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Antagonism of *Bacillus* species (strain BC121) towards *Curvularia lunata*

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A soil bacterium, *Bacillus* sp. strain BC121, isolated from the rhizosphere of sorghum, showed high antagonistic activity against *Curvularia lunata*. A clear inhibition zone of 0.5–1 cm was observed in dual plate assay. After 10 days of incubation, the bacterial strain grew over the fungal mycelial surface and multiplied extensively on it. Scanning electron microscopic observations showed a clear hyphal lysis and degradation of fungal cell wall. In dual cultures, the *Bacillus* strain BC121 inhibited the *C. lunata* up to 60% in terms of dry weight. This strain also produced a clear halo region on chitin agar medium plates containing 0.5% colloidal chitin, indicating that it excretes chitinase. The role of the *Bacillus* strain BC121 in suppressing the fungal growth *in vitro* was studied in comparison with a mutant of that strain, which lacks both antagonistic activity and chitinolytic activity. The extra-cellular protein precipitate from *Bacillus* strain BC121 culture filtrate had significant growth-retarding effect and mycolytic activity on *C. lunata*. The protein extract from the wild strain, when tested on SDS–PAGE gel showed a unique band corresponding to the molecular mass of 25 kDa, which could be the probable chitinase protein.

RHIZOSPHERE bacteria are excellent agents to control soil-borne plant pathogens. Bacterial species like *Bacillus*, *Pseudomonas*, *Serratia* and *Arthrobacter* have been proved in controlling the fungal diseases[1–3]. Earlier reports showed that micro-organisms capable of lysing chitin, which is a major constituent of the fungal cell wall, play an important role in biological control of fungal pathogens[4]. Fungi like *Trichoderma*, and bacteria like *Bacillus*, *Serratia*, *Alteromonos* were reported to have chitinolytic activity[5–8]. Non pathogenic soil *Bacillus* species offer several advantages over other organisms as they form endospores and hence can tolerate extreme pH, temperature and osmotic conditions. *Bacillus* species were found to colonize the root surface, increase the plant growth and cause the lysis of fungal mycelia[9–11]. *Curvularia lunata* is an important seed and soil-borne plant pathogen distributed throughout the world. The infection causes grain mold and leaf spot diseases, resulting in significant economic loss to the dry land crops like sorghum[12]. The species of *Curvularia* along with *Fusarium* pathogen causes viability loss up to 100% in sorghum[13]. Infection by *C. lunata* in human beings results in allergic fungal sinusis and broncho pulmonary fungal diseases[14,15]. The use of synthetic fungicides and crop management strategies was not successful in eradicating these pathogens, besides it resulted in environmental hazards and chronic health problems[16]. Hence, controlling this pathogen using biocontrol agents will help in enhancing the yield of the crop. Keeping this as the objective, an attempt was made to isolate a soil bacterium showing antagonistic activity against *C. lunata*.

Fungal culture of *C. lunata* was obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, India. The fungal culture was maintained on potato dextrose medium at 30°C.

By following serial dilution method, soil bacterial isolates were made from the rhizosphere soil sample of sorghum plants grown at a farm in Osmania University, Hyderabad. All the isolates were subcultured and maintained on nutrient agar (NA) medium. Screening for the antagonistic activity was carried out by following the dual plate assay method[17]. For this, an agar block (1 cm × 1 cm) of freshly grown fungal pathogen was placed at the centre of the nutrient agar plate and allowed to grow for 2–3 cm in diameter. A loopful of bacterial culture was inoculated at the periphery of the plate and incubated at 30°C for 5 days, and development of inhibition zone around the bacterial colony was observed. Out of the 168 isolates tested (BC1–BC168), 12 showed inhibition against *C. lunata*. One bacterial isolate, BC 121, that showed very high level of inhibition was selected for further study. This strain was characterized morphologically and biochemically by following *Bergey's Manual of Systematic Bacteriology*[18] and was found to be a *Bacillus* species. It was grown and maintained on NA at 30°C. When tested against *C. lunata* by the dual plate assay, there was a clear inhibition zone of 0.5–1 cm diameter

