Susceptibility of food-contaminating *Penicillium* genus fungi to some preservatives and disinfectants

Loreta Levinskaitė

Institute of Botany, Nature Research Centre, Vilnius, Lithuania

▌ **Abstract**

Microscopic fungi are able to contaminate and deteriorate various food products and can subsequently cause health problems. Long usage of the same preservatives and disinfectants against spoilage fungi may lead to the development of fungal resistance to those chemicals. The objective of this study was to investigate the susceptibility of 3 *Penicillium* genus fungi, isolated from foodstuffs, to organic acid preservatives and some disinfectants, taking into consideration 2 aspects of their development: spore germination and mycelial growth. Susceptibility of *Penicillium spinulosum*, *P. expansum* and *P. verruculosum* to the preservatives, namely benzoic acid, sodium lactate, potassium sorbate, as well as disinfectants such as Topax DD, Suma Bac D10, Biowash and F210 Hygisept, was investigated. The biocides were used at concentrations of 0.1, 1.0 and 10%. Of the preservatives, benzoic acid and potassium sorbate showed the best inhibition, both on spore germination and mycelial growth. Benzoic acid at a concentration of a 0.1% reduced spore germination by 33-55%, and mycelial growth by 54-97%, whereas at 1% the inhibition was 74-85% and 97-100%, respectively. The effect of the disinfectants at a concentration of 0.1% on spore germination was 25-84% and on colonial growth 68-97%, while at 1.0% the reduction in spore germination reached 53-91% and the inhibition of growth 89-100%. In most cases, the same concentrations added to the media showed higher inhibitory effect on mycelial growth than on spore germination. It was noticed that the fungi responded rather unevenly towards the biocides, showing individual susceptibility.

▌ **Keywords**

Penicillium, preservatives, disinfectants, susceptibility

INTRODUCTION

Microscopic fungi are a very important group of spoilage microorganisms deteriorating various food products, including those processed from cereals. This is because fungi can tolerate reduced water activity resulting from the baking process which removes water from the products [1]. Due to fungal spoilage, about 1-5 % of bakery products could be lost [2]. In addition, some fungi can produce toxins which can cause mycotoxicoses. Many species of *Penicillium* are able to produce toxic substances as well as provoke allergic reactions [3], thus, their presence in foodstuffs is greatly undesirable.

Contamination of food products can occur at various stages of their processing, storage and sale. Fungal spores enter the atmosphere of a processing plant from raw materials, ingredients, containers, etc. and contaminate the products [1, 4]. Cleaning and disinfection of walls and other surfaces can therefore help to reduce the accumulation of fungal spores and minimize contamination [5]. A wide variety of disinfectants suitable for the cleaning of plant premises are offered. The most commonly used disinfectants in the food processing industry are quaternary ammonium compounds (QAC), hypochlorites, amphoteric compounds and peroxides, as well as alcohols, aldehydes and phenolic compounds [4, 6, 7].

Preservatives have been used in the food industry for a long time in order to extend the shelf-life of food products and ensure their good quality. Taking into account the fact that food must be safe for consumption, preservatives added to the food are limited mostly to weak organic acids, e.g. benzoic, sorbic, acetic and propionic, etc. as well as some inorganic acids and salts [8, 9, 10]. Factors such as pH and a_w have a high influence on the activity of the weak acid preservatives [11]. The preservatives are often added as salts as they are more soluble in aqueous solutions [12]. The effectiveness of the preservatives is related to their dissociation level that depends on pH. Undissociated molecules of the chemicals are freely permeable across the plasma membrane into the cell and can affect intracellular processes [12, 13].

Long usage of the same preservatives against spoilage fungi can lead to the development of fungal resistance towards those chemicals [9, 14]. In addition, it has been reported that microbial resistance to biocides depends on their growth phase [15, 16]. It was shown that fungal cell walls are more susceptible to biocides at the logarithmic rather than stationary growth phase during which thickness of the cell wall increases and porosity decreases [17].

Similarly to the response towards preservatives, exposure of microorganisms to sublethal concentrations of disinfectants may also result in the acquisition of resistance [7]. Resistance of microorganisms has been demonstrated towards QAC, halogens, peracetic acid and hypochlorite, etc. [7, 18, 19].

