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A Pictorial Illustration of the Inhibition of Mycelial Growth and Spore Germination of Various Sorghum Fungal Pathogens by a *Bacillus species*

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Abstract

Sorghum is one of the most indispensable cereals for food, fodder, and in brewery, especially in the drier tropics. Recently, sorghum is considered a potential source of biofuel. Globally, the productivity and profitability of sorghum is hampered by biotic stresses, causing anthracnose, grain mold, smuts, and downy mildew. In this study, a bacterium was observed growing on half-strength potato dextrose agar plate containing sorghum seeds. Using both plate and paper disc assays, activity of the determined Gram-positive *Bacillus* (called LP16S) was tested against four destructive sorghum pathogens *Fusarium thapsinum, Colletotrichum sublineola, Curvularia lunata*, and *Bipolaris sp.* Confirmatory *in vitro* analysis showed that LP16S was capable of inhibiting both mycelial growth and spore germination of these pathogens. Identification of the strain using 16S rDNA sequence analysis characterized LP16S as a putative *Bacillus sp*. Work is underway to determine the effectiveness of LP16S in suppressing sorghum diseases.

Keywords: Sorghum, *Fusarium thapsinum, Colletotrichum sublineola, Curvularia lunata, Bipolaris sp*., *in vitro*.

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is the fifth most important crop and its uses ranged from food, feed, fodder, and recently, as a potential source of biofuel [1,2]. The crop is generally tolerant to number of adverse environmental conditions, including moisture stress and high temperature; however, its productivity and profitability across the globe in hampered by various biotic constraints, including pathogens that cause anthracnose, grain mold, smuts, and downy mildew [3-11]. Although management strategies such as the use of resistant sources has been effective in most cases in reducing the impact of these diseases, there is little information on the use of biocontrol agents as a disease management tool.

The use of biocontrol agents to combat plant diseases has long been studied, especially *in vitro* [12-20], but there has been little success in their application under field conditions. Ryder et al. [21] added *Bacillus subtilis* and *B. cereus* to a sodic acid soil planted with wheat in the greenhouse and reported significant reduction in the severity of take-all disease and Rhizoctonia root rot, caused by *Gaeumannomyces graminis* and *Rhizoctonia solani*, respectively. Also, the chytrid fungus (*Gaertneriomyces sp.*) added to soil was shown to reduce the incidence of sorghum downy mildew by 58% [22].

The use of biocontrol agents to control plant infections could be an effective strategy in situations where resistant germplasm or fungicides are not available or effective. So far, the utilization of biocontrol agents to control plant diseases has been limited due to factors such as ineffectiveness or reduced effectiveness in the field when compared to chemical fungicides, as well as commercial availability [23]. The lack of commercial availability of these agents also can be attributed to the fact that these potential biocontrol microorganisms, in particular pseudomonads and many *Bacillus sp.* are devoid of the capacity to produce resting spores [13] and are consequently somewhat labile.

In this communication, we show the antifungal capacity of a bacterial species that was initially observed growing on a culture plate containing sorghum seeds, producing a zone of growth inhibition of fungi. This isolate was investigated for its antagonistic potential against sorghum fungal pathogens and then identified.

Materials and Methods

Bacterial isolation

All work was conducted at the ARS-USDA-Plains Area Research Center, College Station, Texas, USA. Sorghum seeds collected from Texas AgriLife Research Farm, Burleson County, Texas, were placed on half-strength potato dextrose agar (½PDA) plates for routine analysis. After 7-10 d of incubation at 27°C, fungal growth was observed along with an apparent bacterial

colony separated by a zone clear of fungi. The microorganism with the zone of inhibition was subcultured, purified, and stored in a refrigerator. Pure cultures were used for all antifungal activity testing and bacterial classification as noted below.

