2

Review of Literature

Stevia rebaudiana, one of the 950 genera of the Asteraceae family is a genus of more than 200 species (De Oliveira *et al*., 2004). Of this, only *S. rebaudiana* gives the sweetest essence (Savita *et al*., 2004). *Stevia* is composed of several natural, heat-stable steviol glycosides whose intensities of sweetness and flavour profiles differ from each other and vary according to concentration and environment (Geuns, 2003).

Stevia cultivation is affected by several constraints of which root rot disease caused by *Sclerotium rolfsii* (Kamalakannan *et al.,* 2007) and leaf spot disease caused by *Alternaria alternata* are major concerns. Both the diseases reduce the yield of *S. rebaudiana* (Maiti *et al*., 2007b).

Management of these diseases by means of fungicides has been found to be effective in many Stevia growing areas. Extensive fungicide application incurs added costs to producers and leads to development of fungicide tolerant strains of the pathogen and reduces the biodiversity of soil microbes.

Development of fungicide resistance by the pathogen has encouraged the exploitation of non-chemical means of disease management. Some Plant Growth Promoting Rhizobacteria (PGPR) have the capacity to improve the ability of plants to ward off pathogenic infections. Use of biocontrol agents has been shown to be eco-friendly and effective against many plant pathogens. For instance, *Pseudomonas* species and *Bacillus* species were known to induce systemic resistance in a variety of phytopathogens. Amongst the rhizosphere microorganisms, the Plant Growth Promoting Rhizobacteria (PGPR) has been considered important in sustainable agriculture due to their plant growth promotional ability as well as biocontrol potential. PGPR has emerged to be the biggest group of beneficial soil microorganisms involved in the control of a number

of plant diseases and pests by virtue of their ability to synthesize a wide range of antagonistic secondary metabolites. These ubiquitous microorganisms can be a significant component of management practices to achieve sustainable yields.

The review of literature pertaining to the study, "**Bio-management of Root rot and leaf spot diseases in *Stevia rebaudiana* using Plant Growth Promoting Rhizobacteria"** is discussed under the following heading:

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2.1. *Stevia rebaudiana*

Stevia rebaudiana is a South American plant basically from Paraguay and Brazil belonging to the family Asteraceae. Dr. M.S. Bertoni officially discovered the sweet herb Stevia in early 20th century (Kuntal *et al.,* 2007). It perennially grows upto 65 cm tall, with sessile, oppositely arranged lanceolate to oblanceolate leaves, serrate above the middle (Bhosle, 2004).

Natural sweeteners that can substitute for sucrose have caught great attention due to the growing incidence of obesity and diabetes. Stevia (*Stevia rebaudiana*) is an exotic plant in our country. It has both economical and medicinal importance. Now-a-days, it has become a major source of commercial sweetener for the growing natural food market. This plant has gained attention with the rise in demand for low-carbohydrate, low-sugar food alternatives. The sugar or sucrose is the most popular sweetener in the world. However, for adverse health effects of sucrose and known artificial sweeteners, interest in and search for no calorie natural sweeteners has been intensified in recent years. Very fortunately, stevioside was discovered which can satisfy the urge for sweet consumption of diabetic subjects.

The leaves of *Stevia rebaudiana* contain different steviol glycosides, the major constituent being stevioside. Stevioside is a diterpenoid glycoside, comprising of an aglycone (steviol) and three molecules of glucose. In addition to

the stevioside, several other sweet compounds such as steviobioside, rebaudioside A, B, C, D, E and ducoside A, have been isolated from *Stevia rebaudiana* (Seema, 2010).

Stevioside, an abundant component of *S. rebaudiana* leaf, has become well known for its intense sweetness (250-300 times sweeter than sucrose) and is used as a non-caloric sweetener in several countries. A number of studies have suggested that, besides sweetness, stevioside alongwith related compounds, which include rebaudioside A (second most abundant component of *S. rebaudiana* leaf), steviol and isosteviol (metabolic components of stevioside) may also offer therapeutic benefits, as they have anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumor, anti-diarrheal, diuretic and immunomodulatory actions (Chatsudthipong and Muanprasat, 2009).

2.2. Fungal pathogens of Stevia

2.2.1. *Sclerotium rolfsii*

Sclerotium rolfsii Sacc., is a soil-borne pathogen that commonly occurs in the tropics, sub-tropics and other warm temperate regions of the world causing root rot, stem rot, wilt and foot rot on more than 500 plant species including almost all the agricultural and horticultural crops (Fouzia and Saleem, 2009).

The fungus *S. rolfsii* produces abundant white fluffy, branched, septate mycelium with clamp connections only on the main hyphae, which spreads like a fan. Small white tufts are formed on mycelium which later gives rise to smooth, hard and dark brown sclerotia. Sclerotia may be spherical or irregular in shape and at maturity resemble the mustard seeds. These sclerotia survive in soil for long periods, frequently tolerating biological and chemical degradation due to the presence of melanin in the outer membrane. The disease caused by *S. rolfsii* is favored by moist soil conditions and high temperature 77° F. The fungus is spread through irrigation, water and by cultivation equipment (Sharma *et al*., 2002). *Sclerotium rolfsii* infects *Stevia rebaudiana* plants and causes a root rot disease (Kamalakannan *et al*., 2007).

2.2.2. *Alternaria alternata*

The genus *Alternaria* contains ubiquitous species of fungi, including aggressive and opportunistic plant pathogens affecting the majority of cultivated crops. One of the best-known and economically important members of the genus is *Alternaria alternata* (Fr.) Keiss. The fungus produces abundant, branched septate, brownish mycelia. Conidiophores are simple, olive-brown, septate, variable in length with terminal conidia, which were solitary or in short chains. Conidial characteristics from culture are similar to the conidia isolated from infected plants (Ramjegathesh and Ebenezar, 2012).

The genus *Alternaria* contains over 60 species including both parasites on living plants and saprophytes. Some important weed diseases caused by *Alternaria* species e.g., *A. eichhorinae* have been reported as a fungal pathogen on water hyacinth in Egypt, Sudan, Kenya and many other countries (Evans and Reeder, 2001). *Alternaria alternata* has been recorded as a saprophytic or a weak pathogen causing leaf spot on a number of crops (Umamaheswari *et al*., 2008).

In India, Maiti *et al*. (2007b) reported that *A. alternata* causes leaf spot disease in *S. rebaudiana*. The other crops affected by *A. alternata* include cotton, rough lemon glory lily, chickpea and *Aloe barbadensis* (Kamalakannan *et al*., 2008).

2.3. Diseases of *Stevia rebaudiana*

2.3.1. Root rot disease

Root rot caused by soil borne root infecting fungi is found in both indoor and outdoor plants, but more common in indoor plants with poor drainage. The roots of the plant rot usually as a result of overwatering. Soil borne fungal diseases are among the most important factors, limiting the yield of legume crops in many countries worldwide. Root rot caused by *Aphanomyces euteiches, R. solani, Fusarium* spp., *Sclerotium rolfsii* are the most destructive soil-borne diseases of pea, chickpea, lentil, faba bean and lupine (Ahmed *et al*., 2012).

