Rhizobium based formulations for biocontrol of soil borne fungal pathogens of chickpea

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1. Introduction

Chickpea (Cicer arietinum L.) is an important legume food crop grown worldwide and an important protein source in tropic and semi-arid tropic regions. This legume finds an important place in the farming systems adopted by small and marginal farmers in a large number of developing countries, as it restores the soil fertility by fixing atmospheric nitrogen. However, it is attacked by many fungal pathogens like Fusarium oxysporum f. sp. ciceri, Ascochyta rabiei, Botrytis cinerea, Rhizoctonia solani, Sclerotinia sclerotiorum etc. (Mazur et al., 2004). Among them, losses due to Fusarium wilt alone hover around 10-15% each year as a regular feature. In the years of severe epidemics, crop losses have gone as high as 60-70% (Chand and Khirbat, 2009). Management of these diseases is achieved mainly by the use of resistant cultivars and fungicide application. But frequent application of fungicides to the soil has not only caused environmental hazards like water and soil pollution but also destruction of non-target beneficial microorganisms in soil. Recently, biocontrol approaches have been initiated by using antagonistic microorganisms to combat these diseases.

Practice of ‘naturally inoculated’ soil with legume seeds in the 1800s and the subsequent grant of first patent, “Nitragin” (Nobbe and Hiltner, 1896) have led to the development of technologies world-wide for legume inoculation with Rhizobium sp. Inoculation with Rhizobium continues to make significant contributions to the global soybean production, and of other legumes to a lesser extent. Although the basic tenet of Rhizobium inoculation is symbiotic nitrogen fixation in legumes, rhizobia can effectively control various soil-borne plant pathogenic fungi. Fungal pathogens of the genera Fusarium, Rhizoctonia, and Macrophomina are reported to be controlled by Rhizobium leguminosarum, Sinorhizobium meliloti and Bradyrhizobium japonicum (Ozkoc and Deliveli, 2001; Arfaoui et al., 2006; Singh et al., 2010). Potential mechanisms behind rhizobial biocontrol are mycoparasitism, competition for nutrients, production of antifungal metabolites like hydrogen cyanide (HCN), antibiotics, siderophore and induction of plant defense mechanisms (Essalmani and Lahlou 2002; Huang and Erickson 2007; Gopalakrishnan et al., 2015). A number of studies showed accumulation of various phenolic compounds and phytoalexins, de novo synthesis of pathogenesis-related proteins, higher elicitation of different enzymes such as chitinases, β-1-3-glucanases, L-phenylalanine ammonia lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO) in plants pretreated with
rhizobial strains, which play a significant role in induction of systemic resistance against several phyto-pathogens (Arfaoui et al., 2007; Mabrouk et al., 2007; Osdaghi et al., 2011). Induced systemic resistance in presence of *Rhizobium* sp. is due to indirect stimulation of plants to activate the defense mechanisms when challenged with a pathogen (Avis et al., 2008). Even the purified lipopolysaccharide (LPS) of *Rhizobium etli* which was originally isolated from potatoes was found to induce systemic resistance against nematodes (Reitz et al., 2002). Antibiosis, direct parasitism, competition for nutrients and infection sites, and induced host resistance are not necessarily exclusive of one another mechanisms and often found to act synergistically.

Till now, the *Rhizobium* inoculant technologies in India for the pulses are crop specific and the development of *Rhizobium* inoculants with dual purpose of nitrogen fixation and antagonism against pathogens of chickpea can contribute to increased productivity and protection against many pathogens.

The best organism when identified and selected for inoculation requires appropriate formulation, which is an important determinant for the potential success of the inoculant. A biofilm is an assemblage of multiple microbial species associated with a surface or any interfaces, often enclosed in self-produced extracellular polymeric substances (EPS) matrix (Donlan, 2002). Biofilm formation is quite common in natural environments which provide its component microbial cells a certain degree of protection and helps in their growth, survival and successful colonization (Davey and O’toole, 2000). Biofilm comprising bacteria and fungi are known to enhance the growth and survival ability of bacterial inoculants in soil. Root colonization is an important factor in successful establishment of inoculants and biofilms colonizing plant root surface provide better plant growth promotion as well as protection against several soil borne fungal pathogens (Seneviratne, 2003). Hence, biofilmed biofertilizers have the potential to reproduce the beneficial effects consistently at field level under different agro-ecological situations (Prasanna et al., 2014; Triveni et al., 2013, 2015).

The primary goal of any strategy for *Rhizobium* inoculant technology necessitates the search for the best bacteria available since the diversity of rhizobia is enormous and many native species are inefficient nitrogen fixers. The currently available *Rhizobium* germlasm for chickpea has been screened for the nitrogen fixing potential but not for their efficacy as biocontrol agents. Many traits such as antifungal activity, antibiosis, phosphate solubilisation, production of indole acetic
acid (IAA), HCN, and siderophore are to be performed along with plant growth assay for selecting suitable *Rhizobium* species for dual purpose. *Rhizobium* based biofilms using fungal matrix (*Trichoderma* sp.) have never been explored for biocontrol of fungal diseases of chickpea. The development of biofilms needs optimization and evaluation for its biocontrol efficacy. Therefore, the objectives of the proposed study are

**Objectives**

1. To evaluate rhizobial strains for their nitrogen fixing potential and antagonism against fungal pathogens of chickpea
2. To develop *Rhizobium* based biofilmed formulations and evaluate their promise as biocontrol agents in chickpea
3. To understand the plant-microbe interactions with/without fungal challenge, as influenced by inoculation with promising rhizobial formulations
Legume crops play a significant role in providing nutritional security and sustainable agriculture worldwide. They are important to agriculture as they restore the soil fertility by fixing atmospheric nitrogen. In 2006-08, total area covered under food legumes worldwide was 61.5 m ha and the total production was 46.5 m tons. Among all the food legumes, dry beans (all species of *Phaseolus*) cover 46% of total area, followed by chickpea (18%) and cowpea (18%). In terms of production also dry beans dominate at 46%, followed by chickpea, which covers 22% of the total production (Akibode and Maredia, 2011). Globally, total area covered under legume crops is only about one-tenth of that covered under all cereal crops. Most of the legume crops are cultivated under rainfed-low input farming systems, as compared to the cereal crops. In 2008, the average global yields of legume crops (0.86 t/ha) was only about one-fourth the average yields of cereal crops (3.54 t/ha). But, in the last few years, the overall food legume production has increased significantly both in developing and developed countries (Akibode and Maredia, 2011). Chickpea, also known as Bengal gram is one of the most important food legume crops in the world which is widely grown in different states of India and used in multifarious ways in daily uses. South Asia is by far the largest producer of chickpea (76%) in the world with a share of more than 80% of area harvested. India is the largest chickpea growing country in the world. The two South Asian countries—India and Pakistan, together cover more than 75% of total world chickpea area under production.

Nitrogen, a major essential nutrient is required by plants in large quantities and its availability in soil most frequently limits the crop growth and productivity. Although atmospheric nitrogen gas (N$_2$) constitutes 78% of the air, it cannot be directly utilized by the plants for their nutrition. Diazotrophs represent a large group of prokaryotes which can reduce atmospheric N$_2$ to ammonium, a process known as biological nitrogen fixation (BNF). Rhizobia, a group of diazotrophic organisms which establish symbiotic associations with plants of the family *Leguminosae* play a significant role in the sustenance of soil fertility (Vance, 2001; Herridge et al., 2008). The symbiotic association between legumes and rhizobia may contribute up to 13-360 kg N ha$^{-1}$ (Bohlool et al., 1992). However, the efficiency of biological nitrogen fixation varies in different legumes and is also dependent upon the various biotic and abiotic factors like host species, rhizobial efficiency and soil physico-chemical
properties (Gopalakrishnan et al., 2015). Among the food legumes, nitrogen fixation efficiency ranges from 33 to 643 kg N ha\(^{-1}\) in soybean, 126 to 319 kg N ha\(^{-1}\) in groundnut, 125 to 143 kg N ha\(^{-1}\) in black gram and 77 to 92 kg N ha\(^{-1}\) in pigeonpea (Peoples and Craswell, 1992). Beside food legumes, forage legumes and tree legumes also play a vital role in total nitrogen fixation globally (Baddeley et al., 2013).

2.1 Historical perspective of *Rhizobium* utilization and their classification

Hellriegel and Wilfarth (1888) demonstrated the ability of legumes to fix atmospheric nitrogen. Beijerinck (1888) isolated the microorganism from root nodules and named it *Bacillus radiicola*. Later, it was renamed as *Rhizobium leguminosarum* by Frank (1889). Besides biological nitrogen fixation, rhizobia also play a significant role in plant growth promotion (Fig. 2.1).

The practice of ‘naturally inoculated’ soil with legume seeds in the 1800s and the subsequent grant of first patent, “Nitragin,” (Nobbe and Hiltner, 1896) led to the development of technologies worldwide for legume inoculation with *Rhizobium* sp. Commercial production of *Rhizobium* inoculants, mainly for soybean crop was started in 1895 in U.S.A. and it was mostly peat based inoculant (Roughley and Vincent, 1967). Date and Roughley (1977) demonstrated that sterile peat based inoculants are better than non-sterile peat based inoculants, as mortality of rhizobia is higher in unsterilized peat during storage. However, with diversification of carrier materials a number of novel inoculant technologies have been developed and commercialized. There are a number of reviews available on evolution of legume inoculant production and formulations (Smith, 1992; Brockwell and Bottomley, 1995; Lupwayi et al., 2000; Stephens and Rask, 2000). Usually, for inoculant preparation, sufficient amount of fermenter-grown broth culture containing high population of desired rhizobia is mixed with finely powdered sterile carrier material. The success of an inoculant under field conditions also depends on the infectivity and efficiency of nitrogen fixation by the rhizobia.

Rhizobial classification is a complex and ever changing discipline. Until early 1980s different symbiotic nitrogen-fixing bacteria nodulating legume plants were classified in to a single genus *Rhizobium* mainly based on their selective interaction with host plants. It included six recognized species namely *R. leguminosarum*, *R. japonicum*, *R. meliloti*, *R. phaseoli*, *R. trifolii* and *R. lupine* (Zakhia and Lajudie, 2001). In 1984, Jordan introduced a new genus
Bradyrhizobium which mainly included slow growing root nodule bacteria. Later on in 1992, Elkan added two more genera namely Azorhizobium and Sinorhizobium in the rhizobial classification. The classification of rhizobia is still in a transition phase and with introduction of small subunit of ribosomal RNA (SSU rRNA) sequencing technique more and more genera nodulating legumes are being described frequently. According to the present status, these nodulating bacteria have been classified into 14 genera belonging to both the α-subclass (Rhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium, Sinorhizobium (Ensifer), Methylobacterium, Devosia, Phyllobacterium, Ochrobactrum, Shinela, Herbaspirillum, and Microvirga) and the β-subclass (Burkholderia and Cupriavidus) of Proteobacteria (Moulin et al., 2001, Janczarek et al., 2015). In the present review the term ‘rhizobia’ is used to represent all the bacteria forming nodules on the roots and shoots of leguminous plants.

Rhizobia nodulating chickpea plants belong to the genera Mesorhizobium, which was first described by Jarvis et al. (1997). However, several strains of mesorhizobia can establish symbiotic relationship with different legume species from temperate, tropical and arctic areas (Chen et al., 2005). Some of them have very narrow host range like Mesorhizobium ciceri and Mesorhizobium mediterraneum (Nour et al., 1995) or may have broader host range like Mesorhizobium plurifarium and Mesorhizobium tianshanense, which can nodulate legumes from different genera (de Lajudie et al., 1998). A number of studies suggest that there is inherent molecular diversity within the native mesorhizobial population that nodulate chickpea plants (Nandwani and Dudeja, 2009). Nandwani and Dudeja (2009) studied 50 Rhizobium strains from different sites of Haryana district, India and observed that they can be grouped into six different genotypic groups based on PCR-amplified ERIC profiles and RFLP of 16S rDNA. Variability in nodulation has also been reported in different chickpea genotypes which affects the competitiveness and success of the rhizobial inoculant under field conditions (Khurana et al., 1991; Rupela, 1992; Sheoran et al., 1997). Hence, proper screening and evaluation of rhizobial strains is warranted before it can be subjected to inoculant preparation.

2.2 Rhizobia and biocontrol

The practice of Rhizobium inoculation continues to make significant contributions to the global soybean production and other legumes. Although the basic tenet of Rhizobium inoculation is symbiotic nitrogen fixation in legumes,
several species were found not only to promote plant growth but also inhibit the growth of various soil-borne pathogens (Table 2.1) belonging to different genera, in both leguminous as well as non-leguminous plants (Tu, 1978; Antoun et al., 1978; Chakraborty and Purkayastha, 1984; Malajczuk et al., 1984; Ehteshamul-Haque and Ghaffar, 1993; Sharif et al., 2003; Nadia et al., 2007).

Application of Sinorhizobium meliloti, Rhizobium leguminosarum bv. viceae and Bradyrhizobium japonicum as seed coating or as soil drench reduced disease incidence of M. phaseolina, R. solani and Fusarium spp. in okra plants (Ehteshamul-Haque and Ghaffar, 1993). Seed inoculation of Rhizobium leguminosarum bv. phaseoli exhibited significant reduction in root rot disease in bean plants artificially infested with Fusarium solani f. sp. phaseoli (Buonassisi et al., 1986). Several rhizobial and bradyrhizobial isolates have been reported to show antagonistic activity against Macrophomina phaseolina, which causes charcoal rot of groundnut (Arora et al., 2001; Deshwal et al., 2003a). Seed treatment with strain R12 of Rhizobium leguminosarum bv. viceae, isolated from lentil, that exhibited nitrogen fixation attribute in artificial medium was found to significantly increase seedling emergence and reduce incidence of Pythium damping-off of field pea and sugar beet (Bardin et al., 2004). Two other strains R20 and R21 isolated from pea also performed at par against Pythium disease in pea. Rhizobium sp. has also been reported to control chickpea diseases (Huang and Erickson, 2006; Hemissi et al., 2011). Huang and Erickson (2006) reported that seed treatment with Rhizobium leguminosarum bv. viceae can suppress Pythium damping-off of pea and lentil. They observed that disease suppression by the Rhizobium strain was comparable to seed treatment with fungicide Thiram, but it was better in stimulating root nodulation and plant growth promotion.

Arfaoui et al. (2006) observed that among the 21 Rhizobium isolates screened against Fusarium oxysporum f. sp. ciceri, in vitro in dual culture, and in vivo under greenhouse and field conditions, 14 were able to significantly inhibit mycelial growth. They further reported that among those 14 rhizobial isolates, 8 were found positive for the production of volatile antifungal compounds, 6 for cyanide production and 3 were able to solubilise phosphate. Seed treatment with these isolates reduced disease incidence in chickpea plants under greenhouse and field conditions. Similar observations were recorded by Hemissi et al. (2011), where, among the 42 Rhizobium strains efficient for nitrogen fixation and other plant growth
promoting attributes, 24 could effectively control *Rhizoctonia solani* under *in vitro* condition. They were also found positive for phosphate solubilisation and production of volatile antifungal compounds. In pot trials, inoculation of these isolates could control root rot of chickpea.

*Rhizobium japonicum* (*Bradyrhizobium japonicum*) strains were reported to suppress the growth of *Fusarium solani* and *Macrophomina phaseolina* both in culture medium and in soil. Inoculation of these strains not only improved germination of seeds of soybean but also reduced the root rot disease index both in pot and field trials (Al-Ani et al., 2012). In another study, rhizobiotoxine producing strains of *Bradyrhizoium japonicum* protected soybean from infection by *Macrophomina phaseolina* (Chakraborty and Purkayastha, 1984).

### 2.3 Interactive effect of *Rhizobium* with other PGPR in biocontrol

Studies suggest that the combination of PGPR strains (two or more) which have diverse mode of plant growth promotion or antagonism against soil-borne pathogens are more effective than single strain inoculum (Chiarini et al., 1998; Nadia et al., 2007; Mazen et al., 2008; Shaban and EL-Bramawy, 2011). Synergistic effect of culture filtrates of the three wild rhizobial isolates with arbuscular mycorrhizal (AM) fungi were found to reduce the disease incidence of damping-off and root rot diseases of faba bean (El-Batanony et al., 2007; Mazen et al., 2008). Similarly, coinoculation of AM fungi and *Rhizobium leguminosarum* provided protection against *Botrytis fabae* in bean plants (Rabie, 1998). Akhtar and Siddiqui (2008) also reported that the combined application of *Rhizobium* sp. along with *Glomus intraradices* and *Pseudomonas striata* successfully controlled root rot disease in chickpea caused by *Meloidogyne incognita* and *Macrophomina phaseolina*. They also reported significant increase in traits like chlorophyll content, number of pods per plant, nitrogen, phosphorus and potassium contents and over all plant growth. Another report suggests that single inoculation of *Rhizobium leguminosarum* and AM fungi (*Glomus boi* and *Glomus fasciculatum*) were more effective than dual inoculation in controlling *Fusarium* wilt of chickpea (Singh et al., 2010).

Siddiqui and Saukat (2002) reported that the use of *B. japonicum* along with *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* having good antifungal as well as plant growth promoting activity protected tomato roots from root rot and root knot diseases but also increased growth of tomato plants both under greenhouse and field conditions. Kumar et al. (2010) observed that dual inoculation with
Sinorhizobium fredii KCC5 and Pseudomonas fluorescens LPK2 significantly reduced Fusarium wilt in pigeon pea and increased the number of nodules and pods per plant, root and shoot length and plant biomass. They also observed aggressive root colonization, disruption of fungal hyphae and degeneration of fungal conidia which finally lead to suppression of Fusarium udum. Hameeda et al. (2010) also reported that seed priming with antagonistic Pseudomonas sp. CDB 35 and Rhizobium sp. IC 59 not only reduced chickpea collar rot but also increased nodulation and plant biomass.

A number of studies revealed that dual inoculation of Rhizobium with commercial formulations of biocontrol agents like Trichoderma sp. were effective in the suppression of diseases caused by several soil-borne fungal pathogens as well as in plant growth promotion. Ganesan et al. (2007) observed that application of Rhizobium with Trichoderma harzianum not only reduced incidence of collar rot disease in groundnut caused by Sclerotium rolfsii, but also increased root, shoot length and plant biomass. Shaban and El-Bramawy (2011) reported that dual inoculation with Rhizobium and Trichoderma reduced damping off and root rot diseases which resulted in the increase of yield components like branches per plant, pods per plant, seeds per pod, mean seed weight and seed yield of broad bean, chickpea and lupine plants. Recent reports on the use of cyanobacteria as stimulatory partners in legume crops have opened up a new niche for research. Cyanobacterial strains such as Anabaena laxa, Calothrix sp. exhibited improved yields, enhanced nitrogen fixation and leghaemoglobin content when coinoculated with rhizobial strains (Babu et al., 2015; Bidyarani et al., 2016).

2.4 Mechanisms involved in biocontrol

The mechanisms involved in biological control of pathogenic fungi by rhizobia include mycoparasitism, production of antibiotics and antifungal metabolites like hydrogen cyanide (HCN), siderophore production and ensuing competition for iron between pathogens and rhizobia, competition for essential nutrients, induction of plant defense mechanisms and plant growth promotion which avoids susceptibility to pathogenic attack (Fig. 2.2) (Arora et al., 2001; Essalmani and Lahlou, 2002; Huang and Erickson, 2007).

2.4.1 Mycoparasitism

Parasitisation of hyphal tips or inhibition of reproductive structures like sclerotia or zoospores by different groups of rhizobia has been reported (Tu, 1978;
Malajczuk et al., 1984; Kelemu et al., 1995; Sharif et al., 2003). It was suggested that in rhizosphere rhizobia may suppress the invasion of pathogenic fungi through covering the hyphal tips and subsequent lysis of the fungal hyphae through production of antibiotics (Sharif et al., 2003). Inhibition of germination and growth of sclerotia of Sclerotium rolfsii by Bradyrhizobium japonicum was observed by Balasundaram and Sarbhyo (1988). Fast growing rhizobial strains were found more effective in inhibiting the growth of white sclerotia of S. rolfsii. In another report, slow growing Bradyrhizobium has been reported to inhibit mycelial growth, sclerotial formation and germination of Rhizoctonia solani (Kelemu et al., 1995).

Chao (1990) demonstrated reduction in mycelial dry weight of fungal isolates in vitro in the presence of rhizobia. Significant reduction in the survival of zoospores of Phytophthora cinnamoni in vitro was also reported in the presence of rhizobia isolated from nodules of Acacia pulchella. The inhibitory effect on zoospores provided protection to the host plant in non-sterile disease suppressive and conducive soils (Malajczuk et al., 1984).

