3. MATERIALS AND METHODS

The present study was undertaken in the Department of Microbiology, Centre for Post Graduate studies, Jain University, Bangalore on the biocontrol potential and plant growth promotional activity of chitinolytic Bacillus sp. isolated from soils in and around Bangalore, Karnataka state. The materials used and the methods employed in the investigation are outlined below in detail.

3.1 General Procedure

3.1.1 Glassware cleaning

Corning glassware were used for all the experimental studies, wherever required and were kept in the cleaning solution containing 60 g of potassium dichromate, 60 ml of concentrated sulphuric acid in one liter of water for a day. Then they were cleaned by washing with detergent followed by rinsing several times in tap water and finally in distilled water.

3.1.2 Sterilization

All the glassware were sterilized in an autoclave at 1.1 kg/cm² pressure for 20 minutes and all the solid and liquid media were sterilized for 15 minutes at 1.1 kg/cm² pressure.

3.1.3 Chemicals

The analytical reagent grade chemicals of Sigma – Aldrich, Himedia, Qualigens, BDH, E-Merck and Fisher were used for the experiments.
3.1.4 Survey for the collection of rhizosphere soils

Survey was conducted at various districts in and around Bangalore, Karnataka, in the month of June-July, 2010, to collect the rhizosphere soil samples. The samples were collected from chilli rhizosphere from several fields by pulling the chilli plants and gently tapping to dislodge the rhizosphere soil. Such representative samples were collected from chilli growing fields in Chikkaballapur and Bengaluru rural districts. The representative fifteen rhizosphere soils were prepared by pooling the samples collected from different fields’ and stored in refrigerator at 4°C for further studies.

3.1.5 Pathogens

The following six phytopathogens were obtained from Indian Institute of Horticultural Research, Hessarghatta, Bangalore, India: *Alternaria brassica* (OCA1), *Alternaria brassicaceae* (OCA3), *Alternaria alternata* (OTA36), *Fusarium solani* (MTCC 1756), *Colletotrichum gloeosporioides* (OGC1) and *Phytophthora capsici* (98-01). Other cultures viz., *Verticillium theobromae* (MTCC 2066), *Fusarium oxysporum* (MTCC 1755) and *Rhizoctonia solani* (MTCC 4633) were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. All the fungal pathogens were subcultured on Potato dextrose agar and stored at 4°C.

3.2 Isolation of Bacillus sp.

*Bacillus sp.* were isolated from 15 different rhizospheric soils of chilli by serial dilution method. The rhizosphere samples were packed in a sterile polythene bag and labelled properly for further processing (Pandey and Palni, 2007). One gram of soil sample was suspended in 10 ml of sterile distilled water and vortexed for 45s. The suspension was serially diluted up to $10^{-7}$ dilutions. The samples and the dilutions were heat treated in a water bath maintained at 80°C for 20 minutes to ensure that only heat resistant strains
remained (Cook et al., 1997). 100 µl of each dilution was plated on nutrient agar and the plates were incubated at 28± 2°C. The different isolates obtained were screened for chitinase production on plates with minimal salts medium containing (g/L): Dextrose, 1; Ammonium Sulphate, 1; Dipotassium Phosphate, 7; Potassium Monophosphate, 2; Sodium Citrate, 0.5; Magnesium Sulphate, 0.1; pH was adjusted to 7± 0.2, amended with 1% (w/v) chitin (Chernin et al., 1995). The chitinolytic Bacillus sp. were identified based on the production of clearance zones around the colonies and were maintained on nutrient agar amended with 1% chitin, sub cultured and stored at 4°C.

3.2.1 Preparation of colloidal chitin

Colloidal chitin was prepared from crab shell chitin powder (Roberts and Selitrennikoff, 1988). A few modifications were made as described: 10 g of chitin powder was added slowly into 100 ml of concentrated hydrochloric acid under vigorous stirring for 2 h. The mixture was added to 1000 ml of 95% ice-cold ethanol with rapid stirring and kept overnight at 25°C and then stored at -20°C until use. When needed, the filtrate was collected and washed with 0.1 M phosphate buffer (pH 7) until the colloidal chitin became neutral (pH 7), autoclaved and used for further experiments.

