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Effectiveness of Environmentally Safe Food Additives and Food Supplements in an *In Vitro* Growth Inhibition of Significant *Fusarium, Aspergillus* and *Penicillium* species

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Abstract

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We tested 38 legislatively recognised substances such as food additives and supplements for antifungal efficacy, with the aim of providing an alternative to synthetic conventional fungicides. These compounds were tested against 9 significant pathogenic fungal species belonging to the significant genera *Fusarium*, *Penicillium*, and *Aspergillus*. Of these compounds, 6 are proposed as potential candidates to provide a complementary alternative to conventional fungicides. Natamycin provided extreme efficacy expressed as MIC_{50} (5–31 μg/ml), followed by BHA and then BHT, CaNa2EDTA, PABA, and chitosan expressed as MIC_{50} (0.7–1.9 mg/ml). Safety and antifungal activity were discussed in terms of the mode of action and molecular structure, as well as in terms of potential practical use and legislative requirements for the introduction into practice. We presume that food additives and food supplements are definitely a great source of antifungal compounds. In developed areas of the world (e.g. in the EU), they could represent legislatively recognised compounds, so-called basic substances.

Keywords: pathogenic fungi; toxigenic fungi; plant protection; basic substances; health risk; safe antifungals

The most discussed species of pathogenic filamentous fungi in agriculture undoubtedly include the genera *Fusarium*, *Penicillium* and *Aspergillus*. These three genera are most significant in agriculture and the food industry for their ability to produce a majority of very dangerous secondary metabolites, so-called mycotoxins, with detrimental acute or chronic effects on human health (Niessen 2007; Palumbo *et al*. 2008; Potshangbam *et al*. 2017). To a large extent, they are also involved in allergies, unpleasant local or even life-threatening systemic mycoses in man (Chowdhary *et al*. 2016; Muraosa *et al*. 2017).

Depending on the conditions, these pathogenic and toxigenic fungi are eliminated predominantly using synthetic conventional fungicides. However, the benefits of using synthetic fungicides are sometimes debatable. Both currently and historically, many cases of human health damage or environmental damage have been known to occur, precisely due to acute and/or chronic toxicity of active synthetic substances or their residues (Zarn *et al*. 2003; Nakanishi 2007; Costa *et al*. 2008; Scordino *et al*. 2008; Gubbins & Heldenbrand 2010). Moreover, frequent use of these synthetic fungicides has been known to cause the development of more or fully resistant strains (Kim *et al*. 2010; Wang *et al*. 2016). This problem is most noticeable in agriculture, where more sophisticated management of integrated plant protection has been finding increasing support for similar reasons.

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From this point of view, the growing global trend to minimise the use of these artificially synthesised compounds is comprehensible. At the same time, the interest in novel or additional alternative methods of protection against these harmful microorganisms has been rising (Zabka *et al*. 2011, 2014; Carvajal *et al*. 2016). The main criteria applicable to the search for novel alternatives include easy availability, verified safety and low toxicity to mammals or humans, as the case may be. And precisely these criteria are easily satisfied by legislatively recognised food additives commonly used worldwide or other, commonly used substances in the food industry and by food supplements. When any new pesticidal substance is found and introduced, the greatest problem is usually posed by complex and economically demanding verification of its safety, along with other demanding legislative processes. For example, a new approach has recently been established by an amendment of the European Union legislation. The fact that the EU reacts to increasing demands for accelerating the approval process of these safe alternatives, establishing a new term anchored in EU legislation as so-called basic substances (BSs) pursuant to the definition of Regulation (EC) No. 1107/2009, is praiseworthy (Pavela 2016). Such lengthy procedures are more or less avoided in the case of verified and safe BSs, which provide a huge economic and safety advantage.

In this study we try to find alternative and safe antifungal substances that could be used in the development of environment-friendly products for use against pathogenic and toxigenic fungi. The aim of this study fully corresponds to the universal trend of restricting the general or excessive use of synthetic fungicides in the protection of plants and food products during storage. The study evaluated the antifungal efficacy of many commonly used and essentially non-toxic substances of the group of legislatively verified additives utilised in the production of foods, as well as two approved food supplements. A complex of 38 selected individual substances was tested against 9 toxigenic fungal pathogens significant in terms of the food industry and agriculture.

