Biological control of *Phalaris minor* in wheat (*Triticum aestivum* L.) using rhizosphere bacteria

BY

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In

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CERTIFICATE -I

This is to certify that this dissertation entitled, **“Biological control of *Phalaris minor* in wheat (*Triticum aestivum* L.) using rhizosphere bacteria”** submitted for the degree of **Master of Science** in the subject of **Microbiology** to Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Ms. Manisha Phour**, Admn. No. **2010BS107M** under my supervision and that no part of this dissertation has been submitted for any other degree. The assistance and help received during the course of investigation have been fully acknowledged.

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This is to certify that this dissertation entitled, **"Biological control of *Phalaris minor* in wheat (*Triticum aestivum* L.) using rhizosphere bacteria"** submitted by **Ms. Manisha Phour**, Admn. No. **2010BS107M** to Chaudhary Charan Singh Haryana Agricultural University, Hisar in partial fulfillment of the requirements for the degree of **Master of Science** in the subject of **Microbiology** has been approved by the Student's Advisory Committee after an oral examination on the same.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER-I

INTRODUCTION

Weeds pose a serious constraint to agricultural production and losses from weeds alone average almost 12 percent of the world's agricultural output. Weed seedlings emerge yearly from the vast reservoir of viable weed seeds in soils used for cultivation of crops. The resulting weed infestations require repeated weed control treatments with chemical herbicides over a period of years for successful weed management. The common herbicides used for chemical control of weeds include isoproturon, 2, 4-dichlorophenoxyacetic acid (2, 4-D), clodinafop, fenoxaprop, sulfosulfuron, tralkoxydin and tribenuron-methyl etc. (Brar and Walia, 1993). Recently, the herbicide component of all pesticides sold has increased from less than 20% in the 1950s to almost over 60%. These chemical herbicides create spray drift hazards and adversely affect the environment. Moreover, herbicide residues in food commodities directly or indirectly affect human health. These problems necessitated the search for an alternate ecofriendly method of weed management through the biological approach in which microorganisms or their products could be used to suppress the growth or population of the weed species (Templeton, 1988; Kremer and Kennedy, 1996).

The introduction of highly specific indigenous and exotic pathogens as well as natural enemies could be used as weed control agents. These selected microorganisms that specifically inhibit weed seedling development, thereby hinder the establishment of a weed population. Therefore, there are immense possibilities for further increasing the production of cereal crops by application of biological control agents as weedicides (Kremer *et al.,* 1990; Kennedy *et al.,* 1991). Development of bioherbicides will provide cheap and specific biocontrol agents for these biological pollutants. Thus, application of rhizospheric bacteria has reduced dependence on synthetic herbicides, reduced weed seed bank population through environment friendly practices and potentially reduced the costs of weed control in crop production, forestry and aquatic systems (Bailey *et al.,* 2010). The use of rhizospheric bacteria to control weeds has there also decreased chances of developing herbicide resistant in the target weeds due to the multiple mechanisms involved (Omer and Balah, 2011).

Wheat (*Triticum aestivum* L. em. Thell.) is the most important grain crop and is a source of staple food in many countries of the world. It is one of the most important agricultural commodities in the international trade. Though the production of wheat has increased after green revolution but the scarcity of nutrients (N, P and K) in the soil has been found to affect its yield and quality. In addition, a large number of weeds in the wheat-growing field compete with crop plants for light, water and nutrients, and thus further depletes the available nutrients for the crop. Uncontrolled weed growth has been found to reduce yield of wheat ranging from 15-40%, depending upon magnitude, nature and duration of weed infestation (Jat *et al.,* 2003).

Wheat crop is infested with grassy as well as broad leaf weeds. Among grassy weeds, *Phalaris minor* L. Retz. (littleseed carnary grass; mandushi) is an annual grass weed which infests several winter season crops including wheat with a potential to cause significant economic loss (Singh *et al.,* 1999). The adoption of rice-wheat cropping system has further led to the problem of *Phalaris minor* causing yield reduction to the level of 30 to 80% in grain yield of wheat depending upon the intensity (Brar and Walia, 1993). The other important weeds of wheat include *Argemone mexicana* (satyanashi), *Asphodelus tenifolius* (piazi), *Avena ludoviciana* (jangli jai), *Chenopodium album* (bathu), *Convolvulus arvensis* (hirankhuri), *Fumaria parviflora* (gajri), *Lathyrus aphaca* (matri), *Melilotus alba* (metha), *Polypogon monspeliensis* (lomar ghas) and *Rumex retroflex* (jangli palak). Due to crop mimicry, it is difficult to differentiate these weeds from wheat at the seedling stage as their seedlings are identical to wheat seedlings in morphology. Life cycle of these weeds also coincides with wheat. The problem in identification at the early stage impedes in the manual control, hence herbicide application becomes inevitable (Hassan *et al.,* 2005). The evolution of herbicide resistance of *Phalaris minor* in the wheat–rice cropping system of north-west India has been the most serious case of herbicide resistance in the world, resulting in total crop failure and heavy infestation. Many biotypes had become resistant to isoproturon, with resistant biotypes from Haryana requiring up to eleven times the pre-susceptible dose of isoproturon to achieve 50% weed control (Malik and Singh, 1995) and farmers have to use costly herbicides, namely clodinafop and sulfosulfron (Samunder Singh, 2006; Dhaliwal *et al.,* 2007).

Naturally-occurring microbial communities in the rhizosphere have the potential to suppress the weed growth through manipulation of rhizosphere ecosystem (Charudattan and Dinoor, 2000; Mohan Babu *et al.,* 2003a). Although a number of microbial biocontrol agents have been identified, however, very few have been commercially successful. These selected rhizosphere microorganisms colonize the root surfaces of weed seedlings and suppress the growth of weed plant by reducing weed density, biomass and its seed production (Kremer and Kennedy, 1996). Sometimes, plant may not be killed but their competitive ability is much reduced. Many rhizobacteria such as fluorescent and other *Pseudomonas* sp., *Flavobacterium* spp., *Erwinia herbicola, Alcaligenes* spp., strains of *Xanthomonas campestris* pv. *poannua*, *Pseudomonas syringae* pv. *tagetis* and *P. syringae* pv. *phaseolicola* have been exploited as foliar bioherbicides whereas *P. fluorescens, Pseudomonas* spp., *Xanthomonas* spp., *Enterobacter* and *Erwinia herbicola* have been developed as soil application bioherbicides (Kremer, 2000). Currently, fourteen fungi and one bacterium have been registered as bioherbicides in Canada, China, Japan, Netherlands, South Africa and United States of America. These microorganisms have been commercialized as bioherbicides under different trade names.

The mode of action of each biocontrol agent is as varied as the microorganisms themselves (de Luna *et al.,* 2011). They range from simple but effective compounds like cyanide (Kremer and Souissi, 2001; Owen and Zdor, 2001) and organic acids to complex molecules with tertiary structure (Gurusiddaiah *et al.,* 1994; Bouizgarne *et al.,* 2006), and from secondary metabolites (Kroschel and Elzein, 2004) to plant growth regulators, such as auxins and ethylene (de Luna *et al.,* 2005). Bacterial and fungal microbes also produce a wide array of phytotoxins with the potential to be used as herbicides (Duke *et al.,* 1991) and these phytotoxins have a detrimental effect on vegetation (Jobidon, 1991a). One microbial phytotoxin, bialaphos, is currently registered in Japan for agricultural weed control and is marketed under the trade name Herbiace® (Jobidon, 1991a, 1991b). AAL-toxin, a natural metabolite of the pathogen *Alternaria alternata* f. sp. *lycopersici* has been tested on a range of crops and weed species, and has been patented as a herbicide (Abbas *et al.,* 1995). Some deleterious rhizobacteria (DRB) for weed growth induce the damage through the production of phytotoxins that are absorbed by the plant roots.

Various allelochemicals produced by microorganisms such as indole acetic acid (IAA), δ-aminolevulinic acid (ALA) and glycoproteins have also been reported to reduce the germination and development of weeds (Saxena *et al.,* 1996; Barazani and Friedman, 1999; Chon, 2003; Mohan Babu *et al.,* 2003b; Mejri *et al.,* 2010). Reduction in root length was most likely due to production and release of high amounts of auxins causing disruption of the hormonal balance critical to the growth and development of root tissues (Loper and Schroth, 1986). Keel *et al.* (1992) reported that the antibiotic 2, 4-diacetylphloroglucinol produced by *Pseudomonas fluorescens* strain CHA0 showed antifungal, antibacterial and phytotoxic activity. This antibiotic 2,4-diacetylphloroglucinol has also been found to exert herbicidal activity resembling to that of 2,4-dichlorophenoxyacetate (2, 4-D) (Kataryan and Torgashova, 1976).

Suslow and Schroth (1982) suggested that deleterious rhizobacteria (DRB) are ubiquitous and common to rhizosphere of all plant root systems. Selection for rhizobacteria that are specifically detrimental to weed seedling growth could benefit agriculture by contributing to increased crop yields by minimizing weed competition and by reducing the use of chemical herbicides. The presence of soil-borne phytopathogens or rhizobacteria resembling DRB on weed /crop plants has been surveyed; however, the potential of these bacteria as biocontrol agents for weed control was not investigated. Keeping this in view, the present study was undertaken with the following objectives:

(i) To screen rhizobacterial isolates for inhibition of seed germination of *Phalaris minor* weed.

(ii) To study the possible mechanism of bioherbicidal action of selected rhizobacteria.

(iii) To evaluate the effect of selected bioherbicidal bacteria on plant growth of weed and wheat.

CHAPTER-II

REVIEW OF LITERATURE

Wheat is the major winter season crop of India and grown in 27 million hactares. Wheat competes with several grassy and broadleaf weeds in its growth period depending upon the agronomic practices followed, locations (soil types) and weed management practices adopted. Weed flora is always dynamic and weed species adapt more quickly to the management practices followed, for their survival and prevalence. Broadleaf weeds were most common in wheat before the onset of green revolution and fertilizer responsive dwarf varieties were less competitive with grassy weeds that resulted in the dominance of *Phalaris minor* (Singh *et al.,* 1995). It competes with crop for sunshine, space and plant nutrients, and cause lot of damage to crop and also forces the crop to lodge (Walia *et al.,* 2001).

2.1. *Phalaris minor* and its major problems

Phalaris minor (littleseed canary grass) is a monocot plant and graminaceous weed. Locally, it is called Dumbi sitti, Gullidanda, Sitti, Kanki and Mandusi. It grows in rabi (winter) season and found in all parts of the world, especially in tropical and sub-tropical regions. It is mostly observed in wheat, barley and oat crop fields, waste and fallow lands, along roads, streets, near water channels, poultry sheds, dairy farms, residential colony parks and on sand dunes. Its infestation is common in many wheat growing areas and present in every part of the world, except Antarctica and North Pole (Singh *et al.,* 1999). It resembles wheat plants very much until flowering stage and it is very difficult to distinguish it from wheat plant in its early growth stages (Yasin and Iqbal, 2011). Its stem is erect with distinct nodes and internodes. Branches arise from nodes and leaves are long. Ligules are exceptionally long (about 1 cm) and clasp the stem. Panicles are cylindrical erect. Each canary grass produce 200 to 500 shiny, small, black seeds which can easily be identified by its fox-tail type spike. Its panicles commence maturity at about 2 weeks before wheat harvest (Walia, 2006). Aqueous extract of *P. minor* roots and tops cause twisted roots and prevent root hair development. Each plant of canary grass produce about 300 to 460 seeds which contaminate the wheat crop seed (Rammoorthy and Subbain, 2006).

Phalaris minor is a common weed of wheat–rice cropping systems in the north-western Indo-Gangetic plains of India. Rice-wheat cropping system comprises 13.5 m ha (10 m ha in India, 2.2 m ha in Pakistan, 0.8 m ha in Bangladesh and 0.5 m ha in Nepal) of land in South Asia (Woodhead *et al.,* 1994). In India, the area of infestation is mostly contained in the states of Punjab and Haryana. These two states account for around 3 million hactares of wheat–rice cropping land out of India's 10 million hactares cropping system and about 35%

of India’s wheat production. *Phalaris minor* infestation has been a long-standing management problem for farmers in these states. The weed problem dates back to the green revolution of the late 1960s, with the introduction of dwarf wheat varieties and the improved irrigation and fertilizer practices needed to maximize their yield potential. Untreated weed infestation can result in dramatic reductions in wheat yields (the weed does not germinate in the rice part of the rotation). Complete failure of the wheat crop can occur in extreme cases.

Until the early 1990s, *Phalaris minor* was effectively controlled by isoproturon, a substituted urea herbicide first recommended in 1977-78 and widely used since the early 1980s. But continuous use of this single herbicide for 10-15 years coupled with monocropping of rice-wheat led to evolution of resistance in this weed (Walia *et al.,* 1997; Malik and Singh, 1993, 1995). By 1993, the resistance affected area ranged between 0.8 and 1.0 million hactares in North-West India and it also affected other tarai areas. The resistant biotypes from Haryana required 2-8 times (Malik and Singh, 1995), 5-6.5 times (Yadav *et al.,* 1996) and 6.3 to 11.2 times (Malik and Yadav, 1997) more dose of isoproturon compared to prestine/susceptible populations to cause 50% growth reduction. The resistance was also found to be of metabolic in nature (Malik *et al.,* 1995; Singh *et al.,* 1996; Kirkwood *et al.,* 1997). The first case of herbicide resistance in India and for the first time in the world in littleseed canary grass (*P. minor* Retz.) against isoproturon was reported by the scientists of CCS Haryana Agricultural University, Hisar during 1992-93 (Malik and Singh 1993; Malik and Malik, 1994; Malik *et al.,* 1995; Yadav *et al.,* 1996; Balyan *et al.,* 1997). This was the most serious case of herbicide resistance in the world, resulting in total failure of wheat crop under heavy infestation (2000-3000 plants m^{-2}) (Malik and Singh, 1995). The new herbicides brought the *Phalaris minor* infestation under control and restored yields to their previous levels. But red signals of resistance against these alternate herbicides have also been speculated in 2002 and thereafter. It warrants for integration of different weed control methods.

2.2. Development of microbial herbicides

The advantages for developing microbial herbicides include the potential for herbicide resistant weeds; impacts of synthetic pesticides on the environment; chemical herbicides may persist in soil for longer than one growing cycle, thus limiting options for crop rotations; there may be limitations on herbicide registrations for certain crop and finally, there is the potential for injury to non-target organisms (Guske *et al.,* 2004). Due to these concerns, much interest has developed in exploiting microorganisms for weed management. Biological controls for weeds can generally be divided into one of three general types: classical, augmentative or inundative and cultural. Classical biological control by means of pathogens has been used in several parts of the world to control exotic weeds (Bruckart and Hasan, 1991). This approach is fairly simple in its concept, and discover effective and highly host-specific agents from the weed's native geographic range, and confirm their safety as well as effectiveness by rigorous experimental evaluation. One of the most successful examples of classical biological control of weeds is the introduction of a rust fungus, *Puccinia chondrillina*, into Australia to control rush skeleton weed (*Chondrilla juncea*). Following the introduction and establishment, the fungus disseminated rapidly and controlled the most common biotype of the weed. It has been estimated that this highly successful biocontrol project has resulted in a cost-to-benefit ratio of 1:100 in Australia (Cullen and Hasan, 1988). Augmentative or inundative biological control refers to repeated application of a foreign agent with the intent to reduce weed densities to a level where beneficial plant species can compete. An example of Herbicides – Environmental Impact Studies and Management Approaches 136 of an augmentative control is *Colletotrichum gloeosporioides* for control of sicklepod (*Senna obtusifolia* L.; Boyette, 2006). Cultural weed control might include crop rotation, fallow periods, sanitation to prevent the introduction and spread of weed seeds, and maintaining soil fertility to produce healthy crop plants.

