Effect of Nonanal, Citral, and Citrus Oils on Germination of Conidia of Penicillium digitatum and Penicillium italicum

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ABSTRACT

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Suspensions of nonanal in 1% water agar stimulated germination and swelling of conidia of *Penicillium digitatum* Sacc. (33% at 500 μliters/liter, 5 days, control 0%) and *P. italicum* Wehmer (35% at 500 μliters/liter, 3 days, controls 4.5%). Nonanal was the most active of the C6-C12 aldehydes studied. Decanal and dodecanal were very effective in causing swelling of spores. Citral was about as stimulatory as nonanal. Mixtures of citral and nonanal were more stimulatory to germination, or induced more swelling, than either compound alone. Suspensions of oils (1,000 μliters/liter in 1% water agar) from orange, lemon, lime, grapefruit, and tangerine, which contain these compounds along with many related compounds, were more stimulatory than nonanal, citral, or mixtures, and germination was more rapid.

Nonanal was identified as an endogenous germination stimulator of uredospores of *Puccinia graminis* v. *tritici* in 1957 (11). Fries (12, 13) demonstrated stimulation by nonanal of mycelial growth of several species of wood-rotting fungi, including *Stereum sanguinolentum* Fr. Some fungi grew toward sources of nonanal in petri plates. The stimulation of germination of uredospores in pustules of rust fungi has been reported for several species, and varying degrees of species specificity for chemicals related to nonanal have been noted (6, 7, 8, 10). Currently, some 21 species of rust and smut fungi, including seven genera, have been found to be stimulated by nonanal, beta-ionone, or other chemicals known previously for their organoleptic properties as components of natural flavors or fragrances (6, 15). Stimulation of conidial germination of *Penicillium digitatum* Sacc. and *Penicillium italicum* Wehmer by nonanal extends the action of this compound to another class of fungi, the Ascomycetes. Stimulation of pollen germination in several *Pinus* spp. also has been observed (9).

This report concerns stimulation of germination of conidia of *P. digitatum* and *P. italicum* by nonanal and related compounds, and the possible role of naturally occurring compounds of peel oil in the etiology of blue- and green-mold storage diseases in citrus.

MATERIALS AND METHODS

Conidia of *P. digitatum*, originally obtained from a moldy lemon, were harvested from mycelia grown on slants of potato-dextrose agar by agitation with isopentane, or collected by gentle vacuum from infected oranges or grapefruit which previously had been surface-sterilized and inoculated. Chemicals or oils were measured with a Hamilton microsyringe and added to 5.0 ml warm 1% Bacto agar in test tubes. Tubes were mechanically agitated and poured into 5.0-cm diameter plastic petri plates. Suspensions of conidia in isopentane were pipetted to the agar surface. Isopentane (b.p. 30 C) evaporated rapidly. The number of spores per plate was several thousand per ×90 microscopic field. Most chemicals used were obtained from Aldrich Chemical Co. Inc., Metuchen, NJ 08840. Cultures of *P. italicum*, originally obtained from T. T. Hatton, Jr. (U.S. Department of Agriculture, Horticultural Research Laboratory, Orlando, FL 32803) were handled in a similar manner. Spores were observed microscopically through the bottom of unopened petri plates over intervals of 1 to 14 days to 8 wk. Germination counts of at least 400 spores, made by two observers, were averaged and the standard error was calculated. Tests usually were conducted in darkness at 18-25 C.

A crude extract of orange oil was prepared from the peel of an orange obtained from a local grocery. The peel was crushed in a Carver press, suspended in isopentane, and filtered. The extract was washed with distilled water in a separatory funnel and the isopentane layer was dried overnight with anhydrous sodium sulfate. The isopentane was evaporated leaving a viscous orange-colored oil with a characteristic orange fragrance.

Samples of citrus oils also were obtained from Robert Berry (U.S. Department of Agriculture, Citrus and Subtropical Products Laboratory, Winter Haven, FL 33880).

