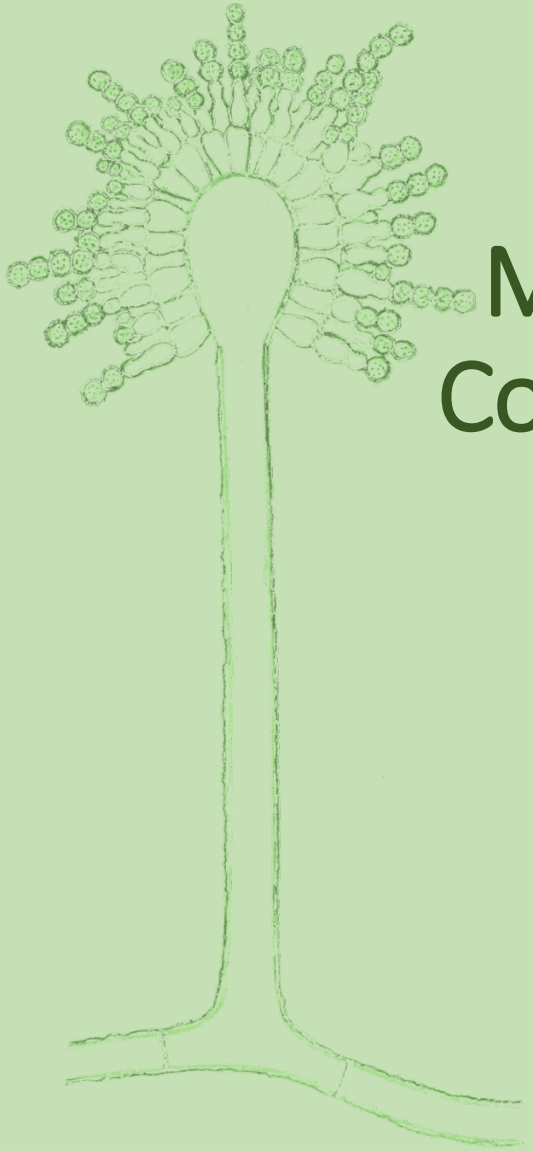
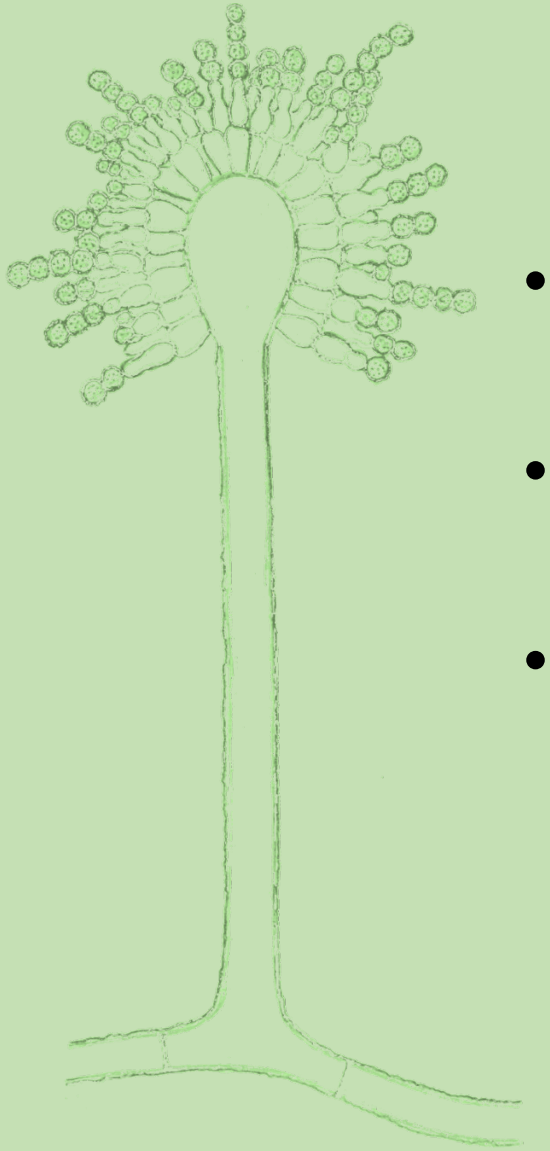


Molecular and Environmental Factors Controlling Aflatoxin Reduction by Non-Toxigenic *Aspergillus* Strains

CRIS Project 6054-42000-026-00D

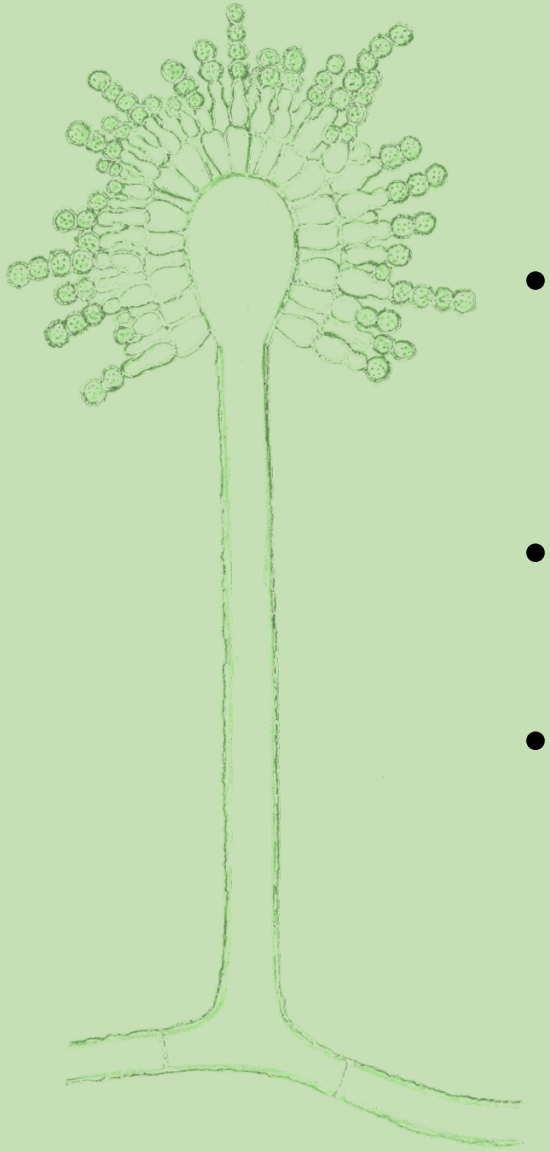
Dr. Geromy G. Moore (LS)

ARS/FSIS Food Safety Meeting 2019



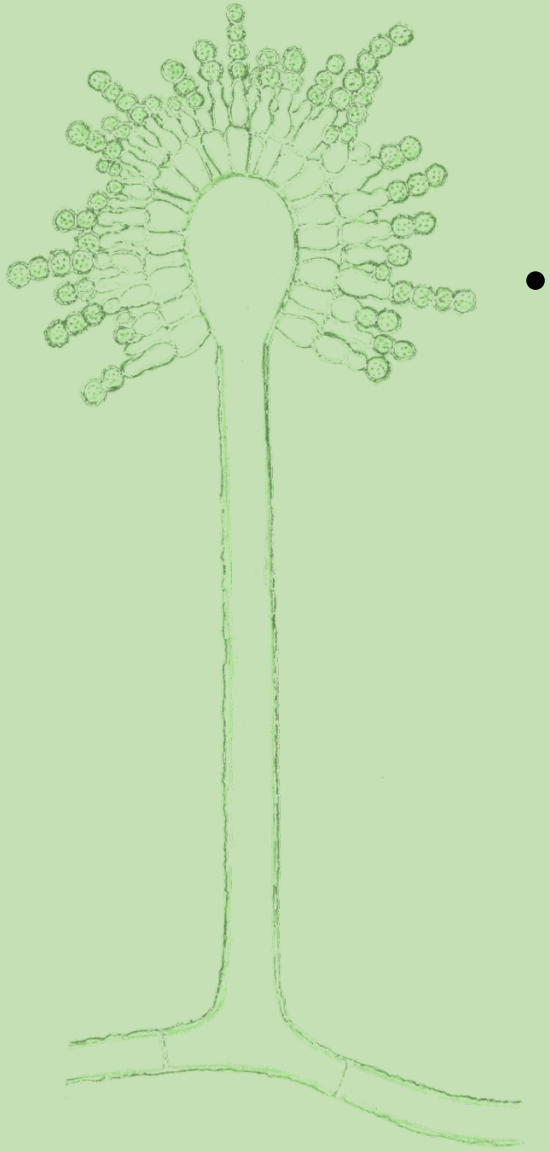
Scientific Background

- Several *Aspergillus* fungi are agriculturally significant
  - *A. flavus*, *A. parasiticus*, *A. nomius*, etc.
- There are multiple mycotoxins to mitigate
  - Aflatoxins (B,G,M), cyclopiazonic acid, etc.
- Biocontrol efforts are earning attention
  - Spray/fumigation involving naturally-derived compounds
  - Pre-harvest application of non-aflatoxigenic *A. flavus* strains
    - Most promising strategy
    - Suggested mechanism = competitive exclusion/displacement



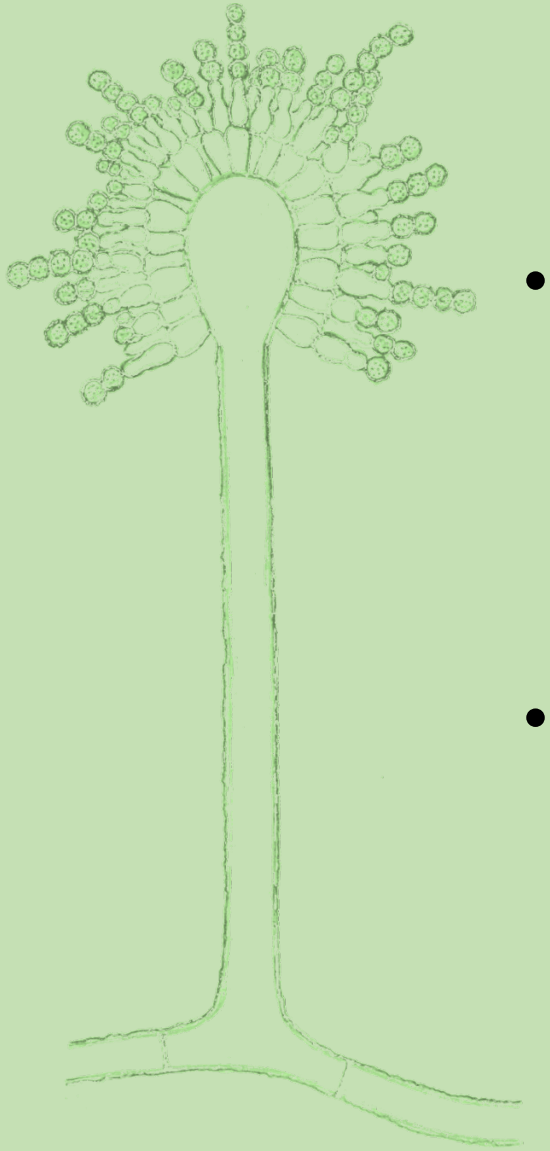
Scientific Background

- Alternative mechanisms suggested
  - Nutrient sequestration (Mehl & Cotty, 2013)
  - Thigmoregulation (Huang et al., 2011)
- What if non-aflatoxigenic *A. flavus* employs more than one mechanism?
- Not yet explored… chemical interactions
  - Chemosensing
    - Extrolites
    - Volatile Organic Compounds



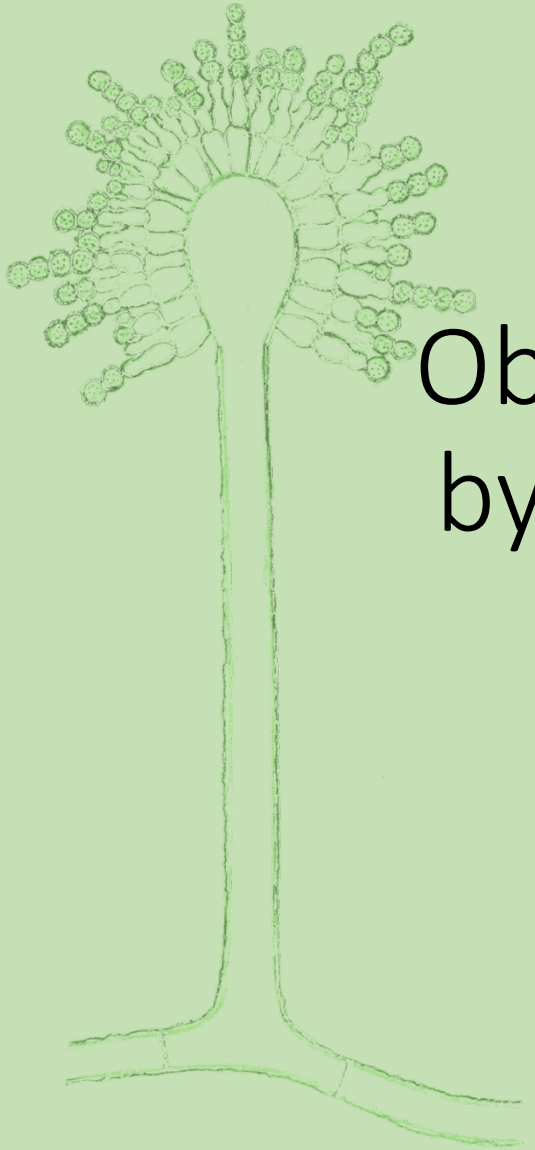
Scientific Background

- What factors could limit or prevent biocontrol efficacy?
  - Intra- and inter-specific sex
    - Non-aflatoxigenic phenotype “repaired”
    - Diversification of mycotoxin profiles
  - Climate stress (elevated CO_2, extreme temps, drought conditions)
    - Facilitate sex/recombination for adaptation
    - Render biocontrol ineffective

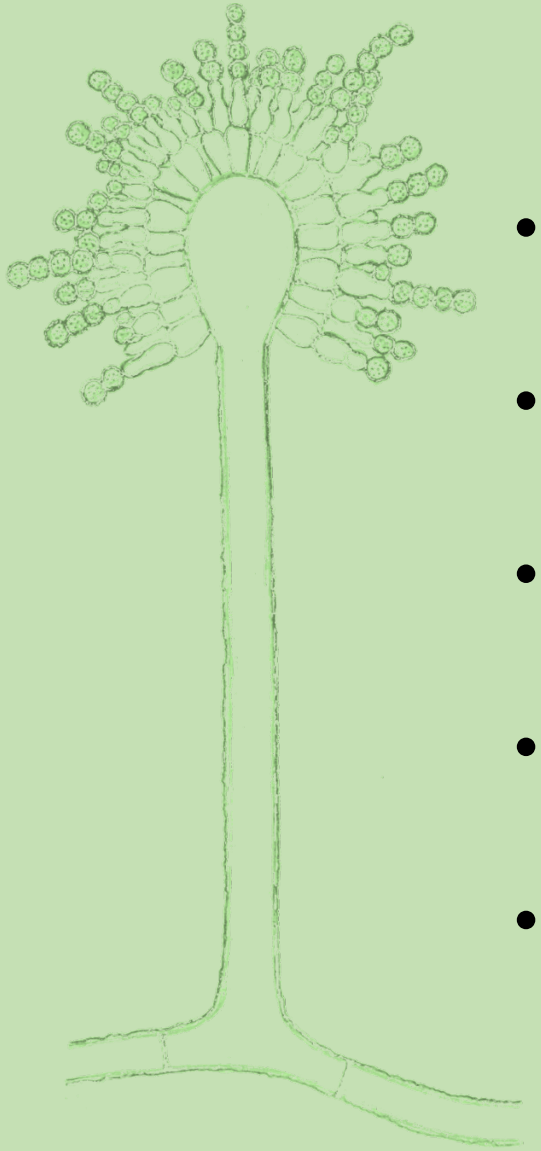


CRIS Project 6054-42000-026-00D

- CRIS Investigators
  - G Moore (LS)
  - M Lebar
  - M Gilbert
  - J Cary
- Two objectives

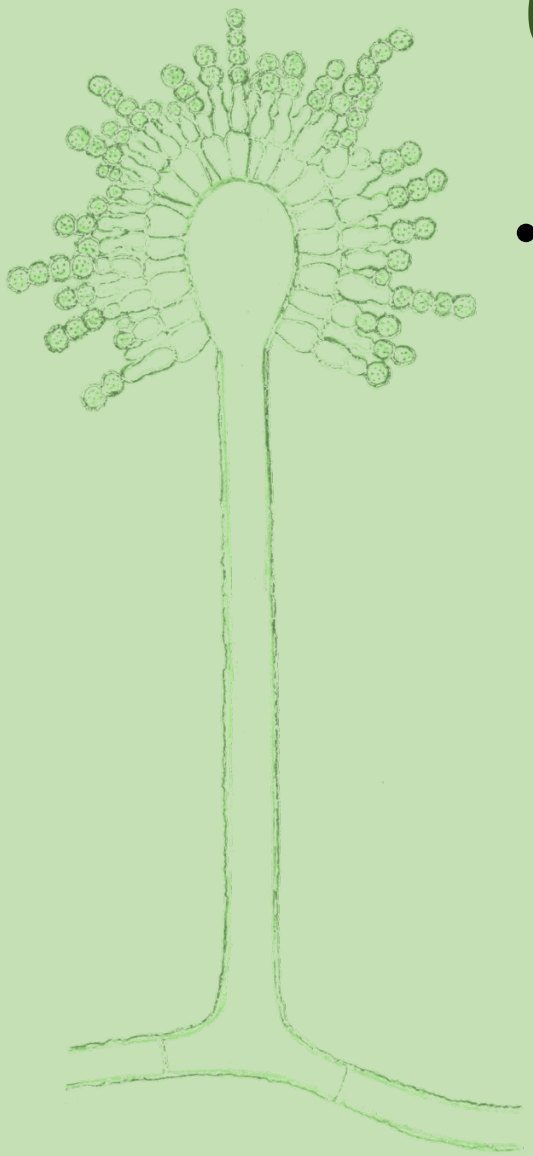


Objective 1: Determine the mechanism by which atoxigenic strains of *A. flavus* reduce pre-harvest aflatoxin contamination by toxigenic strains



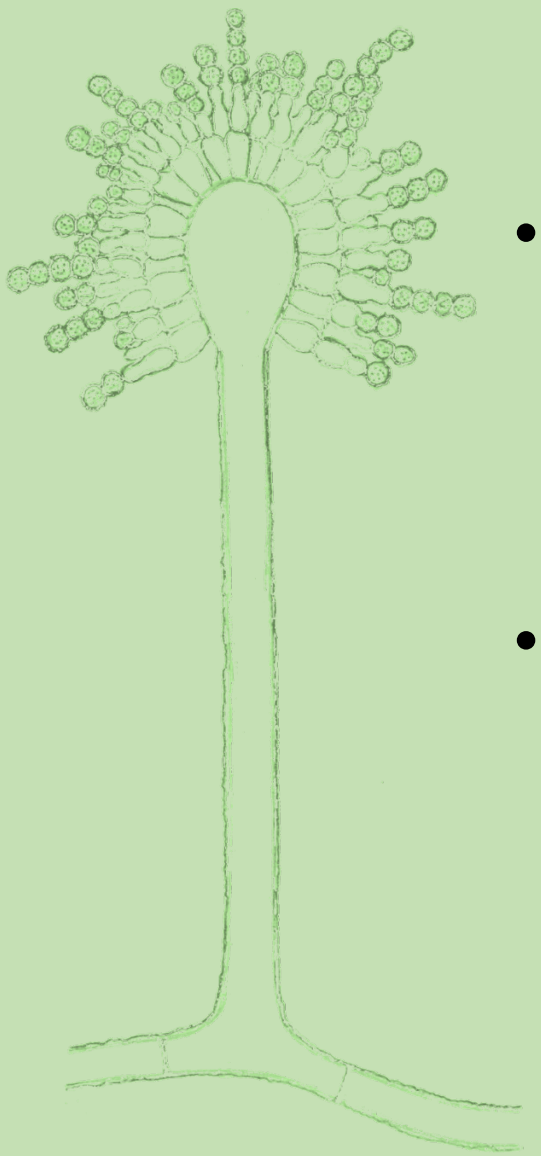
6054-42000-026-00D: Objective 1

- Extrolite study
  - Moore, Lebar
- Unknown VOC study
  - Moore, Lebar
- Known VOC study
  - Moore, Lebar
- Unique VOC Identification study
  - Moore, Grimm (SRRC)
- Thigmoregulation study
  - Moore, Gilbert, Sweany (LSU), Damann (LSU)



6054-42000-026-00D: Objective 1

- Extrolite and VOC studies:
  - LA1 = Atox17; *A. flavus*; AF-/αCPA-
  - LA2 = Tox4; *A. flavus* L-type; AFB_1+/αCPA+
  - LA3 = 07-S-2-1-2; *A. flavus* S-type; AFB_1+/αCPA+
  - LA4 = 07-M-5-1-1-1; *A. parasiticus*; AFB_1+/αCPA+
  - Three types of media: CZ (nutrient poor), CMA (simulate host tissue), YES (good for toxin assays)
  - Three replicates
  - Growth measured as avg. colony diameter in mm
  - Metabolite analysis by TLC, UPLC and LC-MS

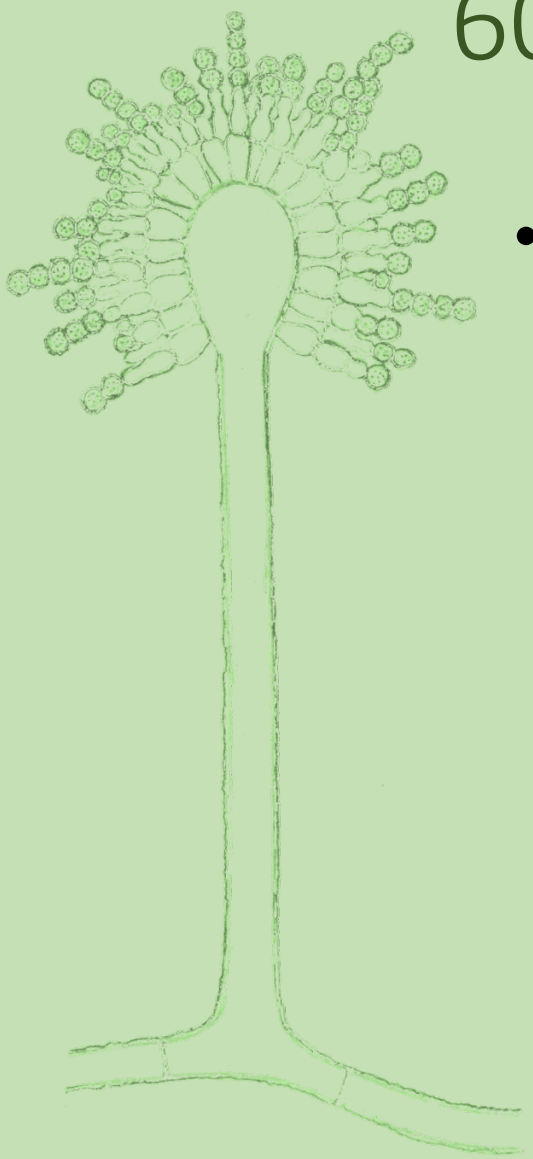


6054-42000-026-00D: Objective 1

- Extrolite study
  - Experiments to observe reductions in growth and/or toxins
  - Identify putative atoxigenic extrolites for further study as biocontrol supplements

- Moore GG, Lebar MD, Carter-Wientjes CH. 2019. The role of extrolites secreted by nonaflatoxigenic *Aspergillus flavus* in biocontrol efficacy. J Appl. Microbiol. doi:10.1111/jam.14175