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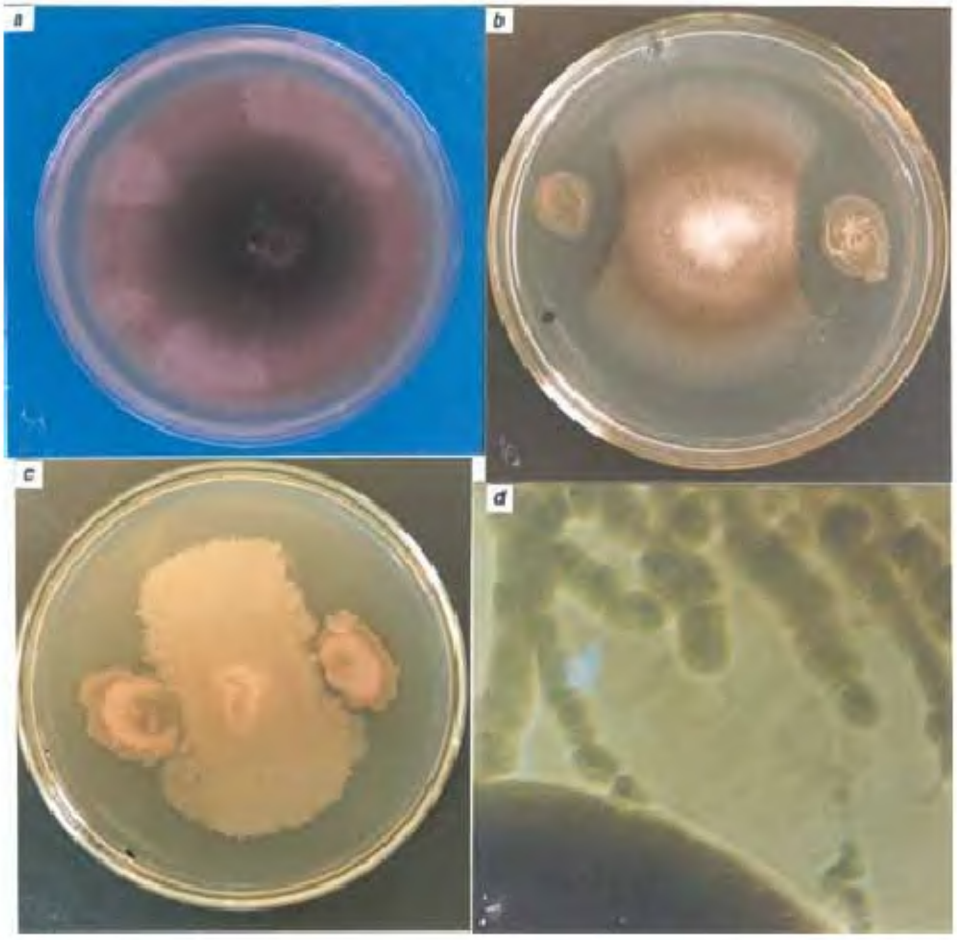

Figure 1. Antagonism of *Bacillus* sp. strain BC121 to *Curvularia lunata*. ***a***, *C. lunata* grown on NA (control); ***b***, Inhibition of *C. lunata* by *Bacillus* sp. strain BC 121 on NA; ***c***, Growth of *Bacillus* sp. strain BC121 over the mycelial mat of *C. lunata*; ***d***, Close-up view of the mycelium from (***c***).

between the fungal culture and *Bacillus* BC121 culture (Figure 1 *b*). The fungal culture grown on NA agar plate without any bacterial culture served as control (Figure 1 *a*). The damage caused by the bacterium to the fungal mycelium 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 inverted microscope. The mycelium present in the inhibition zone was also processed for scanning electron microscopic (SEM) study. For this, the mycelium along with agar disc was fixed with 4% glutaraldehyde, washed with 10 mM sodium phosphate buffer (pH 6.0) and dehydrated with graded alcohol series. This was observed under Jeol scanning electron microscope. Light microscopic study revealed the presence of abnormal hyphae, with condensation and deformation. There were swellings of mycelial tips and cells in between (Figure 2 *a*, *b*). A similar observation has been made in the antagonism of *Arthrobacter* sp. to *Fusarium* sp.[19]. SEM observations showed that the mycelium in the inhibition zone initially grew in a zigzag fashion (instead of growing straight, Figure 3 *c*). This was followed by occurrence of extensive malformation and damages to the mycelium (Figure 3 *b*, *d*) which were not found in the control mycelium (Figure 3 *a*). After ten days of incubation, the bacterial cells grew over the mycelial mat, multiplied and colonized on the mycelial surface (Figure 1 *c*, *d*).

