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1-octanol, a self-inhibitor of spore germination in *Penicillium camemberti*

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**Running title:** Quorum sensing in *Penicillium camemberti*

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Abstract

Penicillium camemberti is a technologically relevant fungus used to manufacture mould-ripened cheeses. This fungal species produces many volatile organic compounds (VOCs) including ammonia, methyl-ketones, alcohols and esters. Although it is now well known that VOCs can act as signaling molecules, nothing is known about their involvement in P. camemberti lifecycle. In this study, spore germination was shown to be self-regulated by quorum sensing in P. camemberti. This phenomenon, also called the "crowding effect", is population-dependent (i.e. observed at high population densities). After determining the volatile nature of the compounds involved in this process, 1-octanol was identified as the main compound produced at high-spore density using GC-MS. Its inhibitory effect was confirmed in vitro and 3 mM 1-octanol totally inhibited spore germination while 100 μM only transiently inhibited spore germination. This is the first time that self-inhibition of spore germination is demonstrated in P. camemberti. The obtained results provide interesting perspectives for better control of mould-ripened cheese processes.

Keywords: Cheese, quorum sensing, Penicillium camemberti, germination, 1-octanol.

1. Introduction

Fungal spores can be dispersed in different ways, namely through air, dust or water, and are important for fungal dissemination and/or survival. Conidia are dispersed asexual spores and can be released in massive numbers by fungi, in particular by *Penicillium* genus members (Dijksterhuis and Samson, 2002) including many food-related species. While conidia are easily disseminated, spore germination only occurs when favorable environmental conditions (i.e. temperature, pH, humidity, light and nutrients) are met. Spore germination is also influenced by spore density and is hindered at high spore densities (Macko et al., 1972). First evidence for this phenomenon, also called "quorum sensing", was shown for genetic competence induction (Felkner and Wyss, 1964; Tomasz and Hotchkiss, 1964) and in light production regulation (Nealson and Hastings, 1979) in Gram-positive bacteria and marine vibrios, respectively. The ability of microbial cells to chemically sense the density of the surrounding population (Fuqua et al., 1994) by extracellular factors has also been found in diverse microorganisms including fungi (Hogan, 2006). This phenomenon actually allows for cell density-dependent growth regulation, hence the term "crowding effect", and appears to be prevalent in diverse fungal species (Hogan, 2006). Previous studies suggested that intercellular signaling via self-inhibitor compound production prevents premature germination and guarantees that spores only germinate at the suitable time, i.e. in favorable environmental conditions.

Self-inhibitors, which can correspond or not to volatile compounds, have been characterized in numerous fungal genera including *Aspergillus, Colletotrichum, Dictyostelium, Fusarium, Glomerella, Penicillium, Puccinia, Syncephalastrum* and *Uromyces* (Bacon et al., 1973; Barrios-González et al., 1989; Chitarra et al., 2004; Garrett and Robinson, 1969; Hobot and Gull, 1980; Lax et al., 1985; Leite and Nicholson, 1992; Lingappa et al., 1973; Macko et al., 1972). Regarding the *Penicillium* genus, Chitarra et al. (2004, 2005) demonstrated that 1-
octen-3-ol influenced different developmental processes during the *Penicillium paneum* lifecycle, including spore germination inhibition.

*Penicillium camemberti*, first described by Thom in 1906 (Raper and Thom, 1949), is mainly used in cheese manufacture and more particularly for mould-ripened soft cheeses such as Camembert, Brie and Coulommiers. *P. camemberti* growth results in the formation of a characteristic white rind (Abbas and Dobson, 2011). During spore germination and mycelial growth, lipase and protease activities are involved in lipid and protein degradation in cheese resulting in fatty and amino acid release (Beresford and Williams, 2004). These latter are then transformed into important taste and aroma compounds such as ammonia, methyl-ketones, primary and secondary alcohols, esters, aldehydes, lactones and sulfur compounds (Abbas and Dobson, 2011) and contribute to overall organoleptic qualities.

Due to the important role of *P. camemberti* in cheese manufacture, there is a clear interest to understand regulatory mechanisms potentially involved in conidia germination to better control soft mould-ripened cheese production. In this context, the aims of this study were to first investigate the effect of spore density on spore germination in *P. camemberti*, then to identify the molecules involved and finally estimate the impact of the identified compound and other volatile compounds on spore germination.

