EFFECT OF *Pseudomonas fluorescens* ON GROWTH OF MEDICINAL PLANTS AND ITS BIOCONTROL EEFECT ON SELECTED PHYTOPATHOGENS

A
Dissertation
Submitted to the **Department of Microbiology,**
Central Campus of Technology, Tribhuvan University,
Dharan, Nepal, in Partial Fulfillment of the Requirements for the
Award of Degree Master of Science in Microbiology
(Agriculture Microbiology)

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This is to certify that **Mrs. Januka Rai** has completed this dissertation work entitled "**EFFECT OF *Pseudomonas fluorescens* ON MEDICINAL PLANTS AND ITS BIOCONTROL EEFECT ON SOME PHYTOPATHOGENS"** as a partial fulfillment of the requirements of M.Sc. degree in Microbiology (Agriculture) under our supervision. To our knowledge this work has not been submitted for any other degree.

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CERTIFICATE OF APPROVAL

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors Mr. Siva Narayan Shah and Mr. Nabin Kumar Dangal for their support, patience, motivation, enthusiasm and immense knowledge and their expertise was invaluable in research writing. Without their support and continual inspiration, it would be impossible to complete my research and writing it.

I am highly thankful to respected teacher Mr. Suman Rai. His expertise was indispensable in choosing the topic in research and in particular methodology. Respectfully, I would like to express my sincere gratefulness to Asst Professor Mr. Hemanta Khanal, HOD of Microbiology, Central Campus of Technology, Dharan, Nepal, for their support throughout the dissertation work and also would like to thank Mr. Prajwal Bhandari and Mr. Ain Bahadur Karki, staff members of Central Campus of Technology.

I owe my special thanks to Nepal Agriculture Research Centre, Tarahara for providing necessary laboratory facilities. I would like to express my deepest appreciation to Miss Shasikala Rai and Mrs. Parbati Joshi Technical officer of RARS, for their direct and indirect support in lab work.

Further, I would like to thank; my seniors Mr. Pradip Rai, for his guidance in need, my colleague; Ms. Jyoti Limbu, Mr Bijay Shrestha, Mr. Jenish Shakya and Ms. Bidhya Dhungana for their regular support, help and inspiration for the completion of this work.

Finally, I must express my very profound gratitude to my parents, my daughter Miss Zawee Rai and my better half Mr. Bijay Rai for providing me unfailing support and continuous encouragement thorough my years of study. This accomplishment would not have been possible without them.

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ABSTRACT

Pseudomonas fluorescens is Plant Growth Promoting Rhizobacteria that aggressively colonize the root zone and promote plant growth are generally termed as Plant Growth Promoting Rhizobacteria (PGPR). *P. fluorescens* act as biocontrol agents as well as plant growth promoter as it produce different types of secondary metabolites likes iron chelating siderophores, hydrogen cynide, exoenzymes, phytohermones, antibiotic and helps to adopts the plants from various stressed conditions. They also protect plants from phytopathogens by controlling or inhibating them, improves soil structure, bioremediates that pollutes soil by sequestering toxic heavy metal species and degrading xenobiotic compounds like pesticides. The aim of study is to isolate and identify *P. fluorescens* from rhizospheric soil of Dhankuta, Sunsari, Morang, Jhapa and Illam to observe its effects on growth of medicinal plants and its biocontrol effect on some phytopathogens. The rhizospheric soil was collected from 5 districts of eastern Nepal, in sterile plastic bag and cultured in king's B media by serial dilution and incubated at 25°C for 48 hrs. Morphological, cultural, biochemical and physiological characterization was done and bacteria were identified. Isolated *P. fluorescens* were cultured on Kings B broth and inoculated on medicinal plants by deep root technique. In vitro effects on selected phytopathogens were observed on Modified Kings B media by dual culture method. It was found that marigold and mint inoculated with *P. fluorescens* was so healthy with increase in height, no. of branches, fresh and dry weight. Increament in height of marigold and mint was found to be around 31% and 28.9% comparing to that of uninoculated plants. Similarly, different isolates were found to suppress different plant pathogens being the D1 most potent in suppressing *S. rolfsii*, JF and DR being most potent in suppressing *R. solani* and *Alternaria spp* respectively. While other isolates were found to moderate potent.

Key words: PGPR, medicinal plants, phytohermones, siderophore

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LIST OF ABBREVIATIONS

PGPR	:	Plant Growth Promoting Rhizobacteria
PDA	:	Potato Dextrose Agar
NA	:	Nutrient Agar
BCA	:	Biological Control Agents
IP	:	Inhibition Percentage
DAPG	:	2, 4- diacetylphloroglucinol
HCN	:	Hydrogen Cyanide
KB	:	King’s B Agar
ISR	:	Induced Systemic Resistance
EO	:	Essentials Oil
IAA	:	Indole Acetic Acid
CMC	:	Carboxy Methyl Cellulose

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CHAPTER I

INTRODUCTION

Background

Plant pathogen refers to any of the organism, such as fungi, bacteria, protists, nematodes, and viruses that cause plant diseases. Among them fungi are most dominantthat over 20,000 fungi cause disease in plants (USEPA 2005). Plants pathogen affects the crop production, so pesticide is generally applied to overcome this problem. However, application of pesticide is often fail and even raiseseveral problems either in human life, environment or agricultural products (Gamliel et al 1997). Recently chemical pesticides has been used widely to improve the crop yield which influence the ecosystem, soil fertility, underground water and its residues remain in soil for many decades which cause environment pollution (Johnsen et al 2001 and Arias-Estevez et al 2008). There is rapid increase in ecotoxicity due to the extensive use of fungicides and pesticides (Chen et al 2015) and development of resistance to fungicides in plant pathogenic microbes (Dzhavakhiya et al 2012; Alghuthaymi et al 2015).

The possible solutions to eradicate these problems are biological control of plant pathogens by using extracts of the plants or Biological Control Agents like *Pseudomonas fluorescens*. Therefore, in twenty-first century biological control has been a significant approach to plant health management and biopesticides markets become more widereespecially in North America and Europe and predicted the growth rate of 10 % per year (Cook 2000, Bailey et al 2006). In the past three decades, numerous strains of fluorescent Pseudomonads have been isolated from the rhizosphere soil and plant roots by several workers and their biocontrol activity against soil-borne and foliar pathogens were studied (Ashrafuzzaman et al 2009). Soil inhabiting *Pseudomonas spp*. had been commonly used for biocontrol, promoting plant growth and bioremediation where 2, 4-diacetylphloroglucinol (DAPG)-producing strains were major group's in biocontrol microorganisms, because of their easy colonization, good competition and broad antimicrobial spectrum (Carroll et al 1995 and Cronin et al 1997).

The different mechanism through which biocontrol of plant disease can occur are antibiosis, competition, suppression, direct parasitism, induced resistance, hypovirulence, and predation (Johnson and Curl 1972; Chaurasia et al 2005). The antagonistic activity has often been associated with the production of secondary metabolites (Haggag and Abdel-latif 2007; Silva et al 2001). The role of siderophores produced by fluorescent pseudomonads in plant growth promotion was first reported by Kloepper et al (1981). The anti-fungal metabolite 2, 4-diacetyl phloroglucinol play a major role in the biocontrol capabilities of *P. fluorescens* (Delany et al 2000). In vitro evaluation of the *P. fluorescens* isolates also confirmed their antagonistic ability against both *Pyricularia grisea* and *Rhizoctonia solani* in dual culture tests (Ahmadzadeh et al 2004). *P. fluorescens* strain played a significant role in disease suppression of *Fusarium oxysporum* in tomato due to the production of HCN (Duffy et al 2003). Good antifungal activity against the plant deleterious fungi, viz., *Aspergillus niger, A. flavus, A. oryzae, F. oxysporum*, and *Sclerotium rolfsii*had been seen by *Pseudomonas* culture and purified siderophores (Manwar et al 2004). Development of resistance in cucumber against *P. syringae* pv. *lachrymans* as as well as to the fungal pathogens *Fusarium oxysporum f. sp. cucumerinum* and *Colletotrichum orbiculare* by application of *P. fluorescens* 89B-27 was reported (Liu et al 1995).

Medicinal plants are source of many effective and potent drugs which are used in many countries for their different therapeutic purposes (Mahesh and Satish 2008). More than 80 % of the world's population relies on traditional medicine for their primary health care needs; according to the World Health Organization (Shettyand Singh 1993; Gotoet al 1998). Some researchers have observed that volatile oils of many plants are known to have antimicrobial activity (Henikoff et al 1995). Many medicinal plants like marigold, mint, has multiple therapeutic effect and used widely as traditional medicine.

Multiple therapeutic effects of mint (*Mentha piperita*) like antioxidant capacity , antitumor activity on different cell lines, anti-allergenic activity, anti-viral activity with significant results on herpes simplex viruses (HSV-1 and HSV-2) and against human immunodeficiency virus-1 (HIV-1) , antibacterial activity against different bacterial strains, including Gram-

positive cocci and rods and Gram-negative rods, modulatory effects on hepatic and renal functions , nervous system actions as analgesic and local anesthetic, and anti-inflammatory actions has been proved in different in vivo and in vivo pharmacological studies (Sato et al 2003, Maliakal et al 2001 and Abiram 2004).

Before the discovery of marigold as medicinal plants it was grown as an ornamental plant for long times and thus, it began to be used as a medicinal plant. The growths of marigold plant began in 17^{th} century in Europe and have been introduced as drugs in some farmacopoeia and are used to cure stomach and intestine diseases (Stary 1991). In Germany, Australia, Czech, Austria, Switzerland, Hungary and recently in Egypt and Syria this plant is used as medicinal plants (Amodbeygi 2005). The pot marigold is used as sudorific, blood refiner, blood sugar reducer and also use as anti-inflammatory in skin (Khavarinejad and Lucia, 2004). Recently, this plant is used in registered homeopathic drugs. Its more and orange kinds are favorable medicinally, since they contain a lot of effective components. To improve the appearance of other medicinal substances, they use the Pot marigold flower's dark orange color (Zaman 2003). In France, they commonly use it brewed to lower body temperature and perspiration as an effective tranquilizer (Mir heydar 2003). Experiments done on the use of the sap of pot marigold laboratory animals have revealed a decrease in collestrol, anti-tumor effects and anti-cytotoxic. In traditional medicine, it is used as anti-spasm, anti-worm and diuretic (Salehi Sormaghi 2006).

Thus, Plant Growth Promoting Rhizobacteria (PGPR) is excellent measure for the better improvement in the growth of these medicinal plants. PGPR are beneficial soil bacteria, which may facilitate plant growth and development both directly andindirectly (Chernin and Chet, 2002). Direct plant growth promotion by microbes is based on improved nutrient acquisition, hormonalstimulation, production of plant growth regulators, and diverse mechanisms involve suppression of plant pathogens by the production of metabolites like antibiotics, siderophore, and so forth that decrease the growth of phytopathogens which is often indirectly connected with plant growth (Barazani and Friedman, 1999; Khalid et al 2004; Ashrafuzzaman et al 2009;

Bertland et al 2001; De Freitas and Germida, 1990; Husen 2003, Glick et al 1995, Kleoepper 2003). Microbial inoculants can be used as an economic input to increase crop productivity and maintain the sustainability of soil is suggested by many researchers (Solanki et al 2011). Bacterial synthesis of plant hormones including indole-3-acetic acid, cytokinin, and gibberellins as well as by increased mineral and nitrogen availability in the soil which help plant growth is triggered by PGPR colonization (Saharan and Nehra, 2011).

Among many PGPR genera, *Pseudomonas* has received the most research attention asit is widely distributed in various environments and easy to culture under laboratory condition (Palleroni et al 2005). Primarily *P. fluorescen*s is identified as an important organism with ability for plant growth promotion and effective disease management properties (Mazzola et al 1992). Interactions of PGPR with plants are exploited commercially and hold great promise for sustainable agriculture (Podile and Kishore, 2006). Applications of these associations have been examined in maize, wheat, oat, barley, peas, canola, soy, potatoes, tomatoes, lentils and cucumber (Gray and Smith, 2005). Hence, the main objective of this experiment is evaluation of antagonistic effect of *P. fluorescens* on selected phytopathogens and growth effect on selected medicinal plants.

1.2 Objective

1.2.1 General objectives:

To study the effect of *P. fluorescens* on growth pattern of some medicinal plants and biocontrol effects on selected phytopathogens.

1.2.2 Specific objectives:

1. To isolate and identify *P. fluorescens.*
2. To determine IAA production by *P. fluorescens.*
3. To determine HCN production by *P. flourescens*.
4. To observe the effects of *P. fluoroscens* on medicinal plants growths.
5. To study the potency of *P. fluorescens* as bio-controlling agent against selected phytopathogens.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction of *Pseudomonas flourescens*

Pseudomonas fluorescens is commonly, nonpathogenic saprophytes that colonize soil, water and plant surface environments. It is a common gram negative, rod-shaped bacterium. As its name implies, under conditions of low iron availability, it secretes a soluble greenish fluorescent pigment called fluorescein. It is an obligate aerobe, except for some strains that can utilize NO_3 as an electron acceptor in place of O_2. Multiple polar flagella facilitate its motility. *P. fluorescens* has simple nutritional requirements and grows well in mineral salts media supplemented with any of a large number of carbon sources (Palleroni et al 1984). Certain member of the *P. fluorescens* have been found to be potential agents for the biocontrol as they inhibit plant diseases by protecting the seeds and roots from fungal infection and promotes plant growth (Hoffland et al 1996). Rapid colonization of rhizosphere by *P. fluorescens* may be the mechanism to control pathogenic microorganisms (Hass et al 2005).

2.2 Taxonomic classification

The taxonomic classification of *P. fluorescens* and soil borne phytopathogens used as model organism in this study are listed in Appendix I.

2.3 Morphological characters

Pseudomonas fluorescens is a Gram-negative, rod-shaped bacterium that belongs to the *Pseudomonas* genus. *P. fluorescens* has multiple flagella. It has an extremely versatile metabolism, and can be found in the soil and in water. It is an obligate aerobe, but certain strains are capable of using nitrate instead of oxygenas a final electron acceptor during cellular respiration. Optimal temperatures for growth of *P. fluorescens* are 25-30°C. It tests positive for the oxidase and catalase test. It is also a nonsaccharolytic bacterial species. On King's B medium, greenish yellow color is highly appeared around the cream

colony with smooth edge, convex, glistening, entire, circular, and fluorescent under the sun light that was wider than colony on nutrient agar.

2.4 *Pseudomonas* as Biocontrol Agents

Pseudomonas affects plants growth in two different ways, directly or indirectly. Directly they promote the plant growth by providing the plant phytohormones, or facilitating the uptake of certain nutrients from the environment and the indirect promotion of plant growth occurs when they suppress or prevent the deleterious effects of one or more phytopathogenic organisms. This can happen by producing antagonistic substances or by inducing resistance to pathogens (Glick 1995). According to Beattie, biocontrol agents are those bacteria that reduce the incidence or severity of plant diseases whereas antagonists are those that exhibit antagonistic activity towards phytopathogens (Beattie 2006).

