Involvement of Gluconic Acid and Glucose Oxidase in the Pathogenicity of *Penicillium expansum* in Apples

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ABSTRACT

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The contribution of gluconic acid secretion to the colonization of apple tissue by *Penicillium expansum* was analyzed by modulation (increase or decrease) of gluconic acid accumulation at the infection court. *P. expansum* isolates that express the most *gox2* transcripts and concomitant glucose oxidase (GOX) activity and that secrete the most gluconic acid cause disease of apple at the fastest rate. Cultures grown under reduced oxygen concentration generated fewer *gox2*

transcripts, produced less gluconic acid, and led to a 15% reduction in disease. Furthermore, the detection of significantly high levels of transcripts of *gox2* and GOX activity at the edge of the decaying tissue emphasize the involvement of GOX in tissue acidification of the decaying tissue. Taken together, these results emphasize the importance of GOX in the production of the gluconic acid that leads, in turn, to host tissue acidification. This acidification enhanced the expression of pectolytic enzymes and the establishment of conditions for necrotrophic development of *P. expansum*.

There is a growing body of evidence indicating that filamentous fungi alter their environmental pH during pathogenicity as a means for modulation of fungal attack in several hosts (14,19). Since pH is a critical factor in its attack strategy, the pathogen tailors ambient conditions to fit its offensive arsenal. For instance, certain fungi, such as *Colletotrichum gloeosporioides* (17) and *Alternaria alternata* (6), alkalize their host tissues by producing significant amounts of ammonia. Other fungi, such as *Sclerotinia sclerotiorum* (14) and *Botrytis cinerea* (27), acidify their host tissues by producing oxalic acid, while *Penicillium expansum*, *P. digitatum*, and *P. italicum* secrete citric and gluconic acids (18).

Previous work of Prusky et al. (18) suggested that production of gluconic and citric acids by *P. expansum* acidifies the infection court, which acts in turn to activate fungal transcription of polygalacturonases leading to an increase in colonization. It was also suggested that acid conditions naturally found in more acid apples or artificially by exogenous treatment with organic acids, enhanced fungal colonization. The objectives in this work were to demonstrate whether the accumulation of organic acid plays a significant role in the pathogenicity of *P. expansum* on apple fruit.

Glucose oxidase (GOX) catalyses the oxidation of β -D-glucose to H_2O_2 and D-glucono-1,5-lactone, which hydrolyses spontaneously to gluconic acid (1). We demonstrate that gluconic acid produced by *P. expansum* strongly contributes to host acidification and enhanced fungal colonization of apple tissue. We have analyzed the relationship between gluconic acid accumulation and the colonization of apples infected by *P. expansum* by either (i) selection of natural isolates with increased GOX activity and gluconic acid accumulation; (ii) down

regulation of GOX resulting in inhibition of the secretion of gluconic acid; and (iii) localizing the capability to secrete gluconic acid and H_2O_2 at the leading edge of the hyphae. Our results support our hypothesis that gluconic acid accumulation by the colonizing pathogen is an important factor for enhanced pathogenicity of *P. expansum* in apple fruit.

MATERIALS AND METHODS

Apple fruits, fungal isolates, growth media, and fruit inoculation. Mature apple fruit cv. Golden Delicious were obtained from local suppliers on the day of the experiment. Single spores of each of three different isolates of *P. expansum* were obtained from decayed apple fruit. Isolates Pe-21 and Pe-16 were obtained from decayed local cvs. Grand Alexander and Golden Delicious apples, respectively, and isolate Pe-23 was obtained from a decayed Golden Delicious apple at Beltsville Agricultural Research Center (BARC), Agricultural Research Service-United States Department of Agriculture (ARS-USDA), Beltsville, MD (29). If not specified otherwise, Pe-21 was used in the various experiments. One-week-old conidia, harvested from potato dextrose agar (PDA) plates (Difco, Detroit, MI) were used for mycelial growth and fruit inoculation assays. The fruit were inoculated by placing 10 μ l of a conidial suspension containing 2×10^6 spores/ml on each of four 2-mm-deep, 2-mm-diameter wounds spaced around a circle on the upper part of the stem side of the fruit. Following inoculation, the fruit were incubated at 25°C in covered plastic containers containing wet paper towels, until decay symptoms developed. In some experiments, the wound sites were treated every 24 h with 50 μ l of 0.25 M citric or gluconic acid. Double-distilled water was used as a control. To analyze the effects of O_2 concentrations on decay development and gluconic acid production, infected fruit were incubated for 24 h at 22 to 23°C and 90% relative humidity and then transferred to

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30-liter containers for 96 h at 25°C. The containers were aerated with different O₂ concentrations at a constant flow rate of 200 ml/min. Each experiment involved at least 10 apples, and the decayed area was averaged over 10 fruits. The experiment was repeated three times and the results of a single representative experiment are presented.