MATERIAL AND METHODS

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

Fungal strains. All target pathogenic and toxigenic fungal strains (Table 2) were obtained from a collection of phytopathogenic fungi maintained at the Crop Research Institute, Prague, Czech Republic. *F. oxysporum*, *F. verticillioides*, *F. culmorum, F. graminearum, F. pseudograminearum, P. brevicompactum*, *P. expansum, A. flavus*, and *A. fumigatus* strains were preserved on slant agar (Potato Carrot Agar) at 4°C. Subcultures on Petri dishes and other manipulations with these strains were carried out in the Biosafety Level Two (BSL 2) laboratory, given the BSL of the *Fusarium* and *Aspergillus* species used in our experiment.

Experimental design used to determine inhibitory effect. The antifungal inhibitory effect of compounds on the growth of fungi was tested using the agar dilution method. Each of the tested compounds was properly dissolved in an equal volume of appropriate solvents (Nanopure water or DMSO in the case of water insoluble ones). The dissolved compounds were properly diluted in Potato dextrose agar (PDA) at a concentration of 1 mg/ml. The final concentration of the solvent in the PDA was 0.25% (v/v). The prepared Petri dishes (9.0 cm in diameter) were aseptically inoculated with assay discs (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 appropriate solvent without tested compounds. Incubation was carried out in the dark at 21°C for seven days. The percent inhibition of the radial growth of the target fungi was calculated according to the following formula: Percent inhibition = [(DC – DT)/DC] × 100, where: DC – colony diameter of the control sets; DT – colony diameter of the treated sets. Compounds whose inhibitory effect on mycelial growth was higher than 50% at a basic concentration of 2 mg/ml were chosen for further testing to evaluate the minimum inhibitory concentration (MIC_{50}). The values MIC_{50} were determined by the method of graded concentration of the compounds (from 0.01 mg/ml to 2.0 mg/ml or from 1 µg/ml to 100 µg/ml in the case of natamycin) in the PDA. Cultivation was carried out in the same way as before (in the dark at 21°C for 7 days). The MIC_{50} was regarded as the concentration of the compound that resulted in a 50% inhibition of visible growth when compared with control sets (Zabka *et al*. 2009, 2013, 2014). The MIC_{50} values were then calculated using statistical analysis.

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Table 1. Compounds used in the study

Compound name	E number	Formula if possible or IUPAC name or other description*
Chitosan LMW (low molecular weight)	–	poly(β-(1,4)-2-amino-2-deoxy-D-glucose)
Para-aminobenzoic acid (PABA)	–	4-aminobenzoic acid
Sodium sulphite	E221	Na_2SO_3
Calcium acetate	E263	$(CH_3COO)_2Ca$
Lactic acid	E270	2-Hydroxypropanoic acid
Sodium ascorbate	E301	sodium 5-[(1S)-1,2-dihydroxyethyl]-3-hydroxy-4-oxo-furan-2-olate
Calcium ascorbate	E302	calcium (2R)-2-[(1S)-1,2-dihydroxyethyl]-4-hydroxy-5-oxo-2H-furan-3-olate
Ascorbyl palmitate	E304	[(2S)-2-[(2R)-4,5-dihydroxy-3-oxo-2-furyl]-2-hydroxy-ethyl] hexadecanoate
Propyl gallate	E310	propyl 3,4,5-trihydroxybenzoate
Octyl gallate	E311	octyl 3,4,5-trihydroxybenzoate
Lauryl gallate	E312	dodecyl 3,4,5-trihydroxybenzoate
Sodium D-isoascorbate	E316	sodium 5-(1,2-dihydroxyethyl)-3-hydroxy-4-oxofuran-2-olate
Butylated hydroxyanisole	E320	2-tert-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole (mixture)
Butylated hydroxytoluene	E321	2,6-bis(1,1-dimethylethyl)-4-methylphenol
Soya lecithin	E322	(2-nonanoyloxy-3-octadeca-9,12-dienoyloxypropoxy)-[2-(trimethyl-azaniumyl)ethyl]phosphinate
Succinic acid	E363	Butanedioic acid
$CaNa_2$ ethylenediaminetetraacetic acid	E385	calcium;disodium;2-[2-[bis(carboxylatomethyl)amino]ethyl-(carboxylatomethyl)amino]acetate
Alginic acid from brown algae	E400	6-(2-carboxy-4,5-dihydroxy-6-methoxyoxan-3-yl)oxy-4,5-dihydroxy-3-methoxyoxane-2-carboxylic acid
Calcium D-gluconate monohydrate	E578	calcium;(2R,3S,4R,5R)-2,3,4,5,6-pentahydroxyhexanoate;hydrate
Nisin	E234	*polycyclic antibacterial peptide from *Lactococcus lactis*
Natamycin	E235	*amphoteric macrolide antifungal antibiotic from *Streptomyces natalensis*
Potassium acetate	E261	CH_3COOK
Sodium acetate	E262	CH_3COONa
Ascorbic acid	E300	(2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2H-furan-5-one
Tocopherol	E306	(2*R*)-2,5,7,8-tetrametil-2-[(4*R*,8*R*)-4,8,12-trimetiltridécil]-3,4-dihidro-croman-6-ol
Erythorbic acid	E315	(5*R*)-5-[(1*R*)-1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5*H*)-one
Potassium L-lactate	E326	potassium 2-hydroxypropanoate
Calcium L-lactate	E327	calcium 2-hydroxypropanoate
Sodium citrate	E331	trisodium 2-hydroxypropane-1,2,3-tricarboxylate
Potassium citrate	E332	tripotassium;2-hydroxypropane-1,2,3-tricarboxylate
Calcium citrate	E333	tricalcium;2-hydroxypropane-1,2,3-tricarboxylate
Guar gum	E412	disodium;[[[5-(6-aminopurin-9-yl)-3-hydroxyoxolan-2-yl]methoxy-hydroxyphosphoryl]oxy-oxidophosphoryl] hydrogen phosphate
Karaya gum	E416	polysaccharide from *Sterculia urens*
Phytagel	E418	polysaccharide from *Pseudomonas elodea*
Pectin from apple	E440	(2S,3R,4S,5R,6R)-3,4,5,6-tetrahydroxyoxane-2-carboxylic acid
Sodium bicarbonate	E500 (ii)	$NaHCO_3$
Calcium chloride	E509	$CaCl_2$
Calcium sulphate	E516	$CaSO_4$