### 2. Materials and methods

#### 2.1 Strains, culture conditions and spore suspension preparations

Two commercial *P. camemberti* strains (coded *P. camemberti* A and B for confidentiality reasons) isolated from mould-ripened cheeses and *P. camemberti* UBOCC-A-101392 and UBOCC-A-108097, and CBS 299.48\(^T\) respectively obtained from the Université de Bretagne Occidentale Culture Collection (UBOCC, Plouzané, France) and the Central Bureau Voor Schimmelcultures (Baarn, Netherlands) were used in this study. Pre-cultures were prepared
on slant Potato Dextrose Agar (Difco PDA, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) by inoculating a spore suspension, conserved at -80°C in 10 % glycerol (v/v), and incubating tubes for 7 days at 25°C. Strains were then sub-cultured on slant PDA and incubated at 25°C for 14 days. After incubation, spores were harvested using 8 mL of sterile distilled water supplemented with 0.1 % Tween 80. Slant agar cultures were scraped using a 1 mL sterile pipette to collect spores. Then, Roux flasks containing 100 mL Malt Extract Agar (MEA, Merck, Darmstadt, Germany) were sowed with 6 mL spore suspension. After incubation for 14 days at 25°C, 25 mL of sterile distilled water supplemented with 0.1 % Tween 80 were added to each Roux flask and the mycelium surface was scraped using a cell scraper (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The resulting spore suspension was transferred into a tube and centrifuged for 5 min at 4000 g at 4°C (CR3i multifunction, Thermo Fisher Scientific Inc., Waltham, MA, USA). After carefully discarding the supernatant, spores were resuspended in 5 mL sterile distilled water supplemented with 0.1 % of Tween 80. Finally, all suspensions of a same strain were pooled together, counted using a Malassez cell and standardized to $2 \times 10^8$ and $2 \times 10^6$ spores.mL$^{-1}$.

### 2.2 Effect of spore concentration on germination kinetics in culture broth

Spore germination kinetics were performed as described by Chitarra et al. (2004, 2005) with some modifications in Malt Extract Broth (MEB, Merck, Darmstadt, Germany) using fresh spore suspensions. To determine the effect of spore density on germination kinetics, 0.75 mL spore suspensions (either $2 \times 10^8$ or $2 \times 10^6$ spores.mL$^{-1}$) were mixed with 0.75 mL MEB 2X to obtain a final spore concentration of $10^8$ or $10^6$ spores.mL$^{-1}$. Suspensions were then incubated horizontally in a rotary shaker set to 25°C and 120 rpm. Germination was followed at 0 h, 8 h, then at 2 h intervals for the following 12 h. A minimum of 100 conidia were counted to determine germination kinetics (i.e. percentage of germinated spore as a function of time).
spore was considered as germinated when the germinal tube length was superior or equal to the spore diameter itself, as previously described (Dantigny et al., 2006). In addition, in order to examine whether spore germination capacity was related to spore density, high density spore suspensions obtained after 20 h incubation were diluted to $10^6$ spores.mL$^{-1}$ in fresh MEB 1X and incubated for another 18 h. Moreover, to assess whether inhibitory molecules were produced during incubation at high spore density, filter sterilized cultures obtained after 20 h incubation were inoculated at $10^6$ spores.mL$^{-1}$ and also incubated for another 18 h. For each spore concentration level, three technical replicates and two biological replicates were performed.

2.3 Effect of spore density on spore germination and radial growth in agar medium

In these experiments, a Petri dish containing Malt Extract Agar (MEA, Merck, Darmstadt, Germany) layers at the bottom and the top (in the lid) was used as described previously (Chitarra et al., 2004). The top MEA layer was surface inoculated with 50 $\mu$L of a $10^6$ or $10^8$ spores.mL$^{-1}$ suspension while 5 $\mu$L of a $10^7$ spores.mL$^{-1}$ suspension were deposited into the center of the plate on the bottom layer. Control cultures were also performed without inoculating the top layer. Colony diameters were measured in two perpendicular directions every 2 days for 14 days (plates were incubated at 25°C). Five replicates per condition were performed. Mean values ± 95 % confidence intervals were calculated for each condition.

