Role of Volatile Compounds from Wounded Oranges in Induction of Germination of *Penicillium digitatum* Conidia

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ABSTRACT

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Volatile compounds from exocarp-wounded oranges induced germination in about 50% of *Penicillium digitatum* conidia on water agar, compared to less than 5% on water agar alone. Limonene, α-pinene, sabinene, β-myrcene, acetaldehyde, ethanol, ethylene, and CO_2 were identified by gas chromatography as the major volatile compounds around wounded oranges. Conidial germination was not stimulated by exposure to any of these compounds alone. A mixture of limonene, acetaldehyde, ethanol, and CO_2 at concentrations measured in the atmosphere around wounded oranges stimulated spore germination on water agar and on silica gel medium to the same degree as the natural mixture of volatile compounds from the fruit. Ethylene alone or in combination with the synthetic mixture of volatile compounds did not increase germination.

Additional keywords: citrus green mold.

Green mold of citrus fruits is initiated by germination of conidia of *Penicillium digitatum* (Pers.:Fr.) Sacc. in wounds in the fruit exocarp (8). This fungus does not cause progressive decay in any other fresh fruit or vegetable. Other *Penicillium* spp. are pathogens on a variety of crops (2) but, with the exception of *P. italicum* Wehmer, do not aggressively infect oranges, lemons, or grapefruit.

P. digitatum spores germinate readily in extracts of citrus fruits and complex media such as potato-dextrose agar (PDA). They also germinate in water containing glucose, ascorbic acid, and mixtures of organic acids and simple sugars (27), but continued hyphal development depends on additional carbon and nitrogen sources. Little or no germination occurs on water agar alone. We assumed that substrate nutrition was the sole determinant of spore development and an important factor in host specificity. Therefore, we were surprised that *P. digitatum* spores germinated on water agar exposed to several wounded oranges in a closed container. The study reported here was undertaken to identify the volatile organic compounds emanating from wounded oranges that stimulated germination in the absence of substrate nutrients.

MATERIALS AND METHODS

Treatment of spores with volatile compounds from oranges. Conidia of *P. digitatum* were brushed from decaying lemons (*Citrus limon* (L.) N.L. Burm. 'Eureka') and stored over silica gel desiccant (Grace Davidson Chemical Co., Baltimore). Spores were suspended in 0.01% (w/v) Triton X-100 and adjusted to $OD_{425} = 0.1$ ($\sim 1 \times 10^6$ spores per milliliter). Spore suspension (0.1 ml) was streaked on solid culture media (3 ml) in 35- × 10-mm glass petri dishes. In most experiments, the spores were streaked on 1% (w/v) water agar (Bacto-Agar, Difco Laboratories, Detroit); PDA and silica gel were used to evaluate the effect of substrate nutrients on spore germination in response to volatile compounds from the peels of oranges (*Citrus sinensis* (L.) Osbeck). Silica gel culture medium was prepared from colloidal silica (Ludox HS-30, E. I. Du Pont De Nemours & Co., Inc., Wilmington, DE) (34). The solidified medium in petri dishes was soaked in 0.001 N HCl for 8 h, followed by sterile distilled water for 24 h, to adjust the medium to pH 5.0–5.5.

Valencia or navel oranges were harvested from local groves and punctured or scratched by a 1- × 3-mm steel tool. In initial tests, inverted petri dish bottoms with spores streaked on water agar were attached with melted paraffin (Parowax, Amoco Oil Co., Chicago) to the surface of oranges, exposing the spores to volatile compounds emanating from the fruit surface. The oranges with attached petri dishes were kept in a humid atmosphere to reduce water loss and cracking of the paraffin seal. The petri dishes were separated from the fruit after 24 h at room temperature (22–24 C), and spore germination was evaluated by observing three groups of 100 spores each.

