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ORIGINAL ARTICLE

Interaction with *Penicillium expansum* enhances *Botrytis cinerea* growth in grape juice medium and prevents patulin accumulation *in vitro*

H. Morales, R.R.M. Paterson, A. Venâncio and N. Lima

IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Braga, Portugal

Significance and Impact of the Study: To our knowledge, this is the first study on the influence of fungal interactions between *Botrytis cinerea* and *Penicillium expansum* on growth parameters and patulin accumulation. The incidence of *P. expansum* in some wine regions is high, but the attack of this fungus to vineyards is rare, being *B. cinerea* the most common disease. In this assay, it was observed that, *in vitro*, the presence of *P. expansum* spores enhanced *B. cinerea* growth, while the latter avoided patulin accumulation.

Keywords
Botrytis cinerea, grape, interactions, patulin, *Penicillium expansum*.

Correspondence
Hector Morales, IBB, Institute for Biotechnology and Bioengineering, Centro de Engenharia Biológica, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
E-mail: hmorales@gmail.com

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Abstract

Interactions between fungi occur when they grow on the same host plant. This is the case of *Botrytis cinerea* and *Penicillium expansum* on grape. *P. expansum* is also responsible for production of the mycotoxin patulin. In this study, the influence of the interaction between both fungi on fungal growth parameters was studied as well as the effect on the accumulation of patulin by *P. expansum*. For that purpose, spores of *B. cinerea* and *P. expansum* were inoculated together (mixed inoculum), and the parameters growth rate, time for growth and patulin accumulation were assessed. The presence of *P. expansum* conidia shortened the time for growth of mixed inoculum colonies which, at the end of incubation, were *B. cinerea*-like. Although some *P. expansum* growth was observed in mixed inoculum colonies, very low levels of patulin were observed. In assays carried out in patulin-spiked medium, *B. cinerea* was capable to metabolize the mycotoxin. The capabilities of *B. cinerea* to shorten time for growth and prevent patulin accumulation are competing abilities that facilitate grape colonization.

Introduction

Grey rot or grey mould is caused by the fungus *Botrytis cinerea*. It is the most common decay during grape ripening and is responsible for severe economic loss. Musts obtained from botrytized grapes are more liable to oxidation because of the polyphenol oxidizing activity of *B. cinerea* laccase and are not suitable for wine production.

Almost 100% of *Penicillium expansum* strains are patulin (PAT) producers (Andersen *et al.* 2004; Morales *et al.* 2008). *Penicillium expansum* is commonly associated with apple rot and PAT contamination in apple derivatives. However, PAT has been reported in grapes (Moake *et al.* 2005), processed grape juice (Scott *et al.* 1977) and fermenting wine (Majerus *et al.* 2008). This fungus has a high incidence in certain wine regions such as bordering regions of North Portugal (Serra *et al.* 2006) and Galiza (Spain).

When individuals of the same or different species develop in either close proximity or contact, interactions arise, frequently as a consequence of common exploitation of a resource. The outcome of competition for resources is detrimental to one of the interacting fungi or both (Cooke and Whips 1993).

The production of geosmin – a well-known compound with a strong earthy smell – by *P. expansum* due to interaction with *B. cinerea* has been studied (La Guerche *et al.* 2005; Morales *et al.* 2011). Although some assays on

the growth of *B. cinerea* and *P. expansum* on grape have been carried out (Judet-Correia *et al.* 2010), the effect of the interaction between both fungi on growth rate and PAT accumulation, which is addressed in the present paper, has not been studied. Also, the capability of *B. cinerea* to metabolize PAT was tested.

Results and discussion

The pure culture of *B. cinerea* had white hyphae at the beginning of incubation time. The hyphae turned into brown from the day 5. At this point, mature conidia could be observed under the microscope. At the end of incubation time, the whole plate was covered by brown *B. cinerea* with typical morphology. The *P. expansum* culture had a blue/green colony with mature conidiophores and conidia from day 2 of incubation. The colonies from the mixed inoculum (Fig. 1) were typical of *B. cinerea* except for the point of inoculation, which were comparable to *P. expansum*. *P. expansum* and *B. cinerea* hyphae could be observed by stereo microscopic observation on day 2. *Penicillium expansum* penicilli and conidia could be observed under the microscope, but not *B. cinerea* conidiophores. At this point, the diameter of the colony was approximately 1 cm. However, from day 3, only *B. cinerea* hyphae surrounded the initial colony, which proceeded to grow. Stereomicroscopic observation revealed large amounts of *B. cinerea* hyphae with immature (white) conidia. On day 7, only *B. cinerea* hyphae were observed covering the whole plate (except at the inoculation point) with mature (brown) conidia at the edge of the colony.

