Quantification of the mortality of conidia of *Botrytis cinerea* and *Penicillium digitatum* in peracetic acid.

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Abstract

Peracetic acid (PAA) is common sanitizer, but information about its fungal toxicity is sparse. PAA toxicity was assessed to conidia of *Botrytis cinerea* and *Penicillium digitatum*, causes of gray mold and citrus green mold, respectively. They were immersed in PAA, entrapped on a glass fiber filter, rinsed with sterile H2O, and placed on potato dextrose agar to observe germination, then the time (LT_{95}) or concentration (LD_{95}) when 95% of the conidia were dead were estimated by probit analysis. Two 100 mg/l (= ppm) solutions were used, one from 4.9% PAA + 26.5% H_2O_2 and the other from 15.0% PAA + 22.0% H_2O_2. The H_2O_2 content in 100 mg/l PAA solutions prepared from these were 540 and 147 mg/l, respectively. Mortality was similar in both therefore due to PAA with little or no H_2O_2 contribution. ET_{95} of *B. cinerea* and *P. digitatum* conidia in 100 mg/l PAA was 5.4 and 2.4 minutes, respectively. LT_{95} of *B. cinerea* and *P. digitatum* conidia in 500 mg/l PAA was 17.2 and 9.8 seconds, respectively. LD_{95} of *B. cinerea* and *P. digitatum* conidia during a 24-hour PAA exposure was 24.7 and 3.3 mg/l, respectively. LD_{95} of *P. digitatum* conidia during 10 or 30 minute PAA exposure was 50 and 41 mg/l, respectively. Although PAA resistance was markedly higher in *B. cinerea* than *P. digitatum*, label rates of PAA are adequate to control conidia of both pathogens. PAA can be mixed with fungicides, label rates are relatively high (600 mg/l preharvest and 85-100 mg/l postharvest), it complies with USDA Organic Program rules, and its disinfection by-products (mostly acetic acid) are of minimal concern.

Populations of conidia of *P. digitatum* and of total natural yeast and mold on the surface of navel oranges were greatly reduced by the application of 500 mg/l PAA to run-off. Expressed as conidia per fruit, *P. digitatum* conidia populations were reduced by PAA application from 480,000 to 3,400 conidia per fruit, a reduction of 99.3%. Natural yeast and mold populations were reduced from 2,760,000 to 66,000 per fruit, a reduction of 97.6%. Expressed as colony-forming-units (CFU), *P. digitatum* conidia populations were reduced from $\log_{10}$ 5.68 CFU to $\log_{10}$ 3.53 CFU, a reduction of $\log_{10}$ 2.15 CFU. Natural yeast and mold populations were reduced from $\log_{10}$ 6.40 CFU to $\log_{10}$ 4.82 CFU, a reduction of $\log_{10}$ 1.61 CFU.

Objectives were to determine:

1) the toxicity of two PAA formulations to conidia of *Pencillium digitatum*, cause of postharvest green mold of citrus, and those of *Botrytis cinerea*, cause of gray mold on many fruits and vegetables both before and after harvest;

2) the contribution of hydrogen peroxide in the formulations to the rate of conidial mortality. One of the PAA formulations contained 4.9% hydrogen peroxide while the other contained 26.5% hydrogen peroxide;

3) the influence of application of 500 mg/l PAA on populations of conidia of *Penicillium digitatum* and of total natural yeast and mold on the surface of navel oranges.

