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Inhibition of Citrus Postharvest Pathogens by Vapor of Citral and Related Compounds in Culture

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The vapors of citral, its isomers geranial and neral, and its related compounds were examined for their effect on *Penicillium digitatum*, *Penicillium italicum*, and *Geotrichum candidum*, the major fungi responsible for postharvest spoilage of citrus. Vapor of citral and its two isomers generated from 15 μL L^{-1} aqueous solutions in Petri dishes inhibited development of the three pathogens, with concentrations of 2−6 μL L^{-1} also being effective against *P. italicum.* Vapors of citral and geranial from 15 μL L^{-1} solutions were fungicidal to *P. digitatum* and *G. candidum*, while neral was fungicidal to *G. candidum.* Citral-related compounds were much less effective, with effectiveness decreasing from citronellal to citronellol and citronellic acid. *R* and *S* isomers of these three citral-related compounds generally had similar effects on the fungi tested.

KEYWORDS: Citral; geranial; neral; vapor; *Penicillium digitatum*; *Penicillium italicum*; *Geotrichum candidum* citrus race; *Geotrichum candidum* var. citri-aurantii

INTRODUCTION

Citral, an acyclic α,β-unsaturated monoterpene aldehyde, is a naturally occurring isoprenoid compound with two isomers, geranial and neral. In citrus fruit, citral is located inside oil glands (cavities) in the flavedo layer of the peel and, therefore, is not distributed uniformly throughout the fruit surface (*1*). Of the citrus essential oil contained in the glands, citral constitutes about 3% of the oxygenated compounds (*2*).

Aldehydes are an intermediate form between primary alcohols and carboxylic acids and are easily oxidized to the corresponding carboxylic acids (*3*). As an α,β-unsaturated aldehyde that contains two C=C double bonds and one carbonyl group, citral is susceptible to several reactions, such as oxidation and reduction. According to Erman (*4*), geranial and neral (citral isomers) are the biosynthetic oxidation products of geraniol, which undergoes reduction to citronellal and further oxidation to citronellic acid, while citronellal is the biosynthetic oxidation product of citronellol. In addition, citral, geranial, neral, citronellal, citronellol, and citronellic acid are known to be components of citrus essential oils (*2*, *5*).

Green mold, blue mold, and sour rot are the most important postharvest diseases of citrus and are caused by *Penicillium digitatum*, *Penicillium italicum*, and *Geotrichum candidum* citrus race (syn. *G. candidum* var. citri-aurantii), respectively (*6*). Citral has been shown to have antifungal properties against these three fungi in culture (*7*−*11*) and in lemon where it is a natural compound in the oil gland (*1*), but its activity seems to vary.

Citral, citronellol, and citronellal have been reported to inhibit the growth of *Botrytis cinerea* and *Monilinia fructicola* (*12*). In addition, vapor of another aldehyde, acetaldehyde, inhibited the growth of *P. digitatum* and *P. italicum* (*13*). Previously, we reported that exposure of spores to citral in the volatile phase completely prevented growth of *P. digitatum*, *P. italicum*, and *G. candidum* when generated from 15 μL L^{-1} aqueous solutions in sealed Petri dishes (*14*).

The activity of the individual citral isomers, geranial and neral, and the related compounds citronellal, citronellol, and citronellic acid against *P. digitatum*, *P. italicum*, and *G. candidum* has not been documented. Therefore, we examined whether the activity of the citral isomers as well as their related compounds in the vapor phase against spores of *P. digitatum*, *P. italicum*, and *G. candidum* differs and, therefore, whether differences in citral composition are likely to influence its antifungal activity.

MATERIALS AND METHODS

Cultures of *P. digitatum*, *P. italicum*, and *G. candidum* were isolated from naturally infected lemon fruit, cultured, and maintained on potato dextrose agar as described previously (*14*). Pathogenicity tests were conducted on Navel oranges, followed by reisolation of the fungi from these fruit. Single spore-derived cultures were then established and used throughout this study.