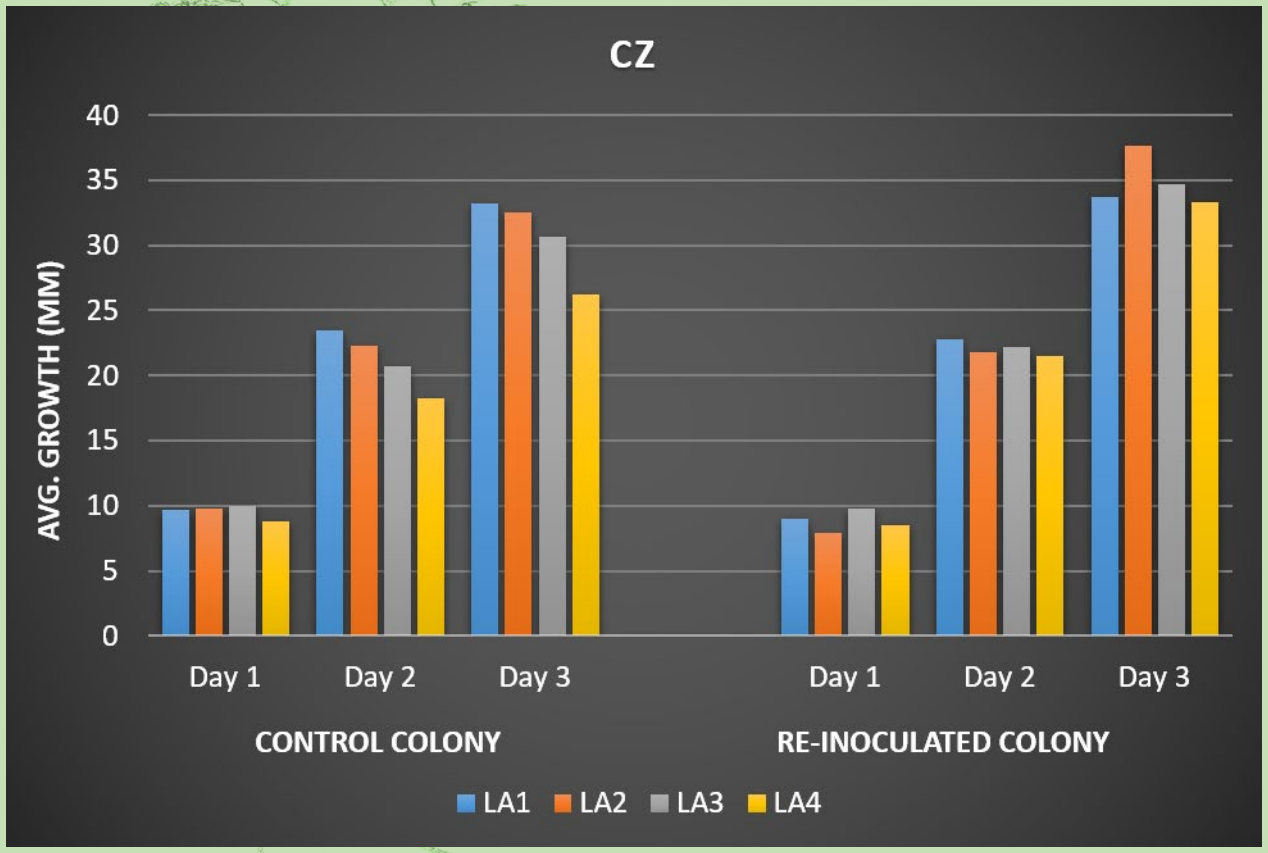
6054-42000-026-00D: Objective 1

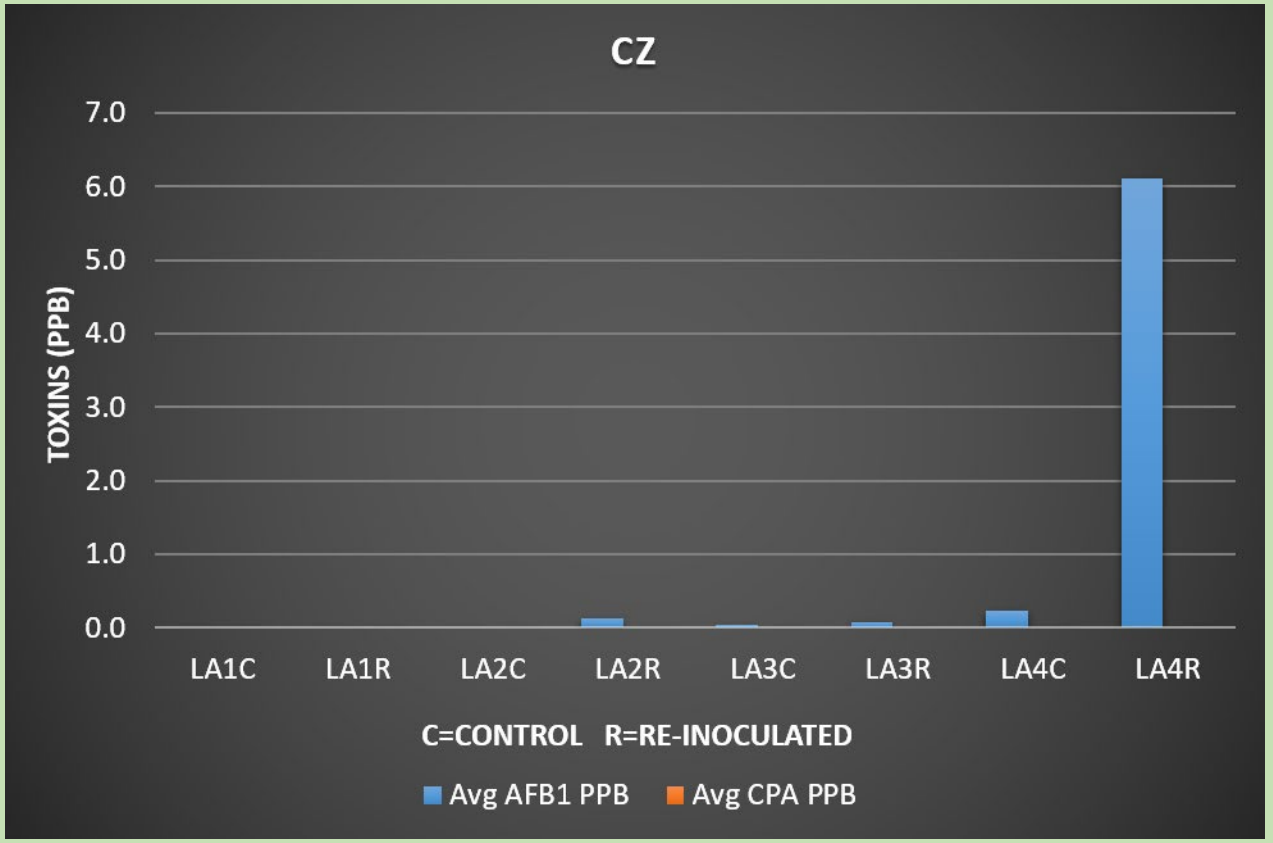


- Extrolite study (solid and liquid media)
  - Control = colonies grown individually
  - Re-inoculated 1 = on solid LA1-infused media
    - Grow LA1 on PES membrane (≤ 0.1 µm pore size)
    - Remove membrane and re-inoculate with experimental strain
  - Re-inoculated 2 = on liquid LA1-infused media (YES only)
    - Grow LA1 on Transwell membrane in 13 ml YES medium
    - Replace insert and re-inoculate with experimental strain
    - Growth measured as avg. colony weight in g
    - Regular amendment of infused YES with fresh YES (≤ 13 ml)

6054-42000-026-00D: Objective 1

- Extrolite study findings: CZ solid media

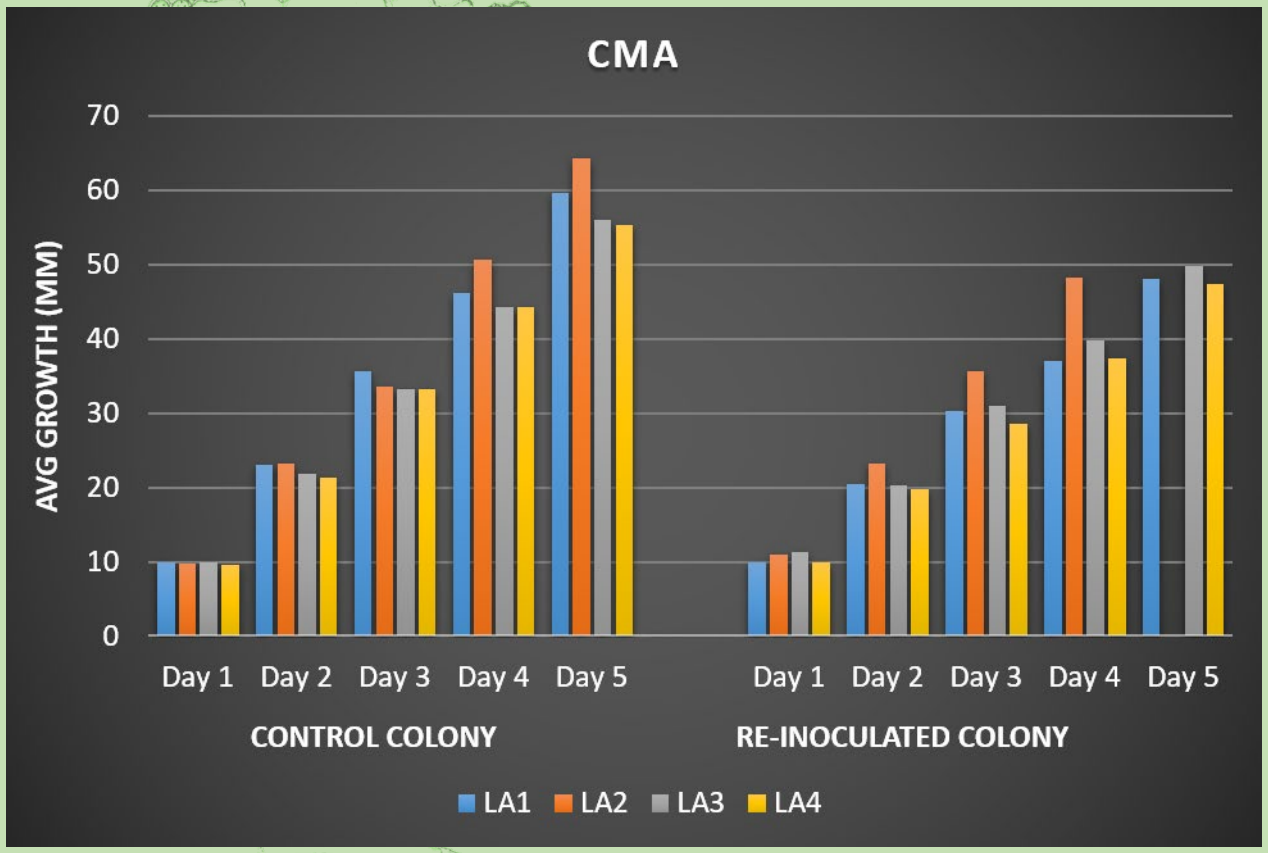


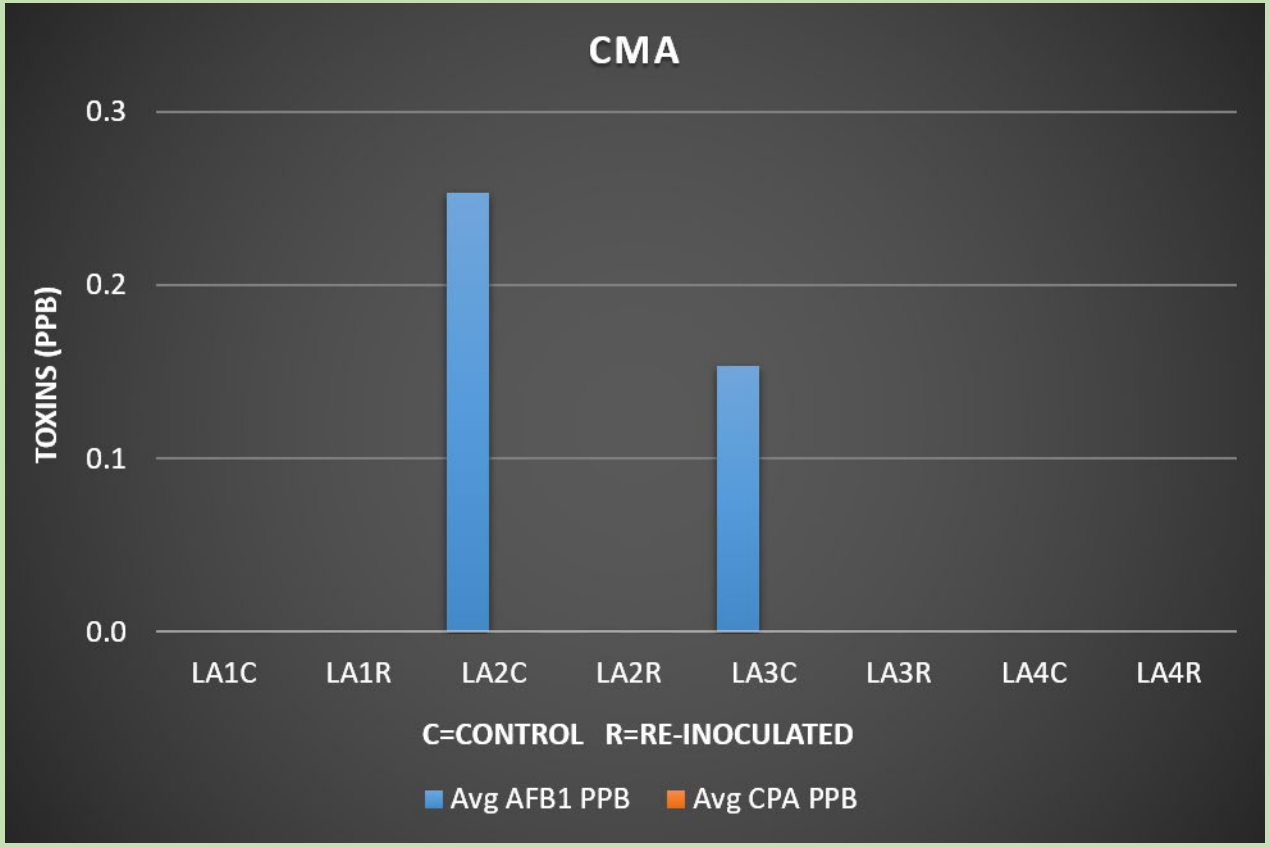


Overall INCREASE in growth (14%) and AFB_1
No detectable αCPA

6054-42000-026-00D: Objective 1

- Extrolite study findings: CMA solid media

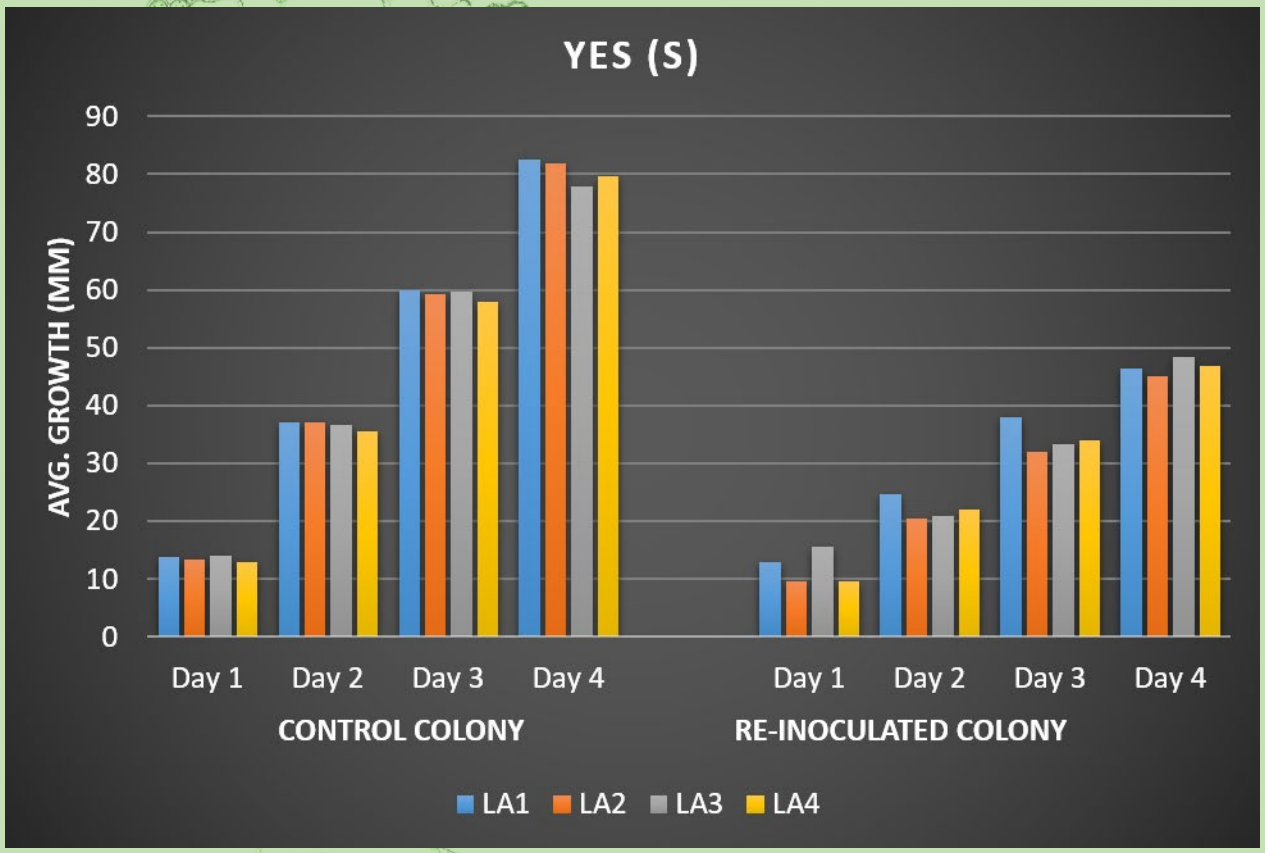


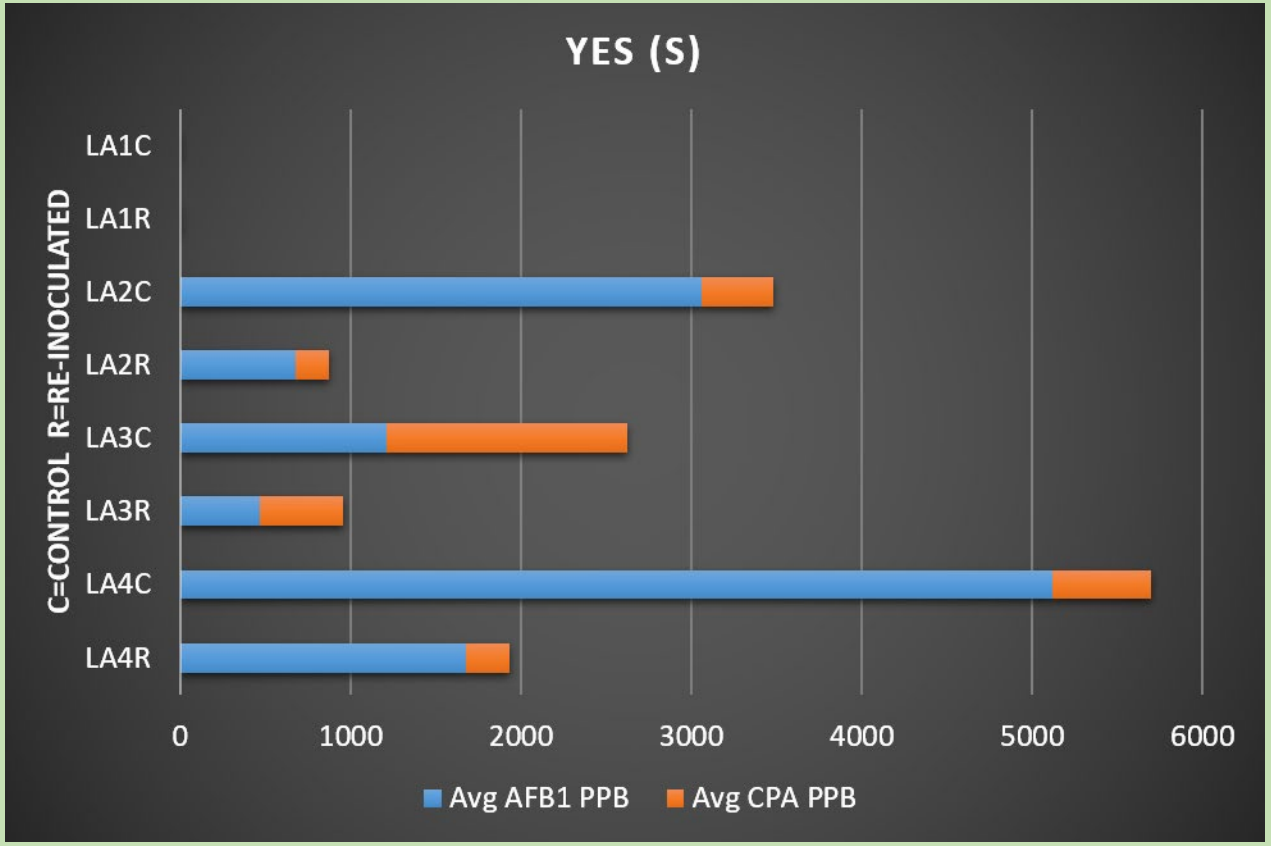


Overall DECREASE (12%) in growth and AFB_1
No detectable αCPA

6054-42000-026-00D: Objective 1

- Extrolite study findings: YES solid media

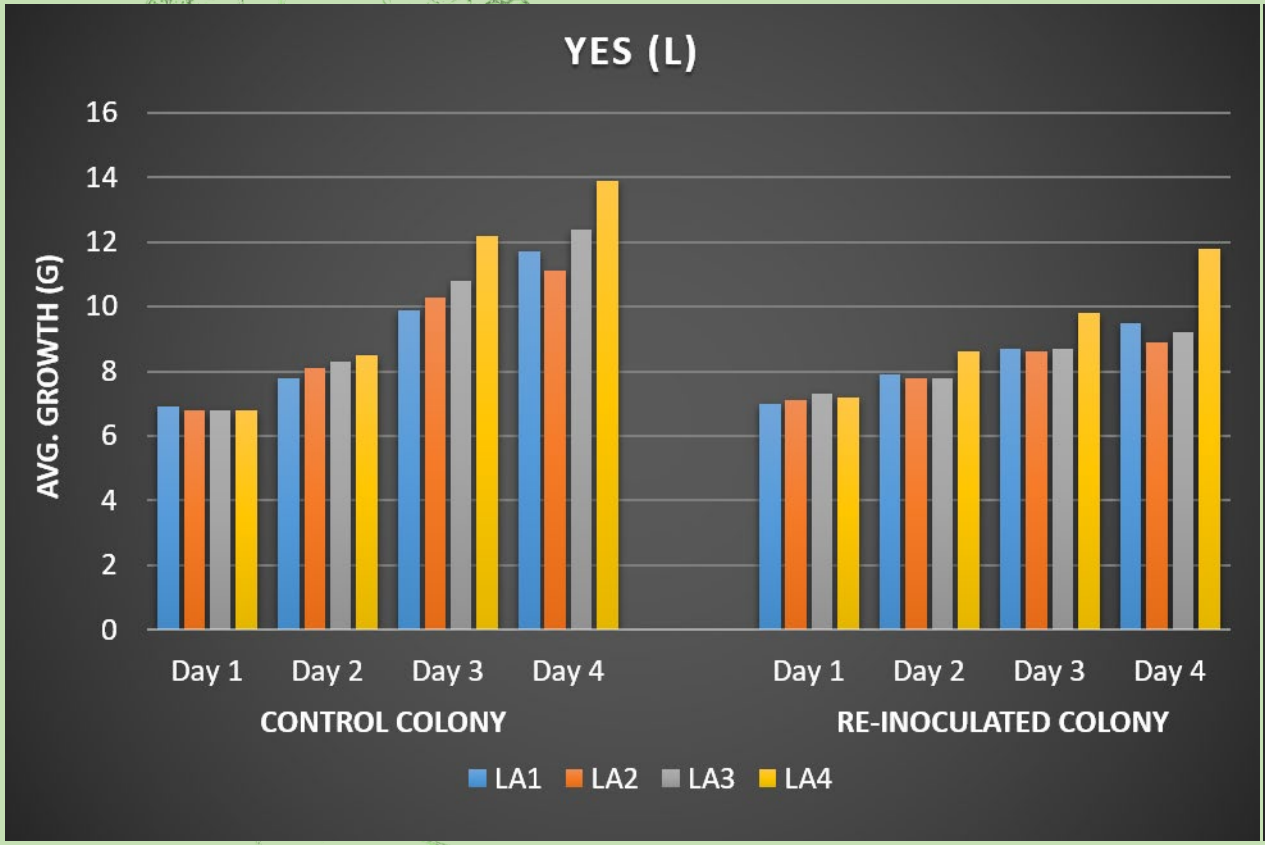


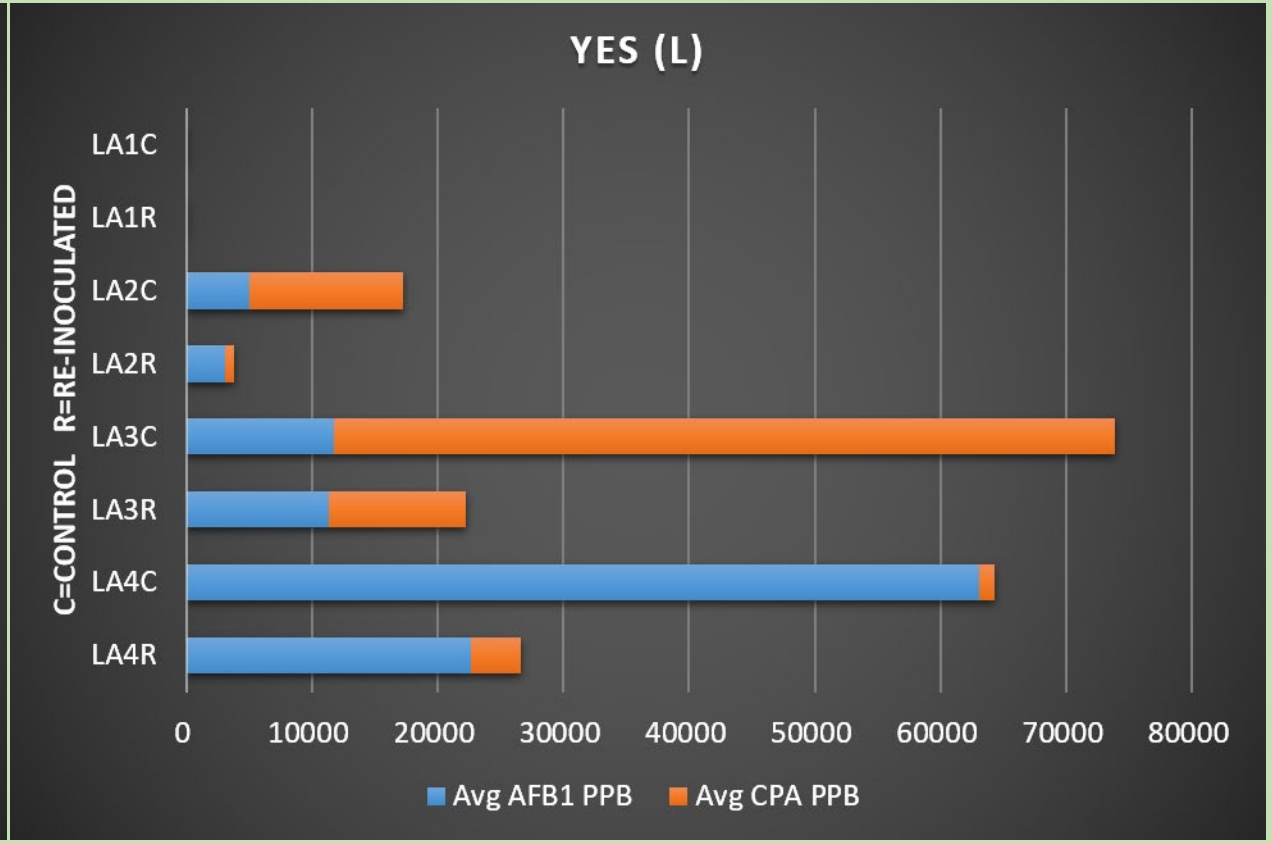


Overall DECREASE in growth (42%) and
AFB_1 (69%) + αCPA (58%)

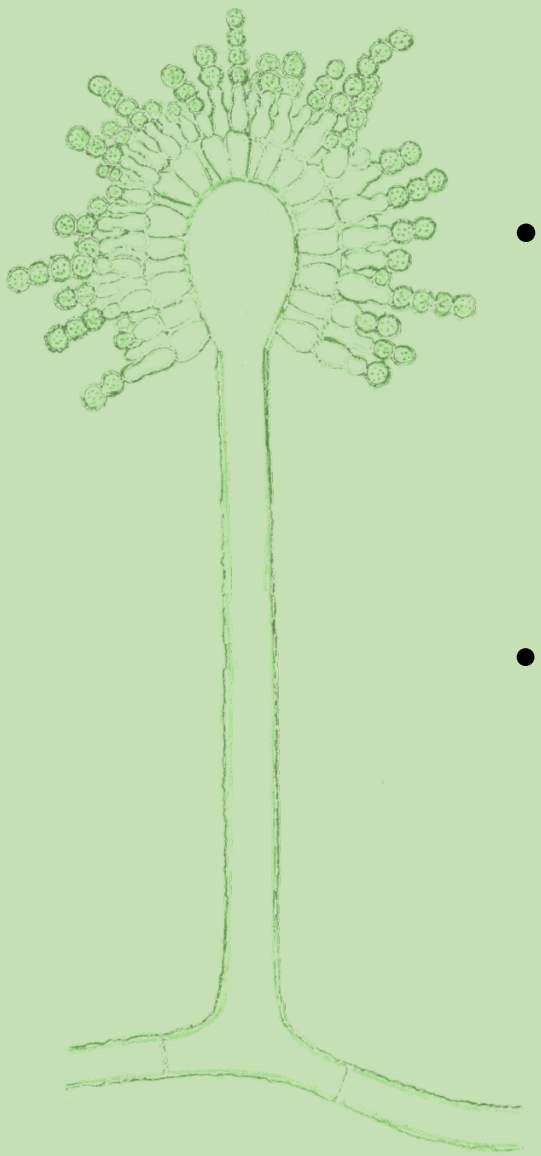
6054-42000-026-00D: Objective 1

- Extrolite study findings: YES liquid media



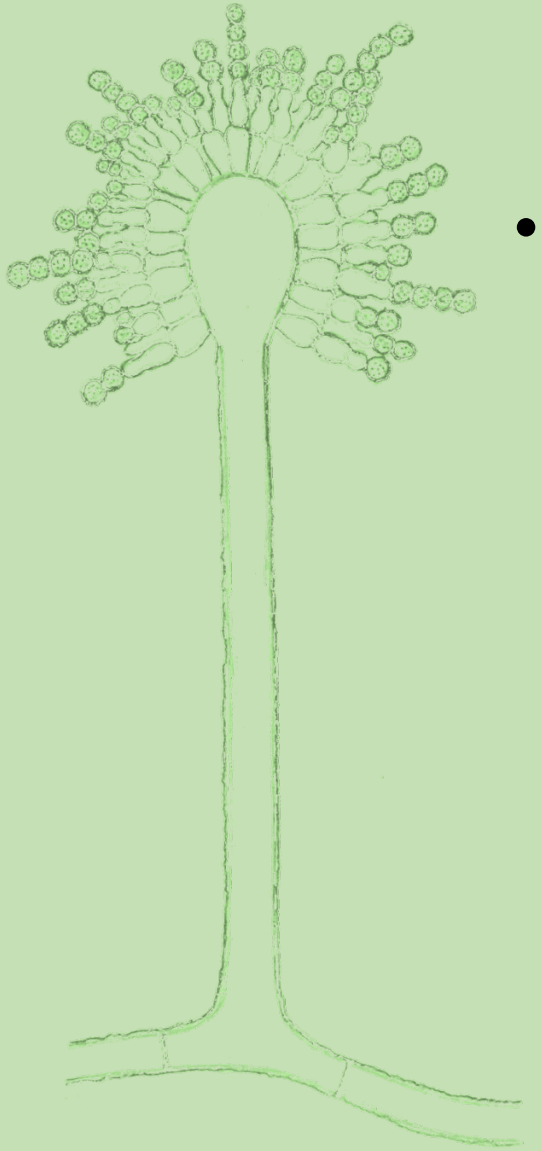


Overall DECREASE in growth (11%) and
AFB_1 (54%) + αCPA (88%)



