Methodology for Determining Relationships Between Inoculum Concentration of *Botrytis cinerea* and *Penicillium expansum* and Stem End Decay of Pear Fruit

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ABSTRACT

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The objective of this research was to determine quantitative relationships between incidence of stem end decay of pear fruit and inoculum concentration of *Botrytis cinerea* and *Penicillium expansum* using dry conidia applied to pear fruit in a settling tower. Five concentrations of conidia were applied to pear fruit, fruit were stored at -1°C for 8 months, and stem end decay was evaluated. In addition, conidia were washed from the surface of inoculated fruit, and DNA was extracted and quantified with real-time polymerase chain reaction (PCR). The linear regression relationships between percent stem end gray mold and *B. cinerea* conidia per liter of air or per square centimeter of fruit surface were significant ($P = 0.01$). At the highest inoculum dose introduced into the settling tower, conidia per liter of air, conidia per square centimeter, and percent stem end gray mold at 8 months after inoculation were 12, 31, and 39, respectively for 2000 and 6, 33, and 67, respectively for 2001. Similarly, the linear regression relationships between percent stem end blue mold and *P. expansum* conidia per liter of air or per square centimeter of fruit surface were significant ($P = 0.01$ and 0.05 , respectively). At the highest inoculum dose introduced into the settling tower, conidia per square centimeter and percent stem end blue mold at 8 months after inoculation were 39 and 26, respectively for 2000 and 66 and 23, respectively for 2003. Real-time PCR provided a rapid, quantitative measure of *B. cinerea* and *P. expansum* DNA on pear fruit surfaces. Because of possible year-to-year shifts in susceptibility of fruit to decay, disease incidence:inoculum dose relationships may be of most value compared within years rather than across years. This would facilitate comparison of decay risk among orchards in order to determine which fruit is most suitable for long-term storage.

The two most important postharvest storage decays of apple and pear fruit are gray mold and blue mold caused by *Botrytis cinerea* Pers. and *Penicillium expansum* Link, respectively (10,14,16). Conidia of these fungi can become airborne and contaminate the surfaces of fruit and bins used for storage of fruit during harvest (11,16,23). Infection of fruit occurs when spores enter wounds or infect through the abscission zone of the pedicel (stem) (3,14,20,21,24,25). This may occur during harvest or later, as fruit float through contaminated dump tank or flume water in the packinghouse (9,22). Stem tissue of d'Anjou pear is susceptible to infection at harvest and remains sus-

ceptible for several months (9,21). A survey of culled pear fruit from packinghouses in the Mid-Columbia region of Oregon and Washington for the period 1994 to 1996 showed that *B. cinerea* is the major pathogen involved in postharvest decay of d'Anjou pear fruit, and that stem-end infections caused significantly higher levels of decay than calyx-end infections (8). The relationships have been determined for inoculum dose of conidia of *B. cinerea* and *P. expansum* in water suspensions (21) and incidence of pear fruit decay originating at wound sites and stem ends. Similarly, relationships also have been determined for conidia in air and decay at wound sites (24) but not for infections originating at the stem end of the fruit.

Recently, real-time polymerase chain reaction (qPCR) protocols have been used to quantify inoculum dose of plant pathogens. Species-specific qPCR primers have been developed for *B. cinerea* (26) and *P. expansum* (6). Airborne spores of several fungi have been quantified with qPCR (12,17). Uses of real-time PCR in epidemiological applications include prediction of storability of potato tubers (1), management decisions for neck rot of onion

(4), and inoculum dispersal dynamics of brown rot of stone fruit (12).

The objective of this research was to determine quantitative relationships between incidence of stem end gray mold and stem end blue mold of pear fruit and inoculum dose using dry conidia applied in a settling tower. In addition, inoculum dose of *B. cinerea* and *P. expansum* applied to fruit was quantified with qPCR.

MATERIALS AND METHODS

Fruit. d'Anjou pear fruit were harvested at commercial maturity with flesh firmness of 67 newtons at the Mid-Columbia Agricultural Research and Extension Center (MCAREC), Hood River, OR. Fruit were stored in air at -1.1°C in cardboard fruit boxes lined with perforated polyethylene bags and used within 3 months of harvest. Fruit surfaces were disinfested with 100 μg of sodium hypochlorite per milliliter of water for 2 min and rinsed with tap water before inoculation.