Concerning growth parameters, the nonlinear regression showed time for growth in mixed inoculum of $\lambda = 2{\cdot}463 \pm 0{\cdot}153$ days for colonies of *B. cinerea* and $\lambda = 1{\cdot}969 \pm 0{\cdot}170$ days for colonies of mixed inoculum. ANOVA tests revealed that time for growth was significantly shorter in mixed inoculum ($P = 0{\cdot}019$). Growth rate was

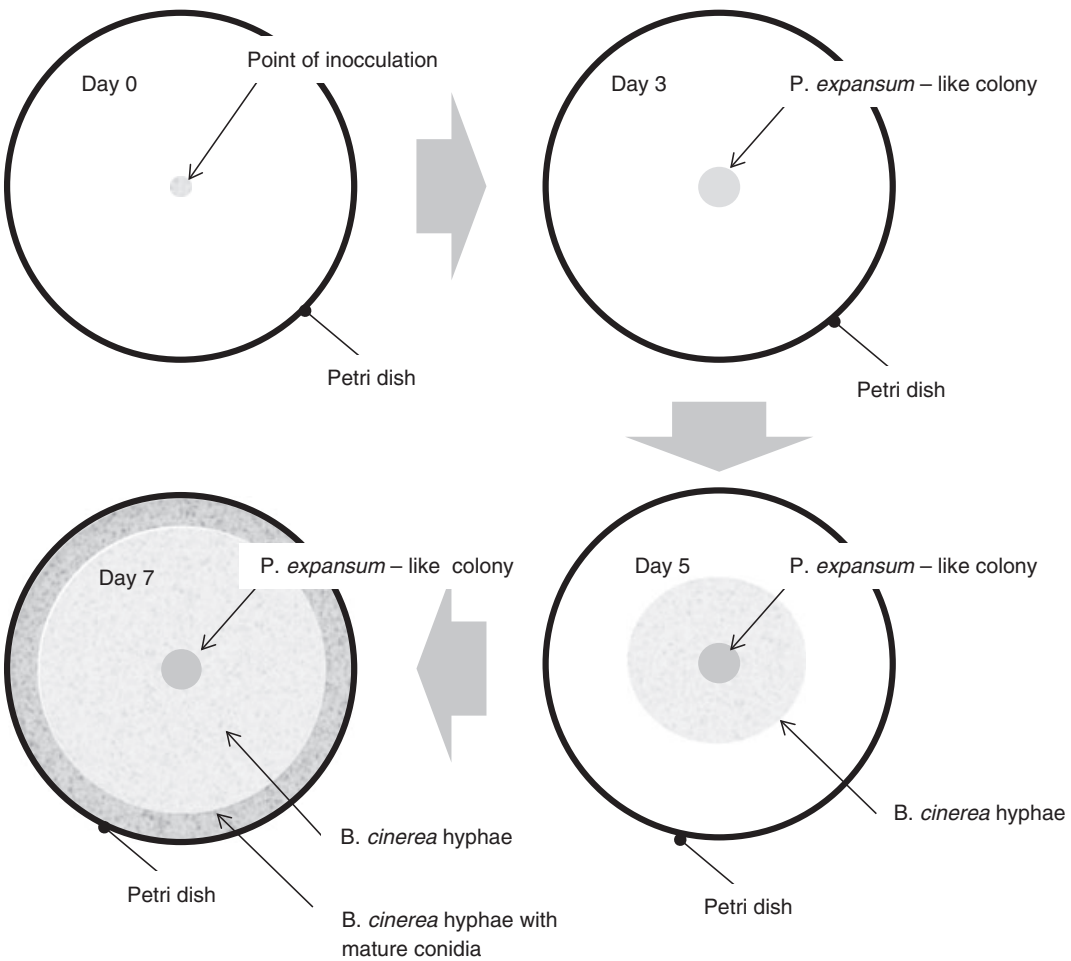

Figure 1 Diagram representing mixed inoculum colony growth.

