Rainforest Expedition and Laboratory (REAL)

Our Search for Microbiological and Chemical Diversity

Program Sponsored by the
Howard Hughes Medical Institute
National Science Foundation

Project Ownership

To have significant impact the program must :

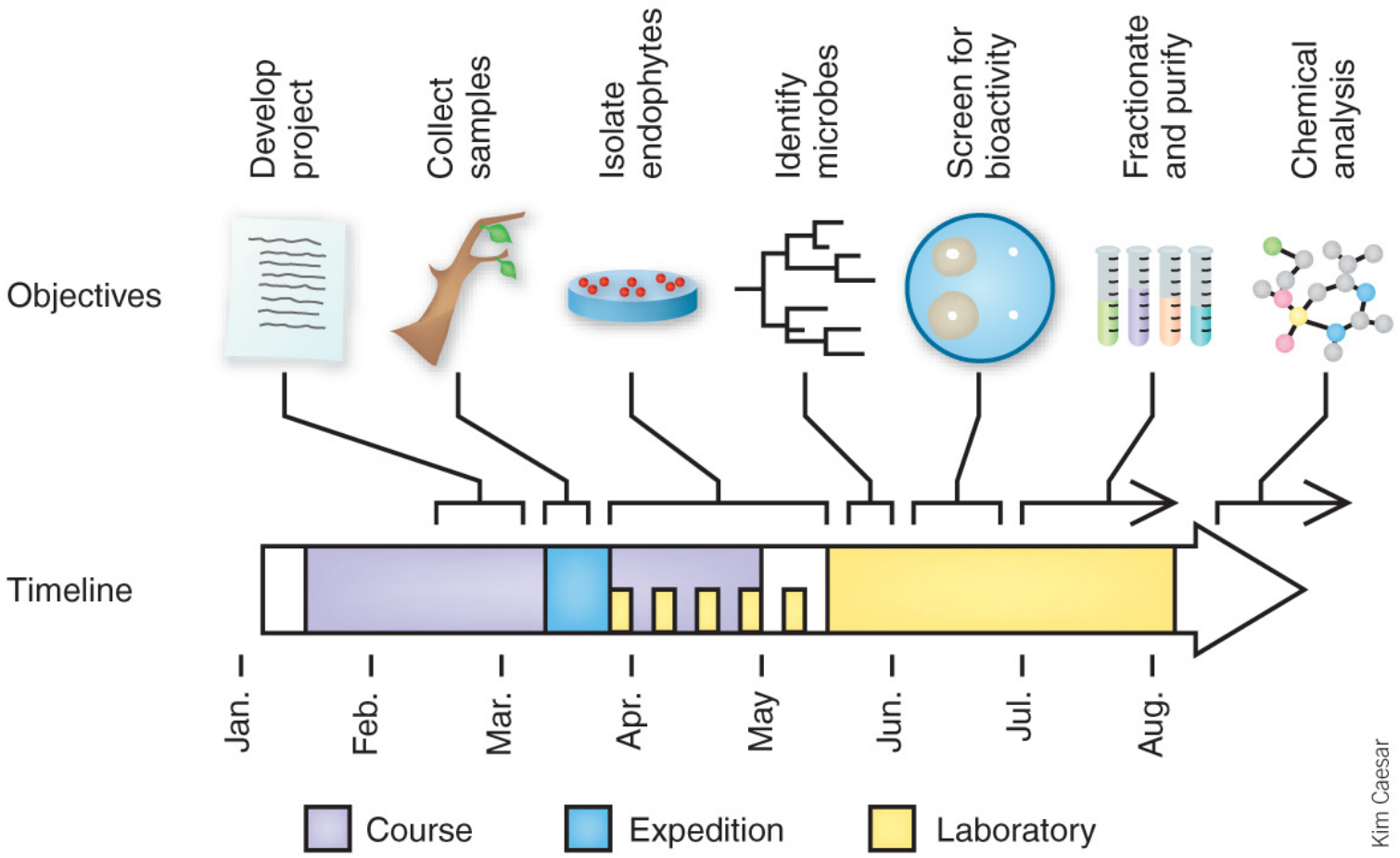
- Give students control over the direction of the project
- Projects with progressively more complex success points
- Provide uncertainty about experimental outcomes
- Provide opportunities to design and explore
- Be large and flexible enough that it can be taken in many different directions

ENDOPHYTES

A microorganism (bacterial or fungal) that is established in a plant and does not cause any overt symptoms or any apparent tissue damage.

1. There are over 1 million plant associated fungi,
2. Majority of these fungi are unknown (>80%)
3. Highest incidence in places of high plant biodiversity
4. This biodiversity provides an easy entrance for students to experience project ownership and develop their own scientific questions

Timeline for Student Objectives



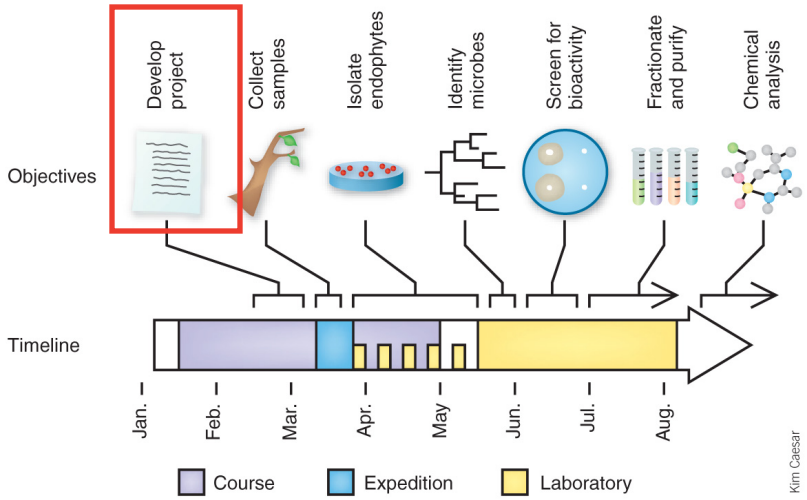


Figure 1 Approximate timeline for the progressive success points of the rainforest expedition and laboratory.

Student Collection Themes based upon Ethnobotany

- Plants used as fish poisons
- Plants used to treat snake bites
- Parasitic plants
- Plants used to ease childbirth
- Plants used in the treatment of infections
- Plants used as aphrodisiacs
- Plants with milky saps
- Plants used to stop bleeding
- Plants used as hallucinogens

Destination 2007: Heath River Peru

Six hours down river from Puerto Maldonado by motorized canoe

Class of 2007

15 Students, 2 Teaching Assistants, 1 High School Biology Teacher

Primarily Sophomores and Juniors


Destination 2008-2012
La Selva Lodge and Yasuni National Forest
Napo River, Ecuador

Class of 2008

18 Students: 3 seniors, 7 juniors, 8 sophomores

Class of 2009

15 Students: 6 juniors, 9 sophomores

Class of 2010

16 Students: 6 juniors, 10 sophomores

Class of 2011

16 Students: 2 seniors, 2 juniors, 11 sophomores, 1 freshman

Class of 2012

15 Students: 1 senior, 2 juniors, 12 sophomores

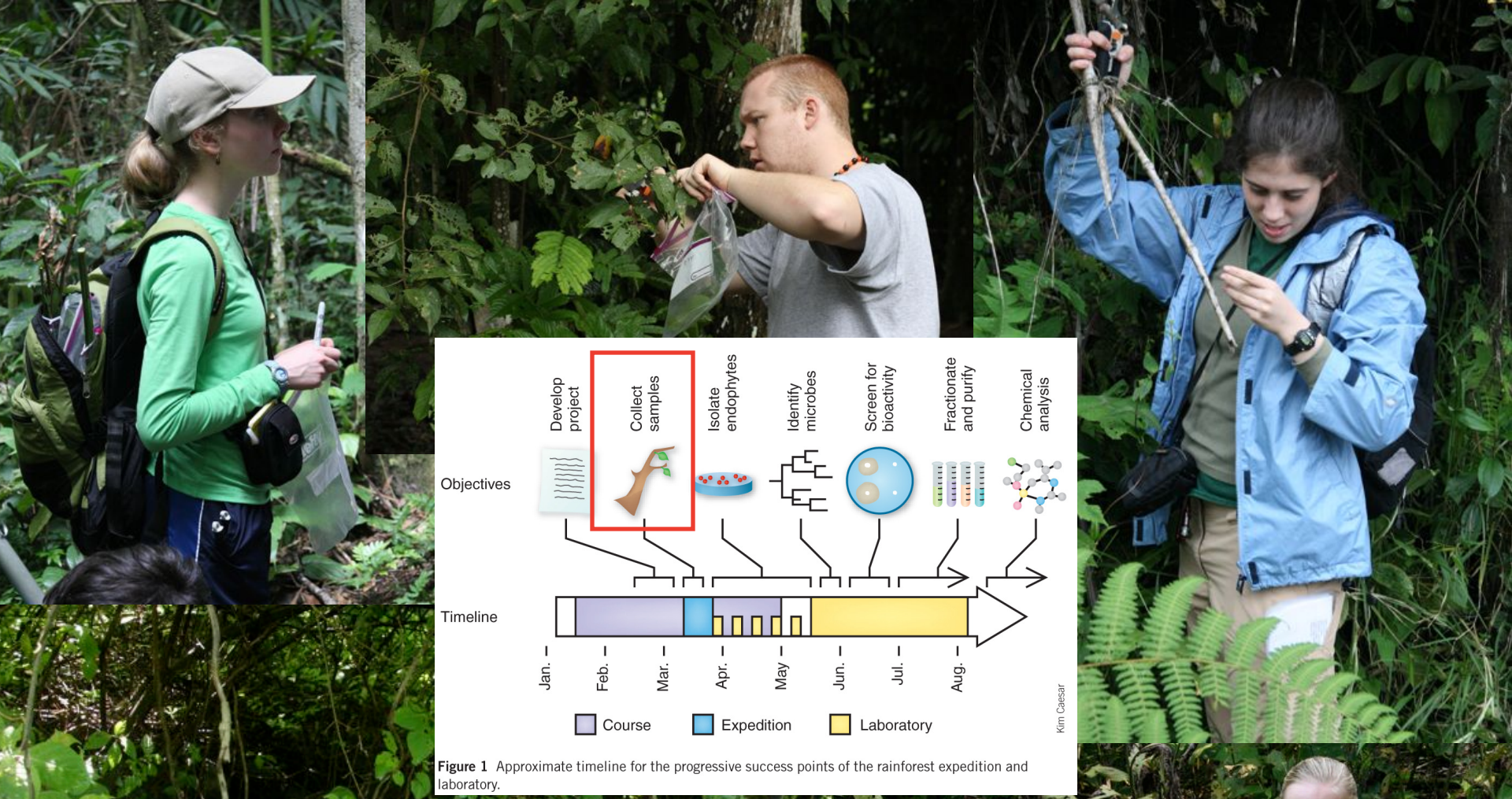


Figure 1 Approximate timeline for the progressive success points of the rainforest expedition and laboratory.

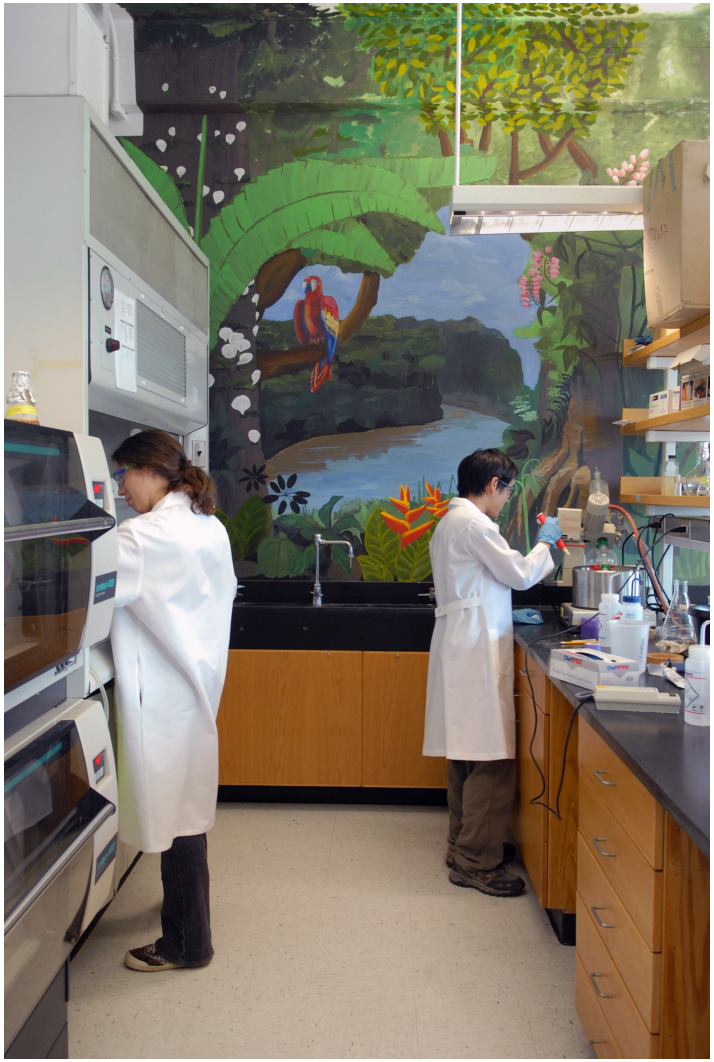
Each student selects 25 plants to sample

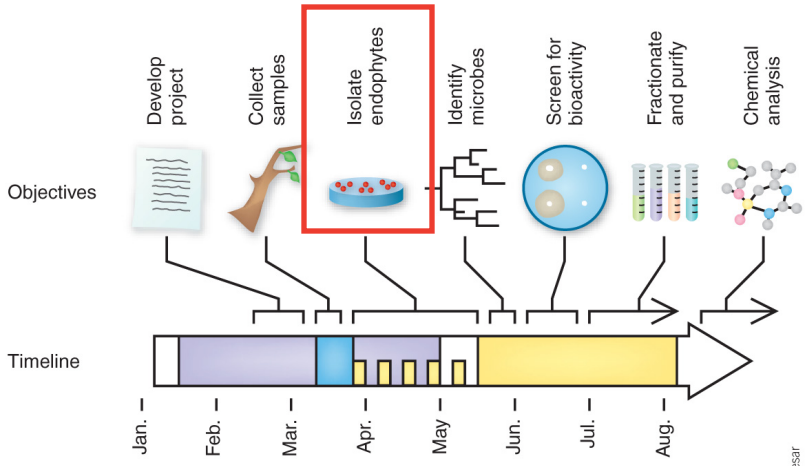
…But how to identify them?

Fer de Lance- The Snake

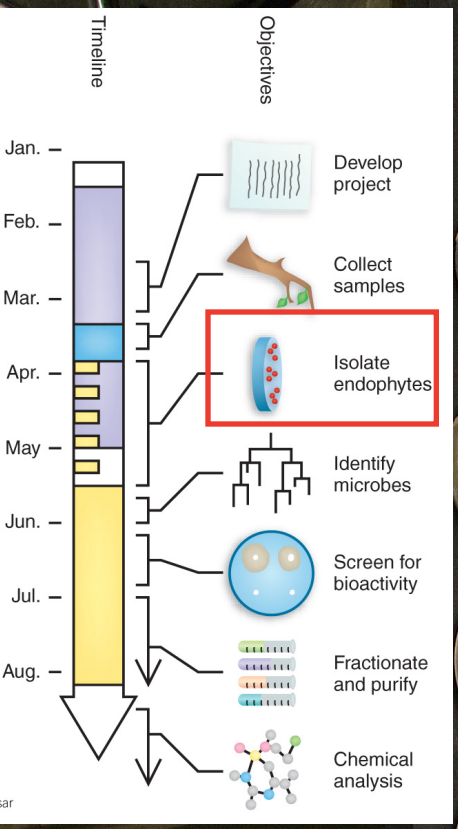
Fer de Lance- The Tree

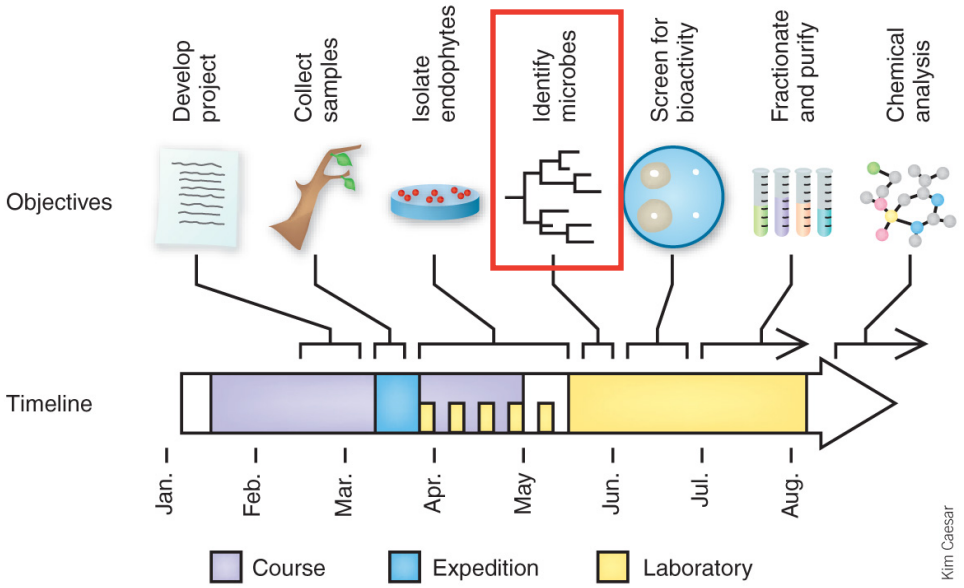
Endophyte Isolation





Endophyte


Timeline
Objectives
Jan.
Feb.
Mar.
Apr.
May
Jun.
Jul.
Aug.
Develop project
Collect samples
Isolate endophytes
Identify microbes
Screen for bioactivity
Fractionate and purify
Chemical analysis

