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Effect of quercetin and umbelliferone on the transcript level of *Penicillium expansum* genes involved in patulin biosynthesis

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Abstract *Penicillium expansum* is commonly associated with patulin accumulation in pome fruits. In *in vitro* studies, two phenolic compounds (quercetin and umbelliferone) proved to be effective in reducing patulin accumulation, particularly when applied in combination, without consistently affecting mycelial growth. To investigate the mode of action of quercetin and umbelliferone, the expression of five genes likely involved in patulin biosynthesis was evaluated using real-time PCR in the presence and absence of the tested phenolic compounds. The relative expression of genes coding isoepoxydon dehydrogenase (*IDH*), 6-methylsalicylic acid synthase (*msas*) and an ATP-binding cassette transporter (*peab1*) proved to be down-regulated when quercetin and umbelliferone were added in combination. Furthermore, the relative expression of two putative cytochrome P450 monooxygenases (*p450-1* and *p450-2*) was reduced by all treatments, although the combination of the two substances was the most effective. These results provide evidence that quercetin and umbelliferone reduce patulin accumulation by acting on the transcription level of the tested genes.

Keywords Blue mould · Gene expression · Patulin · Quercetin · Real-time PCR · Umbelliferone

Abbreviations

AJA	apple juice agar
Ct	cycle threshold
DPI	days post-inoculation
DMRT	Duncan's Multiple Range Test
HPLC	high performance liquid chromatography
RT-PCR	reverse transcriptase PCR
SEM	standard error of mean

Introduction

Blue mould caused by *Penicillium expansum* is one of the most destructive rots of pome fruits in all the producing countries. Besides fruit loss caused during storage and shelf-life, *P. expansum* also has potential public health significance, since it produces the

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mycotoxin patulin, especially in apple products (Neri et al. 2006). Mycotoxins are low-molecular-weight secondary metabolites produced by toxigenic filamentous fungi and can cause disease and death in human beings and other vertebrates (Bennett and Klich 2003). These metabolites constitute a chemically heterogeneous assemblage and are produced only by some species of a genus and only by some strains of a species (Demain 1996). Most of them are formed via biosynthetic pathways and their production is most likely to occur at sub-maximal growth rates. Although in recent years, several efforts have been made to control toxigenic fungi, mycotoxins are still very common in food supplies all over the world, notably in cereal grains, nuts and fruits (Murphy et al. 2006).

Patulin is produced by >60 species of moulds encompassing >30 genera (Lai et al. 2000), with *P. expansum* being generally regarded as the main producer in apples. It was first isolated as an antimicrobial active compound from *Penicillium griseofulvum* in the 1940s. However, during the 1950s and 1960s, it became evident that, in addition to its antibacterial, antiviral, and antiprotozoal activities, patulin was toxic to both plants and animals and therefore it was reclassified as a mycotoxin (Iwahashi et al. 2006). In 2006, the European Commission imposed strict regulatory limits on the patulin content allowed in foods, particularly in baby food (European Commission 2006).

Control of blue mould caused by *P. expansum* is commonly achieved by fungicides, but, the appearance of resistant strains and consumer concern about food and environmental safety are leading to an increasing demand for alternative means of control (Mari et al. 2002). Among these, a number of biocontrol agents and natural compounds have been shown to reduce apple rot during harvest, processing and storage procedures (Neri et al. 2006; Ippolito et al. 2000; Schena et al. 2007) although few of them have been assessed for their effect on patulin production. Furthermore, several reports have highlighted the role of phenolic compounds in protecting plants from competitors, predators, abiotic stresses and pathogens, including postharvest pathogens (Ben-Yehoshua 2003; El-Ghaouth 1997).

Among phenolic compounds, flavonoids and coumarins are known to have useful antimicrobial properties. Quercetin is one of the most abundant flavonoids in apples, known for its antioxidant properties (Nijveldt et al. 2001) and for being a constituent of plant extracts with anti-toxigenic properties (Mossini et al. 2004; Biswas et al. 2002). Coumarin umbelliferone, together with scopoletin and scoparone, is reported to be involved in the resistance of citrus to *Penicillium* spp. (Afek et al. 1999) and to possess anticoagulant, anti-inflammatory and analgesic properties (Repetto and Llesuy 2002).

In a recent study, we found that exogenous applications of quercetin and umbelliferone are effective in controlling blue mould and patulin accumulation in apples (Sanzani et al. 2009). However, no specific information is available about their mode of action.