Introduction

Peracetic acid (PAA) is common sanitizer available from a number of commercial sources. Approved for use by "organic" producers, investigation of PAA for several applications is particularly appropriate at this time. Its primary postharvest use is as a product and equipment sanitizer, with occasional use before harvest. Its toxicity to microbes of food safety concern has been investigated, but specific information about its toxicity to some important fungal pathogens is sparse but reports indicate it is promising. For stone fruit, Mari and coworkers (2004 Postharvest Biol. Technol. 33:319-325) reported excellent results using immersion in 125 mg/l PAA to control postharvest decay caused by *Rhizopus stolonifer* and *Monilinia laxa*. For the citrus industry, sanitation of the the process water used during packinghouse handling is an element in postharvest decay and food safety risk management programs. Fruit are often treated with re-circulated aqueous drenches or passed through tanks containing fungicide solutions. Populations of microbes of food safety concern and spores of pathogens, particularly when fungicide resistant isolates accumulate in them, must be controlled. Hypochlorite is effective in this role, but "organic" rules limit rates that can be used and it rapidly degrades the popular fungicide imazalil and several other fungicides, while PAA does not, so PAA is indicated in this role (2014 Taverner, P. Peracetic Acid. Citrus Postharvest Info Note SARDI 4 pp).

PAA is a mixture of peroxyacetic acid and hydrogen peroxide (H_2O_2), which is added to stabilize the peroxyacetic acid, and acetic acid. A common question concerning PAA use is the contribution of H_2O_2 to PAA solution toxicity. Leggett et al. (2016 Appl Environ. Microbiol. 82: 1035-1040) reported the interaction between PAA and H_2O_2 to inactivate *Bacillus subtilis* to be synergistic, with the sporocidal activity of the combination being largely due to PAA, not H_2O_2. In our work, we compared to PAA formulations with differing H_2O_2 contents on the rate of conidial mortality.

Fungi. *Botrytis cinerea*, isolated from an infected grape, was cultured in petri dishes containing potato dextrose agar (PDA; Cole-Parmer), *Penicillium digitatum*, isolated from a green mold infected orange, was used to infect a navel orange. After two weeks, a lesion with abundant sporulation developed. A sterile stainless steel spatula was used to collect conidia from the resulting lesion or PDA surface and these were placed in sterile water containing 0.01% Triton X-100 (Talas, Inc., 330 Morgan Avenue, Brooklyn, NY) and passed through a single layer of cotton gauze, then adjusted to 4,500,000 conidia/ml in a final volume of 100 ml using a hemacytometer (Reichert Bright-Line). The surfactant was added because the conidia were hydrophobic.

Sporocide assay. For each formulation, the conidial solution (200 ml, 500,000 conidia /ml) and PAA solution (200 ml) were mixed together (T = 0; 23.0°C) and contained 100 ppm PAA and 250,000 conidial/ml. At intervals, a portion of the mixed solution conidia was passed through glass fiber filter (1.5 µm pore size, 47 mm dia.) in a vacuum filter apparatus (Millipore XX10) to entrap them, they immediately rinsed three times in 50 ml volumes of sterile water, and the filter was removed and placed in a sterile disposable polycarbonate test tube. Conidia were filtered from the solution at intervals of 0, 1, 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 minutes. The solution was stirred frequently. One ml of sterile water was added to each filter-containing tube, the filter was crushed with a disposable Pasteur pipet, and 0.1 ml was withdrawn and placed on a petri dish (5-cm dia.) containing PDA. After 15 hours at 23°C, the percentage of germinated conidia was determined by examining the surface of each plate with a compound microscope (Olympus CH-2) at 400x.

Peracetic acid formulations. Jet-Ag from Jet Harvest Solutions (batch number 8255092201, production date 09/22/2015, Longwood, FL 32791) was used. The undiluted Jet-Ag formulation contained 4.9% PAA + 26.5% H_2O_2. The solution applied to fruit contained 500 ppm PAA. The 500 ppm PAA solution was prepared as follows: dispense 10.2 ml of the Jet-Ag formulation to a final volume of 1 L, final contents: 500 ppm PAA + 2,705 ppm H_2O_2.

