International Journal of Food Microbiology 142 (2010) 156–163

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Influence of package, type of apple juice and temperature on the production of patulin by *Byssochlamys nivea* and *Byssochlamys fulva*

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ARTICLE INFO

Article history:
Received 21 February 2010
Received in revised form 19 May 2010
Accepted 19 June 2010

Keywords:
Patulin
Byssochlamys
Apple juice
Mycotoxins
Package

ABSTRACT

Although the production of patulin in apple fruits is mainly by *Penicillium expansum*, there is no information on the ability of heat resistant moulds that may survive pasteurization to produce this mycotoxin in juice packages during storage and distribution. In this study, the production of patulin by *Byssochlamys* spp (*Byssochlamys nivea* FRR 4421, *B. nivea* ATCC 24008 and *Byssochlamys fulva* IOC 4518) in cloudy and clarified apple juices packaged in laminated paperboard packages or in polyethylene terephthalate bottles (PET) and stored at both 21 °C and 30 °C, was investigated. The three *Byssochlamys* strains were able to produce patulin in both cloudy and clarified apple juices. Overall, the lower the storage temperature, the lower the patulin levels and mycelium dry weight in the apple juices ($p<0.05$). The greatest variations in pH and °Brix were observed in the juices from which the greatest mycelium dry weights were recovered. The maximum levels of patulin recovered from the juices were ca. 150 μg/kg at 21 °C and 220 μg/kg at 30 °C. HPLC-UV, HPCL-DAD and mass spectrometry analyses confirmed the ability of *B. fulva* IOC 4518 to produce patulin. Due to the heat resistance of *B. nivea* and *B. fulva* and their ability to produce patulin either in PET bottles or in laminated paperboard packages, the control of contamination and the incidence of these fungi should be a matter of concern for food safety. Control measures taken by juice industries must also focus on controlling the ascospores of heat resistant moulds.

1. Introduction

Patulin is a mycotoxin produced by *Penicillium*, *Aspergillus* and *Byssochlamys* species and represents a major safety concern for apple products (Moake et al., 2005; Sant'Ana et al., 2008). Patulin has been found worldwide in apple juices and products and apple juice processing appears to have limited effects on reducing this mycotoxin (Sant'Ana et al., 2008). Levels of patulin greater than 10 μg/kg (the maximum level set by The European Union for apple-based products for infants) (European Union, 2006) and 50 μg/kg (the maximum level established by Codex and FDA for apple juice) (Codex Alimentarius, 2003; Food and Drug Administration, 2001) can be found in the final product thus posing risks to consumers. These levels depend on the initial levels found in the raw material before fruit processing.

Patulin production by *Penicillium expansum* has been widely studied in culture media (Andersen et al., 2004; Dombrink-Kurtzman and Blackburn, 2005; Kokkonen et al., 2005; Stott and Bullerman, 1975) and in both fruits and fruit juices (Baert et al., 2007a; Baert et al., 2007b; Morales et al., 2008; Paster et al., 1995; Salomão et al., 2009; Sommer et al., 1974; Tournas and Memon, 2009; Vismer et al., 1996; Damaglou et al., 1985; Wilson and Nuovo, 1973). Data on the production of patulin by *Byssochlamys* are available. Studies from the 1970s and 1980s indicated that both *Byssochlamys fulva* and *Byssochlamys nivea* were able to produce this mycotoxin in juices (Rice, 1980; Rice et al., 1977). However recent studies carried out in culture media reported the inability of *B. fulva* strains to produce patulin (Houbraken et al., 2006; Puel et al., 2007).

The ability of *P. expansum* and *Byssochlamys* to produce patulin is linked to the presence of the *idh* (Dombrink-Kurtzman and Engberg, 2006; Puel et al., 2007) and *6msas* genes (Puel et al., 2007), and the presence of the *idh* gene in one *B. fulva* strain isolated from soil has been reported (Paterson, 2004). Paterson and Lima (2009) discussed whether the production of mutagenic secondary metabolites might affect the mycotoxin metabolic pathway in fungi, leading to erroneous

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doi:10.1016/j.ijfoodmicro.2010.06.017

data interpretation. Since secondary metabolites may themselves be potential PCR inhibitors or mutagenic to fungi (Paterson, 2007), false negatives in terms of having the gene for mycotoxin production may lead to the incorrect categorization of a specific strain as safe, with potential implications for food safety.

Numerous factors such as type of carbohydrate, type of juice, pH, soluble solids, temperature, water activity, headspace/oxygen tension and the presence of preservatives may strongly affect mould growth and patulin production in fruits and juices (Damaglou et al., 1985; Rice, 1980; Rice et al., 1977; Roland and Beuchat, 1984; Roland et al., 1984). Currently, cloudy and clarified apple juices filled into different packages (plastic bottles, laminated paperboard or glass bottles) are the main types of apple juice available on the market. There are however no studies describing the growth and mycotoxin production by *Byssochlamys* in different types of either apple juices or packages at common storage and distribution temperatures. Therefore in this study, the production of patulin by *B. nivea* and *B. fulva* strains was studied in experimentally inoculated cloudy and clarified apple juices filled into laminated paperboard and plastic packages, and stored at different temperatures. Data supporting the production of patulin by a Brazilian *B. fulva* strain is also presented.

