Accumulation of the Mycotoxin Patulin in the Presence of Gluconic Acid Contributes to Pathogenicity of *Penicillium expansum*

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Penicillium expansum, the causal agent of blue mold rot, causes severe postharvest fruit maceration through secretion of D-gluconic acid (GLA) and secondary metabolites such as the mycotoxin patulin in colonized tissue. GLA involvement in pathogenicity has been suggested but the mechanism of patulin accumulation and its contribution to *P. expansum* pathogenicity remain unclear. The roles of GLA and patulin accumulation in *P. expansum* pathogenicity were studied using i) glucose oxidase *GOX2*-RNAi mutants exhibiting decreased *GOX2* expression, GLA accumulation, and reduced pathogenicity; ii) *IDH*-RNAi mutants exhibiting downregulation of *IDH* (the last gene in patulin biosynthesis), reduced patulin accumulation, and no effect on GLA level; and iii) *PACC*-RNAi mutants exhibiting downregulation of both *GOX2* and *IDH* that reduced GLA and patulin production. Present results indicate that conditions enhancing the decrease in GLA accumulation by *GOX2*-RNAi and *PACC*-RNAi mutants, and not low pH, affected patulin accumulation, suggesting GLA production as the driving force for further patulin accumulation. Thus, it is suggested that GLA accumulation may modulate patulin synthesis as a direct precursor under dynamic pH conditions modulating the activation of the transcription factor *PACC* and the consequent pathogenicity factors, which contribute to host-tissue colonization by *P. expansum*.

Penicillium expansum is a destructive phytopathogen that causes decay in deciduous fruit during postharvest handling and storage. The pathogen is not considered to be host specific among deciduous fruit and typically causes extensive maceration of the infected tissue, presumably by a common mechanism of tissue acidification (Prusky et al. 2004). Direct tissue acidification is achieved by its secretion of organic acids, mainly D-gluconic acid (GLA) (Hadas et al. 2007; Prusky and Yakoby 2003; Prusky et al. 2004).

Nucleotide sequence data for *GOX2*, *IDH*, and *PACC* of *Penicillium expansum* are available in the GenBank database under accession numbers GQ324948 (*GOX2*), DQ084388 (*IDH*), and AY225524 (*PACC*).

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure is published online and Figure 1 appears in color online.

In *Penicillium* spp., pathogenicity has been attributed to, among other things, the secretion of polygalacturonases which depolymerize pectin, thereby causing tissue maceration (Sánchez-Torres and González-Candelas 2003; Yao et al. 1996). Expression of *PEPG1*, which encodes a polygalacturonase, is upregulated under the acidic conditions resulting from GLA accumulation (Prusky et al. 2004). In *P. expansum*, oxidation of β -D-glucose to D-glucono- δ -lactone and H_2O_2 is catalyzed by glucose oxidase (GOX) (Bruchmann et al. 1987; Kobayashi et al. 1998; Prusky et al. 2004). Previous studies by Hadas and associates (2007) identified two putative genes (*GOX1* and *GOX2*) involved in the production of GLA but concluded that *GOX2* is the most important one for *P. expansum* pathogenicity (Hadas et al. 2007). GLA accumulation by *P. expansum* is pH dependent and is mainly regulated by *GOX2* expression, with highest expression under neutral and alkaline conditions (pH 7.0 to 9.0) compared with acidic conditions (pH 4.0). Barad and associates (2012) used a transgenic approach to analyze the contribution of *GOX2* to the acidification process and pathogenicity. Functional analysis of *GOX2*-RNAi mutants showed that modulation of GLA level strongly affects pathogen interactions with the host: the greater the downregulation of *GOX2* in the *GOX2*-RNAi mutants, the stronger the impairment in GLA production, medium acidification, and apple fruit infection. Those results further supported the hypothesis that *P. expansum*'s ability to acidify the environment results in the expression of genes and secretion of hydrolytic enzymes that contribute to pathogenicity (Prusky et al. 2004).

Other approaches used to functionally analyze the contribution of *GOX2* to pathogenicity involved its heterologous expression in *P. chrysogenum*, a pathogen of nondeciduous fruit. Barad and associates (2012) reported that expression of *P. expansum* *GOX2* in *P. chrysogenum* enhances GLA production, acidification of the medium, surface colonization, and production of conidia on susceptible pear fruit discs; however, fruit colonization was attenuated compared with the wild-type (WT) *P. expansum* strain Pe-21. These results indicated that the observed increase in GLA production is one factor promoting surface colonization and sporulation but complete pathogenicity still requires the expression of other pathogenicity factors which might be lacking in *P. chrysogenum* (Mohammad-Saeid et al. 2010; Qin et al. 2007). Qin and associates (2007) confirmed early work by Yao and associates (1996), in which the expression of three polygalacturonase proteins was found to be repressed under attenuated virulence conditions, suggesting that these enzymes contribute to fungal virulence. However,

there is still only a limited amount of research on the possible secretory proteins contributing to *P. expansum* virulence.

The mycotoxin patulin is a key secreted product that always accumulates in colonized tissue during *P. expansum* colonization, to concentrations of 16,000 to 44,000 $\mu\text{g kg}^{-1}$ (Piemontese et al. 2007; Puel et al. 2010). Patulin is one of a short list of mycotoxins (aflatoxin, ochratoxin A, zearalenone, fumonisins, and trichothecenes) whose levels in food are regulated in many countries around the world because of their toxic effects to people, particularly children (Puel et al. 2010). The biosynthetic pathway of patulin—a polyketide metabolite—includes more than 10 steps (Sekiguchi and Gaucher 1977).

The high accumulation of patulin in decaying tissue during the acidification process raised the question of its biological role in *P. expansum* pathogenicity. Published reports could not reach a conclusion on the importance of patulin as a pathogenicity factor. Although patulin was isolated from more than 30 species belonging to the genera *Penicillium*, *Aspergillus*, *Paecilomyces*, and *Byssosclamyces*, the mycotoxin only occurred in *P. expansum*-colonized fruit. Furthermore, patulin-producing strains of *P. expansum* isolated from a variety of host fruit, including apricot, black mulberry, cherry, kiwi, nectarine, plum, strawberry, and white mulberry, induced levels of patulin accumulation that varied up to sixfold (1,800 to 12,500 $\mu\text{g kg}^{-1}$) (Demirci et al. 2003; Larsen et al. 1998; Leggott and Shephard 2001; Lugauskas et al. 2005; Reddy et al. 2010; Ritieni 2003) in one single apple host. Furthermore, a single isolate inoculated on a different host induced the production of different levels of patulin (from 150 to 11,200 $\mu\text{g kg}^{-1}$), suggesting a differential pattern of production depending on the host and temperature, pH, and other chemical characteristics intrinsic to

fruit-storage conditions. A few reports have described a relationship between colonization and toxin accumulation. McCallum and associates (2002) isolated different *P. expansum* isolates which showed varying levels of patulin production, from 0 to 6,000 $\mu\text{g per gram}$ of dry weight of apple tissue. The highest patulin levels were produced by isolates displaying aggressive growth and rapidly increasing acidity of the medium, accompanied by profuse mycelial development. Furthermore, Sanzani and associates (2012) reported that knock out of 6-methylsalicylic acid synthase (6-MSAS), one of the first genes in the biosynthetic pathway of patulin, reduces pathogenicity by 40 to 50%. All of these results suggest that the association between patulin and pathogenicity under conditions of *P. expansum* colonization in fruit is not entirely clear.