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RESULTS

Nonanal and related compounds.—The effect of nonanal suspensions (10-10,000 μliters/liter) on germination of conidia was determined for *P. digitatum* at 5 days, and for *P. italicum* at 3 days (19 C) (Fig. 1). Maximum germination in this experiment for both species was 34-36% at 500 μgliters/liter. Germination of controls was 0% for *P. digitatum* and 4.5% for *P. italicum*. Some germination (23%) was observed with *P. digitatum* with concentrations (suspensions) as high as 2,500 μliters/liter, or 0.25% nonanal. Maximum germination of *P. digitatum* in experiments with nonanal was about 69%, and little increase was noted after 10 days. Germination in controls always was very low, usually less than 1%, even after 10 days. An occasional germinated spore would grow extensively and produce conidia. Stimulated spores had much reduced germ tube length or mycelial growth. The higher the concentration of nonanal, citral, other stimulatory compounds or oils, the less the growth. After several weeks, some formative effects of compounds were noted, as described later.

The 6- to 12-carbon straight-chain aldehydes were examined for maximum effectiveness on *P. digitatum* germination over the range of 250 to 1,000 μliters/liter. The 7- to 12-carbon aldehydes were active; nonanal was most active at 500 μliters/liter (Fig. 2). In other experiments, hexanal was active at 2,500 μliters/liter.

Orange oil and components.—Conidia of *P. digitatum* were stimulated by suspensions of orange oil from 50 to 10,000 μliters/liter (Fig. 3). The most effective concentration was 250 μliters/liter. Germination of 15% was observed after 24 hr; controls were 0%. Germination reached 70% after 10 days; controls reached 10%. Germination also was observed at 10,000 μliters/liter. Comparable experiments with *P. italicum* showed stimulated germination of 13% at 250 μliters/liter after 1 day (control 0%), and 73% at 2,500 μliters/liter after 10 days (control 6%).

Some chemical components of citrus oils were tested for activity (Table 1). With conidia of *P. digitatum* and *P. italicum*, citral and nonanal were most effective. Geraniol also was effective with *P. digitatum*. In this and other experiments, nonanal and citral were the most effective of the compounds tested.

Mixtures of compounds, particularly of nonanal and citral, (Table 2) were more effective than were single compounds. Nonanal-citral (1:1, v/v) at 500 μliters/liter was more effective (59%) than nonanal (17.8%) or citral (13.5%) alone. At 1,000 μliters/liter nonanal-citral (1:1, v/v) stimulated germination 43%, compared to 0.4% and 0% for nonanal and citral alone, respectively. Similar trends were noted in numbers of swollen conidia. Decanal and dodecanal also induced swelling of spores (Fig. 4-A). Mixtures of decanal and citral were synergistic; decanal and nonanal were not. The combination of an aliphatic aldehyde (e.g., nonanal, which is straight-chain and

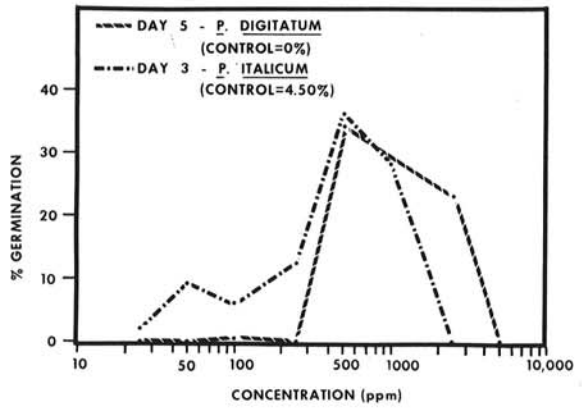

Fig. 1. Effect of nonanal syspensions in 1% water agar on germination of conidia of *Penicillium digitatum* and *P. italicum*.