The pathway of patulin biosynthesis has been established using almost only mutants and by examining the time of appearance of intermediates in the pathway, although ongoing studies continue to produce new intermediaries (White et al. 2006). Biosynthesis is thought to involve a series of condensation and reduction/oxidation reactions, many, if not all, of which are enzyme-catalysed (White et al. 2006). Until recently, only two genes encoding enzymes of the patulin biosynthetic pathway had been cloned and sequenced from *Penicillium urticae*: the 6-methylsalicylic acid synthetase (*msas*) gene (Beck et al. 1990) and an NADPH-dependent isoepoxydon dehydrogenase (*IDH*) gene (Gaucher and Fedechko 2000). In 2006, White et al. (2006) identified and sequenced five genes encoding putative patulin biosynthesis enzymes from *P. expansum,* including *msas* (DQ084387) and *IDH* homologues (DQ084388). Furthermore, they obtained a partial sequence of genes coding two cytochrome P450 monooxygenases (*p450-1* and *p450-2*) and a gene coding an ATP-binding cassette (ABC) transporter (DQ084389, DQ084390 and DQ084391, respectively). These genes are reported to be involved in patulin biosynthesis and release in *Penicillium* spp. (Murphy and Lynen 1975; Gaucher and Fedechko 2000).

In the present study, the mechanisms by which quercetin and umbelliferone reduce patulin production was investigated by means of real-time PCR, i.e. one of the most powerful techniques for studying plant and pathogen responses to biotic and/or abiotic factors (Schena et al. 2004). In particular, the expression levels of five genes (*IDH*, *p450-2, msas*, *p450-1* and *peab1*) likely involved in the toxin biosynthetic pathway, were evaluated in the absence

and presence of the two phenolic compounds, applied singly or in combination.

Materials and methods

Chemicals

Acid-washed glass beads (Ø 5 mm), TRI Reagent® (RNA, DNA and Protein Isolation Reagent), patulin (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one), quercetin (3,3′,4′,5,7-pentahydroxyflavone dihydrate) and umbelliferone (7-hydroxyl-coumarin) were purchased from Sigma (Sigma-Aldrich, Milan, Italy). High performance liquid chromatography (HPLC) grade water was obtained by means of a Milli-Q system (Millipore, Bedford, MA). Solvents were HPLC grade (Mallinckrodt Baker, Milan, Italy).

Fungal cultures and phenolic solutions

The toxigenic *P. expansum* strain 7015, deposited in the 'Toxigenic Fungi Culture Collection' of the Institute of Sciences of Food Production (ISPA – Bari, Italy) and the non-toxigenic *P. expansum* strain FV 268, deposited in the 'Fungi Culture Collection' of the Department of Plant Protection and Applied Microbiology (University of Bari, Italy), were used in this study. Inoculum was produced by culturing fungi for 8 days at 24°C in the dark on a medium (AJA) prepared by dissolving 20 g agar powder (Oxoid, Hampshire, UK) in 1 l of pasteurised commercial apple juice, previously adjusted to pH5.5 with 1 M NaOH. The surface of the culture was rinsed with 6 ml of sterile distilled water containing 0.05% (v v^{-1}) Tween 80. The resulting conidial suspension was filtered through two layers of sterile gauze and spore counts were made in a Thoma counting chamber (HGB Henneberg-Sander GmbH, Lutzellinden, Germany). A diluted conidial suspension with a final concentration of 5×10^4 conidia ml^{-1} was used in all trials.

Phenolic compound stock solutions were prepared at a concentration of 5000 µg ml^{-1} for both quercetin and umbelliferone or at a concentration of 10,000 µg ml^{-1} when combined (1:1 ratio). All solutions were obtained by dissolving the pure standards of each compound in a mixture of phosphate buffer (50 mM, pH7.4) NaOH (1 M, pH 13) $^{-1}$ (9:1 v v^{-1}, pH13).

Patulin determination

Patulin was extracted by adding 3 ml of acidified distilled water (pH adjusted to 4 with pure acetic acid) to the AJA medium and by scraping off the mycelium with a sterile spatula. The rinse water was collected, centrifuged at 10,000*g* for 5 min at room temperature (Beckmann centrifuge, Allegra X 22, Fullerton, CA, USA), filtered through a 0.45 µm syringe filter (Albet, Murcia, Spain) and analysed by HPLC. Results were expressed as µg of patulin ml^{-1} of rinsing water.