Since in many countries more emphasis is placed on additive-free products, there is a danger that some loss of intrinsic preservation will steadily occur [8]. Thus, the

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reasonable and effective use of preservatives in combination with the relevant prevention of microbial contamination still remains a reliable measure to extend the shelf life of various products, and insure food safety.

The objective of this study was to investigate the susceptibility of 3 *Penicillium* genus fungi, isolated from baked products, to organic acid preservatives and some disinfectants, taking into consideration 2 aspects of their development: spore germination and mycelial growth.

MATERIALS AND METHODS

The food preservatives benzoic acid, sodium lactate, and potassium sorbate were chosen for the investigation. The disinfectants Topax DD, Suma Bac D10, Biowash (all based on alkyl dimethyl benzyl ammonium chloride) and F210 Hygisept (based on potassium persulphate) were used in the experiments. The antifungal effect of the biocides was evaluated against the fungi *Penicillium spinulosum, P. expansum* and *P. verruculosum* isolated from baked products (bread and cakes).

To test the effect of the biocides on spore germination, the preservatives and disinfectants were added into Czapek medium to obtain the final concentrations of 0.1, 1.0 and 10%. Spores were harvested from 10-day-old cultures with a sterile needle into the Czapek medium with the biocides. Spore suspensions were filtered through cheesecloth to remove mycelia, and standardized to 10^6 ml by using a hemacytometer. Spore germination was evaluated after 24 h with a microscope, and 200 spores of each replication were counted. The control solution was biocide-free Czapek medium. Three replications for each concentration were conducted. The effect of the biocides on spore germination was expressed as the percentage of inhibition in comparison with spore germination in the control medium.

Radial mycelial growth of fungal colonies was tested on Czapek agar containing the same concentrations of the biocides, as for the above described experiment. Biocide-free Czapek agar plates were used as control. The fungi were inoculated with a needle at 3 points on the agar plates and incubated for 7 days. Radial growth was evaluated by measuring the diameter of fungal colonies. All the experiments were run in triplicate.

Using Excel, the mean values of diameters of the fungal colonies and standard deviations were calculated. Mean values were used to calculate the percentage of mycelial growth inhibition.

RESULTS AND DISCUSSION

The results showed that all the biocides influenced germination of the fungi. Of the food preservatives, the most considerable effect was shown by benzoic acid and potassium sorbate. When concentrations of 0.1 and 1.0% of the preservatives were used, benzoic acid was found to be the strongest inhibitor – 33-55% and 74-83% of spores were inhibited, respectively (Fig. 1). When the preservatives were added at a concentration of 10%, the most efficient inhibitor was potassium sorbate which suppressed spore germination up to 100%, whereas 10% of benzoic acid inhibited this process slightly less. Sodium lactate demonstrated generally

the weakest impact on spore germination, both among the preservatives and all the biocides tested. The percentage of *P. spinulosum* and *P. verruculosum* germination with 0.1% sodium lactate was close to the control variant (in the preservative-free medium), while *P. expansum* was more susceptible. Although 1% and 10% concentrations of the preservative reduced spore germination more evidently in all fungi, the susceptibility differed rather significantly among the cultures. *P. verruculosum* was the most resistant: reduction in spore germination of this fungus was 61% in the presence of 10% sodium lactate, whereas that of *P. spinulosum* – 89%.
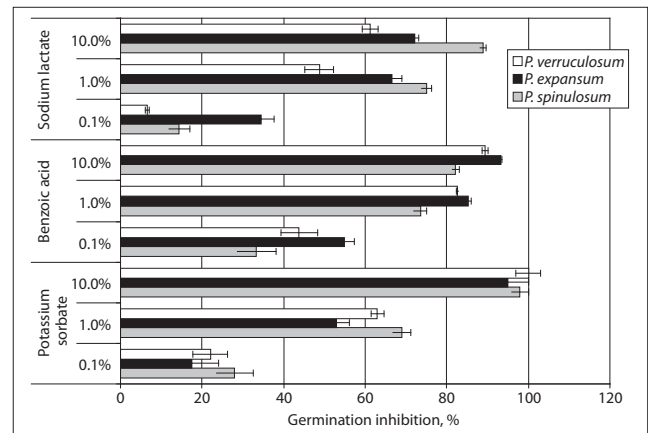

Figure 1. Inhibitory effect of preservatives on spore germination of *Penicillium spinulosum, P. expansum, P. verruculosum*

The results of the experiment with the disinfectants showed that the highest efficiency on spore germination was observed for Suma Bac. The disinfectant inhibited strongly this process of all micromycetes, even at a concentration of 0.1%, which reduced spore germination by 75-84% (Fig. 2). Higher concentrations of the chemical suppressed germination considerably more and at 10% of the disinfectant the inhibition reached 97-100%.

