Assessing the influence of environmental factors on the development and pathogenicity of *Aspergillus flavus*

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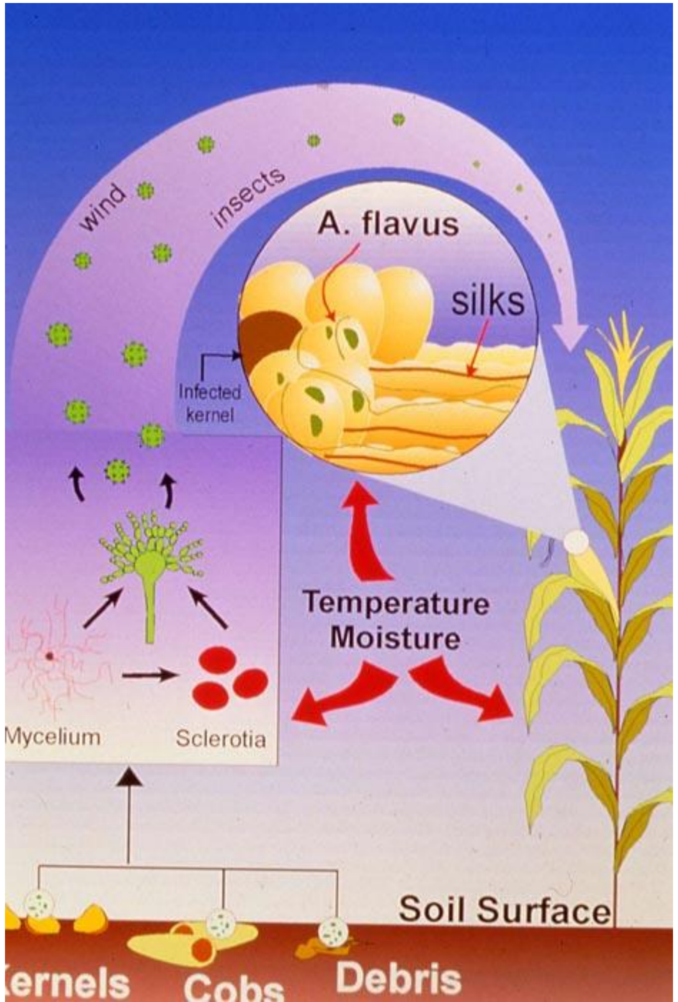
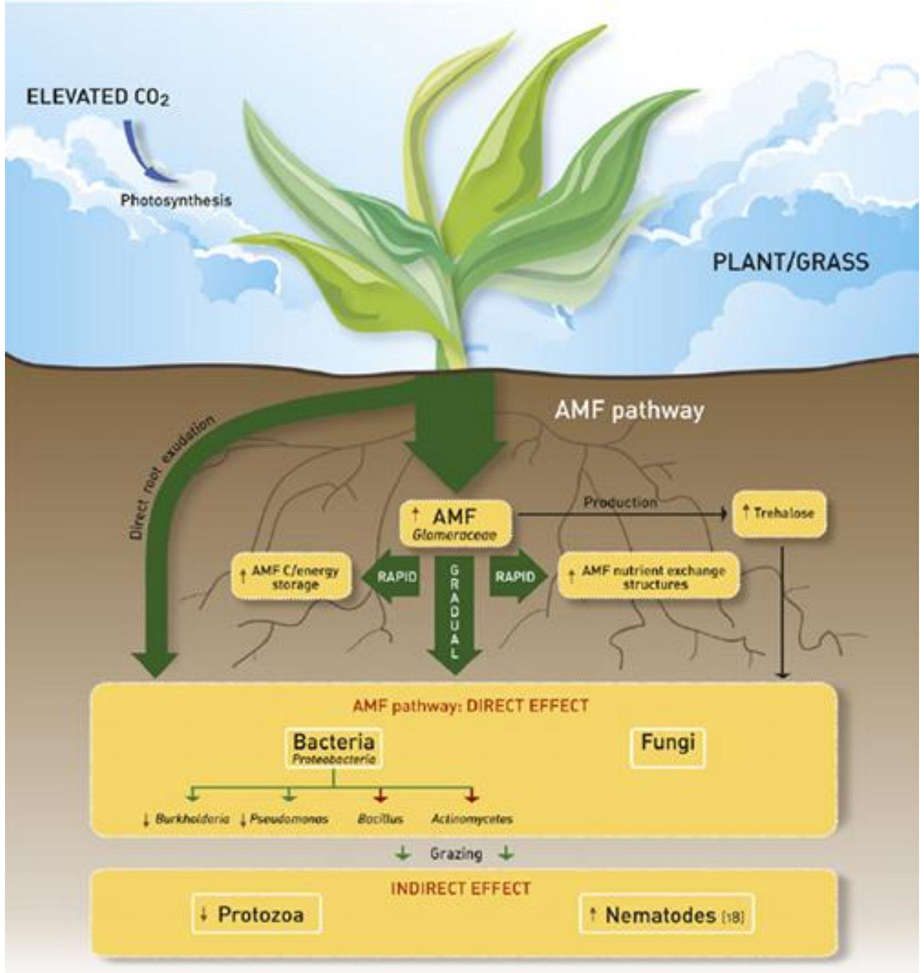
Environmental Factors





Environmental Factors

Water

Temperature

Carbon Dioxide

Water

Temperature

Carbon Dioxide

Previous Research

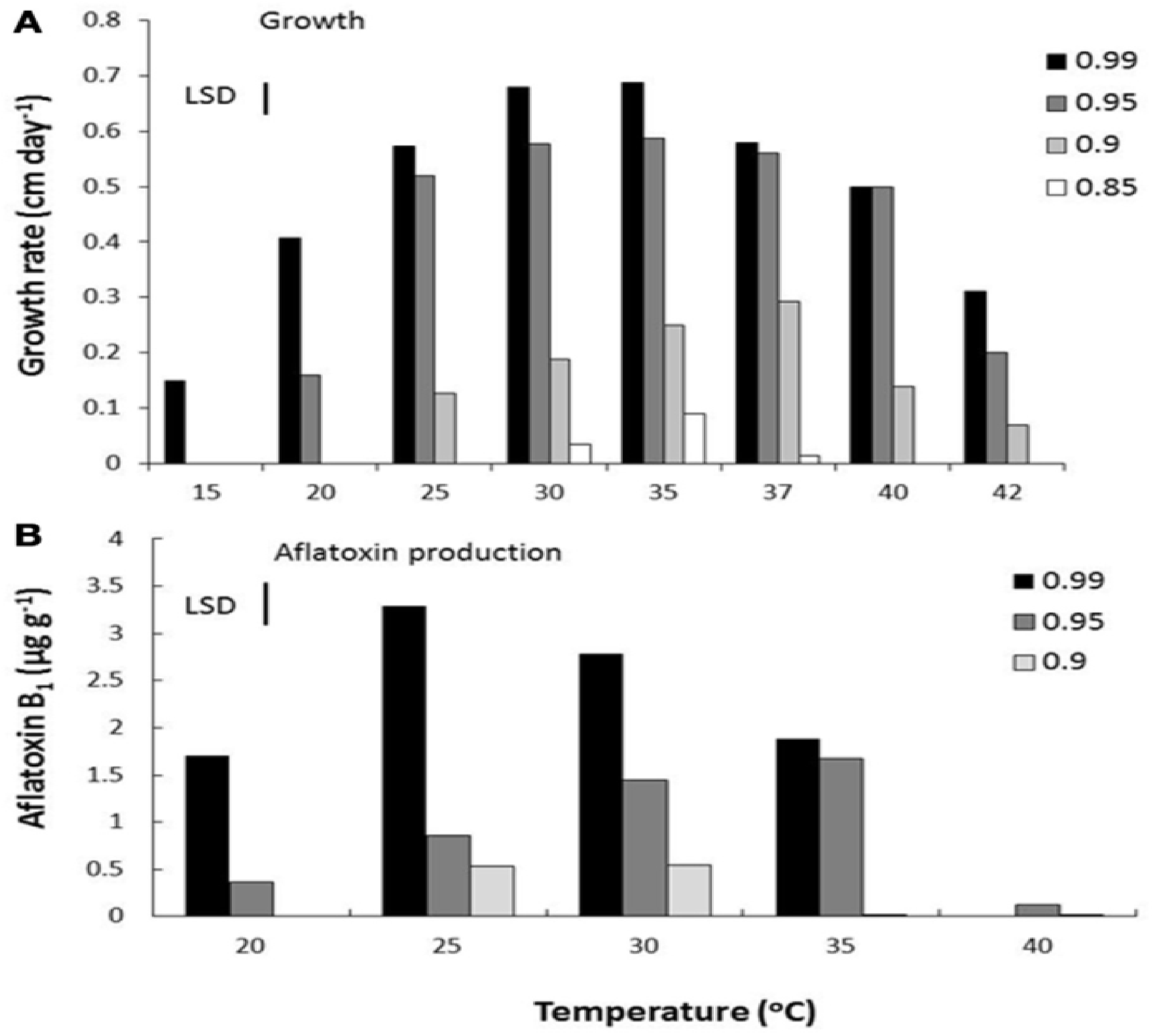
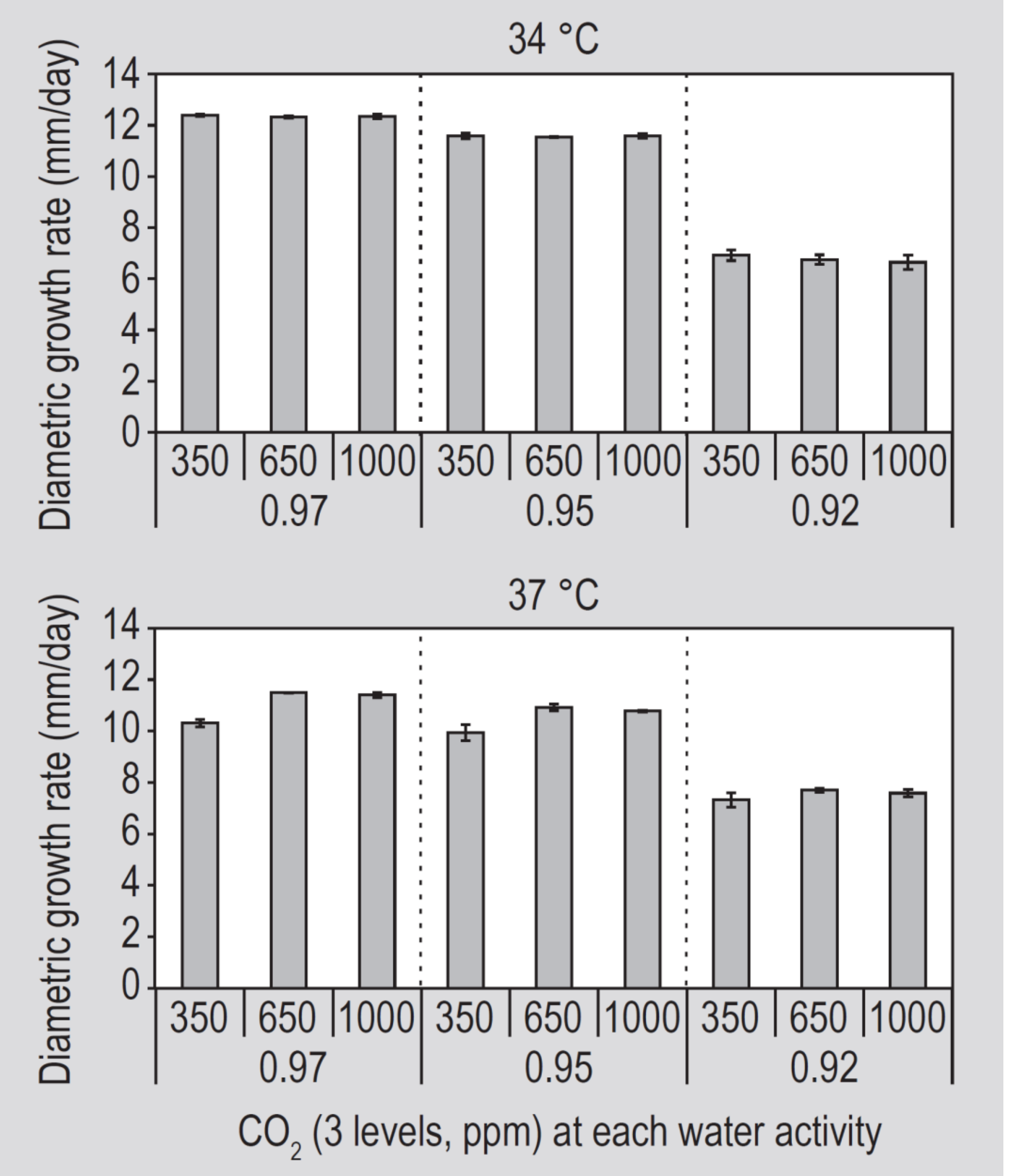


FIGURE 3 | Effect of water activity and temperature on (A) growth and (B) aflatoxin B_1, production by a strain of *A. flavus* (Abdel-Hadi et al., 2012). Bars indicate least Significant Differences.