3.3 Assessing antagonistic potential by dual plate assay

Nine pathogens were used in the present study. The antagonistic activity of Bacillus sp. strains was determined (Huang and Hoes, 1976) using the dual culture technique. The Bacillus sp. was inoculated at 2 opposing corners. Plates were incubated for 7 days, at 28°C and growth diameter of the pathogen was measured and compared to control growth, where the bacterial suspension was replaced by sterile distilled water. Each experiment using a single pathogen isolate was run in triplicate. Results were expressed as the means of the percentage of inhibition of growth of the corresponding pathogen isolate in the presence of
any of the strain of *Bacillus* sp. Per cent inhibition was calculated (Montealegre *et al.*, 2003) using the formula: \(1 - \left( \frac{\text{Fungal growth}}{\text{control growth}} \right) \times 100\).

### 3.3.1 Screening for antagonistic activity in culture filtrate

Further to confirm the antagonist activity of the isolated bacterial isolates, fungal pathogens were inoculated \(10^6\) conidia and grown in 50 ml of potato dextrose broth for one week at 28°C, 200 rev/min in the presence or absence of the culture supernatant (1% v/v) of antagonist bacteria, grown in NB for 48 hrs at 28°C. The biomass was separated, dried at 105°C for 14 h and weighed to obtain the dry weight. The values were expressed in percentage (Zhang *et al.*, 2007).

### 3.4 Morphological and phenotypic characterization of *Bacillus* sp.

These were performed according to Parry et al. (1983) and Holt, (1994) (Fig. 3.1). Parameters investigated were cell and spore morphology, motility, formation of parasporal crystal, growth characteristics (anaerobic growth and growth in the presence of NaCl 6.5%), hydrolysis of starch, casein, nitrate reduction and Voges Proskauer reaction. The isolates were also evaluated for carbohydrate fermentation abilities.

Polymerase chain reaction (PCR) was performed to amplify a partial 16S rRNA gene of the bacteria, and partial 16S rDNA sequencing was used to assist in the identification of the isolate. Isolation of genomic DNA, PCR amplification and sequencing of PCR product for analysis of 16S rRNA were conducted. A similarity search for the nucleotide sequence of 16S rRNA of the test isolate was carried out using a blast search at NCBI (Altschul *et al.*, 1990). The gene sequencing was outsourced and conducted at IIHR.
Bacillus spp. ID Flowchart

**Bacillus spp.**

**Starch Hydrolysis (amylace)**

- B. subtilis
- B. cereus
- B. megaterium
- B. stearothermophilus
- B. polymyxa
- B. mycoides
- B. licheniformis
- B. lentus
- B. altus
- B. anthracis
- B. coagulans

**VP (add creatine as a catalyst or co-solvent)**

- B. subtilis
- B. cereus
- B. polymyxa
- B. mycoides
- B. licheniformis
- B. altus
- B. anthracis
- B. coagulans

**Catalase**

- B. subtilis
- B. cereus
- B. polymyxa
- B. mycoides
- B. licheniformis
- B. altus
- B. anthracis
- B. coagulans

**Citrate**

- B. subtilis
- B. cereus
- B. polymyxa
- B. mycoides
- B. licheniformis
- B. altus
- B. anthracis
- B. coagulans

**Fig. 3.1:** Flowchart for *Bacillus sp.* identification (Holt, 1994)

### 3.4.1 Antibiotic resistance/susceptible pattern

The sensitivity of *B. subtilis* to Streptomycin (10µg), Kanamycin (10µg), Tetracyclin (30µg), and Nalidixic acid (50µg) was determined on Mueller-Hinton agar plates by disc diffusion method (Bauer et al., 1966).
3.4.2 Compatibility study of *B. subtilis* with fungicides

*B. subtilis* was tested for its tolerance against commonly used fungicides. Two fungicides, carbendazim and chlorothalonil were evaluated at field application levels of 1 and 2 gm/l. Respective concentrations of filter sterilized fungicides were dissolved in sterilized distilled water and mixed with nutrient agar just before pouring into the petriplates. Upon solidification, the isolate was streaked onto the plates incubated at 28±2°C for 72 h. Response of the *B. subtilis* isolate to fungicide tolerance were recorded (Papavizas and Lewis, 1981) as follows:

- = no growth; + = growth.