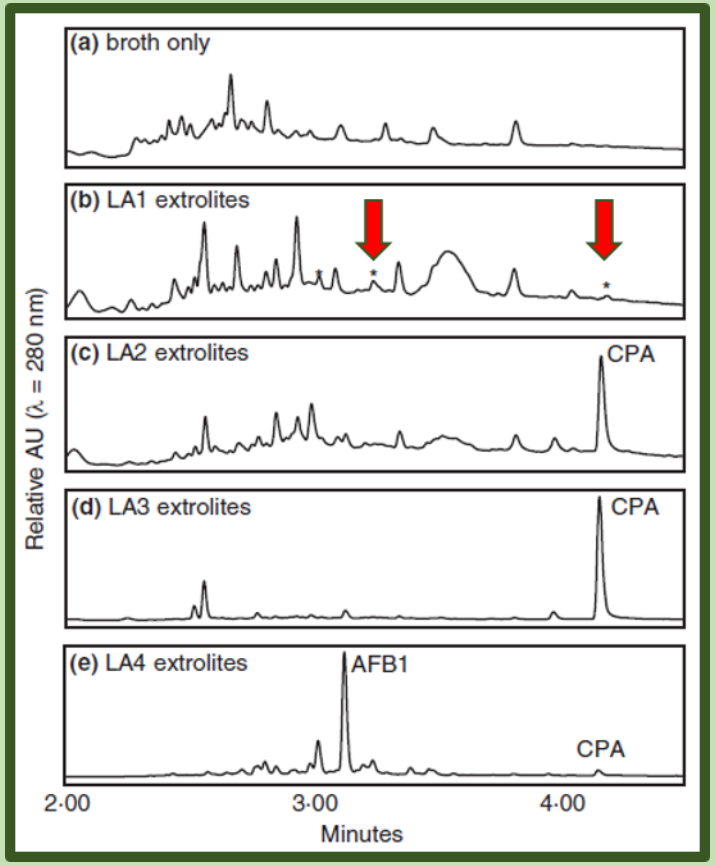
6054-42000-026-00D: Objective 1

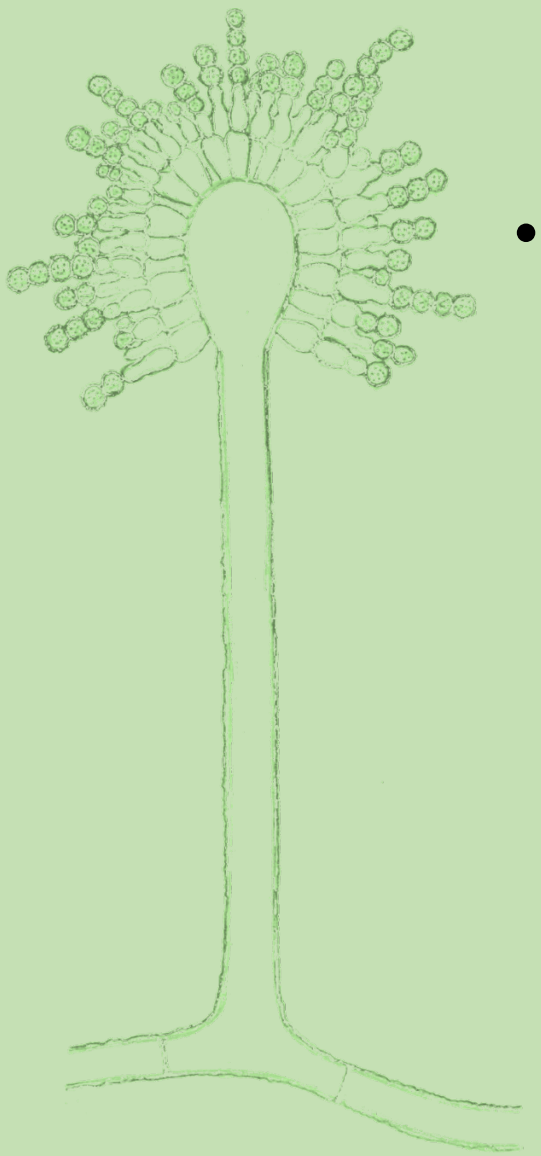
- Extrolite study: conclusions
  - Growth and toxin production are not directly correlated
  - LA1-infused media resulted in reduction of toxins
  - Reductions are not direct result of nutrient depletion
    - Liquid study included regular amendments with fresh medium
- Take Home: At least one extrolite is being secreted onto/into medium by LA1 that can greatly reduce toxin levels



6054-42000-026-00D: Objective 1

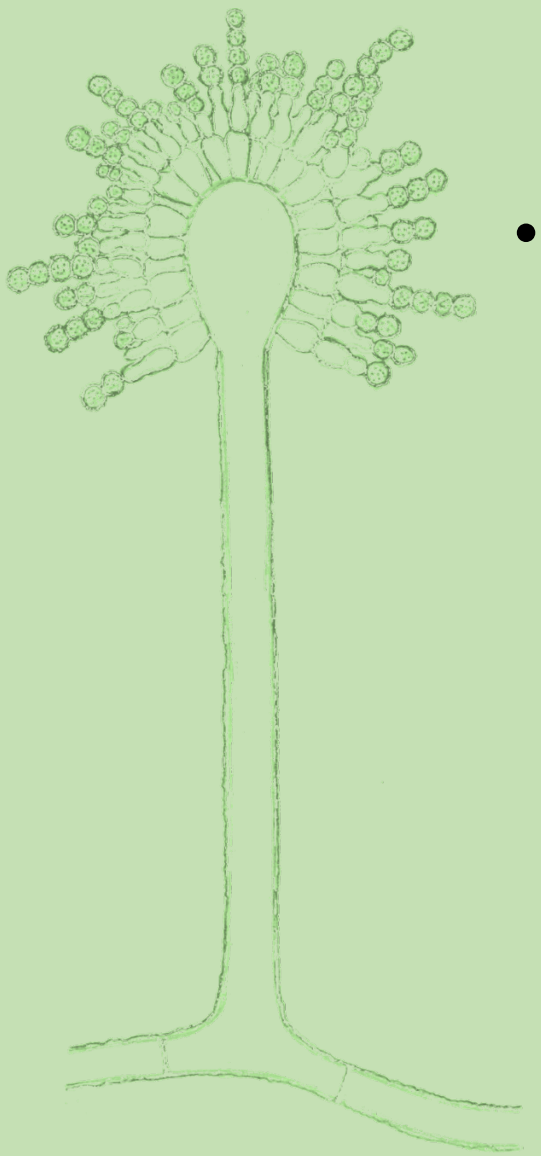
- Extrolite study: ID of unknown extrolites in LA1
  - Unique compounds observed
  - Detected in infused liquid
  - Too little starting material to ID





6054-42000-026-00D: Objective 1

- Extrolite study: future investigations
  - Use strains from different geographic locations
    - Arizona (AF36), Georgia (NRRL 21882), Mississippi (K49)
    - Compare production of toxin-reducing extrolites across different atoxigenic strains, host type preference, and geography
    - Determine if putative extrolite(s) are the same as those in LA1
  - Use different substrates
    - Host tissue; YES medium; A&M medium
    - Substrates that allow for better toxin measurements
    - Observations on host tissue (more like field conditions) compared to synthetic media

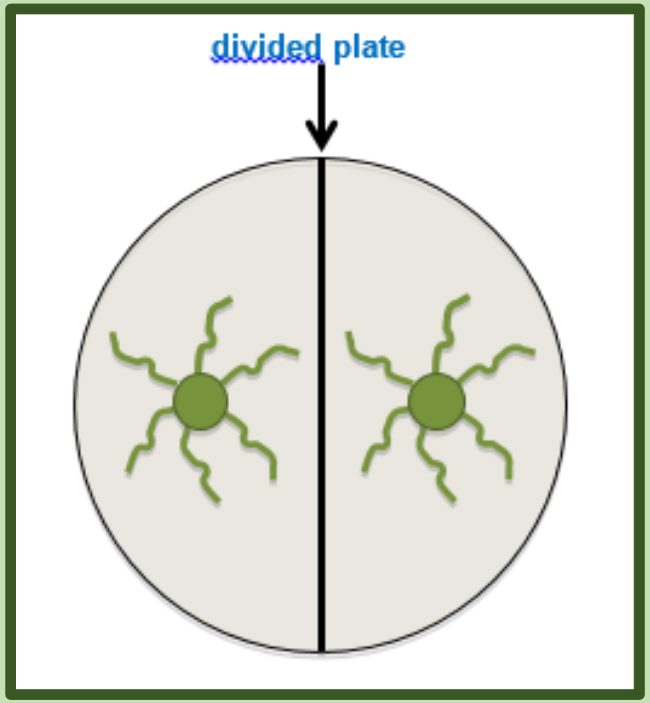


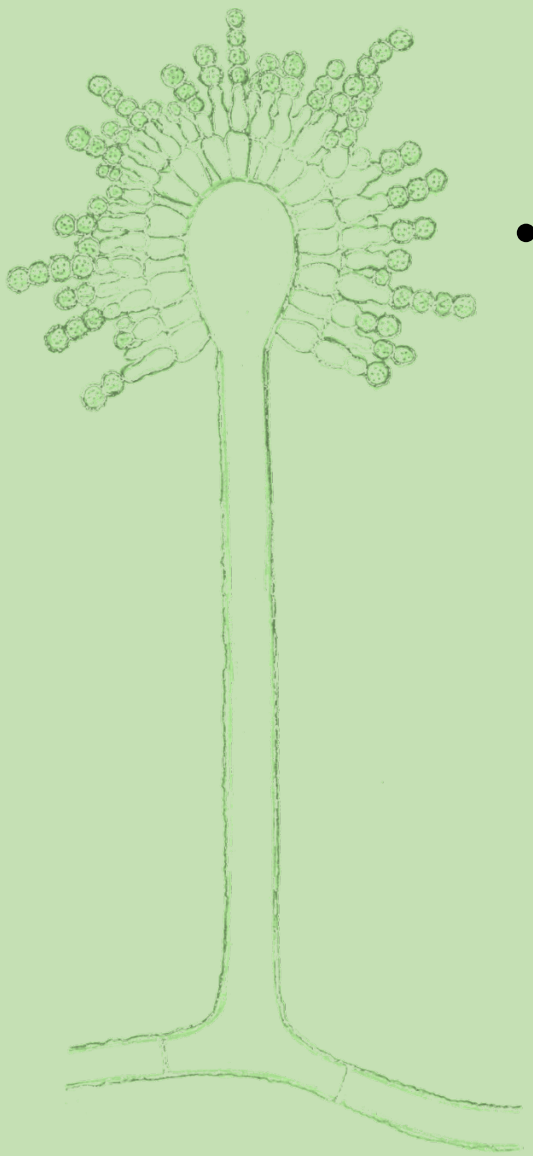
6054-42000-026-00D: Objective 1

- Extrolite study: future investigations
  - Repeat unknown extrolite identifications
    - Identify and isolate compound(s) for supplementation studies to aid biocontrol, and/or
    - To screen candidate strains for naturally-increased production of beneficial extrolites
  - Genomic investigation of LA4 (underway)
    - *A. parasiticus* has never been reported to produce CPA
    - Preliminary data indicates that LA4 could be *A. novoparasiticus*, but…
      - *A. novoparasiticus* is reportedly a clinical species, not agricultural
      - *A. novoparasiticus* is not reported to produce CPA
      - Could be an interspecific hybrid

6054-42000-026-00D: Objective 1

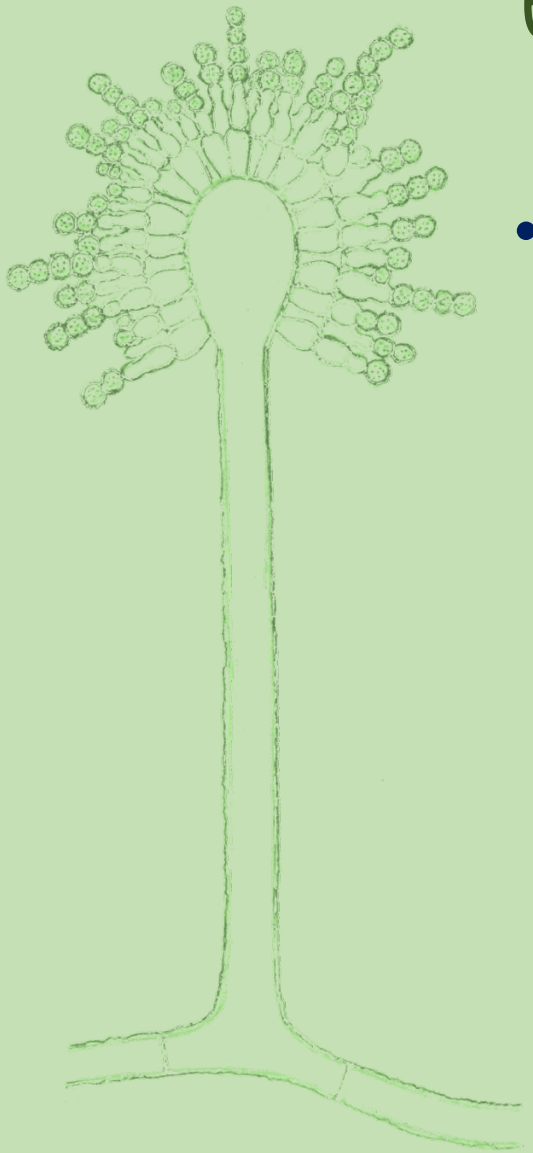
- Unknown VOC study
  - Experiments to observe reductions in growth and/or toxins
  - Divided plate to prevent touch and exposure to putative extrolites
  - LA1 grown for 24h prior to inoculation of toxigenic strain





6054-42000-026-00D: Objective 1

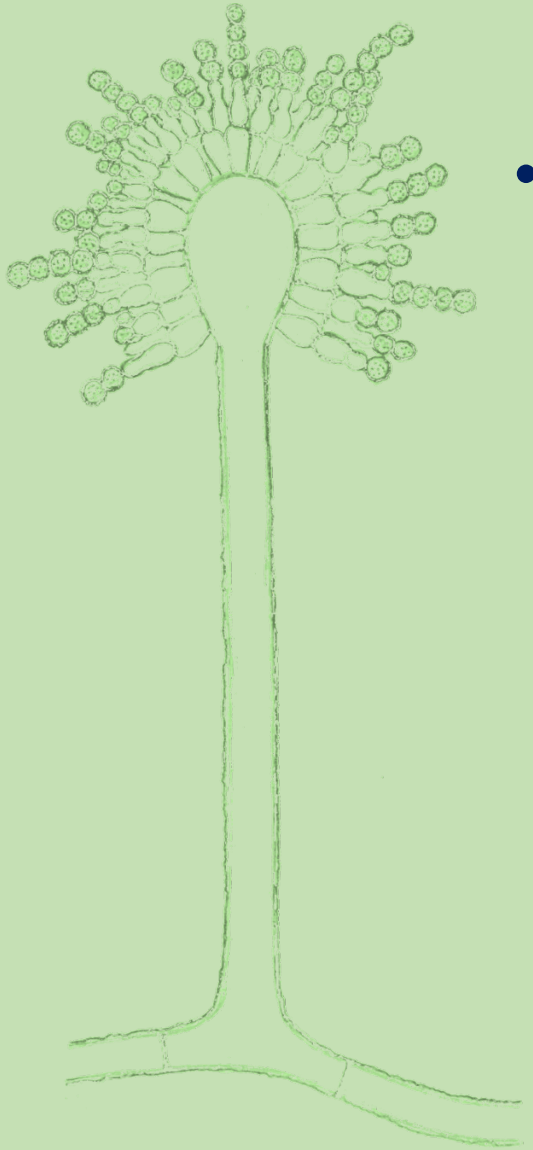
- Unknown VOC study (solid media)
  - Control = colonies grown individually
  - Experimental = toxigenic strains vs. putative LA1 VOCs
    - Grow LA1 one side of plate (24h)
    - Inoculate other side with toxigenic strain



6054-42000-026-00D: Objective 1

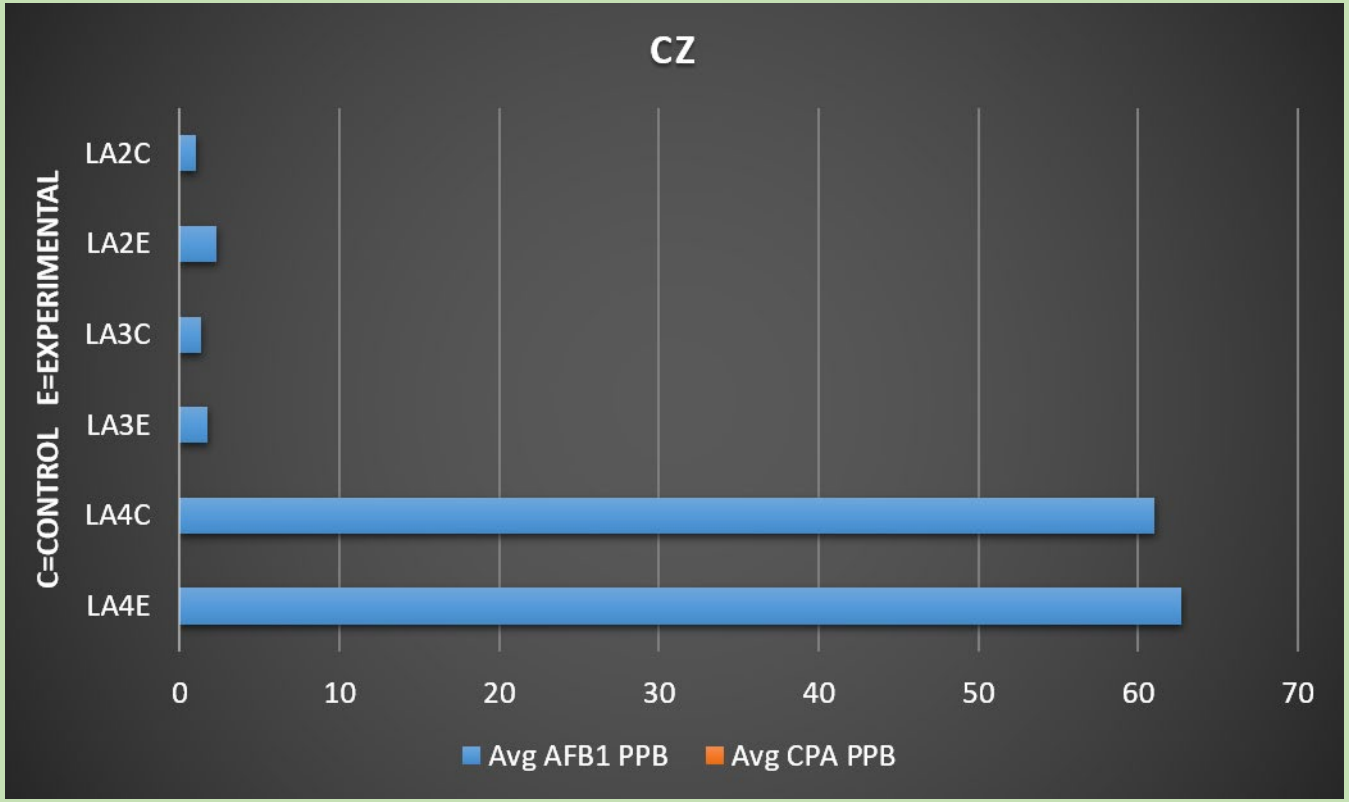
- Unknown VOC study findings: insignificant reductions

Control Colony	Avg. diameter (mm)	Experimental Colony	Avg. diameter (mm)	Percent Reduction
CZ.LA2c	34	CZ.LA2e	30	12
CZ.LA3c	32	CZ.LA3e	28.5	11
CZ.LA4c	27.5	CZ.LA4e	24.25	12
CMA.LA2c	39.5	CMA.LA2e	35	11
CMA.LA3c	33.75	CMA.LA3e	30.25	10
CMA.LA4c	34.5	CMA.LA4e	30.5	12
YES.LA2c	36.75	YES.LA2e	33	10
YES.LA3c	38	YES.LA3e	34	11
YES.LA4c	33.25	YES.LA4e	30	10