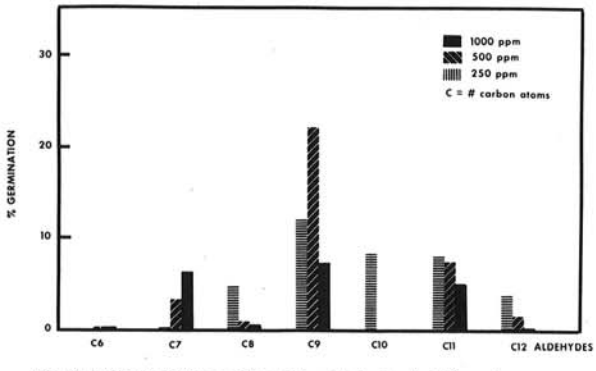

Fig. 2. Effect of linear C_6 to C_{12} aldehydes in 1% water agar on germination of conidia of *Penicillium digitatum* after 3 days.

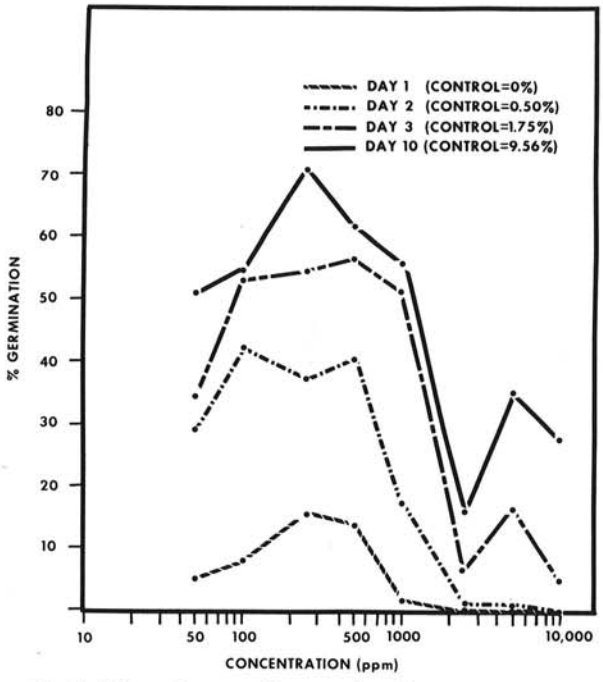

Fig. 3. Effect of orange oil suspensions in 1% water agar on germination of conidia of *Penicillium digitatum*.

saturated) with a terpene aldehyde (e.g., citral, which is branched and unsaturated) was required for enhanced activity. Such combinations occur naturally in citrus oils.

Other citrus oils.—Several citrus oils were tested at 1,000 μliters/liter (Table 3) and found stimulatory after 1 day at 22 C. These included lemon, lime, grapefruit, tangerine, Valencia orange, pineapple orange, and an oil produced from oranges treated with an abscission agent (18). The oils were more active than nonanal and citral at 500 μliters/liter, in which conidia germinated 0% at 1 day, and 1.7% and 18.2%, respectively, at 4 days.

The relative activities of various citrus oils tested (Table 3) can be explained partly by the quantities of straight-chain aldehydes and citral present in the oils (1, 17, 18, 20, 22, 23). Reported quantities of these aldehydes in citrus oils vary, depending on the analytical method used (20), but the quantities listed in Table 4 are representative values and are useful for comparison purposes.

Lime and lemon oils have from four to nine times more citral than the other oils that were tested; this high citral content may account for the relatively high biological activity of these two oils. The quantity of citral present in grapefruit oil does not differ significantly from that present in orange oil. However, quantities of the C_8 to C_{11} straight-chain aldehydes are slightly higher in grapefruit oil than in orange oil. A slight increase in the quantities of these straight-chain aldehydes and citral has been noted in some orange oils from fruit sprayed with abscission agents (18). These limited data suggest that increased levels of straight-chain aldehydes and/or citral in certain oils might explain the increased biological activity. Although nonanal has been identified in lime and tangerine oils, its concentration in these oils has not been determined (20).

