Study of mycolytic enzymes of *Bacillus* sp. against *Colletotrichum gloeosporioides* causing anthracnose in Chilli

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ABSTRACT

Chilli is a universal spice of India and it is cultivated over an area of 9.15 Lakh ha with an annual production of 10.18 Lakh tonnes. Among various fungal diseases, which are severely constraint chilli production world wide, anthracnose disease is one of the major. It is caused by *Colletotrichum* sp. and is one of the most economically important diseases reducing marketable yield from 10 to 80% of the crop production. Of the several biocontrol agents studied, *Bacillus* sp. offers several advantages over other organisms as they form endospores and hence, can tolerate extreme pH, temperature and osmotic conditions. Many members of the Bacillaceae secrete mycolytic enzymes that allow them to degrade extracellular macromolecules. Among the mycolytic enzymes, chitinases and cellulases (β-1,3, β-1,4), which are quite widespread amongst the representatives of saprophytic soil microflora, attract the most attention. In this study, strains of *Bacillus* sp. from 15 chilli rhizosphere soil were screened for chitinolysis on chitin amended plates and their involvement in the suppression of few pathogens was determined. The selected isolate showed broad spectrum antagonism against *Alternaria* sp. (55%), *Colletotrichum gloeosporioides* (57%), *Phytophthora capsici* (62%), *Rhizoctonia solani* (42%), *Fusarium solani* (42%), *Fusarium oxysporum* (40%) and *Verticillium* sp. (36%). The isolate detected positive for chitinase and cellulases (β-1,3 and β-1,4). The induction profile for the enzymes chitinase, cellulases (β-1,4) and β-1,3) with different media, pure substrates as well as with *Colletotrichum* mycelia was determined. Seed treatment with the pathogen and the strain brought significant (65%) reduction in disease incidence as against the untreated control, suggesting the efficacy of this strain as potential biocontrol agent for anthracnose of chilli.

Keywords: *Bacillus* sp., chilli phytopathogens, mycolytic enzymes, antagonism, biocontrol.

INTRODUCTION

Chilli is a universal spice of India. In India it is cultivated over an area of 9.15 lakh ha with an annual production of 10.18 lakh tonnes of dry chilli. Chilli has many culinary advantages. It comprises numerous chemicals including steam-volatile oils, fatty oils, capsaicinoids, carotenoids, vitamins, protein, fibre and mineral elements. Solanaceae represent the third most economically important plant taxon and the most valuable in terms of vegetable crops with agricultural utility representing for more than 3000 species, including the tuber-bearing potato, a number of fruit-bearing vegetables (tomato, eggplant, peppers), ornamental plants (petunias, Nicotiana), plants with edible leaves and medicinal plants. Chilli is being affected by several fungal, bacterial and viral diseases which are the major constraints to its production. Anthracnose disease caused by *Colletotrichum* species, bacterial wilt caused by *Pseudomonas solanacearum* and mosaic disease

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caused by chilli veinal mottle virus (CVMV) or cucumber mosaic virus (CMV) are the most serious destructive diseases of chilli [1-3].

Some of the other diseases that lead to wilting in chilli are phytophthora root rot, verticillium wilt, rhizoctonia root rot and Fusarium wilt. Anthracnose disease caused by *Colletotrichum* sp. is one of the most economically important diseases reducing marketable yield from 10 to 80% of the crop production in some developing countries, particularly in Thailand [4]. Anthracnose is a main problem on mature fruits, causing severe losses due to both pre- and post-harvest fruit decay [1,5]. In the *Colletotrichum* patho-system, different *Colletotrichum* sp. can be associated with anthracnose of the same host [6-8]. *C. acutatum* and *C. gloeosporioides* have been reported to be more prevalent on both young and mature green fruits [9,10]. The effective control of anthracnose involves the use of a combination of cultural, biological, chemical and intrinsic resistance [11]. However management and control of the anthracnose disease are still under extensive research. Health, environmental concern, development of resistance in target populations are also contribute to developing biological control using natural enemies. Nonetheless, the vast array of antimicrobial molecules produced by diverse soil microbes remains as a reservoir of new and potentially safer biopesticides [12]. *Bacillus subtilis, Trichoderma* sp. and *Candida oleophila* are being checked as effective biocontrol agents against *Colletotrichum* sp. [6,9].