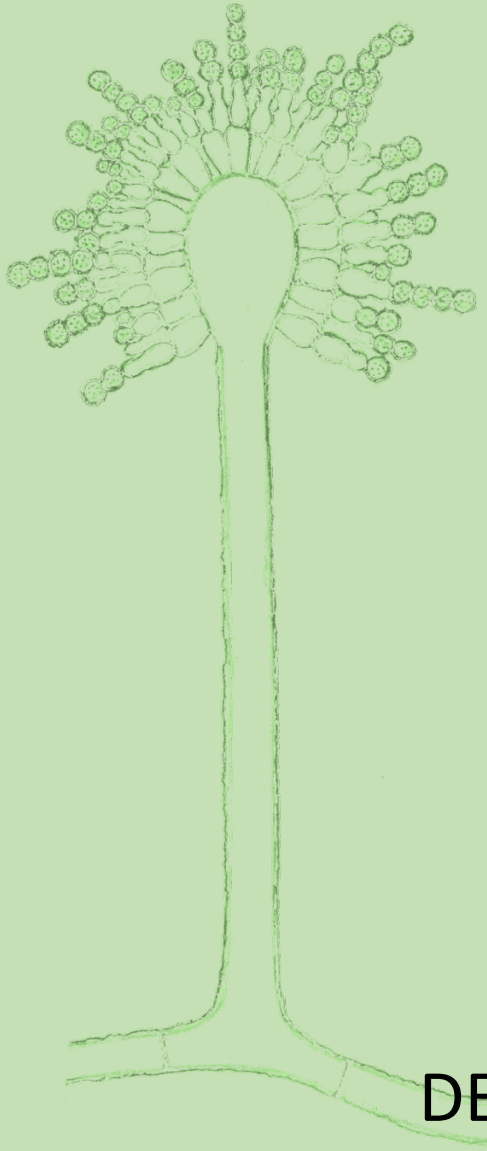


6054-42000-026-00D: Objective 1

- Unknown VOC study findings: toxins on CZ

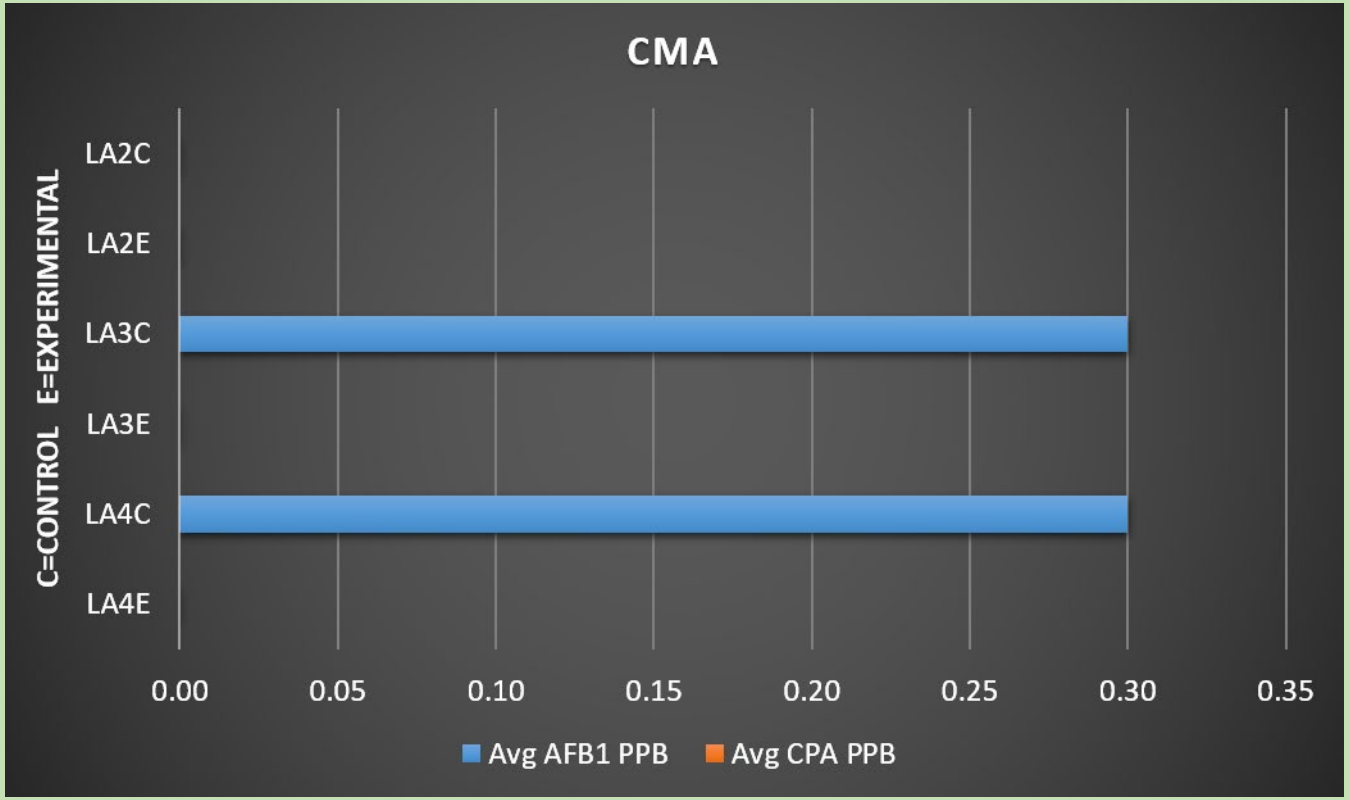


Overall INCREASE in AFB_1… no detectable αCPA

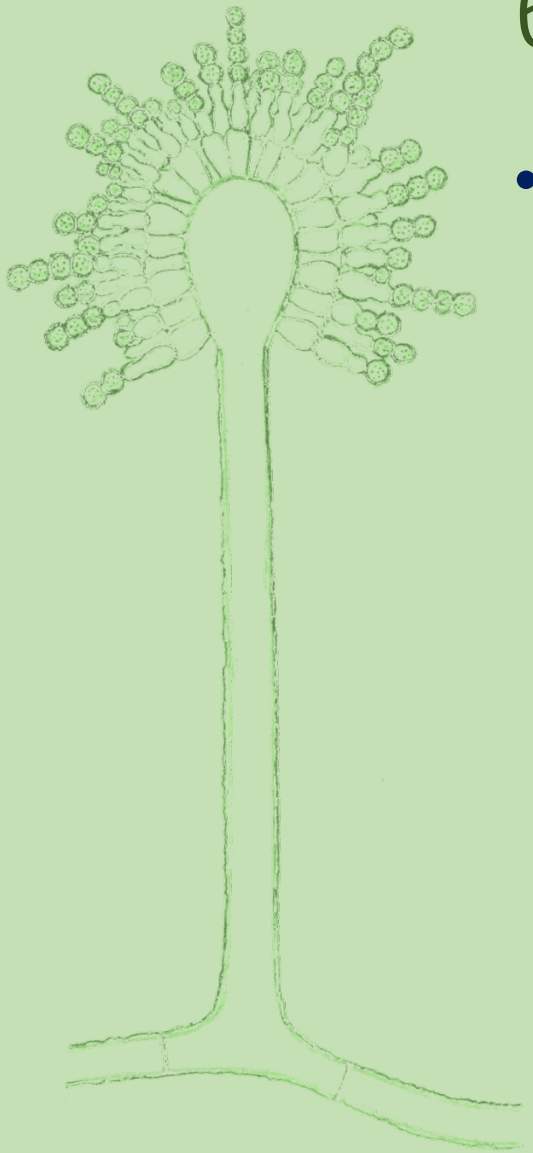


6054-42000-026-00D: Objective 1

- Unknown VOC study findings: toxins on CMA

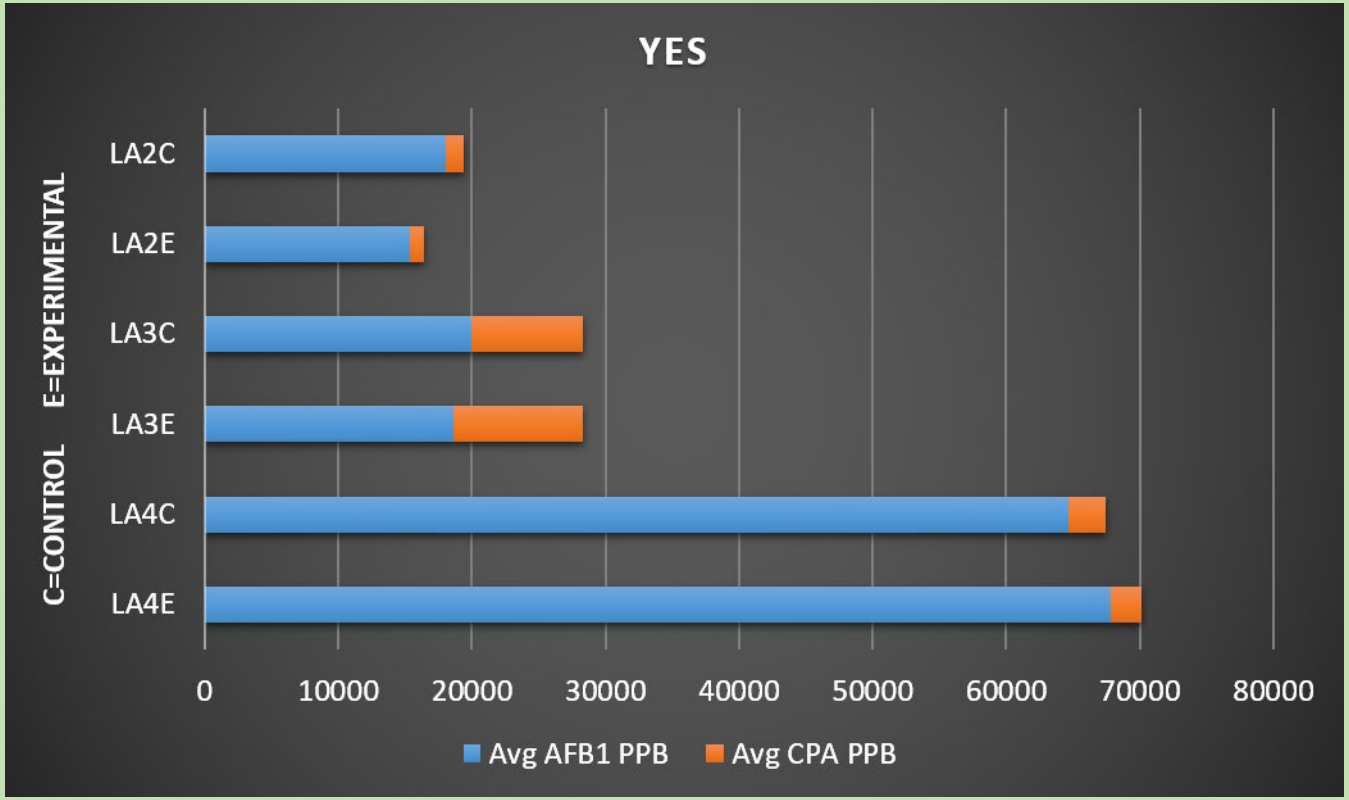


DECREASE in AFB_1 for LA3 and LA4… no detectable αCPA



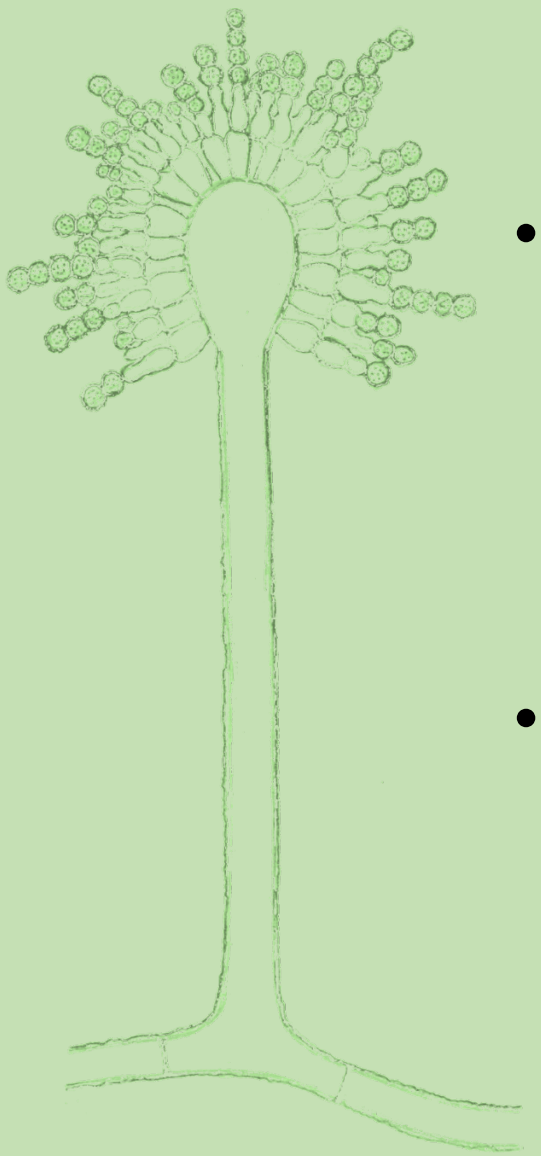
6054-42000-026-00D: Objective 1

- Unknown VOC study findings: toxins on YES



DECREASE in AFB_1 in LA2 and LA3

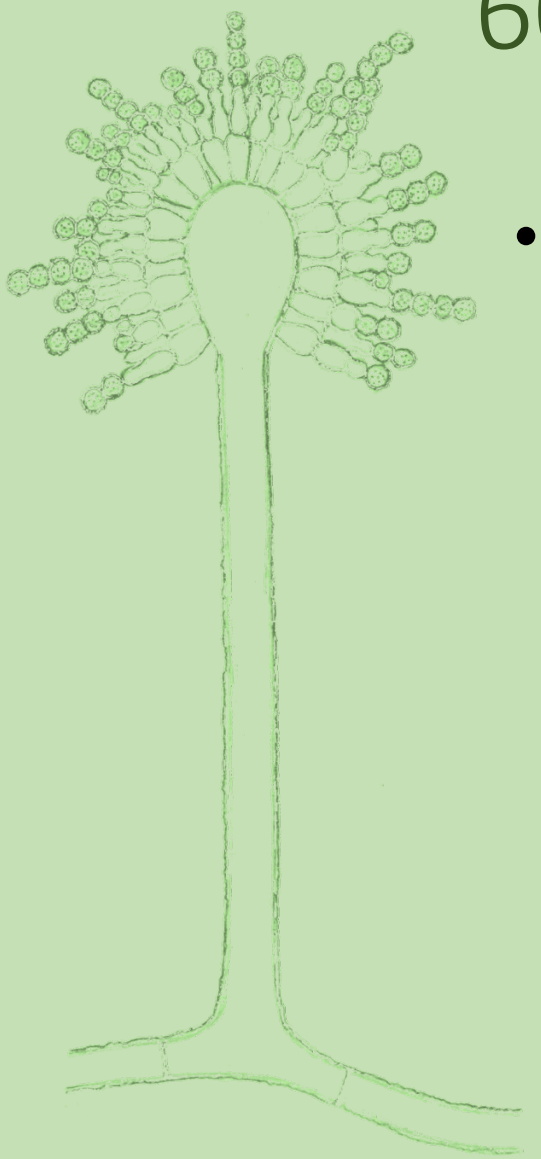
DECREASE in αCPA in LA2 and LA4



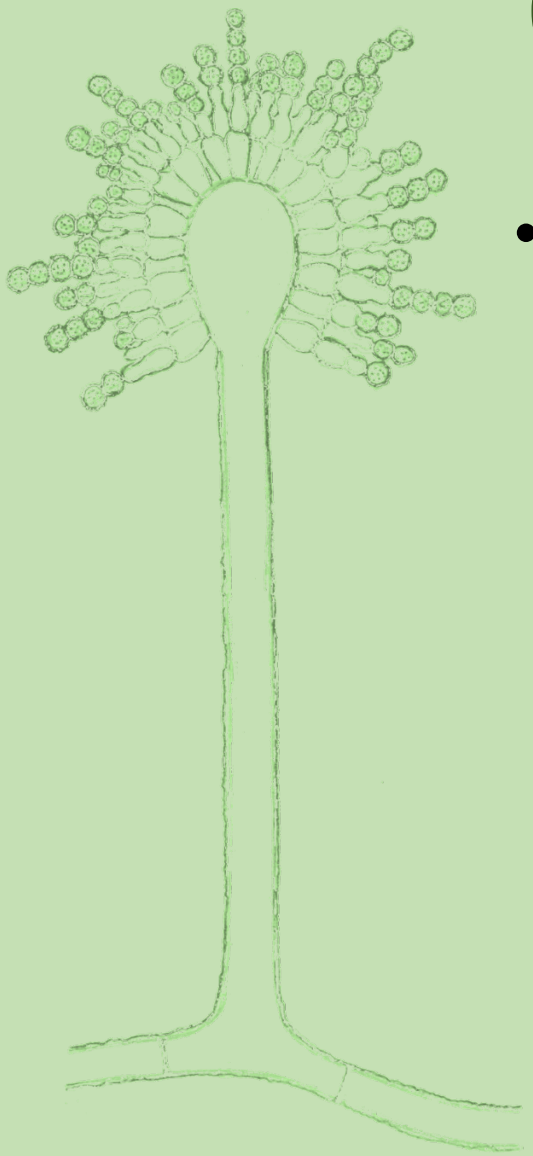
6054-42000-026-00D: Objective 1

- Unknown VOC study: conclusions
  - Some LA1-VOC-induced reductions on growth and/or toxin
    - Strains could not touch (thigmoregulation)
    - No exchange of secreted compounds (extrolite)
    - No nutrient sequestration ahead of inoculation by LA2-LA4
- Take Home: A VOC is being produced by LA1 that has potential to reduce toxin levels

6054-42000-026-00D: Objective 1

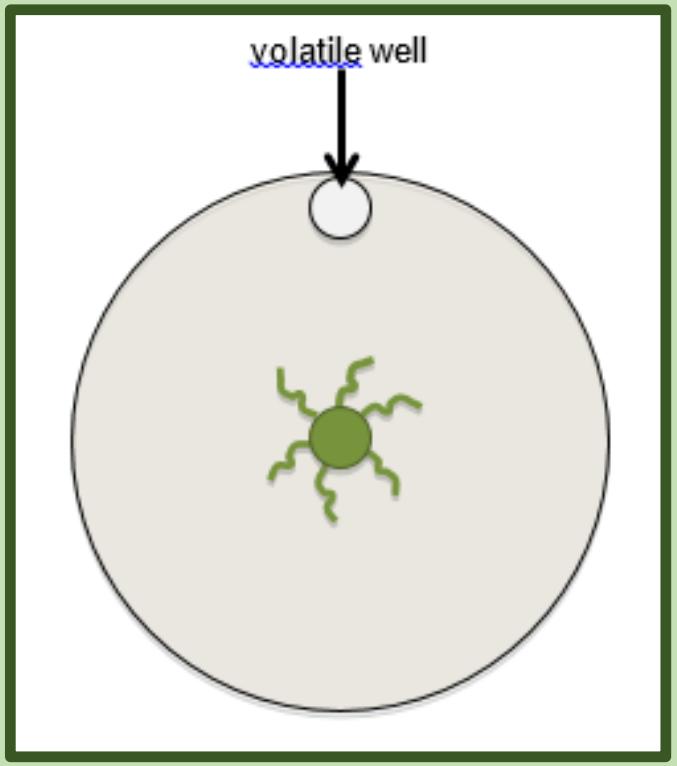


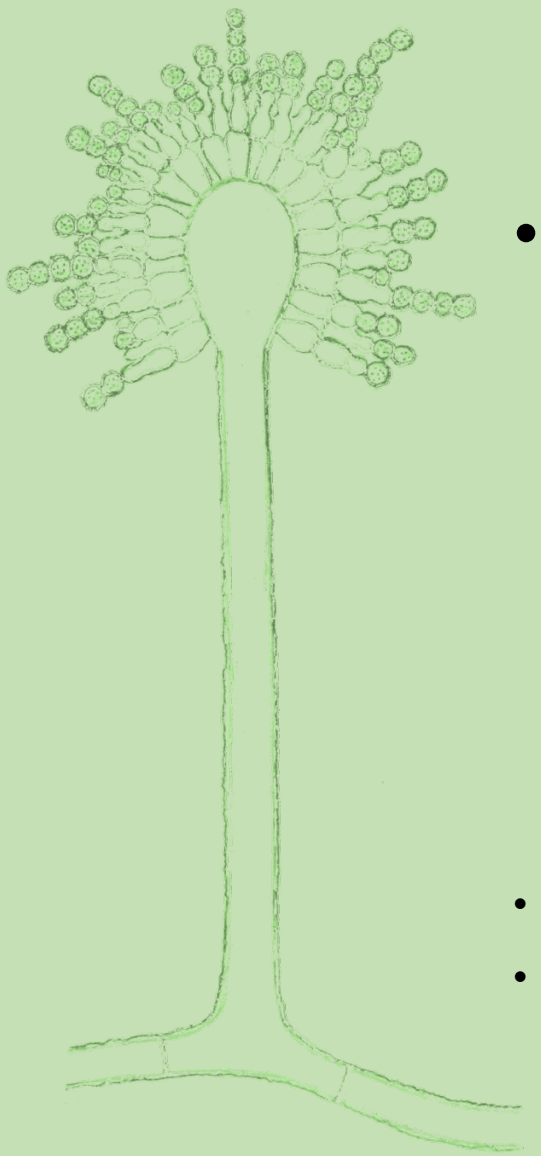
- Unknown VOC study: future investigations
  - Need to identify VOCs unique to LA1
  - Need to scale up the experiment



6054-42000-026-00D: Objective 1

- Known VOC study (ongoing)
  - Experiments to observe reductions in growth and/or toxins
  - 5 VOCs unique to atoxigenic *A. flavus*
  - 5 VOCs unique to toxigenic *A. flavus*
  - 5 µl, 10 µl, 20 µl volumes
  - YES solid medium only

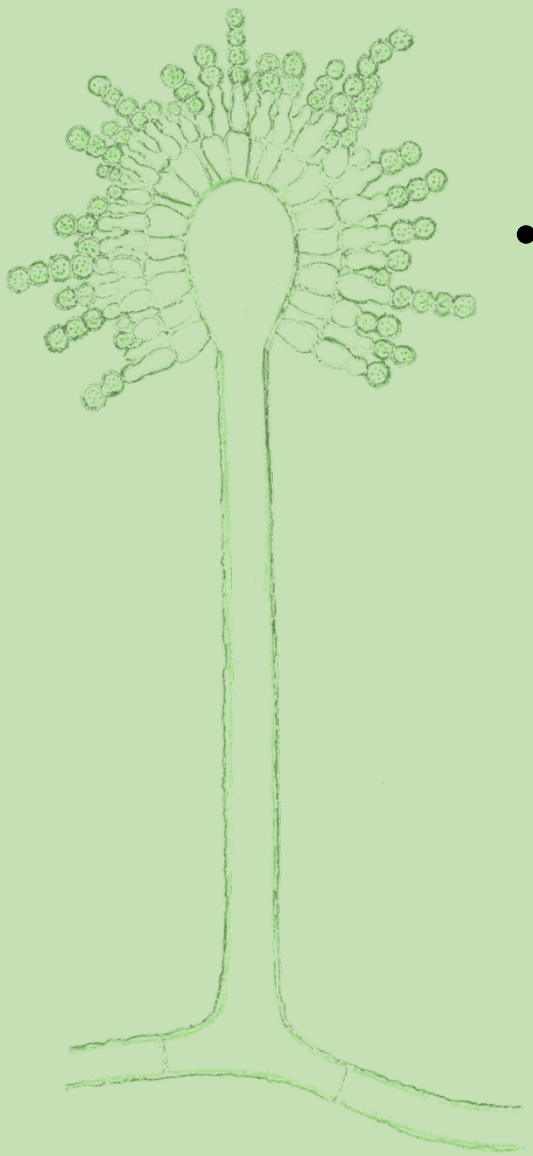




6054-42000-026-00D: Objective 1

- Known VOC study (ongoing)
  - Are atox VOCs from *A. flavus* inhibitory to toxigenic fungi? (NRRLs 5918 and 5565)
    - 3-Octanone, 2,3-Dihydrofuran, Decane, (E)-2-Methyl-2-Butenal, (S)-(−)-2-Methyl-1-Butanol
  - Are tox VOCs from *A. flavus* inhibitory to atoxigenic fungi? (1000E and AF13)
    - Benzaldehyde, Benzyl alcohol, Eucalyptol, Hexane, Isoamyl acetate

- De Lucca, A. J., Boué, S. M., Carter-Wientjes, C., et al. 2010. Ann. Agric. Environ. Med. 17, 301-308.
- De Lucca, A. J., Boué, S. M., Carter-Wientjes, C., et al. 2012. Ann. Agric. Environ. Med. 19, 91-98.

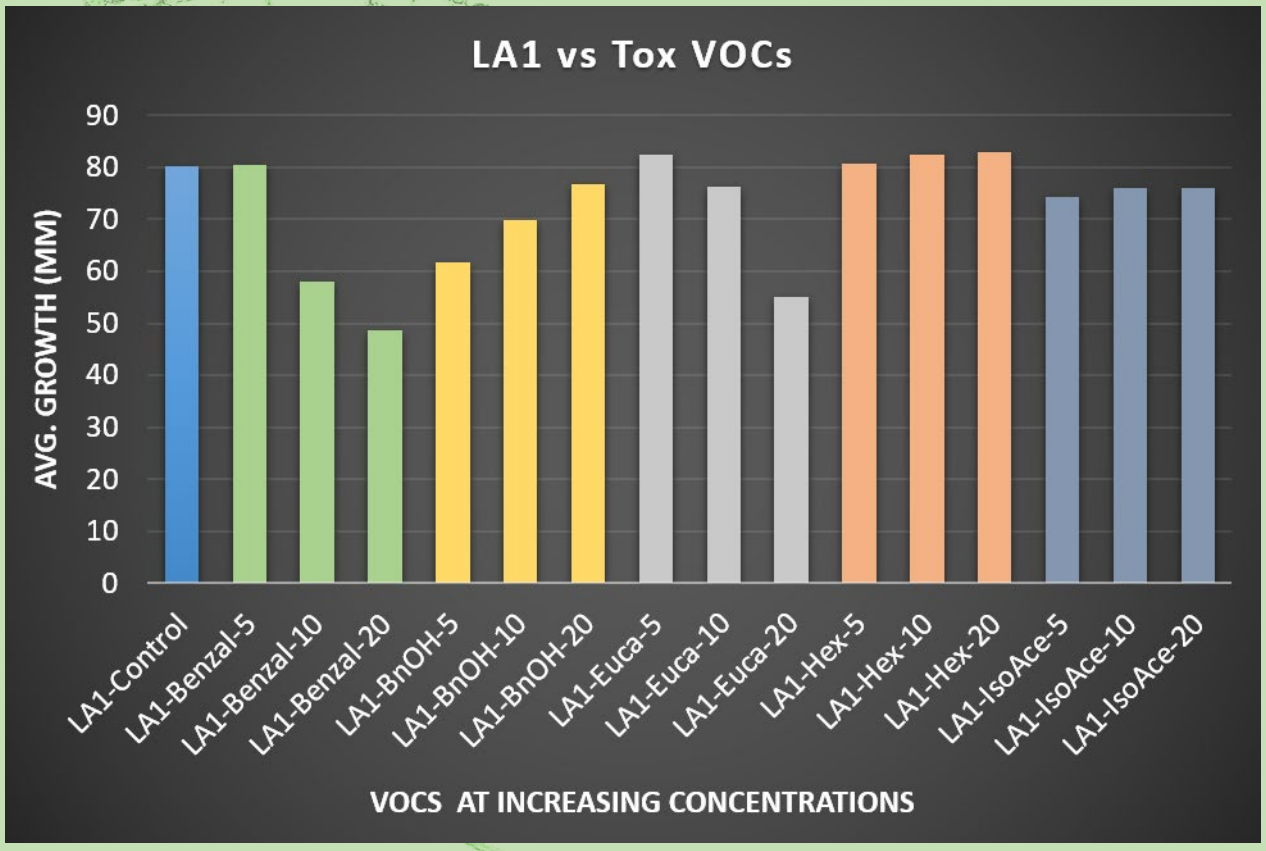


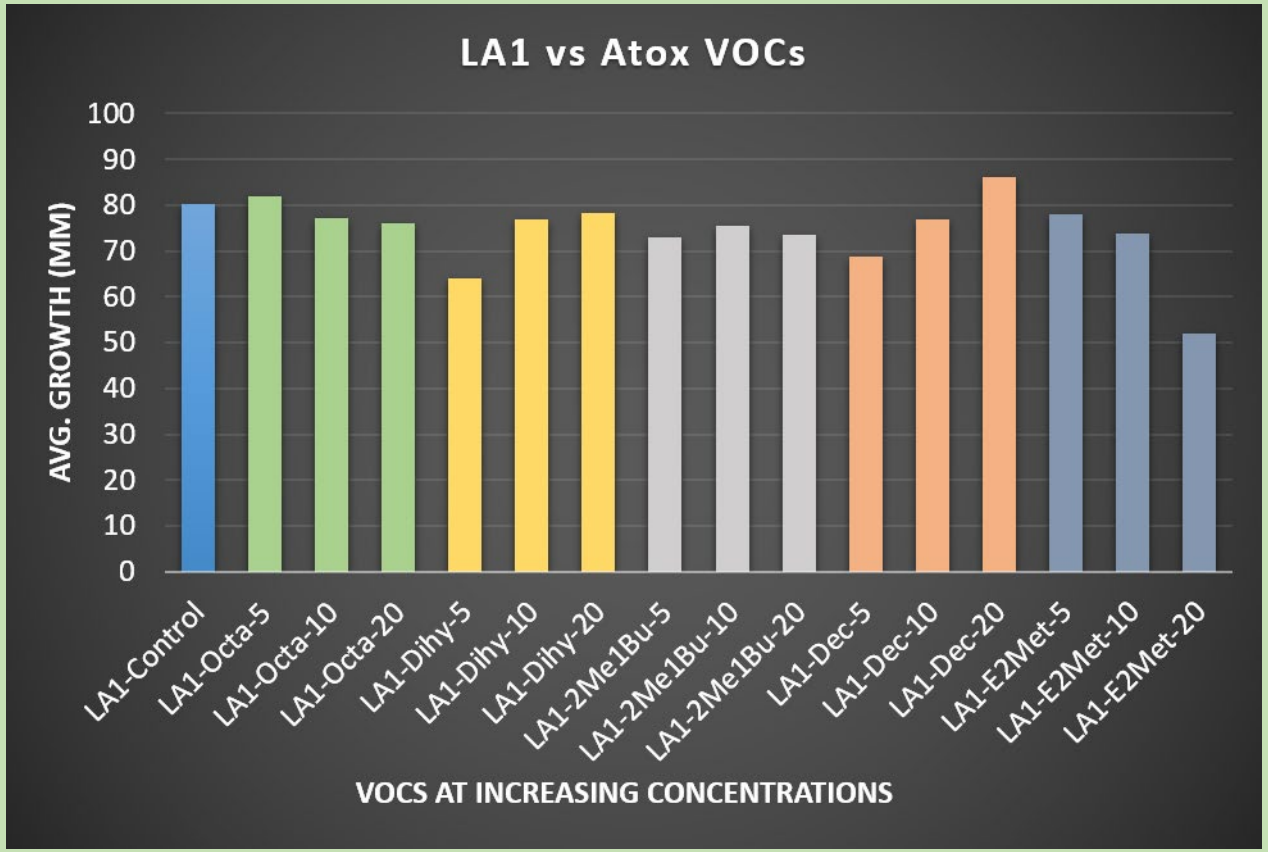
6054-42000-026-00D: Objective 1

- Known VOC study (ongoing)
  - Control = colonies grown individually (no VOCs)
  - Experimental = colonies exposed to single VOC
    - Volume increase = increased exposure to VOC
    - Plates parafilmed to contain VOC while allowing fungus to get O_2
    - These are not necessarily compounds produced by LA strains