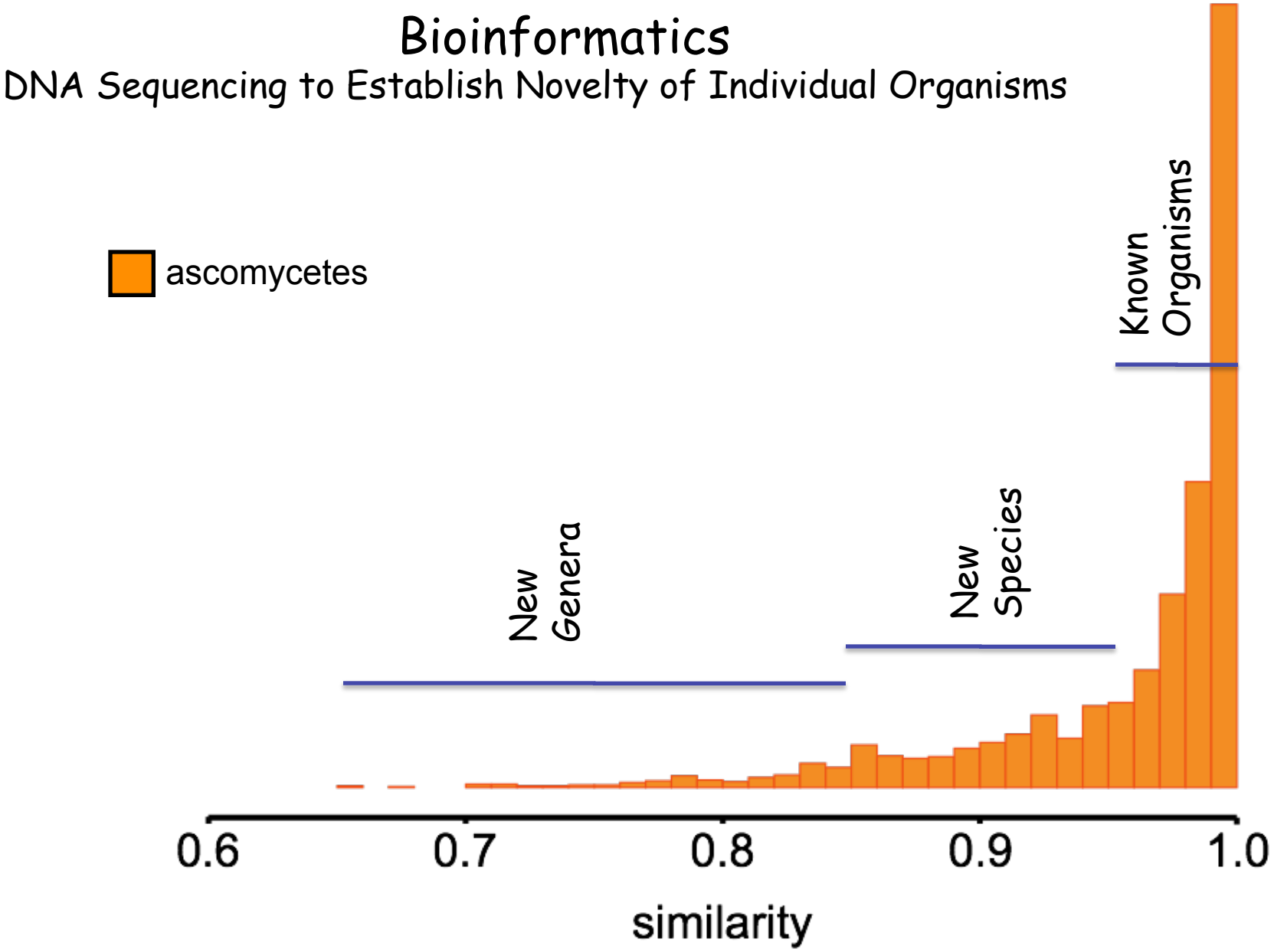


Microbial Characterization

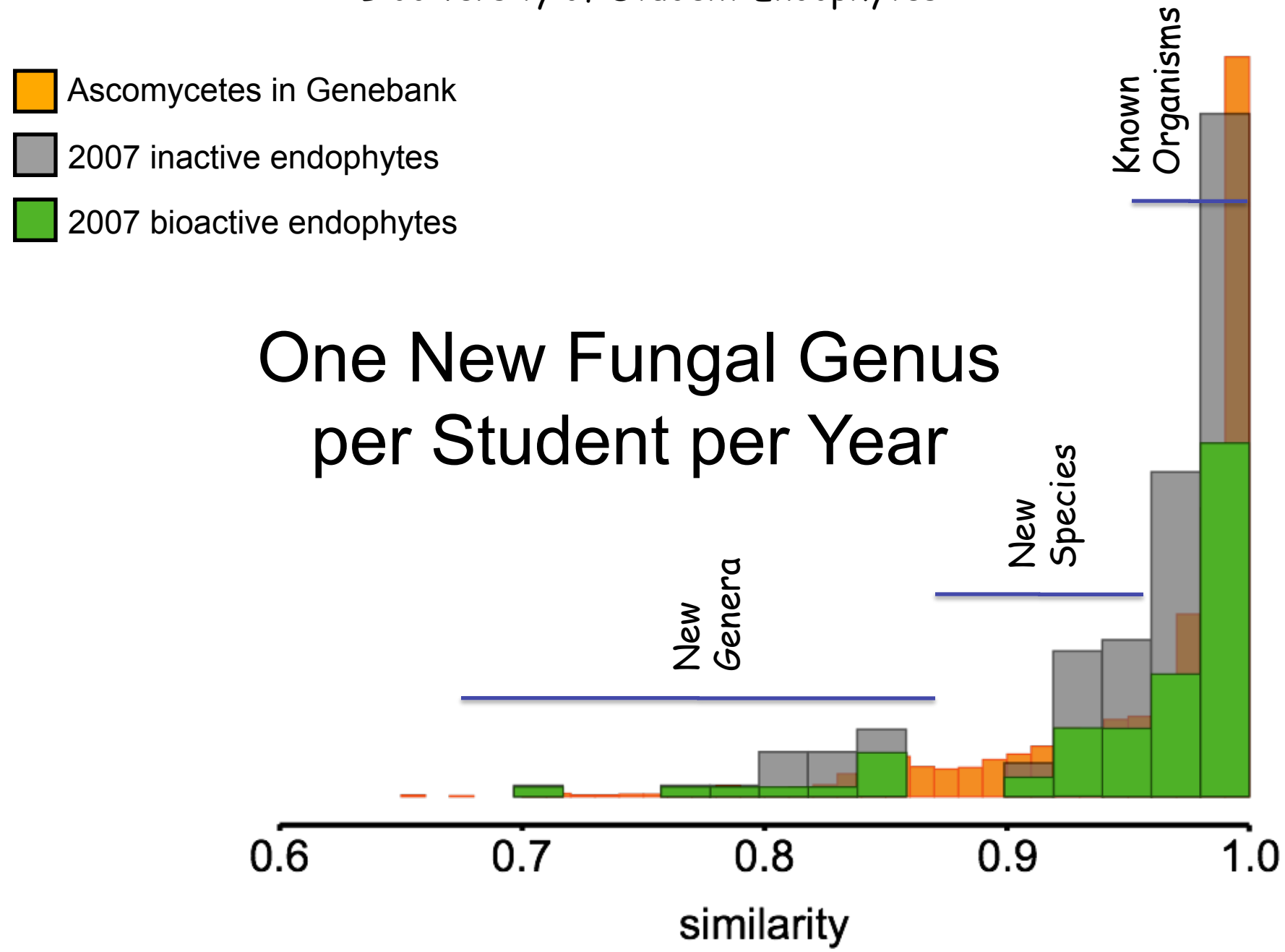
Can Undergraduates Find Something New?

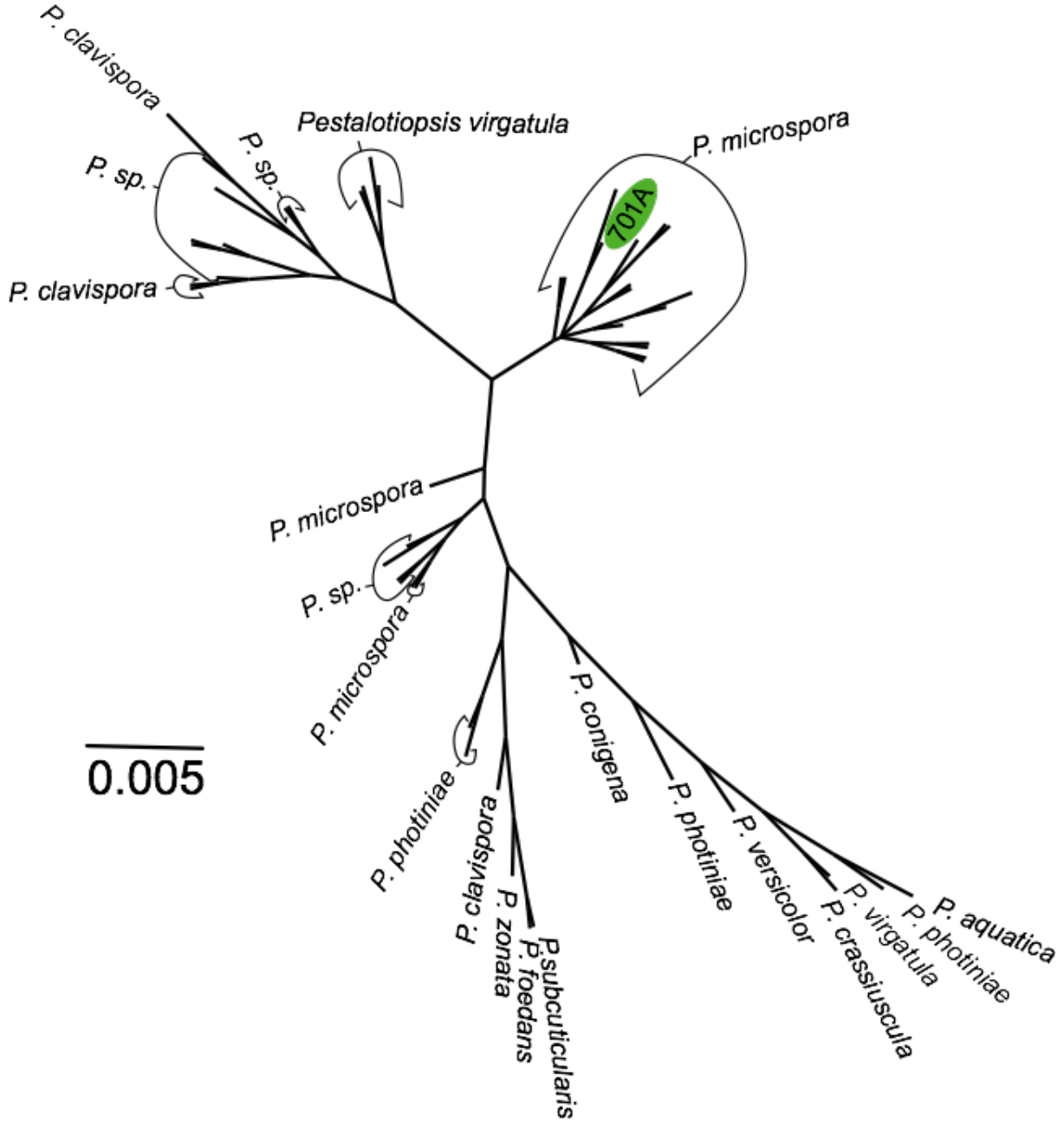
Bioinformatics

DNA Sequencing to Establish Novelty of Individual Organisms

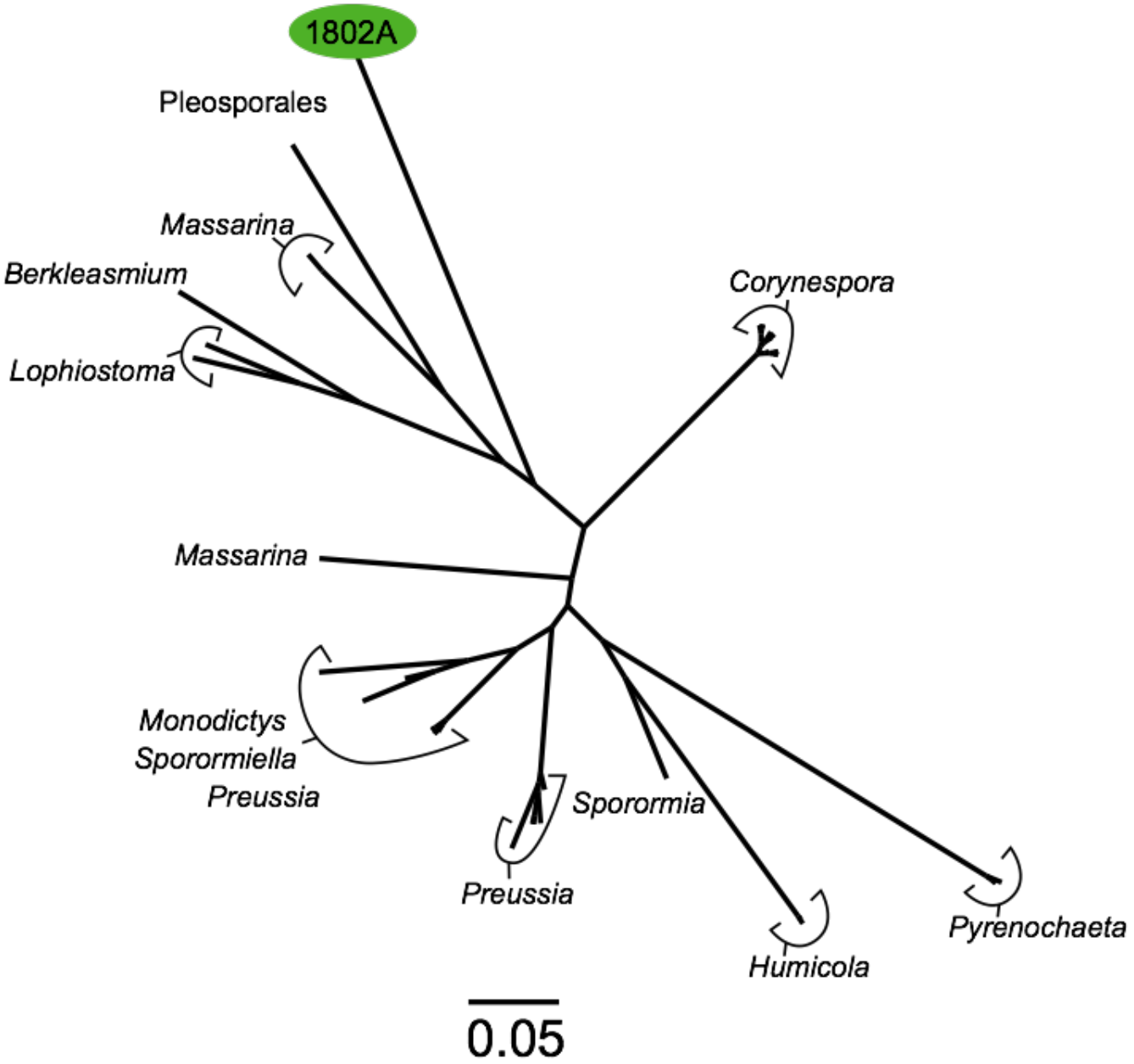


Biodiversity of Student Endophytes

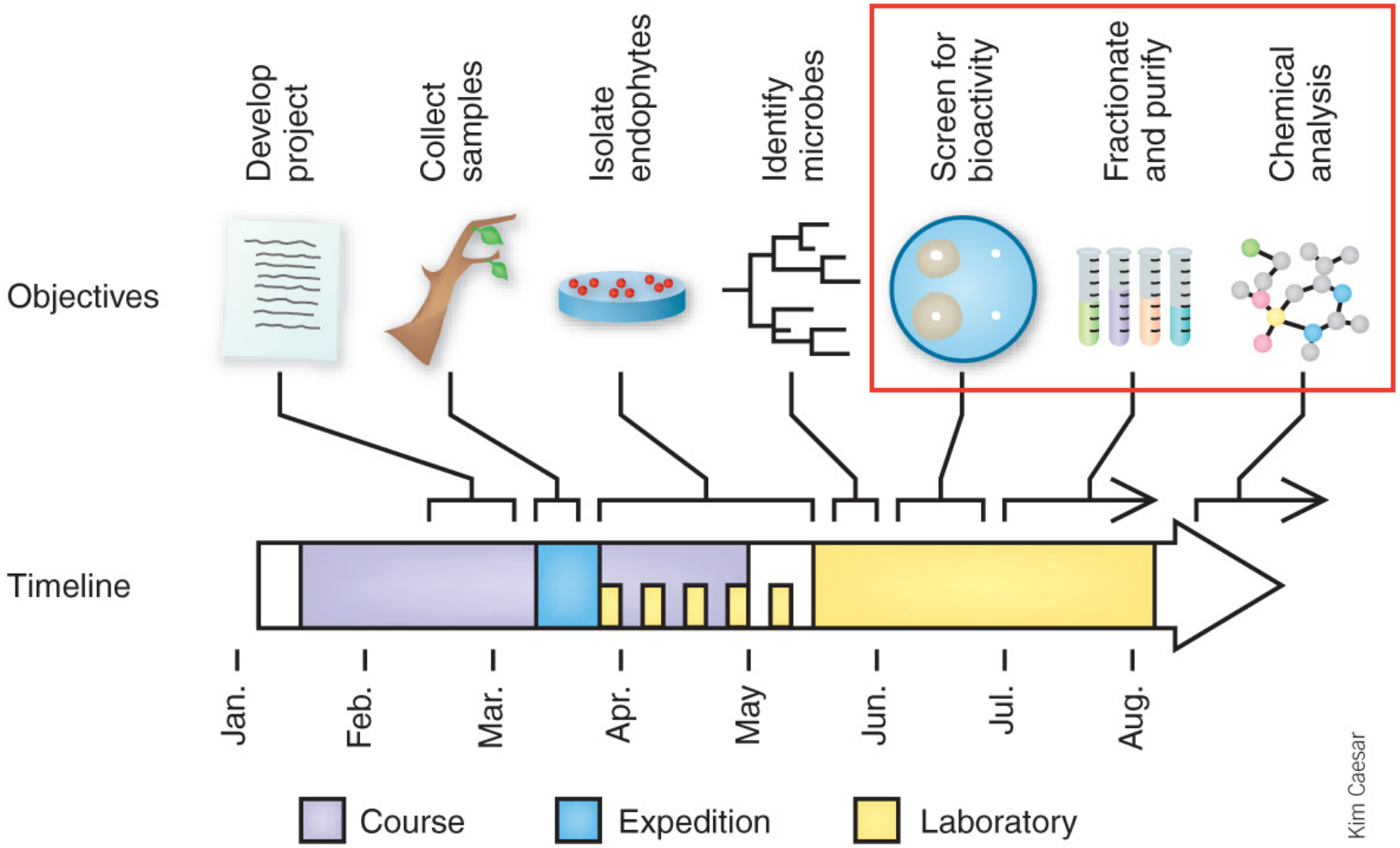



P. clavispora
P. sp.
P. sp.
P. clavispora
Pestalotiopsis virgatula
P. microspora
701A
P. microspora
P. sp.
P. microspora
0.005
P. photiniae
P. clavispora
P. zonata
P. foedans
P. subcuticularis
P. conigena
P. photiniae
P. versicolor
P. crassiuscula
P. virgatula
P. photiniae
P. aquatica