Screening for antifungal activity on mycelial growth

The fungal species *Fusarium thapsinum, Colletotrichum sublineola, and Curvularia lunata* were isolated from infected sorghum kernels and stored in a freezer at -7°C. Using the culture assay, the bacterial isolate called LP16S colony was placed in the center of a Petri dish containing ½PDA medium and agar plugs containing the different fungal species were placed on three equidistant spots (Figure 1a). In Figures 1b and 2, the paper disc assay was utilized in which three 5 mm disc dipped in *Bacillus sp*. spore suspension was placed in equidistant spots on Petri dish containing ½PDA and the fungal species placed between the paper disc. In both assays, the plates were incubated at 27 ± 1°C for 6 d.

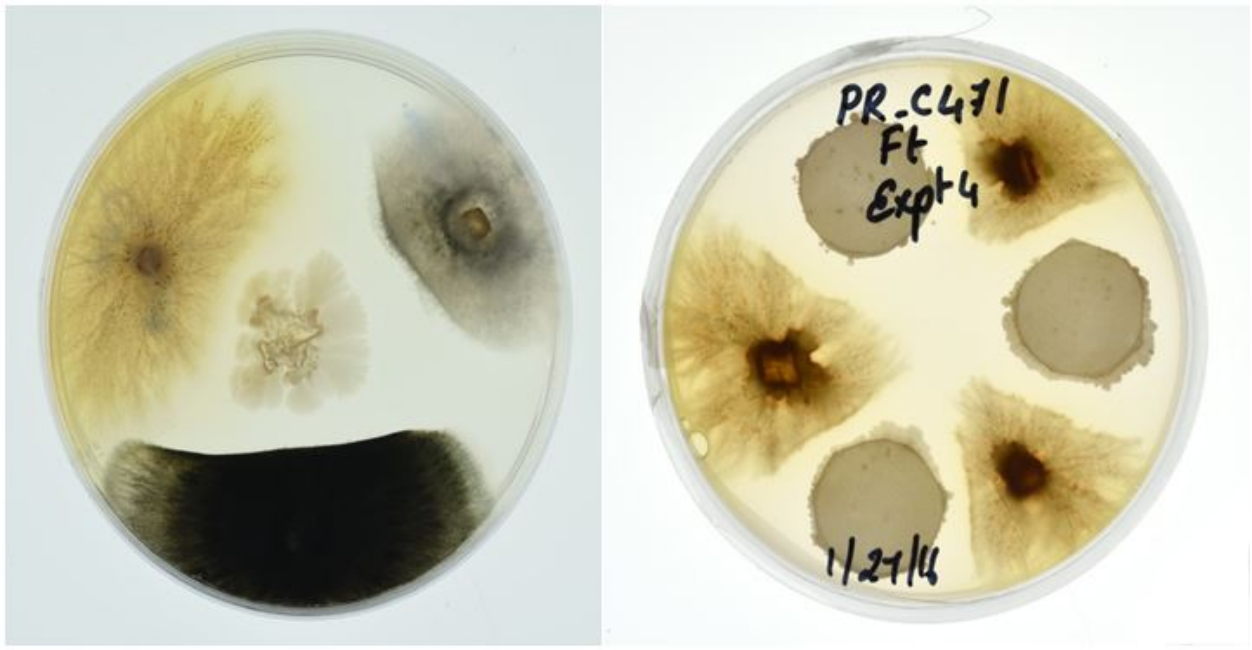

Figure 1: Fungal growth inhibition by bacterial strain LP16S [located at the center of the plate on the left (1a)] of *Fusarium thapsinum, Curvularia lunata*, and *Colletotrichum sublineolum.* The paper disc culture of LP16S [(cream growth on the plate on the right (1b)] with *Fusarium thapsinum.*