2. Material and methods

2.1. Apple juice

Frozen and concentrated cloudy (45°Brix, pH 3.66 and 0.81% acidity — as malic acid) and clarified (70°Brix, pH 3.88 and 1.03% acidity — as malic acid) apple juices were provided by two Brazilian industries. The apple juices were evaluated for the presence of patulin by the manufacturers, in order to ensure that the concentrated juices were free of patulin. The juices were transported and maintained frozen at −20 °C until used. Before the inoculation studies, the apple juices were evaluated to verify the presence of heat resistant moulds (Beuchat and Pitt, 2001). The juices were diluted to 11°Brix with distilled water, pasteurized by autoclaving at 105 °C for 5 min as previously described (Sant'Ana et al., 2009), and filled into plastic bottles. Laminated paperboard packages and bottles containing apple juice (clarified) were obtained from a retail outlet and were also submitted to the quantification of patulin (Section 2.6).

2.2. Packages

Polyethylene terephthalate bottles (PET) (500 mL) and laminated paperboard packages (CT) (200 mL) were used in the studies on the production of patulin by *Byssochlamys* spp. CT packages are the most common type of package used by fruit juice industries in Brazil, but PET bottles were also used because product appearance and package transparency are very important attributes considered by consumers during purchasing (Ragaert et al., 2004). The PET bottles and caps were disinfected with 0.05% and 0.01% v/v of peracetic acid solution for 30 min, as previously described (Sant'Ana et al., 2009).

Oxygen permeation of both types of package was measured to verify its influence on growth and patulin production by *Byssochlamys* spp. The rate of oxygen permeation (RPO_2) (O_2 cc/package/day) was determined in OX-TRAN 2/20 (Mocon Inc, MN). The packages were placed in the apparatus and a coulometric sensor operating at constant efficiency was used to detect oxygen permeation through the packages.

2.3. Identification of Byssochlamys strains

The two *B. nivea* strains and single *B. fulva* strain used were isolated from fruits/fruit-related surfaces. The strains are deposited in culture collections in Brazil (*B. fulva* IOC 4518), Australia (*B. nivea* FRR 4421) and the USA (*B. nivea* ATCC 24008). The ability of *B. fulva* IOC 4518 to produce patulin was investigated due to the previous detection of this mycotoxin after the intentional inoculation of apple juice in the authors' laboratory (data not shown). Furthermore, *B. fulva* IOC 4518 was much more heat resistant than both the *B. nivea* strains (Sant'Ana et al., 2009), surviving pasteurization and producing patulin.

Byssochlamys strains were identified by observation of their macroscopic and microscopic characteristics as described by Tournas (1994) and Pitt and Hocking (1999). In addition, the identities of the *B. nivea* FRR 4421 and *B. fulva* IOC 4518 strains were verified by internal transcribed spacer (ITS) rRNA and 5.8S rRNA gene and partial β-tubulin gene amplification and sequencing. All the procedures were as described by Puel et al. (2007).

2.4. Preparation of ascospore suspensions

Ascospore suspensions were prepared as described by Sant'Ana et al. (2009). Briefly, Roux bottles containing malt extract agar (MEA) were inoculated with *Byssochlamys* strains and incubated at 30 °C for 30 days. Ascospores were then collected after scraping the surface of the MEA using sterile distilled water, followed by filtration to remove hyphal fragments and debris, centrifugation/washing, sonication and storage at 2 ± 0.2 °C for up to 6 months until used. All the suspensions were standardized at 10^7 ascospores/mL.

2.5. Studies on the production of patulin by Byssochlamys spp

PET bottles containing both cloudy or clarified apple juices and CT packages with clarified apple juice were individually and aseptically inoculated with heat activated ascospores of each *Byssochlamys* spp strain studied. *B. fulva* IOC 4518 ascospores were heat activated at 75 °C/5 min, while *B. nivea* ATCC 24008 and *B. nivea* FRR 4421 ascospores were activated at 75 °C/20 min as previously established (Sant'Ana et al., 2009). Each package was inoculated to reach the level of 10^3 ascospores/mL. This inoculum level is typically recommended for microbiological challenge tests (Institute of Food Technologists, 2003).

Polyethylene bottles were aseptically filled with juices in a low laminar cabinet and closed with disinfected caps. Sterile syringes were used to inoculate the apple juice inside the laminated paperboard packages with *Byssochlamys* ascospores. The inoculation was performed after disinfection of the area below the right upper brim, after disinfection with a 70% alcohol solution. The package was then pierced with the syringe and the juice inoculated. The inoculation hole was closed using low density polyethylene (LDPE) pellets melted in a flame. The brim was sealed using LDPE pellets. After inoculation, the juice was mixed and packages incubated at 21 °C and 30 °C for 14 days. Since commercial apple juice is distributed at ambient temperature, these two conditions were chosen to represent an approximation of the mean annual temperature in sub-tropical and tropical regions of Brazil, according to data obtained from the Climate Forecast and Climatic Studies Center (CPTEC — www.cptec.inpe.br). The time of incubation was chosen based on studies in which patulin degradation occurred after 12–14 days (Dombrink-Kurtzman and Engberg, 2006; Puel et al., 2007).

After incubation, the packages were opened and the juice filtered through three sterile, pre-dried and pre-weighed gauze layers. The juice was collected and frozen for the determination of patulin, and the gauzes were washed with sterile distilled water to remove juice residues. The mass of dried fungal mat was determined by drying the gauzes in dishes placed in an incubator at 55 °C for up to 36 h (to constant weight). The fungal mycelium dry weight was determined from the difference in weight of the gauze before juice filtration and after drying.

During the storage period, the pH and °Brix values were determined after 0, 5, 10 and 14 days in one package chosen at random. The pH was determined using a Digimed digital potentiometer, model DMpH-2

(Digimed, Piracicaba, Brazil) and the °Brix values were determined using an Atago HSR model-500 refractometer (Atago, Saitama, Japan). The pH and °Brix values together with the weight of dried mycelia were used to evaluate the extent of growth and spoilage potential of each *Byssochlamys* strain. Nine different packages were inoculated for each temperature condition and type of apple juice. All the experiments were performed in triplicate.

