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Project Title: Characterization of Antifungal Activity of Endophytic Bacteria Associated with Soybean –Charcoal Rot Disease System

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BACKGROUND AND OBJECTIVES

Charcoal rot disease, caused by the fungus *Macrophomina phaseolina*, accounts for an estimated loss of 1.48% of soybean yield in the southern US. No practical management approaches or resistant varieties are available for management of this disease.

Endophytic bacteria occur in all plants and we have demonstrated some differences in endophyte communities of diseased and asymptomatic plants growing adjacent to one another in diseased patches of soybean fields. Our previous MSPB-funded investigations resulted in the identification of a number of bacterial isolates that show antifungal activity against *M. phaseolina*. Preliminary studies indicate that these endophytic bacteria may play a key role in helping plants resist infection by the charcoal rot pathogen and/or inhibit disease development in soybean fields.

The long-range goals of this research are to develop biologically based approaches to plant disease management. The specific objectives for this project are 1) investigation of the effects of inoculation of the representative bacteria on disease development and soybean growth, and 2) characterization of the genes associated with antifungal activity via tansposon-mediatied mutagenesis. Expected results will provide a solid basis for development of charcoal rot disease management for the Mississippi soybean industry.

REPORT OF PROGRESS/ACTIVITY

Objective 1: Investigation of the effects of inoculation of bacteria and fungi on disease development and soybean growth.

***In vitro* assays and Preliminary identification.** To obtain more antimicrobial bacteria, massive screening of the bacterial isolates obtained previously was conducted in the research period. One undergraduate student, who was financially supported by the MSU Undergraduate Student Research program, was involved in the research activities.

Hundreds of the isolates were cultured from the freezer stock and tested using standard plate assays. Endophytic bacteria 3E5-1 and 3E7-1 were both isolated from soybean plants infected with charcoal rot disease. The initial bioassay screening showed that both 3E5-1 and 3E7-1 inhibited growth of *Macrophomina phaseolina* on nutrient broth yeast extract agar (NBY) as well as potato dextrose agar

(PDA). The results indicated that both isolates tested positive for antifungal characteristics, thus calling for further testing (**Fig. 1**).

Further bioassays are under way to evaluate the antifungal spectra of the two bacterial isolates. In order to determine identities of the isolates 3E5-1 and 3E7-1, the bacteria were cultured in NBY broth for DNA extraction. The DNA extraction then allowed polymerase chain reaction (PCR) with 16S rDNA primers, 27F and 1492R. Following PCR, the 16S rDNA sequence analysis of 3E5-1 was performed and found the isolate to be a member of the *Bacillus* genus (Fig. 2). For bacterial isolate 3E7-1 the 16S rDNA sequence analysis revealed the isolate belongs to the *Pseudomonas plecoglossicida* complex (Fig. 3).

Greenhouse assays. The large-scale greenhouse study was set up in June 2018. Prior to setting up the test, a drip irrigation system fed from overhead lines was constructed in the greenhouse. This system prevented overspray and splash when plants were watered, thus providing the greatest assurance that cross-contamination did not occur among the various treatments.

The soil/sand mixture tested in the preliminary assays was prepared and steam-sterilized prior to use. Treatments are as described in **Table 1**. To prepare inoculum of *M. phaseolina* Mp151, 40 flasks of cornmeal sand medium (100g sand, 3g cornmeal, 20 mL distilled water per flask) were prepared and autoclaved. Four 3-mm plugs from plates of Mp151 on PDA were placed into each flask. Flasks were incubated in the dark at 30ºC for 10 days, shaken well by hand at 3-day intervals, at which time the contents of all flasks were combined and mixed.

Each pot containing Mp151 was prepared by hand mixing 20 mL of this inoculum into the sterile soil. Pots for treatments not containing Mp151 were filled with sterile soil into which was mixed 20 mL of sterile cornmeal sand medium. Bacterial inoculum of the three selected endophytic isolates was prepared by inoculating the bacteria into flasks of NBY liquid medium that were then incubated for 18 hours at 28ºC with 220 rpm shaking. Bacteria were extracted from the medium by centrifugation and re-suspended in sterile 0.01M PBS, pH 7.4. Bacteria were inoculated into the seed furrow at the rate of 10^9 cfu in 50 mL PBS per pot.

