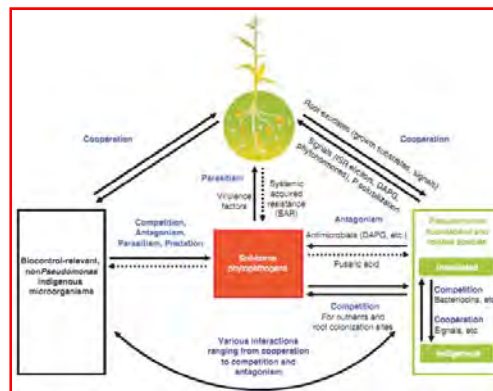


ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
COLLEGE OF NATURAL SCIENCES
DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY



ANTAGONISTIC ACTIVITIES OF *PSEUDOMONAS FLUORESCENS* ISOLATES, AS A BICONTROL OF *BOTRYTIS FABAE* (CHOCOLATE SPOT DISEASE), PLANT GROWTH PROMOTER AND INDUCER OF PHYSIOLOGICAL ACTIVITIES OF FABA BEAN (*VICIA FABAE*)



By

Fekadu Alemu

June 2012

Addis Ababa

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By

Fekadu Alemu

A THESIS SUBMITTED TO GRADUATE STADIES PROGRAM ADDIS ABABA
UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE
DEGREE OF MASTERS OF SCIENCE IN APPLIED MICROBIOLOGY

June 2012

Addis Ababa

Declaration

I, the undersigned, declared that this is my own original work, has not been presented for a degree to any other university and that all sources of materials used for the thesis have been duly acknowledge.

Fekadu Alemu

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Date: _____

The work has been done under my supervision

Advisor: Tesfaye Alemu, Ph.D

Signature: _____

Date: _____

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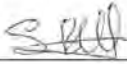


Antagonistic Activities of *Pseudomonas fluorescens* Isolates, as a Biocontrol of *Botrytis fabae* (Chocolate Spot Disease), Plant Growth Promoter and Inducer of Physiological Activities of Faba Bean (*Vicia faba*).

By

Fekadu Alemu

A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Microbiology)

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ABBREVIATIONS

CMC= Carboxymethyl cellulose

DAPG=2,4- Diacetyl phloroglucinol

dia= diameter

FDA= Faba Bean Seed Extract Dextrose Agar

g/l= gram per liter

HCN= Hydrogen Cyanide

IAA= Indole-3-acetic acid

ISR= Induced Systemic Resistance

MIC= Minimum inhibitory concentration

nm= nanometer

PDA= Potato Dextrose Agar

PGPR= Plant Growth-Promoting Rhizobacteria

PCA= Phenazine-1-carboxylic acid

PCN= Phenazine-1-carboxamide

P f1= *Pseudomonas fluorescens* isolate 1

P f2= *Pseudomonas fluorescens* isolate 2

P f3= *Pseudomonas fluorescens* isolate 3

P f4= *Pseudomonas fluorescens* isolate 4

P f5= *Pseudomonas fluorescens* isolate 5

P f6= *Pseudomonas fluorescens* isolate 6

P f7= *Pseudomonas fluorescens* isolate 7

P f8= *Pseudomonas fluorescens* isolate 8

P f9= *Pseudomonas fluorescens* isolate 9

P f10= *Pseudomonas fluorescens* isolate 10

P f11=*Pseudomonas fluorescens* isolate 11

P f12= *Pseudomonas fluorescens* isolate 12

Rpm= Rotation per Minute

SD= Standard Deviation

Spp= species

SPSS= Statistical Products and Service Solutions

UV= Ultraviolet light

ABSTRACT

Plant protection is an important area which needs attention since most of the hazardous inputs added into the agricultural system are in the form of plant protection chemicals. Production of the crop is, however, constrained by several infections including fungal diseases. The present study, *Pseudomonas fluorescens* isolates possess a variety of promising properties which make it a better biocontrol agent. Twelve *Pseudomonas fluorescens* isolates from rhizospheric soil of faba bean were evaluated for their antagonistic activity against *Botrytis fabae* that is known to attack faba bean crops. All *Pseudomonas fluorescens* isolates are employed in controlling chocolate spot diseases of plant. *P. fluorescens*10 (88.1%) showed high antagonistic activity against *Botrytis fabae*. In addition, all isolates were tested for antifungal activity against *Botrytis fabae*. All isolate of *Pseudomonas fluorescens* are indicated successfully employed in controlling chocolate spot diseases of plant due to their antifungal metabolites. The antifungal compounds were extracted from all *P. fluorescens* isolates with equal volume of ethyl acetate, hexane and methanol. The antifungal compounds extracted with ethyl acetate, hexane and methanol from P f3, P f8 and P f3 isolates at 0.1% concentration completely inhibited the pathogen growth respectively. Four fungicides were tested against *B. fabae* and Curzate ® WP and Sancozeb 80% were prevented mycelia growth at higher concentration (80mg/ml per plate). All isolates *Pseudomonas fluorescens* were assessed for their plant growth promoting activity based on their ability to produce hydrogen cyanide (HCN), siderophores, indole acetic acid (IAA), ammonia and phosphate solubilization. The results indicated that all most of the isolates tested possess plant growth promoting traits. Bio-primed faba bean seed with P f9 and P f10 for pathogenicity test in green house was indicated to show positive result. Two isolates of P f9 and P f10 was reduced both disease severity and incidence, increasing faba bean number of leaves per plant, number of branches per plant, height of plants, root length of plant and lateral roots and number of nodule. The present study was also carried out for estimation of total phenols and flavonoids present in fresh faba bean leaf extract with ethanol. Applying the P f9 and P f10 by bio-primed seed of faba bean treatment enhanced the accumulation of total phenols and flavonoids compared to untreated infected and uninfected untreated faba bean. So it could be concluded that the used P f9 and P f10 could resist the detrimental effects of *Botrytis fabae* on the plant growth and yield. These isolates can be used as potential biofertilizers and also as biocontrol agents.

Key words: Antifungal compounds, *Botrytis fabae*, Biocontrol, faba bean, *Pseudomonas fluorescens*, secondary metabolites

1. INTRODUCTION

The diversity and beneficial activity of the plant-bacterial association and its understanding is important to sustain agro-ecosystems for sustainable crop production (Germida *et al.*, 1998). The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, supports large active groups of bacteria (Villacieros *et al.*, 2003) known as plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.*, 1980a). Plant growth promoting rhizobacteria are known to rapidly colonize the rhizosphere and suppress soil borne pathogens at the root surface (Rangajaran *et al.*, 2003) and also be beneficial to the plant by stimulating growth (Bloemberg and Lugtenberg, 2001 and Moeinzadeh *et al.*, 2010). Some *Pseudomonas* spp., especially fluorescent *pseudomonads*, are particularly suitable to be used as agricultural biocontrol agents because they can produce large amounts of secondary metabolites to protect plants from phytopathogens and stimulate plant growth.

The plant growth-promoting ability of these bacteria is generated mainly by the production of indole-3-acetic acid (IAA), siderophores, and some well-known antibiotics (Nagarajkumar *et al.*, 2004), such as phenazine-1-carboxylic acid (PCA) (Mavrodi *et al.*, 1998), pyocyanin (Watson *et al.*, 1986), 2-acetamidophenol (Slininger *et al.*, 2000), pyrrolnitrin (Prn) (Arima *et al.*, 1964), pyoluteorin (Plt) (Howell and Stipanovic, 1980), phenazine-1-carboxamide (PCN) (Chin-A-Woeng *et al.*, 2003), 2,4-diacetylphloroglucinol (DAPG) (Shanahan *et al.*, 1992), viscosinamide, and tesin (Chin-A-Woeng *et al.*, 2003), hydrogen cyanide (HCN) (Voisard *et al.*, 1989), phosphate solubilize (Rodriguez and Fraga, 1999) in various species of *Pseudomonas* (Hu *et al.*, 2005 and Liu *et al.*, 2006).

Pseudomonas fluorescens is a gram-negative, rod-shaped, and non-pathogenic bacterium that is known to inhabit primarily the soil, plants, and water (Peix *et al.*, 2009). It derives its name from its ability to produce fluorescent pigments under iron-limiting conditions (Baysse *et al.*, 2003). These bacteria belong to soil microorganisms that develop one of the very important soil processes of denitrification. Biological control is a promising approach for management of plant diseases (Anderson *et al.*, 2004). Biocontrol agents of *P. fluorescens* are well characterized for their ability to produce antimicrobial compounds (Haas and De'fago, 2005).

Pseudomonas fluorescens is adapted to survival in soil and colonization of plant roots (Kiely *et al.*, 2006) and this applies also to the particular case of biocontrol agents from this species. Biocontrol strains have noticeably been observed at the root surface, (i.e. the rhizoplane) often forming microcolonies or discontinued biofilms in the grooves between epidermal cells (Couillerot *et al.*, 2009).

There is an urgent need to improve *Vicia faba* yield, since this plant remains an important part of the diet of both humans and domestic animals in many parts of the world, because of its high nutritive value in both energy and protein contents. Furthermore, faba bean supplies an important benefit to the crop by fixing atmospheric nitrogen in symbiosis with *Rhizobium leguminosarum* thus, reducing costs and minimizing impact on the environmental, that is why increasing the plant production is one of the major targets of the agricultural policy in several countries (El-Fallal and Migahad *et al.*, 2003 and Mahmoud *et al.*, 2004).

However, this crop is subjected to many abiotic and biotic stresses that seriously compromise the final yields. Among the menacing biotic stresses, chocolate spot, caused by *botrytis fabae*, is a worldwide disease capable of devastating the unprotected faba bean, result in harmful effects on growth, physiological activities and yield. Chocolate spot disease of faba bean is the most widespread and destructive disease in Ethiopia with yield reductions of up to 61% on susceptible cultivars (Dereje and Beniwal, 1987). The problem of adequately protecting plants against the fungus by using fungicides has been complicated by development of fungicidal resistance and many chemicals traditionally used to control chocolate spot disease is less effective (Harrison, 1988), giving only partial disease control, high cost of their use and/or adverse effects on growth and productivity of faba bean as well as on the accompanying microflora (Khaled *et al.*, 1995). Therefore, controlling *B. fabae* by biocontrol agents seemed to better and preferred than the chemical control (El-Fallal and Migahad *et al.*, 2003 and Mahmoud *et al.*, 2004).

2. LITERATURE REVIEW

The microbial inoculants that are used in agriculture include biofertilizers, biocontrol agents and plant growth promoting rhizobacteria. While the biofertilizer organisms make the nutrients available to plants, biocontrol agents protect the plants against the pathogenic organisms and insect pests. The term rhizosphere, proposed by Hiltner one hundred years ago, was initially defined as the soil area under the influence of plant roots (Botelho and Mendonça-Hagler, 2006). Several bacteria thrive on abundant nutrients in the rhizosphere and some of these possess antagonistic action, which safeguard plants from pathogens and stimulate growth (Gray and Smith, 2005). Successful bacterial antagonists often show a synergistic combination of mechanisms responsible for a successful antifungal interaction. *Pseudomonas fluorescens* are important antagonistic bacteria present in soil (Palleroni, 1984).

Plant growth promoting rhizobacteria (PGPR) were first defined by (Kloepper and Schroth, 1978) as the soil bacteria that colonize the roots of plants by following inoculation on to seed and that enhance plant growth. PGPRs enhance plant growth by direct and indirect means, but the specific mechanisms involved have not been well characterized (Glick, 1995 and Kloepper, 1993). Direct mechanisms of plant growth promotion by PGPR can be demonstrated in the absence of plant pathogens or other rhizosphere microorganisms, while indirect mechanisms involve the ability of PGPRs to reduce the deleterious effects of plant pathogens on crop yield. PGPRs have been reported to directly enhance plant growth by a variety of mechanisms, viz., fixation of atmospheric nitrogen that is transferred to the plants, production of siderophores that chelate iron and make it available to the plant roots, solubilization of minerals such as phosphorous and synthesis of phytohormones (Glick, 1995).

Direct enhancement of mineral uptake due to increase in specific ion fluxes at the root surface in the presence of PGPR has also been reported (Bashan and Levanony, 1991 and Bertrand *et al.*, 2000). The indirect means by which PGPRs enhance plant growth is through suppression of phytopathogens by a variety of mechanisms. These include the ability to produce siderophores that chelate iron, making it unavailable to pathogens; the ability to synthesize anti-fungal metabolites such as antibiotics, fungal cell wall lysing enzymes or hydrogen cyanide, which suppress the growth of fungal pathogens. The ability to successfully compete with pathogens for nutrients or to exclude specific niches on the root and the ability to induce systemic resistance in plants are the other mechanisms (Bloemberg and Lugtenberg, 2001, Glick, 1995 and Persello-Carticaux *et al.*, 2003).

2.1. Biocontrol of plant pathogens

The concept of biocontrol of plant diseases includes disease reduction or decrease in inoculum potential of a pathogen brought about directly or indirectly by other biological agencies (Johnson and Carl, 1972). Outside the host, the biocontrol agent may be antagonistic and thereby reduce the activity, efficiency and inoculum density of the pathogen through antibiosis, competition and predation/hyper parasitism. This leads to a reduction in inoculum potential of the pathogens (Baker, 1977).

Biological control is the strategy for reducing disease incidence or severity by direct or indirect manipulation of microorganisms (Papavizas, 1985, Paullitz and Bekanger, 2000, Tesfaye Alemu and Kapoor, 2004 and Zhang *et al.*, 1994). Biological control has attracted great interest because of increasing regulation and restriction of fungicides or unnecessary control attempts by other means. It is especially attractive for soil borne diseases because it needs critical evaluation of economics of the country and the pathogens are difficult to reach with specific fungicides (Montealegre *et al.*, 2003). The application of biological controls using antagonistic microorganisms has proved to be successful for controlling various plant diseases in many countries (Sivan, 1987).

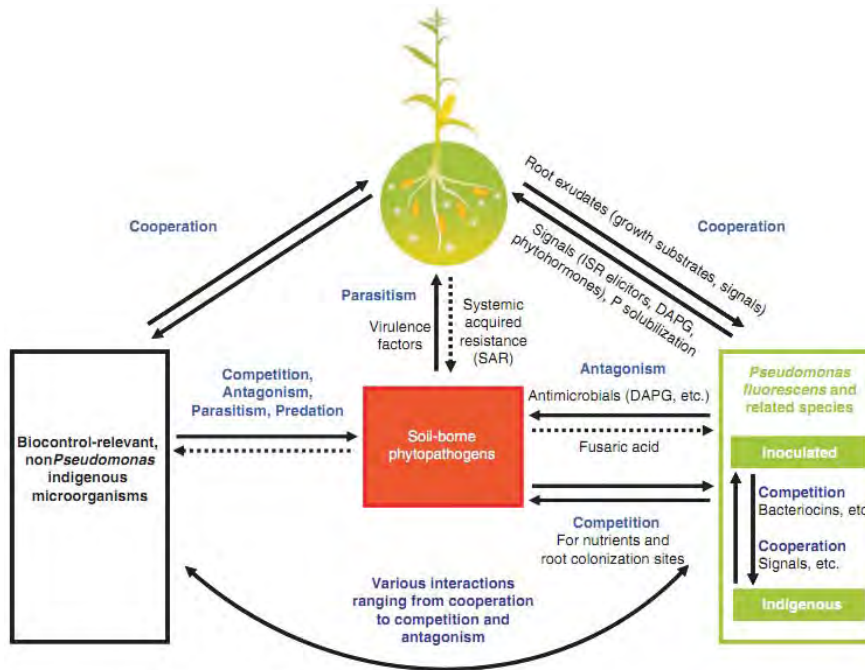
Bio-priming, a seed treatment system that integrates the biological and physiological aspects of disease control, involves coating the seed with fungal or bacterial biocontrol agents (El-Mougy and Abdel-Kader, 2008). The addition of Carboxymethyl cellulose (CMC) or pectin to bio-primed seeds enhanced the antagonists' ability to grow and survive competitively. In addition, they had no effect on seed germination (Elzein *et al.*, 2006). Seed treatment is an attractive delivery system either of fungal or bacterial bioprotectants (Wright *et al.*, 2003). Bioprotectants applied to seeds may not only protect seeds (Sivan and Chet, 1986) but also may colonise and protect roots (Chao *et al.*, 1986) and may increase plant growth (Chang *et al.*, 1986). A successful antagonist should colonise the rhizosphere during seed germination (Weller, 1983).

2.2. *Pseudomonas fluorescens* as biocontrol agents

2.2.1. Inhibition of fungal pathogens by *Pseudomonas fluorescens*

A strain of *Pseudomonas fluorescens* showed antagonistic property against *Rhizoctonia solani* (Howell and Stipanovic, 1979). A number of *Pseudomonas fluorescens* strains isolated from the rhizosphere of potato plants were reported to be antagonistic to *Rhizoctonia solani* in vitro and effectively reduced stem canker under laboratory conditions (Chand and Logan, 1984). Several strains of siderophore producing *P. fluorescens* have been shown to inhibit *Fusarium oxysporum f.sp.cubense*, *Fusarium oxysporum f.sp. vasinfectum*, *Rhizoctonia solani*, *Acrocyndrium oryzae*, *Xanthomonas campestris pv oryzae* and *P. syringiae pv phaseolicola* (Sakthivel *et al.*, 1986).

P. fluorescens isolated from rhizosphere of rape seedlings has been reported to inhibit *Fusarium roseum* and *Pythium ultimum* (Dahiya *et al.*, 1988). Bare root dip treatment or soil drench with *Pseudomonas fluorescens* CHA0 significantly suppressed *Rhizoctonia solani* of tomato (Siddiqui and Shaukat, 2002). *Pseudomonas fluorescens* strain A 506 is a commercially available biological control agent (Available in the name of blight Ban 506; N. farm Americas Inco., Sugar Land, TX) used for the suppression of fire blight on pear and apple trees (Temple *et al.*, 2004).



Overview of plant-protection mechanisms in biocontrol agents from *Pseudomonas fluorescens* and closely-related species of fluorescent *Pseudomonas*. These pseudomonads may act directly on the plant, noticeably via production of various signals (2, 4-diacetylphloroglucinol (DAPG), phytohormones, etc.) and / or by triggering induced systemic resistance (ISR) pathways, and the plant provides them with organic exudates and molecular signals. They may also inhibit the phytopathogens by competition and/or antagonism mediated by secondary metabolites such as DAPG. In addition, these effects are modulated by the action of certain non *Pseudomonas* members of the microbial community, which may also have direct or indirect (i.e. via the plant) biocontrol effects and/or interfere with the functioning of biocontrol agents from *Pseudomonas fluorescens* and related species. As for *Pseudomonas* inoculants, their ecology and plant-beneficial properties can be influenced positively (via signalling and cooperation) or negatively (via competition) by indigenous root-colonizing *pseudomonads*. Dashed lines are used to indicate possible feedback responses of partners subjected to negative interactions, noticeably inhibition of DAPG production in *Pseudomonas* by fusaric acid from *Fusarium oxysporum* phytopathogens, and systemic acquired resistance in plant in response to infection.