3.4.3 Growth promoting attributes of *Bacillus sp.*

3.4.3.1 Indole Acetic Acid (IAA) production

IAA production was detected by the modified method as described by Brick et al. (1991). The cultures were grown in Nutrient broth supplemented with Tryptophan (5mg/ml) and incubated for 2 days. After 48 h of incubation Kovac’s reagent/Salkowski reagent (50ml 35% of perchloric acid, 1ml 0.5M FeCl₃ solution) was added to the broth. The formation of cherry red ring/ pink colour respectively, would indicate the production of IAA.
3.4.3.2 Hydrogen cyanide (HCN) Production (volatile metabolite)

The isolates were screened for the production of hydrogen cyanide by adapting the method of Lock (1948). Nutrient broth was amended with 4.4g glycine/l and Bacillus sp. was streaked on modified agar plate. A Whatman filter paper No.1 soaked in 2% sodium carbonate in 0.5% Picric acid solution was placed at the top of the plate within the lid. Plates were sealed with paraffin and incubated at 28 ±2°C for 3 days. Development of orange red colour on the filter paper indicated HCN production.

3.4.3.3 Detection of diffusible metabolite (agar diffusion method)

For examining antagonism due to diffusible compounds, B.subtilis was grown in Nutrient broth medium amended with CMC at 30°C, 120 rpm for 5 days. At every 24 h interval the supernatant was separated by centrifugation at 10,000 g for 10 min. Supernatant was filtered through a 0.45μm pore size filter (Millipore, India). Test plates were prepared by mixing 9 ml of molten MRBA and 1 ml of supernatant. An actively growing mycelial disc of 9 mm diameter of C.gloeosporioides was inoculated in centre of the petriplate. Plates were incubated for 5 days at 28°C and the results were expressed as mean of percentage inhibition. Plates inoculated with fungal agar plugs alone were used as control (Mukesh et al., 2011) (Delaney et al., 2001).

3.4.3.4 Detection of the Phosphate Solubilizing Activity

Bacterial antagonist was tested for in vitro phosphate solubilizing activity using Pikovskaya agar medium (PVK) (Pikovskaya, 1948). Phosphate solubilization was observed as a zone of clearance around the colony (Qurban et al., 2012).
Composition of PVK medium

<table>
<thead>
<tr>
<th>Chemical</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10.0</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.5</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO₄. 7H₂O</td>
<td>0.1</td>
</tr>
<tr>
<td>Ca₃(PO₄)₂</td>
<td>5.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.2</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2</td>
</tr>
<tr>
<td>MnSO₄. 2H₂O</td>
<td>0.002</td>
</tr>
<tr>
<td>FeSO₄. 7H₂O</td>
<td>0.002</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>

3.4.3.5 Screening for phytase production

The ability of the Bacillus sp. to hydrolyze phytate was checked on phytase screen agar plates containing (g/L) Glucose -10.0; (NH₄)₂SO₄-3.0; KCl-0.5; MgSO₄.7H₂O-0.5; CaCl₂-0.1, Calcium phytate, 5.0; pH 5.5 (Chunshan et al., 2001). After incubating the plates at 30°C for 3 days, phytase production was detected by the presence of a clear zone around a colony.

3.4.3.6 Siderophore Production

The Bacillus sp. isolates were assayed for siderophore production on the chrome azurol S agar medium described by Schwyn and Neilands (1987). Chrome azurol S agar plates were prepared and divided into two equal sectors and spot inoculated with test organism and incubated at 28±2°C for 48-72 h. Development of yellow-orange halo around the growth was considered as positive for siderophore production.

3.4.4 Detection of other hydrolytic enzymes

3.4.4.1 Detection of glucanases

Plates with minimal salt medium containing (1%, w/v) carboxy methyl cellulose (CMC) were prepared. The Bacillus sp. was spot inoculated in the centre of the plate. After an appropriate incubation period at 30°C for 48 h, the agar medium was flooded with an
aqueous solution of iodine for 15 min. The excess Iodine solution was then poured off and plates containing CMC were visualized for zones of hydrolysis detecting β-1,4 glucanase (Teather et al., 1982). Yeast glucan (1%, v/v) containing plates (Chen et al., 1995) were used to detect β-1,3 glucanase activity. Development of a clear zone surrounding the colony indicated enzyme production.

3.4.4.2 Detection of Protease

Protease activity indicated by casein degradation was determined from clearing zones in skim milk agar (Sterilized skimmed milk mixed at 55°C with autoclaved nutrient agar at the rate of 10ml/100ml of NA) after 2 days of incubation at 28°C (Berg et al., 2005).