Three *Rhizobium*-inoculated Pioneer 46A16R soybean seeds were placed in the furrow, and the seeds were covered with soil. Pots that did not receive bacteria were treated similarly by pouring 50 mL sterile PBS into the furrow. Four replicates with three pots for each of the eight treatments were prepared and pots were distributed on greenhouse benches in a randomized complete block design (**Fig. 4**). One 1-gpm pressure-compensating drip emitter was placed into each pot for irrigation. When the plants reach the V1 growth stage, they were thinned to one plant per pot. Plants were destructively sampled at natural senescence. Disease development, pathogen population, plant growth, and disease resistance in Pioneer 46A16R soybeans were recorded. Soybean roots were collected and weighed and disease symptoms were recorded.

The greenhouse trial was terminated on October 9, 2018. The plants were cut at soil-level. Pods were cut from the stems, remaining leaves were removed and discarded, and stems were cut into sections. The soil was washed from the roots and the roots were collected. Roots and stems were examined internally and externally for charcoal rot symptoms. Segments of stem and root were used for cultural isolation to

determine whether *M. phaseolina* was present in the plants. Fresh and dry weights of roots, stems, and pods were obtained and those data were analyzed. As shown in **Table 2**, the three bacterial strains promote soybean growth even though there was no significant difference. The key reason is plant locations in the greenhouse caused significant differences. The plants close to the cooling pad were significantly better than the ones far away from the pad. Significant impact of the bacteria on disease development was not observed based on external and internal symptoms and signs of the disease. However, we have not finished qPCR analysis of the fungal pathogen populations. Once the data are available we will perform analysis statistically.

Objective 2: To characterize the genes associated with antifungal activity production.

We previously showed two endophytic bacterial isolates (A and B), which belong to the genus *Burkholderia*, possess antifungal activities against charcoal rot pathogen *in vitro*, and transposon mutagenesis revealed a non-ribosomal peptide synthetase gene, which is well known for association of biosynthesis of antimicrobial compounds. Based on genomic information obtained previously, it was predicted that the gene of isolate A is required for production of an antifungal oligopeptide.

To purify the antifungal compound, we are optimizing culture conditions for its production. Preliminary data indicate that the antifungal compound is not volatile and the maximum amount of the antifungal compound was observed at Day 6. The antifungal activity remains in the liquid form. Preliminary genetic analysis also indicates that the antifungal compound produced by isolate A is different from the known antifungals produced by *Burkholderia*.

A 57-kb gene cluster has been sequenced, which includes the genes associated with nonribosomal biosynthesis, transcriptional regulation, modification and secretion (**Fig. 5**). Of the genes in the cluster, ORF15 was predicted to encode a LuxR-type regulatory protein that is required for production of the antifungal compound. To study the regulation of the antifungal compound production, one doctoral graduate student, who was financially supported by MSU-MAFES, amplified the ORF15 by PCR and then cloned into the pT vector. After insertion of the Km resistance cassette, the construct was cloned into a suicide vector pBR325. The resulting construction was introduced into competent cells of strain A for mutant generation. A few mutants were obtained and showed elimination of the antifungal activity. However, based on PCR analysis, the mutants are integration mutations, which means the whole plasmid was integrated into the target gene. Nevertheless, the data suggest ORF15 encodes a crucial regulator for antifungal production (**Fig. 6**). Creation of a clean mutant (i.e., a double crossover) is underway.

IMPACTS AND BENEFITS

This research has discovered more bacterial isolates that possess antifungal activities. In vitro assays, these isolates showed significant inhibition to growth of the charcoal rot pathogen *Macrophomina phaseolina*. These bacteria are a very important resource for development of biologically-based management approaches of the soybean disease.

Effects of three of them on development in the greenhouse are under evaluation. The trial will provide important data for possible use of the isolates. The genes responsible for production of antifungal activity could be used to develop genetically-modified organisms for disease control. Elucidation of

chemical structure of the antifungals is extremely useful for development of biopesticides. Collectively, the expected outcomes of the research will provide critical insights into development of products to help soybean plants better resist infection of *Macrophomina phaseolina* and prevent charcoal rot disease development.

END PRODUCTS — COMPLETED OR FORTHCOMING

There have been no solid end products from this project to date. However, one PhD student and three undergraduate students that are supported financially from other funding are gaining degrees from this project. The research data have been presented in a regional meeting and a national conference in 2018. Due to time limit, we did not identify a full-time graduate student working on the project. We anticipate publication of two papers in refereed scientific journals with one published within the next year, and development of a microbial package for charcoal rot disease management.