Inoculum. *B. cinerea* isolate 62, originally obtained from an infected d'Anjou pear fruit in 1982 but inoculated and reisolated annually from pear fruit, was cultured on potato dextrose agar acidified (Difco Laboratories, Detroit) with 1.5 ml of 85% lactic acid per liter (APDA) to prevent bacterial contamination. Conidia were harvested dry from 14-day-old cultures with a cyclone surface sampler (Burkard Manufacturing Co. Ltd., Rickmansworth, Hertfordshire, England). Dry conidia were diluted 1:99 with talc (T-2015; Sigma-Aldrich, St. Louis) that had been sterilized at 140°C for 4 h. The conidia and talc were mixed together for 5 h at 15°C in a twin shell blender (model SB-8; Patterson-Kelly Co., E. Stroudsburg, PA). The conidia/talc mix was stored at 3°C and used as inoculum over a 6-week period. Germination of conidia in the mix was determined prior to each experiment by mixing with sterile distilled water, placing drops on APDA, and observing germination of 100 conidia microscopically at $\times 250$ after 24 h at 20°C .

P. expansum isolate 46, originally obtained from an infected d'Anjou pear fruit in 1980 but inoculated and reisolated annually from pear fruit, was cultured on APDA and spores harvested from 7-day-old cultures as described above. Conidia

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were mixed 1:99 with talc and stored until use as described above.

Inoculation. Pear fruit were inoculated in an aluminum tower 2.06 m high by 0.68 m wide by 0.68 m deep. Prior to each experiment, the room floor was sprayed with 5.25 g of sodium hypochlorite per liter of water, and the room air was sprayed with Lysol (0.1% alkyl dimethyl benzyl ammonium saccharinate plus 79% ethanol; L & F Products, Montvale, NJ). The inside of the tower was sterilized with 95% ethanol. After each inoculation, air was exhausted from the tower for 2 min with a blower vented

outside the building, resulting in 8.8 complete air changes (8.38 m³).

Fruit with stems pointing straight up were placed in two cardboard trays, 22 fruit per tray, in the bottom of the tower. Inoculation temperature was 15 ± 3°C. Conidia/talc mixtures ranging from 1.0 to 5.0 mg were used to obtain inoculum ranging from 0.01 to 0.05 mg of conidia. Each mixture was weighed, transferred to a sterile glass tube of 6-mm internal diameter, and forcefully blown into the top center of the tower with 12 squeezes of an aspirator bulb. Spores were allowed to settle for 20 min, and then fruit were re-

moved. Control fruit were placed in the tower for 20 min but no inoculum was introduced. Each inoculum concentration of *B. cinerea* was replicated five times in 2000 and 2001. Each concentration of *P. expansum* was replicated five times in 2000 and four times in 2003. All replications each year were done within 6 weeks. Fruit were placed in a cardboard fruit box lined with a perforated polyethylene bag. After 3, 6, and 8 months of incubation at -1°C, infection in the flesh of the fruit that originated at the stem end was determined visually. Decayed fruit were removed after each evaluation to prevent secondary spread. Numbers of decayed fruit from all evaluations were totaled and incidence of stem end decay was calculated.

Density of conidia of *B. cinerea* settling onto surfaces was determined by placing three 2.54-by-7.62-cm glass microscope slides coated with a thin layer of silicone grease at different locations on the trays. At the end of each inoculation, slides were removed and stained with cotton blue in lactophenol, and conidia in 40 microscope fields counted at ×250. Density of spores of *P. expansum* on the surface was determined by placing three 100-by-15-mm petri dishes containing APDA on the trays. After exposure, plates were incubated at 22°C for 24 h and colonies were counted with the aid of a stereomicroscope at ×15.