Medina et. al 2015



Medina et. al 2014

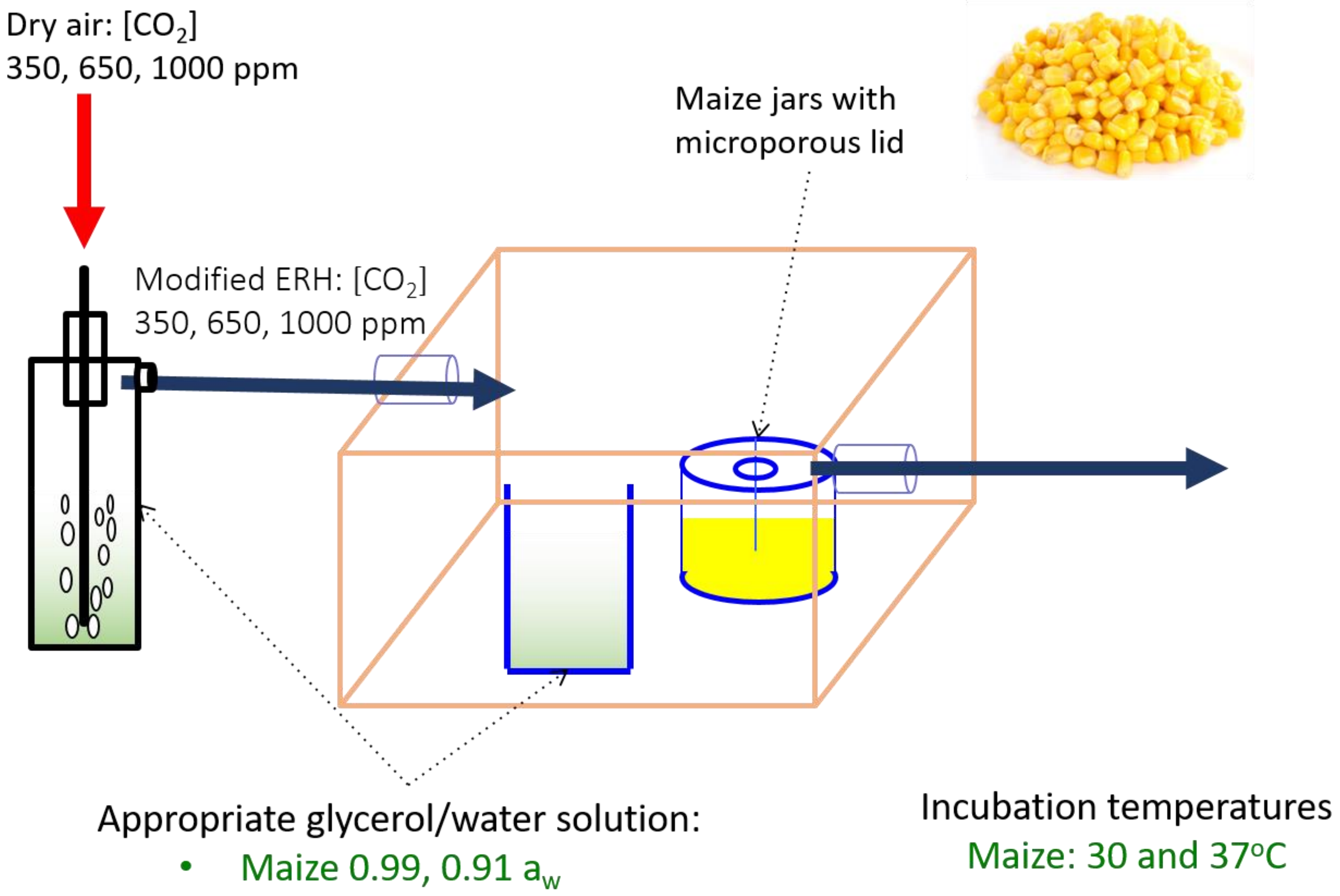
Aflatoxin and CO_2

Temperature (°C)	a_w	CO_2 (ppm)	*aflD*	*aflR*	AFB_1
34	0.97	650	–	–	–
		1000	–	–	–
	0.95	650	–	–	–
		1000	–	↑(×3.6)	–
	0.92	650	–	↑↑(×24.4)	↑(×2.6)
		1000	–	↑(×2.0)	↑(×2.0)
37	0.97	650	↑(×4.6)	–	↑↑(×30.7)
		1000	↑(×6.5)	–	↑↑(×23.8)
	0.95	650	↑(×6.4)	↑↑(×14.6)	↑↑↑(×79.2)
		1000	↑(×3.2)	↑↑(×43.9)	↑↑↑(×78.5)
	0.92	650	–	↑↑(×40.4)	↑↑(×15.1)
		1000	↑↑(×22.5)	↑↑↑(×1680)	↑↑(×23.8)

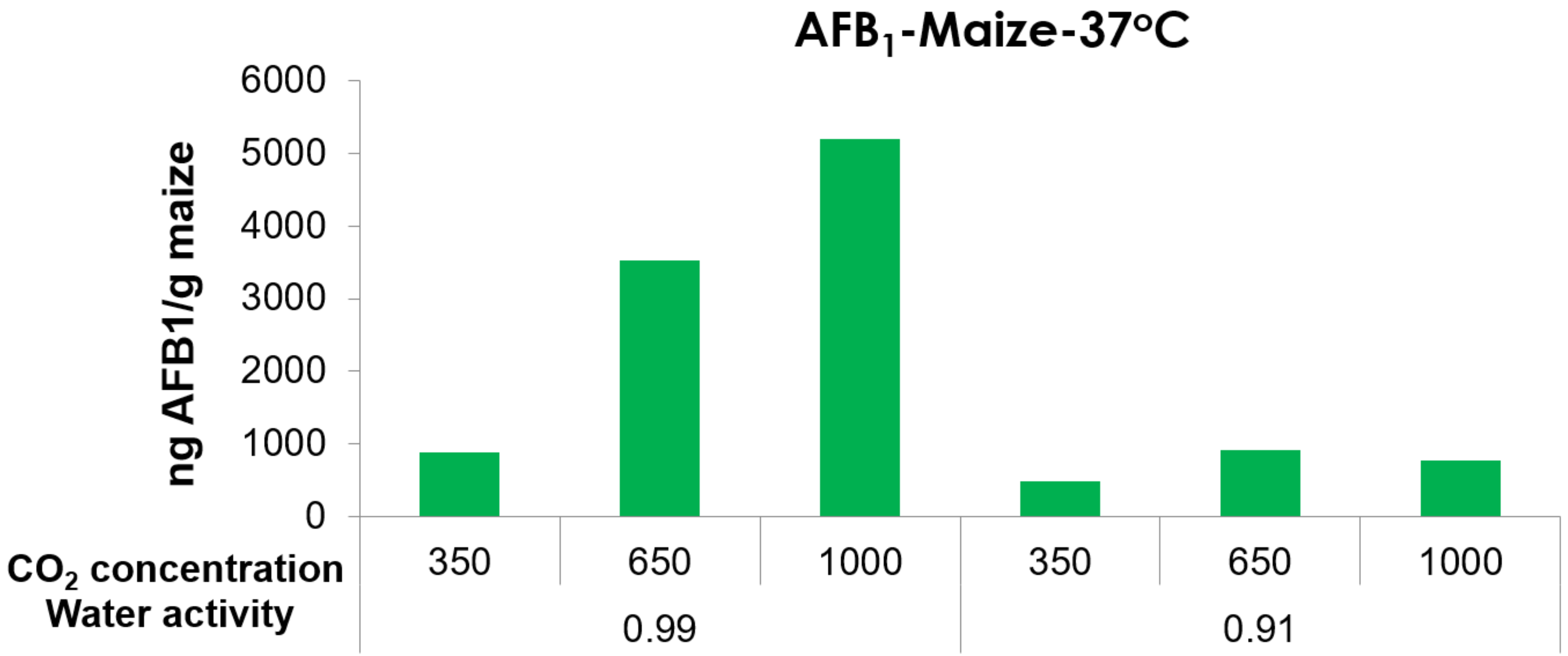
–, variation lower than 2-fold. Numbers between brackets refer to the fold-variation with respect to the control.

Medina et. al 2014

RNA-Seq: Experimental Design



Aflatoxin Analysis of Maize Kernels



RNA Sequencing

- RNA Seq. Results:

  Average of 9.9 million reads per sample obtained.

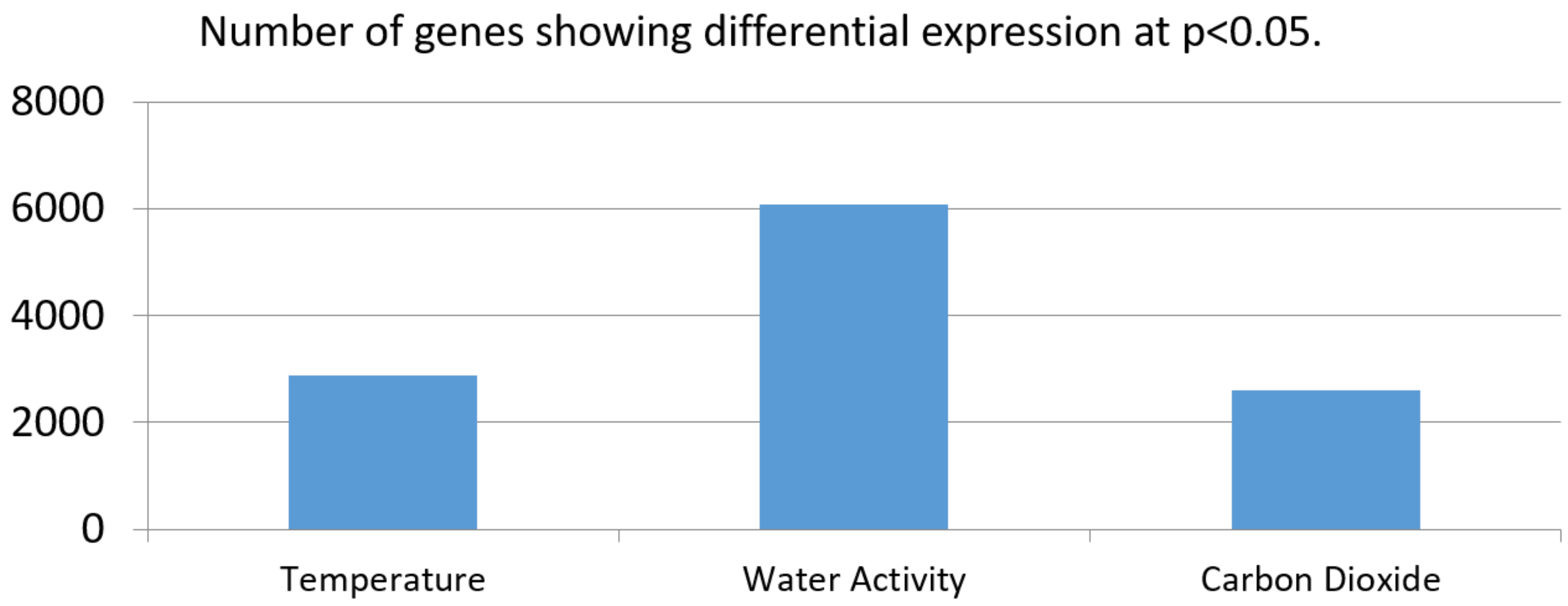
  Average of 53% mapping to exons.

- Mapped to *Aspergillus flavus* strain 3357 genome using Tophat and
- Differential expression and interaction determined by DESeq2.