Statistical analysis. The probit analysis was applied to assess the MIC_{50} values for each effective compound associated with 95% confidence limits (CI^{95}) (Finney 1971). The EPA Probit Analysis Program (Version 1.5) was used for statistical evaluation. The MIC values were statistically calculated and associated with Chi-square values significant at a $P < 0.05$ level. MIC_{50} were assessed for each extract showing the basic fungal growth inhibitory effect higher than 50% at the basic concentration of 2 mg/ml.

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Table 2. List of used species

Species	Strain	Year of isolation	Isolated from	BSL 2
Fusarium oxysporum	MZL/021215	2015	infected maize cob	*
Fusarium verticillioides	MZL/100415	2015		*
Fusarium culmorum	MZL/150514	2014		–
Fusarium graminearum	LS/1208	2008		–
Fusarium pseudograminearum	LS/21702	2002		–
Penicillium brevicompactum	MZL/270215	2015	contaminated stored maize	–
Penicillium expansum	MZL/280912	2012		–
Aspergillus flavus	LS/25702	2002		*
Aspergillus fumigatus	LS/2206	2006		*

BLS 2 – biosafety level 2; *species of moderate risk to humans; in immunocompromised individuals they may cause deep, systemic or superficial mycoses

RESULTS

Antifungal efficacy of all the tested substances expressed as the inhibitory effect percentage in the basic screening concentration (2 mg/ml) is shown in Table 3. Of the total 38 tested substances, 22 showed a measurable inhibitory effect (≥ 10%) in the basic concentration at least for one target fungal pathogen. Of this number, 12 exhibited efficacy against all target pathogenic fungi. However, only 6 substances showed a significant inhibitory effect (≥ 50%) over the entire spectrum of the target pathogens. This group of the most efficient substances includes (ordered according to the descending inhibitory effect percentage): natamycin E235, butylated hydroxyanisole, calcium disodium ethylenediaminetetraacetate ($CaNa_2$ EDTA) E385, butylated hydroxytoluene E321, para-aminobenzoic acid (PABA), and low molecular weight (LMW) chitosan. As expected, the highest inhibitory effect of 100% in all cases was achieved by natamycin E235. Butylated hydroxyanisole E320 can also be considered as a very efficient compound against all fungal pathogens with efficacy approaching 100% in most of the target pathogenic fungi. Significant effects were shown by $CaNA_2$ EDTA E385 with efficacy exceeding 90% in more than one half of the pathogenic fungi. The remaining active compounds such as butylated hydroxytoluene E321 achieved 90% inhibitory effect in less than one half of the cases or – for LMW chitosan and PABA – they only approached this extreme level of inhibition. On the contrary, the total of the 16 remaining food additives were fully or almost fully without any effect against the growth of the target pathogenic fungi. In the basic testing concentration, these substances achieved no or only an insignificant (≤ 10%) inhibitory effect in all of the 9 target pathogens. A much more profound view on efficacy against individual pathogenic fungi is presented using the MIC_{50} values (Table 3). Individual MIC_{50} values could be evaluated in 60 cases, i.e. in all cases where the basic inhibitory effect exceeded 50%. Based on evaluation of MIC_{50} values, natamycin clearly provided the highest efficacy – up to 10 times higher compared to the other efficient substances. For natamycin E235, MIC_{50} values ranged between 4.8 and 31 µg/ml. Lower efficacy, although still with MIC_{50} values of similar order, was achieved only by butylated hydroxyanisole E320 – from 29 to 110 µg/ml. As regards MIC_{50} values of the other active substances, i.e. butylated hydroxytoluene E321, $CaNa_2$ EDTA E385, PABA, and LMW chitosan, their values were on a level 10 times higher, thus 0.2–0.8, 0.17–0.55, 0.7–1.99, and 0.71–1.92 mg/ml, respectively. Obviously, individual inhibition levels and MIC_{50} values were influenced by sensitivity of the species or by resistance within the experimental complex of the used target fungi. According to the MIC_{50} values, most of the efficient substances exhibited the highest efficacy against *A. fumigatus*. On the contrary, most substances exhibited the least efficacy against *A. flavus* where in certain cases, the MIC_{50} values were even several times higher than in the other target fungi.

DISCUSSION

The search for novel alternative methods and safe antifungal substances, in order to reduce the con-

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Table 3. Inhibitory effect of compounds on target fungi at a concentration of 2 mg/ml and MIC_{50}