Source: (Couillerot *et al.*, 2009)

2.2.2. Inhibition of bacterial pathogens by *Pseudomonas fluorescens*

Significant reduction in bacterial wilt disease incidence in tomato was obtained with *Pseudomonas fluorescens* (Pradeepkumar and Sood, 2001). The culture liquid of *Pseudomonas fluorescens* 41 in a dilution of 1:9 is reported to control major cotton diseases caused by *Xanthomonas campestris* pv *malvaceum*, *Rhizoctonia solani*, *Fusarium vasectum* and *Verticillium dahliae*, and also had the stimulating effect on seedling emergence and early growth and yield of cotton (Safiyazou *et al.*, 1995). *Pseudomonas fluorescens* pv *fcp* reduced the incidence of *P. solanacearum* by 50 per cent in banana, 49 per cent in brinjal and 36 per cent in tomato (Anuratha and Gananamanickam, 1990).

2.2.3. Inhibitory activity of *Pseudomonas fluorescens* against phytopathogenic nematodes

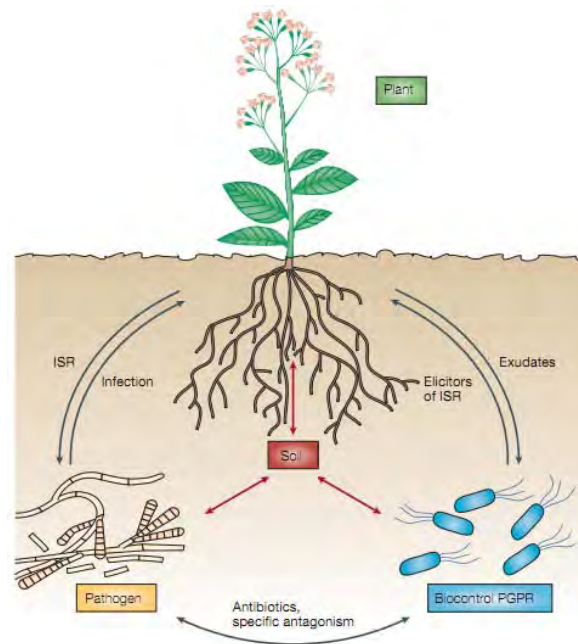
The production of antibiotics like 2, 4-diacetylchloroglucinol by *Pseudomonas fluorescens* which reduce juvenile mobility has been reported by (Cronin *et al.*, 1997). The culture filtrate of *P. fluorescens* strain *PF1* has also been reported to be toxic to *Rotylenchulus reniformis* at all tested concentrations (Jayakumar *et al.*, 2002). Nematode suppressive potential of two high phosphate solubilizing *Pseudomonas fluorescens* strains, *SCEP3* and *SCEP5* was quantified using mortality of second stage juveniles and hatching inhibition in bacterial culture filtrate. Both the strains showed mortality of juveniles and inhibited egg hatching significantly over control (Somasekhar *et al.*, 2003). Jayakumar *et al.*, (2003) evaluated *Pseudomonas fluorescens* isolates of cotton rhizosphere against reniform nematode in cotton and observed significant reduction in soil and root population of all *Rotylenchulus reniformis* and subsequent increase in plant growth parameters in plants inoculated with *Pseudomonas fluorescens*.

A significant increase in plant growth and reduction in root knot galls was observed due to *Pseudomonas fluorescens* in okra plants (Devi and Dutta, 2002). (Sreenivasan and Lakshmanan, 2003) also reported significant reduction in the population of rice root nematode *Hirschmanniella gracilis* in rice plants inoculated with *Pseudomonas fluorescens* isolates of rice rhizosphere. (Jothi *et al.*, 2003) reported tomato plants to give maximum yield (64.3%) and minimum soil population of root knot nematode (*Meloidogyne incognita*) in plants treated with *P. fluorescens*. Similar effect of *P. fluorescens* in tomato and brinjal nurseries against *M. incognita* has been reported by (Santhi and Sivakumar, 1995). (Shanthi *et al.*, 2003) found soil application of *Pseudomonas fluorescens* effective in reducing soil population and root population of lesion nematodes viz., *Radopholus similis*, *Pratylenchus coffeae* and *Helicotylenchus multicinctus*.

2.3. Mechanisms of biocontrol and their production of plant growth promoting substances (PGPS)

Interaction of soil microorganisms and plant in the rhizosphere can be beneficial for plant growth through their production of plant hormones like IAA, fixation of nitrogen and Phosphate solubilization (Bhadbhade *et al.*, 2002).

Biocontrol agents exert a protective effect on the roots through antagonism against phytopathogenic fungi and bacteria (Dwivedi and Johri, 2003). Fluorescent pseudomonads exhibit diverse mechanisms of biocontrol which include antibiosis, HCN production, siderophore production, competition for space and nutrients and induced systemic resistance (Shivakumar, 2007).



Interactions between biocontrol plant growth-promoting rhizobacteria (PGPR), plants, pathogens and soil. These elements interact with one another through biotic and abiotic signals, many of which are still unknown. ISR, induced systemic resistance.

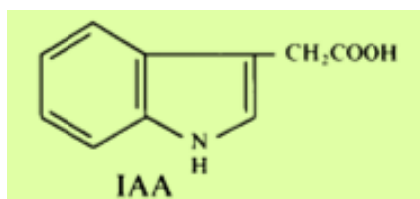
Source: (Weller *et al.*, 2002)

2.3.1. Indole-3-acetic acid (IAA)

IAA is an important phytohormone naturally occurring auxin with broad physiological effects on plants (Davies, 2010). This hormone is commonly produced by PGPR (Barazani and Friedman, 1999). Many plant growth promoting rhizobacteria (PGPR), including *Azospirillum*, *Azotobacter*, *Bacillus*, *Pseudomonas* and *Rhizobium* produce IAA (Dubeikovsky *et al.*, 1993 and Taghavi *et al.*, 2009).

Auxins have been implicated in signaling between microorganism and plants (Spaepen *et al.*, 2007) leading to stimulation of cell division, initiation of lateral and adventitious roots (Malamy and Benfry, 1997), cell enlargement (Salisbury, 1994), phototropism, geotropisms and apical dominance (Ahemad and Khan, 2010b), increase rate of seedling emergence (De Freitas and Germide, 1990) and results into elongation of stems and roots (Yang *et al.*, 1993). It plays a major role in xylem and root formation (Davies, 1995). The stimulation of growth of roots results in enhances uptakes of nutrients by the association plants (Lifshitz *et al.*, 1987). Therefore, promotion of plants growth after inoculation with rhizobacteria has been attributed to IAA production in *Azospirillum brasilense* (Okon and vanderleyden, 1997), *Rhizobium* species (Hirsch and Fang, 1994) and in *Pseudomonas* (Hirsch and Fang, 1994).

Most commonly, IAA producing PGPR strains are believed to increase root growth and length resulting in greater root surface area which enables plants to access more nutrients from soil (Gupta *et al.*, 2002). (Patten and Glick, 1996) demonstrated that bacterial IAA from *P. putida* played a major role in the development of host plant root system. Similarly, IAA production in *P. fluorescens HP72* correlated with suppression of creeping bent grass brown patch (Suzuki *et al.*, 2003).



Source: (Oberha'nsli *et al.*, 1991)

2.3.2. Phosphate solubilization

Phosphorus contributes to the biomass construction of micronutrients, the metabolic process of energy transfer, signal transduction, macromolecular biosynthesis, photosynthesis, and respiration chain reactions (Shenoy and Kalagudi, 2005). Phosphorus is generally deficient in most natural soils, because it is fixed as water-insoluble iron and aluminum phosphates in acidic soils or calcium phosphate in alkaline soils (Singh and Kapoor, 1994).

Involvement of microorganisms in the solubilization of insoluble phosphate was first shown by (Stalstorm, 1903). A large number of heterotrophic and autotrophic microorganisms including bacteria (*Pseudomonas*, *Bacillus*, *Enterobacter*, *Rhizobium*) (Rodríguez and Fraga, 1999), fungi (*Aspergillus* and *Penicillium*) (Illmer and Schinner, 1992, Seshadri *et al.*, 2004 and Wakelin *et al.*, 2004) and cyanobacteria (*Phormidium*) (Mazhar and Hasnain, 2011) have been mechanism of phosphate solubilization studied the for their ability to solubilize hydroxyapatite, tricalcium phosphate, and rock phosphate due to the production of organic acids such as citric, glutamic, lactic, oxalic, malic, fumaric, tartaric, propionic, glycolic and succinic acid (Vazquez *et al.*, 2000 and Bhattacharya *et al.*, 1986), which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate (Kpombrekou and Tabatabai, 1994) by acidification, exchange reactions, and polymeric substances formation to soluble forms HPO_4^{2-} and H_2PO_4^- (Delvasto *et al.*, 2006).

Phosphate solubilize bacteria are important components of soil and directly or indirectly influence the soil's health through their useful activities (Panhwar *et al.*, 2012). Phosphorus is known to improve root growth and nodulation of legumes thereby improve the N content of plants through nitrogen fixation (Pal *et al.*, 2003). Rhizospheric bacteria promote the plant growth by different mechanisms. One of the important mechanisms is the solubilization of mineral phosphates in the rhizosphere and provides soluble P to plants (Zaidi *et al.*, 2009). Phosphate solubilizing bacteria are potential to increase available P for plant, especially in soils with large amounts of precipitated phosphate (Goldstein, 1986). The amount of soluble P in soil is generally very low, normally at levels of mg kg^{-1} or less (Goldstein, 1994). Phosphate solubilizing bacteria play a crucial role in making available solubilized fraction of various phosphate minerals in soils to growing plants.

Application of the phosphate-solubilizing microbes *Agrobacterium*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Aspergillus*, *Trichoderma* and *Glomus* around the roots of plants, in soils and in fertilizers has been shown to release soluble phosphorus, promote plant growth, and protect plants from pathogen infection (Biswas and Narayanasamy, 2006, Ouahmane *et al.*, 2007 Rodríguez and Fraga, 1999, Rudresh *et al.*, 2005 and Zayed and Abdel-Motaal, 2005a, b). Biofertilizers such as microbial inoculants promote plant growth, productivity and increase the nutrient status of the host plant have internationally been accepted as an alternative source of chemical fertilizers (Vessey, 2003).

2.3.3. Ammonia production by *Pseudomonas* species

Nitrogen is one of the most common nutrients required for plant growth and productivity, as it forms an integral part of proteins, nucleic acids and other essential biomolecules (Bockman *et al.*, 1997). About 80 per cent of nitrogen is present in the atmosphere but is unavailable to plants. It needs to be converted into ammonia, a form available to plants and other eukaryotes (Jagadish, 2006). Ammonia production by the PGPB helps influence plant growth indirectly (Ramyasmruthi *et al.*, 2012).

2.3.4. Siderophore mediated biocontrol

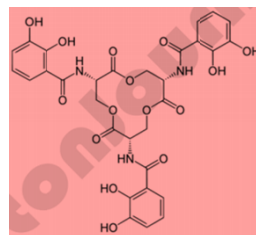
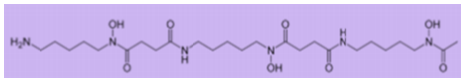
A Siderophore (Greek for iron carrier) is a low molecular weight (500-1000daltons), high affinity ferric iron-chelating compound secreted by organisms (Pal and Gokarn, 2010). Turfnejer (1942) proposed the term 'pyoverdine' for the yellow-green, fluorescent, water-soluble pigment of *P. fluorescens*. *Pseudomonas fluorescens* is one of the fluorescent pseudomonads that secrete pyoverdins (Meyer, 2000) for its essential requirement for iron. Pyoverdin is a yellow-greenish fluorescent siderophore involved in high affinity transport of iron into the cell (Budzikiewicz, 1992). Iron is the most important micronutrient used by bacteria and is essential for their metabolism, being required as a cofactor for a large number of enzymes and iron-containing proteins (Leong and Expert, 1990 and Neilands, 1974).

In the aerobic environment, iron occurs principally as Fe^{3+} and is likely to form insoluble hydroxides and oxyhydroxides, thus making it generally inaccessible to microorganisms. To acquire sufficient iron, the most commonly found strategy in bacteria is the secretion of siderophores, low-molecular weight compounds with high affinity for Fe^{3+} with association constants for complexing iron (Neilands, 1981), which are produced under limiting concentrations of iron. These compounds are able to transport this element inside the iron starved cells for metabolic functions (Press *et al.*, 2001).

Thus, siderophores act as solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation (Indiragandhi *et al.*, 2008). Some PGPR strains produce siderophores that bind Fe^{3+} , making it less available to certain members of native microflora (Kloepper *et al.*, 1980b). Siderophores chelates iron and other metals contribute to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitats (Hofte *et al.*, 1992, Loper and Henkels, 1997). (Duhme *et al.*, 1998) have also demonstrated that catecholates siderophores have been suggested to participate in molybdenum acquisition. Siderophore-producing bacteria promote plant growth indirectly by sequestering the limited iron in the rhizosphere, especially in neutral and alkaline soils, and thereby reduce its availability for the growth of pathogen (Alexander and Zuberer, 1991 and Subba Rao, 1999).

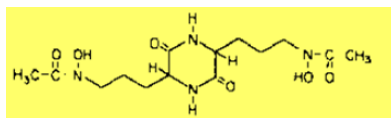
Siderophores are usually classified by the ligands used to chelate the ferric iron. The major groups of siderophores include the catecholates (phenolates), hydroxamates and carboxylates (Saharan and Nehra, 2011). Partial determination of its structure has shown that an unusual amino acid, 6-N-hydroxyornithine, is present in a cyclic peptide chain (Meyer, 1977). This amino acid is also a constituent of several hydroxamate iron-binding compounds: ferrichrome A (Zalkin *et al.*, 1966), rhodotorulic acid (Atkin and Neilands, 1968), coprogen (Keller-Schierlein and Diekmann, 1970), ferribactin (Maurer *et al.*, 1968). These siderophores facilitate iron transport into microorganisms (Neilands, 1974).

(Suryakala *et al.*, 2004) have reported that siderophores exerted maximum impact on *Fusarium oxysporum* than on *Alternaria sp.* and *Colletotrichum capsici*. (Kurek and Jaroszk-Scire, 2003) reported that two *P. fluorescens* strains (resistant to streptomycin and kanamycin) produced Fe^{3+} chelating compounds (including siderophores) and inhibited the in vitro growth of *F. culmorum* (cycoheximide resistant strain) strain by competition for Fe^{3+} . (Hultberg *et al.*, 2000) identified production of pseudobactin/pyoverdine type siderophores by *Pseudomonas fluorescens 5.014*.

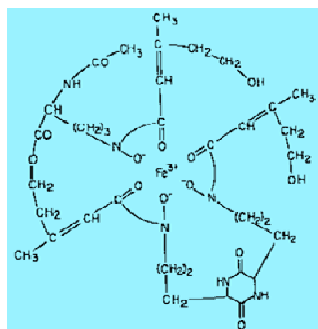


Desferrioxamine B a hydroxamate siderophore. Enterobactin, a catecholate siderophore.

Source: (Saharan and Nehra , 2011).

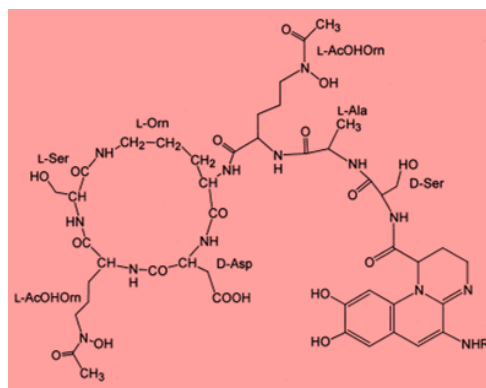


rhodotorulic acid



coprogen

Source: (Neilands, 1981)



Pyoverdins

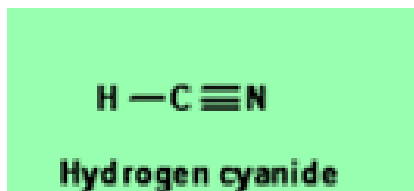
Sources: (Ferna'ndeza *et al.*, 2003)

2.3.5. Hydrogen Cyanide production (HCN)

HCN, a volatile metabolite is thought to play a major role in biological control of some soil borne diseases (Siddiqui *et al.*, 2006). HCN inhibits the electron transport thereby energy supply to the cells is disrupted leading to the death of the organism. It affects the proper functioning of the enzymes and natural receptors by reversible mechanisms of inhibition (Corbett, 1974). It is also known to inhibit the action of cytochrome oxidase (Gehring *et al.*, 1993).

HCN production by *P.fluorescens*, *P.aeruginosa*, *Chromobacterium violaceum* and *Rhizobium* isolates was reported by many researchers (Siddiqui *et al.*, 2003 and Antoun *et al.*, 1998). *P. fluorescens* CHA0 is an aerobic, root-colonizing biocontrol bacterium that protects several plants from root diseases caused by soil borne fungi (Voisard *et al.*, 1989 and Voisard *et al.*, 1994). Cyanide producing strain CHA0 stimulated root hair formation, indicating that the strain induced altered plant physiological activities. Exposing plants to the volatile metabolites of antagonist causes a significant increase in peroxides activity, which may contribute to induction of disease resistance (Wangi *et al.*, 2002). (Keel *et al.*, 1989 and Sacherer *et al.*, 1994) developed a disease assay for *Thielaviopsis basicola* on tobacco using Fe rich clays, which were conducive to biocontrol by strain CHA0.

The release of HCN by rhizospheric bacteria into the soil can be toxic to subterranean animals and phytopathogenic organisms (Guo *et al.*, 2007). Microbial production of HCN has been reported as an important antifungal trait to control root infecting fungi (Ramette *et al.*, 2003). Production of HCN by *Pseudomonads* is associated with biological control of the black root rot of tobacco, but other workers observed that it can have a detrimental effect on plant growth (O'Sullivan and O'Gara, 1992).



2.3.6. Antibiotics of PGPR

Antibiotics are chemically heterogeneous group of organic, low molecular weight compounds produced by microorganisms (Raaijmakers *et al.*, 2002) which at low concentrations result in harmful effects to other microorganisms (Fravel, 2005 and Pal and McSpadden Gardener, 2006).

Antibiosis has been postulated to play an important role in disease suppression by rhizobacteria (Gutterson *et al.*, 1986). Utilization of microbial antagonists against plant pathogens in agricultural crops has been proposed as an alternate to chemical pesticides. Fluorescent *Pseudomonads* species play an active role in suppression of pathogenic microorganisms. These bacterial antagonists enforce suppression of plant pathogens by the secretion of extracellular metabolites that are inhibitory at low concentration (Fernando *et al.*, 2005).

