International Journal of Food Microbiology 162 (2013) 237–244

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

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

Molecular characterization, fitness and mycotoxin production of benzimidazole-resistant isolates of *Penicillium expansum*

Anastasios A. Malandrakis [a,*], Anastasios N. Markoglou [a,1], Sotiris Konstantinou [b], Eleftherios G. Doukas [a], John F. Kalampokis [a], George S. Karaoglanidis [b]

[a] Pesticide Science Laboratory, Agricultural University of Athens, 75 Iera Odos, 118 55 Athens, Greece
[b] Plant Pathology Laboratory, Faculty of Agriculture, Aristotelian University of Thessaloniki, POB 269, 54124, Thessaloniki, Greece

ARTICLE INFO

Article history:
Received 21 November 2012
Received in revised form 15 January 2013
Accepted 23 January 2013
Available online 31 January 2013

Keywords:
Penicillium expansum
Blue mold
Patulin
Citrinin
Benzimidazole resistance

ABSTRACT

Penicillium expansum field-strains resistant to benzimidazole fungicides were isolated in high frequency from decayed apple fruit collected from packinghouses and processing industries located in the region of Imathia, N. Greece. *In vitro* fungitoxicity tests resulted in the identification of two different resistant phenotypes: highly (BEN-HR) and moderately (BEN-MR) carbendazim-resistant. Thirty seven percent of the isolated *P. expansum* strains belonged to the BEN-HR phenotype, carried no apparent fitness penalties and exhibited resistance levels higher than 60 based on EC_{50} values. Cross resistance studies with other benzimidazole fungicides showed that all BEN-HR and BEN-MR isolates were also less sensitive to benomyl and thiabendazole. Fungitoxicity tests on the response of BEN-HR isolates to fungicides belonging to other chemical classes revealed no cross-resistance relationships between benzimidazoles and the phenylpyrrole fludioxonil, the dicarboximide iprodione, the anilinopyrimidine cyprodinil, the QoI pyraclostrobin, the imidazole imazalil and the triazole tebuconazole, indicating that a target-site modification is probably responsible for the BEN-HR phenotype observed. Contrary to the above, some BEN-MR isolates exhibited an increased sensitivity to cyprodinil compared to benzimidazole-sensitive ones. BEN-MR isolates had fitness parameters similar to the benzimidazole-sensitive isolates except for conidia production which appeared significantly decreased. Analysis of mycotoxin production (patulin and citrinin) showed that all benzimidazole-resistant isolates produced mycotoxins at concentrations significantly higher than sensitive isolates both on culture medium and on artificially inoculated apple fruit. Comparison of the β-tubulin gene DNA sequence between resistant and sensitive isolates revealed a point mutation resulting from the E198A substitution of the corresponding protein in most but not all HR isolates tested. Molecular analysis of the β-tubulin gene in moderately resistant isolates did not reveal any amino acid substitution. This is the first report on the existence and distribution of highly mycotoxigenic field isolates of *P. expansum* resistant to the benzimidazoles indicating a high potential risk of increased mycotoxin contamination of pome fruit and by-products.

1. Introduction

Penicillium expansum, the causal agent of blue mold, is considered to be the most important postharvest pathogen of pome fruit causing losses in the field or during harvest, storage and handling of apples and other deciduous fruits (Amiri and Bompeix, 2005; Morales et al., 2008b; Konstantinou et al., 2011). Apart from yield losses from decayed fruit, *P. expansum* represents a health and food safety hazard as it produces a variety of mycotoxins and secondary metabolites toxic to humans and animals (Abramson, 1997; Bullerman, 2000). The most important mycotoxins produced by *P. expansum*, patulin and citrinin, are widely disseminated in rotting pome and stone fruit and their byproducts.