Compound	Target fungal species								
	Fusarium oxysporum	*Fusarium verticillioides*	*Fusarium culmorum*	*Fusarium graminearum*	*Fusarium pseudogram*	*Penicillium brevicompactum*	*Penicillium expansum*	*Aspergillus flavu*	*Aspergillus fumigatus*
	Inhibition (mean) (%) ± SE MIC_{50} (CI^{95})[a] Chi square[b]								
Chitosan	77.4 ± 0.2 0.71 (0.58–0.86) 0.594	72.2 ± 0.5 0.87 (0.71–1.09) 0.316	58.2 ± 0.1 1.14 (0.85–1.76) 0.093	52.4 ± 0.2 1.51 (1.05–3.09) 1.468	63.3 ± 0.1 0.99 (0.81–1.25) 4.703	56.4 ± 0.1 1.28 (0.95–2.01) 1.295	52.0 ± 0.0 1.92 (1.34–3.76) 0.222	55.8 ± 0.2 1.27 (0.97–1.90) 5.746	75.1 ±0.1 0.76 (0.62–0.94) 0.670
PABA	82.2 ± 0.1 0.73 (0.62–0.87) 1.683	88.1 ± 0.0 0.72 (0.62–0.83) 5.018	80.9 ± 0.3 0.69 (0.57–0.83) 1.320	80.2 ± 0.1 0.74 (0.62–0.89) 2.556	73.3 ± 0.3 0.89 (0.74–1.08) 1.478	59.4 ± 0.1 1.41 (1.12–1.96) 1.090	52.0 ± 1.0 1.78 (1.44–2.42) 1.644	50.0 ± 0.1 1.99 (1.56–3.49) 0.792	81.5 ± 0.1 0.70 (0.58–0.83) 2.185
E 221	10.7 ± 0.8 ≥ 2	NI –	NI –	10.1 ± 0.1 ≥ 2	NI –	NI –	NI –	NI –	NI -
E 234	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –
E 235	100.0 ± 0.0 13.6 (10.97–16.77)* 2.151	100.0 ± 0.0 16.5 (13.08–20.90)* 1.679	100.0 ± 0.0 11.5 (9.37–13.83)* 1.667	100.0 ± 0.0 24.1 (19.41–30.49)* 1.234	100.0 ± 0.0 15.2 (12.12–18.96)* 1.537	100.0 ± 0.0 10.9 (8.96–13.19)* 7.058	100.0 ± 0.0 4.8 (3.31–6.34)* 2.723	100.0 ± 0.0 31.0 (23.69–42.83)* 5.501	100.0 ± 0.0 7.0 (6.01–8.12)* 4.280
E 261	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –
E 262	18.4 ± 0.2 ≥ 2	NI –	13.0 ± 0.1 ≥ 2	NI –	NI –	NI –	NI –	NI –	12.6 ± 0.1 ≥ 2
E 263	13.0 ± 0.1 ≥ 2	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –
E 270	28.1 ± 0.1 ≥ 2	15.9 ± 0.4 ≥ 2	19.3 ± 0.6 ≥ 2	22.7 ± 0.6 ≥ 2	NI –	10.2 ± 0.5 ≥ 2	NI	10.3 ± 0.1 ≥ 2	19.9 ± 0.2 ≥ 2
E 300	21.2 ± 1.3 ≥ 2	NI –	NI –	21.3 ± 0.2 ≥ 2	NI –	NI –	NI –	NI –	25.9 ± 0.1 ≥ 2
E 301	18.5 ± 0.2 ≥ 2	11.9 ± 0.5 ≥ 2	NI –	NI –	NI –	12.3 ± 0.1 ≥ 2	NI –	NI –	11.3 ± 0.8 ≥ 2
E 302	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –
E 304	10.4 ± 0.1 ≥ 2	22.0 ± 1.0 ≥ 2	30.7 ± 0.3 ≥ 2	23.8 ± 0.2 ≥ 2	16.8 ± 0.4 ≥ 2	9.1 ± 0.2 ≥ 2	19.0 ± 0.6 ≥ 2	5.9 ± 0.1 ≥ 2	29.9 ± 0.2 ≥ 2
E 306	39.9 ± 0.4 ≥ 2	21.3 ± 0.5 ≥ 2	37.6 ± 0.0 ≥ 2	21.9 ± 0.2 ≥ 2	16.4 ± 0.2 ≥ 2	46.9 ± 0.1 ≥ 2	30.9 ± 0.6 ≥ 2	10.0 ± 0.4 ≥ 2	12.7 ± 1.1 ≥ 2
E 310	64.4 ± 0.0 1.87 (1.51–2.59) 1.387	29.3 ± 0.8 ≥ 2	27.7 ± 0.2 ≥ 2	33.8 ± 0.6 ≥ 2	37.3 ± 0.2 ≥ 2	41.7 ± 0.1 ≥ 2	45.5 ± 0.3 ≥ 2	30.6 ± 0.1 ≥ 2	67.4 ± 0.4 1.81 (1.48–2.39) 1.225