Bacillus sp. have shown promising results for the biological control of various plant pathogens as well as growth promoters of some crops [12,13]. The main features of bacilli that make them attractive agents of biological control of phytopathogens are their wide prevalence, the capacity for rapid growth and endospore formation, relative safety for humans and animals, as well as the production of a broad spectrum of biologically active compounds [14]. A major mechanism involved in the biological control of plant pathogens is parasitism via degradation of the cell wall. The synthesis of extracellular hydrolases capable of destroying fungal cell wall structural polymers is considered to be one of the possible mechanisms. Among the mycolytic enzymes, chitinases (EC 3.2.1.14) and β-1,3-glucanases (EC 3.2.1.6; 3.2.1.39), which are quite widespread among the representatives of saprophytic soil microflora, attract the most attention. However, the active involvement of enzymes in inhibiting the growth of phytopathogenic fungi has been confirmed only for individual well-studied groups of microbial antagonists [15]. The role of extracellular hydrolases of most saprophytic antagonistic bacteria in the biological control of phytopathogens is poorly studied, since their main function is supposed to be the decomposition of organic matter from the dead fungal mycelium for nutrient extraction [16]. β-glucanases, produced by several fungi and bacteria are one of the most potent enzymes for degrading fungal cell walls [17-19]. More recently, intensive efforts have been made to use the biocontrol agents for protecting fruit and vegetable crops from post-harvest diseases [20-22]. Antagonists were selected following evidence of direct interactions or after the demonstration that substances or antibiotics toxic to the potential pathogens were secreted into the growth medium. Strains of *Bacillus subtilis* also have been studied as biocontrol agents of plant pathogens [23-25].

The ability of *B. subtilis* to suppress a wide range of plant pathogens and also its reputation as a safe and friendly microorganism is attractive. Many isolates of *B. subtilis* display antagonistic activity against a number of pre-harvest and post-harvest pathogens on several different crops [26-28]. The present study involves screening and isolation of *Bacillus* sp. from chilli rhizosphere antagonistic to *Colletotrichum gloeosporiodes.*

MATERIALS AND METHODS

Isolation of rhizospheric *Bacillus* sp.

Bacillus sp. was isolated from rhizospheric soils of Chilli grown in and around Bangalore City by soil dilution method. The dilutions were heat treated to ensure that only heat resistant strains

remained. The different isolates obtained were screened for chitinase production on plates with minimal salts medium amended with 1% chitin [28]. The *Bacillus* sp. thus obtained was maintained on nutrient agar amended with 1% chitin.

Preparation of colloidal chitin

Colloidal chitin was prepared from crab shell chitin powder [29]. 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 ice-cold 95% ethanol with rapid stirring and kept overnight at 25ºC and then stored at -20ºC 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) and used for further applications.

Phytopathogens and dual plate assay

The following six phytopathogens were obtained from Indian Institute of Horticultural Research, Hessarghatta, Bangalore, India: *Alternaria brassisicola* (OCA1), *Alternaria brassicae* (OCA3), *Alternaria alternata* (OTA36), *Fusarium solani*, *Colletotrichum gloeosporioides* (OGC1) and *Phytophthora capsici* (98-01); *Verticillium theobromae*, *Fusarium oxysporum* and *Rhizoctonia solani* (MTCC 4633) was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. These nine pathogens were used in the present study. The fungal growth inhibition capacity of *Bacillus* sp. strains was determined [30]. A few modifications were made to suit the need. One 5 mm disk of a pure culture of the pathogen was placed at the centre of a Petri dish containing potato dextrose agar (PDA). The *Bacillus* sp. was inoculated at 2 opposing corners. Plates were incubated for 72 h, at 28ºC 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. Percent inhibition was calculated using the formula: [1 - (fungal growth/control growth)] × 100.

