Characterization of Antifungal Activity of Endophytic Bacteria Associated with Soybean – Charcoal Rot Disease System.

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Annual Report

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Background and Objectives: Soybean yield reduction due to Charcoal rot disease caused by the fungus *Macrophomina phaseolina* in the United States was 20.8 million bushels in 2015. The disease accounts for an estimated loss of 1.48% of soybean yield in Southern states. This disease is particularly problematic due to the lack of fungicides capable of providing effective disease control and lack of genetic resistance in cultivars. 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 disease 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. **Specific objectives for the research project** are 1) to continue investigating the effects of inoculation of the representative bacteria on disease development and soybean growth and 2) to characterize additional genes associated with antifungal activity via transposon-mediated mutagenesis.

Report of Progress/Activity:
Objective 1: to continue investigating the effects of inoculation of the representative bacteria on disease development and soybean growth.

In 2018-2019, we conducted a greenhouse trial to evaluate effects of some bacterial strains on plant growth and disease development. The three endophytic strains of bacteria isolated from soybean plants were chosen for greenhouse testing based on results from previous in vitro bioassays. Statistical analysis of the results show that the bacteria could promote growth and development of root system. Even though disease suppression efficacies of the bacteria were not statistically significant, the endophytic bacteria showed increased resistance to charcoal rot disease. It was hypothesized that the bacteria have disease suppression and soybean growth promotion activity. In 2019-2010, we planned to repeat the greenhouse trial with increase of doses of bacteria inoculum. However, the greenhouse trial was not completed because the key personal left from the project in 2019. In addition, the greenhouse was damaged by a June storm. Later, the big autoclave in the North Farm was broken in fall 2019. Due to the coronavirus pandemic, the research was slow down significantly in the extension period. As of today, the autoclave has not been fixed yet. Currently, we have prepared all the required experimental materials, such as sands and top soils. As we all know, some greenhouse trials with the addition of microbial organisms, sterile soil with autoclaving is essential. We plan to resubmit the

proposal in 2021-2022 and conduct the trials for further investigation. If the big autoclave is not fixed, we plan to autoclave the soils in Stoneville.

Objective 2: To characterize the genes associated with antifungal activity production. We previously showed the endophytic bacterial strain A possesses antifungal activities against charcoal rot pathogen in vitro and transposon mutagenesis revealed that a non-ribosomal peptide synthetase gene, which is well known for association of biosynthesis of antimicrobial compounds is required for antifungal activity production (Fig. 1). To purify the antifungal compound, we are optimizing culture conditions for its production. Preliminary data indicate that production of the antifungal compound on a minimum medium (MM) is equivalent to that on potato dextrose. But the MM makes it much easier to purify the compound because it is composed of known elements. The following are major findings in 2019-2020.

(1) The genome of strain A has been completely sequenced. The genome is composed of three chromosomes, which is totally 7,858,391 bp in size (Table 1; Fig. 2). Surprisingly, we have identified a gene set that shares a high similarity to the *ocf* gene cluster of *Burkholderia contaminans* MS14. The *ocf* gene cluster is responsible for antifungal compound occidiofungin, which is well studied antifungal by our research team. However, strain A is unique and becomes more interesting because it was isolated from inside of a soybean plant.

(2) ORF15 of strain A was predicted to encode a LuxR type regulatory protein that is required for production of the antifungal compound. We have successfully generated a mutant of ORF15 using the *nptII*-insertion approach, and the insertional mutation has been verified using a PCR-based approach. The ORF15 was predicted to code for a LuxR type regulatory protein and complementary assays demonstrated its function in antifungal activity. Total RNAs were extracted from both the wild type strain A and the ORF15 mutant for RNASeq analysis. The RNASeq data show 284 genes were differentially expressed in the mutant MT37 as compared with the wild type strain A ((log2 < 1 or >1, $p < 0.05$). A total of 146 genes were found to be downregulated and 138 genes were upregulated (Figs. 3 and 4). Using a >5-fold change cut off, 16 genes were downregulated expressed, which was predicted to be associated with the biosynthesis, secretion and modification of the antifungal compound. The RNASeq data have been confirmed by quantitative PCR analysis (Fig. 5).