2.6. Patulin determination

The limits of detection and quantification of the HPLC method used to determine patulin in apple juice were 0.5 and 2.0 μg/L respectively (Mallmann et al., 2006). Patulin was extracted from the juice samples (5 mL) with ethyl acetate (20 mL) (Merck, Darmsdat, Germany) in an ultrasonic bath — Ultrasonic Cleaner model 1440D (Odontobrás, Ribeirão Preto, BR) — for 15 min. The efficiency of the extraction procedure was 87% (Mallmann et al., 2006). The supernatant layer was then recovered and evaporated at 40–45 °C. The dried contents were re-suspended in 0.5 mL of acetonitrile/water/acetic acid (840/160/5, v/v/v) (Tedia, Fairfield, USA; Merck, Darmstadt, Germany), and the organic phase was evaporated under a gentle stream of nitrogen. The mobile phase was water–1% acetic acid: acetonitrile (99/1, v/v) at 0.3 mL/min. The HPLC equipment comprised a Synergi 4 μm Fusion-RP80 (250×2 mm) column (Phenomenex, Torrance, USA), UV detector set at 276 nm, liquid chromatographic pump and integrator, all from Agilent (Agilent, Santa Clara, USA). A syringe was used to inject an aliquot of 2 μL into the HPLC apparatus (Agilent, Santa Clara, USA). A stock solution of the patulin standard was prepared from crystallized patulin (Sigma, St. Louis, USA) as described by Trucksess (2000). The determination of patulin was carried out in triplicate. All reagents employed were of analytical grade.

2.7. Confirmation of patulin production by B. fulva IOC 4518

2.7.1. Confirmation by HPLC-UV and HPLC-DAD

Confirmation of the production of patulin by *B. fulva* IOC4518 was achieved by HPLC at a wave length of 280 nm and with diode array detection (DAD) at three different wave lengths (270, 276 and 280 nm). Three dimensional spectral analysis was used by way of the ChemStation® software. Samples of apple juices showing positive for patulin were submitted to this confirmation. Sample purity was checked by comparing the analyte with the patulin standard in the spectral library and by superimposing the sample and patulin standard spectra. The spectra and retention times of the samples and standard patulin were also compared by way of an isoabsorbance scan.

2.7.2. Confirmation by mass spectrometry

Mass spectrometry (MS) was used for the final confirmation of patulin. A specific extraction procedure was used for the MS analysis to minimize suppression or interference by sugars in the juice. Juice (10 mL) contaminated with patulin was placed in centrifuge tubes. The control was 10 mL of apple juice without patulin as previously confirmed by HPLC-DAD. A solution of ethyl acetate/hexane (96/4) (10 mL) was added to the tubes and manually homogenized. The tubes were left in an ultrasonic bath under agitation (45×*g*) for 10 min. The contents of the tubes were then centrifuged for 1 min at 900×*g*. The organic phase was filtered using paper filters with the aid of Pasteur pipettes. Extracts (6.5 mL) were collected in glass tubes and the solvent was evaporated under a gentle stream of N_2 in a thermo block adjusted to 48 °C. Dried samples were re-dissolved in 700 μL of water at pH 4.0 and filtered through a 0.45 μm nylon filter (13 mm).

2.7.2.1. Electrospray ionization mass spectrometry. A Q-TOF mass spectrometer (Micromass, Manchester, UK) was used for ESI-MS analysis. The main general conditions were: source temperature of 100 °C, capillary voltage of 3.0 kV and cone voltage of 20 V. The measurements were made in the negative ion mode via ESI(−)-MS and with direct infusion using a syringe pump (Harvard Apparatus). The flow rate applied was 10 μL/min and the mass spectra were acquired over the 50 to 1000 *m/z* range and accumulated for 60 s. The ESI(−)-MS data were analysed using MassLynx 3.5 (Waters, Manchester, UK).

2.7.2.2. Tandem mass spectrometry. A structural analysis of selected ions from the apple juice samples and patulin standard was performed by ESI(−)-MS/MS. The ion of interest was selected by the first quadrupole and submitted to 15–55 eV collisions with argon in the collision cell. The collision gas pressure was optimized to produce extensive fragmentation of the ion under investigation. The TOF analyzer with 5K resolution and 10 ppm accuracy was used to collect the tandem MS data.

2.8. Data assessment and statistical analysis

Descriptive statistical calculations were performed using Statistica 7.0 for Windows software in order to verify the significant differences amongst the mean values. The data were checked for significant statistical ($p<0.05$) differences using the Analysis of Variance and Duncan's test.

3. Results

3.1. Identification of Byssochlamys strains

The main macroscopic and microscopic characteristics of the *B. nivea* strains (ATCC 24008, FRR 4421) were larger colonies on CYA agar incubated at 25 °C as compared to *B. fulva* IOC 4518. No cleistothecia or gymnothecia were observed and the asci were born in open clusters. The *B. fulva* IOC 4518 ascopores were smooth-walled and ellipsoidal.

The results of the genetic sequencing amplifying ITS and β-tubulin gene fragments indicated 100% similarity of the *B. fulva* IOC 4518 and *B. nivea* FRR4421 with the control strains (*B. fulva*: NRRL1125, NRRL3493, NRRL2975, NRRL2614 and *B. nivea*: NRRL35592, NRRL2615). This confirmation was not carried out with *B. nivea* ATCC 24008 because this strain had already been studied by Puel et al. (2007).