Students Have High Likelihood of Discovering New Fungal Genera



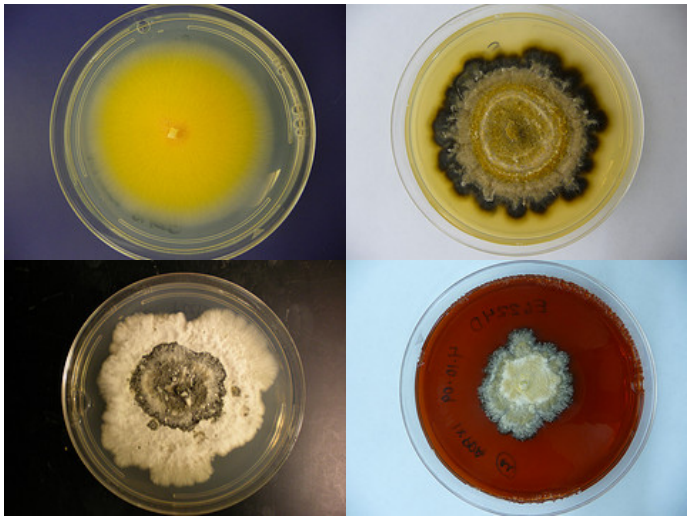
Where there is new Biology there is likely to be new Chemistry



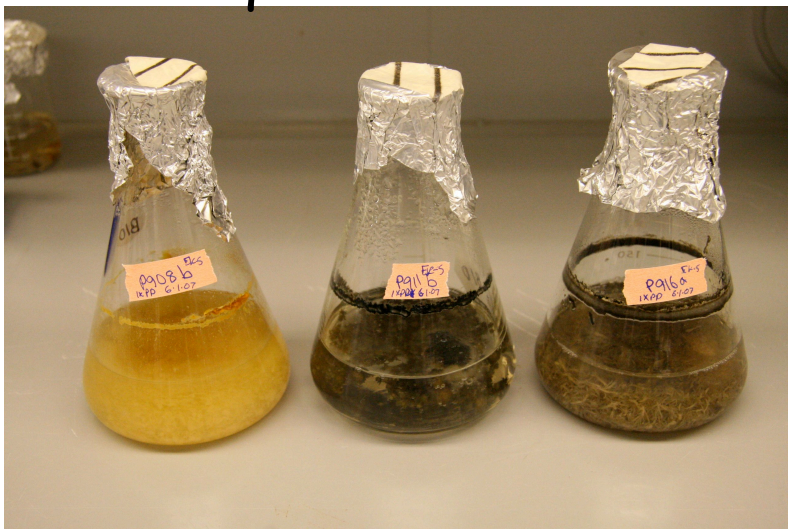
Generating Fungal Extracts for Bioactivity Screening

Plants

Endophytes



Liquid Cultures

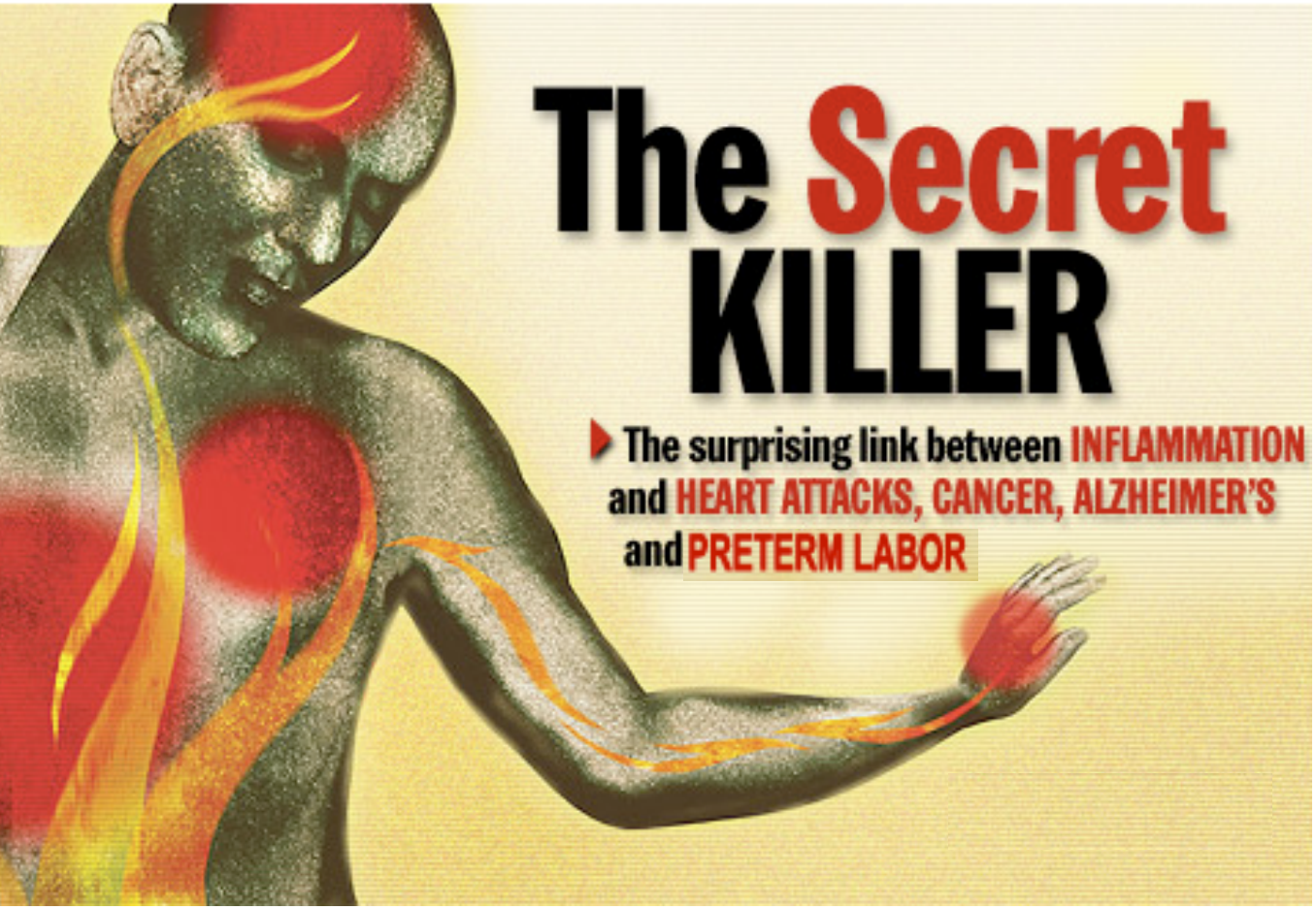


Extracts

~500 fungal extracts generated by the students each year

INFLAMMATION

'The root of all disease'



Time 2004

Sun Jin Lee

(Beckman Scholar)

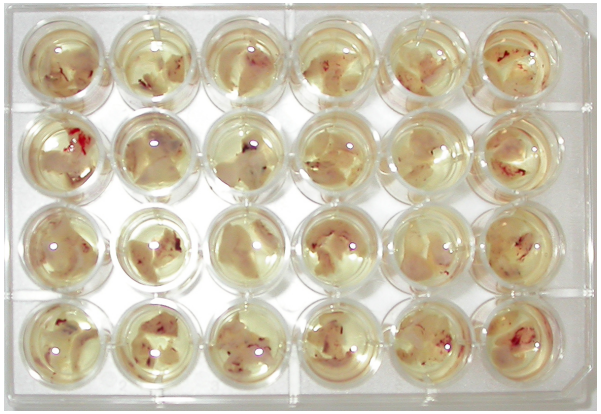
Collaboration with

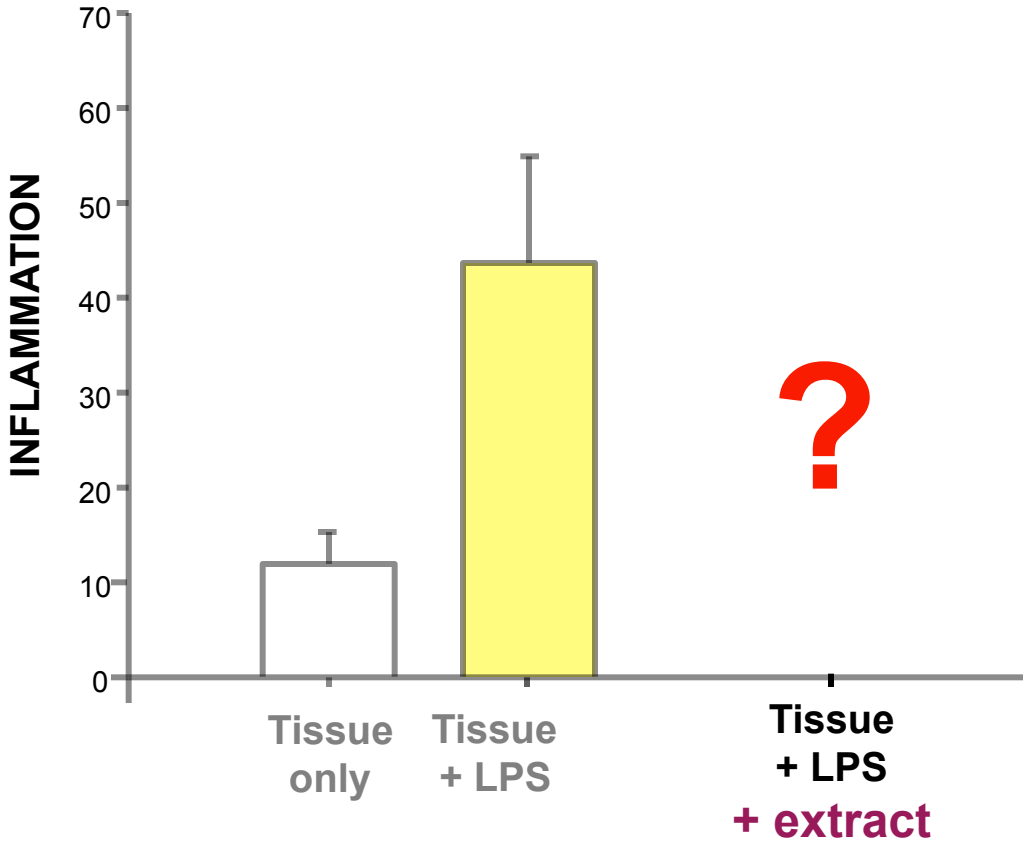
Irina Buhimschi

Search for Anti-Inflammatory Agents Relevant to Pre-term Birth

- Fetal membranes + bacterial protein = inflammation
- Do endophyte extracts reduce inflammation?

Explants of fetal membranes



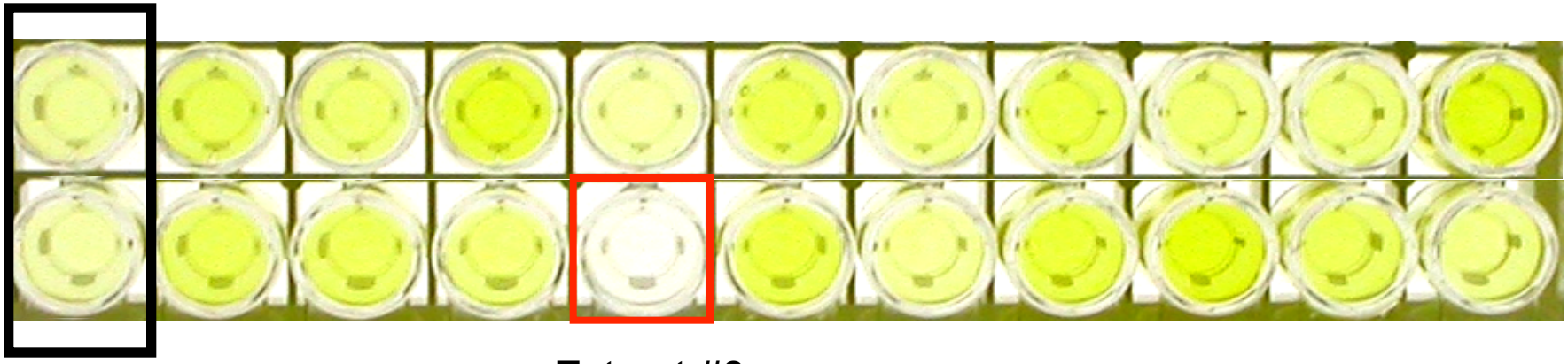


Sun Jin Lee

Search for Anti-Inflammatory Agents Relevant to Pre-term Birth

- ↑ yellow fluorescence = ↑ inflammation (as quantified by IL-8 and IL-6 levels)

Treated with different extracts

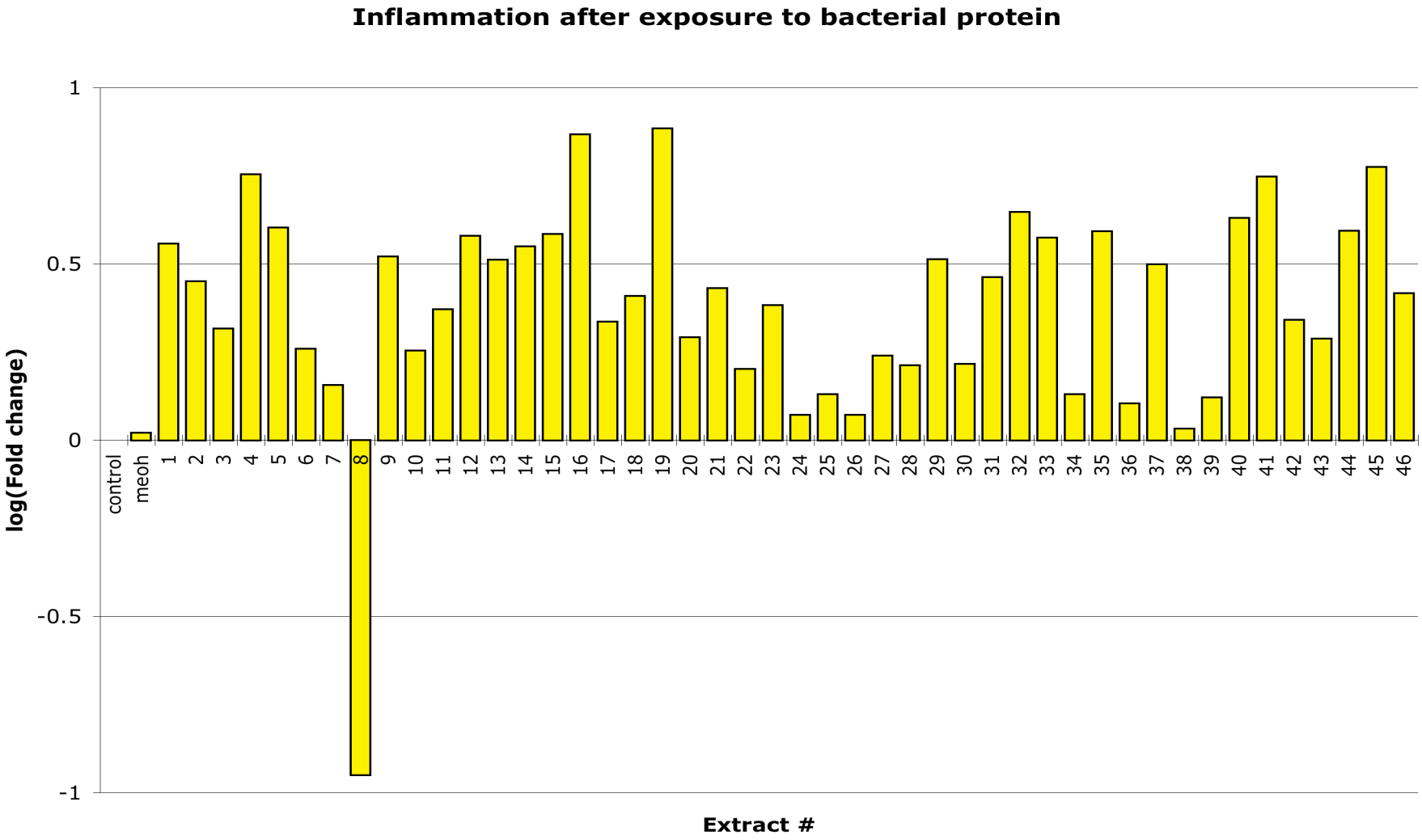


Control

Extract #8

Search for Anti-Inflammatory Agents Relevant to Pre-term Birth

- **Collection of 46 extracts tested**



Active Extract from a Novel Fungus

- **Extract #8 originated from endophyte 404e**

Carnivorous plant *D. montana* from Amazonian savannah

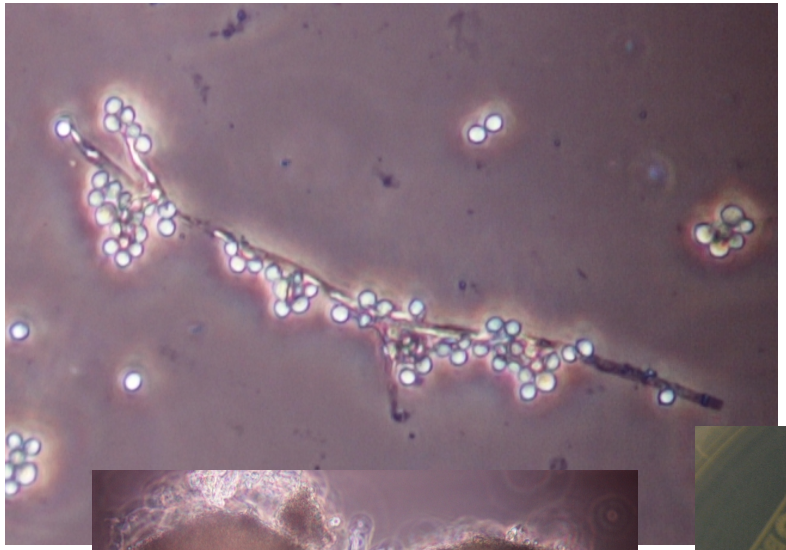
ISOLATION

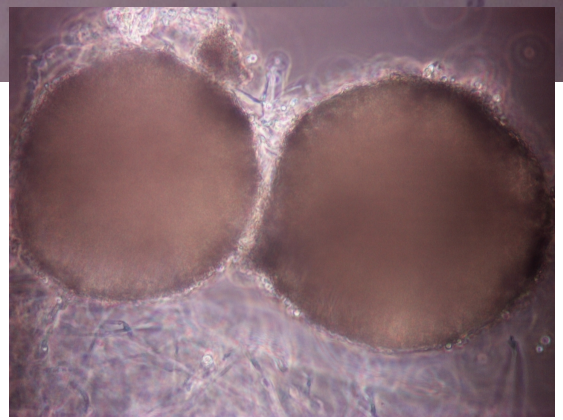
Endophyte '404e'
Collected by TA Kaury Eisenman

