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Methyl Anthranilate and γ-Decalactone Inhibit Strawberry Pathogen Growth and Achene Germination

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ABSTRACT: Plant volatile compounds have been shown to affect microbial growth and seed germination. Here two fruity volatiles found in strawberry (*Fragaria* × *ananassa*), γ-decalactone ("peachlike" aroma) and methyl anthranilate ("grapelike" aroma), were tested for effects on relevant pathogens and seedling emergence. Significant growth reduction was observed for *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Colletotrichum acutatum*, *Phomopsis obscurans*, and *Gnomonia fragariae* at 1 mM γ-decalactone or methyl anthranilate, and 5 mM γ-decalactone or methyl anthranilate supplemented medium resulted in complete cessation of fungal growth. *Phytophthora cactorum* was especially sensitive to 1 mM γ-decalactone, showing complete growth inhibition. Bacteriostatic effects were observed in *Xanthamonas* cultures. Postharvest infestations on store-bought strawberries were inhibited with volatile treatment. The γ-decalactone volatile inhibited strawberry and *Arabidopsis thaliana* germination. These findings show that two compounds contributing to strawberry flavor may also contribute to shelf life and suggest that γ-decalactone may play an ecological role by preventing premature germination.

KEYWORDS: *strawberry, volatiles, antimicrobial, γ-decalactone, methyl anthranilate*

INTRODUCTION

Fresh berries serve as alternatives to popular sources of sweet, empty calories and have additional positive health benefits.[1,2] However, commercial varieties lack exciting flavor and fragrance, as fruit sensory attributes are deprioritized by breeding programs. Additionally, postharvest losses of fresh fruits are significant, and a substantial portion of this is due to spoilage from microbial growth. Both problems may contribute to lower availability or consumption. We hypothesized that the aromatic compounds that could positively influence the human sensory experience may simultaneously suppress microbial growth, specifically in strawberry (*Fragaria* spp.).

Volatile organic compounds are generated by plants to attract pollinators, defend against herbivores, and aid in seed dispersal. To the consumer, volatile compounds are enticing to the senses and can possibly provide information about nutrition.[3] The primary role of fruit volatiles is in the production of aromas and flavors. Recent studies have examined which volatiles contribute best to the human sensory experience in tomato[4] and strawberry.[5] Trained sensory panels identified that the grape-aroma volatile methyl anthranilate has a positive correlation with liking.[6] This grape-flavored volatile imparts a unique flavor to the diploid strawberry *Fragaria vesca* and several *Fragaria* × *ananassa* cultivars, yet it is rare in commercial varieties.[7] γ-Decalactone is the conspicuous aroma in peaches,[8] and it is present in many strawberry accessions.

Such compounds appeal to the human senses. However, over the last two decades, several reports have indicated that volatile organic compounds may be used to retard or in some cases promote[9,10] microbial growth. The antimicrobial properties of certain plant volatiles have been described.[11] Methyl anthranilate has been demonstrated to be effective against seed-borne fungi (*Colletotrichum graminicola*, *Drechslera sorokiniana*, *Fusarium solani*, *Macrophomina phaseolina*, and *Phomopsis sojae*),[12] *Colletotrichum gloeosporioides*,[13] and *Candida albicans*.[14] γ-Decalactone and lactone analogs have been shown to be effective against *Aspergillus* species, *C. albicans*, *Penicillium roqueforti*, and *Staphylococcus aureus*,[15,16] *Erwinia amylovora* and *Pantoae agglomerans*,[17] and even against protozoa.[18] Effective antimicrobial volatiles include lipoxygenase pathway products against *Alternaria alternata* and *Botrytis cinerea*[19] and against *Colletotrichum acutatum*.[20] The mechanism for antimicrobial effects of diverse volatiles seems to be cell membrane destabilization.[20,21] A number of volatiles have been used to slow fungal and bacterial growth in fruits such as strawberry, blackberry, and grape.[22−25]

A series of studies have analyzed the effects of specific volatile compounds on microbial growth in commercial strawberry (*F.* × *ananassa*). Archbold et al.[26] demonstrated that certain volatiles, including (*E*)-hex-2-enal, could inhibit the growth of *B. cinerea* on strawberry fruits. Further, several volatiles, again including (*E*)-hex-2-enal, had a strong effect on the spore germination and growth of *C. acutatum*, and its potential use as a postharvest fumigant has been proposed.[20]

Many review papers have been published recently on plant volatiles and their potential use as herbicides[27−29] and various trophic interactions.[29−33] Volatiles biosynthesized in the lipoxygenase pathway (including *trans*-2-hexenal) inhibited soybean germination.[34] Volatiles from winter cover crop or *Amaranthus palmeri* residues inhibited germination and seedling growth for onion, carrot, tomato, and other crops.[35,36] Volatiles have also been implicated in germination inhibition of weed seeds.[37] Some volatiles have species-specific germination

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inhibition effects[38] and act by inhibiting cell proliferation and DNA synthesis.[39]

In this report, we test the effects of two consumer-desirable fruity volatiles for antimicrobial activity and suppression of seed germination. The findings demonstrate that compounds attractive to human senses also limit microbial growth and may present options to simultaneously enhance fruit flavor and extend product quality.

