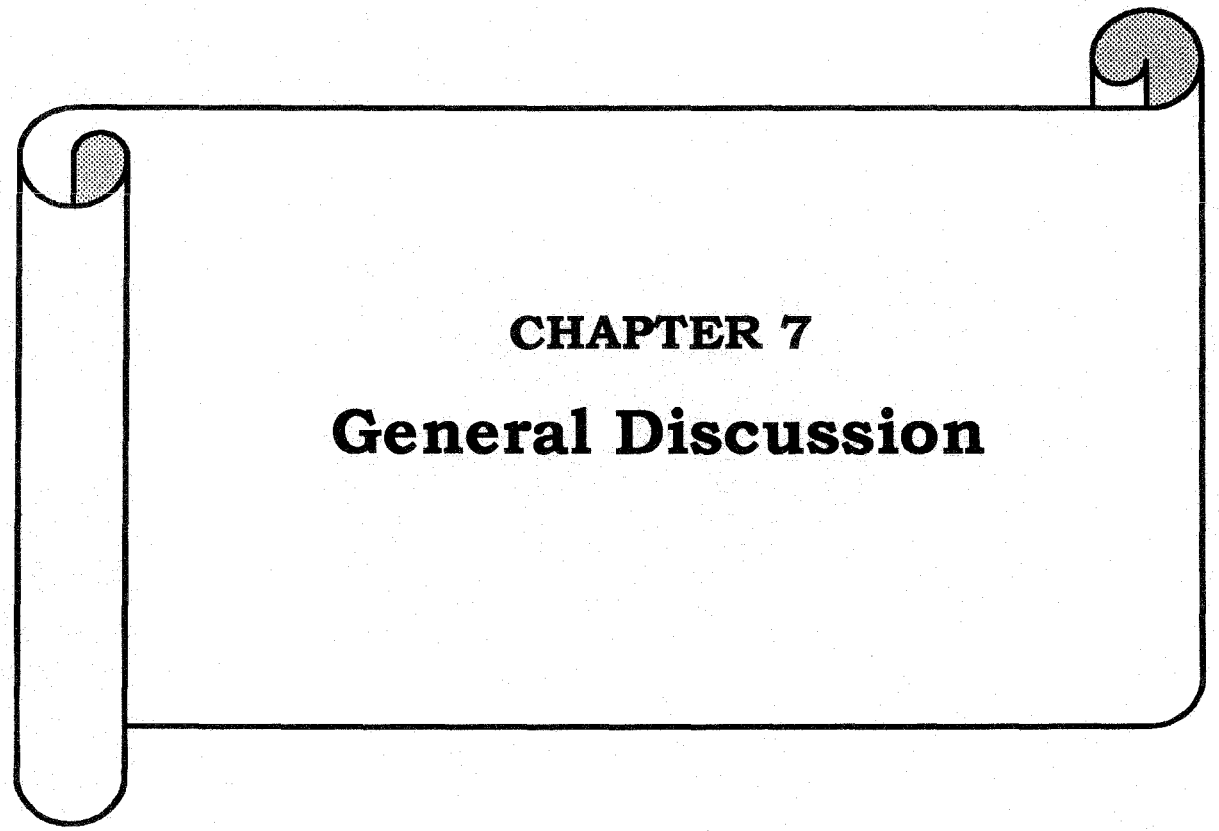

CHAPTER 7

General Discussion

GENERAL DISCUSSION

The ability of soil inhabiting microorganisms to inhibit the growth or metabolic activity of plant pathogens has been studied intensively during the last three decades and continues to inspire research in many fields, such as drug discovery and crop protection (Scher and Baker, 1986; Chet et al., 1990; Boer et al., 1999; Cazorla et al., 2006; Ramette et al., 2011). Biological control of deleterious microbes especially fungi by introducing antagonistic microorganisms onto plant surfaces has been the focus of considerable research partly due to the need to minimise the use of hazardous chemical pesticides or fungicides and thereby enhance the sustainability of agriculture and horticulture and also because biocontrol may provide control of plant diseases that cannot, or only partially, be managed by other strategies (Duffy et al., 2003; Compant et al., 2005).