(3) Gene mutations of strains A and B have been further investigated. The antifungal activities of strains A and B were compared with the know biological control strains, such as MS14 and Lyc2 using plant pathogens *Aspergillus flavus* and *Cochliobolus heterostrophus* (Figs. 6 and 7). The results show that strains A and B have great potentials as biological control bacteria. The two fungal pathogens produce asexual conidia, which are easily sprayed onto agar plates and used for identification of more mutants (Figs. 8, 9, 10, 11). The mutations are been confirmed by complementary plate bioassays.

(4) Efforts to purify the antifungal compounds. We confirmed that production of the antifungal compound by strain B on a minimum medium (MM) is equivalent to that on potato dextrose, which will makes it much easier to purify the compound because all the ingredients of the MM medium is well defined. However, it was surprised to find the antifungal compound was not

produced in a liquid MM medium. Currently, we are investigating to extract the antifungal compound from agar medium.

(5) Five more strains were identified from the collection of the soybean endophytic bacteria. The bacteria are under further investigation.

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*. Strains A and B have showed great potentials as biological control bacteria on soybean. These bacteria are very important resource for development of biologically based management approaches of the soybean disease. More extensive studies on genetics of antifungal production of strains A and B have provided insights to understanding regulations of the antifungal compounds. The genes responsible for production of antifungal activity could be used to develop genetic modified organisms for disease control. Effects of three of them on development in greenhouse will be further investigated. The trial will provide important data for possible use of the isolates. 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 to development of products to help soybean plants better resist infection of *Macrophomina phaseolina* and prevent charcoal rot disease development.

End Products — Completed or Forthcoming

These research results have been presented in 2019 Annual Conference of the Mississippi Entomological Association and Mississippi Association of Plant Pathologists and Nematologists and in the 2020 annual meeting of American Phytopathological Society (APS). Two manuscripts are being prepared for publication on peer-reviewed journals. Disclose documents are being prepared for intellectual property protection.

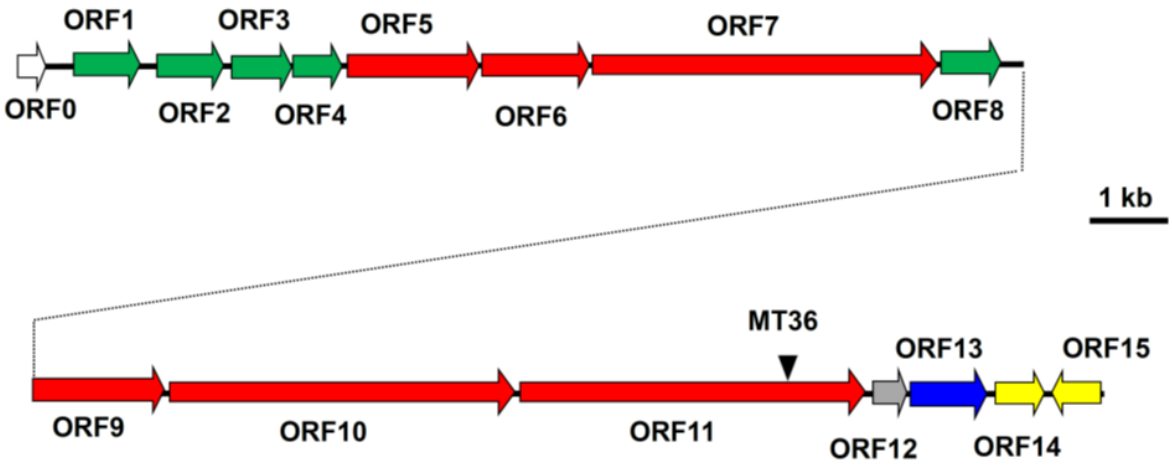

Fig. 1. The 57-kb gene cluster required for antifungal activity of endophytic bacteria *Burkholderia* sp. Strain A. MT36 is a mutant of Strain A and the triangle is the location of transposon.

