Efficacy of organic sulfur compounds from garlic/onion on white rot Sclerotia germination

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Sclerotium cepivorum

- Economic loss can occur at innoculum densities as low as 0.1 sclerotium/liter soil.
- Near total crop loss can occur at innoculum densities of 10 sclerotium/liter soil.

Clockwise: (1) Courtesy of Paul Koepsell. Oregon State University Extension. White Rot (2) Courtesy of F. J. Crowe. Reproduced from Compendium of Onion and Garlic Diseases and Pests, 2nd ed., 2008, American Phytopathological Society, St. Paul, MN. (3) Courtesy of E. A. Kurtz. Reproduced from Compendium of Onion and Garlic Diseases and Pests, 2nd ed., 2008, American Phytopathological Society, St. Paul, MN.

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Methods to Control *Sclerotium cepivorum*

- Crop Rotation.[1]
  - Ineffective due to the ability of sclerotia to remain dormant.
- Fumigation with methyl bromide.[1]
  - EPA prohibits use.
- Treatment with the pesticide metam- sodium.[2]
  - Need for constant re-application.
- Germination Stimulants.[1]
  - Currently being researched.

Source: Watt, Bruce. http://sustainable-farming.rutgers.edu/wp-content /uploads/2014/01/Bruce-WattUMaineGarlicWhiterot.png (accessed Dec 4, 2015).

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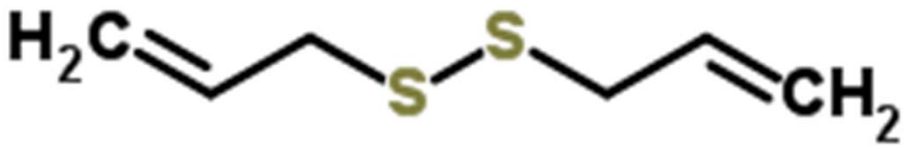
Germination Stimulant in the Form of Diallyl Disulfide (DADS)



- Principle component of the distilled oil of garlic.[5]
- Produced during the decomposition of allicin.[5]

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Figure 1. Some Organosulfur Compounds Derived From Garlic