Organism is Novel at the Genus Level

Aurosphaeria flaviradians

"golden spheres, radiating yellow"

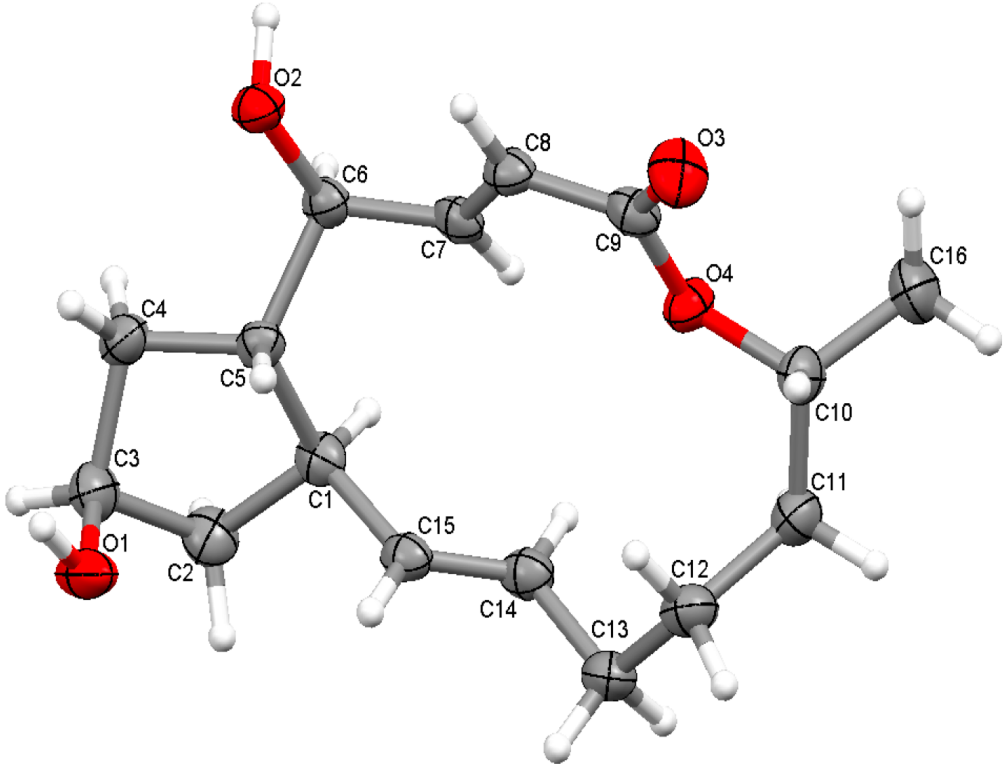


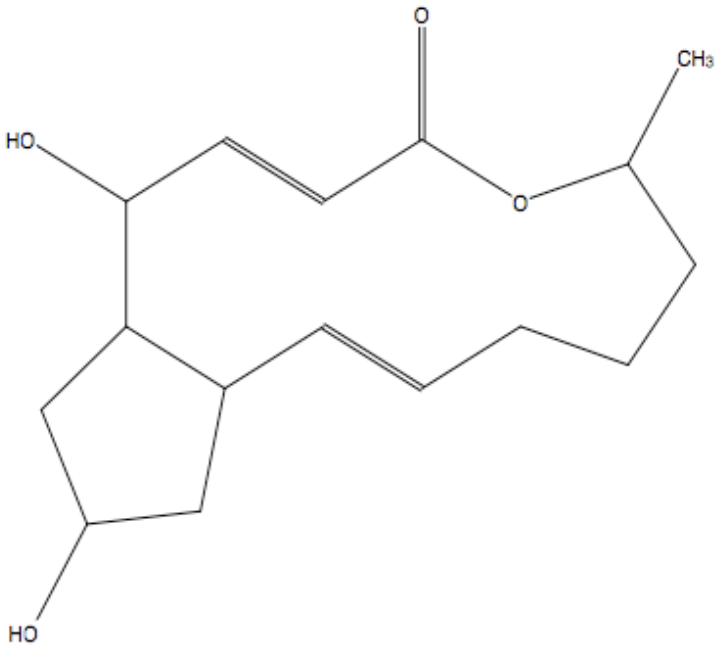


S. J. Lee, G. A. Strobel, K. Eisenman, B. Geary, P. N. Vargas, S.A. Strobel ,*Aurosphaeria,* a novel coelomycetous genus, *Mycotaxon* **107**, 463–472 (2009).

Chemical characterization of anti-inflammatory agent

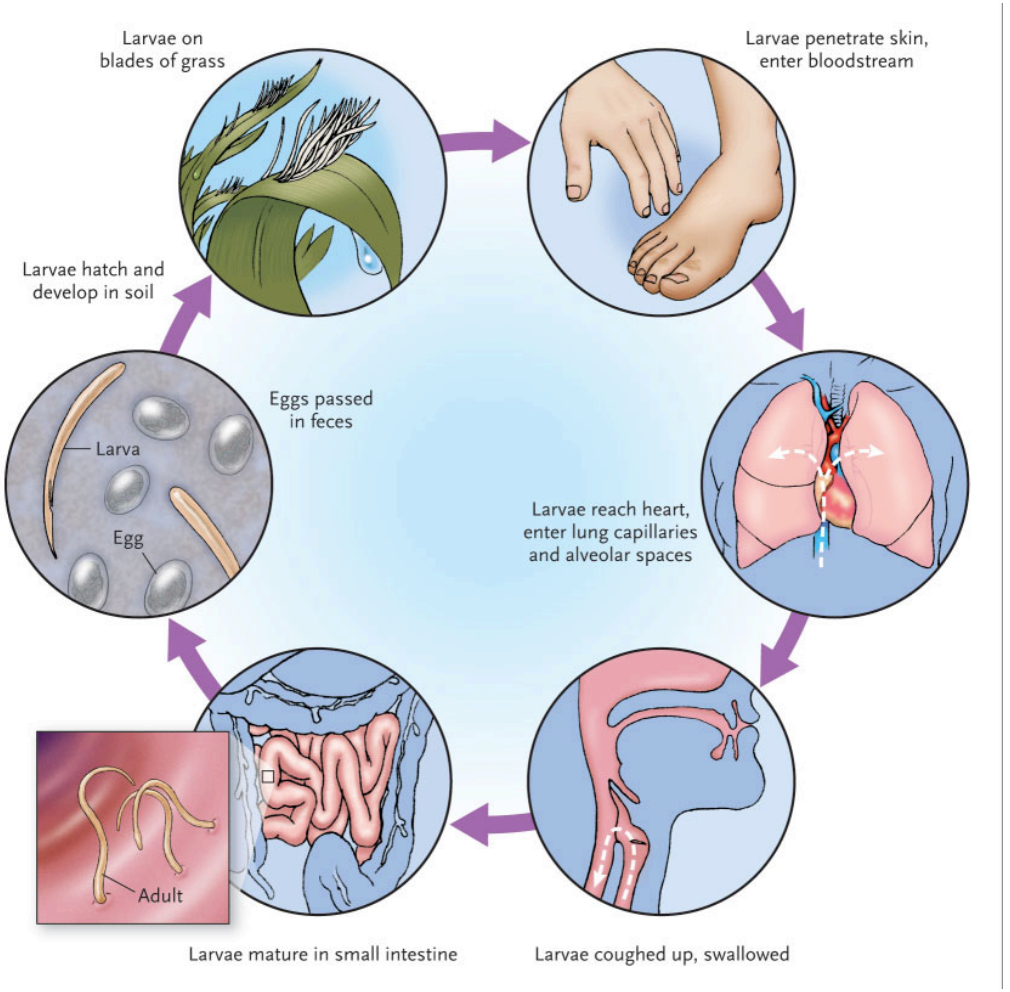
- Purified and crystallized active compound
- Crystal structure revealed it is Brefeldin A
- Compound blocks protein trafficking through the Golgi





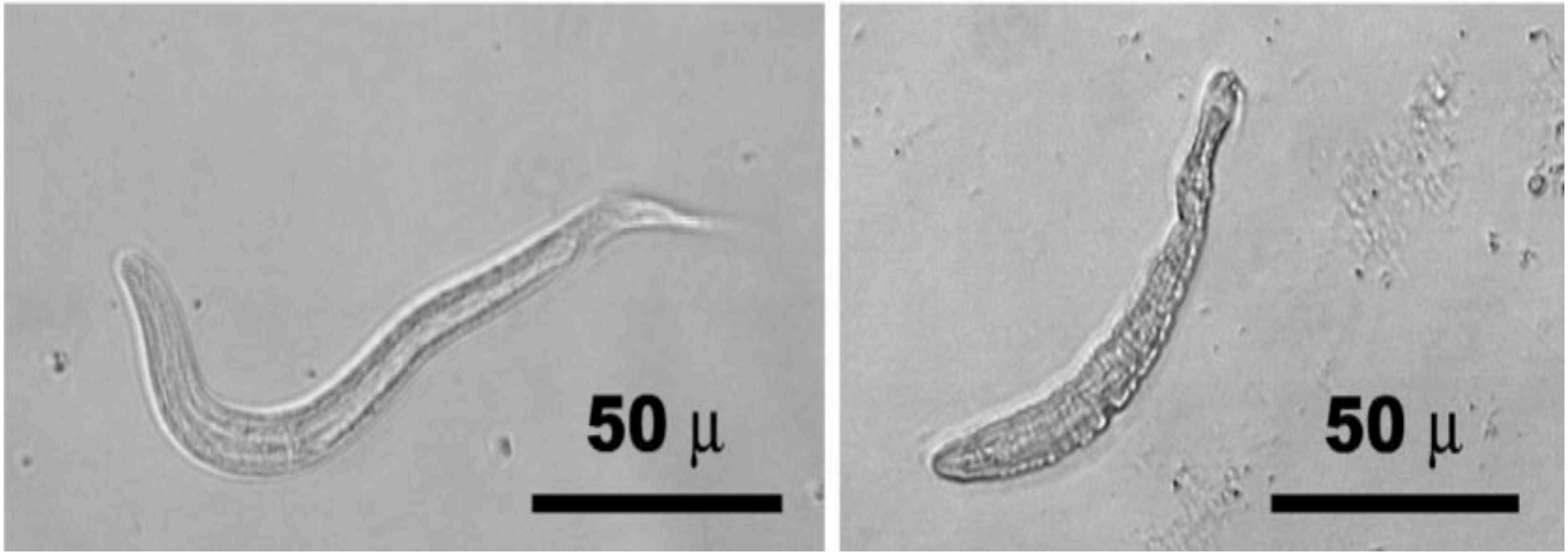
Hookworm Disease

- **Between 750 million and a billion cases worldwide**
- Caused by an infection of the helminth nematode parasites *Necator americanus* and *Ancylostoma duodenale*
- Symptoms:
  - •Adults: headache, fatigue, impotence, decrease in work capacity, iron-deficient anemia
  - • Children: stunted growth and adverse affects on mental development and comprehension and reasoning skills



In Collaboration with Michael Cappello

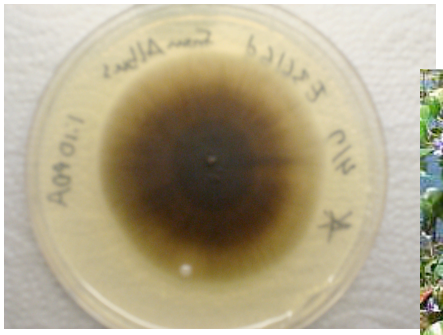
Screening Extracts for Anthelminthic Activity



- Egg hatch test
  - Nematode eggs placed in culture media with or without extracts
  - Monitor hatch rate and larvae motility

- Hit will display
  - Stunted growth
  - Loss of internal organ integrity
  - Decreased motility

Screening Extracts for Anthelminthic Activity

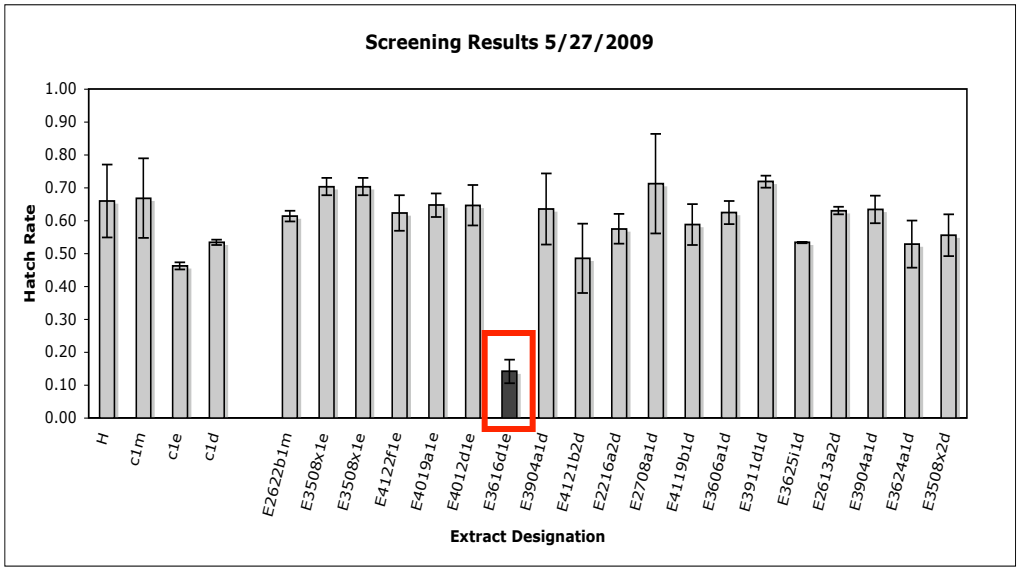


Organism:

Phaeoacremonium sp. (100% sequence homogy)

Plant: *Eichornia azurea*

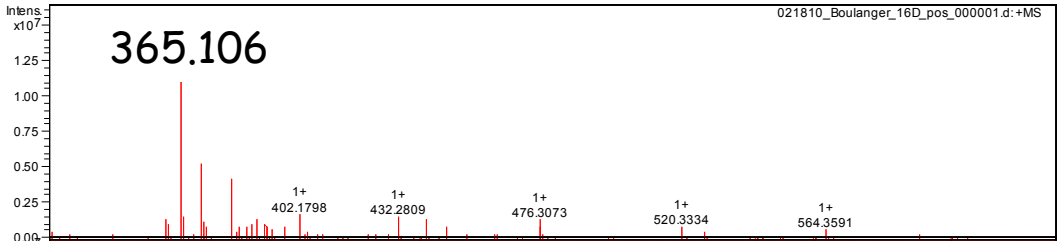
Isolated from Water Hyacinth which grows in large patches on the lakes of the Ecuadorian rainforest.



- Activity found in Egg Hatch Assay (tests for inhibition of Hatching)
- Extract shows strong activity even at 10,000 fold dilution!

Characterization of Active Compound:

Accurate Mass by Mass Spectroscopy

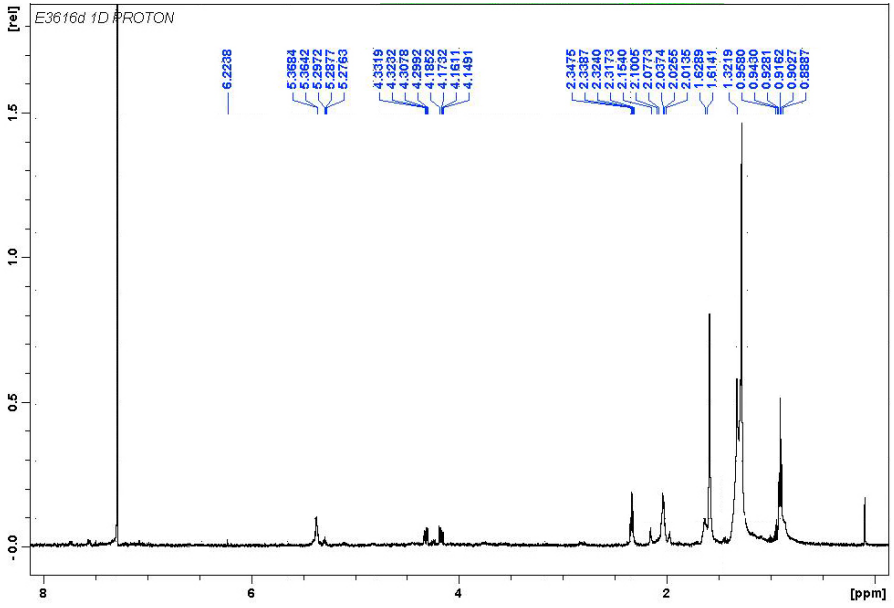


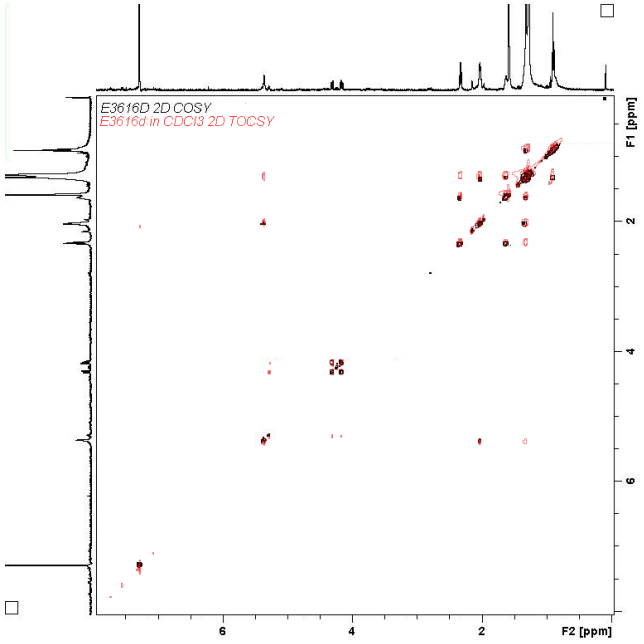
Candidate Chemical Formulas

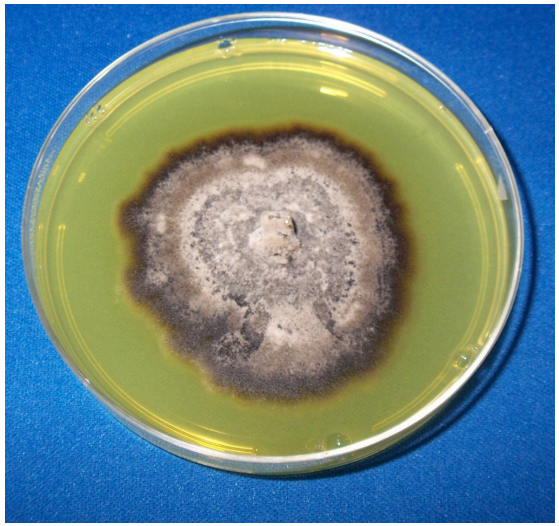
$C_{12}H_{19}N_3O_5$ $C_{18}H_{20}O_8$

$C_{21}H_{18}O_6$ $C_{21}H_{16}O_7$

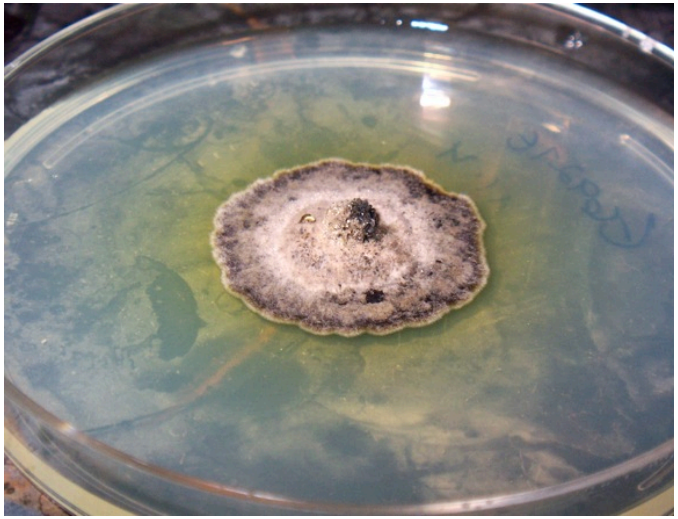
NMR Studies: Revealed that **structure does not match any known compound!**