■ MATERIALS AND METHODS

Microbial Strains. Five fungi, one oomycete, and one bacterial species were included in this work. All strains used in this study were kindly provided by Dr. Natalia Peres from the University of Florida. The following were used in this study: *Colletotrichum gloeosporioides* (isolates 07-120, 13-01, and 98-285), *C. acutatum* (isolates 2-163, 98-324, and 3-37), *Phomopsis obscurans* (isolates 94-35, 94-34, and 97-32B), *Gnomonia fragariae* (isolates 97-34A, and 97-33D, and 99-1C), *B. cinerea* (isolates 05-31B, 12-180, and 134), *Phytopthora cactorum* (isolates 10-115, 11-01, and 12-12), and *Xanthomonas fragariae* (isolates 06-81, 09-24, and 09-33). All fungal/oomycete strains were grown and maintained on potato dextrose agar (PDA; Difco). *X. fragariae* was maintained and grown on WB III medium.[40]

Treatment Compounds. Antimicrobial and/or germination inhibition effects were tested for methyl anthranilate (CAS# 134-20-3, Sigma Aldrich), γ-decalactone (CAS# 706-14-9, Sigma Aldrich), dichloran (CAS# 99-30-9, Sigma Aldrich), copper(II) sulfate (CAS# 7758-98-7, Fisher Scientific), copper(II) chloride (CAS# 7447-39-4, Fischer Scientific), and anthranilic acid (CAS# 118-92-3, Sigma Aldrich). Dimethyl sulfoxide (DMSO) was the solvent for some treatment compounds and was therefore included as a control.

Mycelial Plug Growth Assay. All fungal or oomycete strains were grown on PDA at room temperature (RT) for 2−4 d before inoculations. Six-millimeter circular plugs were taken from the edge of an actively growing culture and placed in the center of PDA supplemented with pure volatile or control compounds. Volatiles were assayed at 0.1, 1, and 5 mM. Anthranilic acid was included in this study, because it differs from methyl anthranilate by a single methyl group. Dichloran, a commercial fungicide, was used as a positive control at 0.01, 0.1, and 1 mM. The no treatment control was PDA without supplements or with DMSO (the solvent for dichloran and anthranilic acid) equal to the highest volume of supplement added to PDA. There was no observable difference in growth between PDA and PDA supplemented with DMSO. Further, preliminary tests showed that there was no significant difference for growth reduction on fresh and week-old supplemented media (data not shown).

Plates were wrapped in Parafilm, and incubated at RT in a dark, vented box. Data were collected after 72 h by measuring the diameter of the growing cultures. All experiments were carried out in triplicate, with three independent biological replicates.

Microbicidal versus Microbistatic Plug Assay. Fungicidal activity was assessed by incubating fungal plugs as described above except for a longer incubation time of 7 d. Three replicates were assayed for each fungi/oomycete and treatment level that showed no growth after 7 d. The fungal plugs were then transferred to PDA without supplements and incubated at RT for an additional 7 days. Compounds were described as fungicidal for a specific microbe and treatment if growth was not apparent after 7 d.

Microbial Growth on Strawberry Tissue Assay. Microbial growth was also assayed on strawberry fruit pieces in the presence and absence of the volatiles. Whole, ripe strawberries were either obtained from the greenhouse (*F.* × *ananassa* accession LF9, a known genotype that does not produce γ-decalactone or methyl anthranilate) or purchased as retail produce with unknown genotypes (volatile profiles were not assessed). Single, unwashed strawberries were sliced laterally to ∼5 mm thick. Multiple slices through the middle of the sections produced triangular strawberry pieces each with pith, cortex, and epidermal tissue types. Randomized berry pieces were then submerged in aqueous solutions of γ-decalactone or methyl anthranilate at 1, 5, or 10 mM and with 1 μL/mL Triton-X 100. Triton-X 100 and water only controls were included. The submerged samples in solutions were then placed in a bell jar and vacuum was applied at 25 mmHg for 10 min. Three or four berry pieces from each treatment were then randomly assigned to individual wells in 12-well plates and sealed with Parafilm. Plates were incubated at RT for 5 d and then imaged and visually scored for fungal growth.

Volatile Spot Assay. The efficacy of vapor phase volatiles against fungal growth on strawberry tissue was investigated. Single, unwashed strawberries were sliced as described above. Five-hundred microliters of distilled water was added to each well to prevent tissue dehydration. One to five microliters of the pure volatile compounds were then applied to the plate lid corresponding to each well, and plates were incubated at RT for 5 d. All treatments were randomly assigned and quantitative data consisted of three independent biological replicates, each composed of three or four technical replicates.

***Xanthomonas* Growth Curve Assay.** *Xanthomonas* was maintained on agar-solidified WB III medium. Five milliliters of starter liquid cultures were grown for 18−24 h at 30 °C. Strains were then prepared to an OD_{600} = 2.0 (×cells/mL) in WB III medium. Inoculum (20 μL) was added to individual wells in 96-well culture plates. The wells were brought up to 200 μL with WB III supplemented with volatiles or control compounds. The final concentrations ranged between 1 and 10 mM. Copper chloride and copper sulfate were included as antibacterial positive controls. Concentrations for these compounds ranged from 0.1 to 1 mM. Both performed similarly in preliminary tests, so only copper chloride is reported.

Data were collected on a BioTek microplate reader using GEN5 software (version 1.09). The cultures were continuously shaken and incubated at 30 °C. The OD_{600} was read every 15 min for 12 h. All strains and treatments were evaluated with three technical replicates and three independent biological replicates. Data are reported for one representative, independent replicate with three technical replicates. OD_{600} data were normalized to 1.0 (100%) based on first readings to enable comparisons between treatments and strains.

***Xanthomonas* Microbicidal Assay.** The microbicidal effects of γ-decalactone and methyl anthranilate were assayed against *X. fragariae*. Suspensions of bacteria (100 μL) were made in liquid WB III medium to OD_{600} = 0.2. Treatments included volatile compounds or $CuCl_2$ at 0.5, 1, and 5 mM final concentrations. DMSO and "no treatment" controls were included. Bacterial suspensions were incubated with shaking at 30 °C for 6 or 24 h. Aliquots were diluted at 1:1000 and plated on solidified WB III medium. The remaining cells were also plated after 24 h. *X. fragariae* was then incubated for 1 week at 30 °C. Microbicidal activity for specific treatments was demonstrated by the absence of bacterial colonies at dilutions where controls were too numerous to count. This assay was repeated three times.