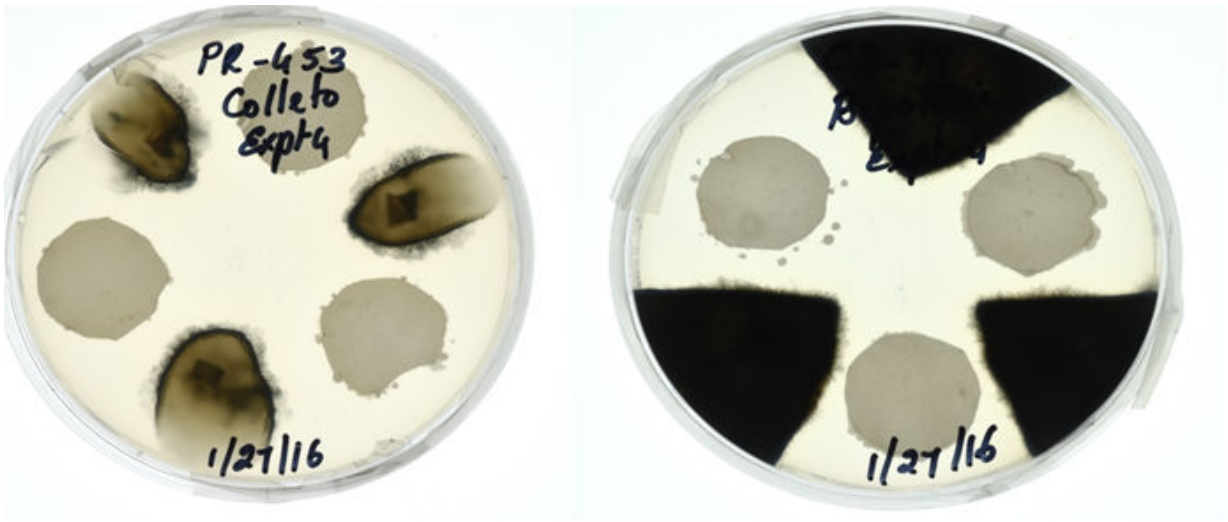

Figure 2: Fungal growth inhibition by bacterial strain LP16S cultured on a paper disc of *Colletotrichum sublineolum* (left Petri dish) and *Curvularia lunata* (right dish).

Inhibition of fungal spore germination

Strain LP16S was grown on Difco nutrient agar whereas the fungal isolates (*F. thapsinum, C. sublineola, C. lunata, and Bioplaris sp*.) were grown separately in Petri plates containing ½PDA medium. Plates were incubated at 27 ± 1°C for 6 d. LP16S spores and fungal conidia for the different isolates were harvested by flooding the plates with 10 ml sterilized water and scraping the agar surface with a rubber spatula to dislodge them. Separately, LP16S spores and the fungal conidial suspensions were filtered through two layers of sterile cheesecloth into separate beakers and diluted with sterile water to final concentrations of 1×10^6 conidia ml^{-1}. Two drops of the LP16S spore suspension were added to four separate vials containing two drops of each fungus and mixed thoroughly. A drop of the different mixtures was spread on separate Petri dishes containing ½PDA (Figures 3-5). The control plates contained only the fungal species, *C. sublineola* and *F. thapsinum*, respectively (Figures 3 and 4). Plates were incubated at 27 ± 1°C for 6 d.

