MINT VARIETAL IMPROVEMENT

Building genomic resources
Assessing sources of Verticillium resistance

Presentation for the 2019 Washington Mint Convention

Kennewick, WA, December 3, 2019

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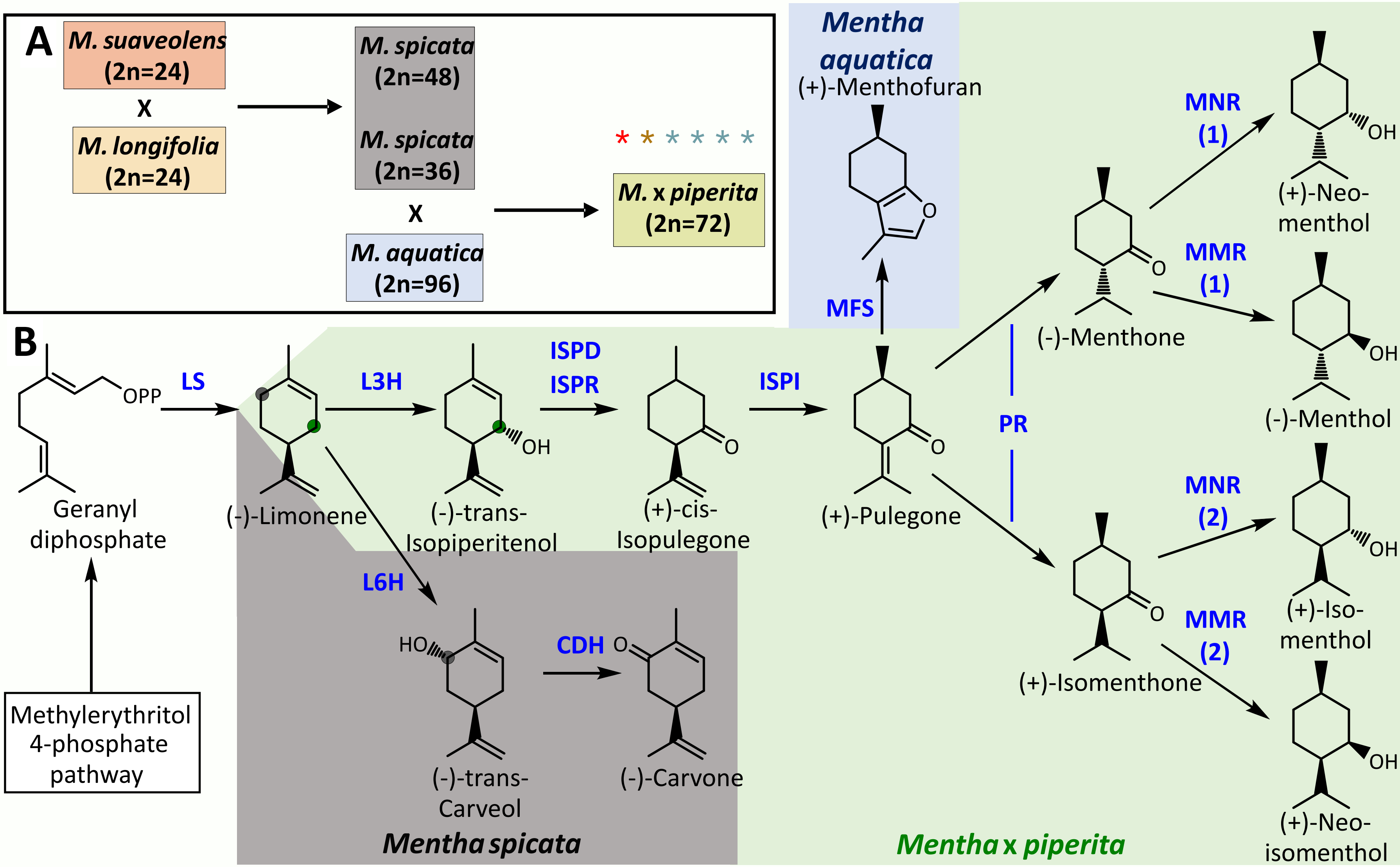
Background

The Mint Family History



2019 Research Objectives

1. Functional evaluation of candidate genes for oil characteristics in *Mentha longifolia*, a diploid species ancestral to both spearmint and peppermint.