Concentration of conidia of *B. cinerea* and *P. expansum* in the air inside the tower was determined by sampling with a portable air sampler for agar plates (Burkard Manufacturing Co. Ltd.). The manufacturer rates impaction efficiency of this sampler at 100% for 5-μm particles at the 20-liters/min sampling rate used herein. Air was sampled through a port on the side of the tower 15 cm above the bottom. Air was sampled onto the same APDA plate for 15 s at 5, 10, 15, and 19 min after inoculation for a total sample volume of 20 liters. Plates were incubated at 22°C and colonies counted after 1 to 2 days.

Fruit washing and DNA isolation. Inoculated fruit were weighed and washed on a rotary shaker followed by sonication, and surface area was calculated as previously described (11). Spores in wash water were concentrated by successive centrifugation starting with 200 ml of original wash water that was concentrated to 10 ml and finally to 100 μl at 12,000 rpm for 10 min, 7,000 rpm for 10 min, and 6,000 rpm for 1 min, respectively. Genomic DNA was isolated using the UltraClean Soil DNA Kit (MoBio, Solana Beach, CA) following the manufacturer's instructions.

Oligonucleotide primer selection. The software package OLIGO 6.3 (MBI, Cascade, CO) was used to design primers used in this study. Both PCR primers for *P. expansum* (Pex-bt-37f 5' TTG AGC CTT ACA ACG CCA CT 3' and Pex-bt-384r 5' TTG ACC TGG CGG TAA GAG GA 3') were selected from β-tubulin sequence

Fig. 1. Relationship between incidence of stem end gray mold of d'Anjou pear fruit and inoculum dose of *Botrytis cinerea* expressed as **A**, number of conidia per liter of air and **B**, conidia per square centimeter of surface. Inoculations were done in 2000 (closed circles) and 2001 (open circles) and decay evaluated after 3, 6, and 8 months of incubation at -1°C.

alignments from another study (18). These *Penicillium* primers produced a PCR product of approximately 350 bp. Design of the *Botrytis* primer Bcin-366r (5'TGA GTC AAC TCT GGA ACG 3') used β -tubulin *B. cinerea* sequences available in GenBank (U27198, X73133, and Z69263). The primer was paired with a more generic primer BT-2M-Up (5'CAA CTG GGC TAA GGG TCA TT 3') (5) to produce a 568-bp product. Primer sequences were screened by performing Blast searches for homology with fungal sequences in the GenBank database. Additionally, to determine if qPCR melting curve analysis could be used as a discriminating tool to differentiate species sharing highly similar sequence homologies, melting temperatures were calculated for any potential amplicon sequences found in GenBank.

Real-time PCR assay. Samples (20 μ l each) were prepared by mixing 1 μ l of DNA solution with 6.8 μ l of sterile distilled water, 10 μ l of DyNAmo HS SYBR Green qPCR Kit (Finnzymes, Espoo, Finland), 1 μ l of each primer mentioned previously (final concentration 200nM), and 0.2 μ l of UltraTherm DNA polymerase (Boca Scientific, Boca Raton, FL). Real-time PCR reactions were run in triplicate on a DNA Engine Opticon 2 System (MJ Research, Inc., Waltham, MA). After a 10-min denaturation step at 95°C, samples were run for 40 cycles of 10 s at 94°C, 20 s at 55.8°C for *B. cinerea* and 20 s at 58°C for *P. expansum*, and 15 s at 72°C for both fungi. A final extension was done at 72°C for 10 min. Melting curves were measured starting at 75°C and increasing by 0.2°C every 1 s until 90°C was reached. Positive controls (standards) consisted of DNA extracted from Bot62 and Pen46 and quantified using a spectrophotometer, reading transmittance at 260 and 280 nm. Five serial 10-fold dilutions of these standards were run in triplicate in the same reaction as the samples. Three negative controls, consisting of 1 μ l of sterile distilled water added to the qPCR mix in place of the DNA solution, were included per run.

Statistics. The relationships between decay incidence and inoculum dose, expressed as both conidia per square centimeter of surface and conidia per liter of air, were plotted and subjected to linear regression analysis. The analysis of variance procedure (Minitab, State College, PA) was used to determine significance among regressions. Linear regression also was used to describe the relationships between DNA on the fruit surface and the quantity of conidia applied as well as conidia per square centimeter of surface (trial 2 only).