In the present study, twenty different siderophore producing bacterial strains was isolated from the rhizosphere of 12 different plants exhibiting broad spectrum antifungal activity against several fungal pathogens which include the soil borne pathogens, *Fusarium solani*, *F. oxysporum*, *F. graminearum* and *Rhizoctonia solani*. The rhizosphere region is heavily populated by a wide array of microorganisms which include both beneficial and harmful ones. The rhizosphere is the first-line defence for roots against attack by pathogenic fungi (Weller, 1988). Therefore, there is an excellent opportunity to find rhizosphere-competent bacteria in the rhizosphere which are potential biocontrol agents (Chet et al., 1990). Most studies on the biocontrol of plant pathogens focus on a multitude of factors related to the microbial antagonist, that is, recovery of appropriate strains from the rhizosphere, correct identification of the strain, how the isolated antagonists affect pathogens; which mechanisms or metabolites are involved and how far the antagonists can function in the specific environment. Consequently, substantial progress has been made in the identification of microbes involved in suppressing plant pathogens, and in identifying microbial traits that contribute to disease suppression and the competence of introduced strains in biological control of diseases in green house and field conditions.

16s rRNA gene remains an important diagnostic marker for prokaryote identification. However, valid species definitions require phenotypic description. While many recent studies describe molecular characterization of prokaryotes for the purpose of phylogenetic analysis, a concerted effort is underway to use this molecular target for routine identification of pathogens microbiology laboratories and to rapidly characterize those organisms that are recalcitrant to identification because of fastidious growth requirements or unusual biochemical patterns (Kolbert and Persing, 1999). However, just as all bacteria can be described with a powerful common framework of their 16S rRNA gene or genomic DNA sequences, it would also be highly desirable and productive to describe all bacteria by their phenotypes, which reflects their physiology (Bochner, 2009). Growth phenotypes are directly and intimately involved in fundamental aspects of cellular genome and organism evolution and they remain a cornerstone of microbial taxonomy (Bochner, 2009).

For identification of the present strains, a polyphasic approach was adopted that included both phenotypic and genotypic studies. The phenotypic studies included a study of the cell size and morphology under microscope and culture morphology in growth media. Additionally, an array of biochemical tests was conducted to characterize the strains by their phenotypes. In the Phylogenetic approach, 16S rRNA gene sequences of all the isolates were determined. Results of all these studies led to the recognition of the isolates; all were identified to the genus level and nine bacteria were identified to the species level. A wide range of bacteria were recovered from the rhizosphere soil which included *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Citrobacter*, *Enterobacter*, *Klebsiella* and *Serratia*. An attempt was made to phylogenetically analyse the seven *Pseudomonas* isolates; these were *P. fluorescens* (one isolate), *P. putida* (two isolates) and the rest were maintained as *Pseudomonas* sp. Despite the efforts, the species of all the strains could not be ascertained. Further analysis with other phylogenetic markers such as *rpo B*, *rpo D* or *gyr B* may help to determine their identity (Ramette et al., 2011).

A successful biocontrol agent should be efficient in suppressesing the pathogen and reduce disease incidence significantly. Biocontrol agents act against pathogens mainly through the process of antagonism in the form of competition, antibiosis and parasitism (Chet et al., 1990; Yasmin et al., 2009; Werra et al., 2009). The activity is not restricted to only one of these, and, in fact, a combination of mechanisms acts in concert in an efficient biocontrol process (Cazorla et al., 2006; Gupta et al., 2006; Bano and Musarrat, 2002; Garbeva et al., 2004; Duffy and De Fago, 1999). Competition between the biocontrol bacteria and the pathogen can lead to dislodgment of the pathogen (Ligon et al., 2000; Weller et al., 2006; Gupta et al., 2002; Kamilova et al., 2008). Experimental evidences in many studies could find a direct correlation between the *in vitro* activities and the biocontrol action in plants (Maurhofer et al., 1994; Jagdeesh et al., 2001). However, some authors reported that production of lytic enzymes, antibiotics or siderophores or even *in-vitro* antagonism could not be linked to disease suppression (Ongena et al., 1999; Pandey et al., 2000; Ahmadzadeh et al., 2006). Microorganisms compete with each other for food and essential elements in the soil (Schippers et al., 1987; Cazorla et al., 2006; Validov et al., 2009). The availability of iron for assimilation by microorganisms in the rhizosphere environment is extremely limiting (O'Sullivan and O'Gara, 1992). Since almost all living organisms require iron for growth, survival in a heterogeneous environment such as the rhizosphere depends largely on the ability to scavenge sufficient iron from a limiting pool. Siderophores mediate the limited amount of iron in the rhizosphere, deprive pathogens of iron and suppress their growth. Many reports have been published showing siderophore involvement in the suppression of plant pathogenic fungi (Loper and Buyer, 1991; Ongena et al., 1999; Saikia et al., 2005; Sayyed et al., 2005; Sayyed and Patel, 2011; Bholay et al., 2012).