To characterize the antagonistic mechanism by this bacterium a mutant of this bacterium was developed, which lost its antagonistic activity. For this the bacterial culture was incubated in 5 ml of nitrosoguanidine solution (1 mg/ml NTG) suspended in 10 mM Tris maleic acid (pH 6.0). The colonies were screened for the loss of antifungal activity. Out of 550 putative mutant colonies tested, one showed no antagonistic property against *C. lunata* (Figure 4 *a*). This mutant isolate also did not grow over the mycelial mat and it was named as BC121M.

To test the antifungal activity of the *Bacillus* strain BC121, dual liquid culture method was employed. One ml of freshly-grown *Bacillus* BC121 culture (containing 10^7 cfu/ml) and 1 ml of 12 h grown *C. lunata* culture

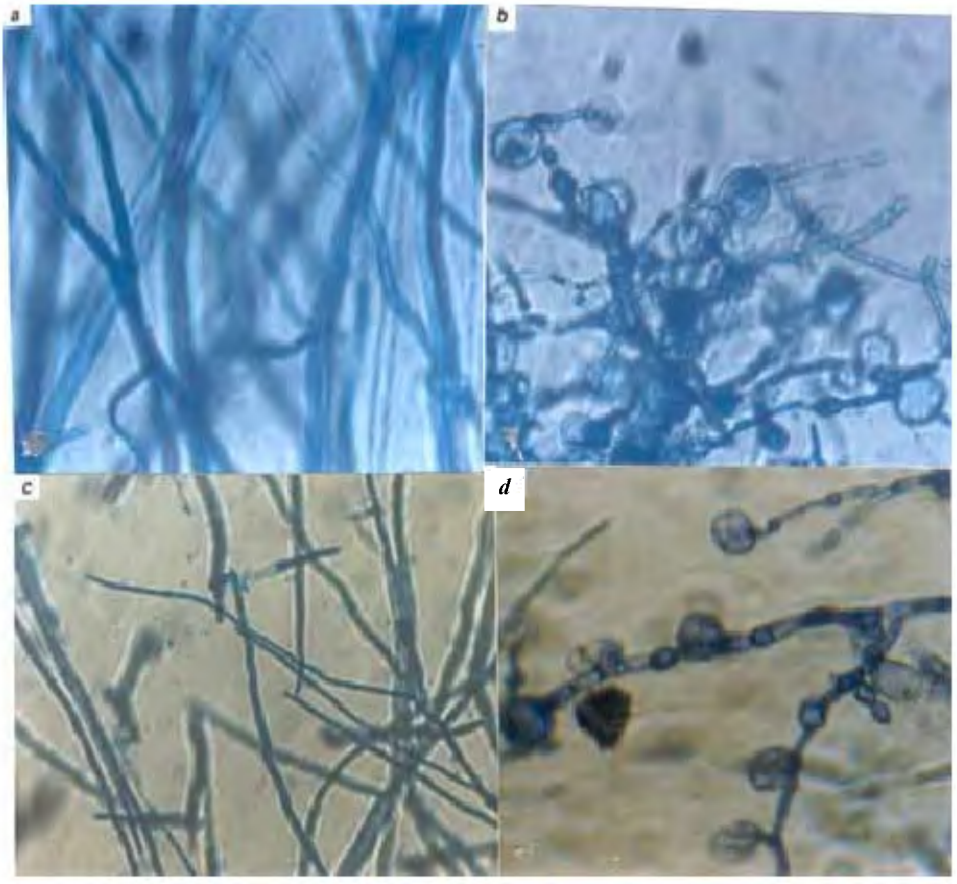

Figure 2. Light microscopic observations of mycelium inhibited by BC121 strain. Mycelium of *C. lunata* (***a***) grown on NA (control), (***b***) present in the inhibition zone, when grown with *Bacillus* strain BC121 on NA, (***c***) grown on nutrient broth medium (control); and (***d***) grown with *Bacillus* strain BC121 on nutrient broth medium.

