0.003414	

Appendix Table 12. Variance analysis of effects of types of isolates and time of application on shoot height on Faba bean *in vivo* condition

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	1501.832120	300.366424	184.81	<.0001
Time	2	105.026626	52.513313	32.31	<.0001
Rep.	2	0.202181	0.101091	0.06	0.9398
Treatment* Time	10	122.491463	12.249146	7.54	<.0001
Error	34	55.260419	1.625306		
Corrected Total	53	1784.812809			
R^2 = 0.969039	MSD=0.74		CV= 3.606568	Root MSE =1.274875	

Appendix Table 13. Variance analysis of effects of types of isolates and time of application on shoot fresh weight on Faba bean *in vivo* condition

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	313.0790815	62.6158163	166.83	<.0001
Time	2	61.6248148	30.8124074	82.10	<.0001
Rep.	2	1.3457370	0.6728685	1.79	0.1819
Treatment* Time	10	72.7541852	7.2754185	19.38	<.0001
Error	34	12.7609963	0.3753234		
Corrected Total	53	461.5648148			
R^2 = 0.972353	MSD=0.35		CV= 5.528286	Root MSE =0.612636	

Appendix Table 14. Variance analysis of effects of types of isolates and time of application on shoot dry weight on Faba bean *in vivo* condition

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	33.63067593	6.72613519	409.31	<.0001
Time	2	3.25471481	1.62735741	99.03	<.0001
Rep.	2	0.02001481	0.01000741	0.61	0.5497
Treatment* Time	10	2.56997407	0.25699741	15.64	<.0001
Error	34	0.55871852	0.01643290		
Corrected Total	53	40.03409815			
R^2 = 0.986044	MSD= 0.07		CV= 6.819335	Root MSE=0.128191	

Appendix Table 15. Variance analysis of effects of types of isolates and time of application on root length on Faba bean *in vivo* condition

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	851.0370370	170.2074074	192.43	<.0001
Time	2	241.3703704	120.6851852	136.44	<.0001
Rep.	2	1.9259259	0.9629630	1.09	0.3481
Treatment* Time	10	199.9629630	19.9962963	22.61	<.0001
Error	34	30.074074	0.884532		
Corrected Total	53	1324.370370			
R^2 = 0.977292	MSD=0.54		CV=3.653723	Root MSE= 0.940495	

Appendix Table 16. Variance analysis of effects of types of isolates and time of application on root fresh weight on Faba bean *in vivo* condition

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	395.6380889	79.1276178	162.01	<.0001
Time	2	114.0844444	57.0422222	116.79	<.0001
Rep.	2	0.2037000	0.1018500	0.21	0.8128
Treatment* Time	10	83.4008000	8.3400800	17.08	<.0001
Error	34	16.6057000	0.4884029		
Corrected Total	53	609.9327333			
R^2 = 0.972775	MSD= 0.40		CV= 6.462267	Root MSE=0.698858	

Appendix Table 17. Variance analysis of effects of types of isolates and time of application on root dry weight on Faba bean *in vivo* condition