HPLC analyses were performed by injecting 20 µl of the filtrate extract into a liquid chromatograph (ThermoQuest Inc. Parkway San José, CA, USA) equipped with a quaternary gradient pump capable of delivering a constant flow rate of 1 ml min^{-1} (Spectraseries gradient pump P4000), a vacuum membrane degasser (SCM 1000), an autosampler injection system with a 50 µl loop (AS 3000), a column oven set at 30°C, a diode array detector (DAD, UV 6000 LP detector) set at 276 nm, and chromatography data system for Windows 2000 (ChromQuest version 2.53). A Phenomenex C_{18} Synergy Hydro column (250 × 4.6 mm, 4 µm particle size) (Phenomenex, Torrance, CA, USA) with a guard filter (3 mm, 0.5 µm pore size) was used. The mobile phase was a slight modification of the one reported by MacDonald et al. (2000) and consisted of a mixture of water, acetonitrile and perchloric acid (96:4:0.1, v v^{-1} v^{-1}).

In vitro effect of phenolics on *P. expansum* growth and patulin accumulation

Tests were conducted in Petri dishes containing 12 ml AJA supplemented or not supplemented (control) with phenolics. In the former, quercetin (5,000 µg ml^{-1}), umbelliferone (5,000 µg ml^{-1}) or their combination (10,000 µg ml^{-1}) were incorporated into melted AJA to obtain a final concentration of 10 µg ml^{-1} of each single compound. Petri dishes were centrally inoculated with 10 µl of a spore suspension (5×10^4 conidia ml^{-1}) of strain 7015 and incubated in the dark for 14 days at 16°C and high relative humidity (RH). All tests were performed in triplicate. Colony diam (mean of the two orthogonal diam) and patulin production (µg ml^{-1}) were recorded at 8 and 14 days post-inoculation (DPI).

The effect exerted by phenolics was expressed by a reduction index (RI, percentage of reduction

of colony diam or patulin accumulation) and calculated using the formula RI (%)=[(A–B) A^{-1}]×100, where A and B correspond to the mean colony diam (mm) or mean patulin level (μg ml^{-1}), measured for not supplemented and supplemented AJA, respectively.

Data were subjected to ANOVA (one-way analysis of variance). Significant differences ($P \leq 0.05$) between mean levels of patulin accumulation and colony diam were identified by the General Linear Model (GLM) procedure applying Duncan's Multiple Range Test (DMRT). Data were processed using the statistical software package Statistics for Windows (StatSoft, Tulsa, OK).

RNA isolation and DNase treatment

Total RNA was extracted from 100 mg of fungal mycelium collected by scraping from AJA plates with a sterile spatula. The mycelium was immediately homogenised for 45 s in microcentrifuge tubes containing 1 ml of TRI REAGENT® and 100 mg of glass beads, using a Fast-prep instrument (Savant Instruments, BIO 101 Savant FP 120, Holbrook, New York, USA) set to a speed of 6 m s^{-1}. The homogenate was chilled in ice for 5 min and centrifuged at 13,000*g* and 4c for 10 min. The supernatant was kept at room temperature for 5 min, supplemented with 200 μl of chloroform, shaken vigorously for 15 s, kept at room temperature for 5 min and then centrifuged at 13,000*g* and 4°C for 10 min. The upper aqueous phase was double-precipitated with isopropanol and 75% ethanol, air-dried and dissolved in 50 μl of nuclease-free water.

To avoid DNA contamination, all RNA samples were subjected to a DNAase treatment. Digestion reactions, containing 6 μl of 10× RQ1 Reaction Buffer (Promega, Milan, Italy), 3 μl of DNAse-Rnase free RQ1 (1 U μl^{-1}, Promega) and 1 μl RNasin® Ribonuclease Inhibitor (40 U μl^{-1}, Promega), were incubated at 37°C for 1 h. Samples were adjusted to a final volume of 200 μl with nuclease-free water, subjected to standard phenol: chloroform extractions and precipitated by adding 0.1 volume of sodium acetate 3 M and 0.7 volume of isopropanol. The pellet was rinsed with 75% ethanol, air-dried, dissolved in 50 μl nuclease-free water and stored at −80°C until needed. RNA yield and purity was determined using a spectrophotometer (Beckman DU 640 Spectrophotometer, Corona, CA, USA) and by electrophoresis in a denaturing agarose gel (1.5%, g v^{-1}).