Toxicological studies have shown that the cyclic tetraketide patulin reacts with essential cellular sulfhydryl (SH, thiol) groups of proteins and glutathione and is highly toxic to plant and animal cells (Wouters and Speijers, 1996). Patulin has been shown to have mutagenic, genotoxic, immunotoxic and neurotoxic effects on rodents and teratogenic effects on chickens (Ciegler et al., 1977; Riley and Showker, 1991; Ritieni, 2003) while it is considered to be a potent carcinogen, a fact though not yet confirmed for humans (Speijers, 2004; Schumacher et al., 2005). The maximum permitted levels of patulin in Europe are 10 μg kg^{-1} for fruit-based baby food, 50 μg kg^{-1} for fruit juice and 25 μg kg^{-1} for solid apple products (European Commission, 2006). In spite the lack of legal requirements about the levels of citrinin in food and feed (Flajs and Peraica,

* Corresponding author. Tel.: +30 2105294546.
E-mail address: tasmal@aua.gr (A.A. Malandrakis).
[1] Sadly, co-author A. Markoglou has passed away.

0168-1605/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.01.014

2009), it is still a concern for human health since it has been shown to be responsible for chronic diseases exerting a strong nephrotoxic, immunotoxic and potential teratogenic action. Exposure of humans to these mycotoxins *via* consumption of infected apple juices and other apple-based products may result in severe toxicosis, including suppression of the immune system, nausea, vomiting and other gastrointestinal traumas that accompany kidney damage (Ciegler et al., 1977; Brackett and Marth, 1979; Hopkins, 1993) which is why the prevention of mycotoxin contamination of agricultural products is one of the major concerns for human and animal safety.

Various physical, chemical and biological detoxification methods to reduce mycotoxin levels in stored and processed agricultural products have been tested as well as measures aiming to reduce disease incidence and development such as pre- and post-harvest sprays with fungicides, careful handling of fruit and storage in controlled temperature/atmosphere chambers (Moake et al., 2005; Conway et al., 2007; Morales et al., 2010). The most effective measure to reduce the incidence of blue mold in most crops, especially under storage conditions, is the frequent application of fungicides (Morales et al., 2007, 2010). Fungicides most commonly used worldwide for postharvest applications against *P. expansum* on fruit belong to benzimidazoles (TBZ) or sterol biosynthesis inhibitors (imazalil) while newer fungicides such as cyprodinil, pyrimethanil, fludioxonil, pyraclostrobin and boscalid have been shown to be effective against the pathogen (Errampali, 2004; Sholberg et al., 2005a; Smilanick et al., 2006; Xiao and Boal, 2009). However, chemical control of the pathogen has suffered heavily from the development of resistance to the intensively used site-specific fungicides such as benzimidazoles and DMIs (Baraldi et al., 2003; Morales et al., 2008a; Rosenberger et al., 1991; Sholberg et al., 2005b; Vinas et al., 1991, 1993). Resistance problems to benzimidazoles have emerged shortly after their introduction in many pathogens worldwide (Ma and Michailides, 2005). In most cases control failure resulted from target site alterations and more specifically resistance mutations at positions 198 and 200 of the beta-tubulin protein even though positions 50 and 240 are also implicated in reduced sensitivity to benzimidazoles (McKay et al., 1998; Albertini et al., 1999). A number of resistance mutations in the β-tubulin gene at positions 167, 198, 200 and 240 have been associated with various benzimidazole resistance levels in *P. expansum* worldwide (Koenraadt et al., 1992; Baraldi et al., 2003; Sholberg et al., 2005b) but no report on benzimidazole resistance concerning the pathogen is available from Greece.

Although sensitivity and benzimidazole resistance are well documented in *P. expansum* (Koffmann et al., 1978; Koenraadt et al., 1992; Baraldi et al., 2003; Sholberg et al., 2005b; Errampali et al., 2006), there is very limited data about the mechanism of benzimidazole resistance of the pathogen in Greece and no information is available regarding the influence of benzimidazole-resistance on mycotoxin production. Thus, the aims of the present study were: i) to determine the sensitivity of *P. expansum* field populations obtained from apples in Greek packinghouses to the benzimidazoles, ii) to investigate the molecular mechanism of resistance, and iii) to assess the impact of benzimidazole resistance on fitness and mycotoxin production of the resistant field isolates.