were inoculated onto 50 ml of Czapeck–Dox medium in 250 ml conical flask. The culture was incubated on a rotary shaker at 30°C for 48 h. For the control experiments, either the fungus alone or the fungus and the mutant BC121M isolate were inoculated. Three replicates for each were grown and the experiment was repeated thrice. A drop of fungal culture from each flask was taken out on a clean glass slide and stained with lactophenol cotton blue and observed under a Nikon inverted microscope. The fungal mycelium grown with *Bacillus* BC121 culture showed damage, swelling and distortions (Figure 2 *d*). The mycelium which grew with *Bacillus* BC121M culture and the control mycelium which was not grown with any bacterial culture did not show these abnormal features (Figure 2 *c*). This clearly indicates the mycolytic activity of the *Bacillus* BC121 culture. Similarly, Podile and Prakash[10] reported the lysis and dissolution of fungal mycelium of *Aspergillus niger* by *Bacillus subtilis* AF1 strain.

The differences in dry weights between the fungal cultures grown with BC121 strain or the mutant BC121M strain or the control culture grown without any bacterium were recorded according to Broekaert *et al.*[20]. For this, 48 h grown dual cultures were passed through the preweighed Whatman No 1 filter paper. It was dried for 24 h at 70°C and weights were measured. There was more than 60% reduction in dry weight of the culture grown with BC121 strain when compared to the control. There was very little reduction in dry weight of the culture when grown with the mutant strain BC121M. This clearly shows that the reduction in dry weight of the fungus when grown with the BC121 strain is due to the antifungal activity of this bacterium. The mutant strain which has lost the antifungal activity could not reduce the dry weight of the fungus.

To test the chitinolytic property of the *Bacillus* BC121, it was inoculated on chitin agar medium[21] supplemented with 0.5% colloidal chitin as principal carbon source. After three days of incubation at 30°C, a clear halo region was observed (Figure 4 *c*). The mutant BC121M strain could not produce any halo region around the colonies. This clearly indicates that the *Bacillus* BC121 secretes chitinase enzymes, while the mutant *Bacillus* BC121M strain has lost the ability to produce chitinase.

The extracellular protein extract was tested for antifungal activity. For this the bacterial cultures were grown