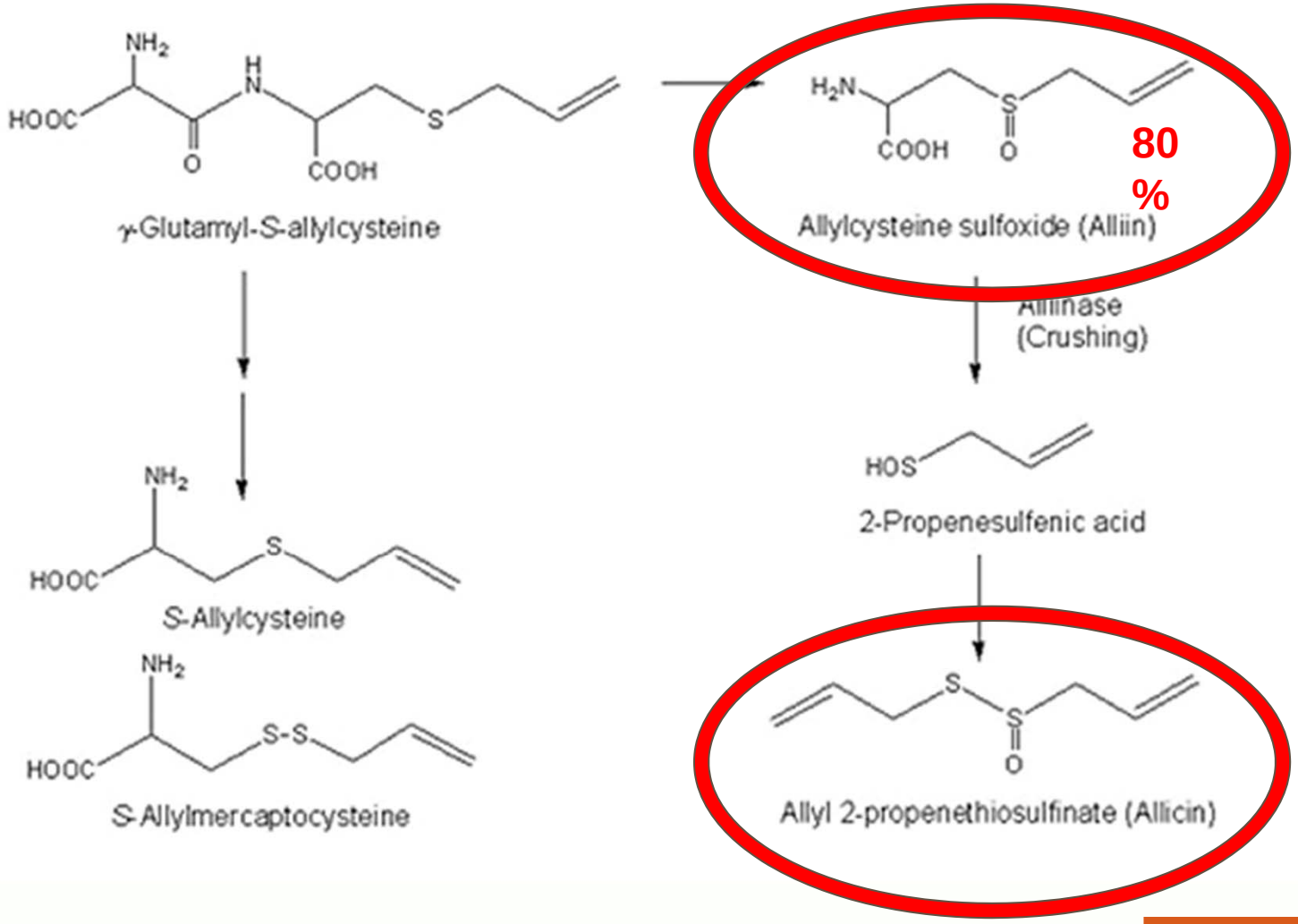

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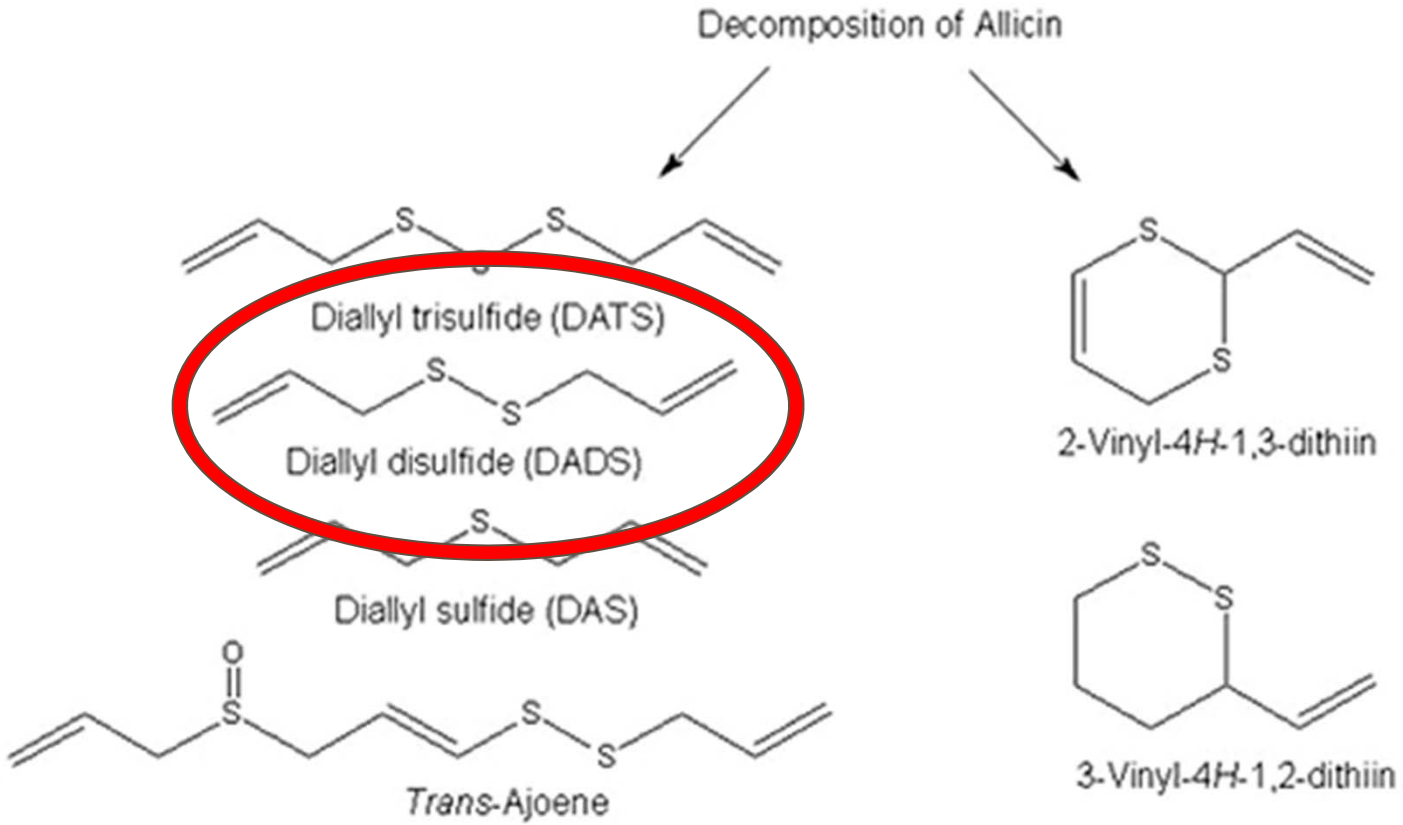
Figure 2. Some Organosulfur Compounds Derived From the Decomposition of Allicin



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Germination Stimulant in the Form of Diallyl Disulfide (DADS)

- DADS soil application reduced the incidence of Allium white rot on onions and garlic within the first 2 or 3 months of treatment.[1]
- Using germination stimulants helped improve root health and yield of garlic.[1]
- Can we use other bio-stimulants (eg. Garlic oil, garlic juice, waste products?)

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Research Results from the white rot team so Far

- Field experiments with garlic juice have limited effect on sclerotia germination
- Soil amendments from garlic/onion waste have no effect
- GC-MS analysis showed the concentration of effective sulfur stimulants were very low

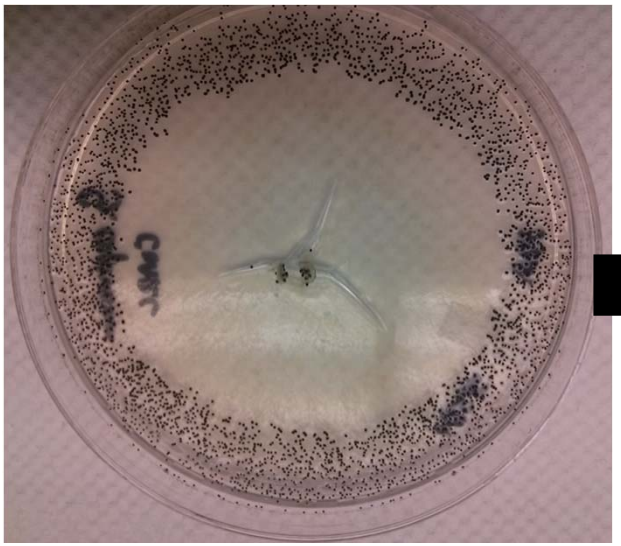
Objectives

- Develop a fast laboratory approach to stimulate sclerotia germination
- Use laboratory approach to screen bio-stimulants
- Provide bio-stimulant input for field trials

Sclerotia isolation (Dung Jeremiah)

- Sclerotia isolation
- Sclerotia preparation and activation
- Sclerotia viability

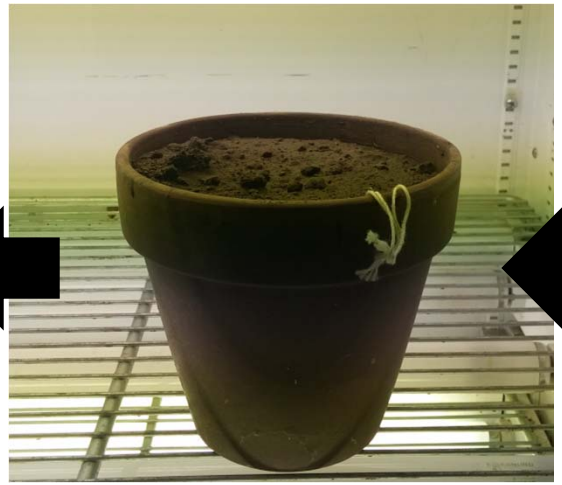
Grow *S. cepivorum* on potato dextrose agar