   Andrew Lefors – bioinformatics

   Iris Lange – bioinformatics, gene cloning

   Narayanan Srividya – functional characterization

   Joel Velasco – functional characterization

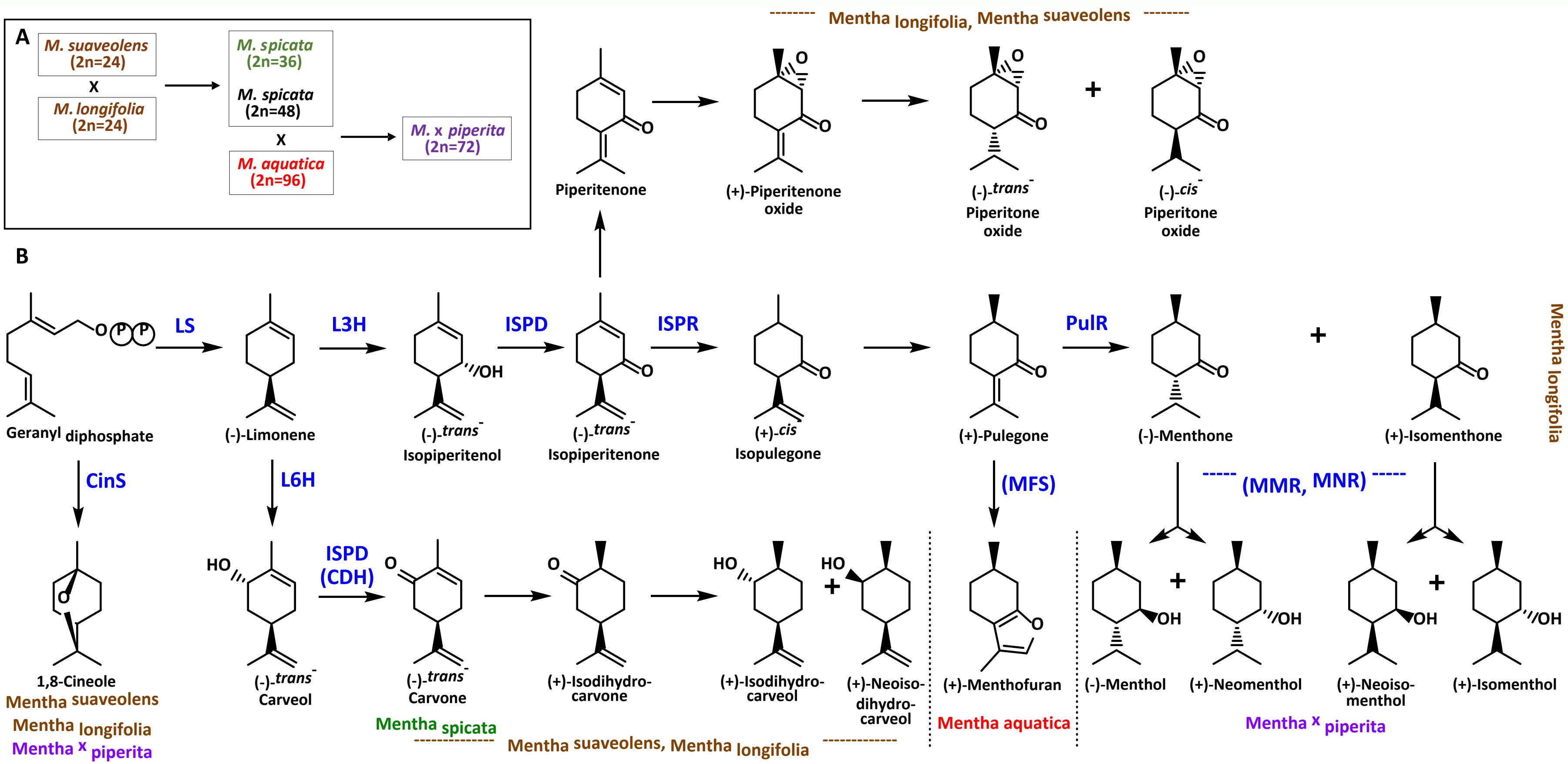
   (Kelly Vining, Iovanna Pandelova, OSU)

2. Develop protocols for the sensitive detection of volatiles produced by Verticillium-infected mint tissues and evaluate if Verticillium infection results in higher volatile emission in resistant spearmint compared to susceptible peppermint.

   Amber Parrish – Analytical chemistry
   (Kelly Vining, Jeremiah Dung, OSU)

Objective 1: Functional evaluation of candidate genes for oil characteristics in *Mentha longifolia*.

Overview of oil biosynthetic pathway



Objective 1: Functional evaluation of candidate genes for oil characteristics in *Mentha longifolia*.

How to find genes putatively involved in oil biosynthesis

Mentha longifolia genome assembly (12 chromosomes)

Automatic annotation with MAKER-P tool kit

Manual annotation by Blast search with sequence of gene with known function from other organisms against *M. longifolia* genome assembly

Generate result table and consolidate data

Note:

Even in diploid genomes, the occurrence of gene families is common, and it is important to decipher which gene copy (or copies) is (are) functional.

Table 1. Characteristics of gene families involved in essential oil biosynthesis in *M. longifolia* .

Gene Annotation	*M. longifolia* Chromosome	Location Identifier	Number of Introns	DNA Strand	Expression Level in Glandular Trichomes [TPM]	Homology to Canonical Genes (Amino Acid Level)
Terpene synthases (E.C. 4.2.3.- or EC 5.5.1.-)						
(-)-Limonene synthase						
LS-1	5	Mlong585_17628	6	(-)	5246	98 % identity to Q40322
LS-2	5	Mlong585_17636	6	(-)	as above	98 % identity to Q40322
Oxidoreductases that incorporate one atom of oxygen (EC 1.14.13.- or EC 1.14.14.-)						
(-)-Limonene 3-hydroxylase						
L3H-1	5	Mlong585_17637	1	(-)	2281	99 % identity to Q9XHE7
L3H-2	5	Mlong585_17629	1	(-)	as above	99 % identity to Q9XHE7
L3H-3	9	Mlong585_30867	1	(+)	2475	97 % identity to Q9XHE7
L3H-4	9	Mlong585_30878	1	(+)	as above	97 % identity to Q9XHE7
(+)-Menthofuran synthase						
MFS-L1	2	Mlong585_05231	2	(+)	1180	90 % identity to Q947B7
Oxidoreductases acting on CH-OH groups of donors (EC 1.1.1.-)						
(-)-Isopiperitenol / (-)-carveol dehydrogenase						
ISPD-1	2	Mlong585_04858	0	(-)	137	98 % identity to Q5C9I9
ISPD-2	2	Mlong585_04874	0	(+)	as above	98 % identity to Q5C9I9
ISPD-L1	2	Mlong585_04859	0	(-)	127	80 % identity to Q5C9I9
ISPD-L2	2	Mlong585_04873	0	(+)	as above	80 % identity to Q5C9I9

Table 1. Characteristics of gene families involved in essential oil biosynthesis in *M. longifolia* .