GOX activity, transcript expression, and gluconic acid accumulation. GOX production, gluconic acid accumulation, and *gox* expression by *P. expansum* were analyzed in liquid medium after placing 500 µl of 2×10^6 spores/ml suspension of the pathogen into 25 ml of yeast sucrose medium (YSM) in 125-ml flasks. The YSM contained 15 g of sucrose, 2 g of yeast extract (Difco), 1 g of K₂HPO₄, and 0.5 g of MgSO₄·7H₂O per liter-medium, at pH 5.5. The cultures were shaken with an orbital shaker (Orbit Environ-Shaker, Lab Line, Barnstead Intl., Dubuque, IA) at 150 rpm and incubated at 25°C for 48 h. Cultures were harvested by vacuum filtration through a sterile Büchner funnel fitted with a filter paper and the remaining mycelia were washed twice over a filter paper with 50 ml of sterile distilled water. The washed mycelia were resuspended in 25 ml of GOX induction medium containing 60 g of sucrose, 7 g of NaNO₃, 3 g of peptone (Difco), 1 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, and 0.5 g of KCl per liter medium. The initial pH value for each flask was determined after the medium had been autoclaved but prior to inoculation, and adjusted to 6.5 with NaOH.

To analyze the effects of O₂ concentration on the in vitro production of GOX and gluconic acid, three flasks containing secondary medium were inoculated for each treatment. The cultures were exposed to a constant flow (50 ml/min) of gas with different O₂ concentrations that penetrated and exited the flask through a rubber stopper in the flask. The cultures were harvested by vacuum filtration as described above after 48 h, and the filtrates were used for determination of pH, quantification of gluconic acid, and determination of GOX activity. The mycelium was frozen in liquid nitrogen and then lyophilized for RNA extraction and dry weight determination. The experiments were repeated four times, and the results of a single representative experiment are presented since there were no significant differences among the experimental repeats. The average values of each treatment (three flasks per treatment) did not differ by more than 2 to 3% between experiments.

To analyze *gox* expression by *P. expansum* in apples, washed mycelia obtained after 48 h of growth in YSM were collected and packed into wounds in the apple tissue that were 6 mm in diameter and 4 mm deep, and were produced by a cork borer. Decay symptoms started to spread around each wound 24 h after inoculation and incubation of the apples at 25°C, and the packed mycelia were removed from the wound 24 or 48 h after the transfer for RNA extraction.

Atmospheric O₂ regulation and determination. The concentration of O₂ in the containers or flasks was adjusted by mixing air (21% O₂, 0.03% CO₂, and 78.97% N₂) with increasing amounts of 100% N₂ to result in the appropriate proportions, and maintained constant in a humidified gas stream flowing at 100 ml/min. The stream was monitored during and at the end of the experiments. Samples of 4 ml of gas from each treatment were injected into a Model 29 gas chromatograph (Fisher-Hamilton, Two Rivers, WI) equipped with a thermal conductivity detector and a CTR 1, 182 cm × 0.61 cm-OD SS column (Alltech, Deerfield, IL) and operated at 22 to 23°C with helium flowing at 30 ml/min as the carrier gas. The data were collected with a 3396 integrator (Hewlett-Packard Co., Avondale, PA).

Analysis of organic acids. For the extraction of organic acids, 0.2-g samples of apple pulp were ground in a Mini Beadbeater (Biospec, Bartlesville, OK) operated at 2,500 rpm in the presence of 0.8 ml of H₂O and 0.5 g of 0.5-mm-diameter glass beads. The ground tissues were then centrifuged for 10 min at 20,800 × g.

The supernatant fractions obtained were filtered through 0.2-µm cellulose acetate filters (Schleicher & Schuell, Dassel, Germany) and kept at -20°C until analysis.

