Detection and Quantification of Bioactive Sulfur Compounds in Onion and Garlic for White Rot Control

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Background

- Can spread quickly and destroy the plant
- Sclerotia can survive more than 40 years in soil
- Once infected, the field can no longer be used for garlic production
Current Challenges for White Rot Control

- **Fumigation with methyl bromide**
  - the most effective treatment
  - killing more than 95% of the sclerotia in the soil
  - phased out and uneconomical

- **Soil treatment with metam-sodium**
  - results have been erratic
  - retreatment is necessary
  - risk to the environment
  - a general biocide.

- **Crop rotation**
  - Ineffective
  - sclerotia are dormant in the absence of specific sulfides exuded from roots of *Allium* crops
Sclerotia Germination

• The fungus will only colonize on *Allium* plants
• Sclerotia germinate only in response to exudation by *Allium* roots
• These exudates contain alkyl and alkenyl-L-cysteine sulphoxides
• Alkyl and alkenyl-L-cysteine sulphoxides are metabolized by the soil microflora to yield a range of volatile thiols and sulfides
• Volatile thiols and sulfides activate the dormant sclerotia.
Research Rationale

• Apply stimulants to the ground in the absence of an Allium crop
• The sclerotia may be “tricked” into germinating.
• In the absence of a host, the mycelium from germinating sclerotia will persist for a few days to several weeks depending on the soil temperature, then die after exhausting nutrient reserves.
• Thus, the total population of sclerotia in a field is susceptible to control with this method (Crowe et al., 1980).
What do we know?

- Diallyl disulfide forced 90-99% of the sclerotia to germinate (Davis et al, 2009).
  - similar to methyl bromide fumigation
  - DADS is no longer commercially available
- We know natural products will work (Davis et al, 2009)

Objectives

- Identify natural sulfur-organic compounds in onion and garlic
- Develop analytical method(s) to quantitate the concentration of volatile sulfur compounds
- Search for potential sources of biostimulates
  - High concentration of volatile sulfur compounds
  - Economical
- Develop a sustainable approach using garlic/onion oil or waste products as biostimulants to manage onion and garlic white rot (long term goal)
Headspace-GC-MS Method

Sample trays

Headspace (HS)
Volatile Sulfur Compounds Analysis

- **Method development**
  - **Sample preparation**
    - *Fresh garlic/onion*: dried with liquid nitrogen, blended into powder → 2g of garlic/onion powder + 6 mL H₂O + 2 mL methanol + 50 µl IS (518 ppm Ethyl Methyl Sulfide, 776 ppm Isopropyl Disulfide) + 2g salt
  - **Instrument analysis**
    - *Headspace sampling*: Samples placed into a 20 ml headspace vial; Equilibrated at 50°C for 50 min; Syringe temp 50°C; Inject 500 µl, 1:5 split
    - *GC-MS Parameters*: HP-Wax Column, 30m length, 250 µm diameter, 0.25 µm film thickness; Oven program: 40°C, 2 min
Calibration curve development

Dia llyl Disulfide

Response Ratio

Concentration Ratio

Coef of Det \( (r^2) = 0.995 \)
# Calibration Curve Development

<table>
<thead>
<tr>
<th>Compound</th>
<th>Average ppm</th>
<th>Stdev</th>
<th>Actual ppm</th>
<th>%recovery</th>
<th>Linear dynamic range (mg/L)</th>
<th>Equation</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allyl Methyl Sulfide</td>
<td>10.2</td>
<td>0.25</td>
<td>10.00</td>
<td>102%</td>
<td>1.25~40</td>
<td>Y=0.9435x</td>
<td>0.998</td>
</tr>
<tr>
<td>Dimethyl Disulfide</td>
<td>4.0</td>
<td>0.10</td>
<td>4.00</td>
<td>101%</td>
<td>0.5~16</td>
<td>Y=1.386x</td>
<td>0.998</td>
</tr>
<tr>
<td>Allyl Sulfide</td>
<td>4.0</td>
<td>0.14</td>
<td>4.00</td>
<td>100%</td>
<td>0.5~16</td>
<td>Y=0.5307x</td>
<td>0.997</td>
</tr>
<tr>
<td>Dimethyl Trisulfide</td>
<td>3.8</td>
<td>0.06</td>
<td>4.00</td>
<td>94%</td>
<td>0.5~16</td>
<td>Y=0.1485x</td>
<td>0.998</td>
</tr>
<tr>
<td>Diallyl Disulfide</td>
<td>11.3</td>
<td>0.72</td>
<td>10.00</td>
<td>113%</td>
<td>1.25~20</td>
<td>Y=0.1485x</td>
<td>0.996</td>
</tr>
<tr>
<td>Diallyl Trisulfide</td>
<td>2.88</td>
<td>0.27</td>
<td>4.00</td>
<td>72%</td>
<td>0.5~16</td>
<td>Y=0.003145x2 +0.01669x</td>
<td>0.992</td>
</tr>
</tbody>
</table>
Volatile sulfur compounds in Fresh Garlic