Table 1. Some worst weeds of the world which are resistant to many herbicides but are controlled with biocontrol agents

Herbicide resistant weed	Herbicide	Biocontrol agent
Eichhornia crassipes (Water hyacinth)	2, 4-D, Glyphosate	*Alternaria alternata, A. eichhorniae, Cercospora piaropi*
Chenopodium album (Common lambsquarters)	Triazine (Atrazine), Nitriles	*Ascochyta caulina, Alternaria alternata*
Salsola kali (Russian thistle)	Sulfonylurea, Sulfometuron	*Uromyces salsolae*
Senecio vulgaris (Common groundsel)	Triazine (Atrazine)	*Puccinia lagenophorae*
Sesbania exaltata (Hemp sesbania)	Glyphosate (Roundup)	*Colletotrichum truncatum*
Cyperus (Purple nutsedge)	Sulfonylurea	*Phyllachora cyperi* *Dactylaria higginsii*
Avena fatua (wild oat)	Glyphosate	*Drechslera avenacea*
Echinochola crus-galli (Barnyard grass)	Dinitroanilin (Trifluralin), Pretilachlor, Quinclorac	*Exserohilum Longirostratum* *Coctilobolus lunatus*

2.3. Successful microbial herbicides

Bioherbicide DeVine, containing a Florida isolate of *Phytophthora palmivora*, is used for the control of *Morrenia odorata* (strangler vine or milkweed vine) in citrus in Florida. Collego, based on *Colletotrichum gloeosporioides* f. sp. *aeschynomene*, is used to control *Aeschynomene virginica* (Northern Joint vetch), a leguminous weed in rice and soybean crops in Arkansas, Mississippi and Louisiana. A stump-treatment product based on the woodinfecting basidiomycete, *Cylindrobasidium laeve*, under the commercial name Stumpout, is registered in South Africa to control resprouting of cut trees in tree plantations and in natural areas. Majority of bioherbicides are mycoherbicides with the exception of Camperico which is a bacterial bioherbicide. An isolate of *Xanthomonas campestris* pv. *poae*, a wilt-inducing bacterium, isolated in Japan from *Poa annua* (annual bluegrass or winter grass), has been registered in Japan as the bioherbicide Camperico to control annual bluegrass in golf coarses. The product is applied after mowing and infects the cut surfaces.

A screening procedure for a bacterial biological control agent has been developed. This screening includes sampling the soil and plant material at the peak of population growth and when the suppression naturally occurs. An initial assay can separate the weed-suppressive microorganisms from the bulk of the native population. Further multiple screenings on various plants are suggested to get rid of those isolates that may also inhibit beneficial plants. While inhibition of the target weed is critical, non-host range must be determined early in the process to ensure the product progresses through the registration process and eventually becomes marketable. Both steps need to be thorough to ensure that no lesser known, but economically important host is detected later in the process. Souissi and Kremer (1998) utilized a multiple-well plate procedure with leafy spurge (*Euphorbia esula* L.) callus to rapidly determine phytotoxicity of rhizobacterial isolates. Vidal *et al.* (2004) developed a successful method to produce yellow starthistle (*Centaurea solstitialis* L.) calli for bioassay screening of biocontrol pathogens such as *Phoma exigua*. A quick substitute bioassay will reduce the length of time needed for the initial screenings. In many cases, a quick screen is not possible (Kennedy *et al.,* 1991) but automating data recording can often make data collection easier (Doty *et al.,* 1994).

2.4. Microbial influences on weed growth

Roots provide plants with water and nutrients. Some biological control agents attach to roots of weeds, live on root surfaces and release toxins that stunt root growth. Many fungi infect roots and disrupt then water transport system, which reduces leaf growth. Beneficial insects and nematodes feed directly on the weed roots causing injury which allows bacteria and fungi to penetrate. Plant leaves capture energy from the sun and store it as sugar. Insects that feed on leaves reduce the leaf surface available for energy capture. Fungi and bacteria that infect leaves reduce the ability of the leaf to make sugars. In either case, there is less energy available for weed growth. Whether through damage on roots or leaves, severe infestations of biological control agents can actually kill weeds, reducing their adverse effects on desirable plants. Many weed species survive from year to year by producing seeds. Fungi or insects that attack seeds can reduce the number of weed seeds stored in the soil, which in turn can reduce the size of future weed populations. This lowers the effort needed to control the remaining emerging weeds. Some bacteria and fungi applied as biological control agents do not survive from year to year. These organisms must be applied on an annual basis. This technique is called the "bioherbicide" strategy. With this tactic, biological agents are used in manner similar to chemical herbicides.

2.5. Occurrence of microorganisms in the soil and rhizosphere

The microbial growth and activity is particularly intense in the rhizosphere. The rhizosphere environment is richer in nutrients and its microbial communities differ from those present in soil (Alexander, 1977). It is now recognized that the 'rhizosphere effect' is due to the availability of root exudates which attract soil microorganisms. This is reflected by the number of bacteria that are found around the roots of plants and that is generally 10 to 100 times higher than in the bulk soil. The physical and chemical properties of soil play a dominant role in multiplication and survival of soil microorganisms. The width of the zone of soil influenced by the living plant root varies with the plant species, its age, cultural conditions and environmental factors (Rangaswami and Bagyaraj, 1998). Bacteria are the most dominant group of microorganisms in the soil and contribute about 300 - 3000 kg wet biomass per hectare of soil. The most abundant bacterial genera present in the soil are *Arthrobacter, Bacillus, Burkholderia, Clostridium, Corynebacterium, Enterobacter, Flavobacterium, Micrococcus, Rhizobium, Pseudomonas, Sarcina* and *Serratia* etc. (Rangaswami and Bagyaraj, 1998; Subba Rao, 2000).

Some bacterial species living in the rhizosphere can effect plant growth in either a positive or in a negative way. Beneficial effects of rhizosphere bacteria have most often been based on increased plant growth and seedling emergence, inhibition of weeds, enhanced nodulation and nitrogen fixation in leguminous crops and suppression of diseases. Sorenson *et al.* (2001) reviewed the factors that control the successful performance of *Pseudomonas* spp. as inoculants and its establishment, proliferation and activity under *in situ* conditions. The use of advanced microscopy using fluorescent antibodies and marker gene technology as tracking methods, have provided new insight into the significance of growth limiting factors in the soil and along the root.

2.6. Isolation of weed inhibiting bacteria

A major group of rhizobacteria with potential for biological control is the pseudomonads (Kennedy *et al.,* 1991). Rhizobacteria and their metabolites have been evaluated as weed control agents in rice systems (Kennedy *et al.,* 1991; Tranel *et al.,* 1993; Norman *et al.,* 1994; Mazzola *et al.,* 1995; Gealy *et al.,* 1996). Live cultures of *Pseudomonas syringae* strain 3366 were found to reduce weed root growth in controlled environment (Johnson *et al.,* 1983) and in field studies (Kennedy *et al.,* 1991).

Kremer *et al.* (1986) reported that the majority of the bacteria which produce toxins are Gram-positive bacteria but there are a small number of Gram-negative bacteria such as *Streptomyces*, *Corynebacterium fasciomonads* and some are nonfluorescent pseudomonads.

Dye *et al.* (1980) reported that there are 41 host range varieties of pseudomonads. *P. syringae* caused disease on characterstic host plant species but induced defence in other plants. Kremer *et al.* (1990) isolated rhizobacteria from seedlings of seven economically important weeds and characterized their potential phytopathogenicity. Fluorescent and nonfluorescent pseudomonads, *Erwinia herbicola*, *Alcaligenes* spp. and *Flavobacterium* spp. were frequently isolated. Only 18% of isolates were potentially phytopathogenic, based on an *Escherichia coli* indicator bioassay. However, the proportion of isolates that inhibited growth in seedling assays ranged from 35 to 65% depending on the weed host. Antibiosis was most prevalent among isolates of fluorescent *Pseudomonas* spp., the activity of which was due to siderophore production in over 75% of these isolates.

Kennedy *et al.* (1991) screened 1000 isolates of pseudomonads for differential inhibition of downy brome (*Bromus tectorum*) and winter wheat. When filtrates of bacterial free culture were tested on agar, 8% inhibited root growth of downy brome, but did not affect root growth of winter wheat. However, when applied to soil (10^8 c.f.u./ml) under nonsterile conditions, only six isolates (~1%) inhibited growth of downy brome. In the field, when sprayed (10^8 c.f.u./m^2), two isolates (0.2%) suppressed downy brome by 31 to 53% and this treatment increased winter wheat yield by 18 to 35%. It suggested that specificity does exist and for an agricultural evaluation, tests in soil are essential. Imaizumi *et al.* (1997) reported that *X. campestris* pv. p*oae* has great potential for controlling annual bluegrass without harming desirable turf grasses such as creeping bentgrass (*Agrostis palustris*) and Kentucky bluegrass (*Poa pratensis*). Zermane *et al.* (2007) reported that *P. fluorescens* has potential for controlling *Orobanche crenata* and *O. foetida* (Broomrape) in Northan Tunisia.

Kremer (2000) evaluated several cover crop species alone or with deleterious rhizobacteria (DRB) for growth suppression of annual weeds. DRB were detected on roots of cover crops. Seed inoculation with DRB further reduced weed biomass in seven of the twelve cover crops and enhanced the weed suppression. Cover crops have also been found to promote proliferation of naturally-occurring weed suppressive microorganisms by releasing allelochemicals that contributed to weed suppression and may disrupt the soil environment to minimize weed establishment (Cardina *et al.,* 1999). Cover crops could also provide habitats for seed predators for reducing weed seedling emergence (Reader *et al.,* 1991). However, development and rapid acceptance of biological control as a practical management option in cropping systems is challenged by factors limiting their spectrum, efficacy and reliability.

2.7. Mechanisms involved in herbicidal activity

The mechanisms by which rhizobacteria inhibit the growth of weed includes: (i) colonization of weed’s root; (ii) antibiotic production; (iii) IAA production; (iv) ALA production; (v) production of other secondary metabolites and (vi) phytotoxin production.

2.7.1. Colonization of weed's root

Wiehe and Hoflich (1995) demonstrated that inoculation of a plant-growth promoting pseudomonad on various crops resulted in colonization of roots of the weeds pigweed (*Amaranthus retroflexus* L.) and barnyard grass (*Echinochloa crus-galli* L. Beauv.) by horizontal migration from the inoculated crops up to 60 cm away. The increase in the specificity of a bioherbicide and the susceptibility of the target organism may allow a selected area of a weed to be controlled without affecting nearby plants of the same species. For example, isolates of *Xanthomonas campestris* pv. *poae* showed some activity against annual meadow grass (*Poa annua*), a lawn weed (Imaizumi *et al.,* 1997).

The multiplication and survival of DRB are greatly affected by environmental factors (Kremer and Kennedy, 1996). For example, soil moisture, soil texture and composition of host plant root exudates influence colonization of the rhizosphere and rhizoplane (Howie and Echandi, 1983) and growth inhibition of grasses by pseudomonads may be influenced by soil type (Horwath *et al.,* 1998). Fredrickson and Elliott (1985) found that root colonization was similar over a range of root zone temperatures but was dependent on soil type, with higher populations developing in soils with lower organic matter content.

2.7.2. Antibiotic production

Antibiotic production by rhizobacteria is one of the major mechanisms postulated for antifungal activity, for suppression of pathogens in the rhizosphere and plant growth promotion. Soil bacteria including rhizobacteria, have been reported to produce antibiotic-like substances in culture media under laboratory conditions, which are effective in inhibiting growth of fungi (Defago and Haas, 1989; Keel et al., 1992; Schimidli- Sacherer et al., 1997; Dunne et al., 1998).

Kataryan and Torgashova (1976) reported that besides the inhibition of phytopathogenic bacteria and fungi, the antibiotic 2, 4-diacetylphloroglucinol also showed phytotoxic activity. It also showed herbicidal activity resembling to that of 2, 4-dichlorophenoxyacetate (2, 4-D). Geldanamycin and nigericin, phytotoxic metabolites obtained from a strain of *Streptomyces hygroscopicus*, were tested for herbicidal activity and selectivity on a range of crop and weed species. Geldanamycin showed significant pre-emergence activity on proso millet, barnyard grass, garden cress and giant foxtail. It had no post-emergence herbicidal effect on any of the species tested. Nigericin had pre-emergence activity on garden cress and large crabgrass and post-emergence activity on garden cress and velvetleaf. The post-emergence effect of nigericin on velvetleaf was especially striking, with leaves showing symptoms of injury within 24–48 h of treatment (Heisey *et al.,* 1990).

Babaczinski *et al.* (1991) reported vulgamycin as phytotoxin against dicotyledonous weeds and grasses if applied post-emergence. Phosphenothrixin that is produced by *Saccharothrix* sp. ST-888 inhibits the germination of gramineous and broadleaved weeds

(Takahashi *et al.,* 1995). Herbimycin represents a potent herbicidal activity when used pre-emergence, against flat-sedge (*Cyperus microiria* Steud.) (Sekizawa and Takematsu, 1983). Lee *et al.* (2003) reported that methoxyhygromycin is an antibiotic having a unique structure produced by *Streptomyces* sp. Methoxyhygromycin compound showed higher activity in the range of 90% at 0.25 kg ha^{-1} against monocotyledonous weeds such as large crab grass (*D. sanguinalis*) and barnyard grass (*E. crus-galli*) than dicotyledonous weeds.

2.7.2.1. Growth inhibition of *Fusarium oxysporum* by rhizospheric bacteria

Sindhu et al. (1999) reported that *Pseudomonas* isolates inhibited the growth of *Fusarium oxysporum*. Yeole et al. (2001) showed that twelve fluorescent pseudomonad isolates obtained from chilli (CHRB2, CHRB3 and CHRB4), cotton (CORB1, CORB2, CORB3 and CORB4), groundnut (GNRB1, GNRB2 and GNRB3) and soybean (SBRB4 and SBRB5) inhibited the growth of twelve soil borne plant pathogens (*Aspergillus* sp., **A. niger**, *Fusarium* sp., *Fusarium oxysporum*, *F. solani*, *Mucor* sp., *Penicillum* sp., *Pythium* sp., *Rhizopus oryzae*, *Rhizoctonia solani* and *Sclerotium rolfsii*) in Fe deficient Kings'B medium. However, the inhibition ranged from 33 to 15%. All *Pseudomonas* strains were found to produce siderophore in iron deficient conditions. The inhibition was curtailed by 24-60% in the presence of iron (50 µm) when sidrophore production abolished.

Bakker et al. (2002) isolated a spontaneous mutant of *P. fluorescens* RS111 that was less sensitive to antagonism by other strains of fluorescent *Pseudomonas*. This mutant, designated as RS111a, appeared to be more effective in suppression of *Fusarium*. Both strains produced an antifungal compound and non-producing mutants were identified. Numerous mechanisms have been proposed including production of antibiotics, siderophore, and hydrolytic enzymes, accumulation of antifungal metabolites, nutrient competition and niche exclusion (Chen *et al*., 1995; Goel *et al.* 2001). Bano and Musarrat (2003) reported the isolation of phorate degrading *Pseudomonas* isolates from agricultural soil. The isolate PS-3 was found to produce siderophore and showed antifungal activity against *Fusarium oxysporum*. Bano and Musarrat (2004) isolated *Pseudomonas* sp. NJ-101 from agricultural soil that exhibited efficient degradation of the insecticide carbofuran. The ability to produce hydrogen cyanide and siderophore stipulated its role in biological control. The growth inhibition of *Fusarium* sp. validated the antagonistic activity of NJ-101 against the common phytopathogens. Ugoji and Laing (2008) tested the effect of *Bacillus* as a biocontrol agent against some *Fusarium* sp..

2.7.3. Indole acetic acid production

The capacity to produce indole acetic acid (IAA) is wide spread among plant-associated bacteria (Gaudin *et al.,* 1994; Patten and Glick, 1996; Glickmann *et al.,* 1998). Estimates of the number of IAA-producing organisms range as high as 80% of total soil bacteria highlighting the enormous contribution potential of these organisms to plant's endogenous pool of IAA. The IAA production ability may allow bacteria to detoxify excess tryptophan or tryptophan analogs that are deleterious to bacterial cell, as some IAA biosynthetic enzymes can convert methylated and halogenated substrates to less toxic compounds (Hutcheson and Kosuge, 1985; Yamada *et al.,* 1985; Bar and Okon, 1992). IAA is also suggested to regulate the expression of important compounds in bacteria such as cAMP and amino acids (Katsy, 1997). Perhaps the most obvious utilization for the prevalence of bacterial production of phytohormones is that it may increase production of plant metabolites that bacteria can utilize for their own growth. Another consequence of enhanced plant growth is production of greater root surface area through which more of these metabolites can be exuded (Gaudin *et al.,* 1994).

Many rhizosphere bacteria produce IAA in culture media specially in the presence of tryptophan (Lynch, 1976; Ek *et al.,* 1983; Sterzelczyk and Pokojska-Burdziej, 1984). Indole-3-acetic acid that stimulate plant growth (coleoptiles tissue) in lower concentrations and in contrast, if the concentration becomes higher, the effect reverses and elongation of root and shoot is inhibited. Natural auxins have modes of action similar to many herbicides that interfere with plant growth such as 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T) (Patten and Glick, 1996).

Omay *et al.* (1993) showed that in liquid culture of *Azospirillum brasilense* Cd, the concentration of IAA increased rapidly with the beginning of stationary phase. This suggested that the increase in IAA production in stationary phase is due to the expression of an overall change in cell metabolism when the carbon source is exhausted. *Bacillus* spp. isolates obtained from the rhizosphere of *Phaseolus vulgaris* 'contender' produced significant amount of IAA when grown in a liquid culture medium supplemented with 100 μg L-tryptophan L^{-1} (Srinivasan *et al.,* 1996). In the absence of L-tryptophan, less IAA was produced.