Citric acid in culture filtrates and apple extracts was analyzed with a high pressure liquid chromatograph apparatus equipped with an L-6200 Intelligent pump (Hitachi Scientific Instrument Co. Ltd., Tokyo). Samples of 20- µl filtrates were injected into a 250 × 4 mm 100-C18 column (Eurospher, Berlin) with a mobile phase comprising 8.8 mM KH₂PO₄, 9.8 mM tetrahydrofuran, and 7.6 mM acetonitrile, adjusted to pH 2.2 with phosphoric acid. The flow rate was 0.7 ml/min. The citric acid was detected at 210 nm with an L-4200 detector (Hitachi), and quantified by comparison with an external standard of citric acid (Sigma-Aldrich Israel Ltd., Rehovot, Israel), with an integrator model 3396 (Hewlett-Packard Co.).

The amount of gluconic acid produced in culture filtrates and apple extracts was measured with a commercial enzyme kit for the determination of D-gluconic acid/D-glucono-δ-lactone (R-Biopharm, Darmstadt, Germany).

Measurement of pH. The pH was measured with a single-pore flat or a double-pore slim electrode (Hamilton, Bonaduz, Switzerland) connected to a ThermoOrion Model 720A Plus pH meter (Orion, Beverly, MA). The pH of the medium was measured in 3-ml aliquots sampled after fungal inoculation, and three replicates were tested for each treatment. The pH of the mesocarp was measured by placing the pH electrode directly against the exposed tissue at several points along a transverse cut through the infection site, made with a scalpel blade. All measurements were repeated on 10 to 12 fruit, for a total of at least 30 measurements.

Assay of GOX activity. GOX activity was measured in culture filtrates of secondary media and in extracts of apple tissues. The extracts were prepared by first homogenizing 10 g of tissue followed by centrifugation at 14,470 × g for 20 min. The supernatant fractions were separated and kept at -20°C until use. The samples were concentrated by filtration through an Amicon Ultra 30,000 MWCO centrifugal filter device (Millipore Corporation, Bedford, MD), and the GOX activity was measured. The control for GOX production in vitro was the inoculated secondary media, used immediately after the addition of the hyphae. Extracts of wounded-healthy tissue of the opposite side of the inoculated fruits were used as a negative control for the inoculated apple tissue. The reaction mixtures contained 20 µg of horseradish peroxidase type 1 (Sigma-Aldrich), 1.8% D-glucose, 0.1% o-dianisidine (Sigma-Aldrich), and 100 µl of the sample in 1.5 ml of 0.1 M potassium phosphate buffer at pH 6.5. The oxidation of o-dianisidine by a peroxidase-coupled reaction was measured by the increase in the absorbance at 460 nm. Measurements of the GOX activities were repeated at least three times. One unit of activity was defined as the amount of enzyme that caused the increase in absorbance at 460 nm by one unit per minute at 25°C and at pH 6.5. Protein concentrations in samples were measured with the Protein-Assay instrument (BioRad Laboratories, Munich), with bovine serum albumin (Sigma-Aldrich) used as the standard. The results of representative measurements are presented.

Cloning of a fragment of *gox* gene from *P. expansum*. Genomic DNA was isolated from the mycelium of *P. expansum* with a DNeasy kit (QIAGEN, Santa Clarita, CA). cDNA was prepared from *P. expansum* mycelia grown in the secondary inducing medium and collected by vacuum filtration. Mycelia collected 10 h after growth were quickly frozen in liquid nitrogen and then lyophilized. The total RNA fractions were prepared with the RNeasy Plant Mini Kit (QIAGEN). Contaminating DNA was removed by digestion with DNase (Turbo DNA Free Set, Ambion Inc., Austin, TX). Reverse transcription reactions were carried out with the Reverse-it 1st strand synthesis kit (ABgene, Surrey, UK). The RNA concentration used in the reaction was 80 ng/µl.

To design degenerate primers, the amino acid sequences encoding for three *gox* genes—*P. amagasakiense* (GenBank accession no. AF012277) (10), *Aspergillus niger* (GenBank accession no. AJ294936) (9), and *Talaromyces flavus* (GenBank accession no. U56240) (16)—were compared by multiple alignment, and primers were designed according to the sequences of the homologous areas of the genes. The primers used were the forward GTYATYGAAARSGGYTTCTAYGAGTC and the reverse CWATGCCAGARTTTCSARGATC. Fragments from the *gox* genes were amplified by PCR of genomic DNA or cDNA with the designed primers. In each case an amplification product of the expected size was cloned into plasmid pGEM-T easy (Promega, Madison, WI), sequenced and compared to *ggox* (GenBank accession no. AJ294936) (9) and *go* (GenBank accession no. J05242) (8) from *A. niger*.