Gene Annotation	*M. longifolia* Chromosome	Location Identifier	Number of Introns	DNA Strand	Expression Level in Glandular Trichomes [TPM]	Homology to Canonical Genes (Amino Acid Level)
Oxidoreductases acting on CH-OH groups of donors (EC 1.1.1.-)						
(-)-Menthone:(-)-menthol reductase						
MMR-L1	11	Mlong585_36607	6	(-)	107	87 % identity to Q9XHE7
(-)-Menthone:(+)-isomenthol reductase-like						
MNR-L1	11	Mlong585_36608	0	(-)	n.a.	pseudogene
Oxidoreductases acting on CH-CH groups of donors (EC 1.3.1.-)						
(-)-Isopiperitenone reductase						
ISPR-1	11	Mlong585_36604	4	(-)	20	94 % identity to Q6WAU1
ISPR-2	11	Mlong585_36605	4	(+)	as above	99 % identity to Q6WAU1
(+)-Pulegone reductase						
PulR-1	3	Mlong585_09229	4	(-)	3116	95 % identity to Q6WAU0
PulR-2	3	Mlong585_09276	4	(-)	as above	95 % identity to Q6WAU0
PulR-3	3	Mlong585_09230	0	(+)	n.a.	pseudogene
PulR-L1	2	Mlong585_06346	4	(-)	153	92 % identity to Q6WAU0

Objective 1: Functional evaluation of candidate genes for oil characteristics in *Mentha longifolia*.

How to characterize the function of genes putatively involved in oil biosynthesis

M. longifolia cDNA library

PCR with gene-specific primers

Subclone amplicon into suitable expression vector

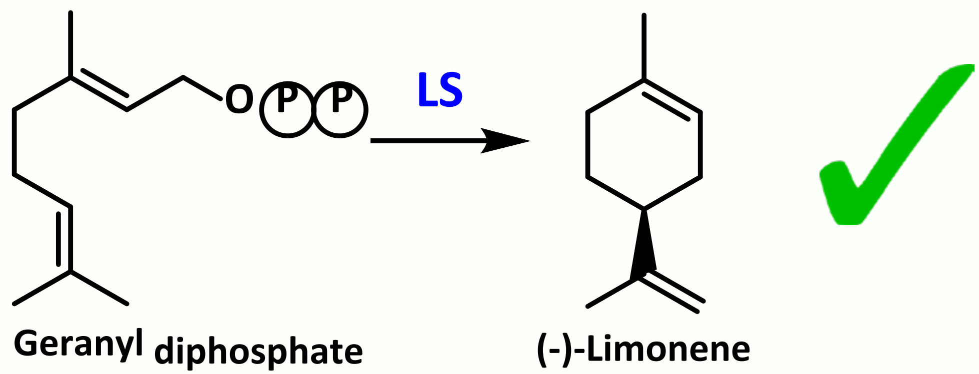
Transform *E. coli*

Induce production of recombinant enzyme

Purify recombinant enzyme

In vitro enzyme assay

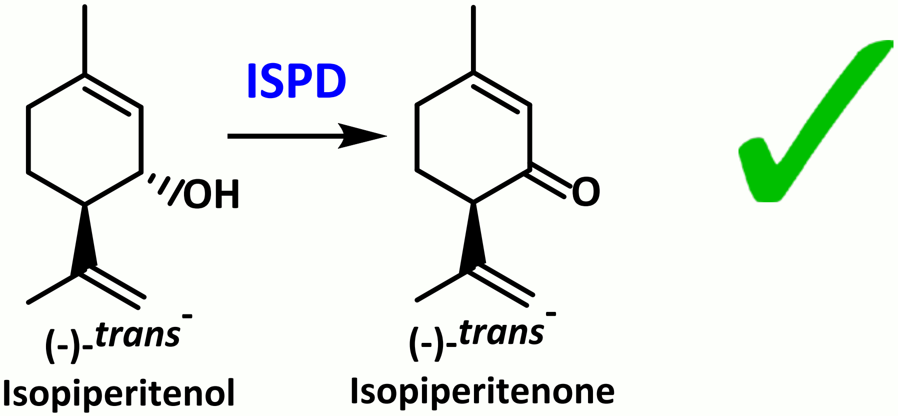
Both limonene synthase candidates catalyze conversion of geranyl diphosphate to (-)-limonene



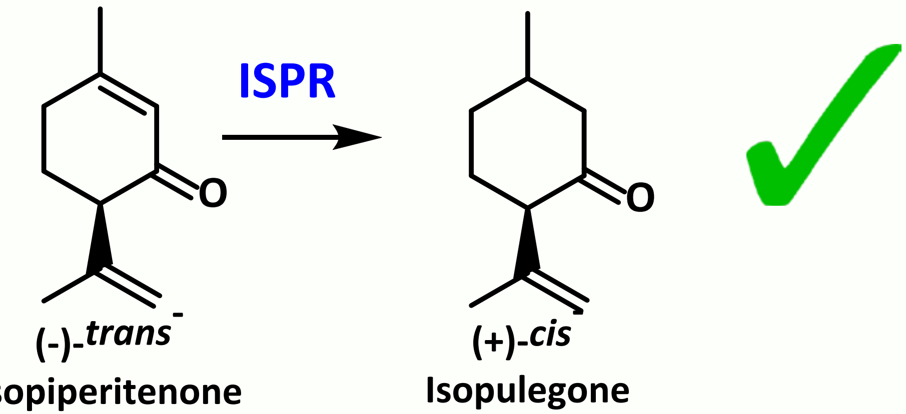
Four limonene hydroxylase candidates still under investigation