2.4.2 Antibiotics production

Production of antifungal antibiotics by different microorganisms plays an important role in biocontrol of fungal pathogens (Weller, 1988; Ligon et al., 2000; Raaijmakers et al., 2002). There are several reports on the production of antibiotics by different rhizobial strains (Deshwal et al., 2003a; Chandra et al., 2007). A number of researchers have reported that several strains of Rhizobium leguminosarum can produce different types of bacteriocin molecules which can be categorised to small, medium to large based on their size (Schwinghamer and Brockwell, 1978; Hirsch, 1979). Hirsch et al. (1980) found that R. leguminosarum contains the symbiotic plasmid pRL1J1, which not only contains genes necessary for nodulation and nitrogen fixation but also the determinants for bacteriocin production. Triplett and Barta (1987) and Robleto et al. (1998), reported production of peptide antibiotic trifolitoxin (TFX) by R. leguminosarum bv. trifolii. Rhizobitoxine produced by Bradyrhizobium has been found to reduce infection of Macrophomina phaseolina in different crops like soybean and groundnut (Chakraborty and Purkayastha, 1984; Deshwal et al., 2003b). Different strains of R. leguminosarum bv. trifolii, R. leguminosarum bv. viciae, S. meliloti and B. japonicum have been reported to secrete antibiotics which can inhibit several phytopathogens (Ozkoc and Deliveli,
2001; Bardin et al., 2004; Hafeez et al., 2005; Chandra et al., 2007; Gopalakrishnan et al., 2015).

2.4.3 Production of antifungal secondary metabolites

2.4.3.1 HCN production

Hydrogen cyanide is a volatile, secondary metabolite which inhibits several metalloenzymes, in particular, copper containing cytochrome C oxidases. Hence, it suppresses the growth and development of many phytopathogenic microorganisms (Martínez-Viveros et al., 2010). Although there are several reports, rhizobia are relatively less efficient in HCN production (Deshwal et al., 2003b). Beauchamp et al. (1991) and Antoun et al. (1998) have found 12.5% and 3% of the total strains screened respectively to be HCN producers. Arfaoui et al. (2006) reported six rhizobial strains which were positive for HCN production and showed antifungal activity towards *Fusarium oxysporum* f. sp. *ciceri* under in vitro and in vivo conditions. *Mesorhizobium loti* MP6, an isolate from root nodules of *Mimosa pudica*, tested positive for HCN production which played significant role in inhibiting the growth of *Sclerotinia sclerotiorum* causing white rot in *Brassica campestris* (Chandra et al., 2007).

2.4.3.2 Siderophore production

Many microorganisms are endowed with the property to produce siderophores (low molecular weight, iron-binding compounds) which help to sequester iron from the environment and release it inside the cells (Matzanke, 1991). Ability of Fe$^{3+}$ acquisition through production of siderophore is crucial as it determines the ability of bacteria to colonize plant roots under iron deficient conditions (Crowley and Gries, 1994; Crowley, 2006).

The production of siderophores by PGPR is a mechanism to prevent multiplication and colonization of pathogenic microorganisms by sequestering Fe$^{3+}$ from the rhizospheric region (Siddiqui, 2006; Martínez-Viveros et al., 2010). Rhizobia have been reported to produce a diverse array of siderophores (Deshwal et al., 2003b). These include Rhizobactin by *S. meliloti* (Smith et al., 1985), citrate type by *B. japonicum* (Guerinot et al., 1990), anthranilic acid by *R. leguminosarum* bv. *viciae* (Riouxi et al., 1986), phenolate type by *Rhizobium leguminosarum* IARI102 (Patel et al., 1988), catechol type by cowpea *Rhizobium* RA-1 (Modi et al., 1985), rhizobactin 1021 by alfalfa symbiont *S. meliloti* (Persmark et al., 1993), cyclic trihydroxamate siderophore, vicibactin by *R. leguminosarum* biovar*viciae* (Dilworth
et al., 1998, Carson et al., 2000), dihydroxamate type by *S. meliloti* (Carson et al., 2000). Siderophore producing strains of *Rhizobium* have been reported to inhibit fungal pathogens both *in vitro* and *in vivo* (Arora et al., 2001; Chandra et al., 2007). *In vitro* inhibition of *Macrophomina phaseolina* by siderophore producing strains of *Rhizobium meliloti* (*Sinorhizobium meliloti*) was reported by Arora et al., (2001). Treatment with these strains also improved groundnut seed germination in the presence of *M. phaseolina*. Deshwal et al. (2003a) also reported three *Bradyrhizobium* strains AHR-2amp+, AHR-5amp+ and AHR-6amp+ which not only produced siderophore, IAA and exhibited phosphate solubilisation *in vitro* but also showed antagonistic activity against *Macrophomina phaseolina* in groundnut. Chandra et al. (2007) reported that seed treatment with *Mesorhizobium loti* MP6, a hydroxamate type siderophore producer, drastically reduced incidence of white rot disease in *Brassica campestris*.

### 2.4.4 Mycolytic enzymes

Chitinases and β-1, 3-glucanases are two main hydrolytic enzymes associated with fungal cell wall lysis. There are reports of rhizobia producing mycolytic enzymes, especially chitinases, which are known to hydrolyze chitin, a major component of fungal cell walls. Sridevi and Mallaiah (2008) reported twelve *Rhizobium* strains isolated from root nodules of *Sesbania sesban* to be chitinase positive. They also found that *Rhizobium* sp. exhibited highest chitinase activity after 36 h of incubation, at neutral pH and showed antagonistic activity against *Aspergillus flavus, Aspergillus niger, Curvularia lunata, Fusarium oxysporum* and *Fusarium udum*. *Rhizobium meliloti* with chitinase producing gene, transferred from *Serratia marcescens* efficiently degrades hyphal tips of *Rhizoctonia solani* (Sitrit et al., 1993). Mazen et al. (2008) observed reduction in damping off disease in faba bean when seed treatment with cultural filtrates of chitinase producing *Rhizobium* spp. was used individually or in combination with AM fungi.

Kumar et al. (2011) reported two isolates of *Ensifer meliloti* and one of *Rhizobium leguminosarum*, which showed good chitinase and β-1, 3-glucanase activity respectively. All the three isolates inhibited growth of *F. oxysporum*, leading to loss of structural integrity of the mycelium, hyphal perforation, lysis, fragmentation and degradation. Smitha and Singh (2014) also observed suppression of growth and development of *Fusarium oxysporum, Macrophomina phaseolina; Sclerotinia sclerotiorum* mycelia by chitinase producing strain *Rhizobium* sp. RS12.
2.4.5 Induction of plant defense mechanisms

**Induced systemic resistance**

Induced systemic resistance has been reported as one of the mechanisms of disease suppression by fungal pathogens by many microorganisms (Mavrodi et al., 2001; Yu et al., 2002). Likewise, rhizobia are also reported to induce the plant to elicit its defense arsenal, when encountered with a fungal pathogen through the production of plant defense enzymes, phenolics, flavonoids or other phytoalexins. Phenolic compounds play a significant role in suppressing the growth and spread of pathogen within the plant. They may act as structural barriers, activators of plant defense genes and modulators of pathogenicity (Ramos et al., 1997). Pre inoculation of broad bean with *Rhizobium leguminosarum* bv. *viceae* led to an increase in total and free phenolics when challenged with *Botrytis fabae* (Rabie, 1998). Mishra et al. (2006) also observed that inoculation of *Rhizobium* to non-legumes, in particular rice led to induction of different phenolic compounds (particularly tannic, gallic, ferulic and cinnamic acids) and was associated with disease suppression of sheath blight caused by *Rhizoctonia solani*.

Induction and accumulation of phytoalexins in plant in response to *Rhizobium* sp. has been reported to protect plants from phytopathogens. Medicarpin and maackiain having profound antimicrobial activity are the major isoflavonoid phytoalexins found in chickpea (Weigand et al., 1986; Weidemann et al., 1991). Induction of isoflavonoid phytoalexins by *Rhizobium* spp. has also been correlated with disease control in alfalfa and common bean (Dakora, 2003). Chakraborty and Chakraborty (1989) reported that production of phytoalexins 4-hydroxy-2, 3, 9-trimethoxy pterocarpan by pea plants in response to *R. leguminosarum* bv. *Viciae* protected the plants from infection of *F. solani* f. sp. *pisi*. Phillips and Kapulnik (1995) reported that cyclic β-glucans produced by bradyrhizobia induced glyceollin, a phytoalexin that could control pathogens in soybean.

Enhanced expression of defense related genes encoding hydrolytic enzymes, anti-oxidant enzymes and other pathogenesis related (PR) proteins are important part of induced systemic resistance in plant. Defense-related enzymes like L-phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), isoflavone reductase (IFR) peroxidase (POX) and chalcone synthase (CHS) play significant roles in controlling pathogenic attack (Arfaoui et al., 2005, Dutta et al., 2008). Pretreatment with selected *Rhizobium* isolate PchDMS, prior to the exposure of

*Fusarium oxysporum*, led to increased levels of peroxidases, polyphenol oxidases, total phenolics and the constitutive isoflavonoids, formononetin and biochanin A in chickpea (Arfaoui et al., 2007). Osdaghi et al. (2011) also reported induction of resistance in common bean when bacterized with *Rhizobium leguminosarum* bv. *phaseoli* against common bacterial blight caused by *Xanthomonas axonopodis pv. phaseoli*. The induction of POX and PAL was also observed in peas inoculated with *R. leguminosarum* and parasitized by *Orobanche crenata* (Mabrouk et al., 2007).

In addition, the purified lipopolysaccharide (LPS) of *Rhizobium etli* was found to induce systemic resistance against nematodes. Reitz et al. (2002) showed that LPS from *R. etli* played a major role in the elicitation of ISR. Purified LPS was shown to induce systemic resistance, but *R. etli* mutants deficient in specific genes involved in production of LPS were deficient in their ability to induce systemic resistance.

### 2.5 Rhizobial Formulations

Once the best rhizobial strain with good nitrogen fixing ability and antifungal activity are identified, the next step is to prepare an appropriate formulation, which is an important determinant for the potential success of the inoculant. The main objective of inoculant formulation is that the selected strain must survive in high number and maintain its properties during long storage time until it is applied in the field. Commercial *Rhizobium* formulations used now-a-days are mainly of two types—solid (carrier based) and liquid. Solid inoculant is prepared by mixing broth culture, containing high rhizobial population with a suitable carrier material. A number of factors determine the choice of carrier material like cost effectiveness, availability, moisture absorbing and pH buffering capacity, survival of rhizobial cells on carrier material etc. (Keyser et al., 1993). A diverse array of soil materials (peat, charcoal, volcanic pumice, clays), organic materials (composts, plant by-products), or inert materials (perlite, vermiculite, bentonite, kaolin, silicates) are used as a carrier all over the world (Smith 1992, Brockwell and Bottomley, 1995; Stephens and Rask, 2000, Temprano et al., 2002, Malus et al., 2012). Liquid inoculants are mainly based on broth cultures, mineral or organic oils, or on oil-in-water suspensions (Albareda et al., 2008).

With the increased popularity of bioinoculants, new technologies have emerged focusing mainly towards cost effectiveness, better stability and longer shelf life. Rebah et al. (2007) reported the use of wastewater sludge with heavy metal
content below legal limits as growth medium and dehydrated sludge as carrier material which can reduce the cost of inoculant production. Polymer based carriers like carboxymethyl cellulose (CMC) with starch; alginate, Polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) are reported to improve stability and shelf life of bio-inoculants (Denardin and Freire, 2000, Ivanova et al., 2005, Júnior et al., 2009).

2.7 Biofilm based formulations

A biofilm is an assemblage of multiple microbial species associated with a surface or any interfaces, often enclosed in self-produced extracellular polymeric substances (EPS) matrix (Donlan, 2002) which provides enhanced survival ability to the species under adverse environmental conditions (Costerton, 1995). The term ‘biofilm’ was coined by the marine microbiologist Claude Zobell, who described the ‘bottle effect’, when referring to the declining levels of floating planktonic bacteria in sea water as they adhere to glass surfaces. Since then it has been well documented that biofilm associated microbes differ from their planktonic relatives in terms of growth and behaviour (Donlan, 2002). Biofilms can be formed on a number of surfaces, such as natural aquatic and soil environments, living tissues, medical devices or industrial piping systems, almost any niche where sufficient moisture and nutrients are available (Flemming and Wingender, 2001).

Biofilm formation helps its partners access to better nutrient availability by syntrophism, protection against environmental stresses (UV radiation, desiccation, acidity, alkalinity and osmotic shock), antimicrobial agents, and acquisition of new genetic traits (Stewart, 2002, Rafique, 2015). Microbial interactions within a soil microhabitat are dynamic processes which often determine the successful establishment of bioinoculants. There are several types of biofilms that can occur in soil. Bacterial, fungal, and fungal-bacterial biofilm are commonly found in diverse habitats, besides cyanobacteria based biofilms which are found widespread particularly in arid areas. The bacterial and fungal biofilms are formed on biotic surface in the soil. In fungal-bacterial biofilms, fungi act as the biotic surface to which the bacteria adhere. Formation of biofilms by bacterial colonization on biotic fungal surface gives the biofilm enhanced metabolic activities, when compared to monoculture (Seneviratne et al., 2008).

Biofilm comprising bacteria and fungi are known to enhance growth and the survival ability of bacterial inoculants in soil. Biofilm formation on fungal surface
may be beneficial in different ways like bacteria can exploit fungi as nutrient source directly or it can degrade complex substrates through production of extracellular enzymes. Biofilm formation on fungal cells enhances bacterial antagonism of fungi by concentrating bacterially derived antifungal compounds. Bacterial cells colonizing fungal hyphae may use it to reach and colonize new microhabitats in soil (Kohlmeier, 2005, Warmink and Elsas, 2009). Simultaneous colonization of bacteria and fungi may be the first step in more complex bacterial-fungal endosymbiont interactions which are critical in root rhizosphere. Biofilm colonizing plant root surface provide effective plant growth promotion as well as protection against several soil borne fungal pathogens (Seneviratne, 2003).

2.7.1 *Trichoderma* as biocontrol agent and its potential as biofilm partner

Members of the *Trichoderma* genus are known as imperfect fungi and they are commonly associated with root, soil and plant debris. Biocontrol potential of these fast growing fungi has long been recognized. In the last decade, it has emerged as the most popular biofungicide used for protecting crops against several plant pathogens worldwide. Biocontrol mechanisms mainly involve direct parasitism and production of antifungal compounds. Weindling (1932) have reported mycoparasitism and subsequent destruction of *Rhizoctonia solani* hyphae, causal organism of damping off disease, by *Trichoderma* sp. Several studies have revealed the potential of antibiotics production by members of genus *Trichoderma*. Howell and Stipanovic (1995) reported the production of gliovirin, an antibiotic by *Trichoderma virens* and also demonstrated its efficiency in inhibiting mycelial growth of *Pythium ultimum* and *Phytophthora* sp. Plant diseases caused by fungal pathogens of different genera like *Phytophthora*, *Fusarium*, *Pythium* and *Rhizoctonia* are reported to be suppressed by *Trichoderma* strains (Duffy et al., 1996; Manjula et al., 2004; Ganesan et al., 2007).

Recent studies have also revealed that certain strains of *Trichoderma* can induce plant defense mechanism and protect plants from several pathogens. Higher elicitation of defense related enzymes like peroxidase, chitinase and accumulation of pathogenesis related proteins have been reported (Howell, 2003; Sharma et al., 2011). They also reported disease suppression and enhancement in plant growth parameters when the seedlings were treated with *Trichoderma* sp.

Strains of *Trichoderma* exhibit biocontrol efficiency in crops like rice, wheat, cotton, tomato etc. (Gomathinayagam et al., 2010; Shanmugaiah et al., 2009;
Maketon et al., 2008; Roesti et al., 2006). Gomathinayagam et al. (2010) reported that the use of *Trichoderma viride* can reduce disease incidence of brown rot of rice both *in vitro* and *in vivo* conditions. Alice and Sundravadana (2012) also reported that both soil and foliar application of commercial formulation of *T. viride* protected *Gloriosa superba*, a medicinal plant from tuber rot disease caused by *Macrophomena phaseolina*. Enhancement in seed germination and growth parameters of cotton plants were observed on treatment with commercial formulations of *T. viride* and other beneficial microbes (Shanmugaiah et al., 2009).

A number of studies have suggested that biocontrol efficiency of *Trichoderma* can be increased by combining it with other potent biocontrol agents (Guo et al., 2004; Huang et al., 2005). Duffy et al. (1996) reported better biocontrol of take all disease of wheat when *Pseudomonas* strain was combined with *Trichoderma koningii*. Zaghloul et al. (2007) have observed better biocontrol of damping off and root rot disease in tomato when the plants were treated with *Bacillus subtilis* and *Trichoderma harzianum* compared to their individual application. Maketon et al. (2008) reported that two biocontrol agents *Bacillus subtilis* AP-01 and *Trichoderma harzianum* AP-001 were more effective in combination in suppressing three tobacco diseases caused by *Ralstonia solanacearum*, *Pythium aphanidermatum* and *Creospora nicotiana*.

Recently biofilm based formulations have been proposed for their utilization as efficient inoculants. Rhizobia can effectively form biofilm with common soil fungi (Seneviratne and Jayasinghearachchi, 2005). Biofilms may grow either on inert supports like carrier materials or in case of fungal-bacterial biofilm; a fungal matrix is used to entrap the bacterial cells. Biofilm formation provides greater stability and protection to rhizobial cells under biotic and abiotic stresses. Better root colonization provided by biofilms enhances the chance of successful establishment of inoculant in rhizosphere. There are several reports of improvement in plant growth parameters when biofilmed inoculants are used compared to their traditional counterpart. The increased cell density within a biofilm provides the chance to exhibit attributes that single cells cannot achieve efficiently (Seneviratne et al., 2008, Jayasinghearachchi and Seneviratne, 2004, Triveni et al., 2013; Prasanna et al., 2014).

Seneviratne et al. (2003) reported that formation and subsequent application of bacterial-fungal biofilm using nitrogen-fixing bacteria and P-solubilizing fungi may enhance plant growth in leguminous as well as non-leguminous crops. The
biofilms exhibited higher biological nitrogen-fixation and organic acid production. Jayasinghearachchi and Seneviratne, (2004) developed fungal-bacterial biofilm using *Bradyrhizobium* under *in vitro* condition and studied its effect on soybean crop. They recorded significant increase in nitrogen-fixing efficiency along with increase in several plant growth parameters.

Enhancement in attributes like seed germination percentage, antifungal activity, production of phytoregulators like IAA and GA and phosphate solubilisation in biofilm formulation as compared to their individual partners, have been reported by several researchers (Jayasinghearachchi and Seneviratne, 2004; Roychowdhury, 2012; Qurashi and Sabri, 2012; Triveni et al., 2013). Bandara et al. (2006) observed improvement in organic acid and IAA production in soil when biofilm formulations were used. These acids were useful in suppressing the plant pathogens, which emphasized that the fungal-bacterial biofilm in endophytic environment plays a significant role in healthy growth and improvement of plants. Triveni et al. (2013) developed three novel *Trichoderma* based biofilms through synergistic interactions with different agriculturally important bacteria (*A. chroococcum, B. subtilis and P. fluorescens*) as partners. These biofilms showed enhancement in different antifungal and PGP attributes as compared to their individual partners and dual cultures, and higher inhibition of growth of fungal mycelium. Increased production of ammonia, IAA and siderophores was found in case of *Trichoderma–Bacillus* and *Trichoderma–Pseudomonas* biofilms, whereas *Trichoderma–Azotobacter* biofilm exhibited highest nitrogen fixation efficiency and 1-aminocyclopropane-1-carboxylic (ACC) deaminase activity.

Seneviratne et al. (2008) reported that as biofilms, rhizobia survive at higher salinity and tannin concentration as compared to rhizobial monoculture. They also observed a significant increase in nitrogen fixation, organic acid production and IAA production. Burmolle et al. (2006) also revealed that, in multispecies biofilms, the synergistic interactions among partners leads to enhancement in biofilm formation besides increased resistance to antimicrobial agents.

Biofilm based formulations, using *Trichoderma* or a cyanobacterium *Anabaena* as their matrix and their combination with different rhizobia and other agriculturally beneficial microorganisms have shown immense potential as PGP and biocontrol agents in several crops, including rice, cotton, soybean, chickpea and lentil (Babu et al., 2015; Bidyarani et al., 2016; Prasanna et al., 2014, 2015; Triveni
et al., 2015). Prasanna et al. (2014) reported that the biofilmed formulations of cyanobacterium *Anabaena torulosa* with *Mesorhizobium ciceri* exhibit higher PGP traits compared to individual bacterial and cyanobacterial partners. They were also able to utilize new saccharides as compared to individual cultures.