Harvest sclerotia

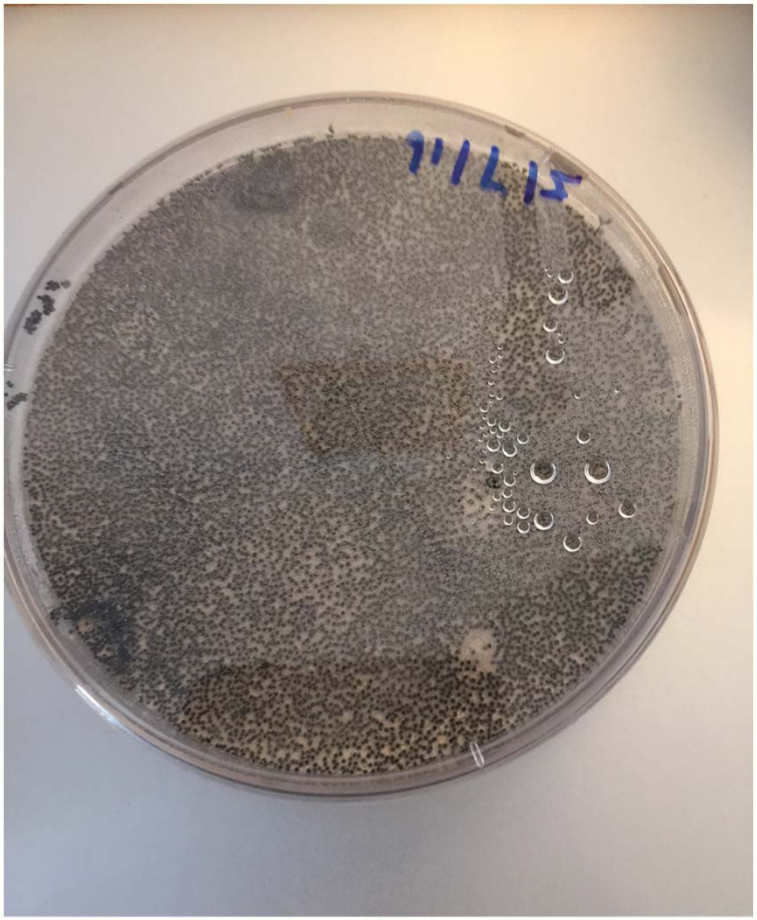
Sclerotia in cloth tea bags

Incubate sclerotia in field soil for 3-6 months in the field, greenhouse, or growth chamber



Recover sclerotia via sieving and sucrose flotation

Sclerotia Gemination on PDA plate



1. Sclerotia can germinate on PDA plate without any stimulants

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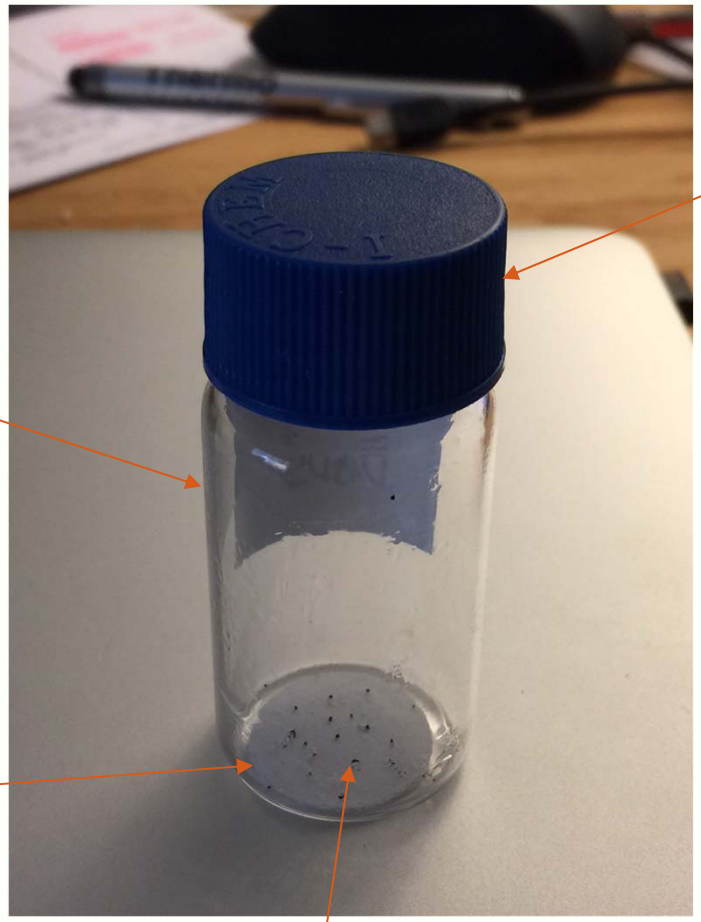
Challenges for germination system

- Sclerotia should not geminate without stimulants
- Need best germination environment
  - Temperature, moisture, oxygen
- Keep stimulants in a defined container
  - Sulfur compounds are volatile
  - Escape
  - Cross-contamination

I-Chem Certified pre-cleaned vial, 20 mL



Teflone lined Silicone Septa

When add PDA agar to the vial, achieve germination

When just filter paper, no germination

Filter paper, maintain moisture

Sclerotia

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How to introduce sample?

- Need clean the sclerotia

  -kill other contaminating bacteria
- Sulfur stimulants must be dissolved in organic solvent
  - Dilute the stimulants
  - Organic solvent should not inhibit sclerotia germination

Experimental Design and Evaluation of Germination Levels

- Autoclave 2 ml PDA agar in 20 ml clean vials, put one small filter paper on the surface of PDA agar as supporting medium, add 100 µl H2O;
- Efficacy Study of different pre-treatments and post-treatments:

Set A: Wash schlerotia with 10% bleach before plating;

Set AM: Wash schlerotia with 10% bleach before plating, 10 µl methanol added after plating;

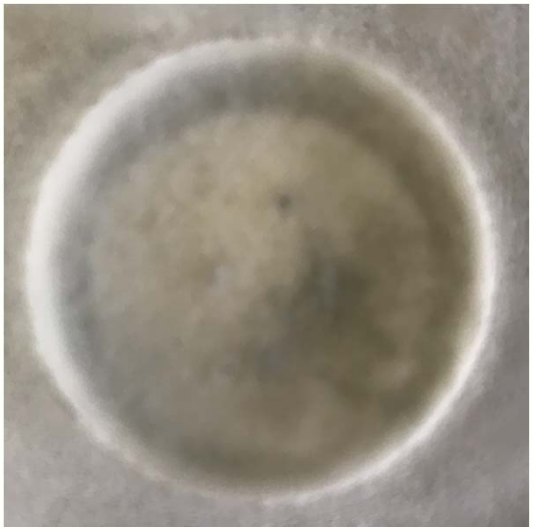
Set B: Wash schlerotia with 70% ethanol before plating;