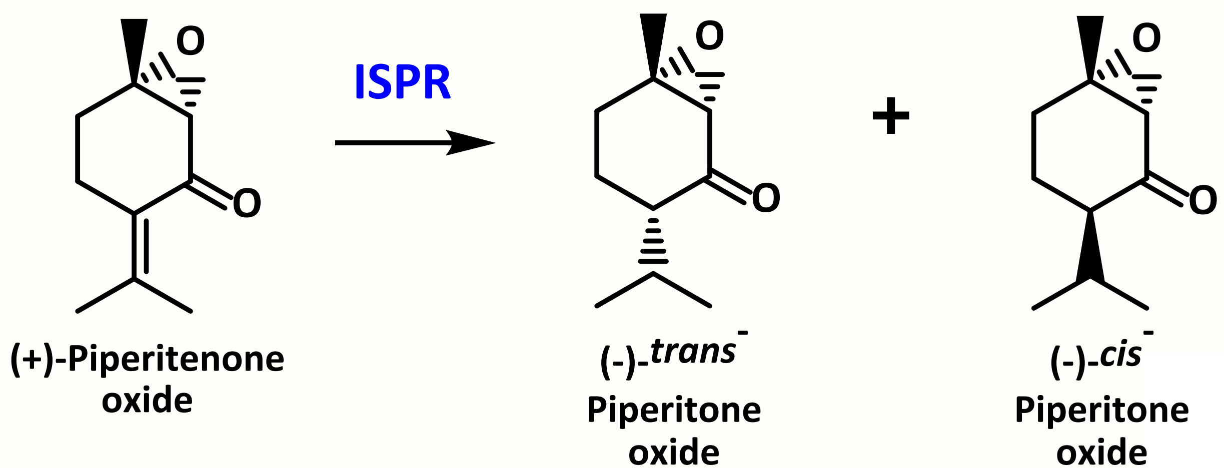
Menthofuran synthase candidate still under investigation

All four isopiperitenol dehydrogenase candidates catalyze conversion of isopiperitenol to isopiperitenone



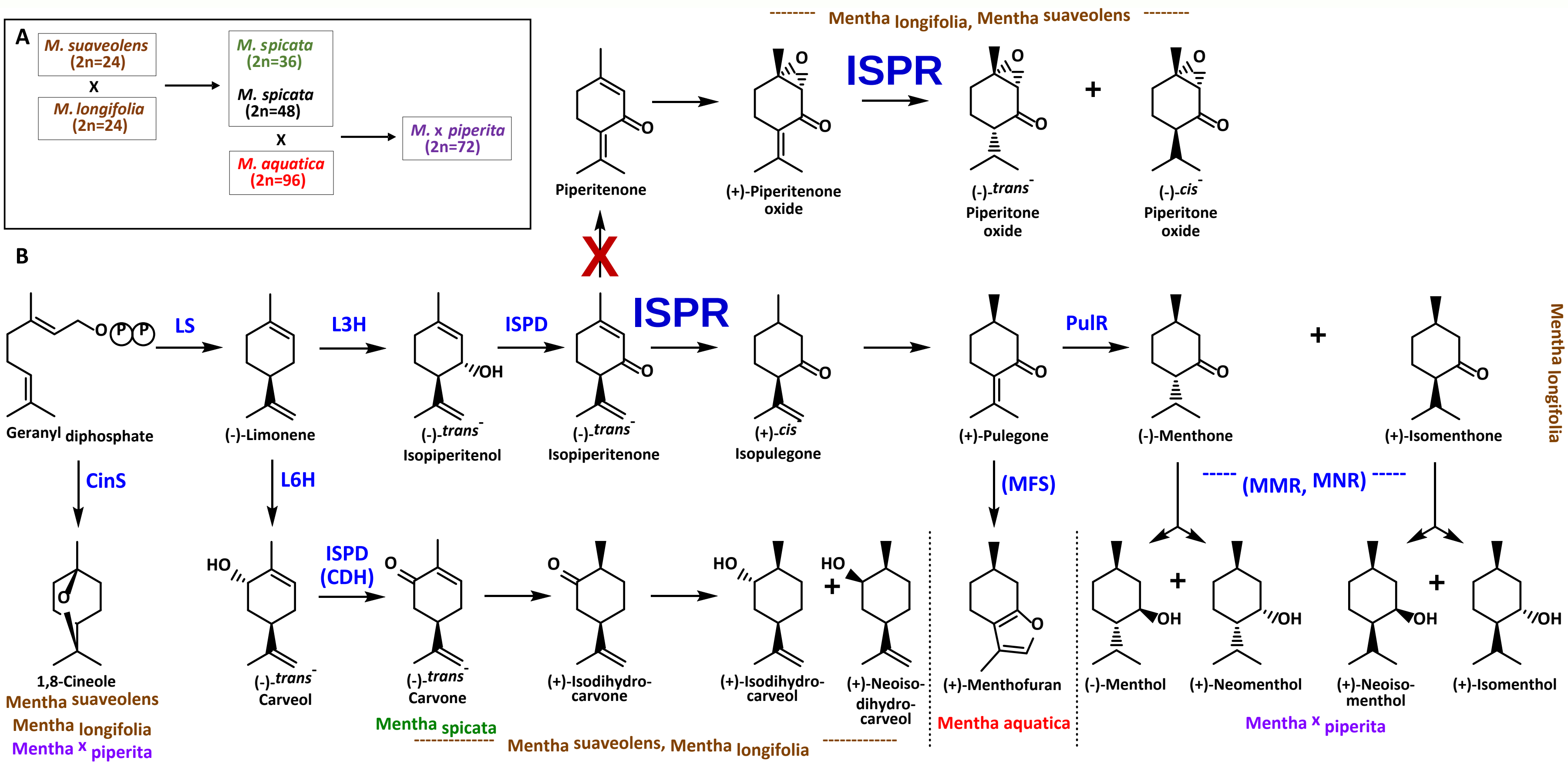
Both isopiperitenone reductase candidates catalyze conversion of isopiperitenone to isopulegone but are also active on other intermediates with double bonds