Achene Germination Assay. *F. vesca* 'Hawaii-4' and 'Baron Solemacher' seeds were used for germination assays. Seeds were surface-sterilized in 70% ethanol for 5 min, agitated in 1% sodium hypochlorite for 20 min, and rinsed six times in sterile, distilled water. Twenty seeds per Petri dish were then transferred to solidified water agar (0.6% agar) supplemented with volatile compounds. γ-decalactone and methyl anthranilate were assayed at 0.1, 0.5, and 0.7 mM. Seeds were incubated for 2 weeks at RT under fluorescent lights (18 h day/6 h night). The number of germinated seeds after 2 weeks was scored for each treatment. Seeds failing to germinate were rinsed in sterile water and transferred to water agar medium without volatile supplementation. Seed viability was assayed by assessing seed germination after 2 weeks.

Strawberry Seedling Phytotoxicity. Seedling phytotoxicity of γ-decalactone and methyl anthranilate was tested. 'Hawaii-4' and 'Baron Solemacher' seeds were surfaced sterilized as described above and plated on water agar. Plates were incubated for 2 weeks at RT under fluorescent lights (18 h day/6 h night). Seedlings with fully emerged cotyledons were transferred to MS medium supplemented with 1, 5, or 10 mM γ-decalactone or methyl anthranilate. A control lacking either volatile compound was included. Twenty seedlings were tested per treatment and visually monitored for seedling health.

Strawberry Mature Plant Phytotoxicity. Phytotoxicity of γ-decalactone and methyl anthranilate was tested by foliar application.

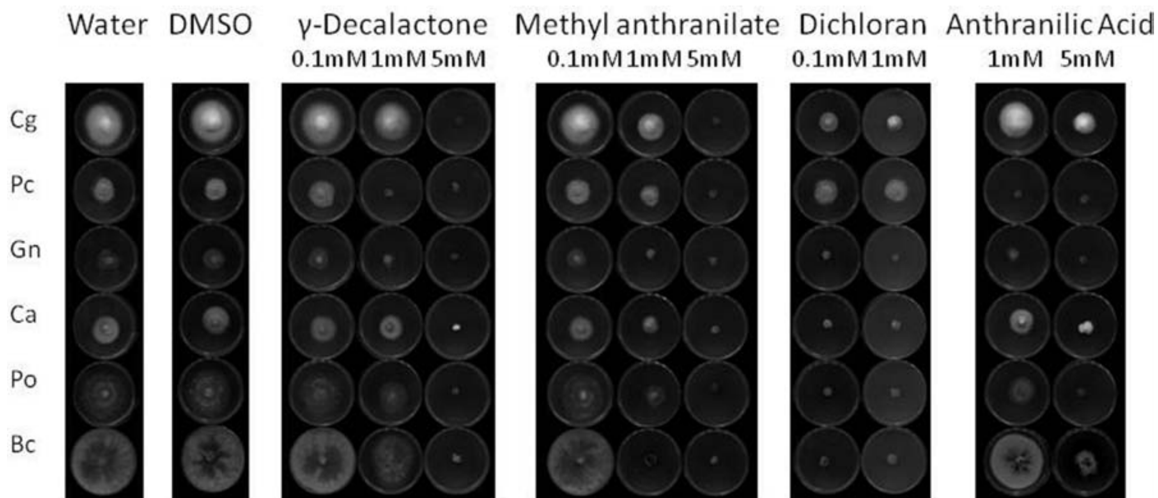


Figure 1. Representative plates from mycelial plug assay on supplemented PDA medium. Six millimeter mycelial plugs were taken from actively growing cultures, placed on supplemented medium, and incubated for 72 h. Cg, *C. gloeosporioides*; Pc, *P. cactorum*; Gn, *G. fragariae*; Ca, *C. acutatum*; Po, *P. obscurans*; Bc, *Botrytis cinerea*.