In later tests, studies were conducted with the atmosphere surrounding wounded oranges in a glass jar (19.4 L) containing 24 medium oranges (~4 kg) each with 24 scratches perpendicular to the fruit equator. Five 90-mm petri dishes filled with perlite saturated with 40 ml of 5 N KOH were placed in the jar to

keep the CO_2 concentration less than 1.0%. The atmosphere in the jar was circulated by a magnet-driven fan beneath the hardware cloth platform that supported the fruit. After 24 h, a sample of the atmosphere surrounding the wounded fruit was withdrawn to evaluate its activity in stimulating spore germination and to analyze organic components. A glass desiccator (2.58 L) containing spores streaked on water agar was partially evacuated with a water aspirator pump and attached by glass tubing to the glass jar with the injured fruit. The atmosphere in the jar was displaced to the desiccator over a 15-min period by admitting air to the bottom of the jar. The desiccator was disconnected from the jar, and after 24 h, the petri dishes were removed, and spore germination was evaluated. In other experiments, a sample (2 L) of the atmosphere surrounding the injured fruit in the glass jar was aspirated by water displacement through two traps in series immersed in a dry ice-isopropanol bath. Essentially all of the terpene compounds were collected in the first trap and were analyzed by gas chromatography or transferred to an evacuated desiccator with spores on water agar by immersing the trap in a boiling water bath.

Analysis of volatile compounds. Organic compounds in the atmosphere surrounding wounded oranges and in the cold trap were analyzed with a Varian 3700 gas chromatograph (Varian Aerograph Co., Sunnyvale, CA) fitted with a flame ionization detector and a 3.175-mm × 3.05-m column of 20% (w/w) Carbowax 20M on Chromosorb W DMCS 60–80 mesh (Varian, Instruments Co., Sunnyvale, CA). The column temperature was isothermal at 120 C for 8 min, increased 6 C/min to 180 C, and isothermal for 2 min. The injector and detector temperatures were 120 and 180 C, respectively. Carrier (N_2) flow was 30 ml/min; H_2 gas flow to the detector was 30 ml/min. The volatile compounds condensed in the cold trap were dissolved in 4 ml of methanol, 6 ml of NaCl-saturated H_2O was added, and the organic compounds were extracted into isooctane. Isooctane solution (2 μl) was injected into the gas chromatograph. The identities of the peaks were confirmed by analysis of the same solution by high-resolution glass capillary gas chromatography (33).

Synthetic mixtures of volatiles. The compounds identified in the atmosphere surrounding wounded oranges were obtained from Sunkist Inc., Ontario, CA; Sigma Chemical Co., St. Louis; or Aldrich Chemical Co., Milwaukee, WI, and were >95% pure by gas chromatography. Mixtures of volatile compounds were prepared by injecting calculated quantities of the pure compounds into a glass desiccator (2.58 L) containing spores on water agar. The atmosphere was stirred for 10 min and analyzed by gas chromatography; the desiccator was held at room temperature (22–24 C) for 24 h. Several-hundred spores were inspected for germination in each desiccator.

RESULTS

Activity and identification of volatile compounds from wounded oranges. Less than 5% of the *P. digitatum* spores on water agar in air or affixed to noninjured oranges germinated. Germination was increased when the exocarp of the fruit was injured, exposing the spores to volatile compounds released from the fruit (Fig. 1). Ten puncture wounds (1 × 3 mm) or three scratches (1 mm × 3 cm) resulted in maximum spore germination (40–60%) in five tests. More or less fruit injury reduced percent germination.

The stimulatory effect of the volatile compounds on germination also was demonstrated when spores on water agar were placed in a jar with wounded oranges or in a glass desiccator with the isolated atmosphere from that jar. The volatile compounds were condensed in a dry-ice trap and transferred to another container without causing a significant loss in spore germination. Ten typical isolates of *P. digitatum* collected from all citrus production areas in California showed the same response to volatile compounds from wounded oranges.

After 24 h, the major terpenes in the atmosphere of the 19.4-L jar containing 24 wounded oranges were limonene (4.25 μg/ml), α-pinene (0.26 μg/ml), sabinene (0.36 μg/ml), and β-myrcene (0.45 μg/ml) (Fig. 2). Similar values were obtained by analysis of several samples on packed and capillary columns. These terpenes were accompanied by ethanol, acetaldehyde, CO_2, and ethylene in concentrations that varied with the conditions in which the wounded oranges were held. When the unventilated atmosphere surrounding the wounded oranges in the 19.4-L glass jar contained KOH that absorbed CO_2, it contained ethanol (0.1 μg/ml), acetaldehyde (0.02 μg/ml), CO_2 (2–20 μl/ml), and ethylene (1–20 μl/L). Samples taken from the petri dish bottoms affixed to injured oranges showed these four compounds at similar concentrations.