2.4 Extraction of volatile compounds

Volatile compounds produced by P. camemberti in liquid cultures after 20 h incubation were extracted as previously described (Husson et al., 2002). Briefly, 0.5 g NaCl and 1 mL diethyl ether were added to 1 mL culture suspension followed by centrifugation at 15,000 g for 2 min
at room temperature. The organic phase (superior phase) was recuperated and Na₂SO₄ was added to remove any traces of water.

2.5 Detection and identification of volatile compound(s) by GC-MS

Detection and identification of volatile compounds were performed by gas chromatography (Agilent Technologies 7820A GC Systems, Agilent Technologies, Santa Clara, CA, USA) coupled with mass spectrometry (Agilent Technologies 5975 Series MSD, Agilent Technologies, Santa Clara, CA, USA) using a HP-5ms (30 m x 250 μm x 0.25 μm) column. The program used for the oven was as follows: 50°C for 5 min, 4°C.min⁻¹ between 50°C and 200°C then 10°C.min⁻¹ from 200°C to 270°C, followed by a final step at 270°C for 10 min. The injector and detector temperatures were set to 250°C and 300°C, respectively. The conditions of the mass spectrometer were mode electronic impact (EI); temperature source 250°C; scanning speed 1 scan.s⁻¹; mass acquisition 50-300 μa. The vector gas was H₂ at a 1.5 mL.min⁻¹ flow rate. For identification, mass spectra were compared to the NIST mass spectral library and were confirmed using the retention index and mass spectrum of pure compounds.

2.6 Effect of pure volatile compounds on spore germination in MEB medium

The impact of the major volatile compound identified by GC-MS on the spore germination process was evaluated in MEB for two strains (P. camemberti A and B) at concentrations ranging between 0 and 4 mM. The effect of other volatile compounds potentially produced by P. camemberti, i.e. ethanol, ammonia, 2-heptanone, 2-nonanone, 1-octen-3-ol, trans-2-octen-1-ol, 2-methyl-1-butanol, 3-methyl-1-butanol and 3-octanone, was also investigated. To perform these assays, after solubilization of the tested compound in propylene glycol and serial dilutions, the percentage of germinated conidia from a 10⁶ spore.mL⁻¹ suspension was
determined in MEB as described above, after 10 and 16h incubation at 25°C with agitation (120 rpm). Control cultures without volatile compound but containing the same amount of propylene glycol were also performed.

2.7 Effect of initial spore concentration on the growth of *P. camemberti* determined by ergosterol analysis in a cheese matrix model

The cheese matrix model was prepared as described previously (Le Dréan et al., 2010). Briefly, to prepare 100 g, 23 g milk protein concentrate, 20 g anhydrous milk fat, 50 mL sterile distilled water, 1.5 g NaCl and 1.26 mL lactic acid were added. Prepared model cheeses were inoculated with either *P. camemberti* A or B spore suspensions to yield a final spore concentration ranging from $10^2$ to $10^6$ spores.g$^{-1}$. Then, 50 g cheese portions were transferred to sterile crystallizing dishes (5.6-cm diameter) and incubated for 9 days at 12°C. After incubation, a 32-mm diameter and ~5 mm depth layer was removed from the cheese surface with a sterile scalpel. Sufficient sterile 2% (w/v) trisodium citrate was added to yield a 1:10 dilution and the resulting suspension was homogenized with an Ultra-Turrax (IKA, Staufen, Germany) at 24,000 rpm.min$^{-1}$ for 1 min. Five mL of the trisodium citrate / cheese mixture were then centrifuged in a 12-mL screw cap tube at 10,000 g for 10 min at 4°C. The supernatant was discarded and the mycelium pellets resuspended in 0.5 mL of methanol. After vortexing, 2.5 mL of 24 % methanolic KOH (24 %) were added and incubated for 2h at 85°C. After cooling in melted ice for 10 min, 2 mL of petroleum ether were added and vortexed 3 times for 20 sec with a cooling step on ice for 2 min after each homogenization treatment. This step was repeated and the extract was centrifuged for 10 min at 3000 rpm at 4°C. The organic phase was collected and evaporated under nitrogen flow for ~15 min and stored at -20°C before use. Prior to HPLC analysis, the dry extract was dissolved in 0.5 mL methanol and filtered through a 0.45-μm acetate filter. Ergosterol was quantified, using
external ergosterol standards (Sigma, St Louis, MO, USA), with a high-performance liquid chromatograph Agilent 1100 series (Agilent Technologies, Santa Clara, CA, USA) equipped with an Interchrom Lichrospher C18 column and an UV detector set at 282 nm. The mobile phase was methanol with a flow rate set to 1.2 mL.min⁻¹ and injection volume was 50 μl. Each sample and standard were analyzed in triplicate. Mean values ± 95 % confidence intervals were calculated for each strain and condition.