The present antagonistic strains were capable of producing multiple extracellular lytic enzymes such as chitinase, protease and lipase and also exhibited biofertilizer traits such as production of the plant growth hormone IAA and the phosphate solubilising enzyme. Most of them recorded robust

siderophore production by the CAS shuttle assay. The *Pseudomonas putida* strain AS04 in particular was able to produce chitinase, protease and lipase and was also recorded chitinase and phosphatase activity. Besides it showed maximum siderophore production among all isolates. Enzymatic dissolution of cell walls leading to loss of fungal protoplasm is one of the main antagonistic mechanisms involved in the activity of biocontrol agents (Lim et al., 1991; Kim and Chung, 2004). Hence, these observations together with electron microscopic evidences suggest that *P. putida* AS04 has excellent potential to act as biocontrol agent that not only limit pathogen proliferation but also promote plant growth.

Since siderophore production was the common antagonistic property of all the strains, the siderophores from each strain was chemically characterized using standardized protocols. Of the 20 isolates, 15 strains were found to produce hydroxamate type of siderophore. Siderophores are considered to be one of the major contributory factors towards the biocontrol action of the antagonistic bacteria because apart from depriving the fungal pathogens of iron and thereby limiting their growth; these molecules also supply iron to the plants and aid in plant growth promotion (Shoda, 2000). The conditions required for maximum siderophore production *in vitro* was optimized for further purification of the siderophore from the strain *P. putida* AS04. Results revealed that siderophore production increased from 59.70 to 89.04% units on addition of 1% sucrose and 0.1% $(NH_4)_2SO_4$ to the original Fiss-glucose minimal media. The production of siderophore began after 8-9 hours and reached maximum after 30 hours resembling secondary metabolite production, which is produced during later stages of growth. The optimum temperature was recorded to be 30°C. For siderophore purification, the strain AS04 was cultured in bulk under the optimized conditions and the acidified culture supernatant was passed through XAD-2 column and the bound siderophore was eluted with methanol. The concentrated siderophore was then passed through a Sephadex LH-20 hydrophobic column and the siderophore containing fractions were pooled and concentrated. This partially purified preparation of siderophore was

subjected to spectral scan and a peak at 430nm indicated that the siderophore was trihydroxamate type.

A literature survey on the use of bacterial inoculums in suppression of plant diseases reveal several reports that have warned against associating *in vitro* inhibition with *in vivo* activity (Paulitz and Loper, 1991; Loper and Buyer, 1991; Ongena et al., 1999). Therefore *in vivo* demonstration of disease control is a prerequisite to establish a potential strain as a biocontrol agent. Brinjal is a major crop grown in sub-Himalayan West Bengal and disease problems are many. Wilt and root rot caused by *Fusarium solani* is one of the major factors that limit brinjal production (Chakraborty and Chatterjee, 2007, 2008; Joseph et al., 2008; Akhtar et al., 2010). Two most potential strains isolated during the present study, namely, AS01 and AS04 both of which were identified as *P. putida* was used for the biocontrol experiments. Before the *in vivo* study, the pathogenicity of the *F. solani* strain was confirmed through the verification of Koch's postulates and growth inhibition studies along with growth kinetic studies of AS01 and AS04 were conducted to evaluate the fungal inhibition in liquid culture. Severe retardation of mycelia growth was found in dual cultures in PDB. *In vivo* biocontrol experiments in brinjal seedlings revealed 73.5% reduction in disease incidence in PPL variety on direct soil application of *P. putida* isolates under sterile conditions. The strain AS04 showed higher disease suppression than AS01 in controlling pathogen infection although the *in vitro* experiments of dual culture in liquid medium did not show any significant difference between these strains in inhibiting the growth of *F. solani*. Lack of correlation between *in vitro* inhibition and biocontrol has been documented in literature (De Boer et al., 2007).

It has long been known that the management of iron availability in the rhizosphere environment, through competition for iron can induce suppressiveness to diseases caused by soil borne pathogens (Scher and Baker, 1982). In the present study it was demonstrated that a particular *Pseudomonas putida* strain AS04 with robust siderophore producing ability was able to suppress wilt caused by *Fusarium solani* in brinjal. The bacteria

possessed multiple plant protecting and plant growth promoting traits such as production of chitinase, protease, lipase, phosphatase and plant growth hormone IAA. But how far these properties are responsible in disease suppression in plants remained unknown. Future studies in determining the exact role of these enzymes and siderophore in limiting pathogen proliferation is warranted.