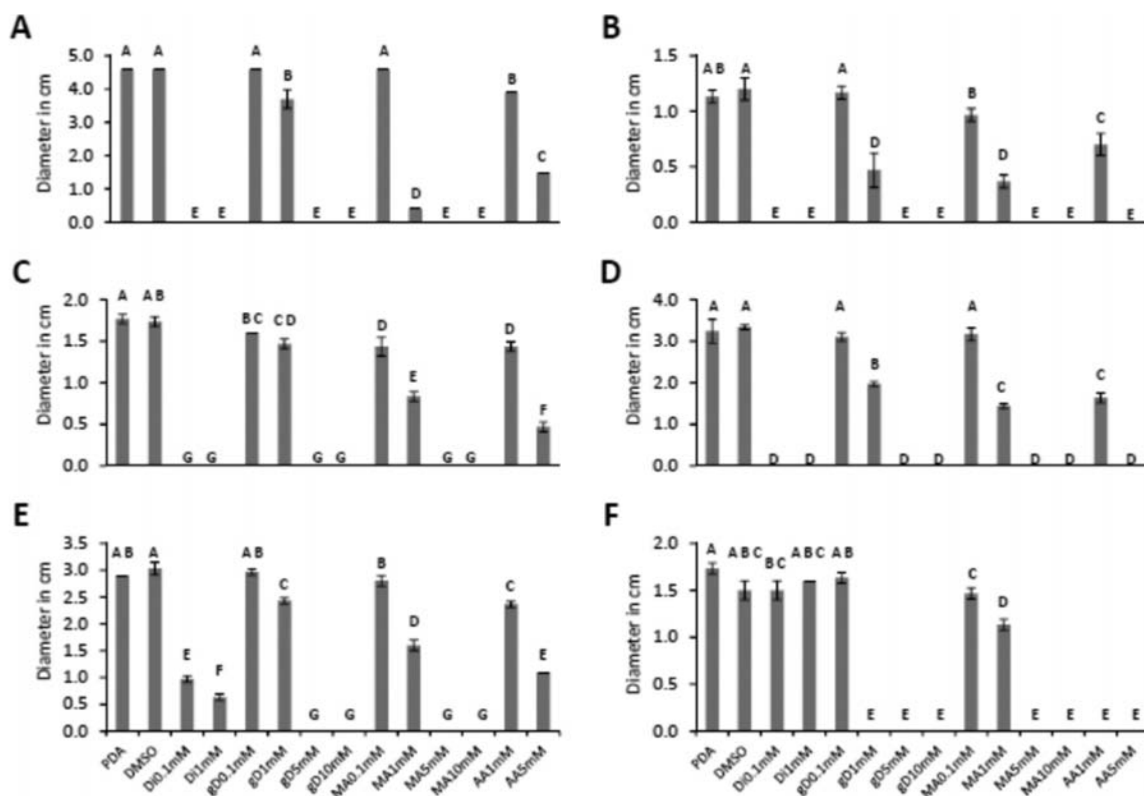


Figure 2. Quantitative mycelial plug assay data for five fungi and one oomycete. Data is shown for millileter diameter growth after 72 h for (A) *B. cinerea*, (B) *G. fragariae*, (C) *C. acutatum*, (D) *P. obscurans*, (E) *C. gloeosporioides*, and (F) *P. cactorum*. PDA, potato dextrose agar (no volatile control); DMSO, dimethyl sulfoxide (diluent control); Di, dichloran; gD, γ-decalactone; MA, methyl anthranilate; AA, anthranilic acid. Data are presented from three technical replicates. Results with the same letter are not significantly different based on a Tukey's HSD test for multiple comparisons ($\alpha < 0.05$).

Mature diploid *F. vesca* 'Baron Solemacher' plants were used for the tests. Fully established plants were maintained in a greenhouse under natural lighting conditions. Treatment conditions included γ-decalactone and methyl anthranilate at 1, 5, or 10 mM in distilled water with 1 μL/mL Triton-X 100. A Triton-X 100 control was included. Treatments were applied by spraying all above-ground plant parts until dripping. Treatments were also directed onto the crown of each plant. Each treatment or control was applied to three plants. The plants were randomly arranged in the greenhouse after application and monitored for evidence of symptomology.

C. acutatum **Light Microscopy.** *C. acutatum* plugs were grown on γ-decalactone supplemented PDA medium as described above. Treatments included PDA only and 2 or 3 mM γ-decalactone or methyl anthranilate. These concentrations were low enough for *C. acutatum* to still grow, but at an inhibited rate. The edge of the fungal colony was then imaged at 25×.

***Arabidopsis* Germination Assay.** *Arabidopsis thaliana* Col-1 seeds were surface-sterilized in 95% ethanol for 5 min and air-dried. Approximately 50 seeds were then spread onto single plates of MES-buffered water agar (0.6% agar, pH 6.0) with or without 1 mM γ-decalactone. Plates were wrapped in Parafilm and vernalized at 4 °C in the dark for 2 d. Seeds were then germinated in bright light at RT. After 5 d, seeds from the γ-decalactone treatment were washed in sterile water and plated on water agar without volatile supplementation. Each treatment included two plates and was repeated three times.

RESULTS

Mycelial Plug Growth Assay. The effect of each volatile compound on microbial growth was measured for five fungi, one oomycete, and one bacterial species commonly associated with strawberry production. Representative, phenotypic results from one experimental replicate are shown in Figure 1. Representative, quantitative results from one independent assay with three technical replicates are presented in Figure 2. Negative controls included PDA medium without a supplement or with DMSO at equal volumes to its level as a volatile diluent. DMSO had no significant effect on growth for any isolate tested. Dichloran was used as a positive control against fungal growth. *B. cinerea*, *P. obscurans*, *G. fragariae*, and *C. acutatum* growth was entirely arrested by 0.1 mM dichloran. *C. gloeosporioides* growth was significantly reduced by dichloran at 0.1 mM and 1 mM relative to PDA controls, but not stopped completely. *P. cactorum* was not significant affected by dichloran at the concentrations tested.

The volatiles γ-decalactone and methyl anthranilate demonstrated significant growth reduction for all species at the concentrations tested: 5 mM γ-decalactone or methyl anthranilate completely inhibited growth of all species, 1 mM γ-decalactone significantly reduced growth of all strains except for *C. acutatum*, 1 mM methyl anthranilate significantly reduced the growth of all strains tested, and *P. cactorum* growth was reduced by methyl anthranilate similar to the other species tested but showed extra sensitivity to γ-decalactone at 1 mM.

Anthranilic acid (AA), the nonvolatile, structurally similar biosynthetic precursor to methyl anthranilate, was also used as a control to illustrate the effect of the volatile compound. When the effects at 1 and 5 mM are compared, methyl anthranilate has a significantly greater effect than AA for all species except for *P. obscurans*, where results were similar.

Microbicidal Versus Microbistatic Plug Assay. The results above were expanded to resolve between microbicidal and microbistatic effects. To demonstrate the difference, the minimal treatments that resulted in complete growth inhibition were again assayed, but with variation. Fungal plugs were grown on the volatile or control compounds for 7 d and then transferred to PDA with no amendments for 7 d. If the effects were microbicidal, no growth would be expected after incubation on PDA for 7 d. If the effects were microbistatic, growth would resume, at least in part, on the PDA only medium. The 5 mM γ-decalactone treatment with *P. cactorum*, *G. fragariae*, or *C. gloeosporioides* was microbicidal; 5 mM anthranilic acid was also microbicidal against *P. cactorum*, and 5 mM γ-decalactone was microbicidal against *B. cinerea* strain 05-31b but not strain 12-180.

Microbial Growth on Strawberry Tissue Assay. In vitro assays demonstrated broad activity against microbial growth, but these tests relied on the use of nutritionally rich and artificial medium. We therefore tested microbial growth inhibition using strawberry tissue as the substrate and the naturally present microbes as the inoculum. Fruit from both the known genotype LF9[41] and store-bought berries were cut into uniform pieces, infiltrated with the volatile dilution, and incubated for 5 d. Both LF9 and store-bought berries performed similarly. Figure 3 shows a panel of fruit pieces after treatment infiltration and incubation. Fungal growth was either completely suppressed or covered the fruit pieces. One-hundred percent fungal coverage was observed on the fruit pieces treated with the water-only control and the 1 mM γ-decalactone and 1 mM methyl anthranilate. Microbial growth was entirely inhibited at 5 mM or 10 mM γ-decalactone or methyl anthranilate.

