Effect of water activity and temperature on competing abilities of common postharvest citrus fungi

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Abstract

The effect of temperature (4–30 °C) and water activity (\(a_w\), 0.995–0.90) on the ‘in vitro’ interactions between Penicillium digitatum, Penicillium italicum and Geotrichum candidum were evaluated. The effect of temperature on growth of green and blue mould decays and their interactions on wounded oranges was also studied. The major competing abilities were observed at optimal conditions of temperature and \(a_w\) for growth (25 °C and 0.995 \(a_w\)), and no differences between growth rates when the fungi were growing alone or paired were observed in the other studied conditions. \(P. italicum\) and \(G. candidum\) were able to reduce the growth rate of \(P. digitatum\) when it was growing paired ‘in vitro’, suggesting that inhibitory metabolites were produced. In the ‘in vivo’ assays, growth rates of green mould were higher than those of blue mould at any temperature studied. However, at 4 °C, \(P. italicum\) began its rot development 1 week before \(P. digitatum\). When these two pathogens were inoculated into the same wound at 25 °C, blue mould was practically inhibited. The difference between the results obtained in ‘in vitro’ and ‘in vivo’ assays suggests that other factors could interact with fungi, favoring the development of one pathogen to the detriment of the others.

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1. Introduction

Green and blue moulds, Penicillium digitatum (Pers.:Fr.) Sacc. and Penicillium italicum Wehmer, respectively, are the main postharvest diseases on citrus fruits. Sour rot, caused by Geotrichum candidum Link, is second in importance to Penicillium decay as a wound-initiated disease of citrus fruits. These pathogens may invade fruit before harvest through injuries made in the field or during harvesting and processing (Eckert and Brown, 1986). If environmental conditions are suitable, they will rapidly grow and compete for the citrus substrate, resulting in interspecific interactions between individual species and groups of species, with some becoming more dominant than others (Marin et al., 1998).

The most frequent determinant of fungal distribution and activity is competition for resources, during which, for any two interactants, the outcome is detrimental to one or both. Two fundamentally different aspects of competition must be distinguished in primary resource capture and antagonism. Primary resource capture describes the process of gaining access to available resources in unoccupied domains. Success depends on characteristics that give the opportunity for rapid arrival and establishment such as quick germina-
tion, high mycelial extension rate, tolerance of stress factors associated with the resource, etc. (Cooke and Whipps, 1993). On the other hand, antagonistic interactions encompass either defense of captured resources or secondary resources capture, resulting in mutual exclusion of individuals from each other’s domain.

Two basic forms of antagonism can be distinguished: in the first, combative interactions are mediated at a distance by means of diffusible or volatile metabolites, giving rise to antibiosis; in the second, combat is initiated upon contact between individual hyphae or mass mycelia, and involves direct physical, rather than chemical, interventions (Cooke and Whipps, 1993). A range of interspecific interactions can occur between contaminant fungi when hyphae meet, including mycelia intermingling, mutual inhibition by antagonism and dominance by one species over another (Cooke and Rayner, 1984). Depending on the type of reaction that occurs, it is possible to compare the competitive capabilities of species under different environmental conditions when organisms interact in pairs.

Species predominance that depends on the nature of their interactions may be altered with temperature, water activity ($a_w$), gas composition and other factors to change the predominant species (Lacey, 1989). Previous research carried out with common cereal fungi (Ramakrishna et al., 1997) showed that $a_w$ and temperature affected species interactions, changing their competitive pattern from neutral to competitive and/or combative interactions when two species met. Temperature and $a_w$ also affected the utilization of different carbon sources for some species of Aspergillus (Lee and Magan, 1999).

Recent studies carried out ‘in vitro’ with P. digitatum, P. italicum and G. candidum growing individually (Plaza et al., 2003) demonstrated that germination and growth were markedly influenced by environmental conditions such as temperature and water activity. However, on the surface of citrus fruits, fungi rarely occur in a monospecific culture but more often as a group of interacting species that compete for the rich substrate. Furthermore, results obtained on culture media cannot be easily extrapolated to natural systems because other factors could affect ‘in vivo’ growth, such as essential oils, volatile compounds and antifungal materials. Understanding the outcome of interactions between these fungi under different environmental conditions may enable more accurate predictions of which individual groups of species may initiate mould development on citrus fruits.