Production of antimicrobial compounds, production of siderophores, and competition with disease-causing microbes for niches and nutrients are different biocontrol mechanisms adopted by *Pseudomonas* spp. (Haas and Keel 2003; Haas and Defago 2005; Morales et al 2010; Ozyilmaz and Benlioglu 2013; Weller 2007). The disease caused by *Gaeumannomyces graminis* var *tritici* was found to be inhibited by antibiotic phenazine-1-carboxylic acid produced by *P. fluorescens* 2-79 (Imran et al 2006). An antibiotic compound, pyrollnitrin produced by *P. fluorescens* inhibited growth of *M. phaseolina* by producing an inhibition zone of 12 mm (Karunanithi et al 2000). Soil borne plant pathogens in the rhizosphere was found to be suppressed by the 2.4-diacetly phloroglucinol by strain CHA0 *P. fluorescens* (Kell et al 1992*).* Additional mechanism for biocontrol properties of *P. fluorescens* is its ability to activate cell wall degrading enzymes of plant pathogenic fungi should be focused (Borowicz et al 1992).

Siderophore production of fluorescent *Pseudomonas* strains and antibiotic like substance production in non-fluorescent strains is also releated to biocontrol activity (Cassinelli et al 1993). Fungistatic properties of *P. fluorescens* against *F. oxysporum* not only depend on the biological properties and age of the bacterial culture but also susceptibility of the fungus to bacterial metabolites

(Grata K 2006). Banana plants pretreated with Fluorescents Pf10 before inoculation were protected from Fusarium wilt caused by *Fusarium oxysporium (*Thangabelu et al 2001). Seedlings of rice showed resistance to *X. oryzae* pv. *pryzae* and the disease incidence decreased from 6.8–1.2, when seed were treated with the formulation of *P. fluorescens* Pf1 before sowing, at sown and at 30 days (Vidhyasekaran et al 2001). Biocontrol of wilt diseases caused by *Fusarium oxysporum* has been indicated by competition of siderophores of fluorescent pseudomonas and pathogen for iron (Kloepper et al 1980 and Scher et al 1980). Pigeon pea wilt disease was controlled by *P. fluorescens* alone or in combination with pesticides where *P. fluorescens* alone increased plant growth, nodulation, phosphorus content and decreased nematode multiplication and wilting in infected plants (Siddiqui et al 1998).

Fungal population in damping off by *P. ultimum* was significantly reduced by *P. fluorescens* Pf 5-014 and its mutant strain 5-214 in *in-vitro* culture (Hultberg et al 2000). In Algeria, significant antagonism against *F. oxysporum* f. sp. *lycopersici* in tomato by *P. fluorescens* was demonstrated in pot culture trials (Botero Ospina & Aranzazu Hernandez 2000). The population of *P. appomidermatum* casual agent of damping off on tomato was decreased along with the increased shoot, root length and dry weight of seedling (Manoranjitham & Prakasam 1999). Soil application of talc-based formulation of *P. fluorescens* was found to control wilt disease in cauliflower effectively, caused by *F. moniliformae* under field conditions (Rajappan and Ramaraj 1999). Larger plant stands and reduced seedling disease symptoms caused by *P. ultimum* and *R. solani*, on surviving plants of cotton was found by application of *P. fluorescens* strain EG1053, in both potting mix with amended pathogens and naturally infected cotton soils (Hagedorn et al 1990).

During In-vitro test, mycelial growth and sclerotial production was notably reduced in *Sclerotium rolfsii* was observed which was the main casual agents of the stem rot in tomato. Induction of systemtic resistance against *R. solani* on *Pseudomonas* treated rice cv.IR50 was developed which resulted on the increase in chitinase and peroxidase activity (Nandakumar et al 2001). Biological control activity of *P. fluorescens* strain RPB14 against major diseases of *Phaseolus vulgaris* cv. was observed both in in-vitro and under

field conditions resulting in the increase in seeed yield by 81.3% over control in Himachal Pradesh (Mondal et al 2004). Prevention of *Fusarium oxysporum*, *Septoria tritici*, *Thielaviopsis basicola*, *Rhizoctonia solani* etc by *P. fluorescens* and its production 2, 4-diacetylphloroglucinol (DAPG) was repored (Keel et al 1992 and Wei et al 2004).

Thus biological control of plant diseases using these beneficial microorganisms can significantly reduce the disease occurance and has been extensively investigated from both, basic and applied points of view (Cheng et al 2010; Schisler et al 2004; Wang et al 2010). Among these beneficial microorganisms, *Pseudomonas* spp have received much recognition as effective biocontrol agents against a broad range of soil-borne diseases (Kim et al 2004; Weller 2007; Whipps et al). Many previous attempts made to search an ideal rhizobacteria with biocontrol activity frequently resulted in the selection of *Pseudomonas* spp. as effective organisms for the suppression of plant fungal diseases (Sang et al 2013).

2.5 *P. fluorescens* as PGPR

Pseudomonas fluorescens is mainly identified as Plant Growth Promoting Rhizobacteria (PGPR) which assertively colonizes the root zone and promote plant growth and has effective disease management properties (Mazzola et al 1992*). P. fluorescens* strain was also found to reduce the use of chemical N, P, and K fertilizer rate (Shaharona et al 2008) as they has ability to produce different secondary metabolites, hydrogen cynide, enzymes and phytohermones, so it is considered as biological control agents against various plants related diseases including root disease (Ursula et al 2000). *P. fluorescens* decrease the use of chemical fertilizers and pesticides in the cultivation plants by promoting plant growth, nutrients uptakes and producing phytochemicals constituents and also protects plants against various soil borne pathogens (Banchio et al 2008).

The role of plant growth promoting rhizobacteria (PGPR) have been extensively studied as biofertilizers to increase the yield of agronomically important crops such as wheat (Khalid et al 2004), corn (Mehnaz and Lazarovits, 2006). Production of indole acetic acid responsible for increasing

root and plant growth promoting activity on rice cultivar ADT by 36 selected isolates of *P. fluorescens* increased the germination of rice seeds along with the induction of the plant growth promotion under in vitro conditions. Among them Pf13 was recorded to have maximum germination, increased the shoot length; root length and vigour index (Dowling et al 1994 and Meera et al 2012). Increase in leaf area and number and shoot and dry weight in pasteurizeed root was observed on blueberry (*Vaccinuim corymbosum)* inoculated with *P. fluorescens* strain Pf 5 and increase in copper and phosphoras uptake was observed by PRA strain (Silva et al 2000). Two strains of *Pseudomonas* GRP3 and PRS9 were inoculated in wheat (*Triticum aestivum*) and enhancement on the plant growth in terms of root-shoot length and weight was observed (Kita et al 2004).

Growth traits and seed yield in chickpea was more effective when *P. fluorescens* was inoculated with microbial fertilizers (Rokhzadi et al 2008). Methi *(Trigonella foenum*) inoculated with *P. fluorescens* was found to have increased dry weight and seed germination (Ratan et al). There was positive effects on fresh and dry weight along with significant increase in strawberry yield were obtained by *P. fluorescens* inoculation which is again moderately increased by use of fungicides (Haggag et al 2012). Reduced water stress and increased plants growth and tropane alkaloids in *H. niger* (chrismas rose) was found by the inoculation of *P. fluorescens* whereas in tomato, remarkable growth of root and shoot weight, length, fruit yield per plant of was recorded in pot culture and field trial (Ghorbanpour et al 2013 and Ahirwar et al 2015). Inoculation of *P. fluorescens* on maize with poor phosphorus level resulted in the increase in plant growth and phosphorus level which conluded the application of *P. fluorescens* on phosphorus poor soil (Krey et al 2013). In this regard, the use of *P. fluorescens* (PGPR) has depicted potential in developing sustainable agriculture system for crop production and protection (Govindasamy et al 2011). Significant higher fresh weight (root and shoot) were observed in Sarpagandha inoculated with *P. fluorescens* as compaired to uninoculated plant which suggest that increament of biomass compaired to other in medicinal plants (Mulla et al 2013).

2.6 Mode of Action

P. fluorescens control different plant pathogens by many different types of mechanism which are explained below.

2.6.1. Microbial Antagonism

Antagonistic activities can be heighlighted by the synthesis of hydrolytic enzymes like chitinases, glucanases, proteases, and lipases that can lyse pathogenic fungal cells (Neeraj et al 2010; Maksimo et al 2011), competition for nutrients and habitat (Stephens et al 1993; Kamilova et al 2005; Validov S 2007), regulation of plant ethylene levels through (Glick and Bashan 1997; Van Loon 2007) and the production of siderophores and antibiotics.

2.6.2 Antibiotics

The biocontrol abilities of *Pseudomonas* strains mainly depend on initiation of systemic resistance in the plant, competitive root colonization and production of antifungal antibiotics (Hass and Keel 2013). The ability of plant growth-promoting bacteria to act as antagonistic agents against phytopathogens is mainly related with the production of antibiotics (Glick et al 2007). In past two decades, the biocontrol of target plant pathogens by the secretion of molecules like antibiotic has been better understood (Dowling and O Gara 1994; Whipps 2001; Lugtenberg and Kamilova 2009). Damping off causing *Rhizoctonia solani* in cotton plants have been cotrolled by the pyrrolnitrin, antibiotic produced by the *P. fluorescens* BL915 strain (Hill et al 1994).

Recently, due to the potential positive effect of lipopeptide biosurfactants produced by *Pseudomonas* and *Bacillus* species on competitive interactions with organisms including bacteria, fungi, oomycetes, protozoa, nematodes and plants, they have been applied in biocontrol (De Bruijin et al 2007; Raaijmakers et al 2010). Phenazine produced by *Pseudomonas* possesses redox activity and can suppress plant pathogens such as *F. oxysporum* and *Gaeumannomyces graminis* (Chin A Woeng 2003).

2.6.3 Siderophore

Along with the production of antibiotics *Pseudomonas* also secretes the low molecular weight iron chelators siderophores to fulfill the nutritional requirements of iron that solubalized iron from their surrounding environments, forming a complex ferric-siderophore that can move by diffusion and be returned to the cell surface (Andrews et al 2003). The major reason in the investigation of PGPR is the ablility of *Pseudomonas* to produce siderophore and metabolites that has remarkable agronomic importance in the inhibition of phytopathogens (Maksimov et al 2011). Siderophore production by different microorganisms play a major role in determining the ability of them to enhance the plant growth and development in soil which by increasing the iron uptake by plants (Masalha et al 2000; Dimpka et al 2009) and also helps in the iron uptake by plants in the presence of other metals such as nickel and cadmium (Burd et al 1998; Dimpka et al 2008).

*Pseudomonas p*rovides competitive advantages by colonizing roots and excluding other microorganisms from this ecological niche by the production of siderophore (Hass and Defago 2005). Pyoverdin produced by *Pseudomonas* is example of potent siderophore that inhibit the growth of bacteria and fungi that present less potent siderophores in iron-depleted media in vitro (Kloepper et al 1980). Recently, iron-chelating siderophores by fluorescent *Pseudomonads* have been investigated for the suppression of soil-borne fungal pathogens through the release of them, making iron unavailable to other organisms (Loper 1998; Dwivedi and Johri 2003).

2.6.4 Bacteriocins

Along with above, production of bacteriocins is another defence mechanism of microorganism which differs from traditional antibiotics in one critical way that they commonly have a relatively narrow killing spectrum and are only toxic to bacteria closely related to the producing strain (Riley and Wertz 2002). They have been shown to inhibit disease by inducing a resistance mechanism in the plant called “Induced Systemic Resistance” (ISR) (Van Loon et al 1998) which is the state of increment in defensive potency developed by plants when appropriately stimulated (Van Loon et al 1998). ISR

was formerly introduced observed in carnation plants which was systematically protected by *P. fluorescens* WCS417r against *F. oxysporum* f. sp. *Dianthi* in carnation plants that was systemically protected by the *P. fluorescens* strain WCS417r against *F. oxysporum* f. sp. *Dianthi* (Van Peer et al 1991) and in cucumber plants, where rhizobacterial strains protected the leaves from *Colletotrichum orbiculare* causing anthracnose (Wei et al 1991).

2.7 Soil Borne Phytopathogens used as Model Organisms

Phytopathogens pose serious problems worldwide in agriculture and food industry which considerably reduce the crop yield worlwide. In addition, many also produce mycotoxins, which are harmful to humans and livestock and cause a number of diseases such as rusts, smuts, rots, downy mildew and may cause severe damage to crops. Outbreak of crop disease depends on the presence of pathogen and complexity of tha soil environment in which pathogen propagules reside. Many pathogens form protective structure to adopt harsh conditions that can survive afor a years and thus remain as the source of infection for very long time (Bruehl 1987). This dissertation focuses on the application of *P. fluorescens* in biological control of following phytopathogens.

2.7.1 *Rhizoctonia solani*

Rhizoctonia solani, the most widely recognized species of *Rhizoctonia* was originally described by Julius Kuhn on potato in 1858, is soilborne plant pathogen with considerable diversity in cultural morphology, host range and aggressiveness. *R. solani* is a basidiomycete fungus that does not produce any asexual spores (called conidia) and only occasionally will the fungus produce sexual spores known as basidiospores. In nature, *R. solani* reproduces asexually and exists primarily as vegetative mycelium and sclerotia. Unlike many basidiomycete fungi, the basidiospores are not enclosed in a fleshy, fruiting body or mushroom. The sexual fruiting structures and basidiospores were first observed and described in detail by Prillieux and Delacroiz in 1891. *R. solani* mainly attacks below ground plant parts like the seeds, hypocotyls, and roots, but is also capable of infecting above ground plant parts like pods, fruits, leaves and stems. The most common symptoms is known as "damping-

off" which is mainly characterized by non germination of severely infected seed whereas infected seedlings are killed before or after they emerge from the soil.

Worldwidely, it is recognized as pathogen to number of plant species including the economically important crops rice, soybean, potato, maize, sugar beet, cabbage, cauliflower, tomato, and lettuce. A number of diseases and pests have attained serious proportions due to the intensive and extensive cropping system along with high yielding crop varieties among them sheath blight of rice caused by *R. solani* is one of them which is transmitted easily in rice (Lee and Rush, 1983; Gangopadhyay and Chakrabarti, 1982). No any variety is known to show complete resistance to *R. solani* (Oard et al 2004; Zuo et al 2010; Srinivasachary et al 2011 and Liu et al 2013). In survey of 6,000 rice cultivars from 40 countries no cultivar was found to have major gene for sheath blight resistance and only partial resistance to ShB was observed (Hashiba 1984).