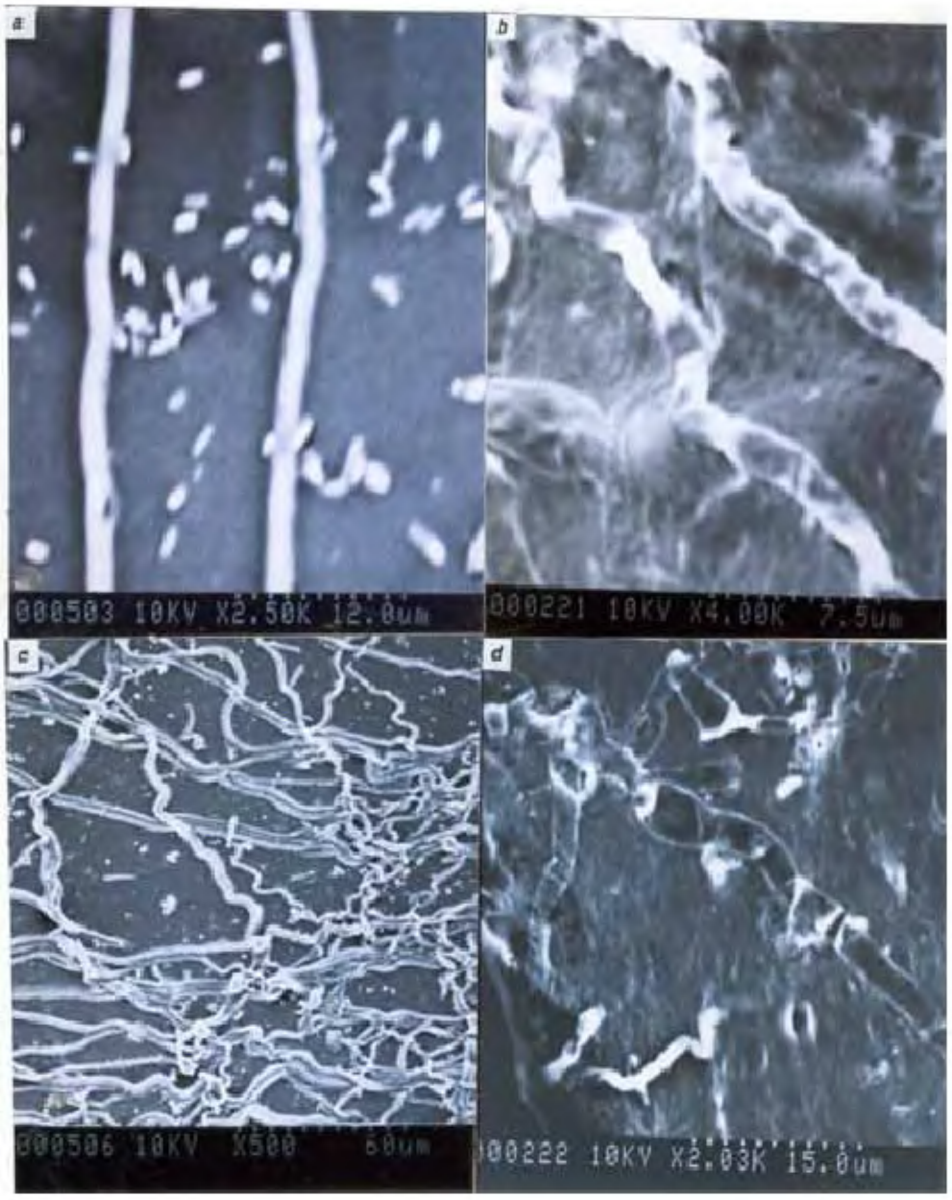

Figure 3. Scanning electron microscopic observations of the mycelium from the inhibition zone. Mycelium of *C. lunata* (***a***) grown on nutrient broth medium (control) and (***b–d***) present in the inhibition zone.

separately in 200 ml of synthetic colloidal chitin medium[21] containing 0.5% colloidal chitin in 1 l conical flask and incubated on a rotary shaker for 3 days at 30°C. After incubation, the culture was taken out and centrifuged at 10,000 *g* for 12 min at 4°C. The supernatant was collected and filter-sterilized using 0.2 micron Millipore filters. The cell-free filtrate was subjected to ammonium sulphate precipitation (80%). After overnight stirring at 4°C, it was centrifuged at 12,000 *g* for 15 min at 4°C. The precipitate was suspended in 10 mM sodium phosphate buffer, pH 6.0 (SP buffer) and was dialysed extensively using 1 kDa molecular weight cut-off dialysis membrane against the same buffer. The protein was concentrated by reducing the water content of the sample by dialysis of the sample against sugar. The concentration of the protein was measured by following the method of Bradford[22].

The antifungal activity of these protein extracts was tested by two methods, i.e. disc plate diffusion assay method and micro titer plate assay using ELISA plates. For the disc plate diffusion assay, 10 µg of protein precipitate suspended in 100 µl of SP buffer was added to filter paper disc and kept against actively growing fungal culture. The 100 µl of SP buffer loaded on separate filter paper served as the control. The plates were incubated at 30°C for 3–5 days. A clear inhibition zone was observed around the filter paper disc loaded with protein precipitate of *Bacillus* BC121 (Figure 5 *b*). There was no inhi-