Amplification of genes likely involved in patulin biosynthesis by conventional RT-PCR

One toxigenic (7015) and one non-toxigenic (FV 268) strain of *P. expansum* were grown on AJA medium at 16°C in the dark and at high RH. Mycelium was collected from both strains at 6 DPI and immediately processed to extract RNA. Specific primers targeting different genes involved in patulin biosynthesis (*IDH*, *p450-1*, *p450-2*, *msas*, and *peab1*) and primers specific to the housekeeping genes beta-tubulin (*β-Tub*) and calmodulin (*Cal*) (Table 1), were designed on deposited cDNA sequences (Table 1) using the Primer3 Software (Rozen and Skaletsky 2000) and synthesised by Invitrogen (Milan, Italy).

For the first strand synthesis, 1 μg of total RNA was mixed with 0.01% Triton X-100 (Sigma), 2.5 μM reverse primer and nuclease-free water (final volume 12.5 μl). The mixture was heated to 95°C for 3 min and quick-chilled in ice for 3 min before adding 7.5 μl of a mixture containing 1 mM DTT (dithiothreitol, Sigma), 1× First-Strand Buffer (Promega), 0.5 mM dNTP Mix (Promega), 20 U RNase Inhibitor (Promega), 7.5 U AMV-RT (Promega) and nuclease-free water. The reaction was incubated for 60 min at 42°C. PCR reactions were conducted in a total volume of 25 μl containing 2 μl cDNA, 1× Reaction Buffer (Sigma), 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.50 μM forward primer, 0.25 μM reverse primer and 1 U Taq polymerase (Sigma). Amplification conditions consisted of 95°C for 2 min, followed by 40 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 30 s, and finally 72°C for 5 min. Reactions were carried out in a thermal cycler (BioRad thermal cycler, MyCycler, Hercules, CA, USA). Amplicons were analysed by electrophoresis on 1.5% agarose gels and ethidium bromide staining.

Set up of real-time quantitative PCR reactions

The same set of primers used in conventional PCR (Table 1) was utilised in real-time PCR reactions to amplify cDNA synthesised, as previously reported, from total RNA of the toxigenic *P. expansum* strain 7015 grown on AJA medium for 6 days at 16°C in the

Table 1 Primers used in the present study to amplify specific fragments from genes involved in patulin biosynthesis (first five pairs) or constituitively expressed (last two pairs)

Primer codes	Primer sequences (5'-3')	Fragment size (bp)	Target genes	Accession N°
Pe 1F Pe 4R	GGATAGCATCCCAAGCGATA CGCTCTACTGTCCACGATGA	337	Cytochrome monooxygenase (*p450-2)*	DQ084390
Pe 5F Pe 6R	TGCTCATCACAGGAGGTACA TAGCAACATCAAATGCCGTG	181	Isoepoxydon dehydrogenase (*IDH*)	DQ084388
Pe 11F Pe 12R	CACTTATTGTGACCCGCAGA CTCGAAGAGGATCCATGAGG	288	6-methylsalicylic acidsynthase (*msas*)	DQ084387
Pe 13F Pe 14R	GAATCTCCGGAAAATGCAAA TTCCCGTTCACGTATCAACA	249	Cytochrome monooxygenase (*p450-1)*	DQ084389
Pe 15F Pe 16R	CAGGAAAACCGAGAAAACCA CACCGCCCACAAGCTATAAT	243	ATP Binding Cassette transporter (*peab 1*)	DQ084391
Tub 1F Tub 2R	AGCGGTGACAAGTACGTTCC ACCCTTGGCCCAGTTGTTAC	150	Beta – tubulin (*β-Tub*)	AY674401
CAL 1F CAL 4R	AGTCGAGGCCACAACAGTCT CGTTGATCATGTCCTGCAAC	208	Calmodulin (*Cal*)	AY678569

dark and at high RH. Amplification mixtures (20 µl) contained 10 µl 2× iQ SYBR Green Supermix (BioRad), 0.5 µl of each primer (10 pM μl^{-1}), 7 µl of nuclease-free water and 2 µl of cDNA. In negative-control samples, cDNA was replaced with sterile water or non-reverse transcribed total RNA to detect possible cross-contamination and ascertain the complete removal of genomic DNA from RNA samples. PCR amplification conditions were 95°C for 5 min and then 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 20 s. Fluorescence was monitored at each PCR cycle during the extension phase at 72°C. Amplifications were performed in 96-well reaction plates using an iCycler iQ thermal cycler (Bio-Rad). Relative normalised fluorescence (DRn) and cycle thresholds (Ct), i.e. the PCR cycles at which fluorescence exceeded threshold fluorescence intensity, were automatically generated by the iCycler associate software (Real Time Detection System Software, version 3.0).