RNA seq Results

66 Different comparisons can be made:

30°C/37°C ----------0.91a_w/0.99a_w-------- 350 ppm/650ppm/1000ppm



Effect of Temperature	Effect of Water Activity	Effect of CO_2
37°C vs 30°C @ 0.99 w_a and 350ppm	0.91 vs 0.99 w_a @ 30°C and 350ppm	1000 ppm vs 350 ppm @ 30°C and 0.99 w_a

Individual comparisons
(based on gene ontology enrichment analyses)

Effect of Temperature

37°C vs 30°C
@ 0.99 w_a and 350ppm

Genes downregulated:
-glycolytic processes
-Energy production
(ie electron transport)

Genes upregulated:
-carbohydrate metabolism
-lipid metabolism

Effect of Water Activity

0.91 vs 0.99 w_a
@ 30°C and 350ppm

Genes downregulated:
-translation
-tubulin complex assy.
-glycolytic processes
-Energy production

Genes upregulated:
-Transcriptional related
(chromatin remodeling,
RNA polymerase)
-Cellular growth (DNA repair
and morphology)

Effect of CO_2

1000 ppm vs 350 ppm
@ 30°C and 0.99 w_a

Genes downregulated:
-secondary metabolic
processes
-response to oxidative
stress

Genes upregulated:
-mitosis/DNA replication
-Cellular growth

$P<0.01$

Individual comparisons

(based on gene ontology enrichment analyses)

37°C vs 30°C @ 0.99 w_a and 350ppm

Genes downregulated:
- -glycolytic processes
- -Energy production (ie electron transport)

Genes upregulated:
- -carbohydrate metabolism
- -lipid metabolism

0.91 vs 0.99 w_a @ 30°C and 350ppm

Genes downregulated:
- -translation
- -tubulin complex assy.
- -glycolytic processes
- -Energy production

Genes upregulated:
- -Transcriptional related (chromatin remodeling, RNA polymerase)
- -Cellular growth (DNA repair and morphology)

1000 ppm vs 350 ppm @ 30°C and 0.99 w_a

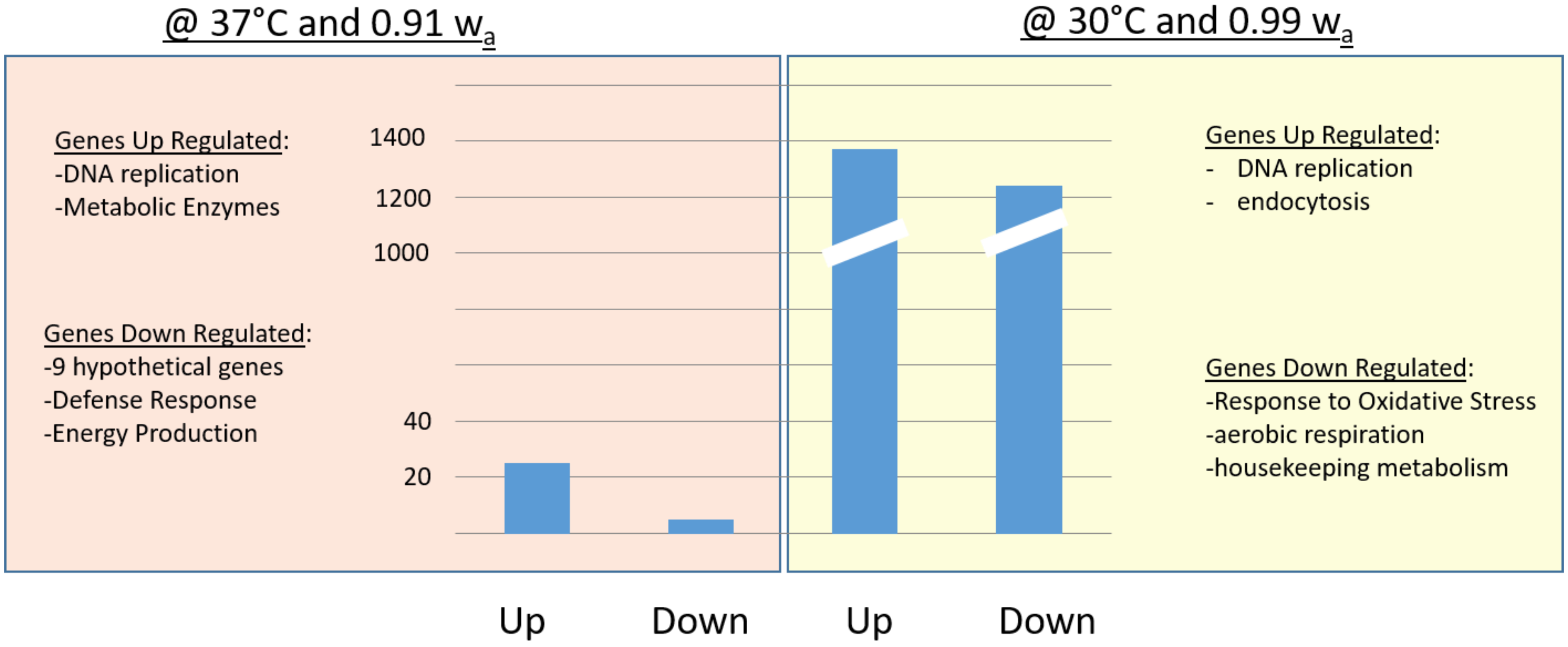
Genes downregulated:
- -secondary metabolic processes
- -response to oxidative stress

Genes upregulated:
- -mitosis/DNA replication
- -Cellular growth

P<0.01

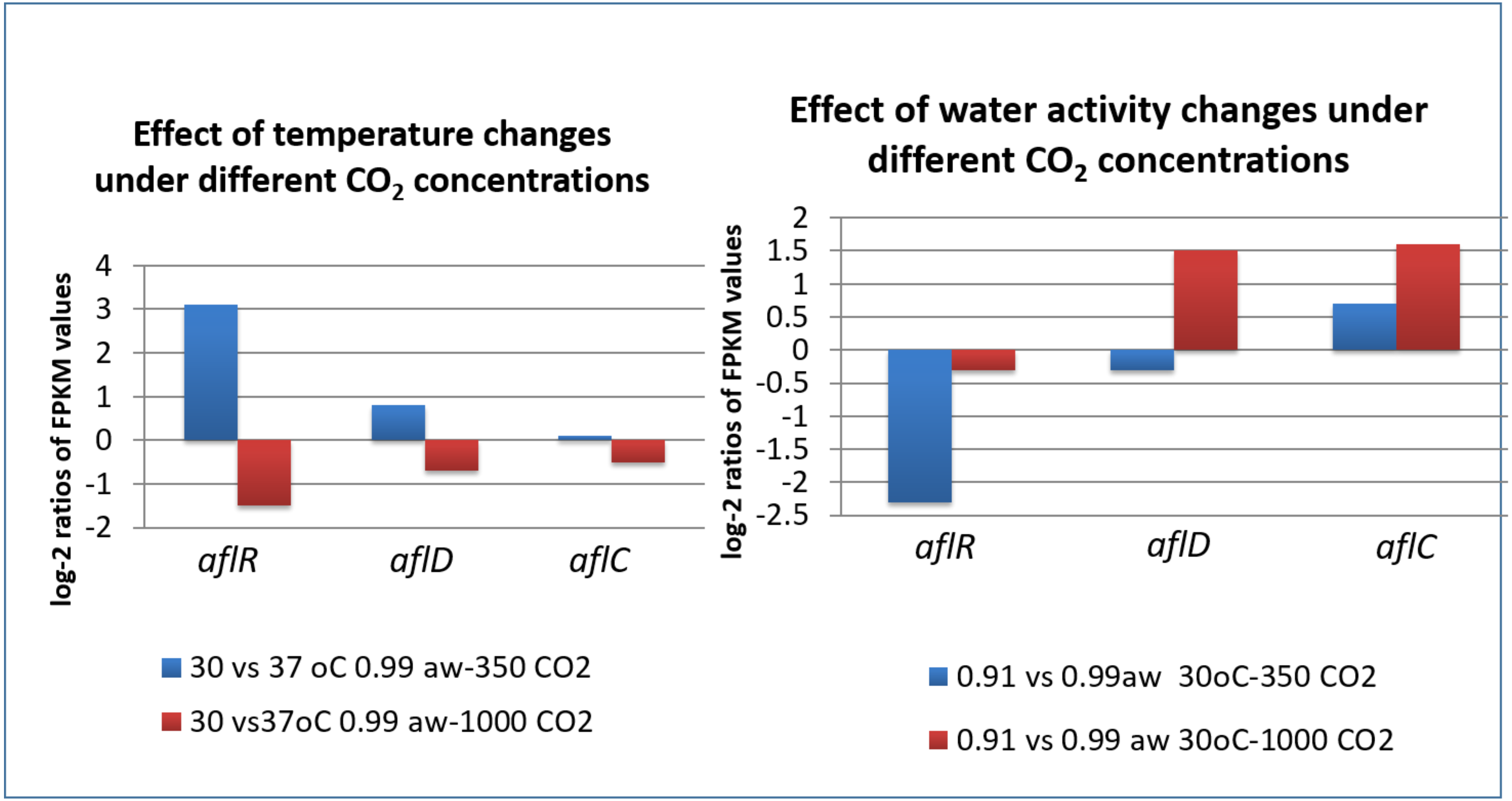
The effect of CO_2: The effect of increasing CO_2 has at higher temperatures And decrease water availability.