Serwar and Kremer (1995) reported that auxins produced in high concentrations in the rhizosphere by deleterious rhizobacteria (DRB) may contribute to reduced root growth of weeds. An *Enterobacter taylorae* isolate with high auxin-producing potential (72 mg L^{-1} IAA-equivalents) was found to inhibit root growth of field bindweed (*Convolvulus arvensis* L.) by 90.5% when combined with 10^{-5}M L-tryptophan compared with non-treated control. Results suggested that providing L-tryptophan to selected auxin-producing DRB may increase phytotoxic activity against emerging weed seedlings and may become a practical biological control strategy.

Suzuki *et al.* (2003) isolated IAA low-producing spontaneous mutant HP72LI after several repeated subculturing of *P. fluorescens* HP72. The IAA low production of the strain HP72LI was due to the low tryptophan side chain oxidase (TSO) activity. The colonization ability of strain HP72 on the bentgrass root is higher than that of strain HP72LI. Moreover, colonization of strain HP72 on the bentgrass root induced root growth reduction, while strain

HP72LI did not induce such growth reduction. The results suggested that IAA production by strain HP72 may play a role in the construction of short root systems and take advantage of root colonization, but does not contribute to the biocontrol properties of *P. fluorescens* HP72.

Kim and Kremer (2005) reported IAA production in *Bacillus japonicum* isolate GD3 which gave suppressive effect on Morning glory growth. Mejri *et al.* (2010) studied the effect of rhizobacterial *Pseudomonas trivialis* strain X33d on growth suppression of weed great brome (*Bromus diandrus* Roth.) The specificity assay, performed on a mixture of soil/sand/peat, highlighted the suppressive activity of *P. trivialis* X33d against great brome and it showed the promoting effect on most of the considered crops, especially durum wheat (*Triticum durum* Desf.). Great brome plants inoculated with X33d and co-seeded with durum wheat showed low root biomass, short root systems and low surface area, volume and number of tips. The production of indole acetic acid by *P. trivialis* X33d was suggested to cause growth suppression of great brome and growth promotion of durum wheat.

2.7.4. Aminolevulinic acid production

The 5-aminolevulinic acid (ALA) is a key intermediate in the biosynthesis of tetrapyrroles, such as porphyrins, vitamin B12, chlorophyll (bacteriochlorophyll) and heme. ALA is a natural photodynamic compound effective as a biodegradable herbicide (Sasikala *et al.,* 1994) as well as having a promoting effect on the growth and photosynthesis of crops and vegetables (Sasaki *et al.,* 1993). In plants, the ALA concentration is strictly controlled at less than 50 nmol/g fresh weight (Stobart and Bukhari, 1984). Herbicidal activity has been reported to increase accumulation of several chlorophyll intermediates, such as protochlorophyllide, protoporphyrin IX and Mg-protoporphyrin IX, when plants are treated with exogenous ALA at relatively high concentrations (5 – 40 mM).

It is assumed that the accumulated chlorophyll intermediates act as photosensitizers for the formation of singlet oxygen, triggering photodynamic damage of ALA-treated plants (Chakraborty and Tripathy, 1992). However, low ALA concentrations, within the range of 0.06 - 0.60 mM, appear to promote rather than damage plant growth by increasing nitrate reductase activity, increasing fixation of CO_2 in the light and suppressing the release of CO_2 in darkness (Hotta *et al.,* 1997 a). The effects of ALA on plant growth at low concentrations have been discovered to be physiologically different from those at high concentrations.

Recently, δ-aminolevulinic acid (ALA) has received great attention as a biodegradable herbicide (Chon, 2003). However, herbicidal activity of ALA on several plants was affected by the application methods. At low concentration (0.01-10 mg/L), ALA showed growth promoting effects on yield of several crops (Hotta *et al.,* 1997 b) whereas, it suppressed plant growth at higher concentrations (> 2mM). Zhang *et al.* (2006) reported that ALA at low concentrations of 0.3 – 3 mg/l promoted development and growth of potato microtubers *in vitro* and enhanced protective functions against oxidative stresses, but ALA at

30 mg/l and higher concentrations may induce oxidative damage. Hyun and Song (2007) reported the production of IAA and ALA by purple nonsulfur bacteria *Rhodopseudomonas* that promoted the seed germination and growth of tomato under axenic conditions.

2.7.5. Production of other secondary metabolites

Pseudomoans fluorescens strain CHA0 has been found to produce several secondary metabolites, notably HCN, 2, 4-diacetylphloroglucinol and pyoluteorin (Oberhansli *et al.,* 1991; Keel *et al.,* 1992). A polyketide secondary metabolite, herboxidiene, produced by *Streptomyces chromofuscus*, showed potent and selective herbicidal activity against weeds but not against wheat (Miller-Wideman *et al.,* 1992).

Two phytotoxic metabolites prehelminthosporal and dihydropore, were isolated from the cultural filtrates of the fungus *Bipolaris* sp. showing herbicidal activity against *Sorghum halepense* (L.) Pers. (Parmar and Devkumar, 1993). Three herbicidal metabolites, 3-hydroxybenzylalcohol, 2-methyl hydroquinone and epiepoformin were isolated from a soil-borne fungus *Scopulariopsis brumptii.* Similarly, a phytotoxic metabolite trans-4 aminoproline isolated from culture filtrates of *Ascochyta caulina* was found to be very effective in controlling *Chenopodium album* (L.) (Evidente *et al.,* 2000). Evidente *et al.* (2005) isolated a new phytotoxic trisubstituted naphthofuroazepinone from the culture filtrates of *Drechslera siccans*, named drazepinone, and characterized as a 3,5,12 a trimethyl-2,5,5a,12a-tetrahydro-1*H*-naphtho[2′,3′:4,5]furo[2,3-*b*] azepin-2-one. Assayed at 2 μg μl^{-1} solution, the novel metabolite proved to have broad-spectrum herbicidal properties. Recently Javaid and Adrees (2009) reported that metabolites of *Alternaria alternata*, *Fusarium oxysporum*, *F. solani*, *Drechslera hawaiiensis*, *D. australiensis* and *D. rostrata* were highly effective in controlling the growth of the noxious weed *Parthenium hysterophorus*.

Gostatin, a product of *Streptomyces sumanensis* (Amagasa *et al.,* 1994), is a potent amino transferase inhibitor that is phytotoxic (Nishino *et al.,* 1984). Pyridazocidin, a cationic compound from soil *Streptomyces* species, caused rapid plant necrosis and chlorosis, much like that of bipyridinium herbicides like paraquat (Oettmeier *et al.,* 1990). Coronatine is a jasmonate analog produced by *Pseudomonas coronafacience* (Gerwick *et al.,* 1997). It usurps jasmonate-controlled signaling pathways (Ichihara *et al.,* 1977), thereby deregulating many essential processes. The typical symptom of this toxin is chlorosis of developing tissues. Cinnacidin, a product of the fungus *Nectria* sp. DA060097, has a similar mode of action to coronatine (Block *et al.,* 2005). The diphenyl ether compound cyperin, a metabolite of *Preussia fleischhakii*, *Phoma sorghina* and *Ascochyta cypericola* (Weber *et al.,* 1988; Feld *et al.,* 1989; Stierle *et al.,* 1992) inhibited plant enoyl (acyl carrier protein) reductase (ENR), which is the target site of synthetic diphenyl ether called triclosan. Inhibition of ENR resulted in light-independent disruption of membrane integrity (Venkatasubbaiah *et al.,* 1992).

2.7.6. Phytotoxin production

Plant pathogens produce a variety of phytotoxins that interfere with plant metabolism, ranging from subtle effects on gene expression to plant mortality (Walton, 1996). The mode of action of a phytotoxin can be direct interaction with a specific plant component (e.g., enzyme or membrane receptor), but if that component is absent or altered, there is no phytotoxic effect.

Bacterial and fungal microbes were found to produce a wide array of phytotoxins with the potential to be used as herbicides (Duke *et al.,* 1991). The isolated phytotoxins may exhibit similar host and non-host specificity to the pathogen. AAL-toxin, a hydroxylated long-chain alkylamine containing a tricarboxylic acid moiety, is structurally related to the fumonisins. It is produced by *Alternaria alternata* f. sp. *lycopersici* and has been found to act as an effective herbicide on a range of crop and weed species. In susceptible varieties of tomatoes, it caused rapid wilting and necrosis (Abbas *et al.,* 1995).

Gealy *et al.* (1996) reported that naturally-occurring rhizobacterium, *Pseudomonas fluorescens* strain D7 produced phytotoxins, which inhibited the root and shoot growth of downy brome by 80% in a seed germination agar assay at 2 ng and 400 ng total solids/ml, respectively. Plasma membranes of isolated protoplasts of downy brome were damaged within 2 h by diluted cell-free broth. Cold-precipitated phytotoxins of strain D7 produced in semi-synthetic and synthetic media reduced dry weight of downy brome seedlings by 100 and 89%, respectively and killed plants rapidly.

A *Pseudomonas syringae* isolate has been registered to control Canada thistle (Johnson *et al.,* 1996). Bender *et al.* (1999) reported that coronatine, syringomycin, syringopeptine, tabotoxin and phaseolotoxin are the most intensively studied phytotoxins of *Pseudomonas syringae* and each contributes significantly to bacterial virulence in plants. Tabotoxin and phaseolotoxin are strongly antimicrobial and function by inhibiting glutamine synthase and ornithine carbamoyltransferase, respectively. Stephen and lydon (1987) reported that phytotoxins from *Streptomyces saganonensis* (herbicidines and herbimycins) are used to control grassy weeds as selective herbicide. Yufen and Zhang (1987) showed that phytotoxins (anismycins) from *Streptomyces actinomycetes* is used to control grassy weeds such as barnyard grass and common crabgrass and broad leaved weeds. Its mechanism is to destroy synthesis of plant chlorophyll.

Rhizobitoxine is a phytotoxin produced by some *Bradyrhizobium* strains (Duke *et al.,* 2011). It inhibits β-cystathionase, which is required for methionine synthesis (Duke *et al.,* 2011). This toxin is phytotoxic enough to have been considered as a commercial herbicide (Giovanelli *et al.,* 1973). Since synthesis of the essential plant hormone ethylene is dependent on methionine, one could assume that ethylene synthesis would be greatly inhibited in plants treated with this compound. Several *Pseudomonas syringae* pathovars produce tabtoxin, a dipeptide prophytotoxin. Tabtoxin is not a glutamine synthetase (GS) inhibitor, but it is hydrolyzed *in planta* to form the potent GS inhibitor tabtoxinine-β-lactam (Preuss *et al.,* 1973; Uchytil *et al.,* 1980).

2.8. Persepective and future aspects

In order to overcome the obstacles associated with development of a microbial herbicide and dramatically improve the chances for success of microbial herbicides, numerous researchers have investigated the combining of biocontrol organisms with a formulation designed to improve application, survivability and efficacy. Zdor *et al.* (2005) studied effects of DRB (*Pseudomonas fluorescens* strain G2-11) in combination with corn gluten meal and semolina flour in soil assays using weed and crop species. Tichich and Doll (2006) examined a novel application approach for the pathogen *Pseudomonas syringae* pv. *tagetis* where the sap of infected Canada thistle is extracted, combined with water and Silwet-77, and sprayed. While there was disease expression in the treated plants, it was not enough to control the Canada thistle and further work is needed on this approach. Zhang *et al.* (2010) studied the stability of pyoluteorin, a polyketide metabolite produced by fluorescent pseudomonads that has shown potential to control weeds among other pests. Various technologies have been used and will continue to be used to enhance biological weed control (Cohen *et al.*, 2002). The protoplast fusion technique was used to create new strains using *Helminthosporium gramineum* subsp. *echinochloae* strain HM1 (high pathogenicity, low spore formation) and *Curvularia lunata* (low pathogenicity, high spore formation) to create strains that effectively control barnyard grass and other weeds in rice production (Zhang *et al.*, 2007). Hypervirulence selection or manipulation may improve efficacy of biological control agents. Sands and Pilgeram (2009) outlined the steps to enhance virulence of the biocontrol agent using amino acid overproduction. They discussed control of the parasitic weeds *Orobanche* and *Striga*, which are especially challenging to control due to the close relationship they develop with their hosts. Economic formulations and genetic manipulations to alter phenotype will assist in the understanding and development of microbial herbicides.

The future advancements in microbial genetics, microbial community analyses and understanding of plant-microbe interactions continue to accumulate and will be instrumental in developing microbial biocontrol of weeds. The area of biological control using soil microorganisms needs further investigations to discover additional isolate-host pairs that are a biocontrol match consisting of a biocontrol agent of highest virulence in contact with the host at its greatest susceptibility. Formulations are needed to increase shelf life of the living organisms to improve survival and efficacy. Research and development of each biocontrol agent are needed so that stakeholders and industry buy in to the marketing, economics and time investments of this approach to weed management. An understanding of microbial community weed, soil quality characteristics and management practices is needed for the development of weed suppressive soils. Investigations of the structure and function of soil microbial communities are needed to advance the area of biological control. Traditional techniques of microbial analyses to describe the composition and diversity of microbial populations in soils has commonly relied on phenotypic characteristics alone and molecular investigations add to the information on structure and function of the soils (Mazzola, 2004). Profiling or fingerprinting of soil and soil microbial community structure using substrate utilization and fatty acid methyl ester analyses may be the first step in targeting weed-suppressive potential.

There are several nucleic acid-based methods that can be used to probe soil and identify those microbes that produce similar compounds to those already known. Probes will assist field studies of known agents to follow survival in soils and explore soil for additional weed-suppressive factors. Nucleic acid technologies provide greater information on genetics and possibly function of a given organism. Array, pyrosequencing and metagenomic investigations can provide information on the microbial community and the biological agent within that community. Selection for hypervirulence; construction of molecular probes; understanding the genetic material of the agent, weed-suppressive compound, and host-microbe interactions can be investigated more thoroughly with these methods. The continual development of novel molecular methods to investigate genetics of a system will provide key information to better understanding of the plant-microbe phenomena. These methods are forever changing and improvments to allow us to have increased knowledge of the microbial portion of the ecosystem and the various interactions that can occur. Soil microbial ecology and the soil microbial community will affect weed ecosystem dynamics, diversity, function and populations. As with soil quality, the compilation of indicators has been attempted often to choose a few indicators of importance. No one approach or method can be used to characterize and follow biocontrol agents, or to isolate and research additional novel plant-microbe interactions have to be explored. The future is bright for continued development of microbial herbicides to reduce herbicide reliance and provide multiple options in weed management.

CHAPTER-III

MATERIAL AND METHODS

The present investigations were carried out on isolation of weed inhibiting rhizobacteria and their inoculation effect on growth of weed and wheat in the Department of Microbiology, Chaudhary Charan Singh Haryana Agriculture University, Hisar. The bacterial cultures were isolated based on their morphological characteristics and purified. The selected bacterial isolates were further examined for their ability to suppress growth of *Phalaris minor* on plates and were tested for their potential to enhance the growth of wheat plants under pot house conditions. The materials used in the study, procedures used and the techniques adopted during the present investigations are detailed in this chapter.

3.1. Microbial cultures

Seventy one bacterial isolates were obtained from the rhizosphere soil of wheat by serial dilution plate method using nutrient agar medium. Twelve reference strains were procured from the Department of Microbiology, Chaudhary Charan Singh Haryana Agricultural University, Hisar. The rhizobacterial strains/isolates were maintained by periodic transfer on Luria Bertani (LB) slants (Sambrook et. al., 1989). These bacterial cultures were stored at 4°C in refrigerator for further use. *Fusarium oxysporum* culture was maintained on PDA medium slants.

3.1.1. Chemicals

All chemicals, reagents and media components used in the present investigation were of AR or MB grade and were obtained from HiMedia, Central drug and E. Merck, respectively.

3.1.2. Host species

Seeds of wheat (*Triticum aestivum* L.) variety WH711 and *Phalaris minor* were obtained from Department of Agronomy, CCS Haryana Agricultural University, Hisar.

3.1.3. Preparation of media

Different media were prepared in single distilled water and autoclaved at 15 lbs pressure for 20 minutes. The following media were used during the present investigations.

(i) Luria Bertani medium (Sambrook *et al.,* 1989)

Composition	**g/l**
Tryptone	10.0
Yeast extract	5.0
NaCl	2.0
Glucose	1.0
Agar-agar	20.0
pH	7.0

(ii) King's B-modified medium (King *et al.*, 1954)

Composition	**g/l**
Peptone	20.0
Glycerol	10.0
Glucose	1.0
K_2HPO_4	1.5
$MgSO_4.7H_2O$	1.5
NaCl	5.0
Agar-agar	20.0
pH	7.0

(iii) Potato dextrose agar medium

Composition	**g/l**
Potato extract	450 ml
Distilled water	450 ml
Dextrose	9.0
Peptone	1.0
Agar-agar	20.0

100 g of sliced potatoes were boiled in 1L of H_2O for about 1 hour and filtered twice through a cloth. The volume was made to 1 liter.