Set BM: Wash schlerotia with 70% ethanol before plating, 10 µl methanol added after plating;

Set C: Wash schlerotia with water before plating;

Set CM: Wash schlerotia with water before plating, 10 µl methanol added after plating;

- **Germination Results -- Set C & CM**



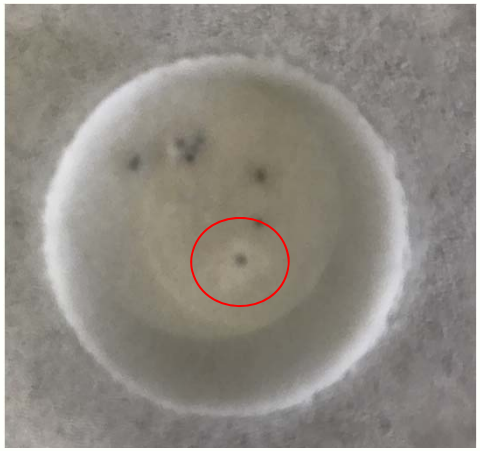
(a) PDA agar, filter paper, 100 µL H2O; wash schlerotia with water before plating.

No germination. Contaminated badly.

(b) PDA agar, filter paper, 100 µL H2O; wash schlerotia with water before plating, add 10 µL methanol after plating.

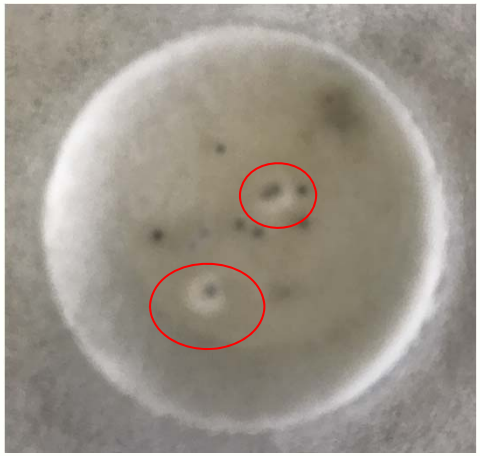
Germination level: (++)

- **Germination Results -- Set A & AM**



(a) PDA agar, filter paper, 100 µL H2O; wash schlerotia with 10% bleach before plating.

Germination level: (+)



(b) PDA agar, filter paper, 100 µL H2O; wash schlerotia with 10% bleach before plating, add 10 µL methanol after plating.

Germination level: (++)

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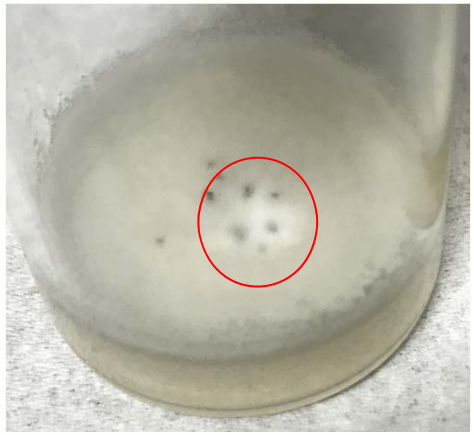
• Germination Results -- Set B & BM

(a) PDA agar, filter paper, 100 µL H2O; wash schlerotia with 70% ethanol before plating.

Germination level: (++)



(b) PDA agar, filter paper, 100 µL H2O; wash schlerotia with 70% ethanol before plating, add 10 µL methanol after plating.

Germination level: (++)

Conclusion:

1. 10% bleach and 70% ethanol both can kill infectious microbe without killing schlerotia, but which pre-treatment has the least suppression effect on schlerotia germination remains to be determined.

2. All treatment added methanol also germinated, proving that methanol actually do not inhibit schlerotia germination.

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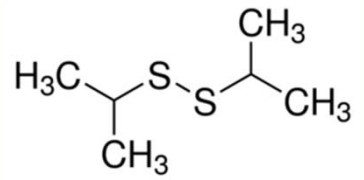
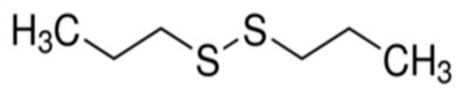
Experimental Design and Evaluation of Germination Levels

- **Germination Stimulants**

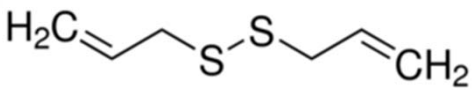
Others: raw sliced garlic, garlic oil blend



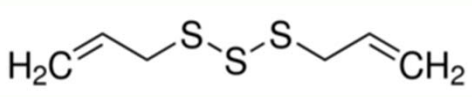
Isopropyl disulfide (IPDS)



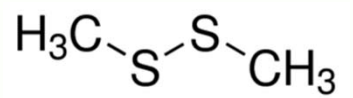
Dipropyl disulfide (DPDS)



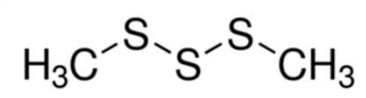
Diallyl disulfide (DADS)



Diallyl trisulfide (DATS)



Dimethyl disulfide (DMDS)



Dimethyl trisulfide (DMTS)

Figure 3. Chemical Structure of Sulfur Stimulants

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Study 1—Efficacy study of individual compounds

Set A: Preliminary test of DADS, raw garlic, garlic oil blend
Set B: filter paper on 1 mL water agar, no nutrient provided, 70 µl H_2O (Control and Test)
Set C: filter paper as supporting medium, no agar, no nutrient, 70 µl H_2O (Control and Test)

Control: 10 µl methanol
Test: Each sulfur compound was added at two concentrations, 10 µl of individual compound (10000 ppm and 1000 ppm in methanol, respectively) was added into 20 mL vial (sealed during incubation) = headspace concentration of 5 ppm and 0.5 ppm, respectively
Triplicates
Incubated at 15 ° C, and observe every day
+++ represent the germination level