27E culture on PDA, 1 month

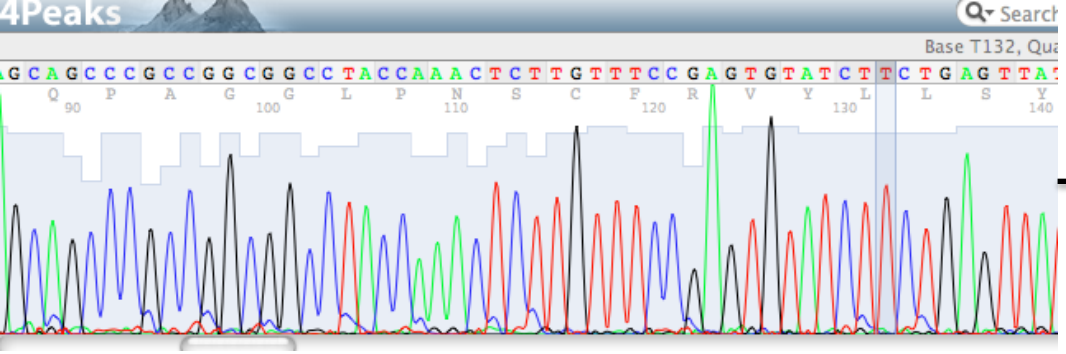


27E culture on PDA, ~2 weeks

Alicia Darnell

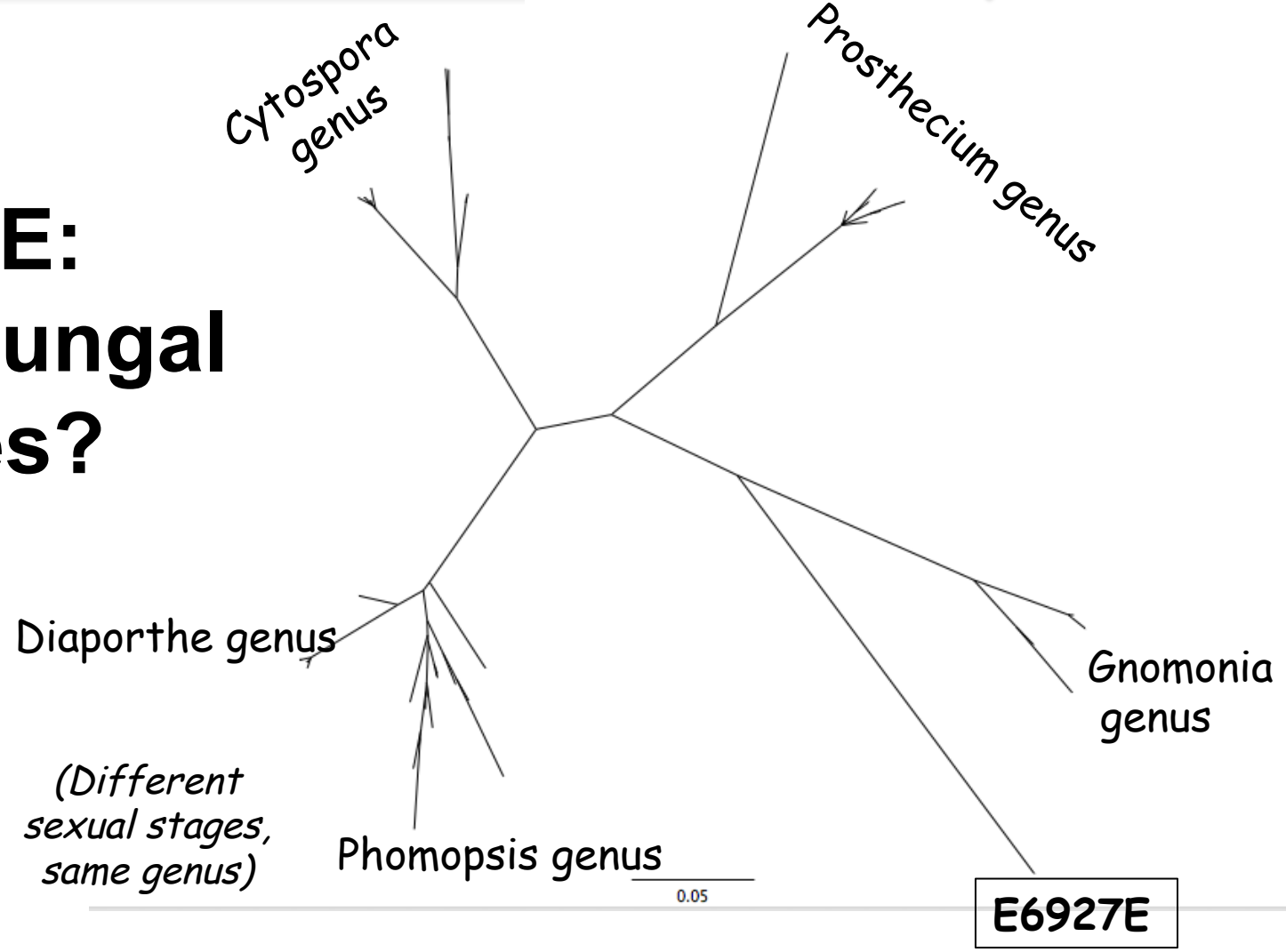
Endophyte: E6927E

Isolated in the Amazon Basin, Ecuador from *Ficus sphenophyllum*

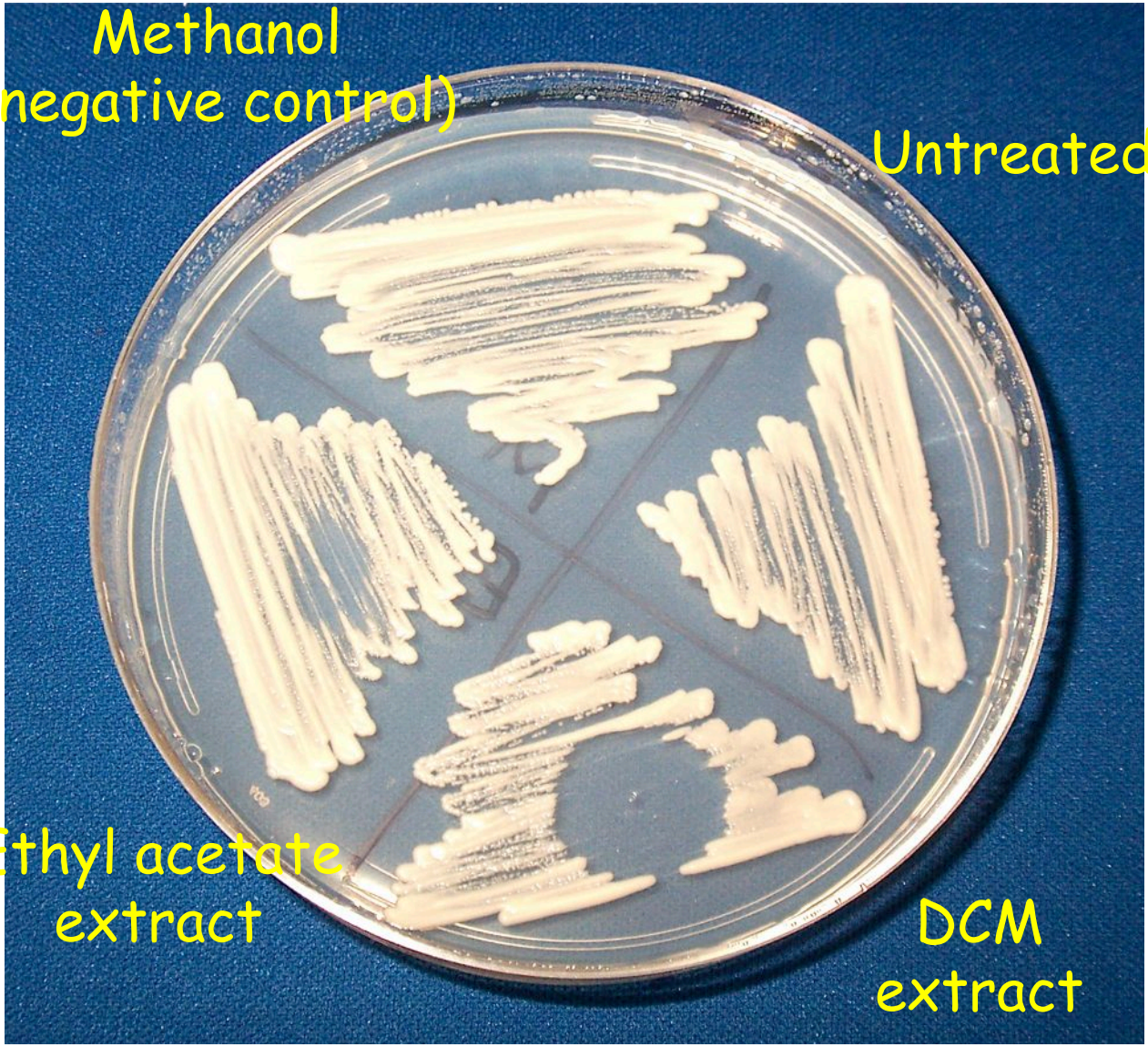


BLAST alignment against database sequences

E6927E: A Novel Fungal Species?



Identification of Strong Candida Inhibition by extract E6927E1d

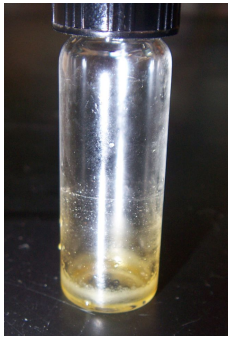


CIDAL Inhibition:
no Candida regrowth after weeks/months on test plate

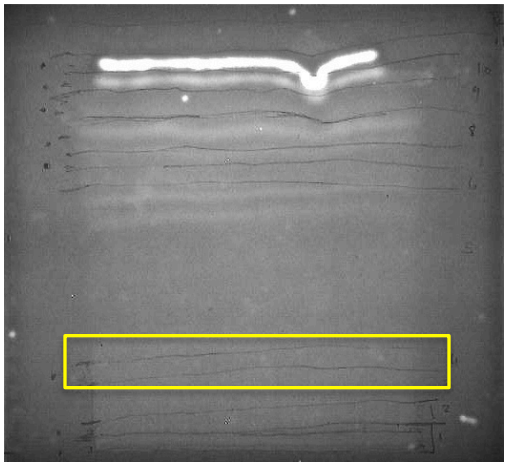
SPECIFIC activity:
extract is NOT active in bacterial screens

Bioassay Guided Purification of The Active Compound

Crude Extract



Fractionation by TLC

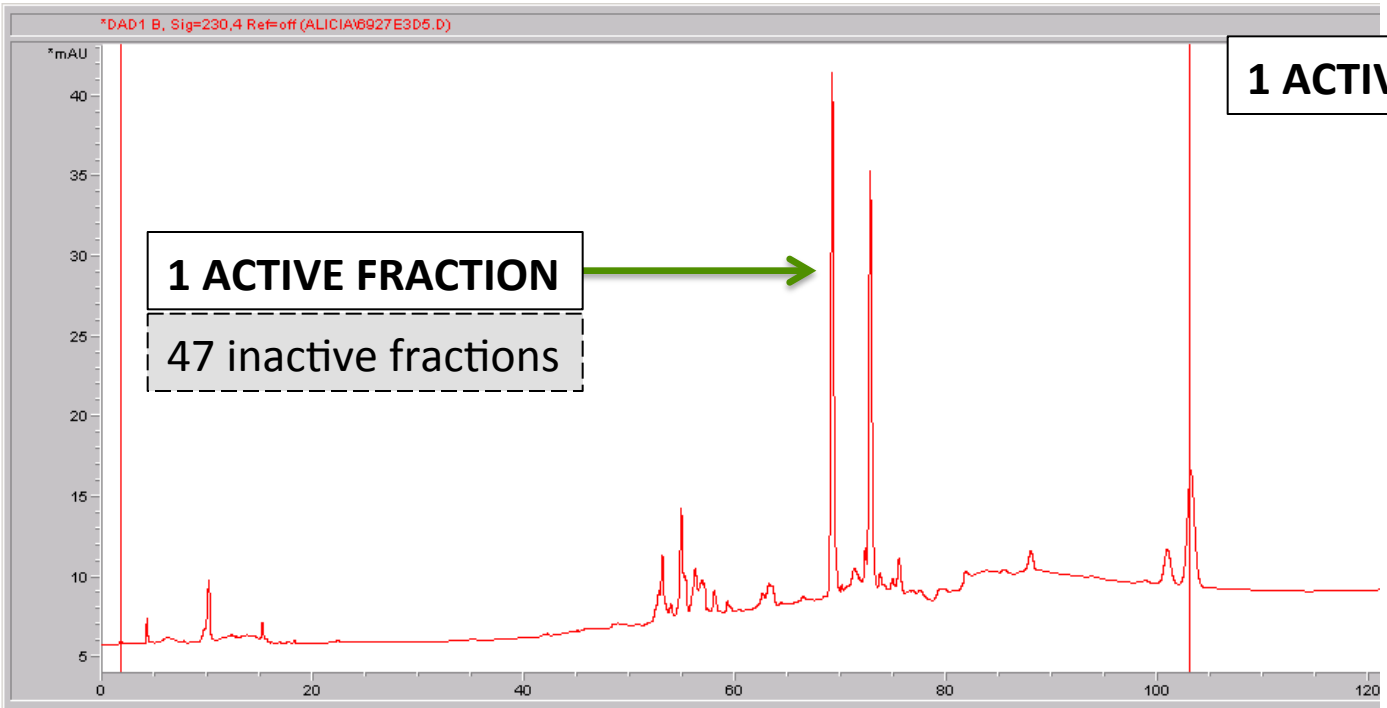


12 fractions

Assay fractions against Candida

1 ACTIVE FRACTION

11 inactive fractions

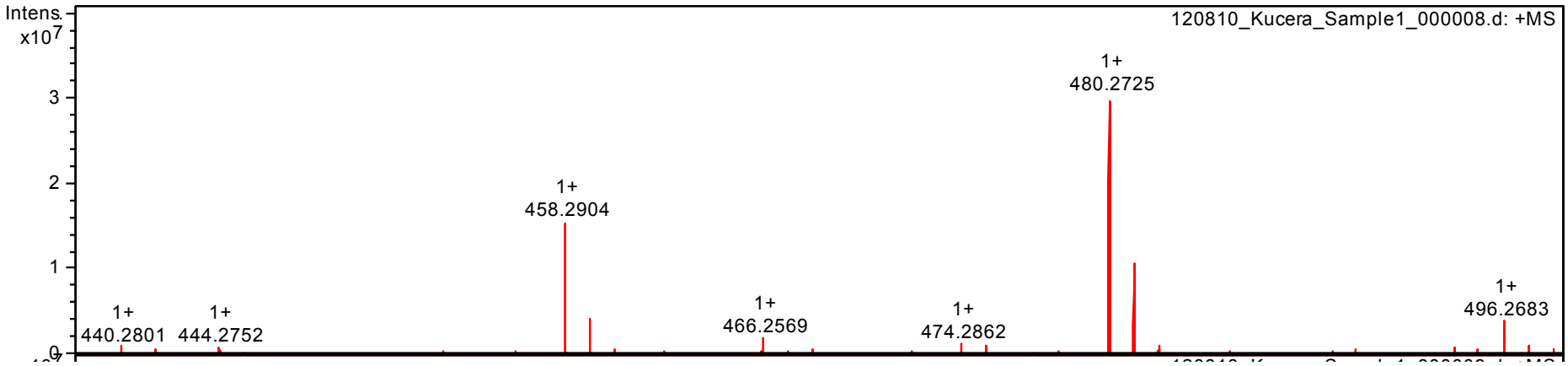


1 ACTIVE FRACTION

47 inactive fractions

HPLC Purification

Chemical Characterization of Pure Active Fraction: High Resolution Mass Spectrometry



Average mass: 457.29010 m/z

Potential Molecular Formulas:

$C_{27}H_{40}NO_5$ (457.29010)

$C_{26}H_{34}N_8$ (457. 29009)

$C_{11}H_{30}N_{20}O$ (457.29060)

$C_{12}H_{36}N_{13}O_6$ (457.29061)

•Some known candida inhibitors:

Fluconazole: 306.271

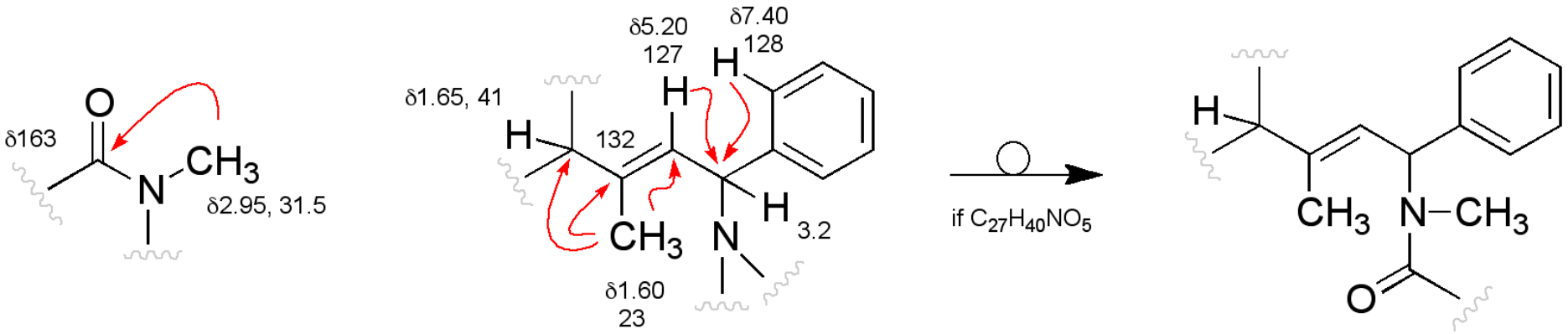
Amphotericin B: 924.084

Rapamycin: 914.172

Na^+ salt of compound gives 480.2725 m/z peak

Sample appears at least 95% pure after purification by TLC and HPLC

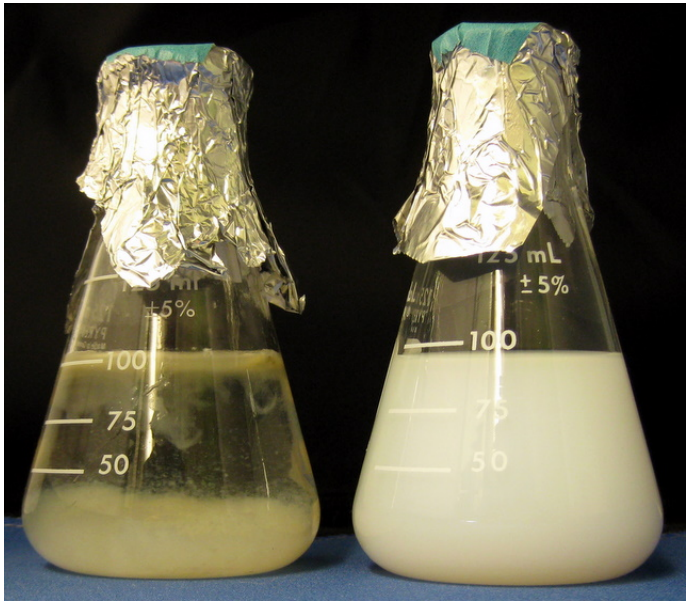
Solved Fragments of the Structure



identifiable fragments

If these are correct, they are novel pieces by "search by structure" function of chemical databases!

Bioremediation: Degradation of Plastic



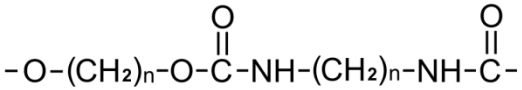
Pria Anand

Jeffrey Huang

Jon Russell

(Beckman Scholar)

Polyurethane



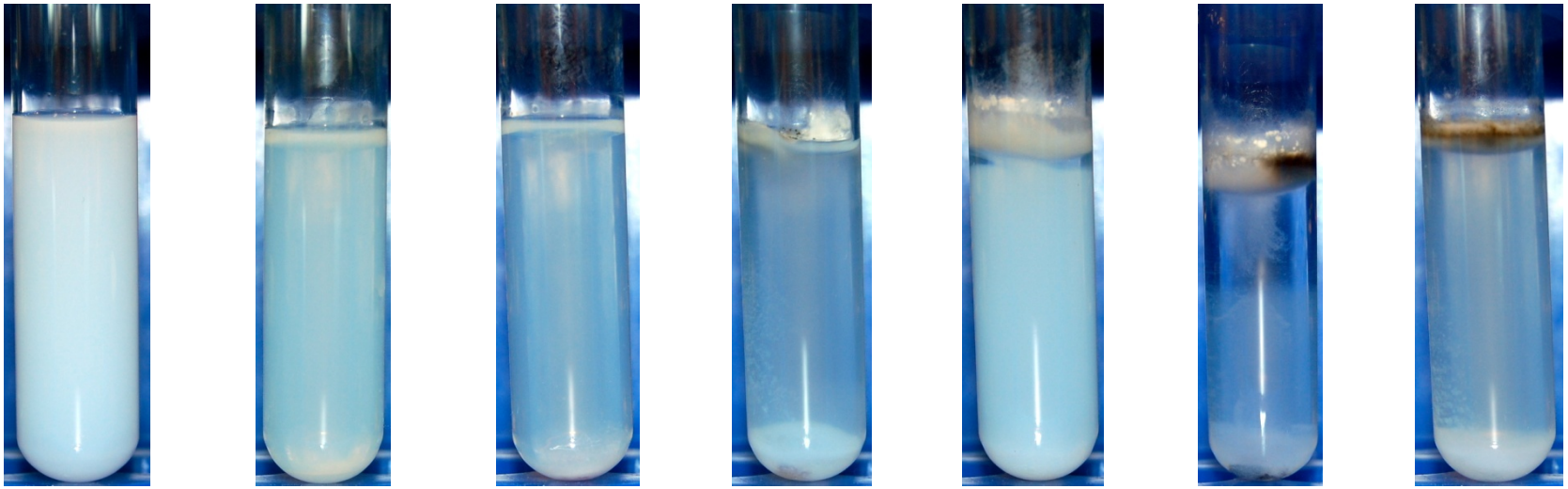
IMPRANIL® DLF

Polyurethane Dispersion

- Uses: varnish, wheels, furniture, automobile seats, houses, sculptures, decorations, construction sealants and firestopping, surfboards, rigid-hulled boats, inflatable boats, tennis grips, electronic components, adhesives, watch-band wrapping, abrasion resistance, filling of spaces and cavities, textiles

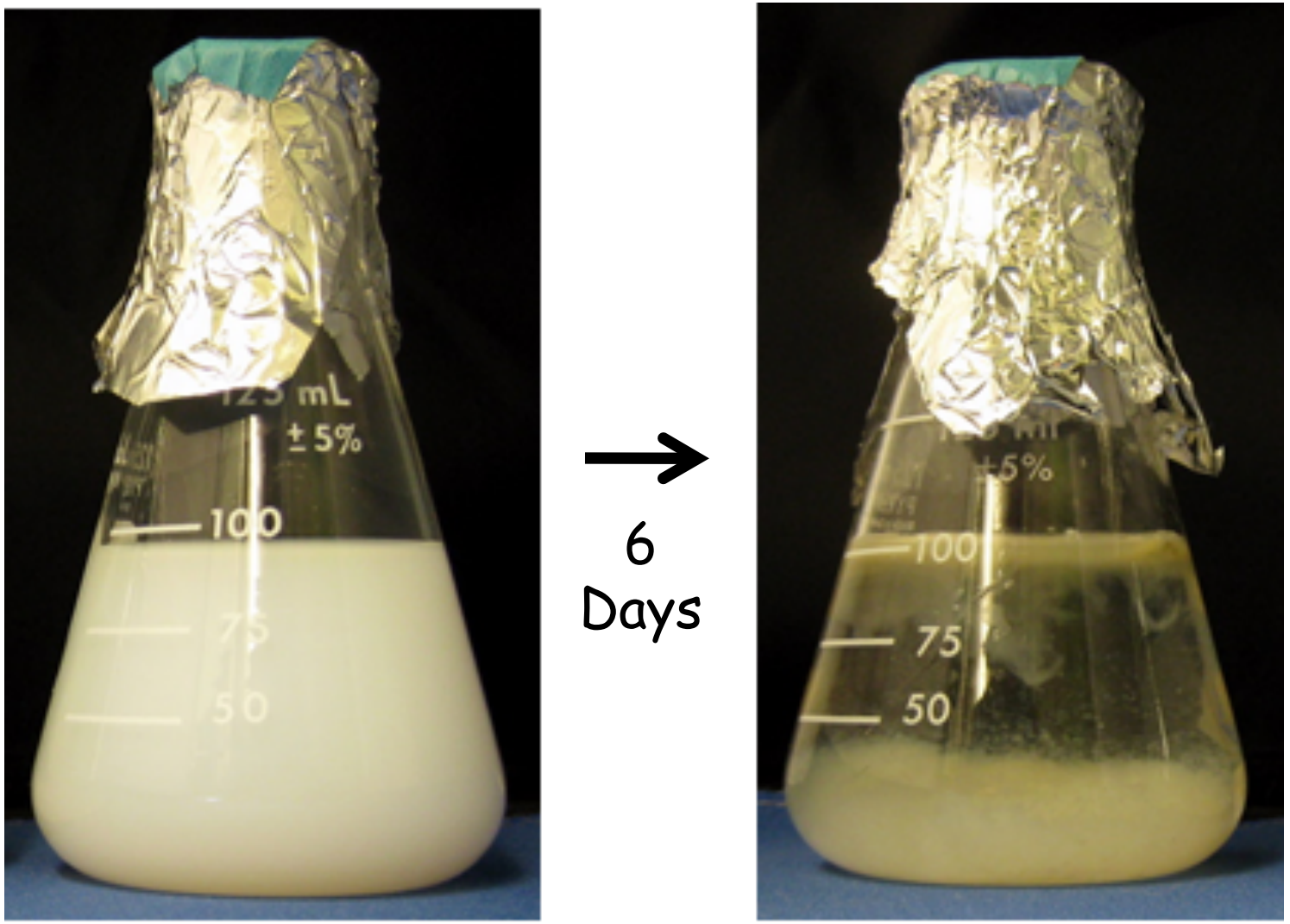
Liquid Cultures

Top 5 Fungi



(-) control *P. chlororaphis* **E2812a** **E2708a** **E2910b** **E2611a** **E3317b**

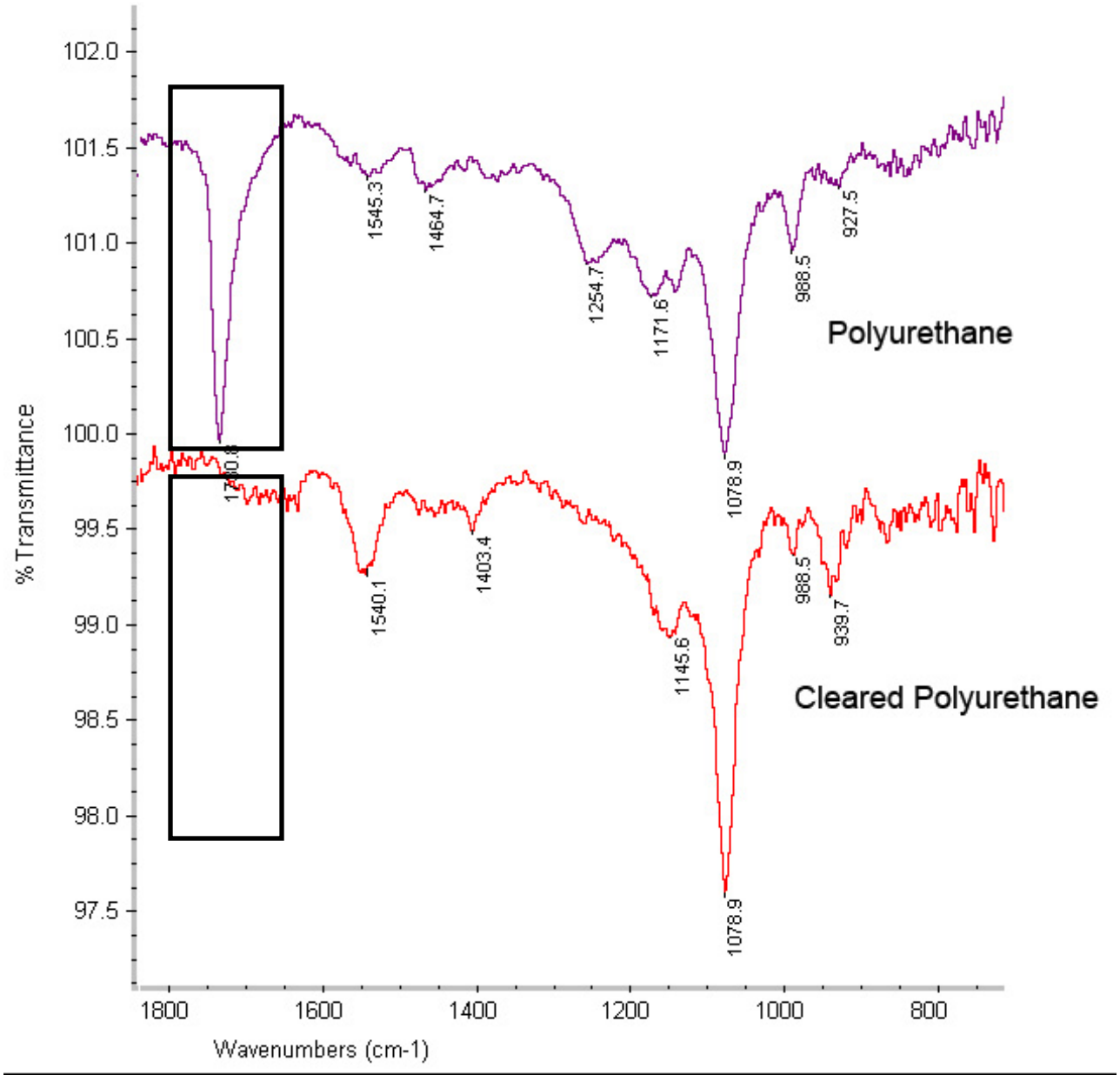
- Can use polyurethane as the sole carbon source
- Can clear the complete solution in six days
- Can grow anaerobically



Control

Pestalotiopsis microspora

What is the degradation pathway?



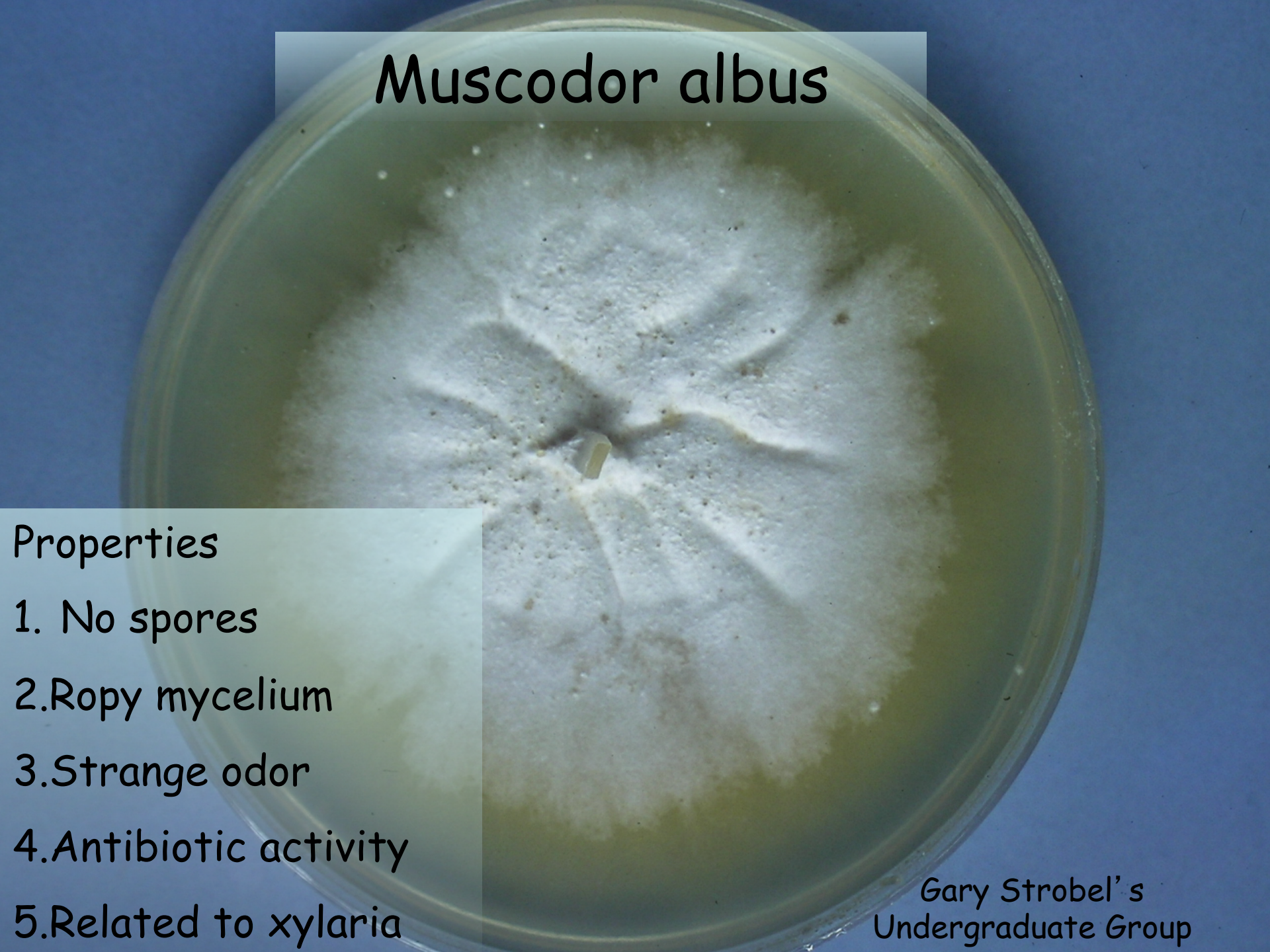
Polyurethane IR spectra before and after degradation