1000ppm vs 350ppm



P<0.05

Effect of CO_2 on aflatoxin genes after 10 days



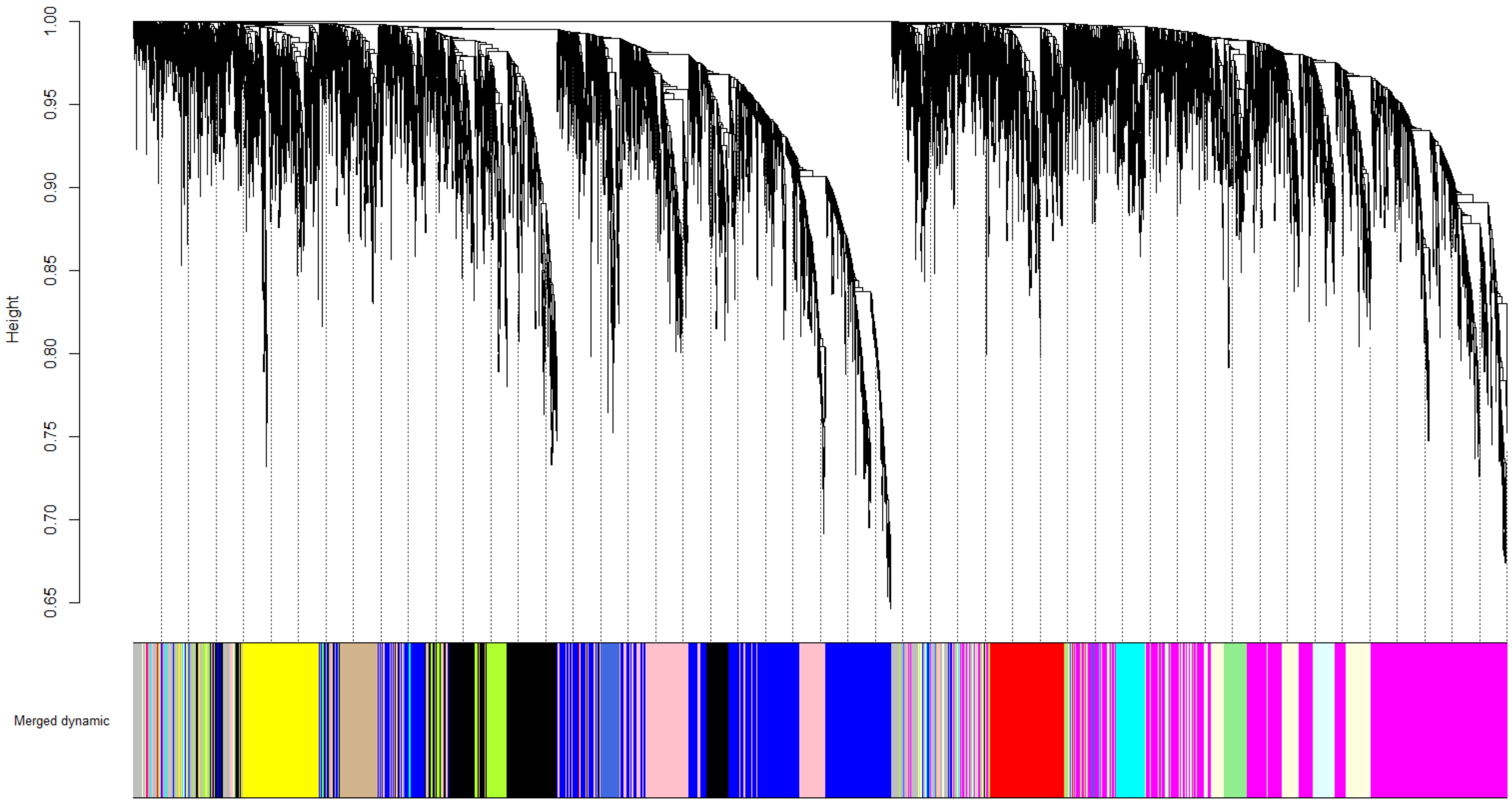
Secondary Metabolite Backbone Genes

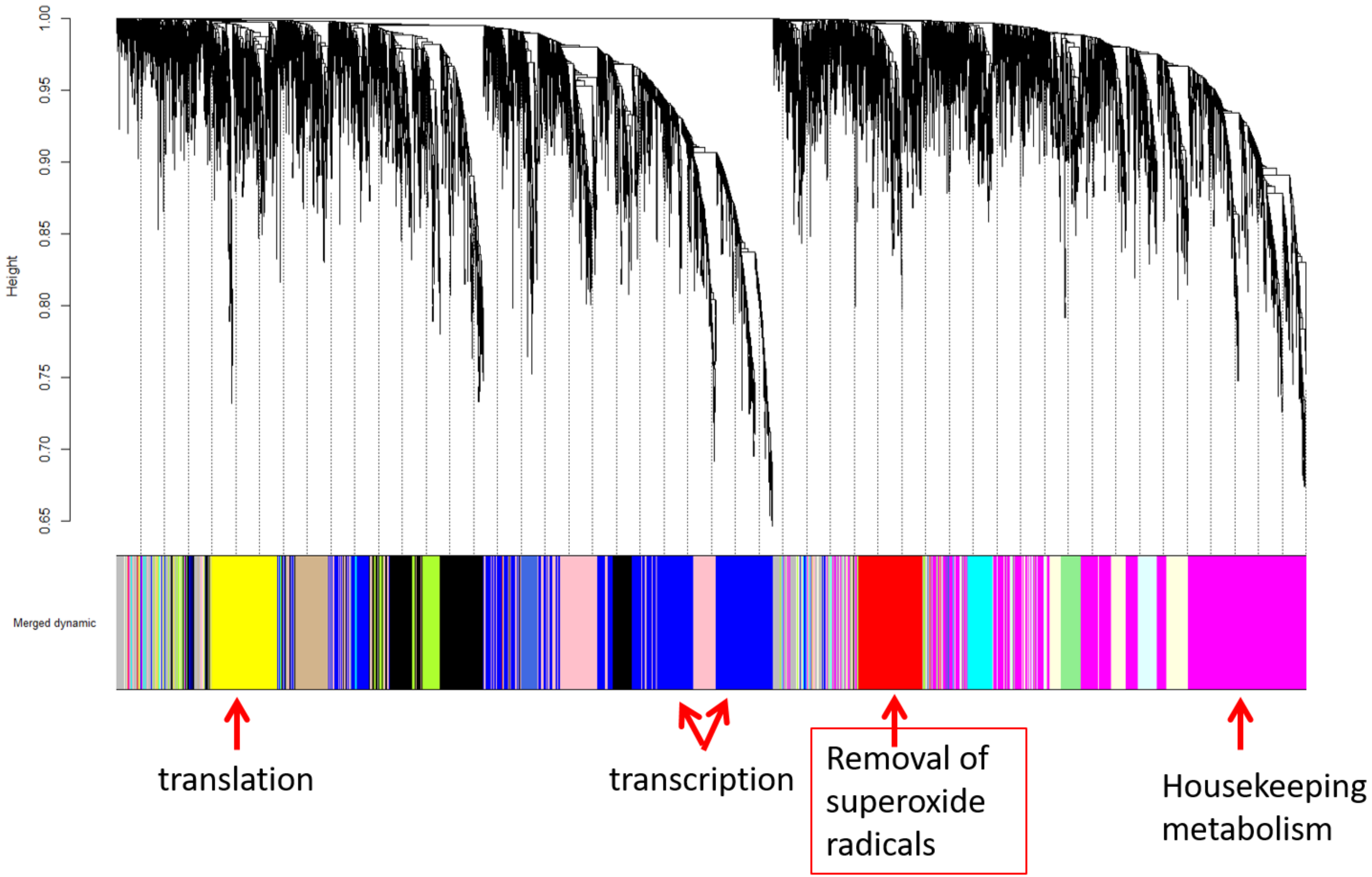
Cluster ID	A. flavus 3357	Name	SM* Product	Effect of Temperature 1000 ppm	Effect of Temperature 350 ppm	Effect of Water activity 1000 ppm	Effect of Water activity 350 ppm	three-way interaction
5	AFLA_006170	polyketide synthetase (PksP)	naphthopyrone	-	1.8	2.1	5.0	3.9
9	AFLA_010580	nonribosomal peptide synthase	siderophore	-	-	-	-	-
10	AFLA_016140	scytalone dehydratase (Arp1) (conidial pigment biosynthesis)	conidial pigment 1,8-dihydroxynaphthalene-melanin	-	-	-2.2	-3.5	-
14	AFLA_041050	enterobactin esterase IroE-like, putative	siderophore	-	-	-	-	-
15	AFLA_045490	dimethylallyl tryptophan synthase, putative	aflatrem, ATM2	-3.6	-	-4.5	-	-
20	AFLA_062860	polyketide synthase (PkfA)	3-(2.4-dihydroxy-6-methylbenzyl)-orsellinaldehyde	-	2.4	-1.8	-	4.5
21	AFLA_064240	nonribosomal peptide synthase (*wykN*)	WYK peptidase inhibitor	-2.5	1.2	-2.7	-	4.9
25	AFLA_070870	isopenicillin N synthetase (*ipnA*)	penicillin	-	-	-	-	-
27	AFLA_082150	polyketide synthase	asparasone	-	-	-2.9	-	-
31	AFLA_095000	Peptidase S41	ustiloxin B	-	-	-	-	-
32	AFLA_096390	aflatrem-geranylgeranyl pyrophosphate synthase (*atmC*)	aflatrem,ATM1	-	-	-	-	-
35	AFLA_101700	NRPS enzyme (*lnaA*)	piperazines	-	2.7	-1.8	-	4.5
36	AFLA_104210	PKS-like enzyme, putative	dihydrocurvularin	-	-2.9	-	-2.9	-
39	AFLA_108550	polyketide synthase	monodictylphenone	-	-	-3.2	-	-
41	AFLA_114820	polyketide synthase (fluP) (pksL2)	6-MSAi	-	-	-2.2	-	2.7
44	AFLA_116890	polyketide synthase (PkiA)	6-hydroxy-7-methyl-3-nonylisoquinoline-5,8-dione	-	-	-	-3.4	-
51	AFLA_127090	polyketide synthase (PkdA)	2-ethyl-4,6-dihydroxy-3-methyl-6-(2-oxopropyl) benzaldehyde et. al.	-	-	-	-	-
54	AFLA_139410	polyketide synthase (*aflC* / *pksA* / *pksL1*)	aflatoxin	-2.1	-	-	0.9	2.9
55	AFLA_139490	hybrid PKS/NRPS enzyme	cyclopiazonic acid	-4.4	-	-8.0	-4.0	8.1
NA**	AFLA_096040	FAD-dependent oxidoreductase (*kojA*)	kojic Acid	-	-	-	-	

Log2 fold change

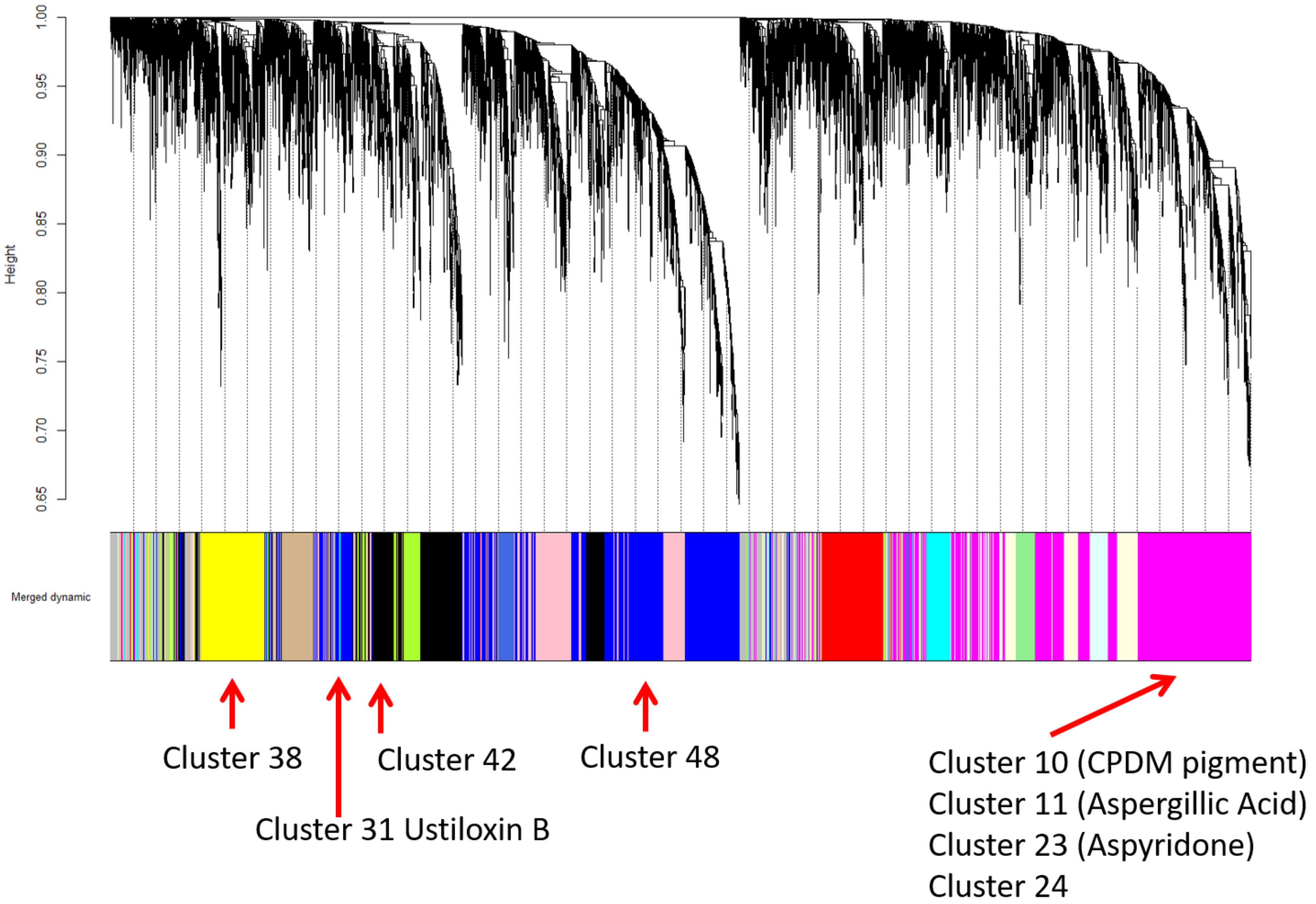
WGCNA Cluster Dendrogram: tool utilized to dissect the data and find biological meaning

- Genes based on co-expression similarity are clustered (shown in bands below)
- Branched tree diagram is based on the statistical relevance of their fit in the cluster. (then biological networks are created)