Biofilm formation is quite common in natural environments which provide its component microbial cells a certain degree of protection and helps in their growth, survival and successful colonization of root rhizosphere (Davey and O’Toole, 2000). Hence, more investigation is warranted to develop *Rhizobium* based biofilmed formulations and evaluate their potential as inoculants, having dual purpose of nitrogen fixation and antifungal attributes.

### 2.7 Future prospects and Challenges

Multifaceted beneficial microorganisms are becoming the most sought after inoculants in global agriculture. Rhizobia, with their nitrogen-fixing and plant growth promoting ability are the most suitable candidates for genetic modification to express antibiotic producing genes from other microbes. Krishnan et al. (2007) have observed that *Rhizobium etli* USDA9032 could be engineered to produce phenazine and exhibit antifungal activity, but the nodulation properties were poor. More research is needed in this area to generate more effective nodulating and disease suppressive strains of rhizobia. Rhizosphere engineering is another area, through which plants are genetically modified to release compounds that encourage the proliferation of beneficial microorganisms (Ryan et al., 2009). Combined application of both strategies transgenic plant and rhizobia may increase root colonization and protection against pathogens and improve plant growth and yield. Another aspect of significance is the need to improve the rhizosphere competence and survival of inoculated rhizobia, and an understanding of their ecophysiology. This will help in developing a robust technology for use by farmers.

Availability of good quality carrier material and improved formulations are equally important for success of inoculants under field conditions. New formulation technologies such as:

- Polymer based formulations, which have shown good storage life (Tittabutr et al., 2007).
- Water-in-oil emulsion technology for developing liquid formulations is beneficial for those organisms which are particularly sensitive to desiccation (Vandergheynst et al., 2007).
✓ Biofilm based formulations, both single and fungal-bacterial have shown good potential as the biofilm formation naturally protects organisms and helps in its survival and growth under adverse soil conditions (Jayasinghearachchi and Seneviratne, 2004).

✓ Application of nanotechnology may open a new class of bioinoculants. Entrapment of bacterial cells in nanostructures leads to improved stability and high surface area. Nanoformulations may contribute to enhanced stability of biofertilizers with respect to desiccation, temperature, and UV inactivation (Khot et al., 2012, Malus et al., 2012).

Further research to address the biosafety issues related to transgenic use and nanoformulations are essential.

There is a definite need for concerted efforts towards improving our understanding of the symbiotic association for more effective use in marginal lands or polluted habitats. The role of other beneficial/PGP microbes as co-inoculants or as elicitors is a challenging area of research in which very less information has been generated.
3. Materials and Methods

The microbiological investigations and biochemical analysis were carried out in the Division of Microbiology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi. The pot experiments were conducted in National Phytotron Facility, IARI, New Delhi and Division of Microbiology, IARI. The field experiments were conducted in the fields of ICAR-IARI, New Delhi.

3.1 Isolation of rhizobium from root nodules of chickpea plants

Healthy 30-40 days old chickpea plants were collected from the fields of ICAR-Indian Agricultural Research Institute, New Delhi and Malda, West Bengal. The root system was washed in tap water to remove the adhering rhizospheric soil. Healthy pink nodules on the tap root were selected. They were separated from root by giving diagonal incisions from two sides of nodules to cut the nodules along with some root portion. Nodules were washed with running water and surface sterilized with 0.1% mercuric chloride and 70% ethyl alcohol for 3 min. and 30 sec. respectively. They were thoroughly washed with sterile water for six times. The nodules were placed on sterile porcelain cavity plate containing drop of sterile water, crushed with a sterile glass rod and the contents were streaked on plates containing yeast extract mannitol agar (YEMA) with Congo red dye (Vincent, 1970). Plates were incubated at 28°C till small, round colonies, colourless or white with central red dot and entire margin develops. Twenty rhizobial isolates were procured from HAU, Hisar.

Congo Red Yeast Extract Mannitol Agar (CRYEMA) (Subba Rao, 1977)

<table>
<thead>
<tr>
<th>Ingredients</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
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<td>Yeast Extract</td>
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</tr>
<tr>
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<td>Sodium chloride</td>
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<tr>
<td>Agar</td>
<td>18.0</td>
</tr>
<tr>
<td>Congo Red dye</td>
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</tr>
<tr>
<td>Distilled water</td>
<td>1000 mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>
All the media used were autoclaved at 15 lb psi (1.06 kg/cm$^2$) pressure for 20 minutes. All the glasswares used were sterilized in hot air oven at 180ºC for 2 hours.

### 3.2 Purification and maintenance of rhizobial isolates

Rhizobial isolates were purified through repeated sub-culturing on plates containing yeast extract mannitol agar with Congo red. Purified cultures were maintained on yeast extract manitol agar slants containing 0.1% calcium carbonate and stored at 4ºC. Glycerol stocks were also prepared and stored at -20ºC, for further use.

### 3.3 Screening of rhizobial isolates against soil borne fungal pathogens of chickpea

Hundred and fifty rhizobial isolates (100 from IARI, 30 from Malda and 20 from Hisar) (Fig. 3.1) specific to chickpea were screened based on their antagonistic behavior against *Fusarium oxysporum* f. sp. *ciceri* (AFC-1), *Ascochyta rabiei* (AR4638), *Botrytis cinerea* (BC6530), *Sclerotium rolfsii* (CR5518) and *Macrophomina phaseolina* (MP). Fungal strains were collected from the Indian Type Culture Collection, New Delhi. The growth inhibition of fungal mycelium by the rhizobial isolates were tested *in vitro* using the dual culture technique as described by Landa et al. (1997). All the isolates were grown in yeast extract mannitol broth in an incubator shaker (120 rpm) at 28ºC for 48 h. An aliquot of 15μl of rhizobia suspension ($10^9$ CFU/mL) were equidistantly placed on the margin of potato dextrose agar (PDA) plates. A 5mm disc cut from the edge of 7 day old fungal culture was placed at the center of the PDA plate and incubated at 28ºC for 7 days. PDA plate containing only fungal disc served as control. The radius of the fungal colony towards the bacterial colony was measured. Percent growth inhibition was calculated using the formula of Whipps (1987).

$$\text{% inhibition} = \frac{(R-r)}{R} \times 100$$

Where R is the maximum radius of the fungal colony in control plate and r is the radius of fungal colony opposite the bacterial colony.

### 3.4 Nodulation test

The identity of ten isolates that exhibited antagonistic activity was confirmed as *Rhizobium* specific to chickpea by nodulation test carried out in pot experiment. Each strain was grown in yeast extract mannitol broth to a logarithmic phase ($10^9$ CFU/mL). Chickpea seeds (variety JG-62) were surface sterilized with 0.1% mercuric chloride and 70% ethyl alcohol for 3 min. and 30 sec. respectively and
thoroughly washed with sterile water for six times. Seeds were then pre-germinated for 48 h on petriplates containing 1% water agar. Pre-germinated seeds were sown in 8 inch diameter plastic pots (3 seeds per pot) containing sterilized sand and vermiculite in 1:1 ratio and inoculated with 1mL of culture per seed at sowing time. Potting mixture was autoclaved at 15 lb psi (1.06 kg/cm$^2$) pressure and 121°C temperature for 1 h on three consecutive days. The experiment was statistically laid out with three replications for each treatment in National Phytotron facility, IARI, New Delhi. Pots with uninoculated seeds served as negative control and with standard *Rhizobium* culture (F75), served as positive control. Plants were watered as and when needed and fertilized with an N-free seedling nutrient solution weekly. They were cultivated in day/night temperature of 22-24/18°C and humidity of 60%. The plants were harvested after 45 days and observed for the presence or absence of nodules.

3.5 Characterization of Antagonistic *Mesorhizobium* isolates for antifungal and plant growth promoting attributes

3.5.1 Production of Hydrogen cyanide (HCN)

HCN production was detected qualitatively by the method of Bakker and Schippers (1987). Log phase cultures ($10^9$ CFU/mL) were streaked individually on plates containing King’s B medium amended with 4.4 g/L glycine. A filter paper (Whatman No.1) soaked in 0.5% picric acid in 2% (w/v) sodium carbonate was placed on the lid of the petri plate. Plates were sealed with parafilm and incubated at 28°C for 72 h. Colour change of the filter paper from yellow to light brown or reddish brown indicated HCN production.

3.5.2 Production of Ammonia

Ammonia production by the cultures was estimated by the method, described by Dye (1962). Cultures were grown in peptone water for 72 h at 28°C on a rotary shaker (120 rpm). After incubation 1 mL of Nessler’s reagent was added to each tube. Development of faint yellow colour indicates small amount of ammonia and deep yellow to brownish colour indicates higher production of ammonia.

3.5.3 Production of Siderophore

Detection of siderophore production by the rhizobial isolates were done by Chrome Azurol Assay (CAS) as described by Schwyn and Neilands (1987). Log phase cultures ($10^9$ CFU/mL) were inoculated on the plates and incubated at 28°C for 5
days. According to Schwyn and Neilands (1987), the development of yellow halo around the bacterial colony is a positive indication of siderophore production. Quantitative assay for siderophore production was done by the method, as described by Triveni et al. (2013).

3.5.4 ACC Deaminase activity

ACC deaminase activity was evaluated qualitatively by the plate assay method as described by Penrose and Glick (2003). The cultures were spotted on plates containing MDF salts minimal medium supplemented with 0.03% ACC. Medium containing ammonium sulphate was used as positive control and medium without any nitrogen source was used as negative control. The experiment was done in triplicate and observations were taken after incubation of 5 days at 28°C.

ACC deaminase enzyme activity was assayed quantitatively by the method of Honma and Shimomura (1978). Enzyme activity is measured as the amount of α-ketobutyrate produced when the enzyme ACC deaminase cleaves ACC (1-aminocyclopropane-1-carboxylate). Cultures were grown in YEM broth at 28°C for 48 h on a rotary shaker (120 rpm). Log phase cultures were centrifuged at 8000 rpm for 10 min and the pellets were washed with 0.1 M Tris-HCl buffer (pH 7.6). Cells were resuspended in 2 mL of M9 minimal medium containing ACC (3.0 mmol/L) as sole source of nitrogen and incubated overnight. After incubation, the cells were collected through centrifugation and washed with 0.1 M Tris-HCl buffer (pH 8.5) for three times and resuspended in 1 mL of the same buffer. Thirty microliter of toluene was added to the cell suspension and mixed thoroughly. Hundred microliter aliquots of these cells were stored at 4°C for protein estimation, while 250 μL was used for ACC deaminase assay. For estimation of ACC deaminase activity, 20 μL of 0.5 M ACC was added to 250 μL of cell suspension, mixed thoroughly and incubated at 30°C for 15 min. After incubation, 1 mL of 0.56 M HCl was added, mixed and centrifuged at 800 rpm for 15 min at 30°C. Then, 800 μL of 0.56 M HCl was added to 1 mL of supernatant and mixed thoroughly. The tubes were incubated at 30°C for 30 min after adding 300 μL of diphenyl hydrazine. Finally, 2 mL of 2N NaOH was added and absorbance was measured at 540 nm.

3.5.5 IAA production

For estimation of IAA production by the rhizobial isolates, nutrient broth amended with tryptophan (50 μg/mL broth) was used. Sterile broth was inoculated with 100 μL of log phase culture suspension (10⁹ CFU/mL) and incubated at 28°C for 72 h on a rotary shaker at 120 rpm. Experiment was designed in triplicate with
appropriate uninoculated control. The amount of IAA produced was estimated by the method as described by Hartmann et al. (1983). Intensity of the pink colour developed was measured at 530 nm using Perkin Elmer spectrophotometer, model Lambda E2201. IAA production was calculated from the standard curve and expressed as μg/mL IAA produced after 72 h.

3.5.6 Phosphate solubilisation

Phosphate solubilisation activity was tested qualitatively on plates containing Pikovskaya’s medium. Distinct clearing zone around the colonies indicated positive result. For quantitative estimation of phosphate solubilisation, Pikovskaya’s broth was inoculated with cultures/biofilms and incubated on a rotary shaker (120 rpm) at 28ºC for 5 days. Experiment was set up with three replicates for each treatment and appropriate uninoculated control. After incubation, broth cultures were centrifuged at 5000 rpm for 10 min. Phosphate solubilisation was estimated by the method as described by King (1932), modified by Jackson (1967). For this, 1 mL of the supernatant was taken in a 50 mL volumetric flask. After addition of 10 mL of chloromolybdic acid the volume was made up to 45 mL. After addition of 0.5 mL of stannous chloride the volume was made up to 50 mL. Intensity of the blue colour developed was measured at 660 nm. The quantity of phosphate solubilized was expressed as μg/mL P solubilized.

3.6 Screening of selected rhizobial isolates for nodulation and nitrogen fixing efficiency

3.6.1 Pot experiment

A pot experiment was conducted at National Phytotron Facility, ICAR-IARI, New Delhi to assess the infectivity and effectiveness in atmospheric nitrogen fixation of the selected rhizobial isolates. The experiment details are same as described in the section 3.4.

3.5.2 Plant growth parameters studied

Nodule number, fresh weight of root and shoot, fresh weight of nodule were recorded 45 days after planting. The intact root system with nodules was used for the measurement of nitrogenase activity. The nodules were then detached and dry weight of nodules recorded after oven drying at 60ºC for three days.

3.5.3 Acetylene Reduction Assay (ARA) to estimate nitrogenase activity

ARA was done to estimate the nitrogen fixation efficiency of the isolates. Roots were taken in 50 mL ARA tubes. The tubes were sealed with gas tight stoppers
(Subba seal). 4 mL of gas phase (10% of the remaining volume in tube) was removed and 4 mL of acetylene (C₂H₂) was injected into the tubes with a hypodermic syringe. After 60 min. of incubation 1 mL of gas mixture was removed with a gas tight syringe and injected into preconditioned NUCON 5765 Gas Chromatograph, housing a two meter long (2mm i.d.) Porapak N column and Flame Ionization Detector (FID). The column temperature was maintained at 80ºC and injector and detector at 100ºC. A flow rate of 30 mL/min of N₂ served as carrier gas. Standard ethylene gas (commercially available) was used for auto-calibration using the software (Company provided) and the amount of ethylene evolved was determined. The nitrogenase activity was expressed in terms of μmole of ethylene produced per gram dry weight of nodules per hour. The equation used to calculate ethylene produced was as follows:

\[
\text{μmole of C}_2\text{H}_4/\text{gram dry weight of nodules/h} = \frac{C \times P_S \times A_S \times V}{P_{\text{STD}} \times A_{\text{STD}} \times T \times P}
\]

Where,

- \(C\) = Concentration of Standard ethylene in μmoles;
- \(P_S\) = Peak area of the sample;
- \(A_S\) = attenuation used for the sample;
- \(V\) = Volume of air space in the test tube in mL;
- \(P_{\text{STD}}\) = Peak area of standard ethylene;
- \(A_{\text{STD}}\) = Attenuation used for standard ethylene;
- \(T\) = Incubation time in hour;
- \(P\) = Dry weight of nodule in g.

### 3.6 Field evaluation of selected rhizobial isolates

A field trial was conducted at the experimental farms of ICAR-IARI, New Delhi. Each rhizobial culture was grown in yeast extract mannitol broth to a logarithmic phase (10⁹ CFU/mL). The inoculants were used to inoculate chickpea seeds (variety JG-62). The experiment was statistically designed with three replicates for each treatment. Plots with uninoculated seeds served as negative control and seeds treated with standard *Rhizobium* strain F75 served as positive control. Fresh weight of shoot and root, Nodule number, fresh weight of nodules were recorded 45 days after planting. Dry weight of nodules was recorded oven drying at 60ºc for three days. Total seed yield from each plot was recorded.

Two strains (Isolate A13 and CR24) were selected based on their anti-fungal activity, nodulation and nitrogen fixing efficiency as evaluated under pot and field conditions.
3.7 Development of *Rhizobium* based biofilmed formulations using *Trichoderma* as fungal matrix

3.7.1 Growth and maintenance of cultures for biofilm development

Two strains of *Mesorhizobium ciceri* A13 and CR24 specific to chickpea were grown in Yeast Extract Mannitol (YEM) broth and incubated in shaking incubator (120 rpm) at 28°C for 48 h. *Trichoderma viride* (ITCC 2211) was procured from Indian Type Culture Collection (ITCC), Plant Pathology Division, ICAR-IARI, New Delhi, India and grown on Potato Dextrose Agar (PDA) plates at 30°C for 7 days.

3.7.2 Compatibility test of *Mesorhizobium* isolates with *Trichoderma* sp.

*Mesorhizobium ciceri* A13 and CR24 and *T. viride* were grown as dual culture on petri plates containing PDA medium. An aliquot of 15μL of log phase *Rhizobium* suspension (10^9 CFU/mL) were spotted on the margin of PDA plates. A 5mm disc cut from the edge of 7 day old *T. viride* culture plate was placed at the center of the PDA plate and incubated at 28°C for 7 days.

3.7.3 Optimization of medium for biofilm development

Six different media- Nutrient broth (NB), Pikovskaya medium (Piko), Yeast extract mannitol broth (YEM), Yeast extract peptone glucose broth (YEPG) Jensen’s medium (JEN) and Jensen’s medium with 1% yeast extract were used to optimize the growth of biofilm. Erlenmeyer flasks (1000 mL) containing 300 mL broth were autoclaved and after cooling inoculated with 2 mL of rhizobial culture (*Rhizobium* strain A13) (10^9 CFU/mL). Experiment was set up in triplicate and flasks were incubated on rotary shaker (120 rpm) at 28°C for 48 h. After incubation, flasks were inoculated with 2 mL of *T. viride* spore suspension (2.2×10^7 spores/mL) and incubated under static condition at 30°C for two weeks. Biofilms were harvested, washed three times with sterile distilled water to remove non adherent cells and centrifuged at 2000 rpm for 5 min. Biofilm fresh weight and dry weight (after oven drying at 70°C for 24 h) were recorded. To get a uniform suspension, biofilms were homogenized by vortexing with glass beads for 10 min. Population counts of each biofilm partner, *Rhizobium* and *T. viride* were recorded on YEMA plates (containing 100 μg/mL cycloheximide) and PDA plates (containing 100 μg/mL streptomycin) respectively.
Nutrient Broth (Rangaswami, 1966)

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<td>Peptone</td>
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<td>Distilled water</td>
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</tr>
<tr>
<td>pH</td>
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</tbody>
</table>

Pikovskaya medium (Pikovskaya, 1948)

<table>
<thead>
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</thead>
<tbody>
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<td>Potassium chloride</td>
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<tr>
<td>Magnesium sulphate</td>
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<tr>
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<tr>
<td>Manganese sulphate</td>
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<tr>
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</table>

Yeast Extract Peptone Dextrose broth (YEPD) (Ausubel et al., 1994)

<table>
<thead>
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<tbody>
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<td>Dextrose</td>
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<tr>
<td>pH</td>
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Jensen’s medium (Jensen, 1954)

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</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>Dipotassium phosphate</td>
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</tr>
<tr>
<td>Magnesium sulphate</td>
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</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Ferrous sulphate  - 0.1  
Sodium molybdate  - 0.005  
Calcium carbonate  - 2.0  
Distilled water  - 1000 mL  
pH  - 7.0  

3.7.4 Development of biofilmed formulations

The mass production of biofilm using *Rhizobium* as partner and *Trichoderma* as matrix was carried out following the procedure described in section 3.7.3 and microscopic observations were recorded on the 14th day of incubation.

3.7.4.1 Visual and Microscopic observations

Different stages of biofilm development were observed at 2 days intervals by visual and microscopic observations (Zeiss Model Axio Scope A1 microscope). After removal of non-adherent cells through repeated washing a thin smear of biofilm was prepared on the slide and air dried. It was stained with Lacto phenol cotton blue for 1 min and after washing counter stained with safranine for 1 min. After the extra stain was washed away slowly, the smear was covered with a coverslip and observed under microscope using 10X, 40X and oil immersion objectives. The observations were made on adherence of *Rhizobium* cells to fungal mycelium and conidiophore and also on the integrity of the biofilm.

3.7.4.2 Population counts of the associated partners

Biofilms were homogenized and the uniform suspension was used for population count using appropriate media. For *Rhizobium* count, the suspension was spread plated on CRYEMA media amended with cycloheximide (100 μg/mL). Likewise, for colony count of *Trichoderma*, the suspension was spread plated on PDA medium amended with streptomycin (100 μg/mL).

3.8 Determination of antifungal and plant growth promoting attributes of the biofilms developed

For biochemical tests, single cultures (10⁹ CFU/mL for *Mesorhizobium ciceri* and 10⁷ CFU/mL for *T. viride*) were compared with the biofilms developed.
3.8.1 Seed germination assay

Chick pea seeds (variety JG-62) were surface sterilized by treating with 0.1% mercuric chloride for 3 min followed by 70% ethanol for 1 min and washed repeatedly with sterile distilled water. This seeds were soaked in different cultures/biofilm for 10 min and placed on 1% water agar plates (10 seeds per plate, 3 replications per treatment) for 3 days. Seeds soaked in sterile distilled water served as control.

