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Growth of *Penicillium verrucosum* and Production of Ochratoxin A on Nonsterilized Wheat Grain Incubated at Different Temperatures and Water Content

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A b s t r a c t

The results of two experiments with wheat grain inoculated with *Penicillium verrucosum* are reported. In Experiment I, wheat grain, containing 10, 20 and 30% water, was incubated for 2 weeks at 10, 15, 21 and 28°C. In Experiment II, wheat grain, containing 14, 16, 18, 20 and 22% water, was incubated for 2 weeks at 10, 15, and 20°C. At initial moisture content (IMC) of the wheat grain up to 16% neither *P. verrucosum* growth nor ochratoxin A (OTA) formation were observed. In the range of IMC from 18% to 22% both the fungal growth and OTA synthesis were distinct, and the parameters were higher at higher temperature in the range 10–21°C. A temperature of 28°C was probably too high for proper metabolism of the fungus, including OTA formation. OTA formation was distinctly related to *P. verrucosum* abundance in the temperature range 10–21°C, expressed both as the counts of fungal colony forming units (CFU) on agar DYSG medium and diameters of the fungal colonies growing around the wheat kernels placed on the surface of DYSG medium. OTA formation and abundance of *P. verrucosum* were negatively correlated with the percentage of wheat kernels, placed on DYSG medium, with growing colonies of fungi different from *P. verrucosum*. CFU counts of *P. verrucosum* on the wheat grain were significantly related to the diameter of the fungal colonies growing around the wheat kernels placed on DYSG medium. The relationship is described by an exponential regression equation.

K e y w o r d s: *Penicillium verrucosum* abundance, ochratoxin A, wheat grain

Introduction

Penicillium verrucosum Dierckx is the only known species of *Penicillium* able to produce nefrotoxic, carcinogenic, teratogenic and immunotoxic ochratoxin A (OTA) on grain and cereal products (Elmholt *et al.*, 1999; Lund and Frisvad, 2003). Growth of *P. verrucosum* appears to be the major cause of grain contamination by OTA in European countries (Arroyo *et al.*, 2005; Lund and Frisvad, 2003; Miller, 1995). *Aspergillus ochraceous* and several related species that are considered rare on grain also produce OTA (Miller, 1995; Pardo *et al.*, 2004; Ramos *et al.*, 1998). There is the general opinion that species of *Aspergillus* genus are important in OTA production in warmer regions, while in colder regions, especially of temperate climates, only the activity of *P. verrucosum* is significant (Elmholt and Hestbjerg, 1999; Lindblad *et al.*, 2004; Park *et al.*, 2005).

Moisture content and temperature are the most important variables in determining growth and rate of mycotoxin production by fungi in stored grain ecosystems (Cairns-Fuller *et al.*, 2005; Pardo *et al.*, 2004; Ramos *et al.*, 1998). *P. verrucosum* is a typical xerophilic storage species that is able to thrive at relatively low water activities (Arroyo *et al.*, 2005; Axberg *et al.*, 1997; Moss, 1996; Ramakrishna *et al.*, 1996). Various other fungal species present on grain differ in their growth responses to temperature and water activity of the substrate (Haasum and Nielsen, 1998; Moss, 1996; Pardo *et al.*, 2004; Ramakrishna *et al.*,

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1996). Ramakrishna *et al.* (1996) reported, on the basis of their own results and results of the cited authors, that some of these fungi might influence the colonization of cereal grains by *P. verrucosum* and subsequent OTA production, but all those studies were conducted with sterilized grain. Therefore, there is a lack of information about growth of *P. verrucosum* and OTA production by the fungus competing with natural microbial contaminants on nonsterilized grain under different environmental conditions.

Development of the selective and diagnostic medium DYSG for the detection of *P. verrucosum* in foods and feeds (Frisvad *et al.*, 1992) offers opportunities for estimating the abundance of the fungus in mixed populations of fungi, using the classical dilution plating technique or direct plating (Elmholt *et al.*, 1999; Czaban and Wróblewska, 2006).

The objective of this study was to determine the effects of different temperatures and the moisture contents on growth of a strain of *P. verrucosum*, isolated from Polish rye, and on its ability to ochratoxin A formation on nonsterile winter wheat grain, inoculated with the fungus.