2.7.2 *Sclerotium roflsii*

Sclerotium roflsii is a very common soil borne fungus infecting a wide range of vegetables, fruits, crops and ornamental plants producing abundant white mycelium on infected plants and in culture. The fungus was named *Sclerotium rolfsii* by Saccardo in 1911. It most active during warm, wet weather in tropical and sub tropical ragions. Advancing mycelium and colonies often grow in a distinctive fan-shaped pattern and the coarse hyphal strands may have a somewhat ropy appearance. Cells are hyaline with thin cell walls and sparse cross walls. Main branch hyphae may have clamp connections on each side of the septum (Aycock 1996). The fungus can cause the rots of the lower stem, root and crown. It can also cause of the rots of the fruits in contact with soil.

Peter Henry Rolfs found the unknown fungus causing tomato blight in 1992 and sent the specimens to Italian mycologist Pier Andrea Saccardo in 1911 who first described the Southern blight disease caused by *S. rolfsi* on wide varety of plants. It has wide host range at least 500 species in 100 families and can be very destructive to numerous vegetable and fruit crops, especially

tomato, pepper, melon, and watermelon (Farral et al 1989 and Mullen 2001). Out of them most common hosts are the legumes, crucifers, and cucurbits (Punja 2005). When inoculum levels are high southern blight is difficult to manage and conditions are conducive to the pathogen. Crop rotation of two years or more to non host crop, selection of fields free of *S. roflsii* are the some methods to solve problem of these disease (Robert et al 2014).

Recently, many research projects involving chemical, biological agents, cultural, soil amendments, disease physiology, nutrition studies and resistant variety is major investigation for many researchers (Singh and Dwivedi, 1991). Dispite of these attempts *S. rolfsii* is difficult to control because of their wide host range, and their capibility to produce insistent sclerotia provide huge economic losses related with the pathogen (Singh 1991).

2.7.3 *Helminthosporium turcicum*

Helminthosporium turcicum is soil borne phytopathogen that causes a foliar disease known as the Northern corn leaf blight (NCLB) was reported by Passeriniin 1876 in Perma. It ia also known as *Exserohilum turcicum*, has characteristic cigar-shaped lesions which starts first as small elliptical spots on leaves, grayish green in color and water soaked lesions. The spot turns greyish with age and get bigger in size, finally attaining a spindle shape. Heavily infected field presents ascorched appearance (Chenulu and Hora 1962). Significant yield loss is observed in susceptible corn hybrids by this pathogen (Welz 2000).

Out of several specific host of *H. turcicum*, corn is the most economically important host, along with other forms like sorghum, Johnson grass, or sudangrass (Smith et al 2004). The most common diagnostic symptom of the disease on corn is cigar-shaped or elliptical necrotic gray-green lesions on the leaves that range from one to seven inches long (Vieira et al 2014*)* lesion number increases and all leaves are covered as the disease is progressed (Reddy 2012).

2.8.*4 Fusarium oxysporium*

Fusarium is a soil fungi which is world widely distributed. Some species like *F. oxysporium*, *F. solani* has an economic importance due to their distructive impact on crops (CANNA 2018). *F. oxysporium* has many restricted form that infect a wide variety of host plants causing various diseases. Though *F. oxysporium* though being soilborne has been outlined being transmitted through seeds (Haware et al 1978). *F. oxysporum* has the capability to survive in most soil frosty, tropical, desert, cultivated and non-cultivated soil (Snyder et al 1940).

The disease is recognizable in death of young seedlings within 25 to 30 days after sowing to wilt of adult plants (Haware et al 1978). *F. oxysporum* affects a wide variety of hosts of any age. Along with the herbaceous plants, *F. oxysporium* mostly infect tomato, tobacco, legumes, cucurbits, sweet potatoes and banana (Netzwerk 2010). The growth of fungus within plant's vascular tissue affects the water supply which results in the lack of water in leaves resulting in the closing of stomata and the leaves wilt which eventually leads to death of plants (Agrios 1988). Though, the vascular wilting being the major symptom, chlorosis, necrosis, premature leaf drop, browning of the vascular system, stunting and damping-off are also generally observed by *F. oxysporium* infection (Agrios 1998). Initially fusarium wilt observed like vein clearing on the younger leaves and drooping of the older lower leaves, which is followed by inhibiting in growrh, yellowing of the lower leaves, defoliation, marginal necrosis and plant death whereas on older plants, symptoms are more distinct between the blossoming and fruit maturation stages (Gerlach 1982).

2.7.5 *Alternaria spp.*

Alternarai is opportunistic, saprophytic soil pathogen which cause leaf spot, rot and blight in many plants producing toxins (Rotem 1994). The genus *Alternaria* includes many saprophytic and endophytic species. It was first described by Nees in 1816 with *Alternaria tenuis* as the only species, which was later renamed as *Alternaria alternata* (Meena et al 2010). The genus *Alternaria* cause diseases on agronomically important plants like

cereals,ornamentals plants, oilcrops, vegetables and fruits (Rahman et al 2002, Thomma 2003; Agrios 2005; Raja et al 2006; Meena et al 2010). They mostly attacks the aerial parts of its host plants and as the disease progress, the circular spot grows to 0.5 inch or more in diameter and usually gray, gray tan, or near black in colour. The pathogens mostly grow in concentric rings without uniform growth rate which is result of fluctuating environmental conditions whereas host having larger leaves allow unrestricted symptom (Laemmlen 2001). Tomatoes are more susceptible to early blight caused by *Alternaria* showing the early symptoms of yellowing and browning of lower leaves followed by development on the leaf tips and along the margin of leaf petiole. Finally the entire leaves are covered in diseased tissue and the leaves fall off (K.P et al 2004).

Alternaria leaf blight of sunflower being the most harmful disease, has been reported to cause a reduction of up to 80% and 33% seed and oil yield, respectively (Calvet et al 2005). Similarly *Alternaria helianthi* is observed as the major cause of Alternaria leaf spot of sunflowers (Westhuizen and Holtzhausen, 1980; Allen et al 1983).

2.7.6 *Fusarium solani*

F. solani is the most important ubiquotous soil-borne fungal pathogens, which develop in both cultured and non-cultured soils, causing the diseases like damping off and root rot diseases in a wide range of vegetables and crop plants including tomato (William 1980).

Mainly citrus fruits like grapes fruits, orange, lemon etc. are infected by *F. solani* which was first isolated from citrus root by Sherbakoff in 1953. The symptoms show the sudden wilt of healthly citrus plant's leaves and turn into yellow followed by death; roots become mlack and rot with brown color, vascular discoloration in stem, drying of fruits which remain on tree (Olsen et al 2000).

2.8 Medicinal Plants Used

Traditionally, plants derived medicines have been used worldwidely in treating of numbers of human diseases for centuries (Chiariandy et al 1999). Harbal product has been major part of traditional medicine systems that have improved our modern knowledge of herbal medicine (Abu et al 2003). Different secondary metabolites, essential oil composition present in medicinal plant determine the potency of herb which is dependent upon the biotic and abiotic factors during its cultivation (Juliani et al 2006). Different medicinal plants used in this research are given below.

2.8.1 Mint

Mint (Mentha piperita) a medicinal plant of Labiatae family, which was commonly used as a local anesthetic agent in cold and cough preparation and liniments for insect bites, eczema, hemorrhoids, toothaches, and musculoskeleton pain (Murray 1995 and Peirce 1999). Powder of mint or its essential oil is used in many food, cosmetics and pharmaceutical product. The active constituents and secondary metabolites depends on the variety, geoghraphical region and processing conditions (Ruizdel et al 2003; Pino et al 2002). Menthol, which isessential oil of mint is responsible for antibacterial activities, digestive system disorder like stimulation of bile flow, facilitates belching and reduces tone in the esophageal sphincter (Fleming 1998; Tyler 1992).

Traditionally, many differents types of digestive complaints like diarrhea, indigestion, nausea and vomiting, morning sickness and anorexia, colic in infants and to reduce gas and cramping were used to be treated by minthus spp. It is also used to treat irritable bowel syndrome (Grigoleit et al 2005). Different antibacterial, antifungal, antiviral, insecticidal and antioxidant properties have been shown by the essential oils of peppermint along with the promising radio protective effects for cancer patients undergoing treatment (Burt 2004 and Baliga & Rao, 2010). Its positive effect against nasea and vomiting in the first few month of pregnancy has been proved clinically (Westfall 2004).

In recent years, mint has been recommended for treating obesity as it has been proven to aid digestion of fats whereas mint tea is also taken asa strongly diuretic (Abbaszadeh et al 2009). Secondary metabolites and its essentials oil of mint is the major reason for its medicinal value which also depends on its biomass. The increase in shoot fresh weight, glandular trichome number, ramification number, root dry weight and essentials oil without alteration in the oil composition has been observed in the mint plants directly inoculated with the native *Pseudomonas* strains in comparison with controls which indicate the clear potentials as bio-inoculants for improving productivity of aromatic and medicinal plants (Santoro et al 2015).

2.8.2 Marigold

Snce 12th century, marigold has been cultivated and used as herbal medicine by Egyptians, Greeks, Hindus and Arabs. In Europe, it was cultivated as ornamental flower in the kitchen garden. Traditionally, it was taken internally to treat fevers, promote menstruation and treat jaundice where as for external use flowers were made into extracts, solutions, balms and salves and applied directly to the skin to help recover wounds and to relieve inflamed and damaged skin.In-vitro flower tincture also showed antiviral activity by suppressing the replication of influenza APR-8, influenza A2 and herpes simplex virus (Silva et al 2007). Marigold has also various anti-bacterial and anti-fungal activities (Rossiter et al 2006; Tonks et al 2007) and has been used for the treatment of abrasions, burns, ulcers, skin inflammations, eczema and wounds (Schulz et al 2004). The potency of marigold against differents disease depends upon the, biomass, secondary metabolites, essentials oil etc present in marigold and it was observed that essential oils yield has been increased by 70% by inoculation of *P. fluorescens* in comparision with control uninoculated marigold plants (Cappellari et al 2013)

CHAPTER III

MATERIALS AND METHODS

This study was carried out at the department of Plant Pathology, Regional Agricultural Research Station, Nepal Agricultural Research Council, Tarahara, Sunsari, Nepal. The laboratory techniques were according to the standard methods.

3.1 Materials Required

The materials, equipments, culture media and reagent used and their application in this study are systematically accounted in Appendix II.

3.2 Soil Sampling

50 soil samples from the rhizosphere of plants were collected randomly from Dhankuta, Sunsari, Morang, Jhapa and Illam, keeping 10 samples from each district for the isolation of *Pseudomonas fluorescens*. The samples were collected from the depth 5-10cm below the ground. The samples were collected from particular field in separate sterile polythene bag lebeled respectively and transported within 1 hrs as possible in lab and in ice bag from long distance fields.

3.3 Isolation of *Pseudomonas fluorescens*

10 gram of soil sample was added to 90 ml of sterilized distilled water to make a dilution of 10^{-1}. Five folds serial dilution of each soil samples were prepared and 0.1 ml of each sample were poured inenriched culture media King's B (Rai et al 2013) contained in a sterile petriplates and spread uniformly with the help of L–shaped dolly rod by spread plate technique. The petriplates were incubated at 27 ± 2^{0}C for 24-48 hrs. The isolated colonies were observed after incubation & colony characters were recorded and then subcultured on Kings B media. They are then subjected to different biochemical tests.

3.4 Identification of *P. fluorescens*

The test isolates were identified on the basis of cultural characterization, microscopic examination, bio-chemical tests and physiological test (Bergey 1939). Colony character includes green pigment producing colony whereas microscopic examination include Grams staining. Gram negative rod shaped bacteria was proceed for physiological test which was done by incubating the inoculated plates in 42^0C and 25^0C for 2 days which differentiated *P. fluorescens* from *P. aeruginosa* and those colonies that showed growth at 42^0C were excluded and only that showed negative growth at 42 ^{0}C and positive at 25^0C was subjected for further biochemical tests. Bio-chemical tests include oxidase test, catalase test, IMViC, nitrate reduction, starch hydrolysis and gelatin hydrolysis. Finally, *Pseudomonas fluorescens* was identified.

3.5 PGPR Characters

3.5.1 Indole acetic acid production

Qualitative analysis of indole acetic acid was done by inoculating *P. fluorescens* in NB amended with 0.5gm/l L-tryptophan and incubating at 27^0C in rotatary shaker at 150 rpm for 2-3 days. Cell free supernatants were then prepared by centrifuging the broth at 5000 rpm for 15 min at 4^0C. 1ml of Salkoski's reagent with 1 ml of cell free broth and 2 drops of orthophospheric acid was added and kept in dark for 20 min in 27 ± 1^0C. Then pink color was noted qualitatively. Those isolates producing highest amount of pink color was selected for plant inoculation.

3.5.2 Hydrogen cynide production

HCN production was detected by inoculating the bacteria on modified nutrient agar media amended with 4.4% glycine/litre according to Lorck (1948). Whatman filter paper no.1 previously soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the lid of the Petri dish and sealed with parafilm to air tight. HCN production was indicated by color orange to brownish red.

3.6 Isolation of plants pathogens

The diseased plant samples showing typical wilt or disease symptom were collected from field and brought to the laboratory, washed rapidly with sterile water. Thereafter, small pieces of diseased portion along with healthy portion were cut using sterile blades and surface sterilized in 1% sodiumhypochloride for 5 min followed by thorough rising in sterile distilled water, three times. The surface sterilized pieces were then inoculated aseptically in a plate containing sterile PDA. The plates were then incubated at 27 ± 2^0C for 24-72 hrs. After 72 hrs, the mycelium growing from the margin of diseased portion was subcultured on fresh PDA plate. In this way pure culture of phytopathogens were isolated.

3.7 Purification and Identificationof Phytopathogens Isolates

The cultures of phytopathogenic fungi were purified by frequent subculture of mycelium by hyphal tip method as described by Nelson P.E (1982). Isolated fungal pathogens were identified according to their morphological character and stored at 4^0C until use.

3.8 Screening of Antagonistic Effect *Pseudomonas* against Phytopathogens by Dual Culture

P. fluorescens was selected for antagonistic effect on the basis of HCN production. Four *P. fluorescens* isolates D1, DT, DR and JF were tested for their potential to antagonized six pathogens viz., *Fusarium oxysporium, Fusarium solani, Exerohilium turcum, Rhizoctonia solani, Sclerotium roflsii and Alternaria alternate* using in-vitro dual culture method.

To test the antagonistic effect of the above phytopathogens and *P. fluorescens* in vitro studies were conducted by adopting the standard methods. Loopful of bacterial culture was placed (5mm in diameter) at one edge on the periphery of modified Kings B media plate supplemented with 2% sucrose and mycelial discs (5mm in diameter) were cut from actively growing 5 days old fungal culture and placed opposite to the bacterial inoculation on plate (Ganesan and Gnanamanickam 1987; Podile et al 1988; Babu et al 2000). In control plate, only fungal pathogens were placed at one edge of petriplate without any

bacterial isolate. The assays of dual culture interaction were conducted in triplicates in Completely Randomized Design. The radius of mycelia growth in treatment and control plate was measured for five days.