3.8.2 In vitro antifungal activity

Cultures/biofilms were tested for their antifungal activity in vitro against *Fusarium oxysporum* f. sp. *ciceri* (AFC-1) using the dual culture technique (Landa et al., 1997), as described in section 3.3 above.

3.8.3 HCN production

HCN was estimated qualitatively by the method of Bakker and Schippers (1987) as described in section 3.4.1

3.8.4 Ammonia production

Cultures/biofilms were tested for their ability to produce ammonia by the method of Dye (1962) as described in section 3.4.2.

3.8.5 IAA production

Biofilms and their individual partners were tested for their IAA production by the method of Hartmann et al. (1983) as described in section 3.4.5.

3.8.6 Phosphate solubilisation

Phosphate solubilisation ability of the biofilms and its individual partners were estimated qualitatively and quantitatively by the method described in section 3.4.6 above.

3.9 Evaluation of biocontrol potential of cultures/biofilms against *Fusarium* wilt under greenhouse condition

3.9.1 Mass multiplication and preparation of fungal inoculum

The phytopathogenic fungus wilt complex (*Fusarium oxysporum* f. sp. *ciceri*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*) was multiplied using sorghum seed
(Sorghum bicolor L. Moench). The inoculum of the fungus was produced on sorghum seeds moistened with water (1:1), which had been autoclaved twice for 90 min. on two consecutive days (Paulitz and Schroeder, 2005). Flasks containing sorghum seeds were inoculated with each fungus (one week old fungal culture grown on PDA medium) separately incubated at room temperature for 15 days and stirred once a week. After incubation, colonized sorghum seeds were collected and mixed in Fusarium oxysporum f. sp. ciceri: Rhizoctonia solani: Sclerotinia sclerotiorum in 1: 1: 1 ratio to produce the fungal inoculum for wilt complex. This fungal inoculum was used at a rate of 5 g/kg of soil to challenge the chickpea seedlings with wilt disease.

3.9.2 Experimental set up

A pot experiment was designed to evaluate the biocontrol potential of the cultures/biofilms against Fusarium sp. (wilt pathogen) challenged chick pea plants in the National Phytotron Facility, with day/night temperature of 22-24/18°C and humidity of 60%. Chickpea seeds (variety JG-62) were surface sterilized and pre-germinated for two days in petridishes containing 1% water agar. The pots were autoclaved at 15 lb psi (1.06 kg/cm²) pressure and 121°C temperature for 1 h on three consecutive days, and then inoculated with Fusarium sp., at the rate of 5 g/kg soil containing 5×10³ spores/kg soil. Basal dose of only phosphate fertilizer (30 kg P₂O₅/ha) in the form of chemical fertilizer single super phosphate (83.7 mg/kg soil) were applied. The pre-germinated seeds were then transplanted into plastic pots (8 inch diameter, 3 seeds per pot) containing Fusarium-inoculated soil mixture (positive control). Seeds were inoculated with 1 mL of either M. ciceri culture (10⁸ CFU/mL) or M. ciceri based biofilm (containing 10⁷-10⁸ CFU/mL of M. ciceri and 10⁶-10⁷ CFU/mL for T. viride) per seedling at sowing time. Seeds treated with Carbendazim (1.5g/kg seeds) were used as chemical control and seeds treated with commercial formulation of Trichoderma viride were used to compare biocontrol potential of the biofilm formulation. Pots with uninoculated seeds and pots without fungal inoculum served as negative controls.

Details of the treatments:
1. Rhizobium isolate A13
2. Rhizobium biofilm 1 (T. viride-A13)
3. Rhizobium isolate CR24
4. Rhizobium biofilm 2 (T. viride-CR24)
The experiment was set up with three replications for each treatment for a period of 8 weeks. Plants were watered as needed and fertilized with 100 mL of nitrogen free seedling nutrient solution weekly.

3.9.3 Measurement of plant parameters

Disease intensity index (DII) was calculated as described by Cachinero et al. (2002). Severity of symptoms on individual plants was rated on a scale from 0–4, according to the percentage of foliage with yellowing or necrosis in acropetal progression: 0 = 0%, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, 4 = dead plant. Incidence and severity data (0–4 scale) within a pot were used to calculate a disease intensity index (DII) by the equation:

\[
\text{DII} = \left( \frac{\sum S_i \times N_i}{4 \times N_t} \right) \times 100
\]

Where; \( S_i = \) symptom severity; \( N_i = \) number of plants with \( S_i \) symptom severity; and \( N_t = \) total number of plants (Hervás et al., 1997).

Plant growth parameters like shoot and root length; shoot and root dry weight as well as nodule dry weight were recorded. Acetylene reduction assay to determine nitrogenase activity of the nodules was performed by the method of Hardy et al. (1973) as described in section 3.5.2.

3.10. Determination of defense-related enzyme activity in host plant

Elicitation and accumulation of defense related enzymes like L-phenylalanine ammonia lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO) in host plant was studied as influenced by seed treatment with *Mesorhizobium ciceri* and its biofilms under pathogen challenge by *Fusarium* wilt. Experiment was done in CRD with seven treatments and three replications. Enzyme assay was done at 7 days interval up to 28 days after sowing (DAS).

3.10.1 L-phenylalanine ammonia lyase (PAL) (EC 4.3.1.5) Activity

Three gram of fresh leaf sample was ground in a pre-cooled (4°C) mortar and pestle containing 9 mL of sodium borate buffer (pH 8.7) mixed with 2-mercaptoethanol (0.8 mL/L of buffer). The extract was then centrifuged at 10,000 rpm for 10 min and the supernatant was used as enzyme extract for the assay.
Phenylalanine ammonia lyase activity was determined spectrophotometrically by following the formation of trans-cinnamic acid which exhibits an increase in absorbance at 290nm. PAL activity was determined according to Sadasivam and Manickam (1991). The reaction mixture contained 0.5 ml borate buffer, 0.2 mL enzyme extract and 1.3 mL of deionized water. Reaction was initiated by adding 1 mL of 0.1 M L-Phenylalanine solution. The test tubes were incubated at 30ºC for 30 min. After incubation, the reaction was stopped by adding 0.5 mL of 1 M trichloroacetic acid and absorbance was measured spectrophotometrically (Perkin Elmer spectrophotometer, model Lambda E2201) at 290 nm against an appropriate blank. The standard curve was drawn with graded amounts of cinnamic acid as described above. Enzyme activity was expressed as μmole trans-cinnamic acid formed per min per gram fresh weight.

3.10.2 Peroxidase (POX) (EC 1.11.1.7) Activity

Three gram of fresh leaf sample was ground in a pre-cooled (4ºC) mortar and pestle containing 9 mL of 0.1M phosphate buffer (pH 7.0). The content was centrifuged at 10,000 rpm for 10 min and the supernatant was used for the enzyme assay. The enzyme activity was assayed using o-dianisidine as hydrogen donor and H₂O₂ as electron acceptor. The rate of formation of yellow coloured dianisidine dehydrogenation product is proportional to peroxidase activity and was measured spectrophotometrically at 430 nm (Thimmaiah, 1999). The reaction mixture contained freshly prepared 1 mL of 0.01 M o-dianisidine in methanol, 0.5 mL of 0.02 M H₂O₂ and 1 mL of 0.1 M phosphate buffer (pH 7.0). In blank, all were included except H₂O₂. The reaction was started by adding 0.2 mL of enzyme extract. The test tubes were incubated at 30ºC for 5min. Following incubation, the reaction was stopped by adding 1mL of 2N H₂SO₄. Absorbance of the yellow colour developed was measured spectrophotometrically (Perkin Elmer spectrophotometer, model Lambda E2201) at 430nm. Specific activity of the enzyme was expressed as units per min per gram of fresh weight of sample considering one unit of enzyme as an increase in OD by 1.0 under standard conditions.

3.10.3 Polyphenol oxidase (PPO) (EC 1.14.18.1) Activity

For this, 3g of fresh leaf sample was ground in a pre-cooled (4ºC) mortar and pestle containing 6 mL of 0.1M sodium phosphate buffer (pH 6.5). The content was centrifuged at 10,000 rpm for 10 min and the supernatant was used for the
enzyme assay. PPO activity was estimated as described by Sadasivam and Manickam (1991). The reaction mixture contained 2.5 mL of 0.1 M phosphate buffer (pH 6.5), 0.5mL of 0.01 M catechol solution in a cuvette. The reaction was started by adding 0.2 mL of enzyme extract. Change in absorbance was recorded spectrophotometrically (Perkin Elmer spectrophotometer, model Lambda E2201) at 495 nm for every 30 second up to 5 min. Enzyme activity was expressed as units per min per gram fresh weight.

3.10.4 Chitinase (EC 3.2.1.14) Activity

Three gram of fresh leaf sample was ground in a pre-cooled (4ºC) mortar and pestle containing 9 mL of 0.1M sodium citrate buffer (pH 5.0). The content was centrifuged at 10,000 rpm for 10 min at 10ºC and the supernatant was used for the enzyme assay. The enzyme is assayed by hydrolysis of chitin as the substrate and the reaction product N-acetylglucosamine (NAG) is determined colorimetrically (Reissig et al. 1955). The reaction mixture contained 1 mL of enzyme extract, 4 mL chitin (Sigma) suspension and 3 mg/mL BSA. It was incubated in water bath at 37ºC for 3h. After incubation 1 mL of reaction mixture was mixed with 1 mL of water and boiled for 10 min. The contents were then centrifuged at 10000 rpm for 10 min. The amount of NAG present in 0.5 mL of aliquot of supernatant was estimated by adding 0.1 mL of K$_2$B$_4$O$_7$ and boiling for exactly 3 min in a water bath. After cooling, 3 mL of p-dimethylaminobenzaldehyde (DMAB) was added and mixed thoroughly. Absorbance of the solution was measured spectrophotometrically at 585 nm against an appropriate blank. Standard curve was prepared similarly using different concentrations of NAG. One unit of enzyme activity is expressed as the amount of enzyme which produces 100 μg NAG/mL chitin solution in 1h at 37ºC at pH 5.2.

3.10.5 β 1,3-glucanase (EC 3.2.1.4) Activity

Three gram of fresh leaf sample was ground in a pre-cooled (4ºC) mortar and pestle containing 9 mL of 25 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and 1 mM phenylmethylsulfonyl fluoride and 10% (w/w) polyvinyl polypyrrolidone. The content was centrifuged at 10,000 rpm for 10 min at 10ºC and the supernatant was used for the enzyme assay after dialyzing overnight against same buffer without NaCl. The β 1,3-glucanase activity was measured spectrophotometrically using laminarin-dinitrosalicylic acid method (Ohtakara, 1988). The reaction mixture contained 500 μL of enzyme extract and 125 μL of laminarin (Sigma) substrate.
After addition of 3 mL of dinitrosalicylic acid the test tubes were incubated in boiling water bath for 15 min. Absorbance was measured spectrophotometrically (Perkin Elmer spectrophotometer, model Lambda E2201) at 530 nm. The enzyme activity was expressed as microgram of glucose released per minute per gram fresh weight of sample.

3.11 Profiling of phenolics, flavonoids and organic acids in root and shoot extracts of *Rhizobium* or biofilms inoculated plant with or without the challenge of *Fusarium oxysporum* f. sp. *ciceri*.

An experiment was designed in hydroponics to evaluate the plant-microbial interaction with/without fungal challenge, as influenced by inoculation with promising rhizobial formulations in the National Phytotron Facility with day/night temperature of 22-24/18 °C and humidity of 60%. Chickpea seeds (variety JG-62) were surface sterilized and pre-germinated for two days in petri dishes containing 1% water agar. Pre-germinated seeds were soaked in 1 mL (per10 seeds) of either *M. ciceri* culture (10^8 CFU/mL) or *M. ciceri* based biofilm (containing 10^7-10^8 CFU/mL of *M. ciceri* and 10^6-10^7 CFU/mL for *T. viride*) for 10 min. Seeds treated with Carbendazim (1.5 g/kg seeds) were used as chemical control and seeds treated with commercial formulation of *Trichoderma viride* were used to compare biocontrol potential of the biofilmed formulation. Treated seeds were then transferred to petri dish containing 1mL of conidial suspension in sterile water (10^7 spores/mL) for 10 min. Finally each seed was transplanted into the cut Eppendorf tubes. The eppendorf tubes were transferred to a thermocol support which was further assembled into the hydroponics set up (Fig. 3.2, 3.3). Seeds treated with only fungal conidia acted as positive control, whereas seeds without any cultural or fungal inoculum acted as negative control. Plants were grown on half strength Hoagland’s nutrient solution (Hoagland and Arnon, 1950).The experiment was statistically designed with three replications per treatment for a period of 10 days.

Profiling of total phenolics, flavonoids and organic acids in shoot and root extracts of *Rhizobium* or *Rhizobium* based biofilms inoculated plants, with or without the challenge of pathogens using HPLC was done after 10 days.
3.11.1 Phenolics

3.11.1.1 Standard preparation for HPLC analysis

Tannic acid, gallic acid, vanillic acid, ferulic acid, salicylic acid, t-cinnamic acid, caffeic acid and coumaric acid were purchased from Sigma-Aldrich (USA). All standards were prepared as stock solution in methanol. Stock solutions were diluted in 62.5% aqueous methanol containing 1 g/L butylated hydroxytoluene (BHT) and 6 M HCl in the range of 0.1 mg/mL to prepare the working standards. Stock and working solutions were stored in darkness at -18 °C.

3.11.1.2 Extraction of Phenolic compounds and sample preparation for HPLC

Fresh shoot and root samples were collected and washed in tap water to clean the adhering soil. Extraction of phenolic compounds was done by the method as described by Proestos et al. (2005). One gram of shoot or root sample was crushed in liquid nitrogen in a pre-cooled (4ºC) mortar and pestle. The powder was transferred to a graduated centrifuge tube. Four milliliters of 62.5% aqueous methanol containing BHT (1 g/L) and 1 mL of 6 M HCl were added. The extraction mixture was vortexed carefully and refluxed at 90ºC for 1h in a water bath. After cooling, the content was filtered and the volume was made up to 10 mL with methanol. Extraction of the polyphenols from plant samples was carried out with boiling alcohol and the subsequent work was done in dark to prevent any undesirable enzymatic oxidation (Harborne, 1998). The samples were filtered quickly through a 0.22 μm membrane filter in clean HPLC vials just before the estimation of phenolic compounds through HPLC.

3.11.1.3 HPLC analysis

Total phenolics were analysed through High Performance Liquid Chromatography (HPLC) (Waters) equipped with 2998 PDA detector and auto sampler using RP-18 column (250 mm × 4.6 mm). The mobile phase consisted of solvent A: 0.1% formic acid and solvent B: methanol, mixed in a gradient of 90:10 to 30:70 during run time of 35 min. Injection volume was 10 μL and the detection of elutes was carried out at 280 nm and phenolic compounds were identified based on retention time of respective compound. The peak areas obtained for the authentic standards for tannic acid, gallic acid, vanillic acid, ferulic acid, salicylic acid, t-
cinnamic acid, caffeic acid and coumaric acid (Sigma-Aldrich, USA) were used as reference to quantify the amount of phenolics in supernatant.

**3.11.2 Flavonoids**

**3.11.2.1 Standard preparation for HPLC analysis**

Flavonoids genistein, formononetine, diadzen and biochanin A was purchased from Sigma-Aldrich (USA). Stock solution for the standards was prepared in methanol based on the solubility of each flavonoid compounds. Stock solution of 0.1 mg/mL, 0.75 mg/mL, 0.25 mg/mL and 0.5 mg/mL for genistein, formononetine, diadzen and biochanin A, respectively was prepared in methanol. The working solution was prepared at the range of 0.1 mg/mL by diluting the stock solution appropriately with methanol: water (50:50) containing 0.5% phosphoric acid. Stock and working solutions were stored in dark at -18 °C.

**3.11.2.2 Extraction of flavonoids and sample preparation for HPLC**

Fresh shoot and root samples were collected and washed in tap water to clean the adhering soil. Extraction of flavonoids was done by the method as described by Li et al. (2010). One gram of shoot or root sample was crushed in liquid nitrogen in a pre-cooled (4°C) mortar and pestle. The powder was transferred to a 15 mL falcon tube. Five milliliters of extraction solvent (methanol:water, 80:20 v/v, containing 0.5% phosphoric acid) was added to each tube and was mixed thoroughly by vortexing. The tubes were then incubated at 30°C for 30 min and then sonicated for 15 min. After that, the volume was made upto 10 mL with the extraction solvent. The content was centrifuged at 10000 rpm for 10 min. Finally, 2 mL of the supernatant was filtered through 0.22 μm membrane filter into clean HPLC vials.

**3.11.2.3 HPLC analysis of flavonoids**

Flavonoids present in the samples were analysed through High Performance Liquid Chromatography (HPLC) (Waters) equipped with 2998 PDA detector and auto sampler with a C18 Column (4.6mm×250mm, 5μm). The mobile phase was acetonitrile: water containing 0.5% phosphoric acid (40:60). Injection volume was 10 μL and the detection of elutes was carried out at 254 nm and flavonoids were identified based on retention time of respective compound. The peak areas obtained for the authentic standards for genistein, formononetine, diadzen and
biochanin A (Sigma-Aldrich, USA) were used as reference to quantify the amount of phenolics in supernatant.

3.11.3 Organic acids

3.11.3.1 Standard preparation for HPLC analysis

Malic acid, formic acid, gluconic acid, propionic acid and succinic acid standards were purchased from Sigma-Aldrich (USA). Stock solution of concentration 1 mg/mL was prepared by dissolving appropriate amount of chemical. Working solution (0.1 mg/mL) was prepared by diluting the stock solution appropriately with methanol: water (80:20) containing 0.1% formic acid. Stock and working solutions were stored in 4°C.

3.11.3.2 Extraction of organic acids and sample preparation for HPLC

One gram of fresh leaf sample was crushed in liquid nitrogen in a pre-cooled (4°C) mortar and pestle. The powder was transferred to a 15 mL falcon tube. The powder was transferred to a 15 mL falcon tube. Five milliliters of extraction solvent (methanol: water, 80:20 v/v, containing 0.1% formic acid) was added to each tube and was mixed thoroughly by vortexing. The tubes were then incubated at 30°C for 30 min and then sonicated for 10 min. After that, the volume was made up to 10 mL with the extraction solvent. The content was centrifuged at 10000 rpm for 10 min. Finally, 2 mL of the supernatant was filtered through 0.22 μm membrane filter into clean HPLC vials.

3.11.2.3 HPLC analysis of organic acids present in shoot extract

Organic acids were analysed through High Performance Liquid Chromatography (HPLC) (Waters) equipped with 2998 PDA detector and autosampler using RP-18 column (250 mm × 4.6 mm). The mobile phase consisted of solvent A: 0.1% formic acid and solvent B: methanol, mixed in a gradient of 90:10 to 30:70 during run time of 35 min. Injection volume was of 10 μL. The detection of elutes was carried out at 210 nm and organic acids were identified based on retention time of respective organic acid. The peak areas obtained for the authentic standards were used as reference to quantify organic acids in supernatant.
4. Results

Rhizobia are well known for their biological nitrogen fixation and plant growth promotion in legumes and play an important role in the nitrogen dynamics in soil. For decades, practice of *Rhizobium* inoculation made significant contributions to the global soybean production and other legumes. Although, the basis of rhizobial inoculant technology is biological nitrogen fixation, many rhizobial strains are reported to exhibit antagonistic activity against several soil-borne phytopathogens. The present study was aimed towards selection of *Rhizobium* strain specific to chickpea that are efficient for nitrogen fixation and exhibit antagonistic activity against fungal pathogens. Further, biofilms were developed with selected strains using *Trichoderma* as the matrix and the different formulations were evaluated for biocontrol of *Fusarium* wilt in pot experiment.

4.1 Isolation of *Rhizobium* from root nodules of chickpea

Isolation of *Rhizobium* was carried out by crushing healthy nodules and streaking the lysate on CRYEMA plates. The plates showing white or translucent colonies with or without central red nuclei were tentatively selected as *Rhizobium*. A total of 100 and 30 isolates were obtained from plants collected from ICAR-IARI, New Delhi and Malda, West Bengal respectively. Twenty rhizobial isolates were obtained from HAU, Hisar (Fig. 3.1).