TABLE 1. Effect of some chemical components of citrus oils on germination of conidia of *Penicillium digitatum* and *P. italicum* in 1% water agar

Penicillium sp. Compound	Conc. (μliters/liter)	Germination (%)
P. digitatum:[a]		
control		0.0
nonanal	500	8.0
nonanol	100	0.2
limonene	100	0.5
geraniol	100	5.2
6-methyl-5-hepten-2-one	500	0.8
citral	500	34.8
β-pinene	100	0.0
β-ionone	1,000	0.0
P. italicum:[b]		
control		14.5
nonanal	500	43.5
nonanol	100	14.0
limonene	500	15.5
geraniol	500	8.5
6-methyl-5-hepten-2-one	1,000	22.5
citral	100	65.5
citronellal	100	29.0

[a]Conidia were observed after exposure for 4 days at 25 C.
[b]Conidia were observed after exposure for 10 days at 24 C.

Formative effects.—In addition to causing swelling of conidia, the various aldehydes appeared to induce the formation of chlamydospores. The aldehyde-stimulated spores always produced rather short germ tubes, particularly compared to the occasional germinated spore found in the controls. The few conidia which did germinate on 1% water agar produced extensive mycelial growth, compared to those on aldehydes or oils, and chlamydospore-like bodies were rare, small, and not swollen. After 7 wk at 22 C, swollen spores had produced short germ tubes, usually about 10 to 20 times the diameter of the unswollen spore. Spherical swollen structures, apparently chlamydospores, were observed, sometimes several in a short chain (Fig. 4-B,C,D). The spherical swellings might also be considered a type of microcycling, in which the original spore swells, germinates, forms another swollen spore, which germinates, then repeats the process several times, without conidiation. At high concentrations of aldehydes or citrus oils, only slight microbial contamination was observed after extensive time periods. The bacteriostatic

TABLE 2. Effect of nonanal, citral, and mixtures on germination of conidia of *Penicillium digitatum* (4 days at 21 C) in 1% water agar

Compound	Germination during exposure to: 500 μliters/liter (%)	1,000 μliters/liter (%)
nonanal	17.8	0.4
citral	13.5	0.0
nonanal-citral 1:1	59.3	43.0
Nontreated water agar control	0.0	0.0

TABLE 3. The effect of suspensions of citrus oils (1,000 μliters/liter) on germination of conidia of *Penicillium digitatum* in 1% water agar

Treatment	Germination[a] (1 day, 22 C) (%)
Water agar control	0.0
Citrus oils:[b]	
lime	42.2**[c]
orange (abscission)	31.8**
grapefruit	29.4**
lemon	11.1**
tangerine	7.7**
Valencia orange	6.1**
pineapple orange	6.1*[d]

[a]Conidia were observed after exposure for 1 day at 22 C.
[b]Obtained from Robert Berry, U.S. Department of Agriculture, Citrus and Subtropical Products Laboratory, Winter Haven, FL 33880.
[c]Asterisks indicate the means were significantly different (P=0.01) from the controls.
[d]Asterisk indicates mean was significantly different (P=0.05) from the controls.

properties of natural essential oils and of some of their chemical components has been reported in the literature (3).

DISCUSSION

This research has shown that nonanal and related compounds, components of citrus oils, and citrus oils stimulate the germination of conidia of two species of *Penicillium* when placed on 1% water agar. In our experiments, some experiment-to-experiment variation was noted in optimum effective concentrations of those materials. In some cases, nonanal appeared most effective at 500 μliters/liter, in others at 1,000 μliters/liter. Variation also was noted in the maximum extent of germination in different experiments, possibly due to differences in spore lot, spore age, or other factors. The spore-stimulating compounds used in this study are volatile, hence transitory, and show biological activity in suspensions far above their low solubility in water [nonanal, 109 μliters/liter (5); 119 μliters/liter (2)]. They may be adsorbed on glass surfaces (2). Hence, exact concentrations are extremely difficult to define. No attempt was made to prepare stabilized emulsions of the compounds that were used, since it was felt the addition of another chemical might bind reactive functional groups and mask biological activity. Efforts to prevent loss of volatiles also would restrict exchange of oxygen and carbon dioxide, which would introduce additional complicating factors.