2. Materials and methods

2.1. Isolates and culture conditions

P. expansum isolates were collected in a survey conducted during the 2008–2009 storage period in 5 packinghouses located in the region of Imathia (Northern Greece). Apple fruit showing decay symptoms were collected from the packinghouses and transferred to the laboratory. Despite the absence of any fungicide postharvest treatment during the years of sampling there was a prolonged history of thiabendazole postharvest treatments in the packinghouses during the recent past. Furthermore, the sampled fruit had originated from apple orchards that, a few days before harvest, had been treated with boscalid, a succinate dehydrogenase inhibitor, aiming to reduce postharvest rots during storage. Isolations were carried out by transferring small fruit pieces from the margin of diseased/healthy tissue to acidified Potato Dextrose Agar (PDA) (Oxoid, Unipath Ltd., Basingstoke, England), in Petri dishes. After 3–4 days incubation at 20 °C, fungal colonies that appeared to be *Penicillium* spp. were single-spored using Tuite's (1969) serial dilution technique. A total of 250 isolates, identified according to Pitt (2002) as *P. expansum*, were used in the study. Yeast Extract Sucrose medium (YES) containing 2 g yeast extract, 15 g sucrose and 1.5 g agar in 100 ml distilled water was used for the *in vitro* study of patulin and citrinin production. For long-term storage, the isolates were maintained on PDA slants at 2 °C.

2.2. Fungicides, mycotoxins and solvents

All fungicides used in *in vitro* experiments were pure technical grade. Carbendazim and tebuconazole were kindly supplied by Bayer CropScience AG (Leverkusen, Germany), benomyl by Du Pont de Nemours and Co. (Wilmington, DE, USA), pyraclostrobin by BASF AG (Limburgerhof, Germany), iprodione by Rhône Poulenc Agro SA, Lyon, France, and cyprodinil and fludioxonil by Syngenta Crop Protection AG (Basle, Switzerland). Thiabendazole (TBZ) and imazalil were purchased from Sigma. Ethanol was used as solvent for all fungicide stock solutions with the exceptions of benomyl and pyraclostrobin which were dissolved in acetone, and TBZ in methanol. Patulin and citrinin standards were purchased from Sigma-Aldrich (St. Louis, USA). Analytical standard stock solutions of patulin and citrinin were made in methanol at various concentrations and stored at −20 °C. Ten standard solutions of each mycotoxin at concentrations from 0.01 to 5 μg/ml were prepared as calibration standards. HPLC grade solvents, methanol and acetonitrile, were purchased from Lab Scan (Dublin, Ireland). Ultrapure-grade HPLC water was obtained by purification of distilled water through a Milli-Q Gradient system (Millipore, Bedford, USA). All fungicides were added aseptically from stock solutions to sterilized growth medium prior to inoculation and the final amount of solvent never exceeded 1% (v:v) in treated and control samples.

2.3. In vitro fungitoxicity bioassays

The measurement of resistance frequency to carbendazim was based on the use of a discriminatory concentration at the minimum inhibitory concentration (MIC) value of the sensitive isolates. PDA plates were amended with carbendazim at the concentration of 1 μg/ml. Control plates were not amended with fungicide. For each isolate three replicate plates were prepared. Cultures were incubated at 20 °C in the dark for 7 days. Isolates exhibiting mycelial growth on the fungicide-amended media were considered as fungicide-resistant while isolates with completely inhibited mycelial growth were considered as fungicide-sensitive.

After determining benzimidazole-resistance profile within the entire pathogen population, 50 isolates of *P. expansum* from all carbendazim-sensitivity phenotypic classes were selected for further fungitoxicity tests in order to precisely determine sensitivity to carbendazim and investigate cross-resistance relationships with other fungicides. Measurement of sensitivity was based on the calculation of EC_{50} values (the concentration causing 50% reduction of mycelial growth) for each fungicide. To measure the EC_{50} values of the 50 selected isolates, L-asparagine-based agar medium (ASP-agar) was amended with 0.01, 0.05, 0.1, 0.5, 1 and 5 μg/ml cyprodinil (Hilber and Schüepp, 1996), whereas PDA medium was amended with 0.005, 0.01, 0.05, 0.1 and 0.5 μg/ml fludioxonil, 0.05, 0.1, 0.5, 1 and 5 μg/ml pyraclostrobin/iprodione and 0.1, 0.5, 1, 5 and 10 μg/ml imazalil/carbendazim/benomyl/tebuconazole. Control media were