Table 1. Chromosome statistics of Strain A

Feature	Chromosome 1	Chromosome 2	Chromosome 3	Total
Size	1,031,707 bp	3,288,546 bp	3,538,138 bp	7,858,391 bp
Genes	1,079	3,123	3,262	7,464
CDS	1,041	3,102	3,230	7,373
Pseudo genes	122	142	161	425
rRNAs	2	2	12	18
tRNAs	2	6	61	69
ncRNA	1	3	0	4
G+C content	68.33%	65.50%	66.79%	66.18%

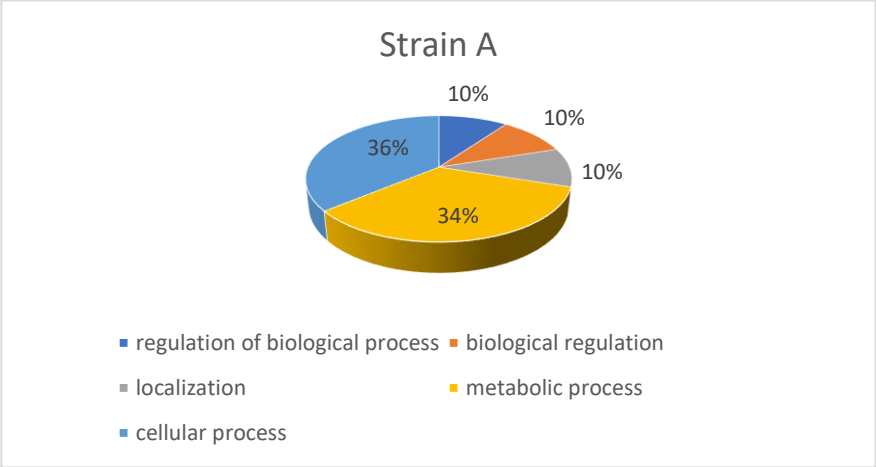

Fig. 2. Gene ontology (GO) analyses of strain A genome predicted by BLAST2GO. GO analysis of the genome corresponding to 7,464 genes as for their predicted involvement in molecular functions, biological processes and cellular component. Data are presented as level 2 GO categorization.

Table 2. Putative genes identified in antifungal gene cluster of Strain A

Gene or ORF[a]	Homolog[b] to MS14	Identity	Homolog[b] to Lyc2	Identity	Function
ORF1	*ocfN*	87.87%	ORF1	97.91%	Thioesterase
ORF2	*ocfM*	96.52%	ORF2	98.73%	SDR family oxidoreductase
ORF3	*ocfL*	92.66%	ORF3	97.48%	Transaminase
ORF4	*ocfK*	90.24%	ORF4	97.26%	Dioxygenase
ORF5	*ocfJ*	91.12%	ORF5	96.67%	AMP-binding protein
ORF6	*ocfI*	93.50%	ORF6	97.38%	oxidoreductase
ORF7	*ocfH*	93.36%	ORF7	97.31%	NRPS-PKS
ORF8	*ocfG*	94.98%	ORF8	98.51%	MBL fold metallo-hydrolase
ORF9	*ocfF*	93.26%	ORF9	96.32%	NRPS
ORF10	*ocfE*	91.66%	ORF10	95.86%	NRPS
ORF11	*ocfD*	89.67%	ORF11	95.67%	NRPS
ORF12	*ocfC*	93.58%	ORF12	96.33%	glycosyl transferase
ORF13	*ocfB*	81.88%	ORF13	97.48%	hypothetical protein
ORF14	*ocfA*	90.30%	ORF14	95.94%	cyclic peptide transporter
ORF15	*AmbR2*	84.42%	ORF15	94.97%	LuxR-type regulator
ORF16	ND	ND	ND	ND	hypothetical protein
ORF17	*AmbR1*	87.23%	ORF16	97.78%	LuxR-type regulator

a, ORF, Open reading frame.
b, homologue to the putative proteins of *Burkholderia contaminans* MS14 and *Burkholderia pyrrocinia* Lyc2, respectively.

ND, not detected.

