Genetic responses and aflatoxin inhibition during interaction between aflatoxigenic and non-aflatoxigenic *Aspergillus flavus*

Rebecca Sweany[1,2], B. Mack[2], G. Moore[2], M. Gilbert[2], J. Cary[2], K. Rajasekaran[2] and Ken Damann Jr.[1]

(1) Louisiana State University, Baton Rouge, LA

(2) U.S. Department of Agriculture-Southern Regional Research Center, New Orleans, LA

Introduction

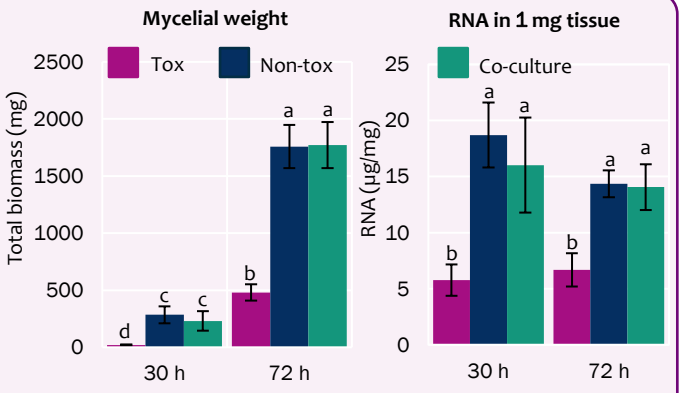
- *Aspergillus flavus* is an opportunistic plant pathogen that infects and contaminates corn with acutely toxic and carcinogenic aflatoxin.
- Non-aflatoxigenic (Non-tox) *A. flavus* isolates are deployed in fields as a biocontrol to mitigate contamination.
- Prevailing mechanism for biocontrol is competitive exclusion via direct replacement of toxigenic (Tox) with Non-tox isolates.
- Non-tox isolates also inhibit aflatoxin production especially when in close or direct contact.
- To understand changes in gene expression during the biocontrol interaction, an *in vitro* RNAseq experiment was conducted.

Methods

- Tox isolate KD53 and Non-tox isolate KD17 were grown alone or co-cultured in liquid glucose salts medium in Petri dishes.
- 3 separate biological replicates (rep) per isolate and co-cultures were grown in the dark at 25°C for either 30, 72 or 96 h.
- Aflatoxin was extracted from medium for 30, 72 and 96 h reps and immediately quantified with high performance liquid chromatography.
- RNA was extracted from all tissue within biological reps at 30 and 72 h.
- 150 bp paired end mRNA libraries were prepared and sequenced using Illumina NextSeq at NC State's Genomic Sciences Laboratory.
- Sequence reads were aligned to the genome of NRRL 3357 and differential expression was determined with DeSeq2. The fraction of each strain present in co-culture was determined by assigning reads to either the Tox or Non-tox isolates using SNPs from FreeBayes.
- SAS version 9.4 was used to generate generalized linear mixed models and compare means or odds for proportional data.

Results

Fungal weight and RNA extracted from isolates grown alone or together

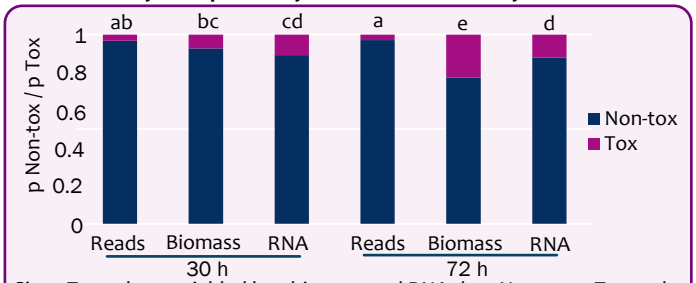


The Tox isolate produced significantly less biomass than both the Non-tox isolate and co-cultures. Significantly less RNA was extracted from equivalent amounts of Tox isolate tissue than Non-tox and co-cultures. Means with the same letter are not significantly different, $\alpha<0.05$.

Aflatoxin production

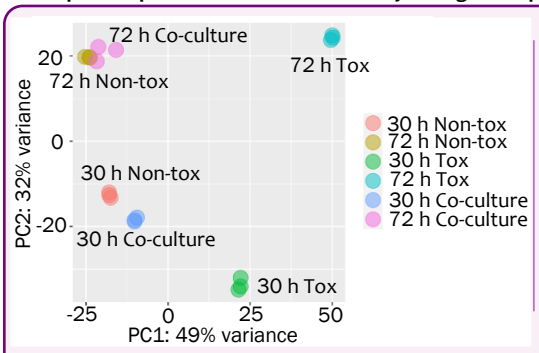
The Tox isolate produced 0 ± 0 s.d. (c), 680 ± 35 (b) and 1902 ± 163 (a) ng/µl aflatoxin B1 at 30, 72 and 96 h respectively. Less than 2 ng/µl AFB1 was detected in co-cultures and Non-tox isolates did not produce AFB1.