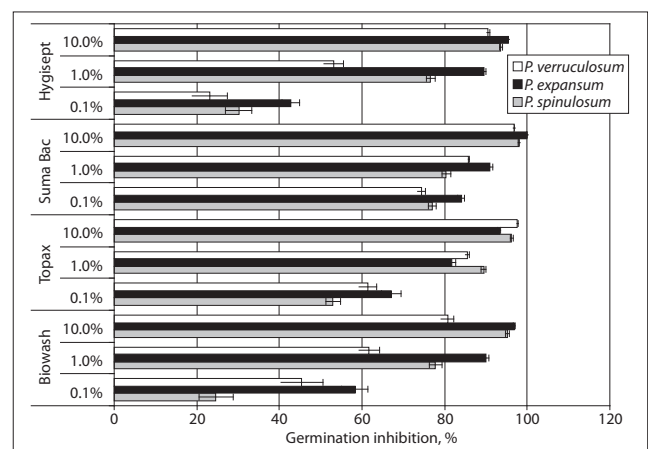**Figure 2.** Inhibitory effect of disinfectants on spore germination of *Penicillium spinulosum, P. expansum, P. verruculosum*

A high inhibitory effect on germination was demonstrated by Topax, especially at concentrations of 1 and 10%. Inhibition at these concentrations of Topax was at the same level as Suma Bac. At the lowest concentration, Topax was less effective in comparison with Suma Bac, with a spore

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inhibition of 53-67%. Biowash and Hygisept demonstrated slightly weaker impacts on spore germination. Under the influence of 0.1% of Biowash, the percentage of germination reduction was 25-58%, whereas higher concentrations inhibited germination more significantly – up to 81-97% at 10% of Biowash. The impact of Hygisept (containing potassium persulphate) on spore germination was rather similar: from 23-42% at the lowest concentration up to 91-95% at the highest concentration. Bore and Langsrud [7] also indicated an inhibitory effect of potassium persulphate-containing the disinfectant Virkon, under which exposure of yeast *Rhodotorula mucilaginosa* for 5 min. showed relatively low resistance to 1% of the inhibitor.

Some other studies point out the efficiency of disinfectants containing quaternary ammonium compounds (QAC), such as benzalkonium chloride. It was demonstrated that benzalkonium chloride (0.25%) showed biocidal activity in ≤5 min contact time against *Aspergillus fumigatus* spores, and caused a 10^4 reduction in viability [20]. In another study, Topax-91 (based on QAC) devitalised *A. niger* spores at a concentration of 1.5% in 60 min. [5].

Investigation of colonial growth of the fungi on agar with the preservatives revealed that sodium lactate was the weakest growth inhibitor. When present at a concentration of 0.1%, the substance slowed down growth of the fungi insignificantly – by only 1-8% (Tab. 1). However, the preservatives benzoic acid and potassium sorbate showed stronger inhibitory effects. All fungi did not grow absolutely with 10%, and two fungi with 1% benzoic acid. Growth of *P. expansum* on the medium containing 1.0% benzoic acid was inhibited by 97%, and reduction of colonial growth at the lowest concentration of this preservative ranged from 54-97%. Potassium sorbate was also very effective against the fungi. Interestingly, *P. spinulosum* showed very high susceptibility towards the preservative, at a concentration of 0.1% inhibition was 95%, and at higher concentrations inhibition of fungal growth was complete. Mycelial growth of the rest of the fungi was reduced from 32-63% at the lowest concentration to 87-91 % at the highest concentration of the disinfectant.