6054-42000-026-00D: Objective 1

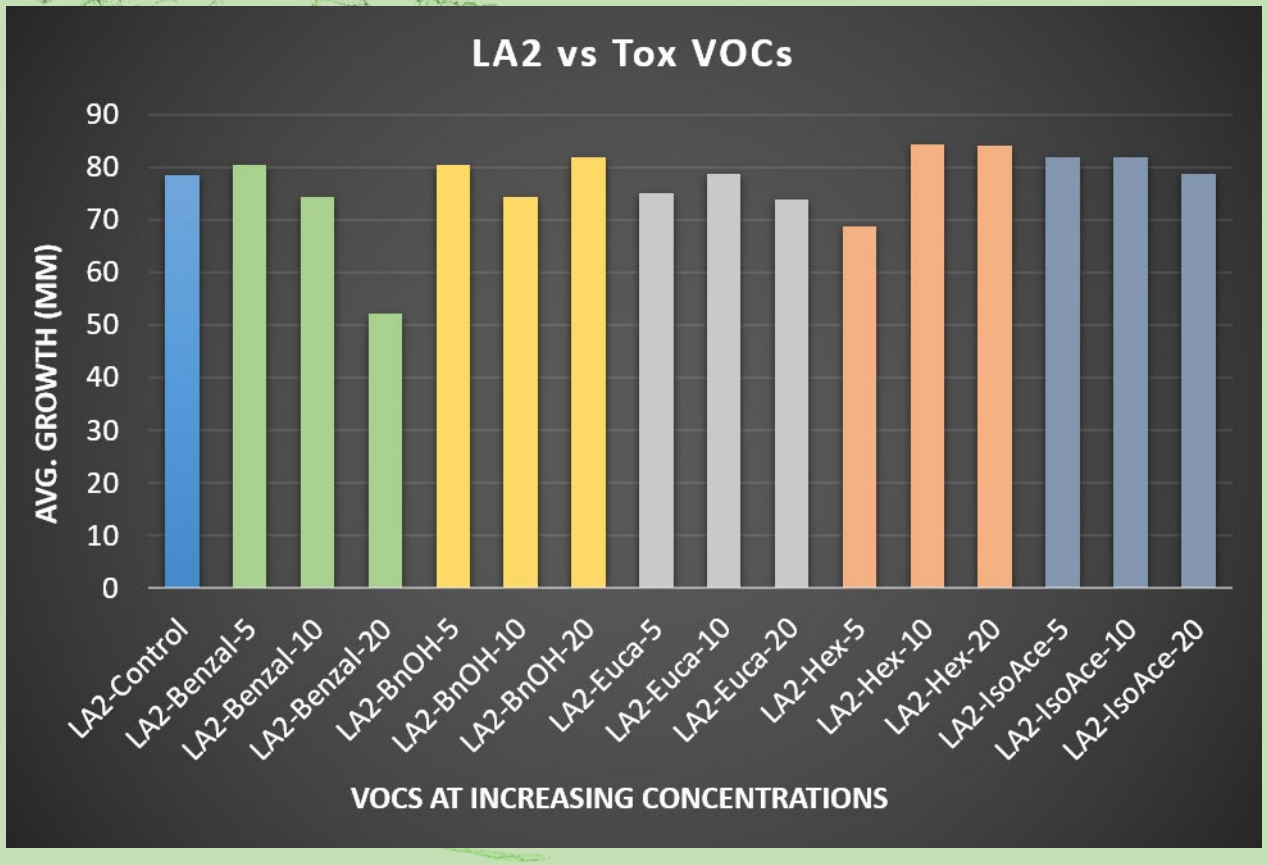
- Known VOC study: findings for LA1 growth

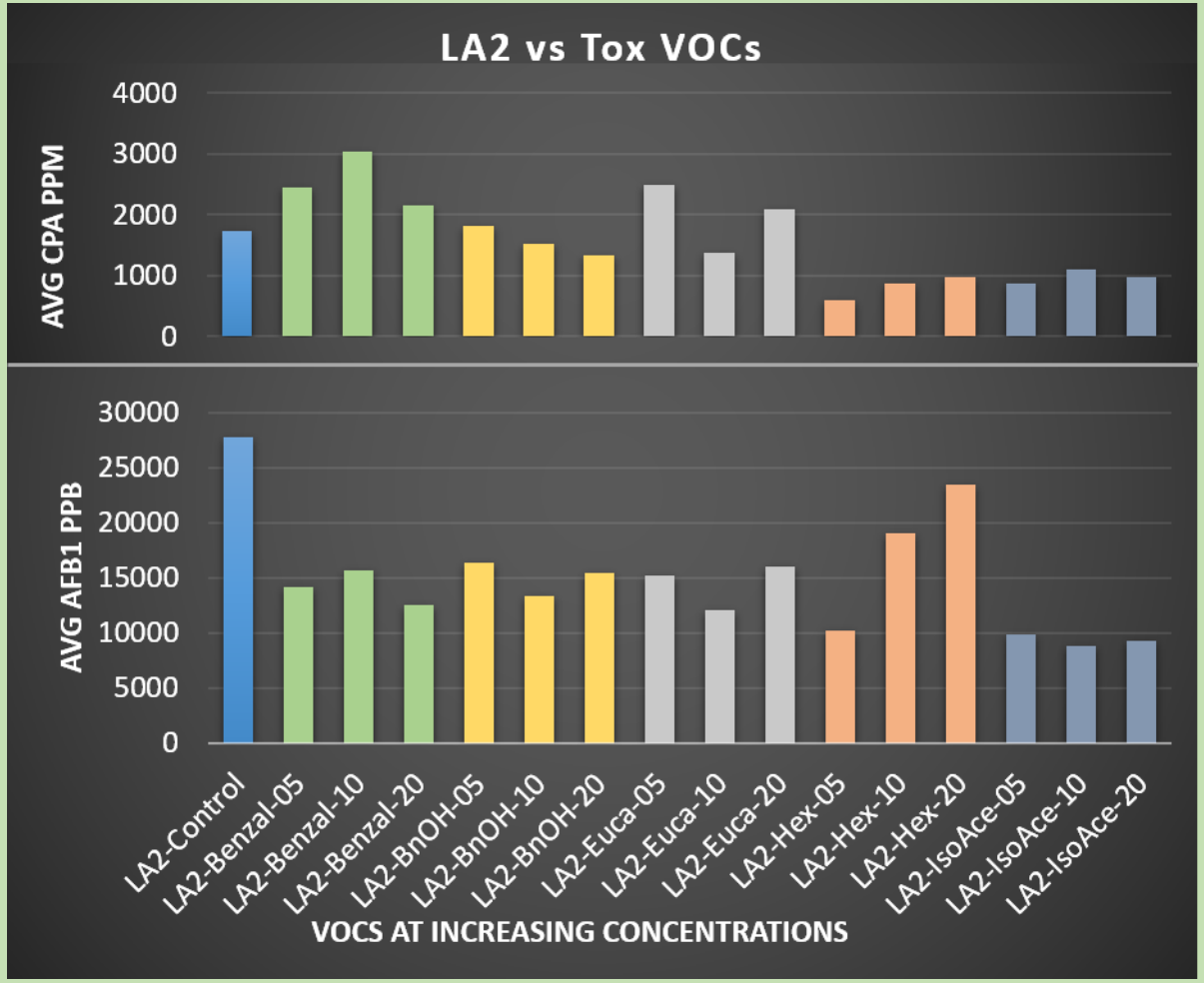




6054-42000-026-00D: Objective 1

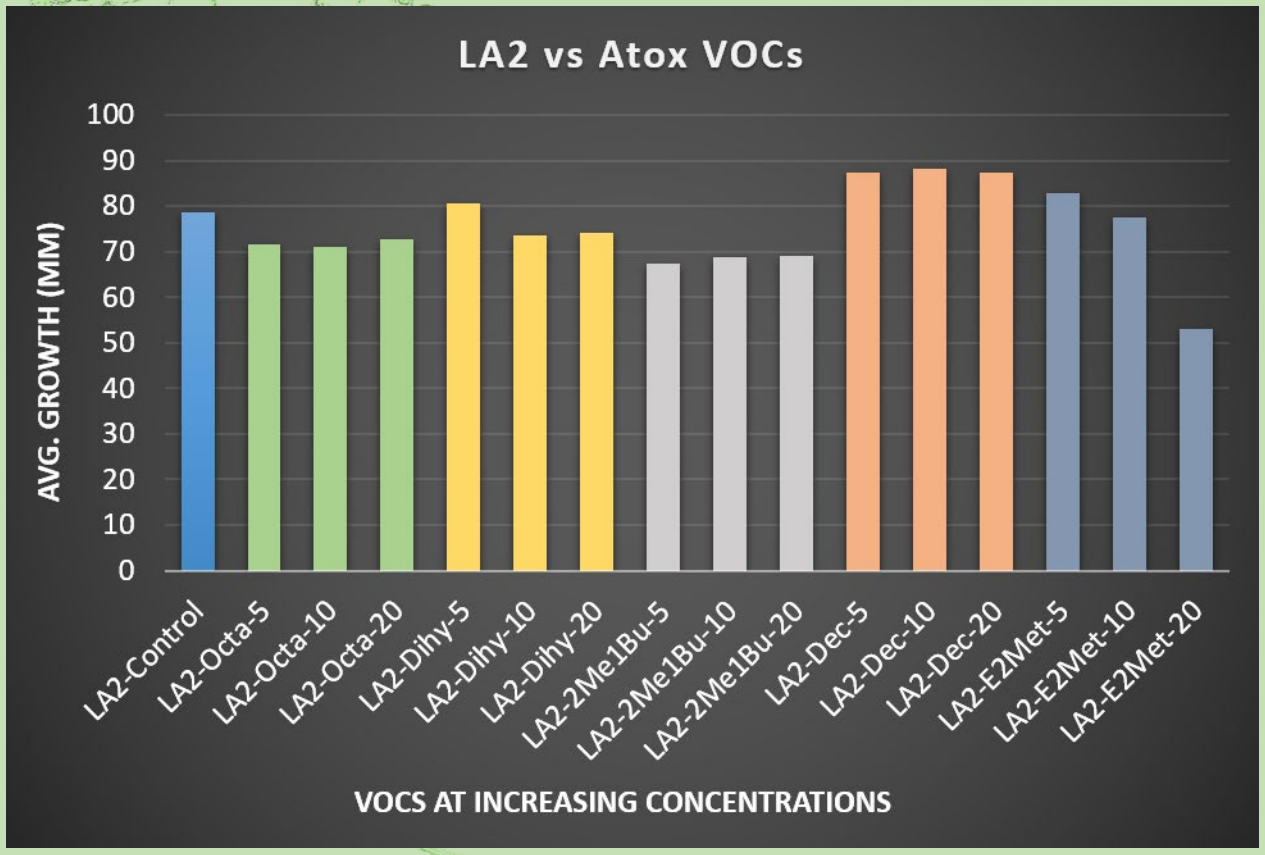
- Known VOC study: findings for LA2 growth & toxins

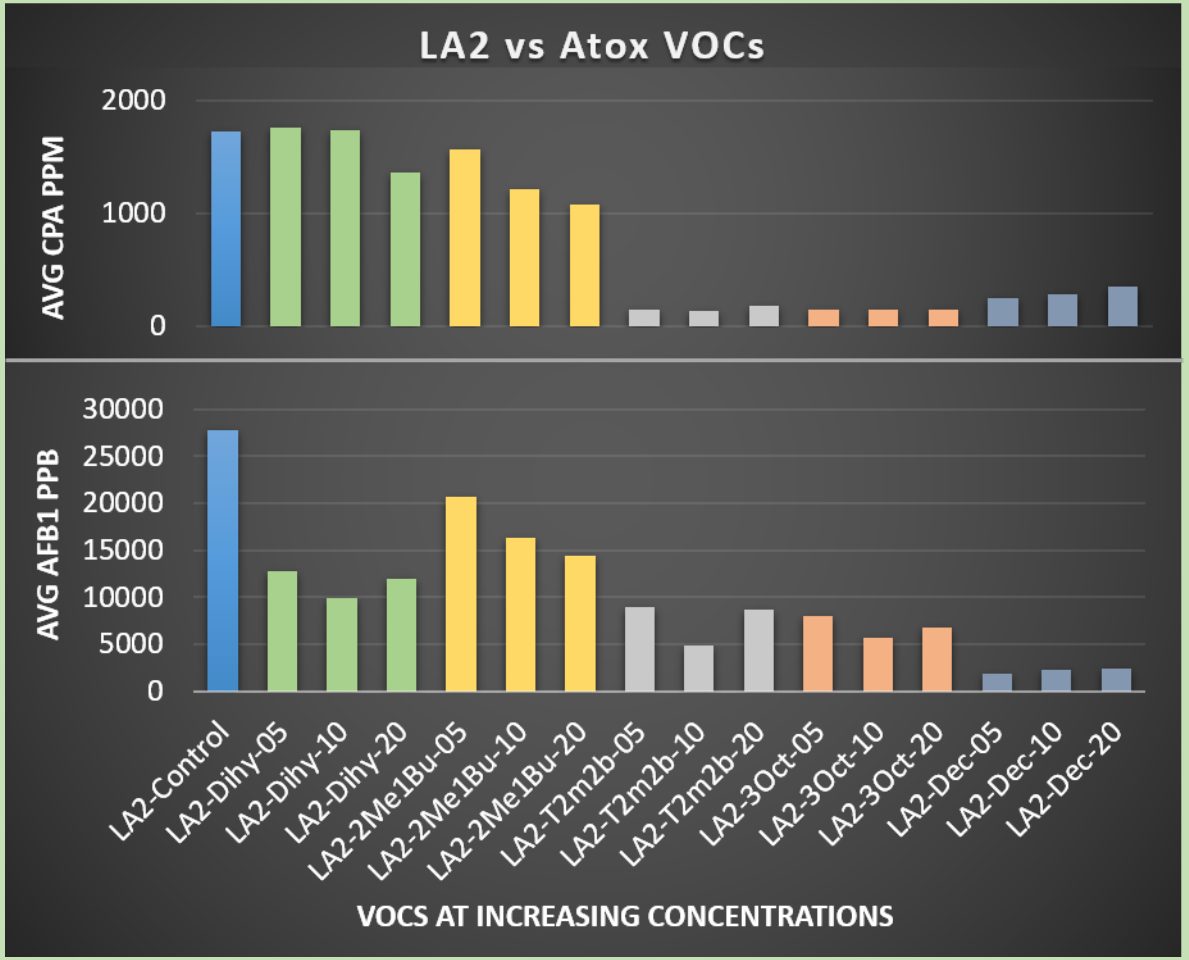




6054-42000-026-00D: Objective 1

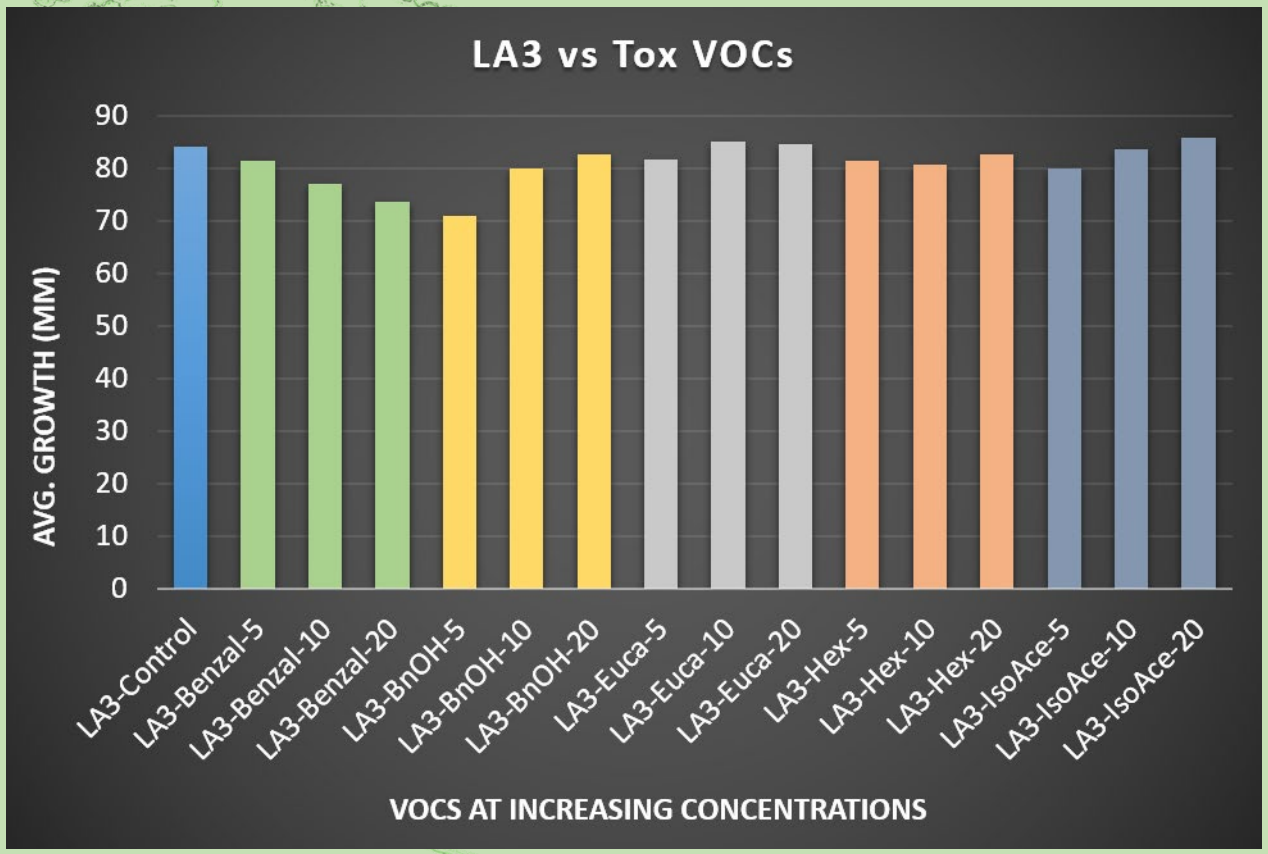
- Known VOC study: findings for LA2 growth & toxins

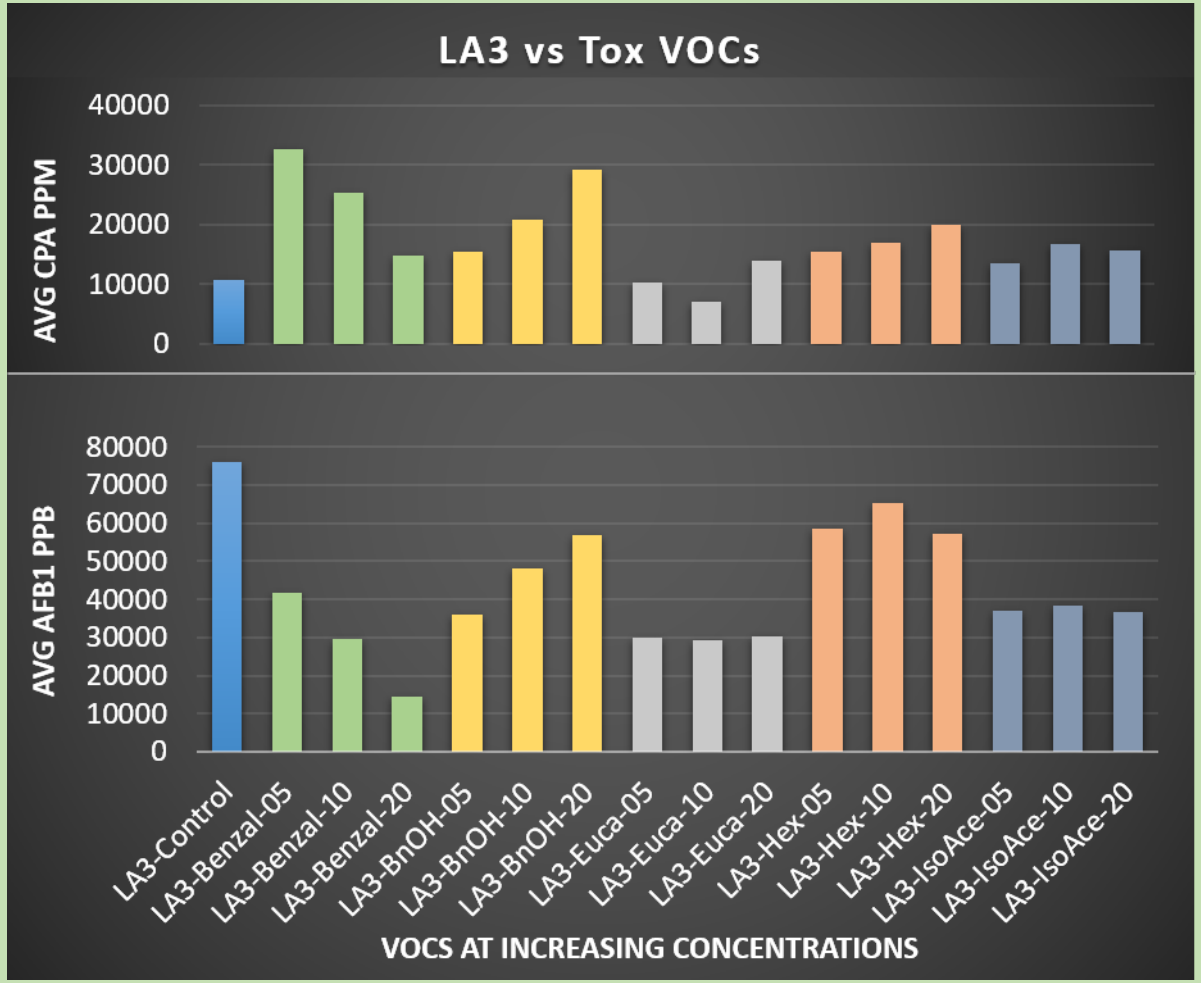




6054-42000-026-00D: Objective 1

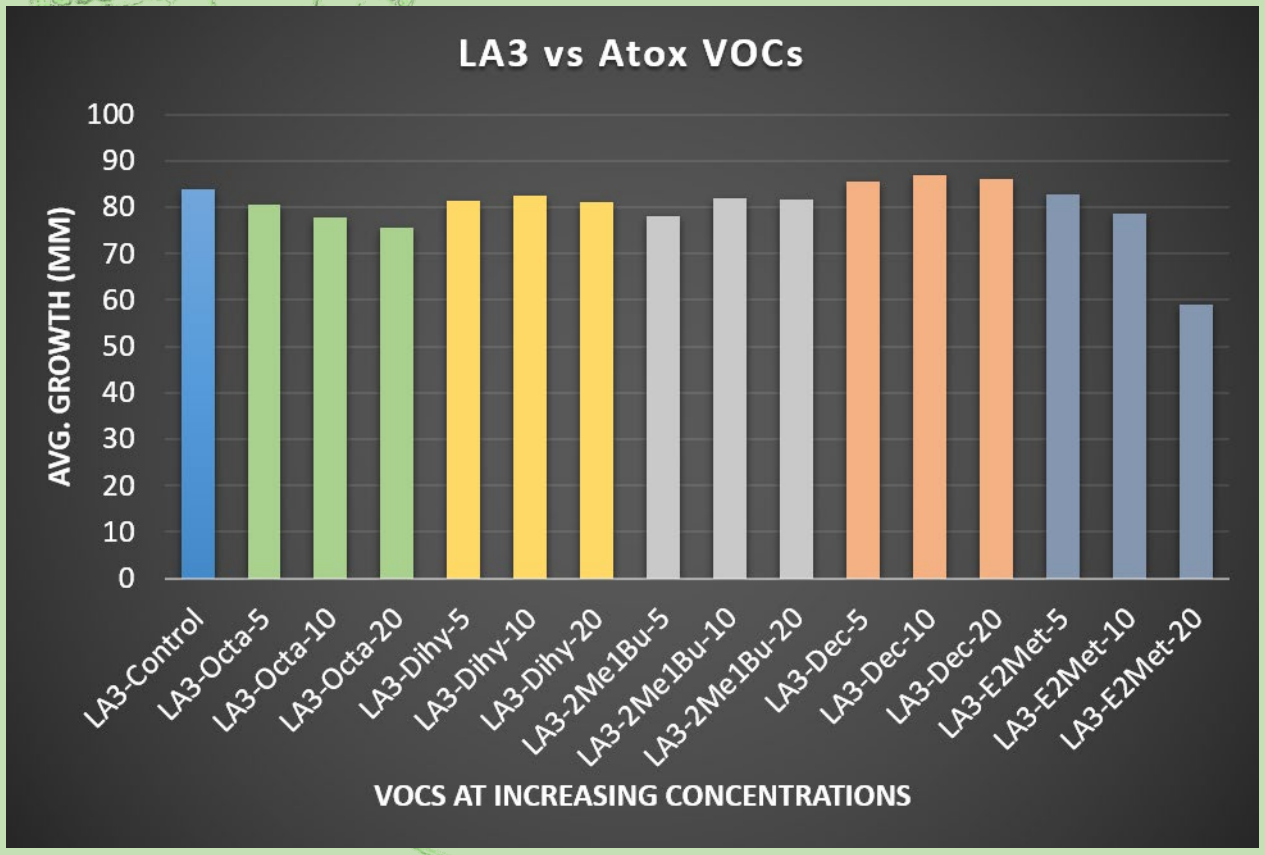
- Known VOC study: findings for LA3 growth & toxins