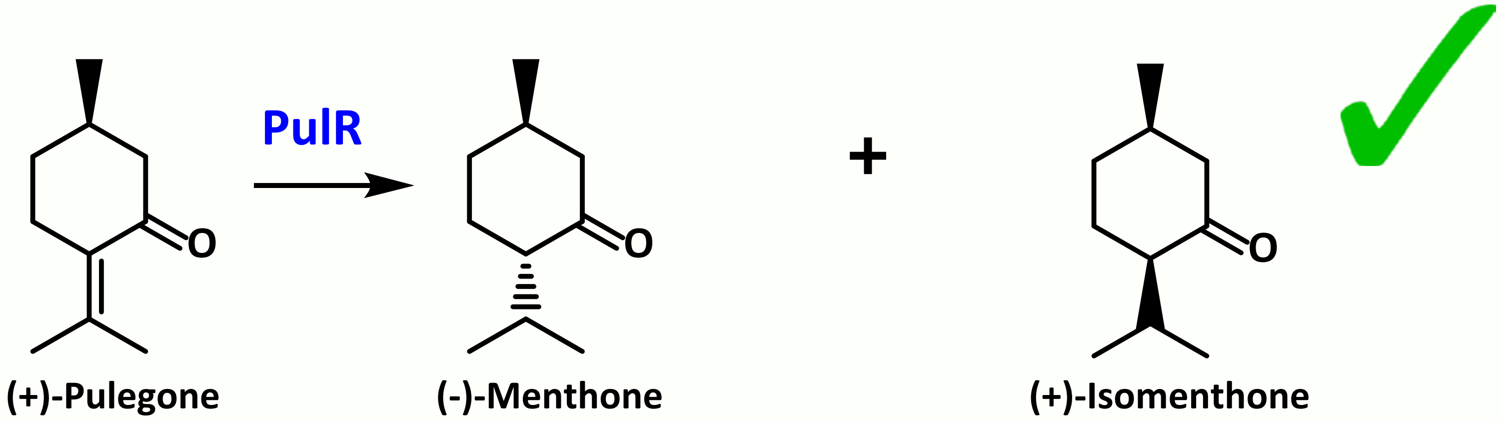


Why is this finding important?

First step toward understanding how to avoid formation of off-pathway products



Two of the four pulegone reductase candidates catalyze the conversion of pulegone to menthone and isomenthone



Objective 2: Evaluate if Verticillium infection results in higher/different volatile emission in resistant spearmint compared to susceptible peppermint

Grow mints on soil

Remove soil and inoculate with *Verticillium dahliae* (controls!)

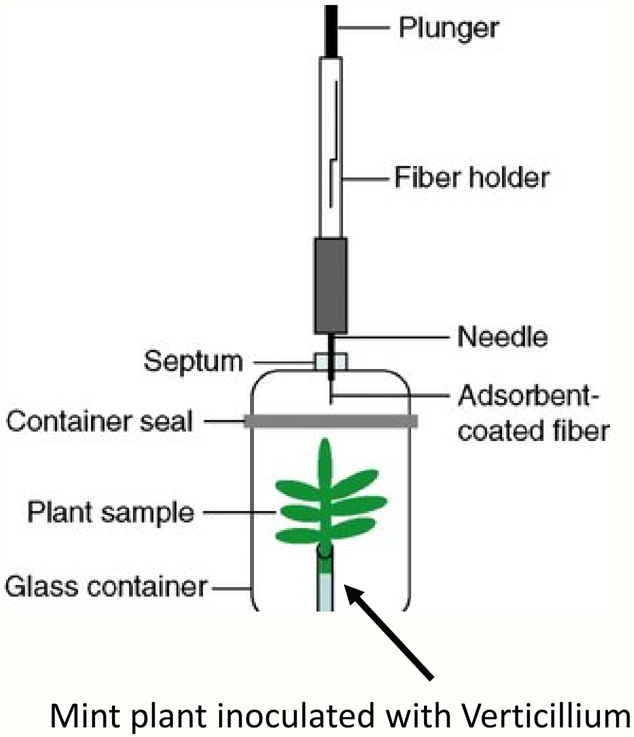
Put plants back into soil

Harvest tissue of interest and place in glass vial (multiple times; also record phenotype)

Perform SPME-GC-MS

Objective 2: Evaluate if Verticillium infection results in higher/different volatile emission in resistant spearmint compared to susceptible peppermint