Our hypothesis, based on our recent publication (Barad et al. 2012) and our present results, is that GLA production is the driving force for activation of patulin synthesis and, together, these compounds contribute to the enhanced pathogenicity of *P. expansum* in fruit. Using three different genetics approaches—i) *P. expansum* *GOX2* mutants that show modulated GLA synthesis; ii) *P. expansum* mutants in *IDH* (encoding isopentenyl pyrophosphate isomerase, in the last stages of patulin synthesis) (Dombrink-Kurtzman 2007) affecting the production of patulin; and iii) *P. expansum* mutants in the transcription factor *PACC*, affecting the biosynthesis of both GLA and patulin—we showed the importance of the acidification process caused by GLA accumulation in the induction of patulin biosynthesis and accumulation and in its contribution to *P. expansum* pathogenicity in colonized fruit. Our results indicate that GLA and patulin are pathogenicity factors of *P. expansum* modulated by the transcription factor *PACC* expressed during fruit colonization.

Fig. 1. D-gluconic acid (GLA) and patulin accumulation and relative *IDH* expression in different areas of the decayed tissue of 'Golden Delicious' apple fruit inoculated with *Penicillium expansum*. The following parameters were measured in different regions of the decayed fruit: **A**, Colonized tissue; **B**, GLA accumulation; **C**, patulin accumulation; **D**, relative expression of *IDH*. Different values presented were extracted from the leading edge of the decayed tissue (LECT), intermediate colonized tissue (ICT), and the center of decay (CCT). Relative expression values of *IDH* are the averages of three replicates from three different RNA extractions from apple tissues compared with the LECT. Average values $\pm$ standard error of three replicates of relative *IDH* expression are presented. Average values of three replicates of total GLA and patulin accumulation are also presented. Columns and symbols with different letters are significantly different at $P \leq 0.05$ according to the Tukey-Kramer multiple comparison test. Experiments were repeated three times, and the results of a single representative experiment are shown.

RESULTS

GLA, pH, and patulin
in apple fruit tissue colonized by *P. expansum*.

Analysis of the decayed tissue colonized by *P. expansum* 5 days after inoculation showed central colonized tissue (CCT)

Fig. 2. Relationship between D-gluconic acid (GLA) and patulin accumulation and relative *IDH* expression in *GOX2*-RNAi mutants of *Penicillium expansum* and their pathogenicity on ‘Golden Delicious’ apple fruit. Solid secondary medium at an initial pH of 7.0 was inoculated with 100 µl of a spore suspension of 10⁶ spores ml⁻¹ and the different parameters evaluated 48 h later by sampling five medium discs from five independent culture plates. **A**, GLA accumulation and final pH values; **B**, patulin accumulation; **C**, relative *IDH* expression of *GOX2*-RNAi mutants compared with the wild-type strain; **D**, colonization diameter. Average values of five replicates are reported for relative *IDH* expression (± standard error), GLA, and patulin accumulation. For colonization, the average values presented were obtained from nine independent inoculation points. Columns and symbols with different letters are significantly different at *P* ≤ 0.05 according to the Tukey-Kramer multiple comparison test. Experiments were repeated three times and the results of a single representative experiment are shown.

Table 1. *Penicillium expansum* strains used in this study

Strain ^a	Genotype	Source
Pe-21	Wild-type <i>P. expansum</i> , Israel	Hadas et al. (2007)
<i>GOX2</i> -RNAi strains: TPe ₁₃₀ , TPe ₁₄₁ , TPe ₁₁₄ , TPe ₁₁₆	RNAi <i>gox1:gox2:hygR</i>	Barad et al. (2012)
<i>IDH</i> -RNAi strains: TPe ₁₇ , TPe ₇₇	RNAi <i>idh:hygR</i>	...
<i>PACC</i> -RNAi strains: TPe ₃₄ , TPe ₁₀₉	RNAi <i>pacC:hygR</i>	...

^a Strains starting with “T” are original transformants.

with initial sporulation, and intermediate colonized tissue (ICT) up to the leading edge of the colony (LECT) followed by the healthy part of the apple (Fig. 1A). GLA concentration in the CCT and ICT was 2.2 to 2.6 g per gram of fresh weight (FW) and declined in the LECT to 1.6 g per gram of FW, compared with no accumulation in the healthy flanking part of the colonized tissue (Fig. 1B). Patulin accumulation was also highest in the CCT and ICT, at 7.5 to 8.1 µg per gram of FW, compared with 0.30 µg per gram of FW and no accumulation in the LECT and healthy tissue, respectively, suggesting the occurrence of both GLA and patulin in the colonized tissue (Fig. 1C). Relative expression of *IDH* showed a sevenfold increase in the CCT compared with the LECT (Fig. 1D). The pH of the healthy part of the apple tissue was 4.2, declining to 3.5 in the CCT and ICT (Fig. 1B). The concomitant occurrence of GLA and patulin during fungal colonization raised the question of the mechanism underlying each metabolite’s accumulation and these metabolites’ contribution to pathogenicity. To answer this, we used a series of *GOX2* mutants (Barad et al. 2012) that differed in their ability to accumulate GLA and in their consequent contribution to pathogenicity.