The inhibition percent of mycelial growth of the pathogens was calculated using the following formula (Perveen and Bokhari 2012)

$$I = \frac{C-T}{C} * 100\%$$

Where, I = Inhibition percentage (%) or antagonistic effect, C = colony diameter of test fungus in control plate and T = colony diameter of the same test fungus in dual culture against Pseudomonas as antagonist. All four isolates of Pseudomonas were tested in vitro for their antagonistic activity against test plant pathogens.

3.9 Application of *P. fluorescens* for PGPR effects

Five isolates of *P. flourescens* one from each districts were selected for inoculating on plants root on the basis on Indole acetic acid production. D1 (Dhankuta), S1 (Sunsari), M3 (Morang), JF (Jhapa) and IM (Illam) were selected.

3.9.1 Inoculums Preparation

The pure colony of *P. fluorescens* isolates were grown in KB broth for 48 hrs on rotating shaker (150 rpm) and centrifuged at 10,000 rpm for 5 min. The pellet was collected aseptically and mixed with sterile carboxy methyl cellulose (CMC) suspension of 10gm/l which work as adhesion for bio-coating the root of medicinal plants.

3.9.2 Bio-priming of Plant's Root

Seedlings of marigold and mint plants were brought which were of equal height, weight and age. Root surface was treated with sodium hypochloride solution for sterilizing the root surface plants and then washed with distilled water. Sterilized roots of plants were then placed in bacterial carboxymethyl cellulose suspension for about 30 min. The control plants were placed in distilled water. The bio coated seedling will be transplanted into pot.

3.9.3 Potting

Sterile soil with equal volume of compost was filled in sterile pot. The bio-coated seedlings were transplanted into pot making four replicates per treatment. One plant per pot was maintained. Control plants were dipped in sterile distilled water.

Result was observed for 60 days. The observations with respect to the growth parameters such plant height, number of branches etc was recorded at the 15 days intervals of plant growth. Plants height, root length, fresh and dry weight, was measured and compared with control plants after 60 days.

4.0 Data Analysis

Data was analyses by using Microsoft Excel and Statistical Tool for Agriculture Research (STAR). Mean comparisions were done using Tukey's Honest Significant Difference (HSD) Test.

RESEARCH DESIGN

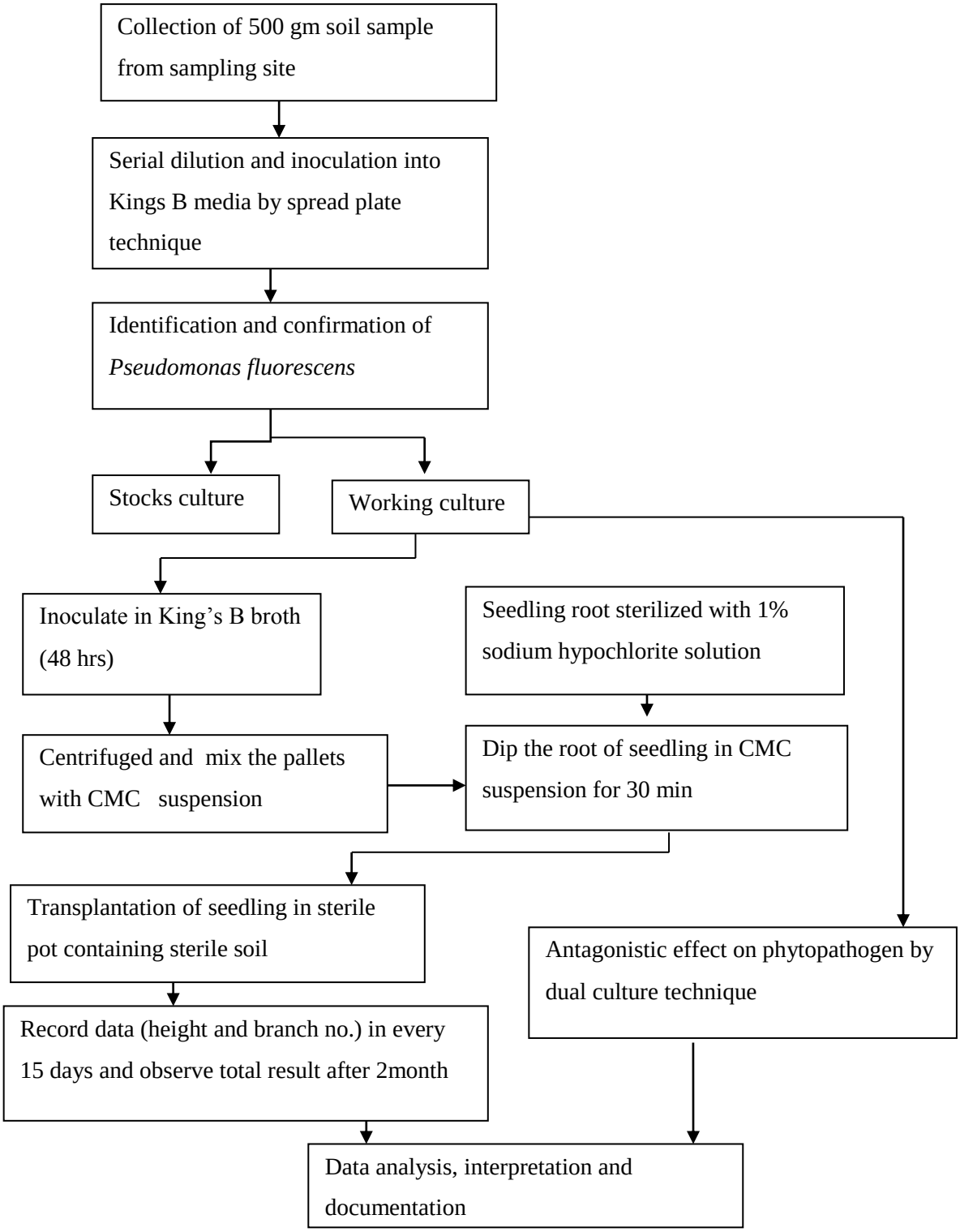


Fig.1.Schematic diagram for biocontrol effects of *P. fluorescens* on phytopathogen and on growth of medicinal plants.

CHAPTER IV

RESULT

4.1 Population of *P. fluorescens*

Out of 50 soil samples collected from five districts (Dhankuta, Sunsari, Morang, Jhapa and Illam)**,** 10 samples from each district, *P. fluorescens* were isolated from 28 samples by serial diulution technique using Kings B media**.** 56% samples were found to be positive for *P. fluorescens.*

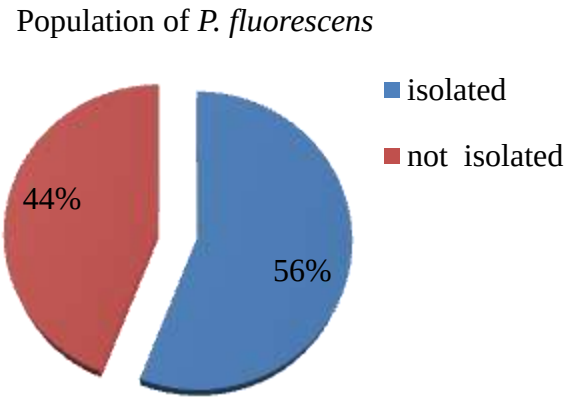


Fig.4.1 Population of *P. fluorescens* from 50 soil samples

4.2 Indole acetic acid and HCN production

Isolates were tested for the indole acetic acid production production qualitatively on the basis of pink color production by cell free supernant after addition of orthophospheric acid. Out of 28 isolates, 5 isolates produced the highest amount of IAA, 8, 4 and 11 produced moderate, poor and none respectively.

Similarly hydrogen cynide production was quantified on the basis of conversion of yellow colored Whatsman filter paper soaked in 2% sodium carbonate in 0.5% picric acid into brownish red. Out of 28 isolates, 3 isolates produced highest amount of HCN 5, 7, 5 and 11 produced moderate, poor and none respectively

Table 4.2 Indole acetic acid and HCN production

Test	High	Moderate	Poor	None
IAA	5	8	4	11
HCN	3	7	5	13

4.3 Isolation of plant pathogens

6 pathogenic fungi were isolated from different types of diseased parts of plants by tissue culture techniques on PDA and CMA then identified by LPCB staining. Diseased plants and their respective pathogenic fungi isolated are shown below in table 4.3.

Table 4.3 Diseased plants and their respective phytopathogens.

S.N	Plants	Plant's parts	Organism	Disease
1.	Banana	Leaf	*F. oxysporium*	Banana Fusarium wilt
2.	Maize	Leaf	*H. turcicum*	Northern Corn Blight
3.	Strawberry	Leaf and stem	*A.alternata*	Early blight of strawberry
4.	Rice	Stem	*R. solani*	Sheath blight
5.	Orange	Stem	*F. solani*	Citrus Dry rot
6.	Rice	Stem and root	*S. rolfsi*	Seedling blight

4.4 Antagonistic effect of *P. fluorescens* against phytopathogens

Antagonistic activities of four isolates against six pathogens (D1, DR, DT and JF) were observed by inoculating the loopful bacterial culture at one edge on the periphery of 2% sucrose supplemented Kings B media plateand mycelial discs of actively growing 5 days old fungal culture was placed opposite to the bacterial inoculation on plate. In control plate, only fungal pathogens were placed at one edge of petriplate without any bacterial isolate.The assays were conducted in triplicates in Completely Randomized Design. The radius of mycelia growth in treatment and control plate was measured for five days.

4.4.1 Antagonistic effect of *P. fluorescens* against *F. oxysporium*

Out of chosen 4 isolates, IP value of DR was significantly higher than DT, JF and D1 in day 3 and day 4 where as there were no significant difference in day 1, day 2 and day 5. Thus, DR isolate was found to be most potent in controlling *F. oxysporium.*

Table no.4.4.1 Antagonistic effect of *P. fluorescens* against *F. oxysporium*

SN	Isolate	Inhibition percentage (IP in %)				
		Day 1	Day 2	Day 3	Day 4	Day 5
1.	D1	25.27	21.33	21.50 [a]	22.17 [b]	22.27
2.	DR	19.70	26.03	27.13 [a]	29.00 [a]	23.93
3.	DT	16.10	19.07	20.50 [b]	22.90 [b]	19.17
4.	JF	16.10	20.13	17.70 [b]	19.90 [b]	20.53
5.	P value	0.357	0.677	0.0090	0.001	0.113
6.	LSD	NS	NS	2.31	2.31	NS
7	CV (%)	35.14	35.15	11.36	7.75	10.28

Note: Mean of three replications. Same letters followed in the columns are not significantly different (P≤0.05) by Turkey's Honest Significant Difference (HSD) test. D1=*P. fluorescens* from maize rhizosphere (Dhankuta)), DR= *P. fluorescens* from raddish rhozosphere (Dhankuta), DT= *P. fluoresc*ens from rhizosphere of tea (Dhankuta), JF= *P. fluorescens* from rhizosphere of fern (Jhapa)

4.4.2 Antagonistic effect of *P. fluorescens* against *R. solani*

Out of chosen 4 isolates, IP value of DT was significantly higher than D1, DR and JF in day 3 where as there were no significant difference in day 1, day 2, day 4 and day 5. Thus, DT isolate was found to be most potent in controlling *R. solani* on the basis of day 3.

Table no.4.4.2 Antagonistic effect of *P. fluorescens* against *R.solani*

SN	Isolate	Inhibition percentage (IP in %)				
		Day 1	Day 2	Day 3	Day 4	Day 5
1.	D1	25.77	24.80	32.10 [b]	33.73	46.53
2.	DR	16.07	27.77	33.0 [ab]	39.33	45.93
3.	DT	25.47	31.93	37.73 [a]	42.07	47.10
4.	JF	9.70	19.47	23.57 [c]	36.53	49.97
5.	P value	0.096	0.138	0.001	0.41	0.801
6.	LSD	NS	NS	2.31	NS	NS
7.	CV (%)	40.66	22.32	8.76	15.77	11.31

Note: Mean of three replications. Same letters followed in the columns arenot significantly different (P≤0.05) by Turkey's Honest Significant Difference (HSD) test. D1=*P. fluorescens* from maize rhizosphere (Dhankuta)), DR= *P. fluorescens* from raddish rhozosphere (Dhankuta), DT= *P. fluoresc*ens from rhizosphere of tea (Dhankuta), JF= *P. fluorescens* from rhizosphere of fern (Jhapa)

4. 4. 3 Antagonistic effect of *P. fluorescens* against *Alternaria spp.*

Out of chosen 4 isolates, IP value of DR was significantly higher than D1, DT and JF in day 3, day 4 and day 5. D1 was at par with DT.Thus, DR isolate was found to be most potent in controlling *Alternaria* (upto 45.57%) followed by JF, DT and D1. No significance difference was found in day 1 and day 2 within the isolates.

Table no.4.4.3 Antagonistic effect of *P. fluorescens* against *Alternaria spp.*

SN	Isolate	Inhibition percentage (IP in %)				
		Day 1	Day 2	Day 3	Day 4	Day 5
1.	D1	21.60	29.13	25.27 [b]	26.77 [b]	28.83 [c]
2.	DR	26.73	35.30	33.67 [a]	38.60 [a]	45.57 [a]
3.	DT	21.40	27.63	25.27 [b]	24.73 [b]	31.20 [c]
4.	JF	29.70	23.07	32.50 [a]	35.60 [a]	40.80 [b]
5.	P value	0.48	0.052	0.01	0.0002	0.000
6.	LSD	NS	NS	2.31	2.31	2.31
7	CV (%)	30.13	15.20	9.91	7.38	4.65

Note: Mean of three replications. Same letters followed in the columns arenot significantly different (P≤0.05) by Turkey's Honest Significant Difference (HSD) test. D1=*P. fluorescens* from maize rhizosphere (Dhankuta)), DR= *P. fluorescens* from raddish rhozosphere (Dhankuta), DT= *P. fluoresc*ens from rhizosphere of tea (Dhankuta), JF= *P. fluorescens* from rhizosphere of fern (Jhapa)

4.4.4 Antagonistic effect of *P. fluorescens* against *Sclerotium rolfsii*

Out of chosen 4 isolates, IP value of D1 was highly significant than DT, JF and DR in day 4 and day 5.Thus, D1 isolate was found to be most potent in suppressing *Sclerotium rolfsii* (upto 42.53%) followed by DT, JF and DR. No significance difference was found in IP value in day 1 and day 3 within the isolates.