3.4.4.3 Detection of Lipase

The method involves measurement of fluorescence caused by the fatty acid released due to the action of lipase on olive oil. A qualitative fluorescence lipase assay is based on the interaction of rhodamine B with fatty acid released during the enzyme hydrolysis of olive oil (Kouker and Jaeger, 1987). The isolates were inoculated on media of the following composition (g/L) Nutrient broth (HiMedia), 8.0; sodium chloride, 4.0; agar, 10.0. The medium was adjusted to pH 7.0, autoclaved and cooled to 60°C. Olive oil (31.25 ml) and 10 ml of rhodamine B solution (0.001%, w/v) was added with vigorous stirring and emulsified by mixing for 1 min. The medium was allowed to stand for 10 min at 60°C to reduce foaming. 20 ml of the medium was poured into sterile petriplates and allowed to solidify. Detection of fluorescence around the colony under UV light indicated the production of lipase.
3.4.5 Assay of hydrolytic enzymes

3.4.5.1 Chitinase

Chitinase activity was measured with colloidal chitin as a substrate. The culture broth was centrifuged at 10,000g for 10 min and enzyme solution (1 ml) was added to 1 ml of substrate solution, which was made by suspending 1% of colloidal chitin in phosphate buffer (pH 7.0). The mixture was incubated at 37°C for 60 min. One ml of Dinitrosalicylic acid (DNS) was added and incubated at 100°C in water bath. The amount of reducing sugar produced in the supernatant was determined by measuring the optical density at 540nm (Miller, 1959). One unit of chitinase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per minute and was calculated from standard curve by using glucose as standard (Monreal and Reese, 1969).

3.4.5.2 β-1,3-glucanase and β-1,4-glucanase

β-1,3-glucanase and β-1,4-glucanase activities were determined by measuring the amount of reducing sugars liberated using DNS (Miller, 1959). The culture broth was centrifuged at 10,000g for 10 min and enzyme solution of 1 ml was added to 1 ml of substrate solution which contained 1 ml of yeast cell wall extract (1%, v/v) for β-1,3-glucanase and carboxy methyl cellulose solution (1%, v/v) for β-1,4-glucanase, respectively. The mixture was incubated in a water bath at 50°C for 30 min and the reaction was terminated by adding 1ml of DNS solution and incubated in boiling water bath for 10-15 min till the development of the colour of the end product. Reducing sugar concentration was determined by measuring the optical density at 540 nm (Gadelhak et al., 2005). The amount of reducing sugars released was calculated from standard curve for glucose and was defined as the amount of enzyme that produced 1 μmol of reducing sugar per minute.
3.5 Optimisation of growth conditions for maximum production of enzymes

3.5.1 Media optimisation

Three different media namely nutrient broth (NB), Luria Bertaini broth (LB) and yeast nitrogen base broth (YNB) amended with 1% colloidal chitin were used to determine the growth and chitinase, glucanases (β-1,3 and β-1,4) production (Shanmugaiah et al., 2008). One ml inoculum of *Bacillus subtilis* with 0.5 OD was inoculated to 100 ml of each medium and incubated at room temperature in a rotary shaker with 100 rpm. The culture filtrate was assayed for the above mentioned enzymes every 24h interval for a period of five days.

3.5.2 Induction of lytic enzyme production with pure substrates and fungal mycelium

*Bacillus* sp. was grown with 1% (w/v) of different substrates such as chitin, glucan and CMC amended in nutrient broth to determine the most suitable substrate for the hydrolytic enzyme production (Shanmugaiah et al., 2008). *B.subtilis* was also separately grown on nutrient broth supplemented with dead fungal mycelium (*C. gloeosporioides* OGC1 and *R.solani*) as inducer for enzyme production at a concentration of 1%,dispensed in Erlenmeyer flasks (250 ml) containing 50 ml of medium. The flasks were autoclaved and inoculated with 1.0 ml of a pure culture of *B. subtilis*. The culture was incubated in shaker (120 rpm), at 28±2°C. To study the induction pattern with pure substrate and fungal mycelium, aliquots from the flask were analyzed daily for chitinase and glucanases (β-1,3 and β-1,4) for a period of 5 days (Moataza, 2006).
3.5.2.1 Preparation of hyphal wall

The pathogenic fungal culture (C. gloeosporioides and R. solani) was inoculated into 50 mL of PDB broth and incubated at 30°C for 5 days under shaking conditions. After incubation, the mycelia were collected by filtration. The mycelia were thoroughly washed with autoclaved distilled water and homogenized on ice with a homogenizer for 5 min. The mycelial suspension was centrifuged at 10,000 g for 20 min at 4°C (Remi-C24, Remi Laboratory Instruments, India). The pellet was resuspended in sodium phosphate buffer (0.1 M, pH 7.0). This preparation was used as substrate for the hydrolytic assay and mycelium induction studies (Moataza, 2006).