**GOX2-RNAi mutants downregulate
IDH expression and patulin accumulation.**

To understand the effect of GLA accumulation on patulin production and pathogenicity, we used *GOX2*-RNAi mutants of *P. expansum*, which were differentially impaired in the secretion of GLA and pathogenicity (Barad et al. 2012). To determine whether downregulation of *GOX2* in the *GOX2*-RNAi mutants (Table 1) modulates *IDH* expression and patulin production, the WT strain Pe-21 and the mutant strains were grown on secondary medium (SM)–agar solid medium adjusted to an initial pH of 7.0 for 48 h (Fig. 2). Functional characterization of *GOX2*-RNAi mutants showed significantly decreased production of GLA and consequent decrease of pH in the medium. In mutant strains TPe₁₃₀ and TPe₁₄₁, which showed 50% (4.3 g per gram of DW) and 45% (3.8 g per gram of DW) decreased production of GLA, respectively, the pH declined from 7.0 to 4.3 and 4.45, respectively, compared with 4.12 in the WT strain, with GLA at 8.7 g per gram of DW. An intermediate level of GLA and only partial decline in pH were observed for RNAi mutants TPe₁₁₄ and TPe₁₁₆ (Fig. 2A). Functional characterization of *GOX2*-RNAi mutants also showed significant downregulation of *IDH* expression and patulin accumulation, by a factor of approximately 10, during growth of TPe₁₄₁ in SM (Fig. 2B and C). These findings indicate that the downregulation by *GOX2*-RNAi significantly decreases GLA and patulin production, suggesting that patulin regulation is largely dependent on the potential for GLA production and the resulting modification in pH (Fig. 2). To determine that the RNAi mutants did not suffer a broader effect on gene expression, we compared the relative expression of other genes from *P. expansum*, including pectin lyase (AY-545054.1), *pacC*, calmodulin (DQ-911134.1), and β-tubulin (GN-872745.1), and their relative expression was not affected compared with the WT strain (results not shown). Analysis of the colonization pattern showed that downregulation of GLA and patulin accumulation significantly reduces the colonization of *P. expansum* mutants (Fig. 2D).

Previous reports have indicated a very low level of GLA accumulation in liquid SM buffered with phthalate buffer to pH 4.5 (Barad et al. 2012; Hadas et al. 2007). These findings were used to clarify the effect of pH change on GLA and patulin accumulation. For this purpose, the WT and *GOX2*-RNAi strains were grown in nonbuffered SM-agar medium at an initial pH of 4.5 compared with 7.0. Growth of the *GOX2*-RNAi mutants at pH 7 resulted in a significant decrease in patulin production as the level of GLA decreased (Fig. 3B). In this case, the initial pH of the medium was decreased from 7 to almost 4 by all the different mutants, suggesting that, after 72 h, differential GLA accumulation leads to differential patulin accumulation, even if the final pH reached by the WT and mutants is approximately 4.0. However, when the fungus was grown at pH 4.5, low levels of patulin were observed in the *GOX2*-RNAi and WT strains with no changes in medium pH (Fig. 3A), suggesting that it is not the low pH but the potential for GLA production that determines the accumulation of patulin.

Dynamics of patulin accumulation

by *GOX2*-RNAi as function of time and *GOX2* expression.

The temporal effect on the differential induction of pH decline by *GOX2*-RNAi mutants was studied to determine the importance of the pH decrease versus *GOX2* downregulation on the induction of patulin accumulation. Growth of the WT strain in nonbuffered solid SM at an initial pH of 7.0 showed a pH decrease to 6.9, 4.3, and 3.8 after 24, 48, and 72 h, respectively (Fig. 4). This differential induction of pH decrease was accompanied by a significant increase in patulin accumulation from 0.78 µg per gram of DW at pH 7 to 64 µg per gram of DW when the pH decreased to 3.7 72 h later. Differential pH decline by the different *GOX2*-RNAi mutants all reduced the level of patulin accumulation. At 24 h after inoculation, when the pH was still approximately 7 and the level of patulin production by the WT was 0.78 µg per gram of DW, 20 and 30% inhibition of patulin accumulation was induced by the *GOX2*-RNAi mutants impaired in GLA secretion (Fig. 4A). However, 48 h after inoculation, when the pH had decreased to almost 4.0 in the WT and 4.5 in the TPe₁₄₁ mutant, the amount of patulin accumulated increased almost 30-fold in the WT but only 2-fold in the TPe₁₄₁ mutant, suggesting the importance of incubation time and pH decline on patulin accumulation and stability. The same pattern was observed when patulin accumulation was determined 72 h after inoculation. In this case, the pH decreased by only 0.4 pH units and patulin increased 313-fold in the WT whereas, in the mutants, there was a delayed increase in patulin accumulation. These results indicate

that lower accumulation of patulin in *GOX2*-RNAi strains is largely dependent on the downregulation of *GOX2* expression.

The pH change induced by the WT and the TPe₁₄₁ *GOX2*-RNAi mutant after 48 h of growth amounted to only 0.3 units compared with the 18-fold increase in patulin production by the WT during the same period, again suggesting that pH alone is not the main factor for patulin induction.

Fig. 4. Dynamics of patulin accumulation by *GOX2*-RNAi mutants of *Penicillium expansum* as a function of time. **A**, Spores of the wild-type Pe-21 strain were inoculated into solid secondary medium adjusted to pH 7.0 and sampled 24, **B**, 48, and **C**, 72 h after inoculation. At each sampling date, five agar discs (10 mm in diameter by 0.5 mm thick) were extracted for analysis of patulin accumulation. Five replicates were sampled from each strain. Average values $\pm$ standard error of five replicates of patulin extractions are presented. Experiments were repeated three times and the results of a single representative experiment are shown.

Fig. 3. Relationship between initial pH and patulin accumulation by the *GOX2*-RNAi mutants of *Penicillium expansum*. **A**, Solid secondary media at an initial pH of 4.5 or **B**, 7.0 were inoculated with 100 µl of a suspension of 10^6 spores ml^{-1} and patulin accumulation and final pH were evaluated 72 h later by sampling five medium discs from five independent culture plates. Average values $\pm$ standard error of five replicates of patulin extractions are presented. Experiments were repeated three times and the results of a single representative experiment are shown.

Analysis of the precursors for patulin production by Grootwassink and Gaucher (1980) indicated the importance of glucose as a carbon source on patulin accumulation. Growth with increasing concentrations of glucose from 25 to 50 mM in 0.2 M phosphate-buffered medium at pH 7.0 (Fig. 5A to C) showed, in all cases, maximal accumulation of GLA by 48 h followed by an increase in patulin 24 h later concomitant with a decrease in GLA concentration. This increase in patulin accumulation was accompanied by an activation of *IDH*. These and previous results of Bork and associates (1998) and Ramachandra and associates (2006) suggested that the β -gluconate, gluconic acid may be metabolized to patulin through glycolysis. To further support this hypothesis, the WT strain was grown in the presence of GLA as sole carbon source. Growth of the WT for 72 h indicated that GLA, under the afore-described conditions, is consumed (reduction in the media) (Fig. 5A) and metabolized to patulin (increase in the media) (Fig. 5B) through the activation of *IDH* expression (Fig. 5C). Interestingly, growth of the WT strain in the presence of sucrose as sole carbon source induced almost 10-fold more patulin than that obtained with a similar concentration of glucose in culture (results not shown). This indicates that GLA and other sugars induce the accumulation of patulin with different efficiencies.