Antibiotics produced by *Pseudomonas* species include 2,4 Diacetyl phloroglucinol (Shanahan *et al.*, 1992b), Phenazine-1-carboxylic acid (Pierson and Pierson, 1996), Phenazine-1-carboxamide (Chin-A-Woeng *et al.*, 1998), Pyoluteorin (Howell and Stipanovic, 1980), Pyrrolnitrin (Thomashow and Weller, 1988), oomycinA (Kim *et al.*, 2000) and viscosinamide (Hokeberg *et al.*, 1998 and Nielsen *et al.*, 1999).

2.3.6.1. Diacetyl phloroglucinol (DAPG)

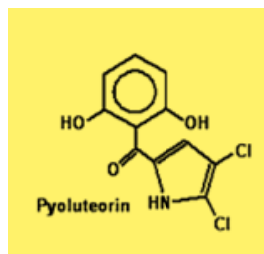
The ubiquitous distribution of fluorescent *Pseudomonads* in the rhizosphere of crop plants has broad spectrum of action in the suppression of fungi, bacteria and nematodes (Haas and Keel, 2003 and Keel *et al.*, 1992). Though several mechanisms are in operation to suppress plant pathogens, the antibiotics produced by fluorescent *Pseudomonads* remain as a crucial factor in checking disease development and pathogens. Among the various extracellular metabolites produced, DAPG is of prime importance in plant protection. Population density of DAPG producers and the antibiotic production was responsible for disease suppression in different soils (Raaijmakers *et al.*, 1999).



Source: (Haas, and Keel, 2003)

2.3.6.2. Pyoluteorin

Pyoluteorin (Plt) is a phenolic polyketide with resorcinol ring. It was first isolated from *P. aeruginosa* (Takeda, 1958) followed by *P. fluorescens Pf-5* and *CHAO* (Bencini *et al.*, 1983 and Bender *et al.*, 1999). Plt has bactericidal, herbicidal and fungicidal properties. Application of Plt to cotton seeds suppressed cotton damping-off (Howell and Stipanovic, 1980).

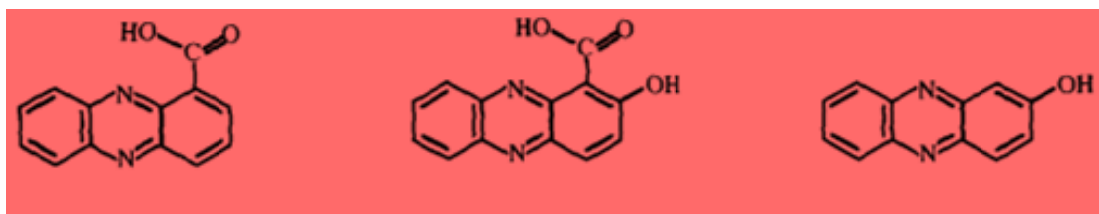


Source: (Raaijmakers *et al.*, 2002)

2.3.6.3. Phenazine

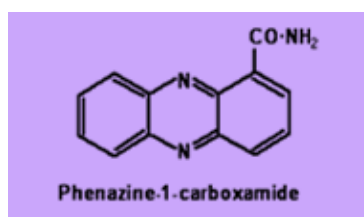
Phenazine is a low molecular weight secondary metabolite, nitrogen containing heterocyclic antimicrobial compound consisting of brightly coloured pigment produced by the *Pseudomonas*, *Burkholderia*, *Brevibacterium* and *Streptomyces* (Anjaiah *et al.*, 1998, Becker *et al.*, 1990, Gealy *et al.*, 1996, Tambong and Hofte, 2001, Thomashow *et al.*, 1990 and Turner and Messenger, 1986). Commonly identified derivatives of phenazine produced by *Pseudomonas* spp. are pyocyanin, phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN) and hydroxy phenazines (Turner and Messenger, 1986).

Both PCA and PCN are produced by *P. fluorescens* 2-79 (Thomashow and Weller, 1988), *P. aureofaciens* 30-84 (Pierson *et al.*, 1995) and *P. chlororaphis* (PCL1391) (Chin A- Woeng *et al.*, 1998). *Pseudomonas chlororaphis* strain PA-23 was effective in controlling Sclerotinia stem rot of canola in greenhouse and field. Priming the seeds with *P. chlororaphis* effectively controlled seed borne diseases of barley and oats (Fernando *et al.*, 2005).



Phenazine I-carboxylate 2-hydroxyphenazine I-carboxylate 2-hydroxyphenazine

Sources: (Handelsman and Stabb, 1996)



Sources: (Haas and Keel, 2003)

2.3.6.4. Pyrrolnitrin

Pyrrolnitrin (PRN) is a chlorinated phenylpyrrole antibiotic produced by several fluorescent and non-fluorescent *Pseudomonads*. It was first isolated from *Burkholderia pyrrocinia* (Arima *et al.*, 1964). *Pseudomonads* species such as *P. fluorescens*, *P. chlororaphis*, *P. aureofaciens*, *B. cepacia*, *Enterobacter agglomerans*, *Myxococcus fulvus* and *Serratia* sp also produce PRN antibiotics (Hammer *et al.*, 1999).

With respect to plant pathogenic fungi, pyrrolnitrin has shown activity against a wide range of *Basidiomycetes*, *Deuteromycetes*, and *Ascomycetes*, including several economically important pathogens like *Rhizoctonia solani*, *Botrytis cinerea*, *Verticillium dahliae*, and *Sclerotinia sclerotiorum* (Ligon *et al.*, 2000). PRN was developed as an agricultural fungicide (Elander *et al.*, 1968). PRN persists actively in the soil for one month and it does not readily diffuse. But it is released after lysis of host bacterial cell, resulting in the slow release. PRN is effective against the post harvest diseases of apple, pear and cut flowers caused by *Botrytis cinerea* (Hammer and Evensen, 1993 and Janisiewicz and Roitman, 1988). It also has strong antifungal action against *R. solani* (El-Banna and Winkelmann, 1988). *P. fluorescens* strains producing PRN reduced take all decline of wheat (Tazawa *et al.*, 2000). *P. chlororaphis* strain PA-23 was effective in controlling *Sclerotinia* stem rot disease of canola in the greenhouse and field (Fernando *et al.*, 2005).



Source: (Raaijmakers *et al.*, 2002)

2.3.7. Competition for infection sites and nutrients

Weller (1985) was of the opinion that *Pseudomonads* catabolize diverse nutrients and have a fast generation time in the root zone. Hence, they are projected as logical candidates for biocontrol by competition for nutrients, more so against slow growing pathogenic fungi. (Mohamad and Caunter, 1995) observed *Pseudomonas fluorescens* to inhibit *Bipolaris maydis* both in vitro and in vivo in infected maize plants but could not detect any inhibitory substances, assayed by a variety of methods, indicating nutrient competition as the operative component of antagonism.

2.3.8. Induced systemic resistance

Induced systemic resistance is broadly defined as activation of latent defense mechanisms in plants prior to pathogenic attack. The mechanism has been hypothesized in recent years to be an operable mechanism in several rhizobacterial systems. Induced resistance is a state of enhanced defensive capacity developed in plants when appropriately stimulated (Kuc, 2001). The bacterial factors involved in ISR induction comprise antibiotics, flagella, lipopolysaccharides, siderophores and salicylic acid (Bakker, 2007 and van Loon *et al.*, 1998).

Induced systemic resistance is associated with increased synthesis of certain enzymes in plant root such as peroxidase, chitinases and phenylalanine ammonia lyase (Lagrimini and Ruthstein, 1987, Nicholson and Hammerschmidt, 1992 and Wojtaszek, 1997), increased levels of certain acid soluble proteins (Zdor and Anderson, 1992), enhanced phytoalexin production (Marley and Hillocks, 1993) and the accumulation of phytoalexins in the induced plant tissue (Vanpeer *et al.*, 1991), strengthening of epidermal and cortical cell walls and deposition of newly formed barriers beyond infection sites including callose, lignin and phenolics (Yedida *et al.*, 1999) and enhanced expression of stress-related genes (Zhang *et al.*, 2002).

The seed bacterization of common bean with *P. fluorescens* S97 suppressed the halo-blight caused by *P. syringe* *pv phaseolicola* through induced systemic resistance mechanism (Alstrom, 1991). *P. fluorescens* induced systemic resistance against *Rhizoctonia solani* causing sheath blight in rice with a twofold increased activity of pathogenesis related peroxidase and chitinase proteins (Nandakumar *et al.*, 1998).

ISR due to *P. fluorescens* has been demonstrated in several species including carnation (Van Peer *et al.*, 1991), radish (Leeman *et al.*, 1995), arabidopsis (Pieterse *et al.*, 1996), cucumber (Wei *et al.*, 1991) and tobacco (Maurhofer *et al.*, 1995).

2.4. Faba bean

Faba bean (*Vicia fabae* L.) is one of the most important food legumes due to its high nutritive value both in terms of energy and protein contents (24-30 %) and is an excellent nitrogen fixer (Sahile *et al.*, 2008a). Ethiopia is the third largest producers of faba bean in the world, next to china and Egypt (Torres *et al.*, 2006) and its share is only 6.96% of world production and 40.5% of Africa (Asfaw *et al.*, 1994). Faba bean is grown on 370,000 hectares in Ethiopia with an annual production of about 450,000 tonnes (ICARDA, 2006). Despite its wide cultivation, the average yield of faba bean is quite low in Ethiopia, because of many biotic and abiotic constraints (Sahile *et al.*, 2008a).

Common names : Ackerbohne (Germany, Austria), Bob obecny (Czech Republic), Broad bean, Faba bean, Field bean, Horse bean, (United Kingdom), Favetta (Italy), Féverole (France), Haba (Spain), Hestebønne (Denmark), Põlduba (Estonia).



Vicia faba (Photos by S. Loss)

2.4.1. Importance of faba bean

In many parts of Ethiopia broad beans are a daily part of the diet of the population. They are an important source of dietary protein, especially valuable during the numerous days of fasting that are observed (Asfaw, 1979) and also providing minerals (iron, zinc, calcium) and vitamins (B1, B2, C) in human diet and livestock rations and a source of biological nitrogen fixation in cereal rotation systems (Khalil and Erskine, 2001). Broad beans may be consumed green, either raw, roasted, or boiled; as dry seed, having been soaked, roasted, or boiled; in a preparation with a hot sauce called "wot"; ground and mixed with barley, wheat, or teff flour to form "injera" (a kind of pancake-type bread); or used in the preparation of various sauces (e.g., in a mixture with mustard and spices that is fermented for 4-5 days) (Asfaw, 1979). Therefore, increasing the crop production is one of the most important targets of agricultural policy in several countries.

2.4.2. Host pathogen interactions

2.4.2.1. Host defence compounds

2.4.2.1.1. Phytoanticipins and Phytoalexins

To arrest the spread of pathogens, plants possess an innate immunity that involves different layers of defence responses. Some of these defences are preformed and others are activated after recognition of pathogen elicitors, and include reinforcement of the cell wall, biosynthesis of lytic enzymes and production of secondary metabolites and pathogenesis related proteins (Lamothe *et al.*, 2009).

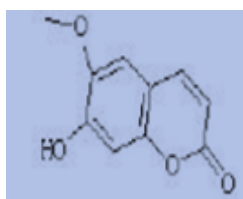
One of the most extensively studied faba bean (*Vicia faba* L.) tissues produce defence responses to pathogen infection is the induced accumulation of secondary metabolites such as furanoacetylenic phytoalexins (Fawcett *et al.*, 1971, Hammerschmidt, 1999, Hargreaves *et al.*, 1977 and Ingham, 1982) and chemical barriers to microbial attack (phytoanticipins). Phytoalexins are a diverse group of low molecular weight anti-microbial compounds that are synthesized and accumulated in appreciable amounts in plants after stimulation by various types of pathogens, and are toxic to pathogens (Mansfield, 2000 and Smith, 1996). Two of the most important of furanoacetylenic phytoalexins are wyerone acid and its methyl ester wyerone, and their induced accumulation in infected tissues causes inhibition of fungal growth (Hargreaves *et al.*, 1977, Letcher *et al.*, 1970, Mansfield and Deverall, 1974b, and Rossall *et al.*, 1980).

Wyerone acid is one of the major furanoacetylenic phytoalexins from broad bean produced in response to infection by *Botrytis fabae* Sard., the causal organism of chocolate spot disease (Letcher *et al.*, 1970 and Soylu *et al.*, 2002). Wyerone is subsequently converted by *B. fabae* to wyerol in broad bean cotyledons (Hargreaves *et al.*, 1976) and to a more anti-fungal wyerone acid in broad bean leaves and pods (Fawcett *et al.*, 1969).

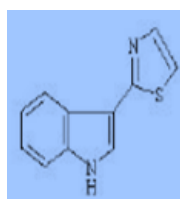
Accumulation of wyerone acid phytoalexin in broad bean leaves infected with *B. fabae* may play an important role in the active defence of broad beans against chocolate spot disease (Fawcett *et al.*, 1971, Mansfield *et al.*, 1980 and Tarrad *et al.*, 1993).

Phytoanticipin are low molecular weight antimicrobial compounds that are present in plants before challenge by microorganisms or are produced after infection solely from preexisting constituents. Some phytoanticipins are found at the plant surface. Others are sequestered as preformed compounds in vacuoles or organelles and released through a hydrolyzing enzyme after pathogen challenge. Because the enzyme involved in the final liberation of the molecule is not formed *de novo* these compounds are not considered as phytoalexins (Lamothe *et al.*, 2009).

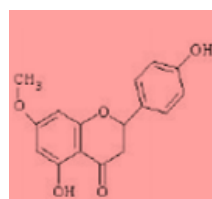
The greater ability of *B. fabae* to colonise faba bean tissues seems to be related to its capacity to detoxify faba bean phytoalexins and to reduce their toxic effects (Hargreaves and Mansfield, 1975, Hargreaves *et al.*, 1977, Madeira *et al.*, 1993, Mansfield and Deverall, 1974b, Rossall *et al.*, 1980, Rossall and Mansfield, 1984 and). Antimicrobial phytoalexin structures:



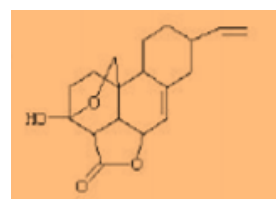
Scopoletin



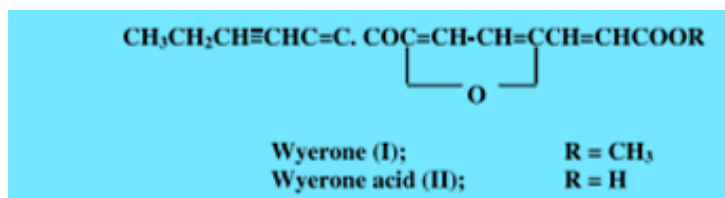
Camalexin



Sakuranetin



Momilactone



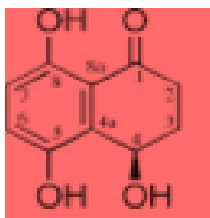
Chemical structures of furanoacetylenic wyerone and wyerone acid phytoalexins

Source: (Nawar and Kuti, 2003)

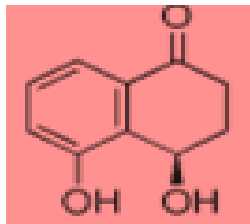
2.4.2.2. Fungal compounds

B. fabae kills plant cells as it grows inside its host. This process probably is carried out by means of the production of enzymes and phytotoxins that degrade plant tissue (Harrison, 1988). *B. fabae* is able to produce pectin degrading enzymes, such as polygalacturonase (PG), during development of chocolate spot (Balasubramani *et al.*, 1971 and Harrison, 1988). These enzymes and phytotoxins that degrade plant tissue were indicated as the principle cause of plant cell death during lesion development (Mansfield and Richardson, 1981).

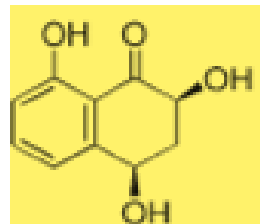
As far as we know, no phytotoxin produced by *B. fabae* has been identified so far. Only Harrison in 1988 reported the presence of some metabolites with phytotoxic activity in extracts from infected leaf tissue and from liquid cultures of *B. fabae*, but did not carry through full isolation and identification of these compounds. The new botrytone, regiolone, and both cis- and trans-3,4-dihydroxy-2,4,8-trihydroxynaphthalenones as phytotoxic metabolites from *B. fabae* (Cimmino *et al.*, 2011).



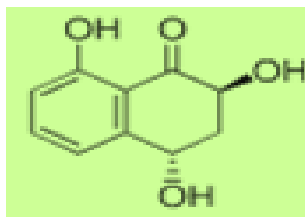
Botrytone



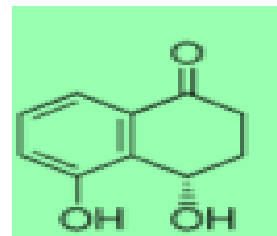
Regiolone



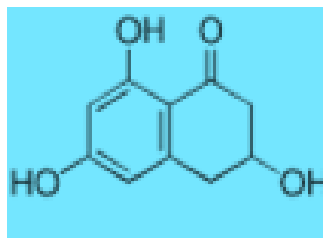
Cis-3,4 dihydroxy-2,4,8- trihydronaphthalenones



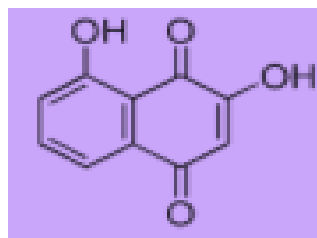
Trans-3,4 dihydroxy-2,4,8-trihydroxynaphthalenones



Isosclerone



Scytalone



3-hydroxyxyjuglone

Structures of phytotoxins, sources: (Cimmino *et al.*, 2011)

2.4.3. Fungal diseases of faba bean

Faba beans are susceptible to a large number of fungal diseases that may cause considerable crop losses. In this respect, (Akem and Bellar, 1999, El-Morsy *et al.*, 1997, Hugar, 2004 and Sepúlveda, 1991) isolated *Fusarium oxysporum* and *F. solani f.sp. fabae*, *Rhizoctonia solani*, *F. oxysporum f.sp. fabae*, *Fusarium oxysporum* and *Macrophomina phaseolina* from wilted and rotten roots of faba bean in different parts of the world as well as considered them the most important and widespread fungal diseases observed at all locations. Meanwhile, all of (Abo-Baker, 2002, Daboor, 2001 and Morsy, 2000) isolated *Ascochyta fabae*, *Botrytis fabae*, *B. cinerea*, *Uromyces fabae [U. viciae-fabae]*, *Alternaria* spp, *Cercospora* and *Stemphylium* spp from spots of the faba bean plants in different parts of the world as well as most of them considered *B. fabae* and *B. cinerea* as the most important and widespread fungi causing chocolate spot disease on faba bean.