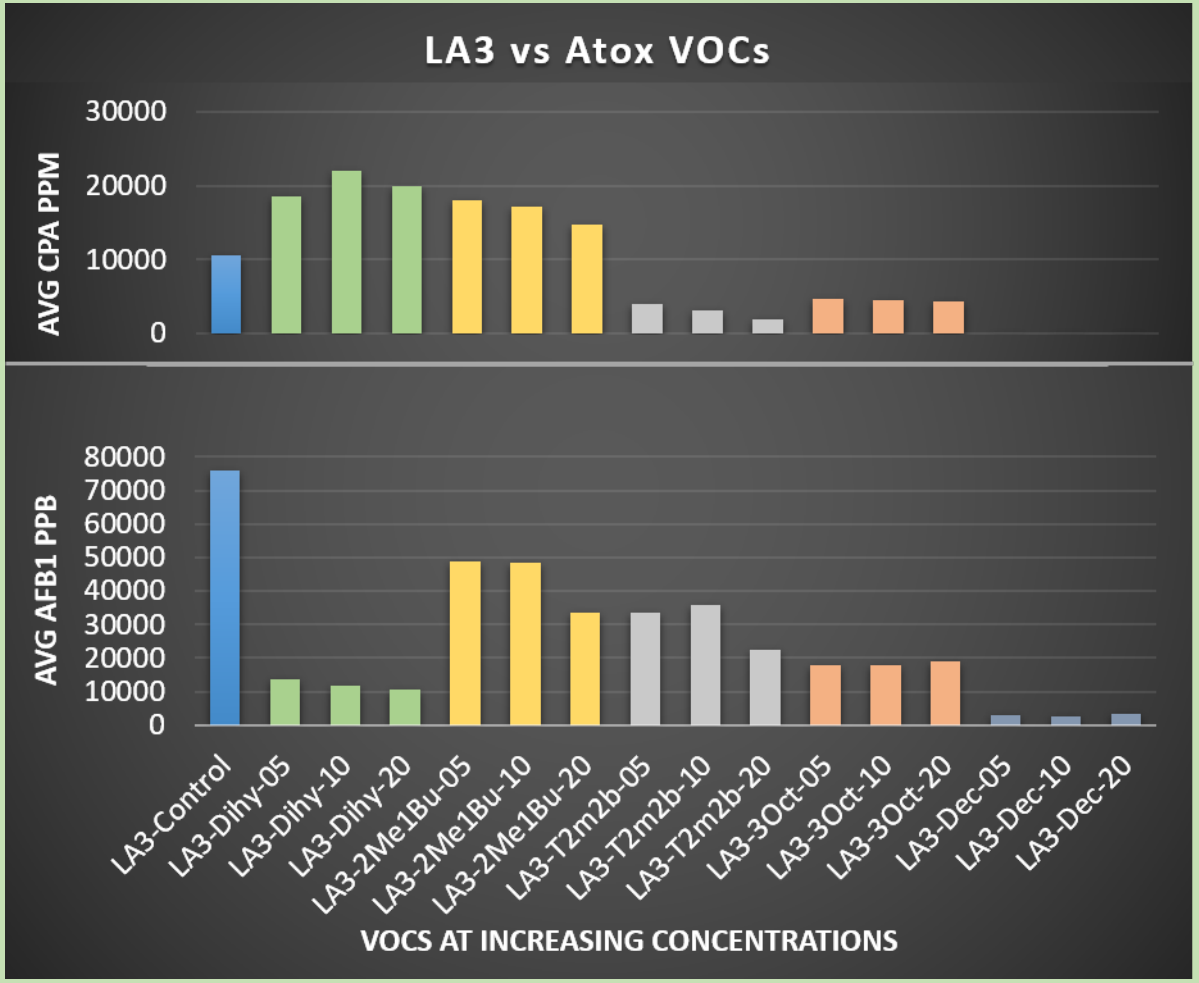




6054-42000-026-00D: Objective 1

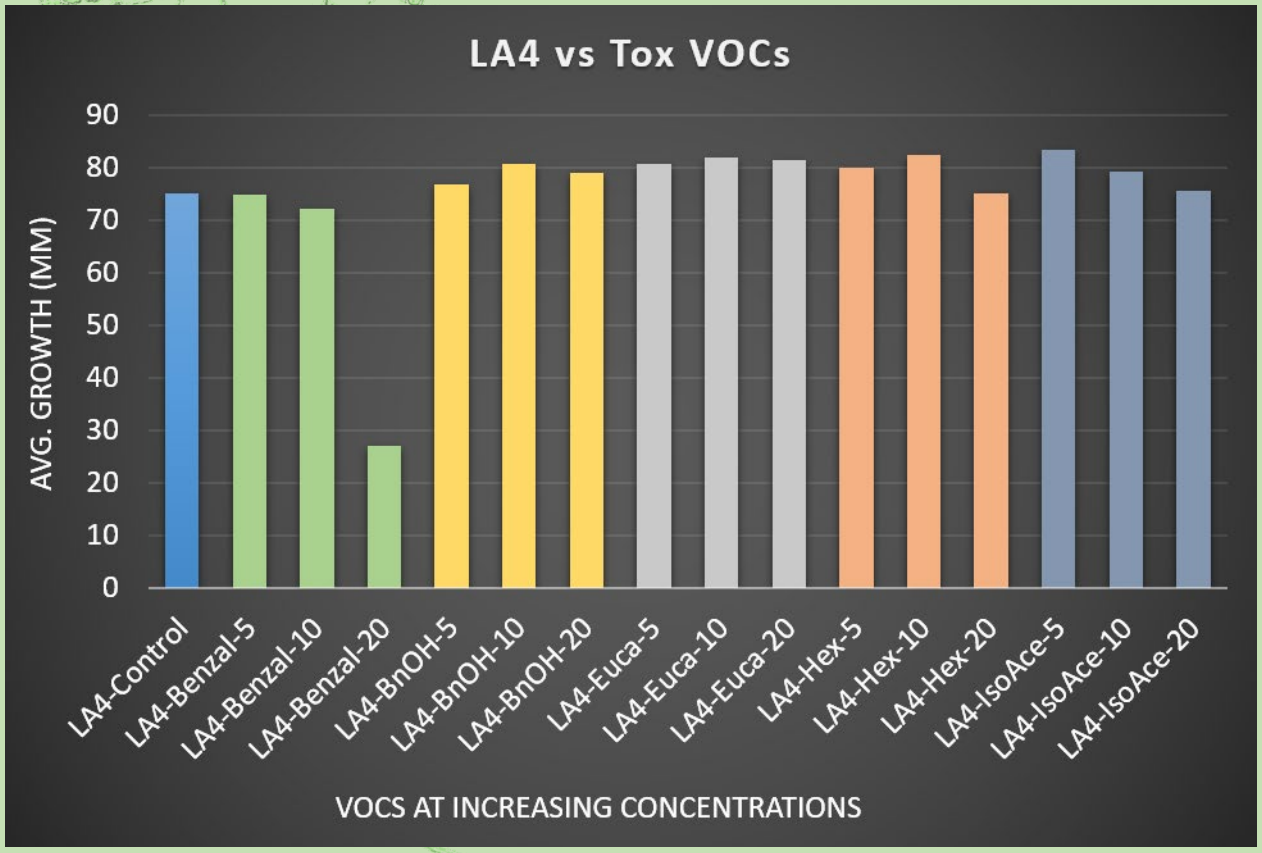
- Known VOC study: findings for LA3 growth & toxins

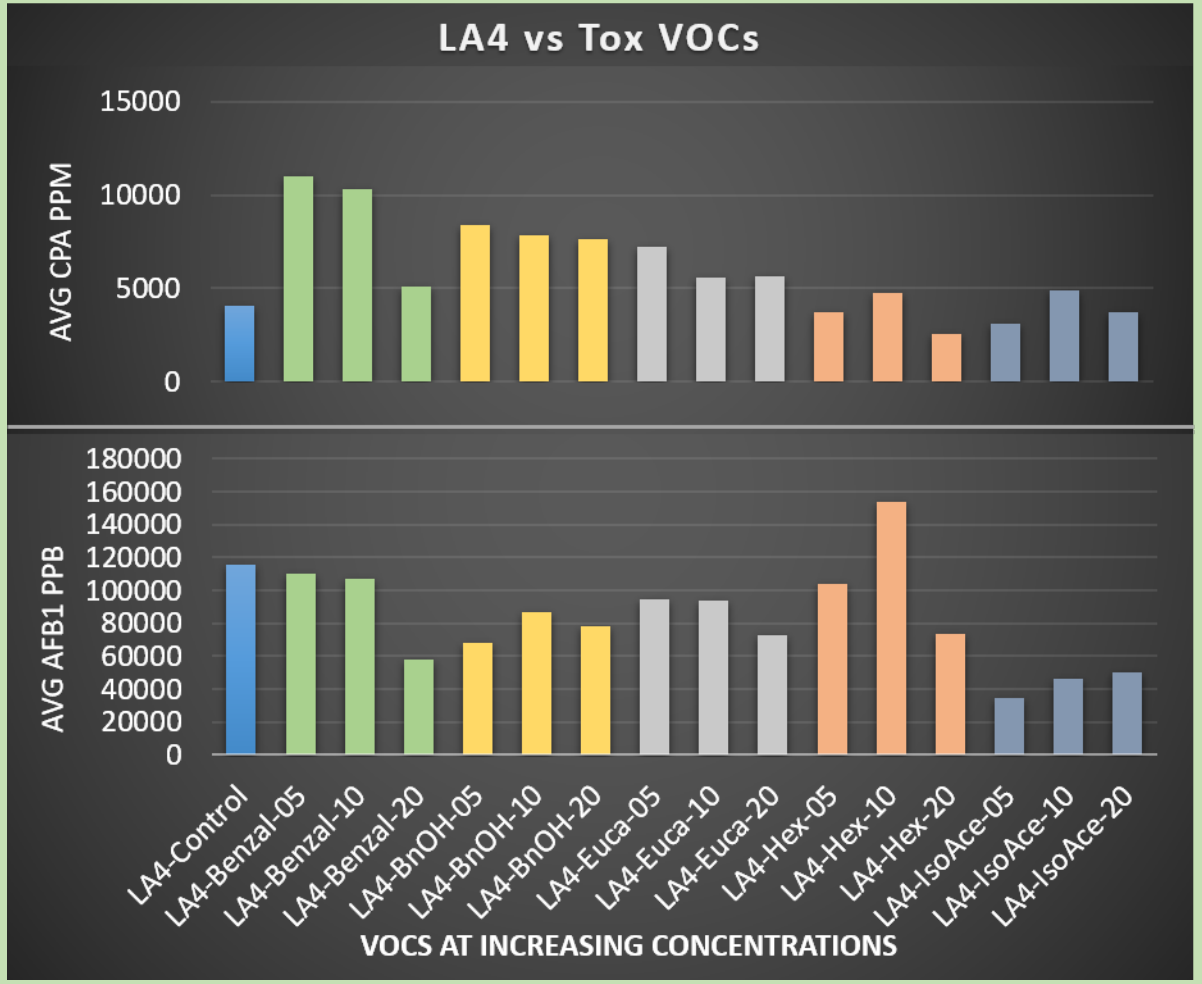




6054-42000-026-00D: Objective 1

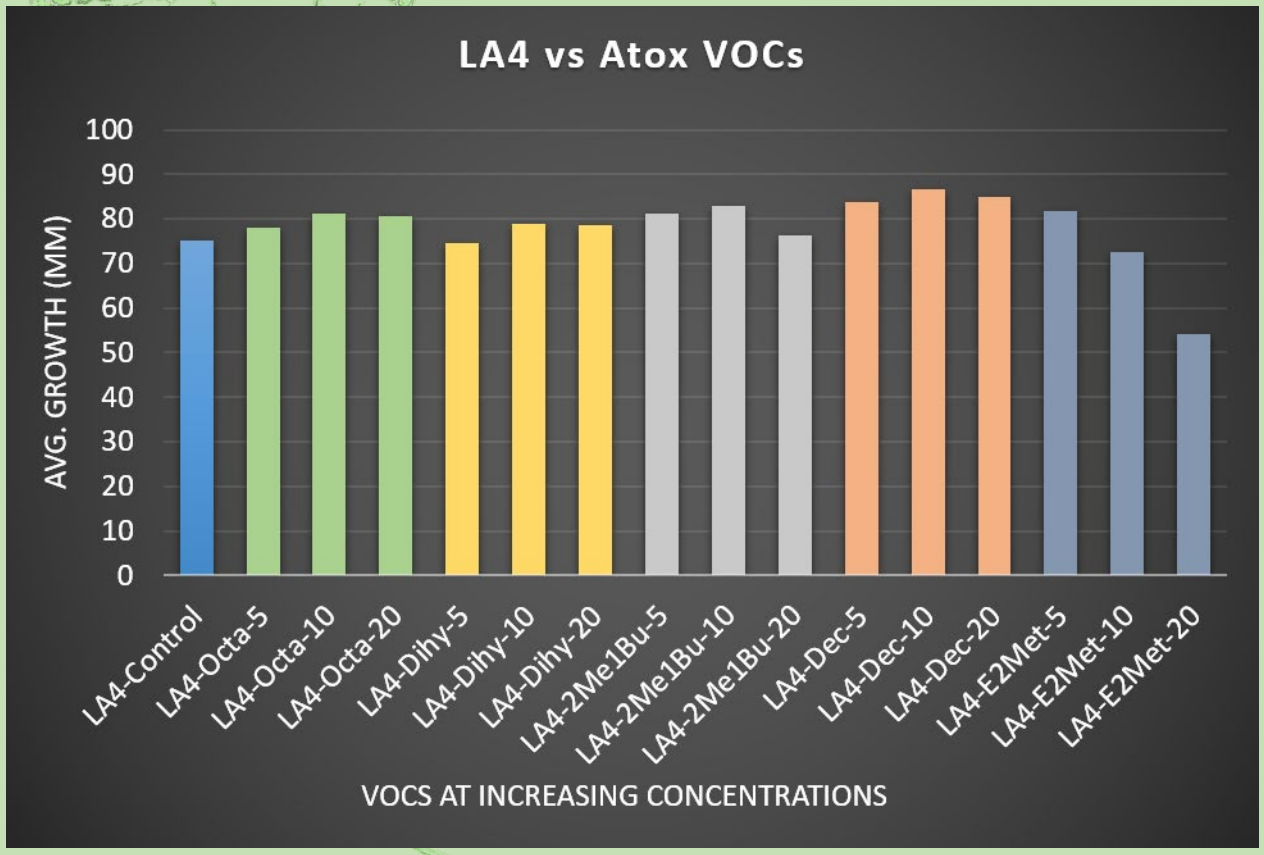
- Known VOC study: findings for LA4 growth & toxins

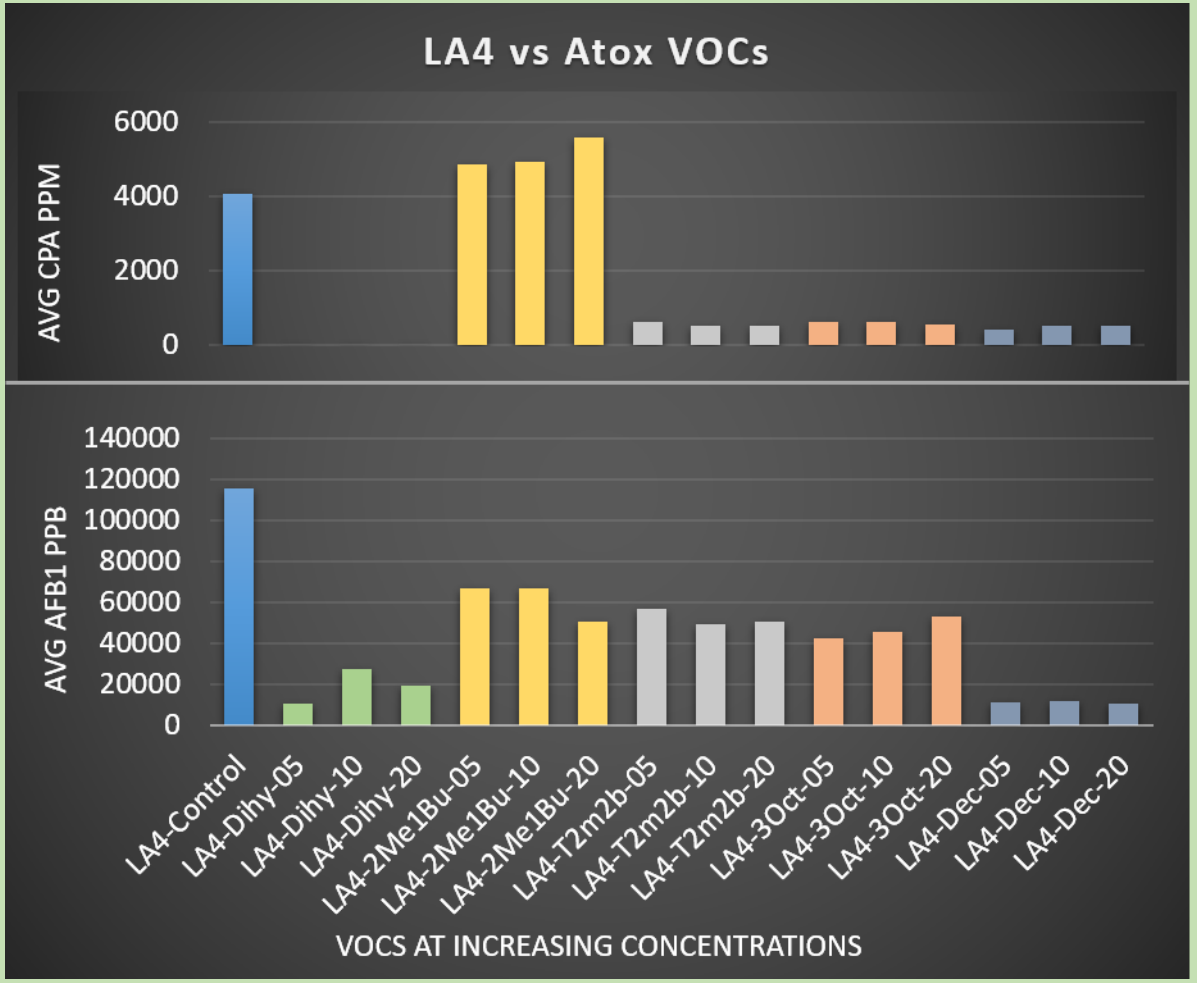


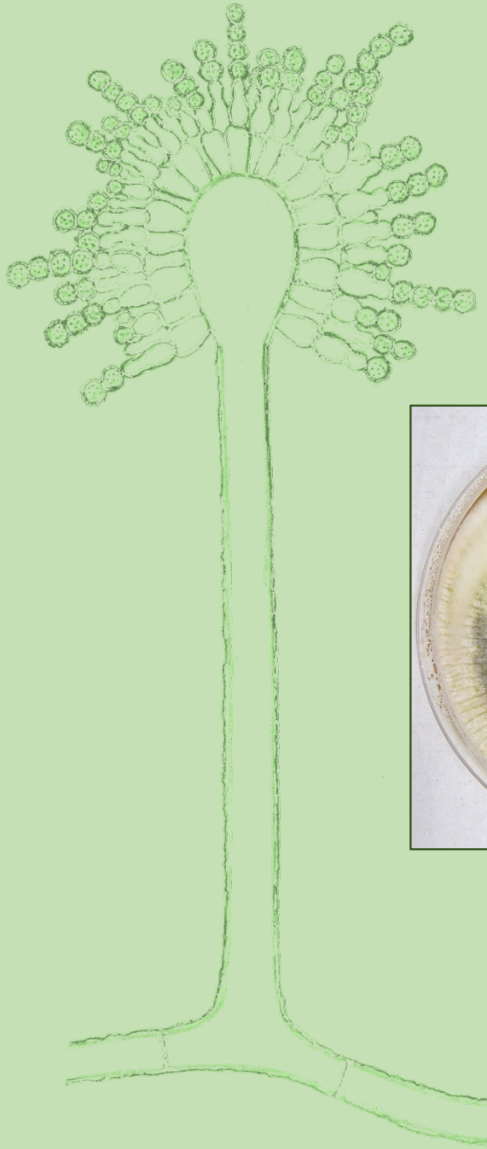


6054-42000-026-00D: Objective 1

- Known VOC study: findings for LA4 growth & toxins

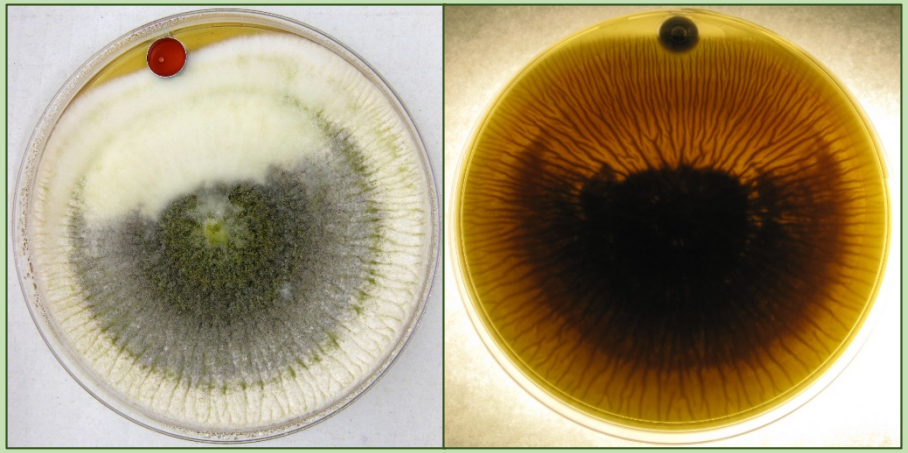


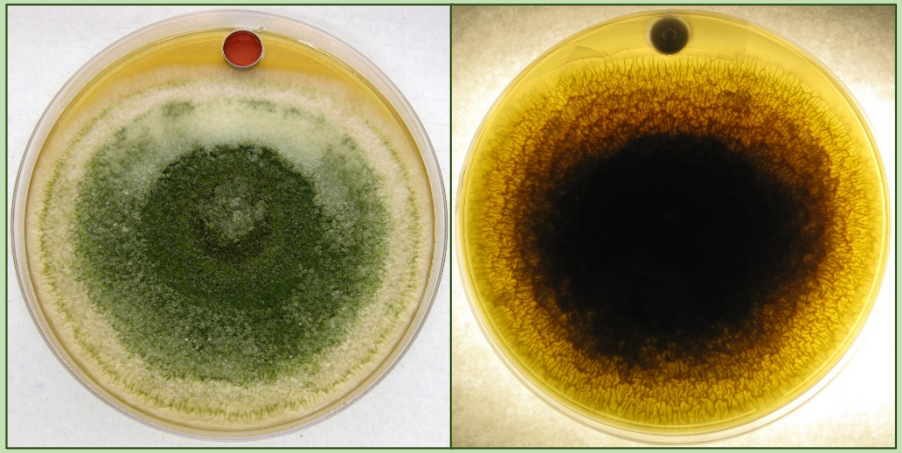




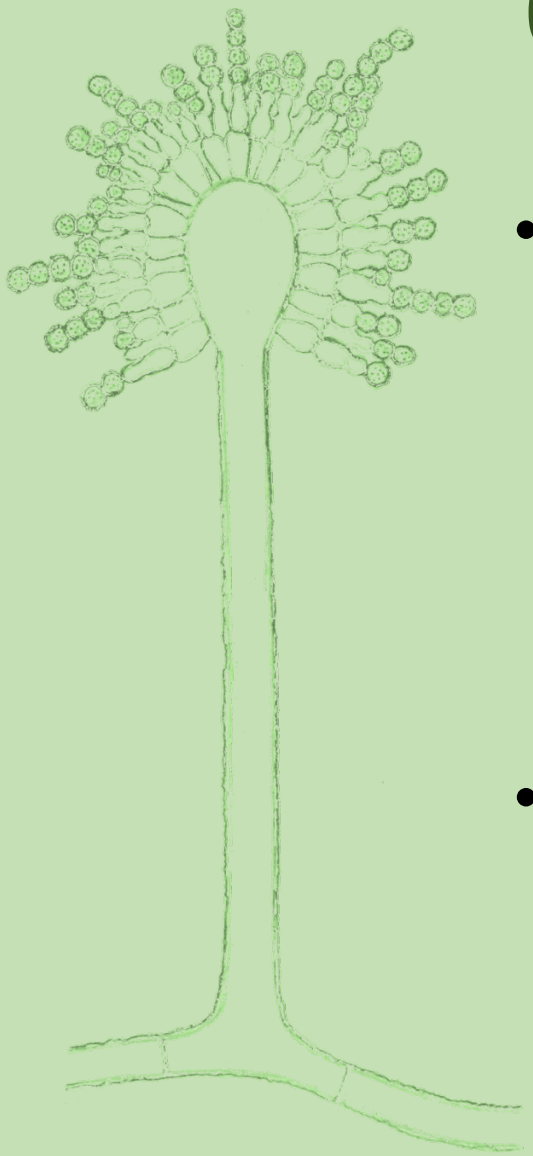
6054-42000-026-00D: Objective 1

- Known VOC study findings: benzyl alcohol impacts morphology for LA3 and LA4



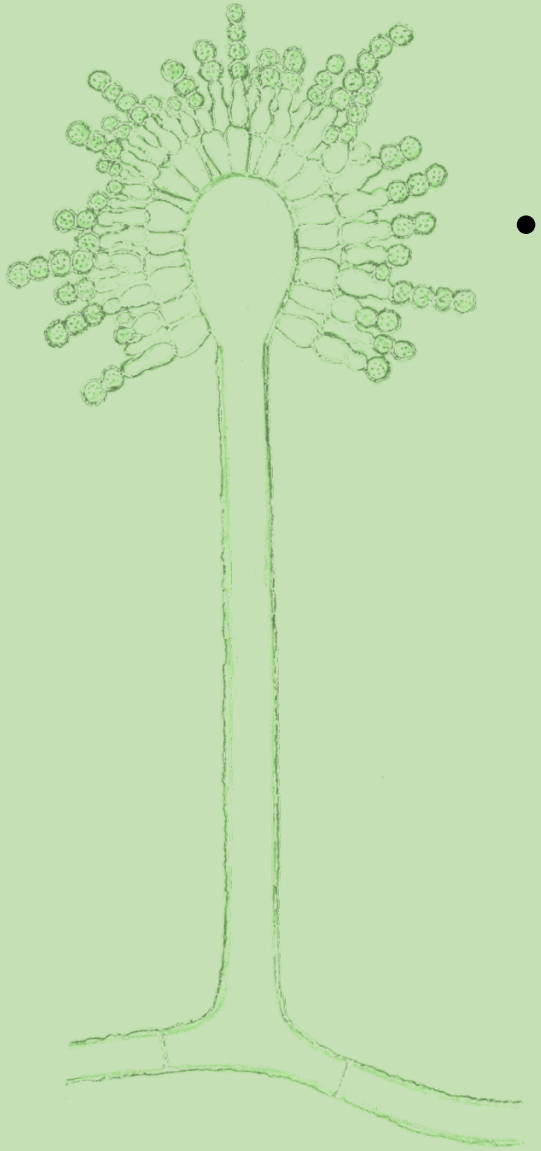


- But not effective at reducing toxin levels!