C-O ester stretch is completely eliminated

What Happened After the Study was Published


Cinnamomum zeylanicum
Gary Strobel's
Undergraduate Group

Muscodor albus

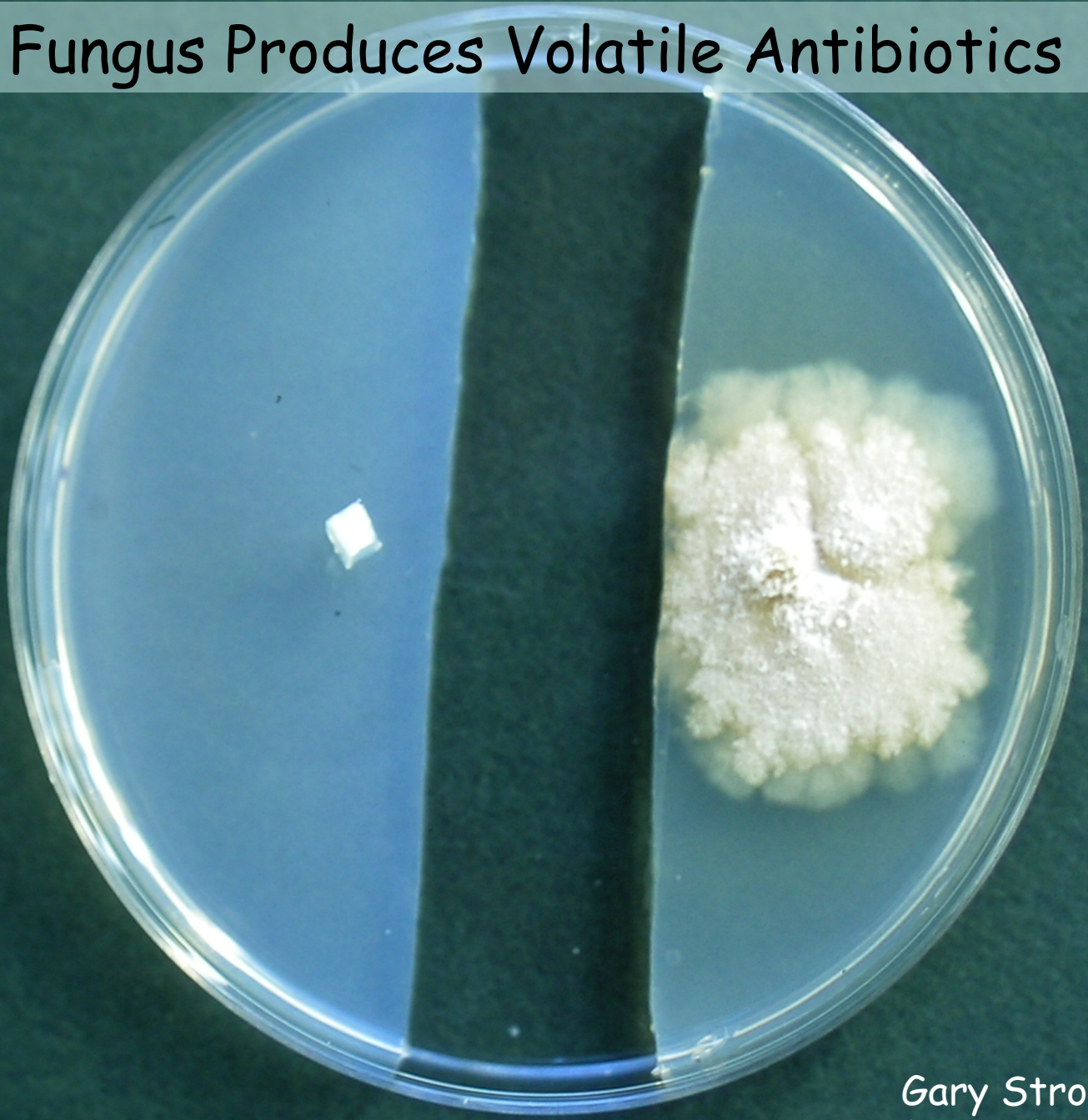


Properties

1. No spores
2. Ropy mycelium
3. Strange odor
4. Antibiotic activity
5. Related to xylaria

Gary Strobel's
Undergraduate Group

Fungus Produces Volatile Antibiotics



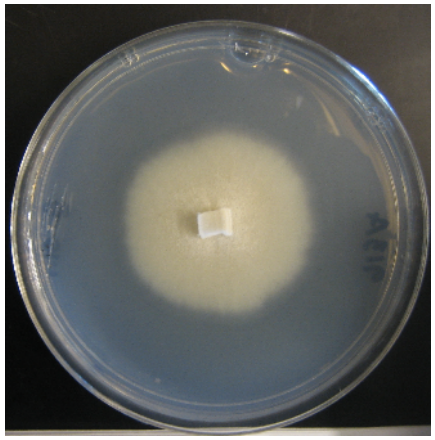
Gary Strobel's
Undergraduate Group

The Collection of Volatiles Produced

Gary Strobel's Undergraduate Group

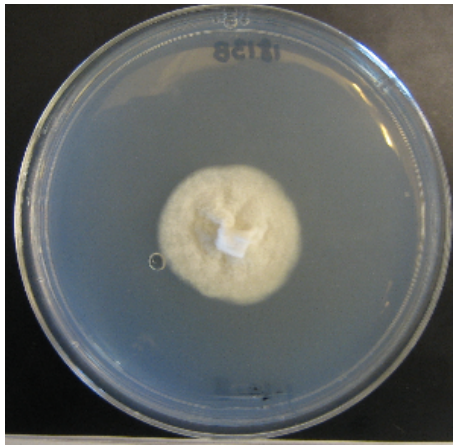
Six New *Muscodor* Isolates

P912B



P913A

P1509A



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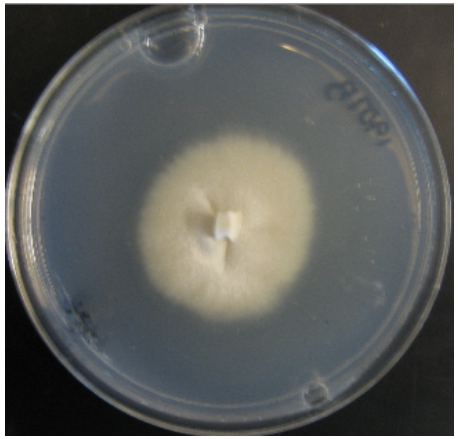
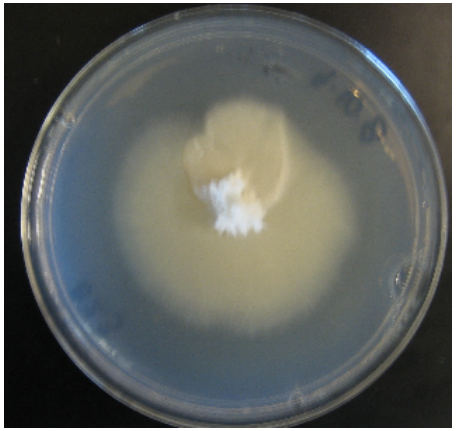


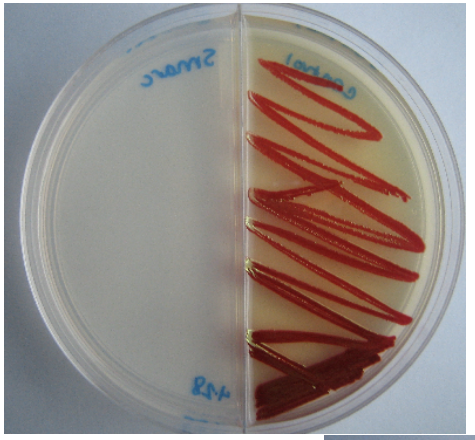
P1907B

M. albus

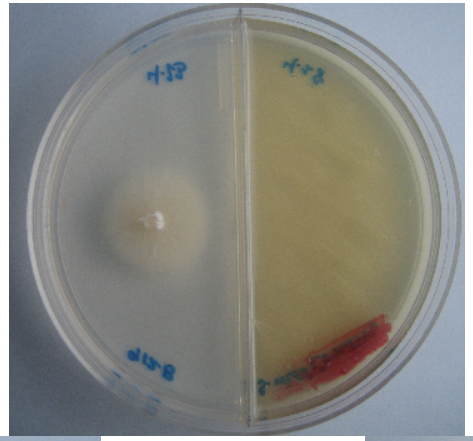


B23

Inhibition of *Serratia marcescens*



control



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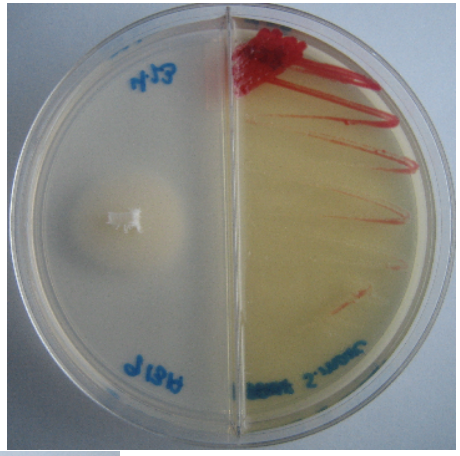

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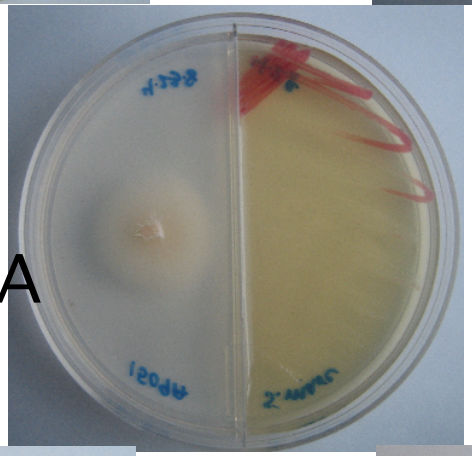

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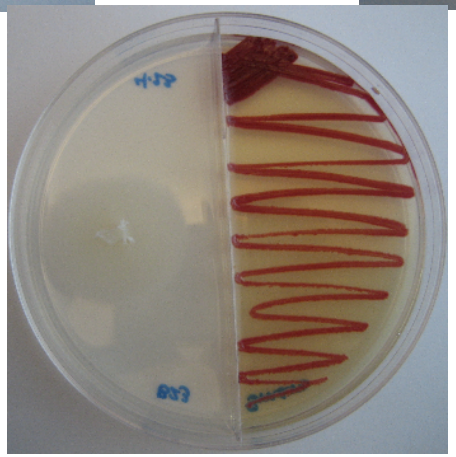
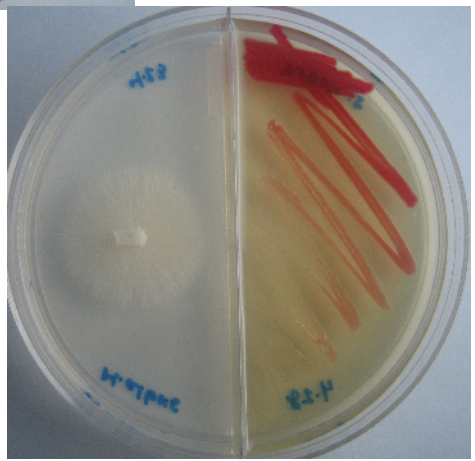
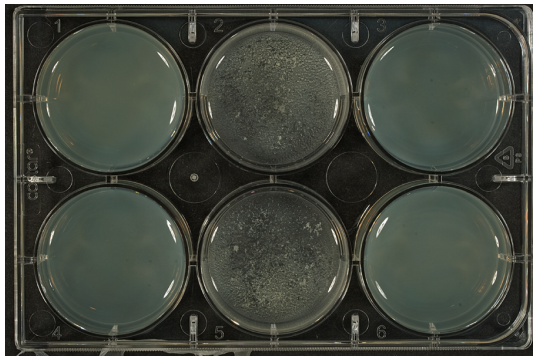


M. albus

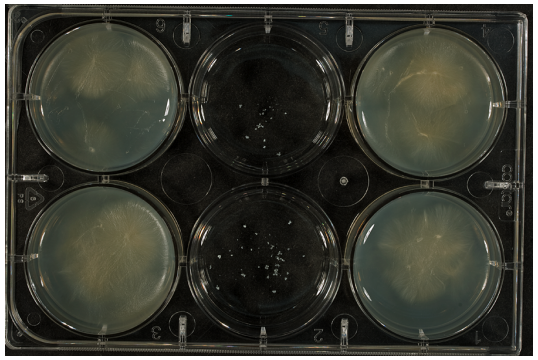


Inhibition of *Mycobacterium tuberculosis*

Control



M.albus



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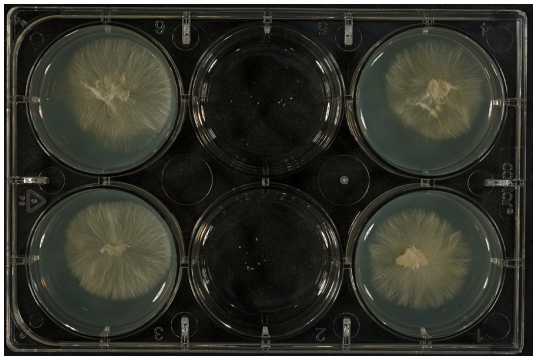

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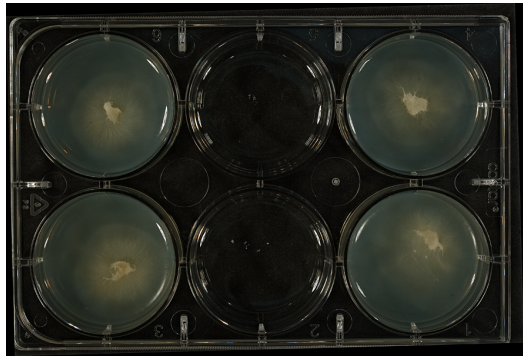

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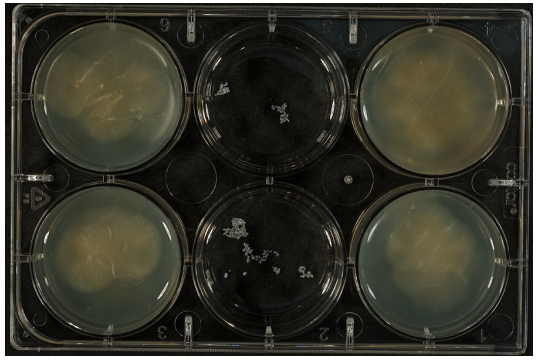

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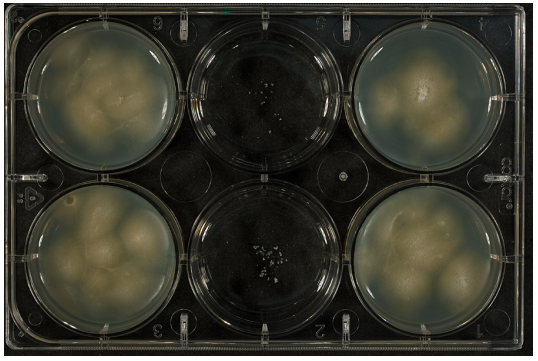

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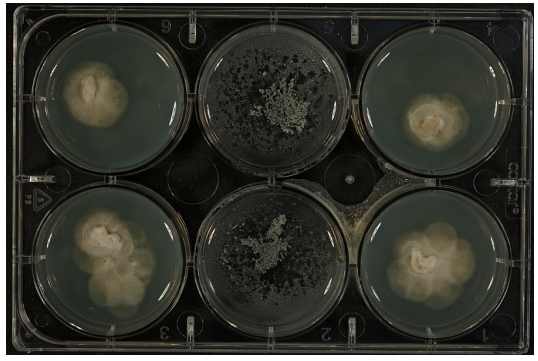

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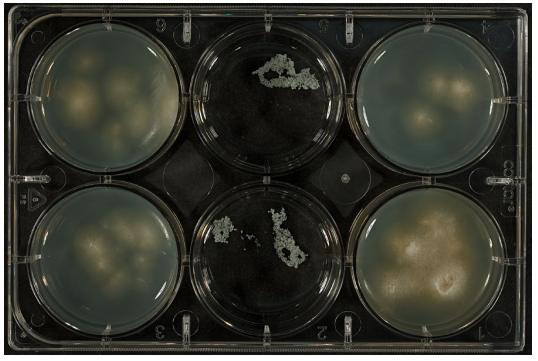
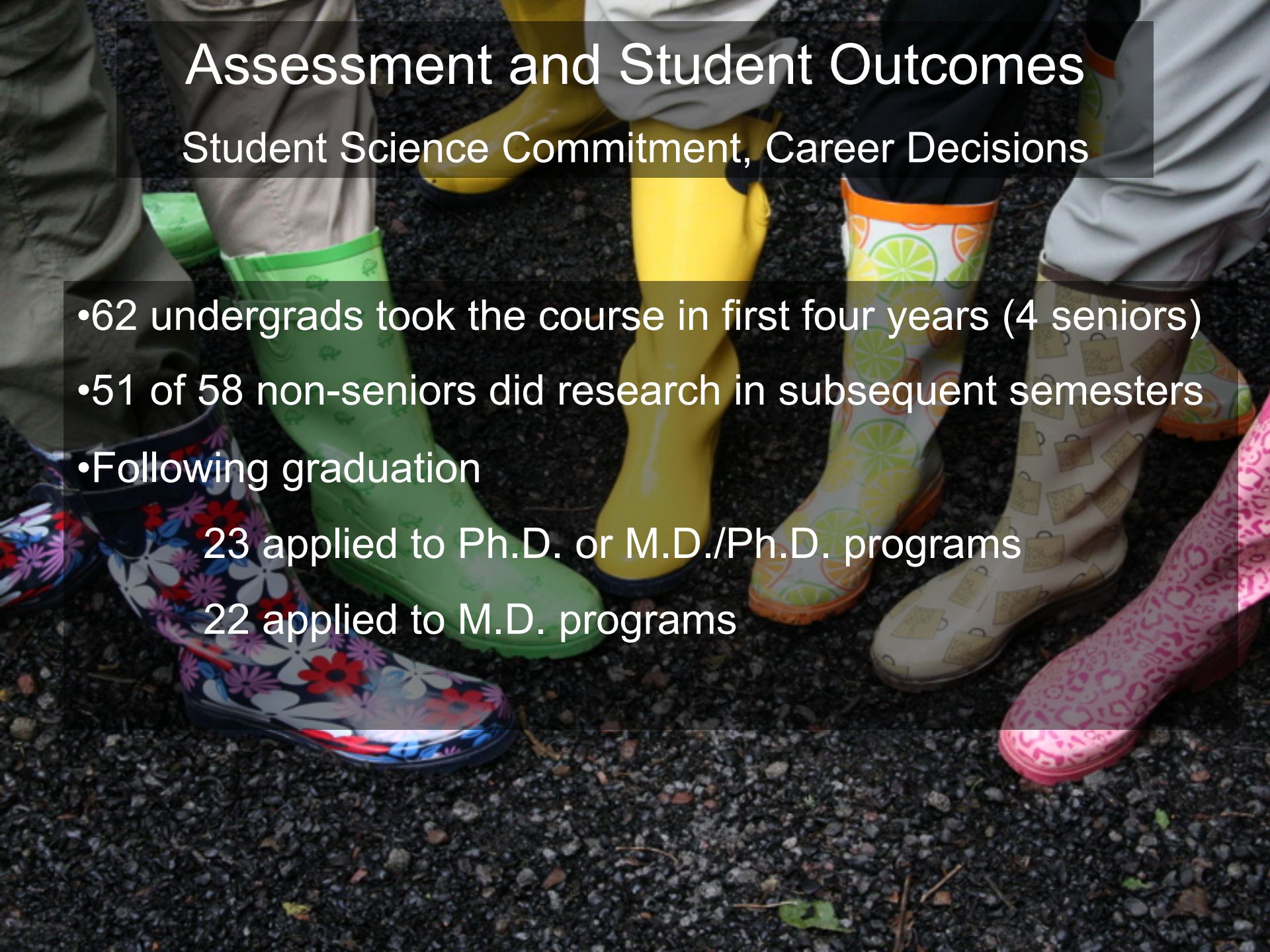


In Collaboration with
Graham Hatfull at the
University of Pittsburgh



Assessment and Student Outcomes

Student Science Commitment, Career Decisions

- 62 undergrads took the course in first four years (4 seniors)
- 51 of 58 non-seniors did research in subsequent semesters
- Following graduation
  - 23 applied to Ph.D. or M.D./Ph.D. programs
  - 22 applied to M.D. programs

Assessment and Student Outcomes

Undergraduate Student Publications

Studies based upon multiple individual observations

•Colutellin A, an immunosuppressive peptide from Colletotrichum dematium.Ren Y, Strobel GA, Graff JC, Jutila M, Park SG, Gosh S, Teplow D, Condron M, Pang E, Hess WM, Moore E. *Microbiology* 154, 1973-9 (2008).