(iv) 0.8% Water agar medium

Components	**g/l**
Agar-agar	0.8 g
Distilled water	100ml

(v) Nutrient agar medium

Composition	**g/l**
Beef extract	3.0
Peptone	5.0
NaCl	5.0
Agar-Agar	20.0

3.2. Methods

3.2.1. Isolation of bacteria from wheat rhizosphere

Soil samples were collected from the rhizosphere of wheat after 60 and 90 days of plant growth. Rhizosphere soil samples were collected randomly from 3 different locations of CCS Haryana Agricultural University, Hisar farm. From each location, samples were collected from six different sites. Five samples were collected from each site (one acre) and pooled together to make the composite sample. The serial dilutions of soil samples (up to 10^{-4}) were made in 9.0 ml sterilized water blank and 0.1 ml of diluted soil suspension was plated on plates King's B medium (Sindhu *et al.,* 1999). The plates were incubated at 28±2°C in BOD incubator for 3-4 days. *Pseudomonas, Bacillus* and other rhizobacterial colonies were selected based on typical morphological and pigment production characteristics.

3.2.2. Effect of bacterial isolates on seed germination of *Phalaris minor*

Effect on seed germination of *P. minor* was studied with all bacterial isolates on water agar medium plates. Seeds were surface sterilized with acid-alcohol (70:30, v/v) for 5 minutes followed by 5-6 successive washings with sterile distilled water. Seeds were treated with different bacterial isolates (10^9 cfu ml^{-1}) for 30 minutes. Inoculated seeds were kept on 0.8 % water agar plates and incubated at 30ºC for 2-5 days. Seeds treated with sterilized LB medium broth alone were kept as control. Root and shoot length was recorded after at 5 and 10 days of germination (Jangu and Sindhu, 2011).

3.2.3. Inhibition of the weed growth by selected bacteria

Bacterial isolates showing inhibition of seed germination were studied for the production of different phytotoxic compounds. The bacteria were grown in LB medium broth for 2-3 days at 28 ± 2°C.

3.2.3.1. Phytotoxicity test

Luria Bertani medium broth was autoclaved at 121ºC in 250 mL conical flasks containing 100 mL medium broth in each flask. Flasks were inoculated with tested cultures from margins of actively growing colonies. Inoculated flasks were incubated under static conditions at 25°C for 2 weeks. After 2 weeks, the cultures were filtered through sterilized muslin cloth followed by Whatman filter paper No. 1. The cultural filtrates were used within 1 week of filtration to avoid any contamination or chemical alteration.

Original culture filtrates of the fifteen selected cultures were sprayed on 1-week and 2-weeks old *Phalaris minor* and wheat seedlings. Both of the sets were sprayed 3-times with an interval of 5 days. Treatment in a similar manner with distilled water spray served as control.

3.2.3.2. Indole acetic acid (IAA) production

Bacterial isolates were tested for their ability to produce indole acetic acid. Cultures were inoculated in duplicate in 30 ml LB broth supplemented with DL-tryptophan @ 100μg ml^{-1} (Hartman *et al.,* 1983) and were incubated at 30ºC for 72 hrs. under stationary conditions of growth. Culture samples were withdrawn after every regular interval of 12 hrs till 72 hrs and centrifuged at 10,000 rpm for 15 min (Remi Instruments, Mumbai India). IAA was determined in the culture supernatant as described by Salkowski (Glickmann and Dessaux, 1995; Jangu and Sindhu, 2011).

Reagents:

Salkowski reagent:

Ferric chloride	0.05 M
Perchloric acid (35%)	1000 ml

Two ml of Salkowski reagent was added to two ml of culture supernatant, mixed and allowed to stand for 30 min for the development of pink colour. Colour intensity was estimated at 500 nm using spectrophotometer (UV-Vis spectrophotometer 117, Systronics Ahemadabad, India) against a reagent blank. Indole acetic acid (100 µg ml^{-1}) was used as standard and results were expressed as µg IAA produced ml^{-1} of culture supernatant.

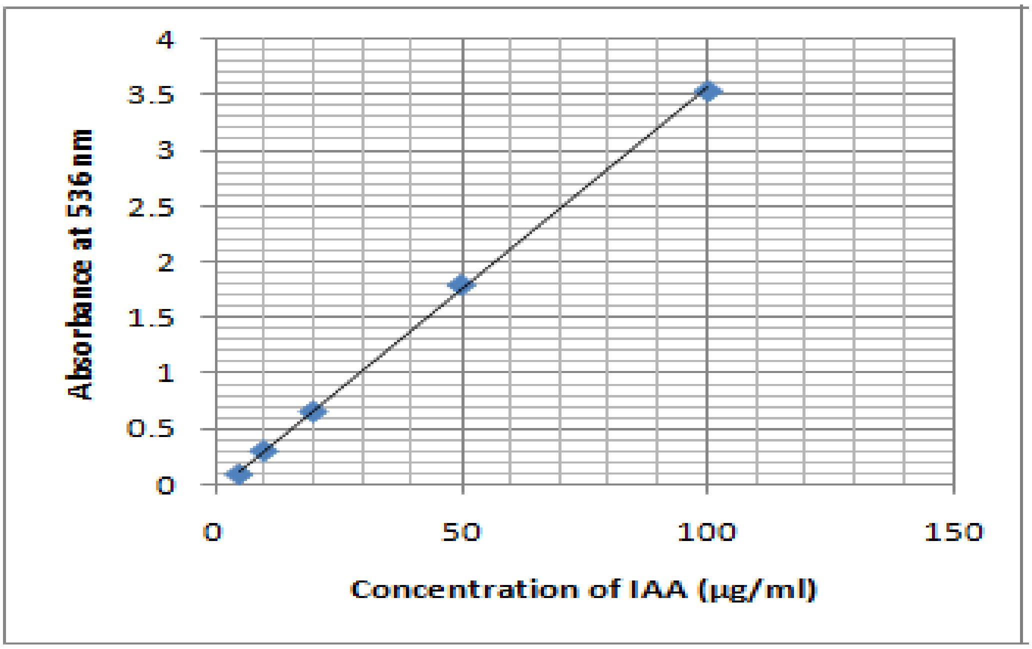

Figure 1. Standard graph for determination of IAA concentration

3.2.3.3. δ-aminolevulinic acid (ALA) production

Bacterial isolates were tested for their ability to produce δ-aminolevulinic acid. Cultures were inoculated in duplicate in 10 ml LB broth supplemented with 15 mM glycine and succinate, and were incubated at 30ºC for 48 hrs under stationary conditions of growth. Culture samples were withdrawn and centrifuged at 10,000 rpm for 15 min (Remi Instruments, Mumbai, India). ALA was determined in the culture supernatant by the method as described by Mauzerall and Garnick (1995).

Reagents:

(i) Modified Ehrlich Reagent:

p-dimethylaminobenzaldehyde	1.0 g
Perchloric acid (70%)	8 ml
Glacial acetic acid	42 ml

(ii) Buffer:

1M acetate buffer of pH 4.6 was made by adding 57 ml of glacial acetic acid (1 M) to 136 gm. of sodium acetate trihydrate (1 M) and diluting to 1liter.

To 0.5 ml of culture supernatant, 50 µl of acetylacetone and 0.5 ml of 1M sodium acetate buffer were added and then tubes were boiled in a water bath for 15 min. After cooling, 3.5 ml of modified Ehrlich reagent were added. The absorbance of the mixture was measured at 556 nm after 20 min at room temperature. The concentration of ALA in the culture supernatant was determined by comparison with standard curve.

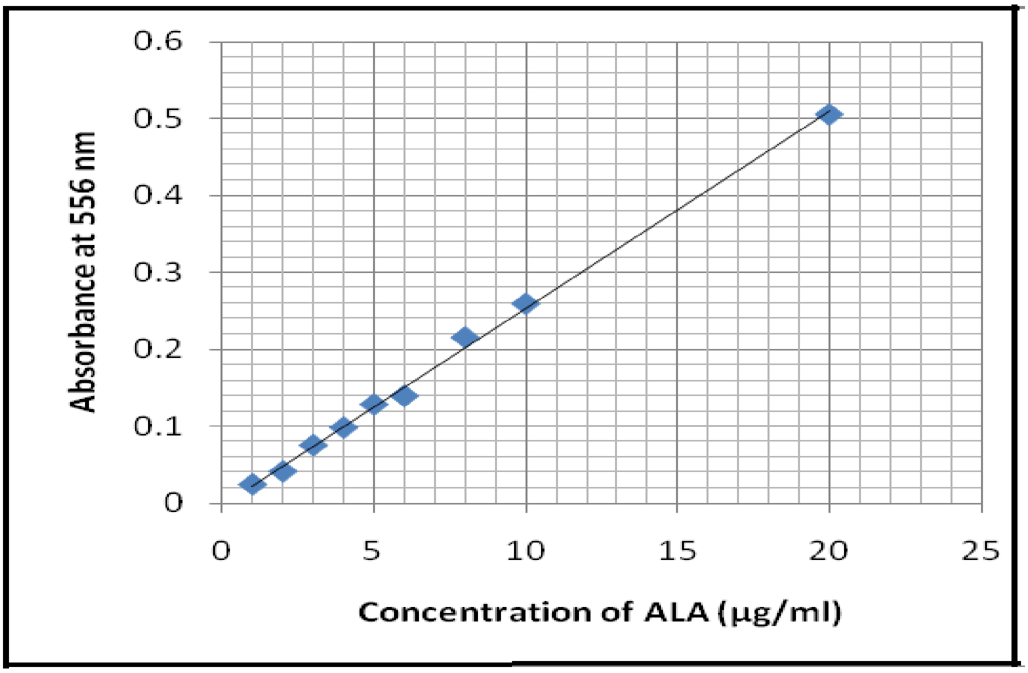

Figure 2. Standard graph for determination of ALA concentration

3.2.3.4. Growth inhibition of *Fusarium oxysporum* by rhizobacterial isolates for antagonistic activity.

The interaction of rhizobacterial isolates with *Fusarium oxysporum* was studied by the spot test method of Sindhu *et al.* (1999) on PDA medium plates. *Fusarium oxysporum* was grown on PDA slants. Spore suspension of fungi was prepared in 3 ml sterilized water. About 0.2 ml of fungal spore suspension was spreaded over PDA medium plates. A loopful of 48-hour old growth of the rhizobacterial isolates was spotted on preseeded plates. Twelve cultures were spotted on each plate. Plates were incubated for 48 hours at 28±2°C and growth inhibition of fungi was recorded after 2-3 days. Detection of antagonistic activity of rhizobcterial isolates was based upon the ability of rhizobacterial strains to inhibit fungal growth on PDA medium plates. The isolates showing zone of inhibition were selected and further tested.

3.2.5. Inoculation effect of weed suppressive bacteria on growth of weed and wheat

A pot house experiment was conducted by inoculation of twelve efficient bacteria selected on the basis of IAA production, ALA production, fungal growth inhibition and effect on *P. minor* seed germination. Their effect on growth of weed and wheat was studied under pot house conditions during the month of November 2011 to February 2012.

3.2.5.1. Treatments

Different treatments given for pot house experiment are presented in Table 3.1. There were nineteen treatments in this experiment as described below. Each treatment had three replications, one replication was used to record observations on plant growth parameters after 25 days, 50 days and 75 days of sowing.

Table 2. Details of the treatments used for pot house experiment

Sr. no.	Treatments
T_1	Control (uninoculated soil) + Wheat (*Triticum aestivum*)
T_2	Control (uninoculated soil + N, P fertilizers) + Wheat
T_3	T_1 + Weed (*Phalaris minor*)
T_4	T_2 + Weed (*Phalaris minor*)
T_5	T_1 + Wheat + Weed
T_6	T_2 + Wheat + Weed
T_7	T_2 + Wheat + Bacterial strain(s)
T_8	T_2 + Weed + Bacterial strain(s)
T_9	T_2 + Wheat + Weed + Bacterial strain(s)

Sandy loam soil was collected from CCS HAU, Hisar farm dry land area (Ludas soil). The physio-chemical properties of the soil used are given in Table 3.2. The earthen pots of 10 kg capacity were filled with sandy loam soil. The seeds of wheat (*Triticum aestivum*) variety WH711 were obtained from Department of Seed Science and Technology, CCS H.A.U., Hisar. The cultures of selected bacterial strains were grown in LB broth for 3 days. Seeds were inoculated with 20 ml growth suspension of selected bacterial strains according to the treatment schedule and grown in the respective pots. About 20 ml growth suspension was added after one week in respective pots according to treatment schedule to check combined effect on weed and wheat plant growth.

Table 3. Physio-chemical properties of Ludas soil

Sr. no.	Characteristics	Values
1.	Sand	63%
2.	Silt	16%
3.	Clay	13%
4.	pH (1:2)	8.5
5.	$Ec_{1:2}$	0.34 (dsm^{-1})
6.	Texture	Sandy loam
7.	Organic – C	0.62%
8.	Available – N	170 Kg/hectare
9.	Available – P	22 Kg/hectare
10.	Available – K	278Kg/hectare
11.	NH_4^+- N	14.4 mg/hectare
12.	NO_3^- - N	69.92 kg/hectare
13.	Total nitrogen	0.056%

Recommended doses of fertilizer used for wheat were N_{150} –P_{60} –K_{30} Kg/hactare in the form of urea, diammonium phosphate and muriate of potash, respectively. Fertilizers were added according to treatment schedule. The plants were grown in the pot house under day light conditions during November 2011 - February 2012. The plants were harvested after 25, 50 and 75 days of sowing and observations were taken for the fresh and dry weight of plants.

3.2.5.2. Plant fresh and dry weight

Plants were carefully uprooted and washed under tap water. Roots were separated from shoots. Shoot and root portions of the plants were weighed. Then, they were dried in oven at 90°C for 24 hrs and weighed again to determine root and shoot dry weight. Data regarding fresh and dry biomass of shoot and root were recorded.

CHAPTER-IV

RESULTS

In this study, bacteria were isolated from rhizosphere soil of wheat plants grown in CCS H.A.U. farm, Hisar. Bacterial isolates were examined for their ability to suppress/inhibit growth of *Phalaris minor* and the mechanisms involved in inhibition/suppression of weed were explored. Some of these rhizosphere bacteria, including *Pseudomonas* and *Bacillus* emerged as important biological control agents which colonized the roots and retarded the root growth of the weed. Highly efficient weed inhibiting/suppressive isolates were tested for their influence on growth of wheat and weed plants under pot house conditions.

4.1. Isolation of rhizobacteria from rhizosphere soil

Seventy one bacterial isolates were obtained from soil samples collected from rhizosphere of wheat, grown in field area of the Department of Plant Breeding, Agronomy and Seed Science and Technology in the farm of CCS Haryana Agricultural University, Hisar at 25, 50 and 75 days of plant growth. The serial dilutions of rhizosphere soil samples were made up to 10^{-5} and dilutions were plated on nutrient agar medium. *Pseudomonas* and *Bacillus* isolates were selected based on morphological and pigment production characteristics (Table 4). Twelve reference strains were obtained from Department of Microbiology, CCS H.A.U., Hisar.

Table 4. List of standard strains/isolates used and their sources

Microbial sp.	Isolates/strain no.	Source
Rhizobacterial isolates	HWM1, HWM2, HWM3, HWM4, HWM5, HWM6, HWM7, HWM8, HWM9, HWM10, HWM11, HWM12, HWM13, HWM14, HWM15, HWM16, HWM17, HWM18, HWM19, HWM20, HWM21, HWM22, HWM23, HWM24, HWM25, HWM26, HWM27, HWM28, HWM29, HWM30, HWM31, HWM32, HWM33, HWM34, HWM35, HWM36, HWM37, HWM38, HWM39, HWM40, HWM41, HWM42, HWM43, HWM44, HWM45, HWM46, HWM47, HWM48, HWM49, HWM50, HWM51, HWM52, HWM53, HWM54, HWM55, HWM56, HWM57, HWM58, HWM59, HWM60, HWM61, HWM62, HWM63, HWM64, HWM65, HWM66, HWM67, HWM68, HWM69, HWM70, HWM 71	Isolated from wheat rhizosphere of CCS H.A.U., Hisar farm

Reference strains	KPM15, SB153, PPM126, NNY60, WPS73, PPM115, CP43, CPA152, CPS67, SYB101, GYB106, P49	Deptt. of Microbiology, CCS H.A.U., Hisar
Fungal culture	*Fusarium oxysporum*	Deptt. of Microbiology, CCS H.A.U., Hisar

4.2. Screening of bacterial cultures for their effect on seed germination of *Phalaris minor*

The observations for retardation/stimulation of root and shoot growth of *Phalaris minor* seedlings were recorded at 5 and 10 days of growth after incubation at 28 ± 2°C. Among 83 strains, ten rhizobacterial isolates i.e., HWM1, HWM7, HWM9, HWM11, HWM17, HWM30, HWM37, HWM47, HWM54 and CP43 showed maximum retardation on 5th and 10th of seed germination of *Phalaris minor* on 0.8 % agar incorporated plates (Table 5). Five rhizobacterial isolates i.e., HWM7, HWM9, HWM24, CP43 and PPM126 showed 100% root growth inhibition on 5th day of seed germination. Maximum root growth redardation was observed in HWM9, HWM30, HWM54, HWM57, HWM58 and CP43 at 10th day of seedling growth.