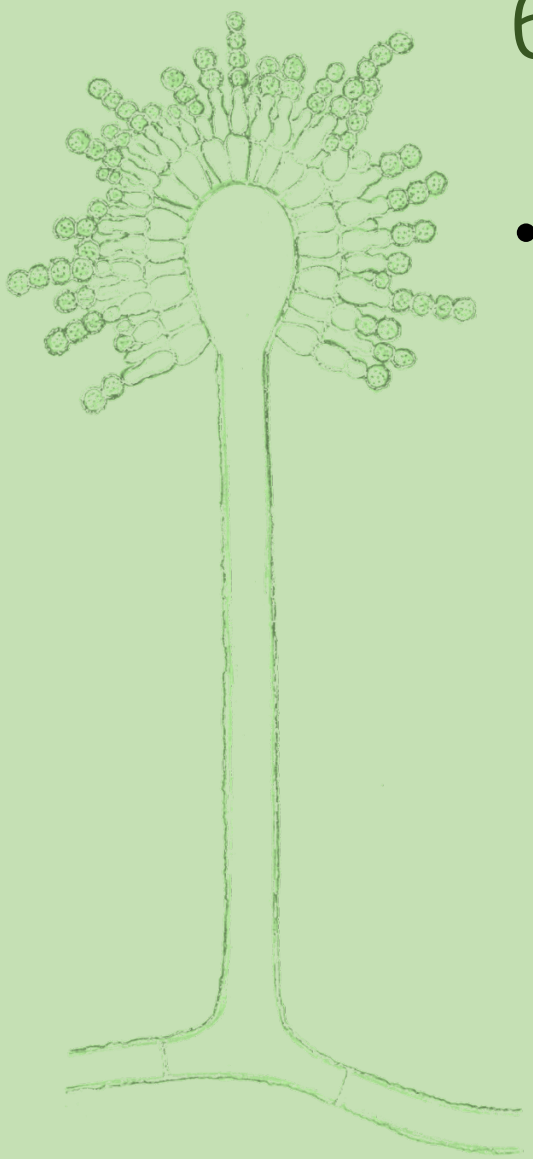
6054-42000-026-00D: Objective 1

- Known VOC study: conclusions (so far)
  - Toxin reduction not directly correlated with growth
  - Tox VOCs most inhibitory to LA1 growth:
    - Hexane, Isoamyl Acetate
    - At high concentrations could impact biocontrol efficacy
  - Atox VOCs inhibitory to AFB_1 and αCPA by LA2-LA4:
    - 3-Octanone and Decane
- Take Home: *A. flavus* VOCs exist that reduce toxin levels



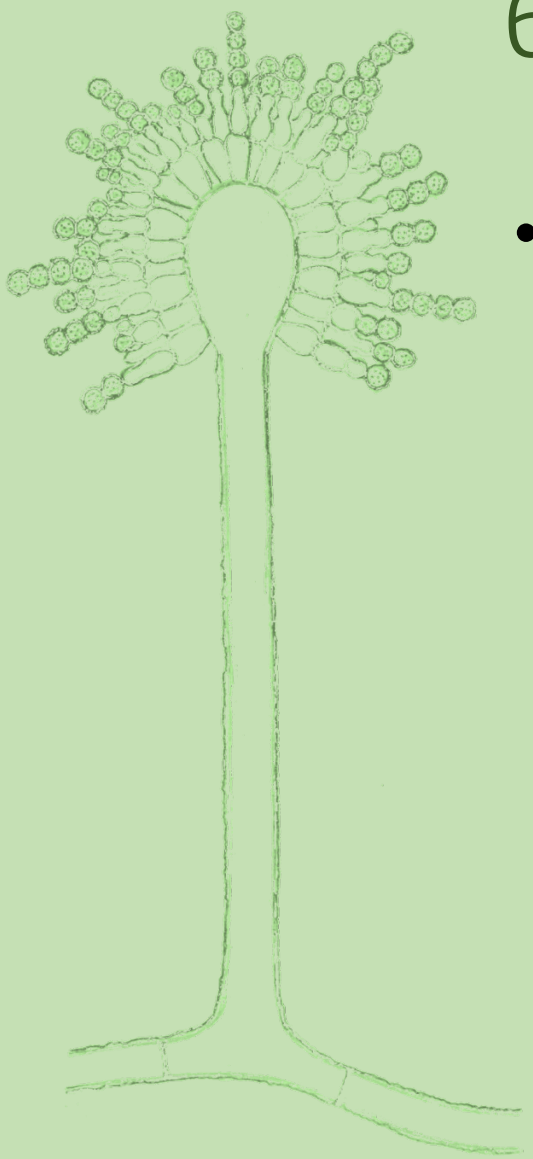
6054-42000-026-00D: Objective 1

- Known VOC study: future investigations
  - Test combination of atox VOCs against toxigenic strains
    - 3-Octanone + Decane; 2-3-Dihydrofuran + Decane



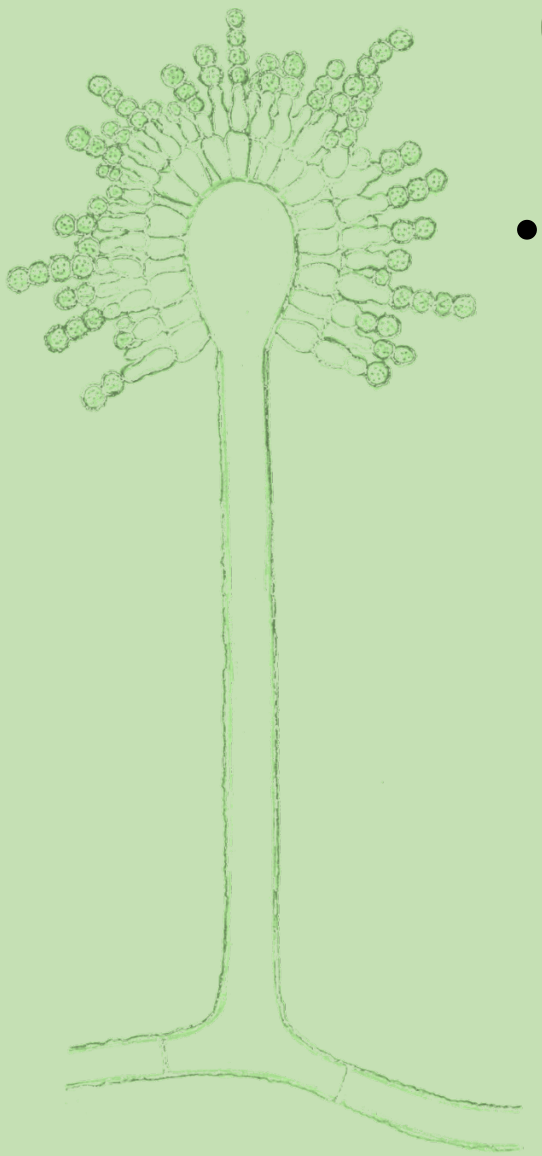
6054-42000-026-00D: Objective 1

- Unique VOC identification study (ongoing)
  - Solid studies involved GC-MS vials (slant cultures)
  - Use of GC-MS to study headspace VOCs
  - Individual strains for unique tox/atox VOCs
    - Repeat Known VOC study
  - Paired strains for interactome headspace VOCs
    - Any new VOCs produced?



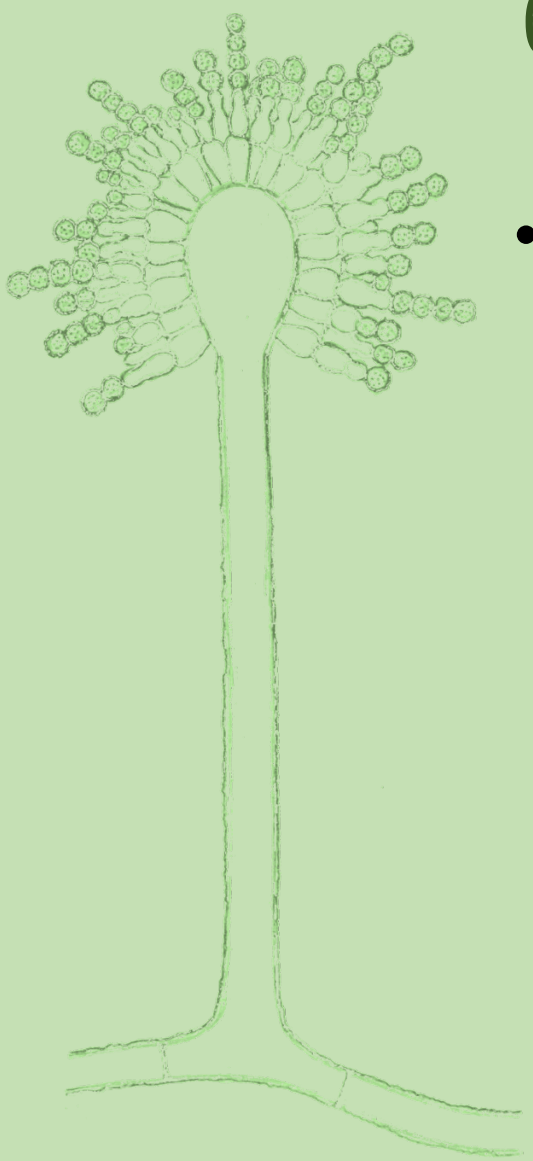
6054-42000-026-00D: Objective 1

- Unique VOC identification study (findings on CMA)
  - LA1: Eicosane; Hexanal; N Heptanal; (E)-2-Decenal
  - LA2: 11-Tricosene; (E)-2-decen-1-ol
  - LA3: Tetradecanal; Tridecane
  - LA4: Nonadecane



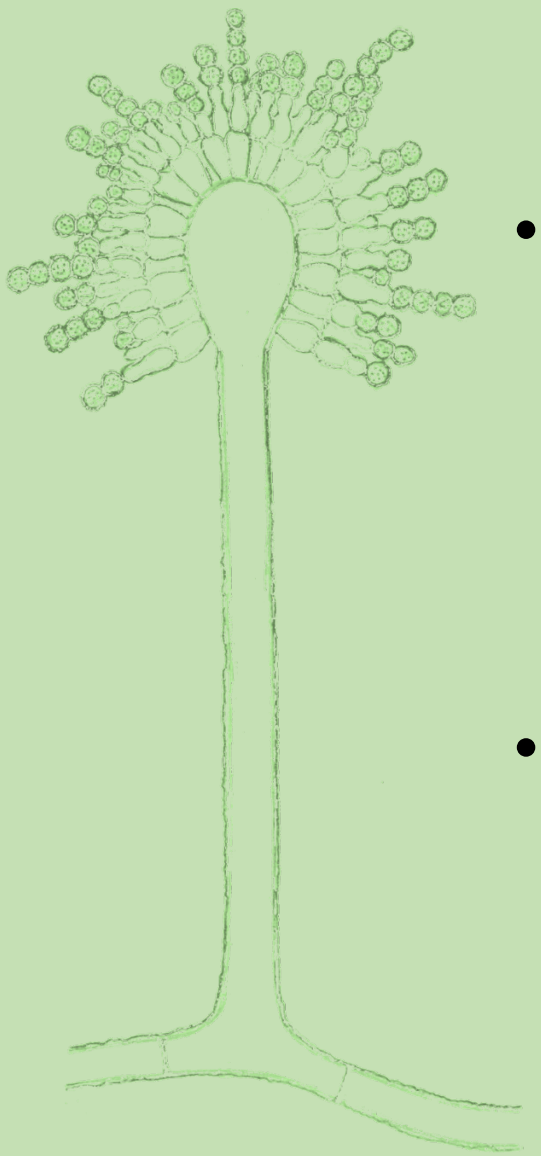
6054-42000-026-00D: Objective 1

- Unique VOC identification study (findings on CZ)
  - LA1: Octyl formate; Selina-3,7(11)-diene; .beta.-Cubebene; Valencene; 7-epi-.alpha.-selinene; Hexadecane; (+,-)-.beta.-Himachalene; Benzene, 1,2,4-trichloro-5-methoxy-; Tetradecane; Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)-; Ethanone, 1,1'-(6-hydroxy-2,5-benzofurandiyl)bis-; Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethylidene)-, (4aR-trans)-
  - LA2: Oxacyclotetradecane-2,11-dione, 13-methyl-; Naphthalene, decahydro-1,6-dimethyl-
  - LA3: Cyclopropane, pentyl-; Cyclopentane, 1-ethyl-1-methyl-; 1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-, [1aR-(1a.alpha.,4a.beta.,7.alpha.,7a.beta.,7b.alpha.)]-; Ledene; Octanal; .delta.-Selinene
  - LA4: Phenol, 3-(1,1-dimethylethyl)-; Phenol, 2-(1,1-dimethylethyl)-



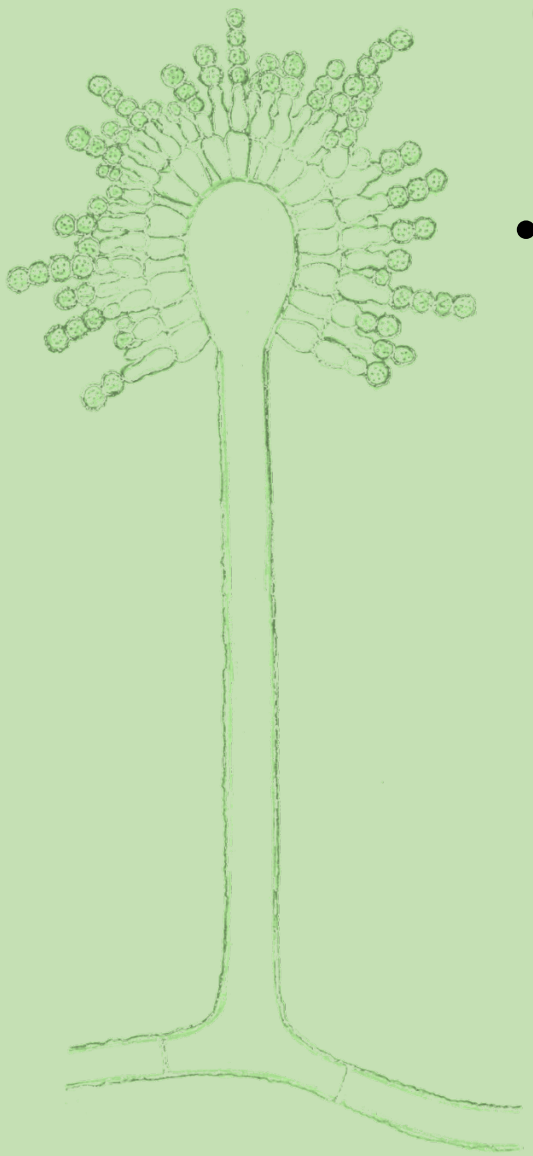
6054-42000-026-00D: Objective 1

- Unique VOC identification study (findings on YES)
  - LA1: Hexadecenoic acid, Z-11-; .alpha.-Gurjunene; 1,3-Octadiene
  - LA2: Cyclopentane, 1-ethyl-2-methyl-; 1-Undecene; 2-Heptanone
  - LA3: 9,12-Octadecadienoic acid (Z,Z)-; Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-, (4aR-trans)-; 2-Heptanone
  - LA4: 2-Heptanone



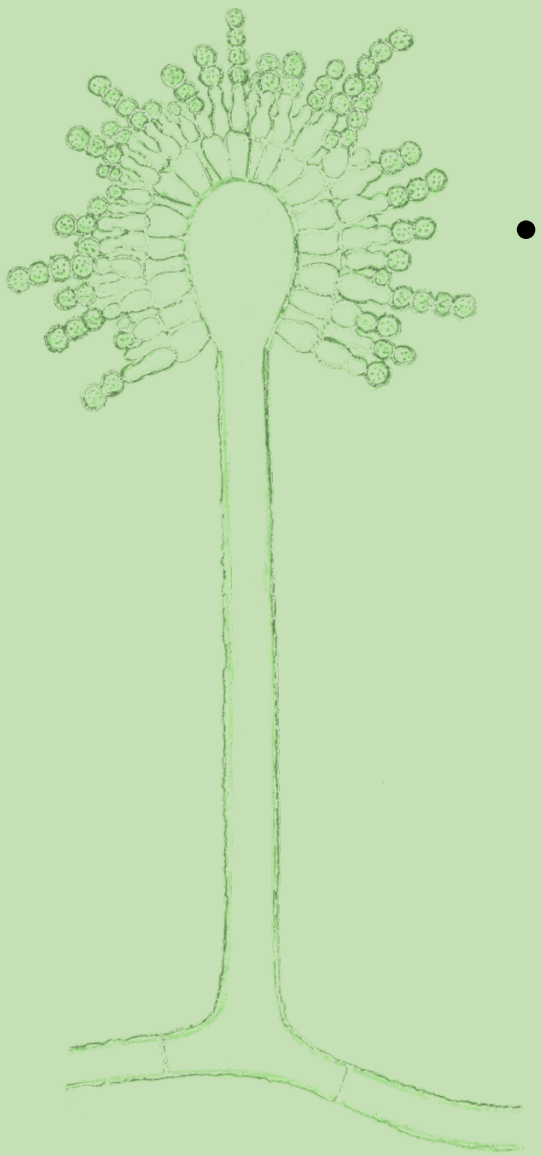
6054-42000-026-00D: Objective 1

- Unique VOC identification study: conclusions for VOCs produced by individual strains
  - VOCs captured vary based on replicate, substrate and isolate
  - More unique VOCs produced while strains growing on CZ
  - LA4 produced fewest unique VOCs
- Take Home: Unique VOCs for LA strains do not appear to be the same as those examined in Known VOC studies from DeLucca et al.



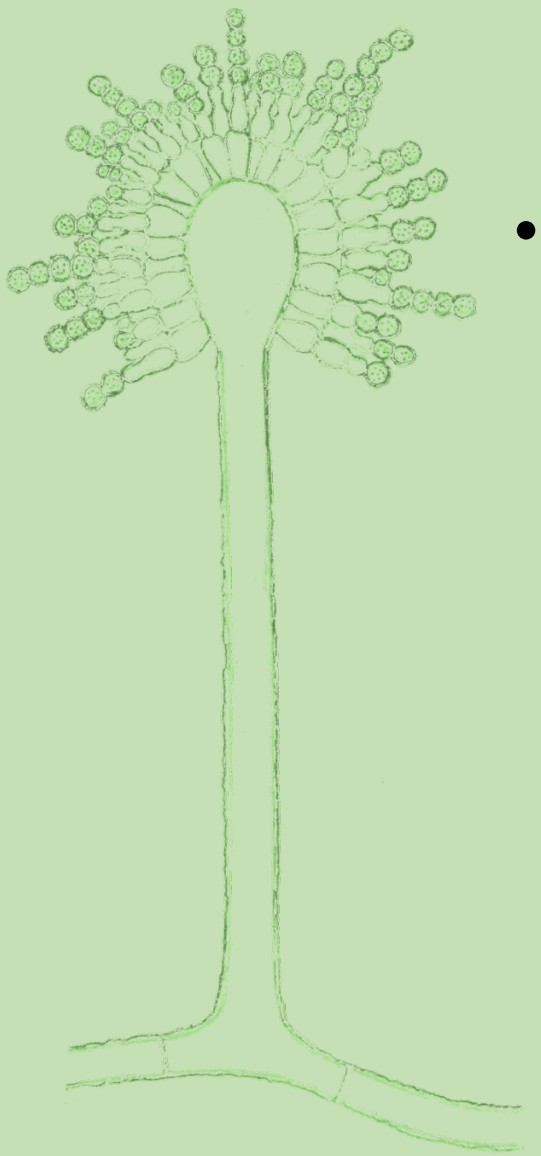
6054-42000-026-00D: Objective 1

- Unique VOC identification study: future investigations
  - Still determining VOCs produced when strains paired
  - Conduct new Known VOC study
    - Compounds from LA strains to test on LA strains
    - Tox VOCs: (E)-2-Decen-1-ol, Tridecane, Nonadecane, 2-Heptanone
    - Atox VOCs: Valencene, Hexadecane, Tetradecane, Eicosane, (E)-2-Decenal



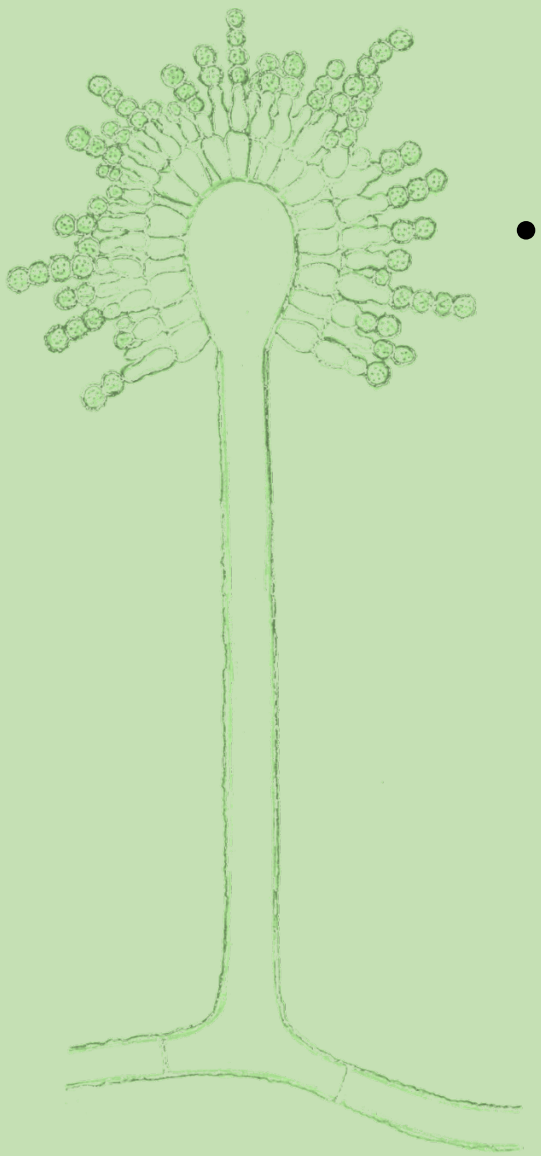
6054-42000-026-00D: Objective 1

- Thigmoregulation by atoxigenic *A. flavus* (ongoing)
  - Atoxigenic strain = Atox17 (LA1)
  - Aflatoxigenic strain = KD53
  - Conditions for gene expression study (RNA-Seq)
    - Atox17 alone
    - KD53 alone
    - Atox17+KD53
    - 30h vs. 72h time points



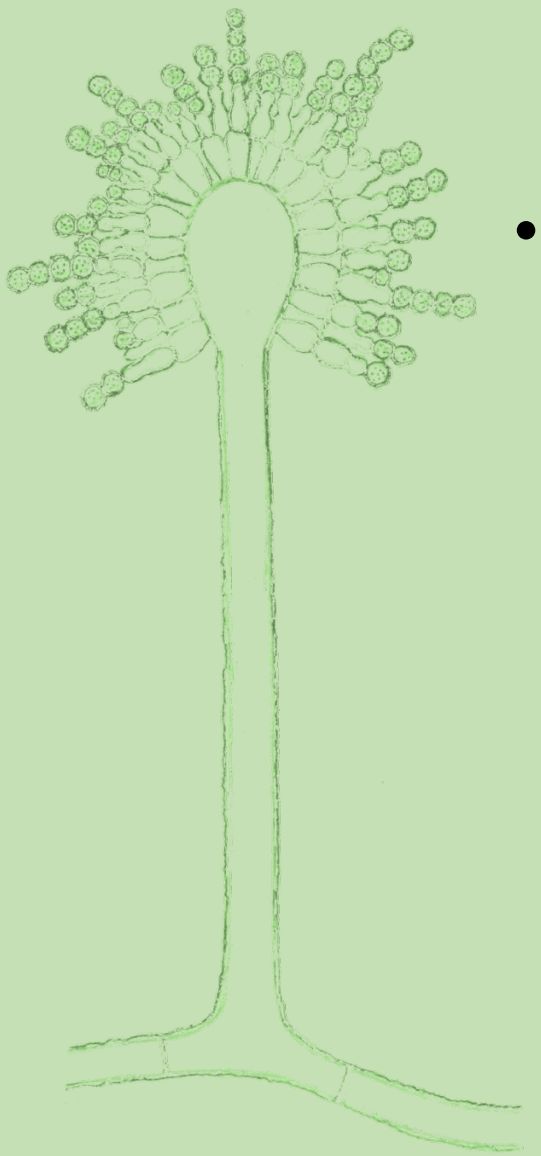
6054-42000-026-00D: Objective 1

- Thigmoregulation by atoxigenic *A. flavus* (ongoing)
  - When looking at increased gene expression after touch, several genes that cluster in the genome are more highly expressed in 17 and when 17 is grown with 53, compared to 53 alone
  - One of these genes is a putative secondary metabolite production gene, two genes are polyketide synthases, one gene is an efflux pump
    - Suggests production of a compound that is excreted out of the cell (i.e. extrolite)



6054-42000-026-00D: Objective 1

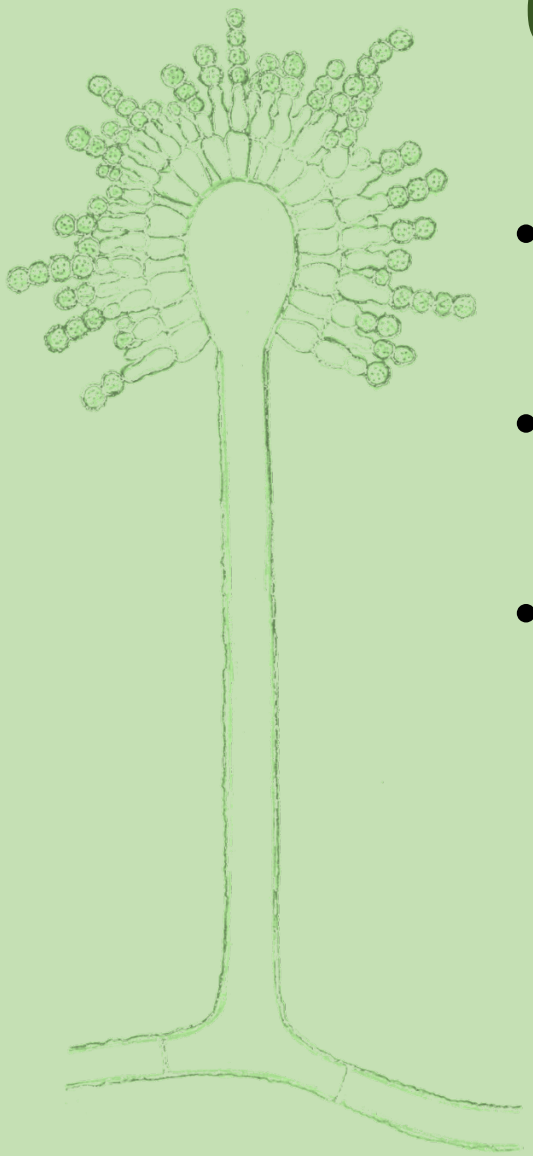
- Thigmoregulation by atoxigenic *A. flavus* (ongoing)
  - Not much clustering observed for highly expressed genes in individual 17, compared to 53
  - Some aflatoxin cluster genes not expressed in Atox17
  - Most aflatoxin cluster genes were expressed (at very low levels) for 17+53 (evidence of growth by 53)
  - At 72h, even less expression of aflatoxin cluster genes for 17+53
    - Possible evidence of greater inhibition after more touch and/or growth by 17



6054-42000-026-00D: Objective 1

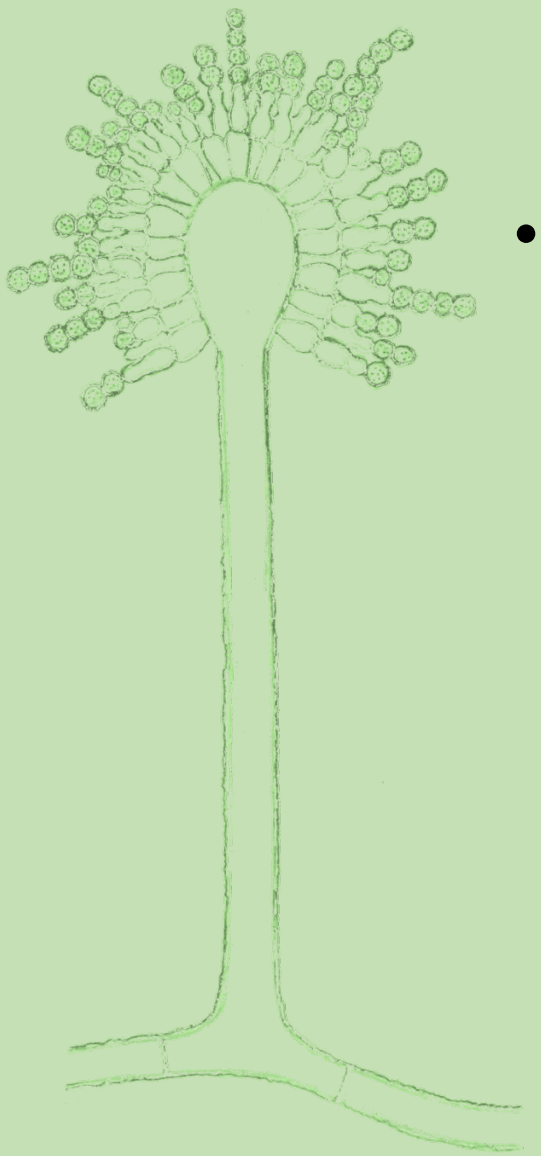
- Thigmoregulation by atoxigenic *A. flavus* (ongoing)
  - However… SNP calling for 17+53 showed none of the 53-unique SNPs present
  - Expression data overall indicates that 17 dominates 53
  - Possible Take Home: Touch inhibition may not just turn off aflatoxin production, it may prevent growth of toxigenic strain altogether