Table no.4.4.4Antagonistic effect of *P. fluorescens* against *Sclerotium rolfsii*

SN	Isolate	Inhibition percentage (IP in %)				
		Day 1	Day 2	Day 3	Day 4	Day 5
1.	D1	34.43	42.47 ^{bc}	44.40	54.03 ^{a}	42.53 ^{a}
2.	DR	20.37	46.17 ^{ab}	44.37	49.77 ^{b}	33.93 ^{d}
3.	DT	37.40	51.23 ^{a}	45.07	45.50 ^{c}	38.47 ^{b}
4.	JF	56.30	37.50 ^{c}	41.57	48.37 ^{b}	35.73 ^{c}
5.	P value	0.14	0.003	0.08	0.000	0.000
6.	LSD	NS	2.31	NS	2.31	2.31
7.	CV (%)	44.89	6.94	3.41	1.68	1.61

Note: Mean of three replications. Same letters followed in the columns arenot significantly different (P≤0.05) by Turkey's Honest Significant Difference (HSD) test. D1= *P. fluorescens* from maize rhizosphere (Dhankuta)), DR= *P. fluorescens* from raddish rhozosphere (Dhankuta), DT= *P. fluoresc*ens from rhizosphere of tea (Dhankuta), JF= *P. fluorescens* from rhizosphere of fern (Jhapa)

4.4.5 Antagonistic effect of *P. fluorescens* against *Fusarium solani*

Out of chosen 4 isolates, IP value of DR was significantly higher than DT, JF and D1 in day 5. DT was at par with D1 and JF. Thus, DR isolate was found to be most potent in suppressing *Fusarium solani* (upto 27.7%). No significance difference was found in IP value in day 1, day 2, day 3 and day 4 within the isolates.

Table no.4.4.5 Antagonistic effect of *P. fluorescens* against *Fusarium solani*

SN	Isolate	Inhibition percentage (IP in %)				
		Day 1	Day 2	Day 3	Day 4	Day 5
	D1	11.93	17.87	20.23	18.20	23.53 [b]
	DR	8.60	8.90	16.17	21.47	27.7 [a]
	DT	14.40	17.77	22.93	14.97	23.53 [b]
	JF	14.70	16.10	20.27	17.20	23.53 [b]
	P value	0.83	0.108	0.094	0.24	0.02
	LSD	NS	NS	NS	NS	2.31
	CV (%)	73.68	29.02	14.01	19.95	6.43

Note: Mean of three replications. Same letters followed in the columns arenot significantly different (P≤0.05) by Turkey's Honest Significant Difference (HSD) test. D1=*P. fluorescens* from maize rhizosphere (Dhankuta)), DR= *P. fluorescens* from radish rhozosphere (Dhankuta), DT= *P. fluoresc*ens from rhizosphere of tea (Dhankuta), JF= *P. fluorescens* from rhizosphere of fern (Jhapa)

4.4.6 Antagonistic effect of *P. fluorescens* against *Exerohilium turcicum*

Out of chosen 4 isolates, IP value of D1 and DR was significantly higher than DT, JF in day 4 and day 5. In day 4, D1 was at par with DR. Similarly DT was at par with JF. Thus, D1 and DR isolate was found to be most potent in suppressing *Exerohilium turcicum* (upto 33.6% and 31.13% respectively).

4.4.6 Antagonistic effect of *P. fluorescens* against *Exerohilium turcicum*

SN	Isolate	Inhibition percentage (IP in %)				
		Day 1	Day 2	Day 3	Day 4	Day 5
	D1	36.10	31.07	34.97	31.97 [a]	33.60 [a]
	DR	24.07	34.47	37.43	27.63 [a]	31.13 [a]
	DT	27.77	36.13	37.43	22.37 [b]	30.33[ab]
	JF	27.30	34.40	33.70	21.27 [b]	27.03 [b]
	P value	0.407	0.404	0.34	0.005	0.017
	LSD	NS	NS	NS	2.31	2.31
	CV (%)	29.55	10.32	7.90	10.83	6.14

Note: Mean of three replications. Same letters followed in the columns are not significantly different (P≤0.05) by Turkey's Honest Significant Difference (HSD) test. D1=*P. fluorescens* from maize rhizosphere (Dhankuta)), DR= *P. fluorescens* from raddish rhozosphere (Dhankuta), DT= *P. fluoresc*ens from rhizosphere of tea (Dhankuta), JF= *P. fluorescens* from rhizosphere of fern (Jhapa)

4.5 In-vivo effects of *P. flourescens* isolates on medicinal plants growth

Five *P. fluorescens* isolates (one from each district) were subjected to in-vivo PGPR effect on medicinal plants growth. Isolates were selected on the basis of highest Indole acetic acid production. Height and branches are measured at 15 days interval for 60 days where as fresh weight and dry weights are measured after harvest of plants on 60 days of inoculation. Then data were compaired with the control where no isolates were inoculated. D1 from Dhankuta (T1), S1from Sunsari (T2), M3 from Morang (T3), JF from Jhapa (T4) and IM from Illam (T5) were different isolates used and four our replicates were maintained for each isolates along with one plant per pot.

4.5.1 Effects of *P. fluorescens* isolates on marigold height

There was high significant difference in height of marigold plants between the treatments and control plants where as no significance difference was found in between the treatments in Day 15, Day 30, Day 45 and Day 60.

Table no. 4.5.1 E ffects of *P. fluorescens* isolates on marigold height

SN	Isolates	Height in cm			
		Day 15	Day 30	Day 45	Day 60
1.	T1	19.50	38.00 [a]	45.25 [a]	51.75 [a]
2.	T2	17.50	36.25 [a]	43.25 [a]	50.75 [a]
3.	T3	19.00	36.00 [a]	44.75 [a]	52.50 [a]
4.	T4	18.25	36.50 [a]	46.00 [a]	52.75 [a]
5.	T5	19.75	40.25 [a]	48.75 [a]	52.50 [a]
6.	Control	16.65	25.25 [b]	32.75 [b]	36.50 [b]
7.	P value	0.042	0.000	0.000	0.0001
8.	LSD	4.49	4.49	4.49	4.49
9.	CV (%)	7.47	8.19	7.31	8.22

Note: Mean of 4 replications. Same letters followed in the columns are not significantly different (P≤0.05) by Turkey's Honest Significant Difference (HSD) test. T1= D1 (Dhankuta), T2= S1 (Sunsari), T3= M (Morang), T4= JF (Morang), T5=IM (Illam), C= Control

4.5.2 Effects of *P. fluorescens* on no.of branches of marigold plants

There was high significant difference in branches no.of marigold plants between the treatments and control plants in Day 60. T3 was at par with T5. Similarly no significance difference was found in between T1, T2 and T4 treatments.

Table no. 4.5.5 Effects of *P. fluorescens* on no.of branches of marigold plants

SN	Isolates	Branches number			
		Day 15	Day 30	Day 45	Day 60
1.	T1	1.00	11.00	14.25	17.00 [ab]
2.	T2	0.75	10.75	13.50	16.25 [ab]
3.	T3	2.25	10.50	14.75	17.25 [a]
4.	T4	1.75	10.25	13.25	16.50 [ab]
5.	T5	3.50	13.00	15.25	18.75 [a]
6.	Control	1.25	10.75	12.00	14.25 [b]
7.	P value	0.318	0.11	0.21	0.005
8.	LSD	NS	NS	NS	4.49
9.	CV (%)	102.5	12.31	10.10	8.0

Note: Mean of 4 replications. Same letters followed in the columns are not significantly different (P≤0.05) by Turkey's Honest Significant Difference (HSD) test. T1= D1 (Dhankuta), T2= S1 (Sunsari), T3= M (Morang), T4= JF (Morang), T5=IM (Illam), C= Control

4.5.3. Effects of *P. fluorescens* on fresh and dry weight of marigold

The fresh weight of marigold (stem) treated with T4 isolate was significantly higher then other isolates as well as control plants. There was significant difference in fresh weight between 5 treatments and control plants. No significant difference was found between T1, T3 and T5. Similarly, dry weight of of marigold treated with T5 isolate was significantly higher then other isolates as well as control plants. T1 was at par with T2, T3 and T4. There was significant difference in dry weight between 5 treatments and control plants.

Table no. 4.5.3 Effects of *P. fluorescens* on fresh and dry weight of marigold

SN	Isolates	Fresh wt	Dry wt
1.	T1	61.12 ^{ab}	10.38 ^{ab}
2.	T2	53.33 ^{bc}	8.38 ^{ab}
3.	T3	62.92 ^{ab}	10.55 ^{ab}
4.	T4	72.15 ^{a}	10.68 ^{ab}
5.	T5	66.95 ^{ab}	12.40 ^{a}
6.	Control	44.90 ^{c}	7.20 ^{b}
7.	P value	0.0003	0.02
8.	LSD	4.49	4.49
9.	CV (%)	11.11	19.89

Note: Mean of 4 replications. Same letters followed in the columns are not significantly different ($P \leq 0.05$) by Turkey's Honest Significant Difference (HSD) test. T1= D1 (Dhankuta), T2= S1 (Sunsari), T3= M (Morang), T4= JF (Morang), T5=IM (Illam), C= Control

4.5.4 Effects of *P. fluorescens* isolates on height of mint plants

The height of mint plants treated with T1, T2, T3 and T4 isolates were significantly higher than the T5 and control plants.

Table no.4.5.4 Effects of *P. fluorescens* isolates on height of mint plants

SN	Isolates	Height in cm			
		Day 15	Day 30	Day 45	Day 60
1.	T1	10.75 [a]	16.25	39.75 [a]	44.75
2.	T2	11.02 [a]	17.75	34.25 [a]	47.50
3.	T3	10.75 [a]	16.75	37.75 [a]	46.75
4.	T4	10.00 [ab]	15.00	34.25 [a]	43.75
5.	T5	11.00 a	17.50	33.25 [ab]	42.25
6.	Control	8.00 [b]	12.25	25.50 [b]	36.00
7.	P value	0.006	0.45	0.007	0.19
8.	LSD	4.49	NS	4.49	NS
9.	CV (%)	10.80	13.92	10.67	14.85

Note: Mean of 4 replications. Same letters followed in the columns are not significantly different (P≤0.05) by Turkey's Honest Significant Difference (HSD) test. T1= D1 (Dhankuta), T2= S1 (Sunsari), T3= M (Morang), T4= JF (Morang), T5=IM (Illam), C= Control

4.5.5 Effects of *P. fluorescens* on branch no.of mint plant

There was no significant difference in branches no.of mint plants between the treatments and control plants till the day of harvesting (60 days).

Table no.4.5.5 Effects of *P. fluorescens* on branch no.of mint plant

SN	Isolates	Branches no.			
		Day 15	Day 30	Day 45	Day 60
1.	T1	7.00	10.75	15.00	23.00
2.	T2	8.00	12.50	15.50	21.50
3.	T3	5.60	12.25	13.25	22.00
4.	T4	6.50	12.00	16.75	19.25
5.	T5	6.50	13.75	18.75	20.50
6.	Control	4.50	8.75	15.25	20.50
7.	P value	0.30	3.00	0.52	0.79
8.	LSD	NS	NS	NS	NS
9.	CV (%)	30.77	25.71	25.40	18.26

Note: Mean of 4 replications. Same letters followed in the columns are not significantly different (P≤0.05) by Turkey's Honest Significant Difference (HSD) test. T1= D1 (Dhankuta), T2= S1 (Sunsari), T3= M (Morang), T4= JF (Morang), T5=IM (Illam), C= Control

4.5.6 Effects of *P. fluorescens* on fresh and dry weight of mint

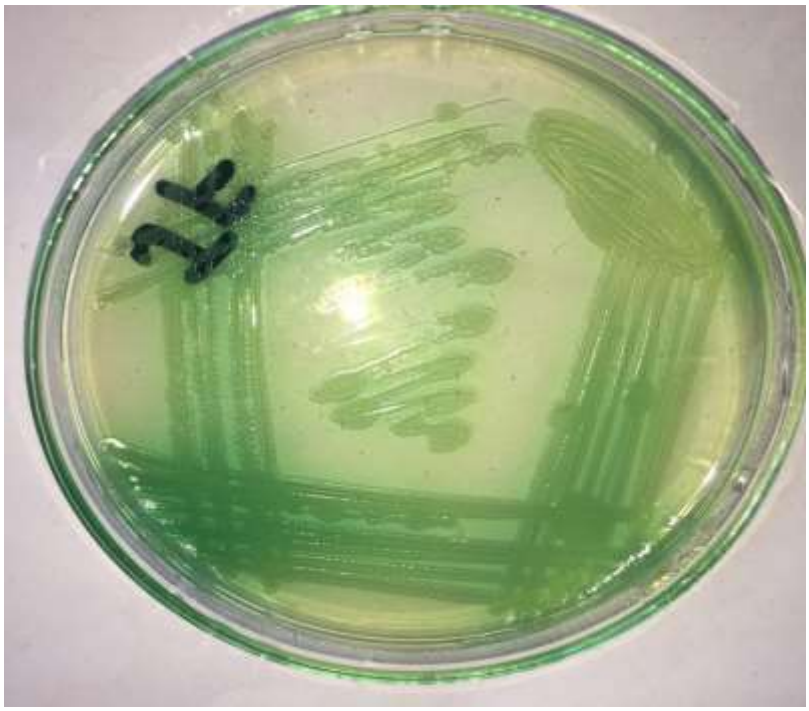
The fresh weight of mint (stem) treated with T1 isolate was significantly higher then other isolates as well as control plants. Fresh weights of all the treatments were significantly higher than control plants. No significant difference was found between T1 and T2. Similarly T3 was at par with T4 and T5. Similarly, dry weight of of mint treated with T1 isolate was significantly higher then other isolates as well as control plants. T2 was at par with T3, T4 and T5.