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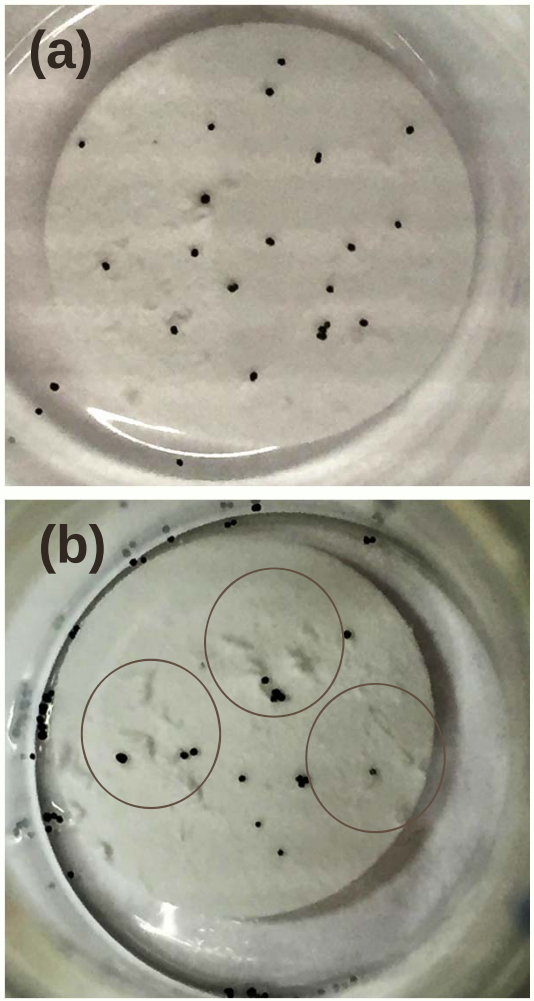
- **Germination Results -- *Set A***



(a) Control
Filter paper, 10 sclerotia, 100 µl H_2O,
10 µl methanol in 20 mL vial

No germination

(b) DADS stimulant (5 ppm in headspace)
Filter paper, 10 sclerotia, 100 µl H_2O
10 µl 1% (10000 ppm) DADS in 20 mL vial

Germination level: (+++)

Figure 4. Germination Result of Set A

- *S. cepivorum* were provided by Dr. Dung; +++ represent the germination level

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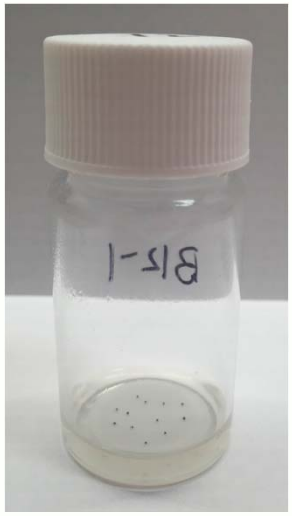
- **Germination Results – *Set B***

Germination Stimulant in Set B

Isopropyl Disulfide (10000ppm, 1000ppm)
Dipropyl Disulfide (10000 ppm, 1000 ppm)
Diallyl Disulfide (10000 ppm, 1000 ppm)
Diallyl Trisulfide (10000 ppm, 1000 ppm)
Dimethyl Disulfide (10000 ppm, 1000 ppm)
Dimethyl Trisulfide (10000 ppm, 1000 ppm)



Set B: filter paper on 1 mL water agar, no nutrient provided, 70 µl H_2O (Control and Test)

- Isopropyl Disulfide treatment showed germination in three days incubation
- No germination was observed in other treatments

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- **Germination Results – *Set C***

Germination Stimulant in Set C

Isopropyl Disulfide (10000ppm, 1000 ppm, 10ppm)
Dipropyl Disulfide (10000 ppm, 1000 ppm, 10 ppm)
Diallyl Disulfide (10000 ppm, 1000 ppm, 10 ppm)

Set C: filter paper as supporting medium, no agar, no nutrient, 70 µl H_2O (Control and Test)