A new root rot disease of Stevia caused by *Sclerotium rolfsii* has been observed in India during 2005. The symptoms appear as yellowing and dropping of

leaves, with wilting of plants and white cottony mycelial growth at collar regions. The mycelial growth spreads to the stem and root, with associated tissue rooting. The pathogen is characterized by cottony mycelial growth on the surface of the root with small (1-3 mm) spherical sclerotia that are brown (Kamalakannan *et al.,* 2007).

Chang *et al*. (1997) observed a stem rot disease of Stevia for the first time in India and identified the causal agent as *Sclerotinia sclerotiorum*. Megeji *et al.* (2005) recorded a stem rot disease in Stevia at Palampur, Himachal Pradesh, India by visual observation without confirming the pathogen.

2.3.2. Leaf spot disease

A leaf spot disease has been first reported in Canada caused by *Septoria steviae* and the disease is characterized by depressed, angular, shiny olive grey lesions, sometimes surrounded by a chlorotic halo, that rapidly coalesced (Reeleder, 1999).

In India, Maiti *et al*. (2007b) has made a first report of leaf spot disease caused by *Alternaria alternata* on Stevia plants. The symptom initially appears as small circular spots, light brown in colour. Later, many become irregular and dark brown to grey, while others remain circular with concentric rings or zones. On severely infected leaves, several spots coalesced to form large necrotic areas. On older leaves concentric spots are more common at the tips. Leaf spots varied from 2-18 mm in diameter.

2.4. Plant growth promoting rhizobacteria (PGPR)

The use of Plant Growth Promoting Rhizobacteria (PGPR) might be an alternative strategy to overcome the biological and environmental hazards posed by the persistent use of synthetic chemicals. The well known PGPR includes *Azospirillum, Azotobacter, Pseudomonas, Bacillus, Azoarcus, Klebsiella, Arthrobacter, Enterobacter, Serratia* and *Rhizobium*. Plant Growth Promoting Rhizobacteria (PGPR) are beneficial soil bacteria, which may facilitate plant growth and development both directly and indirectly. Direct stimulation may include providing plants with fixed nitrogen, phytohormones, iron that has been

sequestered by bacterial siderophores and soluble phosphate, while indirect stimulation of plant growth includes preventing phytopathogens (biocontrol) and thus, promoting plant growth and development (Saharan and Nehra, 2011).

PGPR perform some of these functions through specific enzymes, which provoke physiological changes in plants at the molecular level (Saleem *et al*., 2007). Colonization of the plant root system by PGPRs has been shown to reduce pathogen attack directly through production of antimicrobial substances (e.g. siderophores, β-1,3 glucanase, chitinases, antibiotics, and cyanidric acid), and through competition for space, nutrients and ecological niches. PGPRs also suppress pathogens indirectly through induction of systemic resistance (Mafia *et al*., 2009).

2.5. PGPR as biocontrol agents

Rhizobacterial strains have emerged as potential biocontrol agents for the control of root and foliar diseases of many crops (Earnapalli, 2005).

Slininger *et al.* (2003) have developed such indices, *viz.* relative performance index based on bioagent growth and their antagonistic activity under different conditions. In addition, use of different markers, *viz.* antibiotic production and other regulatory mechanisms by bioagents gained importance in rapid identification of biocontrol agents. This kind of screening and selection offers the system for successful development and commercialization of potential biocontrol agents.

2.5.1. *Pseudomonas fluorescens*

Fluorescent Pseudomonads belong to Plant Growth Promoting Rhizobacteria (PGPR), the important group of bacteria that play a major role in plant growth promotion, induced systemic resistance and biological control of pathogens and so on (Ganeshan and Kumar, 2005). Seed treatment with bacterial isolates like *P. fluorescens* significantly improves seedling stands and plant health and decreases severity of damping off disease compared to untreated seeds in pathogen infested soil (Sharma and Kaur, 2010).

Isolates of fluorescent Pseudomonads like *Pseudomonas fluorescens* P28 and P51 have been evaluated (alone and in combination) under greenhouse and field conditions for studying the efficacy in suppressing *Rhizoctonia* root rot incidence and promoting plant growth in chilli. The results indicate that *P. fluorescens* (P28) is the most effective in reducing disease incidence (Rini and Sulochana, 2006).

Sen *et al.* (2006) reported that in dual culture, significant growth inhibition of *Sclerotium rolfsii* by the strain *Pseudomonas* BRL-1 was observed. Mycelial growth is restricted near the bacterial streak. Increase in incubation period is proportional to growth inhibition of *S.rolfsii* upto six days. Microscopic study of mycelia from the interacting zone has shown hyphal shriveling, deformities like swelling, fragmentation, short branching and lysis.

The biocontrol agents *T. viride* (strains Tv1 and Tv13), *P. fluorescens* (Pf1 and Py15) and *B. subtilis* (Bs16) have been tested individually and in combination for their effectiveness against root rot of green gram caused by *Macrophomina phaseolina*. A combination of Pf1 and Tv1 is most effective in reducing root rot incidence under glass house and field conditions as compared with other single or combined treatments or the untreated control (Thilagavathi *et al.,* 2007). Five strains each of *P. fluorescens* and *Trichoderma* spp. has been found to suppress the root rot of black pepper caused by *Phytophthora capsici* (Diby *et al.,* 2005).

Talc formulation of two biocontrol agents *viz., B. subtilis* and *P. fluorescens* were evaluated against foliar diseases of urdbean. Their efficacy has been compared with that of commonly used fungicide under field conditions. The results revealed that *B. subtilis* and *P. fluorescens* are equally effective as fungicides in reducing the severity of *Cercospora* leaf spot and powdery mildew disease and enhancing the yield of urdbean (Raguchander *et al.,* 2005).

Maurya *et al.* (2008) has reported that application of plant growth promoting rhizobacterial (*P. fluorescens* strain 4 and *P. aeruginosa*) strains has shown high efficacy against collar rot of chickpea *in vitro* as well as in the field.

P. fluorescens and its formulation (corn starch, wheat bran and talc powder formulation) has a potential role in management of ear rot of maize caused by *Fusarium verticilliodes* (Nayaka *et al.,* 2008). Three native strains of *P. fluorescens* UP61.2, UP143.8 and UP148.2 provides effective control against soil-borne pathogens and is applied as potential control agents for *alfalfa* seedling diseases (Quagliotto *et al.,* 2009).

2.5.2. *Bacillus subtilis*

Soil-borne bacteria including species of *Azotobacter*, *Bacillus*, *Clostridium* and *Pseudomonas* have been shown to reduce soil-borne fungal diseases when applied as seed, soil or root inoculants (Ziedan *et al*., 2005). Rhizobacteria *P. fluorescens* CA 05, *P. putida* CA 28 and *Bacillus subtilis* CA 32 showed antagonism when tested singly and in combination for their biological control efficacy against *R. solani* and *S. rolfsii* under green house conditions. The *R. solani* and *S. rolfsii* populations are dramatically reduced after 30 days of transplanting in bacterial treated pots compared to the controls indicating the biocontrol ability of these rhizobacteria (Abeysinghe, 2009).