Spore suspensions were prepared in sterile reverse osmosis water, and the concentration was established using a hemocytometer (*15*). Suspensions of approximately 10^4 spores mL^{-1} of *P. digitatum*, *P. italicum*, and *G. candidum* were used as inoculum. The medium used was Neutral-Dox Yeast agar (NDY), comprising 15 g L^{-1} agar (BiTek, Difco Laboratories, Spark, MD); 30 g L^{-1} sucrose; 2 g L^{-1} $NaNO_3$; 1.0 g L^{-1} KH_2PO_4; and 0.5 g L^{-1} each of yeast extract, KCl, and $MgSO_4$.

The plates were incubated at room temperature (22 °C) for 14 days, and colony-forming units (cfu) were counted. For plates showing no

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obvious growth, microscopic evaluation of the spores for germination was carried out using a compound microscope at 400× magnification after staining the agar using lactoglycerol cotton blue.

Citral, *R*-citronellal, *S*-citronellal, *R*-citronellol, *S*-citronellol, *R*-citronellic acid, and *S*-citronellic acid, di-*tert*-butyl azodicarboxilate (DBAD), fluorobenzene, geraniol, nerol, cuprous chloride (CuCl), Celite, silica gel, and dichloromethane (CH_2Cl_2) were obtained from Sigma Aldrich (Castle Hill, NSW, Australia), and the emulsifier TritonX, used to facilitate mixing of citral with water, was obtained from Ajax Laboratory Chemicals (Philadelphia, PA). K_2CO_3 and phenanthroline were obtained from BDH Laboratory Supplies Merck (Kilsyth, Victoria, Australia).

Geranial and neral were separately synthesized according to the procedure of Marko et al. (*16*) and Marko (personal communication, 2000). A mixture of 8.1 mmol of K_2CO_3 and 1.62 mmol of DBAD was added to a mixture of 1.62 mmol of CuCl and 1.62 mmol of phenanthroline in 100 mL of fluorobenzene. They were mixed for 5 min, and 32.4 mmol of geraniol/nerol dissolved in 60 mL of fluorobenzene was then added to the mixture over 5 min. An oxygen flow was passed through to the reaction mixture and refluxed. The progress of the reaction was monitored by thin-layer chromatography after filtering a sample through a Ciligel (a mixture of Celite and silica gel) pad and washing it four times with 100 mL of CH_2Cl_2. The solvent was evaporated under reduced pressure, and the crude product was purified rapidly by silica gel chromatography. The synthesized geranial and neral were then analyzed by means of gas chromatography, using a Shimadzu GC 14A (Kyoto, Japan) equipped with a BP20 capillary column (50 m long, thickness 0.5 μm, O.D. of 0.43 mm, SGE Scientific Pty Ltd., Australia) and flame ionization detector (FID). The carrier gas used was nitrogen with a head pressure of 123 kPa, the injection temperature was 225 °C with an initial column temperature of 60 °C for 2 min, and the temperature ramping was to 190 °C at 6 °C/min and holding at 190 °C for an additional 20 min. The detection temperature was programmed at 225 °C.

The experiment was conducted in two stages; first, citral was compared to the synthesized isomers, geranial and neral, and then, citral was compared to its related compounds citronellal, citronellol, and citronellic acid.

Initially, 100 μL of spore suspension was spread onto each NDY agar plate using a glass spreader and left to dry. An aqueous solution of each compound was then applied as five equidistant 20 μL aliquots on glass slides on the inside of the lid with the dish placed upside down. The solutions consisted of 2, 6, or 15 μL of each compound diluted to 100 μL with 400 μL L^{-1} aqueous TritonX. The experiment was carried out in four replicates, with one replicated control of water only and another of 400 μL L^{-1} aqueous TritonX applied on the glass slides in the plate lid. Plates were sealed with Sellotape (Scotch, China) to minimize gaseous exchange and incubated as described above. The glass slides and solutions on them were removed from the plate after 14 days, and the plates were then incubated at room temperature for another 8 weeks to determine if any viable spores were present but had not germinated. In addition, agar plugs of 1 cm diameter bearing spores were transferred to fresh NDY plates to eliminate any citral absorbed by the medium, and these were incubated at room temperature for another 2 weeks, while the original plate was also left unsealed at room temperature. This procedure was designed to allow the classification of the vapor as fungistatic or fungicidal.