Experimental

Materials and Methods

Fungal strain of *P. verrucosum* was isolated from grain of rye (LPH63 DE), harvested in 2002.

The fungal growth media and conditions. *P. verrucosum* was isolated and grown on DYSG medium (Lund and Frisvad, 2003; Czaban and Wróblewska, 2006). Grain of winter wheat cv. Mewa and cv. Finezja, harvested in 2004 year, were used in Experiment I and Experiment II, respectively. Basing on of the method described by Lund and Frisvad (2003), the grain was not contaminated with *P. verrucosum* and other ochratoxinogenic fungi. The grain contains 6.1% (after drying at 40°C) and 12.1% water for Mewa and Finezja, respectively. The initial moisture content (IMC) of the grain was adjusted to 10, 20 and 30% in Experiment I, and to 14, 16, 18, 20 and 22% in Experiment II by adding sterile water (and suspension of *P. verrucosum* spores – see below) to beakers, containing 100 g samples of the grain. The samples were mixed, placed into closed plastic bags and kept for 24 hours in a refrigerator at 4°C. The beakers with moistened grain were shaken several times during the cold storage and the grain was mixed again to distribute moisture and the inoculum of *P. verrucosum* before incubation. The grain in each beaker was inoculated with 1 ml of spore suspensions containing 0.80×10^7 or 0.64×10^7 spores, to obtain 2×10^5 or 1.6×10^5 spores per 1 g of wheat grain in Experiment I and Experiment II, respectively. The beakers with inoculated grain were incubated in ventilated plastic bags in darkness at 10, 15, 21 and 28°C in Experiment I, and at 10, 15 and 20°C in Experiment II, for two weeks.

Determination of fungal abundance on the incubated grain. After the incubation, fungi were isolated from the grain by shaking with a solution containing 0.85% NaCl, 0.1% peptone and 0.1% Tween 80 for 30 min (Frisvad, 1986b). The number of colony forming units (CFU) of both *P. verrucosum* and all fungi or only *P. verrucosum* in the resulting suspensions were determined by dilution plating on Martin's (Martin, 1950) and DYSG (Lund and Frisvad, 2003; Czaban and Wróblewska, 2006) media, respectively. The Petri dishes with Martin's medium were incubated at 27°C for 4 days. The plates with DYSG medium were incubated in the dark at 20°C for 5 days. After 7 days *P. verrucosum* colonies had developed their characteristic terracotta-colored pigmentation on the DYSG reverse, caused by synthesis of an anthraquinone (Elmholt *et al.*, 1999; Frisvad *et al.*, 2005). The determinations were done with four replicates. Abundance of *P. verrucosum* on the wheat grain was also determined by the measurement of diameters of *P. verrucosum* colonies growing around the wheat kernels placed on agar DYSG medium, according to Czaban and Wróblewska (2006). Twenty seven incubated wheat kernels were placed on DYSG medium (9 kernels per 1 Petri dish). The diameters of the fungal colonies were measured across the width of the kernels after 5 days of incubation in the dark at 20°C. Although Czaban and Wróblewska (2006) used 22.5°C for incubation of the Petri dishes with wheat kernels contaminated with *P. verrucosum*, temperature of 20°C was chosen in present studies, because it is recommended in standardized method No. 152 of The Nordic Committee on Food Analysis for growth of the fungus (NMKL, 2005).

Competitive relation of *P. verrucosum* and other fungi was assessed on the basis of the percentage of twenty-seven incubated wheat kernels, placed on agar DYSG medium, with growing colonies (beside the kernels) of fungi different from *P. verrucosum*.

Radial mycelial growth of *P. verrucosum* was measured on 9 cm diameter Petri plates with agar DYSG medium, after 3, 5, 7, 9, 11 and 14 days of incubation at 10, 15, 21 and 28°C (Experiment A) and after 14 days at 15, 22.5, 25, 27.5 and 30°C (Experiment B). Each plate was point-inoculated in the center with a suspension of fungal spores. The measurements were done in five replicates.