Detection of hydrolytic enzymes

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 30ºC for 48 h, the agar medium was flooded with an aqueous solution of Congo red for 15 min. The Congo red solution was then poured off and plates containing CMC were visualized for zones of hydrolysis detecting β-1,4 cellulase [31-33]. Yeast glucan containing plates [34] was used to detect β-1,3 cellulase activity. Minimal salts medium with (1%, v/v) yeast cell glucan was prepared and spot inoculated with the isolate. Development of a clear zone surrounding the colony indicated enzyme production.

Assay of hydrolytic enzymes

Chitinase enzyme assay

Chitinase activity was measured with colloidal chitin as a substrate. The culture broth was centrifuged 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 50ºC

for 30 min. 1ml of Dinitrosalicylic acid (DNS) was added and incubated at 100ºC in boiling water bath. The amount of reducing sugar produced in the supernatant was determined by measuring the optical density at 540nm [35]. One unit of chitinase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugars per minute [36].

β-1,3-cellulase and β-1,4-cellulase assay

β-1,3-cellulase and β-1,4-cellulase activities were determined by measuring the amount of reducing sugars liberated using DNS [35]. The culture broth was centrifuged 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-cellulase and carboxy methyl cellulose solution (1%, v/v) for β-1,4-cellulase, respectively. The mixture was incubated in a water bath at 50ºC 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 optical density at 540 nm [37].

Morphological and phenotypic characterization of *Bacillus* sp.

These were performed according to Parry et al. [38]. Parameters investigated were cell and spore morphology, motility, growth characteristics (anaerobic growth and growth in the presence of NaCl 6.5%), hydrolysis of starch, casein, nitrate reduction and Voges Proskauer reaction.

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, cellulases (β-1,3 and β-1,4) production [31]. 1 ml inoculum of *Bacillus* sp. with 0.5 OD was inoculated with 100 ml of each medium and incubated at room temperature at 100 rpm in a rotary shaker. After two days of incubation, the cultures were harvested, centrifuged at 10,000 rpm for 15 min and the supernatant was used for enzyme assay.

Induction with pure substrate and fungal mycelium

Bacillus sp. was grown with 1% of different substrates such as chitin, glucan and CMC amended in nutrient broth to determine the most suited substrate for the hydrolytic enzyme production [31]. The *Bacillus* sp. was separately grown on nutrient broth supplemented with dead fungal mycelium (*Colletotrichum gloeosporoides* OGC1) as inducer for enzyme production at a concentration of 1% and dispensed in Erlenmeyer flasks (250 ml) each flask contained 50 ml of medium. The flasks were autoclaved and inoculated with 1.0 ml of a pure cultured *Bacillus* sp. The culture was incubated in a shaker (120 rpm), at 28±2ºC. Aliquots from the flask were analyzed daily for chitinase and cellulases (β-1,3 and β-1,4) for a period of 5 days [32].

Seed testing

Germination efficiency and antagonism of the *Bacillus* sp. against *Colletotrichum gloeosporoides* was checked on chilli seeds *in vitro*. The water agar plates were seeded with the following: Set 1- Seeds control-plain seeds coated with carboxy methyl cellulose (CMC); Set 2- Seeds coated with CMC and *Colletotrichum gloeosporoides* spores; Set 3- Seeds coated with CMC and *Bacillus* sp.

culture and Set 4 -Seeds coated with CMC and both *Colletotrichum gloeosporoides* spores and *Bacillus* sp. culture. Chilli seeds were 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. The isolate was inoculated into NB medium and incubated for 24 h at 30ºC [32]. *Colletotrichum gloeosporoides* was inoculated onto PDA plates and incubated at 28ºC for 3-4 days. The above three sets of treated seeds were seeded onto 1% 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 against *Colletotrichum gloeosporoides* coated seeds by the isolate.