3.5.3 Hydrolytic activity of B. subtilis culture filtrate

For assessing the hydrolytic activity, the reaction mixture (1 mL) containing 1 mg/mL of fungal mycelia with 1.0 mL of crude enzyme (from B. subtilis grown on NB+CMC) was incubated at 30°C for 24 h. The released total reducing sugars (Miller, 1959) in the control and treated fungal cell wall was estimated using the DNS method. The hydrolytic activity was performed using the culture filtrate of B. subtilis (El-Katatny et al., 2000).

3.5.4 Microscopy

The fungal culture grown on NA agar plate without any bacterial culture served as control. The damage caused by the bacterium to the fungal mycelium by dual plate assay was studied microscopically. The mycelium along with the agar disc present in the inhibition zone and control mycelium was taken, stained with lactophenol cotton blue and observed under a Nikon Trinocular microscope (Basha and Kandasamy, 2002).
3.5.5 Growth of Bacillus subtilis on different nutrient sources

Nutrient broth media including 12 carbon sources, 8 nitrogen sources, 5 surfactants and 9 different metal ions (CaCl$_2$, CoCl$_2$, MgCl$_2$, CuSO$_4$, MgSO$_4$, HgCl$_2$, FeSO$_4$, ZnSO$_4$ and Pb(C$_2$H$_3$O$_2$)$_2$ were screened for their effect on chitinase, β-1,3 glucanase and β-1,4 glucanase production by Bacillus subtilis. Effect of different carbon sources was checked by replacing chitin in the basal media with different carbon sources (equivalent weight) or 5 g/l of different test sugars or 1 g/l of pure sugars (with/without chitin). Nitrogen was screened by replacing the corresponding source in the basal medium in equimolar concentrations (1.0 g/l for peptone) with respect to N. Surfactants and metal ions were screened by supplementing the media with the test component. The concentration of surfactants used while screening was 0.1% while the concentration of metal ions used were 5 mM for macronutrients and 1 mM for micronutrients (Singh, 2010).

3.5.5.1 Enzyme assay with optimised conditions

The culture was grown at 30°C with 120 rpm for 24 h. 200 µL of the culture inoculum was transferred to 250 mL Erlenmeyer flask containing 100 mL of broth medium with alterations made after optimization. All the experiments were performed in triplicates. The culture broth after incubation was centrifuged at 10,000 g for 10 min at 4°C to separate the cells. The cell-free supernatant was analyzed for enzyme activity (Immanuel et al., 2006).

3.6 Mechanism of antagonism

To characterise the mechanism of antagonism of B.subtilis towards the fungal pathogens, mutants with loss of antagonism were obtained using classical mutagenesis and enzyme activity was evaluated to check the relation between loss of antagonism and enzyme activity. Both chemical and physical mutagenesis was used for the study.
3.6.1 EMS mutagenesis

Chemical mutagenesis of *B. subtilis* was carried out with ethyl methane sulfonate by Khattab and Bazaraa (2005). The *B. subtilis* cells (10⁸ cfu/mL) were washed once with 0.1M Tris-HCl buffer (pH 7.5) and resuspended in 1mL of the same buffer. To this 100 μl of EMS was added and incubated in shaker water bath for 45 mins. The cells were washed with 0.1M Tris-HCl buffer (pH 7.5) and serial dilutions were plated on nutrient agar plates. The mutants were tested for their antagonistic activity using the dual plate assay. The mutants with loss or increase in this property were tested for their ability to produce the mycolytic enzymes under shake flask conditions. These mutants were also checked for their hydrolytic activity using *C. gloeosporioides* mycelia as substrate (Gohel *et al.*, 2004).

3.6.2 UV mutagenesis

To characterise the antagonistic mechanism by this bacterium, a mutant of this bacterium was developed, which lost its antagonistic activity. UV mutagenesis of *B. subtilis* was carried out following the procedure of Miller (1992). During UV mutagenesis, the log phase culture of *B. subtilis* was exposed to short wavelength UV light (280 nm, Philips TUV 30 W, G3018, Holland) from a distance of 30 cm for various time intervals (for 0.1 % UV survivors). From the serial dilutions of the mutagenized culture, 0.1 ml was plated on NA plates for isolated mutant colonies. The isolated colonies were further screened for the loss of antifungal activity using dual plate assay. The mutants were also screened for mycolytic enzyme activity and hydrolytic activity with the *Colletotrichum gloeosporioides* mycelia as substrate.