Experimental setup



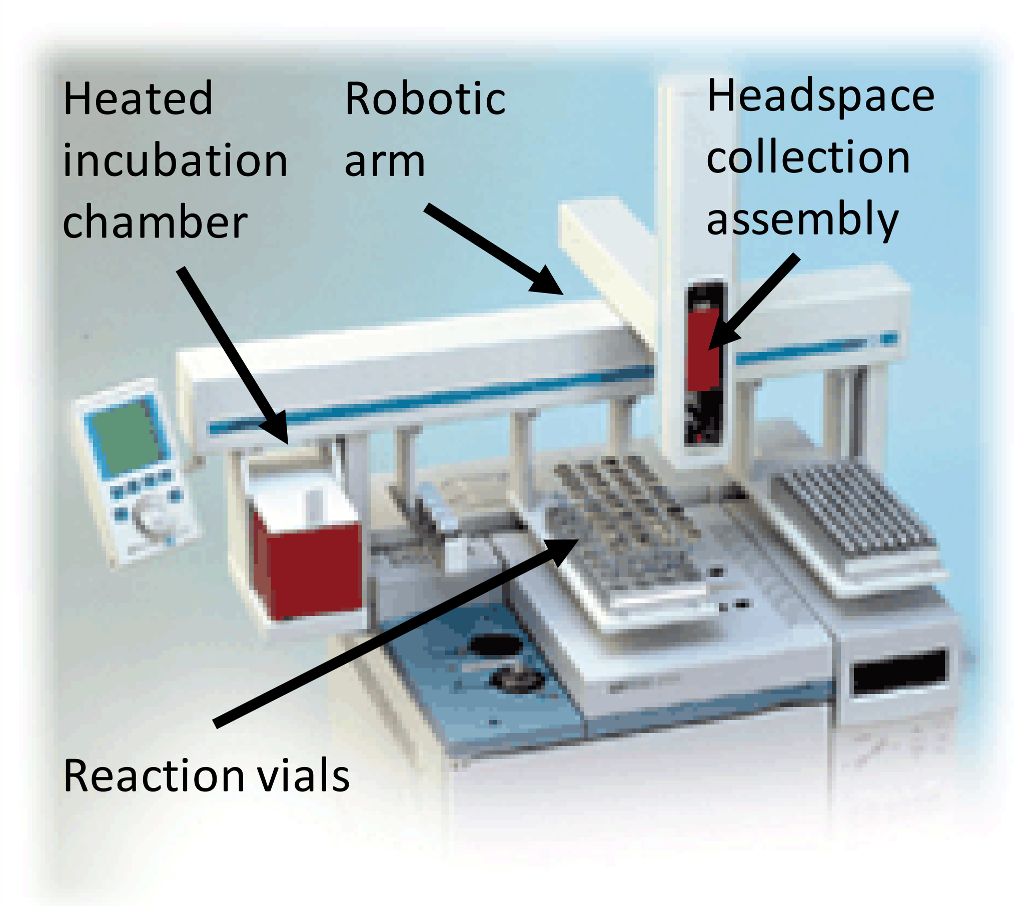
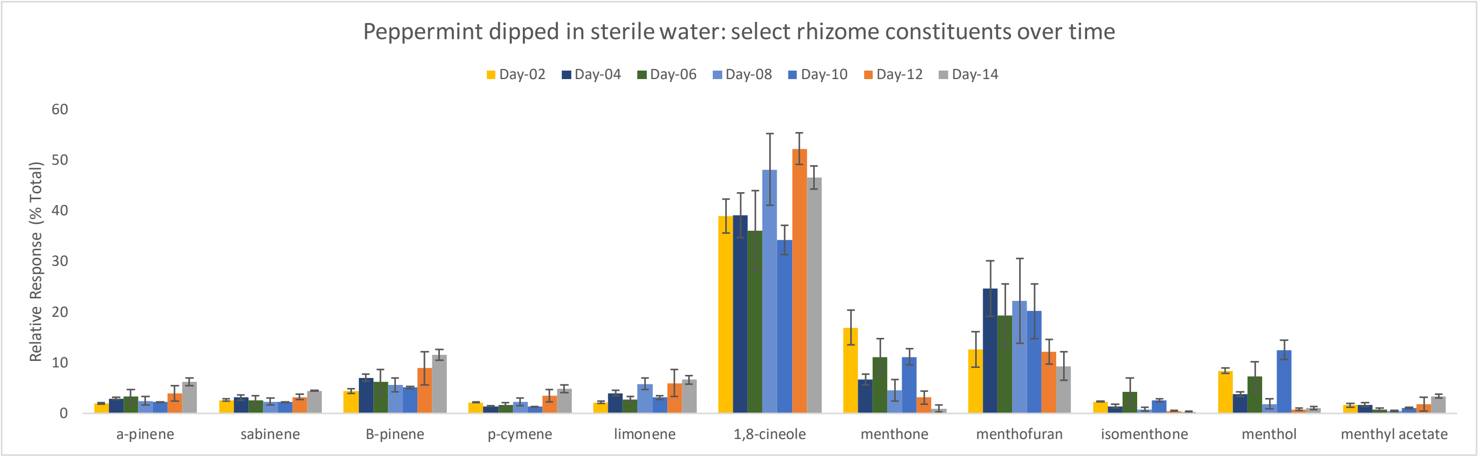
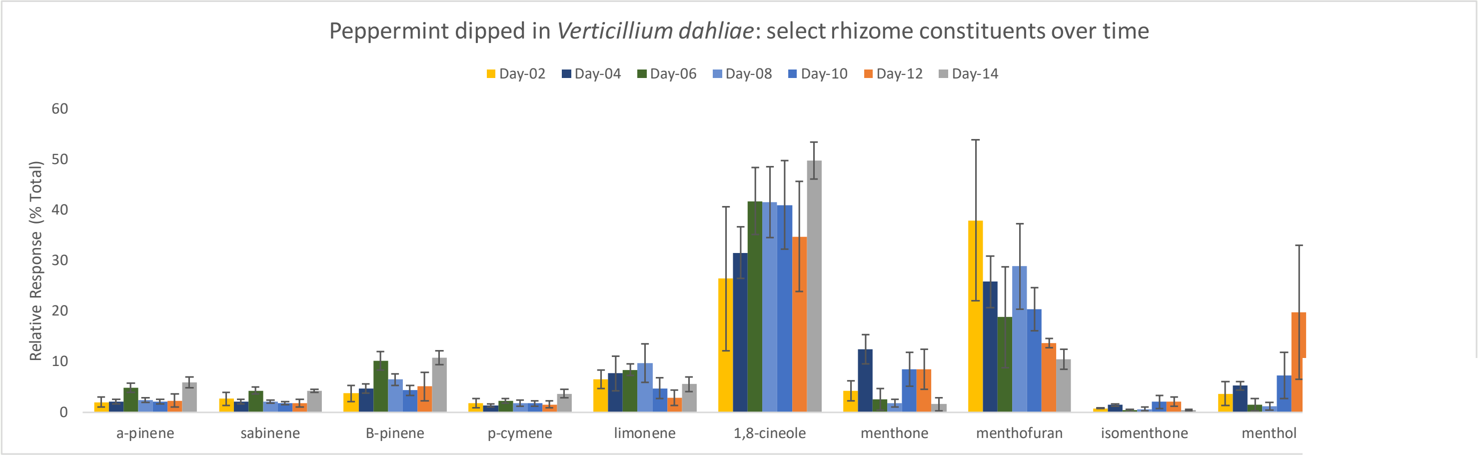


Fig. 1. Volatiles emintted from rhizomes (A, B) and stems (C, D) of Verticillium-inoculated peppermint plants (B, D) and appropriate controls (A, C) (n = 3; standard deviation shown as bars).