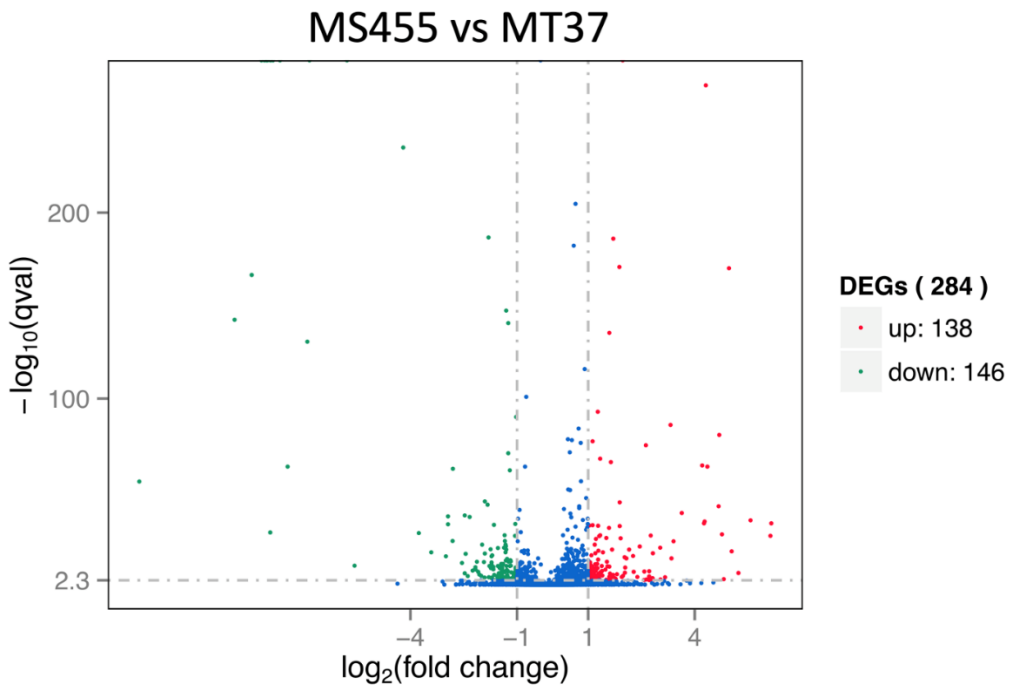

Fig. 3. Transcriptome volcano plots of Strain A (MS455) and its mutant MT37. Significantly up and down regulated genes are highlighted in red and green, respectively. Genes do not show significant differential expression are in blue. The gene expression difference was represented on the axis with a log2 scale.

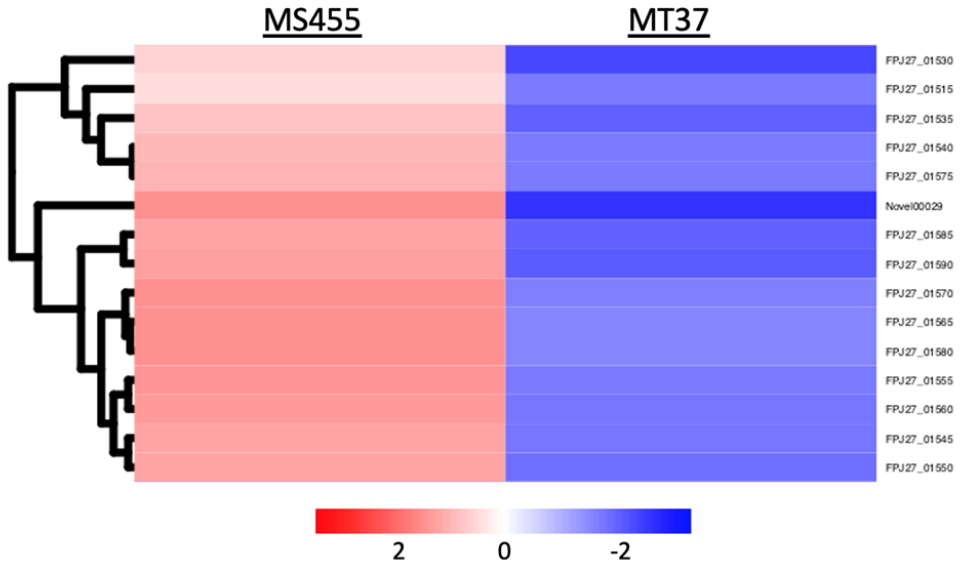

Fig. 4. Heatmap showing the expression level of the genes include 57-kb Gene Cluster of strain A (MS455). Sixteen genes are significant lower expressed in mutant MT37.

