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# Influence of intraspecific variability and abiotic factors on mycotoxin production in *Penicillium roqueforti*

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ABSTRACT

*Penicillium roqueforti* has the ability to produce secondary metabolites, including roquefortine C (ROQC) and mycophenolic acid (MPA). In a previous study, the presence of these mycotoxins, alone or in co-occurrence, has been reported in blue-veined cheese. A high variability of mycotoxin content has also been observed, although the majority of samples exhibited relatively low concentrations. The observed variability raises the question of the factors impacting ROQC and MPA production. In this context, the mycotoxigenic potential of 96 *P. roqueforti* strains (biotic factor) and the effect of some abiotic factors (pH, temperature, NaCl and $O_2$ contents, and C/N ratio) on mycotoxin production were evaluated. A high intraspecific diversity, established via genotypic (RAPD) and phenotypic (FTIR) approaches, was observed. It was associated with mycotoxigenic potential variability and may thus explain part of the observed variability in mycotoxin content of blue-veined cheese. Moreover, a significant decrease of ROQC and MPA production was observed for conditions (temperature, C/N ratio, $O_2$ and NaCl concentrations) encountered during cheese-making compared with optimal growth conditions. The results also highlighted that there was no significant effect of addition of ROQC amino-acid precursor on the production of both mycotoxins whereas a pH increase from 4.5 to 6.5 slightly reduced MPA but not ROQC production.


## 1. Introduction

*Penicillium roqueforti* is a quite common spoilage agent of raw materials and processed food but is also used as a ripening culture for blue-veined cheese manufacturing.

The *P. roqueforti* complex currently comprises four distinct species based on phenotypic and genotypic traits as well as extrolite production, namely, *Penicillium carneum*, *Penicillium paneum*, *Penicillium psychrosexualis* and *P. roqueforti* (Boysen et al., 1996; Houbraken et al., 2010; Houbraken and Samson, 2011). *P. roqueforti* has the potential to produce different secondary metabolites on semi-synthetic media such as isofumigaclavines A and B, roquefortine C (ROQC), mycophenolic acid (MPA), citreoisocoumarin, andrastin A, eremofortin C and PR-toxin, but does not produce penicillic acid, patulin and botryodiploidin (Boysen et al., 1996; Frisvad et al., 2004). Some of these secondary metabolites can be observed in blue-veined cheeses (Fontaine et al., 2015; Kokkonen et al., 2005). However, considering the instability of PR-toxin in cheese (Scott and Kanhere, 1979), ROQC and MPA are considered as the most prevalent mycotoxins in blue-veined cheeses. Beyond the fact that ROQC and MPA can co-occur, the main observation of related studies is that their concentrations are also highly variable, ranging from <11 to 14,125 and from <0.5 to 14,300 μg/kg of cheese for ROQC and MPA, respectively (Fontaine et al., 2015; Kokkonen et al., 2005; Lafont et al., 1979a). Thus, these findings raise the question of the factors that can modulate mycotoxin production in the cheese-making context.

Several biotic and abiotic factors may influence mycotoxin production in *P. roqueforti* during cheese-making and ripening (Hymery et al., 2014). Two biotic factors can be identified, one, the cheese microbial ecosystem (constituted of various bacteria and yeasts) and the other, the *P. roqueforti* strain itself. While the influence of a global ecosystem on *P. roqueforti* metabolism is really complex and difficult to explore, the intrinsic ability of fungal strains to produce mycotoxins can be easily assessed and is likely one of the main factor influencing mycotoxin content in the final product. Flórez et al. (2007) and Ropars et al. (2014) have investigated the genetic diversity of *P. roqueforti* strains isolated

*Abbreviations:* ROQC, roquefortine C; MPA, mycophenolic acid; RAPD, random amplified polymorphic DNA; FTIR, Fourier transform infra-red spectroscopy.

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from blue-veined cheeses using molecular methods such as RAPD-PCR and microsatellites fingerprinting. Using microsatellite markers for genotyping, Ropars et al. (2014) showed the existence of 6 genetically divergent populations among a collection of 114 *P. roqueforti* strains which included 101 cheese isolates. In contrast, there are only a few studies on the pattern of mycotoxin production among a large number of *P. roqueforti* strains in semi-synthetic media (Engel et al., 1982; Finoli et al., 2001; Geisen et al., 2001; Lafont et al., 1979b). As for abiotic factors, several intrinsic factors related to the cheese itself (e.g., pH, $a_w$, NaCl content, C/N ratio and nutrient content) as well as extrinsic abiotic factors (e.g., relative humidity, ripening-time and -temperature or $O_2$), may have an impact on *P. roqueforti* mycotoxin production during cheese production (Hymery et al., 2014). However, while the effects of several of these factors on *P. roqueforti* growth are now well documented (Pose et al., 2007), very little is known on the effects of such factors on mycotoxin production.

The aim of this study was first to evaluate the diversity of 96 *P. roqueforti* isolates including 76 isolates from cheese using RAPD-PCR and FTIR spectroscopy. Then, the mycotoxigenic potential of these isolates was evaluated and compared on two different semi-synthetic media, yeast extract sucrose (YES) agar medium and an in-house "cheese" agar medium. Finally, the impact of abiotic factors, including temperature, pH, NaCl content, $O_2$, C/N ratio and the presence of ROQC precursors, on ROQC and MPA production was assessed for one selected *P. roqueforti* strain.