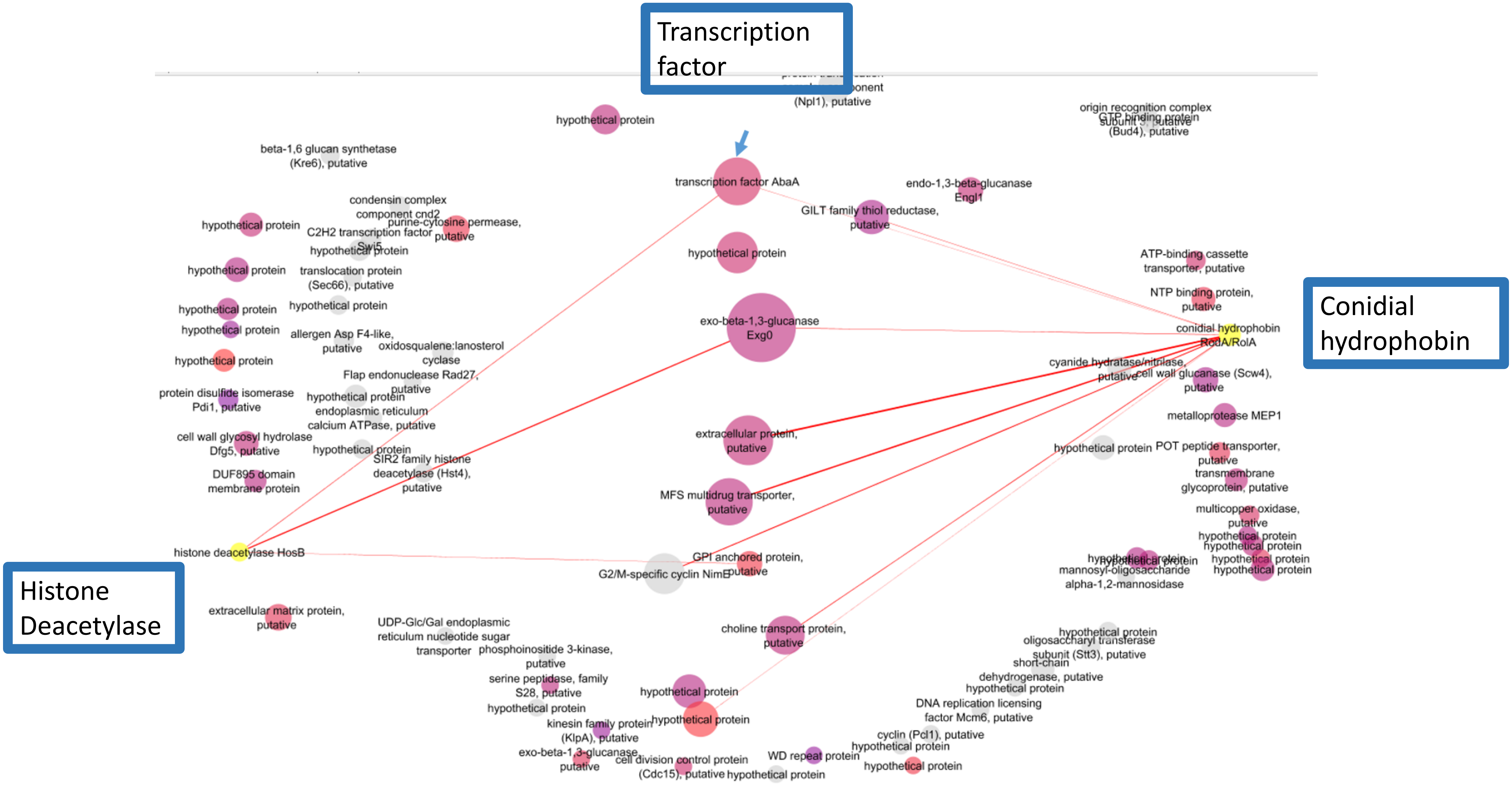




Height
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
Merged dynamic
translation
transcription
Removal of superoxide radicals
Housekeeping metabolism



Height
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
Merged dynamic
Cluster 38
Cluster 42
Cluster 48
Cluster 31 Ustiloxin B
Cluster 10 (CPDM pigment)
Cluster 11 (Aspergillic Acid)
Cluster 23 (Aspyridone)
Cluster 24



Transcription factor
Conidial hydrophobin
Histone Deacetylase
(Npl1), putative
hypothetical protein
origin recognition complex
(Bud4), putative
beta-1,6 glucan synthetase (Kre6), putative
transcription factor AbaA
endo-1,3-beta-glucanase Engl1
condensin complex component cnd2
purine-cytosine permease, putative
C2H2 transcription factor Swi5
GILT family thiol reductase, putative
hypothetical protein
translocation protein (Sec66), putative
ATP-binding cassette transporter, putative
NTP binding protein, putative
exo-beta-1,3-glucanase Exg0
allergen Asp F4-like, putative
oxidosqualene:lanosterol cyclase
conidial hydrophobin RodA/RolA
Flap endonuclease Rad27, putative
cyanide hydratase/nitrilase, putative
cell wall glucanase (Scw4), putative
protein disulfide isomerase Pdi1, putative
endoplasmic reticulum calcium ATPase, putative
metalloprotease MEP1
extracellular protein, putative
cell wall glycosyl hydrolase Dfg5, putative
SIR2 family histone deacetylase (Hst4), putative
POT peptide transporter, putative
DUF895 domain membrane protein
transmembrane glycoprotein, putative
MFS multidrug transporter, putative
multicopper oxidase, putative
histone deacetylase HosB
GPI anchored protein, putative
G2/M-specific cyclin NimE
mannosyl-oligosaccharide alpha-1,2-mannosidase
extracellular matrix protein, putative
UDP-Glc/Gal endoplasmic reticulum nucleotide sugar transporter
phosphoinositide 3-kinase, putative
choline transport protein, putative
oligosaccharyl transferase subunit (Stt3), putative
short-chain dehydrogenase, putative
serine peptidase, family S28, putative
DNA replication licensing factor Mcm6, putative
kinesin family protein (KlpA), putative
cyclin (Pcl1), putative
exo-beta-1,3-glucanase, putative
cell division control protein (Cdc15), putative
WD repeat protein

Conclusions from RNA-seq analysis

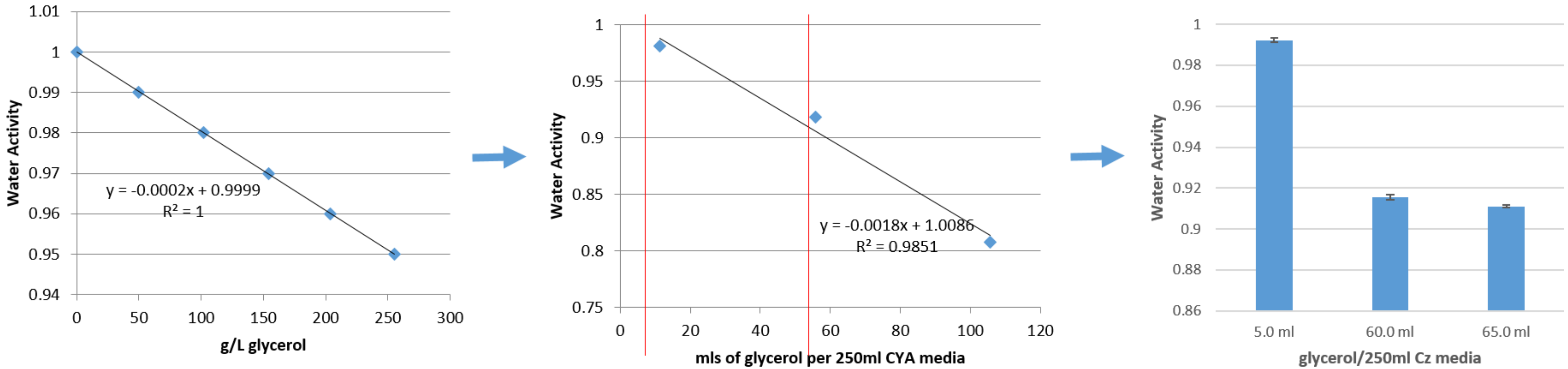
- CO_2 effects aflatoxin production
- CO_2 effects the molecular response to temperature stress and water availability
- Interaction effect of the three variables show altered regulation of core enzymes in secondary metabolic cluster genes.
- WGNCA analysis indicates genes related to oxidative stress response, mitosis and conidia production affected.

Food and Feed Safety Unit

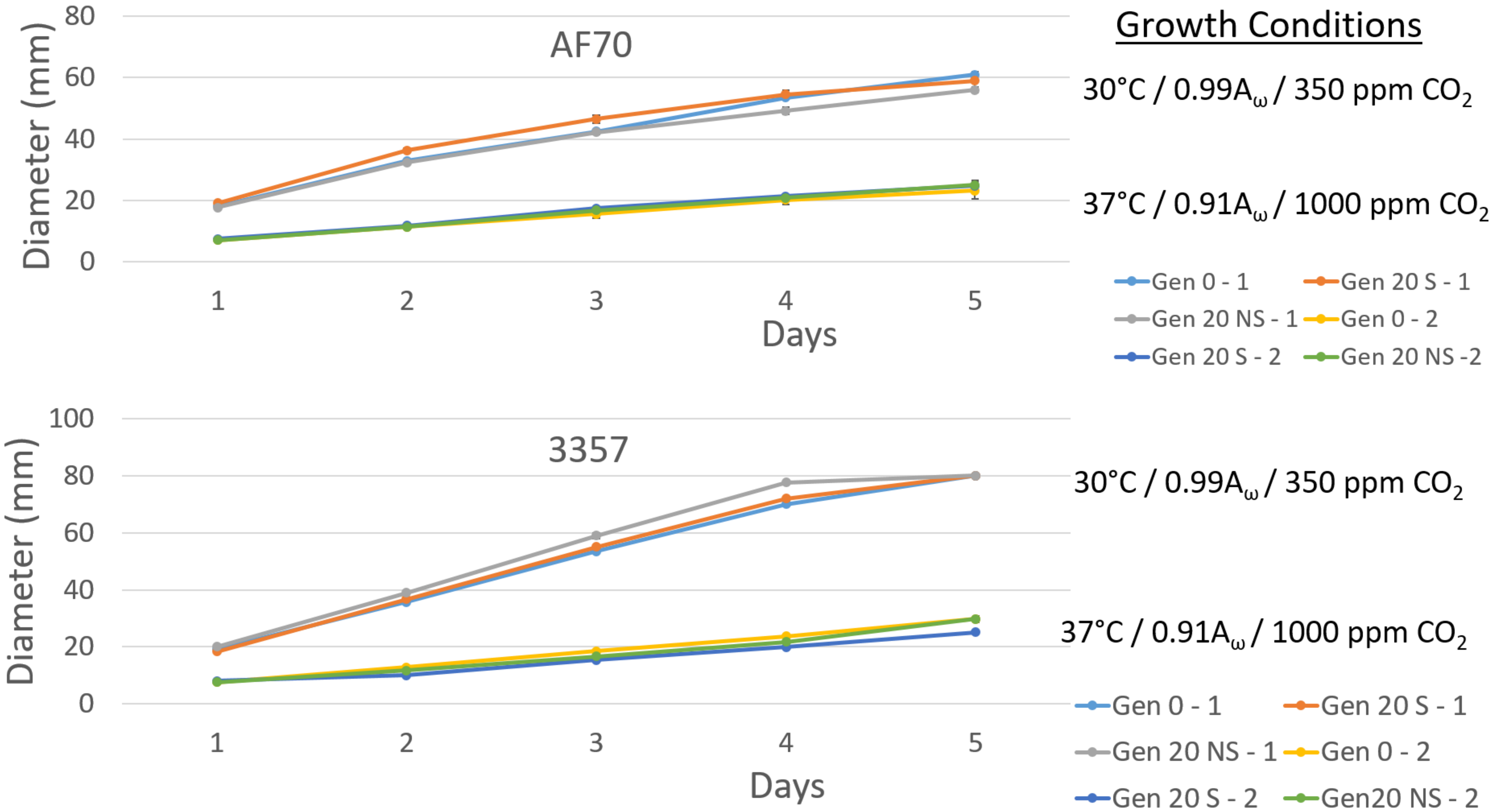
- Goals:
  - Establish acclimatized AF70 and 3357 strains
    - growth
    - spore production
    - toxin production
  - Maize – Fungus interactions.
    - KSA assays using acclimatized strains
      - Determine pathogenicity (GFP) and toxin production

Generating acclimatized strains

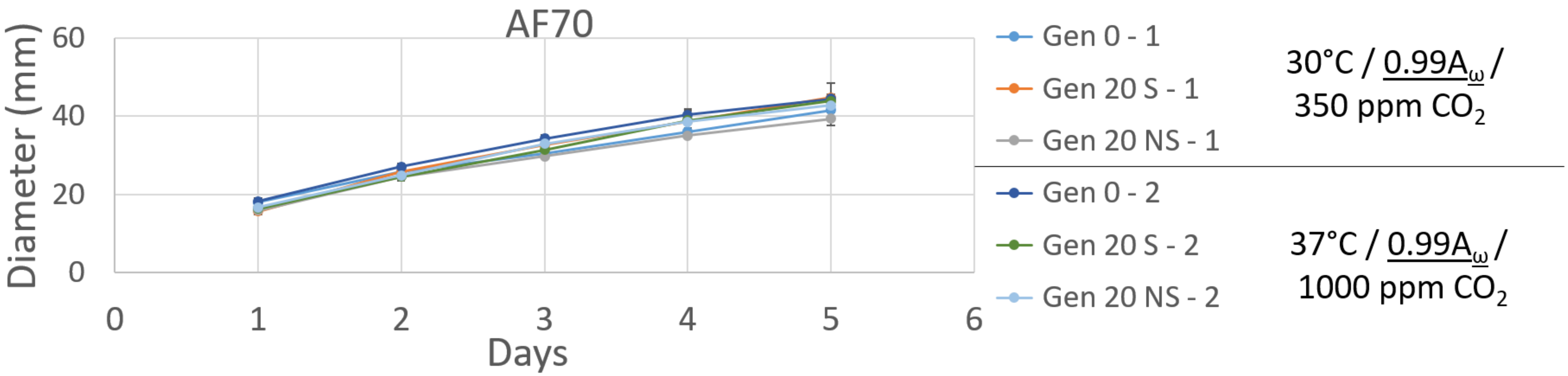
- 20 generations of subcloning conducted at
  - 30°C / 0.99A_ω / 350 ppm CO_2
  - 37°C / 0.91A_ω / 1000 ppm CO_2