Analysis of variance (ANOVA) for a number of cfu was performed using Genstat version 5.1 (Genstat 5, Release 4.1, 4th ed., Lawes Agricultural Trust, 1998) and general ANOVA (with blocking) with treatment formulated as control/(chemical × concentration). Data for each fungus and each method were analyzed separately.

RESULTS

The composition of the synthesized geranial and neral was 94:2% and 2:92% geranial to neral, respectively. The effects of citral vapor, its isomers, and its related compounds on *P. digitatum*, *P. italicum*, and *G. candidum* are presented in **Tables 1**−**3**, respectively. In comparison to the water control for each fungus, treatment with 400 μL L^{-1} aqueous TritonX reduced the number of cfu by 8−13, 13−17, and 22−26% for *P. digitatum*, *P. italicum*, and *G. candidum*, respectively.

Table 1. Effect of Citral Vapor, Its Isomers, and Related Compounds on the Growth of *P. digitatum* at Different Concentrations, Expressed as cfu[a]

	cfu per plate above solutions of				growth over plate (%) after removing 15 μL L^{-1} solution
compd	control	2 μL L^{-1}	6 μL L^{-1}	15 μL L^{-1}	
		Experiment 1 (LSD = 4)			
H_2O only	119	*b*			
TritonX + H_2O	104				
citral		40	7	0	0
geranial		42	10	0	0
neral		43	4	0	50
		Experiment 2 (LSD = 9)			
H_2O only	131				
TritonX + H_2O	120				
citral		9	1	0	
R-citronellal		56	25	5	
S-citronellal		40	28	5	
R-citronellol		41	38	37	
S-citronellol		50	34	34	
R-citronellic acid		68	48	14	
S-citronellic acid		73	32	18	

[a] All data represent the mean of four replicates and LSD ($P < 0.001$) for each experiment. [b] Not part of the experimental design.

Effects on *P. digitatum*. No growth of *P. digitatum* was observed in the presence of 15 μL L^{-1} solutions of either citral or its isomers (**Table 1**). In the presence of 2 μL L^{-1} solutions of the compounds, the number of cfu of *P. digitatum* was reduced by 66, 65, and 64% for citral, geranial, and neral, respectively, as compared to the water control. The vapor of citral, geranial, and neral from 6 μL L^{-1} solutions reduced the number of cfu by 94, 92, and 97% as compared to the water control.

Vapors of all of the citral-related compounds tested reduced cfu in comparison with the water controls. Exposure to vapor of citronellic acid provided the least inhibition of growth of *P. digitatum* in the presence of 2 μL L^{-1}, 44−48% as compared to the water control (**Table 1**). In the presence of 6 μL L^{-1} solutions of citronellic acid, the *R* isomer was more inhibitory than the *S* isomer, but there was no difference between the isomers in the presence of 15 μL L^{-1} solutions. As compared to the water control, vapor of citronellal reduced cfu by 57−69, 79−81, and 96% in the presence of 2, 6, and 15 μL L^{-1} solutions, respectively. This effect was similar to that of citral. The *R* isomer of citronellal was more inhibitory than the *S* isomer only at 2 μL L^{-1}.

Spores of *P. digitatum* previously exposed to vapors of citral and geranial from 15 μL L^{-1} solutions showed no germination, either from the transferred plug or on the remainder of the original plate. This suggests that citral and geranial at this concentration were fungicidal to *P. digitatum*. However, some spores previously exposed to vapor of neral at this concentration germinated on the original plate (**Table 1**). Therefore, vapor of neral from 15 μL L^{-1} solutions can be classified as fungistatic for *P. digitatum*, even though it did appear to have killed most of the spores.