Melting curves of real-time PCR products were evaluated from 55°C to 95°C to confirm the amplification of single PCR bands. The following cycling conditions were utilised: initial denaturation for 5 min at 95°C, cooling to 55°C and melting from 55°C to 95°C with a 0.5°C transition rate every 10 s. Moreover, to further confirm the amplification of single PCR bands, an aliquot (15 µl) of products from each primer pair was subjected to 1.5% agarose gel electrophoresis.

Finally, to assess the range of concentrations at which target RNA and Ct values were linearly correlated and to determine reaction efficiency, specific real-time PCR reactions were conducted using cDNA synthesised from 10-fold serially diluted RNA samples. In particular, 1000, 100, 10 and 1 ng of total RNA were reverse-transcribed to cDNA and used in specific reactions. Standard curves and linear equations were determined using the iCycler associate software by plotting Ct values (y-axis) against logs of total RNA (x-axis).

Relative expression of genes likely involved in patulin biosynthesis in response to phenolic application

Total RNA was extracted from *P. expansum* strain 7015, grown for 6 days at 16°C in the dark and at high RH on AJA supplemented with one or both phenolics (10 µg ml^{-1} of each single compound) or with solving buffer (control). Extractions were performed in triplicate. Each RNA sample was reverse-transcribed and amplified, as reported above. The relative expression of *IDH*, *p450-1*, *p450-2*, *msas*, and *peab1* genes was evaluated by using the ΔΔCt method (Livak and Schmittgen 2001) with beta-tubulin (*β-Tub)* as the housekeeping non-regulated reference gene. In particular, the relative expression was calculated according to the following formula: $2^{(-\Delta\Delta Ct)}$, where ΔCt=(average Ct of house-

keeping gene - average Ct of target gene) and ΔΔCt=(average ΔCt of phenolic compound treated sample - average ΔCt of untreated sample). Data were transformed to $\log_2$ and levels of change (i.e. either increases or decreases) were categorised as follow: 'low' ≥−1.0 to ≤1.0; 'medium' ≥−2.0 to <−1.0, or >1.0 to ≤2.0; 'high' <−2.0, or >2.0 (Kim et al. 2008). The relative expression values were automatically generated by entering Ct values from housekeeping and target genes into Gene Expression Relative Quantification spreadsheets (BioRad).

Results

In vitro effect of phenolics on patulin production and *P. expansum* growth

Patulin accumulation was significantly reduced by phenolics at all assessment times. In particular, at 8 DPI patulin was reduced by 42, 45 and 68% in dishes supplemented with quercetin, umbelliferone and their combination, respectively (Table 2). Similar results were achieved at 14 DPI (Table 2). The *in vitro* effect of phenolics on *P. expansum* mycelial growth was less evident. A slight inhibition (≤11%) was observed after 14 DPI in plates supplemented with quercetin, both singly (10 μg ml^{-1}) or in combination with umbelliferone (20 μg ml^{-1}) (Table 2).

Amplification of genes involved in patulin biosynthesis by conventional RT-PCR

The total RNA used in all reactions was of good quality since it was undegraded and free from protein and DNA contamination. Extraction yields were on average 2 μg mg^{-1} of mycelium.

RT-PCR reactions performed using total RNA extracted from a toxigenic (7015) and a non-toxigenic (FV 268) strain of *P. expansum,* both grown on AJA medium at 16°C in the dark and at high RH, confirmed the involvement of the five selected genes (Table 1) in patulin-biosynthesis. In particular, three PCR bands, specific to genes *IDH*, *p450-2* and *msas,* were amplified only from the patulin-producing strain (Fig. 1a). The remaining two bands, specific to genes *p450-1* and *peab1,* were amplified from the total RNA of both strains, although the bands were much more marked in the toxigenic strain 7015 (Fig. 1a). The housekeeping genes *β-Tub* and *Cal* were similarly expressed in both *P. expansum* strains (Fig. 1a, b); however, for subsequent quantitative real-time PCR reactions the *β-Tub* gene was preferred, because it seemed to be better expressed than *Cal* gene in both *P. expansum* strains (Fig. 1b).