2.4.3.1. Chocolate spot (*Botrytis fabae* Sard.)

The first description of chocolate spot disease came from Berkley during 1849 to 1875 in a series of articles in Britain. However, he was unable to associate a pathogen to the disease (Gaunt 1983). (Sardina, 1930 and 1932) described the pathogen *Botrytis fabae* during 1928-1929 when the faba bean crops were attacked by the disease in different areas of Spain. The disease has been reported from Tunisia, Algeria, Morocco, Libya, Ethiopia, England, Spain, Norway, Germany, Scotland, Russia, Japan, China, Canada, North and South America, and Australia (Abdelmonem, 1981, Conner, 1967, Hebblethwaite, 1983, Ikata, 1933, Mengistu, 1979, Sardiña, 1929, Tupenevich and Kotova, 1970 and United States Dept. of Agri., 1960).

Losses caused by chocolate spot are due mainly to a decreased number of pods per plant (Williams, 1978), damage the foliage, limit photosynthesis activity and reduce faba bean production globally (Torres *et al.*, 2004). Other workers showed that faba bean leaves approaching maturity are more susceptible than the younger ones (Deverall and Wood, 1961 and Mansfield and Deverall, 1974).

The chocolate spot disease (*B. fabae*) occurs mainly on leaves, but stems and flowers may also be infected under sever conditions. Disease severity is favoured between 92-100% relative humidity and 15-20 °C (Harrison, 1980 and 1984), other factors including inoculum density, waterlogging, high plant density and host physiology (Griffiths and Amin, 1977, Ingram and Hebblethwaite, 1976 and Moore and Leach, 1968) have been shown to be closely related with disease development. Also, under prolonged wet conditions, the disease may be epidemic with heavy crop losses (Bernier *et al.*, 1993). The spots on leaves and stems enlarge and develop a grey, dead centre with a red-brown rim or margin. Chocolate spot can kill flowers and stems. Spores will form on this dead tissue ((Noorka, and El-Bramawy, 2011).



Photo: Chocolate spot on leaves and flowers (SARDI).



Chocolate spot on leaves

2.4.3.2. Rust (*Uromyces viciae fabae* (Pers.) Schroet)

Rust caused by *Uromyces viciae fabae* (pers.) Schroet, is one of the most widely distributed diseases of faba bean around the world (Guyot, 1975 and Hebblethwaite, 1983). The pathogen has been reported from all over West Asia and North Africa (Hawtin and Stewart, 1979). In general, rust appears late in the season and causes an estimated 20% loss in faba bean production (Bekhit *et al.*, 1970 and Mohamed, 1982).

The rust disease occurs on the leaves where numerous, small, orange-brown pustules, each surrounded by a light yellow halo, are observed symptoms which first appear as minute, slightly raised, white to cream colored spots on leaves and to a lesser extent on stems. As spots enlarge the epidermis ruptures, releasing masses of brown spores (urediospores) to form characteristic pustules (uredia). The pustules are often surrounded by a ring of yellow tissue ((Noorka and El-Bramawy, 2011).



Photo: Bean rust (SARDI).

2.4.3.3. *Ascochyta fabae*

Ascochyta fabae Speg is the causal agent of leaf, stem and pod spot disease of faba beans, *Vicia faba* L. Various methods of assessing disease reaction have been proposed (Jellis *et al.*, 1984, Lockwood *et al.*, 1985, Pritchard *et al.*, 1989, Tivoli *et al.*, 1987 and van Breukelen, 1985).

Ascochyta blight symptoms appear on plant leaves, stems and pods (Beaumont, 1950). On the leaves spots are larger, circular to oblong 2-22 x 2-16 mm in diameter with light coloured centres and a dark or red border (Yu, 1947). Leaf symptoms may be oval shaped with grey centres and red margins while stem lesions may be circular or elongate (Yu, 1947). Stem lesions are darker than the leaf lesions, elongate, sunken and spreading, with pale centers and dark margins and in severe attacks, stems may break from the infection point (Gaunt 1983). On the pods spots are circular or oval, dark brown with black edges and deeply sunken in host tissues (Yu, 1947).



Photo: *Ascochyta* blight leaf lesion (SARDI).



Ascochyta blight leaf lesion



Photo: Bean grain with *ascochyta* blight damage (Pulse Australia).

2.4.4. Control of fungal diseases

Diseases are the major cause of reduction in yield and quality (Vanderplank, 1963) and the most important factors which determine the yield level and stability in the production of faba bean (Robertson, 1993). In general, there are four ways to control fungal diseases: such as (1) Disease free seed, (2) Use of fungicides, (3) Cultural methods and (4) Resistant cultivars.

2.4.4.1. Control of chocolate spot

Botrytis fabae is transmitted through seed (Geard, 1962) and by spores which usually spread during heavy rains, but in dry weather spore dispersal is reduced (Creighton *et al.*, 1985). Wind speed also affects the spread of the pathogen (Harrison and Lowe, 1987). Disease spreads from one field to the adjacent field by airborne conidia usually produced on lesions during high humidity. During warm and humid conditions air borne conidia are readily produced on dead leaves and disease spreads very quickly (Ellis and Waller, 1971, MacLeod and Sweetingham, 1999). Crop rotation, adjustments of sowing date, proper drainage, use of potash and fungicides reduce the disease incidence (Liang, 1993). Fungicides, Thiabendazole, Prochloraz and Iprodione provided a degree of disease control but were not as effective as Benomyl (Bainbridge *et al.*, 1985).

Chemical application provides partial control (Bouhassan *et al.*, 2004 and Tivoli *et al.*, 1986) and is costly for the farmers, which reduces profit margins as well as being harmful to the environment (Bouhassan *et al.*, 2004). Therefore, the least expensive and best practical method for the control of the chocolate spot disease is the use of resistant cultivars (Makkouk and Hanounik, 1993). The faba bean lines L82009, L82007, L82011 and L82010 have been rated as resistant to chocolate spot (ICARDA, 1987).

2.4.4.2. Control of Rust

Rust occurs mostly late in the season and therefore, chemical control may not be economical. However, when rust occurs with chocolate spot in the same field, Mancozeb (Dithane-M45) can be used (Mansour *et al.*, 1975). Removal of infected plant debris (Prasad and Verma, 1948), destruction of other host species and rotating faba bean with non-host crops (Conner and Bernier, 1981), should play an important role in reducing chances of survival and primary infections in the field. Several rust-resistant lines have been reported. The faba bean lines BPL 1179, 261, 710, 8, 406, 417, and 484 have been found to be resistant in Syria, Egypt and Canada. The faba bean lines L82009, L82007, L82011 and L82010 have been rated as resistant to rust (ICARDA, 1987).

2.4.4.3. Control of Ascochyta blight

A. fabae attacks leaves, stems and pods of the plant and in severe attacks the pathogen can infect the seeds (Tivoli *et al.*, 2006). *A. fabae* is significantly transmitted through seed across the world (Kharrat *et al.*, 2006, Punithalingam, 1993, Punithalingam and Holliday, 1975 and Wallen and Galway, 1977) and locally by infected debris (Geard, 1962 and Tivoli *et al.*, 1987), rain splash and wind (Kimber and Davidson, 2004 and Tivoli, 2007). Disease control by rotation, use of clean seed and chemical treatments has not been completely effective in combating Ascochyta blight (Sillero *et al.*, 2001 and Tivoli *et al.*, 2006). However, the use of resistant cultivars is considered the most successful method of disease control (Roman *et al.*, 2003 and Tivoli *et al.*, 2006).

A number of fungicides with different modes of action and application rates have been applied to control pathogens of faba bean (Hampton, 1980, Hanounik, 1980, Rahat *et al.*, 1993 and Wallen and Galway, 1977). (Liew and Gaunt, 1980) compared the effectiveness of foliar fungicides Chlorothalonil, Captafol/Mancozeb and RH 2161 along with water spray as control.

Chemical control does not provide complete protection and is costly which reduces profit margins (Egan *et al.*, 2006) as well as being a danger to the environment (Agrios, 2005, Bouhassan *et al.*, 2004 and Brown, 2006) and human health (Leppik, 1970).

The most suitable method of plant disease control for sustainable production is the use of resistant varieties (Bouhassan *et al.*, 2004 and Roman *et al.*, 2003).

2.5. Major Groups of Antimicrobial Compounds from Plants

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins, and phenol compounds, which are synthesized and deposited in specific parts or in all parts of the plant.

2.5.1. Phenols

Medicinal plants are an important source of antioxidants (Rice-Evans, 2004). Natural antioxidants increase the anti-oxidant capacity of the plasma and reduce the risk of certain diseases (Prior and Cao, 2000). Polyphenols are the major plant compounds with anti-oxidant activity. Typical phenolics that possess anti-oxidant activity are known to be mainly phenolic acids (Demiray *et al.*, 2009). Phenolic compounds are a class of antioxidant agents which act as free radical terminators (Om Prakash and Yamini, 2007). It is reported that the phenolics are responsible for the variation in the antioxidant activity of the plant (Luo *et al.*, 2004). Phenolic constituents of plants have an anti-oxidant activity and offer protection against oxidative damage (Rice-Evans *et al.*, 1997). They exhibit anti-oxidant activity by inactivating lipid free radicals or preventing decomposition of hydro peroxides into free radicals (Pitchaon *et al.*, 2007 and Pokorny *et al.*, 2001). Phenols have been recorded to offer resistance to diseases and pests in plants, and grains containing high amount of polyphenols are resistant to several plant diseases (Malick and Singh, 1980).

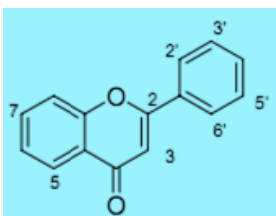
Phenolics are oxidised to phenoxy radicals (Lakshmanan *et al.*, 2007). This phenoxy radical reduces the AA into monodehydroascorbate. Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenyl propane derived compounds which are in the highest oxidation state. Caffeic acid is effective against viruses, bacteria, and fungi. Catechol and pyrogallol both are hydroxylated phenols, shown to be toxic to microorganisms. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. More highly oxidized phenols are inhibitory.

The mechanisms for phenolic toxicity to microorganisms include Substrate deprivation, Membrane disruption, and enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Cowan, 1999) The increased oxidation of phenolics due to the fungicide application may therefore contribute to the acceleration of the oxidative damage (Jaleel *et al.*, 2008). Activities of oxidative enzymes in any infected plant tissues are known to contribute to disease resistance mechanisms through the oxidation of phenols (Tarrad *et al.*, 1993).

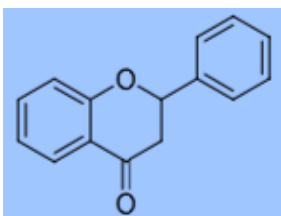


2.5.2. Flavonoids

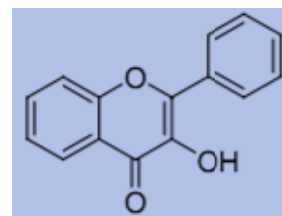
Flavonoids are hydroxylated phenolic substances. Since they are synthesized by plants in response to microbial infection, they have not been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes. Flavonoid compounds exhibit inhibitory effects against multiple viruses (Cowan, 1999). Based on the skeleton of flavonoids some subgroups are: flavones, flavanones, isoflavones, chalcones, aurones (Dewick, 2001).



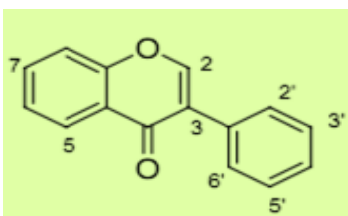
Flavone



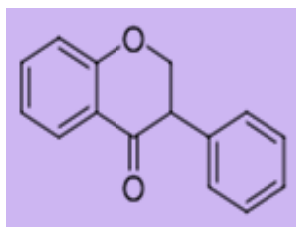
Flavanone



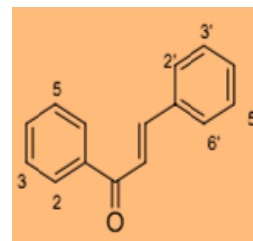
Flavonols



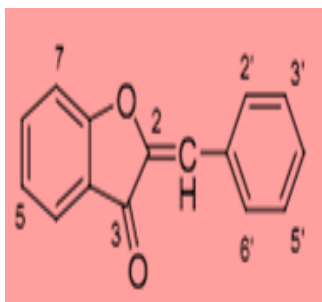
Isoflavone



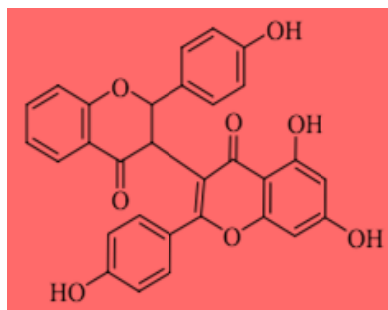
Isoflavanone



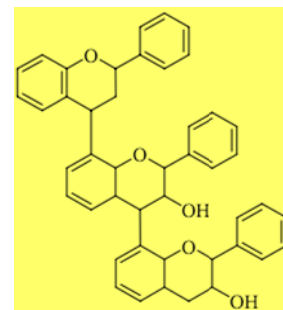
Chalcones



Aurones



Biflavone



Procyanidin

Flavonoids

Source: (Dewick, 2001)

3. OBJECTIVES OF THE STUDY

3.1. General objective

The present study was designed to isolate certain rhizospheric bacteria of *Pseudomonas fluorescens* for their antagonistic potential and their antifungal compounds against *B. fabae*, assess their potential in producing plant growth promoting substances, their potential in disease reducing, their effects on growth parameters and their stimulate biochemical in faba bean leaf.

3.2. Specific objectives

- To isolate and identify *Pseudomonas fluorescens* isolates and their antagonistic effect against *B. fabae*
- To evaluate the effectiveness of extracts of secondary metabolites of *Pseudomonas fluorescens* isolates and determine the MIC (minimum inhibitory concentration) of fungicides against *B. fabae*
- To assess in vitro production of plant growth promoting substances IAA, phosphate solubilizer, ammonia, siderophore (Fe-III chelating agent) and HCN production in vitro
- To evaluate bio-primed faba bean seeds varieties (NC 58, Moti and ILB 938) with *Pseudomonas fluorescens* isolates 9 and *Pseudomonas fluorescens* isolates 10 for their disease severity and incidence reduction, their growth promotion capable and estimate the total phenol and flavonoids faba bean plants under greenhouse condition

4. MATERIALS AND METHODS

4.1. Soil sample collection

The rhizospheric soil samples were collected in an envelope from fields growing faba bean (*Vicia faba* L.) from five Kebeles: Mechale wartsu at altitude of 2560 meters above sea level, Wachale at altitude of 2540 meters above sea level, Gore kateme at altitude of 2590 meters above sea level, Eveno at altitude of 2510 meters above sea level and Gago at altitude of 2520 meters above sea level of North Showa of Oromia Region of Salele Zone, Ethiopia. The soils were brought to Mycology Laboratory, Department of Microbial, Cellular and Molecular Biology, College Natural Sciences, Addis Ababa University.

4.2. Isolation of *Pseudomonas fluorescens*

Isolation of *Pseudomonas fluorescens* isolates studies were carried out on King's B medium (King *et al.*, 1954). 1g of rhizosphere soil sample was suspended in 99 ml of sterile distilled water. Samples were serially diluted and 0.1 ml of sample was spreaded on King's B medium plates. After incubation at 28°C for 48 h the plates were exposed to UV light at 365 nm for few seconds and the colonies exhibiting the fluorescence were picked up and streaked on to the slants for maintenance, purified on King's B medium plates and also designated as P fl1to12 which stands for *Pseudomonas fluorescens* isolates used for further studies.

4.3. Pathogens

One isolate of *Botrytis fabae* was obtained from Holeta Agricultural Research Centre, Ethiopia. This pathogenic fungus was isolated from the Leaf of infected faba bean leguminous crops.

4.4. Plant material

Faba bean seed used in the present work was obtained from Holeta Agriculture Research Centre, Ethiopia. They were provided three varieties of faba bean seed (such as: NC 58, Moti and ILB 938).

4.5. In vitro tests of fungal antagonism

All *Pseudomonas fluorescens* isolates were assessed for potential antagonistic activity against *Botrytis fabae* on King's B agar using dual culture technique (Rangeshwaran and Prasad, 2000). An agar disc (4 mm dia.) was cut from an actively growing (96 hr) phytopathogen, *B. fabae* culture and placed on the surface of fresh King's B agar medium at the center of the Petri plates. A loopful of actively growing *Pseudomonas fluorescens* isolates (each) was placed opposite to the fungal disc and streaking the *Pseudomonas fluorescens* isolates on the plate at four locations, approximately 3 cm from the center. Plates inoculated with phytopathogen and without bacteria were used as control. All in vitro tests of antagonism were performed triplicates, with new coinoculations used each time. Plates were incubated at room temperature for 7 days. Degree of

antagonism was determined by measuring the radial growth of pathogen with bacterial culture and control and percentage inhibition calculated by the following equation (Riungu *et al.*, 2008).

$$\text{Percent Inhibition} = \frac{100 (C - T)}{C}$$

Where,
C = Radial growth of fungus in control plates (mm)
T = Radial growth of fungus on the plate inoculated with Antagonist (mm)

4.6. Culturing of *Pseudomonas fluorescens* isolates for extraction of secondary metabolites

All *Pseudomonas fluorescens* isolates were grown in 100ml of King's B media in 250 ml conical flask in orbital shaker at 28⁰C, 120 rpm, for 96 hours. The culture was centrifuged at 10,000 rpm for 15min to get the cell-free filtrate (Tripathi and Johri, 2002).