4.2 Screening of rhizobial isolates against soil borne fungal pathogens of chickpea

In the present investigation, among a set of 150 isolates screened, 10 isolates showed good antagonistic activity against all five different fungal pathogens of chickpea tested (Table 4.1, Fig. 4.1). Large amount of variation were recorded among the isolates in their ability to inhibit the mycelial growth. Among the pathogens, maximum inhibition of mycelial growth was observed for *Botrytis cinerea* (49.14%), in the presence of isolate A13. *Rhizobium* isolate A13 exhibited maximum antifungal activity followed by isolate CR24 all the fungal cultures used in the study. However, percent mycelial inhibition of *Fusarium oxysporum* f. sp. *ciceri* (35.71%), *Sclerotium rolfsii* (33.33%) and *Macrophomina phaseolina* (35%) fungal growth was statistically at par (Table 4.1). Isolate CR24 also exhibited good antagonistic activity towards *Botrytis cinerea* (45.71%) and *Fusarium oxysporum* f. sp. *ciceri* (32.14%).
4.3 Nodulation test

The identity of the ten isolates as *Rhizobium* specific to chickpea was confirmed through nodulation test. All the ten isolates formed nodule on the roots of chickpea plants. Hence they were further selected for biocontrol characterization.

4.4 Characterization of Antagonistic *Rhizobium* isolates for antifungal and plant growth promoting attributes

In this study, among all the isolates tested for HCN production isolate CR18 and CR24 exhibited HCN production (Table 4.2; Fig. 4.2) and were higher in case of isolate CR18 as compared to CR24.

Among all the cultures tested, isolate A3, A10, A13, A16 and CR24 were detected positive for ammonia production (Table 4.2). Isolates A3 and A16 exhibited much higher production of ammonia as compared to other isolates.

None of the isolates were found positive for siderophore production or ACC demainase activity in both the plate and quantitative assays.

All the cultures used in this study were found positive for IAA production, with values ranging from 2.39 to 47.34 μg/mL (Table 4.2, Fig. 4.3). However, IAA production by isolates A16 and CR14 was found to be negligible as compared to other isolates. Maximum IAA production of 47.34 μg/mL was recorded for isolate A15 followed by A13 (42.72 μg/mL).

Among all the isolates tested, only two isolates A13 and CR24 exhibited distinct clearing zone around colony in plate assay with Pikovskaya medium, which reveals their potential for phosphate solubilisation (Table 4.2; Fig. 4.4). Quantitative estimation of solubilized phosphorous revealed that A13 was superior to CR24 and found to solubilize 9.84 μg PO₄³⁻/mL as compared to 5.63 μg PO₄³⁻/mL by CR24 (Fig. 4.16).

4.3 Effect of antagonistic *Mesorhizobium* isolates on growth parameters, nodulation and nitrogen fixing efficiency in chickpea plants under greenhouse condition

In this experiment, all the cultures showed good nodulation and nitrogenase activity (Table 4.3; Fig. 4.5). Nodule number per plant was significantly higher in plants treated with rhizobial isolate A13, CR24 and A10, whereas uninoculated plants failed to show any nodules.
All the isolates exhibited good nitrogenase activity as revealed by acetylene reduction assay. Observations revealed that nitrogenase activity in terms of µmole C₂H₄ produced/gram dry weight of nodule/h was highest in treatment inoculated with rhizobial isolate A13, followed by isolate CR24 (Table 4.3). Nitrogenase activities recorded due to inoculation of isolates A10, CR18 and CR20 were statistically at par to the treatment inoculated with standard rhizobial culture F75 (Table 4.3).

A differential effect was observed in all the plant growth parameters with the inoculation of different isolates. Among the isolates, A13 was superior to all and could stimulate the root and shoot length, root and shoot biomass and nodule number statistically. For all the plant growth parameters, isolate A13, CR24, A3 and A10 were statistically at par (Table 4.3). Among the rhizobial isolates, inoculation with CR9 could not influence any of the plant growth parameters.

4.4 Field evaluation of selected rhizobial isolates

The ten isolates showing antagonistic activity against fungal pathogens were evaluated for their influence on growth and yield of chickpea under field conditions (Fig. 4.6). Plots inoculated with isolate A13 recorded the maximum shoot biomass while treatment inoculated with CR24 recorded the maximum root biomass after 60 days of growth (Table 4.4). Maximum grain yield of 1017 kg/ha was recorded again for plots treated with A13, However the yield was statistically at par with plots treated with A3, A15, CR24, CR18, CR20, CR24 and standard strain F75 (Table 4.4).

Based on antifungal activity, plant growth promoting attributes, nodulation and nitrogen fixing ability under pot and field conditions, the two best rhizobial isolates A13 and CR24 were selected for biofilm development and further studies.

4.5 Development of Rhizobium based biofilmed formulations using Trichoderma as fungal matrix

4.5.1 Compatibility test of Mesorhizobium isolates with Trichoderma sp.

Both the rhizobial isolates - Mesorhizobium ciceri A13 and Mesorhizobium ciceri CR24 were able to grow simultaneously with Trichoderma viride on PDA plates and no zone of inhibition were observed (Fig. 4.7). Hence, both the bacterial cultures were compatible with the fungal culture and could be used as partners for biofilm development.
4.5.2 Optimization of medium for biofilm formation

Biofilm development in nutrient broth (NB) and Pikovskaya media (Piko) was poor, as compared to other media (Table 4.5). Biofilm in Yeast extract mannitol (YEM) medium showed higher sporulation by the fungal partner, hence less attachment of bacterial cells to the fungal matrix was observed (Fig. 4.8). Although biofilm development was low in Jensen’s medium, but addition of 1% yeast extract stimulated the biofilm formation significantly. The extent of formation of biofilm was monitored through the CFU counts of the associated partners and the fresh and dry weight of biofilm developed. The population count of bacteria and Trichoderma was 1.20×10^7 and 1.62×10^8 CFU/mL respectively was found highest in biofilm developed in the Jensen’s medium amended with 1% yeast extract. Accordingly, the fresh weight (28.29 g) and dry weight of biofilm developed (13.10 g) was also significantly higher in modified Jensen’s medium, as compared to those obtained in other tested media (Table 4.5; Fig. 4.9). Hence, Jensen’s medium amended with 1% yeast extract was used for development of biofilm with Rhizobium as partner and Trichoderma as matrix.

4.5.3 Development of *Rhizobium* based biofilmed formulations

Jensen’s medium amended with 1% yeast extract was used for mass production of biofilm (Fig. 4.10). Erlenmeyer flasks (1000 mL) containing 300 mL broth were autoclaved and after cooling inoculated with 2 mL of 48 h grown rhizobial culture (10^9 CFU/mL). *Rhizobium* strain A13 and CR24 were used. Flasks were incubated at rotary shaker (120 rpm) at 28ºC for 48 h. After incubation, flasks were inoculated with 2 mL of *T. viride* spore suspension (2.2×10^7 spores/mL) and incubated under static condition at 30ºC for two weeks. After 14 days, biofilm mats were collected, washed three times with sterile water to remove non adherent cells and centrifuged at 2000 rpm for 5 min. Biofilms were homogenized and the uniform suspension was used for population count and further biochemical analysis.

4.5.4 Visual and Microscopic observations of biofilm development

The microscopic observation of the biofilm showed the attachment of *Rhizobium* cells to the mycelium and conidiophore of Trichoderma (Fig. 4.11). Even after washing of the attached cells, a large number of *Rhizobium* cells adhered to the fungal matrix. The observations at different time intervals revealed that maximum attachment was achieved after 14 days of growth in Jensen’s medium amended with 1% yeast extract. The biofilm remain stable for 20 days following which the
disintegration of the biofilm started and the Rhizobium cells got detached from the fungal matrix.

4.5.5 Population count of partners in biofilms

In this study, individual rhizobial cultures *Mesorhizobium ciceri* A13 (1.4×10^9 CFU/mL), *Mesorhizobium ciceri* CR24 (1.2×10^9 CFU/mL) and *T. viride* (2.2×10^7 spores/mL) were used for biofilm preparation as well as for biochemical tests. In biofilm1 (*T. viride*-*Mesorhizobium ciceri* A13), population counts of 1.62×10^8 CFU/mL for *Mesorhizobium ciceri* sp. A13 and 1.2×10^7 CFU/mL of *T. viride* were recorded, whereas biofilm 2 (*T. viride-* *Mesorhizobium ciceri* CR24) exhibited higher population count of *Mesorhizobium ciceri* sp. CR24 (1.92×10^8 CFU/mL) and *T. viride* (1.58×10^7 CFU/mL).

4.6 Determination of antifungal and plant growth promoting attributes of the biofilms developed

4.6.1 Seed germination assay

Germination percentage in chickpea seeds was taken as an index of plant growth promoting activity of the cultures individually and their biofilmed counterparts (Fig. 4.12). Treatment of seeds with individual cultures of rhizobia or *Trichoderma* did not influence the germination significantly, whereas biofilms of both the rhizobial cultures were found to be significantly superior to water soaked seeds (control) in terms of percent germination.

4.6.2 Biochemical tests

All the cultures individually and their biofilms exhibited antagonistic activity against *Fusarium oxysporum* f. sp. *ciceri* (Table 4.6, Fig. 4.13). Biofilmed formulations showed higher antifungal activity than their individual partners. Biofilm 1 (*T. viride*-IsolateA13) and Biofilm 2 (*T. viride*-IsolateCR24) exhibited 9.87% and 10.58% increased inhibition of growth of fungal mycelium, as compared to individual partners, respectively.

In this study, all the cultures and their biofilms, except *Mesorhizobium ciceri* isolate A13 exhibited HCN production (Table 4.6), with biofilms showing higher production than individual cultures. Both the cultures individually and their
biofilms were found positive for ammonia production and biofilms showed increase in ammonia production, as compared to individual cultures (Table 4.6)

All the cultures used in this study were found positive for IAA production, with values ranging from 2.03 to 19.50 μg/mL (Fig. 4.14). Individual rhizobial cultures showed higher production of IAA as compared to their biofilmed formulation. The highest IAA production was found in rhizobial isolate A13 (19.50 μg/mL) followed by its respective biofilm (17.34 μg/mL).

All the individual cultures and biofilms were found to be efficient solubilizers of inorganic phosphate (Fig. 4.15). Distinct clearing zone was observed on Pikovskaya’s medium plates. Phosphate solubilizing activity ranged from 2.42 μg/mL (T. viride) to 15.50 μg/mL (Biofilm1). Biofilms exhibited significantly higher solubilisation potential than their individual partners.

4.7 Evaluation of biocontrol potential of cultures/biofilms against *Fusarium* wilt under greenhouse condition

4.7.1 Disease intensity index (DII)

Disease intensity index (DII) was calculated as described by Cachinero et al. (2002). Single inoculations of *Rhizobium* strains, A13 or CR24 performed better as compared to their respective biofilms, with regard to disease control. The DII (%) of rhizobial isolate A13 was 5.5, whereas that of its biofilm was 10.0. Similarly the DII (%) of rhizobial isolate CR24 was 8.3 while its biofilm was 11.11 (Table 4.7; Fig. 4.16). The DII (%) for both the *Rhizobium* strains was significantly lower than the chemical control treatment.

4.7.2 Effect of *Rhizobium* and *Rhizobium* based biofilms on chickpea plant growth under challenge of *Fusarium* wilt

In the present study, all the treatments exhibited enhancement in growth parameters of chickpea plants as compared to the control treatment challenged with *Fusarium oxysporum* f. sp. *ciceri* (Table 4.7; Fig. 4.16). *Mesorhizobium ciceri* isolate A13 showed highest increase in shoot length (83.11%) and shoot weight (119.66%) as compared to control. It also showed highest increase in root length (101.8%) and root weight (104.8%). Both the rhizobial isolates showed better performance than their biofilmed counterpart in terms of shoot and root length and
biomass; although observations revealed that Biofilm1 and rhizobial isolate CR24 were statistically at par. Seed treatment with *T. viride* and carbendazim performed fairly well as compared to control under fungal infection and were found statistically at par. Root nodules were formed only in plants treated with rhizobial isolates and biofilms and nitrogenase activity was found perfectly correlated with nodulation. Plants grown in pots without fungal inoculum were better than plants from *T. viride* and carbendazim treated plants in terms of growth parameters, but found less vigorous than rhizobia/biofilm inoculated plants.

4.7.3 Determination of defense-related enzyme activity in host plant

4.7.3.1 L-phenylalanine ammonia lyase

L-phenylalanine ammonia lyase activity in shoots of chickpea plant was low initially (7 DAS), but it increased sharply at 14 DAS and after that it gradually decreased for all the treatments (Fig. 4.17A). Both *Mesorhizobium ciceri* strains and their biofilms treated plants exhibited higher elicitation of enzyme as compared to control (uninoculated) plants. Enzyme activity was found significantly higher in biofilm treated plants than their rhizobial counterpart. Seeds treated with *T. viride* and Carbendazim showed lower amount of PAL activity. Lowest enzyme activity was observed in plants without any inoculation or fungal challenge.

4.7.3.2 Peroxidase

The peroxidase activity in the shoot increased gradually from 7 to 21 days and at 28 days showed a decline for all the treatments. In general, biofilm inoculated plants exhibited higher peroxidase activity at all stages of plant growth as compared to single *Rhizobium* inoculation. The activity of peroxidase enzyme was significantly lower in control plants (Fig. 4.17B).

4.7.3.3 Polyphenol oxidase

Polyphenol oxidase activity in plants inoculated with biofilms and their individual partners increased upto 14 DAS and decreased thereafter till 28 DAS (Fig. 4.17C). Maximum enzyme activity was observed in biofilm 1(*T. viride- Rhizobium* isolateA13) treated plants on 14 DAS. Observations revealed that enzyme activity in *Rhizobium* isolate CR24 and biofilm 2 (*T. viride- Rhizobium* isolate CR24) treated plants was statistically at par. Although the total enzyme activity was lower, Plants treated with *T. viride* carbendazim also showed an increase in activity till 14 DAS and decreased thereafter. Lowest enzyme activity was observed in plants without any inoculation or fungal challenge.
4.7.3.4 Chitinase (EC 3.2.1.14) Activity

In the present study, all the cultures individually and their biofilmed formulations were found positive in terms of elicitation of chitinase activity in host plant under challenge of Fusarium wilt (Fig. 4.18). Although, the enzyme activity observed was quite low and it ranged from 0.01 to 0.24 units/mL. Enzyme activity was low initially at 7 DAS, but sharply increased on 14 DAS and gradually decreased thereafter till 28DAS. Both the Rhizobium isolates A13 and CR24 and their biofilms exhibited higher elicitation of enzyme activity, as compared to the plants without any inoculation. Biofilms treated plants recorded higher enzyme activity than their respective partners and maximum activity was observed in plants inoculated with biofilm 1 (T. viride-Rhizobium isolate A13) on 14 DAS. Traces of enzyme activity were also observed in plants inoculated with T. viride or carbendazim. Lowest enzyme activity was found in plants without any inoculation or fungal challenge.

4.7.3.5 β 1,3-glucanase (EC 3.2.1.4) Activity

The activity of β 1,3-glucanase in the plants followed a trend similar to PAL activity. In general, for all treatments, the activity increased sharply from 7 to 14 days but afterwards decreased and lower activity was observed at 21 days for plants harvested from all the treatments (Fig. 4.19).

Elicitation of enzyme activity was observed to be much higher in plants treated with either single Rhizobium isolate or biofilm as compared to plants without any inoculation. Biofilm treated plants recorded higher enzyme activity (80.68 and 73.62μg glucose released/ min/ g fresh tissue for biofilm 1 and biofilm 2 respectively) as compared to plants treated with their rhizobial partners (73.52 and 70.68μg glucose released/ min/ g fresh tissue for treatments inoculated with M. ciceri A13 and M. ciceri CR24 respectively). Enzyme activity was much lower in plants treated with commercial formulation of T. viride or chemical formulation of carbendazim. Plants without any inoculation or pathogen challenge exhibited lowest amount of β 1, 3-glucanase activity.

4.8 Profiling of phenolics, flavonoids and organic acids in root and shoot extracts of Rhizobium or biofilms inoculated plants with or without the challenge of Fusarium oxysporum f. sp. ciceri.

4.8.1 Phenolics

Total phenolics were analysed through High Performance Liquid Chromatography (HPLC) and quantified by comparing with the peak areas obtained
for the authentic standards run under same condition. Accumulation of tannic, ferulic, salicylic and cinnamic acid was quantified both in the root and shoot extracts. Variations were observed in the accumulation level among the root and shoot extracts. For example, accumulation of salicylic acid was higher for single inoculation treatment with either of the *Rhizobium* strains as compared to their respective biofilms in the shoot extracts. However, in root extracts, a reverse trend was recorded (Fig. 4.20). The accumulation of tannic, ferulic and t-cinnamic acid followed an identical pattern both in roots and shoots. In general, plants inoculated with biofilm formulations showed higher accumulation of these acids as compared to single inoculation of *Rhizobium* strains in both roots and shoots. Surprisingly, in shoots, the concentration of salicylic acid and tannic acid was significantly higher in control plants challenged with fungal pathogen. For tannic acid, a similar trend was observed in root extracts also. In case of both root and shoot extracts, in general, the accumulation of phenolics was significantly lower in plants from the control treatments (Biological control, chemical control and absolute control) (Fig. 4.20).

4.8.2 Flavonoids

In the present investigation, all the treatments exhibited accumulation of flavonoid compounds in shoot and root. Accumulation of genistein, formononetin and biochanin A was observed in all the treatments; although diadzein was not detected in any of the treatments (Fig. 2.21, Fig. 2.22). In case of shoot extracts, plants treated with either rhizobial isolates or their biofilmed formulations showed higher accumulation of genistein and biochanin A, as compared to plants without any inoculation (Fig. 2.21A). Accumulation of formononetin was higher in control plants. Observations revealed that plants treated with biofilms accumulated higher amount of flavonoids compared to plants treated with individual rhizobial isolates. Plants treated with *T. viride* and carbendazim showed lower accumulation of flavonoids in shoots.

In case of root, the overall accumulation of genistein and formononetin was lower compared to plants without inoculation under pathogen challenge (Fig. 4.21B). Plants treated with biofilms were observed to accumulate higher amounts of flavonoids as compared to plants treated with their rhizobial counterpart. Accumulation of biochanin A was much higher compared to other flavonoids in all the treatments. Plants treated with either single culture or biofilm showed higher accumulation of isoflavones as compared to control plants under pathogen stress.
Plants treated with commercial formulation of *T. viride* and carbendazim also recorded accumulation of flavonoids in variable amounts (Fig. 4.21).

**4.8.3 Profiling of organic acids in shoot extracts of of *Rhizobium* or *Rhizobium* based biofilms inoculated plants, with or without fungal challenge**

Organic acids were analysed through High Performance Liquid Chromatography (HPLC) (Waters) equipped with 2998 PDA detector and autosampler using RP-18 column (250 mm × 4.6 mm) and quantified by comparing with the peak areas obtained for the authentic standards run under same condition. In the present investigation, all the treatments showed accumulation of malic, succinic, propionic and formic acid in variable amounts (Fig. 4.23). Plants treated with either individual *M. ciceri* cultures or their biofilms exhibited much higher accumulation of organic acids compared to plants without any inoculation. Biofilm treatment enhanced accumulation of malic, succinic and propionic acid in plants compared to their individual counterpart (Fig. 4.23 A- Fig. 4.23C). In case of formic acid individual *M. ciceri* A13 and CR24 exhibited higher accumulation (22.56 and 26.75 μg/mL respectively), as compared to their biofilmed formulation (Fig. 4.23D). Plants treated with of *T. viride* and carbendazim recorded lesser accumulation of flavonoids in shoot extracts. Plants without any inoculation or pathogen challenge exhibited lowest accumulation of all the organic acids studied.
5. Discussion

*Rhizobium* is a symbiotic nitrogen fixing bacterium which develops nodules in leguminous plants through its association with root. Since the turn of this century, the importance of *Rhizobium* as inoculants for legume crops has increased as means of providing nitrogen for growth and metabolic activity. During the mid-sixties, commercial production of *Rhizobium* inoculants was started in India. With the advancements in science and further characterization of this organism, several other agriculturally important attributes were unveiled. However, there are few reports which reveal the biocontrol potential of *Rhizobium* against fungal pathogens. The selection of *Rhizobium* strains that exhibit dual function i.e. nitrogen fixation and biocontrol potential against fungal pathogens can be a gainful proposition; particularly for several leguminous crops like chickpea, pea and lentil that suffer severe losses due to the infection of soil borne pathogenic fungi. The use of *Rhizobium* as a biocontrol agent will provide an alternate, eco-friendly strategy to combat pathogens without application of hazardous chemical pesticides. The present study was aimed to identify chickpea specific *Rhizobium* strains that exhibit dual functional activities i.e. biocontrol for wilt disease and also contribute to nitrogen pool of plant. Furthermore, the mechanism involved in biocontrol activity against fungal pathogen in both host and *Rhizobium* was also elucidated.