The relationship between patulin and pathogenicity.

Previous findings indicated that acidification of the fruit host environment via the accumulation of GLA enhances

Pepg1 gene expression in *P. expansum*, resulting in enhanced maceration and colonization of the fruit by the fungus (Barad et al. 2012; Hadas et al. 2004). Our present findings indicate that *GOX2*-RNAi mutants with reduced pathogenicity show reduced GLA and patulin accumulation. These results raise the question of the possible contribution of patulin to *P. expansum* pathogenicity. To analyze this, we developed *IDH*-RNAi mutants (Fig. 6A). In total, 87 hygromycin-resistant transformants were purified to single spores on hygromycin B selection medium, and several rounds of hyphal transfer from the edge of the colony were performed. The transformants were tested for relative expression of *IDH* 48 h after transfer to GLA-inducing SM at pH 7.0.

Physiological analysis of the *IDH*-RNAi mutants revealed growth and sporulation patterns similar to those of the WT strain when they were grown on potato dextrose agar (PDA). Putative *IDH*-RNAi transformants were confirmed by polymerase chain reaction (PCR) screening with FHyg and RHyg primers (Tables 1 and 2; Fig. 6B). Characterization of the WT and *IDH*-RNAi mutants showed that they did not differ significantly in GLA accumulation, and pH reduction after 48 h was similar for the WT, control vector strain, and *IDH*-RNAi mutants TPe₁₇ and TPe₇₇ (Fig. 6D); however, the mutants showed an 87.5% decrease in patulin accumulation (Fig. 6E). Analysis of the colonization pattern in fruit showed 28 and 12% inhibited colonization by TPe₁₇ and TPe₇₇, respectively (Fig. 6F), suggesting that patulin also contributes to *P. expansum* patho-

Fig. 5. Accumulation of D-gluconic acid (GLA), relative *IDH* expression, and patulin in solid secondary medium (SM) using increasing concentrations of A to C, glucose and D to F, GLA as carbon sources. Spores of isolate Pe-21 were inoculated into 0.2 M phosphate-buffered solid SM-agar adjusted to pH 7.0, and disc samples were taken 0, 24, 48, and 72 h later. Five replicates were sampled for each substrate. Average values $\pm$ standard error of five replicates of GLA, relative *IDH* expression, and patulin extractions are presented. Experiments were repeated three times and the results of a single representative experiment are shown.

genicity. Patulin's ability to directly induce cell death and, in this way, contribute to fungal colonization was detected when apple fruit were treated with the mycotoxin at 50 and 100 $\mu\text{g ml}^{-1}$ (Supplementary Fig. S1). Results indicated a three- to fourfold increase in cell death on the apple tissue, confirming early findings by Riley and Showker (1991) in animal tissue.

Differential modulation of GLA and patulin accumulation and pathogenicity by PACC-RNAi mutants.

To confirm the global regulation of GLA and patulin accumulation under changing pH, the relative expressions of *P. expansum* PACC, GOX2, and IDH were analyzed under nonbuffered conditions at pH 4 and 7. Growth of the WT strain on solid GLA-inducing medium at pH 7 resulted in overexpression of PACC, GOX, and IDH compared with *Penicillium* spp. grown at pH 4.0 (Fig. 7). These results suggest a dependence on alkaline conditions for activation of GOX2 and IDH for the accumulation of GLA and patulin.

Functional analysis of the downregulation of GOX2 and IDH and its effect on GLA and patulin accumulation and pathogenicity was carried out following downregulation of the transcription factor PACC, involved in the activation of genes

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence 5'→3'
F28S	GGAACGGGACGTCATAGAGG
R28S	AGAGCTGCATTCCCAACAAC
RGOX2 371	GGGCGTGTCAGGAACC
FGOX2 290	TTCACAACCGCACCGAGA
FIDH-RT	TACTGTGCTCGAACCGTCAC
RIDH-RT	TCTTTGCTAGCGACATCGAA
F_Idh_SnaBI+SphI	TGGGTACGTAAGCAATGGCATGCCATA AGTTCC
R_Idh_StuI+XhoI	TCAATCGCTGTTCTCGAGCAGGCCTCA TTCCG
FPACC-RT	ACGAATTCTTTGGCGACTTG
RPACC-RT	CATTGAGGAAGGGAAGCTGA
F_pacC_SnaBI+SphI	TGTACGTAAAAGTACCAACAACCTCAG CATGCCCTG
R_pacC_StuI+XhoI	GACCCTCGAGGAAGGGAAGCTGAAAGG CCTGCAAAAC
Fhyg	GTCTGCGGGTAAATAGCTG
Rhyg	ATTTGTGTACGCCGACAGT
gox Fwd single	GAACGAGGTACCTGGACTCGAGCACA CAAGGC
gox Rev single	CCACAAGTCAAGCTTTAGCATGCCAGCG GT

Fig. 6. Patulin and D-gluconic acid (GLA) accumulation, relative IDH expression, and pathogenicity on 'Golden Delicious' apple fruit induced by IDH-RNAi mutants of *Penicillium expansum*. **A**, Development of IDH-RNAi mutants using the psilent-1 plasmid to generate silenced IDH gene by using the inverted-repeat transgene of the IDH sequence; **B**, downregulated IDH TPe mutants were confirmed by amplification of a 0.75-kb polymerase chain reaction product of the hygromycin (Hyg) resistance gene; **C**, comparative relative IDH expression; **D**, GLA accumulation; **E**, patulin accumulation; **F**, colonization by the wild-type, the IDH-RNAi mutants TPe₁₇ and TPe₇₇, and the control vector strain ps6. Spores of the different strains were inoculated onto solid secondary medium–agar adjusted to pH 7.0, and samples were taken 48 h later. Five agar discs (10 mm in diameter by 0.5 mm thick) were extracted for analysis of GLA and patulin accumulation. Five replicates were sampled for each strain. Average values $\pm$ standard error of five replicates of relative IDH expression are presented. Comparisons of inhibition of patulin and GLA culture extractions, and colonized diameter from three inoculations of Pe-21 and of the IDH-RNAi mutants, were carried out on the same apple. Columns and symbols with different letters are significantly different at $P \leq 0.05$ according to the Tukey-Kramer multiple comparison test. Experiments were repeated three times and the results of a single representative experiment are shown.

modulated by pH, using RNAi technology (Fig. 8A). In total, 120 hygromycin-resistant transformants were purified to single spores on hygromycin B selection medium, and several rounds of hyphal transfer from the edge of the colony were performed. The transformants were tested for relative *PACC* expression 3 h after transfer to GLA-inducing SM at pH 7.0. Two *PACC*-RNAi transformants—T_{Pe₃₄} and T_{Pe₁₀₉}—that were found to downregulate relative *PACC* expression, were used for further characterization. Putative *PACC*-RNAi transformants were confirmed by PCR screening with FHyg and RHyg primers (Tables 1 and 2; Fig. 8B). PCR analysis of the *PACC*-RNAi transformants T_{Pe₃₄} and T_{Pe₁₀₉} (Table 1) generated the expected 750-kb PCR fragment (Fig. 8B).