Table no.4.5.6 Effects of *P. fluorescens* on fresh and dry weight of mint

SN	Isolates	Fresh wt	Dry wt
1.	T1	44.27 [a]	7.00 [a]
2.	T2	43.92 [a]	4.45 [ab]
3.	T3	41.67 [ab]	6.70 [ab]
4.	T4	35.12 [ab]	5.65 [ab]
5.	T5	39.42 [ab]	6.17 [ab]
6.	Control	32.48 [b]	4.95 [b]
7.	P value	0.009	0.01
8.	LSD	4.49	4.49
9.	CV (%)	11.72	13.08

Note: Mean of 4 replications. Same letters followed in the columns are not significantly different (P≤0.05) by Turkey's Honest Significant Difference (HSD) test. T1= D1 (Dhankuta), T2= S1 (Sunsari), T3= M (Morang), T4= JF (Morang), T5=IM (Illam), C= Control

Color Plates

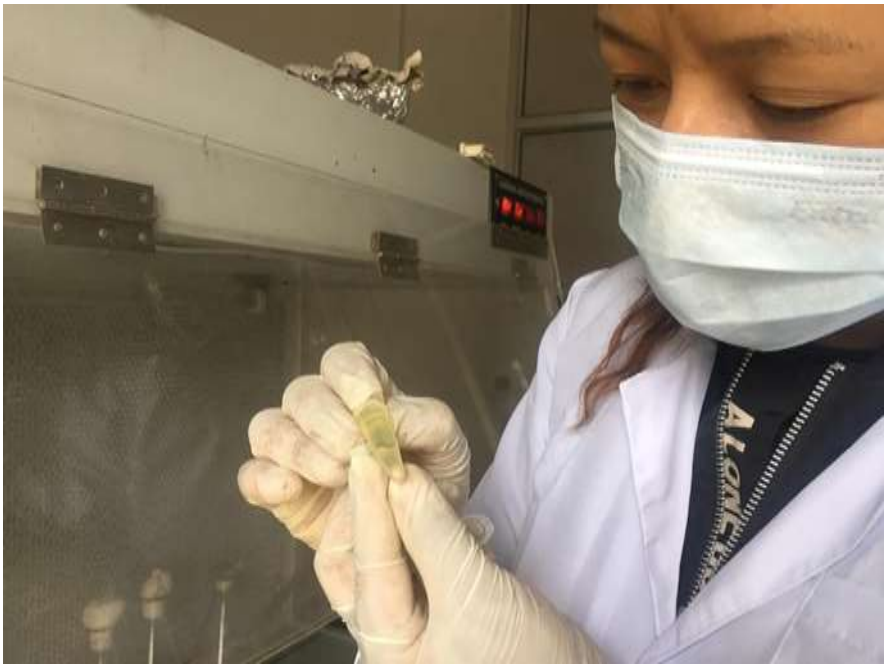


Photograph 1: *Pseudomonas fluorescens*

Photograph 2: Antagonist effect of *P. fluorecens* against *R. solani*

Photograph 3: Treatment marigold vs Control

Photograph 4: Measurement of height and branches



Photograph 5: Pallet collection for root inoculation

Photograph 6: Root inoculation

CHAPTER V
DISSCUSSION

Result obtained from the investigation of "Effects of *Pseudomonas fluorescens* on Medicinal Plants and Biocontrol of Some Phytopathogens" stated in chapter iv have been examined critically and discussed here with appropriate interpretation, facts and comparison with previous works are mention in this chapter.

In this investigation different isolates of *Pseudomonas fluorescens i*solated from 5 differents districts were subjected to Dual culture method against 6 differents phytopathogens and root inoculation was done to observe PGPR effects.

Many studies have been conducted on the application of antagonistic microbes, such as *Pseudomonas spp*. for the control of Fusarium wilt (Tu & Chang 1983). In my research Biocontrol order of potential (BOP) for *Fusarium oxysporium* was found to be DR≥D1 ≥DT ≥ JF. While control plates showed regular radial growth covering the whole plates on 5 days. Antagonistic effects were observed every day but growths and antagonistic effects were more prounced by 4 days after inoculation. The present study showed the in-vitro potential of 4 isolates of *P. fluorescens.* This result was supported by Saravanan et al (2013) showing two isolates Pf5 and Pf6 being most potent. *P. fluorescen*s also showed the greatest inhibition against *R. solani* and *F. oxysporum (*Montealegre et al 2003 and Rini & Sulochana 2007). Althought in-vitro assay do not always provide the reliable data on potential of boicontrol agents, they provides provides guidelines to apply them in vivo assay for controlling the phytopathogens (Chaves et al 1991).

Biocontrol order of potential (BOP) for *Rhizoctonia solani* was found to be DT≥DR≥D1≥JF. Isolate DT could suppress the growth by 47.10% at 5 days of inoculation. An inhibitory halo was observed suggesting the presence of fungistatic metabolites secreted by the bacteria. Similarly, change in mycelial color was observed closed to the colony end of *R. solani*, being this one of a darker brown than the one observed at the center of colony. Same experiment

was done by Manoj Kumar Maurya in 2014 and found that the maximum inhibition of 68.23% was exhibited by PF 07 and minimum, 55.8% was recorded with the P.f.05 isolate. This result was also supported by Rai Dinesh (2018) where in-vitro condition and inhibition of mycelial growth and sclerotial germination of *R. solani* was suppressed ranging from 48%-92% and 29% -87% respectively. While comparing this experiments it seems that potency is of present isolates seems to be less.

In 2014, Manoj Kumar Maurya had found out the maximum inhibition (48.13%) of *Alternaria alternate* by P.f.07 and minimum 44.45% was recorded with isolate P.f.05 in a dual culture. Following that research, invitro biocontrol effects of 4 isolates were conducted and found the BOP DR ≥ JF ≥ DT ≥ D1. Isolates from Dhankuta (DR) suppressed the growth of *Alternaria*in maximum amount with IP value 45.57 % which is nearly equal to the previous researcher Manoj Kumar Maurya.

BOP for *Sclerotium roflsi* was D1 ≥ DT ≥ JF ≥ DR. Plates were fully covered mycelia on day 5 in control plates where as others mycelia were inhibited by *P. fluorescens* secreating antifulgal compound resulting the brownish zone of inhition. D1 had highest IP value 54.03% and DR being the least potent with 45.5% IP value. Present stuty is supported by the earlier worker Mahato and Mondal (2014) who had conducted a reaserchand found *Pseudomonas* had highest inhibitory activity 74.25% out of four isolates *Azotobacter chroococcum*, FYM, *Pseudomonas* and *Trichoderma viride.* Karthikeyan et al (2006) reported that P. *fluorescens* inhibited stem rot of groundnut caused by *Scleretium rolfsii* by 64.40% in in-vivo test.

BOP for *Fusarium solani* was DR ≥ JF ≥ D1 ≥ DT. All the isolate caused little inhibition. Taking day 5 reference inhibition range was 23.53%-27.7%. This was supported by previous research by Karima et al (2012) who carried out comparision research on antagonistic effect of fungal biocontrol agents and bacterial biocontrol agents and found that the fungal bio-control agent of T. *harizanum* and *T. Viride* reduced the mycelial growth of *F.solani* from 48.8% to 76.7% and 27.7 to 82.2% respectively while *P. fluorescens* reduced the mycelia growth from 19.7 to 20.9 % only. Out of chosen 4 isolates, DR being

the most potent suppressed the growth of *Fusarium solani* by 27.7% followed by DT, JF and DR by 23.53% being same.

For *Exerohilium turcicum* (Northern Corn Blight), order of sequence of BOP was D1 ≥ DR ≥ DT ≥ JF. All the isolates showed nearly equal equal percentage of inhibition ranging from 27.03 % to 33.6%. Similar experiment was conducted by T.A Wani and found that inhibition percentage of *P. flurescens* against *E. turcicum* be 37.95%. Antagonistic effect was observed with *Bacillus subtilis* and *P. fluorescens* against *E. turcicum* in dual culture *in vitro* and concluded that Pseudomonads and bacilli have been shown to play a key role in the suppression of plant pathogens in different cropping systems (Harlapur S et al 2007).

Effects of 5 differents *P. fluorescens* isolates (D1, S1, M3, JFand IM) on growth of marigold has been observed in present study and found that there was high significant difference in height of marigold plants between treated and control plants. T4 isolates has highest effect on plants (52.75cm height) followed by T5, T3, T1 and T2 with height 52.5cm, 52.5cm,51.75cm and 50.75cm respectively whereas control plant has only 36.50cm height in 60 days. This result has been supported by Kita et al (2004), who used two strains of *Pseudomonas* GRP3 and PRS9 were inoculated in wheat (*Triticum aestivum*) and found enhanced plant growth in terms of root-shoot length and weight. In present study, there was significant difference in branches no. of marigold plats between treated plant and control plants in during harvesting time (60 days). Whereas T5 (IM) and T3 (M3) treatments had the highest number of branches compairing to other isolates and control plant. Similarly, increased no.of leaf area, leaf no.and dry and fresh weight was obtained by inoculation of *P .fluorescens* strain Pf in blueberry was observed by Silva et al (2000) supporting present study.

In present study, mint inoculated with *P. fluorescens* has been observed that T2 isolates has highest effect on plants (47.50 cm height) followed by T3, T1, T4 and T5 with height 46.75 cm, 44.75cm, 43.75cm and 42.25 cm respectively whereas control plant has only 36.00cm height. Significance difference in fresh and dry weight of treated and untreated plants were observed. Similar experiment was conducted by Santoro et al (2015) in mint where direct

inoculation of native *Pseudomonas* strains showed increased shoot fresh weight, glandular trichome number, ramification number, root dry weight and essentials oil without alteration in the oil composition in comparison with controls which indicate the clear potentials as bio-inoculants for improving productivity of aromatic plants**.** Previous worker Mulla et al (2013) inoculated *P. fluorescens* in Sarpagandha and compaired with uninoculated plant and observed significant increased in total fresh weight and dry weight supporting present study PGPR effect of *P. fluorescens* on medicinal plants.

CHAPTER VI

CONLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The biological control approach of plant pathogens and diseases has been studied for many years, and the introduction of beneficial microorganisms into soil or the rhizosphere has been proposed for the biological control of soilborne crop diseases. Biologicsl control agents have emerged as new strategies of managing plant disease by inducing systematic resistance and growth promotion of plants against diseases. From present study, it can be concluded that *P. fluorescens* isolates can serve as good option as plant growth promoter as well as biocontrol against phytopathogenic fungi. In vitro experiment was conducted to observe antagonistic effect in dual culture and from that fact it can be concluded that different isolates has different potency and potency of same isololates varied with the plant pathogens. From in vivo experiment it can be concluded that *P. fluorescens* act as good plant growth promoter increasing plant height, branch numbers, fresh weight and dry weight which can be used as biofertilizer replacing the chemical fertilizer.

6.2 RECOMMENDATIONS

Based on the results and findings of the experiments, the recocommendation madeare as follow:-

1. *Pseudomonas fluorescens* and phytopathogens showed various degree of interaction and reveal the different degree of inhibition of phytopathogens. From this farmer can be recommended to farmers to use *P.fluorescens* as boicontrol agents replacing chemical pesticides.
2. In some phytopathogens, there were only few percent of suppression and found show resistance to *P. fluorescens* in such cases farmers are recommended to use biopesticides in combination with small dose chemical fungicides.
3. *P. fluorescens* not only acts as biocontrol agents but it is also a good plant growth promoter. It promotes plant growth by secreating many different types of plant growth promoters like Indole acetic acid, gibberelic acid etc.Thus *P. fluorescens* is recommended to use as biofertilizer.
4. Medicinal plants cultivating farmers are specially recommended to use *P. fluorescens* as biocontrol as well as plant growth promoter replacing chemical fertilizer as wel as chemical pesticides as chemicals residues may be present in medicines after processing too.
5. Isolate D1 is recommended to use in controlling seedling blight caused by *Sclerotium roflsii.*
6. Biopsticides industries are recommended for mass production of these biocontrol agents having high host range and increase the knowledge in farmer about biopesticide and organic farming.

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APPENDIX I

Scientific classification of *P.fluorescens* and phytopathogen used as model organisms

Kingdom	**Bacteria**
Phylum	Proteobacteria
class	Gammaproteobacteria
Order	Pseudomonadales
Family	Pseudomondaceae
Genus	Pseudomonas
Species	Fluorescens

Position	***Alternaria***	***Exerohilium***	***Sclerotium***
Kingdom	Fungi	Fungi	Fungi
Division	Ascomycota	Ascomycota	Ascomycota
Class	Dothideomycetes	Dothideomycetes	Pezizomycotina
Order	Pleosporales	Pleosporales	Dothideomycetes
Family	Pleosporaceae	Pleosporaceae	Pleosporomycetidae
Genus	Alternaria	exerohilium	

Position	***Rhizoctonia***	***Fusarium***
Kingdom	Fungi	Fungi
Division	Basidomycota	Ascomycota
Class	Agaricomycota	Sordariomycetes
Order	Cantharellales	Hypocreales
Family	Ceratobasidiaceae	Nectriaceae
Genus	Rhizoctonia	Fusarium

APPENDIX II

Culture Media Used in Research

1. Kings B media

Ingredients	Gms / Litre
Proteose peptone	20.000
Dipotassium hydrogen phosphate	1.500
Magnesium sulphate heptahydrate	1.500
Agar	20.000
Final pH (at 25°C)	7.2±0.2

Suspend 42.23 gm og dehydrated media into 1000mlof water containing 15 ml of glycerol. Heat to boiling,and autoclave at 121 ^{0}C for 15 min.

2. Potato dextrose agar

Ingridient	Gms/litre
Potato	200gm
Dextrose	20gm
Distilled water	1000ml
pH	6.5±2

Required amount of water was cut into small pieces and boiled in 500 ml og distilled water for 15 min and iltered through muslin clothes to have desired ectract. Thereafter 20 gm of dextrose and 20 gm of agar was added and mixed and autoclaved at 121 ^{0}C for 15 min

3. Starch agar

Ingridients	gm/litre
Beef extract	3 gm

Soluble starch	10 gm
Agar	12 gm
pH	7.3 ±2

Suspend 25 gm of powder in 1 L of purified water and mix thoroughly. Heat and boil for 1 min, autoclave at 121^{0}C for 15 min

4. Modified Kings B.

Ingredients	Gms / Litre
Proteose peptone	20.00
Dipotassium hydrogen phosphate	1.500
Magnesium sulphate heptahydrate	1.500
Agar	20.00
Sucrose	20.00
Final pH (at 25°C)	7.2±0.2

Suspend 42.23 gm og dehydrated media into 1000mlof water containing 15 ml of glycerol ans 2% sucrose. Heat to boiling and autoclave at 121 ^{0}C for 15 min.

APPENDIX III

Statistical Analysis

Marigold and mint plant

Statistical Tool for Agricultural Research (STAR)
Sun Jul 07 14:45:17 2019
Analysis of Variance
Completely Randomized Design
ANALYSIS FOR RESPONSE VARIABLE: Mg.Ht.15
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Trt	6	1, 2, ..., 6

Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: Mg.Ht.15

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Trt	5	27.7083	5.5417	2.91	0.0425
Error	18	34.2500	1.9028		
Total	23	61.9583			

Summary Statistics

CV(%)	Mg.Ht.15 Mean
7.47	18.46

Standard Errors

Effects	StdErr
Trt	0.9754

Pairwise Mean Comparison of Trt
Tukeys's Honest Significant Difference (HSD) Test

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	1.9028
Critical Value	4.4944
Test Statistics	3.0998

Summary of the Result:

Trt	means	N	group
1	19.50	4	a
2	17.50	4	a
3	19.00	4	a
4	18.25	4	a
5	19.75	4	a
6	16.75	4	a

Means with the same letter are not significantly different.