3.6.3 Dual plate assay

The wild and mutant strains of *B.subtilis* were evaluated for their antagonistic potential against *C gloeosporioides* by dual culture technique of Huang and Hoes (1976). Each experiment using a single pathogen isolate was run in triplicates.
3.6.4 Hydrolytic activity of *B. subtilis* (Wild Type and Mutants) culture filtrate

For assessing the hydrolytic activity, the reaction mixture (1 ml) containing 1 mg/ml of fungal mycelia with 1.0 ml of crude enzyme (from *B. subtilis* grown on NB+CMC) was incubated at 30°C for 24 h. The released total reducing sugars (Miller, 1959) in control and treated fungal cell wall was estimated using the DNS method.

3.6.5 Sensitivity of the culture supernatant of *B. subtilis* to proteolytic enzymes, TCA and heat treatment

The crude supernatant (1 mL) was subjected to treatments for 1 h at 37°C (for enzymes) or room temperature (for TCA). The proteolytic enzymes (Sigma) were used at a final concentration of 1 mg mL⁻¹ in 10 mmol⁻¹ potassium phosphate buffer, pH 7.0. The crude supernatant in buffer without enzymes as well as the enzyme solutions was exposed to the same conditions. For the heat treatment, the preparations were incubated at 70°C, 80°C and 90°C and autoclaving (121°C) for 20 min. Antifungal activity of the culture filtrate was checked before and after all treatments on a test plate made with *C. gloeosporioides* as mentioned in the dual plate assay (Tendulkar *et al.*, 2007).
3.7 Assessing antagonistic potential of *B. subtilis* against selected pathogens in chilli seeds

3.7.1 *In vitro* seedling assay

Germination efficiency and antagonism of the *B. subtilis* against *C. gloeosporioides* and *R. solani* was checked on chilli seeds *in vitro* using water agar. The water agar (1%, w/v) plates were seeded with the following (Leben, 1983):

- Set 1- Seeds (Control): plain seeds coated with carboxy methyl cellulose (CMC);
- Set 2- Seeds coated with CMC and *Colletotrichum gloeosporioides/ R. solani* spores;
- Set 3- Seeds coated with CMC and *B. subtilis* culture and
- Set 4 - Seeds coated with CMC and either *C. gloeosporioides/ R. solani* spores with *B. subtilis* culture.

Chilli seeds (Arka shweta variety) were procured from IIHR, surface sterilized successively with sterile distilled water and 0.1% HgCl$_2$. To remove the residual HgCl$_2$, the seeds were washed with sterile distilled water. *B. subtilis* was inoculated into NB medium and incubated for 24 h at 30ºC (Moataza, 2006). *C. gloeosporioides/ R. solani* was inoculated onto PDA plates and incubated at 28ºC for 3-4 days. The above three sets of treated seeds were seeded onto 1% (w/v) water agar plates. Plain CMC coated seeds on water agar were used as control. The four sets were monitored regularly for germination and growth. After one week, the sets were observed for germination and biocontrol effect against *C. gloeosporioides* and *R. solani* coated seeds by the isolate. The seeds were observed for germination, incidence of pathogen attack of the seedling and severity of fungal infestation.
3.7.2 In Vitro Root Colonization

The antagonists were tested for their ability to colonize chilli roots in vitro, using a modification of the methods by Patten and Glick (2002) and Montealegre et al. (2003). Chilli seeds were surface sterilized with 70% ethanol for 5 min and subsequently with 1% sodium hypochlorite for 1 min and rinsed two times in sterile distilled water. For each treatment, 15 seeds were transferred to a sterile moist chamber i.e. discs of filter paper placed in 9 cm. diameter Petri dishes and moistened with sterile distilled water. The antagonistic bacteria were grown in NB on a rotary shaker at 28±2°C and 180 rpm for 24 h. The culture was serially diluted and plated on Nutrient agar to give a final concentration of 100 cfu/ml using the viable plate count method. One ml aliquot of each inoculum was added to the seeds in the moist chamber and the plates were incubated at room temperature for 1 h to allow binding of the bacteria to the seed coat. Both treated seeds and controls were then incubated at 30°C for 10-12 days in the dark for root development. One centimeter of root from each treatment was aseptically excised, one seed per treatment, and transferred to sterile double distilled water, shaken and serially diluted. From each dilution, 0.1ml aliquot was plated on nutrient agar media for bacteria and the plates were incubated at 30°C for colony counts. The number of antagonist isolates colonizing the root was calculated as colony forming units/cm root (cfu/cm root).