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Table 3 to be continued

Compound	Target fungal species								
	Fusarium oxysporum	*Fusarium verticillioides*	*Fusarium culmorum*	*Fusarium graminearum*	*Fusarium pseudogram*	*Penicillium brevicompactum*	*Penicillium expansum*	*Aspergillus flavu*	*Aspergillus fumigatus*
	Inhibition (mean) (%) ± SE MIC_{50} (CI ^{95})[a] Chi square[b]								
E 311	55.5 ± 0.2 1.98 (1.43–2.92) 1.633	48.8 ± 0.8 ≥ 2	50.0 ± 0.1 ≥ 2	63.8 ± 0.2 1.79 (1.20–2.25) 0.429	66.0 ± 0.2 1.72 (1.54–2.12) 1.793	49.4 ± 1.2 ≥ 2	48.0 ± 0.1 ≥ 2	32.9 ± 0.9 ≥ 2	61.3 ± 0.1 1.57 (1.23–2.51) 0.819
E 312	30.4 ± 0.1 ≥ 2	NI –	13.9 ± 0.4 ≥ 2	NI –	NI –	NI –	NI –	NI –	37.2 ± 0.2 ≥ 2
E 315	39.0 ± 0.0 ≥ 2	22.8 ± 0.5 ≥ 2	44.0 ± 0.0 ≥ 2	38.6 ± 0.1 ≥ 2	28.0 ± 0.2 ≥ 2	26.4 ± 0.3 ≥ 2	24.2 ± 0.2 ≥ 2	12.3 ± 1.0 ≥ 2	36.6 ± 0.6 ≥ 2
E 316	20.7 ± 0.1 ≥ 2	NI –	12.5 ± 0.3 ≥ 2	NI –	NI –	NI –	NI –	NI –	20.0 ± 0.3 ≥ 2
E 320	97.8 ± 0.1 37 (29–45)* 0.032	96.1 ± 0.1 53 (43–64)* 4.870	98.5 ± 0.1 42 (34–51)* 2.079	98.1 ± 0.1 45 (37–55)* 1.445	98.4 ± 0.0 37 (29–49)* 2.710	91.9 ± 0.3 45 (36–54)* 1.218	71.3 ± 0.8 97 (80–121)* 0.498	75.9 ± 0.1 110 (85–124)* 1.681	98.3 ± 0.0 29 (24–34)* 2.145
E 321	61.9 ± 0.1 0.55 (0.44–0.66) 0.622	80.2 ± 0.2 0.21 (0.01–0.43) 0.197	88.6 ± 0.1 0.17 (0.02–0.36) 0.214	90.0 ± 0.3 0.19 (0.04–0.32) 0.816	88.0 ± 0.0 0.45 (0.36–0.53) 0.850	80.3 ± 0.5 0.21 (0.23–0.26) 0.078	75.2 ± 0.1 0.30 (0.23–0.39) 1.795	74.1 ± 0.0 0.38 (0.31–0.47) 3.348	98.6 ± 0.0 0.21 (0.17–0.25) 2.605
E 322	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –
E 326	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –
E 327	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –
E 331	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –
E 332	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI -
E 333	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –	NI –
E 363	22.2 ± 0.2 ≥ 2	NI –	11.4 ± 1.0 ≥ 2	NI –	NI –	NI –	NI –	NI –	15.4 ± 0.2