Real-time PCR analysis. Mycelia of Pe-21 and Pe-16 were obtained from secondary cultures by vacuum filtration after various periods of incubation in the induction medium. RNA was extracted and cDNA was synthesized as described above. Expression levels of *gox1* and *gox2* were assayed by real-time PCR analysis and were normalized to the expression level of 28S rRNA. PCR amplification of *gox* genes was carried out in 15- μ l aliquots of reaction mixtures containing 7.5 μ l of cDNA template (when the expression of *gox1* or *gox2* was measured the sample was diluted 1:10, and dilutions of 1:1, 1:10, 1:100, and 1:1,000 were used for standard curves; when the expression of 28S rRNA was measured the sample was diluted to 10^{-4} and dilutions of 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} were used for standard curves), 1.875 μ l of each primer as described below, and 3.75 μ l of Absolute QPCR Green ROX Mix (ABgene, Rochester, NY). The PCR assays were run on a Rotor-Gene 3000 Real-Time Cycler (Corbett-Research,

Mortlake, Australia) with the following cycling conditions: 95°C for 15 min, followed by 40 cycles of 95°C for 3 s, 60°C for 15 s, and 72°C for 10 s. The Syber Green signal was measured at the end of every cycle, and a final melting step from 72 to 95°C for 2 min was used for generating a melting curve. The primers for each gene were designed with the Primer Express version 2 software (Applied Biosystems, Foster City, CA). To optimize the concentrations of the primers, several reactions with different concentrations of each of the primers were performed. Primer efficacy was examined by running template solutions of various concentrations and verifying a slope of -3.2 ± 0.2 in a curve of cycle threshold (Ct) versus log (template concentration). For the optimization of *gox2* and 28S rRNA primers, a mixture of cDNAs of the two isolates Pe-21 and Pe-16 was used as a template. For the optimization of *gox* primers a pGEM-T plasmid carrying a fragment of the gene was used as a template. The primer sequences, final concentrations, and product size were as follows: for *gox1* gene 5′CGGAACAACGGAGAACCTTA 400 nM and 5′GGGACCCACAGTGGAAATC 400 nM, 100 bp; for *gox2* gene 5′ACAACCGCACCGAGATTGTT 100 nM and 5′AGGTTGACCCTCCAGTCCTT 175 nM, 50 bp; and for 28S rRNA gene 5′GGAACGGGACGTCATAGAGG 175 nM and 5′AGAGCTGCATTCCCAACAAC 175 nM, 100 bp.

The standard curves and gene concentrations were calculated with the Rotor-Gene software, version 6 (Corbett-Research) using the absolute quantitation analysis. The amount of the GOX cDNA relative to that of 28S (constitutively expressed) is referred to as “*gox* relative expression”; the reported values of *gox* relative expression are averages of two replicates. Each experiment was repeated three times, and the results of a single representative experiment are presented.