3. Results

3.1 Effect of spore concentration on germination kinetics in broth medium

Germination kinetics according to initial spore population for the 2 studied P. camemberti strains in MEB are presented in Fig. 1. Independently of the studied strains, cultures containing 10⁶ spores.mL⁻¹ presented higher germination percentages compared to 10⁸ spores.mL⁻¹ cultures. Indeed, after 20h incubation, >90 % and <3% spores had germinated at 10⁶ versus 10⁸ spores.mL⁻¹. Similar results were also obtained on P. camemberti strains UBOCC-A-101392, UBOCC-A-108097 and CBS 299.48T (data not shown). To determine whether germination inhibition was the consequence of higher spore density, 10⁸ spores.mL⁻¹ cultures obtained after 20 h incubation were diluted to 10⁶ spores.mL⁻¹ and further incubated for 18 h. Germination percentages for both strains were ~ 90 % indicating that a "crowding-effect" was responsible for this inhibition.

In order to check for self-inhibitory molecules in the high density spore suspension, 20-h 10⁸ spores.mL⁻¹ culture filtrates were inoculated with a new spore suspension (10⁶ spores.mL⁻¹) and germination kinetics were compared to a control (Fig. 2). While 98 % of spores had germinated in the control, germination rates in the culture filtrates were only 13 % and 8 % for P. camemberti A and B, respectively (Fig. 2), thus, highlighting that one or several self-inhibitors were present in the culture medium previously containing 10⁸ spores.mL⁻¹.
3.2 Effect of spore density on spore germination and radial growth in agar medium

To examine if spore density could impact spore germination and radial growth in solid medium, a Petri dish containing MEA layers at both the bottom and top (in the lid) was used as previously described by Chitarra et al. (2004, 2005). As shown in Fig. 3, growth in the bottom of the Petri dish was significantly delayed as compared to the control (Fig. 3A) when the lid was inoculated with 50 μL of a spore suspension containing $10^6$ (Fig. 3B) or $10^8$ (Fig. 3C) spores mL$^{-1}$, the latter having the strongest effect. Indeed, mean radial growth rates were 4.14, 0.57 and 0.47 mm d$^{-1}$ for the control and the lid agar inoculated with $10^6$ and $10^8$ spores mL$^{-1}$, respectively. Overall, these results strongly suggested that volatile compounds, able to move from the MEA layer of production to the MEA layer of action, could be responsible for the observed effects.

3.3 Detection and identification of volatiles compound(s) by GC-MS

The volatile compounds present in MEB cultures containing $10^8$ spores mL$^{-1}$ of $P.\ camemberti$ strain A or B were analyzed by GC/MS and compared to those of the non-inoculated MEB medium (Fig. 4). Major differences in volatile compound profiles between the inoculated media (Fig. 4A) and control (Fig. 4B) were observed. Noteworthy, the most abundant volatile compound produced at high spore density exhibited a retention time of 10.997 min. The nature of this compound was assigned to 1-octanol based on its mass spectrum (Fig. 4C) and confirmed after comparison of its mass spectrum with that of pure 1-octanol (Fig. 4D). This compound was also verified to have a different retention time and mass spectrum from those of 1-octen-3-one, 3-octanone, 2-octanone, octanal, trans-2-octen-1-ol and 1-octen-3-ol (retention times were 7.237, 8.03, 8.201, 8.488, 11.935 and 12.796 min, respectively) in the tested conditions (data not shown).
3.4 Effect of pure volatile compounds on germination efficiency in MEB