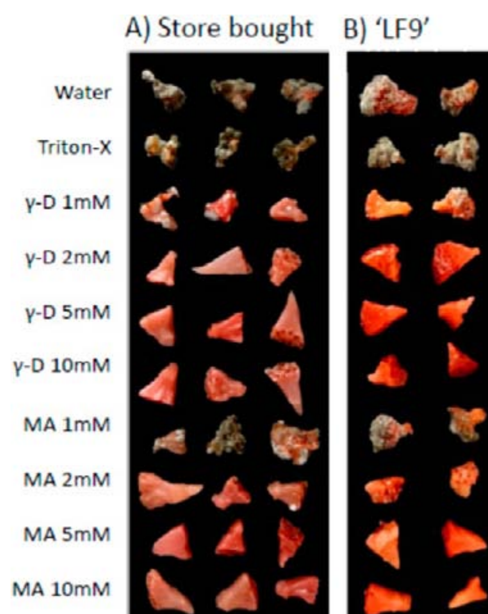


Figure 3. Strawberry tissue infiltration assay. Strawberry pieces from a single (A) store bought, unknown genotype or (B) greenhouse-grown LF9 berry were infused with volatile or control solutions and incubated for 5 d in semisealed 12-well plates. Fungal growth was visually scored, with both water and Triton-X controls reaching 100% fungal coverage. Technical replicates are shown for a single experiment.

Volatile Spot Assay. A variation of the infiltration assay tested the vapor-phase effects of the volatiles on reducing fungal growth. Volatiles were applied to the lid of a semisealed 12-well culture plate with strawberry pieces and sterile water placed in the bottom of each well. Representative results are shown in Figure 4. γ-Decalactone or methyl anthranilate at 10 mM was sufficient to completely control fungal growth in most cases. Lower applications of volatiles completely reduced fungal growth more often than the water only and DMSO controls. Methyl anthranilate was overall more effective than γ-decalactone at completely controlling fungal growth at each of the three levels tested.

***Xanthomonas* Growth Curve Assay.** *X. fragariae* cultures were grown in the presence and absence of methyl anthranilate or γ-decalactone to assess their effect. Liquid cultures were inoculated to OD_{600} = 0.2, and then culture density was measured every 15 min for 12 h. The assay was performed on three *Xanthomonas* isolates. Copper(II) chloride was used as a control. The culture density without amendments grew by ∼10% after 12 h (Figure 5), which is characteristic of this slow-growing microbe. Panel A shows the growth curves for γ-decalactone. Complete inhibition of growth was caused by 3

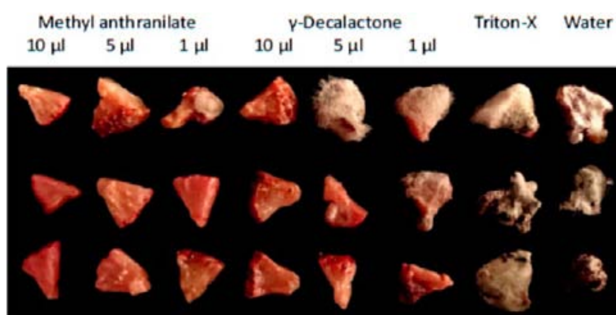


Figure 4. Strawberry tissue volatile spot assay. Strawberry (*F.* × *ananassa* 'LF9' and a retail-purchased genotype) pieces were incubated in semisealed 12-well plates with pure volatile compounds applied to the plate lid. Controls contain water and Triton-X at identical concentrations as volatile-containing replicates. All three technical replicates are shown for a single experiment.

and 5 mM γ-decalactone, and 1 mM γ-decalactone reduced growth by ~70%. Methyl anthranilate growth data are shown in Panel B. Methyl anthranilate had no effect on growth at 1 or 3 mM, but it completely inhibited growth at 5 mM. The volatile effects can be compared to that of copper(II) chloride (Figure 5, panel C), which shows no significant effect at 0.1 or 0.15 mM after 12 h but complete inhibition at 0.2 mM.

Xanthomonas Microbicidal Assay. The *Xanthomonas* growth curve assays showed a slight decrease in OD_{600} after 12 h for some treatments. These were then assayed for microbicidal activity. Treatments included both 6 and 24 h incubations with *Xanthomonas* in liquid WB III medium. Microbicidal activity was shown by the absence of *Xanthomonas* colonies when dilutions were plated on solidified WB III medium after treatments. The 5 mM γ-decalactone or methyl anthranilate treatment was microbicidal (displaying no colonies) on WB III plates when diluted 1:1000 for both 6 and 24 h incubation times. Colonies on the no treatment and DMSO controls were both too numerous to count at this dilution. The copper(II) chloride treatment was ~10 times more effective, showing no colonies at 0.5 mM for both the 6 and 24 h treatments. The largest difference between treatments was shown when plating 24 h incubated cultures without dilution. The 0.5 mM copper(II) chloride treatment was almost entirely microbicidal, but the volatile treatments displayed a lawn similar to the no treatment and DMSO controls (data not shown).