Davis and Smoot (4, 5) observed inhibition of germination of conidia of *P. digitatum* when placed in solutions of C_5 to C_9 aldehydes in aqueous 2.5% sucrose plus mineral salts. In tests with nonanal in 2.5% sucrose in 1% water agar, we observed over 90% inhibition of germination with concentrations of nonanal as low as 25 μliters/liter (23% germination in sucrose controls) which is in agreement with the results of Davis and Smoot (4, 5).

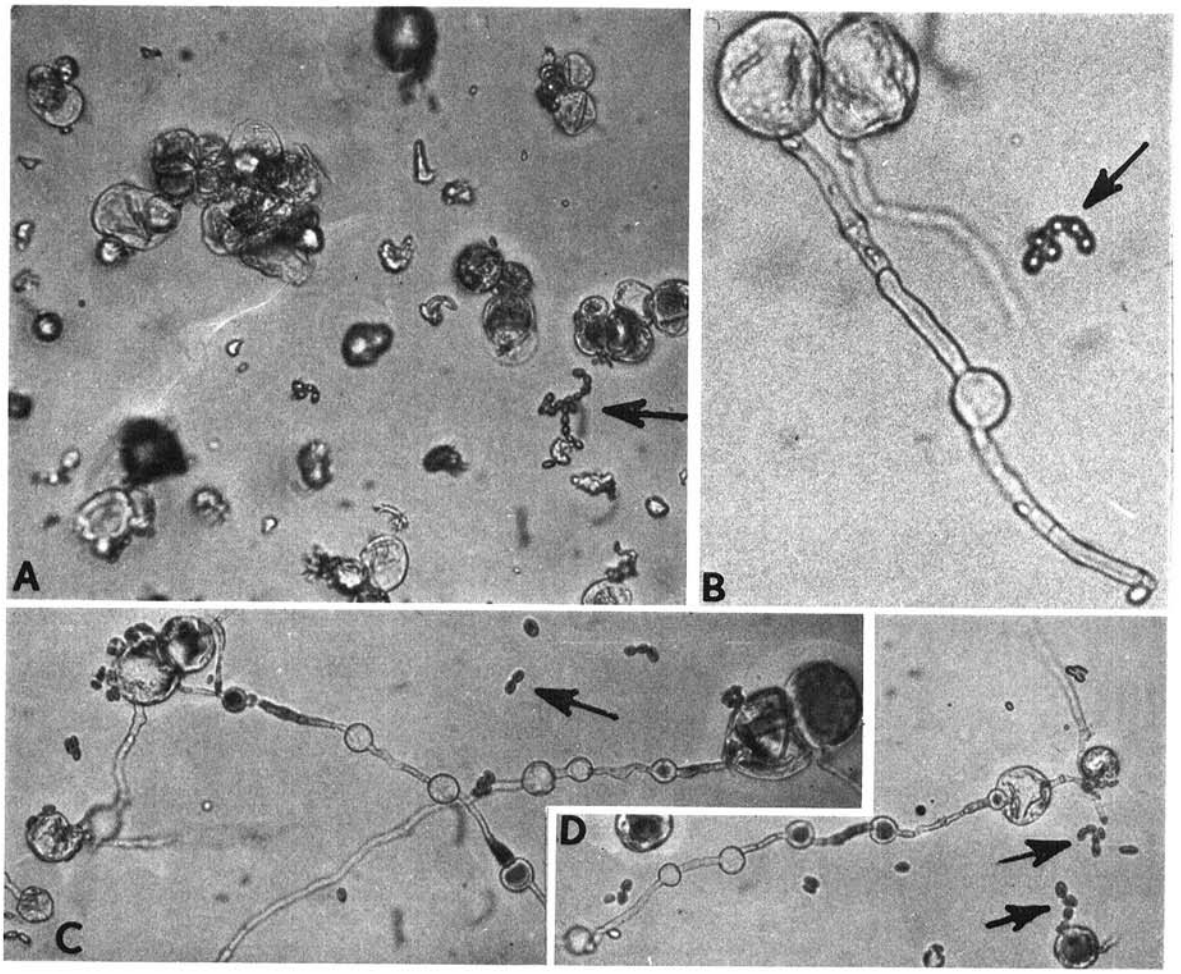

Fig. 4-(A to D). Formative effects of aldehydes on conidia of *Penicillium digitatum*. **A)** Effect of dodecanal (1,000 μliters/liter) showing massive swelling of conidia, many collapsed. (× 222). **B)** Nonanal (1,000 μliters/liter) showing germ tube with chlamydospore (× 333). **C)** and **D)** Octanal (1,000 μliters/liter) showing chains of chlamydospores (× 277). All at 7 wk. Arrows indicate nonswollen, nongerminated conidia.

TABLE 4. Quantities of aldehydes in citrus peel oils

Aldehyde	Aldehydes in peel oil of: Lime (μliters/liter)	Lemon[a] (μliters/liter)	Grapefruit[a] (μliters/liter)	Tangerine[b] (μliters/liter)	Orange[a] (μliters/liter)
Octanal	P[c]	960	5,660	P	4,630
Nonanal	P	1,460	1,040	P	650
Decanal	P	730	7,070	1,650	5,020
Undecanal	P	550	410	P	220
Citral[d]	53,000[e]	24,200[e]	6,000[e]	720[f]	5,000[e]

[a]Calculated from the percent of each aldehyde [Smoot et al. 1971. U.S. Dep. Agric. Handb. 398 (21)] times the aldehyde content (1).
[b]Calculated from M. G. Moshonas and P. E. Shaw. 1974. J. Agric. Food Chem. 22:282-284 (17).
[c]P = present, but not quantitated [P. E. Shaw. 1977. Pages 427-462 *in* S. Nagy et al. eds. Citrus science and technology, Vol. I. AVI Publishing Co., Westport, Connecticut. (20)].
[d]Citral is neral plus geranial.
[e]Yokoyama et al. 1961. J. Assoc. Off. Anal. Chem. 44:535-541 (23).
[f]Geranial only.