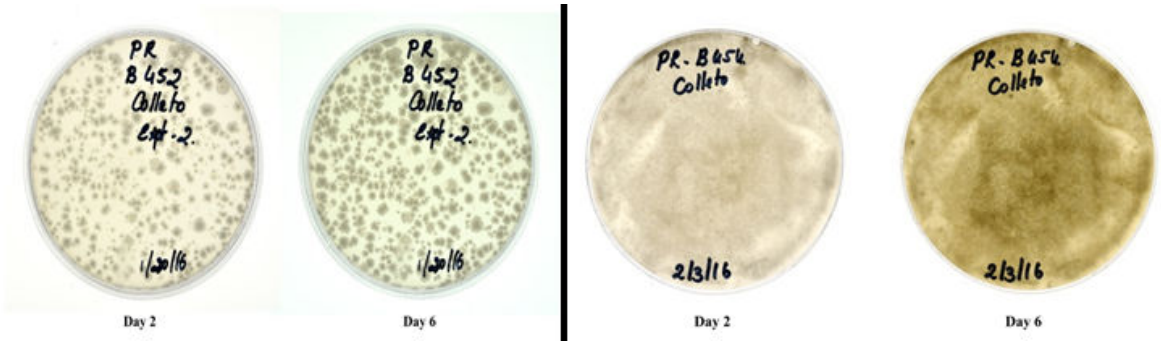

Figure 3: Inhibition of spore germination of *Colletrichum sublineolum* by bacterial strain LP16S. Control plates (two plates on the right) show complete germination of the fungal spores as compared with plates (two plates on the left) containing both LP16S and fungal spores. Plates containing both microorganisms show clear areas where fungal spore germination was absent.

Figure 4: Inhibition of spore germination of *Fusarium thapsinum* by the bacterial strain LP16S. Control plates on the right show complete germination of the fungal spores as compared with plates containing both LP16S and fungal spores. Plates containing both microorganisms (plates on the left) show clear areas where fungal spore germination was absent.

Bacterial identification

The unknown bacterium designated as LP16S was Gram stained using standard methods. The 16S rDNA sequence was generated as part of a separate whole genome sequencing study using 454 Roche Technology. Strain LP16S was characterized using the 16S rDNA bacterial sequence comparisons with both Gram positive and negative representatives. The evolutionary history generated was inferred using the Neighbor-Joining method [24]. The evolutionary distances were computed using the Jukes and Cantor [25] method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7 [26].

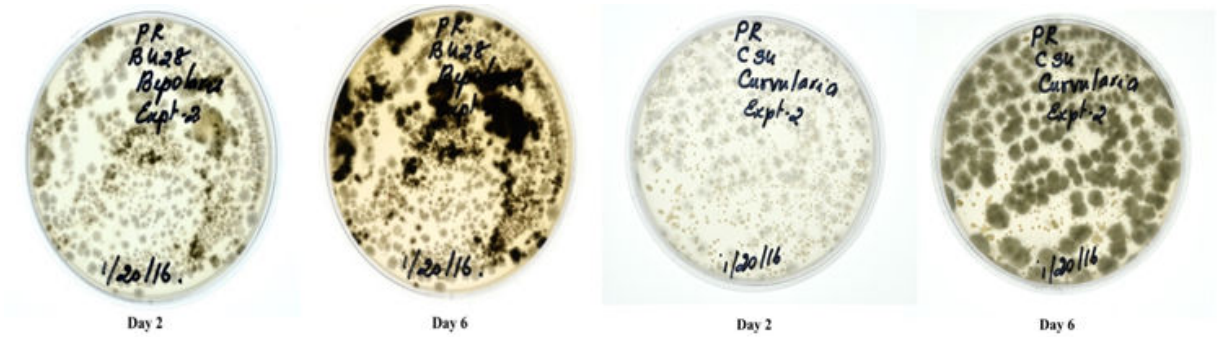

Figure 5: Plates seeded with both the bacterial strain LP16S and fungi i.e., *Bipolaris sp.* (top plates) and *Curvularia lunata* (bottom plates). Clear areas in the plates indicate where fungal spore germination is inhibited.

Results

Strain LP16S was shown to inhibit mycelial growth of *F. thapsinum, C. sublineola,* and *C. lunata*, that cause both sorghum grain mold and anthracnose. Inhibition of the mycelial growth was indicated by a clear zone between the LP16S and the fungal spp. as shown in Figures 1 and 2. Control plates containing only the fungal spp. (not shown) colonized the entire plates within the same time period. Similarly, LP16S inhibited spore germination as indicated by clear zones of the aforementioned sorghum pathogens; as well as, *Bipolaris sp.* (Figures 3-5). In the control plates containing only *C. sublineola* and *F. thapsinum*, the fungi grew rapidly and colonized the entire plates as shown in Figures 3 and 4.