3.2. Studies on patulin production by Byssochlamys spp

Fig. 1 shows that the three *Byssochlamys* strains (*B. nivea* FRR 4421, *B. nivea* ATCC 24008 and *B. fulva* IOC 4518) were able to produce patulin in apple juice. Overall, the lower the storage temperature, the lower the patulin levels in the apple juice samples ($p<0.05$). The maximum levels of patulin recovered from juices stored at 21 °C and 30 °C were ca. 150 μg/kg and 220 μg/kg, respectively, after 14 days of storage. Patulin production was significantly higher ($p<0.05$) in clarified apple juice packaged in PET bottles, whereas cloudy juice packaged in laminar paperboard packages yielded low amounts of patulin.

In cloudy juice packaged in laminated paperboard packages, patulin production by the three *Byssochlamys* strains was only observed at 21 °C (LOD 0.5 μg/kg; LOQ 2 μg/kg), but the mycotoxin production was only significantly different for *B. nivea* ATCC 24008 ($p<0.05$). However, at 30 °C *B. fulva* IOC 4518 was the only strain able to produce patulin at approximately 150 μg/kg.

Patulin production was significantly different at 21 °C and 30 °C in cloudy juices packaged in laminated paperboard package as compared to that in PET bottles ($p<0.05$). In clarified juice packaged in PET bottles, although no significant difference ($p>0.05$) was found amongst the three strains grown at 30 °C, at 21 °C patulin production by *B. fulva* was significantly lower ($p<0.05$) when compared to the

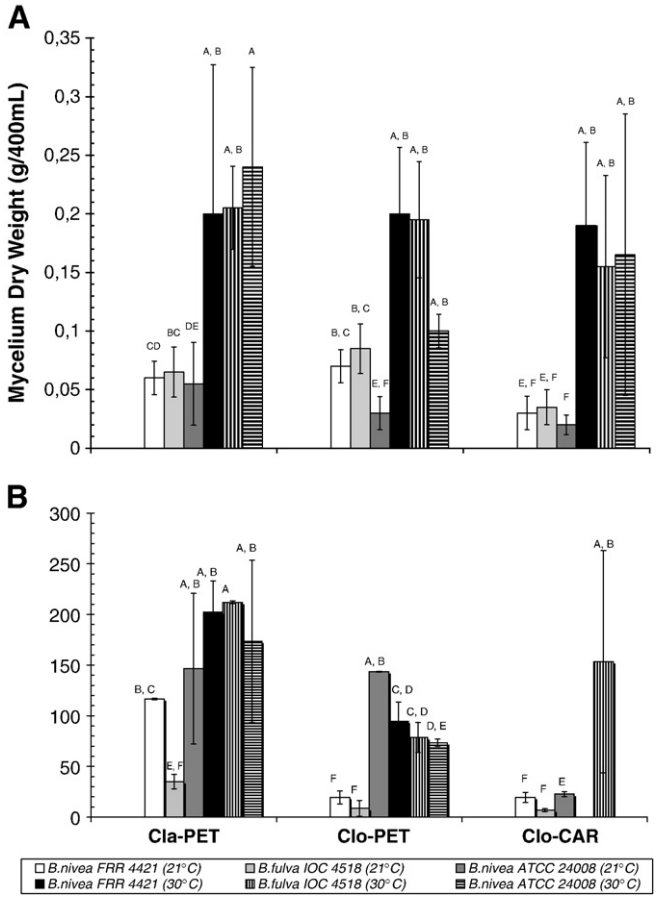

Fig. 1. Mycelium dry weight (A) and patulin production (B) by *Byssochlamys* spp. grown in different types of package and apple juice at 21 °C and 30 °C. Cla-PET = clarified apple juice packaged in PET bottles; Clo-PET = cloudy apple juice packaged in PET bottles and clo-CAR = cloudy apple juice packaged in laminated paperboard packages.

amounts produced by the *B. nivea* strains (ATCC 24008 and FRR 4421). Patulin production by *B. nivea* FRR 4421 was significantly higher ($p<0.05$) in clarified apple juice packaged in PET bottles at 21 °C but not in cloudy juice packaged in PET bottles or cloudy juice in laminated paperboard packages.

B. fulva IOC4518 yielded higher levels of patulin in all types of juice and package at 30 °C than at 21 °C. *B. nivea* ATCC 24008 achieved its highest levels of patulin production at 21 °C in clarified and cloudy juices packaged in PET bottles ($p>0.05$), but significant difference ($p<0.05$) was only found in cloudy juice packaged in laminated paperboard. For *B. nivea* FRR 4421, patulin production was significantly different between clarified and cloudy juices packaged in PET bottles at 30 °C, whereas for *B. fulva* IOC 4518, no significant difference ($p>0.05$) was found between the clarified juice packaged in PET bottles and cloudy juice packaged in laminated paperboard packages. *B. nivea* ATCC 24008 produced significantly lower ($p<0.05$) patulin levels in cloudy juice packaged in PET bottles when compared to clarified juice packaged in PET bottles.

The lower the temperature, the lower the mycelium dry weight recovered from the juices (Fig. 1). The mycelium dry weights were not significantly different ($p>0.05$) at 30 °C considering the type of package, strain and juice. At 21 °C, for the three strains, the mycelium dry weight was not significantly different ($p>0.05$) in the cloudy and clarified juices packaged in PET bottles, but a significant difference was found in cloudy juice packaged in laminated paperboard packages. At 21 °C, *B. nivea* FRR 4421 and *B. fulva* IOC4518 produced significantly greater ($p<0.05$) mycelium dry weight in cloudy and clarified juices packaged in PET bottles than *B. nivea* ATCC 24008.