RESULTS

Germination of conidia of *B. cinerea* was between 93 and 100% for all experiments. The linear regression relationships between percent stem end gray mold and

B. cinerea conidia per liter of air or per square centimeter of surface were significant at $P = 0.01$, except for the regression in year 2000 of decay versus conidia per liter of air, which was significant at $P = 0.05$ (Fig. 1A and B). At the highest inoculum dose introduced into the settling tower, conidia per liter of air, conidia per square centimeter, and percent stem end gray mold at 8 months after inoculation were 12, 31, and 39, respectively, for 2000 and 6, 33, and 67, respectively, for 2001. For both decay regressed on conidia per liter and per square centimeter, the slopes of the regression lines were significantly different for year 2000 versus 2001.

Germination of conidia of *P. expansum* in the talc powder was 96 to 100% during the 4 weeks it took to complete the inoculation experiments. The regression of stem end blue mold on conidia per liter of air was significant at $P = 0.01$ (Fig. 2A). There was no difference in slope or Y intercept of regression lines of stem end blue mold versus conidia per square centimeter for 2000 and 2003, and data for the 2 years were combined. The resulting regression was significant at $P = 0.05$ (Fig. 2B). At the highest inoculum dose introduced into the settling tower, conidia per square centimeter and percent stem end blue mold at 8 months after inoculation were 39 and 26,

Fig. 2. Relationship between incidence of stem end blue mold of d'Anjou pear fruit and inoculum dose of *Penicillium expansum* expressed as **A**, number of conidia per liter of air (2003 only) and **B**, conidia per square centimeter of surface (data from 2000 and 2003 combined). Decay was evaluated after 3, 6, and 8 months of incubation at -1°C.

Table 1. Quantification of fungal DNA on the surface of d'Anjou pear fruit inoculated with dry conidia of *Botrytis cinerea* and *Penicillium expansum* using a real-time polymerase chain reaction assay^a

Conidia applied (mg) ^b	<i>B. cinerea</i>			<i>P. expansum</i>		
	Trial 1	Trial 2		Trial 1	Trial 2	
	DNA (pg/cm ²) ^c	DNA (pg/cm ²) ^d	Conidia/cm ^{2e}	DNA (pg/cm ²) ^f	DNA (pg/cm ²) ^g	Conidia/cm ^{2h}
0.00	0.10	0.09	0.7	0.08	0.07	0.0
0.01	0.47	0.72	3.7	0.25	0.07	5.2
0.02	0.64	1.40	6.1	0.24	0.08	13.2
0.03	1.17	1.57	12.3	0.35	0.17	23.8
0.04	1.35	1.74	16.4	0.36	0.24	26.3
0.05	2.44	2.94	28.0	0.39	0.28	32.9

^a Mean of three replications of five fruit per replicate per trial.

^b Conidia mixed 1:99 in talc and applied to fruit in settling tower.

^c Regression equation is $y = 42.5x - 0.034$ where y = picograms of DNA/cm² and x = milligrams of conidia applied, $P = 0.003$, $R^2 = 0.916$.

^d Regression equation is $y = 50.0x + 0.162$ where y = picograms of DNA/cm² and x = milligrams of conidia applied, $P = 0.002$, $R^2 = 0.930$.

^e Regression equation is $y = 0.093x + 0.371$ where y = picograms of DNA/cm² and x = conidia/cm², $P = 0.002$, $R^2 = 0.923$.

^f Regression equation is $y = 5.78x + 0.135$ where y = picograms of DNA/cm² and x = milligrams of conidia applied, $P = 0.007$, $R^2 = 0.869$.

^g Regression equation is $y = 4.55x + 0.039$ where y = picograms of DNA/cm² and x = milligrams of conidia applied, $P = 0.004$, $R^2 = 0.893$.

^h Regression equation is $y = 0.007x + 0.037$ where y = picograms of DNA/cm² and x = conidia/cm², $P = 0.005$, $R^2 = 0.882$.

respectively for 2000 and 66 and 23, respectively for 2003. Conidia per liter of air was 215 in 2003 but was not determined in 2000.