Small proportion (p) of sequence reads aligned to Tox in co-culture cannot be fully be explained by low biomass and RNA yields



Since Tox cultures yielded less biomass and RNA than Non-tox, pTox and pNon-tox in co-cultures were predicted by dividing individual biomass and total RNA by the sum of Tox and Non-tox grown apart. These were compared to prop reads aligned to Tox and Non-tox during co-culture. Except for biomass at 30 h, fewer reads aligned to Tox than would be expected by the relatively low biomass and RNA yields of the Tox isolate.

Principle component and differential analysis of gene expression between isolates and co-culture



Differential gene expression	↑	↓
30 h		
Non-tox vs Tox	2092	1732
Co-culture vs Tox	1952	1609
Co-culture vs Non-tox	125	185
72 h		
Non-tox vs Tox	1553	1525
Co-culture vs Tox	1565	1289
Co-culture vs Non-tox	98	6

Biological reps clustered together. 30 and 72 h expression patterns were different. Regardless, co-cultures and Non-tox cultures clustered closely and had few differentially expressed genes, possibly due to limited Tox in co-culture. Compared to 72 h, 30 h Tox clustered closer to the Non-tox isolates and co-cultures but had more significant differentially expressed genes.

Genes highly overexpressed by Non-tox and co-cultures compared to Tox at 72 h

Non vs Tox	Co vs Tox	Co vs Non	Chrm	Pred SM	Putative function
4.7	4.8	-	1[a]	0, 0[b]	Peroxisome biogenesis
7.8	7.8	-	2	0, 0	Uncharacterized protein family UPF0047
9.6	9.4	-	2	0, 0	Protein glycosylation
7.3	7.1	-	5	0, 0	Perforin domain for causing holes in cell membranes
6.1	6.5	-	5	0, 0	Unknown
10.3	10.2	-	5	3, 0	Zn(2)-C6 fungal type DNA binding transcription factor
9.8	9.7	-	5	3, 0	Crotonase activity involved in metabolism
8.4	8.3	-	5	3, 0	1-aminocyclopropane-1-carboxylate oxidase
2.3	2.9	-	5	0, 0	Unknown
2.6	3.0	-	5	0, 0	Ankyrin repeat domain protein-protein interactions
6.1	5.7	-	5	0, 0	Short-chain reductase
8.7	8.8	-	6	0, 0	Phosphorylation
-	-	-	8	0, 0	Unknown
7.9	-	-	8	0, 0	2-methylcitrate dehydratase-catabolism

Genes up-regulated by Non-tox to Tox and further up-regulated during co-cultivation at 72 h

Non vs Tox	Co vs Tox	Co vs Non	Chrm	Pred SM	Putative function
1.7	3.9	2.3	2	0, 0	Cutinase/acetylxylan esterase
1.3	3.0	1.7	2	0, 0	Hsp30-like heat shock protein
2.8	2.3	-	2	0, 0	Fatty acid repression
4.8	7.0	2.2	2	0, 0	Major facilitator-membrane transport
2.1	3.6	1.5	2	0, 0	4-carboxymuconolactone decarboxylase
3.9	5.9	2.0	2	0, 0	Major facilitator-membrane transport
3.9	7.2	3.3	4	0, 0	Unknown-NAD(P) binding
6.0	6.8	-	4	0, 0	NAD(P)H-dependent FMN reductase LOT6
-	3.1	2.4	6	0, 0	Unknown-NAD(P) binding
-	4.2	2.6	8	4, 0	(S)-2-hydroxy-acid oxidase
4.6	5.4	-	8	5, 2	Polyketide synthase
6.2	7.4	-	8	5, 2	Hydrolase
4.8	6.0	-	8	5, 2	Polyketide synthase
5.2	6.5	-	8	5, 2	monooxygenase-FAD dependent oxidoreductase
2.9	4.1	-	8	0, 2	Mitochondrial carrier protein
7.3	9.0	-	8	0, 2	Efflux pump, major facilitator
9.7	11.3	-	8	0, 2	O-methyl transferase
2.8	5.0	2.2	8	0, 0	Haem bifunctional catalase-peroxidase

Selection of genes with significant fold changes of $\log_2$ (gene counts) between Non-tox and Tox isolates grown alone, co-culture and Tox and co-culture and Non-tox isolate grown alone.
[a] Chromosome genes are located. [b] If gene is part of a predicted secondary metabolite (SM) cluster, 1st number predicted by Smurf, 2nd number predicted by anti-smash. 0 means not in SM cluster. Putative function based on interpro predictions and gene descriptions.

Conclusions

- Only 3% reads uniquely aligned to Tox during co-culture, significantly fewer than would be expected due to the slow growth of Tox, indicating Tox growth and/or gene expression was inhibited in response to Non-tox.
- Few reads aligned to the aflatoxin gene cluster during co-culture. (Supplemental)
- 18 genes expressed during Non-tox mono-culture were further up-regulated during co-culture, indicating a response to contact. Of those genes, 7 belong to a putative secondary metabolite (SM) cluster, suggesting a potentially inhibitory compound is produced.
- Multiple genes with reductive and peroxisome activity were up-regulated by Non-tox and co-cultures suggesting Non-tox lowered oxidative potential. Since aflatoxin is reported to alleviate oxidative stress, the Non-tox may reduce need for aflatoxin production.
- This study demonstrates a potential role of inhibitory SMs and reducing agents in the biocontrol mechanism and deserves further exploration to improve biocontrol formulations.