Fig. 2 shows the variations in pH and °Brix in the juices inoculated with *Byssochlamys* spp. and stored at 21 °C and 30 °C. The greatest variations in pH and °Brix were observed in the juices in which the largest mycelium dry weights were found. Regardless of the strain, juice, package and temperature, a reduction in pH and °Brix was always observed. The variations in pH were between 0.1 and 0.5 at 21 °C and 30 °C, respectively, whereas the variations in °Brix were about 0.1–0.8 units at 21 °C and 0.6–0.8 units at 30 °C. Despite these variations, at some points (cla-PET and clo-CAR at 21 °C and clo-PET at 30 °C), a slight increase in the pH value was observed (about 0.05 pH units), but this change was not considered a pattern.

3.3. Confirmation of patulin production by *B. fulva* IOC 4518

Patulin production in apple juices by *B. fulva* IOC 4518 was confirmed at three different wavelengths by HPLC using DAD ($\lambda = 270$, 276 and 280 nm) and UV ($\lambda = 280$ nm) detection. Fig. 3 shows the HPLC of the juice spiked with the patulin standard (0.5 μg/mL) (A) and for the juice inoculated with *B. fulva* IOC 4518 (B), with the characteristic patulin peak.

The results presented above were also supported by the ESI(−)-MS analysis (Fig. 4) of the control and juice inoculated with *B. fulva* IOC 4518 samples. Many ions detected in the control sample were also detected in the contaminated juice, but the juice inoculated with *B. fulva* IOC 4518 showed several ions with $m/z<200$ as a unique feature and, most importantly, an ion with m/z 153 that could correspond to the target molecule: that is, the deprotonated patulin $[M-H]^-$ ion. To probe for patulin detection, ESI (−)-MS/MS experiments (Fig. 5) were carried out under the same conditions for this m/z 153 ion, and using a solution of standard patulin as well. The nearly identical spectra with a diversity of fragment ions firmly confirmed patulin detection: deprotonated patulin $[M-H]^-$ with m/z 153, $[M-H-CO_2]^-$ with m/z 109, $[M-H-CO_2-CO]^-$ with m/z 81, $[M-H-CO_2-2\times CO]^-$ with m/z 53, $[M-H-H_2O]^-$ with m/z 135, $[M-H-CO]^-$ with m/z 125 and $[M-H-H_2CO]^-$ with m/z 123.

4. Discussion

The levels of patulin found in the initial stages of apple juice processing are of considerable food safety concern (Sydenham et al., 1995; Sydenham et al., 1997) since much of this mycotoxin remains in the final products (Sant'Ana et al., 2009). Although *P. expansum* is the main species associated with the production of patulin during the refrigerated storage of apples (Baert et al., 2007c; Salomão et al., 2009), the low heat resistance of its spores ($D_{60\,°C}<1$ s) (Shearer et al., 2002) leads to its elimination during apple juice pasteurization (95–105 °C/15–30 s). In addition to the levels of patulin found in the initial stages of juice processing, moulds such as *Byssochlamys* spp. could play an important role as patulin producers in pasteurized juice. This is because of their known heat resistance (Sant'Ana et al., 2009) and their ability to grow at low pH values and in low oxygen atmospheres (Tournas, 1994; Taniwaki et al., 2009). Despite this potential risk, the production of patulin by *Byssochlamys* spp in different packages and types of apple juice stored under normal commercial conditions has not yet been investigated.

It has been shown that the production of mycotoxins is highly dependent on the substrate (Kokkonen et al., 2005). Studying the ability of a specific fungal strain to produce mycotoxin in a target food can therefore be the best strategy to understand the implications for food safety of a mould–food–mycotoxin interaction.

In this study, the three *Byssochlamys* spp. tested were isolated from fruit or fruit-related surfaces. The microscopic and macroscopic characteristics of these strains agreed with those previously described for *B. nivea* and *B. fulva* species (Pitt and Hocking, 1999). The genetic sequencing of ITS and β-tubulin fragments also supported the identities of the *Byssochlamys* strains used in this study. ITS and β-tubulin