Achene Germination Assay. The results from the assays above show that both γ-decalactone and methyl anthranilate have a profound effect in suppressing microbial growth. These findings presented the hypothesis that the volatiles could affect plants themselves. The first test was a seedling germination

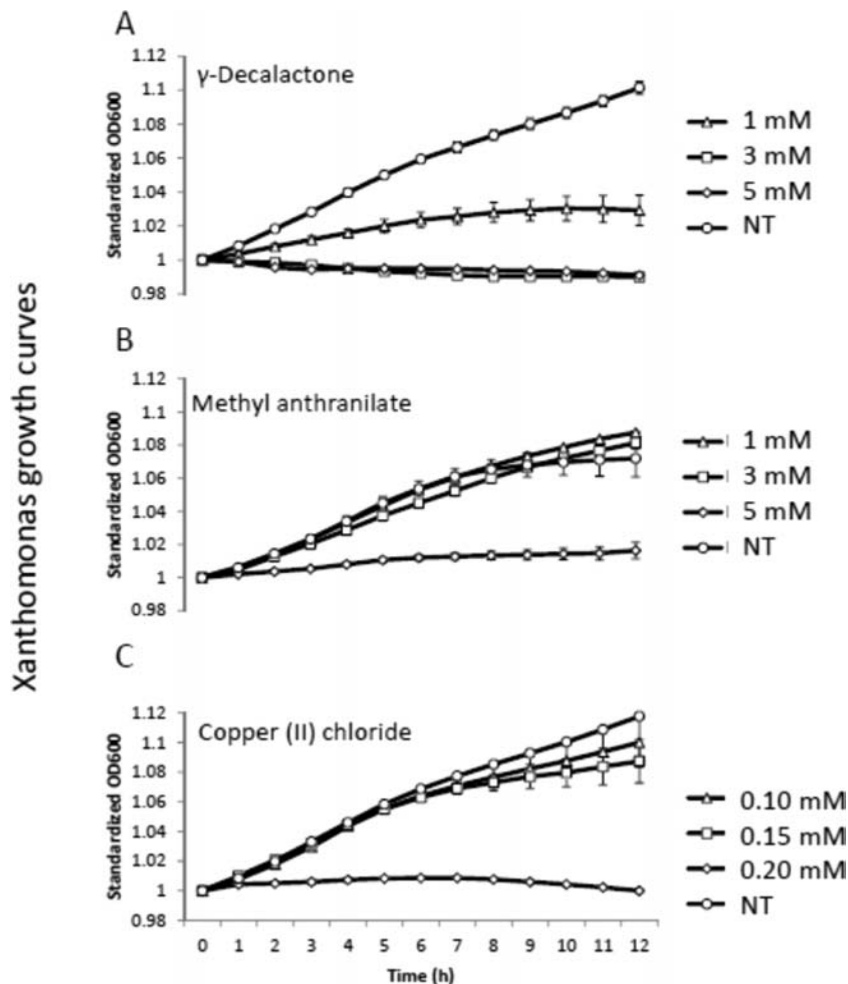
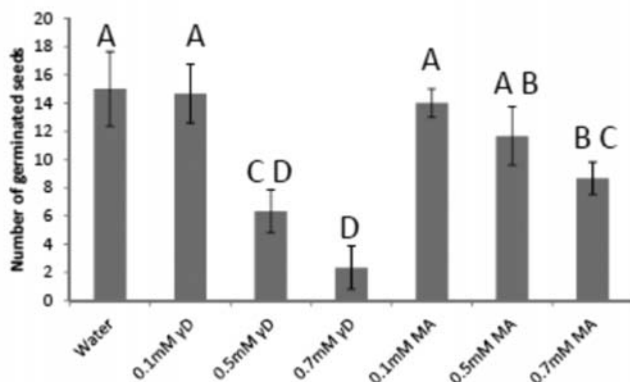


Figure 5. Xanthomonas growth curves in WB III medium supplemented with volatiles: (A) γ-decalactone, (B) methyl anthranilate, or (C) copper(II) chloride supplemented medium. Combined data from three technical replicates are shown. "NT" indicates no treatment.

assay. Germination is a complex hormonal, enzymatic, and physiological process whereby the embryonic plant initiates growth and emerges from the seed. There are potentially many nodes where a biologically active agent might affect the process. To test this hypothesis, strawberry achenes were surface-sterilized and placed on water agar with or without volatile supplementation at 0.1, 0.5, or 0.7 mM for 2 weeks. The results for two genotypes are shown in Figure 6. The results for the *F. vesca* 'Baron Solemacher' and 'Hawaii-4' genotypes show that concentrations that are ~10% of antimicrobial levels can suppress, but not completely inhibit, germination (Figure 6). For γ-decalactone and 'Baron Solemacher', 0.1 mM has no effect, yet significant inhibition is achieved at 0.5 and 0.7 mM. Only 7 mM γ-decalactone had a significant effect for inhibiting 'Hawaii-4' germination. The effects of methyl anthranilate are not as dramatic, with a downward trend with higher concentrations leading to a significant difference only between 0.7 mM γ-decalactone and the water control. This phenomenon was reversible, as inhibited seeds are able to germinate post-treatment when moved to standard media without supplemental volatiles (data not shown).



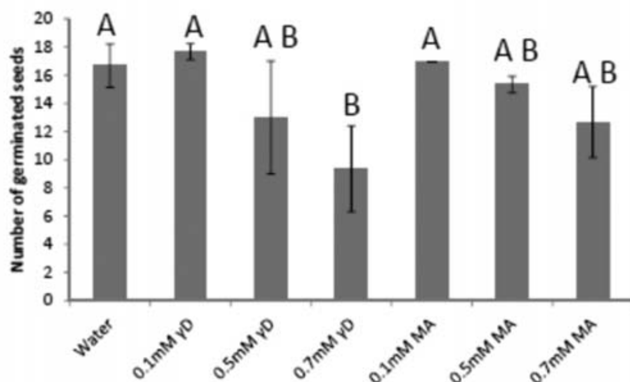


Figure 6. Volatile suppression of germination for two *F. vesca* genotypes: (A) 'Baron Solemacher' and (B) 'Hawaii-4'. Each genotype and treatment included 20 achenes and three technical replicates. Representative data from a single experiment with three technical replicates is shown. Results with the same letter are not significantly different.

Strawberry Seedling Phytotoxicity. Seedlings with expanded cotyledons were used to test phytotoxicity post-germination. Seeds were surface-sterilized and germinated on water agar. Germinated seedlings were then transferred to water agar with or without volatile supplementation. γ-Decalactone or methyl anthranilate at 1 mM was not phytotoxic, as cotyledons remained green and appeared healthy, but the presence of the volatiles resulted in decreased root growth compared to water agar controls (data not shown), and 5 mM γ-decalactone or methyl anthranilate treatments led to seedling browning and senescence after 2 weeks (data not shown).