==========
ANALYSIS FOR RESPONSE VARIABLE: MgHt.30
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MgHt.30
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 542.3750 108.4750 12.91 0.0000
Error 18 151.2500 8.4028
Total 23 693.6250

Summary Statistics
CV(%) MgHt.30 Mean
8.19 35.38
Standard Errors
Effects StdErr
Trt 2.05
Pairwise Mean Comparison of Trt
Tukeys's Honest Significant Difference (HSD) Test
Alpha 0.05
Error Degrees of Freedom 18
Error Mean Square 8.4028
Critical Value 4.4944
Test Statistics 6.5141

Summary of the Result:
Trt means N group
1 38.00 4 a
2 36.25 4 a
3 36.00 4 a
4 36.50 4 a
5 40.25 4 a
6 25.25 4 b
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: MgHt.45
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MgHt.45

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Trt	5	616.2083	123.2417	12.21	0.0000
Error	18	181.7500	10.0972		
Total	23	797.9583			

Summary Statistics

CV(%)	MgHt.45 Mean
7.31	43.46

Standard Errors

Effects	StdErr
Trt	2.25

Pairwise Mean Comparison of Trt
Tukeys's Honest Significant Difference (HSD) Test

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	10.0972
Critical Value	4.4944
Test Statistics	7.1408

Summary of the Result:-

Trt	means	N	group
1	45.25	4	a
2	43.25	4	a
3	44.75	4	a
4	46.00	4	a
5	48.75	4	a
6	32.75	4	b

Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: MgHt.60
==========
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Trt	6	1, 2, ..., 6

Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MgHt.60

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Trt	5	816.7083	163.3417	9.89	0.0001
Error	18	297.2500	16.5139		
Total	23	1113.9583			

Summary Statistics

CV(%)	MgHt.60 Mean

8.22 49.46
Standard Errors
Effects StdErr
Trt 2.87

Pairwise Mean Comparison of Trt
Tukeys's Honest Significant Difference (HSD) Test
Alpha 0.05
Error Degrees of Freedom 18
Error Mean Square 16.5139
Critical Value 4.4944
Test Statistics 9.1321
Summary of the Result:
Trt means N group
1 51.75 4 a
2 50.75 4 a
3 52.50 4 a
4 52.75 4 a
5 52.50 4 a
6 36.50 4 b
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: MgBr15
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MgBr15
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 20.5000 4.1000 1.27 0.3184
Error 18 58.0000 3.2222
Total 23 78.5000

Summary Statistics
CV(%) MgBr15 Mean
102.57 1.75
Standard Errors
Effects StdErr
Trt 1.27
Table of Means
Trt MgBr15 Means
1 1.00
2 0.75

3 2.25
4 1.75
5 3.50
6 1.25
==========
ANALYSIS FOR RESPONSE VARIABLE: MgBr30
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MgBr30
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 19.7083 3.9417 2.13 0.1079
Error 18 33.2500 1.8472
Total 23 52.9583

Summary Statistics
CV(%) MgBr30 Mean
12.31 11.04
Standard Errors
Effects StdErr
Trt 0.9610
Table of Means
Trt MgBr30 Means
1 11.00
2 10.75
3 10.50
4 10.25
5 13.00
6 10.75
==========
ANALYSIS FOR RESPONSE VARIABLE: MgBr.45
==========
Summary Information-
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MgBr.45
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 16.0000 3.2000 1.60 0.2106
Error 18 36.0000 2.0000

Total 23 52.0000

Summary Statistics
CV(%) MgBr.45 Mean
10.10 14
Standard Errors
Effects StdErr
Trt 1
Table of Means
Trt MgBr.45 Means
1 14.25
2 13.50
3 14.75
4 13.25
5 15.25
6 13.00

==========

ANALYSIS FOR RESPONSE VARIABLE: MgBr.60

==========

Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MgBr.60
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 43.3333 8.6667 4.87 0.0054
Error 18 32.0000 1.7778
Total 23 75.3333

Summary Statistics
CV(%) MgBr.60 Mean
8 16.67
Standard Errors
Effects StdErr
Trt 0.9428
Pairwise Mean Comparison of Trt
Tukeys's Honest Significant Difference (HSD) Test
Alpha 0.05
Error Degrees of Freedom 18
Error Mean Square 1.7778
Critical Value 4.4944
Test Statistics 2.9963
Summary of the Result:
Trt means N group

1 17.00 4 ab
2 16.25 4 ab
3 17.25 4 a
4 16.50 4 ab
5 18.75 4 a
6 14.25 4 b
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: MiHt.15
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MiHt.15
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 28.7083 5.7417 4.64 0.0067
Error 18 22.2500 1.2361
Total 23 50.9583

Summary Statistics
CV(%) MiHt.15 Mean
10.80 10.29
Standard Errors
Effects StdErr
Trt 0.7862

Pairwise Mean Comparison of Trt
Tukeys's Honest Significant Difference (HSD) Test
Alpha 0.05
Error Degrees of Freedom 18
Error Mean Square 1.2361
Critical Value 4.4944
Test Statistics 2.4985

Summary of the Result:
Trt means N group
1 10.75 4 a
2 11.25 4 a
3 10.75 4 a
4 10.00 4 ab
5 11.00 4 a
6 8.00 4 b
Means with the same letter are not significantly different.

==========
ANALYSIS FOR RESPONSE VARIABLE: MiHt.30
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MiHt.30
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 25.8333 5.1667 0.99 0.4515
Error 18 94.0000 5.2222
Total 23 119.8333

Summary Statistics
CV(%) MiHt.30 Mean
13.92 16.42
Standard Errors
Effects StdErr
Trt 1.62
Table of Means
Trt MiHt.30 Means
1 16.25
2 17.75
3 16.75
4 15.00
5 17.50
6 15.25
==========
ANALYSIS FOR RESPONSE VARIABLE: MiHt.45
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MiHt.45
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 479.8750 95.9750 7.24 0.0007
Error 18 238.7500 13.2639
Total 23 718.6250
Summary Statistics
CV(%) MiHt.45 Mean
10.67 34.12

Standard Errors
Effects StdErr
Trt 2.58

Pairwise Mean Comparison of Trt
Tukeys's Honest Significant Difference (HSD) Test
Alpha 0.05
Error Degrees of Freedom 18
Error Mean Square 13.2639
Critical Value 4.4944
Test Statistics 8.1843

Summary of the Result:
Trt means N group
1 39.75 4 a
2 34.25 4 a
3 37.75 4 a
4 34.25 4 a
5 33.25 4 ab
6 25.50 4 b
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: MiHt.60
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MiHt.60
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 344.0000 68.8000 1.65 0.1985
Error 18 752.0000 41.7778
Total 23 1096.0000
Summary Statistics
CV(%) MiHt.60 Mean
14.86 43.50
Standard Errors
Effects StdErr
Trt 4.57
Table of Means
Trt MiHt.60 Means
1 44.75
2 47.50

3 46.75
4 43.75
5 42.25
6 36.00
==========
ANALYSIS FOR RESPONSE VARIABLE: MiBr.15
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MiBr.15
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 26.0000 5.2000 1.30 0.3076
Error 18 72.0000 4.0000
Total 23 98.0000

Summary Statistics
CV(%) MiBr.15 Mean
30.77 6.50
Standard Errors
Effects StdErr
Trt 1.41

Table of Means
Trt MiBr.15 Means
1 7.00
2 8.00
3 6.50
4 6.50
5 6.50
6 4.50
==========
ANALYSIS FOR RESPONSE VARIABLE: MiBr.30
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MiBr.30
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 59.3333 11.8667 1.32 0.3005

Error 18 162.0000 9.0000
Total 23 221.3333

Summary Statistics
CV(%) MiBr.30 Mean
25.71 11.67
Standard Errors
Effects StdErr
Trt 2.12

Table of Means
Trt MiBr.30 Means
1 10.75
2 12.50
3 12.25
4 12.00
5 13.75
6 8.75
==========
ANALYSIS FOR RESPONSE VARIABLE: MiBr.45
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MiBr.45
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 68.5000 13.7000 0.86 0.5287
Error 18 288.0000 16.0000
Total 23 356.5000

Summary Statistics
CV(%) MiBr.45 Mean
25.40 15.75
Standard Errors
Effects StdErr
Trt 2.83
Table of Means
Trt MiBr.45 Means
1 15.00
2 15.50
3 13.25
4 16.75
5 18.75

6 15.25
==========
ANALYSIS FOR RESPONSE VARIABLE: MiBr.60
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: MiBr.60
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 34.8750 6.9750 0.47 0.7944
Error 18 267.7500 14.8750
Total 23 302.6250

Summary Statistics
CV(%) MiBr.60 Mean
18.26 21.12
Standard Errors
Effects StdErr
Trt 2.73

Table of Means
Trt MiBr.60 Means
1 23.00
2 21.50
3 22.00
4 19.25
5 20.50
6 20.50
==========
ANALYSIS FOR RESPONSE VARIABLE: Mg.Fresh.wt.
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: Mg.Fresh.wt.
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 1911.9871 382.3974 8.54 0.0003
Error 18 805.5225 44.7512
Total 23 2717.5096
Summary Statistics

CV(%) Mg.Fresh.wt. Mean
11.11 60.23

Standard Errors
Effects StdErr-
Trt 4.73
Pairwise Mean Comparison of Trt
Tukeys's Honest Significant Difference (HSD) Test
Alpha 0.05
Error Degrees of Freedom 18
Error Mean Square 44.7512
Critical Value 4.4944
Test Statistics 15.0330

Summary of the Result:
Trt means N group
1 61.12 4 ab
2 53.33 4 bc
3 62.92 4 ab
4 72.15 4 a
5 66.95 4 ab
6 44.90 4 c
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: Mg.Dry.wt.
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: Mg.Dry.wt.
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 68.1138 13.6228 3.50 0.0219
Error 18 69.9925 3.8885
Total 23 138.1062
Summary Statistics
CV(%) Mg.Dry.wt. Mean
19.89 9.91
Standard Errors
Effects StdErr
Trt 1.39

Pairwise Mean Comparison of Trt
Tukeys's Honest Significant Difference (HSD) Test

Alpha 0.05
Error Degrees of Freedom 18
Error Mean Square 3.8885
Critical Value 4.4944
Test Statistics 4.4313

Summary of the Result:

Trt	means	N	group
1	10.28	4	ab
2	8.38	4	ab
3	10.55	4	ab
4	10.68	4	ab
5	12.40	4	a
6	7.20	4	b

Means with the same letter are not significantly different.

==========

ANALYSIS FOR RESPONSE VARIABLE: Mint.Fresh.wt.

==========

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Trt	6	1, 2, ..., 6

Number of Observations Read and Used: 24

ANOVA TABLE

Response Variable: Mint.Fresh.wt.

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Trt	5	462.4283	92.4857	4.32	0.0093
Error	18	385.1250	21.3958		
Total	23	847.5533			

Summary Statistics

CV(%)	Mint.Fresh.wt. Mean
11.72	39.48

Standard Errors

Effects	StdErr
Trt	3.27

Pairwise Mean Comparison of Trt

Tukeys's Honest Significant Difference (HSD) Test

Alpha 0.05
Error Degrees of Freedom 18
Error Mean Square 21.3958
Critical Value 4.4944
Test Statistics 10.3946

Summary of the Result:

Trt means N group
1 44.27 4 a
2 43.92 4 a
3 41.67 4 ab
4 35.12 4 ab
5 39.42 4 ab
6 32.48 4 b
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: Mint.Dry.wt.
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Trt 6 1, 2, ..., 6
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: Mint.Dry.wt.
Source DF Sum of Square Mean Square F Value Pr(> F)
Trt 5 12.1888 2.4378 3.98 0.0132
Error 18 11.0375 0.6132
Total 23 23.2263

Summary Statistics
CV(%) Mint.Dry.wt. Mean
13.08 5.99
Standard Errors
Effects StdErr
Trt 0.5537
Pairwise Mean Comparison of Trt
Tukeys's Honest Significant Difference (HSD) Test
Alpha 0.05
Error Degrees of Freedom 18
Error Mean Square 0.6132
Critical Value 4.4944
Test Statistics 1.7597

Summary of the Result:
Trt means N group
1 7.00 4 a
2 5.45 4 ab
3 6.70 4 ab
4 5.65 4 ab
5 6.17 4 ab
6 4.95 4 b
Means with the same letter are not significantly different.

APPENDIX IV

Statistical analysis for antagonistic effect

Analysis of Variance
Completely Randomized Design
==========
ANALYSIS FOR RESPONSE VARIABLE: Al.D1
==========
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Al.D1

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	148.6025	49.5342	0.88	0.4897
Error	8	448.7467	56.0933		
Total	11	597.3492			

Summary Statistics

CV(%)	Al.D1 Mean
30.13	24.86

Standard Errors

Effects	StdErr
Isolate	6.12

Table of Means

Isolate	Al.D1 Means
1	21.60
2	26.73
3	21.40
4	29.70

==========
ANALYSIS FOR RESPONSE VARIABLE: Al.D2
==========
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Al.D2

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	229.7767	76.5922	4.00	0.0518
Error	8	153.1000	19.1375		
Total	11	382.8767			

Summary Statistics

CV(%)	Al.D2 Mean
15.20	28.78

Standard Errors

Effects	StdErr
Isolate	3.57

Table of Means

Isolate	Al.D2 Means
1	29.13
2	35.30
3	27.63
4	23.07

==========

ANALYSIS FOR RESPONSE VARIABLE: Al.D3

==========

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE

Response Variable: Al.D3

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	185.3425	61.7808	7.38	0.0108
Error	8	66.9400	8.3675		
Total	11	252.2825			

Summary Statistics

CV(%)	Al.D3 Mean
9.91	29.18

Standard Errors

Effects	StdErr
Isolate	2.36

Pairwise Mean Comparison of Isolate

Least Significant Difference (LSD) Test

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	8.3675
Critical Value	2.3060
Test Statistics	5.4464

Summary of the Result:

Isolate	means	N	group
1	25.27	3	b

2 33.67 3 a
3 25.27 3 b
4 32.50 3
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: Al.D4
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS

Isolate 4 1, 2, 3, 4
Number of Observations Read and Used: 12
ANOVA TABLE
Response Variable: Al.D4
Source DF Sum of Square Mean Square F Value Pr(> F)
Isolate 3 406.1692 135.3897 25.16 0.0002
Error 8 43.0533 5.3817
Total 11 449.2225

Summary Statistics
CV(%) Al.D4 Mean
7.38 31.42
Standard Errors
Effects StdErr
Isolate 1.89
Pairwise Mean Comparison of Isolate
Least Significant Difference (LSD) Test
Alpha 0.05
Error Degrees of Freedom 8
Error Mean Square 5.3817
Critical Value 2.3060
Test Statistics 4.3679

Summary of the Result:
Isolate means N group
1 26.77 3 b
2 38.60 3 a
3 24.73 3 b
4 35.60 3 a
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: Al.D5
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Isolate 4 1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Al.D5

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	562.5667	187.5222	64.74	0.0000
Error	8	23.1733	2.8967		
Total	11	585.7400			

Summary Statistics

CV(%)	Al.D5 Mean
4.65	36.60

Standard Errors

Effects	StdErr
Isolate	1.39

Pairwise Mean Comparison of Isolate
Least Significant Difference (LSD) Test

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	2.8967
Critical Value	2.3060
Test Statistics	3.2045

Summary of the Result:

Isolate	means	N	group
1	28.83	3	c
2	45.57	3	a
3	31.20	3	c
4	40.80	3	b

Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: Fs.D1
==========
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Fs.D1

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	71.8425	23.9475	0.29	0.8338
Error	8	668.0067	83.5008		
Total	11	739.8492			