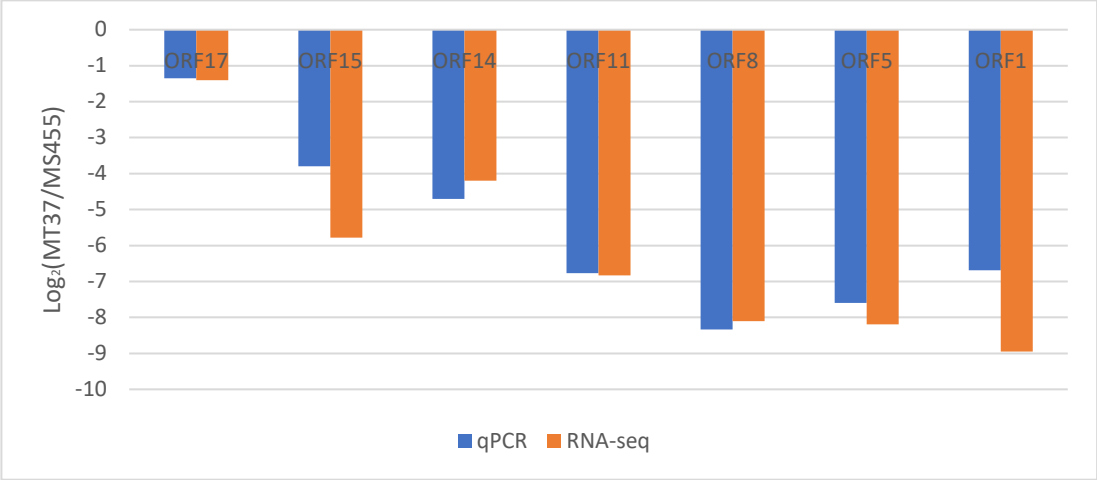

Fig. 5. Validation of RNA-seq data by quantitative RT-PCR. Fold change differences of mutant MT37 to Strain A. The fold difference was calculated by qPCR using comparative quantification method, and log 2 ratio of obtained values was compared with log2 ratio of MT37/Strain A NPKM values. Blue bars indicate their fold change differences from qPCR; Orange bars indicate their fold change differences from RNA-seq.

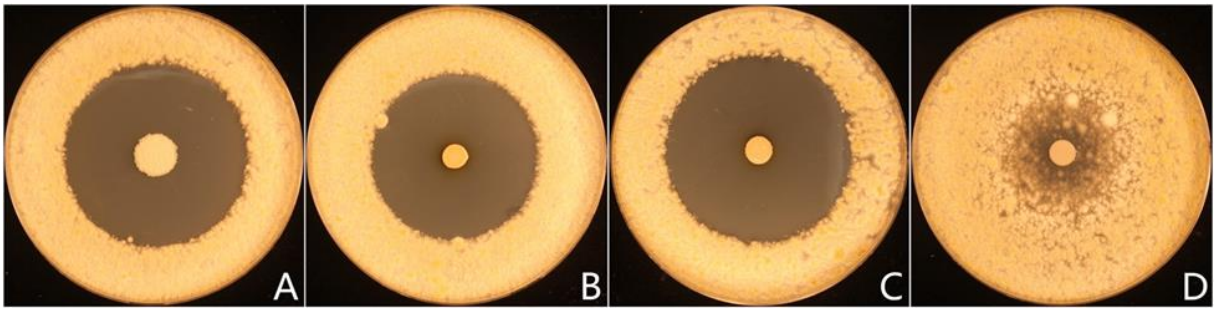

Fig. 6. Plate bioassays of antifungal activities of strain MS14 (A), Lyc2 (B), Strain A (C), Strain B (D) against *Aspergillus flavus*.