A. niger ggox	1	SGFYES—DRGPLVEDLNAYGEIFGSEVDHAYQTVELATNNLTTELIRSGNG	49
A. niger go	1	SGSYES—DRGPITIEDLNAYGDIFGSSVDHAYETVELATNNLTTELIRSGNG	49
P. expansum gox1	1	SGFYES—SEGDIIEDLNDYGDIFGTTVDHAYETVSQAINNRTENIRSGNG	49
P. expansum gox2	1	KGVSTSRSTSLSLKNLNHYGDIFGTSVDQATEFTIPLATHRTEIVRSKGK	50
A. niger ggox	50	LGGSTLVNGGWTWRPHKQVDSWEITVFGNEGWNWENVAAYSLAERARAP	99
A. niger go	50	LGGSTLVNGGWTWRPHKAQVDSWEITVFGNEGWNWENVAAYSLQAERARAP	99
P. expansum gox1	50	LGGSTLVNGGWTWRPHKAQVDSWEKVTGNKDINWDLFPYMQKAEIARPP	99
P. expansum gox2	51	LGGSTLVNGGWTWRPHKAQVDSWEITVFGNEGWNWENVAAYSLPYMKKIEARAP	100
A. niger ggox	100	NAKQVAACHYFDPSCCHGTNGTVHVGPRDTGDDYTPITDALMTTVENMGVP	149
A. niger go	100	NAKQIAACHYFNPSCCHGTNGTVHAGPRDTGDDYSPITKALMSAVEDRGVP	149
P. expansum gox1	100	SDVEIAACHYFYNSSCHGTNGTVHAGPRNNGEPYSPITKTLIDSAKERGVP	149
P. expansum gox2	101	NAEQIAACHYFDPSCCHGTGTVHVGPRDTGSEFSPMIKSLMKNNANSGLP	150
A. niger ggox	150	TKKDLGCGDPHGVSMFPNTLHEDQVRSDAAREWLLPNYQRPNLQVLTGQL	199
A. niger go	150	TKKDFGCEDPHGVSMFPNTLHEDQVRSDAAREWLLPNYQRPNLQVLTGQY	199
P. expansum gox1	150	TQLDFHCCTPRGISMIPNALHEDQVRSDAAREWLLPNYKRPNLQVLTGQF	199
P. expansum gox2	151	VQKDLGCGDPHGISMLINDVHEDQTRSDAAREWLLPNYQRPNLKILTGQM	200
A. niger ggox	200	VGKVLLDQ---NNTVP---KAVGVVEFGTHKANTFNVAKHEVLLAAGSAVS	244
A. niger go	200	VGKVLLSQ---NGTTP---RAVGVEFGTHKGNTHNVAKHEVLLAAGSAVS	244
P. expansum gox1	200	VGKVLIQTATSGAIPGYKAVGVNFGTKNVNFNVAKHEVLLAAGSAVS	249
P. expansum gox2	201	VGKVLEDT---TTTTP---KAVGVNFGTHAKVNFVHARHEVLLAAGSAVS	245
A. niger ggox	245	PQILEHSGI	253
A. niger go	245	PTILEYSGI	253
P. expansum gox1	250	PRILEHSGI	258
P. expansum gox2	246	PQILEHSGI	254

Fig. 1. Comparisons of predicted amino acid sequences encoded by *gox1* and *gox2* of *Penicillium expansum* with each other and with the amino acid sequences of two GOXs of *Aspergillus niger*.

Macroscopic and microscopic detection of H₂O₂ in fruit tissue. H₂O₂ was detected macroscopically by diaminobenzidine (DAB) (Sigma fast DAB tablets) staining. DAB polymerizes instantly, and develops a localized dark color as soon as it comes into contact with H₂O₂ in the presence of peroxidase (26). A dark color was observed in 1-mm slices of *P. expansum*-infected Golden Delicious apples after dipping the tissues in 0.07% solution of DAB. Similar infected tissues were immersed in distilled water as control. Sliced tissues were photographed immediately before and 20 h after staining.

For microscopic detection of oxidative changes produced by mycelia of *P. expansum*, 2-mm slices of infected Golden Delicious apples were immersed in 50 mM 2-[N-morpholino]ethanesulfonic acid assay buffer, pH 6.2, containing 10 μ M dichlorodihydrofluorescein diacetate (H₂DCFDA) (Molecular Probes, Eugene, OR) for 30 min at room temperature. Slices were then washed with the same buffer. Oxidation of the H₂DCFDA by H₂O₂ was detected as the highly green fluorescent 2',7'-dichlorofluorescein that was monitored by fluorescence spectroscopy using excitation and emission wavelengths of 502 and 523 nm, respectively. Images were acquired using an Olympus IX81 confocal laser-scanning microscope system (Olympus, Tokyo). Confocal images were obtained via UPlanApo 10X0.40 objective lens at an excitation wavelength of 488 nm (Argon laser), BA515-525 emission filter for H₂DCFDA, and BA660 IF emission filter for autofluorescence. Transmitted-light images were acquired using Nomarski differential interference contrast.

RESULTS

gox regulation of gluconic acid production in *P. expansum*.

To determine the relative expression of the two genes encoding putative proteins with GOX activity, we first cloned DNA fragments of *gox* genes from the fungus based on published *gox* sequences (9,10,16). Fragments from two different *gox* genes of *P. expansum* were generated and cloned by standard PCR technology. One fragment, designated *gox1*, was 784 bp (accession no. AY669127) and was isolated from an amplicon generated from genomic DNA. The second fragment, designated *gox2*, was 761 bp (accession no. DQ157372) and was isolated from an amplicon generated from cDNA produced from RNA after growth on GOX inductive medium. The two putative GOX genes shared 64% identity and 85% similarity (Fig. 1). A pairwise comparison to the *A. niger* *gox* genes *ggox* and *go* indicated that *gox2* shared a similar degree of identity (i.e., 64 and 69% identity and 82 and 84% similarity, respectively). Between the two GOX fragments of *A. niger* there was an identity of 87% and a similarity of 94%.