3.7.3 Preparation of talc/lignite based formulation of B.subtilis

200µL of B.subtilis was inoculated into the Nutrient broth and incubated on rotary shaker at 150 rpm for 48 h at room temperature (28°C). After 48 h of incubation, the broth containing 9x10⁸ cfu/mL was used for the preparation of talc/lignite based formulation. To the 400 mL of bacterial suspension, 1 kg of the talc powder (sterilized at 105°C for 12 h), calcium carbonate 15 g (to adjust the pH to neutral) and carboxymethyl cellulose 10 g (as
adhesive) were mixed under sterile conditions (Vidyasekaran and Muthamilan, 1999). After overnight shade drying under sterile conditions, it was packed in polypropylene bag and sealed. At the time of application, the moisture content of the formulation was 10% and the population of bacteria in talc formulation was 2.5 to 3 x 10^8 cfu/g.

3.7.3.1 Study of Shelf Life Stability of B. subtilis in Talc/Lignite Formulation

The prepared formulation was stored under different temperatures viz., 30°C and 4°C. One g samples were taken from three randomly selected covers and serial dilutions were made and plated on Nutrient agar medium to determine the average concentration of viable cells of strain in the delivery medium. For monitoring the shelf life of the formulation, samples were taken monthly for a period of 6 months and subjected to the same plate count procedure (Anitha and Rabeeth, 2010).

3.8 Assessing antagonistic potential of B. subtilis against selected pathogens in chilli seedlings under glass house conditions

A pot culture experiment was conducted to assess the biocontrol potential of B. subtilis against C. gloeosporioides anthracnose and R. solani root rot by dual inoculation of the pathogen and the biocontrol agent.

3.8.1 Pathogen culture

C. gloeosporioides (OGC1) and R. solani were cultured on PDA (Difco) at 28°C for 10 days. The conidial suspensions were collected by scraping the colony surface with a sterile scalpel and 10 mL of sterile distilled water, after which they were filtered through four layers of cheese cloth to remove any mycelial debris and spores were counted with a hemocytometer and adjusted to 2.1 × 10^6 conidia/mL.

3.8.2 Biocontrol agent preparation

The talc based bioformulation of the biocontrol agent, B. subtilis with a population of 2.5 to 3 x 10^8 cfu/g was used for the pot studies.
3.8.3 Soil analysis and preparation for sowing

The medium red soil was obtained from a vegetable growing field in Bengaluru district for the pot culture studies. The soil was mixed with sand and well decomposed farm yard manure in the ratio 1:1:1 and filled into plastic pots of 5 kg capacity at the rate of 4.5 kg/pot. The potting mixture was analysed for soil texture, pH (measures acidity / alkalinity), electrical conductivity (measures salinity / alkalinity), available P content (mmols⁻¹), exchangeable K content (C mols/kg soil), soil organic matter content (%) (Walkley and Black, 1934) content of calcium, magnesium, copper, Iron and sulphur. (Jackson, 1960; Page et al., 1989; Hunter, 1984).

Chilli cultivar Arka shweta seeds (Obtained from IIHR, Hessaraghata, Bangalore) were surface sterilized and then sown in a plastic tray (55 cm × 35 cm × 15 cm) containing potting medium (red soil : sand : farm yard manure at 1 : 1 : 1 w/w/w). Seedlings at the two-leaf stage at the rate of 2 seedlings per pot were transplanted to plastic pots (5 cm × 15 cm × 10 cm) containing the same potting medium. Chilli plants were raised and subsequently used for the disease incidence and biocontrol assay (Kabir Lamsal et al., 2012).
Treatments

The experiment consisted of twelve treatments and the treatment for soil and seed application method details are given below.

- T1 = \textit{B. subtilis}
- T2 = \textit{Bacillus subtilis} + \textit{Colletotrichum gloeosporioides}
- T3 = \textit{B. subtilis} + \textit{Rhizoctonia solani}
- T4 = Carbendazim + \textit{B. subtilis}
- T5 = Chlorothalonil + \textit{B. subtilis}
- T6 = Carbendazim + \textit{C. gloeosporioides}
- T7 = \textit{C. gloeosporioides}
- T8 = Carbendazim
- T9 = \textit{R. solani}
- T10 = \textit{R. solani} + Chlorothalonil
- T11 = Chlorothalonil and
- T12 = Control (untreated).