*IS1: Ethyl Methyl Sulfide
1: Allyl Methyl Sulfide
*IS2: Isopropyl Disulfide
2: Dimethyl Disulfide
3: Allyl Sulfide
4: Methyl Allyl Disulfide
5: Diallyl Disulfide
6: Diallyl Trisulfide

*IS represents for Internal standard, which is added manually for quantitative analysis purpose.
Fresh Garlic (3 samples)

CE: California Early  CL: California Late  FTR: Forked Tree Ranch
fresh onion

![Graph showing concentration of various onion compounds](image-url)
Onion Waste

1: Ethanol  2: Propanol  3: S-Methyl thioacetate  4: Isobutyl alcohol  5: Acetic acid
Soil amendment

Concentration (ppm)

- Allyl Methyl Sulfide
- Dimethyl Disulfide
- Allyl Sulfide
- Dimethyl Trisulfide
- Diallyl Disulfide
- Diallyl Trisulfide

Soil amendment
Garlic juice 1 (n=3)-2015

Concentration (ppm)

GJ1(2+6+2)

- Allyl Methyl Sulfide
- Dimethyl Disulfide
- Allyl Sulfide
- Dimethyl Trisulfide
- Diallyl Disulfide
- Diallyl Trisulfide

GJ1: LOT # 4555-315 (the Garlic Company, CA)
Garlic juice 2 (n=3)-2015

Concentration (ppm)

GJ2(2+6+2)

GJ 2: Garlic Juice Crop App, Formula #2, LOT # 4466-315 (the Garlic Company, CA)
### Concentration of sulfur compounds (mg/kg)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>GC 1522903</th>
<th>K 032651</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allyl Methyl Disulfide</td>
<td>5.50 ± 0.26</td>
<td>0.57 ± 0.03</td>
</tr>
<tr>
<td>Dimethyl Disulfide</td>
<td>0.50 ± 0</td>
<td>0.15 ± 0</td>
</tr>
<tr>
<td>Allyl Disulfide</td>
<td>4.13 ± 0.23</td>
<td>0.35 ± 0</td>
</tr>
<tr>
<td>Dimethyl Trisulfide</td>
<td>0.32 ± 0.03</td>
<td>0.05 ± 0</td>
</tr>
<tr>
<td>Diallyl Disulfide</td>
<td>3.22 ± 0.20</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>Diallyl Trisulfide</td>
<td>0.00 ± 0</td>
<td>0.00 ± 0</td>
</tr>
</tbody>
</table>
Commercial DADS sample

- Allyl sulfide (45.1%) at 451,000 ppm
- DADS (24.8%) at 247,000 ppm
- DATS (9.2%) at 91,500 ppm
How to make more DADS?

H₂C≡CH₂
Formation of VOC in Garlic

Alliin (S-allyl-L-cysteine sulfoxide)
(mesophyll cells)

S-methyl and S-trans-1-propenylcysteine sulfoxides

ALLINASE (vascular bundle cells; released upon crushing)

Allyl thiosulfinates

Allicin (diallyl thiosulfinate) (67-81%)

Allyl methyl thiosulfinates (16-26%)

Allyl trans-1-propenyl thiosulfinates (4-7%)

Allyl sulfides

Diallyl sulfides, n=1-3
(83-92%)

Allyl methyl sulfides, n=2-3
(8-17%)