The objectives of this study were to determine the influence of $a_w$ and temperature on competing abilities of P. digitatum, P. italicum and G. candidum ‘in vitro’ and the effect of temperature on the competitiveness of P. digitatum and P. italicum on wounded oranges.

2. Materials and methods

2.1. Isolates

P. digitatum isolate PDM-1, P. italicum PIM-1 and G. candidum GCTA were isolated from decayed citrus fruits. These isolates were the most aggressive in our collection (Pathology Laboratory, UdL-IRTA, Lleida, Catalonia, Spain).

Actively growing, 7- to 12-day-old colonies of P. digitatum, P. italicum and G. candidum grown on PDA were used to obtain spore suspensions in sterile distilled water containing a drop of a wetting agent (Tween 80) per litre and modified with glycerol to different $a_w$ (0.995, 0.95 and 0.90). Spore suspensions were adjusted to $1 \times 10^6$ spores ml$^{-1}$ using a haemocytometer.

2.2. Medium

The basic medium used for ‘in vitro’ studies was Orange Serum Agar (OSA) with a pH of 5.5. The water activity of this basal medium was 0.995, and this $a_w$ was modified by the addition of different amounts of glycerol to obtain $a_w$ levels of 0.95 and 0.90. The $a_w$ of all media was confirmed by measurement in an Aqua Lab Water Meter (Decagon Devices, Washington, USA).

2.3. Fruit

For competition studies on fruits, ‘Valencia’ oranges without any commercial postharvest treatments were used. Fruits were disinfected by dipping in a 1% NaOCl solution for 5 min and in a 70% ethanol solution for 2 min, and finally rinsing with sterile distilled water.
2.4. Competition in ‘in vitro’ studies

Growth rates for each species were obtained by inoculating OSA Petri plates centrally with a 10-μl spore suspension adjusted at 10^6 spores ml⁻¹ with the same a_w of the plate. For the interactions, two species were inoculated 4 cm apart on 9-cm Petri plates for each treatment. After inoculation, Petri plates of the same a_w treatment were enclosed in polyethylene boxes and incubated at 4, 10, 25 and 30 °C for a maximum of 60 days. All experiments were carried out with three replicates per treatment.

Periodically, depending on treatment, two radial measurements were made of each colony in two directions at right angles to each other for growing alone, and colony radius was measured on the line forming the two inoculation points for interaction studies. At the end of the experiment, the temporal increases in radius were plotted against time and the linear regression was calculated in order to estimate the growth rate in millimeter per day under each set of environmental conditions for each species.

2.5. Type of interactions between species in ‘in vitro’ studies

The interaction of each dual culture was examined macro- and microscopically, and each fungus was classified into one of the following groups depending on the reaction when hyphae met: intermingling of the hyphae of one fungus over the other, antagonism on contact stopping the growth when hyphae contact and antagonism at a distance of more than 2 mm between both hyphae.

2.6. Competition studies on fruits

In order to determine the growth rate of rot, 10 μl of the spore suspension of P. digitatum and P. italicum, adjusted to 1 × 10^6 spores ml⁻¹, was individually inoculated in wounds previously made with a steel rod of 3 × 3 × 3 mm on the surface of disinfected oranges. Fruits inoculated with the same fungus were sealed in polyethylene boxes and incubated at 4, 10, 25 and 30 °C.

For interaction studies, two types of experiments were carried out. In the first, a 10-μl aliquot of spore suspension of each species was inoculated 4 cm apart on the fruit surface previously wounded at two points with a steel rod. In the second one, 10 μl of a mixed conidia suspension containing 1 × 10^6 spores ml⁻¹ of each fungus was inoculated in wounded oranges (one inoculation per fruit). After inoculation, fruits with the same treatment were incubated at 4, 10, 25 and 30 °C. All experiments were carried out with three replicates per treatment.