RESULTS AND DISCUSSION

The importance of chitinase in biological control of fungi, nematodes and insect pests is an emerging field of research [39]. Detection of chitin degrading bacteria from natural sources such as rhizosphere soil is useful in the isolation of bacteria that produce antifungal or other novel compounds [32,40-42]. Out of 15 samples plated, 9 chitinolytic colonies were isolated based on the clearance zones that they formed. These chitinolytic bacteria were subjected to dual plate assay with nine different chilli fungal pathogens, one particular isolate designated as isolate 2 showed inhibitions of all the nine pathogens and this was chosen for further work. The selected isolate showed broad spectrum antagonism against *Alternaria alternate* (45%), *Alternaria brassicae* (52%), *Alternaria brassicicola* (58%), *Colletotrichum gloeosporioides* (57%), *Phytophthora capsici* (55%), *Rhizoctonia solani* (42%), *Fusarium solani* (42%), *Fusarium oxysporum* (40%) and *Verticillium* sp. (36%), the range of percentage inhibition varied from 40-62 (Figure 1).

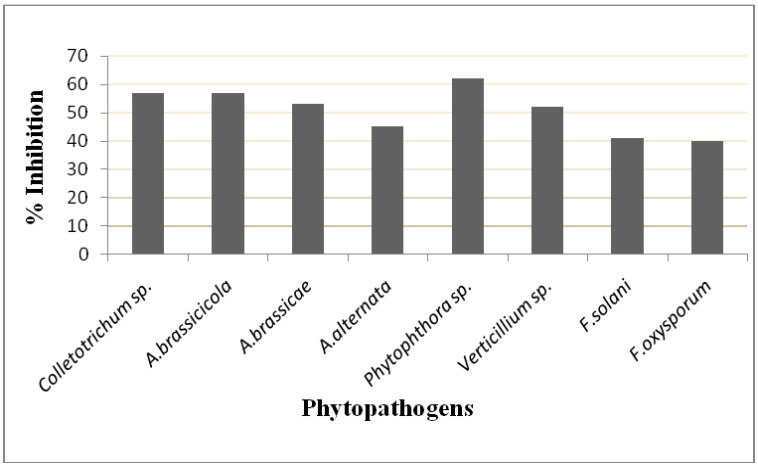

Figure 1. Dual plate assay showing *in vitro* inhibition of fungal phytopathogen growth by *Bacillus* sp.

Mycolytic enzymes produced by antagonistic microorganisms are very important in biocontrol technology. There are many reports on production of lytic enzymes by microorganisms [43-45]. In recent years microbial lytic enzymes such as chitinase, and β-1,3-glucanase have been exploited for the management of plant diseases. β-glucanases, produced by several fungi and bacteria are one of the most potent enzymes for degrading fungal cell walls [16-19]. Hence the isolate was checked for other hydrolytic enzyme production and was found to produce both cellulase β-1,3 and β-1,4 as it produced clearance zones on yeast glucan plates as well as CMC plates respectively.

This strain was characterized morphologically and biochemically by Bergey's manual of systematic bacteriology. It was grown and maintained on NA at 30ºC. The isolate upon Gram's staining was identified as a gram positive, spore forming rod. The hanging drop preparation also confirmed it to be a motile rod. It answered positive for catalase, nitrate reduction, Voges Proskauer, starch hydrolysis and growth on 6.5% NaCl medium. It answered negative for parasporal crystal formation and citrate utilisation. Hence, it was tentatively identified as *Bacillus* sp. Culture medium is a key factor for the growth as well as metabolite production by microorganisms. Among three media tested, only colloidal chitin amended NB supported chitinase production (10 U/ml), while all 3 media supported the production of cellulase (β-1,3 and 1,4). As evident from the graph (Figure 2) NB supported maximum activity of all three hydrolytic enzymes with peak activity on day 3 (10 U/ml for chitinase, 8 U/ml for cellulase β-1,3 and 13 U/ml for cellulase β-1,4). Hence nutrient broth was chosen as the best basal medium for optimization study.