Screening of isolates is a prerequisite to identify effective strains with dual functional attributes. The screening experiments need to be carried out on a larger dataset so as to obtain the right candidate for the desired function. In this regard, large number of isolations was made from the nodules of chickpea plants collected from two different locations- experimental farms of Indian Agricultural Research Institute, New Delhi and Malda, West Bengal. In addition, *Rhizobium* strains were also procured from the culture collection of Hisar Agricultural University, Hisar, Haryana. A total of 150 distinct isolates were used which were further subjected to screening for antagonistic activity against five fungal pathogens namely *Fusarium oxysporum* f. sp. ciceri (AFC-1), *Ascochyta rabiei* (AR4638), *Botrytis cinerea* (BC6530), *Sclerotium rolfsii* (CR5518) and *Macrophomina phaseolina* (MP). In earlier studies also, germplasm collections have been screened to siphon out potent antagonists against fungal pathogens (Nautiyal, 1997; Antoun et al., 1998). Among 150 isolates, only 10 isolates showed antagonistic activity against all the 5 fungal pathogens tested. A number of studies revealed the potential of rhizobial strains as
biocontrol agents against soil-borne pathogens of legume crops such as *Rhizoctonia* spp., *Fusarium* spp., *Sclerotium rolfsii*, *Macrophomina phaseolina* and *Pythium* spp. (Deshwal et al., 2003a; Arfaoui et al., 2006; Hemissi et al., 2011; Shaban and EL-Bramawy, 2011). Among the isolates, isolate A13 and CR24 showed 45 to 49% growth inhibition of fungal mycelium in dual culture plate assay (Table 4.1). Studies suggested that in vitro suppression of fungal growth and formation of inhibition zones were presumably due to the metabolites released by the bacteria into the culture medium (Chandra et al., 2007; Martínez-Viveros et al., 2010; Kumar et al., 2011). In our investigation, cultures showing good antifungal property were also found to produce antifungal metabolites like HCN and ammonia (Table 4.2). Arfaoui et al. (2006) reported six rhizobial strains, positive for HCN production which showed antagonistic activity towards *Fusarium oxysporum* f. sp. *ciceri* under in vitro and in vivo conditions. Although there are several reports, rhizobia are relatively less efficient in HCN production (Deshwal et al., 2003b). Beauchamp et al. (1991) found that 4 out of 32 rhizobial strains produced HCN. Antoun et al. (1998) have found 12.5% and 3% of the total strains screened respectively to be HCN producers. *Mesorhizobium loti* MP6, an isolate from root nodules of *Mimosa pudica*, positive for HCN production played a significant role in the inhibition of growth of *Sclerotinia sclerotiorum*, causal organism of white rot in *Brassica campestris* (Chandra et al., 2007).

In contrast, the production of ammonia has been commonly detected among rhizobium isolates (66%) (Singh et al., 2013). In our study, 40% of isolates (4 out of 10) were also ammonia producers (Table 4.2). Among the isolates with antagonistic activity, none of them were found positive for siderophore production. It is well known that many microorganisms are endowed with the property to produce siderophores (low molecular weight, iron-binding compounds) which help to sequester iron from the environment and release it inside the cells (Matzanke, 1991). The ability of Fe$^{3+}$-Fe acquisition through production of siderophores is crucial as it determines the ability of bacteria to colonize plant roots under iron deficient conditions (Crowley and Gries, 1994; Crowley, 2006). The production of siderophores by PGPR is a mechanism to prevent multiplication and colonization of pathogenic microorganisms, by sequestering Fe$^{3+}$ from the rhizospheric region (Siddiqui, 2006; Martínez-Viveros et al., 2010). A number of reports are available on diverse array of siderophores produced by rhizobia (Deshwal et al., 2003b).
All the rhizobial isolates with antagonistic activity were further characterized for their nodule forming and nitrogen fixing efficiency. It is pertinent to conduct nodulation assay to confirm the identity of an isolate as *Rhizobium* as some reports are available on presence of non-nitrogen fixing nodule endophytes (Mhamdi et al., 2005, Meyer et al., 2015). Some of the non-nitrogen fixing nodule endophytes show similarity with morphological and cultural characteristics of *Rhizobium* (Saini et al., 2015).

In our investigation, all the tested strains were able to infect their host plant and fix atmospheric N\(_2\). Good nodulation was observed in all the treatments and *M. ciceri* isolate A13 and isolate CR24 exhibited better nitrogenase activity compared to other isolates and control. The nitrogenase activity among the isolates varied from 0.98 to 2.36 \(\mu\) moles C\(_2\)H\(_4\) produced/g dry weight of nodule/h. Earlier studies have reported variable results and isolates exhibiting 0.06 to 1.52 \(\mu\) moles C\(_2\)H\(_4\) produced/gram dry weight of nodule/h activity have been reported (Soussi et al., 1999). All the treatments, except CR9 were also found to enhance chickpea plant growth parameters as compared to un-inoculated control and standard *Rhizobium* F75 under pot and field conditions. Bardin et al. (2004) reported that seed treatment with certain strains of *Rhizobium leguminosarum* Frank bv. *viceae* could enhance nitrogen fixation in artificial medium in field pea and sugar beet. Shaban and EL-Bramawy (2011) reported that dual inoculation with *Rhizobium* and *Trichoderma*, not only reduced disease incidence of damping off and root rot, but also increased yield parameters like pods per plant, seeds per pod, mean seed weight and seed yield of broad bean, chickpea and lupine plants. The increase in the dry biomass due to inoculation was directly correlated with the nodule number and ARA activity. Similar results have been reported in many different studies (Akhtar and Siddiqui, 2008; Al-Ani et al., 2012).

It is worthwhile to mention that isolate CR9 showed unusual characteristics and resulted in the stunting of plants. However, in-depth characterization of such a strain was beyond the purview of the objectives of the thesis. The unusual characteristics of CR9 isolate may be due to secretion/production of rhizobitoxins, as described in previous published reports (Chakraborty and Purkayastha, 1984; Ozkoc and Deliveli, 2001).

The interest in *Rhizobium* strains exhibiting plant growth promoting attributes gained momentum since its first report as PGPR by Noel et al. (1996). Therefore, the isolates showing antagonistic activity were also screened for some of
the plant growth promoting attributes, for their effective use as inoculants in future. The direct effects of PGPR have been most commonly attributed to the production of plant hormones such as auxins, gibberellins and cytokinins by supplying biologically fixed nitrogen, solubilization of phosphorus or production of ACC deaminase enzymes (Noel et al., 1996; Glick et al., 1994).

In general, IAA produced by rhizobacteria promotes root growth by directly stimulating plant cell elongation or cell division. A low level of IAA produced by rhizosphere bacteria promotes primary root elongation, whereas a high level of IAA stimulates lateral and adventitious root formation but inhibits primary root growth (Buddhika et al., 2014). The ability to produce IAA among Rhizobium appears to be more common (Gopalakrishnan et al., 2015). In our study, almost 80% of the isolates screened showed the production of IAA to varying levels in the range of 2.39 to 47.34 µg/mL (Fig. 4.3). In contrast, there are also reports of very few isolates showing IAA production (Bhattacharyya et al., 2012). From the published literature, it is difficult to conclude that the production of IAA among Rhizobium is a group specific character or a location specific character.

Rhizobia, including R. leguminosarum, R. meliloti, M. mediterraneum, Bradyrhizobium sp. and B. japonicum (Afzal and Bano, 2008; Egamberdiyeva et al., 2004; Rodrigues et al., 2006; Vessey, 2003) are potential P solubilizers. These bacteria synthesize low molecular organic acids which act on inorganic phosphorous. For instance, 2-ketogluconic acid with a phosphate-solubilizing ability has been identified in R. leguminosarum (Halder et al., 1990) and R. meliloti (Halder and Chakrabarty, 1993).

A large number of strains of Rhizobium were able to solubilize the insoluble phosphate compound, hydroxyapatite, in liquid culture. Abd-Alla (1994) reported that the R. leguminosarum bv. viceae strains employ two different mechanisms for phosphate solubilization, one requiring presence of NH$_4^+$ and the other without its presence. In our study, we identified Rhizobium strains which solubilize phosphorus with values ranging from 5.63 to 9.84 µg/mL, using tri-Calcium phosphate as an insoluble source of phosphorus (Fig. 4.16).

In our study, none of isolates showed ACC deaminase activity in both qualitative and quantitative assays. Ma et al. (2003) has also reported that ACC deaminase activity was not exhibited by chickpea microsymbiont M. ciceri under free living conditions. However, there are few reports on ACC deaminase activity in
different groups of rhizobia (Duan et al., 2009; Nascimento et al., 2012; Bhattacharyya et al., 2012).

In recent years, innovative technologies have been developed to enhance the efficiency of the selected microbial cultures and their effective delivery in the soil. Different options are now commercially available like carrier based inoculants, liquid formulations, oil-based formulations, single or dual or multiple (consortium) culture formulations. An ideal technology would involve the synergistic interactions among two organisms, each with its specific functional attributes. Working in this direction, the biofilms of selected strains (two *Rhizobium* strains - A13 and CR24) were developed using *T. viride* as the matrix. There are few reports available on the use of biofilms in agriculture (Seneviratne, 2003; Jayasinghearachchi and Seneviratne, 2004; Triveni et al., 2012, 2013). Biofilms using *Bradyrhizobium-Penicillium* sp. combination have been developed earlier, wherein mycelia colonization by *Bradyrhizobium* and *Azorhizobium* has been demonstrated (Jayasinghearachchi and Seneviratne, 2004). In the present study, we selected *T. viride* to serve as a matrix for development of biofilm. *Trichoderma* sp. represents the commercially successful biocontrol agent besides their ability to promote the growth of plants (Harman, 2006). Biofilm forming potential of several fungi including *T. viride* was carried out and it was observed that light and time significantly influence biofilm formation (Bahata et al., 2008). Earlier studies have shown that combining of two or more organisms in the form of biofilm provide an additive effect in their mode of action (Guetsky et al., 2002; Triveni et al., 2013).

The protocols for the development of *Trichoderma* based biofilms with *Bacillus subtilis, Pseudomonas fluorescens, Azotobacterchroococcum* were optimized by Triveni et al. (2012). Since, the nutritional requirement of all microorganisms are varied, therefore, it was pertinent to optimize a suitable medium for development of biofilm using *Trichoderma viride* and *Mesorhizobium ciceri*. Among the media tested, Jensen’s medium amended with 1% yeast extract supported maximum growth of biofilm. Similar observations were made by Triveni et al. (2012) while developing biofilms using *T. viride* and *A. chroococcum*. They also reported that addition of 1% yeast extract enhanced the growth of *T. viride*. Elsewhere, it has been documented that yeast extract is a perfect combination of both carbon and nitrogen sources, which can stimulate growth and cellulose production by *Trichoderma viride* (Gautam et al., 2010). The sequential incubation of *Rhizobium* two days prior to *Trichoderma* and further incubation under static conditions resulted
in higher biomass of the biofilm produced. This was further confirmed by the higher values of CFU counts for the partners (Table 4.5). Microscopic observations also revealed adherence of *Rhizobium* cells in greater numbers on the mycelium and conidiophore of *T. viride*, when incubated under static condition. The static condition permitted higher biomass of biofilms, as it allowed gradual attachment of bacteria and colonization of fungal mycelia. Similar observations have been made earlier on natural biofilms developing on the air water surface of stagnant pool or on the rocks at the bottom of most streams (Lynch et al., 2005).

Studies suggest that the combination of PGPR strains (two or more) which have diverse mode of plant growth promotion or antagonism against soil-borne pathogens are more effective than single strain inoculum (Chiarini et al., 1998; Mazen et al., 2008; Nadia et al., 2007; Shaban and El-Bramawy, 2011). There are several reports on rhizobia exhibiting antifungal activity against pathogenic fungi and reducing the disease incidence in plants (Kelemu et al., 1995; Deshwal et al., 2003b; Bardin et al., 2004). In our study, both the *M. ciceri* isolates and *T. viride* alone exhibited antifungal property, in terms of inhibition of fungal mycelium (32.5, 30.14 and 26.57% respectively). But observations revealed that there was a significant increase in inhibition, when biofilms were applied (35.71% and 33.33% respectively) (Table 4.6). Biofilms also showed increase in HCN and ammonia production as compared to their individual partners, which correlated with the antifungal activity. Similar results were observed by Triveni et al. (2013) in case of *B. subtilis–T. viride* and *P. fluorescens–T. viride* biofilms. Arfaoui et al. (2006) reported suppression of *Fusarium* wilt of chickpea under in vitro and in vivo conditions by six rhizobial strains positive for HCN production. Chandra et al. (2007) also observed growth inhibition of *Sclerotinia sclerotiorum* causing white rot in *Brassica campestris* by HCN producing *Rhizobium loti* MP6. In case of biofilms, a significant increase in plant growth promoting traits was also observed. In this study, chickpea seeds treated with biofilms exhibited higher germination percentage compared to seeds treated with individual cultures (Fig. 4.12). Biofilm1 and 2 showed 13 and 21.7% increase in germination percentage respectively, as compared to control. Similar type of observations were recorded by Triveni et al. (2013). This signifies that biofilms, as compared to their individual cultures, may be providing a better growth promoting environment in terms of phytohormones and vitamins, which enhances seed germination. Qurashi and Sabri (2012) reported that biofilmed formulations of *Halomonas variabilis* (HT1) and *Planococcus rifietoensis* (RT4)
stimulated germination of wheat by 152% and 178% respectively as compared to their individual inoculation. Biofilmed biofertilizers have already shown immense potential as inoculants in several crops like rice, cotton, wheat, tea, soybean and mung bean (Prasanna et al., 2015; Seneviratne et al., 2008; Seneviratne and Jayasinghearachchi, 2004; Triveni et al., 2015). Biofertilizers mediated increase of plant growth and yield is often attributed to production of growth regulators like IAA, gibberellins and cytokinins (Chauhan et al., 2009; Roychowdhury, 2012). Cassán et al. (2009) reported an increase in the growth of maize and soybean by a strain of Azospirillum brasilense and Bradyrhizobium japonicum producing IAA. Besides its role as phytostimulator, IAA acts as signalling molecule in plant–microbial interactions and biofilm development (Spaepen et al., 2007; Lee et al., 2007). In this study, individual rhizobial isolates showed higher IAA production compared to their respective biofilms (Fig. 4.14). Similar observations were recorded by Buddhika et al. (2014). They also observed that although IAA production by the biofilms was lower than monoculture, it significantly increased percent seed germination. In our study also, biofilms showed reduction in IAA production, but increased seed germination when compared to individual rhizobial cultures.

A number of studies revealed that rhizobia can efficiently solubilize inorganic phosphates through the production of organic acids (Halder and Chakrabarty, 1993; Vessey, 2003; Afzal and Bano, 2008). Seneviratne (2003) have reported that biofilm formation may enhance phosphate solubilisation ability. In our study also, phosphate solubilisation was enhanced from 57.5 to 152.2% in biofilmed formulations, as compared to individual cultures (Fig. 4.15). Similar type of enhancement was observed by Jayasinghearachichi and Seneviratne (2006) in case of Pleurotus ostreatus-Bradyrhizobium elkanii SEMIA 5019 biofilm.

Several published reports illustrating the potential of rhizobia in biocontrol of disease causing plant pathogens and improving plant growth are available. Application of rhizobial isolates as seed coating or as soil drenching reduced disease incidence of M. phaseolina, R. solani and Fusarium spp. in okra plants (Ehteshamul-Haque and Ghaffar, 1993). In our study, both the biofilmed and their counterpart rhizobial isolates were found to reduce disease incidence and increase plant growth parameters like shoot and root length and biomass in Fusarium inoculated pots (Table 4.7). Similar type of observations were recorded by Ganesan et al. (2007), where dual inoculation of Rhizobium with Trichoderma harzianum not only reduced incidence of collar rot in groundnut caused by Sclerotium rolfsii, but also increased
several growth parameters like root, shoot length and plant biomass. Dual inoculation of *Rhizobium* and *Trichoderma* have also been reported to reduced damping off and root rot diseases and increase yield components in broad bean, chickpea and lupine plants (Shaban and EL-Bramawy, 2011).

A number of studies have revealed the role of defense related enzymes like L-phenylalanine ammonia lyase, peroxidase and polyphenol oxidase in disease resistance (Sivakumar and Sharma, 2003; Arfaoui et al., 2007). In our study, increase in activity of PAL, POX and PPO enzymes was observed in culture/biofilm inoculated plants as compared to non-inoculated control as well as plants without any inoculation or fungal challenge. Enzyme activity was observed much higher in case of biofilms treated plants compared to single inoculation throughout the study period (Fig. 4.17). This suggests that biofilms are much efficient in elicitation of induced systemic resistance in plants. Similar type of increase in defense enzyme activity has been reported by Mabrouk et al. (2007) and Dutta et al. (2008) in pea and pigeon pea against *Fusarium* wilt. Osdaghi et al. (2011) also reported induction of resistance in common bean when bacterized with *Rhizobium leguminosarum* bv. *phaseoli* against common bacterial blight caused by *Xanthomonas axonopodis* pv. *phaseoli*.

Induced systemic resistance (ISR) in plants is a common response to nonpathogenic bacteria, viruses, and fungi and is accomplished by the inoculation of a plant with an avirulent or non-pathogenic isolate prior to or concomitantly with a virulent pathogen. Once resistance is induced, the plant expresses a number of inducible defense responses including the production of cell wall lytic enzymes such as chitinases and β-1,3-glucanases (Lawton and lamb, 1987). Stimulation or induction of chitinase gene expression by pathogen attack is often observed (Collinge et al., 1993). The character of this expression can be systemic or local (Bishop et al., 2000). It depends on the infecting pathogen and its virulence. In *Arabidopsis thaliana* plants, infection by an incompatible pathogen causes a rapid accumulation of class IV chitinase mRNA (Bishop et al., 2000). The chitinases’ induction in *in vitro* cultures revealed a very complicated pattern. In a *Nicotiana* sp. culture, the presence of auxin and cytokinin in the medium repressed chitinase genes. After passage of the explants on the medium without these phytohormons, the induction of chitinases was triggered immediately (van Buuren et al., 1992).
A number of studies have revealed the potential of rhizobacteria to induce chitinase and β-1, 3-glucanase activity in plants, when challenged with a virulent pathogen. Mauch et al. (1984) reported that infection of immature pea pods with *Fusarium solani* f.sp. *phaseoli* (a non-pathogen of peas) or *Fusarium solani* f. sp. *pisi* (a pea pathogen) resulted in the induction of chitinase and β-1,3-glucanases. Within 30 h, the activities of the two enzymes increased 9-fold and 4-fold, respectively. Chitinase and β-1,3- glucanase were also induced by autoclaved spores of the two *F. solani* strains.

Xue et al. (1998) also observed that inoculation of bean hypocotyls with a non-pathogenic binucleate *Rhizoctonia* (BNR) species induced systemic resistance and protected plants from infection of root rot pathogen *Rhizoctonia solani*. Bean seedlings, treated with BNR 48 h prior to their challenge with *R. solani* showed few necrotic lesions and reduced disease severity as compared with seedlings not treated with BNR. Significant increase in peroxidases (2 fold) and β-1, 3-glucanase (8 fold) activity was observed in inoculated plants as compared to uninoculated control. Similar type of induction of chitinase and β-1, 3-glucanase enzymes was reported by Cachinero et al. (2003). They observed that inoculation of kabuli chickpea with incompatible race 0 of *Fusarium oxysporum* f. sp. *ciceri* protected the plants through induction of higher chitinase, β-1, 3 -glucanase and peroxidase activity when challenged with virulent *Fusarium oxysporum* f. sp. *ciceri* race 5. In our study, plants treated with either individual *M. ciceri* isolates or their biofilms exhibited higher chitinase activity as compared to plants without any inoculation. Chitinase and β-1, 3-glucanase activity peaked in the shoots at 14 DAS. Beyond 14 days, there was a gradual dip in the activity of both the enzymes (Fig. 4.18; Fig. 4.19). Similar type of observation was reported by Viera et al. (2010), where, induction of defense proteins like peroxidase and β-1, 3-glucanase was recorded during the early period of infection by the fungi *Fusarium oxysporum* f. sp. *cubenses* and *F. oxysporum* f. sp. *phaseoli*.

Plants have the ability to synthesize a diverse array of secondary metabolites like simple phenols, phenolic acids, flavones, flavonoids alkaloids etc. Many of these compounds play significant role in plant defense mechanism against herbivory and pathogen infection (Cowan, 1999; Beckman et al., 2000). A number of studies have reported marked increase in synthesis of these phenolic compounds in plants infected with pathogenic fungi (Cherif et al., 1992; Fernandez et al., 1998; De et al., 2003).
Mostly these compounds are synthesized in different branches of the general phenylpropanoid pathway, leading to the elaboration of various hydroxycinnamic acids and derivatives with antifungal activity. Inhibition of disease development and resistance against phytopathogens by accumulation of polyphenols may be attributed to nutrient deprivation (metal complexation, protein insolubilisation), inhibition of fungal oxidative phosphorylation, inhibition of extracellular fungal enzymes (cellulases, pectinases), and antioxidant activity in plant tissues (Scalbert, 1991). Esterification and subsequent deposition of polyphenols on cell wall also act as a physical barrier against the pathogen invasion. Among the different phenolic compounds, the presence of gallic, ferulic, caffeic, coumaric, chlorogenic and cinnamic acids are well recognized and found widely distributed in plants, in combination with other compounds usually in form of esters. Many of these compounds possess good antifungal activity (Sarma et al., 2002).