Ochratoxin A (OTA) extraction. After the incubation, the wheat grain, collected from the beakers, was ground and carefully mixed. Samples (10 g) of the mixture were homogenized for 1 min with 80 ml of 80% acetonitrile and then extracted for 16 h at room temperature with occasional shaking. The extract was filtered through filter paper. The residual material was incubated for 1 h with 50 ml of 80% acetonitrile, and then it was sonicated for 3 min and filtered through filter paper. The both filtrates were combined and evaporated at a temperature not exceeded 40°C to remove acetonitrile. The condensed extract was diluted with water and cleaned-up by a SEP-PAK C18 microcolumn, which was rinsed with water and then with 100% methanol. The extracts were evaporated to dryness and solubilized in 1 ml of 100% methanol. The samples were kept in dark vials at 5°C.

OTA content determination. Measurements with a HPLC Waters system equipped with a 474 fluorescence detector (excitation 230 nm, emission 470 nm) were done. The samples were separated using a C18 Eurospher-100 column (5 µm, 250×4 mm) at 35°C. Sample volume was 50 µl. The analysis was performed under isocratic conditions at a flow rate of 1 ml/min of the mobile phase (CH_3CN:H_2O:H_3PO_4; 54:45:1) for 15 min. Ochratoxin A (Sigma) was used as a standard. The retention time of OTA was approximately 7.8 min. The measurement was done in triplicates.

Statistical evaluations. The data were subjected to one-way analysis of variance and the means were separated with Student's t-test (at $P = 0.0001$ for OTA concentration and fungal colony determinations, and at $P = 0.05$ for fungal CFU number determina-

tions). Although concentrations of OTA and CFU numbers are shown on a logarithmical scale in Fig. 1 and 2, only basic values were used in these statistical analyses. Statistical evaluation of significant differences between the percentages of wheat grains surrounded by colonies of fungi different from *P. verrucosum* on DYSG medium were calculated according to Czaban and Wróblewska (2006). For estimation of all examined relationships, linear correlation analysis as well as linear and exponential regression analyses were applied. Together with correlation coefficients (r), the probabilities (P) and the number of replicates (n) are presented. The determination coefficient (R^2) is presented with linear and exponential regression equations.

Results and Discussion

In the presented studies, the wheat grain was inoculated with a large number of *P. verrucosum* spores, 10 times greater than the CFU number of other fungi on the grain, to obtain a distinct dominance of *P. verrucosum* at the beginning of the incubation period to be sure that *P. verrucosum* would grow and produce OTA on nonsterile grain.

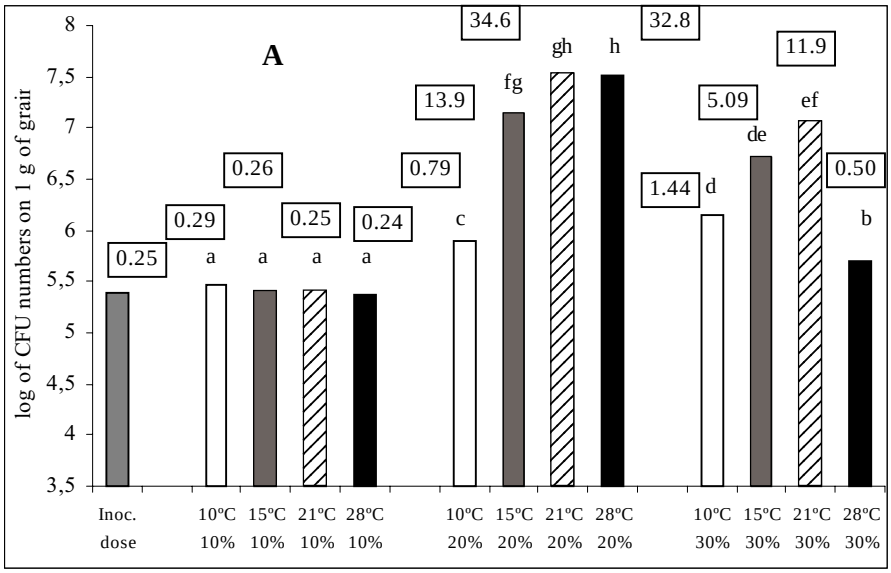
In **Experiment I**, three levels of IMC – initial moisture content (10, 20 and 30%) of the incubated wheat grain (after inoculation with *P. verrucosum*) were chosen.