Other studies also indicate benzoic and sorbic acids as well as their salts as being the most effective among preservatives of organic acids. Benzoic acid in comparison with lactic and citric acids has manifested as a good inhibiting agent. Benzoic acid (100µl of 1% acid/15ml PDA) produced 75-100% inhibition of food-associated fungi, including *Penicillium oxalicum* [21]. Suhr and Nielsen [12] discovered that sorbate and benzoate were more effective against spoilage fungi than propionate. In comparison with ascorbic and citric acids, benzoic and sorbic acids including their salts (sodium benzoate and potassium sorbate) had a very good effect – 1% potassium sorbate and benzoic acid reduced colonial growth of fungi *P. digitatum and P. italicum* over 60%, and complete inhibition was observed at concentrations of 2% and 4% of these acids and their salts [22]. It has also been reported that a mixture of potassium sorbate and sodium lactate, together with sodium nitrite, considerably restricted yeast growth [23].

Analysis of the inhibitory effect of the disinfectants showed that all disinfectants inhibited fungal growth absolutely at a concentration of 10%. At lower concentrations, the most effective inhibitors were Suma Bac and Biowash. Even 2 fungi under the influence of both disinfectants did not grow completely at a concentration of 1%, and the growth of *P. expansum* with Suma Bac and *P. verruculosum* with Biowash was significantly slow. The effect of 0.1% of these 2 disinfectants also was considerable: inhibition of growth reached 88-97%. A high antifungal effect was demonstrated by Hygisept. When growing with 1% Hygisept, the fungi were inhibited by 92-95%. Development of the fungi on the medium with 0.1% Hygisept was also evidently affected – growth reduction was 72-89 %. Topax inhibited growth of the fungi similarly to Hygisept, with exception of *P. expansum,* which did not grow absolutely with 1% Topax.

The data showed that individual susceptibility of the fungi to the biocides was uneven. In the case of spore germination, the most susceptible fungus towards most of the biocides was *P. expansum* (benzoic acid, Biowash, Suma Back and Hygisept). Interestingly, *P. expansum* germinated better than the other fungi under the influence of potassium sorbate. In

Table 1. Inhibitory activity of preservatives and disinfectants on colonial growth of *Penicillium* genus fungi.

Biocides	Concentration, %	*P. spinulosum*		*P. expansum*		*P. verruculosum*	
		diameter of colonies, mm	growth inhibition, %	diameter of colonies, mm	growth inhibition, %	diameter of colonies, mm	growth inhibition, %
Potassium sorbate	0.1	2.0±0.3	95	23.4±1.3	32	12.0±1.2	63
	1.0	0	100	14.1±0.8	59	3.8±0.4	88
	10.0	0	100	4.4±0.5	87	2.8±0.4	91
Benzoic acid	0.1	14.7±0.7	64	15.9±0.9	54	0.9±0.3	97
	1.0	0	100	1.1±0.3	97	0	100
	10.0	0	100	0	100	0	100
Sodium lactate	0.1	37.9±1.5	8	33.9±0.8	1	30.4±1.0	5
	1.0	22.4±1.5	46	21.3±1.0	38	23.0±1.0	28
	10.0	1.8±0.4	96	2.9±0.6	92	6.7±0.9	79
Biowash	0.1	1.1±0.3	97	1.7±0.5	95	3.8±0.4	88
	1.0	0	100	0	100	1.5±0.5	95
	10.0	0	100	0	100	0	100
Topax	0.1	10.3±1.1	75	4.1±0.8	88	10.3±0.7	68
	1.0	3.8±0.7	91	0	100	3.4±0.7	89
	10.0	0	100	0	100	0	100
Suma Bac	0.1	2.0±0.5	95	3.4±0.5	90	1.1±0.3	97
	1.0	0	100	1.0±0	97	0	100
	10.0	0	100	0	100	0	100
Hygisept	0.1	7.0±0.7	83	9.6±0.7	72	3.6±0.7	89
	1.0	3.3±0.9	92	2.0±0.5	94	1.6±0.5	95
	10.0	0	100	0	100	0	100

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comparison with the other fungi, *P. verruculosum* showed more tolerance towards sodium lactate, Hygisept, Biowash and Suma Bac. Spore germination of *P. spinulosum* was evidently less affected by benzoic acid, when compared with the other fungi, but more significantly inhibited by higher concentrations of sodium lactate.