Bacillus subtilis ME488 has been shown to suppress wilt disease caused by *F. oxysporum* f.sp *cucumerinum* on cucumber when applied as a drench to germinating cucumber seeds. ME488 also suppresses the disease caused by *P. capsici* on red pepper (Chung *et al.,* 2010). *Bacillus subtilis* BN1 exhibits strong antagonistic activity against *Macrophomina phaseolina* and other phytopathogens including *Fusarium oxysporum* and *Rhizocotonia solani*. BN1 resulted in vacuolation, hyphal squeezing, swelling, abnormal branching and lysis of mycelia. Pot trial study resulted in statistically significant increase in seedling biomass besides reduction in root rot symptoms in chir-pine seedlings. These attributes of *Bacillus subtilis* BN1 verifies it as a potent biocontrol agent against root rot of chirpine (Singh *et al.,* 2008).

The inhibitory effect of *T. viride, T. harzianum, B. subtilis* and *P. fluorescens* against the incidence of faba bean root rot incidence is significantly higher than *T. hamatum* and *B. cereus* respectively (El- Mougy and Abdel-Kader, 2008).

Okigbo (2005) demonstrated that *Bacillus subtilis* from yam farm soil has the potential to control the rot of yam.

A mixture consisting of *P. fluorescens* Pf1 plus *B. subtilis* plus *T. viride* has been proved to be most effective in reducing onion leaf blight (*Alternaria palandui*) disease and in promoting plant growth and bulb yield in greenhouse and field tests (Karthikeyan *et al.,* 2008).

2.6. Characterisation of PGPR

2.6.1. Biochemical characterization

Various phenotypic and biochemical methods have been developed and used for characterizing fluorescent pseudomonad isolates. The genus *Pseudomonas* is characterized by gram-negative rod shaped aerobic cells and are associated with plants.

Ramamoorthy *et al*. (2002a) identified twenty isolates of fluorescent pseudomonads from the rhizosphere soil of different crops based on the biochemical characterization. All the isolates showed fluorescence on King's B medium and all the tested isolates did not grow at 41°C indicating that they did not belong to *P. aeruginosa*. Most of the bacterial isolates showed a positive response for gelatin liquefaction and trehalose utilization tests except two isolates. Hence, these two isolates are grouped under *P. putida* and the remaining 18 isolates have been classified as *P. fluorescens.*

Mallesh (2008) has identified 7 effective antagonistic bacteria as *Pseudomonas*. All these were gram negative, rod shaped and had the ability to produce water soluble yellow green pigment. Further, they fluoresced under UV were tested positive for gelatin liquefaction, casein hydrolysis, lipid hydrolysis, catalase activity, acid and gas production, hydrogen sulphide production, urease activity and grows at 41°C. They have showed negative response for spore formation, starch hydrolysis and growth at 4°C.

Two isolates of Plant Growth Promoting Rhizobacteria (PGPR) isolated from the rhizosphere soil of Pyrethrum (*Chrysanthemum cineraefolium*) were designated as MA-2 and MA-4 and identified as *Bacillus subtilis* and *Pseudomonas fluorescens* on the basis of cultural as well as biochemical testing (Mishra *et al*., 2010).

Thus, rapid identification of potentially and economically viable bioagents is possible through various methods of biochemical characterization (Weller *et al*., 2002).

2.6.2. Molecular characterization

P. aeruginosa can be confirmed by polymerase chain reaction based on molecular analysis of 16S rRNA amplicon size of ~ 622-bp on 1 per cent gel electrophoresis by using universal bacterial primers (Ayyadurai *et al.,* 2005). In another study, Aysun (2009) has confirmed the isolated bacteria as *Pseudomonas fluorescens* by polymerase chain reaction with universal bacterial primers such as E334F and E1115R and obtained an amplicon size of ~ 780 bp on 1 per cent gel electrophoresis. Geetha *et al*. (2007) has proved a strain of *Bacillus subtilis* VCRC B471 exhibiting mosquito larvicidal and pupicidal activity to be *B. subtilis* by polymerase chain reaction. PCR amplification of 16s rRNA gene of another strain of *B. subtilis* VCRC B471 yielded an amplicon of size -600 bp.

2.7. Screening and selection of PGPR strains

It has been suggested that microorganisms isolated from the root or rhizosphere of a specific crop adopted better to that crop and provide effective control of diseases than organisms originally isolated from other plant species. Such plant associated microorganisms serve as better biocontrol agents because they are already closely associated and adopted to the plant or plant part as well as to the particular environmental condition in which they are supposed to function. The screening of such locally adopted strains has yielded improved biocontrol strains in some cases (Khalid *et al.,* 2004).

Identification of effective antagonists strains represents only the first step towards the development of effective biological control. After identification of

several organisms as potential antagonists, it is advisable to continue to work with selected strains to determine the specific mechanisms, interactions, conditions and requirements responsible for effective biological control. Consummate understanding of these characteristics makes it possible to establish the limitations as well as the full potential of biocontrol to develop strategies for management and implementation.

Slininger *et al.* (2003) has developed such indices, *viz.* relative performance index based on bioagents growth and their antagonistic activity under different conditions. In addition, use of different markers, *viz.* antibiotic production and other regulatory mechanisms by bioagents gained the importance in rapid identification of biocontrol agents. This kind of screening and selection offer the system for successful development and commercialization of potential biocontrol agents.

Sen *et al.* (2006) has reported that in dual culture, significant growth inhibition of *Sclerotium rolfsii* by *Pseudomonas* BRL-1 has been observed. Mycelial growth is restricted near bacterial streak. Increase in incubation period is proportionate to growth inhibition of *S.rolfsii* upto six days. Microscopic study of mycelia from interacting zone showed hyphal shrivelling mycelial deformities like swelling, fragmentation, short branching, and lysis.

2.7.1. *In vitro* screening of *Sclerotium rolfsii* by *Pseudomonas fluorescens*

A number of strains of *P. fluorescens* suppress plant diseases by protecting the seeds and roots from fungal infection. This effect is the result of production of a number of secondary metabolites including antibiotics, siderophores and hydrogen cyanide. Competitive exclusion of pathogens as a result of rapid colonization of the rhizosphere by *P. fluorescens* may also be an important factor in disease control (O' Sullivan, and O'Gara, 1992).

El-Mougy and Abdul-kader (2008) screened some antagonistic fungal and bacterial agents against *Rhizoctonia solani, Fusarium solani and Sclerotium rolfsii* under *in vitro* conditions. The isolates of *T. viride, T. harzianum, B. subtilis and P. fluorescens* were found to have inhibitory effect which is significantly higher than

T. hamatum and B. cereus. Ten isolates of *P. fluorescens* obtained from leaf and fruit surfaces of apple were evaluated as potential biocontrol agents for the control of grey mold on apple under *in vitro* and *in vivo* conditions (Mikani *et al.,* 2008).