The effects of 1-octanol, as well as other volatile compounds potentially produced by *P. camemberti*, on germination efficiency were studied *in vitro*. The germination inhibitory effect of 1-octanol was confirmed in MEB. Indeed, total inhibition of spore germination was achieved in the presence of 3 mM 1-octanol while a concentration as low as 100 μM transiently delayed spore germination after 10h but not after 16h. Indeed, at the latter concentration, the percentage of germinated spores was reduced by 10 % as compared to the control. It is worth mentioning that 1-octen-3-ol, a compound previously identified to act as a self-inhibitor in *P. paneum* (Chitarra et al., 2005, 2004), and its isomer trans-2-octen-1-ol had a similar minimum inhibitory concentration (MIC) than 1-octanol. Other compounds, namely, ethanol, ammonia, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-heptanone and 3-octanone did not inhibit spore germination in the tested conditions (MIC>4 mM).

3.5 Effect of spore concentration on *P. camemberti* growth as determined by ergosterol analysis in a cheese matrix model

Fungal biomass obtained after 9 days incubation at 12 °C as a function of the initial spore concentration (10^2 to 10^6 spores.g^-1 of cheese) is shown in Fig. 5. Independently of the tested strain, there was no significant difference in fungal biomass whatever the initial spore concentration used.

4. Discussion

Self-regulation of spore germination as a function of spore density has been previously characterized in a wide range of fungal species including *Penicillium* species (Chitarra et al., 2004, 2005). In the present study, we demonstrated for the first time that this phenomenon,
which was reversible, also occurred in *P. camemberti*, an industrially relevant fungus used in
the dairy industry. We also showed that 1-octanol, a volatile compound, was produced at high
spore density and that this compound hindered spore germination *in vitro*. In nature,*
*Penicillium* spp. produce high spore numbers which are directly exposed to air, thus
explaining why volatile self-inhibitors may be more widespread than non-volatile ones. Thus,
as suggested by Chitarra et al. (2004) for 1-octen-3-ol, we can hypothesize that 1-octanol is
produced by *P. camemberti* conidia and released into the air in order to inhibit germination
until appropriate environmental conditions are met. Likewise, as previously described for 1-
octen-3-ol in *P. paneum* and *Aspergillus nidulans* (Chitarra et al., 2005; Herrero-Garcia et al.,
2011), conidia treated with 3 and 4 mM 1-octanol did not enter into isotropic growth,
meaning that 1-octanol prevented the initiation of the spore swelling process which precedes
polarized growth and the formation of a germ tube. Thus, this volatile compound may have an
effect on fungal membrane permeabilisation which controls water entry into conidia (Chitarra
et al., 2005).

More generally, eight-carbon (8-C) volatiles are ubiquitous among fungi and characteristic of
fungal aromas (Combet et al., 2006). They result from the oxidation and cleavage of fatty
acids, in particular linoleic acid. Such products constitute for 44.3-97.6% of the total amount
of volatiles produced, depending on the extraction method used (Maga, 1981; Tressl et al.,
1982; Venkateshwarlu et al., 1999). The well-known 1-octen-3-ol presenting a mushroom-
like aroma is the most abundant VOC while 1-octanol is characterized by a detergent, soap
and orange-like aroma (Combet et al., 2006). In the present study, 1-octanol was the only 8-C
volatile compound identified in cultures with high conidial density while 1-octen-3-ol was not
detected. Likewise, *P. commune*, which is considered as an ancestral form of the domesticated
*P. camemberti* (Pitt et al., 1986), was not found to produce 1-octen-3-ol at high spore density
(Chitarra et al., 2005). Nevertheless, 1-octen-3-ol was found, here, to block the germination
process in vitro. Given that the role of this compound as a self-inhibitor of germination has also been demonstrated in several fungal genera and species (Chitarra et al., 2004; Herrero-Garcia et al., 2011), it could be considered as a cross-talk molecule. In addition, 1-octen-3-ol is produced at high levels by *P. camemberti* during growth, especially in cheese (Abbas and Dobson, 2011), therefore its role in controlling other steps of the *P. camemberti* growth cycle cannot be excluded and should be further studied. Finally, 3-octanone, a methyl-ketone derived from linoleic acid which is also produced by *P. camemberti* (Adda et al., 1989), also deserves further attention since this compound was identified as a conidiogenic compound in *A. nidulans* (Herrero-Garcia et al., 2011).

