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Antifungal efficacy of some natural phenolic compounds against significant pathogenic and toxinogenic filamentous fungi

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HIGHLIGHTS

- The antifungal efficacy of the 21 different phenols on 6 filamentous fungi was investigated.
- MIC50 and MIC100 revealed different pathogen interspecies sensitivities.
- Thymol and carvacrol showed the highest antifungal efficacy on target pathogens.
- Most of the phenolic acids possess little or no antifungal activity.
- The antifungal efficacy depends mainly on chemical structures and OH group position.

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ABSTRACT

In terms of food safety, species of the *Fusarium*, *Aspergillus* and *Penicillium* genera are considered the most significant because they produce the great majority of known mycotoxins. Developing resistance against commonly used fungicides have become a critical problem in area such as agriculture, the storage and production of food and even in human medicines. The need for research and development of new alternative antifungal treatment based on natural antifungal substances is obvious. Here, the antifungal efficacy of 21 phenolic components of essential oils and plant substances were tested against these filamentous fungi with respect to their different molecular structures. Minimum inhibitory concentration values MIC_{50} and MIC_{100} were successfully estimated for 15 substances by means of probit analysis. Thymol and carvacrol were evaluated as the most effective. The MIC_{50} values for thymol ranged from 30 to 52 μg mL^{-1}. The MIC_{100} values for thymol ranged from 76 to 255 μg mL^{-1}, respectively. For carvacrol, the MIC_{50} values ranged from 37 to 76 μg mL^{-1}, and the MIC_{100} ranged from 131 to 262 μg mL^{-1}. The results also revealed differences in the efficacy of phenols depending on molecular structures and different interspecies sensitivity.

1. Introduction

Pathogenic and toxinogenic fungi, which are constantly developing resistance against commonly used fungicides, have become a critical problem in many areas such as agriculture, human and animal health and safety, the storage and production of foods and human medicines (Howard et al., 2010; Ahmad et al., 2011). Besides their potential to cause yield losses and food decay, many of them produce dangerous secondary metabolites, extremely hazardous to consumers. This topic is also of current concern in relation to the safety of food production. In terms of food safety, species of the *Fusarium*, *Aspergillus* and *Penicillium* genera are considered the most significant because they produce the great majority of known mycotoxins (Edwards et al., 2002; Niessen, 2007; Palumbo et al., 2008). In addition, *Aspergillus* and *Fusarium* genera include species that are able to cause very serious human mycoses. Their treatment is most problematic and questionable due to the toxicity and side effects of the medicines used, which are based on synthetic fungicides (Howard et al., 2008; Maertens et al., 2009; Mazzei and Novelli, 2009; Gubbins and Heldenbrand, 2010). Given the speed at which resistance is currently developing, the supply of new, more effective synthetic fungicides is becoming insufficient. This results in excessive use and increased dosing of fungicides, leading to harmful loading of the natural ecosystem with non-biodegradable residues. Paradoxically, these omnipresent sublethal fungicide concentrations may be one of the causes of the accelerated occurrence of resistant forms; moreover, they may have a harmful effect on human health (Caldas et al., 2001; Zarn et al., 2003; Jiang et al., 2005; Cus et al., 2010; Mullin et al., 2010). In light of these problems, there is a growing need to research and develop new, environmentally safe substances which will undergo quick and natural degradation in the environment. Alternative methods of suppressing pathogenic and toxinogenic fungi, based on the use of natural plant substances, often result

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in research on the development of highly effective essential oils. Plant essential oils have been suggested as alternative sources for antifungal treatment (Singh et al., 2009; Zabka et al., 2009; Kumar et al., 2010). Natural phenolic substances are among the most antifungal active substances present in plant essential oils. In spite of their high antifungal, antibacterial and insecticidal efficacy, they show a very low toxic effect on homeothermic animals (Xu et al., 2008; Ahmad et al., 2011). Phenolic compounds are characterized by the presence of a hydroxy (–OH) group, attached to a benzene ring or other complex aromatic ring structures, e.g., pyrogallol, catechol, or resorcinol. They range from simple phenols to polyphenols, such as flavonoids and tannins (Bruneton, 1999). The hydrophobic nature of phenolic compounds ensures their preferential partition into the lipid membrane. The mechanism of the antifungal effect thus depends predominantly on the ability to affect the function of cellular lipoprotein membranes, causing an impairment of cellular ionic homeostasis, acidification of vacuolar and cytosolic pH, and even the destruction of structural cellular integrity (Xu et al., 2008; Rao et al., 2010). In spite of the known facts and intensive research on these substances, few comprehensive studies have been published on how antifungal efficacy against important pathogenic and toxinogenic fungi may be dependent on the molecular structure of natural phenols; in fact, the structure seems to be quite an essential element of biological activity (Ultee et al., 2002). Our previous research indicates that, for example, the insecticidal efficacy of natural phenolic substances shows a dependence precisely on the molecular structure of the tested compound (Pavela, 2011). In previous research on the antifungal and insecticidal properties of plant essential oils (Pavela, 2008, 2009; Zabka et al., 2009), essential oils with high thymol content proved to be very effective and suitable for the development of botanical pesticides. However, it is not known whether structurally similar phenols and their acids may exhibit similar efficacy.