The optimal tree with the sum of branch length=0.42228418 is shown in Figure 6. Positions containing gaps and missing data were eliminated. There were a total of 1358 positions in the final dataset. Based on the analysis, our unknown bacterium (LP16S) was characterized by 16S rDNA gene sequencing and identified as belonging to the *Bacillus* genus most similar to *Bacillus subtilis* and to a lesser degree to *Bacillus amyloliquefaciens* (Figure 6). The phylogenetic tree based on 16S rDNA of LP16S, two *Bacillus sp.,* and *Paenibacillus sp.* showing the highest nucleotide sequence similarities is noted in Figure 6. A section of the sequencing dataset from LP16S when placed on the BLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blastho me supported similarities of the protein profile to those of *B. subtilis* and *B. amyloliquefaciens,* respectively (Figure 6).

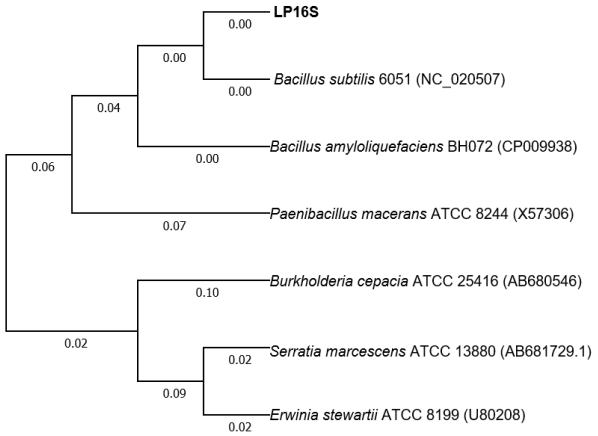

Figure 6: Evolutionary relationships of taxa based on 16S rDNA bacterial sequence comparisons from both Gram positive and negative representatives. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [27]. The NCBI Genbank accession numbers are in parenthesis.

Discussion

In the case of diseases that cannot be easily or economically controlled by host resistance or fungicides such as soilborne pathogens [12,20,13] or mycotoxin contamination [16], biological control may be a potential alternative.

In this study, our antagonistic bacterium (LP16S) was identified as a *Bacillus sp*. closely similar to *B. subtilis* and *B. amyloliquefaciens*. Antifungal activity of *B. subtilis* and *B. amyloliquefaciens* had been documented in several studies [13,15-19,21].

A number of enzymes, chemicals and antibiotics such as protease, chitinase, acetylbuanediol, iturin A, fengycin, bacillomycin, mycosubtilin, non-ribosomal lipopeptides, etc., produced by *Bacillus sp*. have been shown to inhibit the growth of several fungal pathogens, including *C. gloeosporioides*, *Glomerella cingulata*, *Aspergillus niger*, *A. flavus*, *Rhizoctonia solani Fusarium oxysporum f. sp. cubense*, and *Rhizopus stolonifer* [15-19]. Notably, LP16S was shown to possess similar inhibitory activity on mycelial growth and spore germination of four sorghum pathogens, *F. thapsinum*, *C. sublineola*, *C. lunata*, and *Bipolaris sp*. However, the nature of the antifungal compounds of LP16S has not yet been determined and are potentially novel. Another species possibly related to LP16S, *Paenibacillus sp*. obtained from the root zone of sorghum plants was shown in vitro to reduce the mycelial growth, sporangium production, zoospore germination, and germ elongation of *Phytophthora parasitica* [12]. In addition, the bacterium

inhibited the hyphal development of *F. oxysporum*, *F. culmorum*, *Aphanomyces euteiches*, *Chalara elegans*, *Pythium sp*. and *Rhizoctonia solani* [12].

The pictorial data presented here clearly showed growth inhibition *in vitro* of sorghum pathogens by strain LP16S. Thus, further work towards determining the effectiveness of the putative *Bacillus sp*. as an antifungal biocontrol agent is ongoing. Whole genome sequencing of strain LP16S is being conducted to gain both information of probable antimicrobial products and species classification.

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