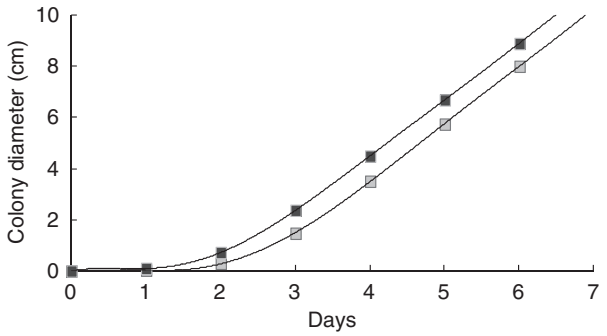

Figure 2 Mould growth (expressed as increase in colony diameter) against time obtained by nonlinear regression of Baranyi model (Baranyi and Roberts 1994). ■ Mixed inoculum of *B. cinerea* and *P. expansum,* ■ Pure inoculum of *B. cinerea*

$\mu = 2{\cdot}253 \pm 0{\cdot}142$ cm day^{-1} for *B. cinerea* colonies and $\mu = 2{\cdot}197 \pm 0{\cdot}137$ cm day^{-1} for colonies of mixed inoculum with no significant differences. The growth curves are presented in Fig. 2.

Concerning PAT accumulation, plates with *P. expansum* as pure culture yielded significantly ($P < 0{\cdot}0001$) higher amounts of the mycotoxin [average, $113{\cdot}7 \pm 43{\cdot}2$ μg (g agar)$^{-1}$], whereas PAT concentrations in plates with the colonies formed by mixed inoculum were much lower [average $1{\cdot}2 \pm 1{\cdot}5$ μg (g agar)$^{-1}$]. No PAT was observed in pure cultures of *B. cinerea*, as expected.

Concerning metabolism of PAT by *B. cinerea*, concentration of PAT in control plates (with no inoculation) after 7 days of incubation was the same as in the spiked medium. On the other hand, after incubation, no PAT was detected in PAT-spiked plates inoculated with *B. cinerea*. Thus, PAT seems not to be degraded due to chemical or physical factors in the present conditions and may be metabolized by *B. cinerea*.

Competition for resources involves the process of gaining access to unoccupied domains. Success depends on characteristics that give the opportunity for rapid arrival and establishment, such as a) quick germination of dispersal spore or b) high mycelial extension rate (Cooke and Whips 1993). The shorter time for growth observed may permit *B. cinerea* to colonize the whole Petri dish in mixed inoculum plates. Because primary resource capture competition is restricted to early stages of community development, the shorter time for growth of mixed inoculum spores may be determinant in the ability of *B. cinerea* to colonize the grape.

Judet-Correia *et al.* (2010) reported that the optimum growth rate of *B. cinerea* on grape is strain dependent and that *P. expansum* seemed to be more adapted to low water activity (a_w) than *B. cinerea*. Tolerance to any stress factors associated with the resource is also a variable that influences in competitive interactions between fungi (Cooke and Whips 1993). Further studies with different strains of *B. cinerea* at different a_w should be carried out to clarify whether the behaviour observed in this study depends on some stress factors like water availability.

Concerning PAT accumulation in mixed inoculum, it is remarkable that initial growth of *P. expansum* was observed in such colonies (Fig. 1), and much or the entire of this initial colony was collected for PAT analysis. Interestingly, these samples yielded very little amounts of PAT compared to those from colonies of pure *P. expansum*. The role of mycotoxins is not clearly established but, in some cases, they seem to play a role in eliminating other micro-organisms that compete in the same environment (Thomma 2003). In the case of grape, both *B. cinerea* and *P. expansum* compete for the same host (the grape) and it seems that *B. cinerea* enhanced growth prevents PAT accumulation even when *P. expansum* has started growing. In view of the results with PAT-spiked plates, it seems that the lack of accumulation of PAT is not only because of weak growth of *P. expansum* in mixed inoculum colonies but because *B. cinerea* is capable to metabolize the mycotoxin thus detoxifying the medium.

In summary, some of the competing abilities of *B. cinerea* against *P. expansum* are the capability to decrease time for growth in the presence of *P. expansum* spores and metabolizing PAT for detoxification of the substrate. Further studies should focus on assessing the effect of higher concentrations of PAT on *B. cinerea* together with other abiotic factors such as water activity or temperature. Also, the implications of these results on geosmin accumulation should be assayed.

Materials and methods

The medium used for all assays was grape extract agar (GEA). Grape extract was prepared as follows: 1 kg of grapes was crushed in 1 l of distilled water, macerated during 2 h at 70–80°C and filtered. The volume was brought to 1 l and sterilized at 115°C for 10 min (Santos *et al.* 2002). This extract can be stored at 5°C for several weeks or frozen. To prepare 1 l of GEA, 800 ml of distilled water with 20 g of agar was sterilized at 121°C for 15 min. When the temperature decreased to about 70°C, the grape extract was added (200 ml) and plated in 9-cm Petri dishes.