Summary Statistics

CV(%)	Fs.D1 Mean
73.64	12.41

Standard Errors

Effects	StdErr
Isolate	7.46

Table of Means

Isolate	Fs.D1 Means
1	11.93
2	8.60
3	14.40
4	14.70

==========

ANALYSIS FOR RESPONSE VARIABLE: Fs.D2

==========

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE

Response Variable: Fs.D2

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	162.5758	54.1919	2.80	0.1085
Error	8	154.7533	19.3442		
Total	11	317.3292			

Summary Statistics

CV(%)	Fs.D2 Mean
29.02	15.16

Standard Errors

Effects	StdErr
Isolate	3.59

Table of Means

Isolate	Fs.D2 Means
1	17.87
2	8.90
3	17.77
4	16.10

==========

ANALYSIS FOR RESPONSE VARIABLE: Fs.D3

==========

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Fs.D3

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	70.1533	23.3844	3.01	0.0945
Error	8	62.1467	7.7683		
Total	11	132.3000			

Summary Statistics

CV(%)	Fs.D3 Mean
14.01	19.90

Standard Errors

Effects	StdErr
Isolate	2.28

Table of Means

Isolate	Fs.D3 Means
1	20.23
2	16.17
3	22.93
4	20.27

==========
ANALYSIS FOR RESPONSE VARIABLE: Fs.D4
==========
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Fs.D4

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	65.6758	21.8919	1.71	0.2427
Error	8	102.7133	12.8392		
Total	11	168.3892			

Summary Statistics

CV(%)	Fs.D4 Mean
19.95	17.96

Standard Errors

Effects	StdErr
Isolate	2.93

Table of Means

Isolate	Fs.D4 Means
1	18.20
2	21.47

3 14.97
4 17.20
==========
ANALYSIS FOR RESPONSE VARIABLE: Fs.D5
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Isolate 4 1, 2, 3, 4
Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Fs.D5---
Source DF Sum of Square Mean Square F Value Pr(> F)
Isolate 3 39.0625 13.0208 5.22 0.0275
Error 8 19.9600 2.4950
Total 11 59.0225
Summary Statistics
CV(%) Fs.D5 Mean
6.43 24.57

Standard Errors
Effects StdErr
Isolate 1.29
Pairwise Mean Comparison of Isolate
Least Significant Difference (LSD) Test
Alpha 0.05
Error Degrees of Freedom 8
Error Mean Square 2.4950
Critical Value 2.3060
Test Statistics 2.9741

Summary of the Result:
Isolate means N group
1 23.53 3 b
2 27.70 3 a
3 23.53 3 b
4 23.53 3 b
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: Sr.D1
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Isolate 4 1, 2, 3, 4
Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Sr.D1

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	1967.5292	655.8431	2.36	0.1473
Error	8	2221.4333	277.6792		
Total	11	4188.9625			

Summary Statistics

CV(%)	Sr.D1 Mean
44.89	37.12

Standard Errors

Effects	StdErr
Isolate	13.61

Table of Means

Isolate	Sr.D1 Means
1	34.43
2	20.37
3	37.40
4	56.30

==========
ANALYSIS FOR RESPONSE VARIABLE: Sr.D2
==========
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Sr.D2

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	303.4492	101.1497	10.70	0.0036
Error	8	75.6600	9.4575		
Total	11	379.1092			

Summary Statistics

CV(%)	Sr.D2 Mean
6.94	44.34

Standard Errors

Effects	StdErr
Isolate	2.51

Pairwise Mean Comparison of Isolate
Least Significant Difference (LSD) Test

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	9.4575

Critical Value 2.3060
Test Statistics 5.7903

Summary of the Result:

Isolate	means	N	group
1	42.47	3	bc
2	46.17	3	ab
3	51.23	3	a
4	37.50	3	c

Means with the same letter are not significantly different.

==========

ANALYSIS FOR RESPONSE VARIABLE: Sr.D3

==========

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE

Response Variable: Sr.D3

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	21.7900	7.2633	3.25	0.0813
Error	8	17.9000	2.2375		
Total	11	39.6900			

Summary Statistics

CV(%)	Sr.D3 Mean
3.41	43.85

3.42 Standard Errors

Effects	StdErr
Isolate	1.22

Table of Means

Isolate	Sr.D3 Means
1	44.40
2	44.37
3	45.07
4	41.57

==========

ANALYSIS FOR RESPONSE VARIABLE: Sr.D4

==========

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE

Response Variable: Sr.D4

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	113.6367	37.8789	54.90	0.0000
Error	8	5.5200	0.6900		
Total	11	119.1567			

Summary Statistics

CV(%)	Sr.D4 Mean
1.68	49.42

Standard Errors

Effects	StdErr
Isolate	0.6782

Pairwise Mean Comparison of Isolate
Least Significant Difference (LSD) Test

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	0.6900
Critical Value	2.3060
Test Statistics	1.5640

Summary of the Result:

Isolate	means	N	group
1	54.03	3	a
2	49.77	3	b
3	45.50	3	c
4	48.37	3	b

Means with the same letter are not significantly different.

==========

ANALYSIS FOR RESPONSE VARIABLE: Sr.D5

==========

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Sr.D5

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	126.0000	42.0000	114.03	0.0000
Error	8	2.9467	0.3683		
Total	11	128.9467			

Summary Statistics

CV(%)	Sr.D5 Mean
1.61	37.67

Standard Errors
Effects StdErr
Isolate 0.4955
Pairwise Mean Comparison of Isolate
Least Significant Difference (LSD) Test
Alpha 0.05
Error Degrees of Freedom 8
Error Mean Square 0.3683
Critical Value 2.3060
Test Statistics 1.1427

Summary of the Result:
Isolate means N group
1 42.53 3 a
2 33.93 3 d
3 38.47 3 b
4 35.73 3 c
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: Ex.D1
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Isolate 4 1, 2, 3, 4
Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Ex.D1
Source DF Sum of Square Mean Square F Value Pr(> F)
Isolate 3 237.0358 79.0119 1.09 0.4072
Error 8 579.6533 72.4567
Total 11 816.6892

Summary Statistics
CV(%) Ex.D1 Mean
29.55 28.81
Standard Errors
Effects StdErr
Isolate 6.95

Table of Means
Isolate Ex.D1 Means
1 36.10
2 24.07
3 27.77

4 27.30
==========
ANALYSIS FOR RESPONSE VARIABLE: Ex.D2
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Isolate 4 1, 2, 3, 4
Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Ex.D2
Source DF Sum of Square Mean Square F Value Pr(> F)
Isolate 3 40.5967 13.5322 1.10 0.4047
Error 8 98.6400 12.3300
Total 11 139.2367

Summary Statistics
CV(%) Ex.D2 Mean
10.32 34.02
Standard Errors
Effects StdErr
Isolate 2.87

Table of Means
Isolate Ex.D2 Means
1 31.07
2 34.47
3 36.13
4 34.40
==========
ANALYSIS FOR RESPONSE VARIABLE: Ex.D3
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Isolate 4 1, 2, 3, 4
Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Ex.D3
Source DF Sum of Square Mean Square F Value Pr(> F)
Isolate 3 31.2367 10.4122 1.30 0.3407
Error 8 64.2800 8.0350
Total 11 95.5167
Summary Statistics
CV(%) Ex.D3 Mean
7.90 35.88

Standard Errors
Effects StdErr
Isolate 2.31
Table of Means
Isolate Ex.D3 Means
1 34.97
2 37.43
3 37.43
4 33.70
==========
ANALYSIS FOR RESPONSE VARIABLE: Ex.D4
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Isolate 4 1, 2, 3, 4
Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Ex.D4
Source DF Sum of Square Mean Square F Value Pr(> F)
Isolate 3 221.1825 73.7275 9.43 0.0053
Error 8 62.5467 7.8183
Total 11 283.7292

Summary Statistics
CV(%) Ex.D4 Mean
10.83 25.81
Standard Errors
Effects StdErr
Isolate 2.28

Pairwise Mean Comparison of Isolate
Least Significant Difference (LSD) Test

Alpha 0.05
Error Degrees of Freedom 8
Error Mean Square 7.8183
Critical Value 2.3060
Test Statistics 5.2647

Summary of the Result:
Isolate means N group
1 31.97 3 a
2 27.63 3 a
3 22.37 3 b

4 21.27 3 b
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: Ex.D5
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Isolate 4 1, 2, 3, 4
Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Ex.D5
Source DF Sum of Square Mean Square F Value Pr(> F)
Isolate 3 66.1625 22.0542 6.27 0.0170
Error 8 28.1200 3.5150
Total 11 94.2825

Summary Statistics
CV(%) Ex.D5 Mean
6.14 30.52
Standard Errors
Effects StdErr
Isolate 1.53

Pairwise Mean Comparison of Isolate
Least Significant Difference (LSD) Test
Alpha 0.05
Error Degrees of Freedom 8
Error Mean Square 3.5150
Critical Value 2.3060
Test Statistics 3.5300

Summary of the Result:
Isolate means N group
1 33.60 3 a
2 31.13 3 a
3 30.33 3 ab
4 27.03 3 b
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: Fo.D1
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Isolate 4 1, 2, 3, 4
Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Fo.D1

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	170.6600	56.8867	1.24	0.3573
Error	8	366.8667	45.8583		
Total	11	537.5267			

Summary Statistics

CV(%)	Fo.D1 Mean
35.15	19.27

Standard Errors

Effects	StdErr
Isolate	5.53

Table of Means

Isolate	Fo.D1 Means
1	25.27
2	19.70
3	16.10
4	16.00

==========
ANALYSIS FOR RESPONSE VARIABLE: Fo.D2
==========
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Fo.D2----

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	84.8625	28.2875	0.52	0.6773
Error	8	431.0867	53.8858		
Total	11	515.9492			

Summary Statistics

CV(%)	Fo.D2 Mean
33.92	21.64

Standard Errors

Effects	StdErr
Isolate	5.99

Table of Means

Isolate	Fo.D2 Means
1	21.33

2 26.03
3 19.07
4 20.13
==========
ANALYSIS FOR RESPONSE VARIABLE: Fo.D3
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Isolate 4 1, 2, 3, 4
Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Fo.D3
Source DF Sum of Square Mean Square F Value Pr(> F)
Isolate 3 141.0025 47.0008 7.72 0.0095
Error 8 48.6867 6.0858
Total 11 189.6892

Summary Statistics
CV(%) Fo.D3 Mean
11.36 21.71
Standard Errors
Effects StdErr
Isolate 2.01

Pairwise Mean Comparison of Isolate
Least Significant Difference (LSD) Test
Alpha 0.05
Error Degrees of Freedom 8
Error Mean Square 6.0858
Critical Value 2.3060
Test Statistics 4.6449
Summary of the Result:
Isolate means N group
1 21.50 3 b
2 27.13 3 a
3 20.50 3 b
4 17.70 3 b
Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: Fo.D4
==========
Summary Information
FACTOR NO. OF LEVELS LEVELS
Isolate 4 1, 2, 3, 4
Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Fo.D4---

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	136.0425	45.3475	13.69	0.0016
Error	8	26.5067	3.3133		
Total	11	162.5492			

Summary Statistics

CV(%)	Fo.D4 Mean
7.75	23.49

Standard Errors

Effects	StdErr
Isolate	1.49

Pairwise Mean Comparison of Isolate
Least Significant Difference (LSD) Test

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	3.3133
Critical Value	2.3060
Test Statistics	3.4273

Summary of the Result:

Isolate	means	N	group
1	22.17	3	b
2	29.00	3	a
3	22.90	3	b
4	19.90	3	b

Means with the same letter are not significantly different.
==========
ANALYSIS FOR RESPONSE VARIABLE: Fo.D5
==========
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Fo.D5

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	40.2833	13.4278	2.74	0.1133
Error	8	39.2467	4.9058		
Total	11	79.5300			

Summary Statistics

CV(%)	Fo.D5 Mean
10.28	21.55

Standard Errors

Effects	StdErr
Isolate	1.81

Table of Means

Isolate	Fo.D5 Means
1	22.57
2	23.93
3	19.17
4	20.53

==========

ANALYSIS FOR RESPONSE VARIABLE: Rs.D1

==========

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE

Response Variable: Rs.D1

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	547.3500	182.4500	2.98	0.0965
Error	8	490.2000	61.2750		
Total	11	1037.5500			

Summary Statistics

CV(%)	Rs.D1 Mean
40.66	19.25

Standard Errors

Effects	StdErr
Isolate	6.39

Table of Means

Isolate	Rs.D1 Means
1	25.77
2	16.07
3	25.47
4	9.70

==========

ANALYSIS FOR RESPONSE VARIABLE: Rs.D2

==========

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Rs.D2

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	247.3492	82.4497	2.45	0.1384
Error	8	269.3400	33.6675		
Total	11	516.6892			

Summary Statistics

CV(%)	Rs.D2 Mean
22.32	25.99

Standard Errors

Effects	StdErr
Isolate	4.74

Table of Means

Isolate	Rs.D2 Means
1	24.80
2	27.77
3	31.93
4	19.47

==========
ANALYSIS FOR RESPONSE VARIABLE: Rs.D3
==========
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Rs.D3

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	313.0867	104.3622	13.62	0.0016
Error	8	61.2933	7.6617		
Total	11	374.3800			

Summary Statistics

CV(%)	Rs.D3 Mean
8.76	31.60

Standard Errors

Effects	StdErr
Isolate	2.26

Pairwise Mean Comparison of Isolate
Least Significant Difference (LSD) Test

Alpha	0.05
Error Degrees of Freedom	8

Error Mean Square 7.6617
Critical Value 2.3060
Test Statistics 5.2117
Summary of the Result:

Isolate	means	N	group
1	32.10	3	b
2	33.00	3	ab
3	37.73	3	a
4	23.57	3	c

Means with the same letter are not significantly different.

==========

ANALYSIS FOR RESPONSE VARIABLE: Rs.D4

==========

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE
Response Variable: Rs.D4

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	115.9300	38.6433	1.08	0.4109
Error	8	286.1867	35.7733		
Total	11	402.1167			

Summary Statistics

CV(%)	Rs.D4 Mean
15.77	37.92

Standard Errors

Effects	StdErr
Isolate	4.88

Table of Means

Isolate	Rs.D4 Means
1	33.73
2	39.33
3	42.07
4	36.53

==========

ANALYSIS FOR RESPONSE VARIABLE: Rs.D5

==========

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Isolate	4	1, 2, 3, 4

Number of Observations Read and Used: 12

ANOVA TABLE

Response Variable: Rs.D5

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	3	28.7367	9.5789	0.33	0.8017
Error	8	229.7800	28.7225		
Total	11	258.5167			

Summary Statistics

CV(%)	Rs.D5 Mean
11.31	47.38

Standard Errors

Effects	StdErr
Isolate	4.38

Table of Means

Isolate	Rs.D5 Means
1	46.53
2	45.93
3	47.10
4	49.97