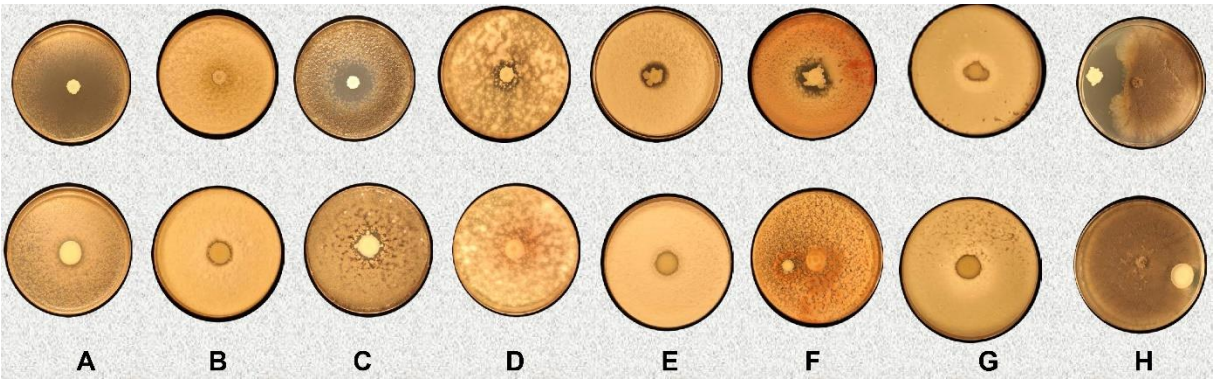

Fig. 1. Plate bioassays of antifungal activity of isolates 13SE_838 (Upper) and 13SE_841 (Lower). The plates were inoculated with 10 µl of the bacteria (OD420: 0.3) and then incubated at 28oC for four days. The plates A-G were oversprayed with the fungi and the plates H were inoculated with agar disk with the tested fungus, and further incubated 1-3 days. Tested Fungi: (A), *Alternaria brassicola*, (B), *Aspergillus flavus*, (C) *Cochliobolus heterosporus*, (D) *Fusarium oxysporum* (E) *Geotrichum candidum*, (F) *Glomerulla singulata*, (G) *Penicillum digitatum*, (H) *Macrophomina phaseolina*