Physiological analysis of the *PACC*-RNAi mutants revealed growth and sporulation patterns similar to those of the WT strain when they were grown on PDA. Functional characterization of *PACC*-RNAi mutants during growth on SM adjusted to

pH 7.0 showed significant downregulation of *PACC* (by 44 and 72% for T_{Pe₁₀₉} and T_{Pe₃₄} mutants, respectively) compared with the WT Pe-21 and the transformant control vector (Fig. 8C). The downregulation of relative *PACC* expression by the T_{Pe₃₄} and T_{Pe₁₀₉} mutants reduced disease development by 46 and 40%, respectively, 5 days after inoculation of ‘Golden Delicious’ apple fruit (Fig. 8F). Moreover, *PACC*-RNAi mutants T_{Pe₃₄} and T_{Pe₁₀₉} showed a 63 and 27% reduction, respectively, in GLA production (Fig. 8D) and a 92 and 95% reduction, respectively, in patulin accumulation 2 days after inoculation of SM-solid plates adjusted to pH 7 (Fig. 8E). These results indicate that, whereas downregulation of *IDH* only reduces patulin accumulation without affecting GLA levels, downregulation of *GOX2* reduces both GLA and patulin accumulation and has an additive effect on the mutants’ reduction in pathogenicity.

DISCUSSION

pH and patulin production.

Patulin is a common secondary metabolite present in decayed tissue colonized by *P. expansum* and other *Penicillium* spp. (Puel et al. 2010). Following inoculation of the *P. expansum* isolate Pe-21, we detected the accumulation of GLA at 2.5 µg per gram of FW in the colonized tissue, expression of the gene encoding the last enzyme of patulin biosynthesis (*IDH*), and patulin accumulation to a concentration of 8.0 µg per gram of FW decayed tissue. Barad and associates (2012) suggested that pathogenicity of *P. expansum* is modulated by changes in pH mediated by GLA accumulation as a factor for activation of polygalacturonase under acidic conditions. However, the joint accumulation in the colonized tissue of two fungal metabolites, GLA and patulin, raised the question of their contribution, either separately or synergistically, to *P. expansum* pathogenicity.

Our first approach to analyze the contribution to pathogenicity of the accumulated GLA and patulin was to modulate GLA accumulation by *GOX2*-RNAi mutants. Using a series of *GOX2*-RNAi mutants, Barad and associates (2012) demonstrated that downregulation of *GOX2* is accompanied by impairment in the ability to produce GLA and apple colonization. Using the same mutants, we observed that the higher the impairment in GLA accumulation, the higher the inhibition of relative *IDH* expression and patulin accumulation. Impairment in the ability to produce GLA by 98% (T_{Pe₁₄₁} compared with the WT) was accompanied by 90% downregulation in the relative expression of *IDH* and 94% reduction in patulin accumulation (from 11.6 to 0.5 µg per gram of DW) (Fig. 2B). The question was whether the low pH or the dynamic process of GLA accumulation that enhances the pH decrease is the factor that activates *IDH* overexpression and patulin accumulation. To answer this question, we cultured the *GOX2*-RNAi mutants of *P. expansum* with differential capabilities of acidification (Barad et al. 2012) in solid media at initial pH of 4.5 and 7.0. Growth at pH 4.5 was previously shown by Barad and associates (2012) and Hadas and associates (2007) to inhibit the accumulation of GLA. In our experiments, growth at pH 4.5 eliminated the differential expression of *GOX2* in the *GOX2* mutants and reduced patulin accumulation to similar low levels in all mutants. In contrast, if the *GOX2*-RNAi mutants were cultured at an initial pH of 7.0, the differential activation of *GOX2* led to differential accumulation of GLA and patulin (Fig. 2), confirming the earlier reports indicating enhanced expression of *GOX2* at high pH (Barad et al. 2012; Hadas et al. 2007). These results suggest that the concurrent dynamic pH regulation by GLA accumulation and not low pH per se is the critical factor in the induction of this secondary metabolism of patulin production and fungal colonization.

Fig. 7. Relative gene expression (RE) of *GOX2*, *IDH1*, and *PACC* of *Penicillium expansum* determined by quantitative reverse-transcription polymerase chain reaction at pH 4 and 7. Average $\pm$ standard error of relative expression of three selected *P. expansum* genes on the second day of growth on solid secondary medium. Experiments were repeated three times and the results of a single representative experiment are shown.

To demonstrate the cumulative effect of differential *GOX2* expression on patulin accumulation as a function of time, the *GOX2*-RNAi mutants were cultured at an initial pH 7 for 24, 48, and 72 h. Under these conditions, differential *GOX2* expression was directly related to patulin accumulation in each of the three sampling periods. In all of the sampling periods, 98% decreased accumulation of GLA in the *GOX2*-RNAi mutant TPe₁₄₁ reduced patulin production by 56% after 72 h. Interestingly, only a 0.2 pH unit difference was observed between the WT and the mutant with the most reduced pathogenicity, where a 100% increase in level of patulin was observed. This supports our previous finding that it is not the final pH of the medium but fungal exposure to the dynamic GLA-accumulation process that is important for the induction of patulin production.