Set up of quantitative real-time PCR reactions

Preliminary trials made it possible to set optimal real-time PCR amplification conditions using the primers tested in conventional PCR and the SYBR Green as fluorescent dye. Melting curve analyses showed the presence of a single melting peak for all target genes and for the *β-Tub* housekeeping gene, thus indicating that each primer pair amplified a single product with a distinct melting temperature (data not shown). Negative-control samples in which reverse transcribed RNA was replaced by water or non-reverse transcribed RNA, did not produce any increase in fluorescence, thereby proving the absence of cross-contamination and

Table 2 *Penicillium expansum* colony diam (mm) and patulin production (μg ml^{-1}) on AJA supplemented with quercetin (QUE) and umbelliferone (UMB) singly (10 μg ml^{-1}) or in combination (QUE+UMB, 20 μg ml^{-1})

Treatment	Colony diam±SEM (mm)		Patulin accumulation±SEM (μg ml^{-1})	
	8 days	14 days	8 days	14 days
Control (no phenolics)	49.0±1.5^{a}	79.2±3.2^{a}	3.1±0.3^{a}	3.7±0.2^{a}
QUE	45.3±1.9^{a}	70.5±0.9^{b}	1.8±0.2^{b}	2.6±0.2^{b}
UMB	45.0±1.2^{a}	74.7±3.0^{a}	1.7±0.3^{b}	2.2±0.1^{bc}
QUE+UMB	43.8±1.8^{a}	70.3±0.5^{b}	1.0±0.1^{c}	1.8±0.1^{c}

Each value corresponds to the mean of three replicates±standard error of mean (SEM). In each column, data with the same letters are not significantly different ($P \leq 0.05$) according to DMRT

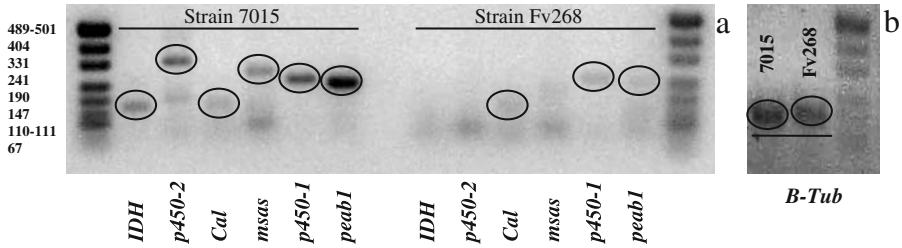

Fig. 1 RT-PCR amplification products obtained from *P. expansum* strain 7015 (patulin producer) and FV 268 (patulin non - producer) with primers targeting genes involved in patulin biosynthesis (*IDH*, *p450-1, msas, p450-2* and *peab1*). The calmodulin (*Cal*) (A) and the beta-tubulin (*β-Tub*) (B) housekeeping genes were also amplified and used as controls. Lane 1: Marker pUC19 DNA ladder (67-501 bp) digested to completion with MspI

the absence of DNA contamination. Furthermore, agarose gel electrophoresis of real-time PCR amplification products obtained for genes *IDH, p450-2, msas, p450-1, peab1* and *β-Tub* confirmed the presence of single PCR bands of the expected size (data not shown).

To evaluate reaction efficiency and to determine the range of concentrations at which target RNA and Ct values were linearly correlated, specific standard curves were constructed for each gene. Ct values and RNA concentrations proved to be linearly correlated in the range 1000-1 ng for all examined genes with determination coefficients (R^2) ranging from 0.98 to 0.99 (Table 3). Since 100 ng of RNA were efficiently amplified for all target genes, this concentration was utilised in the subsequent real-time PCR reactions. Reaction efficiencies for *p450-2, p450-1,* and *peab1* were high (>90%) and similar to that of the housekeeping *β-Tub* gene (93.4%) (Table 3). Lower levels of reaction efficiency were achieved for *IDH* and *msas* genes (Table 3).

Table 3 Linear equations, determination coefficients (R^2) and reaction efficiencies obtained by plotting serially-diluted RNA concentrations (log scale) and corresponding Ct values experimentally determined in real-time PCR reactions for genes *β-Tub*, *p450-2, IDH, msas, p450-1* and *peab1*

Gene	Linear equation	R^2	Reaction Efficiency
β-Tub	$y=-3.49x+26.56$	0.99	93.4
p450-2	$y=-3.45x+29.16$	0.98	94.9
IDH	$y=-3.79x+22.21$	0.96	83.6
msas	$y=-3.62x+32.67$	0.98	88.9
p450-1	$y=-3.49x+30.02$	0.99	93.4
peab1	$y=-3.32x+31.96$	0.99	100

Relative expression of genes likely to be involved in patulin biosynthesis in response to quercetin and umbelliferone application