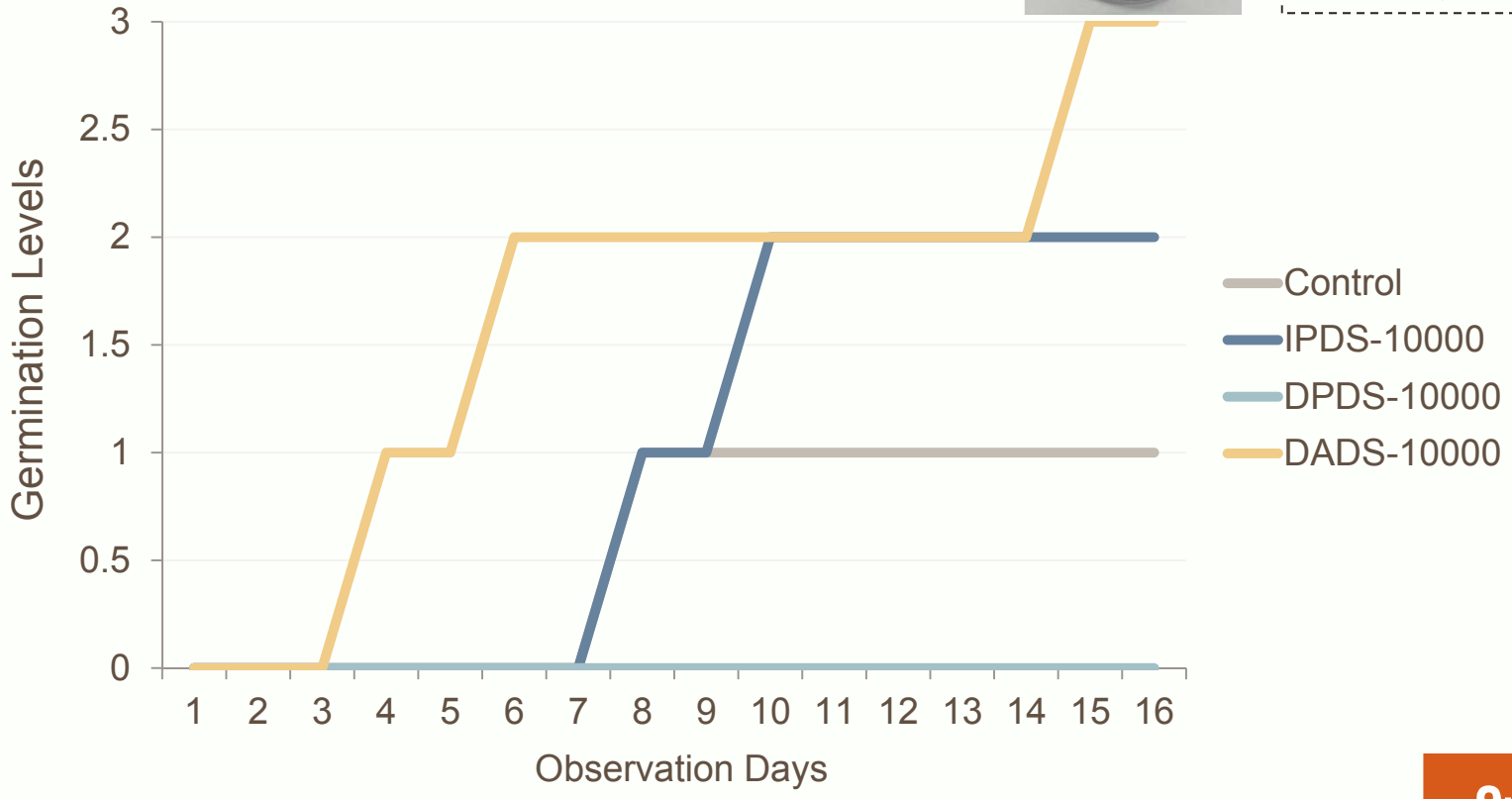

Figure 5. Germination Result of Set C

(0 -- no germination, 3 – germination level +++)

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Study 2—Efficacy study of individual compounds by comparing new and old sclerotia

- **Efficacy Study of Individual Compound:**

Set D: Use sclerotia received on 06/02/2016. filter paper as supporting medium, no agar, no nutrient, 50 µl H_2O (Control and Test)
Set E: use sclerotia received from Dr. Dung on 10/26/2016, other treatments were the same as Set D

D1,E1: Control,
D2,E2: [DADS] 10µl 1% Diallyl Disulfide (10000 ppm)
D3,E3: [DATS] 10µl 1% Diallyl Trisulfide (10000 ppm)
D4,E4: [DMDS] 10µl 1% Dimethyl Disulfide (10000 ppm)
D5,E5: [DMTS] 10µl 1% Dimethyl Trisulfide (10000 ppm)
D6,E6: [garlic oil] 10µl garlic oil blend
D7,E7: [DADS sample 1] 10µl 1% DADS sample 1(10000 ppm)
D8,E8: [DADS sample 2] 10µl 1% DADS sample 2 (10000 ppm)
D9,E9: [AMS] 10µl 1% Allyl Methyl Sulfide (10000 ppm)
D10,E10: [AS] 10µl 1% DADS (10000 ppm)
D11,E11: [DPDS] 10µl 1% Dipropyl Disulfide (10000 ppm)
D12,E12: [IPDS] 10µl 1% Isopropyl Disulfide (10000 ppm)

Duplicates, Incubated at 15 ° C, and observe every day
+++ represent the germination level