Rhizobacterial isolates i.e., HWM1, HWM10, HWM18, HWM37 and NNY60 caused 50-70% shoot inhibition and HWM11, HWM20, HWM30, HWM54, SB153, P49, WPS73 caused 20-50% shoot inhibition on 5th day of seed germination. Rhizobacterial isolates i.e., HWM1, HWM25 and HWM30 caused 50-75% root inhibition and HWM10, HWM16, HWM37, HWM42, HWM47, HWM68, CPS67, GYB106 and PPM115 caused 20-50% root inhibition on 5th day of seed germination.

Rhizobacterial isolates i.e., HWM11, HWM30, HWM54, HWM57 and HWM58 caused 50-70% shoot inhibition and HWM17, HWM47, CP43, P49, SB153, NNY60 and PPM115 caused 20-50% shoot inhibition on 10th day of seed germination. Rhizobacterial isolates i.e., HWM30, HWM54, HWM57, HWM58, HWM70 and CP43 caused 50-70% root inhibition and HWM17, HWM29, HWM31, HWM42, HWM68, HWM69, P49, PPM115, PPM126 and SYB101 caused 20-50% root inhibition on 10th day of seed germination.

Rhizobacterial isolates i.e., HWM12, HWM31, HWM35 and HWM58 showed maximum stimulatory effect on 5th day of seed germination. On 10th day of seed germination eleven rhizobacterial strains i.e., HWM4, HWM10, HWM13, HWM18, HWM23, HWM31, HWM46, HWM50, HWM67, CPA152 and WPS73 showed maximum stimulatory effect on shoot growth of *Phalaris minor*. Rhizobacterial isolates i.e., HWM13, HWM23, HWM31 and

CPA152 showed maximum shoot growth stimulation at both 5th and 10th days of seeed germination.

Table 5. Effect of selected rhizobacterial isolates on growth of *Phalaris minor* seedlings

Sr. No.	Rhizobacterial Isolates	Observations at			
		5th day		10th day	
		Shoot (cm)	Root (cm)	Shoot (cm)	Root (cm)
	Control	2.4	2.0	5.2	4.6
1.	HWM 1	0.8	0.8	5.6	3.9
2.	HWM 4	2.	2.0	9.5	3.6
3.	HWM 7	NG	NG	2.0	2.0
4.	HWM 9	NG	NG	NG	NG
5.	HWM 10	1.0	1.6	9.7	4.6
6.	HWM 11	1.4	1.8	2.6	2.6
7.	HWM 12	3.5	3.0	4.5	4.9
8.	HWM 13	2.7	2.5	8.9	4.6
9.	HWM 16	2.4	1.6	4.3	3.9
10.	HWM 17	2.4	1.7	3.7	3.3
11.	HWM 18	1.0	1.7	9.6	4.4
12.	HWM 20	1.4	1.8	7.5	3.6
13.	HWM 23	2.6	2.3	8.9	4.0
14.	HWM 24	NG	NG	7.6	4.1
15.	HWM 25	0.7	0.6	5.4	6.3
16.	HWM 29	3.0	2.2	4.2	3.0
17.	HWM 30	1.6	0.8	2.4	1.1
18.	HWM 31	3.8	2.5	10.1	3.4
19.	HWM 35	3.2	3.1	5.2	5.1
20.	HWM 37	1.0	1.2	4.3	3.9
21.	HWM 42	3.4	1.4	6.5	3.2
22.	HWM 46	2.4	2.1	10.8	4.6
23.	HWM 47	2.0	1.5	3.2	2.4
24.	HWM 49	2.4	2.5	9.3	5.2
25.	HWM 50	2.6	2.0	11.8	3.9
26.	HWM 54	1.7	2.3	1.9	1.6
27.	HWM 55	2.8	2.0	5.6	4.7
28.	HWM 57	2.6	2.3	2.6	1.8
29.	HWM 58	4.7	3.1	2.6	1.8
30.	HWM 59	2.2	1.7	5.1	3.5
31.	HWM 67	2.3	1.9	9.4	4.4
32.	HWM 68	1.4	1.4	7.6	3.0
33.	HWM 69	3.3	1.8	4.9	2.4
34.	HWM 70	2.2	1.6	4.7	2.1
35.	CPS67	1.9	1.4	4.3	3.9
36.	KPM 15	2.4	1.9	7.6	4.6
37.	CP43	NG	NG	3.6	1.1
38.	P49	1.7	1.6	3.6	2.8
39.	SB153	1.5	1.8	3.7	4.3
40.	NNY60	1.2	1.8	3.0	3.5
41.	CPA152	2.5	2.8	10.7	3.9
42.	PPPM115	2.5	1.3	3.6	2.8
43.	SYB101	2.2	2.5	4.5	3.3
44.	PPM126	NG	NG	7.5	2.8
45.	GYB106	1.2	1.2	6.1	4.5
46.	WPS73	1.8	2.0	8.8	3.7

NG : no growth

4.3. Mechanism of weed growth inhibition by selected bacteria

Based on stimulation/retardation effect on seedling growth of *P. minor*, forty four bacteria were selected to understand the mechanism of weed inhibition. Bacterial isolates showing inhibition of seed germination were studied for the production of different metabolic compounds. Bacteria were grown in LB medium broth for 2-3 days at 28 ± 2°C.

4.3.1. Phytotoxicity test

Culture filtrates of fifteen selected cultures were sprayed on 1-week and 2-weeks old *Phalaris minor* and wheat seedlings. Both of the sets were sprayed 3-times with an interval of 5 days. Treatment in a similar manner with spray of distilled water served as control. Out of fifteen strains, three rhizobacterial isolates i.e., HWM11, P49 and SYB101 only caused yellowing of leaves whereas eight rhizobacterial strains HWM10, HWM25, KPM15, SB153, PPM126, WPS73, CPA152 and GYB106 caused appearance of disease spot (Table 6; Figures 3, 4).

Table 6. Phytotoxicity of the cultural filtrates of selected bacterial strains on weed's leaf

Sr. No.	Bacterial isolates	Observation
1.	HWM10	Appearance of disease spot
2.	HWM11	Yellowing of leaves
3.	HWM25	Appearance of disease spot
4.	CPS67	-
5.	NNY60	-
6.	SYB101	Yellowing of leaves
7.	P49	Yellowing of leaves
8.	GYB106	Appearance of disease spot
9.	PPM126	Appearance of disease spot
10.	CP43	-
11.	SB153	Appearance of disease spot
12.	CPA152	Appearance of disease spot
13.	PPM115	-
14.	WPS73	Appearance of disease spot
15.	KPM15	Appearance of disease spot

4.3.2. Screening of rhizobacterial isolates for production of indole acetic acid

Production of phytohormones has been a dominant mechanism of plant growth promotion by rhizobacteria. However, when these hormones are produced in high concentration, they may inhibit growth of root and shoot. Selected rhizobacterial isolates were tested for production of IAA at 4 days of growth. Nine isolates i.e., HWM7, HWM11, HWM18, HWM23, HWM24, HWM37, HWM42, HWM57 and PPM115 produced IAA ranging from 7.0 to 10.0 µg/ml (Table 7; Figure 5). Two isolates HWM49 and HWM35 produced 11.10 and 14.07 µg/ml IAA, respectively. Maximum production of IAA (> than 25 µg/ml) was observed in isolates CPS67, CP43 and HWM13. The production of IAA was further studied on the basis of different incubation time and at different concentration of tryptophan i.e., 50, 100, 500 and 1000 µg/ml.

Table 7. Production of indole acetic acid by selected rhizobacterial isolates

Sr. No.	Rhizobacterial Isolates	Concentration of IAA (µg/ml)
1.	HWM4	2.62
2.	HWM7	8.47
3.	HWM9	3.22
4.	HWM10	2.98
5.	HWM11	8.41
6.	HWM13	31.94
7.	HWM16	4.70
8.	HWM17	1.44
9.	HWM18	7.90
10.	HWM20	4.97
11.	HWM23	7.20
12.	HWM24	7.60
13.	HWM25	3.83
14.	HWM31	0.15
15.	HWM35	14.07
16.	HWM37	9.53
17.	HWM42	8.05
18.	HWM49	11.10
19.	HWM54	4.22
20.	HWM55	6.75
21.	HWM57	7.66
22.	HWM67	6.48
23.	CP43	25.97
24.	PPM115	9.62
25.	WPS73	3.92
26.	CPS67	50.25
27.	NNY60	1.41
28.	P49	6.72
29.	PPM126	3.61
30.	KPM15	0.30
31.	SYB101	2.77
32.	GYB106	2.14
33.	SB153	1.77
34.	CPA152	0.06

Figure 3. Yellowing of leaves by application of bacterial isolate SYB101

Figure 4. Appearance of disease symptoms on leaf of *Phalaris minor* by application of bacterial isolate HWM25

Six isolates were further screened for their ability to produce IAA at different concentrations of tryptophan and incubation time. A significant increase in the production of IAA was recorded in these cultures when grown in presence of higher concentration of tryptophan i.e., 500 and 1000 µg/ml (Table 8). CPS67 and CP43 strains showed maximum IAA production than other isolates. In the presence of 100 µg/ml tryptophan, CPS67 strain produced significantly higher amount of indole acetic acid i.e., 210.8 µg/ml at 36 hrs than CP43 strain 29.36 µg/ml. When 1000 µg/ml tryptophan was added to the medium and incubated at 48 hrs, strains CPS67 and CP43 produced 1.8 mg/ml and 79.62 µg/ml IAA, respectively. The highest concentration of 1.8 mg/ml in the culture CPS67 was achieved after 48 h of incubation.

Table 8. Production of IAA by bacterial isolates at different incubation periods and tryptophan concentrations

Sr. no.	Bacterial isolates	Concentration of tryptophan (µg/ml)	Incubation time (Concentration of IAA in µg/ml)					
			12 hrs	24 hrs	36 hrs	48 hrs	60 Hrs	72 hrs
1.	CPS67	50	14.38	29.13	22.25	9.16	13.02	23.94
		100	16.64	67.98	210.8	12.93	14.35	12.09
		500	33.56	170.1	50.94	31.12	22.62	39.35
		1000	16.55	174.3	244.8	1819.0	126.0	77.20
2.	CP43	50	14.65	19.78	22.80	11.34	20.35	15.56
		100	21.80	26.90	29.36	19.18	34.50	24.97
		500	49.76	49.16	69.95	65.96	57.36	65.38
		1000	52.20	81.73	87.67	79.62	100.6	130.4
3.	HWM35	50	1.96	7.41	5.38	6.18	0.301	6.66
		100	3.16	7.84	6.42	6.36	6.96	8.86
		500	8.53	10.97	12.27	14.23	17.8	16.55
		1000	9.95	12.24	9.47	17.46	7.54	6.75
4.	HWM13	50	4.31	6.42	11.24	12.75	13.33	12.36
		100	7.93	10.58	16.28	16.91	17.25	16.79
		500	11.06	10.85	17.70	19.87	21.50	19.18
		1000	9.07	9.28	19.72	20.47	24.15	22.92
5.	HWM49	50	1.8	0	10.37	9.16	11.85	15.53
		100	0	0.93	12.69	12.93	14.41	18.24
		500	0.33	1.86	4.55	6.90	7.35	8.20
		1000	1.5	2.38	13.51	9.95	8.80	14.77
6.	HWM37	50	5.27	5.60	8.11	7.90	8.86	8.86
		100	6.42	7.78	8.08	8.11	0.63	17.64
		500	10.97	9.53	10.08	12.60	3.19	14.77
		1000	18.12	8.05	12.12	4.52	3.98	16.76

4.3.3. Screening of rhizobacterial isolates for production δ-aminolevulinic acid.

ALA has been reported to promote the growth and yield of agricultural crops at low concentration and also used as biodegradable herbicide when produced at high concentration by rhizobacteria. Overall, 73% of total isolates were found to produce ALA. Maximum ALA production (>9 µg/ml) was observed by bacterial isolates HWM59, HWM69 and strain CP43

(Table 9). Other 22 isolates produced ALA more than 2.0 µg/ml. Five bacterial isolates i.e., HWM9, HWM13, HWM20, HWM29 and HWM67 produced less than 1.0 µg/ml of ALA.

Table 9. Production of δ-aminolevulinic by rhizobacterial isolates

Sr. No.	Rhizobacterial isolates	Concentration of ALA (µg/ml)
1.	HWM4	3.40
2.	HWM7	2.56
3.	HWM9	0.76
4.	HWM10	2.24
5.	HWM11	1.28
6.	HWM12	3.88
7.	HWM13	0.24
8.	HWM17	0.56
9.	HWM18	5.32
10.	HWM20	0.88
11.	HWM23	2.16
12.	HWM24	2.96
13.	HWM25	3.68
14.	HWM29	0.04
15.	HWM31	3.76
16.	HWM46	5.04
17.	HWM47	1.48
18.	HWM49	5.84
19.	HWM50	3.72
20.	HWM55	1.20
21.	HWM58	2.0
22.	HWM59	9.54
23.	HWM67	0.60
24.	HWM68	7.00
25.	HWM69	11.91
26.	HWM70	4.00
27.	CP43	10.25
28.	PPM115	3.16
29.	CPA152	2.08
30.	CPS67	7.50
31.	NNY60	2.64
32.	P49	4.64
33.	PPM126	3.92
34.	SYB101	1.12
35.	GYB106	4.28
36.	SB153	4.40

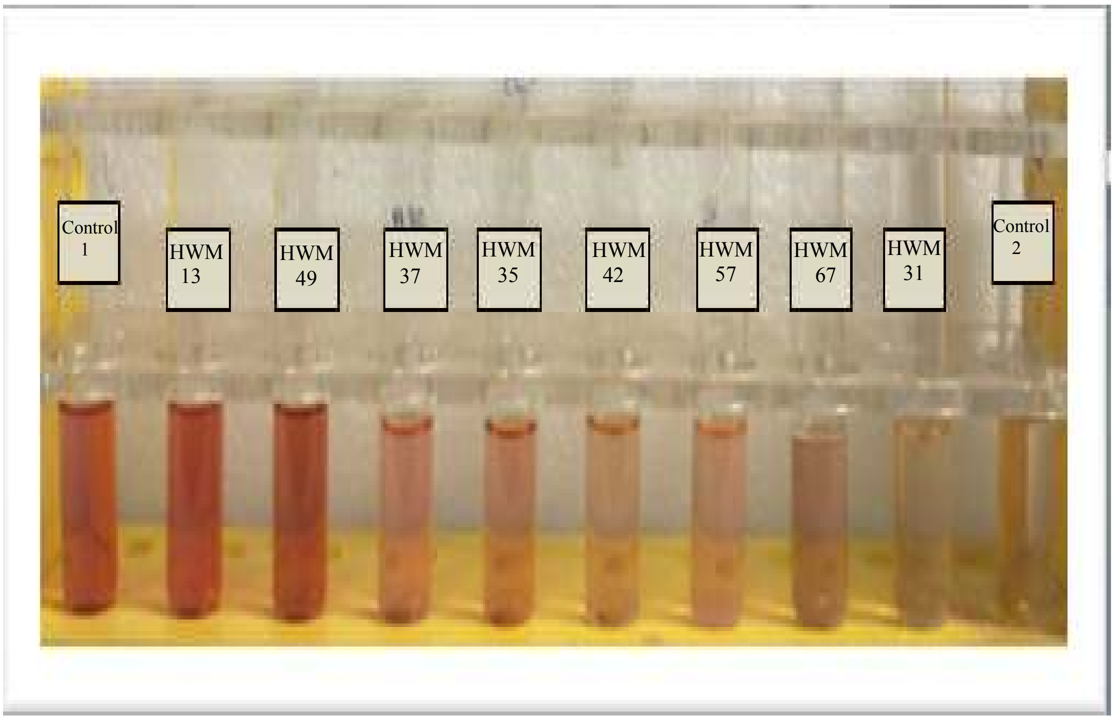

Figure 5. **Colour developed in broth inoculated with different bacterial isolates after addition of Salwoskii reagent for IAA measurement**

4.3.4. Inhibition of fungal growth by rhizobacterial isolates

Some strains of *Pseudomonas* and *Bacillus* produce antibiotics, siderophores, hydrolytic enzymes and other secondary metabolites which are responsible for growth inhibition of fungi. Overall, 20.5% of total isolates tested were found to inhibit the growth of *Fusarium oxysporum* on plates (Table 10). Large zone of inhibition was formed by bacterial isolates HWM13, WPS73, SB153, HWM25, HWM31, HWM37 and HWM57 (Figures 6a, b). Two bacterial isolates HWM18 and HWM54 showed little inhibition of fungal growth.