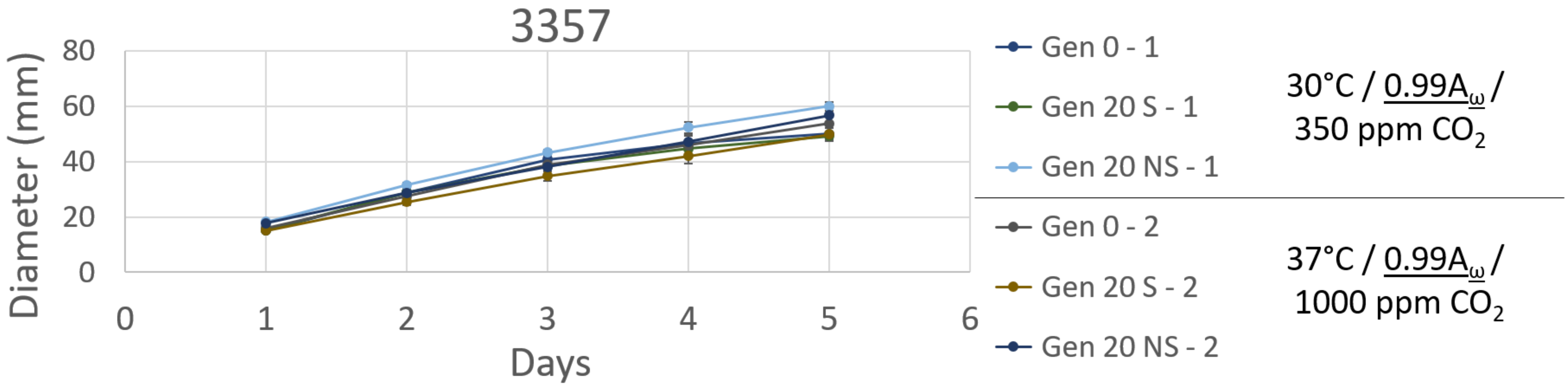


Mogensen et. al., 2009



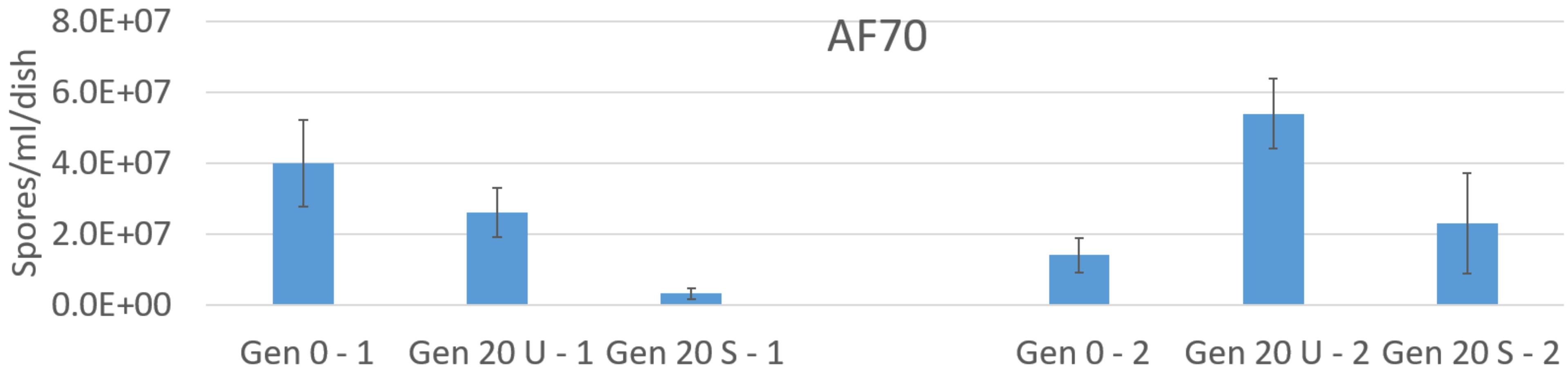
Growth Conditions
AF70
Diameter (mm)
80
60
40
20
0
1
2
3
4
5
Days
30°C / 0.99Aω / 350 ppm CO2
37°C / 0.91Aω / 1000 ppm CO2
Gen 0 - 1
Gen 20 S - 1
Gen 20 NS - 1
Gen 0 - 2
Gen 20 S - 2
Gen 20 NS -2
3357
Diameter (mm)
100
80
60
40
20
0
1
2
3
4
5
Days
30°C / 0.99Aω / 350 ppm CO2
37°C / 0.91Aω / 1000 ppm CO2
Gen 0 - 1
Gen 20 S - 1
Gen 20 NS - 1
Gen 0 - 2
Gen 20 S - 2
Gen20 NS - 2

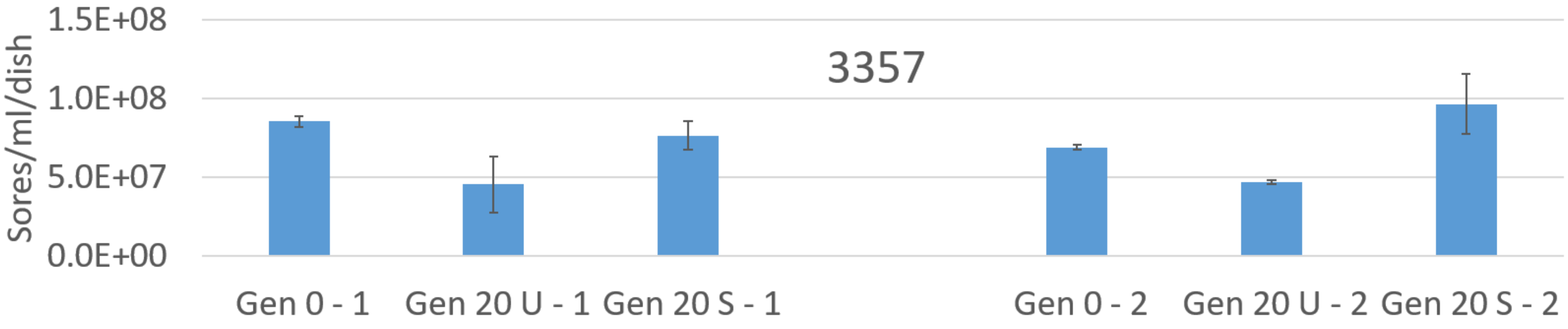

AF70
Diameter (mm)
Days
Gen 0 - 1
Gen 20 S - 1
Gen 20 NS - 1
Gen 0 - 2
Gen 20 S - 2
Gen 20 NS - 2
30°C / 0.99Aω / 350 ppm CO2
37°C / 0.99Aω / 1000 ppm CO2


3357
Diameter (mm)
Days
Gen 0 - 1
Gen 20 S - 1
Gen 20 NS - 1
Gen 0 - 2
Gen 20 S - 2
Gen 20 NS - 2
30°C / 0.99Aω / 350 ppm CO2
37°C / 0.99Aω / 1000 ppm CO2

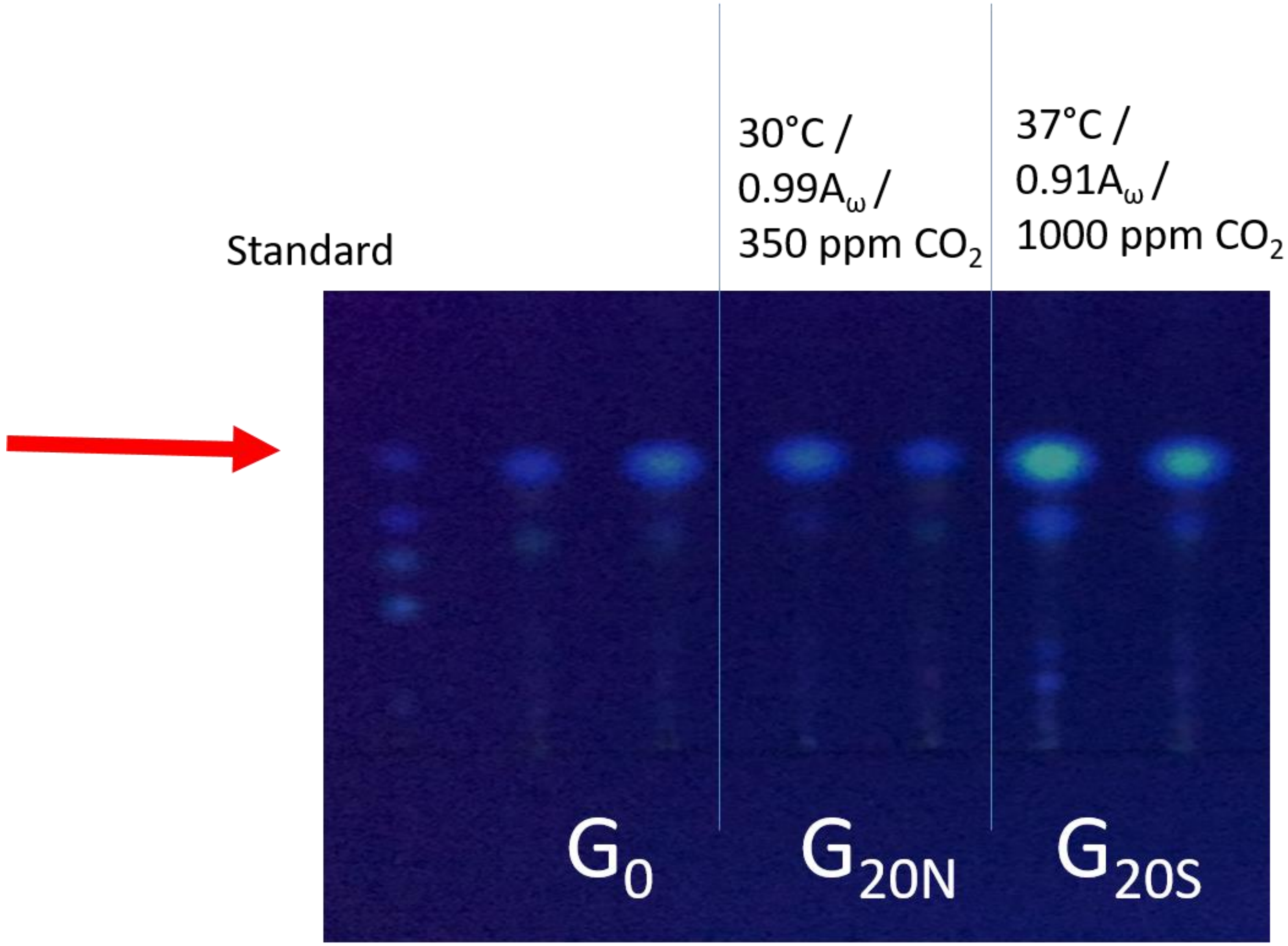
Spore Production

30°C / 0.99A_ω / 350 ppm CO_2 37°C / 0.91A_ω / 1000 ppm CO_2





Aflatoxin Production



Acclimitized strains grown In liquid YGT media for 5 days.