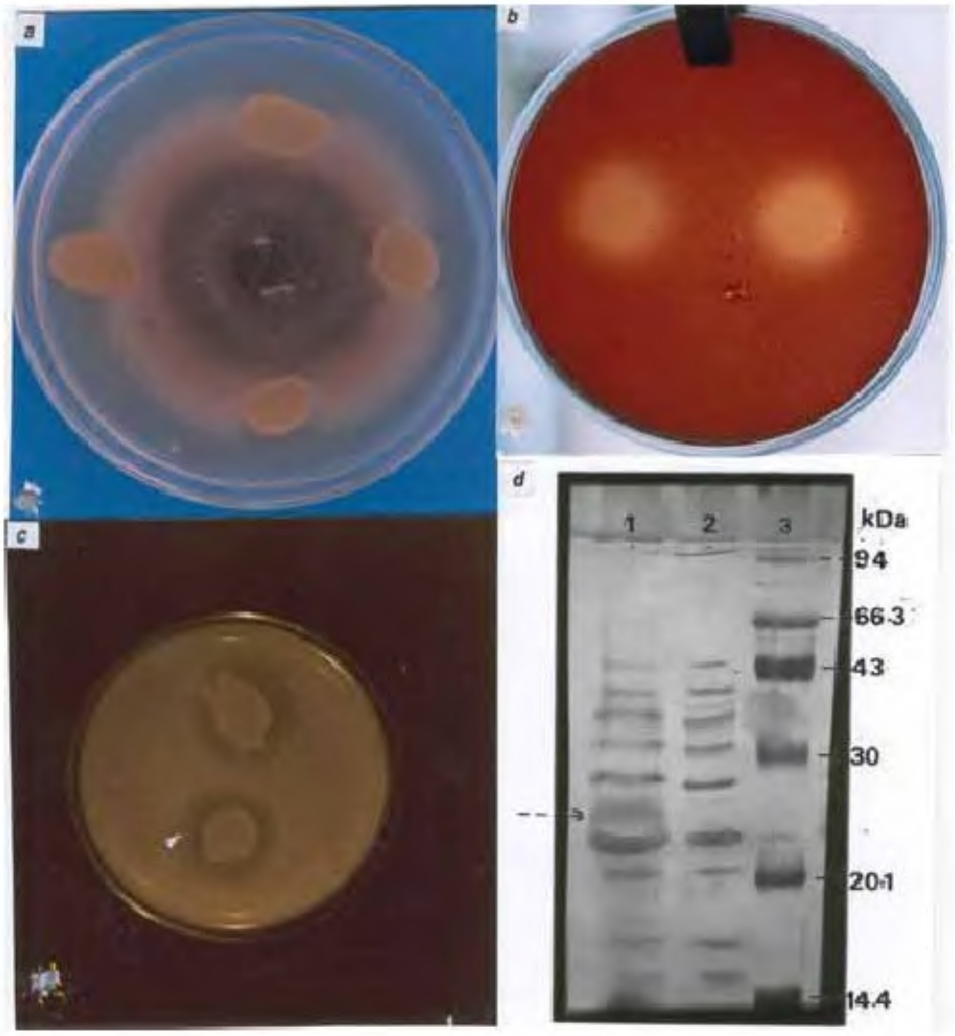

Figure 4. ***a***, Bacillus strain BC121M (mutant) not showing inhibition to *C. lunata* on NA; ***b***, Clear halo zones produced by the extracellular protein extract on chitin agar medium (plates were stained with 0.1% Congo red solution); ***c***, Clear halo zones produced by *Bacillus* sp. BC121 strain on chitin agar medium; and ***d***, SDS–PAGE analysis of extracellular protein extracts: Lanes 1 and 2, Extracellular protein extract of strain BC121 and strain BC121M; lane 3, Molecular weight markers (phosphorylase B, 94 kDa; bovine serum albumin, 66.3 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; lysozyme, 14.4 kDa).

bition of fungal culture around the filter paper disc loaded with SP buffer or protein precipitate of *Bacillus* BC121M culture (Figure 5 *c*). The microscopic observations of the mycelium present in the inhibition zone showed abnormal features similar to that from the inhibition zone, when the strain BC121 was inoculated. For micro titer plate assay, the spore suspension (300–500 spores/ml) suspended in anti fungal assay medium, prepared according to Duvick *et al.*[23] was used. From this, 80 µl of spore suspension was taken in a well of a 96-well ELISA plate. To each well, 20 µl of protein sample (10 µg) or 20 µl of SP buffer was added and incubated overnight at 30°C. After 16–24 h, the spore germination or hyphal extension was observed under a Nikon inverted microscope.