•Purification, identification and activity of phomodione, a furandione from an endophytic Phoma species.Hoffman AM, Mayer SG, Strobel GA, Hess WM, Sovocool GW, Grange AH, Harper JK, Arif AM, Grant DM, Kelley-Swift EG. *Phytochem.* 69 , 1049-56 (2008).

•S. A. Smith, D. C. Tank, L. A. Boulanger, C. A. Bascom-Slack, K. Eisenman, D. Kingery, B. Babbs, K. Fenn, J. S. Greene, B. D. Hann, J. Keehner, E. G. Kelley-Swift, V. Kembaiyan, S. J. Lee , P. Li, D. Y. Light, E. H. Lin, C. Ma, E. Moore, M. A. Schorn, D. Vekhter, P. V. Nunez, G. A. Strobel, M. J. Donoghue, S. A. Strobel. Bioactive endophytes warrant intensified exploration and conservation. *PLoS ONE* **3**, e3052 (2008).

•S. J. Lee, G. A. Strobel, K. Eisenman, B. Geary, P. N. Vargas, S.A. Strobel, *Aurosphaeria,* a novel coelomycetous genus. *Mycotaxon* 107: 463–472 (2009).

•C. A. Bascom-Slack, C. Ma, E. Moore, B. Babbs, K. Fenn, J. S. Greene, B. D. Hann, J. Keehner, E. G. Kelley-Swift, V. Kembaiyan, S. J. Lee, P. Li, D. Y. Light, E. H. Lin, M. A. Schorn, D. Vekhter, L. A. Boulanger, W. M. Hess, P. Nunez Vargas, G. A. Strobel and S. A. Strobel, Multiple, novel biologically active endophytic actinomycetes isolated from upper Amazonian rainforests. *Microbial Ecology* 58, 374-383 (2009).

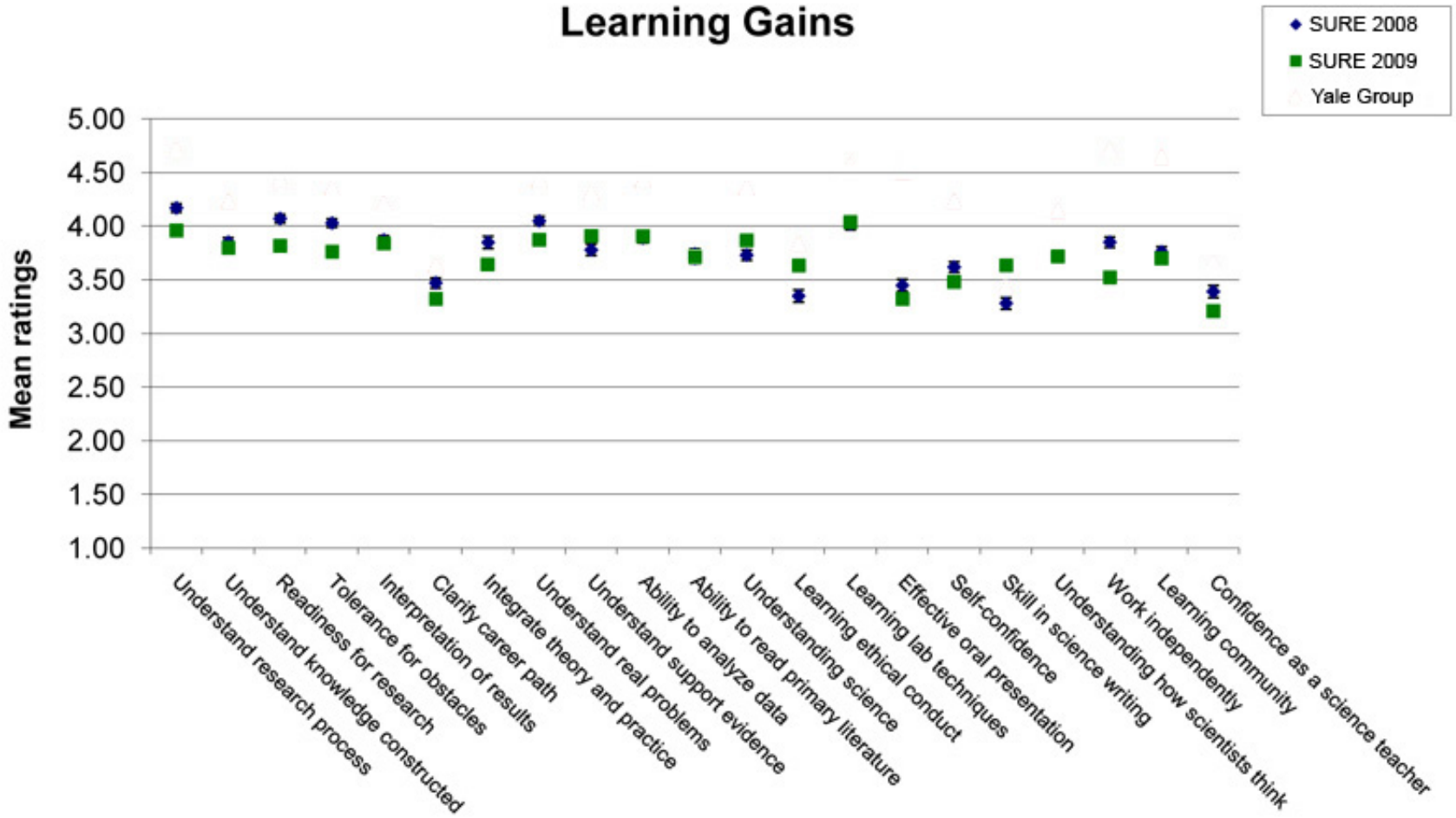
• Kharwar RN, Verma VC, Kumar A, Gond SK, Harper JK, Hess WM, Lobkovosky E, Ma C, Ren Y, Strobel GA. Javanicin, an antibacterial naphthaquinone from an endophytic fungus of neem, Chloridium sp. *Curr Microbiol.*;58,:233-8 (2009).

J. R. Russell, J. Huang, P. Anand, K. Kucera, A. G. Sandoval, K. W. Dantzler, D. Hickman, J. Jee, F. M. Kimovec, D. Koppstein, D. H. Marks, P. A. Mittermiller, S. J. N. Gastélum, M. Santiago, M. A. Townes, M. Vishnevetsky, N. E. Williams, P. N. Vargas, L. Boulanger, C. Bascom-Slack, and S. A. Strobel, Biodegradation of polyester polyurethane by endophytic fungi, *App. & Environ. Microbio.* 77, 6076-6084 (2011).

B. W. Benham-Pyle, D. J. Spakowicz, B. Geary, G. A. Strobel, M.P. Nunez Vargas, and S. A. Strobel, *Vietor*, a novel endophytic genus of Hypocreales producing manumycin A (submitted).

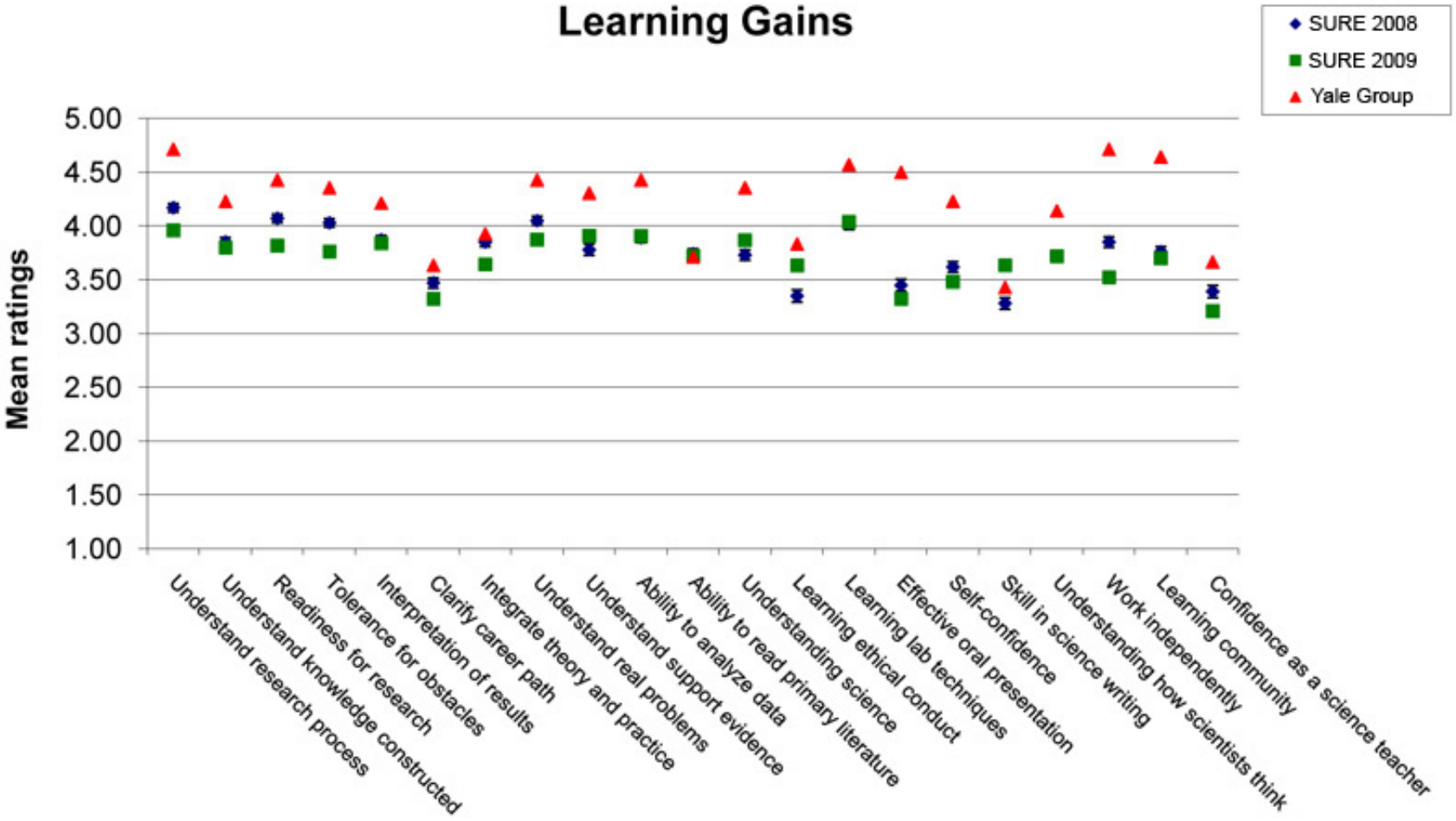
Assessment and Student Outcomes

Summer Undergraduate Research Experience (SURE) Survey



Assessment and Student Outcomes

Summer Undergraduate Research Experience (SURE) Survey



Assessment and Student Outcomes

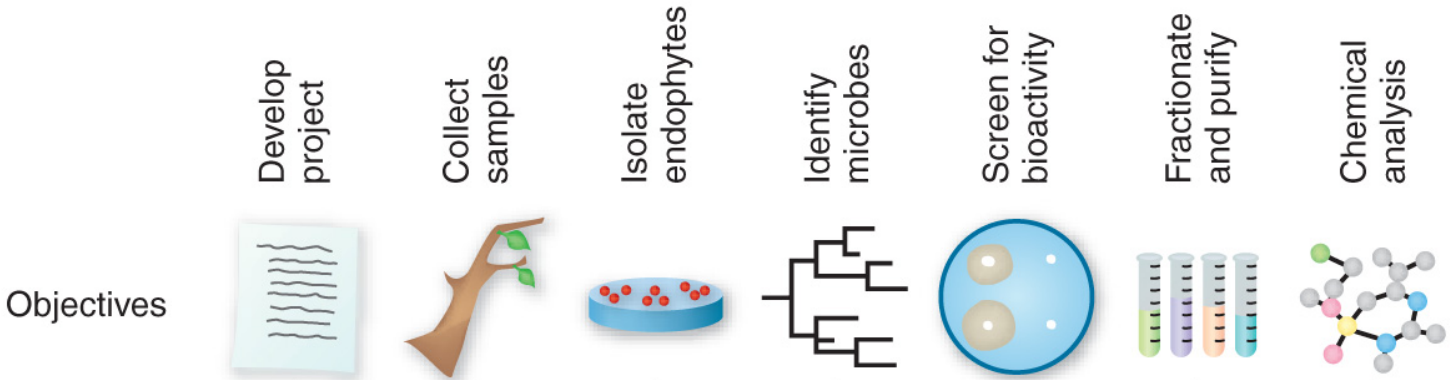
Student Interviews-Quantification of Project Ownership

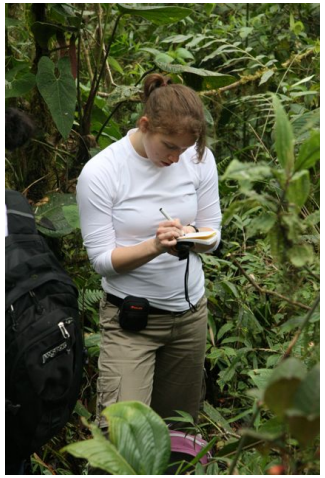
Professor David Hanauer-Indiana University of Pennsylvania

- Interviewed 15 students doing endophyte research and 15 students doing traditional summer research
- Asked six basic questions related to their projects
- Performed a linguistic analysis looking for criteria that connote ownership

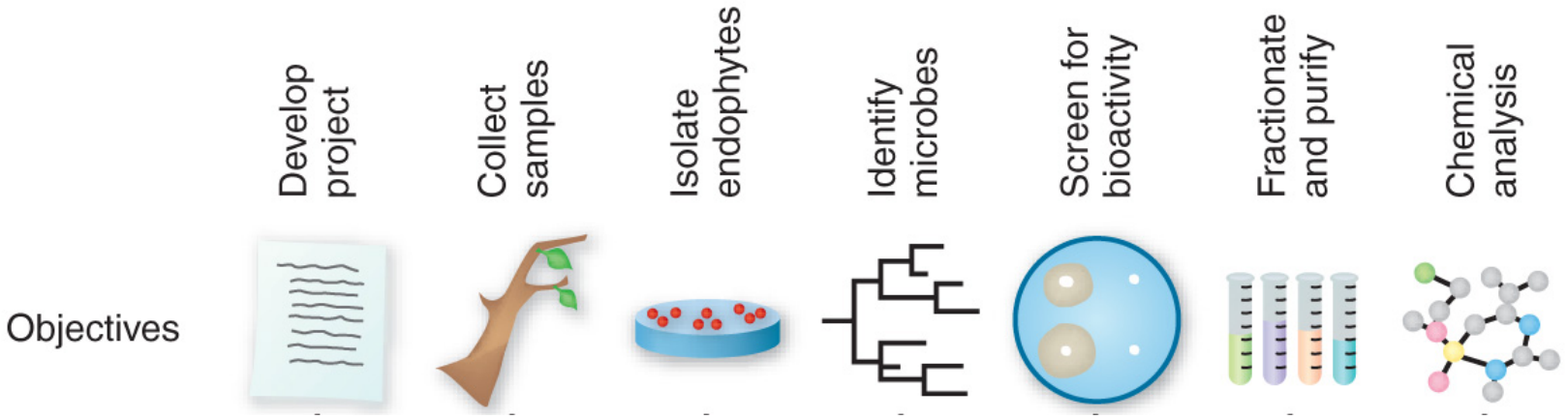
	First Person Personal Pronouns	Affective Lexicon	Cognitive Lexicon	Insight
Group A High autonomy	5.03 (1.33)	4.3 (0.97)	21.42 (1.5)	3.4 (1.14)
Group B Mid autonomy	3.91 (1.12)	3.88 (1.1)	21.54 (2.2)	3.23 (0.94)
Group C Low autonomy	2.96 (1.53)	3.37 (1.06)	22.93 (2.82)	3.32 (1.58)

Several students completed all experimental objectives
Others are in various stages of compound purification





Integration of Research and Educational Objectives



- This pathway of scientific discovery is accessible to novice scientists
- Initial objectives are simple and inspire confidence and curiosity
- Endophyte biodiversity is sufficiently unexplored that many observations will be novel
- Integrated training in biological and chemical disciplines

Thanks also to:

Gary Strobel

Percy Nunez

Carol Bascom Slack

Lori Boulanger

Kaury Kucera

David Kingery

Lauren Saunders

Dan Spakowicz

Meghan Griffin

Cambria Alpha

94 undergraduates