Table 10. Formation of inhibition zone by bacterial isolates/strains against *Fusarium oxysporum*

Zone of inhibition	Bacterial isolates/strains
+ +	HWM18, HWM54
+ + +	HWM25, HWM31, HWM37, HWM57
+ + + +	HWM13, SB153, WPS73
–	HWM1, HWM4, HWM7, HWM9, HWM10, HWM11, HWM12, HWM17, HWM20, HWM23, HWM24, HWM29, HWM30, HWM35, HWM42, HWM46, HWM47, HWM49, HWM50, HWM55, HWM58, HWM59, HWM67, HWM68, HWM69, HWM70, CPS67, NNY60, P49, PPM126, KPM15, CP43, PPM 115, CPA152
NG (no growth)	M16, SYB101, GYB101

Antifungal activity of bacterial isolates/strains was tested on the basis of zone of inhibition formed on PDA plates containing *Fusarium oxysporum* spores after 3-4 days of incubation using spot test method (Sindhu *et al.,* 1999).

4.4. Effect of inoculation of selected rhizosphere bacteria on growth of wheat and weed under pot house conditions

Six bacterial isolates/strains i.e., HWM10, HWM11, HWM25, NNY60, CPS67 and SYB101 were tested in the pot house to check their effect on growth of wheat and weed. Seeds of wheat var. WH711 and *Phalaris minor* were grown in pots containing 10 kg soil and sand mixture. The seeds were inoculated with 20 ml growth suspension of selected bacterial isolates/strains. Inoculated plants were grown under day light conditions in the pot house during the months of November 2011 to Feburary 2012. The plants were harvested at 25, 50 and 75 days after sowing and observations for root and shoot growth were recorded.

Application of recommended doses of fertilizers (RDF) in wheat resulted in 60% increase in shoot dry weight (SDW) and 12.2% increase in root dry weight (RDW) at 25 days after sowing (Table 11). At 50 days, application of RDF in wheat resulted in 51.5% increase in shoot dry weight and 17.8% increase in root dry weight (Table 12). After 75 days of sowing, application of RDF in wheat resulted in 76.9% increase in shoot dry weight and 60.41% increase in root dry weight (Table 13). Rhizobacterial strains/isolate i.e., SYB101, CPS67 and HWM11 were found to stimulate maximum growth of wheat alongwith inhibiting the growth of *Phalaris minor*. Bacterial isolates HWM10 and HWM25 were found to enhance growth of wheat and *Phalaris minor*. Rhizobacterial strain NNY60 was found to inhibit wheat growth whereas it enhanced growth of *Phalaris minor*.

At 25 days of wheat growth, inoculation of bacterial strain SYB101 showed 35.5% increase in root dry weight and 61.6% increase in shoot dry weight of wheat and caused 33.3% decrease in root dry weight and 25% decrease in shoot dry weight on growth *Phalaris minor* (Table 11). Similarly, inoculation with bacterial strain CPS67 showed 32.25% increase in root dry weight and 13.1% increase in shoot dry weight of wheat growth and caused 13.3% decrease in root dry weight and 20% decrease in shoot dry weight on growth of *Phalaris minor*. Inoculation with bacterial isolate HWM11 showed 36.19% increase in root dry weight and 44% increase in shoot dry weight of wheat growth and showed no effect on growth of *Phalaris minor*. Inoculation of bacterial isolates HWM10 showed 30.46% increase in root dry weight and 45% increase in shoot dry weight of wheat growth and showed 25% increase in root dry weight and 60% increase in shoot dry weight of *Phalaris minor*.

At 50 days of wheat growth, inoculation of bacterial strain SYB101 showed 12.77% increase in root dry weight and 49% increase in shoot dry weight of wheat and caused 33.3% decrease in root dry weight and 7.5% decrease in shoot dry weight of *Phalaris minor* (Table 12). Similarly, inoculation with bacterial strain CPS67 showed 21.9% increase in root dry weight and 8.4% increase in shoot dry weight of wheat growth and caused 37% decrease in root dry weight and 7.5% decrease in shoot dry weight on growth of *Phalaris minor*. Inoculation with bacterial isolate HWM11 showed 33.5% increase in root dry weight and 57.4% increase in shoot dry weight of wheat growth and showed 18.2% decrease in root dry weight and 40.3% decrease in shoot dry weight on growth of *Phalaris minor*. Inoculation of bacterial isolates HWM10 showed 60% increase in root dry weight and 31.4% increase in shoot dry weight of wheat growth and showed 46% increase in root dry weight and 72% increase in shoot dry weight of *Phalaris minor*.

At 75 days of wheat growth, inoculation of bacterial strain SYB101 showed 40% increase in root dry weight and 50.57% increase in shoot dry weight of wheat and caused 47.1% decrease in root dry weight and 55.6% decrease in shoot dry weight of *Phalaris minor* (Table 13; Figure 7a). Similarly, inoculation with bacterial strain CPS67 showed 10.6% increase in root dry weight and 12.1% increase in shoot dry weight of wheat growth and caused 56.74% decrease in root dry weight and 20.3% decrease in shoot dry weight on growth of *Phalaris minor*. Inoculation with bacterial isolate HWM11 showed 49.6% decrease in root dry weight and 51.5% increase in shoot dry weight of wheat growth and showed 56.2% decrease in root dry weight and 30% decrease in shoot dry weight on growth of *Phalaris minor* (Figure 7b). Inoculation of bacterial isolates HWM10 showed 40.3% increase in root dry weight and 26.6% increase in shoot dry weight of wheat growth and showed 22.7% increase in root dry weight and 28.6% increase in shoot dry weight on growth of *Phalaris minor*.

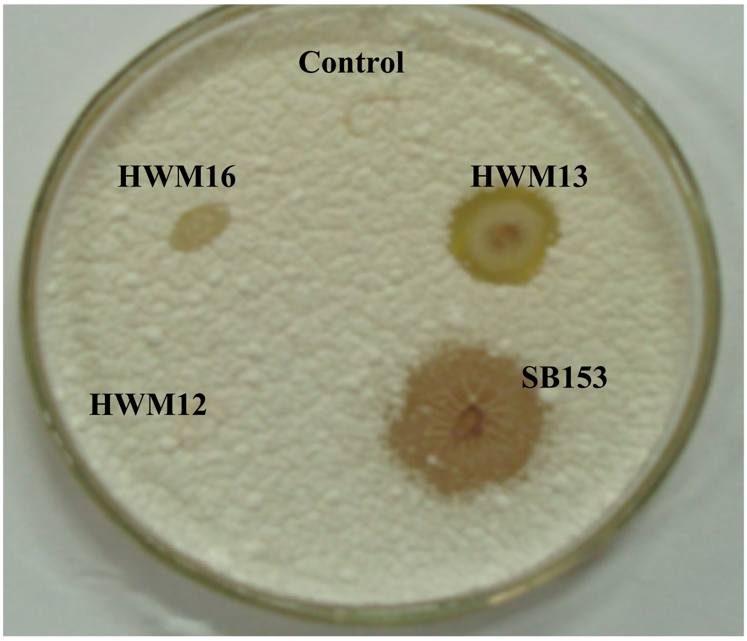

Figure 6(a). Fungal growth inhibition by bacterial isolates HWM13, HWM16 and strain SB153

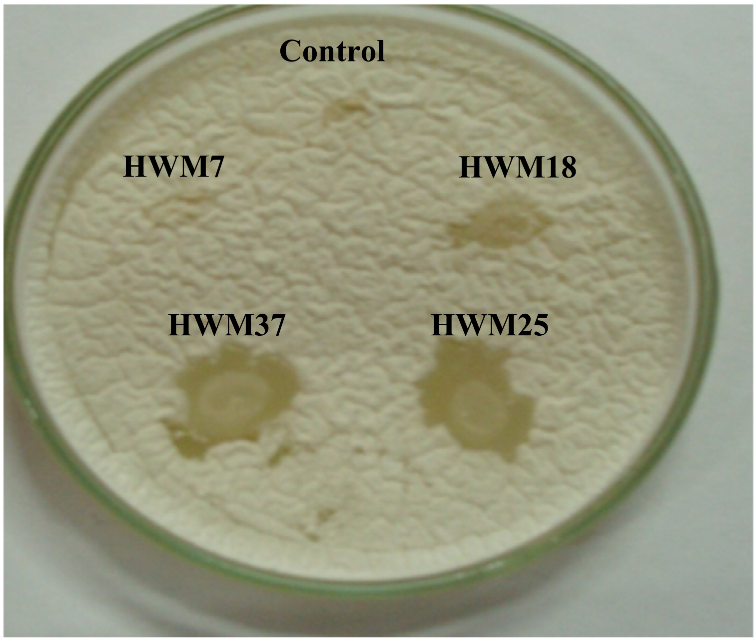

Figure 6(b). Fungal growth inhibition by bacterial isolates HWM7, HWM18, HWM25 and HWM37

Table 11. Effect of bacterial inoculation on plant biomass at 25 days after sowing

Sr. no.	Treatment		Wheat				Weed			
			Root fresh wt (mg)	Root dry wt (mg)	Shoot fresh wt (mg)	Shoot dry wt (mg)	Root fresh wt (mg)	Root dry wt (mg)	Shoot fresh wt (mg)	Shoot dry wt (mg)
1	T_1	Control (uninoculated soil) + Wheat (*Triticum aestivum*)	390	137	206	36	-	-	-	-
2	T_2	Control (uninoculated soil + N, P fertilizers) + Wheat	465	139	509	90	-	-	-	-
3	T_3	T_1 + Weed (*Phalaris minor*)	-	-	-	-	11	3	76	10
4	T_4	T_2 + Weed (*Phalaris minor*)	-	-	-	-	8	2	62	8
5	T_5	T_1 + Wheat + Weed	816	195	318	58	9	3	29	5
6	T_6	T_2 + Wheat + Weed	333	105	178	33	7	3	18	2
7	T_7	T_2 + Wheat + CPS67	231	67	503	102	-	-	-	-
8	T_8	T_2 + Weed + CPS67	-	-	-	-	18	5	64	11
9	T_9	T_2 + Wheat + Weed + CPS67	470	155	189	38	6	2.6	14	1.6
10	T_7	T_2 + Wheat + NNY60	223	71	312	66	-	-	-	-
11	T_8	T_2 + Weed + NNY60	-	-	-	-	15	4	51	8
12	T_9	T_2 + Wheat + Weed + NNY60	258	86	394	69	11	3	48	7
13	T_7	T_2 + Wheat + SYB101	475	144	442	79	-	-	-	-
14	T_8	T_2 + Weed + SYB101	-	-	-	-	14	4	52	8
15	T_9	T_2 + Wheat + Weed + SYB101	530	162	400	86	4.2	2	12	1.5
16	T_7	T_2 + Wheat + HWM11	304	107	349	62	-	-	-	-
17	T_8	T_2 + Weed + HWM11	-	-	-	-	13	3	63	10
18	T_9	T_2 + Wheat + Weed + HWM11	536	143	270	59	10	2	13	2
19	T_7	T_2 + Wheat + HWM25	605	221	372	85	-	-	-	-
20	T_8	T_2 + Weed + HWM25	-	-	-	-	15	3	106	11
21	T_9	T_2 + Wheat + Weed + HWM25	252	74	453	79	15	2.9	57	8
22	T_7	T_2 + Wheat + HWM10	214	55	700	134	-	-	-	-
23	T_8	T_2 + Weed + HWM10	-	-	-	-	20	2	68	9
24	T_9	T_2 + Wheat + Weed + HWM10	510	151	272	60	22	4	33	5
25	T_9	T_2 + Wheat + Weed + CP43	403	155	501	89	4.1	1.4	13	1.3
26	T_9	T_2 + Wheat + Weed + SB153	322	106	407	73	26	6	57	6.6
27	T_9	T_2 + Wheat + Weed + CPA152	243	66	538	80	14	2.5	71	2.5
28	T_9	T_2 + Wheat + Weed + PPM115	214	26	505	86	16	3	84	3.1
29	T_9	T_2 + Wheat + Weed + WPS73	23	53	426	75	10	3	60	3.1
30	T_9	T_2 + Wheat + Weed + KPM15	333	88	374	67	23	0.1	57	0.1

Inoculation effect on growth of wheat and weed at 25 days of growth was tested on the basis of fresh and dry weight of root and shoot using pot house experiment (Sindhu *et al.,* 2002).
The values of plant fresh and dry weight are calculated as per plant basis.

Table 12. Effect of bacterial inoculation on plant biomass at 50 days after sowing

Sr. no.		Treatment	Wheat				Weed			
			Root fresh wt (mg)	Root dry wt (mg)	Shoot fresh wt (mg)	Shoot dry wt (mg)	Root fresh wt (mg)	Root dry wt (mg)	Shoot fresh wt (mg)	Shoot dry wt (mg)
1	T_1	Control (uninoculated soil) + Wheat (*Triticum aestivum*)	378	71	447	102	-	-	-	-
2	T_2	Control (uninoculated soil + N, P fertilizers) + Wheat	518	140	1168	136	-	-	-	-
3	T_3	T_1 + Weed (*Phalaris minor*)	-	-	-	-	370	66	871	95
4	T_4	T_2 + Weed (*Phalaris minor*)	-	-	-	-	248	42	356	36
5	T_5	T_1 + Wheat + Weed	1125	193	908	296	89	224	220	22
6	T_6	T_2 + Wheat + Weed	518	157	1167	218	152	27	388	40
7	T_7	T_2 + Wheat + CPS67	747	150	1593	275	-	-	-	-
8	T_8	T_2 + Weed + CPS67	-	-	-	-	386	82	1086	128
9	T_9	T_2 + Wheat + Weed + CPS67	884	201	1448	238	1318	17	397	37
10	T_7	T_2 + Wheat + NNY60	774	196	1700	295	-	-	-	-
11	T_8	T_2 + Weed + NNY60	-	-	-	-	774	196	776	11
12	T_9	T_2 + Wheat + Weed + NNY60	626	15	107	190	128	27	326	39
13	T_7	T_2 + Wheat + SYB101	661	132	1778	234	-	-	-	-
14	T_8	T_2 + Weed + SYB101	-	-	-	-	293	26	813	106
15	T_9	T_2 + Wheat + Weed + SYB101	666	180	1866	428	142	18	244	37
16	T_7	T_2 + Wheat + HWM11	1120	236	2242	602	-	-	-	-
17	T_8	T_2 + Weed + HWM11	-	-	-	-	276	29	612	131
18	T_9	T_2 + Wheat + Weed + HWM11	1200	236	2246	720	155	21	148	23
19	T_7	T_2 + Wheat + HWM25	1300	244	3194	512	-	-	-	-
20	T_8	T_2 + Weed + HWM25	-	-	-	-	284	37	785	124
21	T_9	T_2 + Wheat + Weed + HWM25	672	132	2272	282	297	35	638	87
22	T_7	T_2 + Wheat + HWM10	680	235	1820	281	-	-	-	-
23	T_8	T_2 + Weed + HWM10	-	-	-	-	364	50	920	94
24	T_9	T_2 + Wheat + Weed + HWM10	1420	392	2278	318	390	50	819	143
25	T_9	T_2 + Wheat + Weed + CP43	580	183	1680	390	121	19	262	32
26	T_9	T_2 + Wheat + Weed + SB153	534	122	1480	318	342	41	693	86
27	T_9	T_2 + Wheat + Weed + CPA152	892	218	1720	288	265	70	443	49
28	T_9	T_2 + Wheat + Weed + PPM115	588	160	1628	248	285	35	686	70
29	T_9	T_2 + Wheat + Weed + WPS73	445	98	753	103	194	36	448	45
30	T_9	T_2 + Wheat + Weed + KPM15	755	440	1197	210	332	52	666	30

Inoculation effect on growth of wheat and weed at 50 days of growth was tested on the basis of fresh and dry weight of root and shoot using pot house experiment (Sindhu *et al.,* 2002). The values of plant fresh and dry weight are calculated as per plant basis.