The metabolic capability for patulin accumulation by *P. expansum* under conditions of GLA accumulation and decreasing pH suggests that GLA might be one of the precursors for patulin's biosynthetic accumulation. This might explain McCallum and associates' (2002) results where, without knowing the cause for the pH decrease, they found that more aggressive isolates of *P. expansum* greatly reduce the pH of the

growth medium and produce detectable levels of patulin, whereas the weaker growers produce a smaller reduction in pH and no detectable patulin. To further determine the relationship between GLA and patulin, *P. expansum* was grown in the presence of sucrose, glucose, or GLA as sole carbon source, with sucrose being the most efficient precursor for patulin accumulation. Increasing concentrations of glucose increased the accumulation of GLA followed by patulin 1 day later. Growth on glucose yielded accumulation of higher amounts of patulin than growth on GLA, indicating that, whereas different substrates might behave as metabolic precursors for patulin, they differ in their efficiencies. Interestingly, few reports have referred to the sources for carbon metabolism for patulin biosynthesis (Puel et al. 2010). Like aflatoxin, glucose is an optimal carbohydrate source for patulin production but it is unclear whether the carbon source directly induces patulin production or modulates its biosynthesis through general metabolism. The present results indicate that glucose and GLA act as substrate precursors for patulin production with differential efficiency. Previously, Ramachandra and associates (2006) suggested that the GLA may be metabolized to patulin through glycolysis to pyruvate and, from there, probably via general metabolism to

Fig. 8. Patulin and D-gluconic acid (GLA) accumulation, relative *PACC* expression, and pathogenicity on 'Golden Delicious' apple fruit induced by *PACC*-RNAi mutants of *Penicillium expansum*. **A**, Development of *PACC*-RNAi mutants using the psilent-1 plasmid to generate silenced *PACC* using the inverted-repeat transgene of the *PACC* sequence; **B**, downregulated *PACC* TPe mutants were confirmed by amplification of a 0.75-kb polymerase chain reaction product of the hygromycin (*Hyg*) resistance gene; **C**, comparative relative *PACC* expression; **D**, GLA accumulation; **E**, patulin accumulation; **F**, colonization of wild-type, *PACC*-RNAi mutants TPe₃₄ and TPe₁₀₉, and the control vector TPe₆. Spores of the different strains were inoculated onto solid secondary medium-agar-containing plates adjusted to pH 7.0 and samples were taken 48 h later. Five agar discs (10 mm in diameter by 0.5 mm thick) were extracted for analysis of GLA and patulin accumulation. Five replicates were sampled for each strain. Average values $\pm$ standard error of five replicates of *PACC* expression are presented. Comparisons were carried out of inhibition of patulin and GLA culture extractions and colonization by three inoculations of Pe-21 and the *PACC*-RNAi mutants on the same apple. Columns and symbols with different letters are significantly different at $P \leq 0.05$ according to the Tukey-Kramer multiple comparison test. Experiments were repeated three times and the results of a single representative experiment are shown.

patulin, suggesting the importance of GLA accumulation during early stages of *P. expansum* colonization of fruit as a precursor for patulin accumulation (Barad et al. 2012; Hadas et al. 2007).

GLA, patulin and pathogenicity.

Our second approach to differentiating the contributions to pathogenicity of the accumulated GLA and patulin was to modulate patulin accumulation using *IDH*-RNAi mutants. No differences were found in GLA accumulation or pH modulation by the *IDH*-RNAi mutants versus the control strains when grown at an initial pH of 7.0. However, whereas the WT, control vector, and *IDH*-RNAi mutants all produced a similar level of GLA (8.5 to 11.0 g per gram of DW) and similarly reduced the pH from 7 to almost 3.6, the *IDH*-RNAi mutants reduced patulin accumulation by almost 85% and pathogenicity by 12 to 28% (Fig. 6). These results confirmed the claim of Sanzani and associates (2012) that knockout of 6-MSAS, an enzyme involved in the first committed step of patulin biosynthesis, inhibits fungal colonization. Those mutants, obtained by gene-disruption technique, continued to produce 68 to 59% of the patulin produced by the WT strain, leading to a 34 and 54% reduction in disease incidence and severity, respectively. In our work, downregulation of *IDH* by almost 92% reduced patulin production by almost 89% but reduced colonization by only 12 to 28%; this suggested that, although patulin contributes to the process of colonization, it has a lower effect than the level of colonization inhibition reported by the knockout of 6-MSAS in the *P. expansum* strain used by Sanzani and associates (2012). The phenotypic response of the host to patulin accumulation is reasonable considering the previously reported cytotoxic cell response, which was thought to indicate that patulin contributes to fungal colonization by enhancing cell death (Barhoumi and Burghardt 1996; Riley and Showker 1991), and this was confirmed in apple tissue. These results indicate that the observed accumulation of patulin is an independent factor modulating pathogenicity of *P. expansum*. However, the contribution of patulin to pathogenicity is enhanced under increased accumulation of GLA, as observed during colonization of the fruit tissue.

The effect of acidification by GLA accumulation on patulin and pathogenicity of *P. expansum*.

Several reports have already indicated that the production of secondary metabolites is determined by generic factors and associated cellular regulatory systems, such as the global nitrogen, carbon, and pH regulatory systems common to most fungi (Yu and Keller 2005). Nitrogen and carbon source and low pH play key roles in the regulation of sterigmatocystin or aflatoxin production in *Aspergillus* spp. (Kachholz and Demain 1983; Keller et al. 1997a; Woloshuk et al. 1997). In *Aspergillus nidulans*, low pH is required for sterigmatocystin production, whereas alkaline pH is required for penicillin production (Keller et al. 1997a; Shah et al. 1991). Low pH is also conducive to the production of fumonisin by *Fusarium proliferatum* (Keller et al. 1997b) and ochratoxin by *A. ochraceus* (O'Callaghan et al. 2006). There are no published reports on pH modulation as a factor in *P. expansum* pathogenicity. However, the higher expression of *GOX2* and *IDH* at pH 7 compared with pH 4 suggested that pathogenicity factors that modulate *P. expansum* colonization might be regulated by the transcription factor PACC. At acidic pH, the PACC protein is in an inactive conformation and is unable to bind to the promoter sites of the target genes but, under alkaline growth conditions, activated PACC acts as both an activator of alkaline-expressed genes and a repressor of acid-expressed genes (Penalva et al. 2008). Loss-of-function mutations in PACC