E 385	80.1 ± 0.1 0.68 (0.57–0.79) 1.608	88.1 ± 0.0 0.65 (0.57–0.74) 2.561	95.0 ± 0.2 0.44 (0.37–0.51) 3.079	97.3 ± 0.1 0.20 (0.14–0.26) 0.944	97.7 ± 0.1 0.27 (0.20–0.33) 2.576	80.5 ± 0.1 0.75 (0.64–0.88) 0.198	90.1 ± 0.1 0.56 (0.48–0.63) 0.755	79.0 ± 0.1 0.80 (0.69–0.945) 0.671	99.7 ± 0.0 0.38 (0.34–0.42) 2.485
E 400	22.2 ± 0.0 ≥ 2	13.0 ± 0.2 ≥ 2	14.6 ± 0.1 ≥ 2	29.7 ± 0.2 ≥ 2	18.8 ± 0.4 ≥ 2	32.2 ± 0.1 ≥ 2	24.3 ± 0.2 ≥ 2	15.4 ± 0.5 ≥ 2	32.7 ± 0.2 ≥ 2

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Table 3 to be continued

Compound	Target fungal species								
	Fusarium oxysporum	*Fusarium verticillioides*	*Fusarium culmorum*	*Fusarium graminearum*	*Fusarium pseudogram*	*Penicillium brevicompactum*	*Penicillium expansum*	*Aspergillus flavu*	*Aspergillus fumigatus*
	Inhibition (mean) (%) ± SE MIC_{50} (CI ^{95})[a] Chi square[b]								
E 412	NI	NI	NI	NI	NI	NI	NI	NI	NI
	–	–	–	–	–	–	–	–	–
E 416	NI	NI	NI	NI	NI	NI	NI	NI	NI
	–	–	–	–	–	–	–	–	–
E 418	NI	NI	NI	NI	NI	NI	NI	NI	NI
	–	–	–	–	–	–	–	–	–
E 440	NI	NI	NI	NI	NI	NI	NI	NI	NI
	–	–	–	–	–	–	–	–	–
E 500 (ii)	NI	NI	NI	NI	NI	NI	NI	NI	NI
	–	–	–	–	–	–	–	–	–
E 509	NI	NI	NI	NI	NI	NI	NI	NI	NI
	–	–	–	–	–	–	–	–	–
E 516	NI	NI	NI	NI	NI	NI	NI	NI	NI
	–	–	–	–	–	–	–	–	–
E 578	12.6 ± 0.2	NI	NI	NI	NI	NI	NI	NI	NI
	–	–	–	–	–	–	–	–	–

[a]minimum inhibitory concentration (MIC_{50}) with 95% confidence intervals; [b]Chi-squared value, significant at $P < 0.05$ level; *values in µg/ml; NI – no inhibition

sumption of commonly used synthetic fungicides, has been gaining importance in the production of safe foods. As indicated by this study using a model of many common food additives and two additional natural substances with verified safety, applicable representatives can be found that offer safety verified by practice, namely that they have been rigorously monitored and used in the food industry over the long term. Their potential use as alternative plant protection substances is feasible due to their high antifungal efficacy against a broad spectrum of model fungi. Based on the results presented by this study, these substances can be considered to have a sufficient antifungal potential.