4.6.1. Extraction of secondary metabolites of *Pseudomonas fluorescens* isolates of with selected organic solvents

Secondary metabolites were extracted from the effective growth medium (King's B medium) by partitioning with organic solvents viz., Ethyl acetate, Hexane and methanol the three solvents were being tried for extraction of secondary metabolites (Tripathi and Johri, 2002). The antifungal compounds were extracted from cell-free broth with equal volume of ethyl acetate, hexane and methanol and separated the extract and aqueous by using separating funnel and then evaporated in a rotary evaporator at 45⁰C, 60⁰c and 65⁰c at 121 rpm to ensure complete solvent removal respectively. The extracted secondary metabolites without concentration were tested for their efficacy against pathogens by poison food technique (Nene and Thapliyal, 1971). The concentrations of extracted secondary metabolite (0.1%) 25µm were prepared and poured on King's B agar medium with mixing, before a 4 mm disc of *Botrytis fabae* culture was inoculated at the center of each plate, three replications were maintained for each treatment and the petridishes were incubated at 28⁰C. King's B medium plates with only solvent served as control. After full growth of the control plate's size of colony diameter measured in mm and percentage inhibition of mycelial growth was calculated using the formula (Mohana and Raveesha, 2007).

$$\text{Percentage inhibition} = \frac{C-T}{C} \times 100$$

Where C, average increase in mycelial growth in control plate and T, average increase in mycelial growth in treatment plate

4.7. Determination of minimum inhibitory concentration (MIC)

B. fabae was tested in this study to see the in vitro antifungal activity of five types of fungicides Bayleton® 50% WP, Curzate® WP (Cymoxanil 4.2%, copper oxychloride 39.75 and rest inert), Ridomil Gold MZ (metenoxam 4%, mancozeb 64% and inert 32%) and Sancozeb 80% WP (mancozeb 80% and inert 20%) at different concentration ranges from 10 to 80mg/ml.

Then independently each of fungicides were dissolved with 1ml of sterilized distilled water in test tubes to obtain 10 to 80mg/ml concentration. Different concentration of fungicide was transferred to King's B medium and mixed uniformly. After complete solidification, 4mm mycelial disc of *B. fabae* inoculated at the center. They were incubated at 28°C for seven days. The plate containing the least concentration of fungicides showing no visible sign of growth was considered as MIC (Andreus, 2001).

4.8. Biochemical characterization of *Pseudomonas fluorescens* isolates for plant growth promoting (PGP) traits

4.8.1. Assay for Siderophore Production

Siderophore production was tested by growing *Pseudomonas fluorescens* isolates on the king's B medium at 28°C for 48 hours. The plates were exposed to UV light for few seconds and the colonies exhibiting the fluorescence (Ramyastruthi *et al.*, 2012).

4.8.2. Assay for hydrogen cyanide production

Hydrogen cyanide production was assayed by the method suggested by (Castric, 1977) and Lorck, 1948). For the production of HCN, *Pseudomonas fluorescens* isolates were streaked into King's B agar plates supplemented with glycine (4.4 g/l). After this, petriplates were inverted and a piece of filter paper impregnated with 0.5% picric acid and 2% of sodium carbonate was placed on the lid. Petri plates were sealed with parafilm and incubated at 28° C for 96 h. Detection of HCN production was performed by the method of Bakker and Schippers (Bakker, and Schippers, 1987). Production of cyanide was determined by a color shift from yellow to orange in the filter paper (Castric, 1975).

4.8.3. Assay for indole acetic acid (IAA) production

All the *Pseudomonas fluorescens* isolates were tested for IAA production (Loper and Schroth, 1986). The test *Pseudomonas fluorescens* isolates culture were inoculated in the nutrient broth with L-tryptophan 500 mg/l at 28 ± 2 °C for 1 week. Fully grown cultures were centrifuged at 3000 rpm for 30 min. 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 FeCl₃ solution). Development of pink color indicates IAA production (Bric *et al.*, 1991).

4.8.4. Assay for ammonia production

Pseudomonas fluorescens isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10ml peptone water in each tube and incubated for 48-72h at 28°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive for ammonia production (Cappuccino and Sherman, 2005).

4.8.5. Assay for Phosphate-Solubilization

Phosphate-solubilization test was conducted qualitatively by plating the *Pseudomonas fluorescens* isolates in agar containing precipitated tricalcium phosphate. The medium was a modification of Pikovskaya medium (Subba Rao, 1999), consisted of 10 g glucose, 5 g tribasic phosphate (Ca₃HO₁₃P₃), 0.5 g (NH₄)₂SO₄, 0.1 g MgSO₄.7H₂O, 0.2 g KCl, trace of MnSO₄ and FeSO₄, 0.5 g yeast extract, and 15 g agar, in 1,000 ml distilled water. *Pseudomonas fluorescens* isolates culture were streaked on the surface of agar plates and incubated at 28°C for 3 days. After 3 days, the colonies showing the clear zones around them were considered as positive for positive P-solubilization.

4.9. Greenhouse experiments

4.9.1. Preparation of fungal inoculum

The inoculums of *Botrytis fabae* was prepared from old culture grown on faba bean seed dextrose agar at 28°C. Conidia were harvested by scraping the surface of the colonies with a spatula and then transferring them to sterilised distilled water and filtering them through nylon mesh. Spore suspensions of *B. fabae* were then adjusted to 2.5×10^5 spores mL⁻¹ with sterile distilled water using a hemocytometer, as detailed by (Derckel *et al.*, 1999).

4.9.2. Preparation of bacteria inoculum

Pseudomonas fluorescens isolates was grown for 48 h in King's B (KB) broth medium, and then cells were harvested by centrifugation. *Pseudomonas fluorescens* isolates were resuspended in sterile distilled water and the concentration adjusted to give 10^9 - 10^{10} cells/ml (El-Mougy and Abdel-Kader, 2008).

4.9.3. Bio-priming of faba bean seeds

Carboxymethyl cellulose (CMC) and pectin were used as adhesive polymers for the bio-priming process of three varieties of faba bean seeds with antagonistic bioagent. Two isolates of *Pseudomonas fluorescens* were resuspended in sterile distilled water and the concentration adjusted to give 10^9 - 10^{10} cells/ml. Ten grams of either CMC or pectin was suspended in 1 L of each of the individual *Pseudomonas fluorescens* isolates bioagent suspensions in conical flasks, which were shaken for 10min on a magnetic stirrer plate. Seeds of faba bean (at the ratio of 500 g/L) were imbibed in each of the prepared priming solutions for 16 h (Jensen *et al.*, 2004).

The bio-primed seeds were then air-dried on filter paper for 1 h in a laminar flow hood and packed into glass jars sealed with a 45-mm membrane and stored in a refrigerator at 5° C until required. Another group of surface-sterilized faba bean seeds (using 70% ethanol for 2min, then air-dried on sterilized filter paper) were prepared as control treatments (El-Mougy and Abdel-Kader, 2008).

4.9.4. Pot experiments

Antifungal activity of *Pseudomonas fluorescens* isolates against *Botrytis fabae* pathogen was evaluated in pot by bio-primed faba bean seeds. The experiment were designed under greenhouse conditions in Ecology and Ecophysiology Greenhouse, Department of Microbaial, Cellular and Molecular Biology, College Natural Sciences, Addis Ababa University in the month of March 2012, using pots (21cm-diameter) containing 4 kg of sterilized loamy clay soil. First, soils were infested 20 ml of *B. fabae* spore suspension (Haggag *et al.*, 2006) by soil drenching, containing 2.5×10^5 spores/ml and mixed thoroughly to ensure equal distribution of fungal inoculums in different pots and then the pots were irrigated for 7 days before bio-control agent inoculation. Next, four of bio-primed faba bean seeds were sown in each pot. The experiment included the following treatments; 1) non-infested soil (control), 2) soil treated with *Botrytis fabae* only, 3) *Botrytis fabae* + each *Pseudomonas fluorescens* isolates, separately. Pots were kept under greenhouse conditions till the end of the experiment (Abd-El-Khair *et al.*, 2010).

4.10. Disease Assessment

The plants were rated for disease incidence (DI) and disease severity (DS), the former as the presence or absence of disease (percentage of infected leaves on the plant) and the latter as the severity percentage of disease damage.

The disease severity of chocolate spot disease was estimated at 50 and 70 days from sowing under natural infection by using the scale of Bernier *et al.* (1993) as follow:

- 1= No disease symptoms or very small specks (highly resistance).
- 3= few small discrete lesions (resistant).
- 5= some coalesced lesion with some defoliation (moderate resistant)
- 7= large coalesced sporulating lesions, 50% defoliation and some dead plant (Susceptible)
- 9= Extensive lesions on leaves, stems and pods, severe defoliation, heavy sporulation, stem girdling, blackening and death of more than 80% of plants (Highly susceptible)

Chocolate spot disease severity was assessed according to the scale of Bernier *et al.* (1984).

$$\text{Disease severity \%} = \frac{(n \times v)}{9 N} \times 100$$

Where:

- (n)= Number of plants in each category.
- (v)= Numerical values of symptoms category.
- (N)= Total number of plants.
- (9)= Maximum numerical value of symptom category.

The disease incidence of chocolate spot as a disease percentage was determined after 50 and 70 days from sowing the first treatment according to the following formula:

$$\text{Disease incidence} = \frac{\text{Number of infected leaflets}}{\text{Total number of tested leaflets}} \times 100$$

The efficacy percentage (E %) of each of two *Pseudomonas fluorescens* (P f9 and P f10) in reducing disease, severity percentage of faba bean was assessed according to the equation adapted by Rewal and Jhooty (1985) as follow:-

$$E \% = \frac{\% \text{disease severity in control} - \% \text{disease severity in treatment}}{\% \text{Disease severity in control}}$$

4.11. Studied Characters

At 70 days of the cultivation period, growth parameters (plant height (cm), number of leaves, branches, nodule number (Alemayehu Workalemahu, 2009) and root length (cm) per plant) were determined (El-Ghamry *et al.*, 2009).

4.12. Re isolation of the pathogen

Pathogenicity was confirmed by inoculating isolates onto faba bean plants. The pathogen *B. fabae* was re isolated from the leaf lesion of the control plants in the in vivo experiment. Leaf lesions were cut in to pieces and surface sterilized with 70% ethanol for 2 min and rinsed thrice with sterile water in Petri plates. Pieces dried with sterile filter paper and plated on Faba bean seed extract dextrose agar (FDA) medium and incubated at 28^oc for 7 days. The fungus was subculture to purify, and identification was by Comparison with the previous culture.

4.13. Biochemical parameters of test faba bean leaves

4.13.1. Collection of plant material

The leaves of the plant faba bean collected from Ecology and Ecophysiology Greenhouse, Addis Ababa University in the month of May 2012. The faba bean leaves were brought to Mycology Laboratory, Department of Microbial, Cellular and Molecular Biology, College Natural Sciences, Addis Ababa University.

4.13.2. Estimation of total phenol

The measurement of total phenol is based on Mallick and Singh (1980). For preparation of the calibration curve, 1 ml aliquots of 0.2, 0.4, 0.6, 0.8 and 1.00 mg/ml ethanolic catechol solutions were combined with 0.5ml Folin-ciocalteaus reagent. After 3 min, 2ml 20% sodium carbonate was added and the contents were mixed thoroughly. The final color change was measured colorimetrically at 650 nm and the calibration curve was drawn. 0.5 g of fresh plant tissue was ground using a pestle and a mortar with 10 ml of 80% ethanol and centrifuged at 10 000 rpm for 20 minutes. The supernatant was evaporated to dryness and preserved. The residue was dissolved in 5 ml of distilled water and used as the extract. To 2 ml of the extract, 0.5 ml of Folin Ciocalteau reagent was added. After 3 min, 2 ml of 20% Na₂CO₃ solution was mixed in thoroughly. The mixture was kept in 40^oc for exactly 1 min and after cooling the absorbance was read at 650 nm in a spectrophotometer. The total phenol was determined using a standard curve prepared with different concentrations of gallic acid (C₇H₆O₅ H₂O).

The content of phenolic compounds in plants ethanolic extracts was calculated as the following equation:-

$$T = \frac{c \times V}{m} \times 100$$

where, T is total content of phenolic compounds, in mg of catechol/100g of fresh weight material, c is concentration of established from the calibration curve, in mg ml⁻¹, v is the volume of extract in ml and m is the weight of pure plant ethanolic extract in g.

4.13.3. Estimation total flavonoids

0.5 g of fresh plant tissue was ground using a pestle and a mortar with 10 ml of 80% ethanol and centrifuged at 10000 rpm for 20 minutes. The supernatant was evaporated to dryness and preserved. The residue was dissolved in 5 ml of distilled water and used as the extract. To 2 ml of the extract, 0.3 ml of 5% sodium nitrate was added to the tubes. After 5 min, 0.3 ml of aluminium chloride (AlCl₃) (10%) was added to all the tubes. At the 6 min, 2 ml of sodium hydroxide (1 M) was added to the mixture. Immediately, the contents of the reaction mixture were diluted with 2.4 ml of distilled water and mixed thoroughly. Absorbance of the mixture was determined at 510 nm versus a prepared blank immediately. Gallic acid was used as the standard compound for quantification of total flavonoids (Zhisen *et al.*, 1999).

4.14. Data analysis

All the measurements were replicated three times for each assay and the results are presented as mean ± SD and mean ± SE. IBM SPSS 20 Version statistical software package was used for statistical analysis of percentage inhibition and disease incidence and disease severity in each case.

5. RESULTS

5.1. Isolation of *Pseudomonas fluorescens*

During this research work, 12 *Pseudomonas fluorescens* were isolated from rhizospheric soil of healthy faba bean from five Kebales of Salale zone of Oromeyia region on King's B medium and observed under UV light at 365 nm for few seconds (Fig. 1). Then it was purified again on same medium and observed under UV light (Fig. 2). All the rhizospheric isolates were tentatively named as in Table 1 and maintained on Nutrient Agar slants for further testing and biochemical production test.

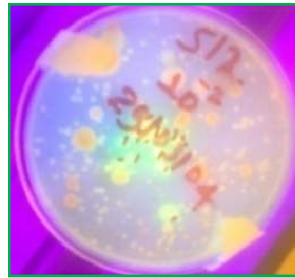


Fig.1. *Pseudomonas fluorescens* showing pigment under UV light



Fig.2. *Pseudomonas fluorescens* isolates confirmed again under UV light.

5.2. Pathogens

Spore morphology of *Botrytis fabae* attachment to mycelia when slide culture was observed under microscope (Fig.3 and Fig.4).



Fig. 3. Conidiophore of *Botrytis fabae* (Branched dichotomously)

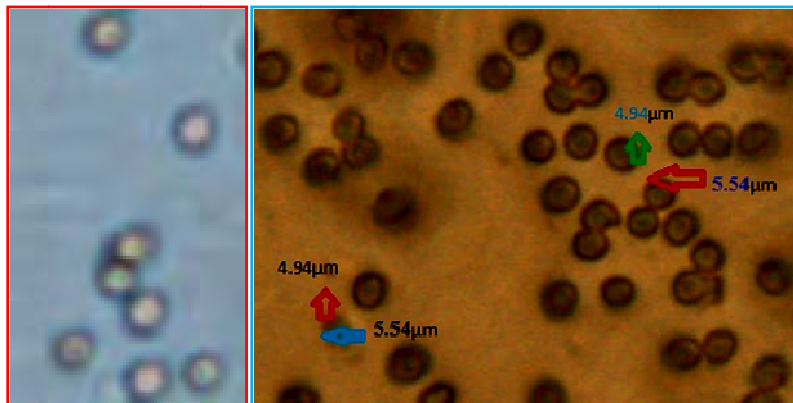


Fig.4. Conidia of *Botrytis fabae* (Ovoid shape) with measure horizontal and vertical diameter at 400x magnifications

5.3. In vitro antagonism

The results of in vitro tests of antagonism toward the plant pathogen *B. fabae* were shown in Table 1. Inhibition was clearly discerned by very limited growth of fungal mycelium in the inhibition zone surrounding a bacterial colony. The antagonistic effects of *P. fluorescens* isolates against *B. fabae* were in the range of 84.1- 88.1%. P f10 gave the maximum inhibition about 88.1 %, followed by P f9 (88.0 %). Control plates not treated with *P. fluorescens* isolates were completely covered by the *B. fabae* showing no inhibition.

Table 1. Effect of *Pseudomonas fluorescens* isolates treatments against the linear mycelial growth of *Botrytis fabae* in vitro tests.

<i>Pseudomonas fluorescens</i> isolates	Antagonistic effect against <i>Botrytis fabae</i>	
	Mycelial diameter (cm) Mean \pm SD	Inhibition (%)
P f1	2.30 \pm 0.26458	87.0
P f2	2.63 \pm 0.32146	85.4
P f3	2.43 \pm 0.20817	86.5
P f4	2.86 \pm 0.11547	84.1
P f5	2.73 \pm 0.30551	84.8
P f6	2.63 \pm 0.49329	85.4
P f7	2.23 \pm 0.25166	87.6
P f8	2.40 \pm 0.36056	86.7
P f9	2.20 \pm 0.00000	88.0
P f10	2.13 \pm 0.15275	88.1
P f11	2.46 \pm 0.50332	86.3
P f12	2.80 \pm 0.20000	84.8
Control	9.00 \pm 0.00000	-

5.4. Antifungal activity of ethyl acetate extracts of secondary metabolites of *Pseudomonas fluorescens* isolates against *B. fabae*

The results was indicated in Table 2 and that the complete inhibited by P f3 and the maximum inhibition of mycelia growth of *Botrytis fabae* was brought out by 0.1% concentration of extracts of P f9 (86.30%) followed by P f10.

Table 2. Percent of inhibition of ethyl acetate extract of *Pseudomonas fluorescens* isolates metabolites at 0.1 % concentration against *B. fabae*

Antifungal compounds of <i>Pseudomonas fluorescens</i> isolates	<i>B. fabae</i>	
	MG (mm) Mean \pm SE	INH %
P f1	15.33 \pm 1.45297	82.96
P f2	18.00 \pm 2.08167	80.00
P f3	No growth	100
P f4	14.67 \pm 1.45297	83.70
P f5	16.33 \pm 0.88192	81.85
P f6	14.67 \pm 1.76383	83.70
P f7	15.33 \pm 1.45297	82.96
P f8	14.83 \pm 0.60093	83.52
P f9	12.33 \pm 0.88192	86.30
P f10	13.33 \pm 0.88192	85.20
P f11	15.67 \pm 1.20185	82.60
P f12	16.67 \pm 1.20185	81.48
Control	90.00 \pm 0.0000	-

Key: MG= Mycelial growth; INH= inhibition over control; SE=Standard error of mean

5.5. Antifungal activity of hexane extracts of secondary metabolites of *Pseudomonas fluorescens* isolates against *B. fabae*

The result shown in Table 3 as indicated that P f8 at 0.1% concentration was totally inhibited the growth of myceia and the highest percent of inhibition on the growth of *B. fabae* was obtained with extracts of P f9 (85.60%) and and followed by P f10.