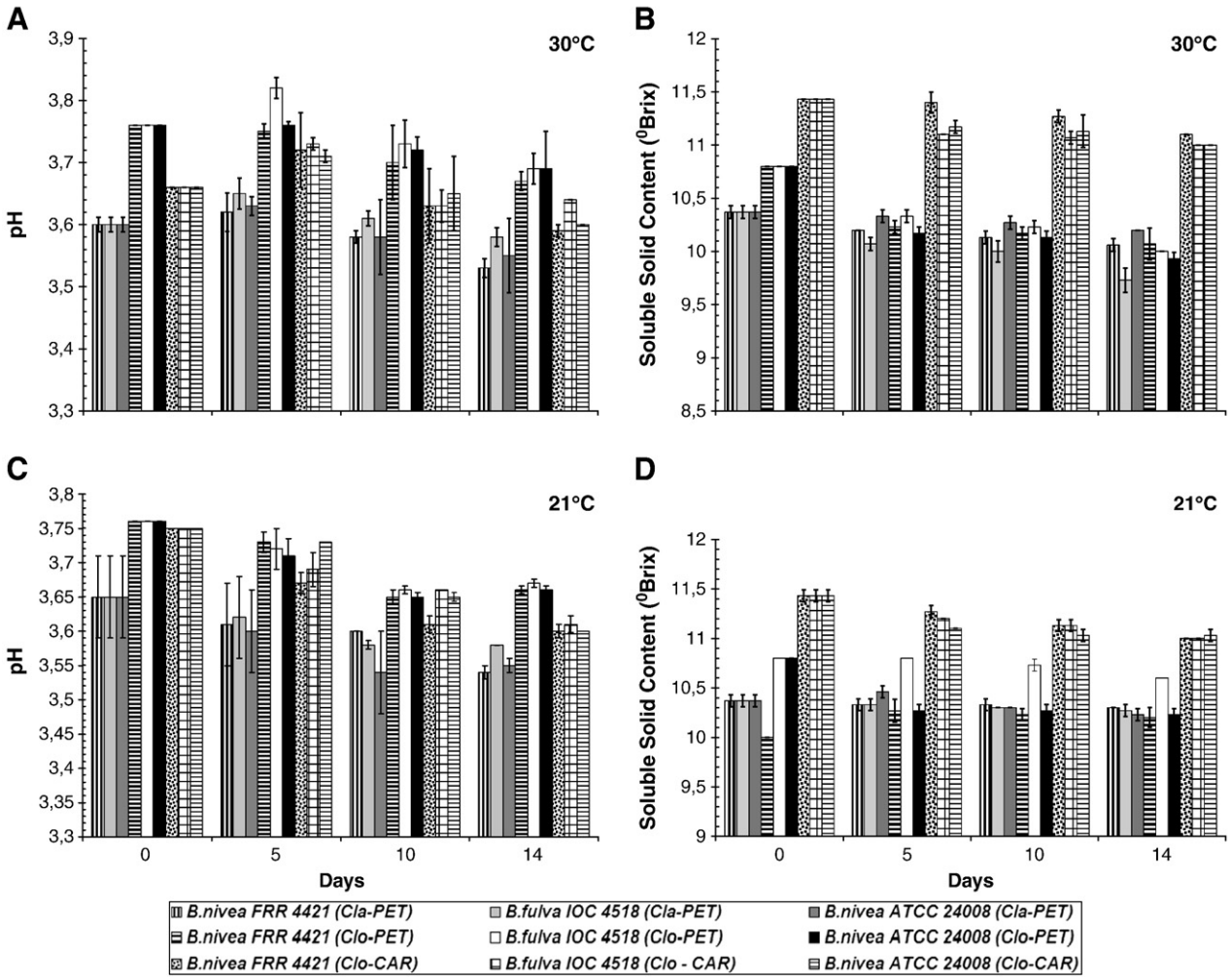

Fig. 2. Variation of pH (A and C) and °Brix (B and D) due to the growth of *Byssochlamys* spp. in different types of package and apple juice at 21 °C and 30 °C. Cla-PET = clarified apple juice packaged in PET bottles; Clo-PET = cloudy apple juice packaged in PET bottles and clo-CAR = cloudy apple juice packaged in laminated paperboard packages.

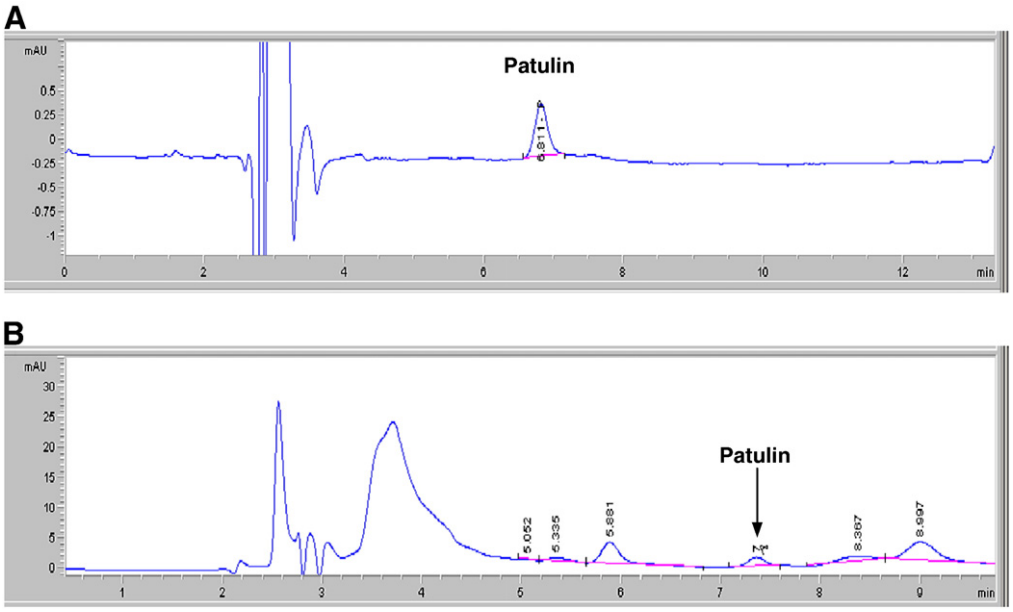

Fig. 3. HPLC chromatograms showing the characteristic peak of patulin as detected using a UV detector (λ = 276 nm). (A) Peak refers to the standard solution (apple juice) spiked with 0.5 μg/kg of patulin; (B) peak refers to the patulin quantified in apple juice (211 μg/kg) inoculated with *B. fulva* IOC 4518 and incubated for 14 days at 30 °C.