393 strains were tested against 8 fungal pathogens of groundnut including 5 necrotrophic fungi, *Aspergillus flavus*, *Aspergillus niger*, *Rhizoctonia bataticola*, *Rhizoctonia solani*, and *Sclerotium rolfsii* and 3 biotrophic fungi, *Cercospora arachidicola*, *C. personata* and *Puccinia arachidis*. *Pseudomonas* spp. GRS 175, *Pseudomonas aeruginosa* GPS 21, GSE 18, GSE 19, and GSE 30 and their cell-free culture filtrates were found to be highly antagonistic to all the test fungi (Kishore *et al.,* 2005).

Abeysinghe (2009) screened rhizobacteria, *P. fluorescens* CA 05, *P. putida* CA 28 and *B. subtilis* CA32 as biocontrol agents to control *S. rolfsii* and *R. solani* in *Capsicum annum.* All of them showed antagonism against *S. rolfsii* and *R. solani.*

Marine isolates of fluorescent Pseudomonads were studied for their siderophore production and antagonistic action against phytopathogenic fungi. Studies carried out *in vitro* revealed that purified siderophore and *Pseudomonas* culture have showed good antifungal activity against the fungi, namely, *A. niger, A. flavus* and *A. oryzae, F. oxysporum* and *S. rolfsii* (Manwar *et al.,* 2004).

Fluorescent Pseudomonads PGPR1 and PGPR4 from a pool of 233 rhizobacterial isolates obtained from the peanut rhizosphere showed strong *in vitro* inhibition to *Sclerotium rolfsii* (Dey *et al.,* 2004). Of all the sixty bioagents screened for its antagonistic activity, the potential antagonistic isolates ANT5 (100%), ANT11 (100%), KDP6 (100%), TPT15 (100%) and TPT17 (100%) completely inhibited the mycelial growth of the pathogen and were found to be superior compared to other isolates against the test pathogen, *S.rolfsii* in dual culture (Basha *et al*., 2012).

2.7.2. *In vitro* screening of *Sclerotium. rolfsii* by *Bacillus subtilis*

The use of antagonistic species of *Bacillus* as biocontrol agents has been extensively studied. Their potential as biocontrol agents is investigated because they produce several kinds of antimicrobial compounds, including peptide

antibiotics and hydrolytic enzymes viz., gluconases, produced by several bacteria are one of the most potent enzymes for degrading fungal cell walls (Mawadza *et al*., 2000).

Bacillus subtilis CA 32r, a stable spontaneous kanamycin resistant isolate, has been found to show antagonism in a petriplate assay against *Sclerotium rolfsii*, the causal agent of collar rot and root rot of chilli (Abeysinghe, 2007). A total of 137 bacterial isolates from surface sterilized root, stem and nodule tissues of soybean were screened for their antifungal activity against major phytopathogens like *Rhizoctonia bataticola, Macrophomina phaseolina, Fusarium* and *Sclerotium rolfsii*. Nine bacterial endophytes in which eight of them belonged to the *Bacillus* sp. suppressed the pathogens under *in vitro* plate assay (Senthilkumar *et al.,* 2009).

2.7.3. *In vitro* screening of *Alternaria alternata* by *Pseudomoans fluorescens*

Ramyasmruthi *et al*. (2012) screened 18 chitinolytic *Pseudomoans fluorescens* strains against three different *Alternaria* species such as *Alternaria alternata, Alternaria brassicola* and *Alternaria brassiceae*. Among them, *P. fluorescens* strain 6 was found to be most effective against the mycelial growth of three *Alternaria* species.

Mishra *et al*. (2011) noticed that six-day old culture filtrate of *Pseudomonas fluorescens* can completely inhibit the growth of *Alternaria alternata* and *Curvularia andropogonis* at 10% concentration. The *Pseudomonas fluorescens* isolates viz., AP4 and AP8 has been shown to have good inhibition activity against *Alternaria alternata* by dual culture method (Ramanujam *et al*., 2011).

Pseudomonas putida IsoF was found to colonize tomato roots and produce *N*-acyl-L-homoserine lactone in the rhizosphere which also increases the systemic resistance of tomato plants against the fungal leaf pathogen, *A. alternata* (Schuhegger *et al*., 2006). *P. fluorescens* has been found to be more efficient in inhibiting the colony growth of *A. alternata* by 64.9 per cent (Indira *et al.,* 2006). Out of 18 isolates of *Pseudomonas* tested against *A. triticina,* nine isolates showed inhibitory effects (Siddiqui, 2007).

Antifungal activity of different strains of *Pseudomonas fluorescens* were tested against some plant pathogens such as *Alternaria cajani*, *Curvularia lunata*, *Fusarium sp.*, *Bipolaris sp.* and *Helminthosporium sp.* in *in vitro.* Different concentrations (1000, 2000, 3000, 4000 and 5000 µg/mL) of *Pseudomonas fluorescens* were used and maximum spore germination of the fungus has been found to be inhibited at 4000 and 5000 µg/mL. The results indicated that all the strains of *Pseudomonas fluorescens* presented a most significant value against *Alternaria cajani* and *Curvularia lunata* (Rachana and Shalini, 2008).

2.7.4. *In vitro* screening of *Alternaria alternata* by *Bacillus subtilis*

Dragana *et al*., (2011) observed that *Bacillus* strain Q3 isolate caused a high percent of inhibition (61.75) on *Alternaria alternata* growth. 4 day old culture filtrate of *Bacillus subtilis* MA-2 completely inhibited the growth of *Alternaria alternata* at 10 per cent concentration (Mishra *et al*., 2011).

Sid *et al*. (2003) has documented antagonistic activity of *B. subtilis* (HS93) against *A. alternata*. Hou *et al*. (2006) reported that *B. subtilis* strain LEV-006 was antagonistic to four major fungal pathogens of Canola including *A. brassicae*.

Culture filtrates of *B. subtilis* completely arrested the growth of *A. daturae*, a serious pathogen of leaf spot of *Datura fastulosa*. The bacterial metabolite was thermostable and fungistatic and caused abnormally abundant septations and formation of chlamydospore like swollen bodies in the hyphae (Rai, 1975). Application of an antifungal complex (Antibiotic F), derived from a local strain of *B. subtilis* AECL 69, on the cut stem ends (2 cm radius) of citrus before storage showed a significant control of black rot caused by *A. citri* in Valencia oranges and Kinnow mandarins (Farooqi *et al.,* 1982).

Basim and Katricioglo (1990) studied the antagonistic activity of 12 isolates of *B. subtilis* against *A. alternata* and *A. solani* by dual culture technique. Among the isolates tested, *B. subtilis* AB-2 and AB-27 isolates were the most antagonistic against the pathogen tested.

2.8. Plant growth promotion

The use of Rhizobacterial *Bacillus* isolates like OSU 142 and M3 in sugar beet and barley (Cakmakci *et al.,* 2001), corn (Ataoglu *et al.,* 2004) and tomatoes (Turan *et al.,* 2004) was found to stimulate the yield and quality parameters tested. In addition, floral and foliar application of *Bacillus* OSU-142 showed increased yield and growth and decreased shot-hole disease in apricot (Esitken *et al*., 2003).

Plant growth benefits due to the addition of PGPR include increase in germination rates, root growth, yield including grain, leaf area, chlorophyll content, magnesium, nitrogen and protein content, hydraulic activity, tolerance to drought and salt stress, shoot and root weights and delayed leaf senesence (Lucy *et al.*,2004).