## 2. Materials and methods

### 2.1. P. roqueforti isolates

Ninety-six strains of *P. roqueforti* were investigated in the present study. Fifty-seven isolates had been isolated between November 2011 and December 2011 from 34 blue-veined cheeses collected from six European countries (Denmark, France, Italy, Ireland, Netherlands and Scotland) which had also been analyzed for their mycotoxin content (Fontaine et al., 2015). Thirty-nine strains were obtained from the UBO Culture Collection (UBOCC, Brest). Among these, 19 isolates corresponded to blue-cheese ripening cultures, 19 were obtained from spoiled dairy products and fruit preparations and one was of unknown origin.

### 2.2. DNA extraction

Each isolate was grown for 7 days in potato dextrose broth (PDB) at 25 °C, under shaking at 120 rpm. Total DNA extraction was performed using the FastDNA Spin Kit (MPBio, Illkirch, France), according to the manufacturer's instructions. DNA quantification was performed using a Nanodrop spectrophotometer (Nanodrop Technology, Labtech, Palaiseau, France).

### 2.3. Confirmation of species affiliation

Affiliation to the *P. roqueforti* species was confirmed for all the isolates after amplification and sequencing of part of the β-tubulin gene using the Bt2a and Bt2b primers (Glass and Donaldson, 1995). Sequences were compared to the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) and phylogenetic analyses were performed using sequences from the NCBI database and the MEGA6 software (Tamura et al., 2013).

### 2.4. Intraspecific diversity assessment using RAPD-PCR

Three different primers, M13 (Kure et al., 2002) and ari1 and omt1 (Geisen et al., 2001) were used to evaluate the intraspecific diversity of *P. roqueforti* isolates. The PCR-reaction mixture consisted of 1× reaction buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 μM of RAPD primer, 1.25 U of GoTaq Polymerase (Promega, Charbonnière, France) and 1 μL of DNA at 40 ng $\mu L^{-1}$. Cycling conditions were performed as described previously by Kure et al. (2002) for the M13 and Geisen et al. (2001) for ari1 and omt1 primers using a FlexCycler (Analytik Jena, Jena, Germany).

RAPD-PCR products were analyzed by electrophoresis on a 1.25% agarose gel (w/v) in 1× TBE buffer (Promega, France), at 150 V for 5 h at 7 °C. The DNA ladder 100 bp (Promega) was used as molecular size standard. Reproducibility of RAPD-PCR and electrophoresis separation were assessed for the three different primers using in each experiment a common DNA extract as a control.

The analysis of RAPD-PCR pattern was conducted using BioNumerics software, version 2.0 (Applied Maths). The normalization and band matching were performed automatically and verified visually. The reproducible part of the three different patterns obtained with each primer was used to assess the intraspecific diversity of the tested isolates. A data matrix based on the band positions of RAPD-PCR patterns was constructed using the Sorensen–Dice's coefficient with clustering parameters (optimization 2%, band matching 2%, tolerance change 0%) for cluster determination. The genetic profiles generated by each primer were concatenated and a dendrogram of similarity of RAPD-patterns was constructed by the unweighted pair group method using arithmetic averages (UPGMA). A limit of 90% of similarity was chosen to determine genotypic differences between strains on the base of reproducibility results and in agreement with Flórez et al. (2007) and Marcellino et al. (2001).

### 2.5. Intraspecific diversity assessment using FTIR

Culture preparation, spectral acquisition and spectral analysis were performed as described previously (Lecellier et al., 2014) on a FTIR high-throughput system comprising a spectrometer (Tensor 27, Bruker Optics, Champs sur Marne, France) coupled to a high-throughput module (HTS-XT, Bruker Optics).

Spectra obtained from three biological and 5 technical replicates, passing the quality tests, were averaged for further analysis. A baseline correction was performed for each averaged spectrum, followed by the computation of second derivative spectra and vector normalization according to the procedure described by Lecellier et al. (2014). Principal component analysis was applied to discriminate the average spectra of the different strains of *P. roqueforti*.

### 2.6. Mycotoxin production and mycotoxigenic potential of P. roqueforti isolates

#### 2.6.1. Media

Two semi-synthetic media were used to determine the effect of medium composition on mycotoxin production and to assess the intraspecific variability of mycotoxin production (concentration per g of medium) and mycotoxigenic potential (concentration per g of medium per g of biomass) among *P. roqueforti* isolates. YES agar medium (Frisvad and Filtenborg, 1983), characterized by a high C/N ratio supposedly favoring mycotoxin production, and a "cheese" agar (CA) medium (modified from Le Dréan et al., 2010) characterized by a low C/N ratio, were used. For approximately 400 mL of CA medium, 20.4 g milk protein concentrate (MPC, Lactalis, Retiers, France), 26.7 g anhydrous milk fat and 2.8 mL DL-lactic acid (Sigma-Aldrich, France) were dissolved in a sterile solution containing 257.8 mL distilled water, 6 g agar, 8 g NaCl, 2.6 g lactose and 0.65 g yeast extract (bioMérieux, France) adjusted to pH 5 with HCl. The obtained mixture was homogenized for 1 min using an Ultraturrax T25 Basic (IKA, Heidelberg, Germany), heated at 80 °C for 30 min in a water bath and transferred into Petri dishes. The microbiological quality (sterility) of CA medium was checked on plate count agar medium prior to use.