Allyl trans-1-propenyl sulfides, n=2-3
(not detected)
Sample preparation

- Garlic cloves were dried with liquid nitrogen and blended into a powder.
  + 2.0 g of frozen garlic powder
  + 6.0 mL of 0.01 M phosphate buffer (pH 2, 4, 6, 7, or 8)
- Samples were incubated:
  - Room temperature, 35°C and 45°C degrees.
  - GC/MS analysis at 5 hours, 1 day, 3, 7, 14 days.
- Preparation for analysis:
  + small stir bar
  + 2.0 mL methanol (MeOH)
  + 50 μL internal standard (538 ppm ethyl methyl sulfide, 760 ppm isopropyl disulfide.
  + 2.0 g salt
Effect of pH on the formation of DADS in garlic paste

Conclusion:

(1) DADS formation is more favorable at higher pH (i.e. pH 7 and pH 8)

(2) Acidic condition will inhibit the alliinase activity,

(3) DADS is also not stable in acidic conditions.
Effect of incubation time on the formation of DADS at pH 7

Conclusion:

1. After 1 day incubation, the DADS concentration will increase about 2.5 times compared to no incubation treatment at pH 7.

2. Mild heating (35°C) will improve enzyme activity compared to room temperature.
Other compounds- Allyl Methyl Sulfide

Concentration (mg/kg) highest concentration is labeled in red

RT

35C
Other compounds- Dimethyl Disulfide

RT

35C

45C
Other compounds - Allyl Sulfide

**RT**

- **35C**
- **45C**
Other compounds - Dimethyl Trisulfide

**RT**

**35C**

**45C**
Other compounds - Diallyl Trisulfide

RT

35C

45C
Limits of the Sky

• Dynamic conversions of various forms of volatile sulfur compounds
• Can achieve 250 ppm of DADS, 10-100x of the garlic juice products
• All forms of volatile sulfur compounds were about 1000 ppm
• 1000x less than the commercial DADS
Homemade garlic juice (revised protocol)

Sample preparation

- Garlic cloves were dried with liquid nitrogen, blended into powder.
  - 50 g of frozen garlic powder
  - 100g of 0.05 M phosphate buffer (pH 7, 8, 9)

- Samples were incubated:
  - 35°C water bath, gentle shaking, 1 day.
  - Centrifuge at 4°C, 7000 rpm, 10 min

- Save the supernatant in a plastic tube and keep in 4°C refrigerator (Garlic Juice)

- Instrument analysis
  - HS-GC-FID
  - 50 µL internal standard (538 ppm ethyl methyl sulfide, 760 ppm isopropyl disulfide).
  - 2 ml Garlic Juice + 6 ml Salt Water + 2 ml MeOH + 2 g Salt
Concentration of sulfur compounds in homemade garlic juice during storage

AMS: Allyl Methyl Sulfide, DMDS: Dimethyl Disulfide, AS: Allyl Sulfide, DMTS: Dimethyl Trisulfide, DATS: Diallyl Trisulfide, DADS: Diallyl Disulfide

AMS: Allyl Methyl Sulfide, DMDS: Dimethyl Disulfide, AS: Allyl Sulfide, DMTS: Dimethyl Trisulfide, DATS: Diallyl Trisulfide, DADS: Diallyl Disulfide
New Directions

• Waste from garlic processing plants
  – Waste water?
  – Effluent from drying?
  – Other materials
Garlic powder?

- Study showed the efficacy of using garlic powder, effluent (garlic waste) at 500 liter/ha, DADS at 0.5 ml/m², and Methyl bromide at 448 kg/ha

Numbers of sclerotia of *Sclerotium cepivorum* in 500 cm³ of soil sampled immediately before treatments were applied (the first sampling date in each trial) and periodically thereafter in two locations. (RM Davis *et al.* 2007)
Garlic powder?

- Soil-applied garlic powder/paste could be a better option compared to the garlic juice product.

- DADS at 0.5 ml/m², and methyl bromide at 448 kg/ha

Numbers of sclerotia of *Sclerotium cepivorum* in 500 cm³ of soil sampled immediately before treatments were applied (the first sampling date in each trial) and periodically thereafter in two locations. (RM Davis *et al*. 2007)
Efficacy Study

- We do not know the minimum concentration of volatile sulfur compound required for sclerotia generation
- Jeremiah has prepared some viable sclerotia (~150) for us
- We want to develop a device to study sclerotia generation in lab
- Screen various volatile sulfur compounds and products in lab before field trial