The relationship between increased *gox* expression, gluconic acid accumulation, and pathogenicity of *P. expansum*.

To examine the relationship between increasing secretion of organic acids and pathogenicity we selected natural isolates of *P. expansum* that exhibited increasing levels of aggressiveness. The rates of decay development caused by three isolates, when inoculated into apple wounds, increased in the order Pe-21 < Pe-23 < Pe-16: 5 days after infection, the decayed areas associated with the three isolates were 200, 235, and 290 mm², respectively (Fig. 2A). The increase in the decayed area was proportional to the increase in gluconic acid accumulation (Fig. 2B) and to the decrease in tissue pH (Fig. 2C). The most aggressive isolate (isolate Pe-16) secreted the largest amount of gluconic acid (8,000 μ g/g fruit weight [fw]) and caused a decrease of 0.85 units in the pH of the plant tissue. The least aggressive isolates, Pe-23 and Pe-21, secreted 6,400 and 3,100 μ g/g fw gluconic acids, respectively, and reduced the pH by 0.7 and 0.5 units, respectively. All three isolates caused a significant

increase in the concentration of citric acid relative to the control but the amount of acid that accumulated was similar in all three isolates (Fig. 2B). When gluconic acid or citric acid (at 0.25 M) was added independently to an infected wound, fungal colonization increased by 43 and 23%, respectively (data not shown).

To assess whether *gox* transcript levels and activity of GOX were correlated with the accumulation of gluconic acid with fungal colonization (Fig. 2), we compared these parameters during colonization and fungal growth in vitro in the highly and less aggressive Pe-16 and Pe-21 strains, respectively. The expression of *gox1* and *gox2* under GOX-inducing conditions in both isolates indicated that transcripts of *gox2* were expressed both in vivo and in vitro, whereas *gox1* was expressed only in vitro at a level approximately 80 times lower than the expression of *gox2* (data not shown). Overall GOX activity in Pe-16 was more than threefold greater than in Pe-21 12 h after transfer to inducing media (1.70 compared with 0.55 units/g protein). The relative expression of *gox2* at 9 and 12 h after transfer was 250- and 14-fold greater in Pe-16 compared to Pe-21 (Fig. 3).

Effect of reduced levels of oxygen concentration on reduced expression of *gox2*, gluconic acid accumulation, and pathogenicity of *P. expansum*. The reduction of percent oxygen in culture from 21 to 15% or to 10% resulted in a 15- and 250- fold decrease in *gox2* expression, a 2.6- and 19-fold reduction in GOX activity, and a 1.5- and 5.5-fold reduction in gluconic acid concentration (Fig. 4). Overall growth at either 15 or 10% oxygen did not appear affected, as fungal dry weights were similar to those found at 21% oxygen. Reduction in percent oxygen to 5 or 3% led to even fewer *gox2* transcripts, less GOX activity and gluconic acid accumulation, and also led to a significant reduction in fungal growth (Fig. 4).

Fig. 2. Effect of *Penicillium expansum* isolates on the pH and on the production of organic acids in infected cv. Golden Delicious apples. **A**, Decayed area 5 days after inoculation with *P. expansum*; **B**, Amounts of organic acids (dark column indicates citric acid and bright column indicates gluconic acid); and **C**, pH of the infected tissue. Average values of 10 replicates ($\pm$ SD) are presented.

Fig. 3. Differential expression of *gox2* of *Penicillium expansum* isolates with different levels of aggression. RNA from mycelia of highly aggressive (Pe-16) and less aggressive (Pe-21) *P. expansum* strains were extracted at different lapsed times after transferring into the secondary inducible medium. *gox2* expression was detected by real-time PCR analysis. The *gox2* relative expression values are the averages $\pm$ SD of two replicates. The experiment was repeated three times and the results of a single representative experiment are shown.

Exposure of *P. expansum*-infected apples to an oxygen concentration of 10% reduced gluconic acid accumulation at the leading edge of colonized tissue from 2,710 to 962 mg/liter, and reduced tissue colonization by 15%, i.e., from 410 to 350 mm². A further decrease in oxygen concentration to 3% reduced gluconic acid accumulation and host colonization by 84 and 26%, respectively, as compared to their levels at 21% O₂ (Fig. 5).