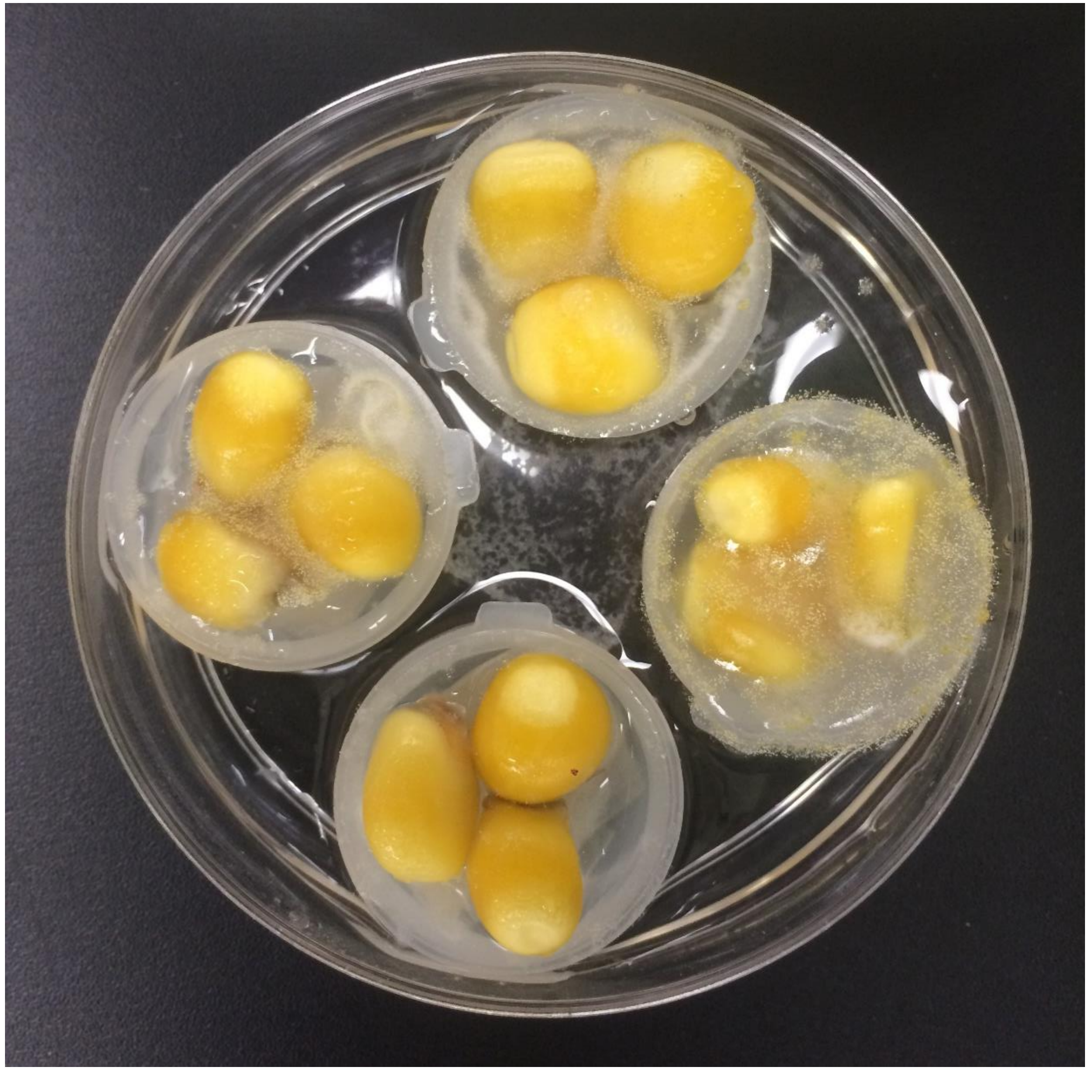
Kernel Screening Assay

Environmental Conditions

Corn Line

Fungal Strains

Water Activity



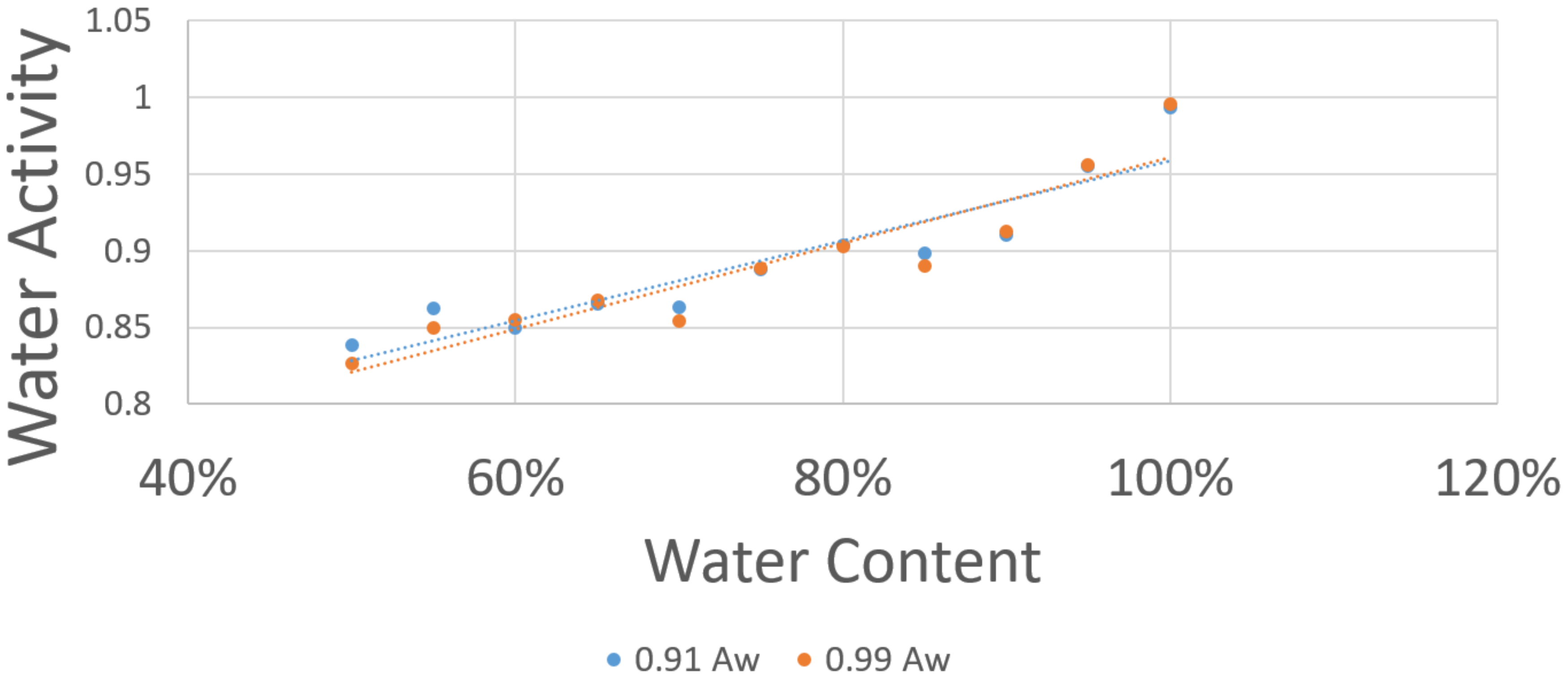
Growth

Toxin Production

Gene Expression

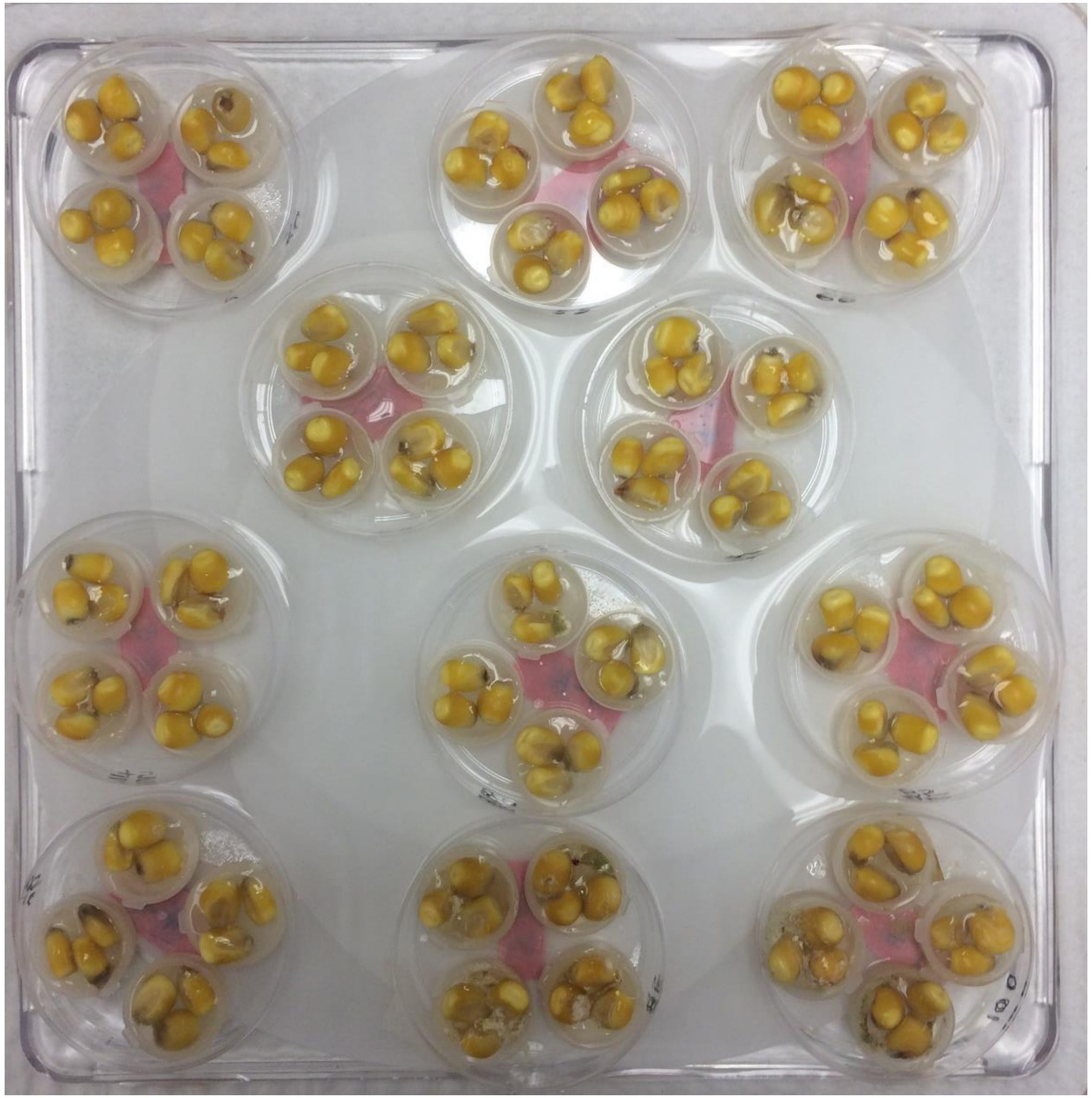
Establish KSA assays – interaction between kernel and fungus under different environmental conditions.