Growth parameters of B. cinerea and mixed inoculum

Suspensions in Tween 80 (0·005% v/v) of *B. cinerea* (a) and *P. expansum* (b) were made at concentration 10^6 spores ml^{-1}. The mixed inoculum (c) was made by mixing 1 ml of each spore suspension. For growth parameters assessment, 4 GEA plates were inoculated with 20 μl of

B. cinerea spore suspension (a) and 4 were inoculated with 40 μl of the mixed suspension (c) so the quantity of spores for each species was again 10^6 spores ml^{-1} in all cases. The plates were inverted and incubated for 7 days at 25°C. The average of two perpendicular diameters of the colony was recorded daily. The same diameters were measured during the whole assay.

Growth parameters were assessed using the model of Baranyi and Roberts (1994) in which mould growth (expressed as increase in colony diameter) is empirically modelled against time using a model originally developed for bacterial growth. This model has been successfully used to fit growth of some filamentous fungi (Marin *et al.* 2008).

Diameter values of *B. cinerea* colonies and mixed inoculum colonies were adjusted by nonlinear regression to the primary model of Baranyi (eqn 1) (Baranyi and Roberts 1994) in which the logarithmic term Dmax (maximum diameter) was deleted to omit the upper asymptote, as suggested by Valik *et al.* (1999).

$$D = \mu\left[t + \frac{1}{\mu}\right] \mathrm{In}\left[e^{-\mu t} + e^{-\mu\lambda} - e^{-\mu t-\mu\lambda}\right] \qquad 1$$

where D: colony diameter (cm), μ: growth rate (cm day^{-1}), t: time (days), λ: time for growth (days), *metabolism of PAT by B. cinerea, PAT accumulation and degradation.*

Two different assays involving PAT were carried out. In the first assay, the accumulation of PAT in colonies of mixed inoculum was assessed and compared to that of colonies of *P. expansum*. The Petri dishes of mixed inoculum used for assessment of growth parameters were used. Parallel, 4 more GEA plates had been inoculated with spore suspension of *P. expansum* (b) and had been incubated under the same conditions as mixed inoculum. Three plugs of agar (0·5 cm diameter) were removed from all the colonies after incubation. The three plugs were removed from the centre, middle and edge of each of the colony, placed in a 4-ml amber vial and weighed. These underwent PAT extraction and analysis, which is addressed later on.

As commented in the results section, the colonies of mixed inoculum yielded very low amounts of PAT, so a second assay was performed to determine whether *B. cinerea* was capable to metabolize PAT. PAT-spiked GEA was inoculated with the fungus and incubated. The levels of PAT were analysed and compared to those in spiked medium kept under the same conditions of time and temperature. The purpose was to assure that the decrease in PAT was due to metabolism of *B. cinerea* and not to chemical or physical degradation of the mycotoxin. Grape extract agar medium was spiked with PAT at concentration 2 μg ml^{-1}. Three hundred and twenty microlitres of PAT standard at concentration 1 mg ml^{-1} was spiked in 240 ml of GEA medium, which was kept at temperature of 50°C. The flask was capped and gently shaken to assure complete dissolution of the mycotoxin. With the aid of a sterile pipette, 8 plates were prepared with 20 ml of spiked GEA each. Four plates with spiked GEA were inoculated with 20 μl of *B. cinerea* spore suspension (a). The remaining 4 plates were not inoculated as control. The plates were incubated at 25°C during 7 days. As in the previous assay, 3 plugs of agar (0·5 cm diameter) were removed from the centre, middle and edge of the *B. cinerea* colony or from the agar (in the case of control plates) after incubation and underwent PAT extraction and analysis (see below).

Extraction and analysis of PAT

The extraction was made with ethyl acetate (2 ml). The vials were vortexed for 1 min and left for 1 h at room temperature. Then, the vials were vortexed again and the ethyl acetate was transferred to a 2-ml vial with the aid of a syringe. The ethyl acetate was evaporated under a stream of nitrogen, and the pellet was resuspended in 2 ml of water adjusted to pH 4 with glacial acetic acid. This was filtered through a 0·45-μm nylon filter and transferred to an HPLC vial (Morales *et al.* 2008). PAT content was determined by reversed-phase HPLC with UV detection (Brause *et al.* 1996). Briefly, acetonitrile water (5% v/v) was the mobile phase. The column was a 4·6 × 250 mm Water Spherisorb 5 μm ODS2. PAT was detected under UV at 276 nm wavelength. PAT was expressed in μg PAT (g agar)$^{-1}$. Differences in PAT accumulation were tested by ANOVA at $\alpha < 0{\cdot}05$. All statistical assays were performed with the software SPSS (SPSS Inc. Chicago, IL, USA).

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