Localization of GOX activity, H₂O₂ production and gluconic acid secretion during decay development by *P. expansum*. GOX activity could not be detected in the extracts of healthy tissue. GOX-specific activities of 0.11 and 0.04 units/g protein were measured, respectively, at the leading edge and in the middle of the area decayed by *P. expansum*. The pH of healthy tissue was approximately 4.2, in contrast to that of leading edge (3.7) or that in the middle of the colonized area (3.5).

H₂O₂, a byproduct of GOX activity and other oxidative changes, was detected in decayed apple tissues by the brown color following staining with DAB (Fig. 6B) in comparison with the light brown color of the water control (Fig. 6A). Oxidative change produced by mycelia was also detected mostly inside (but also outside) the fungal hyphae by the fluorescence of dichlorodihydrofluorescein diacetate at the leading edge of the decaying area (Fig. 6C), suggesting that H₂O₂ is released to the decaying surrounding area.

Fig. 4. Effects of atmospheric O₂ concentration on the transcript expression of *gox2*, GOX activity, and gluconic acid accumulation by *Penicillium expansum*. **A**, Relative expression of *gox2* and GOX activity in culture media 10 h after *P. expansum* hyphae were transferred to inductive conditions and exposed to mixtures of gases containing reduced O₂ levels; and **B**, Concentration of gluconic acid, medium pH, and weight of dry hyphae. Average values $\pm$ SD of three replicates of the relative expression of *gox2*, GOX activity, concentration of gluconic acid, pH, and fungal dry weight are presented. Dry weight of the fungal hyphae as affected by reducing atmospheric oxygen levels was also analyzed statistically by Student's *t* test; different letters indicate significant differences at $P \leq 0.05$.

DISCUSSION

Gluconic and citric acids are the main organic acids produced by *P. expansum*, *P. digitatum*, and *P. italicum* during these fungi's necrotrophic colonization in fruits (18). It was suggested that changes in external pH during fruit infection due to these acids in turn favors the production of fungal pectolytic enzymes (18). The addition of exogenous gluconic acid or citric acid at the *P. expansum* infection site significantly enhanced decay development, supporting the hypothesis that these acids contributed to disease development (18). However, the data of the present work suggest that gluconic acid is the main organic acid contributing to the colonization of *P. expansum*: (i) aggressive isolates of *P. expansum* secreted significantly higher amounts of gluconic acid, whereas the amounts of citric acid accumulated were much lower and did not change correlatively with the increase in the aggressiveness of the isolates; (ii) addition of 0.25 mM gluconic acid to the infected fruit tissue increased *Penicillium* colonization twofold compared with similar concentrations of citric acid and (iii) GOX activity was detected at the leading edge of the decaying tissue, thereby localizing the production site of gluconic acid to the decay development front. Thus, we suggest that gluconic acid is the main acid contributing to *P. expansum* host acidification and that this strengthens the association between gluconic acid accumulation and virulence of *P. expansum*. Gluconic acid may further facilitate fungal colonization by chelating Ca^{2+} ions, weakening the host cell wall (14,15), or by directly affecting cell death (5,25).

GOX activity could not be detected in healthy apple tissues but was readily measured in the middle of the decayed area in apples infected by *P. expansum*. At the leading edge of the decayed tissue, GOX activity was nearly three times higher than in the middle of the decayed area. Genes encoding GOX have been cloned from several strains of *Aspergillus* and *Penicillium* sp. as well as from *T. flavus* and *B. cinerea* (8,9,10,12,16,20). Two putative genes (*gox1* and *gox2*) encoding GOX were detected in *P. expansum* and shared significant nucleotide and pre-dicted amino acid similarity with GOX genes from *A. niger* (8,9). In the present study the expression of *gox1* could not be detected in vivo, whereas *gox2* was highly expressed both in vivo and in vitro. It is possible that the two *gox* genes of *P. expansum* are expressed under different conditions: *gox2* is expressed during the pathogenic process, whereas *gox1* is expressed under conditions not encountered in the present study.

Fig. 5. Effects of atmospheric O_2 concentration on gluconic acid accumulation (expressed as micrograms per gram of fresh weight [fw] of fruit) and virulence (expressed as decay area) in apples inoculated with *Penicillium expansum* at 25°C. Average values $\pm$ SD of seven replicates of gluconic acid concentration and decayed area are presented. Effect on the decayed area of reducing atmospheric oxygen levels was analyzed statistically by Student's *t* test; different letters indicate significant differences at $P \leq 0.05$.