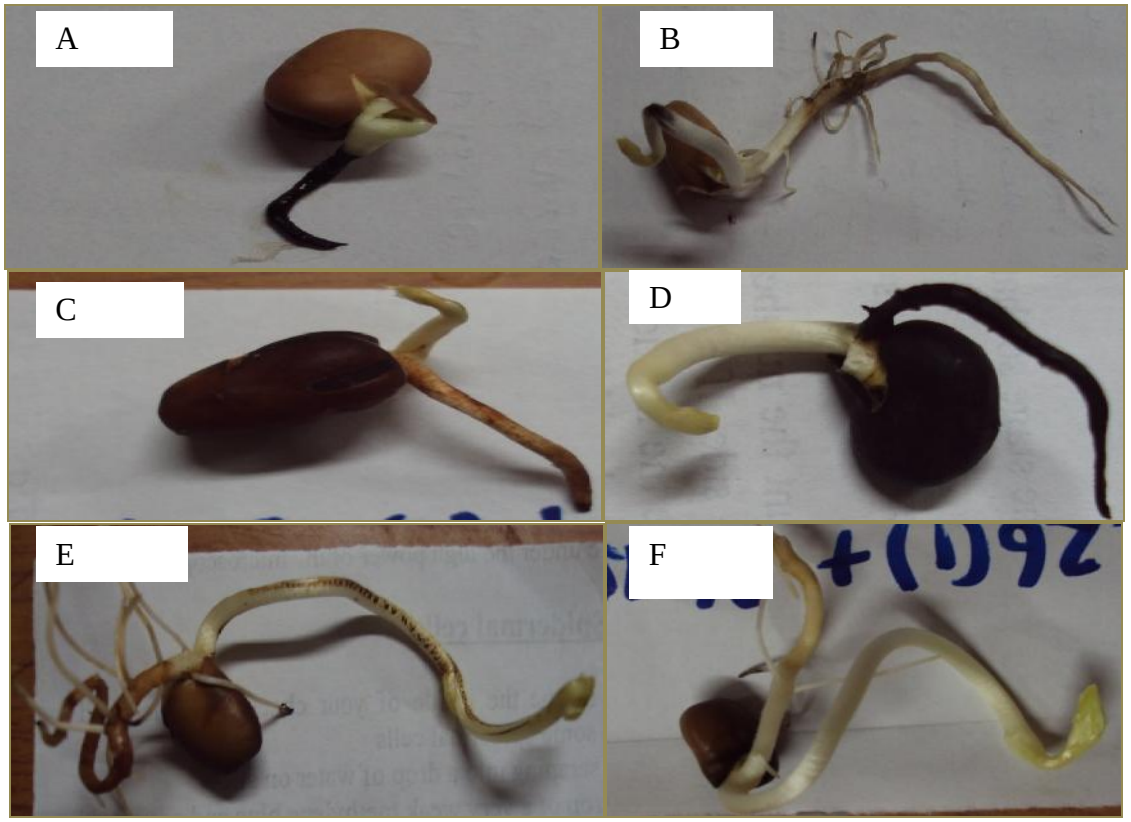
Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	10.56585370	2.11317074	1338.32	<.0001
Time	2	3.27093704	1.63546852	1035.78	<.0001
Rep.	2	0.00424815	0.00212407	1.35	0.2740
Treatment* Time	10	5.23001852	0.52300185	331.23	<.0001
Error	34	0.05368519	0.00157898		
Corrected Total	53	19.12474259			
R^2 = 0.997193	MSD= 0.02		CV=3.499856	Root MSE=0.039736	

Appendix Table 18. Variance analysis of effects of types of isolates and time of application on flower number on Faba bean *in vivo* condition

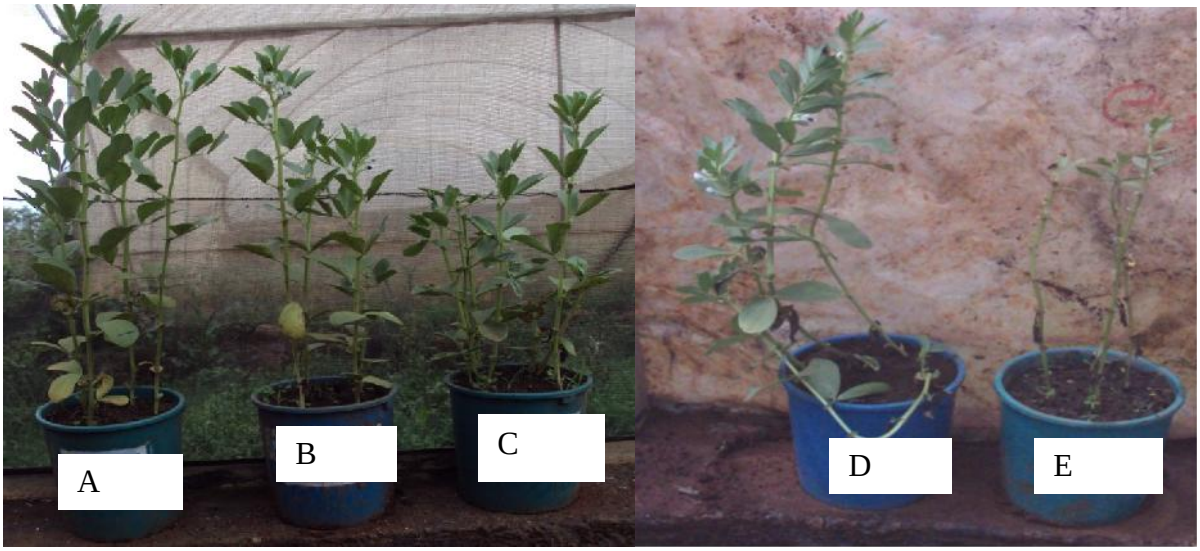
Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	1194.981481	238.996296	698.72	<.0001
Time	2	163.592593	81.796296	239.14	<.0001
Rep.	2	4.037037	2.018519	5.90	0.063
Treatment* Time	10	97.074074	9.707407	28.38	<.0001
Error	34	11.629630	0.342048		
Corrected Total	53	1471.314815			
R^2 = 0.992096	MSD=0.34		CV= 5.020958	Root MSE=0.584849	