It was observed that only few spores had germinated and the hyphae showed abnormal swelling and lysis (Figure 5 *d*). A similar result was obtained with crude protein extract of *B. subtilis* AF1 strain inhibiting *Aspergillus niger* and was proved in the biological control of crown rot disease in groundnut[10]. The damages of the mycelial cell wall clearly indicate the chitinolytic activity of the extracellular proteins. The chitinolytic activity of the protein precipitate was checked on the chitin plates. For this, 25 µg of the protein sample suspended in 100 µl of the SP buffer was placed on 1.5% agar plates containing 0.5% colloidal chitin. The plates were incubated at 30°C for 16 h. To detect the halo region around the protein sample, the plates were stained with 0.1% Congo red solution, according to Teather and Wood[24]. A clearing

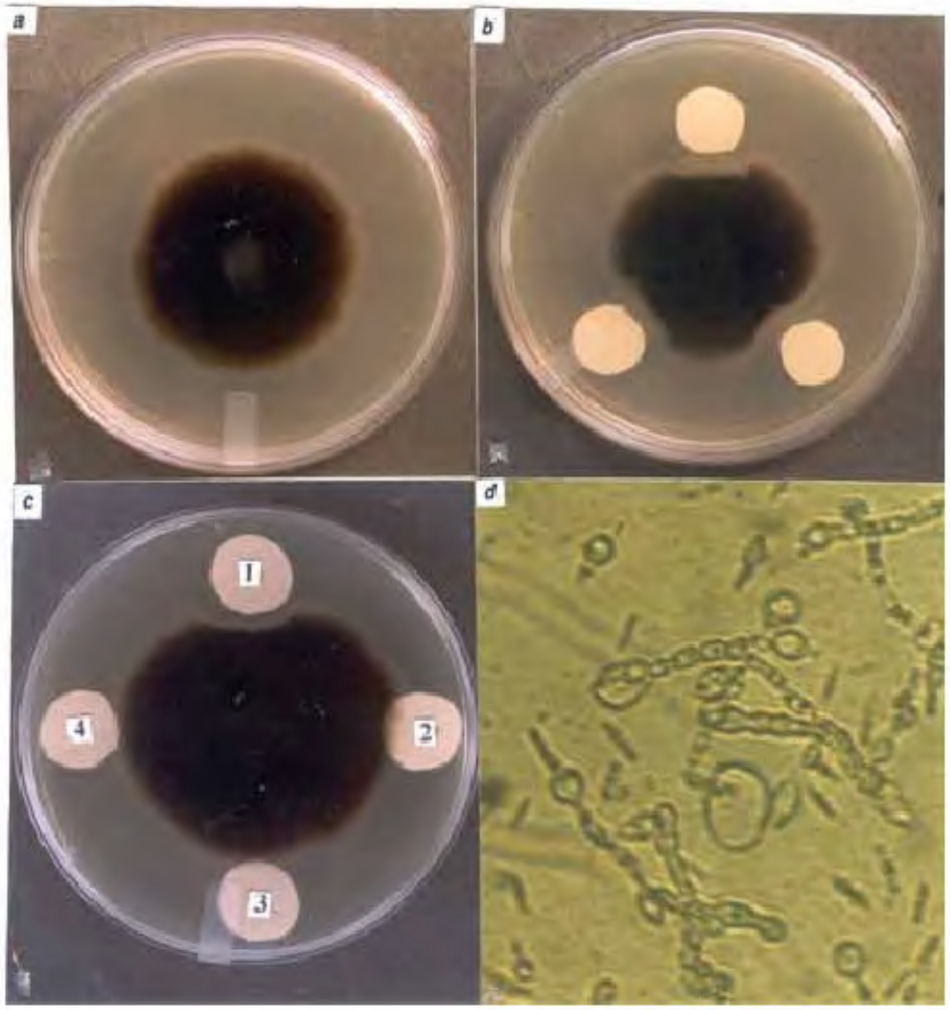

Figure 5. Anti-fungal activity of the extracellular extract of *Bacillus* sp. ***a***, *C. lunata* grown on NA (control); ***b***, Inhibition of *C. lunata* by the extracellular extract of *Bacillus* sp. (loaded on to filter paper discs); and ***c***, *C. lunata* grown along with protein sample and buffer: 1, Protein extract of BC121; 2, Protein extract of BC121M; 3, SP buffer alone; 4, Boiled extracellular protein extract of BC121.

zone of hydrolysis of chitin was observed in the region where the protein precipitate of BC121 was placed (Figure 4 *b*). This test proves the hydrolysis of chitin by the chitinolytic activity of the proteins. The protein extracts of the wild (BC121) and the mutant (BC121M) strains, when analysed on SDS–PAGE gel, showed difference in the presence of one particular band of 25 kDa size (Figure 4 *d*; in strain BC121) which is probably the chitinase band as it is absent in the extracellular extract of the mutant BC121M strain.