Begum *et al.* (2003) studied the effectiveness of plant growth promoting rhizobacterial isolates against some seed borne fungal diseases. Among them, *B. pumilus* (SE-34), *B. pasteurii* (T4), *B. subtilis* (IN 937-6) and *B*. *subtilis* (GB-03) strains stood first in the improvement of crop, both in greenhouse and field conditions. Potential strains increased the biomass of plants, total number of leaves, fruits, length, girth and biomass of the fruit. The colonization of these bacterial strains reduced the incidence of seed mycoflora which indirectly enhanced the per cent seed germination and vigour index of seedlings.

Minakshi *et al.* (2005) isolated a total of 113 rhizobacteria from different rhizotic zones of pigeon pea. Seed treatment using four isolates of rhizobacteria, *viz*. RS29, RS39, RS41 and RP3 resulted in 90 per cent seed germination in contrast to 50 per cent obtained in untreated control after 72 h of incubation and the isolates RS34, ER17, RP7 and RS41 increased shoot height and shoot dry biomass as compared to uninoculated control, whereas, isolates RS45, RS36, RS37, ER23, RP24 influenced root dry biomass significantly.

2.9. Antimicrobial compounds of PGPR

Production of secondary metabolites such as siderophores, indole acetic acid, salicylic acid and antibiotics such as phenazine, pyocyanine and 2, 4 diacetyl phloroglucinol by *Pseudomonas* spp. associated with induced systemic resistance

activity in sugarcane against red rot disease were assessed. Many of the strains were found to produce these metabolites and antibiotics in the culture medium. Most of the purified metabolites completely arrested conidial germination and mycelial growth of the fungus. These results suggest that the metabolite production may play a role in antagonism against the pathogen (Viswanathan and Samiyappan, 2004).

2.9.1. Hydrogen cyanide (HCN) production

Volatile compounds such as ammonia and hydrogen cyanide produced by a number of rhizobacteria were reported to play an important role in biocontrol. HCN expression and production by *Pseudomonas* is strongly dependent on iron availability. Moreover, the antifungal activity of *Pseudomonas* and others (*Bacillus* and *Azotobacter*) may be due to the production of HCN and siderophores or synergistic interaction of these two or with other metabolites. Some members of the *Pseudomonas* strains were found to antagonize certain plant pathogens and produced hydrogen cyanide (HCN) that inhibited the growth of infected plants and reduced their yield. That was due to the ability of HCN to interfere with cytochrome oxidation of the infected plants (Hassanein *et al*., 2009).

In search of efficient PGPR strains with multiple activities, a total of 72 bacterial isolates belonging to *Azotobacter*, fluorescent Pseudomonads, *Mesorhizobium* and *Bacillus* were isolated and screened *in vitro* for their plant growth promoting traits like production of indole acetic acid, ammonia, hydrogen cyanide, siderophore, phosphate solubilization and antifungal activity. More than 80% of the isolates of *Azotobacter*, fluorescent Pseudomonads and *Mesorhizobium ciceri* were found to produce indole acetic acid. Siderophore production and antifungal activity of these isolates except *Mesorhizobium* were exhibited by 10 - 12.77% isolates. HCN production was also found to be a more common trait of *Pseudomonas* (88.89%) and *Bacillus* (50%) (Ahmad *et al.,* 2008).

Kumar *et al.* (2009) demonstrated that *Pseudomonas aeruginosa* LES4, an isolate of tomato rhizosphere was positive for several plant growth-promoting attributes like production of indole acetic acid, HCN and siderophore, solubilization

of inorganic phosphate alongwith urease, chitinase and β-1,3 glucanase activity. In addition, it showed strong antagonistic effect against *Macrophomina phaseolina* and *Fusarium oxysporum*.

Six potential isolates of *Bacillus* and *Pseudomonas* (out of 20 isolates screened under greenhouse assay) were evaluated under pot and field conditions for the biocontrol of wilt disease complex of pigeon pea caused by *Heterodera cajani, Meloidogyne* spp. and *Fusarium udum*. Isolate Pa324 was found to be the best for the biocontrol of wilt disease of pigeonpea which produced greater amount of siderophores, hydrogen cyanide and indole acetic acid than other isolates (Siddiqui *et al.,* 2008). Jamali *et al.* (2009) demonstrated that the production of hydrogen cyanide (HCN) and 2, 4-diacetylphloroglucinol (DAPG) are major factors in the control of soil-borne diseases by *Pseudomonas fluorescens* CHA0.

2.9.2. Siderophore production

Siderophores are small, high-affinity iron chelating compounds secreted by microorganisms such as bacteria, fungi and grasses. Production of secondary metabolites like antibiotics, iron - chelating siderophores, and cyanide are most often associated with fungal suppression by fluorescent pseudomonads in the rhizosphere of several crops (Hohnadel and Meyer,1998).

Siderophores are usually classified by the ligand used to chelate the ferric iron. The major groups of siderophores include the catecholates (phenolates), hydroxamates and carboxylates. Fluorescent pseudomonads produce yellow-green pigments (pyoverdines), which fluoresce under UV light and function as siderophores. They deprive the iron, required for their growth and pathogenesis. Siderophores sequester ferric ions in the environment and the ferric siderophores are taken up in the microbial cells after specific recognition by membrane proteins. The production of siderophores is an important trait of PGPR in their ability to suppress soil-borne pathogens. Competition for ferric iron between the PGPR and the plant deleterious microorganisms is considered to be the mode of action of these siderophores (Varma and Chincholkae, 2007).

Fifteen rhizobacterial fluorescent pseudomonads isolates were obtained from rice growing region of Andhra Pradesh, India. Among them, 10 strains of *Pseudomonas fluorescens* were selected based on preliminary screening for antifungal activity against rice fungal pathogens (*Pyricularia oryzae* and *Rhizoctonia solani*). The strains of *Pseudomonas fluorescens* inhibited the growth of rice fungal pathogens. Among these Pf 003 strain completely inhibited the mycelial growth of two rice pathogens (*P.oryzae* and *R.solan*i) both in the presence and absence of ferric chloride which indicated the siderophore mediation alongwith antifungal metabolites (Battu and Reddy, 2009).

The effects of *Pseudomonas putida, Pseudomonas alcaligenes* and a *Pseudomonas* isolate (Ps28) on root colonization, antifungal activity against *Macrophomina phaseolina* and the production of siderophore, hydrogen cyanide (HCN) and indole acetic acid (IAA) were estimated by Akhtar and Siddiqui (2009). They reported that *P. putida* had the greatest inhibitory effect on *M. phaseolina* and produced the highest amount of siderophores, IAA and HCN compared to *P. alcaigenes* and Ps28.

Pseudomonas aeruginosa PUPa3 was shown to exhibit a broad spectrum of antifungal activity towards phytopathogenic fungi. The antifungal metabolite by PUPa3 was extracted, purified and characterized using nuclear magnetic resonance (NMR) and mass spectroscopy (MS). The results revealed that strain PUPa3 produce indole-3-acetic acid (IAA), siderophore, phosphatase and protease (Kumar*,* 2005).