Plant growth-promoting rhizobacteria (PGPR)-mediated induced systemic resistance has gained considerable importance recently in controlling a wide spectrum of fungal diseases in a number of crop plants, both in glasshouses as well as in fields (Wei et al., 1991; Hoffland et al., 1996; Singh et al., 2000). In the present study, the experiment was designed to look for induction of compounds of phenylpropanoid pathway in chickpea plants challenged with fungal pathogen and inoculated with rhizobia with antagonistic property. Efforts were made to carry out comparative profiling of phenolics, flavonoids and organic acids in plants grown under different treatments.

McKeehen (1999) studied the phenolic acid profiles of six cultivars of wheat with known tolerance to Fusarium head blight and found that p-coumaric and ferulic acid were the two principal phenolic compounds present, which demonstrated significant reductions (p < 0.05) of Fusarium species growth at all concentrations tested. Sarma and Singh (2003) have also reported significant increase of ferulic acid in stem portions in Sclerotium rolfsii infected plants compared to healthy seedlings. In vitro studies of ferulic acid revealed significant antifungal activity against S. rolfsii. In our study, marked increase in tannic, ferulic and t-cinnamic acid was observed in plants inoculated with either M. ciceri strains or their biofilms (Fig. 4.20). In general, induction of phenolic compounds was higher in treatments with biofilm compared to individual rhizobial cultures in both root and shoot under pathogen challenge. In a similar report by Sarma et al. (2002), chickpea plants
treated with PGPR strains *Pseudomonas fluorescens* (Pfs1-7) showed an increase in gallic, ferulic, chlorogenic and cinnamic acids when challenged with *Sclerotium rolfsii* under field condition. Maximum accumulation of cinnamic acid was observed in plants treated with Pfs3 strain (1660 ng g$^{-1}$ fresh wt.), which was almost 19.5 times higher than untreated control plants. PAL enzyme catalyses the reductive deamination of phenylalanine to form cinnamic acid in same phenylpropanoid pathway. Ferulic acid is also produced in the same pathway. Hence, simultaneous increase of PAL activity and accumulation of these phenolics have been demonstrated in plant tissues (Jones, 1984, McKeehen, 1999). In our study also, a sharp increase in PAL activity was observed on 14 DAS which correlates with the accumulation of ferulic acids and t-cinnamic acids in the inoculated plants.

Many studies have indicated that salicylic acid plays an important role in plant defense response against pathogen attack and is essential for the development of SAR (Ryals et al., 1996; Leeman et al. 1996). Leeman et al. (1996) found that salicylic acid produced by selected *Pseudomonas fluorescens* (Trevisan) Migula strains is involved in the induction of systemic resistance against *Fusarium* wilt in radish. In our investigation, significant accumulation of salicylic acid was observed in both root and shoot portion of the plants challenged by fungal pathogen (Fig. 4.20). In general, plants treated with either rhizobial cultures or their biofilms exhibited higher accumulation of salicylic acid in root as compared to plants without any inoculation. Chen et al. (1999) have also reported an increase in salicylic acid levels in cucumber roots after treatment with two PGPR strains against *Pythium aphanidermatum* causing root and crown rot of cucumber.

The isoflavonoids, on the other hand show a very limited distribution in the plant kingdom and mostly confined to the legumes. Isoflavonoids are synthesized as part of the phenylpropanoid pathway and they show a wide range of biological activities. Isoflavonoids play a key role in plant-microbe interactions, serving as the signal molecule for establishing the symbiotic relationship between plants and rhizobia. They are also the precursors to the major phytoalexins in legumes, indicating that the activation of their synthesis during the interaction of resistant plants with the pathogen provides a large array of defense compounds. The four most common isoflavones found in legume plants are daidzein (1,7,4’-trihydroxysoflavone), formononetin (2,7-hydroxy-4’-methoxyisoflavone), genistein (3,5,7,4’-tridydroxyisoflavone) and biochanin A (4,5,7-dihydroxy-4’-
methoxyisoflavone). Most of these compounds are synthesized in response to infection by pathogens (Cherif et al., 2007).

Arfaoui et al. (2006) reported that pretreatment of chickpea seedlings with selected *Rhizobium* isolates before challenge with *Fusarium oxysporum* f. sp. ciceri, increased significantly the levels of peroxidases, polyphenoloxidases, total phenolics and the constitutive isoflavonoids, formononetin and biochanin A. Analysis revealed that maximum levels of formononetin and biochanin A were recorded in chickpea roots after 10 days and 20 days, respectively of challenge with Foc. Wegulo et al. (2005) observed that isoflavones, daidzein, genistein and glycitein, in soybean were strongly increased after infection by *Sclerotinia sclerotiorum*. In a similar study, Morkunas et al. (2005) recorded marked increase in synthesis of isoflavonoids like genistein, wighteone and luteon in *Lupin luteus* after infection with *Fusarium oxysporum*. In our study, accumulation of isoflavones like genistein, formononetin and biochanin A was observed in plants treated with antagonistic rhizobial cultures and their biofilms (Fig. 4.21). In general, accumulation of genistein and formononetin was higher in shoot portion, whereas, significant accumulation of biochanin A was observed in the roots of the infected plants. Cahinero et al. (2002) also observed increase in production of phytoalexins medicarpin and maachiain and related isoflavones formononetin and biochanin A in chickpea plants induced by incompatible race 0 of *Fusarium oxysporum* f. sp. against virulent race 5 of the pathogen.

In the present study profiling of organic acids was also carried out. There was a significant increase in the contents of malic, succinic, propionic and formic acid in treatments inoculated with rhizobial isolates or their respective biofilms (Fig. 4.23). There are no reports available on the accumulation of organic acids in response to challenge with pathogen and inoculation with antagonist. Almost 100% increase in the content of formic acid in *Rhizobium* or biofilm inoculated treatment suggests its involvement in providing resistance to the chickpea plant. However, detailed further study is required to decipher the role of these organic acids in inducing resistance against phytopathogenic fungi.
6. Summary and Conclusions

In the last few decades, excessive application of chemical fertilizers and fungicides to control plant diseases and improve plant productivity has led to severe damage to soil microflora and soil health. Integrated use of bioinoculants as a nutrient management strategy has emerged as potential solution towards sustainable agriculture and crop productivity. Rhizobia which are well recognised for their symbiotic nitrogen fixation and plant growth promotion are also reported to exhibit antagonistic activity towards a number of fungal pathogens. Although, the basic tenet regarding the use of *Rhizobium* inoculants is biological nitrogen fixation, inoculants with dual function of nitrogen fixation and antagonistic activity can be better options in sustainable agriculture. Commercial *Rhizobium* formulations used currently are mainly of two types-solid (carrier based) and liquid. With the increased popularity of bio-inoculants, new technologies have emerged focusing mainly towards cost effectiveness, better stability and longer shelf life. Biofilmed biofertilizers have emerged as one such novel option. The increased cell density within a biofilm provides the opportunity to exhibit attributes that single cells cannot achieve efficiently. The present study was aimed to identify chickpea specific *Rhizobium* strains that exhibit dual functional activities i.e. biocontrol for wilt disease and also contribute to nitrogen pool of plant. Furthermore, the mechanism involved in biocontrol activity against fungal pathogens in both host and *Rhizobium* was also illustrated. *Rhizobium* based biofilms were prepared using *Trichoderma viride* as fungal matrix and their efficiency as inoculant was evaluated.

The salient features of the research undertaken are summarised below:

1. A total of 150 distinct isolations were made from different locations like IARI experimental farm, New Delhi, Malda, West Bengal and HAU, Hisar.
2. They were screened for antagonistic activity against five fungal pathogens namely *Fusarium oxysporum* f. sp. *ciceri* (AFC-1), *Ascochyta rabiei* (AR4638), *Botrytis cinerea* (BC6530), *Sclerotium rolfsii* (CR5518) and *Macrophomina phaseolina* (MP). Among the 150 isolates, only 10 isolates showed antagonistic activity against all the five fungal pathogens and percent growth inhibition of fungal mycelium varied from 5 to 49%.
3. All the isolates showing good antifungal activity were able to form nodules in chickpea host (confirming their identity as *Rhizobium*) and showed high
nitrogenase activity, detected through Acetylene Reduction Assay. Nodule number ranged from 8-16 per plant and the isolate A13 exhibited highest nodule number. The nitrigenase activity among the isolates varied from 0.98 to 2.36 µ moles C$_2$H$_4$ produced/gram dry weight of nodule/h. The isolate A13 and isolate CR24 exhibited higher nitrogenase activity compared to other isolates and control.

4. Ten selected *M. ciceri* isolates were evaluated for production of antifungal metabolites such as HCN, ammonia and siderophores. Among the ten isolates, 2 isolates showed HCN production and 4 were found positive for ammonia production. However, none of the isolates exhibited any siderophore activity.

5. In our study, almost 80% of the isolates screened showed production of IAA to varying levels in the range of 2.39 to 47.34 µg/mL. Two *M. ciceri* strains out of ten were found to solubilize phosphorus, with values ranging from 5.63 to 9.84 µg/mL, when tri-calcium phosphate was used as insoluble source of phosphorus.

6. In pot experiment, all the cultures except CR9 exhibited enhancement in plant growth parameters, including shoot/ root length and biomass as compared to uninoculated control.

7. Under field conditions, these cultures were also found to significantly enhance nodulation, nitrogen fixation and other plant growth parameters such as shoot and root length and yield. Two cultures - *M. ciceri* isolate A13 and *M. ciceri* isolate CR24 were identified as most promising for the development of bioinoculant.

8. *M. ciceri* based biofilms using isolate A13 or CR24 as the partner with *Trichoderma viride* as fungal matrix were developed. The protocol for biofilm development was optimized in terms of medium composition and population count of individual partners.

9. Biofilms exhibited significant increase in antifungal activity in terms of inhibition of growth of fungal mycelium, compared to its individual partners. Biofilms also showed an increase in HCN and ammonia production as compared to their individual partners, which are correlated to antifungal activity.

10. In case of biofilms, a significant increase in plant growth promoting traits was also observed. In this study, chickpea seeds treated with biofilms
exhibited higher germination percentage (13-21.7%) compared to control. Significant increase in phosphate solubilisation (57.5-152.2%) was also observed.

11. In pot experiment, both the *M. ciceri* biofilms and the individual rhizobial isolates were found to reduce disease incidence and increase plant growth parameters like shoot and root length and biomass when challenged with *Fusarium* wilt.

12. Elicitation of plant defense related enzymes like L-phenylalanine ammonia lyase, peroxidase and polyphenol oxidase was found to be higher in *M. ciceri*/biofilm treated plants, as compared to uninoculated plants under pathogen challenged soil. Induction of pathogenesis related proteins like chitinase and β 1, 3- glucanase was also observed.

13. Profiling of phenolics in root and shoot of *M. ciceri*/biofilm treated plants revealed significant accumulation of tannic, ferulic, salicylic, and t-cinnamic acids as compared to uninoculated plants. Induction and role of these phenolic compounds are well documented in development of resistance in plants against virulent pathogens.

14. Greater accumulation of isoflavonoids like genistein, formononetin and biochanin A and organic acids like malic, succinic, propionic and formic was observed in plants inoculated with rhizobia or their biofilms as compared to uninoculated plants, which signifies better induction of plant defense mechanisms under pathogen challenge.

*M. ciceri* strains with dual attributes of nitrogen fixation and antagonistic activity against soil borne fungal pathogens can be selected. Additionally, they can be characterised for their ability to produce HCN or ammonia, which is prevalent while production of siderophore or ACC deaminase is rare among rhizobia. Inoculation with rhizobial isolates is also beneficial as they possess plant growth promoting attributes like IAA production and P solubilisation. Biofilms of *Rhizobium* with *Trichoderma viride* as matrix could be prepared using Jensen’s medium amended with 1% yeast extract. These biofilms exhibit synergistic effect and enhancement in almost all the PGP and biocontrol attributes were observed as compared to individual *Rhizobium* isolates. *Rhizobium* based biofilms using fungal matrix (*Trichoderma* sp.) have never been explored for biocontrol of fungal diseases of chickpea. Our study illustrates
the promise of *Mesorhizobium ciceri* based biofilms with *T. viride* having both the potential of plant growth promotion and biocontrol of plant pathogens. Biofilmed inoculant having dual attributes can therefore be a novel means of contributing towards increased plant growth and productivity in an environment-friendly manner.
*Rhizobium* based formulations for biocontrol of soil borne fungal pathogens of chickpea

**Abstract**

Chickpea (*Cicer arietinum* L.) is an important legume food crop grown worldwide and plays significant role in restoring soil fertility and sustainable agriculture. However, it is prone to diseases caused by several fungal pathogens which reduce the crop yield. In a pursuit to identify *Rhizobium* cultures that are efficient for nitrogen fixation and can inhibit some of the soil borne fungal pathogens, 150 chickpea specific *Mesorhizobium ciceri* isolates developed through isolation from the root nodules and procured from culture collection, HAU, Hisar were screened against *Fusarium oxysporum* f. sp. *ciceri*, *Ascochyta rabiei*, *Botrytis cinerea*, *Sclerotium rolfsii* and *Macrophomina phaseolina* for their antifungal activity. Among them, 10 isolates showed good antagonistic activity and percent inhibition of fungal mycelia ranged from 5-49 %. Promising cultures also exhibited important anti-fungal and plant growth promoting traits such as production of HCN, ammonia, IAA and phosphate solubilisation. In pot experiment, all the cultures having antagonistic potential showed good nodulation and nitrogen fixing ability. *M. ciceri* isolate A13 followed by CR24 were significantly superior with regards to nodule numbers, nitrogen fixing potential and dry weight of nodules, roots and shoots. In a field trial also seed treatment with rhizobial isolates A13 and CR24 improved different plant growth parameters and yield. Based on the above data two cultures *M. ciceri* isolate A13 and *M. ciceri* isolate CR24 were identified as most promising and used for further studies.

*Rhizobium* based biofilms using isolate A13 or isolate CR24 as partners and *Trichoderma viride* as fungal matrix were developed. The medium and culture conditions were optimized to produce higher biomass of biofilms. Jensen’s N free medium amended with 1% yeast extract, sequential inoculation of the cultures and incubation under static conditions after inoculation of fungus for 14 days yielded maximum biomass of biofilm. These biofilms showed 9 to 11% increase in antifungal activity towards *Fusarium oxysporum* f. sp. *ciceri* compared to respective rhizobial isolates. These biofilms also exhibited significant enhancement in several plant growth promoting attributes like seed germination, production of ammonia,
IAA and phosphate solubilisation, when compared to their individual partners. The enhancement in PGP activities indicates positive interactions among the partners. The effect of selected *M. ciceri* cultures individually and their respective biofilms on growth parameters of chickpea under pathogen challenged soil illustrated that the biofilms performed at par with the *M. ciceri* strains for most of the plant biometrical and disease related attributes.

Elicitation of defense related enzymes like L-phenylalanine ammonia lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO) was found to be higher in *M. ciceri* isolates or their respective biofilms treated plants as compared to uninoculated plants under pathogen challenged soil. Induction of pathogenesis related proteins like chitinase and β 1, 3- glucanase was also observed. Profiling of phenolics in root and shoot of *M. ciceri/biofilm* treated plants revealed significant accumulation of tannic, ferulic, salicylic, and t-cinnamic acids as compared to uninoculated plants. Larger accumulation of isoflavonoids like genistein, formononetin and biochanin A and organic acids like malic, succinic, propionic and formic acid was also observed in plants inoculated with rhizobia or their biofilms as compared to uninoculated plants, which signifies better induction of plant defense mechanisms under pathogen challenge. This study reveals the potential of *Rhizobium-Trichoderma viride* biofilms to be used as inoculant having dual purpose of nitrogen fixation and antifungal activity.
चने की फसल के मूदाजनित फफूंद रोगजनकों के जैवनियंत्रण हेतु
राइजोबियम आधारित फॉर्मलशंस
सारांश
चना (सयसर अरियकिलुम) विश्व भर में एक महत्त्वपूर्ण दलहन खाद्य फसल है और इसकी मिठी की उर्वरता सुधारने तथा स्वास्थ्य कृप्ति में महत्त्वपूर्ण भूमिका है। तथापि अनेक फफूंद रोगजनकों से रोग प्रुत्त होने के कारण फसल उत्पाद घट जाता है। ऐसे राइजोबियम बैक्टेरिया जो नत्रजन स्थरीकरण में दक्ष/कुशल तथा मूदाजनित फफूंद रोगजनकों के नरीधन करने में सक्षम हों की पहचान करने के लक्ष्य से १५० चना विशेष राइजोबियम (मिजोराइजोबियम सीसेरी) वियोजन का उपयोग रोगजनकों से पूर्वीकरण विधि तथा हिसार कृप्ति विश्वविद्यालय, हिसार के कल्चर संग्रह से उपार्जित कर दिया गया। इन की सूंख्य क्षमिकर इंजन आक्रायोमॉल्स एंफ प्रजाति सीसेरी, अस्कोकायेटा रबिईइ, बोट्रिक्स सिनेरेया, स्कलेरोहटयम रोलफ्सी, एवं मक्रोफोमीना फासेओलोमलना के प्रति फफूंद रोग निरोधन गुण के लिये की गयी। इन में से १० वियोजनों द्वारा उच्च प्रतिरोधिता प्रदर्शित की गई तथा फफूंद तंत्रों का ५ - ४९% प्रवीण था। आशाजनक वियोजनों द्वारा महत्त्वपूर्ण फफूंदरोधी तथा पादप वृधिकारक लक्षण उद्धारणार्थ अमोलिया, हाइड्रोजन साइनाएड, इण्डोल एम्सहटक अम्ल का उत्पादन व फास्फोरस पुलनशील करने की विशेषता दर्शाई गयी। गमला विश्व परिस्थिति में सभी फफूंदरोधी वियोजनों द्वारा उच्च जडग्रूंथ विकास तथा वायुमंडल की नाइट्रोजन स्थरीकरण क्षमता प्रदर्शित की गई। मिजोराइजोबियम सीसेरी ए १३ तथा उसके बाद सी आर २४ वियोजन उल्लेखनीय रूप से जड ग्रूंथियाँ की संख्या, नाइट्रोजन स्थरीकरण क्षमता, जड ग्रूंथियाँ का शुष्कभार, जडों व तनों/ टहनी के विकास में सक्षम पाए गए। मिजोराइजोबियम सीसेरी ए १३ तथा सी आर २४ वियोजन उल्लेखनीय रूप से जड ग्रूंथियाँ की संख्या, नाइट्रोजन स्थरीकरण क्षमता, जड ग्रूंथियाँ का शुष्कभार, जडों व तनों/ टहनी के विकास में सक्षम पाए गए। इन परिक्षणों के आधार पर मिजोराइजोबियम सीसेरी ए १३ तथा सी आर २४ वियोजन समृद्ध आशाजनक पाये गये तथा इन का उपयोग विस्तृत अध्ययनों के लिये किया गया।
ट्राइकोडरमा विरिडी की फफूंद आव्यूह में राइजोबियम बैक्टेरिया वियोजन ए १३ अथवा वियोजन सी आर २४ का उपयोग सहयोगी के रूप में कर बायोफिल्म विकसित की गई। बायोफिल्म के उच्च बायोमास उत्पादन हेतु आदर्श/ इष्टिमाध्यम व संवर्धन परिस्थितियों की फसलन की गई। एक प्रतिशत(1%)यीस्ट एक्स्ट्रेक्ट युक्त जेनसेन नाइट्रोजन विनियन संवृद्धि माध्यम में फफूंद के डीकारण के १४ दिन उपरांत
राइजोबियम बैक्टेरिया वियोजन का क्रमबद्ध संचारण तथा स्थिर उत्पादन अवस्था में अधिकतम बायोफिल्म बायोमास प्राप्त हुआ। इन बायोफिल्मों द्वारा ए 43 तथा सी आर 24 वियोजन की तुलना में फ़्रैज़रियम आक्सीस्पोरियम एफ़ प्रजाति सीसेरी के प्रति क्रमशः ६% - २६% अधिक फफूंद रोधिता दर्शाई गयी। इन बायोफिल्मों द्वारा एकल साथी ए 43 तथा सी आर 24 वियोजन की तुलना में अनेक पादप वृद्धिकारक गुणों जैसे बीज अनुकूलण, अमोनिया उत्पादन, इलेक्ट्रोड एसिटिक अम्ल का उत्पादन व फास्फोरस घुलनशील करने की क्षमता में महत्वपूर्ण वृद्धि दर्शाई। पादप वृद्धिकारक गुणों में बढ़ोतरी बायोफिल्म के घटकों/ साथियों में धनात्मक अन्योन्यक्रया का संकेत देता है।