On the basis of these studies it is concluded that the *Bacillus* BC121 isolate is showing antagonistic property probably through the chitinolytic mechanism and hyper parasitism, which has been proved to be an effective mechanism in controlling the fungal pathogens[25]. These observations and further studies will help in developing the *Bacillus* BC121 isolates as a potential biological control agent against *C. lunata*.

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Genotyping of antifungal compounds producing plant growth-promoting rhizobacteria, *Pseudomonas fluorescens*

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Plant growth-promoting rhizobacterial strains belonging to fluorescent pseudomonads were isolated from the rhizosphere of rice and sugarcane. Among 40 strains that were confirmed as *Pseudomonas fluorescens*, 18 exhibited strong antifungal activity against *Rhizoctonia bataticola* and *Fusarium oxysporum*, mainly through the production of antifungal metabolites. Genotyping of these *P. fluorescens* strains was made by PCR–RAPD analysis, since differentiation by biochemical methods was limited.

PLANT growth-promoting rhizobacteria (PGPR) improve plant growth in two different ways, directly or indirectly. The direct promotion of plant growth by PGPR is through production of plant growth-promoting substances[1] or facilitation of uptake of certain nutrients from the soil[2]. On the other hand, PGPR can also prevent the proliferation of phytopathogens and thereby support plant growth. One of the mechanisms involved here is through their ability to produce siderophores for sequestering iron[3]. The secreted siderophores binds to the Fe^{3+} that is available in the rhizosphere, and thereby effectively prevent growth of pathogens in that region. For example, *Pseudomonas fluorescens* belonging to the PGPR class produces siderophores and control *Pythium ultimum*[4]. Some other PGPR synthesize antifungal antibiotics, e.g. *P. fluorescens* produces 2,4-diacetyl phloroglucinol, which inhibits growth of phytopathogenic fungi[5]. Certain PGPR degrade fusaric acid produced by *Fusarium* sp. causative agent of wilt and thus prevents the pathogenesis[6]. Some PGPR can also produce enzymes that can lyse fungal cells. For example, *Pseudomonas stutzeri* produces extracellular chitinase and laminarinase which could lyse the mycelia of *Fusarium solani*[7]. In recent years, fluorescent *Pseudomonas* has been suggested as potential biological control agent due to its ability to colonize rhizosphere and protect plants against a wide range of important agronomic fungal diseases such as black root-rot of tobacco[8], root-rot of pea[9], root-rot of wheat[10], damping-off of sugar beat[11,12], etc.

Soil samples were collected from 31 different locations representing rhizosphere of mainly rice and sugarcane around Madurai. The rhizosphere samples were plated on King's B-medium (*Pseudomonas* isolation agar medium, Hi-Media, Mumbai) and the plates were incubated at 37°C for 24 h. Colonies that fluoresced under UV light (λ= 356 nm) were selected and further purified on the same medium. Among the isolates, 40 were confirmed as *P. fluorescens* based on biochemical tests such as arginine hydrolysis, catalase activity, production of fluorescing compounds, gelatin liquefaction and growth at 4°C and 42°C. Plant growth-promoting properties of the selected 18 strains were confirmed with their ability to produce indole acetic acid, phosphate solubilization and inhibition of fungal phytopathogens.

Preliminary screening for antifungal activity was performed on PDA medium without supplementation of $FeCl_3$. Under this condition, fungal growth inhibition could be either due to production of siderophores or antifungal metabolites. Pathogen used were *Fusarium oxysporum* Fox1 and *Rhizoctonia bataticola* Rba1 (obtained from Tamil Nadu Agricultural University, Coimbatore) and antagonism was tested on PDA with and without $FeCl_3$ (100 µg ml^{-1}).

An agar plug (4 mm dia) taken from an actively growing fungal culture was placed on the surface of the PDA plate. Simultaneously, *P. fluorescens* strains were streaked 3 cm away from the agar plug at sides towards the edge of petri plates. Plate inoculated with fungal agar

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