Daily, or as necessary depending on the treatment, for a maximum of 60 days, radius rot was measured at two right angles to determine lesion size increment of either fungi growing alone or mixed in the same wound, and radius rots at the line forming two wounds for lesion size increment when the fungi were growing separately on the same fruit.

Competition studies were not carried out on fruit with G. candidum because it did not grow on oranges in the studied conditions.

2.7. Statistical analysis

A general linear model (GLM) procedure of the Statistical Analysis System (SAS Institute, Cary, NC) was performed on growth rates, lesion size increment and data on the period previous to rot development. Statistical significance was judged at the P<0.05 level. When analysis was statistically significant, Duncan’s Multiple Range Test for separation of means was performed.

3. Results

3.1. Effect of temperature, water activity and interspecific interactions ‘in vitro’ on growth rates of individual species

In general, growth rates of P. digitatum, P. italicum and G. candidum were affected more by the presence of the other tested species at 25 °C and unmodified a_w than at the other studied conditions.

At unmodified a_w (0.995 a_w) medium, the growth of P. digitatum was significantly decreased by interaction with P. italicum or G. candidum at 25 and 10 °C, but no significant effects were observed at 4 and 30 °C (Fig. 1). In contrast, when P. italicum was plated out in the presence of P. digitatum or G. candidum, its growth rate increased over the temperature range studied, especially
at 25 °C, from 3.5 mm day\(^{-1}\) when growing alone to 5.1 and 4.4 mm day\(^{-1}\) in the presence of \textit{P. digitatum} and \textit{G. candidum}, respectively. A similar pattern was observed with \textit{G. candidum} at 25 °C, which increased its growth rate by more than 2 mm day\(^{-1}\) when growing in the presence of \textit{P. digitatum} or \textit{P. italicum}. In these conditions, \textit{G. candidum} was the fungus that grew fastest, reaching growth rates of more than 6 mm day\(^{-1}\). On the other hand, at 30 °C, the growth rate of \textit{G. candidum} was significantly reduced by the presence of \textit{P. italicum}.

At 0.95 \(a_w\), no significant differences were observed between the growth rates of \textit{P. digitatum}, \textit{P. italicum} and \textit{G. candidum} when they were growing alone or paired at 25 and 10 °C (Fig. 2), even though \textit{P. italicum} increased its growth rate by more than 0.75 mm day\(^{-1}\) in the presence of \textit{P. digitatum} at 25 °C. Generally, \textit{P. italicum} grew faster than the other studied isolates at all temperatures studied, except at 30 °C at which it grew at a similar rate to \textit{P. digitatum}.

In the driest condition studied (0.90 \(a_w\)), no growth was observed for \textit{G. candidum}. \textit{P. digitatum} and \textit{P. italicum} were only able to grow at 25 °C, but their growth rates were very low (less than 1.2 mm day\(^{-1}\)) and were not affected by the presence of each other (data not shown).

### 3.2. Water activity and temperature effects on the type of interaction between species 'in vitro'

When \textit{P. digitatum} and \textit{P. italicum} grew paired, mycelia of both fungi stopped their growth before...
contact, for each aw and temperature studied, keeping a distance greater than 2 mm for all tested time periods. In contrast, when P. digitatum or P. italicum grew in the same plate with G. candidum, they did not stop their growth until hyphae interacted with each other.

3.3. Effect of temperature on lesion size increment of P. digitatum and P. italicum and their interactions on citrus fruits

As in the ‘in vitro’ studies, growth of P. digitatum and P. italicum on citrus fruits was greatest at 25 °C, and lesion size increment decreased as the temperature decreased (Fig. 3). No growth was observed at 30 °C in either P. digitatum or P. italicum.

At the temperature range studied, P. italicum rot decay growth was slower than that of P. digitatum. At 25 °C, P. digitatum decay developed at a rate of 20 mm day⁻¹ and that of P. italicum only at 6 mm day⁻¹. Although at low temperatures this difference was less marked, the lesion size increment of P. digitatum was more than twice that of P. italicum. When these two isolates were growing paired in the same fruit, but separated from each other, the lesion size increments were not significantly different to when they were growing alone, regardless of temperature.