Synthetic mixture of volatile compounds. The terpenes alone in several concentrations and combinations failed to stimulate spore germination significantly above the level of spore germination on water agar in air. Limonene, the major terpene (~90%) in the wounded-fruit atmosphere, was combined with ethanol and acetaldehyde in approximately the concentrations analyzed in the atmosphere surrounding wounded oranges (designated 1× concentrations: 4.25, 0.20, and 0.025 μg/ml, respectively). The combination of these three compounds induced germination of ~15% of the spores on water agar. Addition of CO_2 (10 μl/ml, 1× concentration) to this mixture further increased germination to 38%, the maximum level attained in the CO_2-varying experiment (Fig. 3). This 1× mixture of limonene (4.25 μg/ml), ethanol (0.20 μg/ml), acetaldehyde (0.025 μg/ml), and CO_2 (10 μl/ml) resulted in the highest level of germination in the four experiments shown. A 50% reduction in the concentration of any of the components reduced spore germination compared to the 1× mixture (Fig. 3). Higher concentrations (2× and 4×) of the components, especially limonene and acetaldehyde, reduced germination significantly. Addition of ethylene (0.1, 3.0, 15, or 50 μl/L) to the

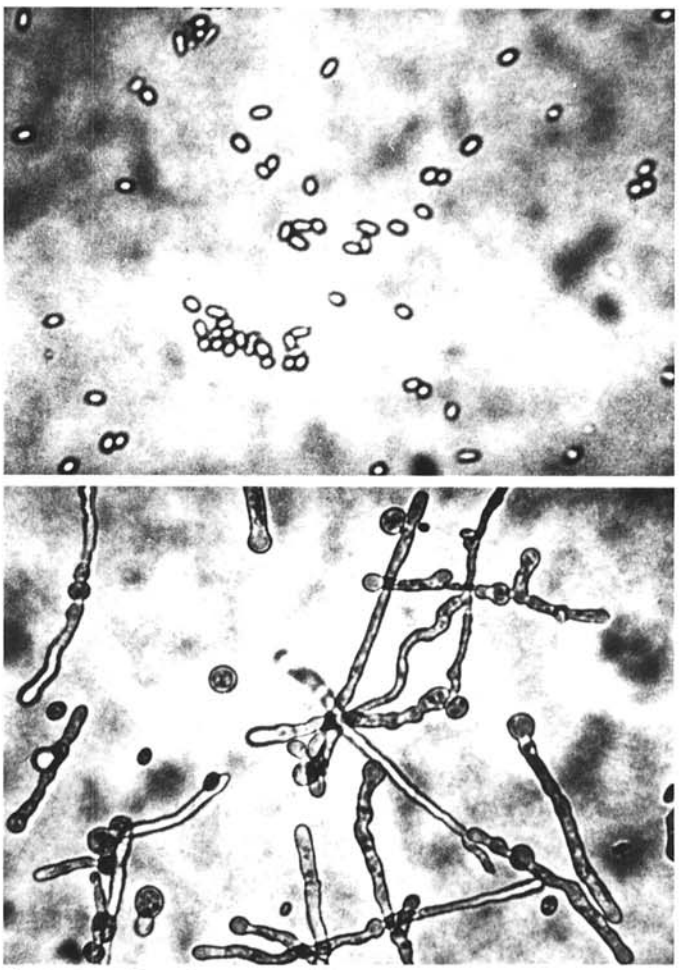

Fig. 1. *Penicillium digitatum* conidia on water agar after incubation for 24 h at 22–24 C in a petri dish affixed to a noninjured orange (top) or a wounded orange (bottom). Nongerminated conidia are ~5 × 7 μm.