Table 13. Effect of bacterial inoculation on plant biomass at 75 days after sowing

Sr. no.	Treatment		Wheat				Weed			
			Root fresh wt (mg)	Root dry wt (mg)	Shoot fresh wt (mg)	Shoot dry wt (mg)	Root fresh wt (mg)	Root dry wt (mg)	Shoot fresh wt (mg)	Shoot dry wt (mg)
1	T_1	Control (uninoculated soil) + Wheat (*Triticum aestivum*)	478	228	1058	332	-	-	-	-
2	T_2	Control (uninoculated soil + N, P fertilizers) + Wheat	1796	576	6850	1940	-	-	-	-
3	T_3	T_1 + Weed (*Phalaris minor*)	-	-	-	-	1367	323	1934	432
4	T_4	T_2 + Weed (*Phalaris minor*)	-	-	-	-	1157	362	2518	485
5	T_5	T_1 + Wheat + Weed	1342	432	2324	626	281	69	614	110
6	T_6	T_2 + Wheat + Weed	1170	330	2346	682	631	178	796	187
7	T_7	T_2 + Wheat + CPS67	586	164	1050	434	-	-	-	-
8	T_8	T_2 + Weed + CPS67	-	-	-	-	449	115	1216	267
9	T_9	T_2 + Wheat + Weed + CPS67	1.223	295	2675	776	350	77	816	149
10	T_7	T_2 + Wheat + NNY60	1678	582	3960	944	-	-	-	-
11	T_8	T_2 + Weed + NNY60	-	-	-	-	618	129	1098	217
12	T_9	T_2 + Wheat + Weed + NNY60	600	156	2916	882	1097	314	1153	219
13	T_7	T_2 + Wheat + SYB101	910	212	2024	476	-	-	-	-
14	T_8	T_2 + Weed + SYB101	-	-	-	-	418	90	976	261
15	T_9	T_2 + Wheat + Weed + SYB101	1866	550	3640	1380	477	94	380	83
16	T_7	T_2 + Wheat + HWM11	2520	691	6195	1631	-	-	-	-
17	T_8	T_2 + Weed + HWM11	-	-	-	-	634	166	1712	330
18	T_9	T_2 + Wheat + Weed + HWM11	570	166	3764	1406	345	78	591	131
19	T_7	T_2 + Wheat + HWM25	1812	568	3714	730	-	-	-	-
20	T_8	T_2 + Weed + HWM25	-	-	-	-	952	244	1888	566
21	T_9	T_2 + Wheat + Weed + HWM25	668	196	3151	1056	1008	290	1434	284
22	T_7	T_2 + Wheat + HWM10	1436	438	4864	1062	-	-	-	-
23	T_8	T_2 + Weed + HWM10	-	-	-	-	965	156	2992	568
24	T_9	T_2 + Wheat + Weed + HWM10	1626	554	3702	930	849	229	1572	262
25	T_9	T_2 + Wheat + Weed + CP43	1374	486	2646	866	484	126	537	132
26	T_9	T_2 + Wheat + Weed + SB153	770	175	2452	647	434	122	795	152
27	T_9	T_2 + Wheat + Weed + CPA152	802	282	3226	998	565	147	1100	245
28	T_9	T_2 + Wheat + Weed + PPM115	718	248	1184	272	574	138	1055	224
29	T_9	T_2 + Wheat + Weed + WPS73	420	130	2900	770	372	101	633	193
30	T_9	T_2 + Wheat + Weed + KPM15	570	161	2896	850	385	89	795	146

Inoculation effect on growth of wheat and weed at 75 days of growth was tested on the basis of fresh and dry weight of root and shoot using pot house experiment (Sindhu *et al.,* 2002).
The values of plant fresh and dry weight are calculated as per plant basis.

4.5. Identification of bacterial isolates using standard morphological and biochemical tests

Selected bacterial isolates were identified by colony morphology and Gram staining. Bacterial isolates were grown at 28±2°C for 24 h on LB medium slants. For the identification, various morphological and biochemical characteristics of the cultures were studied as per procedures described in Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994). Morphological characters used were colony morphology, pigment production, Gram reaction, bacterial morphology, bacterial arrangement and endospore production (Table 14). Bacteria were found to belong to the genera of *Bacillus, Pseudomonas* and *Serratia*.

Table 14. Growth and morphological characteristics of bacterial isolates/strains

Isolates/ Strains	Growth and morphological characteristics	Gram reaction
HWM13	Round, L-form colonies with bulged margin, creamish yellow	Gram –ve, small rods
HWM18	Irregular, erose margin, colorless	Gram –ve, rod shaped
HWM25	Irregular with erose margin, gummy pinkish white colonies	Gram –ve/+ve, small rods
HWM31	Round with scalped margin, white colonies	Gram +ve, rod shaped spore former
HWM35	Round with smooth margin, white colonies	Gram +ve, cocci/very small rods
HWM37	Round smooth entire margin, gummy creamy colonies	Gram +ve, cocci shaped
HWM42	Round with raised margin, white colonies	Gram –ve, cocci/very small rods
HWM49	Irregular shape with smooth entire margin, whitish colonies	Gram –ve, cocci/small rods
HWM50	Round, raised margin, yellow colonies	Gram +ve, oval shaped cocci
HWM54	Small, round (L- form), yellow colonies	Gram -ve, small rods
HWM57	Round, raised, gummy white colonies	Gram +ve, rod shaped spore former
HWM67	Small round, raised, white colonies	Gram +ve, cocci
SB153	Irregular shape with wavy margin,	Gram +ve, rod shaped
CPS67	Round, creamish colonies	Gram –ve, small rods
WPS73	Gummy and fluorescent colony	Gram -ve, small thin rods
PPM115	Round with smooth margin, creamy creamy colonies	Gram –ve, cocci/very small rods

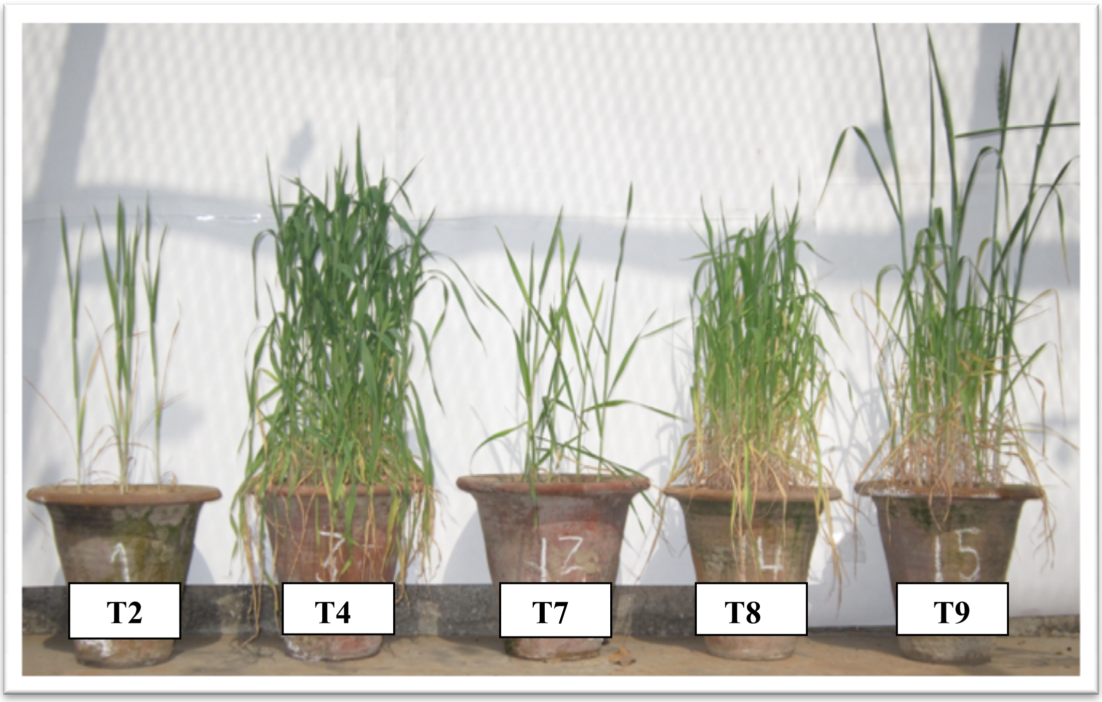

Figure 7(a). Effect of inoculation with bacterial isolates on the growth of wheat and weed plants with different treatments at 75 days after sowing

T2: Control (uninoculated soil + N, P fertilizers) + Wheat; **T4:** T_2 + Weed (*Phalaris minor*); **T7:** T_2 + Wheat + SYB101; **T8:** T_2 + Weed + SYB101; **T9:** T_2 + Wheat + Weed + SYB101

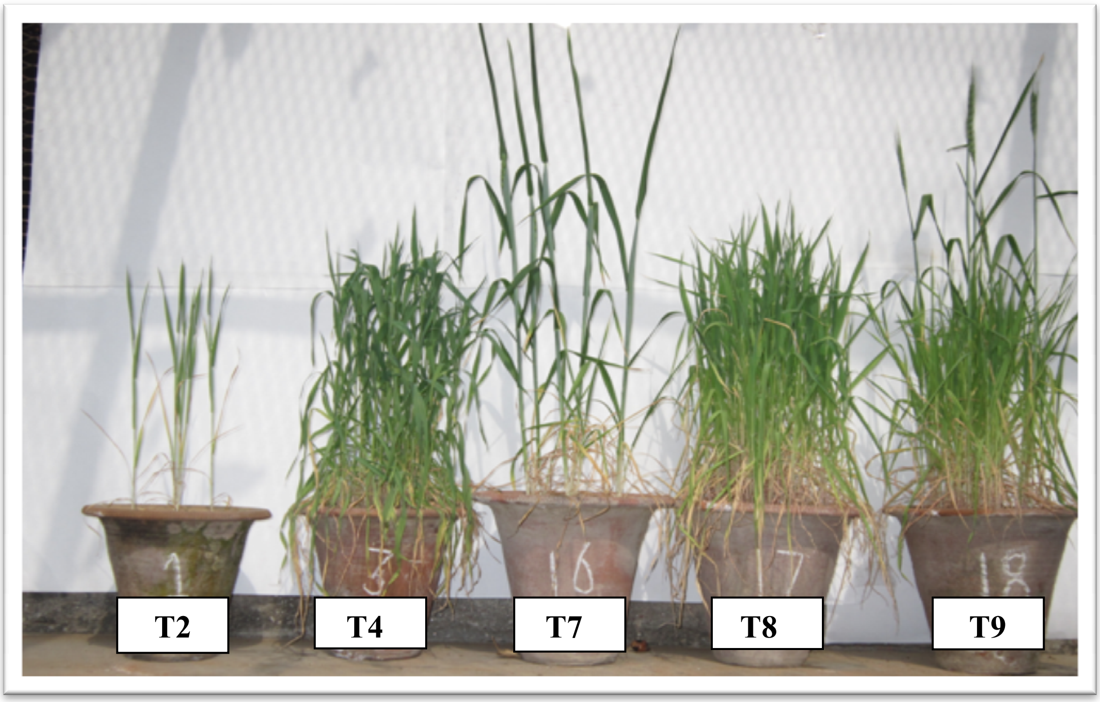

Figure 7(b). Effect of inoculation with bacterial isolates on the growth of wheat and weed plants with different treatments at 75 days after sowing

T2: Control (uninoculated soil + N, P fertilizers) + Wheat; **T4:** T_2 + Weed (*Phalaris minor*); **T7:** T_2 + Wheat + HWM11; **T8:** T_2 + Weed + HWM11; **T9:** T_2 + Wheat + Weed + HWM11

CHAPTER-V

DISCUSSION

In the present investigation, isolation of weed inhibiting rhizobacteria from the wheat rhizosphere was made and the mechanism involved in weed inhibition was studied. The effect of inoculation of bacterial isolates was evaluated on the inhibition or stimulation of weed and wheat growth under pot house conditions.

5.1. Isolation of rhizobacteria from wheat rhizosphere

Seventy one rhizosphere bacteria were obtained from the rhizosphere of wheat and twelve strains of bacteria were procured from the department of Microbiology (Table 4). Isolated bacterial colonies were picked from 10^{-4} and 10^{-5} dilutions of rhizosphere samples. The bacteria isolated in this study from the wheat rhizosphere were mainly selected on the basis of morphological characteristics to those resembling to *Bacillus* and *Pseudomonas* species. In earlier studies, Kennedy *et al.* (1991) screened 1000 isolates of pseudomonads for inhibition of downy brome (*Bromus tectorum*). Similarly, Gupta *et al*. (1998) isolated 16 strains of PGPR from rhizosphere zone of green gram which were identified as *Enterobacter, Pseudomonas* and *Bacillus* spp. These PGPR were found to increase shoot and root length, and biomass of mungbean plants. Kennedy and Stubbs (2007) screened 10000 bacterial isolates taken from of wheat, jointed goatgrass (*Aegilops cylindrical*) and downy brome (*Bromus tectorum*) rhizospheres for inhibition of jointed goatgrass and downy brome weed.

5.2. Screening of bacterial cultures for their effect on seed germination of *Phalaris minor*

Preliminary studies showed that out of 83 rhizobacterial isolates/strains tested, only 36 isolates/strains suppressed the growth of *Phalaris minor* seedlings on 0.8% agar containing medium plates (Table 5). Among 83 isolates/strains, ten rhizobacterial isolates i.e., HWM1, HWM7, HWM9, HWM11, HWM17, HWM30, HWM37, HWM47, HWM54 and CP43 showed maximum retardation on 5th and 10th of seed germination of *Phalaris minor* (Table 5). Five rhizobacterial isolates i.e., HWM7, HWM9, HWM24, CP43 and PPM126 showed 100% root growth inhibition on 5th day of seed germination. Maximum root growth redardation was observed in bacterial isolates HWM9, HWM30, HWM54, HWM57, HWM58 and strain CP43 at 10th days of seedling growth.

Rhizobacterial isolates i.e., HWM1, HWM10, HWM18, HWM37 and NNY60 caused 50-70% shoot inhibition on 5th day of seed germination. Five rhizobacterial isolates i.e., HWM11, HWM30, HWM54, HWM57 and HWM58 caused 50-70% shoot inhibition and isolates HWM17, HWM47, CP43, P49, SB153, NNY60 and PPM115 caused 20-50% shoot inhibition on 10th day of seed germination. Rhizobacterial isolates i.e., HWM30, HWM54, HWM57, HWM58, HWM70 and CP43 caused 50-70% root inhibition and HWM17, HWM29, HWM31, HWM42, HWM68, HWM69, P49, PPM115, PPM126 and SYB101 caused 20-50% root inhibition on 10th day of seed germination.

Some of the rhizobacterial isolates i.e., HWM12, HWM31, HWM35 and HWM58 showed maximum stimulatory effect on 5th day of seed germination. Ten rhizobacterial strains i.e., HWM4, HWM10, HWM13, HWM18, HWM23, HWM31, HWM46, HWM50, HWM67, CPA152 and WPS73 showed maximum stimulatory effect on shoot growth of *Phalaris minor* at 10 days of growth. Rhizobacterial isolates i.e., HWM13, HWM23, HWM31 and CPA152 showed maximum shoot growth stimulation at both 5th and 10th days of seed germination.

Earlier, Kennedy *et al.* (2007) found 23-90% root inhibition of joint goatgrass by seven bacterial isolates. Loper and Schroth (1986) found that two bacterial strains belonging to Enterobacteriaceae were capable to reduce plant root elongation and increased shoot/root ratio in sugarbeet, when applied as seed inoculants. It is also possible that phytoalexins produced by seedlings as a host defense response after inoculation (infection) of rhizobacteria could be inhibitory for seedling growth initially. The production of toxic metabolites by other non-fluorescent *Pseudomonas* strains with an inhibitory effect on wheat root growth has also been reported (Fredrickson *et al.,* 1987).

5.3. Mechanism of weed growth inhibition by selected bacteria

5.3.1. Phytotoxicity test

Twelve reference strains and three rhizobacterial isolates were tested for phytotoxicity test (Table 6). Out of these, eight strains showed disease spots and rest of three strains caused yellowing of leaves of *Phalaris minor*. All the reference strains, except, CPS67 and NNY60 were able to infect the plant and produce disease symptoms. Disease started as small necrotic spots. SYB101, HWM11 and P49 caused yellowing of *Phalaris minor* leaves. Similarly, Shukorjuraimi *et al.* (2005) showed that application of three aqueous extract concentrations (at full, half, and quarter-strength) prepared from the fungus *Exserohilum longirostratum* produced necrotic, chlorosis and wilting in *Echinochloa crus-galli* seedlings within 48 hours after immersion. At full strength, the solution reduced living leaf area by a disease incidence of 92.5% as compared to controls and was significantly different compared to the other two treatments. Half strength and quarter strength aqueous extracts caused a disease incidence of 85.0% and 57.5%, respectively. The results indicated that higher aqueous extract concentration increased seedling mortality. EI-Morsy *et al.* (2006) found that application of *Alternaria alternata* strain produced typical brown necrotic lesions on the leaves of water hyacinth and caused extensive defoliation.