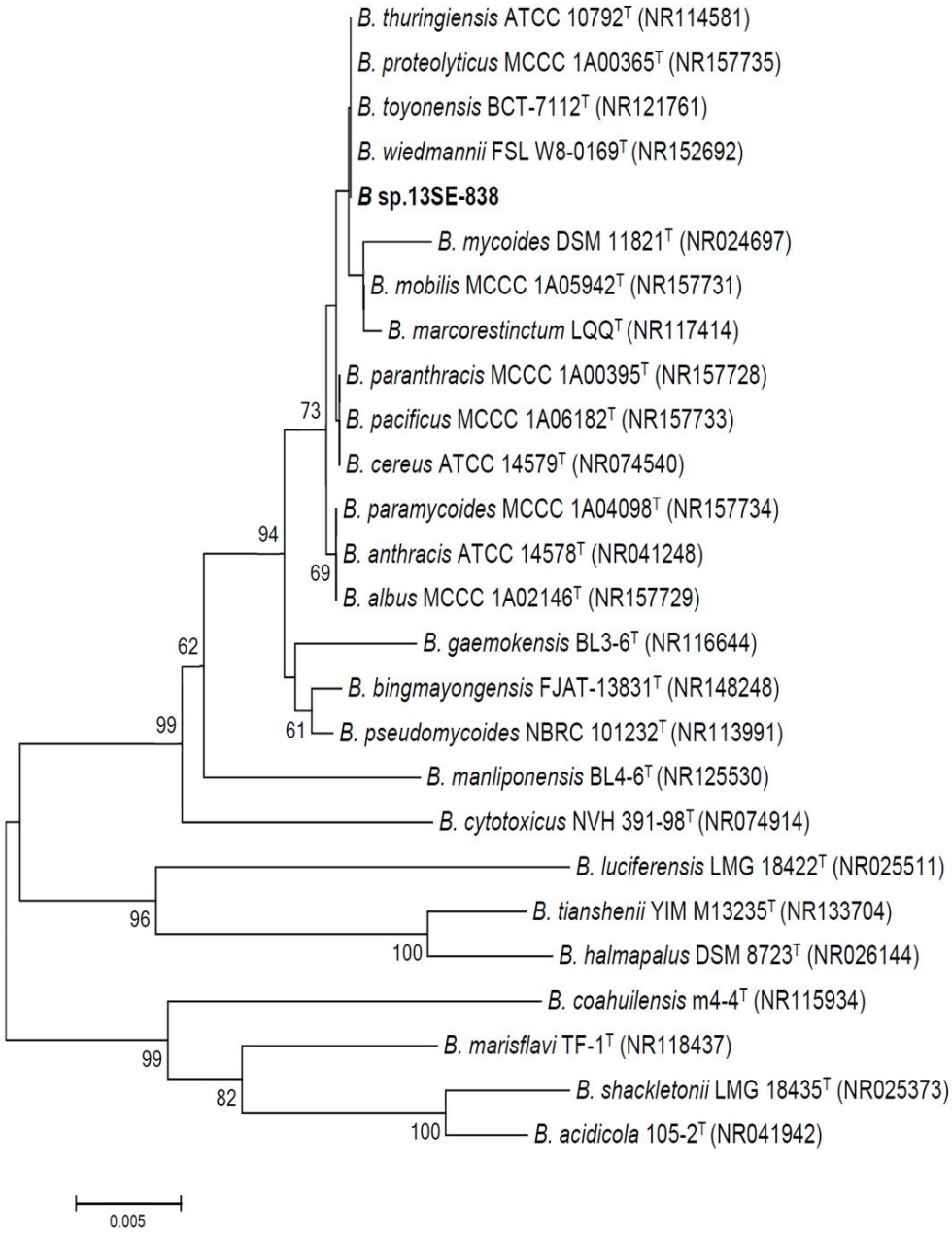

Fig. 2. Neighbor-joining tree illustrating the phylogenetic position of strain 13SE-838 and related members of the genus Bacillus based on partial 16S rRNA gene sequences. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Only bootstrap values above 50% are indicated.