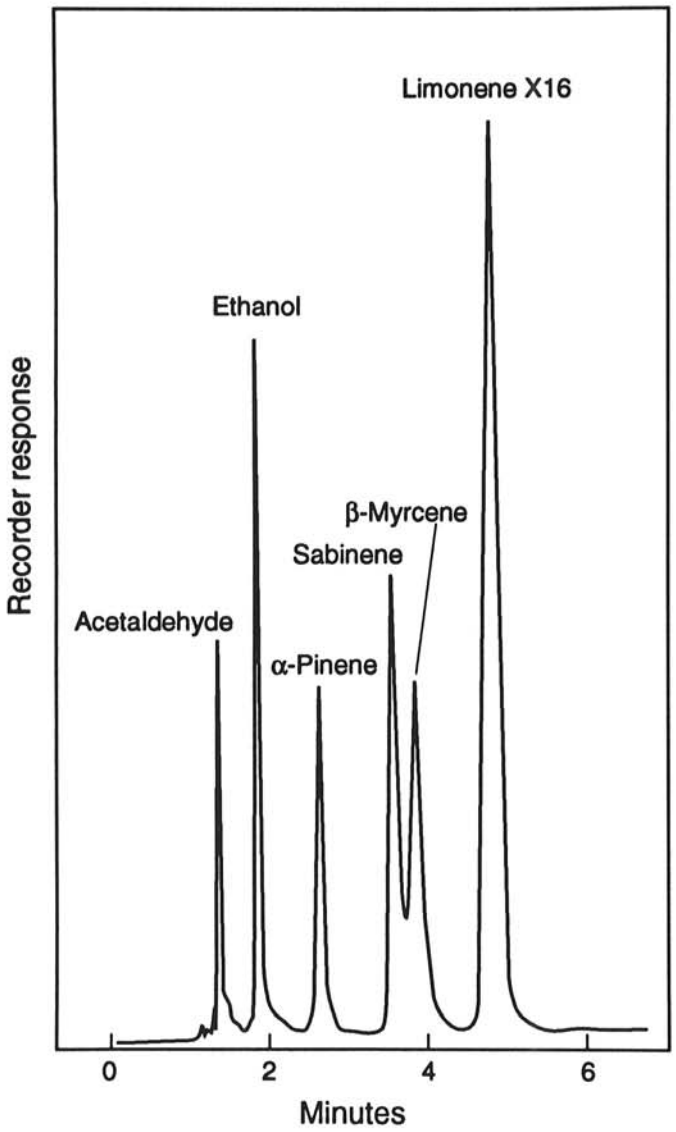

Fig. 2. Gas chromatogram of volatile organic compounds in the atmosphere surrounding wounded oranges. Limonene peak shown was attenuated 16 times.

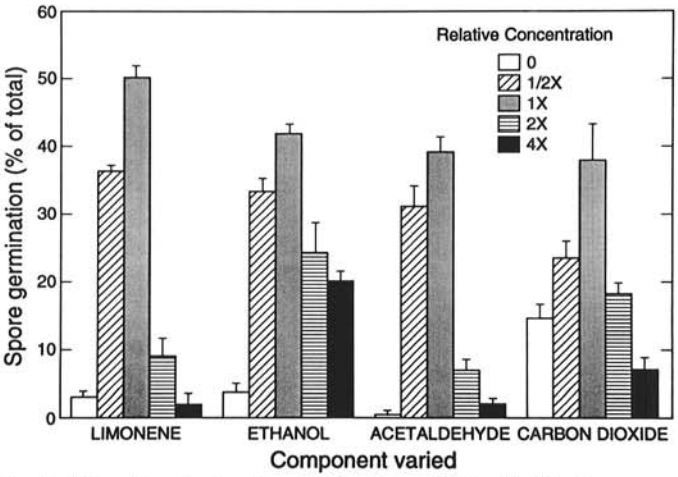

Fig. 3. Effect of synthetic mixtures of compounds identified in the atmosphere surrounding wounded oranges on the germination of *Penicillium digitatum* spores on water agar. Relative concentrations are expressed as proportions of those in the natural atmosphere (1×). The 1× concentration for each component was limonene, 4.25 μg/ml; ethanol, 0.20 μg/ml; acetaldehyde, 0.025 μg/ml; and CO_2, 10 μl/ml. Mixture components other than those identified on abscissa were 1×. Bars indicate means and standard errors for four observations.