In the last part of the present study, we investigated whether the initial spore inoculum could impact the *P. camemberti* colonization process in model cheeses. We found that estimated biomass by ergosterol measurements was similar regardless of initial inoculum, suggesting that the germination and growth coordination phenomena could also occur in cheese. In addition, low inoculum levels containing \(10^2\)-\(10^3\) spores.g\(^{-1}\) of cheese could be sufficient to maximize *P. camemberti* growth. In the cheese industry, the inoculation level of *P. camemberti* is empirically determined as a function of the organoleptic properties desired in final product (surface appearance, texture and flavor). Better knowledge of the phenomena governing the *P. camemberti* colonization process could be useful to better control growth and metabolic activities during cheese ripening.

**Conclusions**

In the present study, self-regulation of spore germination by quorum sensing in *P. camemberti* was shown. The volatile nature of the involved compounds was demonstrated and 1-octanol was found to be the main volatile compound produced at high spore density. Its inhibitory effect was verified in vitro, showing that 3 mM 1-octanol totally inhibited spore germination.
while 100 μM only transiently inhibited this process. This is the first time that spore germination self-inhibition is detected in *P. camemberti* and such knowledge could be useful to better control the ripening process of mould-ripened cheeses.

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**References**


**Figure legends**

**Fig. 1.** Germination of *Penicillium camemberti* strain A (A) and B (B) conidia at 25°C in malt extract broth as a function of the tested spore density (10^6 spores.mL^-1, □ and 10^8 spores.mL^-1, ▲). The arrows represent the time (20h) at which the spore suspensions were diluted to 10^6 spores.mL^-1. Two biological replicates and three technical replicates were performed for each strain. Error bars represent standard deviations.

**Fig. 2.** Germination kinetics (inoculum level of 10^6 spores.mL^-1) of *Penicillium camemberti* strain A (□) and B (▲) in filter-sterilized 10^8 spores.mL^-1 cultures obtained after 20-h incubation (doted lines) as compared to germination of strain A (□) and B (▲) in fresh malt extract broth (solid lines). Error bars represent standard deviations.

**Fig. 3.** Inhibition of spore germination and radial growth of *Penicillium camemberti* cultivated on malt extract agar in the absence (A) or in the presence of a 50 μL at 10^6 (B) or 10^8 (C) spores.mL^-1 inoculated in the top layer (in the lid) of a Petri dish. CI95%: 95% confidence interval.

**Fig. 4.** Chromatograms showing the volatile compounds found in malt extract broth containing 10^8 spores.mL^-1 of *Penicillium camemberti* strain A after 20 h incubation at 25°C (A) in comparison with non-inoculated MEB (B) and the mass spectra of the molecule presenting a retention time of 10.997 min (C) in comparison with pure 1-octanol (D).

**Fig. 5.** Fungal biomass (mean ± 95 % confidence interval) of *Penicillium camemberti* strain A (black bars) and B (grey bars) after 9 days incubation at 12°C in a cheese matrix model as a function of initial spore concentration ranging from 10^2 to 10^6 spores.g^-1 cheese.
Fig. 1.

A

B
Fig. 2.

![Graph showing germinated conidia percentage over time.](image)

Fig. 3.

![Images of petri dishes with diameters](image)

**A** Diameter = 58 ± 1.22 mm  
  CI95% = 1.52 mm  

**B** Diameter = 8 ± 0 mm  
  CI95% = 0 mm  

**C** Diameter = 6.6 ± 0.54 mm  
  CI95% = 0.68 mm
Fig. 4.
Fig. 5.
Highlights:

- Spore germination in *Penicillium camemberti* is self-regulated by quorum sensing.
- Quorum sensing is observed when spore densities are high.
- Compounds involved in quorum sensing are volatile.
- 1-octanol was found to be the main volatile compound produced at high-spore density.