Initial Blast searches of the GenBank database with Bcin-366r/ BT-2M-Up sequences showed the primer pair to be specific for *B. cinerea*; however, later conformational searches revealed potential cross-reactions with several other species (*B. fabae*, *Monilinia fruticola*, *M. laxa*, and *Sclerotinia sclerotiorum*). Blast search using Pex-bt-37f /Pex-bt-384r sequences indicated the primer pair to be specific for *P. expansum*.

The qPCR melting curve analysis for *P. expansum* control and sample DNA (using Pex-bt-37f/Pex-bt-384r) produced an amplicon with a melting point (T_m) of 86.0°C, and amplification products from control and sample DNA of *B. cinerea* (using Bcin-366r/ BT-2M-Up) gave a T_m value of 82.8°C. Melting curve analysis was conducted using 0.2°C incremental steps. Calculated T_m values for closely related β-tubulin sequences from GenBank (*P. commune*, *P. solitum*, and *P. auran-tiogriseum*) differed from *P. expansum* by 0.4 to 1.1°C and sequences of *M. fruticola*, *M. laxa*, and *S. sclerotiorum* from *B. cinerea* by 0.3 to 0.5°C.

Quantities of DNA of *B. cinerea* and *P. expansum* on the surfaces of inoculated pear fruit were determined with qPCR (Table 1). The relationships between fungal DNA and inoculum dose were highly significant ($P \leq 0.01$), as were relationships between fungal DNA and conidial surface density. Quantities of DNA of *B. cinerea* on the fruit surfaces exceeded those of *P. expansum* at all inoculum concentrations (Table 1).

DISCUSSION

In a previous study, the relationships between disease incidence at puncture-wound sites on pear fruit and inoculum dose of dry airborne conidia of *B. cinerea* and *P. expansum* were determined (24).

The relationships described herein are extensions of that study to the infection court at the abscission zone of the stem. Interestingly, similar concentrations of dry inoculum caused similar incidences of infection in both studies. For example, 200 conidia/liter of air of *P. expansum* caused infection at 29% of punctures (24) and at 21% of stems herein.

Pear decay originating at stem ends is of commercial significance (10), and a better understanding of the disease incidence:inoculum dose (DI:ID) relationship is an important component of an integrated control strategy. Integrated decay control systems involving nutrition, harvest maturity, biocontrol, fungicides, and sanitation have been developed (27,28,30). Several of these components, particularly removal of fallen fruit, fungicides, and sanitation, lower the inoculum dose in the orchard. The density of fungal propagules on the fruit surface at harvest appears to be a good predictor of the incidence of postharvest decay (9). Furthermore, biocontrol agents are more effective in low-inoculum situations (7,15).

The fungicide ziram often is applied 2 to 3 weeks prior to harvest to pome fruit trees in the Pacific Northwest (30) and reduces total decay by about 25%. Because we did not include fruit treated with ziram in our study, the effect of ziram or other preharvest fungicides on the DI:ID relationships requires further research. A second factor that requires additional study is host susceptibility. We found that the relationship between stem end gray mold and inoculum dose was significantly different for 2000 versus 2001. Year-to-year shifts in susceptibility of fruit to decay may be related to seasonal temperature and moisture fluctuations, resulting in altered levels of polygalacturonase inhibitors, phenolics, and phytoalexins as well as physical changes such as cuticle thickness (2,13,19,20,29). Thus, the DI:ID relationship may differ for each cropping season and be of most value within years rather than across years. This

would facilitate comparison of decay risk among orchards in order to determine which fruit is most suitable for long-term storage.

Research is in progress to predict the amount of gray mold and blue mold decay developing in long-term cold storage based on populations of *B. cinerea* and *P. expansum* on the fruit surface as determined by standard dilution plating and DNA quantification with qPCR. We demonstrated herein that inoculum on the fruit surface can be rapidly and accurately determined with qPCR and that picograms of DNA per square centimeter of fruit surface is a good measure of the surface density of conidia. Previously, qPCR has been used to quantify airborne spores of *M. fruticola* (12) and *Fusarium circinatum* (17). Extensive orchard sampling will be necessary to obtain a large data base of naturally occurring DNA concentrations of *B. cinerea* and *P. expansum* on pear fruit surfaces at harvest before thresholds can be determined for use in a decay risk prediction model.

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