Table 3. Percent of inhibition of hexane extract of secondary metabolites of *Pseudomonas fluorescens* isolates at 0.1 % concentration against *B. fabae*

Antifungal compounds of <i>Pseudomonas fluorescens</i> isolates	<i>B. fabae</i>	
	MG (mm) Mean \pm SE	INH %
P f1	18.67 \pm 2.33333	79.26
P f2	17.00 \pm 1.73205	81.11
P f3	18.67 \pm 1.20185	79.26
P f4	20.67 \pm 1.45297	77.04
P f5	22.67 \pm 0.88192	74.81
P f6	21.33 \pm 1.85592	76.30
P f7	23.00 \pm 1.52753	74.44
P f8	No growth	100
P f9	13.00 \pm 0.57735	85.60
P f10	16.00 \pm 2.51661	82.22
P f11	20.33 \pm 1.20185	77.41
P f12	18.00 \pm 1.52753	80.00
Control	90.00 \pm 0.00000	-

5.6. Antifungal activity of methanol crude extracts of extracellular metabolites of *Pseudomonas fluorescens* isolates against *B. fabae*

The effect of extracellular metabolites extracts of the test *Pseudomonas fluorescens* isolates on the growth of *B. fabae* is indicated in Table 4. The two effective extracts of P f3 and P f10 were showed complete inhibition and highest percent of inhibition of the mycelial growth of *B. fabae* respectively.

Table 4. Percent of inhibition of methanol extract of secondary metabolites of *Pseudomonas fluorescens* isolates at 0.1 % concentration against *B. fabae*

Antifungal compounds of <i>Pseudomonas fluorescens</i> Isolates	<i>B. fabae</i>	
	MG (mm) Mean \pm SE	INH %
P f1	19.33 \pm 1.20185	78.52
P f2	20.00 \pm 1.52753	77.78
P f3	No growth	100
P f4	18.00 \pm 0.57735	80.00
P f5	17.33 \pm 1.66667	80.74
P f6	22.00 \pm .57735	75.56
P f7	20.00 \pm 1.73205	77.78
P f8	22.33 \pm 0.88192	75.19
P f9	14.00 \pm 2.08167	84.44
P f10	13.67 \pm 2.51661	84.82
P f11	25.00 \pm 1.52753	72.22
P f12	16.00 \pm 1.52753	82.22
Control	90.00 \pm 0.00000	-

5.7. Determination of minimum inhibitory concentration (MIC) for fungicide

Botrytis fabae plant pathogen showed differences in their colony diameter growth patterns (mm) at different concentration of Bayleton ® 50% WP. The colony diameter of *B. fabae* was ranges from 54.67-27.67mm and the lowest mycelia growth showed at 80mg/ml concentration. Whereas the colony diameter of *B. fabae* was range from 48.33-24.33mm and the complete mycelia growth inhibited at 80mg/ml concentration of Curzate ® WP. On the other hand, Ridomil Gold MZ showed difference mycelial growth ranges from 28.33-14.00mm at difference concentration of *B. fabae* and the lowest growth at observed at 80mg/ml concentration. While using different concentration of Sancozeb 80% WP on mycelial growth of *B. fabae* was ranges 61.67-29.33mm and at 80 mg/ml prevented the mycelial growth of *B. fabae* as indicated in Fig.5

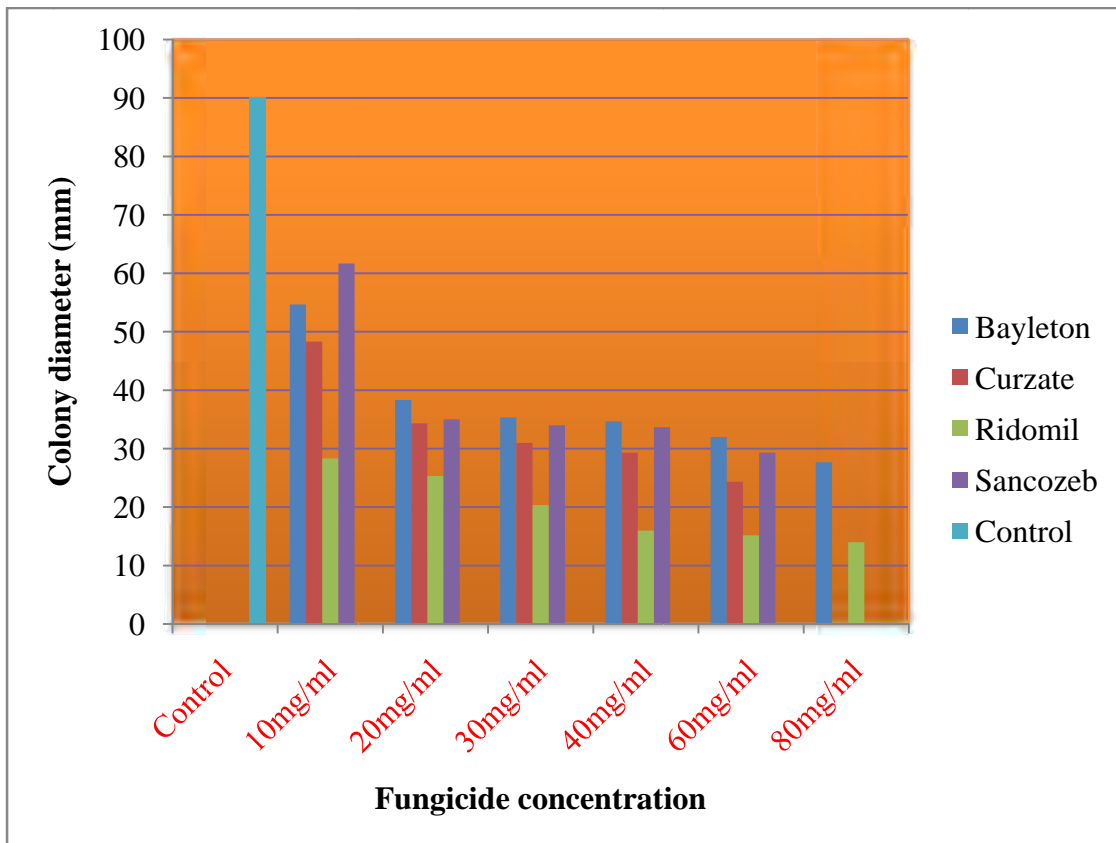


Fig.5. MIC for Bayleton ® 50% WP, Curzate ® WP, Ridomil Gold MZ and Sancozeb 80% WP fungicides

5.8. In vitro production of plant growth promoting substances

All of *Pseudomonas fluorescens* isolates were positive for produced plant growth promoting substances as showed qualitatively.

5.8.1. Assay for siderophore Production

Production of Siderophore was exhibited by all the isolates of *Pseudomonas fluorescens* and colonies were exhibited yellowish green pigment production on King's B agar plates was indicated siderophore production.

5.8.2. Assay for hydrogen cyanide (HCN) production

Hydrogen cyanide production was tested to all *Pseudomonas fluorescens* isolates as well as was determined by a color shifted from yellow to orange in the filter paper indicated hydrogen cyanide production.

5.8.3. Assay for indole acetic acid (IAA) production

IAA production was shown in all the isolates of *Pseudomonas fluorescens* and the development of pink colour was indicated the IAA production.

5.8.4. Assay for ammonia production

Ammonia production was detected in all *Pseudomonas fluorescens* isolates. The results indicated the ability of all the isolates to produce ammonia as yellow color was a positive for ammonia production.

5.8.5. Assay for Phosphate-Solubilization

Twelve (12) isolated *Pseudomonas fluorescens* isolates were tested for Phosphate solubilizing activity in Pikovskaya media plates, a clear halo zone was indicated P-solubilizing activity.

5.9. Pot experiments

Evaluation of bio-primed seeds of faba bean treatments with *Pseudomonas fluorescens* isolates were suppression of *B. fabae* disease incidence and severity investigated under artificial inoculation conditions in Table 5. Disease symptoms attributed to *Botrytis fabae* were observed slightly on faba bean plants grown in soil artificially infested with bio-primed seeds of faba bean with two *Pseudomonas fluorescens* isolates (P f9 and P f10) in pot experiment as compared with control.

Disease severity of bio-primed seeds of faba bean Moti or ILB 938 with P f9 and P f10 showed lowest compared with the untreated (Infected untreated plants) after 50 days.

In general, two isolates of *Pseudomonas fluorescens* effectively reduced the disease on the susceptible (NC 58), moderately resistant (Moti) and relative resistant (ILB 938).

Whereas, disease incidence of bio-primed seeds of faba bean Moti and ILB 938 with P f9 and P f10 were showed lowest compared with the untreated after 50 day.

Two isolates of *Pseudomonas fluorescens* (P f9 and P f10) effectively reduced the disease on the NC 58, Moti and ILB 938 varieties. Disease severity was constantly delayed on NC 58, Moti and ILB 938 varieties during the observation period after 70 day

Disease incidence after 70 day, were lowest on 16.67% ILB 938 varieties with P f9 and P f10 compared with the untreated.

Table 5. Disease severity and incidence of chocolate spot of *Botrytis fabae* on faba bean leaves treated with *Pseudomonas fluorescens* isolate 9 and *Pseudomonas fluorescens* isolate 10 of bio-primed seed of faba bean under greenhouse condition

Treatments and Control	After 50 day			After 70 day		
	DS (%)	efficacy (%)	DI (%)	DS (%)	efficacy (%)	DI (%)
P f9 NC 58	11.11	40.01	25.00	11.11	57.14	41.67
P f9 Moti	3.70	66.70	8.33	11.11	40.01	33.33
P f9 ILB 938	3.70	66.70	8.33	3.70	80.02	16.67
P f10 NC 58	3.70	80.02	16.67	3.70	85.73	33.33
P f10 Moti	3.70	66.70	8.33	3.70	80.02	16.67
P f10 ILB 938	3.70	66.70	8.33	3.70	80.02	16.67
Negative Control NC 58	18.52		66.67	25.93		75.00
Negative Control Moti	11.11		33.33	18.52		66.67
Negative Control ILB 938	11.11		16.67	18.52		58.33
Positive Control NC 58	3.70		-	3.70		-
Positive Control Moti	3.70		-	3.70		-
Positive Control ILB 938	3.70		-	3.70		-

Key: NC 58, Moti, ILB 938, are varieties of faba bean seeds

5.10. Plant growth parameters

5.10.1. Effect of *Pseudomonas fluorescens* isolates (P f9 and P f10) on growth parameters under greenhouse conditions

5.10.1.1. Number of leaves per plant

The use of bio-priming seeds of faba bean of NC 58, Moti and ILB 938 varieties with P f9 and P f10 treatments were showed significantly increase number of leaves/plant over untreated (negative control or infected untreated plant) and positive control (Uninfected untreated plants) as indicated in Table 6 after 70 days.

5.10.1.2. Number of branches per plant

Two isolates of *Pseudomonas fluorescens* (P f9 and P f10) enhanced the number of branches/plant significantly over negative and positive control. Bio-primed seed of faba bean with P f9 increased the number of branches per plants on NC 58, Moti and ILB 938 varieties, while bio-primed seed of faba bean with P f10 increased the number of leaves per plants on NC 58, Moti and ILB 938 varieties as indicated in Table 6 after 70 days.

5.10.1.3. Plant height

As result indicated bio-primed seed of faba bean with P f9 increase height of plants on of Moti, NC 58 and ILB 938 varieties and with P f10 increased the number of leaves per plants on NC 58, ILB 938 and Moti varieties as, compared negative and positive control in Table 6 after 70 days.

Table 6. Effect of two *Pseudomonas fluorescens* isolates (P f9 and P f10) on growth parameter of faba bean plants

Treatments and Control	After 70 day		
	No of leaves per plant Mean \pm SD	No of branches per plant Mean \pm SD	Plant height(cm) Mean \pm SD
P f9 NC 58	65.33 \pm 8.92732	1.75 \pm 1.05529	87.25 \pm 3.72034
P f9 Moti	75.25 \pm 21.23944	2.25 \pm 0.96531	86.92 \pm 9.62439
P f9 ILB 938	67.83 \pm 13.11372	2.25 \pm 1.35680	88.25 \pm 6.25409
P f10 NC 58	70.58 \pm 9.81148	1.67 \pm 0.65134	84.67 \pm 2.80692
P f10 Moti	83.08 \pm 11.26102	2.42 \pm 0.79296	91.92 \pm 7.57338
P f10 ILB 938	79.50 \pm 34.25970	2.58 \pm 0.99620	90.08 \pm 16.14963
Negative Control NC58	61.67 \pm 7.60781	1.17 \pm 0.93744	84.42 \pm 6.62582
Negative Control Moti	66.33 \pm 11.37248	1.58 \pm 0.51493	82.17 \pm 8.84033
Negative Control ILB 938	60.42 \pm 13.78707	2.00 \pm 0.60302	83.75 \pm 8.08056
Positive Control NC 58	62.00 \pm 8.22413	1.42 \pm 1.16450	84.25 \pm 6.98212
Positive Control Moti	72.08 \pm 10.13059	1.83 \pm 0.83485	81.00 \pm 4.76731
Positive Control ILB 938	61.50 \pm 8.45845	2.08 \pm 1.16450	81.01 \pm 4.76730

5.10.1.4. Nodule per plants

The result showed that bio-primed of faba bean seeds with P f9 were increase the nodule number average per plant of NC 58, ILB 938 and Moti, while bio-primed with P f10 also increased the nodule number average per plant of NC 58, Moti and ILB 938 as indicated in Table 7 after 70 day.

5.10.1.5. Root length of faba bean

Bio-primed faba bean seeds with P f9 were increase the root length per plant of NC 58, ILB 938 and Moti, while bio-primed with P f10 also increased the root length per plant of NC 58, ILB 938 and Moti in Table 7 after 70 day.

Table 7.The effect *Pseudomonas fluorescens* isolates (P f9 and P f10) on nodulation and Root length of faba bean

Treatments	After 70 day	
	Nodule number/plant root system	Root length (cm)
P f9 NC 58	33.25 ±3.30404	32.50 ± 2.08167
P f9 Moti	61.50 ±1.91485	49.75 ±15.96611
P f9ILB 938	50.00 ±17.04895	44.75 ±5.50000
P f10 NC 58	35.00 ±10.89342	34.75 ±13.59841
P f10 Moti	68.25 ±9.21502	53.00 ±11.51810
P f10 ILB 938	81.25 ±12.06579	45.00 ±3.91578
NC 58 Negative control (B. f)	28.50 ± 13.66997	21.00 ±7.25718
Moti Negative Control (B. f)	48.25 ± 8.88351	22.75 ±13.04799
ILB 938 Negative control (B. f)	33.50 ± 10.66146	30.25 ± 7.76209
NC 58 Positive control	29.00 ± 11.57584	24.25 ± 3.77492
Moti Positive control	56.00 ±18.31211	35.13 ±18.99287
ILB 938 Positive Control	40.75 ±15.64981	35.25 ±12.55322

5.11. Assessment of secondary metabolites induced by two isolates of *Pseudomonas fluorescens* (P f9 and P f10) on faba bean plants

5.11.1. Total phenol of faba bean

Bio-priming faba bean seeds with P f9 and P f10 were increased the faba bean total phenols in NC 58, Moti and ILB 938 varieties as compared negative and positive control. The lowest amount of total phenol content (106.42 mg/g) was recorded in NC 58 variety with P f9 and 100.50 mg/g was recorded in NC 58 variety with P f10 as indicated in Table 8 after 70 day.

5.11.2. Total flavonoids of faba bean

Bio-priming faba bean seeds with P f9 and P f10 were increased the faba bean total flavonoids in NC 58, Moti and ILB 938 varieties with range 450.58 to 770.19 mg/g compared negative and positive control (). The lowest amount of flavonoids content 450.58 mg/g was recorded in NC 58

variety with P f9 and 489.81mg/g was recorded in NC 58 variety with P f10 as indicated Table 8 after 70 day.

Table 8. The total phenolic and flavonoids content of leaf extract of faba bean (*Vicia fabae*)

S.No	Bio-priming of faba bean seed	Varieties of faba bean	Parameter analyzed	
			Total Phenols (mg cat/100g fresh wt)	Total flavonoids (mg /100g)
1	<i>P. fluorescens</i> 9	NC 58	106.42	450.58
		Moti	152.25	770.19
		ILB 938	122.32	666.35
2	<i>P fluorescens</i> 10	NC 58	100.50	489.81
		Moti	123.03	617.65
		ILB 938	123.70	595.15
3	Negative control	NC 58	84.80	383.66
		Moti	90.60	457.85
		ILB 938	102.93	532.27
4	Positive control	NC 58	94.52	358.62
		Moti	99.29	359.42
		ILB 938	109.55	447.12

6. DISCUSSION

All *P. fluorescens* isolates treatments reduced the mycelial growth of *B. fabae* on King's B medium. These might be due to producing secondary metabolites which inhibited growth of *B. fabae*. Similarly, the antimicrobial activity of *Pseudomonas fluorescens* had reported against numerous fungi (Khan and Zaidi, 2002 and Sivamani and Gnanamanickam, 1988). *Pseudomonas fluorescens* was shown to effectively inhibit *R. solani* and *P. oryzae* by agar plate method (Rosales *et al.*, 1995). (Vidhyasekaran and Muthamilan, 1995) recorded that *Pseudomonas fluorescens* strains showed inhibitory action against the chickpea (*Cicer arietinum*) wilt pathogen *Fusarium oxysporum* f. sp. *ciceris* under in vitro studies.

As present results showed that all *P. fluorescens* isolates were success on the inhibition *B. fabae* myceial growth with highest inhibition 88.1%. On other study, according to Sherga (1997), *Bacillus* isolates can be used as a biocontrol agent against *Botrytis fabae* and *Botrytis cinerea*. The highest reduction was caused by *Bacillus* isolate 115y (64%) against *Botrytis fabae* (Sahile *et al.*, 2009). *Pseudomonas fluorescens* isolated from rhizosphere of organic farming area is an effective against *Rhizoctonia solani* (Anitha and Das 2011). *Pseudomonas fluorescens* strain 003 was found to effectively inhibit (85%) the mycelial growth of fungal pathogens tested *Rhizoctonia solani* (Reddy *et al.*, 2007).

And also *Pseudomonas fluorescens* 003 was found to be highly effective in controlling *Rhizoctonia solani* with inhibition 58% (Reddy *et al.*, 2010). *Pseudomonas flourescence* showed highest antifungal activity against *Penicillium italicum* (94%) and was moderately effective against *Aspergillus niger* (61%) (Mushtaq *et al.*, 2010). Isolate of *Pseudomonas fluorescens* on co-inoculation with fungal pathogens showed maximum inhibition for phytopathogens of *Collectotrichum gleosporioides* (58.3%), *Alternaria brassicola* (50%), *Alternaria brassicae* (12.5%), *Alternaria alternate* (16.66%), *Fusarium oxysporum* (14.28%) and *Rhizoctonia solani* (50%) (Ramyasmruthi *et al.*, 2012).

The antifungal assay of the three solvent extracts of secondary metabolites of P f1 revealed that the ethyl acetate extract showed the highest antifungal activity and followed by hexane extract, suggesting that the antifungal compound is better extracted with ethyl acetate than other solvents. Similarly, (Battu and Reddy, 2009) the metabolites extracted from *P. fluorescens* with ethyl acetate was effectively inhibited (89-90%) both the pathogens (*P.oryzae* and *R. solani*) tested at 5 per cent concentration. The culture filtrates obtained from *Pseudomonas fluorescens* showed the inhibition 55.2% against *Stenocarpella maydis* (Petatán-Sagahón, 2011).