216 bacterial isolates were tested for their siderophore production and the effectiveness in inhibiting the mycelial growth of fungi such as *Alternaria* spp., *Fusarium oxysporum* and *Sclerotium* spp. In dual culture, the siderophore producing rhizobacteria showed a strong antagonistic effect against all these fungi (Chaiharn *et al.,* 2009).

2.9.3. Salicyclic acid production

Salicylic acid is a natural phenolic compound present in many plants and is an important component in the signal transduction pathway and is also involved in

local and systemic resistance to pathogens. Production of chitinase, *β*-1, 3 glucanase, siderophores, salicyclic acid (SA) and hydrogen cyanide (HCN) by *P. fluorescens* strains were evaluated. The highest *β*-1, 3 glucanase activity, siderophore production, SA production and HCN production were recorded by the *P. fluorescens* strain PfMDU2. A significant relationship between the antagonistic potential of *P. fluorescens* against *R. solani* and its level of *β*-1, 3 glucanase, SA and HCN was observed (Nagarajkumar *et al.,* 2004).

Mandal *et al.* (2009) demonstrated that exogenous application of salicylic acid through root feeding and foliar spray could induce resistance against *Fusarium oxysporum* f. sp. *lycopersici* (Fol) in tomato. The results indicated that the induced resistance observed in tomato against *Fusarial* wilt might be due to salicylic acid dependent systemic acquired resistance. Esmailzadeh *et al.* (2008) also reported that SA pre-treatment plants caused an increase in the endogenous free SA levels in tomato leaves which resulted in systemic resistance induction against *Alternaria alternata*, the causal agent of tomato stem canker.

Selected isolates of *Pseudomonas fluorescens* (*Pf*1–94, *Pf*4–92, *Pf*12–94, *Pf*151–94 and *Pf*179–94) and chemical resistance inducers (salicylic acid, acetylsalicylic acid, DL-norvaline, indole-3-carbinol and lichenan) were examined for growth promotion and induced systemic resistance against *Fusarium* wilt of chickpea. Among chemical inducers, SA showed the highest protection of chickpea seedlings against wilting. 52-64% reduction of wilting was observed in soil treated with isolate *Pf*4–92 alongwith chemical inducers. All the isolates of *P. fluorescens* produced SA in synthetic medium and in root tissues. HPLC analysis revealed that *Pf4–92* produced comparatively more SA than the other isolates (Saikia *et al.,* 2003).

Investigations were conducted to determine the role of salicylic acid (SA) in Induced Systemic Resistance (ISR) against blue mold disease of tobacco elicited by Plant Growth Promoting Rhizobacteria (PGPR). When plants were treated with *Bacillus pumilus* strain SE34 and challenged with *Peronospora tabacina*, SA level increased markedly one day after the challenge, compared to the non-bacterized and challenged control. These observations indicate that

SA accumulation in tobacco plants may play a role in ISR against tobacco blue mold by PGPR (Zhang *et al.,* 2002).

2.9.4. Indole- 3- acetic acid (IAA) production

Indole- 3- acetic acid (IAA) is the most abundant, naturally occurring auxin produced by bacteria (Bloemberg and Lugtenberg, 2001). The proportion of IAA producing bacteria in the rhizosphere may be of relevance for plant growth and previous studies have shown that IAA producing bacteria can be stimulated in the rhizosphere (Weisskopf *et al*., 2005).

P. fluorescens AK1 and *P. aeruginosa* AK2 were shown to have the best plant growth promoting activity. These isolates were tested for their ability to produce indole acetic acid in pure culture in the presence and absence of L-tryptophan. For both strains, indole production increased with increase in tryptophan concentration. Inoculation of rice seeds *P. fluorescens* AK1 and *P.aeruginosa* AK2 showed a good level of indole acetic acid compared to uninoculated seeds (Karnwal, 2009). Dubey and Maheshwari (2002) reported that out of the 10 Fluorescent pseudomonads isolated from the rhizophere which showed biocontrol potential, all of them displayed the production of siderophore, HCN and IAA.

Bacillus megaterium DE BARY TRS-4 was isolated from tea rhizosphere and tested for its ability to promote growth and cause disease reduction in tea plants. The results revealed that the ability of *Bacillus megaterium* to promote plant growth and reduce the brown root rot disease in tea plants is due to a combination of several mechanisms such as induction of defense related enzymes and production of IAA, siderophore and antifungal metabolites (Chakraborty *et al.,* 2006).

7 Plant Growth Promoting Rhizobacterial (PGPR) strains which were isolated from the rhizoplane and rhizosphere of wheat from four different sites of Pakistan were analyzed for production of indole acetic acid (IAA), phosphorous solublization capability and inhibition of *Rhizoctonia solani* on rye agar medium.

The results shows that these three strains belonging to *Azotobacter* and *Azospirillum* produced IAA ranging from 19.4 to 30.2 µg/ml and a mixture of all three strains showed maximum inhibition of *R. solani* (Fatima *et al.,* 2009).

Among 628 bacterial strains, strain P94 of *P. corrugata* exhibited the most obvious antagonistic activity against *Botrytis cinerea*. It showed a positive reaction for HCN, protease, phosphatase and indole acetic acid tests. Therefore, the secondary metabolite producing novel *P. corrugata* strain P94 exhibited an innate potential of biocontrol activities *in vitro* (Guo *et al.,* 2007).

2.9.5. Chitinase production

Chitinase is one of the major pathogenesis-related proteins, which is a polypeptide that accumulates extracellularly in infected plant tissues. An attempt was made to isolate and purify the chitanase enzyme using moth beans as an enzyme source (Garg and Gupta, 2010).

Chang *et al.* (2010) had isolated a bacterial strain *Bacillus subtilis* NPU 001 from soil and found that this strain excreted a chitinase which was purified by sequential chromatography which inhibited hyphal extension of the fungus *Fusarium oxysporum.*

Ramyasmruthi *et al*. (2010) had isolated 18 bacterial strains from the rhizosphere of Solanaceae family, namely, brinjal, capsicum, chilli and screened for the production of chitinase enzyme. A total of 10/18 isolates are the most potent chitinolytic bacterial species. Of these, 6 isolates showed zone size higher than 5 mm.These isolates also showed varied levels of PGPR traits like siderophore, HCN, phosphate solubilisation and IAA.

The reports of Radjacommare *et al.* (2004) had mentioned the induction of 28 and 35 kDa chitinase isoforms in rice plants treated with *P. fluorescens* and challenge inoculated with sheath blight pathogen *Rhizoctonia solani*.

Chitinase produced by *Serratia plymuthica* C48 inhibits spore germination and germ-tube elongation in *Botrytis cinerea*. The ability to produce extracellular

chitinases is considered crucial for *Serratia marcescens* to act as antagonist against *Sclerotium rolfsii*, and for *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 to suppress *Fusarium oxysporum* f. sp. *cucumerinum*. It has also been demonstrated that extracellular chitinase and laminarinase synthesized by *Pseudomonas stutzeri* digests and lyses mycelia of *F. solani* (Saikiar *et al*., 2006).