Effects on *P. italicum*. As for *P. digitatum*, total inhibition of *P. italicum* was observed in the presence of 15 μL L^{-1} solutions of citral, geranial, and neral (**Table 2**). Also, no growth was observed in the presence of geranial at any of the concentrations tested or for the 6 μL L^{-1} solution of citral. Vapor

Table 2. Effect of Citral Vapor, Its Isomers, and Related Compounds on the Growth of *P. italicum* at Different Concentrations, Expressed as cfu[a]

compds	cfu per plate above solutions of				growth over plate (%) after removing 15 μL L^{-1} solution
	control	2 μL L^{-1}	6 μL L^{-1}	15 μL L^{-1}	
	Experiment 1 (LSD = 4)				
H_2O only	102	*b*			
TritonX + H_2O	89				
citral		14	0	0	30
geranial		0	0	0	10
neral		13	7	0	100
	Experiment 2 (LSD = 12)				
H_2O only	109				
TritonX + H_2O	90				
citral		0	0	0	
R-citronellal		100	55	18	
S-citronellal		85	61	13	
R-citronellol		78	31	42	
S-citronellol		84	37	47	
R-citronellic acid		109	105	93	
S-citronellic acid		99	90	79	

[a] All data represent the mean of four replicates and LSD ($P < 0.001$) for each experiment. [b] Not part of the experimental design.

Table 3. Effect of Citral Vapor, Its Isomers, and Related Compounds on the Growth of *G. candidum* at Different Concentrations, Expressed as cfu[a]

compds	cfu per plate above solutions of				growth over plate (%) after removing 15 μL L^{-1} solution
	control	2 μL L^{-1}	6 μL L^{-1}	15 μL L^{-1}	
	Experiment 1 (LSD = 8)				
H_2O only	112	*b*			
TritonX + H_2O	80				
citral		4	2	0	0
geranial		6	4	0	0
neral		12	9	0	0
	Experiment 2 (LSD = 4)				
H_2O only	81				
TritonX + H_2O	60				
citral		3	1	0	
R-citronellal		38	2	0	
S-citronellal		38	5	0	
R-citronellol		45	42	27	
S-citronellol		49	40	27	
R-citronellic acid		68	51	35	
S-citronellic acid		61	53	34	

[a] All data represent the mean of four replicates and LSD ($P < 0.001$) for each experiment. [b] Not part of the experimental design.

of neral from 2 and 6 μL L^{-1} reduced the cfu of *P. italicum* by 87 and 93% as compared to the water control (**Table 2**). All treatments except for *R*-citronellal and citronellic acid (*R* and *S*) at 2 μL L^{-1} and *R*-citronellic acid at 6 μL L^{-1} reduced cfu as compared to the water control. The vapor of citronellal showed the greatest inhibitory effect of the citral-related compounds at 15 μL L^{-1}, where cfu were reduced by 84−88% as compared to the water control (**Table 2**). The vapor of citronellic acid was the least inhibitory, and the number of cfu as compared to the water control was reduced by 0−9, 4−17, and 15−28% in the presence of 2, 6, and 15 μL L^{-1} citronellic acid, respectively. At the higher concentrations tested, the two isomers of citronellic acid differed significantly, with the *S* isomer providing more inhibition.

No germination of *P. italicum* spores was observed on plugs previously exposed to vapor of citral, geranial, and neral from 15 μL L^{-1} solution. However, some spores on the remainder of the original plate exposed to 15 μL L^{-1} solutions of citral, geranial, or neral germinated (**Table 2**). This suggests that the citral, geranial, and neral vapors at this level were fungistatic for *P. italicum*.

Effects on *G. candidum*. Again, all treatments of citral, geranial, and neral reduced cfu as compared to the water control and total inhibition of growth was observed in the presence of 15 μL L^{-1} solutions (**Table 3**). Vapor of 15 μL L^{-1} solutions of citral and the two isomers of citronellal inhibited growth of *G. candidum* completely, but lower concentrations did not (**Table 3**). Of the citral-related compounds, the vapor of citronellal was most inhibitory to *G. candidum*, and there were no differences between the two isomers at any of the concentrations tested. Citronellol and citronellic acid reduced cfu as compared to the water control but did not inhibit *G. candidum*, even at the highest concentration.