Strawberry Mature Plant Phytotoxicity. The strong effect of volatiles on germination and seedlings led to tests on mature strawberry plants. Greenhouse grown, healthy 'Baron Solemacher' plants were used in this experiment, and 50 mL solutions were prepared for water, Tween-20 only, and 1, 5, or 10 mM γ-decalactone or methyl anthranilate. The 50 mL solutions were applied to all above-ground plant parts including the crown. Plant health was visually monitored after two applications, 1 week apart. No visible effects for any treatment were detected 2 weeks after the last application (data not shown).

***C. acutatum* Light Microscopy.** Light microscopy showed morphological differences between control and volatile treatments for *C. acutatum*. Panels A and D of Figure 7 are typical PDA control cultures that showed long spreading hyphae at the colony edge. Treatment with either 2 or 3 mM γ-decalactone (7, panels B and C) or methyl anthranilate (7, panels E and F) resulted in denser hyphae. This result was most pronounced for 3 mM methyl anthranilate, where numerous, dense hyphae are shown as *C. acutatum* attempted to grow out onto the volatile supplemented medium.

***Arabidopsis* Germination Assay.** *Arabidopsis* seeds were germinated on MES-buffered water agar with and without 1 mM γ-decalactone supplementation. Figure 8 shows seed germination after 2 d under bright light for (A) water agar control and (B) 1 mM γ-decalactone supplemented water agar. Panel C of Figure 8 shows the recovery germination after 5 d of 1 mM γ-decalactone treatment followed by 2 d on water agar medium with volatile supplementation. After 2 d, the *Arabidopsis* seeds on the water agar control achieved >90% germination, but 0% of the seeds on 1 mM γ-decalactone germinated (data not shown). After 5 d, the water agar grown seedlings had fully expanded and green cotyledons with long roots, while ~3% of the 1 mM γ-decalactone treated seeds had only just germinated. The γ-decalactone-treated seeds achieved similar germination rates after being washed and transferred to water agar for an additional 5 d.

DISCUSSION

In this report, we demonstrate the antimicrobial properties of two fruit volatiles, namely, γ-decalactone and methyl anthranilate. These two volatiles are shown to have effects on suppressing the growth of important strawberry pathogens. Figures 1 and 2 show mycelial growth on PDA alone or supplemented with volatile compounds. All microbes in Figure 2 were significantly inhibited by 1 mM γ-decalactone or methyl anthranilate, and fungal growth was completely suppressed at 5 mM for either volatile. Lower concentrations of volatiles had little effect, although reproducible and significant effects were observed. The results for methyl anthranilate were always stronger than the effects of anthranilic acid, a nonvolatile, probable biosynthetic precursor to methyl anthranilate. This result supports the hypothesis that the volatile compound is more effective in suppressing microbial growth than similar

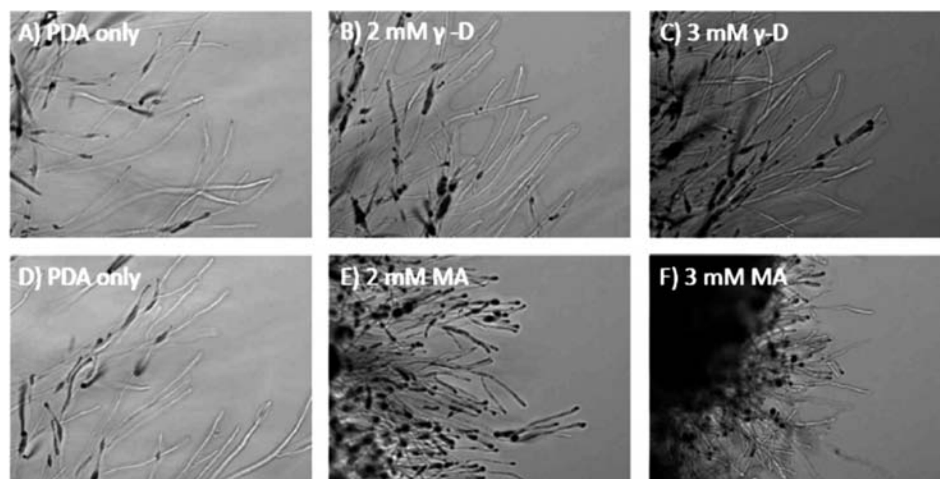


Figure 7. Light microscopy of *C. acutatum* treated at sublethal levels of γ-decalactone (γ-D) and methyl anthranilate (MA). Mycelial plugs were taken from actively growing edges of colonies maintained on PDA and transferred to PDA or PDA with volatile supplementation. Representative images are shown at 25× magnification.

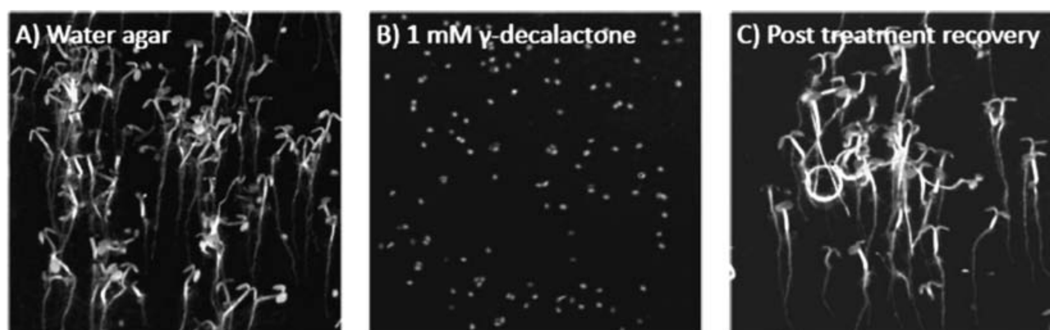


Figure 8. *Arabidopsis* seed germination assay. *Arabidopsis* seeds were germinated for 5 d on (A) water agar or (B) 1 mM γ-decalactone supplemented water agar. Seeds treated for 5 days with γ-decalactone were subsequently transferred to water agar and incubated for 5 d, as shown in panel C.

nonvolatiles diffused in agar-based media. The effects of 1 mM γ-decalactone on *P. cactorum* led to complete growth inhibition. While none of these compounds at any concentration achieved the efficacy of the commercial fungicide dichloran (with the exception of *P. cactorum*, for which dichloran was ineffective), all showed at least some capacity to inhibit growth at 5 mM, with sensitivity noted at 1 mM in certain cases.