2.10. Bioformulations of PGPR strains

Ardakani *et al*., (2010) isolated two strains of *Pseudomonas fluorescens* from different rhizosphere soils and plant roots in the Iranian cotton fields and developed formulations like talc-based powder and bentonite-based powder as mineral carriers and peat and rice bran as organic carriers for increasing stability in interaction between PGPR and cotton plants. These products were applied to cotton seeds at intervals of 15, 30, 45 and 60 days after sowing to control seedling damping-off. The results showed that all treatments except TAL-B2 formulation were effective (up to 62.5% control) as compared to untreated seeds.

A talc-based formulation of *P. fluorescens* isolated from the rhizosphere of different crops had been developed and tested against root rot disease caused by *Macrophomina phaseolina* in Coleus (Kamalakannan, 2004).

Bio-priming of plants with some PGPB can also provide systemic resistance against a broad spectrum of plant pathogens, insects and nematodes can be reduced by application of PGPB (Compant *et al*., 2005).

2.10.1. Talc-based bioformulation

Commercial application of PGPR for control of soil-borne diseases depends upon the development of commercial formulations in which bacteria can survive for a considerable length of time, on the development of a suitable method of application to control pathogen establishment and disease development and assessment of their efficacy under field conditions. The efficacy of various carriers in sustaining the population of a strain of *B. subtilis* BSCBE4 and *P. chlororaphis* PA23 during storage have been assessed. The results demonstrated that *Bacillus*

and *Pseudomonas* strains survived upto 180 days in peat and talc formulation (Nakkeeran *et al.,* 2006).

Among the various bioformulations tested as seed treatment and foliar application, the talc-based formulations of *Pseudomonas fluorescens* (Pf 1 and Py 15) and *Bacillus subtilis* with Zimmu extract (*Allium cepa* L.x.*Allium sativum* L.) was found to be superior in reducing the early blight disease incidence in tomato when compared to other treatments (Latha *et al.,* 2009). The application of talc formulation through seed, seed treatment plus foliar spray and foliar spray alone significantly reduced the leaf blight incidence both under green house and field conditions (Chitra et *al.,* 2006).

Singh and Sinha (2005) carried out the investigation to find out the effect of different formulations and rates of *P. fluorescens* strains 1 and 5 against sheath blight disease of rice caused by *R. solani* on yield parameters of rice, under glasshouse conditions. The results showed that Talc + CMC based formulation of the bio-agent exhibited maximum reduction of disease severity (56.6%) and increased grain yield (31.2%).

Sallam *et al*. (2009) developed powder formulations of *Bacillus subtilis* and tested their effect against onion white rot disease. They found that soil application of the formulation at the time of planting and two weeks before transplanting significantly reduced the incidence of white rot on onion cultivars.

2.11. Rhizobacteria in the management of plant diseases

PGPR has the ability to protect above ground plant parts against fungal, bacterial and viral diseases by Induced Systemic Resistance (ISR). Kloepper *et al.* (1992) reported that among the PGPR, Fluorescent pseudomonads are the most exploited bacteria for biological control of soil borne and foliar plant pathogens.

Pseudomonas fluorescens isolates are effective bacterial antagonists for the management of soil borne and foliar diseases. Among the various isolates tested, *P. fluorescens* isolate Pf1 effectively inhibited mycelial growth of the pathogen *in*

vitro conditions and decreased the fruit rot incidence under greenhouse conditions (Ramamoorthy *et al*., 2002b).

PGPR strain *B. pumilus* INR-7 effectively protected pearl millet against downy mildew under greenhouse and field experiments (Niranjan Raj *et al.*, 2003).

PGPR mediated resistance in mango trees infected with *Colletotrichum gloesporiodes* significantly reduced the anthracnose infection besides enhancing fruit yield under field conditions (Vivekananthan *et al.*, 2004). These studies clearly indicate the PGPR to have diverse mechanisms to operate to combact the pests and pathogens and work efficiently in both greenhouse and field conditions.

2.12. Induced Systemic Resistance (ISR)

Induced resistance is defined as an enhancement of the plants defensive capacity against a broad spectrum of pathogens and pests that is acquired after appropriate stimulation. Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR) are two forms of induced resistance wherein plant defenses are preconditioned by prior treatment that results in resistance against subsequent challenge by a pathogen or parasite (Choudary *et al*., 2007). Induced Systemic Resistance by antagonistic bacteria has earlier been reported by several workers (Bakker *et al*., 2007). The level of defense-related enzymes is known to play a crucial role in the degree of host resistance. Increase in activity and accumulation of these enzymes depends not only on the inducing agent but also on the plant genotype, physiological conditions and the pathogen.

2.12.1. Induction of defense related compounds

The defense enzymes peroxidases (PO) and polyphenol oxidases (PPO) which catalyse the formation of lignin and phenylalanine ammonia lyase (PAL) is involved in the synthesis of phytoalexins and phenolics (Karthikeyan *et al*., 2005).

Van Loon *et al.* (2005) suggested that rhizobacteria-induced resistance could be involved in the production of phytoalexins, synthesis of pathogenesis related proteins and expression of defense related enzymes against various types of pathogens. Dutta *et al.* (2008) demonstrated that seed treatment with *Bacillus*

cereus (BS02) and *Pseudomonas aeruginosa* strain PRLJ 04 increased the level of defense enzymes *viz.,* PAL, PO and PPO against *Fusarium udum* wilt disease.

Jourdan *et al.* (2009) demonstrated that multiple strains of *Bacillus subtilis* stimulated plant defense responses and cyclic lipopeptides that may be involved in the elicitation of induced systemic resistance phenomenon.

2.12.1.1. Phenylalanine ammonia lyase

Phenylalanine ammonia lyase is the first enzyme in the phenylpropanoid pathway that catalyses the conversion of L-phenylalanine to trans-cinnamic acid which in turn enters different biosynthetic pathways leading to lignin synthesis, a major product of phenylpropanoid metabolism. This defense mechanism is used for protection against pathogen invasion. Since changes in PAL activity are key events controlling the synthesis of phenylpropanoids, PAL has become one of the most extensively studied enzymes in plants. Induction of PAL as a response to pathogen infection was well documented in various host-pathogen interactions (Geetha *et al*., 2005).

Biocontrol agents mainly bacterial inoculants were believed to induce systemic defense responses in plants besides other mechanisms including direct antagonism, antibiosis and siderophore production. Induction of defense responses by plant growth-promoting bacteria (PGPB) is largely associated with the production of pathogenesis related (PR) proteins like β-1,3 glucanase and the defense enzyme phenylalanine ammonia lyase and oxidative enzymes like peroxidase and polyphenol oxidase (Compant *et al.,* 2005). Groundnut plant showed significantly enhanced germination and induction of PO, PPO, PAL and β-1, 3 glucanase when seeds were treated with *Methylobacterium* spp and challenged with *Aspergillus niger/Sclerotium rolfsii.* Five isozymes of PPO and PO could be detected in these plants (Madhaiyan *et al.,* 2006). Recent research work showed that *P. fluorescens* strains might stimulate the production of biochemical compounds associated with host defense mechanism (Kavino *et al.,* 2007). Of these, the early induction of PAL was more important as it is the first enzyme in the polypropanoid pathway which leads to the formation of lignin with the help of peroxidases.