Natamycin was intentionally included in the study. It is the only substance in our study that is designed to directly suppress fungi, particularly on the surface of foods, especially of cheeses (Resa *et al.* 2014). Furthermore, it is commonly used for the treatment of mycoses in human medicine (Prajna *et al.* 2010). Its use in plant protection has not been sufficiently tested. The low MIC_{50} values of natamycin were expected, but its efficacy also had to be verified in a model of economically important pathogens. In our opinion and based on the exceptionally low MIC_{50} values, natamycin fulfils the preconditions for use in modern plant protection, namely in the production of plant products safe for the environment and health. The higher price of natamycin is offset by its very high efficacy even against agriculturally important pathogens, as shown in our study, and in particular, by its extreme safety confirmed by both legislation and practice, such as by its general authorisation in the EU under the E code E235, for example (Woodward 2012). Unlike commonly used fungicides, usually of the group of azoles as shown by Zarn *et al.* (2003), natamycin is not absorbed from the gastrointestinal tract or through the skin, and oral or topical administration of natamycin is not associated with any harmful side effects (Aparicio *et al.* 2000; Juneja *et al.* 2012). The natural origin of natamycin is another advantage. It is classified among

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the polyene macrolide antibiotics produced by the strains *Streptomyces natalensis* or *Streptococcus lactis*, with a strong specific bond to ergosterol, without causing any changes in the permeability of the plasma membrane (Te Welscher *et al.* 2008, 2010). Natamycin may also offer a potential in plant protection as a complementary ingredient to reduce the dose of the main fungicide while preserving the necessary antifungal effect. In this regard, natamycin has been known to form synergistic complexes with some medically important azole fungicides of the second generation (Al-Hatmi *et al*. 2015). However, its synergism has not been described for common azole fungicides used in agriculture. In our study, the antifungal efficacy of natamycin was approached only by butylated hydroxyanisole BHA (E320). This synthetic phenolic substance is used primarily as an antioxidant, just like the next one in terms of efficacy, butylated hydroxytoluene BHT (E321). Their high antifungal efficacy is conditioned by the presence of the hydroxyl group, higher affinity to the lipid component of cell membranes, and disturbance of their natural permeability. A difference in the efficacy of BHA and BHT is due to different arrangements and the presence of different functional groups. The presence and position of the hydroxyl group with respect to other functional groups in the molecule of phenolic compounds influences antifungal activity, as confirmed by previous studies (Zabka & Pavela 2013; Zabka *et al*. 2014). Although this study demonstrated a relatively high efficacy against filamentous pathogenic fungi, some research studies indicated potential, rather serious health effects of higher exposure or in sensitive individuals (Goodman *et al*. 1990; Kahl & Kappush 1993; Race 2009). Given these contradictions, we believe that the use of BHA and BHT in plant protection against pathogenic fungi in the process of producing safe foods may be debatable and less acceptable by the lay public, even if, according to their parameters, they can be classified among BSs in the EU. On the other hand, the use of both these substances as supportive additives in plant protection could be beneficial, given the ascertained synergism with commercial fungicides (Simonetti *et al*. 2002, 2003). Moreover, both BHA and BHT are still used in the food industry for preservation and emulsification of edible oils, fats and other foods (Fan & Eskin 2015).

$CaNa_2$ EDTA was the next most efficient compound against the tested filamentous pathogenic fungi in our study. It is a calcium disodium salt of ethylenediaminetetraacetic acid (EDTA). $CaNa_2$ EDTA is authorised in the EU under the E code E385 and is used as a favourite stabilizer and preservative agent. Given that it is a strong chelator, in medicine it is used in conditions of heavy metal poisoning. Based on recent research, this compound is safe and exhibits minimal harmful effects, which moreover occur only in high doses (Ernst 2000; Flora *et al*. 2008; Van de Sande *et al*. 2014). The mechanism of action of $CaNa_2$ EDTA against pathogenic fungi can be attributed primarily to its chelation capacity to bind divalent ions, particularly Mg^{2+}, in cell membranes, increased permeability and overall energy destabilisation as indicated by some studies (Hancock & Wong 1984; Alakomi 2006). Based on some findings, the efficacy of commercial fungicides increases when mixed with non-modified EDTA on account of the increased permeability of cell membranes (Hachem *et al*. 2006). Considering that the legislatively approved form $CaNa_2$ EDTA is also a permeabiliser, a similar effect could also be achieved here. For this reason, $CaNa_2$ EDTA could be considered not only as a primary, but also as a complementary substance, offering the potential to increase antifungal efficacy in an attempt to reduce the dose of commercial fungicides.