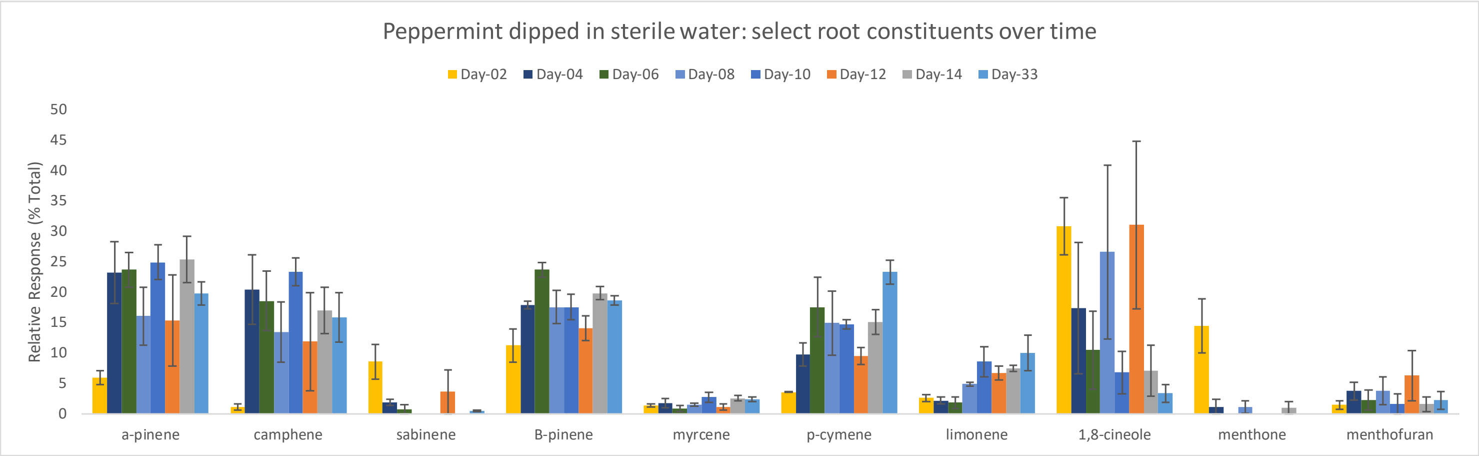
A



B

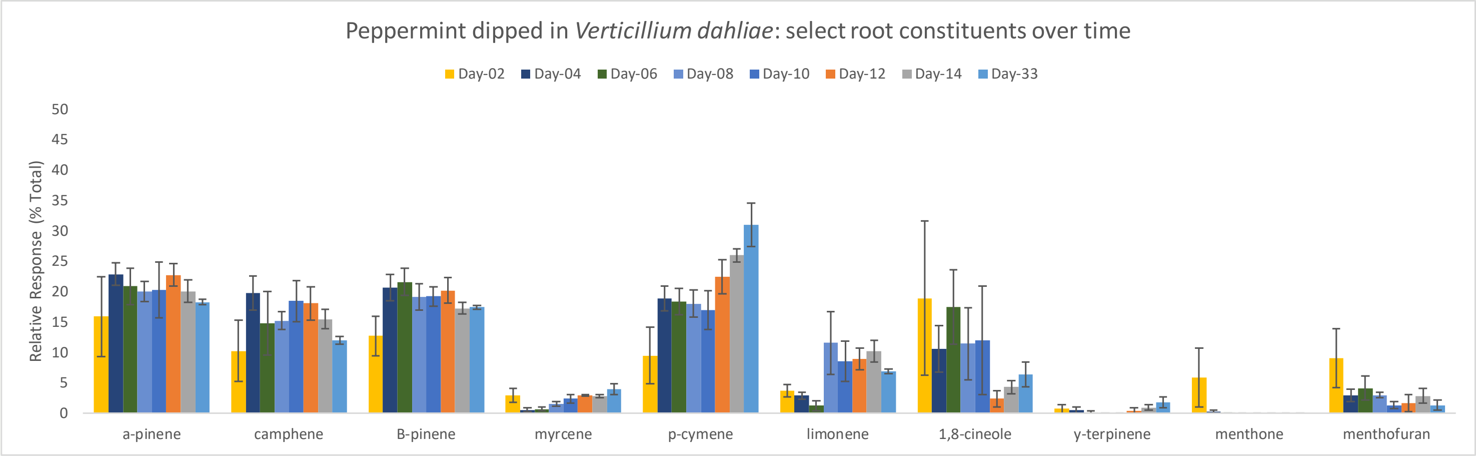


E



Peppermint dipped in sterile water: select root constituents over time
Day-02 Day-04 Day-06 Day-08 Day-10 Day-12 Day-14 Day-33
Relative Response (% Total)
0
5
10
15
20
25
30
35
40
45
50
a-pinene
camphene
sabinene
B-pinene
myrcene
p-cymene
limonene
1,8-cineole
menthone
menthofuran

F



Peppermint dipped in *Verticillium dahliae*: select root constituents over time
Day-02 Day-04 Day-06 Day-08 Day-10 Day-12 Day-14 Day-33
Relative Response (% Total)
0
5
10
15
20
25
30
35
40
45
50
a-pinene
camphene
B-pinene
myrcene
p-cymene
limonene
1,8-cineole
y-terpinene
menthone
menthofuran

Conclusions from peppermint experiment

- Rhizomes and stems of control plants emit similar blends of volatiles, with 1,8-cineole and (+)-menthofuran being the most abundant metabolites.

- The volatile blends emitted by rhizomes and stems of Verticillium-inoculated plants are very similar to those emitted by appropriate controls.