The results of mycelial growth on the media with the biocides also demonstrated differences in susceptibility among the fungi. It was noted that the biocides inhibited colonial growth with rather unequal efficiency, namely Suma Bac was the most effective against *P. spinulosum* and *P. verruculosum*, Topax more severely inhibited *P. expansum*, potassium sorbate – *P. spinulosum,* and benzoic acid – *P. verruculosum*. On the other hand, it was also noted that *P. expansum*, in comparison with the other fungi, was the most tolerant to potassium sorbate and benzoic acid, whereas *P. verruculosum* to Biowash, Topax and sodium lactate.

Variations in susceptibility towards biocides among fungal species have also been reported by other authors. *P. roquefortii* and *P. commune* showed increased tolerance towards 0.3% sorbate at pH6 in comparison with other fungi [12]. Lactic acid (100µl of 1% acid/15ml PDA) expressed antifungal activity against food-associated fungi *Aspergillus luchuensis* and *A. flavus*, whereas it did not affect *P. oxalicum* and other fungi [21].

Tolerance and resistance of microorganisms to biocides could occur for several reasons - known to be intrinsic and acquired. Microbial adaptation to a sublethal dose of an inhibitor can result in the ability to survive in higher concentrations of the inhibitor, and/or in more rapid growth at sublethal inhibitor concentration [8]. *Penicillium roquefortii* and other species were demonstrated to possess high tolerance or resistance to weak acid preservatives, e.g. calcium propionate stimulated fungal growth of *P. roquefortii* after an extended lag-phase [12], *P. roquefortii* and some other *Penicillium* species, isolated from sorbate-treated cheeses, were capable of metabolizing sorbate and developing at 9,000 ppm inYM broth [9]. Adaptation mechanisms to antimicrobial substances following Russel and Gould [8] could be divided into 3 strategies: destruction/removal of the inhibitor, prevention of the access to the cell by the inhibitor, and amelioration of the damage caused by inhibitors. Little is known about the mechanisms of resistance towards biocides in fungi. It has been reported that resistance to disinfectants depends on the cell wall as a barrier, which excludes or lessens the entry of disinfectants, as has been shown with *Saccharomyces cerevisiae* affected with chlorexidine [17]. It is indicated that decreases in the relative porosity and thickness of cell walls of *S. cerevisiae* could possibly reduce chlorexidine uptake into the cells [24]. McDonell and Russel [17] proposed possible mechanisms of fungal resistance towards disinfectants and antiseptics, which include exclusion, enzymatic inactivation and phenotypic modulation. The study on *Penicillium roquefortii* susceptibility towards potassium sorbate indicated that the preservative decreases Δ12 desaturase activity which controls the adaptive response of mycelial fungi [25]. Resistance to weak organic acids in yeasts involves induction of pumping of the preservative anion from the cell, enzymes allowing preservative degradation and changes to the cell envelope, minimizing diffusion of the preservative into the cell [23].

Analysis of the obtained results on the inhibitory activity of the biocides against germination and colonial growth revealed that there were some similarities and differences in the impact on these 2 phases of fungal development. It was discovered that in many cases a more significant effect of the preservatives and disinfectants was observed on mycelial growth than on spore germination. This phenomenon was most clearly manifested at a concentration of 0.1% of the biocides. At this concentration, inhibition of colonial growth reached 97% by both preservatives and disinfectants, while the highest reduction in spore germination was 55% by the preservatives and 84% by the disinfectants.

In case of the preservatives, it was discovered that both germination and mycelial growth of the fungi was most strongly inhibited by benzoic acid and potassium sorbate, while sodium lactate was the weakest preservative in most cases. Comparison of the inhibitory effect of the disinfectants showed that Suma Bac was the strongest inhibitor on spore germination, followed by Topax which had a slightly weaker effect (depending on the concentrations). Weaker inhibition was shown by Biowash and Hygisept, and both of them influenced germination similarly, whereas colonial growth of the fungi was most evidently inhibited by both Suma Bac and Biowash.

CONCLUSIONS

Of the preservatives, benzoic acid and potassium sorbate showed the best inhibition, while the most effective antifungal disinfectant was Suma Bac, followed by Topax, in the spore germination phase, and by Biowash in mycelial growth phase.

The preservatives and disinfectants mostly had a higher inhibitory effect on mycelial growth than on spore germination, and this phenomenon was the most conspicuous at a concentration of 0.1% of the biocides.

It was found that fungi responded rather unevenly towards the biocids – there were noticeable variations in the individual susceptibility of the fungi towards both the preservatives and disinfectants.

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