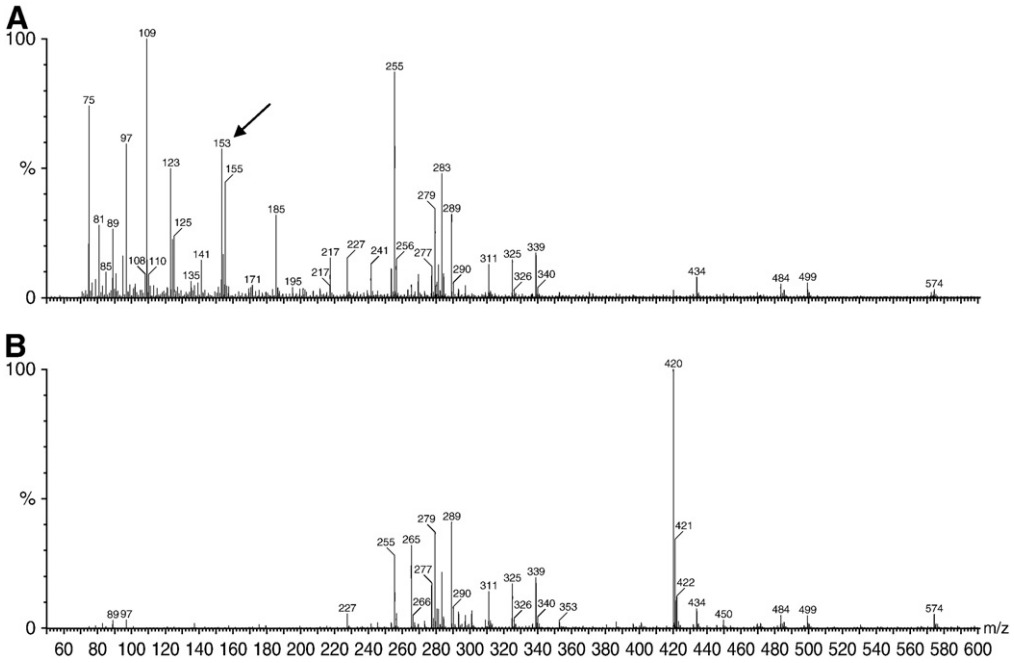

Fig. 4. ESI(−)-MS of apple juice (A) contaminated and (B) uncontaminated with patulin. The contaminated juice was inoculated with *B. fulva* IOC 4518 (10^3 spores/mL).

fragments have commonly been used as genetic markers to study the genetic diversity of fungi, since these regions have a higher evolutionary rate than rRNA codification regions, and may contain polymorphism sites that permit one to discriminate between populations or species from a genus (Homan et al., 1997; Singer and Berger, 1991; Tsuchiya et al., 2003).

The period during which patulin production occurs may vary between 3 and 21 days depending on the microorganism, substrate, headspace in the packages and temperature (Puel et al., 2007; Rice, 1980; Roland and Beuchat, 1984). However an incubation time between 10 and 14 days has been considered suitable to detect patulin (Dombrink-Kurtzman and Engberg, 2006; Houbraken et al., 2006). It is known that after this period the mycotoxin may be degraded by enzymes synthesized during the synthetic patulin pathway (Forrester and Guacher, 1972). Thus a period of 14 days was chosen for the storage of the apple juices inoculated with *Byssochlamys*, aiming to avoid patulin degradation and false negatives.

Patulin production was significantly affected by the strain, type of apple juice, package and temperature ($p<0.05$). Mycotoxin production by *Byssochlamys* was characterized by considerable variability, more evident at 30 °C (see standard deviation in Fig. 1). This variability could be explained by intrinsic differences related to secondary metabolic pathways in each fungal strain, leading to patulin production and degradation at different stages of growth.

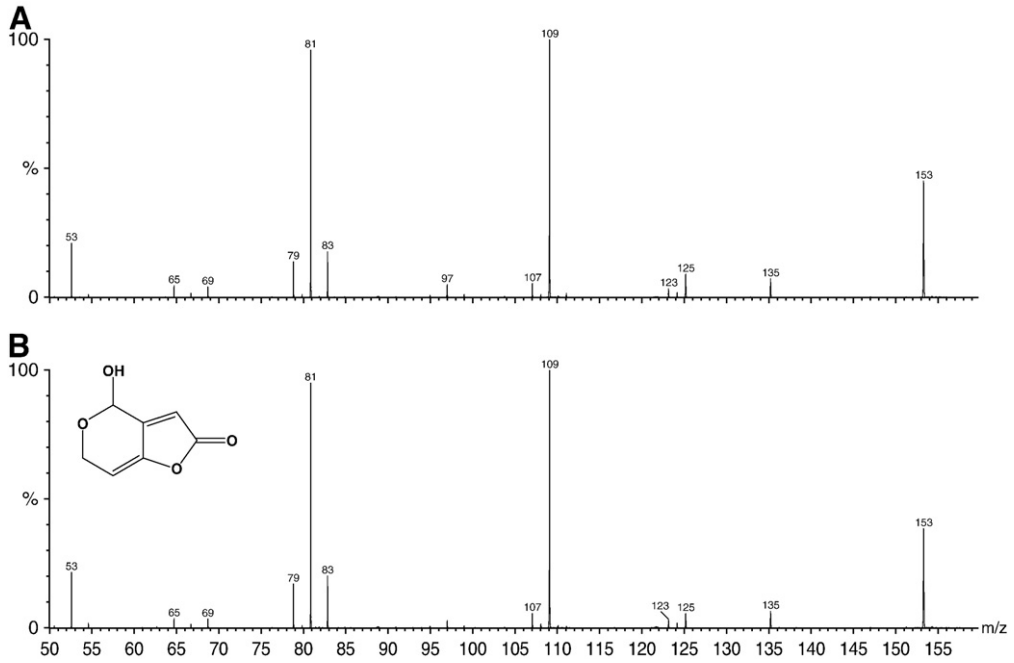

Fig. 5. ESI(−)-MS/MS of the ion of *m/z* 153 from A) the contaminated juice and B) from a patulin standard.

Variability in mycotoxin production by different fungi has already been reported (Baert et al., 2007c) but this subject still needs further investigation. The characterization of mycotoxin variability is of major relevance in the context of risk assessment, in order to design and strengthen control measures to reduce or avoid unacceptable patulin levels in apple products.