PAA application before harvest. Water alone or the conidial suspension containing 4,500,000 conidia/ml were applied to the fruit to run-off before harvest with a handheld pressure sprayer (Delta Sprayer, Delta Industries, King of Prussia, PA) on March 18, 2016. Two days later, on March 20, 2016, 500 ppm PAA or water were applied to run-off using the same sprayer, after it was disinfected with chlorine bleach. Both water and PAA solutions contained 0.01% Triton X-100 surfactant, a common agricultural surfactant. Approximately one ml of liquid was retained per fruit. The fruit were mature 'Atwood' navel oranges, approximately 65-70 mm in diameter and 150 grams in weight. Twelve fruit were labeled and half (6) were sprayed with PAA and the other half (6) were sprayed with water. After one additional day (March 21, 2016) the fruit were harvested, and the microbes were extracted and their populations determined on a selective medium.

Conidia and yeast and mold population extraction. Five PAA-treated and five water-treated fruit were harvested March 21, 2016 by clipping the fruit with a alcohol sterilized clippers to drop them directly into 1-gallon capacity sterile zip bags. To each bag containing five oranges, 500 ml of sterile water containing 0.01% Triton X-100 was added and the bag sealed. The bags were moved rapidly from side to side with a displacement of 20 cm ten times initially, then at intervals of 10 minutes for the next thirty minutes. A corner of each bag was cut open with an alcohol-sterilized scissors, and an aliquot of 1 ml was removed and used in a serial dilution series with 10-fold dilution steps in sterile water.

Conidia and yeast and mold population determinations. The medium consisted of a mixture of Potato Dextrose Agar (PDA; Alpha Biosciences, Inc. Baltimore, MD) and Dichloran Rose-Bengal Chloramphenicol Agar (DRBC; EM Science, Gibbstown, NJ) in a ratio (by weight) of 3 parts PDA to 1 part DRBC. This medium was used to minimize interference from the growth of bacteria on the medium surface, and to restrict colony size of yeasts and molds to facilitate their enumeration. DRBC is the medium indicated by the Food and Drug Administration protocol "spread plate" method of the Bacteriological Analytical Manual for quantification of yeasts and molds (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071435.htm). Preliminary tests conducted indicated the plating efficiency of DRBC was poor for *Pencillium digitatum* unless the medium was used in mixture with PDA.

An aliquot of 0.25 ml from the extraction solution within the zip bag and the first two tubes of the 10-fold dilution series was placed and spread with a sterile glass rod on a selective medium. Duplicate plates were prepared. The plates surface dried in air and the lids placed on the dishes and the number of colonies examined after 3 days in darkness at 22°C. *P. digitatum* appeared cream-colored when viewed from the bottom of the petri plates, white initially when viewed from the top of the plates until the colonies sporulated producing a characteristic pale green color.

Expression of population sizes of *P. digitatum* conidia and of natural yeasts and mold populations per fruit were calculated as follows: where A = colonies per plate, B = 4, 1 ml/0.25 ml the volume applied to each plate adjusted to 1 ml, C = reciprocal of dilution of the extract plated; the number would be 1, 10, or 100, for the undiluted, 10-fold, or 100-fold diluted extract; D = 500, the volume of extract used for the five fruit; and F = the number of fruit within the extract solution. The limit of detection (LOD) is 2000 CFU per fruit ($\log_{10}$ 3.30) defined as the appearance of 5 colonies on plates prepared with the undiluted extract.

Botrytis cinerea — 100 mg/l PAA + 540 mg/l H_2O_2 — 100 mg/l PAA + 147 mg/l H_2O_2

Exposure time (min)	Jet-Ag (4.9% ai) Germinated	Total exam	% Germinated	Jet-Ag 15 (15% ai) Germinated	Total exam	% Germinated
0	50	50	100.0	50	50	100.0
1	89	116	76.7	160	200	80.0
2.5	84	337	24.9	49	192	25.5
5	24	361	6.6	48	836	5.7
7.5	6	154	3.9	14	695	2.0
10	1	150	0.7	6	235	2.6
12.5	1	325	0.3	7	425	1.6
15	0	500	0.0	0	500	0.0