5.3.2. Production of indole acetic acid by bacterial isolates

Maximum IAA production was observed in bacterial isolates HWM13 and strains CPS67 and CP43 (Table 7). Other 31 isolates produced IAA in low concentration. Selected bacterial cultures were allowed to grow at different concentrations and incubation period to find out the effect of concentration of tryptophan on production of IAA. It was found that IAA production by bacterial isolates/strains was more at 1000 μg/ml tryptophan concentration (Table 8). IAA production decreased after 60 hrs of incubation in strains CP43 and CPS67. Table 8 illustrated that concentration of indole acetic acid in CPS67 and CP43 reached to 1.8 mg/ml and 130 μg/ml, respectively. Earlier reported that different bacterial strains have been found to produce IAA in varying amounts (Prikryl *et al.,* 1985; Keyeo *et al.,* 2011). Similarly, Mejri *et al.* (2010) reported tha production of indole acetic acid by *Pseudomonas trivialis* X33d that caused growth suppression of great brome and growth promotion of durum wheat. Serwar and Kremer (1995) showed that an *Enterobacter taylorae* isolate with high auxin-producing potential (72 μg/ml) was found to inhibit growth of *Convolvulus arvensis*. The inhibitory effect of some deleterious rhizobacteria (DRB) was also related to their high amount of IAA excretion in *Enterobacter taylorae* (Sarwar and Kremer, 1995). Similar concentration dependent effect of IAA on stimulation or inhibition of root/shoot growth has been reported in earlier studies (Arshad and Frankberger, 1991). Loper and Schroth (1986) observed a significant linear relationship between IAA accumulation of the rhizobacterial strains and decreased root elongation of sugar beet seedlings. The initial stunting effect on seedlings could be due to contact of bacterial cell with legume seeds or due to synthesis or secretion of excessive amount of IAA or by production of some inhibitory agent/toxin by the bacterium when grown in synthetic medium or in root exudates of legumes (Gealy *et al.,* 1996; Bolton and Elliott, 1989).

5.3.3. Production of δ-aminolevulinic acid

Bacterial isolates HWM69, HWM59 and CP43 showed more than 10 μg/ml ALA production (Table 9). Other 33 isolates produced ALA in low concentration (0.2 - 7.5 μg/ml). Strain CP43 inhibited seed germination of *Phalaris minor*. Two bacterial isolates HWM46 and HWM50 stimulated shoot growth of *Phalaris minor*. Bacterial strains CPS67 and CP43 produed significant amount of IAA and ALA (Table 7, 9) and retarded the growth of *P. minor* (Table 5). On the other hand, isolate HWM13 which produced only IAA, led to significant stimulation of shoot only at 10 days. These results indicated the tissue specific stimulation effect of IAA.

Similar results have been reported by Hotta *et al.* (1997 b) demonstrating that low concentration (0.01-10 mg/l) of ALA showed growth promoting effect on crops whereas, it suppressed plant growth at higher concentrations (>2 mM). Koh and Song (2007) reported that two strains of *Rhodopseudomonas* sp. KL9 and BL6 produced an efficient growth enhancement of tomato seedlings under axenic conditions, together with the production of indole-3-acetic acid and ALA. Solubilization of insoluble phosphate may also be responsible for the growth promotion of tomato seedlings. Lee *et al.* (2008) reported that application of *Rhodopseudomonas* sp. KL9 can enhanced growth, fruit formation, yield and the quality of fruit in tomato plants in greenhouse. Other research workers have also shown that ALA could be used in agricultural applications as a herbicide, insecticide and growth promoting factor including salt and cold temperature tolerance in plants (Watanabe *et al.,* 2000). Tanaka *et al.* (1992) found that low concentration of ALA application increased the chlorophyll content and accelerated the growth of plant tissue and rice seedling.

5.3.4. Fungal growth inhibition by rhizobacterial isolates

It was found that out of 83 rhizobacterial isolates/strains tested, only 17 isolates/strains inhibited *F. oxysporum* on PDA containing medium plates using (Table 10). Among 17 strains, three cultures i.e., HWM13, SB153 and WPS73 showed maximum zone of inhibition. Isolates HWM25, HWM31, HWM37, HWM18, HWM54 and HWM57 showed moderate zone of inhibition. Selected antifungal bacterial isolates HWM5, HWM37 and HWM57 suppressed *in vitro* growth of *Phalaris minor*. Majority of the isolates/strains did not inhibit the growth of *F. oxysporum* on PDA plates (Table 10). Earlier, Siddiqui *et al.* (2001) showed that *Pseudomonas aeruginosa* and *Bacillus subtilis* strains produced growth inhibition zones by inhibiting the radial growth of *Macrophomina phaseolina*, *Fusarium oxysporum* and *Rhizoctonia solani*. Karuppiah and Rajaram (2011) showed that eight *Bacillus* sp., out of 63 different *Bacillus* isolates, exhibited plant growth promoting activities and six of these *Bacillus* isolates, also inhibited the growth of *Penicillium* sp., *Cercospora* sp. and *Fusarium oxysporum*. Heisey and Putnam (1986) reported that strain of *Streptomyces hygroscopicus*, produced geldanamycin and nigericin antibiotics in culture broth that strongly inhibited germination and growth of garden cress.

5.4. Inoculation effect of weed inhibiting bacteria on growth of wheat and *Phalaris minor*

Plant growth-inhibitory bacteria associated with plant roots may exert their harmful effects on plant growth and nutrition through a number of mechanisms such as production of phytotoxins, production of phytohormones (IAA, ALA) and antibiotics, and may inhibit/block mechanism involved in formation of nutrient and cellular mechanisms when they are either applied to seeds or incorporated into the soil. Moreover, rhizospheric bacteria with inhibitory properties for weed growth have also been found to enhance the growth of wheat plant.

Twelve bacterial isolates/strains were tested in the pot house to check their effect on growth of wheat and weed. Seeds of wheat var. WH711 and *Phalaris minor* were grown in pots containing 10 kg soil and sand mixture. The seeds were inoculated with 20 ml growth suspension of selected bacterial islates/strains. Inoculated plants were harvested at 25, 50 and 75 days after sowing and observations for root and shoot growth were recorded.

Application of recommended doses of fertilizers (RDF) in wheat resulted in 60% increase in shoot dry weight (SDW) and 12.2% increase in root dry weight (RDW) at 25 days after sowing (Table 11). At 50 days, application of RDF in wheat resulted in 51.5% increase in shoot dry weight and 17.8% increase in root dry weight (Table 12). After 75 days of sowing, application of RDF in wheat resulted in 76.9% increase in shoot dry weight and 60.41% increase in root dry weight (Table 13). At 25 days of wheat growth, inoculation of bacterial strain SYB101 showed 35.5% increase in root dry weight and 61.6% increase in shoot dry weight and caused 33.3% decrease in root dry weight and 25% decrease in shoot dry weight on growth *Phalaris minor* (Table 11). Similarly, inoculation with bacterial strain CPS67 showed 32.25% increase in root dry weight and 13.1% increase in shoot dry weight of wheat growth and caused 13.3% decrease in root dry weight and 20% decrease in shoot dry weight on growth of *Phalaris minor*. Inoculation of bacterial isolates HWM10 showed 30.46% increase in root dry weight and 45% increase in shoot dry weight of wheat growth and showed 25% increase in root dry weight and 60% increase in shoot dry weight of *Phalaris minor*.

At 50 days of wheat growth, inoculation of bacterial strains SYB101 and CPS67 showed 12.77% and 21.9% increase in root dry weight and 49% and 8.4% increase in shoot dry weight and caused 33.3% and 37% decrease in root dry weight and 7.5% decrease in shoot dry weight of *Phalaris minor* (Table 12). Bacterial isolate HWM11 showed 33.5% increase in root dry weight and 57.4% increase in shoot dry weight of wheat growth and showed 18.2% decrease in root dry weight and 40.3% decrease in shoot dry weight on growth of *Phalaris minor*. Inoculation of bacterial isolate HWM10 showed 60% increase in root dry weight and 31.4% increase in shoot dry weight of wheat growth and showed 46% increase in root dry weight and 72% increase in shoot dry weight of *Phalaris minor*. At 75 days of wheat growth, inoculation of bacterial strain SYB101 showed 40% increase in root dry weight and 50.57% increase in shoot dry weight and caused 47.1% decrease in root dry weight and 55.6% decrease in shoot dry weight of *Phalaris minor* (Table 13). Inoculation with bacterial isolate HWM11 showed 49.6% decrease in root dry weight and 51.5% increase in shoot dry weight of wheat growth and showed 56.2% decrease in root dry weight and 30% decrease in shoot dry weight on growth of *Phalaris minor*. Inoculation of bacterial isolate HWM10 showed 40.3% increase in root dry weight and 26.6% increase in shoot dry weight of wheat growth and showed 22.7% increase in root dry weight and 28.6% increase in shoot dry weight on growth of *Phalaris minor*.

Javaid and Akbar (2012) showed maximum herbicidal activity against *Phalaris minor* due to metabolites produced by *Drechslera hawaiiensis* where 65% and 64% reduction in length and dry biomass of shoot was recorded. Regular application of certain bioherbicides has been reported to provide long-term control of the target weed. For example, Kadir and Charudattan (2000) reported that *Dactylaria higginsi* provided excellent long-term control of purple nutsedge (*Cyperus rotundus* L.) when it was applied regularly. Shukorjuraimi *et al.* (2005) showed that *Exserohilum longirostratum* reduced growth and dry weight of barnyard grass ecotypes PK-04, L-01 and B-04 and the range of dry weight reduction was 37.58 to 86.34%.

These results suggested that effective *Phalaris minor* inhibiting and wheat-growth promoting bacterium-plant systems must be tested in controlled vegetation experimental designs with specific consideration of practical applications such as soil type, plant types grown and the environmental factors. Although, many bacterial isolates/strains inhibited the growth of *Phalaris minor* under pot house conditions, the extent of inhibition by tested bacterial strains under actual field conditions remain unclear. Moreover, plant type/variety has also been reported to influence the root colonization ability of the inoculated strains (Sheng, 2005) and therefore, competitive and effective bacterial strains must be selected from the pool of indigenous soil bacteria which could be adopted to the particular conditions of the inoculation site (Paau, 1989; Sindhu and Dadarwal, 2000). Thus, experiments concerning stimulation of wheat and inhibition of *Phalaris minor* in pot house studies must be followed by investigations under field conditions. *Phalaris minor* inhibiting and wheat stimulating bacteria isolated in this study may have use in the area where herbicide resistant *Phalaris minor* exists and further research could lead to an alternative mean of herbicide for use in sustainable agriculture.

CHAPTER-VI

SUMMARY AND CONCLUSIONS

Wheat is an important staple food crop of India. The yield of wheat in India is severely affected by weeds. More than 40 weed species have been reported from various wheat growing areas of India. Weeds compete with the crop plants not only by occupying the space but also compete for other resources such as water, light and nutrients. Littleseed canarygrass (*Phalaris minor* Retz.) is one of the most problematic, frequently occurring and densely populated weed. It is a narrow-leaf highly competitive weed that caused 33–68% reduction in yield in different wheat varieties when there is 1:1 ratio of wheat. It is also a troublesome weed of wheat fields in Pakistan, USA, Canada, Africa, Australia, France, Iran, Iraq and Mexico. Due to introduction of high-yielding dwarf wheat varieties along with increased irrigation and fertilizer facilities, *P. minor* has became aggravated pest. In addition, rice–wheat crop rotation also stimulated its emergence, growth and development. Although synthetic herbicides are very effective in controlling weeds of wheat, however, the use of these agro-chemicals has increased consumer's concern due to environmental pollution and other health related issues as well as numerous cases of herbicide resistance in many weeds world over. For more sustainable systems, there is an increasing trend towards the search for alternatives of these chemicals which are based on natural compounds. Use of fungal and bacterial cultures with herbicidal activity is one of the alternative strategies to manage the weeds. The present study was carried out to investigate the herbicidal activity of rhizobacterial isolates for the management of *P. minor.*

In the present study, seventy one bacterial cultures were isolated from rhizosphere soil collected from CCS H.A.U., Hisar farm at 60 and 90 days of wheat growth. These bacterial isolates along with twelve reference strains were screened for their effect on seed germination of *Phalaris minor* on water agar plates. Nine rhizobacterial isolates i.e., HWM1, HWM7, HWM9, HWM11, HWM17, HWM30, HWM37, HWM47, HWM54 and CP43 showed maximum retardation on 5th and 10th of seed germination of *Phalaris minor*. At 10th day of seed germination, 14.5% bacterial isolates showed retardation of shoot growth and 19.3% bacterial isolates retarded root growth. Out of fifteen rhizobacterial isolates/strains tested for phytotoxicity effect on *Phalaris minor*, three rhizobacterial isolates/strains i.e., HWM11, P49 and SYB101 only caused yellowing of leaves whereas eight rhizobacterial isolates/strains HWM10, HWM25, KPM15, SB153, PPM126, WPS73, CPA152 and GYB106 caused appearances of disease spot.

Screening of rhizobacterial isolates for production of indole acetic acid showed that nine isolates i.e., HWM7, HWM11, HWM18, HWM23, HWM24, HWM37, HWM42, HWM57 and PPM115 produced IAA ranging from 7.0-10.0 µg/ml. Two isolates HWM49 and HWM35 produced 11.10 and 14.07 µg/ml IAA, respectively. Maximum production of IAA (> than 25 µg/ml) was observed in isolates CPS67, CP43 and HWM13. Rhizobacterial isolates/strains HWM59, HWM69, CP43 and CPS67 showed >7.0 µg/ml production of δ-aminolevulinic acid (ALA). Other 22 bacterial isolates produced ALA ranging from 2 µg/ml to 7 µg/ml. Fungal growth inhibition studies showed that 20.5% of total rhizobacterial isolates inhibited the growth of *Fusarium oxysporum* on PDA medium plates. Large zone of inhibition was formed by bacterial isolates HWM13, WPS73, SB153, HWM25, HWM31, HWM37 and HWM57.

Twelve rhizobacterial isolates/strains were tested for their effect on growth of wheat and weed under pot house conditions. Rhizobacterial strains/isolates i.e., SYB101, CPS67 and HWM11 were found to stimulate maximum growth of wheat and inhibited the growth of *Phalaris minor*. Bacterial isolates HWM10 and HWM25 were found to enhance growth of wheat and *Phalaris minor*. Rhizobacterial strain NNY60 was found to inhibit wheat growth whereas enhanced growth of *Phalaris minor*. These selected rhizobacterial isolates may specifically inhibit seedling germination of weed under field conditions. Thus, there are immense possibilities for further increasing the production of cereal crops by application of these biological herbicides.

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ABSTRACT

Title of the thesis	:	**Biological control of *Phalaris minor* in wheat (*Triticum aestivum* L.) using rhizosphere bacteria**
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Key words: Rhizosphere bacteria, Indole acetic acid, δ-aminolevulinic acid, Wheat, *Phalaris minor*

In the present study, seventy one bacterial cultures were isolated from wheat rhizosphere soil. These bacterial isolates along with twelve reference strains were screened for their effect on seed germination of *Phalaris minor* on water agar plates. Ten rhizobacterial isolates i.e., HWM1, HWM7, HWM9, HWM11, HWM17, HWM30, HWM37, HWM47, HWM54 and CP43 showed maximum retardation on 5th and 10th of seed germination of *Phalaris minor*. At 10th day of seed germination, 14.5% bacterial isolates showed retardation of shoot growth and 19.3% bacterial isolates retarded root growth. Out of fifteen rhizobacterial isolates/strains tested for phytotoxicity effect on *Phalaris minor*, three rhizobacterial isolates/strains i.e., HWM11, P49 and SYB101 only caused yellowing of leaves whereas eight rhizobacterial isolates/strains HWM10, HWM25, KPM15, SB153, PPM126, WPS73, CPA152 and GYB106 caused appearance of disease spot. Screening of rhizobacterial isolates for production of indole acetic acid showed that nine isolates i.e., HWM7, HWM11, HWM18, HWM23, HWM24, HWM37, HWM42, HWM57 and PPM115 produced IAA ranging from 7.0-10.0 μg/ml. Two isolates HWM49 and HWM35 produced 11.10 and 14.07 μg/ml IAA, respectively. Maximum production of IAA (> than 25 μg/ml) was observed in isolates CPS67, CP43 and HWM13. Rhizobacterial isolates/strains HWM59, HWM69, CP43 and CPS67 showed >7.0 μg/ml production of δ-aminolevulinic acid. Other 22 bacterial isolates produced ALA ranging from 2 to 7 μg/ml. Fungal growth inhibition studies showed that 20.5% of total rhizobacterial isolates inhibited the growth of *Fusarium oxysporum* on PDA medium plates. Large zone of inhibition was formed by bacterial isolates HWM13, WPS73, SB153, HWM25, HWM31, HWM37 and HWM57. Twelve rhizobacterial isolates/strains were tested for their effect on growth of wheat and weed under pot house conditions. Rhizobacterial strains/isolates i.e., SYB101, CPS67 and HWM11 were found to stimulate growth of wheat and inhibited the growth of *Phalaris minor*.

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