1× synthetic mixture of volatile compounds did not increase germination further.

The overall concentration of the synthetic mixture of volatile compounds was increased or reduced relative to the 1× mixture in an effort to increase spore germination above 50%. At the 1× concentration, 45% of the spores germinated on water agar (Fig. 4). Germination was reduced by higher and lower concentrations of the mixture of volatile compounds. More than 95% of the spores germinated on PDA in air.

Combination of volatile compounds and substrate nutrients. Water agar (1%) contains nutrients (e.g., galactose) at a level that does not support germination of *P. digitatum* spores but that could interact physiologically with volatile compounds from oranges. Spores were streaked on 1% water agar and silica gel medium, and the cultures were affixed to wounded oranges or exposed to the 1× synthetic mixture in a desiccator. The level of germination was the same on water agar and silica gel medium, indicating that nutrients in the water agar did not play an important role in the stimulation of germination by the volatile compounds. The influence of substrate nutrients on the response of spores to volatile compounds was evaluated further by adding galactose to the spore suspension streaked on silica gel medium. Less than 0.01% galactose in the spore suspension had no effect on spore germination on silica gel in the presence or absence of the volatile compounds. Galactose (0.01–0.08%) in the spore suspension resulted in 5–10% germination on silica gel medium in air and increased germination in cultures sealed to wounded oranges from 35 (no galactose) to 51% (0.08% galactose). Low concentrations of PDA (10^{-6}–10^{-2} times the Difco label recommendation) added to water agar resulted in 10–30% germination without volatile compounds from oranges and 65–100% germination in an atmosphere of volatiles. Volatile compounds and substrate nutrients appeared to have an independent and additive effect on spore germination. Each experiment was performed on two or more occasions with similar results.

DISCUSSION

P. digitatum spores germinated on media derived from plant products and on defined media containing glucose and certain organic acids (27). Spores on water have been induced to germinate by adding citrus oils, citral, nonanal, and geranial to the substrate (14). However, nonanal and homologous aldehydes were toxic to spores on medium containing sucrose (7,14). Other components of citrus oils (e.g., limonene, terpinolene, α-pinene, and β-myrcene) had no effect on spore germination on water agar or PDA (7,14,35). Variability in published accounts of the germination-

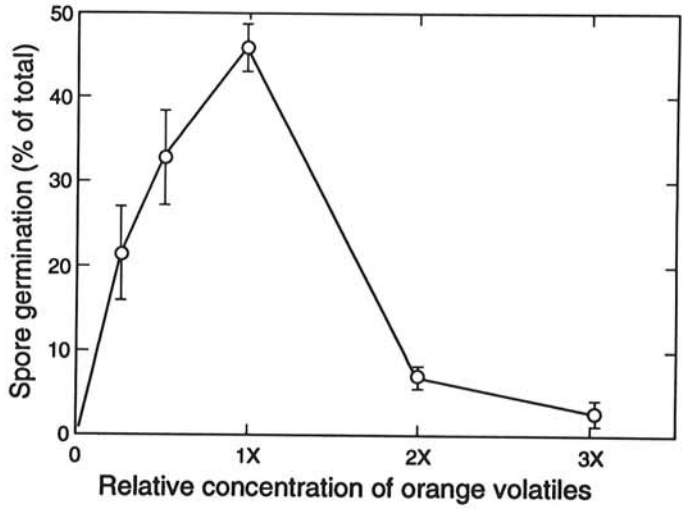

Fig. 4. Effect of concentration of the synthetic mixture of volatile compounds on the germination of *Penicillium digitatum* spores. 1× is the concentration typical of the natural mixture measured surrounding wounded oranges. Bars indicate means and standard errors for four observations.

stimulating activity of essential oils may be due to their low water solubility, volatility, interaction with nutrients, and the possibility of both stimulatory and inhibitory action dependent on concentration in the medium (1,4,5,12,14,21,22).