Volatile-mediated growth inhibition results demonstrate that the volatiles are actually microbicidal for some species under the described treatment regimes. The mechanism is unknown but could potentially involve a direct physiological disruption or slowing growth to the point where the organism cannot gain sufficient nutrition to survive. Microscopic examination showed that hyphae failed to elongate on volatile-containing media.

The microbes resident on fresh berries could also be suppressed using these volatiles. Greenhouse and store-bought strawberry pieces were placed in culture after treatment. Indigenous fungi grew rapidly on controls (Figure 3). Similar results were observed when fruit pieces were incubated with 1 mM γ-decalactone or methyl anthranilate. However, when strawberry pieces were treated with 5 or 10 mM of either volatile, no visible growth was present. These tests demonstrate that volatiles can limit microbial growth on the strawberry fruit itself.

Similar antimicrobial results were observed in the growth of liquid cultures of *Xanthamonas*. Cultures were incubated for 6 or 12 h in the presence of γ-decalactone or methyl anthranilate, or water or copper(II) chloride as controls. The results approximate those seen for fungal growth on solid medium. Again, both volatiles exhibited antimicrobial activity at 5 mM, even after the shorter 6 h incubation. This concentration of volatile is about 10-fold higher than that of the copper(II) chloride control.

These results show that the growth of a wide cross section of strawberry pathogens may be significantly curtailed using either of these two strawberry flavor volatiles. While the concentrations required are 10−20 times more than the effective levels of commercial fungicides and antibiotics, these volatile compounds are naturally produced by the fruit and may be conferring antimicrobial effects in addition to a positive effect on human sensory experience. While the concentrations seem unrealistic with respect to what may be naturally produced by the fruit (γ-decalactone <0.035 mM; methyl anthranilate <0.039 mM; A. Chambers, unpublished data), it is important to remember that the compounds may be localized to specific fruit regions or cell types, and a germinating spore may be met with a proximal defense of fruit volatiles. Future studies will assess the localization of volatile production in response to pathogen attack, as well as the synergistic or additive effects of multiple volatiles. The observation that these two volatiles increase with ripening, a time when pathogens are more likely to become problematic, is further correlative evidence that these compounds may be serving an antimicrobial function in addition to attraction of animal consumers.

The effects on fungi led to tests of inhibitory tests on plants. Phytotoxic effects may limit development of these compounds as natural antimicrobials in horticultural or postharvest contexts. If γ-decalactone or methyl anthranilate was interfering with fundamental processes common to actively growing and developing eukaryotic cells, perhaps germination would be inhibited. Previous reports have demonstrated stimulatory or inhibitory effects of volatiles on germination, mostly in the context of allelopathic effects on weeds.[42,43] Volatiles released by *Amaranthus palmeri* breakdown products, including short-chain alcohols like 2-heptanol, 1-hexanol, and 1-pentanol (among others) or acetaldeyde inhibited horticultural seed germination in onion, tomato, and carrot.[36] Others studies have examined volatile compounds produced by plants as potential herbicides.[25] Another report showed that *Salvia* monoterpenes specifically affected cell proliferation in the root apical meristems of neighboring *Brassica campestris* seedlings.[39] Some of the same terpenoids, namely pinenes, have been identified in certain strawberry genotypes.[44] While this phenomenon has been observed, it is not mechanistically understood.

Arabidopsis was used to further investigate germination inhibition, because it has a more uniform and rapid germination. *Arabidopsis* seedling germination was also reversibly inhibited by 1 mM γ-decalactone. This shows the broad effect of γ-decalactone on two distinct plant species. Future work may investigate if this phenomenon results from an active mechanism of germination suppression. A mutant screen in the *Arabidopsis* model may yield clues to the mechanism for inhibition.

Breeding for specific fruity volatiles may produce cultivars that appeal to the senses and extend shelf life or limit fungicide applications. Transgenes controlling rate-limiting steps in biosynthesis may also be added and may both increase flavors and pathogen resistance. Most volatile treatments on microbes have proven detrimental to microbial growth and raise the hypothesis that these volatiles could be added to packaging to slow postharvest fruit infestations. These volatile compounds are naturally found in fruit, so their presence in a modified atmosphere may enhance shelf life and find reasonable consumer acceptance. It is possible that the compounds may even influence fruit flavors. For example, exogenous application of (*E*)-2-hexenal was absorbed by the fruit and converted to esters, compounds associated with fruity/floral aromas.[22] Others have suggested that aromatic compounds could serve as substrates for in planta formation of antimicrobial compounds.[34] Further evaluation of their use in this application along with the effect on fruit flavor and aroma is currently being evaluated.

γ-Decalactone and methyl anthranilate suppress fungal growth, bacterial growth, and seedling germination at millimolar concentrations. While it is interesting to speculate on the potential evolutionary and ecological roles of strawberry fruit volatiles, it is also exciting to look forward to possible applications. These findings may now be extrapolated to production and postharvest contexts. Future tests will assess the possibility of using natural fruit volatiles to retard fungal infestation or disease spread within a production canopy. It is possible that fumigation of a greenhouse or other controlled environment might be achieved using fruity volatile compounds, and the same concept may be extended to packaging or retail display. The fundamental findings described in this work open these possibilities and may be especially welcome if they impart their favorable essence onto the fruits themselves.

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