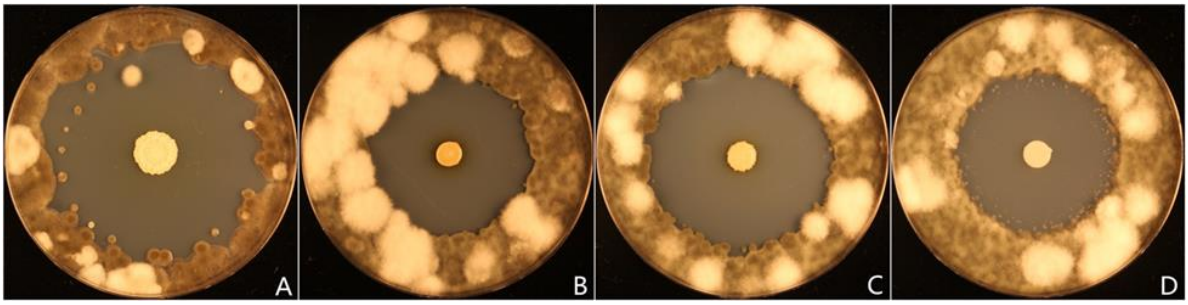

Fig. 7. Plate bioassays of antifungal activities of strain MS14 (A), Lyc2 (B), Strain A (C), Strain B (D) against *Cochliobolus heterostrophus.*

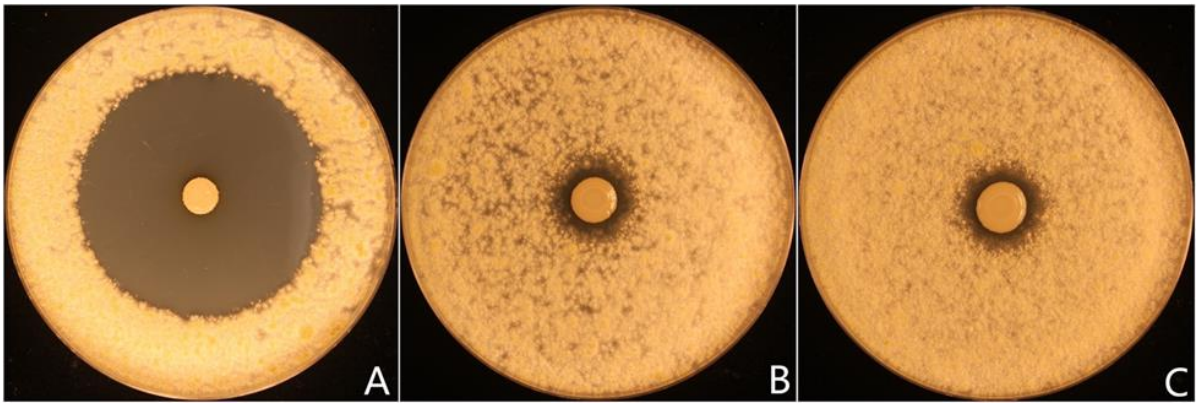

Fig. 8. Plate bioassays of antifungal activities of strain A and its mutant against *Aspergillus flavus*. A: the wild type strain of strain A; B: StrainAMT36 (*ORF11*::*Tn5*); C: StrainAMT37 (*ORF15*::*nptII*)

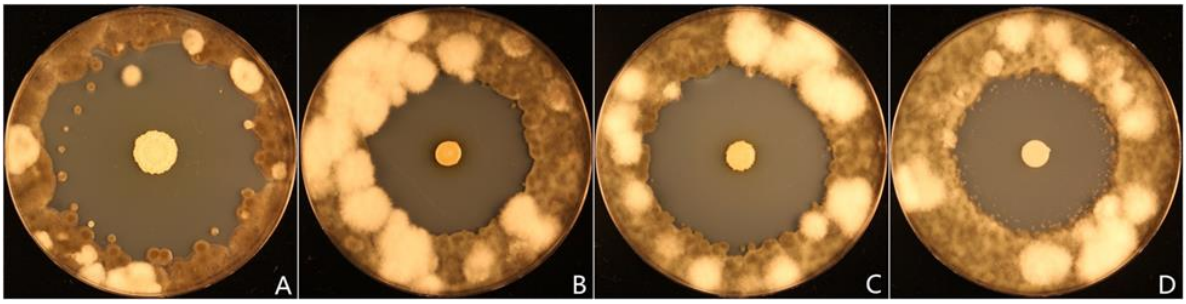

Fig. 9. Plate bioassays of antifungal activities of strain MS14 (A), Lyc2 (B), Strain A (C), Strain B(D) against *Cochliobolus heterostrophus.*