In most cases, the five tested genes were differentially expressed when *P. expansum,* strain 7015, was grown for six days on AJA supplemented with phenolics. In particular, the relative expression of *IDH* and *msas* genes was up-regulated at a medium-low level in the presence of quercetin and umbelliferone (Fig. 2); by contrast, the two substances did not significantly modify the expression of gene *peab1*. However, when the two phenolics were applied in combination, *IDH*, *msas* and *peab1* genes were markedly down-regulated (Fig. 2). A different expression profile was observed for *p450-2* and *p450-1* genes, which were down-

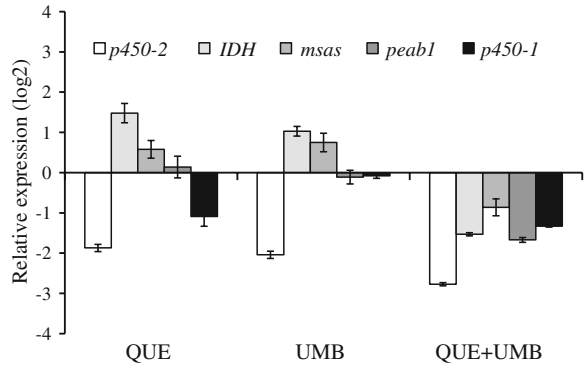

Fig. 2 Relative expression ($\log_2$ transformed) of *p450-2, IDH, p450-1, msas* and *peab1* genes in the toxigenic strain 7015 of *P. expansum* grown for six days on AJA supplemented with phenolics. Data were analysed using the $2^{-\Delta\Delta Ct}$ method, and normalised for differences in the amount of total RNA added to each reaction using the *β-Tub* housekeeping gene. Data represent means of 3 replicates±standard error of mean (SEM)

regulated by all treatments. In particular, quercetin caused a 'medium' reduction of gene expression (−1.87 and −1.09 for *p450-2* and *p450-1,* respectively), as compared to the untreated samples. Similar results were obtained in the presence of umbelliferone. However, the combination of the two phenolics proved to be the most effective treatment, with a 'high' reduction (−2.77) for gene *p450-2* and a 'medium' reduction (−1.33) for gene *p450-1* (Fig. 2).

Discussion

In *in vitro* trials the addition of quercetin and/or umbelliferone to the growth medium significantly reduced patulin accumulation with only a small effect on the radial growth of *P. expansum* at 14 days of incubation. These results are in agreement with Mossini et al. (2004) who reported that neem leaf extracts containing quercetin and other phenolics inhibited the *in vitro* production of patulin but did not affect *P. expansum* growth. Apparently, quercetin and umbelliferone did not affect the primary fungal metabolism responsible for fungal growth, but reduced toxin accumulation, which is commonly associated with secondary fungal metabolism (Calvo et al. 2002). Similarly, Demain (1996) reported that the reduction or elimination of a secondary metabolite did not stop fungal growth. Moreover, a greater reduction of patulin production (up to 68%) was achieved when a combination (1:1, 20 μg ml^{-1}) of the two compounds was applied. This improved effect could be ascribed to the double concentration used, although a synergic action cannot be ruled out. Indeed, recently a synergic action between quercetin and umbelliferone in controlling patulin accumulation was demonstrated when the two phenolics were applied on apples by dipping (Sanzani et al. 2009).

Based on these results, the main objective of the present investigation was to gain insight into the mechanisms by which quercetin and umbelliferone reduce patulin accumulation. In particular, an attempt was made to verify if their effect was a consequence of the down-regulation of genes coding enzymes likely involved in the crucial steps of toxin biosynthesis. This assumption was also supported by previous evidence that the expression of genes of the secondary metabolism is strictly controlled by nutrients, inducers, products, metals and growth rate, and in most cases, regulation is at transcription levels (Demain 1996). To verify this hypothesis, the expression level of five *P. expansum* genes *IDH*, *p450-1, msas, p450-2* and *peab1* (White et al. 2006) was analysed in the presence and absence of the two phenolics. The involvement of the selected genes in patulin biosynthesis and release was confirmed in the present study by amplifying their specific cDNAs from a producing and non-producing strain of *P. expansum*. As expected, specific bands were exclusively amplified by RT-PCR from the patulin-producing strain 7015 (*IDH*, *p450-2* and *msas* genes) or were amplified from both strains (genes *p450-1* and *peab1*), but with a significantly higher band intensity for the toxigenic one. The crucial role of *msas* and *IDH* genes in patulin biosynthesis highlighted by our results was also established by Puel et al. (2007), who reported that the absence of these genes in the patulin-producing fungus *Byssochlamys fulva* resulted in its inability to produce the toxin.