Without sucrose, however, nonanal in water agar was stimulatory, as indicated. The differential action of the aldehydes in the presence or absence of sucrose presents an interesting metabolic problem, perhaps of some importance in disease establishment.

The induction of chlamydospore formation shows additional interesting biological activity of this group of compounds. Nyman (19) showed nonanal to be effective in stimulating the accelerative phase of growth in the yeast, *Dipodascus aggregatus*. Fries (14) has shown that hexanal, and to a lesser extent, nonanal, induce the formation of coremia in *Ceratocystis picea*.

The synergistic effect of citral and nonanal is of considerable interest since both occur in various citrus oils. Citrus oils, which contain many compounds stimulatory to uredospores of *Puccinia graminis* var. *tritici* (the pathogen of stem rust of wheat) were much more active on conidia of *P. digitatum* than was the mixture of nonanal and citral. Synergistic effects of compounds such as these also have been shown in insect pheromone studies. Nonanal and undecanal, for example, have been reported to have pheromone activity in the greater wax moth *Galleria mellonella* (16). In a previous publication (6), reference was made to the fact that many of the compounds that stimulate fungal spore germination also act as insect pheromones.

The stimulation of conidia of *P. digitatum* and *P. italicum* by nonanal and citral, and by many citrus oils which contain these compounds, may implicate them in the infection of the host. Mechanical injury to the rind has been cited as the chief factor for development of molds (21), and the release of citrus oil during injury may be an important factor in spore germination. Further study of the release of these allelopathic compounds during injury of the host may lead to a better understanding of the infection process, and hopefully to better ways to control blue-mold and green-mold rots of citrus.

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