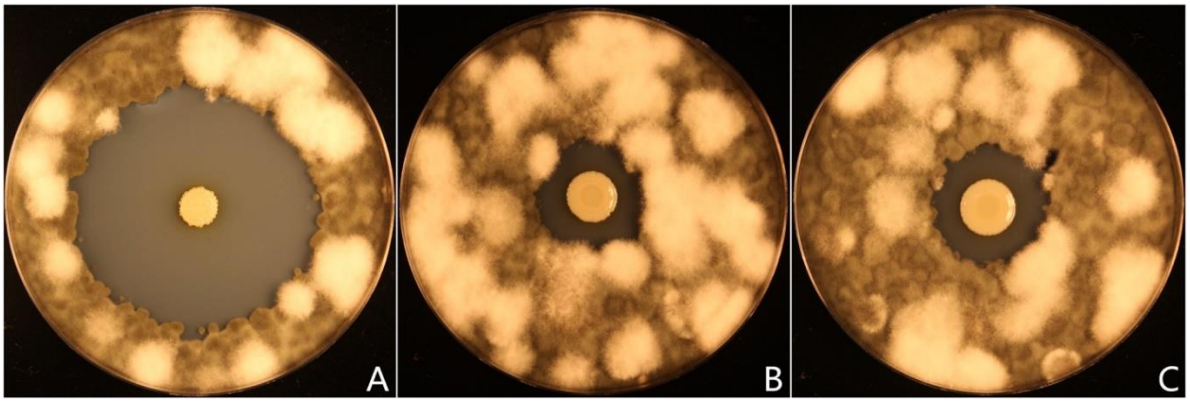

Fig. 10. Plate bioassays of antifungal activities of strain A and its mutant against *Cochliobolus heterostrophus.* A: the wild type strain of Strain A; B: StrainAMT36 (*ORF11*::*Tn5*); C: StrainAMT37 (*ORF15*::*nptII*)

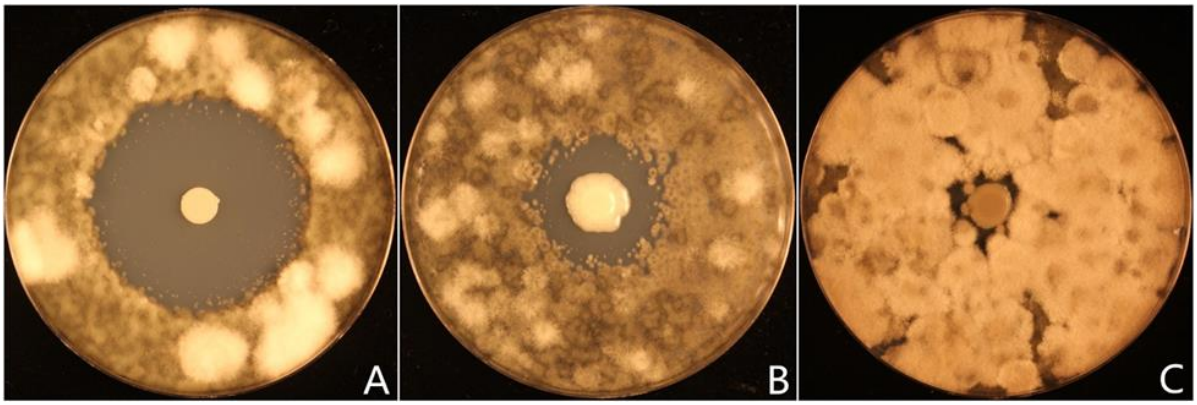

Fig. 11. Plate bioassays of antifungal activities of strain B and its mutant against *Cochliobolus heterostrophus*. A: the wild type strain of B; B: StrainBMT3 (*TatC*::*Tn5*); C: StrainBMT8 (*LysR*::*Tn5*)