Although other points of control not considered in this work may occur, on the whole, real-time RT-PCR results revealed with a reasonable degree of certainty that both quercetin and umbelliferone reduce *P. expansum* patulin accumulation by acting on gene transcription. In fact, the expression of all five investigated genes was down-regulated by the two phenolics applied in combination. The real-time PCR reaction efficiencies for the selected genes were sometimes not found within the optimum range (90–110%); however, the number of genes analysed and their similar expression patterns seem to be self-validating.

Single applications of quercetin and umbelliferone did not significantly modify the expression of gene *peab1* and up-regulated *IDH* and *msas* genes to a medium-low level. However, in combined applications, the expression of these genes was significantly down-regulated as compared to the control samples, thus confirming the above mentioned HPLC results. The *IDH* gene encodes for the enzyme isoepoxydon dehydrogenase which catalyses the conversion of isoepoxydon to phyllostine. This gene has been extensively utilised to detect the presence of toxigenic strains of *Penicillium* spp. in apple juice (Paterson et al. 2003). The *msas* gene belongs to the polyketide synthase family. Although polyketide biosynthesis has been the focus of intensive research over the past decade, relatively few polyketide synthase gene

clusters have been described in fungi (Desjardins and Proctor 2007). The best understood system is the one responsible for the biosynthesis of 6-MSA, which is the first step in the patulin synthetic pathway.

The *peab1* gene encodes an ATP-binding cassette (ABC) transporter that functions as an efflux pump. Its function is of particular interest since it can play a significant role in protecting plant pathogens from synthetic fungicides or from plant defence compounds, such as the phenolics proposed in the present study. Schoonbeek et al. (2001) demonstrated that the ABC transporter *BcatrB* affects the sensitivity of *Botrytis cinerea* to phytoalexin resveratrol. Similarly, Burse et al. (2004) found that ABC transporter (*acrB*)-deficient *Erwinia amylovora* mutants are more susceptible towards apple phenolics, including quercetin. Moreover, transporters may prevent suicidal effects by releasing patulin in the surrounding tissues or in the growth medium. For instance, *TOXA* gene in *Cochliobolus carbonum* encodes an efflux pump which contributes to self-protection against its own toxin and/or is involved in toxin secretion (Roohparvar et al. 2007). The modification of a self-defence mechanism in *P. expansum* could be involved in the mode of action of quercetin and umbelliferone since unreleased patulin might interfere with the normal secondary fungal metabolism.

The cytochrome monooxygenases *p450-1* and *p450-2* seemed to be the best candidates for explaining the effect of quercetin and umbelliferone on patulin biosynthesis since they were down-regulated by both phenolics, either singly or in combination. This finding seems of particular interest considering that *p450-1* and *p450-2* proved to be very strongly induced (250–1127 fold) under patulin-permissive conditions (White et al. 2006). The key role of cytochrome monooxygenases in oxygen activation during the hydroxylation of m-cresol to m-hydroxybenzyl alcohol by m-cresol 2-hydoxylase has been reported by Murphy and Lynen (1975). They are also involved in the biosynthesis of other mycotoxins such as trichothecenes in *Fusarium sporotrichioides* (Meek et al. 2003), aflatoxin B1 in *Aspergillus parasiticus* (Udwary et al. 2002) and *Aspergillus flavus* (Keller and Hohn 1997). The down-regulation of *p450-1* and *p450-2* by quercetin and umbelliferone is in agreement with their reported antioxidant properties (Nijveldt et al. 2001; Repetto and Llesuy 2002), which could interfere with the pro-oxidant activity of both monooxygenases. Natural antioxidants such as flavonoids, including quercetin, are known to inhibit the activity of various oxidant enzyme systems such as cyclooxygenase and lipoxygenase (Sellappan and Akoh 2002). Moreover, Kim et al. (2008) reported that the application of caffeic acid, a naturally occurring antioxidant phenolic which reduces aflatoxin production, also reduced P450 monooxygenase gene expression.

It can be concluded that quercetin and umbelliferone do not seem to affect primary fungal metabolism, but reduce patulin production by acting on its biosynthetic pathway. Since both phenolics also reduce blue mould incidence and severity on apples (Sanzani et al. 2009), the results of the present study are particularly interesting in the light of the increasing development of pathogen strains resistant to synthetic fungicides. However, further studies on quercetin and umbelliferone safety and their mode of action in controlling blue mould on apples are needed to encourage their commercial utilisation in integrated control strategies.

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