The antifungal bioassay of the three solvent extracts of secondary metabolites of P f2 against chocolate spot disease of faba baen confirmed the presence of significant inhibitory effect which extracted with ethyl acetate, hexane and methanol. This indicated that the antifungal compound was soluble in ethyl acetate and hexane and slightly soluble in methanol. According to (Petatán-

Sagahón, 2011) the culture filtrates obtained from *Pseudomonas* spp. showed a low inhibition 5.0% against *Stenocarpella maydis*.

The in vitro evaluation of antifungal activity of ethyl acetate and methanol extracts of secondary metabolites of P f3 at 0.1% concentration revealed that are completely inhibited the tested pathogen *B. fabae* compared to hexane solvents, suggesting that the antifungal compound are completely extracted with ethyl acetate, methanol and slightly extracted with hexane. Similarly, according to (Reddy *et al.*, 2007) had reported that the crude compounds from *P. fluorescens* isolates metabolites completely inhibited the growth in all pathogens (*Magnaporthe grisea*, *Dreschelaria oryzae*, *Rhizoctonia solani* and *Sarocladium oryzae* at 5%.

The antifungal activity of the three solvent extracts of secondary metabolites of P f4 revealed that ethyl acetate extracts showed highest antifungal activity, suggesting that the antifungal compound is better extracted with ethyl acetate than methanol and hexane. According to (Maleki *et al.*, 2010) antifungal activity of *Pseudomonas fluorescence CV6* was showed higher mycelial inhibition against *Colletotrichum gloeosporioides*.

The antifungal assay of the three solvent extracts of secondary metabolites of P f5 indicated that the ethyl acetate extract showed the highest antifungal activity and followed by methanol and hexane extract, suggesting that the antifungal compound is better soluble with ethyl acetate and methanol and slightly soluble with hexane. On other study, (Rovera *et al.*, 2000) had reported that the antibiotic 2, 4- DAPG produced by *P.aurantica* and their antifungal activity against *Macrophomina phaseolina*. And also (Shalini and Srivastava, 2008) was screened the antifungal activity of *P. fluorescence* against phytopathogenic fungi.

The antifungal bioassay of the three solvent extracts of secondary metabolites of P f6 against chocolate spot disease of faba baen tested the inhibitory effect which extracted with ethyl acetate, hexane and methanol. This indicated that the antifungal compound was soluble in ethyl acetate and hexane and slightly soluble in methanol. On other study, the maximum inhibition of conidial germination of *Fusarium oxysporum* was brought out by 2% *Pseudomonas fluorescens* (83.15 %) and the inhibition of radial mycelial growth of pathogen was effected by 2% concentration of culture filtrate of *Pseudomonas fluorescens* (60.0 %) (Rajeswari and Kannabiran, 2011)

The antifungal activity of the three solvent extracts of secondary metabolites of P f7 showed that ethyl acetate extracts showed highest antifungal activity, suggesting that the antifungal inhibitory compound is better extracted with ethyl acetate than methanol and hexane. On other study, the filtrates obtained in logarithmic phase from the *P. fluorescens* 16 inhibited 54% the growth of *Stenocarpella maydis* (Petatán-Sagahón *et al.*, 2011).

The in vitro evaluation of antifungal activity of hexane extracts of secondary metabolites of P f8 revealed that is completely inhibited the tested pathogen *B. fabae* compared to hexane and methanol solvents, suggesting that the antifungal compound are completely extracted with

hexane and slightly with ethyl acetate and methanol. On other study, *Pseudomonas* spp. showed antifungal activity against *Verticillium dahliae* var. *longisporum* in vitro and were evaluated as potential biocontrol agents by (Berg *et al.*, 1998).

The antifungal activity of ethyl acetate extracts of secondary metabolites of P f9 against chocolate spot disease was show the highest inhibition followed by hexane and methanol extracts. Therefore, this indicated the secondary metabolite of P f9 is highly soluble with ethyl acetate and relatively less soluble with hexane and methanol. According to (Thomashow *et al.*, 1990) showed that the production of phenazine-1-carboxylic acid antibiotics by *P. fluorescens* in correlated with disease control.

The ethyl acetate methanol and hexane extracts of secondary metabolites of P f10 showed the highest antifungal activity in the in vitro test and this is suggesting due to the secondary metabolites of P f10 is better extracted with ethyl acetate methanol and hexane solvents. On other study, Antibiotics induced by *P. fluorescens* inhibit *P. ultimum* (Howie and Suslow, 1991).

The antifungal assay of the three solvent extracts of secondary metabolites of P f11 indicated that the ethyl acetate extract showed the highest antifungal activity and methanol and hexane extracts wereless inhibitory effect against *B. fabae*, suggesting that the antifungal compound is better soluble with ethyl acetate and partially soluble with methanol and hexane. According to (Maleki *et al.*, 2011) had reported that antifungal activity of *Pseudomonas fluorescence CV6* was tested against *Rhizoctonia solani AG4* cause bean damping-Off disease.

Bioassay activity of the three solvent extracts of secondary metabolites of P f12 showed that methanol extracts showed highest antifungal activity, suggesting that the antifungal inhibitory compound is better extracted with methanol than ethyl acetate and hexane. According to (Maleki *et al.*, 2010) had reported that antifungal activity of *Pseudomonas fluorescence CV6* was showed highest mycelial inhibition against *Magnaporthe grisea*.

Bayleton ® 50% WP fungicide was slightly suppressed the radial growth of *B. fabae*. On other hand, Ridomil Gold MZ fungicide was gradually decreased the radial growth of *B. fabae*. While, Curzate ® WP and Sancozeb 80% WP were reduced mycelial growth and finally inhibited the mycelia growth with high concentration.

On other study, (Kimber *et al.*, 2007) reported Procymidone, Copper oxychloride and Captan were more effective than other fungicides to control the disease in field trials during 2005 and 2006.

In this study, the *Pseudomonas fluorescens* isolates exhibited the PGP traits like Phosphate-Solubilization, Siderophore production, Hydrogen cyanide production, ammonia production, and indole acetic acid (IAA) production. Hence, they have been advocated as biocontrol agents and plant growth promoting rhizobacteria. Similarly, PGPR colonize roots of plant and promote plant growth and development through a variety of mechanisms. The exact mechanism by which

PGPR stimulate plant growth is not clearly known, although several mechanisms such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved in plant growth promotion (Glick, 1995 and Lalande *et al.*, 1989). They are known to inhibit the growth of plant pathogens by diverse mechanisms such as antibiotic production (Hill *et al.*, 1994), siderophore production (Loper, 1988), HCN release (Voisard *et al.*, 1989) and competitive colonization of plant roots (Weller, 1985).

In this study, the qualitative estimation of siderophores by *Pseudomonas fluorescens* isolates showed that they were powerful producer of siderophores under limited iron on King's B medium. The production of siderophores by *Pseudomonas fluorescens* isolates indicated that these bacteria isolates can be used as a bio-control against soil borne phytopathogens. Similarly, (Ramyaasmruthi *et al.*, 2012) reported that *Pseudomonas fluorescens* as siderophore producer on King's B medium. *P. fluorescens* NCIM 5096 was able to give higher yields of siderophores under iron stress conditions (Sayyed *et al.*, 2005). Siderophores provide a competitive advantage to producer organism over fungal pathogens for the absorption of available iron (Jeffrey *et al.*, 1999). The role of siderophores in the control of diseases has been well documented by Baker *et al.* (1986).

In the present work, all *Pseudomonas fluorescens* isolates were positive for HCN production, which acts as an inducer of plant resistance. On other study, HCN is a secondary metabolite produced by gram negative *P. fluorescens*, *P. aeruginosa* and *Chromobacterium violaceum* (Askeland and Morrison, 1983) and is postulated to play a role in biological control of pathogens (Defago *et al.*, 1990). *Pseudomonas* spp. that showed remarkable inhibitory effects against the test fungal pathogens produced HCN (Muleta *et al.*, 2007). This compound, although reported as a potential inhibitor of enzymes involved in major plant metabolic processes (Bakker and Schippers, 1987) is currently attracting remarkable attention and wide applications in areas of biocontrol methods. HCN production by rhizobacteria has been postulated to play an important role in the biological control of pathogens (Voisard *et al.*, 1989).

In the present study, IAA production in all *Pseudomonas fluorescens* isolates were positive and agreement with earlier reports. Similarly, PGPR traits showed positive for the traits of IAA (Ramyaasmruthi *et al.*, 2012). The ability of bacteria to produce IAA in culture depends on the availability of precursors. In same way as, it has been reported that IAA production by PGPR can be influenced by substrate availability (Mirza *et al.*, 2001). When the media were supplemented with tryptophan, the IAA production increased substantially (Suresh *et al.*, 2010).

It is expected that inoculation with *Pseudomonas fluorescens* isolates containing PGPR characteristics consequently promote root, shoot growth and lateral roots as well as nodulation as indicated in Fig. 17. The ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant. Microbial biosynthesis of IAA in soil is enhanced by tryptophan from root exudates or decaying cells (Benizri *et al.*, 1998 and

Frankenberger and Arshad, 1991) and also improves plant growth by increasing the number of root hairs and lateral roots (Okon and Kapulnik, 1986). IAA is one of the most important phytohormone and function as important signal molecule in the regulation of plant development during the onset of symbiosis in legumes (Barker and Tsgu, 2000).

In present work, mostly all isolates of *Pseudomonas fluorescens* were able to produce ammonia. Similarly, *Pseudomonas fluorescens* isolate was produced ammonia as reported according to (Ramyaasmruthi *et al.*, 2012). Ammonia production by rhizobacteria strains is reported (Wani *et al.*, 2008). Rhizosphere microorganisms mediate many soil processes such as decomposition and nitrogen fixation (Pradhan and Sukla, 2005). It has been assumed that inoculation with bacteria like *Pseudomonas fluorescens* may enhance the plant growth as a result of their ability to fix nitrogen (Joseph *et al.*, 2007). Production of inhibitory volatiles may increase the survival rate of bacteria in soil, by eliminating potential competitors for nutrients (Mackie and Wheatley, 1999).

In the present work, all isolates of *Pseudomonas fluorescens* bacteria showed zone of phosphate solubilization. Isolate 1, 3 and 9 of *Pseudomonas fluorescens* showed the clearer zone in PVK medium. Similarly, (Pandey and Palni, 1998) reported *Pseudomonas corrugata* as phosphate solubilizer. Highest phosphate solubilization zone was also recorded by *Pseudomonas* spp. as reported by (Kumar *et al.*, 2012) on PVK medium. The microorganisms capable to form a halo zone due to organic acids production in the media plates (Singal *et al.*, 1991) and are selected as potential phosphate solubilizers (Das, 1989). Production of phosphatase enzyme by Phosphate Solubilize bacteria and microbial phytases activity was reported by (Ponmurugan and Gopi, 2006). This solubility of P might be the activity of certain microbes in preferable phosphate sources or due to the activity of phosphatase enzyme.

Application of bio-primed faba bean seed (Moti or ILB 938) with P f9 gave the maximum reduction of chocolate spot severity at 50 days, but at 70 days the highest reduction was recorded on ILB 938 variety. Whereas bio-primed faba bean seed (NC 58) with P f10 gave the highest reduction of chocolate spot severity at 50 and 70 days. Generally, it may be related to the ability of P f9 and P f10 to stimulate the phenol and flavonoids in faba bean plants associated with increased the protection against disease. Data clearly indicated that in untreated plants, chocolate spot infection gradually increased on leaves during growth periods and great differences were obtained among treatments of P f9 and P f10 and untreated control.

On other study, as the report showed that that the bio-priming of seeds with bacterial antagonists increases the population load of the antagonist 10-fold on the seeds and thus protected the rhizosphere from the invasion of plant pathogens (Callan *et al.*, 1990) and also the use of bio-priming seeds could be considered a safe, cheap and easily applied biocontrol method to be used against soil borne plant pathogens and physiological aspects of disease control, involves coating the seed with fungal or bacterial biocontrol agents (El-Mougy and Abdel-Kader, 2008).

Pseudomonas fluorescens strain possessing multiple mechanisms of broad spectrum antagonism and PGP activities which can be explored as one among the best biocontrol agent (Ramyastruthi *et al.*, 2012). (Maleki *et al.*, 2010) as reported *Pseudomonas fluorescens* CV6 had a broad spectrum antifungal activity against phytopathogens that can be used as an effective biological control candidate against devastating fungal pathogens that attack various plant crops. (Agarry and Osho, 2005) reported in vitro and in vivo inhibition of *Aspergillus fumigatus* by *Pseudomonas fluorescens* using as a microbial antagonist. (Elad and Stewart, 2004) have also reported that *Trichoderma*, *Gliocladium* and *Ulocladium* have greatest potential for *Botrytis* diseases and commercial success has been achieved in glasshouse and post-harvest environments for disease control. Earlier also *Penicillium brevicompactum* and *Cladosporium cladosporioides* isolated from faba bean leaves were found to have significant antagonistic activity against *B. fabae* in vitro and in vivo (Jackson *et al.*, 1997).

Growth parameters of faba bean plants recorded significant increases with P f9 and P f10 treatments. The increment in growth parameter may be due to that P f9 and P f10 are extremely important component because they constitute a stable fraction of carbon, thus regulating the carbon cycle and release of nutrients, including nitrogen and phosphorus which decreasing the need for inorganic fertilizer for plant growth. Similarly, the use of *Tichoderma viride tag4* in combined with *Rhizobium leguminosarum* is an effective strategy for an integrated management of chocolate spot disease as well as increasing growth and productivity of faba bean (Saber *et al.*, 2009).

Bio-primed seed of faba bean with P f9 increased the number of leaves per plants on NC 58, Moti and ILB 938 varieties. The highest number of leaves per plants (75.25 leaves/plant) observed on ILB 938 variety, while bio-primed seed of faba bean with P f10 increased the number of leaves per plants on NC 58, ILB 938 and Moti varieties and gave maximum number of leaves per plants (83.08leaves/plant) on Moti variety. On other study, *Trichoderma harzianum* significantly increased number of leaves in treated bean plants were 15.2 leaves/plant, while in untreated plants were 9.5 leaves/plant (Abd-El-Khair *et al.*, 2010). Application of humic acids (HA) at 2000+ amino acids (AA) at 2000 ppm came in the top of other treatments in increasing 46.00 leaves number per plant (El-Ghamry *et al.*, 2009). (Mahmoud *et al.*, 2011) as reported that seed treatment with Bion and Salicylic acid achieved heighest faba bean shoot length at 0.36 and 0.34 D.F.D. 26.0 leaves per plants respectively.

P f9 showed the maximum number of branches of Moti or ILB 938 varieties 2.25 branches/plant and bio-primed with P f10 was 2.00 branches/plant of ILB 938 varieties. On other study, *Trichoderma harzianum* significantly increased the branches number average 6.3 branch/plant, compared to 3.7 branch /plant in control treatment (Abd-El-Khair *et al.*, 2010). Application of HA at 2000+ AA at 2000 ppm came in the top of other treatments in increasing 4.67 branches number per plant (El-Ghamry *et al.*, 2009).

The highest shoot length 88.25 cm was recorded on faba bean plants of ILB 938 variety inoculated with P f9 and 91.92 cm was recorded on faba bean plants of Moti variety by P f10.

On other study, *Trichoderma hamatum* gave the highest increase of plant height 49.8 cm compared to 37.3 cm in the control plants (Abd-El-Khair *et al.*, 2010). Application of AA at 3000 ppm came in the top of other treatments in increasing plant height 74.33 cm (El-Ghamry *et al.*, 2009). And also reported by (Mahmoud *et al.*, 2011) seed treatment with Bion achieved highest faba bean shoot length at 0.36 D.F.D 54.3cm.

The highest numbers of nodule per plant 61.50 and 81.25 nodule/plant were recorded in faba bean Moti and ILB 938 bio-primed with P f9 and P f10 respectively. On other study, (Alemayehu Workalemahu 2009) was report that highest average nodule number was observed 96 nodules/ plant. *Rhizobium leguminosarum var viceae* was identified as fast growing bacteria nodulating faba bean in several studies (Aynabeba *et al.* 2001and Jordan, 1984).

The highest root length per plant 49.75 and 53.00 cm root length/faba bean were recorded in faba bean Moti bio-primed with P f9 and P f10 respectively. On other study, as reported that *Pseudomonas sp.* produced significant levels of IAA and caused shoot and root elongation in soybean (Xie *et al.*, 1996).

The application of bio-primed faba bean seed with P f9 increased total phenols in NC 58, ILB 938 and Moti varieties as well as bio-primed with P f10 was increase total phenols in NC 58, Moti, ILB 938 varieties. The maximum amount of total phenol was recorded 152.25 mg/g in Moti variety with P f9 isolate and 123.70 mg/g was recorded in ILB 938 variety with P f10. Phenolic compounds are secondary metabolites which synthesize in plants. They possess some biological properties such as: antioxidant, anti-apoptosis, anti-aging, and anti-inflammation. These results indicate that there is a correlation between the disease incidences and severity due to the concentration of total phenols in faba bean tissue.

Similar way, Mahmoud *et al.* , (2011) as reported that total phenole increased in treated faba bean followed by uninfected untreated plants and infected untreated plants. The total phenols content of infected faba bean was significantly increased due to the effects of the pathogens. It resulted in 238.80 mg catechol/100g fresh weight compared with the control treatment, which had 149.21mg catechol/100g fresh weight (Elwakil *et al.*, 2009). IAA leads to increase in total phenol content, this material protect plants against pathogen stress (Chowdhury, 2003). Total phenols and phenolics have long been considered as important defence-related compounds whose levels are naturally high in the resistant varieties of many crops (Onyeneho and Hettiarachchy, 1992 and Saini *et al.*, 1988).

The value of phenolic content indicates that the plant has antioxidant activity (Rani *et al.*, 2011). The level of polyphenols in the ethanol extract was 5.1 mg/g which was higher when compared to methanol, chloroform, hexane and aqueous extracts of *Tinospora cordifolia* leaves (Premanath and Lakshmidevi, 2010). The ethanol extracts of *Leptadenia pyrotechnica*, *Haloxylon salicornicum* and *Ochradenus baccatus* have high total phenolic content 158.3, 164.4 and 145.3 mg/g extract (Alqasoumi *et al.*, 2012) respectively. The total Phenol content in *Acacia concinna* has been found to be 650mg/100ml (Raja and Sama, 2012).