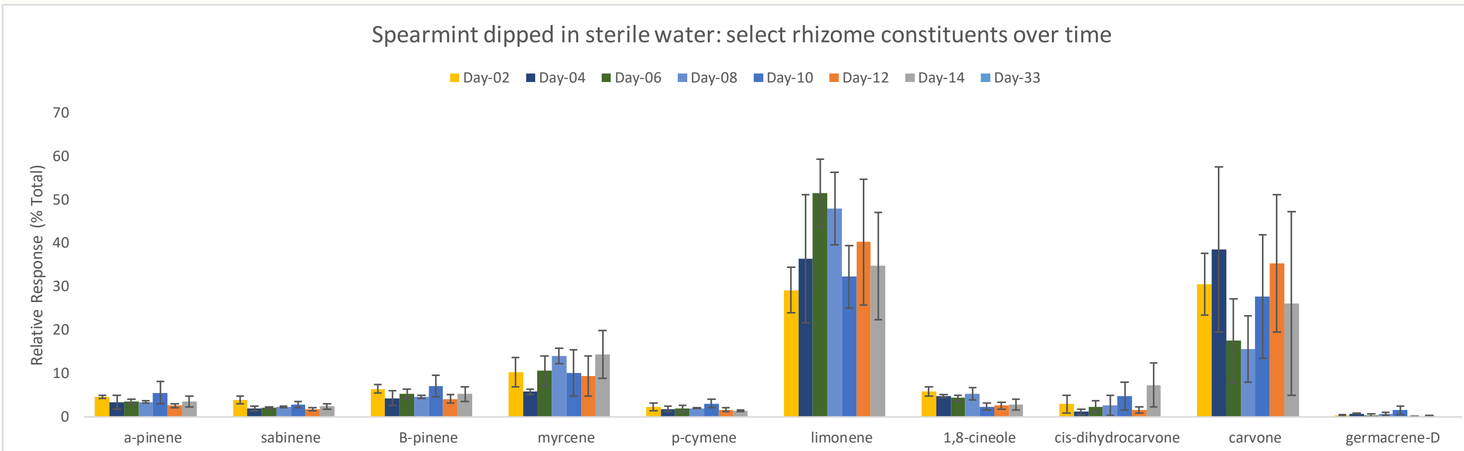
- The volatile blends emitted by roots of control plants are significantly different from those of rhizomes and stems, with cyclic hydrocarbons (α-pinene, β-pinene, camphene, *p*-cymene) dominating the profile.

- The volatile blends emitted by roots of Verticillium-inoculated plants are very similar to those emitted by appropriate controls.

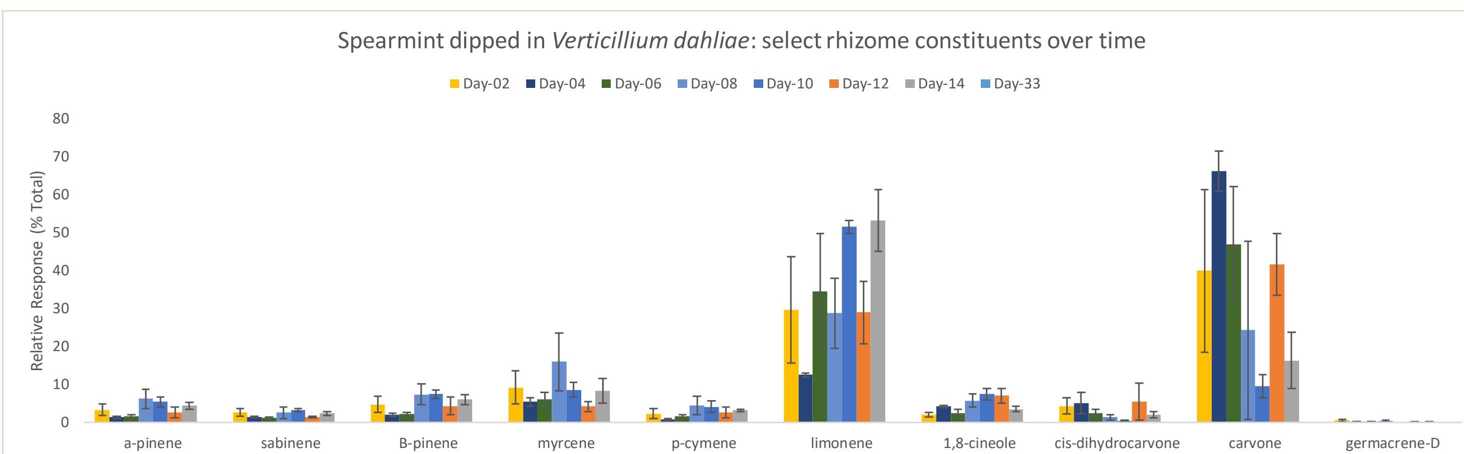
- The volatile blends of all organs investigated here by headspace SPME analysis are significantly different from those distilled from leaves, where (-)-menthone and (-)-menthol are the signature metabolites. This observation could be due to actual differences in volatile profiles or bias of the analytical technique, and requires further investigation.

Fig. 2. Volatiles emitted from rhizomes, stems, and roots of Verticillium-inoculated spearmint plants (B, D, F, respectively) and appropriate controls (A, C, E, respectively) (n = 3; standard deviation shown as bars).

A



B



Conclusions from spearmint experiment

- Rhizomes and stems of control plants emit similar blends of, with (-)-limonene and (-)-carvone being the most abundant metabolites.

- The volatile blends emitted by rhizomes and stems of Verticillium-inoculated plants are very similar to those emitted by appropriate controls.

- The volatile blends emitted by roots of control plants are significantly different from those of rhizomes and stems, with cyclic hydrocarbons (α-pinene, β-pinene, *p*-cymene) dominating the profile. Surprisingly, the root volatile profile of spearmint is similar to that of peppermint.

- The volatile blends emitted by roots of Verticillium-inoculated plants are very similar to those emitted by appropriate controls.

2020 Research Goals

1. Continue molecular marker development for Verticillium wilt resistance and monoterpene profiles.
   (Vining lab)

2. Continue testing if mint oils or oil constituents are effective in achieving *Verticillium dahliae* growth inhibition.
   (also assess possibility that some metabolites may promote Verticillium growth)
   (Lange, Dung labs)

3. Assemble polyploid genome sequences of Black Mitcham peppermint and Native spearmint.
   (build on experience with *M. longifolia* and harness novel technologies)
   (Vining, Lange labs)

Q&A time