cause an acidity-mimicking phenotype and result in increased expression of “acid” genes and reduced expression of “alkaline” genes, as was found for *Colletotrichum gloeosporioides* (Miyara et al. 2012). Under neutral or alkaline conditions, PACC has been reported to regulate many genes in *A. nidulans*, *P. chrysogenum* (Denison 2000; Eisendle et al. 2004; Espeso and Arst 2000), *C. gloeosporioides* (Miyara et al. 2008, 2010), and *Sclerotinia sclerotiorum* (Kim et al. 2008), as was found for *P. expansum*. In *S. sclerotiorum*, which regulates acidification of the environment with oxalic acid, site-directed mutagenesis of *pac1* created a dominant activating *pac1^c* mutation that resulted in the activation of *pac1*, leading to oxalic acid accumulation that was largely independent of ambient pH (Kim et al. 2008). This indicated a positive role for *pac1* in oxalic acid regulation. Functional analysis of PACC-RNAi mutants of *P. expansum* was used as our third approach to determining the contributions of GLA and patulin during pathogenicity and pH regulation by PACC. PACC-RNAi mutants reduced GLA and patulin accumulation and produced a 45% reduction in pathogenicity, while the pH decreases in the WT and mutants were similar. These results suggest that, in *P. expansum*, PACC has a significant role in both factors that modulate pathogenicity (i.e., GLA and patulin accumulation). These results are similar to those obtained with *Sclerotinia* spp., whose oxalic acid production increased with increasing ambient pH (Maxwell 1973; Rollins 2003; Rollins and Dickman 2001). In *P. expansum*, PACC also displayed pH-regulated expression, its steady-state levels increasing with growth medium pH. How GLA and patulin accumulation are regulated during fungal pathogenicity in fruit is still not clear. However, aside from the effect of GLA on patulin accumulation, previous work has indicated that nitrogen level may regulate the expression of patulin metabolism. Grootwassink and Gaucher (1980) suggested that a distinct metabolic profile can be expressed in cultures containing ammonium as the nitrogen source. When ammonium ions were added to cultures producing patulin, rapid downregulation of *IDH* and 6-MSAS transcripts and patulin accumulation was observed (Rollins and Gaucher 1994). Maceration of fruit tissue by pectolytic enzymes may lead to differential accumulation of nitrogen precursors that affect pathogen metabolism, as was reported for the downregulation of sterigmatocystin and ochratoxin A (Abbas et al. 2009; Feng and Leonard 1998). These changes might explain, in part, the significant differences in patulin accumulation, given the differential nitrogen contents of host fruit. Further research is needed to fully understand the mechanism governing the downregulation of genes modulating patulin biosynthesis. The presence of several putative GATA sites along the upstream regions of both the 6MSAS and *IDH* genes of *P. griseofulvum*, which might interact with a global nitrogen regulatory factor encoded by the *nrfa* gene (an *AreA*-orthologous gene), was suggested as a possible mechanism for the modulation of patulin accumulation (Ellis 1996).

In summary, GLA appears to play a central role in pH responsiveness. Its production is a pH-regulated process and its accumulation, by virtue of environmental acidification, may serve as a regulator of acid pH-regulated processes such as *PGI* expression (Hadas et al. 2007; Prusky et al. 2004). On the other hand, *P. expansum* uses GLA accumulation during modification of the ambient pH environment as a regulatory cue for disease and accumulation of secondary metabolites such as patulin.

MATERIALS AND METHODS

Fungal strains and culture conditions.

The WT *P. expansum* isolate Pe-21 was obtained from decayed apple fruit (*Malus domestica* ‘Golden Delicious’) pur-

chased from a local market in Israel (Hadas et al. 2007). Cultures were grown at 27°C in the dark and maintained on PDA plates (Difco, Detroit) unless otherwise indicated. Conidia were harvested with 10 ml of sterile distilled water supplemented with 0.01% (vol/vol) Tween 80 (Sigma-Aldrich, Copenhagen, Denmark). When required, hygromycin B (Roche, Branchburg, NJ, U.S.A.) at 250 µg ml⁻¹ was added to the growth medium. Cells were visualized with a model BX60F-3 microscope and a model SZ-60 stereoscope (both from Olympus America, Inc., Melville, NY, U.S.A.).

Assay for colonization and disease development.

To assess disease development, 'Golden Delicious' apple fruit were inoculated by placing 5 µl of a conidial suspension containing 10⁶ spores per milliliter on each of six 2-mm deep, 2-mm diameter wounds spaced around a circle on the upper part of the stem end of the fruit. Following inoculation, the fruit were incubated at 25°C in covered plastic containers containing wet paper towels until decay symptoms developed.

Analysis and quantification of total GLA production by *P. expansum*.

To evaluate total GLA (i.e., free GLA and D-glucono-δ-lactone) level in all of the transformant cultures, 10⁶ fungal spores per milliliter were inoculated into 40 ml of a primary medium, (i.e., glucose minimal medium [GMM]) in 125-ml flasks containing (per liter) 10 g of glucose, 5 g of yeast extract (Difco), 50 ml of nitrate salts, and 1 ml of trace elements, at pH 6.5. The cultures were incubated at 25°C with shaking at 150 rpm for 48 h. Cultures were harvested by vacuum filtration through a sterile Büchner funnel fitted with a filter paper (Whatman No. 1), and the remaining mycelia were washed twice with 50 ml of sterile distilled water. The washed mycelia were resuspended in 50 ml of GLA-inducing SM containing (per liter) 60 g of sucrose, 7 g of NaNO₃, 3 g of tryptone (Difco), 1 g of KH₂PO₄, 0.5 g of MgSO₄ · 7H₂O, and 0.5 g of KCl, adjusted to pH 7.0. The culture was incubated at 25°C on a rotating shaker at 150 rpm for 48 h, unless otherwise specified. For experiments with modified pH, the initial pH of the SM was adjusted to the desired level with either 10 N NaOH or concentrated HCl. After 48 h of growth, aliquots of the suspensions were collected and the amounts of GLA produced were measured with an enzymatic method for measuring total GLA content (free GLA and D-glucono-δ-lactone) (Boehringer Mannheim, Tokyo) according to the manufacturer's instructions. Similarly, mycelium was frozen in liquid nitrogen and lyophilized for dry weight determination or RNA extraction. To check the GLA concentration in SM-agar media, five 1-cm²-diameter discs were placed in 5 ml of sterilized water and homogenized with an HG-300 homogenizer (MRC, Calgary, Canada). A 1-ml aliquot of the solution was sampled into a 1.5-ml Eppendorf tube and was centrifuged for 5 min at 20,800 × g. The supernatant was taken for GLA analysis.

Analysis and quantification of patulin production by *P. expansum*.