Essential oils often are involved in the flavor or odor characteristics of plant products (19,25,32). The participation of a compound in the odor characteristic of a fruit indicates that it exists, often in a very low concentration, in the enveloping atmosphere. The concentration of such compounds around citrus fruits is influenced by fruit maturity, temperature, ethylene treatment, and surface injury (23–25,31). Many of these volatile compounds have been identified and tested in vitro for physiological activity against microorganisms (1,12,14,22). In some cases, the pure compound or mixture has been incorporated directly into the substrate at some arbitrary concentration that cannot be related to the in vivo response of the microorganism to the atmosphere surrounding the fruit.

Earlier we reported that *P. digitatum* spores on water agar were induced to germinate by volatile compounds emanating from diced peels of oranges, lemons, grapefruits, tangerines, kumquats, and chopped leaves of *Citrus* spp., rosemary (*Rosmarinus officinalis*), and lemon grass (*Cymbopogon citratus*) (10). Exposure to volatile compounds from citrus fruit stimulated spore germination in 10 isolates each of *P. digitatum* and *P. italicum* but not in eight other *Penicillium* spp. In fact, most *Penicillium* spp. were inhibited by a concentration of volatiles that stimulated germination of *P. digitatum* and *P. italicum*. Volatile compounds from orange peel inhibited germination of *Botrytis cinerea*, whereas apple pieces stimulated germination (4). Volatile compounds may play a role in host selectivity of some postharvest pathogens.

The major components of the atmosphere surrounding wounded oranges, identified and quantified by gas chromatography, were the terpene hydrocarbons, limonene, β-myrcene, α-pinene, and sabinene accompanied by acetaldehyde, ethanol, CO_2, and ethylene. Nonanal, citral, and other oxygenated terpenes associated with orange oil were not detected. The same pattern of volatile compounds was found above wounded oranges at 20–27 C (24). The average quantity of volatile compounds from wounded fruits was 75 times greater than that from noninjured fruit. This explains the low germination of *P. digitatum* spores on water agar exposed to sound oranges. Our synthetic mixture of limonene, acetaldehyde, ethanol, and CO_2 at concentrations measured in the atmosphere around wounded oranges stimulated spore germination on water agar to the same degree as the external fruit atmosphere. This mixture of volatile compounds accounts for the stimulatory effect of wounded citrus fruits on the germination of *P. digitatum* and *P. italicum* in the absence of substrate nutrients (10).

The stimulation of microorganisms, especially fungi, by volatile organic compounds has been reviewed comprehensively (12,15). Most studies have dealt with the role of volatile compounds in the germination of fungal spores and sclerotia in soil. Relatively little research has focused on their effect on fruit and foliar pathogens.

Remoistened hay (alfalfa, grasses, sugar beet, peanuts) emitted volatile compounds that stimulated germination of fungal propagules suppressed by soil stasis. Volatile organic compounds from alfalfa hay were identified as methanol, acetaldehyde, isobutyraldehyde, and isovaleraldehyde (26). A synthetic mixture of volatile compounds from alfalfa stimulated soil microbial activity (26) and eruptive germination of sclerotia of *Sclerotium rolfsii* (21). Each component alone, except for butyraldehyde, stimulated germination to some extent, although less than the complete mixture (21). Lower alcohols and aldehydes stimulated sclerotial germination and other alcohols (C_1–C_8), and aldehydes (C_1–C_7) also were effective (3,16,21,30). Ethylene did not stimulate sclerotial germination (3). Volatile exudates from aged pea seeds stimulated germination of spores in soil-induced stasis (18). In this case, the active compounds might be carbonyl compounds arising from lipid peroxidation. 2,4-Hexadienal and octanal were most active in releasing soil stasis in *Alternaria alternata* conidiospores; nonanal and other aldehydes (C_3–C_{14}) were inactive.