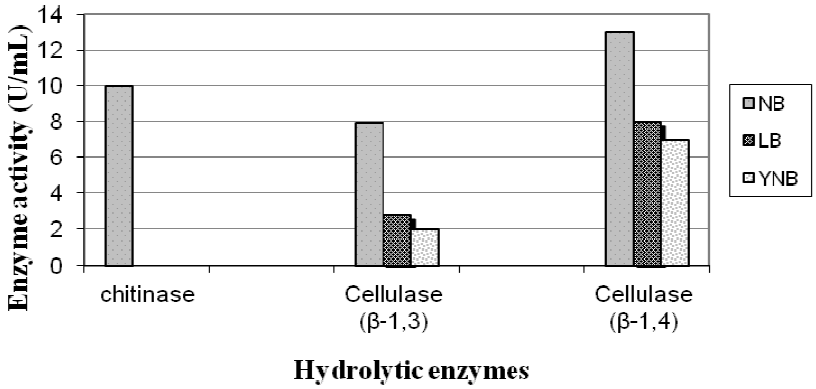

Figure 2. Production of chitinase, cellulases (β-1,3 and β-1,4) by *Bacillus* sp. in different media [Nutrient broth (NB), Luria Bertanni broth (LB) and yeast nitrogen base broth (YNB)] amended with 1% colloidal chitin.

The induction profile of the three mycolytic enzymes with pure substrates such as colloidal chitin, yeast cell glucan, carboxy methyl cellulose (CMC) supplemented in NB showed higher levels of enzyme production with addition of each of the substrates as compared to NB alone. The addition of CMC to NB proved to be the best for all the three enzymes, giving activity of 20 U/ml for chitinase, 18 U/ml for β-1,3 cellulase and 33 U/ml for β-1,4 cellulase (Figure 3). This is in accordance with the fact that most of the mycolytic systems reported in the literature are inducible [45-47]. In *T. harzianum*, the β-1,6-glucanase activity was secreted into the growth medium containing chitin, pustulan, nigeran, fungal cell walls [48] or autoclaved mycelia as the sole carbon source, rather than the nutrient medium alone.

Mycolytic enzymes, which are key enzymes in the lysis of cell walls of higher fungi, are produced by other organisms that are known to attack and parasitize fungi. Media containing fungal cell wall fragments as carbon source supported high production of chitinases and β -1,3-glucanases [49-53]. The induction profile of the *Bacillus* sp. was checked with autoclaved *Colletotrichum gleosporiodes* mycelium used as the carbon source in the medium. Data represented in figure 4, showed that the lysis of dead mycelia of *Colletotrichum gloeosporoides* was very efficient by the *Bacillus* sp. Similar levels of all the three enzymes were observed in the presence of autoclaved mycelia; chitinase (2.84 U/ml by day 1), β-1,3-cellulase (4.21 U/ml by day 2) and β-1,4-cellulase (13.21 U/ml by day 3) suggesting the possible role of these enzymes in antibiosis of the mycelia.

Moataza [32] also reported varied levels and types of mycolytic enzymes by different *Pseudomonas* strains with different pathogens like *P. capsici* and *R. solani*.

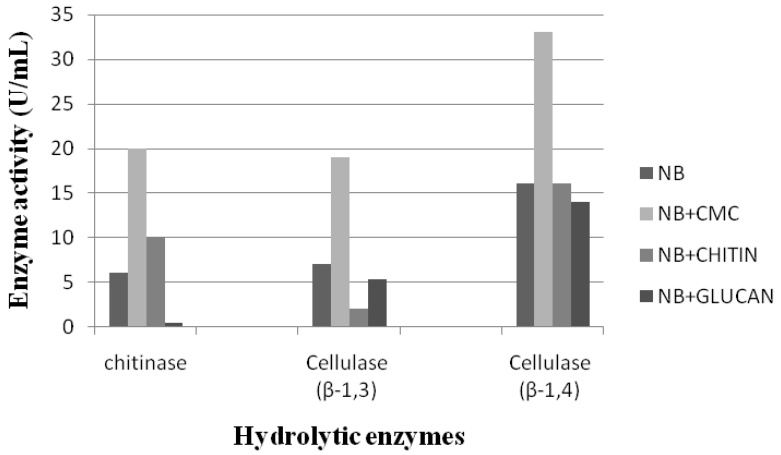

Figure 3. Chitinase, β-1,3 cellulase and β-1,4 cellulase production by *Bacillus* sp. with different substrates.