PABA was evaluated as the next most efficient substance in our study, based on MIC_{50} values with respect to individual pathogenic filamentous fungi. Although this substance is not classified as a food additive, it is a type of food supplement. In medicine, it is used in diagnostic tests to determine the state of the gastrointestinal tract and occasionally in the treatment of irritable bowel syndrome, to treat its associated gastrointestinal symptoms (Sonwalkar *et al*. 2003; Sardesai 2011). PABA is a natural substance, chemically similar to sulphonamides, and is essential for the functioning of metabolic processes, even though the human organism is not able to synthesize it (Gaby 2006; Singh *et al*. 2011). Sometimes, PABA is called vitamin Bx, for which a positive effect of increasing plant resistance has also been described (Song *et al*. 2013; Boubakri *et al*. 2016). Although, according to Wong and Orton (2011), it may cause photosensitive reactions in susceptible individuals, or allergies, predominantly upon apical application, PABA is considered a very safe substance in terms of toxicology, which has been demonstrated even in very high concentrations (Chang & Hu 1996; Correa-Basurto *et al*. 2005). Given this information and its efficacy against filamentous pathogenic fungi in

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our experiments, we believe that the use of PABA, e.g. in the BS mode as a new alternative antifungal substance, associated with environmentally friendly plant protection and the production of safe foods and other products, could be highly beneficial.

In our study we also tested and evaluated the direct antifungal effect of LMW chitosan, which can already be partially encountered in plant protection. Chitosan showed the lowest, but still significant, antifungal efficacy against all filamentous pathogenic fungi in our study. It is a polysaccharide, a copolymer of glucosamine and *N*-acetylglucosamine (Younes & Rinaudo 2015). The efficacy of chitosan is due to many mechanisms, such as electrostatic interactions of the positively charged chitosan molecule with the cell membranes, disturbance of the osmotic balance of the cytosol, and the direct destabilisation of membranes. Due to its strong chelation activity, important metal ions are blocked, particularly Ca^{2+} (Goy *et al*. 2009; Lee *et al*. 2016). Some studies mention an indirect secondary effect of chitosan applied to plants, such as increased resistance of plants through the elicitation of defence mechanisms in plant tissues, together with the mechanical barrier of the chitosan layer (Amborabé *et al*. 2008; El Hadrami *et al*. 2010). Neither mode of action excludes the other, and they offer suitable complementarity in terms of practice. The virtually non-toxic chitosan is already being tested in practice as an alternative substance for plant protection, with the benefit of being environmentally safe (Thanou *et al*. 2001; Alves & Mano 2008; Keen & Thanou 2010). Our experiments confirmed and evaluated efficacy on the level of MIC_{50} values for a broad spectrum of filamentous fungi important for agriculture, medicine and the food industry, and this reinforces our conviction that the general use of chitosan has an enormous potential in the protection of plants, food and agricultural products against pathogenic fungi.

In our study we performed tests of 38 commonly used substances classified as food additives or food supplements, authorised and used in the advanced countries of the world. Among these known substances we found six potential candidates, with antifungal activity, for the development of new, safe antifungal products. The environmental trend of reducing the consumption of synthetic conventional fungicides and seeking other alternatives has been accepted worldwide. However, in light of this universal trend, we should emphasise the above-mentioned advantage of authorising these and similar substances, for example, in the EU. Given that all the active substances that we tested satisfy the conditions for classification as BSs pursuant to the definition of Regulation (EC) No. 1107/2009, their authorisation should be most feasible precisely in EU countries. However, we assume they will have a potential for worldwide application in the protection against harmful filamentous fungi. On the basis of these and similar substances, we can expect the portfolio of hygienically and environmentally safe antifungal substances on the market to expand. We expect their principal potential to be in the field of the production of safe foods and environmentally friendly systems of agriculture, and generally in the possibility of reducing the need to apply conventional fungicides in all debatable areas of the suppression of harmful fungi.

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