Despite the obvious aroma of many fruits and vegetables and the recognized physiological role of ethylene and CO_2, relatively few publications have appeared on the effect of these volatile compounds on plant pathogens. Germination of teliospores of *Puccinia punctiformis* was stimulated by chopped onions and garlic but not by orange peel or geranium leaf (13). Known constituents of onion and garlic did not stimulate germination, but the related compounds nonyl-, decyl-, and dodecylisothiocyanate were active. Spores of *Botrytis cinerea* and *Penicillium glaucum* (=*P. expansum*) were stimulated by volatile compounds from apple fruit slices but were inhibited by orange peels, potatoes, and onions; CO_2 was not involved (4). Volatile compounds from the flesh of apples and nectarines stimulated spore germination of *P. expansum* and *Monilinia fructicola*, but not *P. digitatum*, in our earlier experiments (10). The activity of apples in promoting germination of fungus spores has been attributed, in part, to ethylene. Spores of *B. cinerea* and *P. expansum* in water germinated during exposure to ethylene at 10 and 100 μl/L, respectively (20). In another report, ethylene (1–1,000 μl/L) did not affect germination of spores of *P. expansum* or *B. cinerea* on PDA but induced almost 20% germination in *P. digitatum* (11). The low germination (42%) observed for *P. digitatum* on PDA without added ethylene suggests a problem with spore vitality or composition of the medium in these experiments. Teliospores of *Uromyces agropyri* on water were induced to germinate by exposure to ethylene sources (ripening bananas, lemons, and ethephon) (17). In our experiments, *P. digitatum* spores on water agar exposed to a synthetic mixture of volatile compounds of oranges were not further stimulated by ethylene at 1–50 μl/L.

Ethanol and acetaldehyde are common volatile products of fruits and other plant tissues, especially in a low-oxygen environment (6,25). At low concentrations, each compound stimulated germination of sclerotia and fungus spores (3,15,16,21,26); at higher concentrations, acetaldehyde was fungitoxic (16,26) and has been evaluated as a fumigant to control postharvest diseases (28). Ethanol and acetaldehyde were released by ripening apricots and stimulated germ tube growth of *M. fructicola* in vitro (6). Acetaldehyde vapor concentrations above 10 μg/ml were fungitoxic. Exposure of green apricots with latent infections of *M. fructicola* to acetaldehyde vapors resulted in active brown rot lesions. In our experiments, *P. digitatum* spores on water agar appeared to be more sensitive to acetaldehyde than to ethanol.

Volatile compounds that stimulate fungal development probably do so by altering membrane permeability or regulating metabolism (12,15). *P. digitatum* spores on water agar are not self-inhibited and contain all nutrients required to support germination. Germination can be induced by glucose (25 mg/ml), ascorbic acid (50 μg/ml), or citrus fruit volatiles. Trehalose and polyols disappear from the spores during germination (9). Germination stimulants may induce an essential enzyme or overcome a metabolic block in the utilization of reserve nutrients as proposed for nonanal stimulation of urediospores (15).

The possible role of volatile compounds from citrus fruits in postharvest disease development has been suggested by their stimulatory action on germination of *P. digitatum* spores. We showed that volatile compounds evolved by wounded fruits of several *Citrus* spp. could induce germination of *P. digitatum* on water agar (10). Lemons infected with *Phytophthora citrophthora, Geotrichum candidum,* or *P. digitatum* also emanated volatiles that induced germination of *P. digitatum* on water agar. The observation that nonanal and geranial in the substrate did stimulate spore germination is irrelevant to this discussion because these compounds are not components of the atmosphere surrounding oranges (25). *P. digitatum* spores in water condensate on oranges may be induced to germinate by volatile compounds emanating from an adjacent wound, as suggested by our experiments demonstrating the additive effect of substrate nutrients and volatile compounds on germination. Volatile compounds might also elicit a chemotrophic response in the hypha as described for the action of alfalfa volatiles on *S. rolfsii* (29).

The significance of volatile compounds in postharvest pathology

clearly requires further investigation. Factors, such as wounding, disease, temperature, and low O_2, that increase the release of natural volatile compounds from citrus fruits (10,23–25) may stimulate germination of pathogen spores and increase the incidence of postharvest diseases. Brown (4), in his classic paper on the stimulation of *Botrytis* spore germination by volatile compounds from apples, concluded ". . .one may confidently state that the atmosphere of an apple storage is very favorable to *Botrytis* germination."

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