Objective 2: Determine the role of mating-type genes and climatic (environmental) stressors on the ability of *A. flavus* biocontrol strains to compete, survive and recombine, thereby impacting the persistence and efficacy of these strains



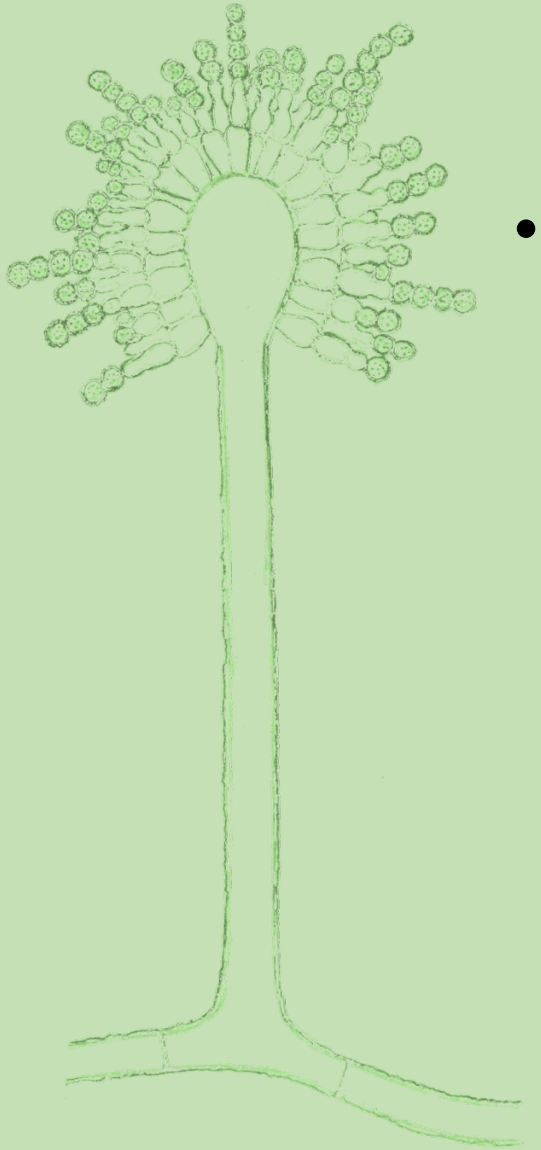
6054-42000-026-00D: Objective 2

- *A. flavus* MAT gene manipulation study
  - Moore, Cary, Lebar, Uka (U Ghent), Di Mavungu (U Ghent)
- Impact of climate stressors on biocontrol strains
  - Moore, Gilbert
- Persistence of biocontrol strains
  - Moore, Weaver (ARS-Stoneville, MS)



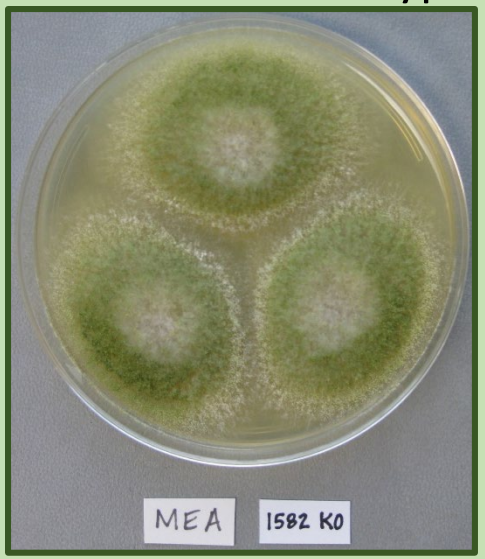
6054-42000-026-00D: Objective 2

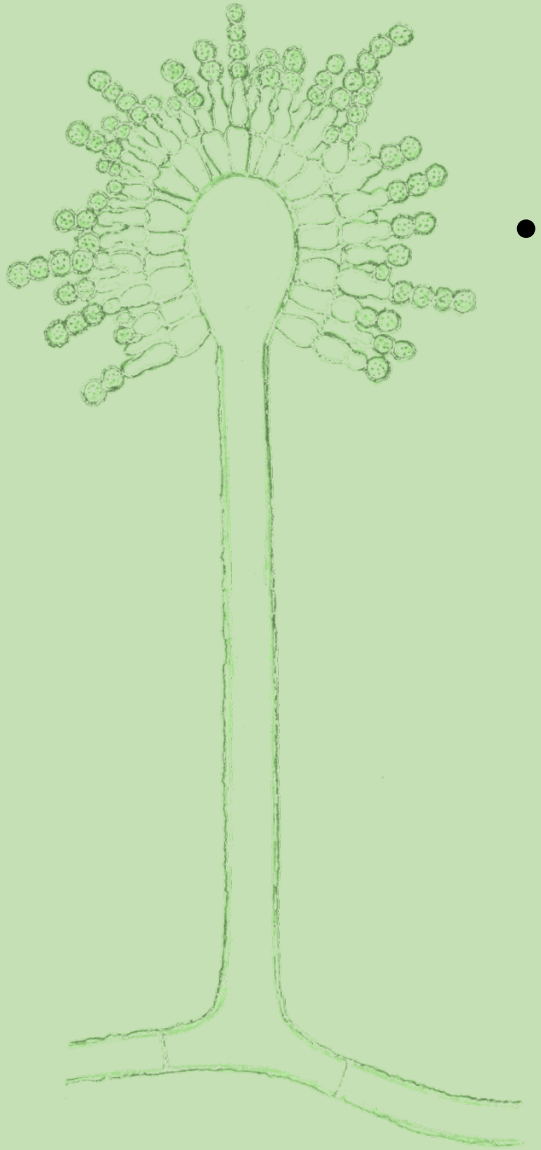
- *A. flavus* MAT gene manipulation study (ongoing)
  - SRRC 1582 (MAT1-1; AF+/CPA+); AF36 (MAT1-2; AF-/CPA+)
    - MAT KO mutants
    - MAT swap mutants
    - Isogenic controls (phl+, ptr+)
  - Growth and morphology studies
  - Mating studies (MCA vs. CZ @ 6mo)
  - Metabolite studies (individual strains; YES vs. MCA)
  - Metabolite studies (mixed cultures; YES vs. MCA)



6054-42000-026-00D: Objective 2

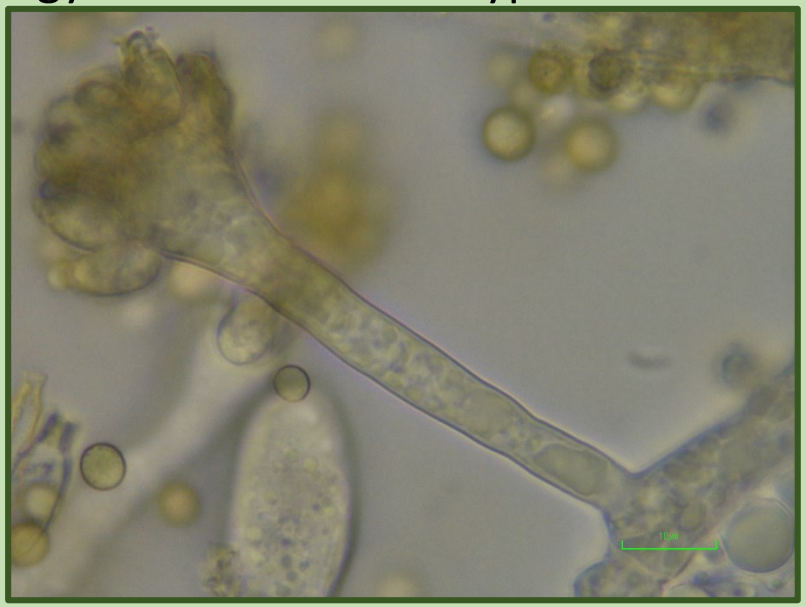
- *A. flavus* MAT gene manipulation study findings
  - Growth and morphology (so far)
    - 1582 KO mutants seem consistent with wild type

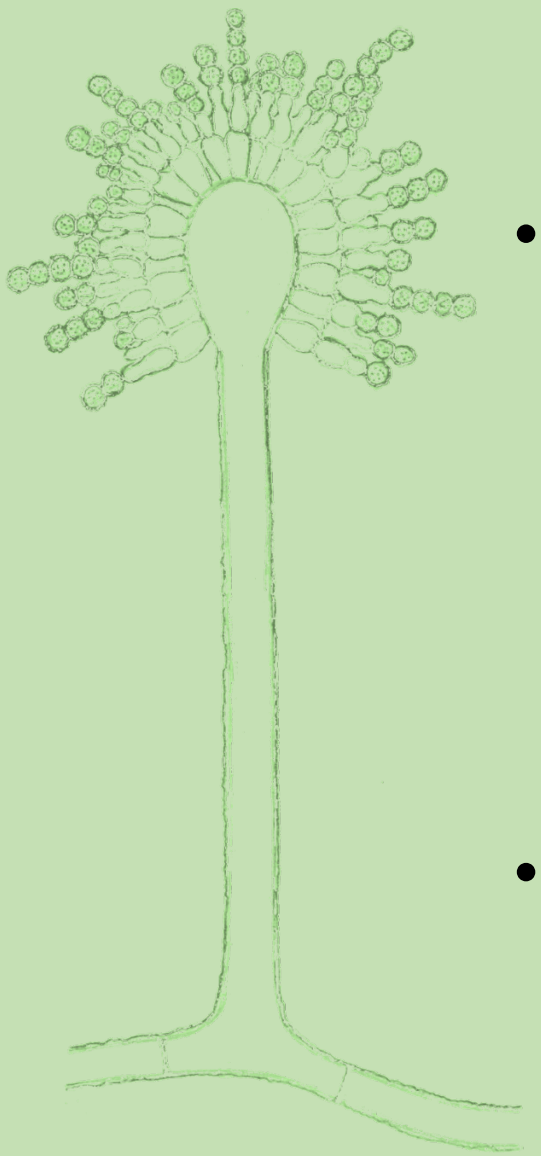




6054-42000-026-00D: Objective 2

- *A. flavus* MAT gene manipulation study findings
  - Growth and morphology
    - 1582 S mutant growth and morphology differs from wild type

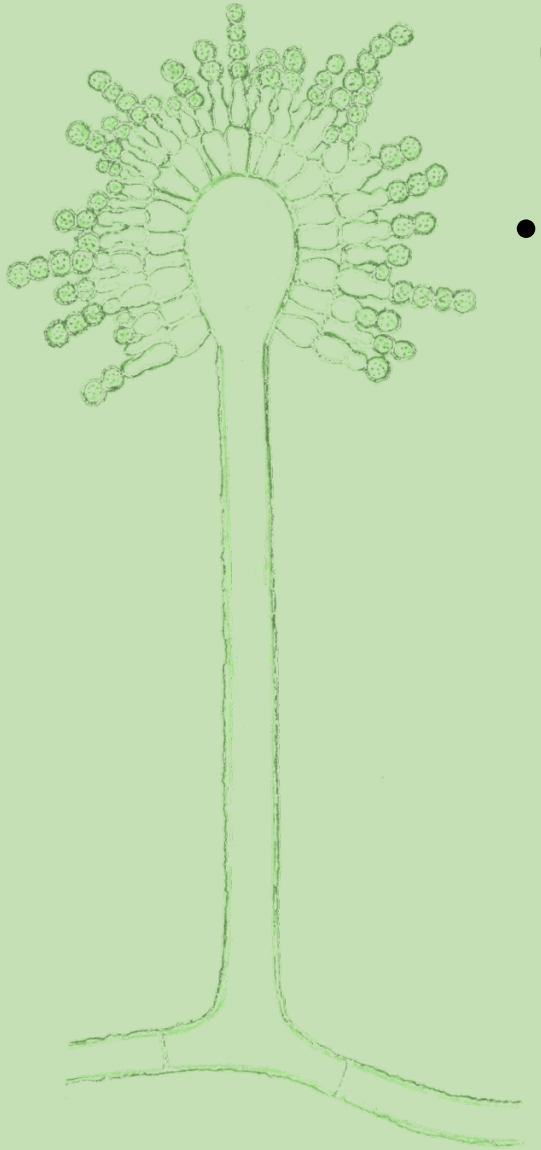




6054-42000-026-00D: Objective 2

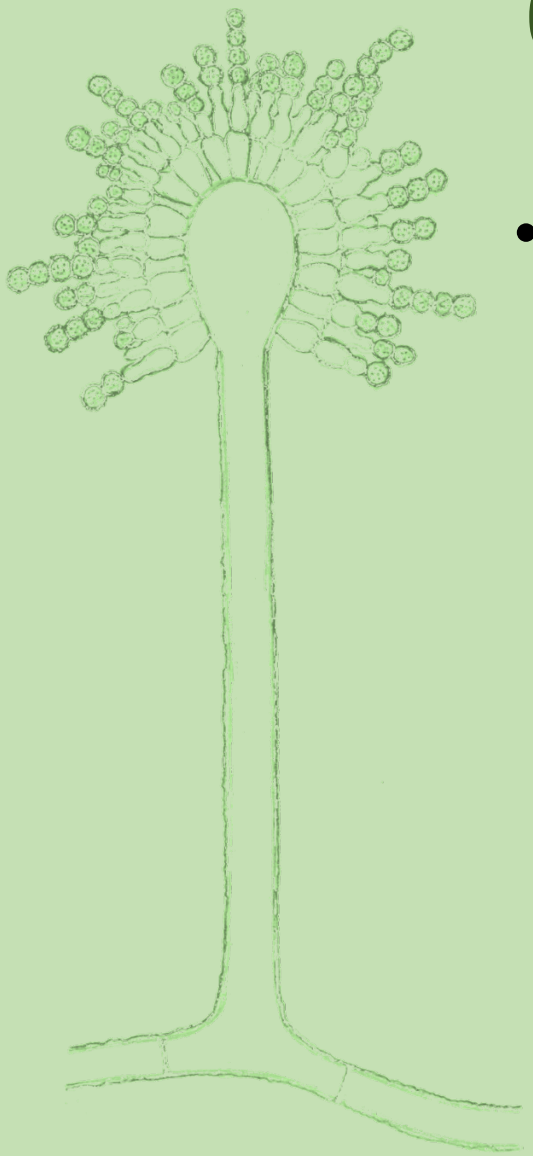
- *A. flavus* MAT gene manipulation study findings
  - Mating studies
    - Crosses involving KO and S mutants yielded no evidence of sex
    - Successful crosses yielded more evidence on MCA than CZ
    - Mostly immature or aborted cleistothecia observed
    - Only WTxWT on MCA yielded healthy, mature cleistothecia
  - Metabolite studies (underway)

- Take Home: *MAT1-1* gene not integral to growth and morphology for 1582; swap w/ AF36's *MAT1-2* gene does impact 1582 morphology



6054-42000-026-00D: Objective 2

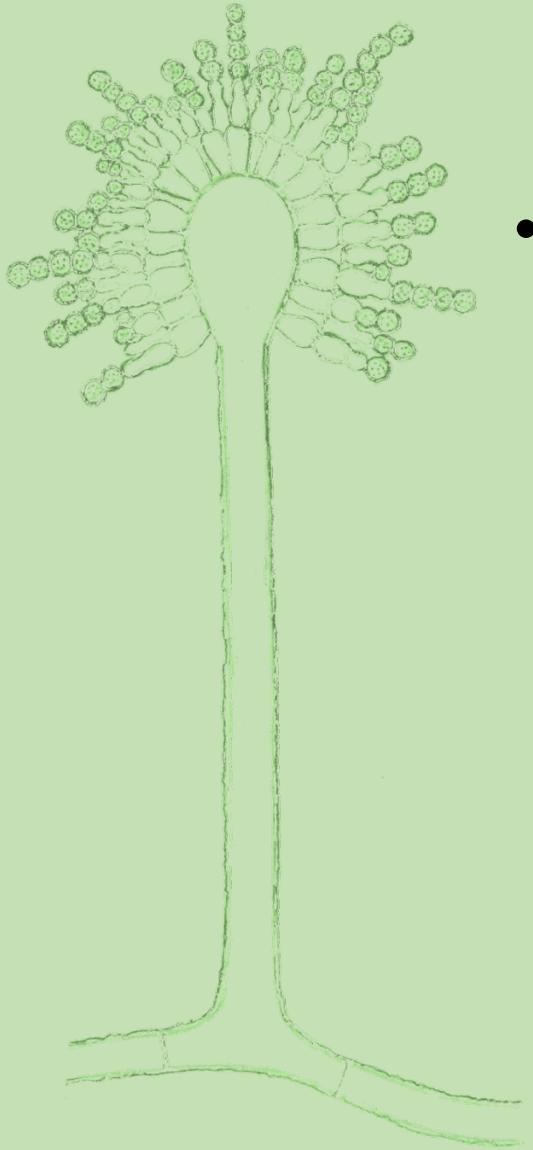
- Impact of climate stressors on biocontrol strains
  - AF+ strains paired with AF- strains (mixed spore suspension)
    - AF70, NRRL 3357, SRRC 1582 vs. Atox17, AF36, K49
  - Colonies incubated under conditions involving
    - Low (0.91 a_w) vs high (0.99 a_w) water activity in substrate
    - Low (350 ppm) vs high (1000 ppm) CO_2 levels
    - Low (25_n-30_d ^oC) vs high (32_n-37_d ^oC) temperature
  - Growth, aflatoxin and qPCR analyses underway
    - Some AFB_1 reduction observed



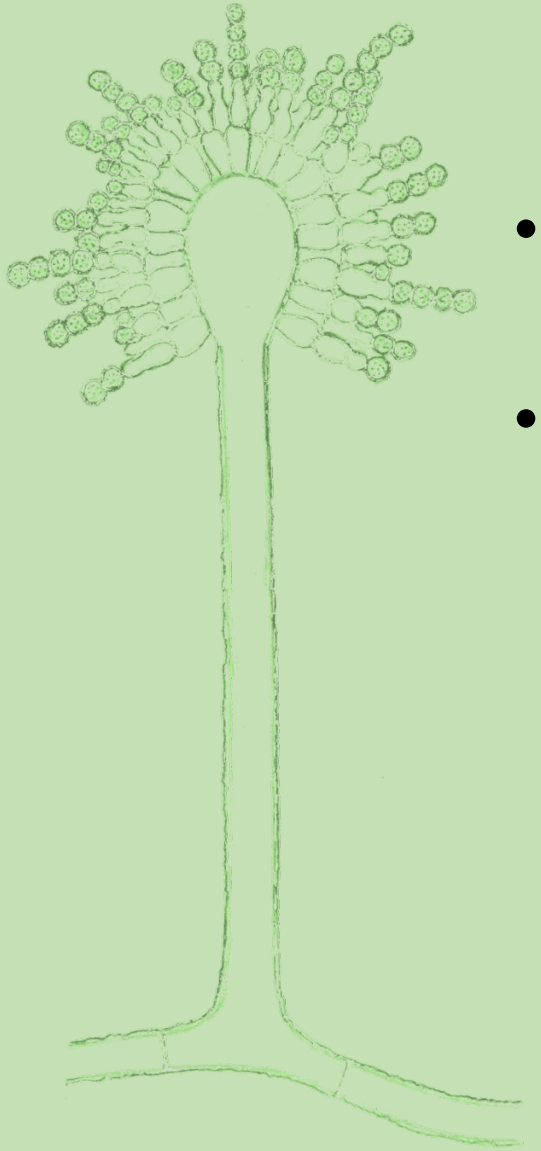
6054-42000-026-00D: Objective 2

- Impact of climate stressors on biocontrol strains
  - Mating experiments
  - Incubated under conditions involving
    - Low (0.91 a_w) vs high (0.99 a_w) water activity in substrate
    - Low (350 ppm) vs high (1000 ppm) CO_2 levels
    - Low (25_n-30_d ^oC) vs high (32_n-37_d ^oC) temperature
  - Will climate stress increase frequency of sex/recombination?
  - Will climate stress decrease time scale of sex/recombination?
  - Will more healthy/mature fruiting bodies and ascospores result?

6054-42000-026-00D: Objective 2

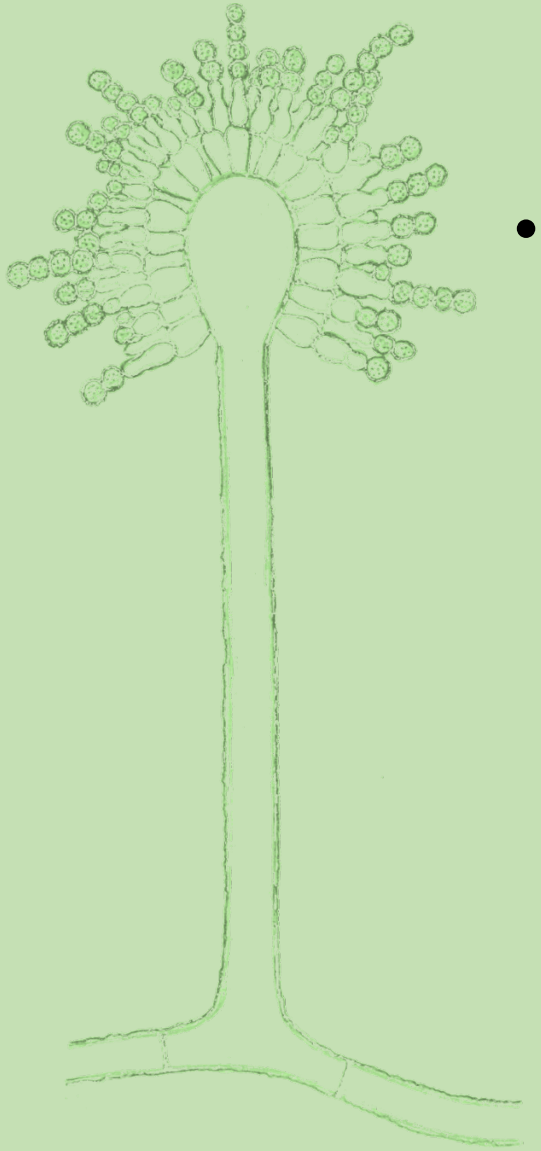


- Persistence of biocontrol strains (eGFP)
  - Serial culture of K49-GFP
    - Persistence of fluorescence up to 80 weeks
  - Growth chamber and greenhouse studies



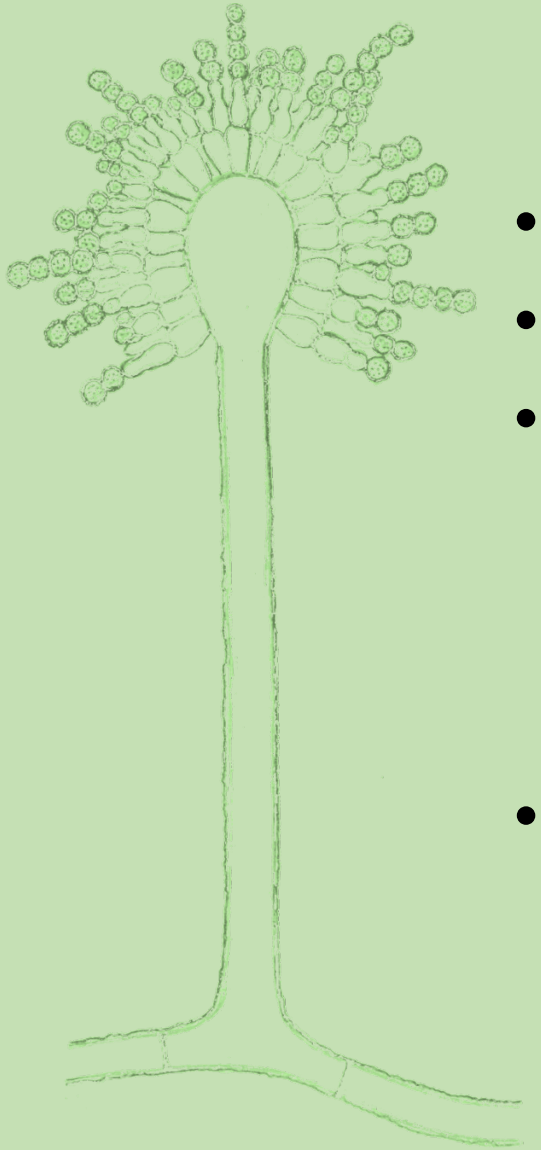
Other Ongoing Studies

- Recombination study with *A. flavus* familial sample
  - Gilbert, Uka (U Ghent), Di Mavungu (U Ghent)
- Genome sequencing for aflatoxigenic species
  - M Gilbert
  - 15 Type strains; several *A. flavus*



Next CRIS Cycle

- Microbiome study
  - Rajasekaran, Cary, Gilbert, Rivers (ARS-Gainesville, FL), Chalivendra (LSU)
  - Corn silk; kernel
  - eGFP tagged biocontrol strain (K49)
  - mCherry (RFP) tagged toxigenic strain (Tox4; LA2)
  - Impacts on microbiome (and vice verse)



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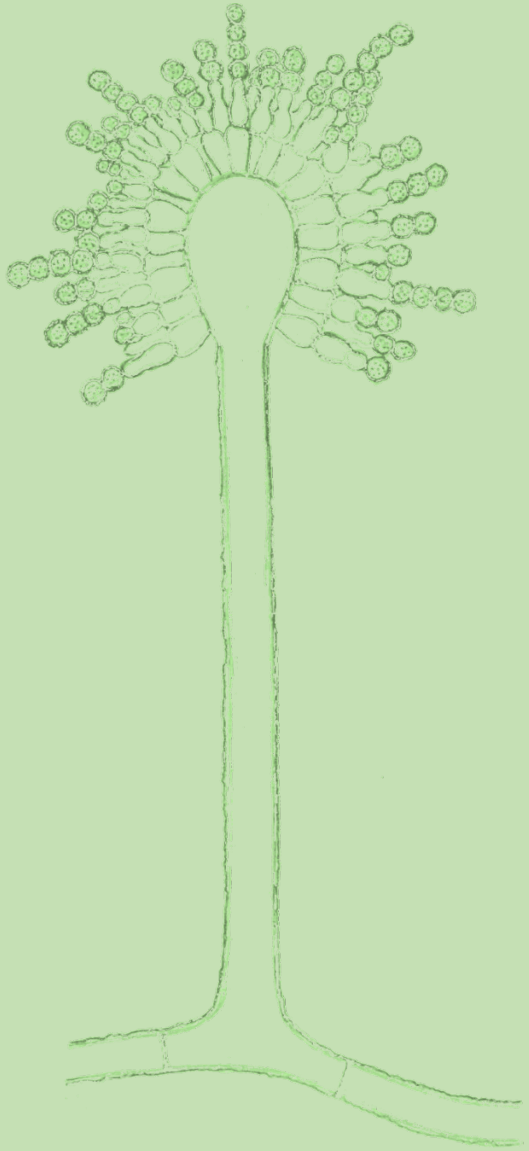
  - Grimm, Lloyd (ARS-SRRC)
  - Uka, Di Mavungu (U Ghent)
  - Weaver (ARS-Stoneville, MS)
- Summer Student Workers
  - Gauthier (2016), Warfield (2018)



Questions
and/or
Wisdom to convey?