Azoxystrobin and *Pseudomonas fluorescens* were evaluated for their efficacy in inducing defense enzymes in tomato against *Alternaria solani* and *Septoria lycopersici*. The activity of defense enzymes peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), β-1,3 glucanase, chitinase, catalase and defense-inducing chemicals (total phenols) were found to be increased in azoxystrobin and *P. fluorescens* treated tomato plants. The activity of these defense enzymes and chemicals were higher in azoxystrobin and *P. fluorescens* treated tomato plants challenge inoculated with the pathogens compared to other treatments. Increased expression of specific isoforms of PO and PPO has been observed due to ISR induction (Anand *et al.,* 2007).

Umesha and Hariprasad (2010) reported the various PGPR application as seed treatment, root dipping and foliar spray treatments in field. Among the PGPR strains, *Bacillus subtilis* strain GB3 was the most effective in providing significant suppression of bacterial spot and was well correlated with increased activity of defense related enzymes, *viz.* peroxidase and phenylalanine ammonia lyase.

2.12.1.2. Peroxidase

Peroxidase is a key enzyme in the biosynthesis of lignin and other oxidized phenols. PO catalyses the oxidation of hydroxyl-cinnamyl alcohols into free radical intermediates, which subsequently are coupled to lignin polymers (Gross, 1980).

Peroxidases are defense-related enzymes with broad action spectrum activity. They play key roles in plant-pathogen interactions and are believed to be one of the most important factors of the plant's biochemical defense against pathogenic microorganisms, and actively involved in the self-regulation of plant metabolism after infection (Saravanan *et al*., 2004).

Biocontrol strains stimulate the activities of defense enzymes PO, PPO and PAL in plants that could be involved in the synthesis of phytoalexins. More specifically PO is involved in the production and modulation of active oxygen species, which could play various roles directly or indirectly in reducing pathogen viability and spread (Van Loon and Bakker, 2005). Karthikeyan *et al.* (2006b), from

their pot culture experiment, concluded that enhanced activities of defense-related enzymes *viz.,* peroxidase (PO) and polyphenol oxidase (PPO) were noticed in groundnut treated with *P. fluorescens* or *T. viride* and challenged with *S. rolfsii.*

The PO activities are linked to lignification and generation of hydrogen peroxide at later stages of infection, which inhibit pathogens directly, or generating of other free radicals with antimicrobial effects that restrict the development of challenging phytopathogenic bacteria (Silva *et al.,* 2004). Increased accumulation of oxidative enzymes PO, PPO, PAL, pathogenesis related PR proteins, chitinase, β-1,3 glucanase and phenolics were observed in banana plants which were treated with *P. fluorescens* CHA0 bioformulation amended with chitin and challenged with Banana bunchy top virus (BBTV) under glass house conditions (Kavino *et al.,* 2008).

Pseudomonas fluorescens isolates *viz,* Pf1, PfCBE, PfPOL and PfBSR has been found to protect the groundnut from root knot nematode, *Meloidogyne arenaria.* Higher level of peroxidase activity in bacterized groundnut inoculated with nematodes was observed. Isoform analysis revealed the induction of unique PO1 isoform and higher level of PO2 isoform in bacterized groundnut inoculated with nematodes (Kalaiarasan *et al.,* 2006). Soil application of biocontrol formulations (*Pseudomonas fluorescens*, *Trichoderma viride* and *T. harzianum)* in combination with chitin induced a significant increase in the activities of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), chitinase and β-1, 3 glucanase in coconut roots infected with *Ganoderma lucidum*, the causal agent of *Ganoderma* disease (Karthikeyan *et al.,* 2006a).

2.12.1.3. Polyphenol oxidase

PPO usually accumulates upon wounding in plants. Biochemical approaches to understand PPO function and regulation are difficult because the quinonoid reaction products of PPO covalently modify and cross-link the enzyme. The increased activation of PPO could be detected in cucumber leaf in the vicinity of lesions caused by some foliar pathogens. PO and PPO are reported to be capable of oxidizing phenols to quinones and they were associated with disease resistance in plants (Mansfield, 1983).

Plant growth promoting Fluorescent pseudomonads strains Pf1, TDK1 and PY15 were evaluated for their efficacy against leaf folder (*Cnaphalocrocis medinalis*) pest in rice plants under field conditions individually and in combinations. The results showed the higher activity of polyphenol oxidase (PPO) and lipoxygenase (LOX) in plants treated with *P. fluorescens* mixtures (Pf1 + TDK1 + PY15) than the plants treated with individual strains, chemical and untreated controls and revealed that PGPR strain mediated induction of PPO and LOX in rice plants could be involved in resistance mechanisms against leaf folder attack (Saravanakumar *et al.,* 2008).

The treatment with *Pseudomonas fluorescens* PfB13 enhanced the activity of defense enzymes responsible for induction of systemic resistance *viz*., peroxidase, polyphenol oxidase and phenylalanine ammonia lyase in banana plants for the management of nematode, *Rodopholus similis* (Senthilkumar *et al.,* 2008).

2.12.1.4. Phenols

Plant phenolics are well-known antifungal, antibacterial and antiviral compounds that play an important role in determing resistance or susceptibility of a host to parasitic infection (Galeotti *et al*., 2008).

Bacillus subtilis GBO3, *Bacillus amyloliquefaciens* 1M937a and *Brevibacillus brevis* recorded maximum protection of tomato from bacterial canker disease under green house conditions. The level of PAL and total phenol contents increased significantly upon the PGPR treatment. The rate of reduction in diseases was directly proportional to the amount of increased level of PAL and total phenol content (Girish and Umesha, 2005).

Karthikeyan *et al.* (2009) demonstrated that the pre inoculation of black gram plants with *Pseudomonas fluorescens viz*., Pf 1 and CHAO were found to reduce urdbean crinkle virus (ULCV) infection significantly. Soil and foliar application of *P. fluorescens* (Pf1) induced the accumulation of phenolics and enhanced the activities of phenylalanine ammonia lyase, peroxidase and

polyphenol oxidase. The induced defense mechanisms might have played a role in reducing the disease.

Two plant growth-promoting rhizobacteria (PGPR), *viz*., *P. fluorescens* strain *Pf4* and *P. aeruginosa* strain *Pag*, protected chickpea (*Cicer arietinum*) plants from *S. rolfsii* infection when applied singly or in combination as seed treatment. The two PGPR strains induced the synthesis of specific phenolic acids, salicylic acid (SA) as well as total phenolics at different growth stages of chickpea seedlings with varied amount. In the presence of a culture filtrate of *S. rolfsii*, the two *Pseudomonas* strains induced more phenolic acids in treated than in untreated and control plants. Resistance in chickpea plants by *Pseudomonas* strains through induction of phenolic compounds as well as induced systemic resistance through SA-dependent pathway was also evident (Singh *et al*., 2003).

With this background information, the experimental design for the study was formulated as given in the following chapter.