1) Soak kernels 48 hours in appropriate glycerol:water

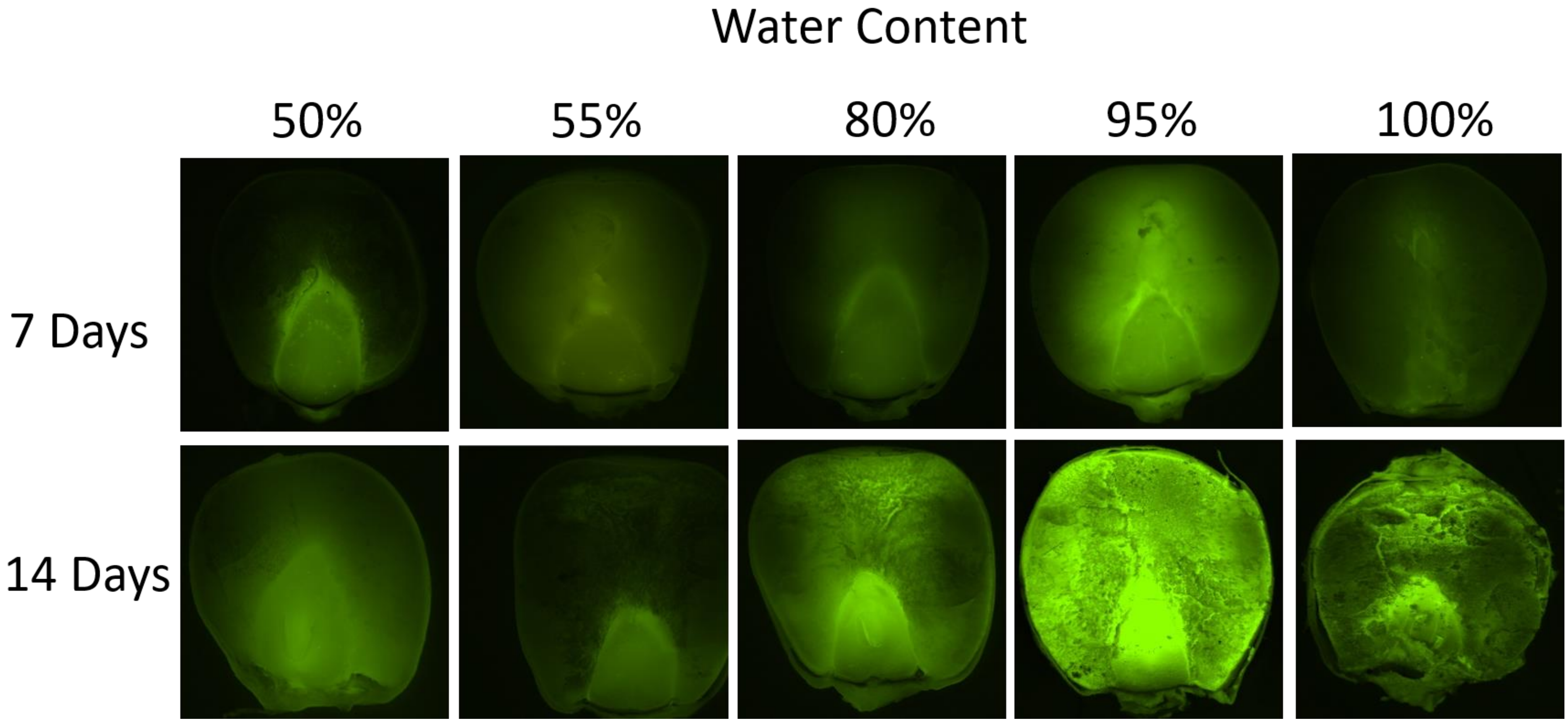


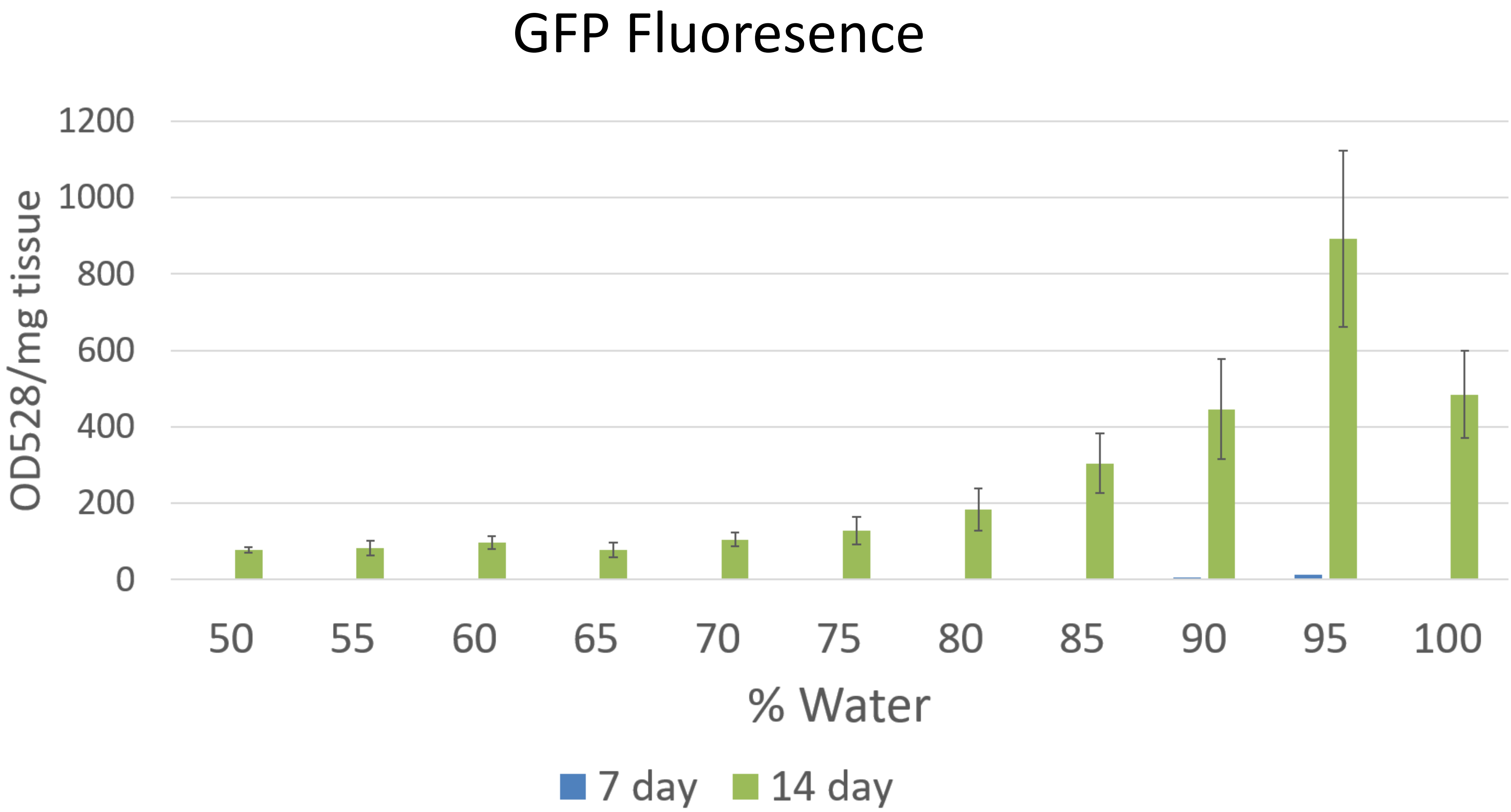
2) Inoculated in CO_2 incubators with AF70 strains:

1) GFP transformed
2) Acclimitized



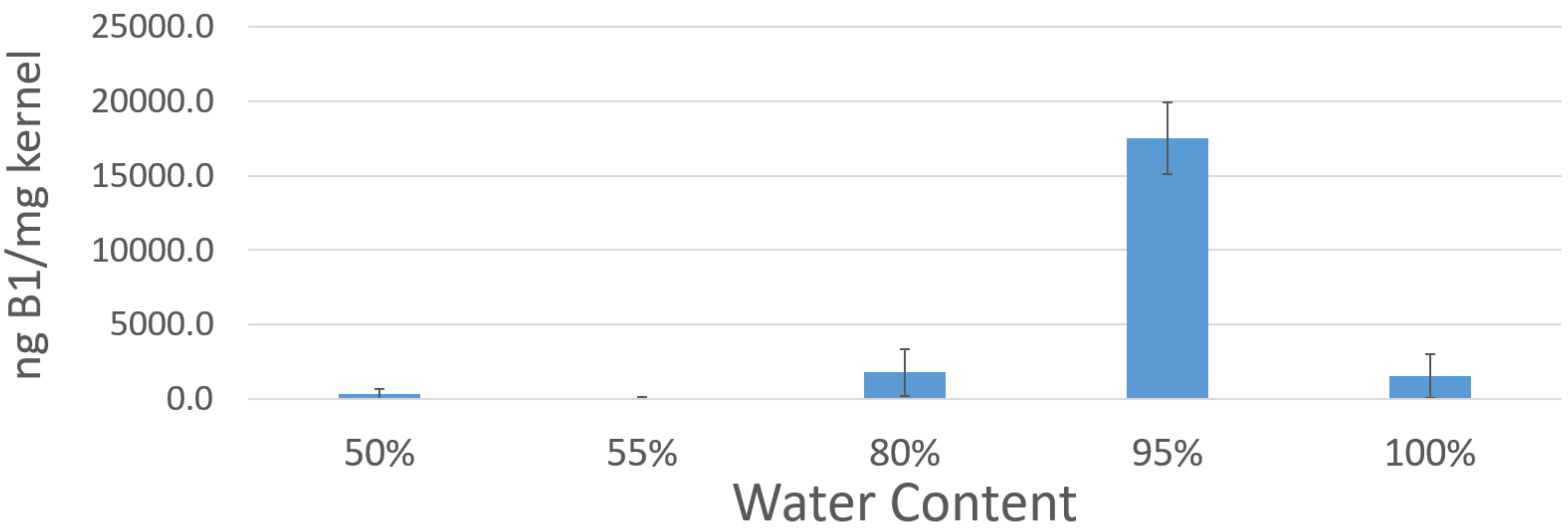
GFP-fluorescence to measure fungal growth





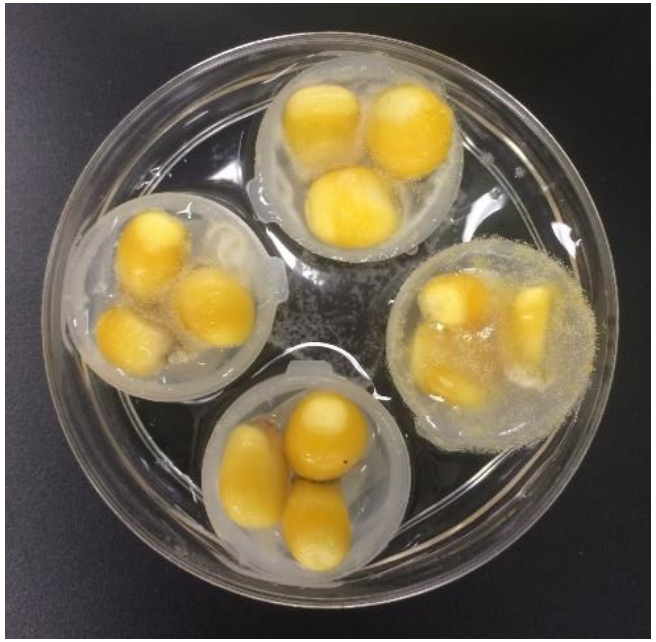
GFP Fluoresence
OD528/mg tissue
0
200
400
600
800
1000
1200
50
55
60
65
70
75
80
85
90
95
100
% Water
7 day
14 day

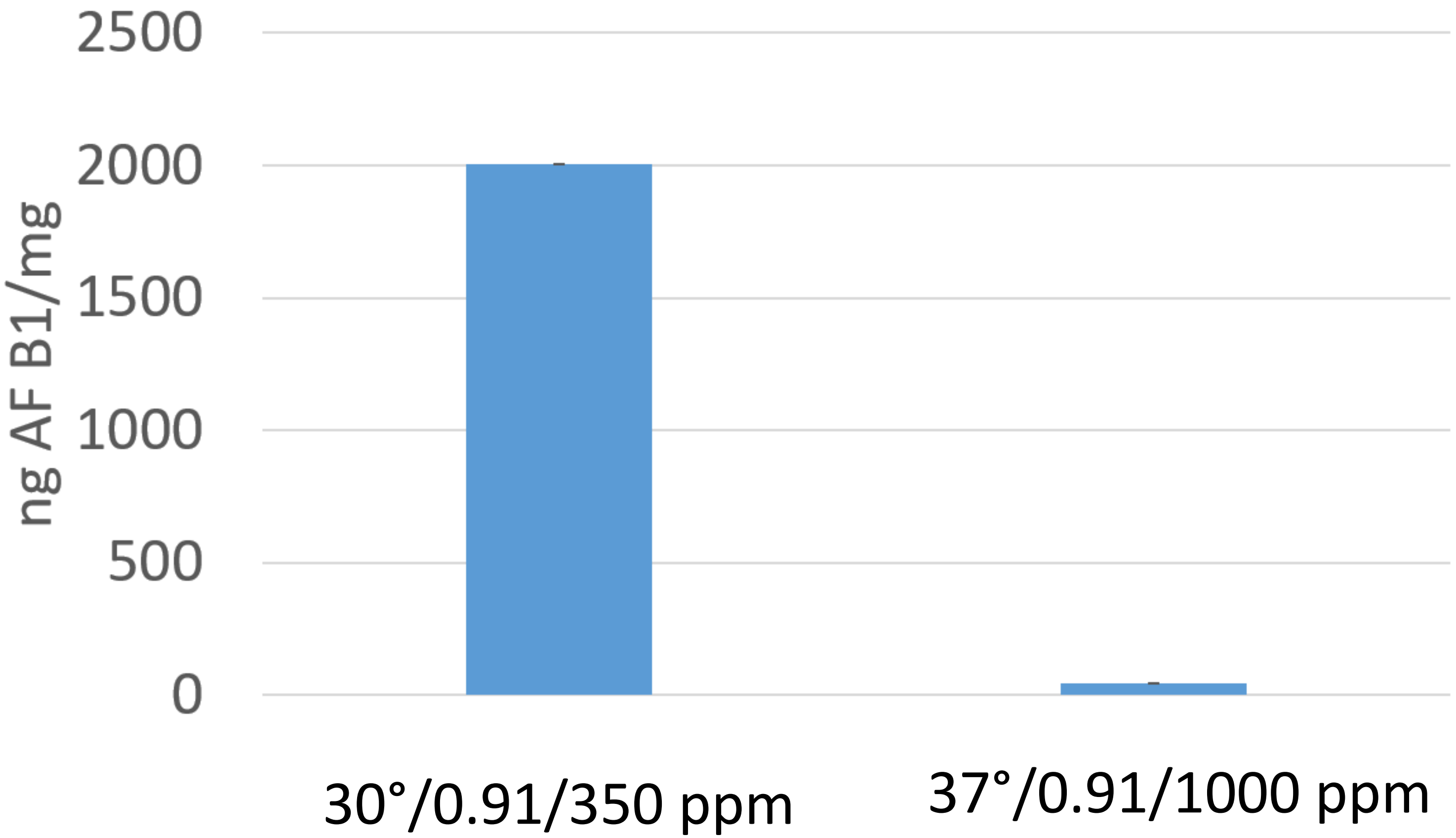
Aflatoxin Production



50% 80% 95% 100%

Acclimatized Lines – KSA Assay





Conclusions

- Development of Acclimatized strains indicate changes in spore production and aflatoxin production
- Changes in development (i.e.; spore production) and toxin profile are being explained and assessed in conjunction with RNA-Seq data.
- Development of a modified kernel screening assay is permitting the identification of key virulence genes affected by environmental factors.

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