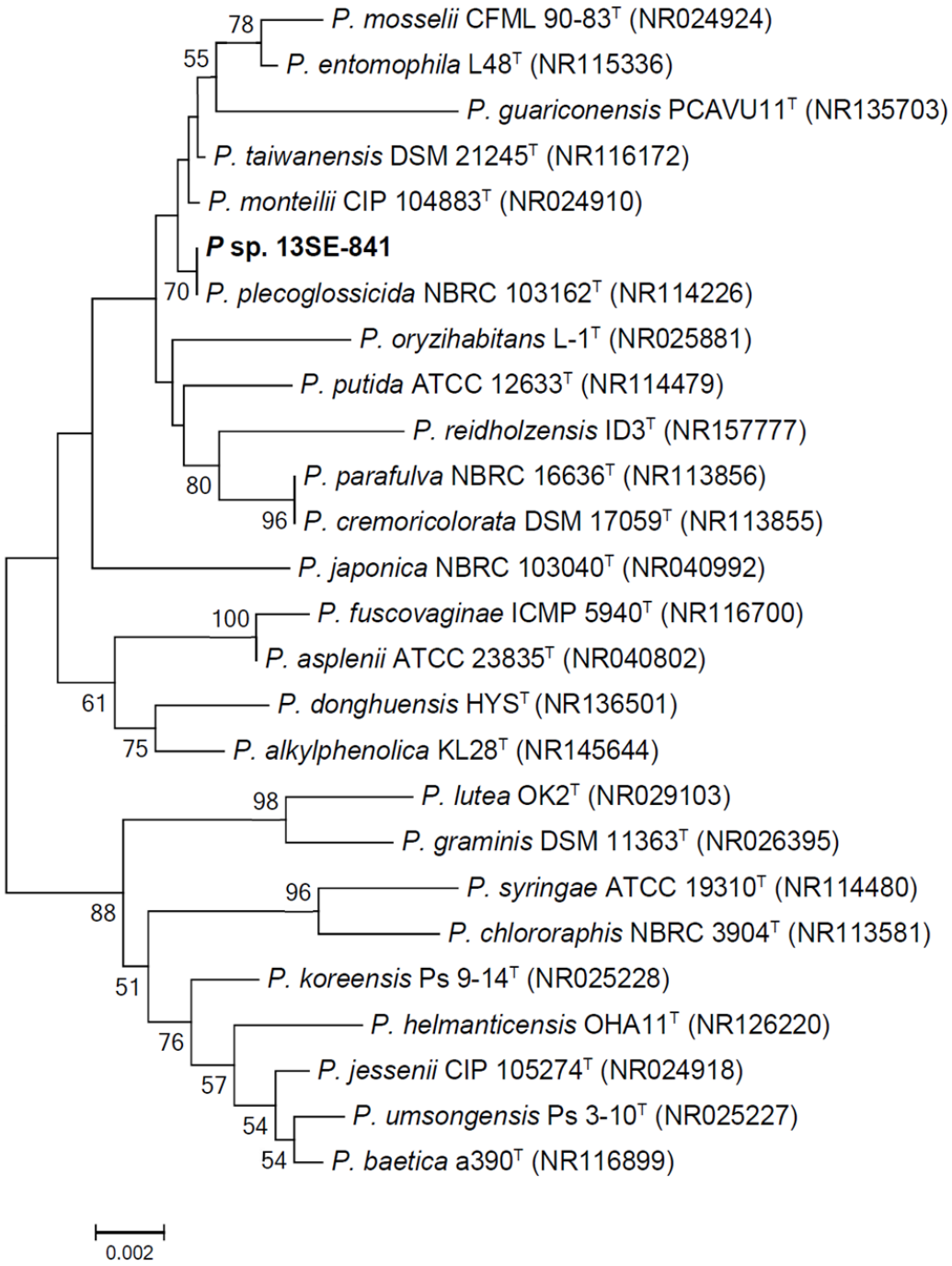

Fig. 3. Neighbor-joining tree illustrating the phylogenetic position of strain 13SE-841 and related members of the genus Pseudomonas based on partial 16S rRNA gene sequences. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Only bootstrap values above 50% are indicated.

Table 1. Greenhouse assay treatments.

Treatment Name	Fungal Inoculum	Bacterial Inoculum
Negative Control	20mL sterile cornmeal sand medium	50mL sterile buffer
Mp151 Control	20mL Mp151 inoculum	50mL sterile buffer
Isolate A Control	20mL sterile cornmeal sand medium	10^9 cfu Isolate A in 50mL buffer
Isolate B Control	20mL sterile cornmeal sand medium	10^9 cfu Isolate B in 50mL buffer
Isolate C Control	20mL sterile cornmeal sand medium	10^9 cfu Isolate C in 50mL buffer
Isolate A + Mp151	20mL Mp151 inoculum	10^9 cfu Isolate A in 50mL buffer
Isolate B + Mp151	20mL Mp151 inoculum	10^9 cfu Isolate B in 50mL buffer
Isolate C + Mp151	20mL Mp151 inoculum	10^9 cfu Isolate C in 50mL buffer

Fig. 4. Greenhouse trial of endophytic bacteria to suppress charcoal rot disease of soybean.

Table 2. Effects of endophytic bacteria on soybean plant growth and disease development

Treatment	Seedlings Emerged[1]	Measures at Conclusion of Test					
		Plant Height (cm)[1]	Pods per Plant[1]	Dry Weight (g)			
				Pod[1]	Stem[1]	Root[1]	Total Plant [1]
Buffer Only	2.9 A	58.19 A	69.7 A	38.95 A	7.25 A	24.27 A	70.47 A
Macrophomina phaseolina Only	2.9 A	56.52 A	59.6 A	33.78 A	5.93 A	13.91 ABC	53.62 A
Strain A Only	2.5 A	60.01 A	78.3 A	45.28 A	7.34 A	23.33 AB	75.94 A
Strain B Only	2.9 A	56.42 A	63.8 A	37.22 A	6.14 A	23.58 AB	66.95 A
Strain C Only	2.9 A	56.34 A	62.5 A	36.85 A	6.19 A	14.68 ABC	57.73 A
Strain A + MP	2.8 A	56.60 A	59.3 A	34.33 A	6.12 A	12.07 BC	52.52 A
Strain B +MP	2.9 A	56.89 A	70.9 A	39.35 A	7.03 A	12.13 BC	58.51 A
Strain C +MP	2.9 A	56.29 A	62.4 A	35.26 A	5.78 A	10.99 C	52.03 A

[1]Least squares mean of 12 plants. Means in the same column bearing the same letter(s) are not significantly different by LSD using the Holm-Tukey grouping for treatment at the 5% level of probability.