The production rate of GOX and the synthesis of gluconic acid has been shown to be affected by various conditions such as glucose levels, pH values (1,22), and oxygen levels (7,23,28). Glucose accounts for almost 50% of the concentration of the total soluble solids (TSS) in fruits, a factor that describes fruit sweetness. Apple TSS generally increases from between 7 and 8% to between 13 and 14% during fruit growth and postharvest ripening, conditions under which *P. expansum* commonly attacks (13). Semashko et al. (24) showed that GOX activity by *P. funiculosum* showed higher catalytic activity at pH above 6.0 than at lower pH values. This is in contrast to our work where we found that the pH of most of the infected fruits was below 6.0 (18).

One other factor modulating the rate of GOX activity and the synthesis of gluconic acid that was tested was the O_2 concentration (7,23,28). Oxygen-sensing and oxygen-responsive genes have been studied in yeasts (2,11), but there have been only a few reports on the effect of O_2 concentration on the expression of virulence genes in filamentous fungi. The reduction in the accumulation of gluconic acid under anoxia supports the

Fig. 6. Detection of oxidative changes in Golden Delicious apples inoculated with *Penicillium expansum*. Slices of inoculated apples were **A**, immersed in water; **B**, stained with diaminobenzamine (DAB) solution for 20 h; or **C**, immersed in dichlorodihydrofluorescein diacetate for microscopic detection of oxidative changes at the decay edge (marked by the square in A). Oxidative changes were detected mostly inside the hyphae (see arrows) and around the fungal hyphae. No fluorescence was observed in the healthy tissue (upper part of C). Images were acquired using a confocal laser-scanning microscope.

hypothesis that the reduction of atmospheric O₂ inhibits GOX activity and the biosynthesis of this organic acid by *P. expansum*. Reduction of oxygen concentrations from 21 to 10% inhibited the catalyzed oxidation of glucose by GOX at four different levels: (i) at the level of gene expression; (ii) GOX activity; (iii) gluconic acid accumulation in culture; and (iv) gluconic acid accumulation in apples. Reduction of environmental O₂ level from 21 to 10%, however, did not affect *P. expansum* growth but inhibited colonization of the host. This suggests that the limitation of fungal colonization is not a result of restriction of respiration and growth, but is probably caused by the down-regulation of *gox2* and the reduction in gluconic acid accumulation at the leading edge. It is possible that the inhibition of fungal colonization by only 15% when gluconic acid accumulation is inhibited by 63% is the result of compensation by the pathogen inducing the synthesis of other organic acids such as citric acid. Nevertheless, since reduction in GOX activity in vitro by reduced O₂ concentration levels was not accompanied by a decrease in the culture pH (Fig. 4), it is probable that citric acid or other organic acid is not accumulated. In *B. cinerea*, down-regulation of GOX by gene knockout resulted in mutants that displayed similar virulence to that of the wild-type strain (21). In this case, it was suggested that other oxidative reactions or even alternative unidentified GOX genes provide products that contribute to fungal attack and necrosis (21).

To summarize, we have presented data that support the hypothesis that gluconic acid is a key factor contributing to fungal disease of apple. The lower exogenous pH likely favors the production of other fungal pathogenic factors (e.g., cell wall degrading enzymes) that contribute to tissue necrosis (18). Reactive oxygen species (ROS) detected in the infected plant tissues that are likely produced from the oxidation of glucose by GOX may also contribute to the weakening of apple tissue and securitization, in a manner similar to plant infection by *Botrytis cinerea* (21). Since *P. expansum* penetrates the host only through wounds, it is possible that the ROS at the host's wound are critical for initial *gox* activation and the initiation of *Penicillium* attack. In this relation, Castoria et al. (4) reported that ROS generation was detected during the first 4 h immediately after apple fruit is wounded. Superoxide and subsequently generated ROS may act as second messengers that control the activity of the factor(s) that regulate the transcription of hypoxic genes such as *gox* (3,7). The fact that ROS generation in wounded tissue is more rapid and more pronounced in apple fruits stored for long period of time is consistent with the general occurrence of enhanced levels of oxidant species in senescent tissues (4,13) and the susceptibility of fruits to fungal attack via their senescent tissue during storage.

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