Treatments

For Foliar spray and root dip application method

- T1 = Control (untreated).
- T2 = \textit{B. subtilis}
- T3 = Carbendazim
- T4 = Chlorothalonil
The talc-based formulation of \textit{B. subtilis} was applied as seed treatment (20g/kg of seeds), seedling root dip (20g/L water for 1 hr), soil treatment (10 g/pot) and foliar spray (20g l\(^{-1}\)). The soil treatment was given at the time of transfer of the seedlings to the pot. The foliar spray was given thrice, at vegetative growth, pre flowering, fruit maturation stages.

Carbendazim was applied as seed treatment (1-2g/kg of seeds), seedling root dip (1gL\(^{-1}\)) and soil treatment (1g/pot) and foliar spray (1gL\(^{-1}\)).

Chlorothalonil was applied as seed treatment (1-2g/kg of seeds), seedling root dip(1gL\(^{-1}\)) and soil treatment (2g/pot) and foliar spray (2gL\(^{-1}\)).

### 3.8.4 Method for seedling dipping

Solution was prepared @ 1Kg biopesticide for 40 litres of water, poured into a tray and roots of the seedlings were dipped for required time duration. Later seedlings were removed from the solution and used for transplanting into the pots. The plants were grown and disease incidence was recorded as per standard protocols.

The Chilli seedlings treated with the seedling root dip and foliar spray application methods were subjected to natural infection whereas the soil and seed application methods were challenge inoculated with the potent pathogen (Rini and Sulochana, 2006). Pathogen inoculated and un-inoculated controls were maintained. Watering was done regularly; disease incidence was recorded 90-120 days after sowing.

Parameters recorded to study growth enhancement included plant height, fresh weight, dry weight and length of shoot and root and yield, whereas, number of diseased plants and the root and fruit quality were recorded and assessed to study disease control. The dry matter content of the plant was recorded after drying the plants to constant weight at 60\(^{\circ}\)C.
3.8.5 Challenge inoculation with Pathogen/s

To incite the disease, 25 day-old seedlings transplanted into the pots were inoculated with *C. gloeosporioides/ R. solani* isolate at concentration of $10^6$ conidia/ml, 5 days after transplanting. The inocula were sprayed over the canopy (2 ml/plant).

The plants were allowed to grow for 100 days and observation on the per cent infection was calculated by using the formula given below (Viqar Sulatana *et al.*, 2006).

\[
\text{No. of plants infected} \\
\% I = \frac{\text{No. of plants infected}}{\text{No. of plants infected in control}} \times 100
\]

All treatments were replicated 6 times in factorial completely randomized design (CRD) (Table 3.1).

<table>
<thead>
<tr>
<th>T12</th>
<th>T1</th>
<th>T4</th>
<th>T6</th>
<th>T9</th>
<th>T7</th>
<th>T5</th>
<th>T2</th>
<th>T8</th>
<th>T3</th>
<th>T10</th>
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</thead>
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<td>T9</td>
<td>T2</td>
<td>T12</td>
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<td>T7</td>
<td>T9</td>
<td>T8</td>
<td>T10</td>
<td>T1</td>
</tr>
</tbody>
</table>

Table 3.1. CRD of the treatments for pot culture studies

3.8.6 Experimental design and statistical analysis

The experiment was carried out following Randomized Block Design (RBD) with three replicates. Data on growth yield and yield contributing characters were recorded and statistically analyzed with the help of computer package program SPSS (IBM SPSS Statistics 20.INK) for DMRT test at 5% ($p = 0.05$) level of significance.
3.9 Post harvest studies for anthracnose

The severity of anthracnose disease on chilli fruits caused by *C. gloeosporioides* was studied by using 30 wounded chilli fruits (one wound per fruit) inoculated with *C. gloeosporioides*. One group of 30 chilli fruit samples was thoroughly sprayed with talc formulation (40g of *B. subtilis* talc formulation/L) in distilled water. Control fruits were treated with sterile distilled water. All chilli fruits were put on 17 x 11 x 4 cm plastic trays wrapped with high density polyethylene sleeve. Sterile water was used to maintain relative humidity (RH) in the fruit trays. The trays were stored in the dark at 28 °C for 10 days. The experiments were repeated three times and each treatment was conducted in triplicate of wounded control and experimental chilli fruits (Chanchaichaovivat *et al.*, 2007). The efficiency of *B. subtilis* was monitored by growth and productivity of the chilli that showed against anthracnose disease.

3.10 Statistical analysis

The entire study was carried out with three replicates. Data was recorded and statistically analyzed with the help of computer package program SPSS (IBM SPSS Statistics 20.INK) for DMRT test at 5% (*p* =0.05) level of significance.