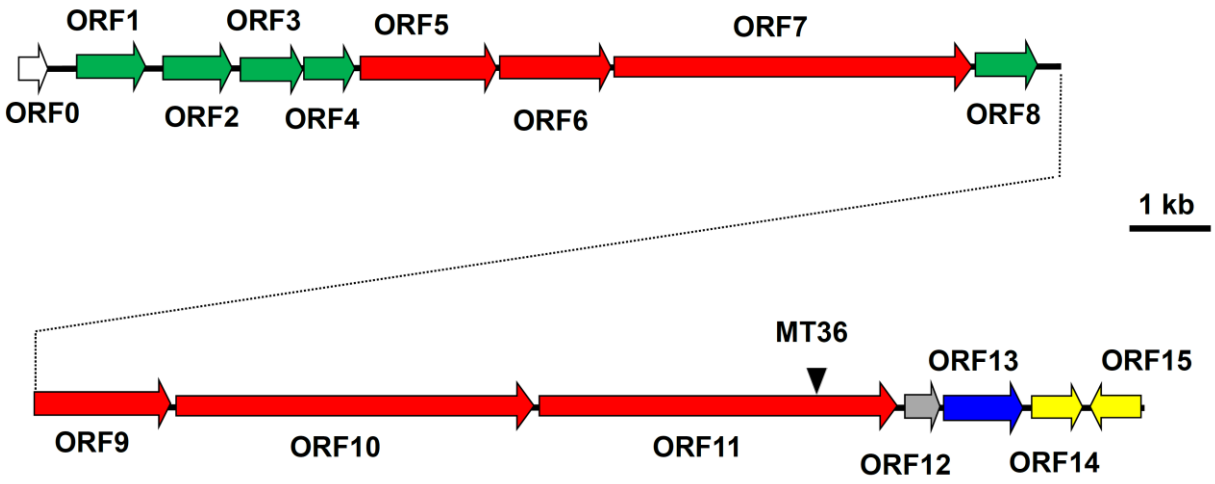

Fig. 5. The 57-kb gene cluster required for antifungal activity of endophytic bacteria *Burkholderia* sp. isolate A. MT36 is a mutant of isolate A and the triangle is the location of transposon.

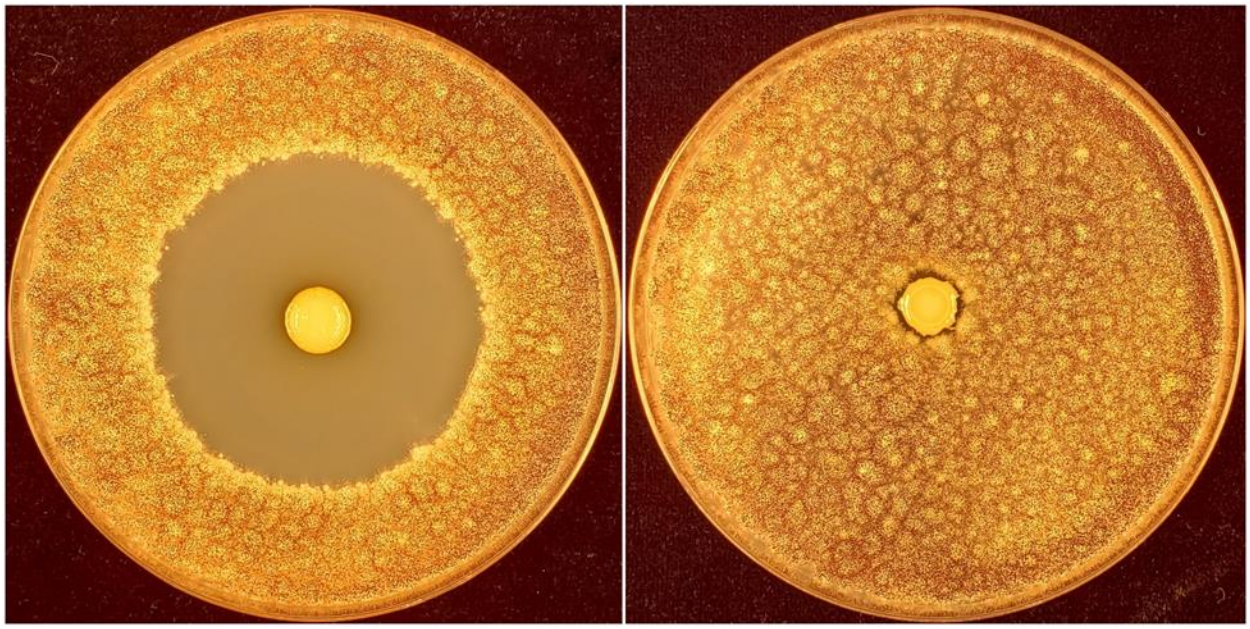

Fig. 6. Plate bioassays of strain MS455 (left) and its mutant MT37 (right) against *Aspergillus niger*