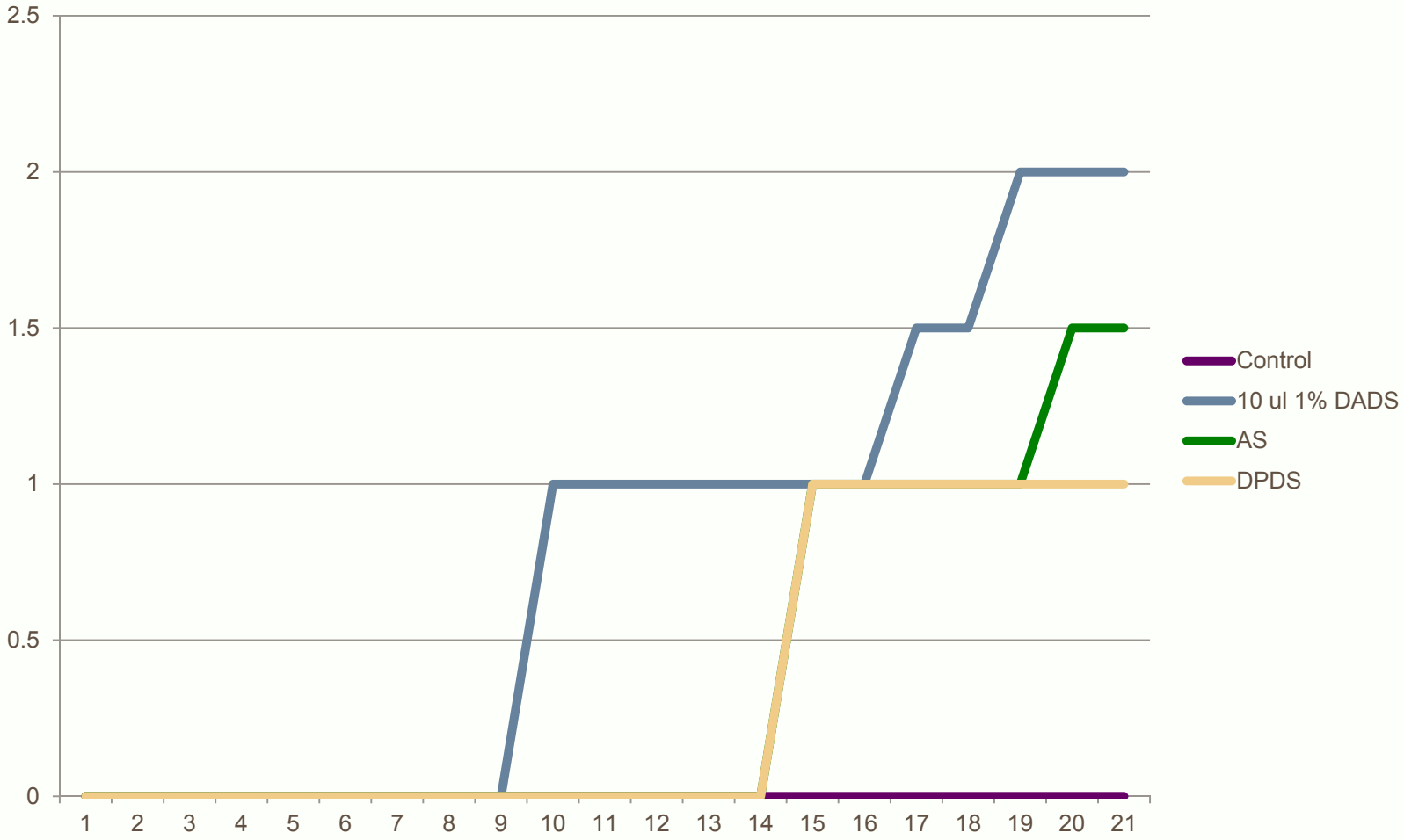

Figure 6. Germination Result of Set D

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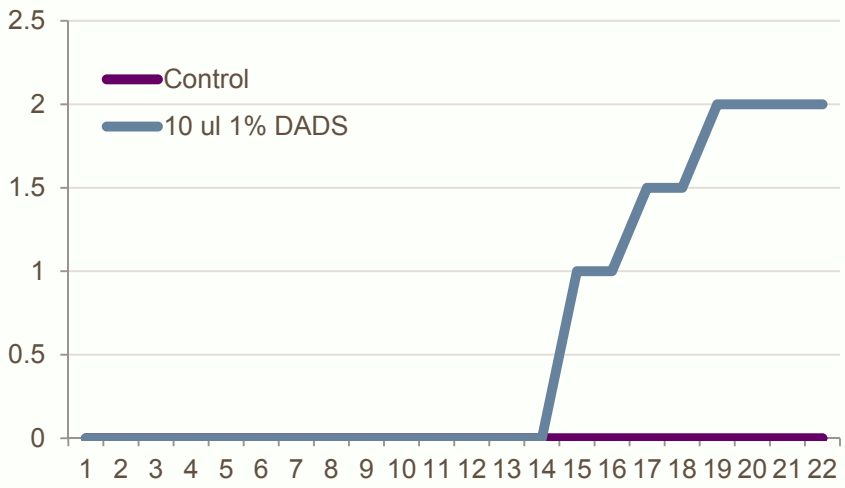

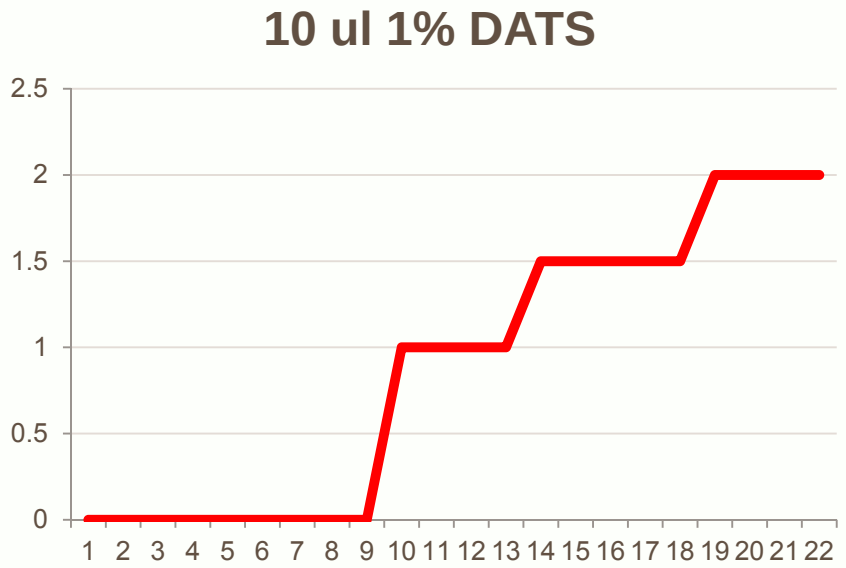

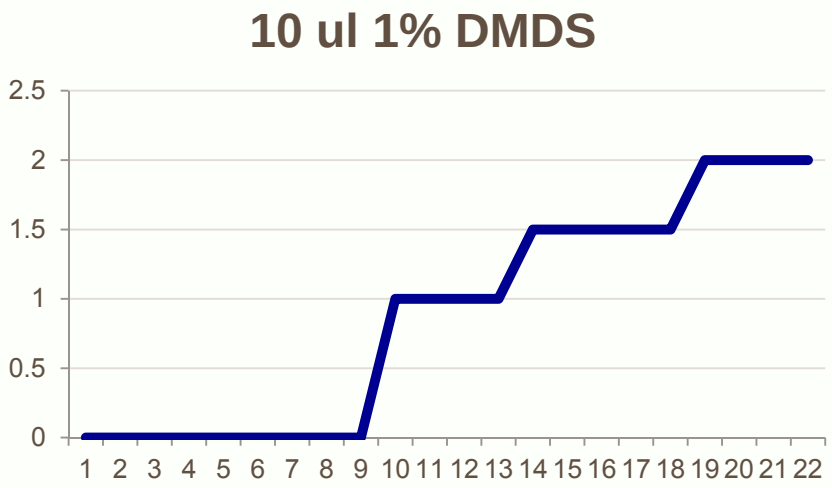

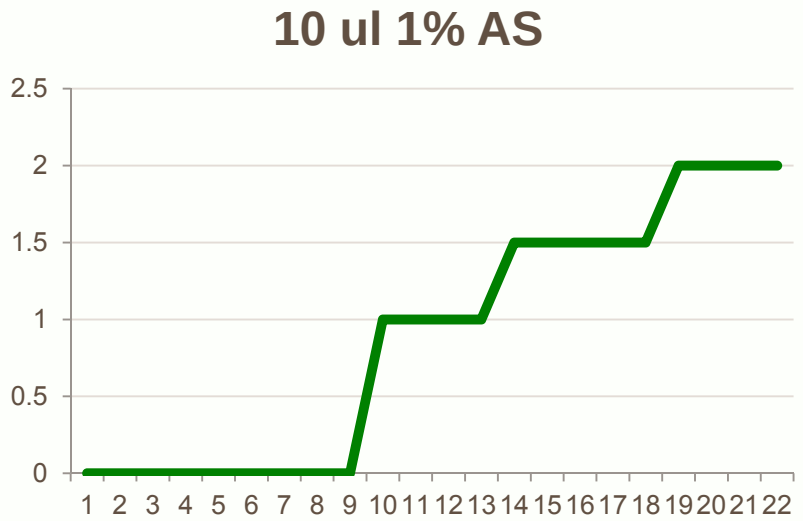

Figure 7. Germination Result of Set E

Conclusion

- Beside Diallyl Disulfide (DADS), Isopropyl Disulfide (IPDS) could also be an effective germination stimulant for sclerotia
- Current effective dosage: 10 µl of sulfur compound added into 20 mL vial (5 ppm in the vial)
- Sclerotia activity changes during long term storage by comparing germination rate using fresh sclerotia and aged sclerotia, fresh sclerotia is more sensitive to more compounds including DADS, AS, DMDS, DMTS

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Study 3—screening of germination stimulants