Appendix Table 19. Variance analysis of effects of types of isolates and time of application on leaf number on Faba bean *in vivo* condition

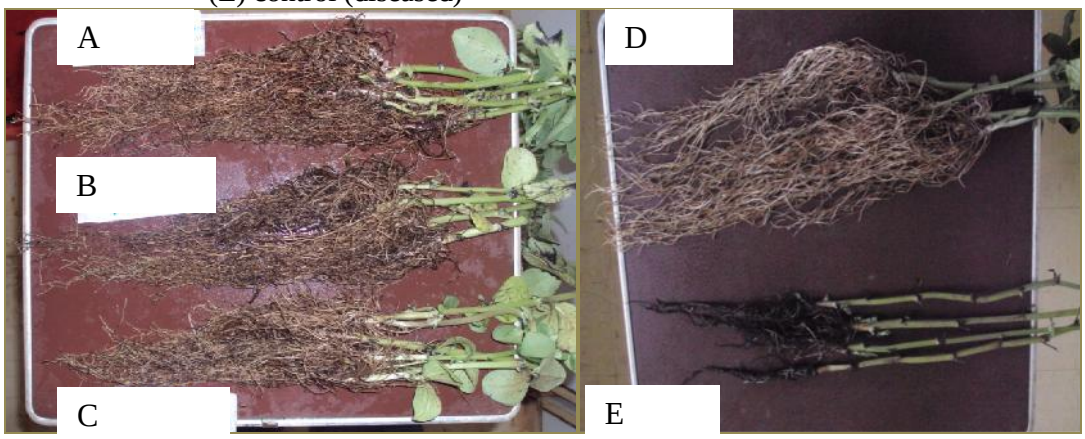
Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	5	836.9780093	167.3956019	301.79	<.0001
Time	2	114.8981481	57.4490741	103.57	<.0001
Rep.	2	0.6412037	0.3206019	0.58	0.5664
Treatment* Time	10	86.9907407	8.6990741	15.68	<.0001
Error	34	18.858796	0.554670		
Corrected Total	53	1058.366898			
R^2 = 0.982181	MSD=0.4		CV= 2.702311	Root MSE=0.744762	



Appendix fig. 1. Effect of *Rhizobium* isolates on Faba bean seed plumule and radicle growth on 10th day of incubation. (A) Control, (B) health, (C) Rh26(1), (D) Rh15(2), (E) fb-1WG and (F) Combination



Appendix fig. 2**.** Effect of different inoculation time of *Rhizobium* isolate (Combination) on plant height of Faba bean: (A) before, (B) at time (C) after, (D) Health (uniniculated) and (E) control (diseased)



Appendix fig. 3. Effect of different inoculation time of *Rhizobium* isolate (combination) on root length of Faba bean: (A) before, (B) at time, (C) after (D) Health (uniniculated) and (E) control (diseased)