फफूंद रोगग्रस्त मृदा में चयनित मिजोराइजोबियम सीसेरी वियोजन का एकल रूप से व उनकी बायोफिल्मों द्वारा उपचारित चने की फसल पर परिक्रमण से स्पष्ट हुआ कि अधिकांश पादप जीव मापी उ रोग संबंधी गुणों हेतु बायोफिल्मों का प्रदर्शन मिजोराइजोबियम सीसेरी प्रजातियों के समस्त मृदा में मिजोराइजोबियम सीसेरी वियोजन का एकल रूप से अथवा उनकी क्रमशः बायोफिल्मों द्वारा उपचारित चने के - पौधों में रक्षामूलक उत्प्रेरक/ एंजाइम जैसे अल्फा-फिनाइलअलानीन अमोनियालायेस(पाल), पेरोक्सिडेस व पालीफिल्लाओक्सिडेस की मात्रा अन उपचारित चने के - पौधों के तुलना में अधिक था। रोगग्रस्त प्रोटीनों जैसे काइटिनेस व बीटा १-३ ग्लूकेस का विप्रेरर्न भी देखा गया। बायोफिल्मों द्वारा उपचारित चने के जड़ व तनी/ तहरी की फिनोलिक प्रोफाइल में टेनिक, फेरुलिक, सलीसाईलिक तथा टी- सिनाइक अम्ल अन उपचारित चने के - पौधों के तुलना में अधिक एकत्र था। आइसो प्लेवोलोइड्स जैसे की जनिस्तेंन, फोरमोनों नीतिन व बायोचार्मीन ए तथा कार्बनिक अम्ल जैसे की मैलिक, सुक्राशिक, प्रोपोइनिक, फोममैन अम्ल का संचय भी मिजोराइजोबियम सीसेरी प्रजातियों अथवा उनकी बायोफिल्मों द्वारा उपचारित चने की फसल में अन उपचारित चने के - पौधों के तुलना में अधिक था। यह दर्शाता है कि रोगग्रस्त की चुनौती में रक्षा तन्त्र का बेहतर विप्रेरर्न हुआ है। इस परिक्रमण से स्पष्ट है कि राइजोबियम- ट्राइकोडरमा विरिडी़ की बायोफिल्म टीका का उपयोग नाइट्रोजन स्थिरीकरण व फफूंदरोधी क्रिया के दोहरे कार्य के लिये किया जा सकता है।


Fig. 2.1 Diverse roles of *Rhizobium*-Legume association
Fig. 2.2 Potential mechanisms involved in biocontrol of fungal pathogens by *Rhizobium*
**Fig. 3.1** Distribution of rhizobial isolates from different sources

**Fig. 3.3** Growth of chickpea plants in hydroponics assembly for collection of shoot and root extract after 10 days of sowing
Fig. 4.1 Antifungal activity exhibited by *Mesorhizobium* isolates against different pathogenic fungi. (A, a) *Fusarium oxysporum* f. sp. *ciceri* (AFC-1); (B, b) *Sclerotium rolfsii* (CR5518); (C, c) *Botrytis cinerea* (BC6530)
**Fig. 4.2** HCN production by the *Mesorhizobium* isolates. (A) Control; (B) Isolate CR18

**Fig. 4.3** Production of IAA by *Mesorhizobium* isolates. Superscripts in the histogram, denoted by common letter are not significantly different at 1 % level of probability by Duncan’s Multiple Range Test (DMRT)
**Fig. 4.4** Phosphate solubilisation by the *Mesorhizobium* isolates in Pikovskaya medium after 48 h

**Fig. 4.5** Screening of selected *Mesorhizobium* isolates for nodulation and nitrogen fixing efficiency at National Phytotron Facility after 45 days after sowing
Fig. 4.6 Field evaluation of ten selected rhizobial isolates
Fig. 4.7 Compatibility test of *Rhizobium* cultures with *Trichoderma viride* in dual culture plate assay
Fig. 4.8 Biofilm development in different medium

Fig. 4.9 Optimization of medium for biofilm development
Fig. 4.10 Development of *Rhizobium* based biofilmed formulation using *Trichoderma viride* as fungal matrix. Biofilm growth at (A) Day 2; (B) Day 7; (C) Day 14. (D) Harvested biofilm after 14 days of incubation.
Fig. 4.11 Microphotographs of biofilms showing attachment of bacteria to *T. viride* maycelia
Fig. 4.12 Percent germination of chickpea seeds, as influenced by biofilms and their partners
Fig. 4.13 Antifungal activity shown by biofilms and its partners against *Fusarium oxysporum* f. sp. *ciceri*. (A) Control; (B) Isolate A13; (C) Biofilm 1 (*T. viride*-*Rhizobium* isolate A13); (D) Biofilm 2 (*T. viride*-*Rhizobium* isolate CR24)
Fig. 4.14 IAA production exhibited by individual cultures and biofilms
Fig. 4.1 Phosphate solubilisation exhibited by individual cultures and biofilms.

(A) Qualitative assay; (B) Quantitatively assay
**Fig. 4.16** Effect of biofilm and its partners on growth of chickpea plants challenged with wilt complex under greenhouse condition. (A) Absolute control (without *Rhizobium* or fungal inoculum); (B) Control without *Rhizobium* Inoculum; (C) *Rhizobium* isolate A13; (D) Biofilm 1 (*T. viride - Rhizobium* isolate A13); (E) *Rhizobium* isolate CR24; (F) Biofilm 2 (*T. viride - Rhizobium* isolate CR24)
Fig. 4.17 Role of biofilms and their partners in elicitation of different defense enzymes in chickpea plants. (A) PAL activity, (B) POX activity, (C) PPO activity. Treatments denote Rh1, isolate A13; Bf1, *T. viride*-isolate A13 biofilm; Rh2, Isolate CR24, Bf2, *T. viride*-isolate CR24 biofilm; Control, seeds without *Rhizobium* inoculation; Tv. treated, seeds treated with commercial formulation of *T. viride*; Chem. control, seeds treated with carbendazim (1.5g/kg seed); Ab. Control, without any *Rhizobium* or fungal challenge.
Fig. 4.18 (A) Standard curve for calculating Chitinase activity; (B) Chitinase activity exhibited by plants treated with Rhizobium and Rhizobium based biofilms
Fig. 4.19 (A) Standard curve for calculating $\beta$-1, 3-glucanase activity (B) $\beta$-1, 3-glucanase activity exhibited by plants treated with *Rhizobium* and *Rhizobium* based biofilms

\[ y = 0.0033x + 0.024 \]

\[ R^2 = 0.9807 \]
Fig. 4.20 Accumulation of phenolics in (A) shoot and (B) root extracts of *Rhizobium* or *Rhizobium* based biofilms inoculated plants, with or without the challenge of *Fusarium* wilt.
Fig. 4.21 Accumulation of flavonoids in (A) shoot and (B) root extracts of *Rhizobium* or *Rhizobium* based biofilms inoculated plants, with or without the challenge of *Fusarium* wilt
Fig. 4.22 Comparison of chromatograms of isoflavones detected through HPLC in root extract of (A) Plants without inoculation (Control); (B) Plants inoculated with *Rhizobium* isolate A13; (C) Plants inoculated with Biofilm1(*T. viride-Rhizobium* isolate A13). X-axis: Retention time in minutes, Y-axis: Absorption Units
Fig. 4.23 Profiling of organic acids in *Rhizobium* or *Rhizobium* based biofilms inoculated plants, with or without the challenge of *Fusarium* wilt. (A) Malic acid; (B) Succinic acid; (C) Propionic acid; (D) Formic acid
<table>
<thead>
<tr>
<th>Organism</th>
<th>Plant pathogen</th>
<th>Host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizobium sp. NBR19513</td>
<td>Fusarium spp.</td>
<td>Chickpea</td>
<td>Nautiyal (1997)</td>
</tr>
<tr>
<td></td>
<td>Rhizoctonia bataticola</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pythium sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizobium sp.</td>
<td>Macrophomina phaseolina</td>
<td>Soybean,</td>
<td>Omar and Abd-alla (1998);</td>
</tr>
<tr>
<td>Bradyrhizobium sp.</td>
<td></td>
<td>Sunflower,</td>
<td>Siddiqui et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Okra</td>
<td></td>
</tr>
<tr>
<td>Rhizobium meliloti</td>
<td>Macrophomina phaseolina</td>
<td>Ground nut</td>
<td>Arora et al. (2001)</td>
</tr>
<tr>
<td>Bradyrhizobium japonicum</td>
<td>Macrophomina phaseolina, Fusarium solani</td>
<td>Tomato</td>
<td>Siddiqui and saukat (2002)</td>
</tr>
<tr>
<td></td>
<td>Rhizoctonia solani</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizobium sp.</td>
<td>Fusarium solani f. sp. phaseoli</td>
<td>Bean</td>
<td>Estevez de Jensen et al. (2002)</td>
</tr>
<tr>
<td>Rhizobium sp.</td>
<td>Fusarium oxysporum f. sp. ciceri</td>
<td>Chickpea</td>
<td>Arfaoui et al. (2005)</td>
</tr>
<tr>
<td>Mesorhizobium loti MP6</td>
<td>Sclerotinia sclerotiorum</td>
<td>Indian mustard</td>
<td>Chandra et al. (2007)</td>
</tr>
<tr>
<td>Rhizobium leguminosarum bv. viceae</td>
<td>Pythium spp.</td>
<td>Pea, Lentil</td>
<td>Huang and Erickson (2007)</td>
</tr>
<tr>
<td>Rhizobium sp.</td>
<td>Sclerotium rolfsii</td>
<td>Ground nut</td>
<td>Ganesan et al. (2007)</td>
</tr>
<tr>
<td>Sinorhizobium frediiKCC5</td>
<td>Fusarium udam</td>
<td>Pigeon pea</td>
<td>Kumar et al. (2010)</td>
</tr>
<tr>
<td>Rhizobium leguminosorurum</td>
<td>Fusarium oxysporum f. sp. ciceri</td>
<td>Chickpea</td>
<td>Singh et al. (2010)</td>
</tr>
<tr>
<td>Ensifer meliloti, Rhizobium leguminosorum</td>
<td>Fusarium oxysporum</td>
<td>Fenugreek</td>
<td>Kumar et al. (2011)</td>
</tr>
<tr>
<td>Rhizobium sp.</td>
<td>Fusarium oxysporum, Fusarium solani,</td>
<td>Faba bean,</td>
<td>Shaban and El-Bramawy (2011)</td>
</tr>
<tr>
<td></td>
<td>Macrophomina phaseolina,</td>
<td>Chickpea,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rhizoctonia solani, Sclerotium rolfsii</td>
<td>Lupine</td>
<td></td>
</tr>
<tr>
<td>Rhizobium sp.</td>
<td>Cylindrocladium parasiticum</td>
<td>Soybean</td>
<td>Gao et al. (2012)</td>
</tr>
<tr>
<td>Rhizobium japonicum</td>
<td>Fusarium solani, Macrophomina phaseolina</td>
<td>Soybean</td>
<td>Al-Ani et al. (2012)</td>
</tr>
<tr>
<td>Rhizobium sp. RS12</td>
<td>Fusarium solani, Macrophomina phaseolina</td>
<td>Chickpea</td>
<td>Smitha and Singh (2014)</td>
</tr>
</tbody>
</table>
Table 4.1 Antifungal activity in terms of percent inhibition of mycelia by rhizobial isolates against different fungi in dual culture plate assay

<table>
<thead>
<tr>
<th>Isolate Number</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Foc</td>
</tr>
<tr>
<td>A3</td>
<td>21.43</td>
</tr>
<tr>
<td>A10</td>
<td>25.00</td>
</tr>
<tr>
<td>A13</td>
<td>35.71</td>
</tr>
<tr>
<td>A15</td>
<td>28.57</td>
</tr>
<tr>
<td>A16</td>
<td>28.57</td>
</tr>
<tr>
<td>CR9</td>
<td>10.71</td>
</tr>
<tr>
<td>CR14</td>
<td>10.71</td>
</tr>
<tr>
<td>CR18</td>
<td>7.14</td>
</tr>
<tr>
<td>CR20</td>
<td>32.14</td>
</tr>
<tr>
<td>CR24</td>
<td>32.14</td>
</tr>
<tr>
<td>LSD (p=0.01)</td>
<td>2.17</td>
</tr>
</tbody>
</table>

Foc: *Fusarium oxysporum* f. sp. *ciceri* (AFC-1); Ar: *Ascochyta rabiei* (AR4638); Sr: *Sclerotium rolfsii* (CR5518); Be: *Botrytis cinerea* (BC6530); Mp: *Macrophomina phaseolina*
Table 4.2 Characterization of antagonistic *Mesorhizobium* isolates for antifungal and plant growth promoting attributes

<table>
<thead>
<tr>
<th>Isolate Number</th>
<th>HCN production</th>
<th>Ammonia production #</th>
<th>Siderophore Production*</th>
<th>IAA Production*</th>
<th>Phosphate solubilisation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>A10</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>A13</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>A15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>A16</td>
<td>-</td>
<td>++++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CR9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CR14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CR18</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>CR20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>CR24</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>

*Degree of activity (+++ > ++ > +)*

# Colour, ranged from yellow (+) to dark brown (++++)
**Table 4.3** Effect of antagonistic *Mesorhizobium* isolates on growth parameters, nodulation and nitrogen fixing efficiency in chickpea plants under greenhouse condition at 45 days after sowing

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Length (cm)</th>
<th>Dry weight (g)</th>
<th>Nodule number</th>
<th>ARA (µ moles ( \text{C}_2\text{H}_4 ) produced/gram dry weight of nodule/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot</td>
<td>Root</td>
<td>Shoot</td>
<td>Root</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CR9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CR14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CR18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CR20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CR24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F75</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*ND= Not Detected*
Table 4.4 Field evaluation of antagonistic *Mesorhizobium* isolates at 45 days after sowing

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot fresh weight (g)</th>
<th>Root fresh weight (g)</th>
<th>Yield (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.53</td>
<td>15.81</td>
<td>538.15</td>
</tr>
<tr>
<td>A3</td>
<td>46.11</td>
<td>16.72</td>
<td>870.15</td>
</tr>
<tr>
<td>A10</td>
<td>42.07</td>
<td>14.72</td>
<td>722.89</td>
</tr>
<tr>
<td>A13</td>
<td>53.24</td>
<td>18.65</td>
<td>1017.40</td>
</tr>
<tr>
<td>A15</td>
<td>47.81</td>
<td>18.60</td>
<td>856.76</td>
</tr>
<tr>
<td>A16</td>
<td>41.86</td>
<td>16.91</td>
<td>763.05</td>
</tr>
<tr>
<td>CR9</td>
<td>28.87</td>
<td>13.55</td>
<td>591.70</td>
</tr>
<tr>
<td>CR14</td>
<td>31.31</td>
<td>15.97</td>
<td>512.31</td>
</tr>
<tr>
<td>CR18</td>
<td>42.40</td>
<td>16.16</td>
<td>923.69</td>
</tr>
<tr>
<td>CR20</td>
<td>46.60</td>
<td>17.69</td>
<td>947.79</td>
</tr>
<tr>
<td>CR24</td>
<td>47.36</td>
<td>19.79</td>
<td>963.86</td>
</tr>
<tr>
<td>F75</td>
<td>48.52</td>
<td>18.05</td>
<td>950.47</td>
</tr>
<tr>
<td>LSD (p=0.01)</td>
<td>8.49</td>
<td>2.26</td>
<td>208.67</td>
</tr>
</tbody>
</table>
### Table 4.5 Optimization of biofilm formation using different medium

<table>
<thead>
<tr>
<th>Medium</th>
<th>Biofilm fresh weight (g)</th>
<th>Biofilm dry weight (g)</th>
<th>Population count (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bacteria</td>
</tr>
<tr>
<td>NB</td>
<td>10.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.10x10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Piko</td>
<td>10.34&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.20&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.62x10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>YEM</td>
<td>19.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.50&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.78x10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>YPG</td>
<td>23.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.31x10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>JEN</td>
<td>22.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9 x10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>JEN+1% Yeast extract</td>
<td>28.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.20x10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>LSD (0.01)</td>
<td>1.23</td>
<td>0.560</td>
<td></td>
</tr>
</tbody>
</table>

Details of medium NB, Nutrient Broth; Piko, Pikovskaya medium; YEM, Yeast Extract Manitol broth; YPG, Yeast Extract Peptone broth, JEN, Jensen’s medium; JEN+1% yeast extract.
<table>
<thead>
<tr>
<th>Treatments</th>
<th>Antifungal activity against <em>Fusarium oxysporum</em> f. sp. <em>ciceri</em> (%) inhibition</th>
<th>HCN production *</th>
<th>Ammonia Production #</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td><em>Rhizobium</em> isolate A13</td>
<td>32.50</td>
<td>++</td>
</tr>
<tr>
<td>T2</td>
<td><em>Rhizobium</em> isolate CR24</td>
<td>30.14</td>
<td>++</td>
</tr>
<tr>
<td>T3</td>
<td><em>Trichoderma viride</em></td>
<td>26.57</td>
<td>+</td>
</tr>
<tr>
<td>T4</td>
<td>Biofilm1</td>
<td>35.71</td>
<td>++</td>
</tr>
<tr>
<td>T5</td>
<td>Biofilm2</td>
<td>33.33</td>
<td>+++</td>
</tr>
</tbody>
</table>

*Degree of activity (+ + + + + > + + + > + + > + > +)

# Colour, ranged from yellow (+) to dark brown (+ + + + +)

Table 4.6 Antifungal attributes exhibited by biofilms and their individual partners
Table 4.7: Effect of biofilms and their partners on growth of chickpea plants under fungal challenge

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DII (%)</th>
<th>Shoot length (cm)</th>
<th>Shoot weight (g)</th>
<th>Root length (cm)</th>
<th>Root weight (g)</th>
<th>Nodule dry weight (g)</th>
<th>ARA (μmol C₃H₇/gram dry weight of nodule/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizobium1 (A13)</td>
<td>5.5</td>
<td>26.13 ± 0.67</td>
<td>9.16 ± 0.25</td>
<td>14.61 ± 0.20</td>
<td>5.08 ± 0.15</td>
<td>0.35 ± 0.01</td>
<td>3.17 ± 0.04</td>
</tr>
<tr>
<td>Biofilm 1</td>
<td>10</td>
<td>24.32 ± 0.46</td>
<td>8.55 ± 0.15</td>
<td>13.30 ± 0.26</td>
<td>4.43 ± 0.14</td>
<td>0.28 ± 0.01</td>
<td>2.80 ± 0.02</td>
</tr>
<tr>
<td>Rhizobium2 (CR24)</td>
<td>8.3</td>
<td>24.30 ± 0.62</td>
<td>8.26 ± 0.10</td>
<td>13.34 ± 0.50</td>
<td>4.64 ± 0.12</td>
<td>0.24 ± 0.02</td>
<td>2.10 ± 0.01</td>
</tr>
<tr>
<td>Biofilm 2</td>
<td>11.11</td>
<td>22.77 ± 0.38</td>
<td>7.87 ± 0.08</td>
<td>11.70 ± 0.26</td>
<td>3.57 ± 0.07</td>
<td>0.21 ± 0.01</td>
<td>1.94 ± 0.02</td>
</tr>
<tr>
<td>Control (without Rhizobium)</td>
<td>19.4</td>
<td>14.27 ± 0.30</td>
<td>4.17 ± 0.35</td>
<td>7.24 ± 0.22</td>
<td>2.48 ± 0.15</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>T. viride treated seed</td>
<td>13.8</td>
<td>19.47 ± 0.30</td>
<td>7.43 ± 0.14</td>
<td>8.49 ± 0.17</td>
<td>3.40 ± 0.04</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chemical control</td>
<td>10</td>
<td>18.49 ± 0.29</td>
<td>7.37 ± 0.10</td>
<td>8.66 ± 0.15</td>
<td>3.43 ± 0.10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Absolute Control (without Rhizobium and fungal inoculum)</td>
<td>0.0</td>
<td>21.48 ± 0.70</td>
<td>8.38 ± 0.13</td>
<td>10.50 ± 0.10</td>
<td>3.48 ± 0.03</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not Detected; Means in the columns followed by same superscript letters indicate no significant difference (p = 0.05) by Duncan’s multiple range test.