In mixed inoculation assays, no significant reduction of lesion size of P. digitatum and P. italicum was observed at 10 and 4 °C, but at 25 °C, both pathogens reduced their development by more than 5 mm day⁻¹ due to the presence of the other fungus, and P. italicum was almost inhibited to 1.1 mm day⁻¹ by the interaction with P. digitatum.

3.4. Effect of temperature on the period previous to rot development of P. digitatum and P. italicum on citrus fruits

The period previous to rot development increased as the temperature decreased (Fig. 4). At 25 and 10 °C, these previous periods of both P. digitatum and P. italicum were not significantly different, but at 4 °C, P. italicum started its rot development 16 days after

Fig. 3. Lesion size increment of P. digitatum and P. italicum, when growing alone (●) and interacting with the other pathogen in different wounds (□), and in the same wound (△) at different temperature levels on ‘Valencia’ oranges. Error bars show standard error. Asterisks show significant differences on lesion size increment at temperature levels.

Fig. 4. Previous period to rot development of P. digitatum (●) and P. italicum (■) at different temperature levels on wounded ‘Valencia’ oranges. Error bars show standard error. Asterisks show significant differences on previous period to rot development at temperature levels.
inoculation occurred, while *P. digitatum* delayed its rot growth 1 week more (after 23 days of inoculation).

4. Discussion

The ‘in vitro’ study showed that growth rates, and subsequently competing abilities of *P. digitatum*, *P. italicum* and *G. candidum*, are dependent on environmental conditions such as *a_w* and temperature and on interactions between species.

At 0.95 and 0.90 *a_w*, growth rates of main post-harvest pathogens on citrus were not significantly affected by the presence of the other studied species. It has previously been reported (Marín, 1998) that under stress conditions, primary resource capture prevails over antagonism, so high growth rates are determinant for rapid arrival and establishment in the niche and to achieve initial primacy. In contrast, when environmental conditions are favorable and allow similar rates of germination and growth for each species, the most predominant competitive strategy will be antagonism.

The fact that *P. italicum* was able to reduce the growth rate of *P. digitatum* at a distance on OSA medium at 25 and 10 °C suggests that competition may involve the production of inhibitory metabolites. Moreover, interactions between *P. digitatum* and *P. italicum* resulted in antagonism at a distance. It is known that *Penicillium* spp. are able to export antifungal metabolites, and previous research carried out with *P. digitatum* and *P. italicum* (Faid and Tantaoui-Elaraki, 1989) showed that these fungi could produce metabolites toxic to bacteria, plants and animals. Previous studies (Lacey, 1989) carried out with other species of *Penicillium* growing in cereal grains showed that *Penicillium brevicompactum* was strongly antagonistic to several *Aspergillus* and *Penicillium* spp. on malt extract agar medium, and Magan and Lacey (1984) concluded that although *P. brevicompactum* grew very slowly, it was the dominant species because of the production of metabolites such as mycophenolic acids.

Unlike in the previous case, possible metabolites produced by *P. italicum* did not reduce *G. candidum* growth at 25 °C but increased it, indicating that fungitoxic activity of metabolites was dependent on the species. In these conditions, the growth rate of *P. italicum* also increased significantly, so positive interactions between *P. italicum* and *G. candidum* could occur. It has been reported that positive interactions are based on combined physical and metabolic capabilities that enhance growth and/or survival rates (Atlas and Bartha, 1993).

The growth rates of *G. candidum* at 25 and 30 °C were the highest when it was growing in the presence of *P. digitatum* or *P. italicum*. This fact may provide *G. candidum* with a competitive advantage in comparison to *P. digitatum* and *P. italicum*, because it may rapidly colonize citrus substrate to capture as much as possible of the available resources before physical interactions occur.

Under drought conditions, populations that can best tolerate and survive desiccation can displace less tolerant populations because they are better adapted or because they are more effective competitors under these conditions (Atlas and Bartha, 1993). For these reasons, at 0.95 and 0.90 *a_w*, *P. italicum* became the most competitive species because of its higher growth rates in comparison with other tested fungi.