Higher levels of patulin were recovered from clarified apple juice than from cloudy juice when packaged in PET bottles. At 21 °C, *B. nivea* ATCC 24008 produced 146.5 μg/kg of patulin in clarified apple juice, whereas at 30 °C up to 212.5 μg/kg of this mycotoxin was recovered from clarified apple juice inoculated with *B. fulva* IOC 4518. These contrasting levels may be explained either by differences in available substrates for mycotoxin metabolism, or by underestimation during analysis. It is already known that patulin production is reduced when the size of the carbohydrate molecule increases (Damaglou et al., 1985). In addition, the binding of patulin with juice proteins may lead to underestimation of this mycotoxin during the analysis (Baert et al., 2007b). Since cloudy apple juice is richer in pectin and proteins than clarified juice, this may be the main contributing factor to the low patulin recovery from cloudy apple juices.

The influence of the type of package on the production of patulin by *Byssochlamys* can be observed when data for cloudy juice packaged in PET bottles and in laminated paperboard packages are compared. Although growth of *B. nivea* strains was observed at 30 °C in both PET and laminated paperboard packages (Fig. 1A), patulin was not detected (LOD 0.5 μg/kg) in the laminated paperboard packages but was detected in the PET bottles, showing the inability of these strains to grow in the environment found in laminated paperboard packages. Differences in the headspace and oxygen permeability between PET bottles and laminated paperboard packages may have influenced the production of patulin by *B. nivea* strains. A headspace of 4 cm was found in the PET bottles, but in the laminated paperboard packages, the headspace was minimal or absent. In addition PET bottles have shown greater oxygen permeability (0.055 cc/package day atm) than laminated paperboard packages (0.0021 cc/package day atm). According to Rice (1980), patulin production increases with the size of the headspace found in the packages. The production of patulin by *B. fulva* IOC 4518 in cloudy apple juice packaged in laminated paperboard packages (153 μg/kg), demonstrated the potential of this strain to produce this mycotoxin under the conditions found in laminated paperboard packages. This is of concern, since this strain has also synthesized large amounts of patulin in clarified apple juice contained in PET bottles at both 21 °C and 30 °C.

The mean mycelium dry weight produced by *Byssochlamys* strains at 30 °C was about 6 times higher than that produced at 21 °C. This increase is in agreement with the data presented by Roland and Beuchat (1984), Roland et al. (1984) and Rice et al. (1977), who reported that mycelium dry weight was inversely proportional to temperature. More mycelium material was produced at 30 °C than at 18 °C or 21 °C (Rice et al., 1977; Roland and Beuchat, 1984; Roland et al., 1984). However no correlation was observed in the present study between mycelium dry weight and patulin, whereas the pH value and °Brix were observed to correspond to the extent of mycelium dry weight accumulation, corroborating previously published data (Rice, 1980; Roland and Beuchat, 1984; Roland et al., 1984). The small variations in pH and °Brix found in the present study indicated that the *Bysschlamys* strains preferentially used sugars as their source of energy instead of organic acids, which would result in greater variations (Roland et al., 1984). Slight increases in pH (about 0.05 units) during apple juice storage could be explained by small amounts of ammonium produced by the *Byssochlamys* strains (Roland and Beuchat, 1984).

Regarding the confirmation of the identity of *B. fulva* IOC 4518 based on the genetic sequencing of ITS and β-tubulin fragments, in comparison to the other four *B. fulva* (NRRL1125, NRRL3493, NRRL2975, and NRRL2614), and according to the ESI(−)-MS/MS (Fig. 4) and HPLC results, patulin production by this *B. fulva* strain was firmly established. Several flaws should be considered when considering the previously reported inability to produce patulin, such as: a restricted number of strains tested for patulin production (Puel et al., 2007), and the non-use of an internal control in fungal PCR, which may lead to false negatives in patulin gene detection (Paterson, 2007). The production of mutagenic secondary metabolites by moulds in the culture may directly affect its DNA or may lead to enzyme inhibition causing decreased stability of the nucleic acid (Paterson et al., 2008). Thus the statement that all *B. fulva* strains are unable to synthesize patulin should be carefully re-interpreted, and it should be stated that *B. nivea* strains are more likely to produce patulin than *B. fulva* strains.

The data showed that if, on one hand, the *B. nivea* strains (FRR 4421 and ATCC 24008) stood out as the main patulin producers at 21 °C, on the other hand, *B. fulva* IOC 4518 was the main producer at 30 °C. Despite the recognition of *B. nivea* strains as the main patulin producers in the *Byssochlamys* genus, it must be considered that the incidence of a species may vary from region to region and may be governed by conditions that are not well known (Jesenská et al., 1992; Tournas, 1994). *B. fulva* presents marginally superior heat resistance, which suggests a greater possibility to survive apple juice pasteurization than *B. nivea* (Beuchat and Rice, 1979; Tournas, 1994). These effects must be considered to avoid an overestimation of one species as compared to the other. The control of contamination and incidence of these fungi must therefore be carried out for both *Byssochlamys* species with a view to food safety. Finally, the low variation in pH and °Brix, and the low mycelium dry weight and high patulin production in apple juice under the conditions studied, indicated the potential for mycotoxin production in the various packages of pasteurized apple juice during commercialization. Considering the conditions assessed here, patulin production may occur within 10 days after juice pasteurization, posing a risk to consumers. This possibility reinforces the need to strengthen the control measures so as to avoid or reduce contamination of the raw materials and juices by ascospores of heat resistant fungi such as *Byssochlamys*.

Acknowledgements

The authors are grateful to the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPQ), *Fundacao de Amparo a Pesquisa do Estado de São Paulo* (FAPESP), *Coordenação de Aperfeiçoamento de Pessoal de Nível de Superior* (CAPES), *Fundo de Apoio Ao Ensino, à Pesquisa e à Extensão* (FAEPEX) (Processes 282/06 and 129/07) and to *Prodetab* (Embrapa), for providing funds for this research. The authors are also grateful to Dr. O. Puel for sequencing the *B. fulva* and *B. nivea* strains.

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