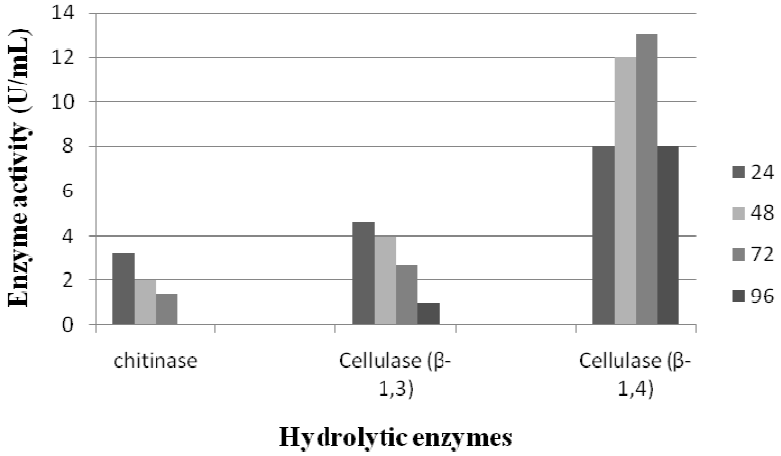

Figure 4. Hydrolytic enzymes induction with autoclaved mycelium of *Colletotrichum gloeosporiodes* containing medium.

Treatment of the chilli seeds with *Bacillus* sp. culture showed 100% germination index similar to the untreated seeds (Figure 5). The treatment of the seed with co-inoculation of the pathogen with *Bacillus* sp. culture showed 65% reduction in disease incidence by the treatment as compared to the seed treated with pathogen alone (87.5%). Kamil et al. [54] reported that the seed coat treatment of sunflower seeds with *Bacillus licheniformis*, induced high reduction in percentage of infection of *R. solani* damping off (from 60 % to 25 %) as compared with the pathogen alone. Our observations also comply with these reports. The selection of effective antagonistic organisms is the first and foremost step in biological control.

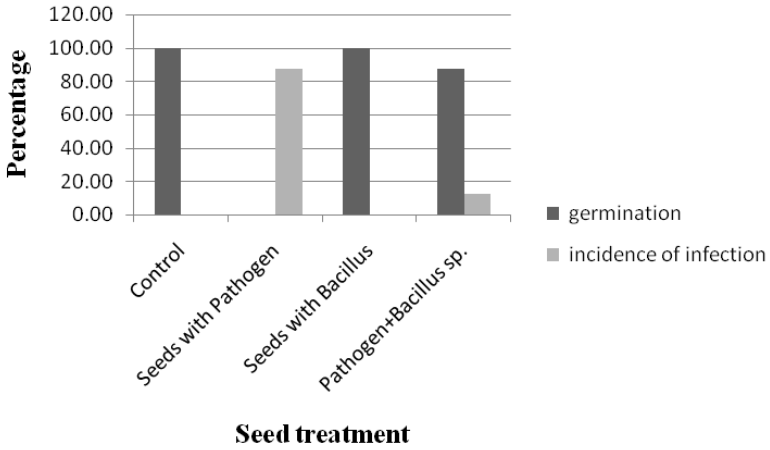

Figure 5. *In vitro* Chilli seed assay to determine germination percentage and disease incidence in the presence of the fungal pathogen *Colletotrichum gloeosporiodes* and the antagonist *Bacillus* sp.

Our investigations have been successful in isolating a *Bacillus* sp. capable of producing mycolytic enzymes inducible with pure substrates as well as fungal mycelium, paving way for a novel biocontrol agent which would not only be effective in controlling anthracnose caused by *Colletotrichum gloeosporiodes* but also other chilli fungal pathogens.

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