In fruit studies, lesions caused by *P. digitatum* expand faster than those of *P. italicum* regardless of temperature, resulting in an advantage due to rapid establishment on citrus substrate and to primary access to available resources. These results differ from those of ‘in vitro’ studies, in which the growth rates of *P. italicum* were the same as or slightly higher than *P. digitatum* at all studied temperatures and *a_w*. A possible explanation could be that on citrus fruits, and particularly in the wound, there are more parameters interacting with fungi apart from temperature and *a_w*, such as volatile compounds, essential oils of the peel and nutrient substances, and they may affect germination and growth of the fungus, favoring *P. digitatum* to the detriment of *P. italicum*. Eckert and Ratnayake (1994) identified several volatile compounds such as limonene, acetaldehyde, ethanol and CO₂ from exocarp-wounded oranges that induce germination of *P. digitatum* and *P. italicum*. Although this assay cannot explain the different growth rates of *P. digitatum* and *P. italicum*, it suggests that other substances could stimulate germination or growth of *P. digitatum* or inhibit *P. italicum*.

At 25 and 10 °C, the time period prior to rot development was very similar for *P. digitatum* and *P. italicum*. However, at 4 °C, *P. italicum* initiated its
rot development 7 days before *P. digitatum*. Although *P. italicum* growth rate is lower than that of *P. digitatum*, the former initiates blue mould rot 7 days before, and in this period, it is able to capture first the available resources in the wound, hindering green mould development. This could explain why at the low temperatures used in long storage by the citrus industry, *P. italicum* is the major postharvest pathogen (*Tuset, 1987*).

It is important to note that at 30 °C, no growth was observed on orange fruits by either *P. digitatum* or *P. italicum*, while on OSA medium at this temperature, these fungi grew at a rate equal to or greater than 1 mm day\(^{-1}\) at 0.99 and 0.95 \(a_w\). As in the previous case, this could be due to other parameters than \(a_w\) and temperature. Ben-Yehoshua et al. (1989) showed that by keeping citrus fruits for several days after harvest at 30 °C and 90–100% RH, *Penicillium* decay could be reduced. They found these conditions to elicit the biosynthesis of lignin and its phenolic precursors in the outer pericarp of the fruit while reducing the growth of the pathogen.

In contrast to ‘in vitro’ studies, the growth rates of *P. digitatum* and *P. italicum* on oranges were not different when they were growing alone or separated on the same fruit. Although the ‘in vitro’ results suggested that *P. italicum* could be an antibiotic producer that affects *P. digitatum* growth, it can also be argued that ‘in vivo’ antibiotics may not persist in sufficient quantity to have any perceptible effect (Cooke and Whipps, 1993) or are not diffusible in the same way.

When *P. digitatum* and *P. italicum* were inoculated together in the same wound, their pattern was very different. At 25 °C, their growth rates decreased by almost 5 mm day\(^{-1}\) in comparison with their growth alone, *P. italicum* being practically inhibited. Moreover, it was observed that sporulation of *P. italicum* occurred earlier. It has been reported by Cooke and Whipps (1993) that when mycelium of two species meet or intermingle, a frequent result is the stimulation of sporulation in one or both fungi. Accelerated or enhanced reproduction can be viewed as being beneficial, but is obviously achieved at the expense of curtailed vegetative development (Cooke and Whipps, 1993). The highest rot growth of *P. digitatum* decay and the great inhibition caused to *P. italicum* could explain why under ambient conditions *P. digitatum* is the main postharvest pathogen affecting the Spanish citrus industry (*Tuset, 1987*).

The present work gives a general impression of how the main postharvest pathogens affecting citrus fruits interact with each other, and studies their potential competitiveness and the possible outcome during hyphal growth under different environmental conditions. Since the results of ‘in vitro’ studies do not directly reflect what occurs in the citrus fruit, this suggests that other factors apart from temperature and \(a_w\) may affect fungal growth, and probably their competing abilities. Moreover, possible interactions of these fungi with other microorganisms present on the surface of citrus fruit, such as bacteria, yeasts and other moulds, may be highly complex and could impact the manner in which the three studied fungi interrelate.

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References