To evaluate the patulin level in culture, 100 µl of a 10⁶ fungal spores per milliliter solution was grown on 55-mm-diameter petri dishes containing 10 ml SM-agar for 48 h at 27°C (unless otherwise specified). Five discs of 1 cm² in diameter were added to 5 ml of sterilized water and homogenized by HG-300 homogenizer. Patulin was extracted twice with 10 ml of ethyl acetate by shaking for 1 min using a vortex mixer and centrifuging for 5 min at room temperature at 3,200 × g. The organic phase was extracted with 10 ml of a 1.5% sodium carbonate solution by shaking for 1 min using a vortex mixer and centrifuging for 5 min at room temperature, 3,200 × g. The

sample was completely dried, redissolved in 0.5 ml of the elution solution 0.02 M ammonium acetate and acetonitrile (9:1, vol/vol), and filtered through a 0.2-µm Minisart filter (Sartorius Stedim, Goettingen, Germany) before injecting into the column. Quantitative analysis of patulin was performed by a high-performance liquid chromatograph (HPLC) (Hitachi-Merck, Dartford, U.K.) equipped with a UV-VIS detector at 280 nm, with an RP-18 5-µm column (Merck, Amsterdam). The concentrate (50 µl) was injected to the HPLC equipped with a C18, 250-by-4.6-mm, Microsorb-MV-100-5 column, at a rate of 0.8 ml/min. The patulin fraction was eluted with 0.02 M ammonium acetate and acetonitrile (9:1, vol/vol) with a retention time of approximate 7 min. Results were compared with a commercial patulin standard (Sigma-Aldrich).

pH measurements.

pH was measured with a double-pore slim electrode (Hamilton, Reno, NV, U.S.A.) connected to a Thermo Orion Model 720A Plus pH meter. The pH of the medium was measured in the homogenized disc plate or in the Erlenmeyer with liquid media.

Nucleic acid analysis.

Extraction of DNA from fungi, restriction-enzyme digestion, and gel electrophoresis were performed by standard methods (Sambrook et al. 1989). DNA extraction was carried out using the MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, U.S.A.). RNA extraction was carried out using the SV Total RNA Isolation kit (Promega, Madison, WI, U.S.A.). Purity of the extracted RNA was assayed by the ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.), and the RNA was then stored at -80°C until further analysis.

Construction and characterization of *PACC*- and *IDH*-RNAi mutants.

To assess the functions of *pacC*, we developed strains in which *PACC* or *IDH* was silenced by RNAi technology using the inverted-repeat transgene of the *PACC* or *IDH* sequence (Barad et al. 2012). The silencing vector pSilent-1 (Nakayashiki et al. 2005) was used to generate the *PACC*-RNAi or *IDH*-RNAi constructs. A 580-bp fragment of *pacC* and 647 bp of *IDH* with no predicted introns were amplified using the primers F_pacC_SnabI+SphI, R_pacC_StuI+XhoI and F_idh_SnabI+SphI, R_idh_StuI+XhoI, respectively. The resulting fragments contained the engineered restriction sites 5' *pacC* *SnabI/SphI* and 3' *pacC* *StuI/XhoI* or 5' *idh* *SnabI/SphI* and 3' *idh* *StuI/XhoI*, respectively. First, the restriction sites *SphI* and *StuI* were used to insert the fragment in the forward orientation into pSilent-1. Next, the restriction sites *SnabI* and *XhoI* were used to insert the fragment in the reverse orientation. The resulting plasmid was used for Pe-21 transformation.

Fungal transformation of protoplasts was performed essentially according to Miller and associates (1985). In short, spores (5 × 10⁷) originated from 7-day-old cultures were germinated in GMM for 8 h and filtered through Whatman No. 1 paper. The collected spores were then transferred to a filtered (0.45-µm filter) enzyme mixture prepared in 15 ml of osmotic medium (147.9 g MgSO₄ in 10 mM NaPO₄ buffer adjusted to pH 5.8 in 500 ml) containing 0.1 g of lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich), 0.05 g of cellulose from *A. niger* (Fluka Japan, Tokyo), 0.1 g of novozyme (InterSpex Products, Inc., Foster City, CA, U.S.A.), and 0.1 g of Yatalase (Takara, Shiga, Japan). The spores were shaken overnight at 27°C and 50 rpm and transferred to osmotic buffer that enabled the protoplast collection. The protoplasts were then transformed as reported by Miller and associates (1985). Strains exhibiting

impaired expression of the gene were tested by PCR for the presence of the hygromycin fragment: a 0.75-kb PCR fragment was obtained by using forward and reverse primers from the hygromycin cassette (Fhyg and Rhyg, respectively) (Table 2).

Gene-expression analysis by quantitative reverse-transcription PCR analysis.

To examine gene expression, cultures of the WT isolate Pe-21 and RNAi mutants were cultured as indicated above for measurement of GLA level. RNA was extracted from 80-mg samples of frozen mycelia with the SV Total RNA isolation kit. The reverse-transcription reaction was performed on 1 µg of total RNA with the Reverse-it First-Strand Synthesis Kit (Abgene, Surrey, U.K.), and cDNA samples were diluted 1:10 (vol/vol) with ultrapure water.

Real-time quantitative PCR was performed with the Rotor-Gene 3000 system (Corbett Research, Cambridgeshire, U.K.). PCR amplification was performed with 3.4 µl of cDNA template in 10 µl of a reaction mixture containing 6.6 µl of mix from the SYBR Green Amplification Kit (ABgene) and 300 nM primers: the forward and reverse primers for the *GOX 2* amplicon were FGOX2-290 and RGOX2-371, respectively; those for the *IDH* amplicon were FIDH-RT and RIDH-RT, respectively; those for the *PACC* amplicon were FPACC-RT and RPACC-RT, respectively; and those for the 28S rRNA of *P. expansum* were F28S and R28S, respectively (Table 2). Results were analyzed with the Rotor-Gene 6 software. The 28S rRNA gene served as an endogenous control and the calibration sample varied according to the experiment, as indicated. A mixture of all cDNAs was used in all treatments as a template for calibration curves designed for each pair of primers, to verify that a single PCR product had been formed. Relative quantification was calculated with the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). The ΔC_T value was determined by subtracting the cycle threshold (C_T) results for the target gene from those for the endogenous control gene and normalized against the calibration sample to generate the $\Delta\Delta C_T$ values. Each experiment was performed in triplicate, and three different biological experiments were conducted. One representative set of results is presented. Mean values of $2^{-\Delta\Delta C_T} \pm$ standard error of each treatment were subjected to mean comparisons by least significant difference (LSD), according to the Tukey-Kramer multiple comparison test at $P < 0.05$.

Statistical analysis.

Data were analyzed with the JMP software package (version 3.2.6; SAS Institute, Cary, NC, U.S.A.). Mean comparisons of *GOX2* expression, GLA and patulin production, and ambient pH measurements were analyzed according to LSD, with the Tukey-Kramer multiple comparison test at $P \leq 0.05$. Frequencies of germination were pooled for each set of three replications per treatment or isolate, after application of Student's *t* test to determine homogeneity of variance among the three independent experiments, and no statistically significant ($P \leq 0.05$) differences among experiments were found.

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