The aim of our work was to investigate whether any antifungal phenolic compounds can be found that provide even higher efficacy than that of thymol. In our study we therefore explored 21 simple phenols and their acids, differing in their chemical structure, to test their antifungal properties against important toxinogenic and pathogenic filamentous fungi, particularly *Fusarium oxysporum*, *F. verticillioides*, *Penicillium brevicompactum*, *P. expansum*, *Aspergillus flavus*, and *A. fumigatus.*

2. Materials and methods

2.1. Chemicals

All the compounds (Table 1) used in our experiments were purchased from Sigma Aldrich Chemical Co., Czech Republic. All pure chemicals were used without further purification. Methanolic solutions of each phenolic compound were used immediately after preparation.

2.2. Fungal strains

All target pathogenic and toxinogenic fungal strains were obtained from a collection of phytopathogenic fungi maintained at the Crop Research Institute, v.v.i., Prague, Czech Republic. *F. oxysporum* and *F. verticillioides* strains were isolated originally from an infected corn cob, whereas *P. brevicompactum*, *P. expansum*, *A. flavus* and *A. fumigatus* were isolated from contaminated stored corn. Strains were preserved on slant agar (Potato Carrot Agar) at 4 °C. Subcultivations on Petri dishes and other manipulations with these strains were carried out in the Bio Security Level Two (BSL 2) laboratory, given the BSL of the *Fusarium* and *Aspergillus* species used in our experiment.

2.3. Experimental design used to determine inhibitory effect

The inhibitory effect of phenolic compounds on the growth of fungi was tested using the agar dilution method. Investigated phenolic compounds were dissolved in pure methanol. The dissolved phenols were properly diluted in Potato Dextrose Agar (PDA) at a concentration of 1000 μg mL^{-1}. The final concentration of the solvent (methanol) in the PDA was 0.25% v/v. The prepared Petri dishes (9.0 cm diameter) were aseptically inoculated with assay disc (0.4 cm) cut from the periphery of a 7-day-old culture of the target fungi. The control sets were subsequently prepared using an equal volume of methanol without phenols. Incubation was carried out in the dark at 21 °C for seven days. Assays were carried out in quadruplicate. The percent inhibition of the radial growth of the target fungi was calculated according to the following formula: percent inhibition = $(DC - DT)/DC \times 100$, where DC is the colony diameter of the control sets and DT is the colony diameter of the treated sets. Phenolic compounds whose inhibitory effect on mycelial growth was higher than 50% at a basic concentration of 1000 μg mL^{-1} were chosen for further testing to evaluate the minimum inhibitory concentration (MIC_{100}) and median inhibitory concentration (MIC_{50}). The values of MIC_{100} and MIC_{50} were determined by the method of graded concentration of the compounds (from 15 μg mL^{-1} to 1000 μg mL^{-1}) in the PDA. Cultivation was carried out in the same way as before (in the dark at 21 °C, for 7 days). The MIC_{100} was regarded as the lowest concentration of compound that did not permit any visible growth when compared with control sets. The MIC_{50} was regarded as the concentration of compound that resulted in a 50% inhibition of visible growth when compared with control sets. The MIC_{100} and MIC_{50} values were then calculated using statistical analysis (Zabka et al., 2009, 2011).

2.4. Statistical analysis

Probit analysis was applied to assess the MIC_{100} and MIC_{50} values and 95% confidence intervals (Finney, 1971). The EPA Probit Analysis Program (Version 1.5) was used for statistical evaluation. The MIC_{100} and MIC_{50} values with 95% confidence intervals were statistically evaluated for each phenolic compound showing initial fungal growth inhibition higher than 50%. The final MIC values with 95% confidence intervals are listed in Table 3.

3. Results

The efficacy of all 21 tested phenols on the fungal growth of six target fungal species is provided in Table 2. The highest inhibitory effect on all fungi, 100% at a basic experimental concentration of 1000 μg mL^{-1}, was observed in the case of 10 compounds (thymol, carvacrol, isoeugenol, eugenol, 2-ethylphenol, 4-ethylphenol, salicylaldehyde, 2-methoxy-4-methylphenol, 4-ethylguaiacol and salicylic acid). Guaiacol, vanillin and p-coumaric acid caused 100% growth inhibition in only a few cases. The lowest antifungal effect was shown by six substances (phloroglucinol, caffeic acid, vanillic acid, gallic acid, syringic acid and sinapic acid), where the growth inhibition ratio did not exceed 50% in any case. Little or no inhibitory effect was observed. MIC_{50} and MIC_{100} values were successfully estimated for 15 substances by means of probit analysis (Table 3). Thymol and carvacrol clearly showed the highest efficacy. The MIC_{50} values for thymol ranged from 30 μg mL^{-1} to 52 μg mL^{-1} across the whole spectrum of target fungi. The MIC_{100} values for thymol ranged from 76 to 255 μg mL^{-1}. For carvacrol, MIC_{50} values ranged from 37 to 76 μg mL^{-1} and MIC_{100} ranged from 131 to 262 μg mL^{-1}. Isoeugenol, eugenol, 2-ethylphenol, 4-ethylphenol, salicylaldehyde and 4-ethylguaiacol exerted a good effect, but the MIC values were not as significant as in the case of