Bio-priming faba bean seeds with P f9 were increase the faba bean of total flavonoids in NC 58, ILB 938 and Moti varieties, where as P f10 showed increase total flavonoids in the faba bean varieties NC 58, ILB 938 and Moti. The height amount of flavonoids content 770.19 mg/g was recorded in Moti variety with P f9 and 617.65 mg/g was recorded in Moti variety with P f10. The antioxidant activity of flavonoids is due to their ability to reduce free radical formation and to scavenge free radicals. These results indicate that there is a correlation between the disease incidences and severity due to the concentration of total flavonoids in faba bean tissue.

On other study, reported as indicate flavonoids are phenolic acids which serve as an important source of anti-oxidants found in different medicinal plants and related phytomedicines (Pietta, 1998). Ethanol extract of the leaves had a flavonoids content of 0.52 mg/g which was higher when compared to methanol, chloroform, hexane and aqueous extracts of *Tinospora cordifolia* (Premanath and Lakshmidevi, 2010). *Haloxylon salicornicum* and *Leptadenia pyrotechnica* were found to have the highest total flavonoid content 92.0 and 89.0 mg/g extract respectively, while *Ochradenus baccatus* displayed lower total flavonoid content 85.0 mg/g extract (Alqasoumi *et al.*, 2012). Many studies have shown that flavonoids play important pharmacological roles against various human diseases, such as cardiovascular diseases, cancer, inflammation and allergies (Vinson *et al.*, 1998). The anti-oxidant activity of flavonoids is due to their ability to reduce free radical formation and to scavenge free radicals or chelating process (Kessler *et al.*, 2003). It acts as scavengers of various oxidizing species i.e. super oxide anion (O_2^-), hydroxyl radical or per oxy radicals, they also act as quenchers of singlet oxygen (Das and Ratty, 1986).

7. CONCLUSION AND RECOMMENDATION

7.1. CONCLUSION

- ❖ Application of fungicides for disease control are largely affecting human health, normal flora of soil and environment and also pathogenic fungi became very fast resistant to them.
- ❖ For this reason, seed inoculation with *Pseudomonas fluorescens* isolates as a bio-primed seed showed antagonistic activities against *B. fabae* is an acceptable alternative to chemical fungicides application.
- ❖ Based on present studies, *Pseudomonas fluorescens* isolates under investigation possess a variety of promising properties which make them better biocontrol agents that are capable of producing plant growth promoting substances, antifungal substances, enhance the accumulation of antifungal (antioxidant) compound in faba bean leaf and subsequent enhancement of yield of the crop.
- ❖ The uses of *P. fluorescens* 9 and 10 (P f9 and P f10) are an effective strategy for management of chocolate spot disease as well as increasing growth parameters of faba bean.
- ❖ Bio-primed faba bean seed with P f9 and P f10 are reducing disease severity and incidence in faba bean in green house during pathogenicity test.
- ❖ The biochemical analysis of the test bio-primed faba bean seed with P f9 and P f10 showed the maximum flavonoids content compared to phenol components.
- ❖ The use of environmental friendly *Pseudomonas fluorescens* isolates also for increase soil fertility and production of faba bean crop through management of chocolates spot disease.

7.2. Recommendation

- The present study contribute to the understanding and utilization of *P.fluorescens* isolates as biocontrol agent. Therefore, further investigations are needed to investigate which type of biochemical production are making *P.fluorescens* isolates as one of the most suitable candidate in suppressing the phytopathogenic fungi.
- More investigations are needed to investigate this regard for isolation and characterization of antifungal compounds.
- *Pseudomonas fluorescens* isolates must be evaluated and tested against *B.fabae* in field condition in near future.
- After field application was succeeded, it must be further exploited for the commercial production of an inoculum to use a bio-primed seed as biocontrol and biofertilizers are an efficient approach to replace chemical fertilizers, fungicides and its incorporation in the production system of *Vicia faba*.

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9. APPENDICES

Appendix 1. *Pseudomonas fluorescens* isolates on agar media



1

P f1



2

P f2



3

P f3



4

P f4



5

P f5



6

P f6



7

P f7



8

P f8



9

P f9



10

P f10



11

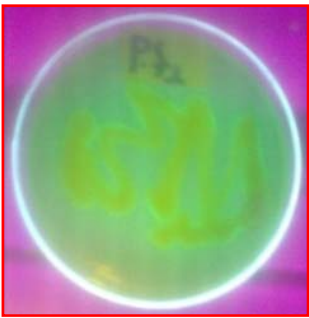
P f11



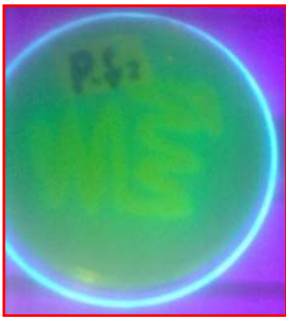
12

P f12

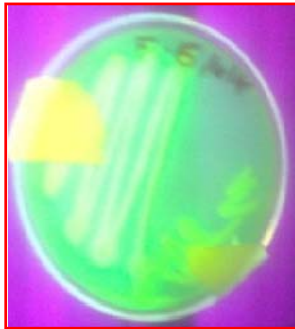
Appendix 2. *Pseudomonas fluorescens* isolates under VU light



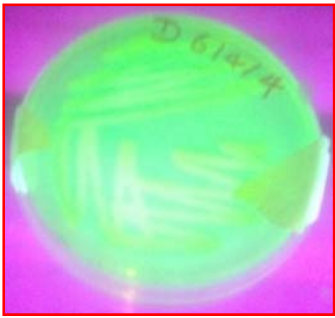
P f1



P f2



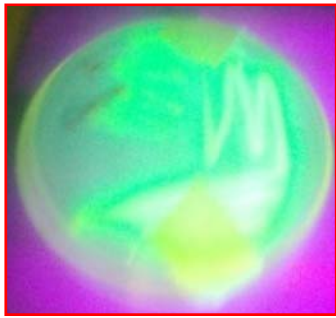
P f3



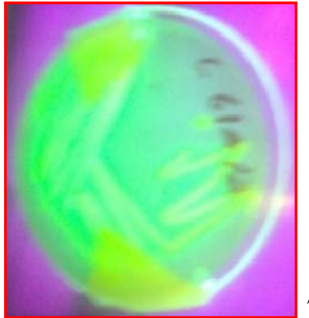
P f4



P f5



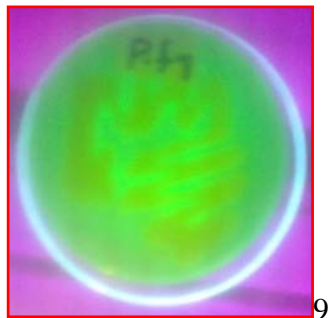
P f6



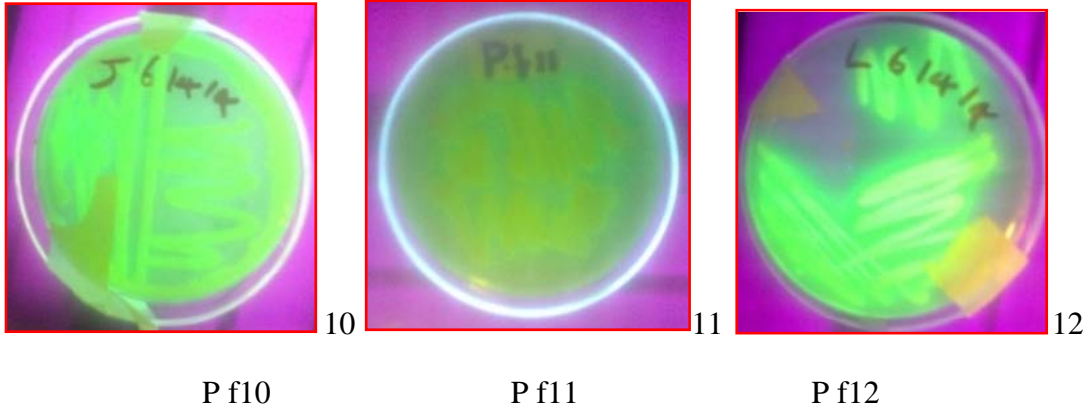
P f7



P f8



P f9



Appendix 3. Microscopic observation of *B. fabae* magnification



Appendix 4. Dual culture of *Pseudomonas fluorescens* isolates against *B. fabae*



Pseudomonas fluorescens 10 against *B. fabae*



Control

Appendix 5. Antifungal activity of ethyl acetate extracts of secondary metabolites of *Pseudomonas fluorescens* isolates against *B. fabae*



Control

Appendix 6. Antifungal activity of hexane extracts of secondary metabolites of *Pseudomonas fluorescens* isolates against *B. fabae*



Hexane extracts of secondary metabolites of *Pseudomonas fluorescens* (8)



Control

Appendix 7. Antifungal activity of methanol extracts of secondary metabolites of *Pseudomonas fluorescens* isolates against *B. fabae*



Methanol extracts of secondary metabolites of *Pseudomonas fluorescens* 3

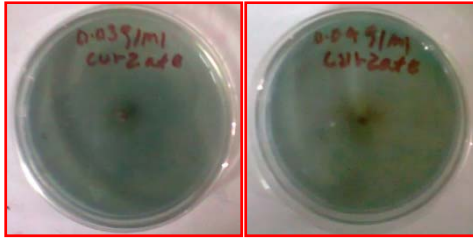


Control

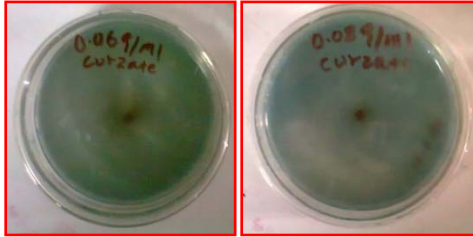
Appendix 8. Fungicides effects on *B. fabae* mycelia growth at different concentration



Curzate ® WP against *B. fabae* at 10mg/ml Curzate ® WP against *B. fabae* at 20mg/ml



Curzate ® WP against *B. fabae* at 30mg/ml Curzate ® WP against *B. fabae* at 40mg/ml

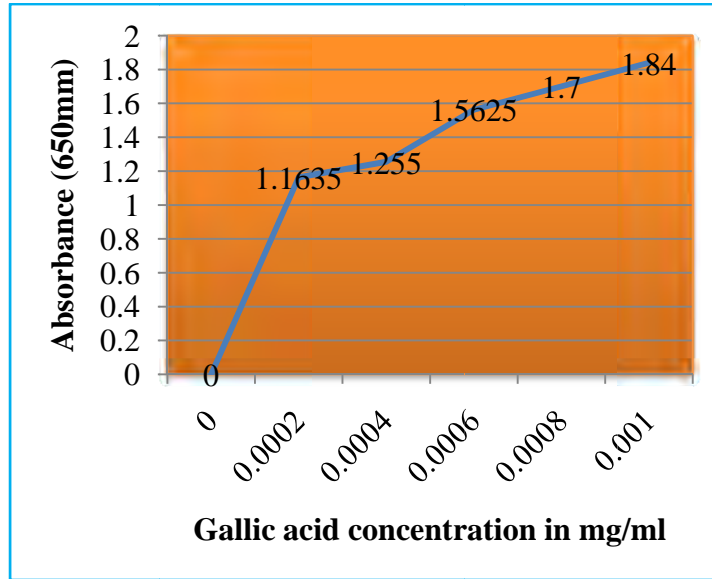


Curzate ® WP against *B. fabae* at 60mg/ml Curzate ® WP against *B. fabae* at 80mg/ml



Control

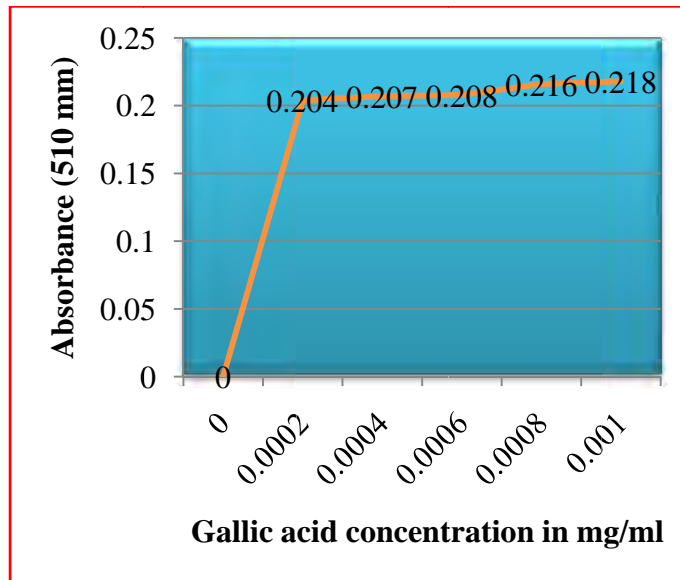
Appendix 9. Standard graph for phenol



Gallic acid standard curve for Phenol estimation

Absorbance verse gallic acid concentrationm graph which used to compared the faba bean leaf extract to estimate total phenol.

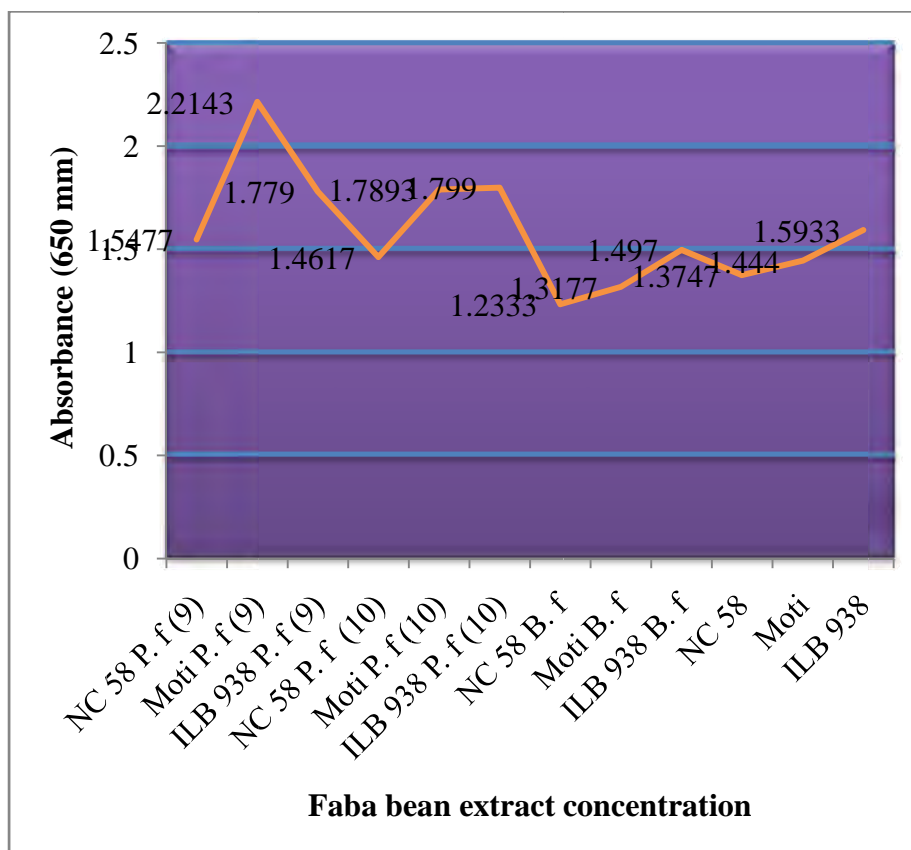
Appendix 10. Standard graph for flavonoids



Gallic standard curve flavonoids estimation

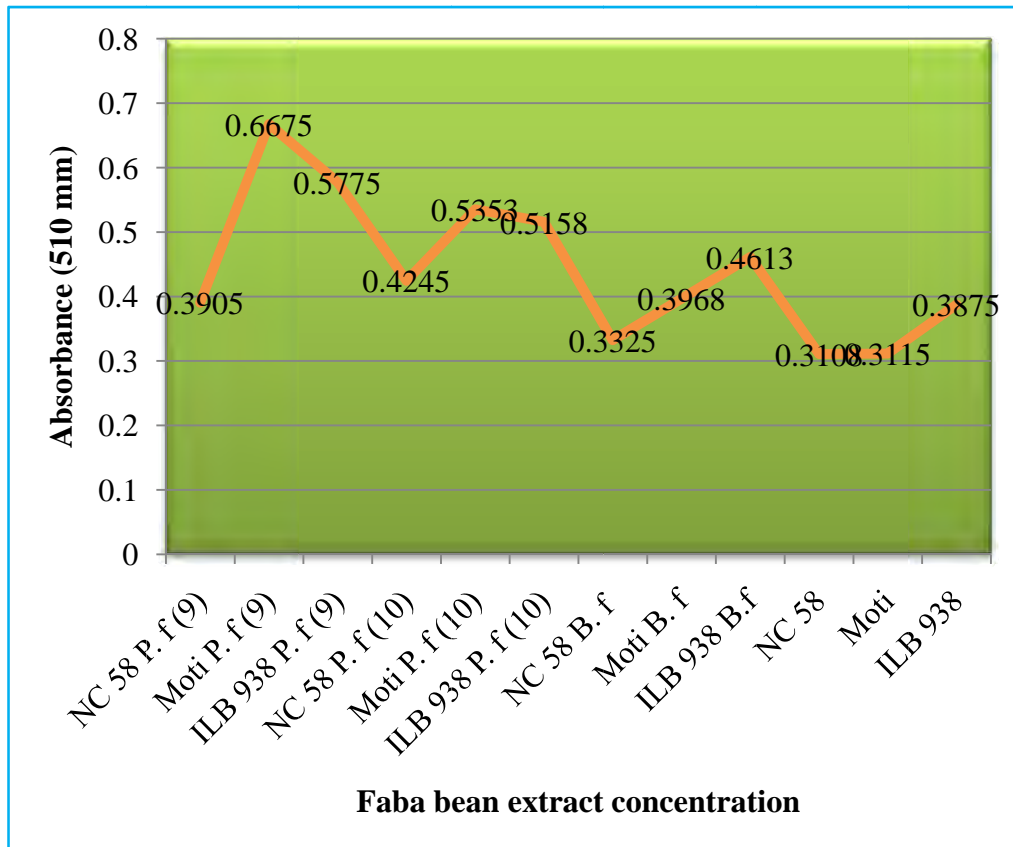
Absorbance verse gallic acid concentration graph which used to compared the faba bean leaf extract to estimate total flavonoids.

Appendix 11. Graph for faba bean extract absorbance verse extract concentration for phenol and flavonoids



Graph for bio-primed faba bean seed (NC 58, Moti and ILB 938) with P f9 and P f10, negative and positive control for determination of phenol

The graph was obtained after concentration of phenol in faba bean leaf extract was calculated with compared of standard curve.



Graph for bio-primed faba bean seeds with P f9 and P f10 and negative and positive control for determination of flavonoids

The graph was obtained after concentration of flavonoids in faba bean leaf extract was calculated with compared of standard curve.