No germination of *G. candidum* spores was observed from transferred plugs after removal of 15 μL L^{-1} solutions of citral, geranial, or neral nor on the remainder of the original plates (**Table 3**). This suggests that vapors of citral, geranial, and neral at this concentration were fungicidal toward *G. candidum*.

DISCUSSION

In our previous paper (*14*) on the exposure of spores of *P. digitatum*, *P. italicum*, and *G. candidum* to volatile citral, complete inhibition of growth in the presence of 6 and 15 μL L^{-1} citral solutions was noted. In the present study, the result for 15 μL L^{-1} citral solutions was confirmed; however, for 6 μL L^{-1} solutions, complete inhibition was achieved only for *P. italicum*, while *P. digitatum* and *G. candidum* were inhibited by 94 and 98%, respectively. Both studies used the same batch of citral, and the composition was identical; this difference may, therefore, be due to this concentration being borderline for complete control.

The inhibitory effect of volatile citral and its isomers at all concentrations tested was similar for *P. digitatum* and *G. candidum*, while for *P. italicum* neral had less of an effect than geranial and citral. At 15 μL L^{-1}, the effectiveness was similar against the three fungi; however, at 6 μL L^{-1}, the effectiveness of citral and its isomers was less for *P. digitatum* and *G. candidum* than for *P. italicum*. The effectiveness of citral against *G. candidum* is of interest, as guazatine is the only synthetic fungicide that is currently effective against sour rot of citrus and is not permitted in all countries around the world (*17*).

Among the citral-related compounds tested, the vapor of citronellal provided the most inhibition of the three fungi tested, but it was less effective than citral. This can be explained by considering the vapor pressure of the compounds, which was 3902, 169, 29, and 2 kPa for citral, citronellal, citronellol, and citronellic acid, respectively (*18*). The effectiveness was generally greater for compounds with higher vapor pressure, since a higher vapor pressure results in a higher headspace concentration. Wolken et al. (*19*) also found that citral was more toxic to *P. digitatum* than the related alcohols, citrol/geraniol, or geranic acid, and Skog et al. (*11*) found that citral had a similar activity to acetaldehyde. The effectiveness of *R* and *S* isomers of these compounds in some cases varied, but overall, no trend was observed.

The more inhibitory effect of citral against the fungi can also be explained by the fact that citral is a member of the α,β-

unsaturated aldehyde class, in which the carbonyl group is adjacent to α- and β-carbons. Because of their position, the α- and β-carbons are conjugated with the carbonyl group, making the β-carbon positively polarized and able to react easily with nucleophiles (nuclephilic attack) (*20*). According to Witz (*21*), the chemical nature of α,β-unsaturated aldehydes and some of their toxicological effects is based on their ability to function as direct alkylating agents. These alkylating agents are capable of covalent binding to cellular nucleophile groups, which means that they are capable of modifying cellular processes and are potentially toxic.

Additional work is needed to develop citral as a fumigation agent, particularly since concentrations that have been shown to be effective in dips can cause peel injury in the fruit (*10*).

In conclusion, the inhibitory effect of citral, its isomers, and its related compounds differed among the three fungi and the nature of its activity (fungistatic or fungicidal) may be concentration-dependent. Citral was the most effective and completely inhibited the growth of *G. candidum*, *P. digitatum*, and *P. italicum* at a concentration of 15 μL L^{-1} and, in some cases, at lower concentrations. Because citral is available commercially but the two isomers are not, using citral as a mixture to control spoilage fungi is the best option.

More work is needed to determine the most appropriate fumigation method and concentration for fruit in storage to control disease, such as continuous fumigation, which can be applied within packages, or short period fumigation, which can be applied in a chamber.

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