Penicllium digitatum — 100 mg/l PAA + 540 mg/l H_2O_2 — 100 mg/l PAA + 147 mg/l H_2O_2

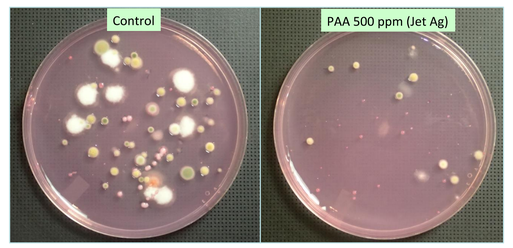
Exposure time (min)	Jet-Ag (4.9% ai) Germinated	Total exam	% Germinated	Jet-Ag 15 (15% ai) Germinated	Total exam	% Germinated
0	340	347	98.0	340	347	98.0
1	85	241	35.3	66	193	34.2
2.5	9	534	1.7	56	568	9.9
5	0	500	0.0	9	515	1.7
7.5	0	500	0.0	0	500	0.0
10	0	500	0.0	0	500	0.0
12.5	0	500	0.0	0	500	0.0

Figure 1. The number of germinated conidia, total number examined, and percentage of germinated conidia after exposure to 100 mg/l (ppm) peracetic acid (PAA) with either a higher concentration of H_2O_2 (540 mg/l "Jet-Ag") or a lower concentration of H_2O_2 (147 mg/l "Jet-Ag 15") in the 100 mg/l PAA solution.

PAA concentration throughout 24 hr	*Penicillium digitatum* Germinated	Total exam	% Germinated	*Botrytis cinerea* Germinated	Total exam	% Germinated
0	26	105	24.8	78	88	88.6
5	3	314	1.0	70	92	76.1
10	1	500	0.2	109	146	74.7
15	0	500	0.0	95	110	86.4
20	0	500	0.0	68	121	56.2
30	0	500	0.0	1	500	0.2
40	0	500	0.0	0	500	0.0
50	0	500	0.0	0	500	0.0

Table 1. Mortality of *P. digitatum* and *B. cinerea* conidia after exposure for 24 hours in various concentration of PAA. The LD95 of the conidia was 3.3 ppm and 24.7 ppm, respectively, for *P. digitatum* and *B. cinerea*.

Effect of PAA on *Penicillium digitatum* and natural yeast & mold populations



	Control cfu/fruit	PAA-treated cfu/fruit	Reduction by PAA Log_{10}	Percentage
P. digitatum	480,000 cfu ($\log_{10}$ 5.68)	3,400 cfu ($\log_{10}$ 3.53)	2.15	99.3%
Yeast & molds	2,760,000 cfu ($\log_{10}$ 6.40)	66,000 cfu ($\log_{10}$ 4.82)	1.61	97.6%

LOD = 3.30 $\log_{10}$

Results & Discussion

- PAA resistance was about 2-3 fold higher in *B. cinerea* than *P. digitatum* in brief exposures (minutes), *B. cinerea* was much more resistant in longer exposures (hours).
- Although PAA resistance was higher in *B. cinerea* than *P. digitatum*, label rates of PAA are adequate to control conidia of both pathogens.
- The rate of mortality of conidia of both fungi in PAA formulations with either 4.9% or 26.5% hydrogen peroxide was similar, indicating the contribution of hydrogen peroxide to conidial mortality was minimal.
- Application of PAA to navel oranges before harvest reduced *P. digitatum* and natural yeast and mold populations by about 99% or 2 $\log_{10}$ units.
- For decay management in fresh products, PAA at label rates has adequate potency to control conidia of the green mold and gray mold pathogens. If used at registered rates it warrants thorough evaluation for preharvest and postharvest use. A suggested application is to apply PAA just before harvest, so microbe populations and pathogen propagules would be greatly reduced and their would be insufficient time for them to re-populate the surface of the product.