- **Efficacy Study of Individual Compound:**

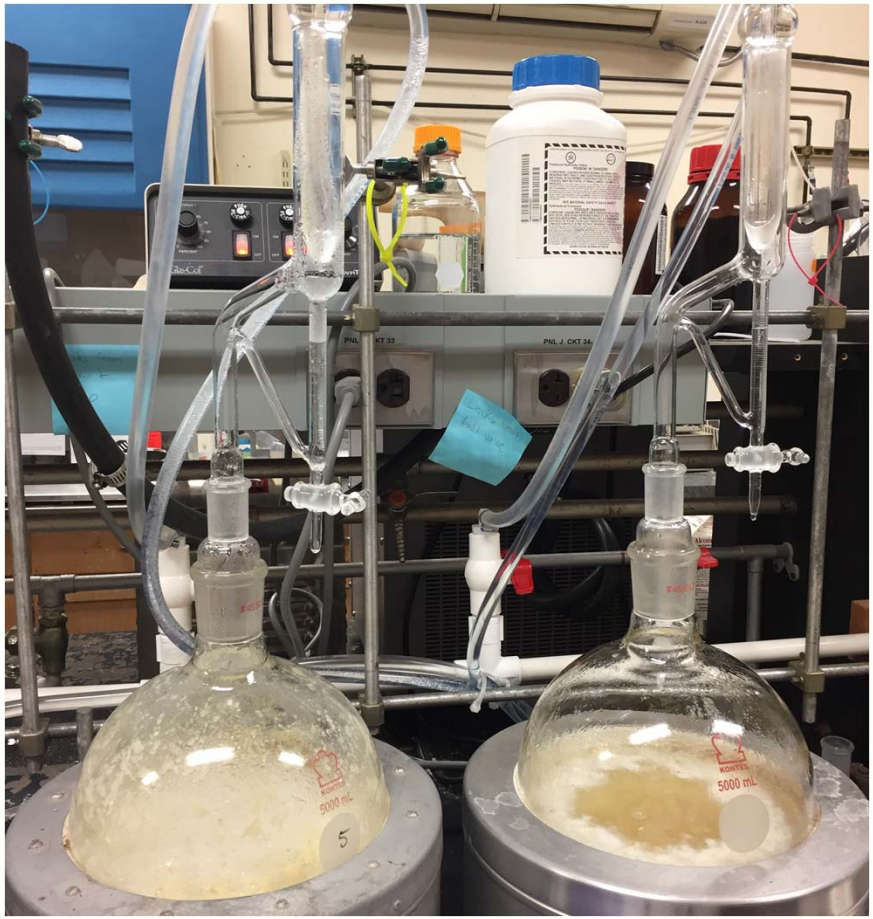
Set F: use sclerotia received from Dr. Dung on 10/26/2016

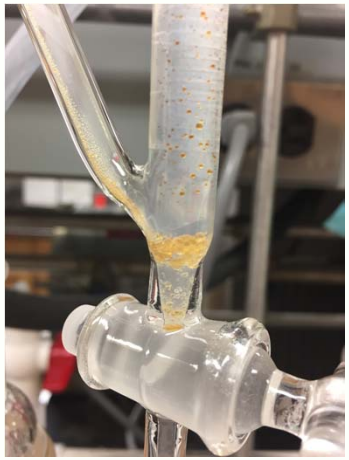
F0: Control, 10 µl MeOH + 50 µl Milli-Q
F1: [DATS] 10µl 10% DATS (100mg in 1mL acetone), 50 µl Milli-Q
F2: [DMDS] 10µl 1% Dimethyl disulfide (10000 ppm), 50 µl Milli-Q
F3: [AMS] 10µl 1% Allyl methyl sulfide (10000 ppm), 50 µl Milli-Q
F4: [DPDS] 10µl 1% Dipropyl disulfide (10000 ppm), 50 µl Milli-Q
F5: [IPDS] 10µl Isopropyl disulfide, 50 µl Milli-Q
F6: [commercial garlic oil] 10µl commercial garlic oil, 50 µl Milli-Q
F7: [distilled garlic oil 1] 10µl California early garlic oil, 50 µl Milli-Q
F8: [distilled garlic oil 2] 10µl California late garlic oil, 50 µl Milli-Q
F9: [commercial DADS] 10µl 1% commercial DADS (10000 ppm), 50 µl Milli-Q
F10: [DADS sample 1] 10µl 1% DADS sample 1 (10000 ppm), 50 µl Milli-Q
F11: [DADS sample 2] 10µl 1% DADS sample 2 (10000 ppm), 50 µl Milli-Q
F12: [garlic juice] 10 µl GJ 4555-315, 50 µl Milli-Q

Duplicates, Incubated at 15 ° C, and observe every day
+++ represent the germination level

Result: no germination after 2 weeks, all vials are dried

Garlic oil distillation





- 400g garlic (California early/California late)
- 1.5 L Milli-Q water
- Blend garlic with water, and let it sit in the hood for 2 hrs
- Distillation and collect the oil phase

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Study 3—screening of germination stimulants

- **Efficacy Study of Individual Compound:**

Set G: use sclerotia received from Dr. Dung on 10/26/2016

G0: Control, 20 µl MeOH + 100 µl Milli-Q
G1: [Commercial DADS] 20µl 1% DADS (10000 ppm) + 100 µl Milli-Q
G2: [DADS sample 1] 20µl 1% 20µl 1% DADS (10000 ppm) + 100 µl Milli-Q
G3: [DADS sample 2] 20µl 1% 20µl 1% DADS (10000 ppm) + 100 µl Milli-Q
G4: [DPDS] 20µl 1% Dipropyl disulfide(10000 ppm) + 100 µl Milli-Q
G5: [IPDS] 20µl 1% Isopropyl disulfide (10000 ppm) + 100 µl Milli-Q
G6: [agar control] on agar, 20 µl MeOH + 100 µl Milli-Q
G7: [control commercial DADS] agar, 20µl 1% DADS (10000 ppm) + 100 µl Milli-Q
G8: [agar DADS sample 1] agar, 20µl 1% 20µl 1% DADS (10000 ppm) + 100 µl Milli-Q
G9: [agar DPDS] agar, 20µl 1% Dipropyl disulfide(10000 ppm) + 100 µl Milli-Q
G10: [agar IPDS] agar, 20µl 1% Isopropyl disulfide (10000 ppm) + 100 µl Milli-Q
Duplicates, Incubated at 15 ° C, and observe every day
+++ represent the germination level

- After two week's observation, no germination

Summary

- Developed a laboratory protocol for biostimulant screening
- Achieved sclerotia germination with sulfur-containing biostimulants
- Identified several other bio-active sulfur compounds that can geminate sclerotia
- However, we are unable to achieve reproducible germination

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Next step

- Better environment control (temperature, moisture)
- Evaluate California soil as germination media
- Screen biostimulants, including garlic oil
- Study stimulant efficacy

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