The first level (10%) of IMC in the grain was chosen as too low for growth of all fungi. As expected, *P. verrucosum* and other fungi did not reveal any symptoms of growth or metabolic activities on the grain with IMC 10%. In this case, the numbers of the fungal CFU were at similar level as the inoculation dose (Fig. 1A, 1B), and *P. verrucosum* colony diameters around the kernels placed on the DYSG medium were small (Fig. 1C). Also, no OTA was detected in the grain (Fig. 1E).

The next level (20%) of IMC in the grain was chosen as close to "the boundary value", which enables xerophilic storage moulds (including *P. verrucosum*) to grow, but it prevents the growth of field fungi (French *et al.*, 1989). Indeed, except for the grain incubated at 10°C, the intensive growth of *P. verrucosum* was noted in all series with this moisture content, expressed as high CFU numbers of the fungus (Fig 1A and 1B) and its big colony diameters (Fig. 1C). However, distinct growth of fungi different from *P. verrucosum* on the grain was not observed (Fig. 1B). Only single colonies could be detected on plates with Martin's medium in the case of lower dilutions, so their CFU numbers could not be determined, because it was masked by high numbers of *P. verrucosum*. The low percentage (in comparison to the series with 10% and 30% IMC) of the wheat kernels (placed on DYSG medium) with colonies of fungi different from *P. verrucosum* (Fig. 1D) also confirms the distinct domination of *P. verrucosum* in the series with 20% IMC at 15, 21 and 28°C. In these series, very high numbers of CFU of *P. verrucosum* (Fig. 1A and 1B) and large diameter of the fungal colonies (Fig. 1C) were related to very high production of OTA by the fungus at 15 and 21°C, but not at 28°C (Fig. 1E). This temperature was probably too high for efficient metabolism of the fungus. This assumption was confirmed by radial mycelial growth in Experiment A (Fig. 1F) and in Experiment B (Fig. 1G) on agar DYSG medium. Although in Experiment A the diameters of *P. verrucosum* colonies were the largest at 28°C, the characteristic for the fungus terracotta dye production on the reversal side of its colonies was inhibited to a certain degree. At this temperature, the obverse of the fungal colony was also changed. In radial mycelial growth Experiment B the terracotta dye production was also decreased at 27.5°C and completely inhibited at 30°C.

Data of Experiment I are consistent with results of Myslivec and Tuite (1970) and Myslivec *et al.* (1975). They reported that *P. cyclopium* could not grow at temperatures higher than 30°C. Similarly, Northolt *et al.* (1979) found that strains of *P. viridicatum* and *P. cyclopium* could not produce OTA at 31°C. The correct present taxonomic classification of these strains most likely should be *P. verrucosum* (Domsch *et al.*, 1986; Frisvad, 1986a; Frisvad and Filtenborg, 1989). Results of some studies (Ramakrishna *et al.*, 1996) with a strain of *P. verrucosum* obtained from the Rothamsted Experimental Station culture collection confirm that a temperature of 30°C is not favourable to OTA formation by this fungus and its growth.

After 2-weeks of incubation of wheat grain with the highest level of IMC (30%) distinct increases of CFU number of molds differing from *P. verrucosum* was noted (Fig. 1B). Good growth of these fungi reduced growth of *P. verrucosum*. CFU numbers of *P. verrucosum* were lower in comparison to series with 20% IMC (Fig. 1A and 1B). It was particularly noticeable in the case of the wheat grain incubated at 28°C. Probably, the competition effect of *P. verrucosum* on the grain was weak at this temperature. Although the inoculum dose of *P. verrucosum* was 10 times greater than the CFU number of other fungi, no one colony of this fungus was detected on Martin's medium (Fig. 1B) and diameters of *P. verrucosum* colonies surrounding wheat kernels placed on DYSG medium were even smaller than those of the series with 10% IMC (Fig. 1C). Also, just as in the case of 20% IMC, OTA concentration in the grain incubated at 28°C was much lower than in the grain incubated at 15°C and 21°C (Fig. 1E). As mentioned in the previous paragraph,


A
log of CFU numbers on 1 g of grain
8
7,5
7
6,5
6
5,5
5
4,5
4
3,5
0.25
0.29
0.26
0.25
0.24
0.79
13.9
34.6
32.8
1.44
5.09
11.9
0.50
a
a
a
a
c
fg
gh
h
d
de
ef
b
Inoc. dose
10ºC 10%
15ºC 10%
21ºC 10%
28ºC 10%
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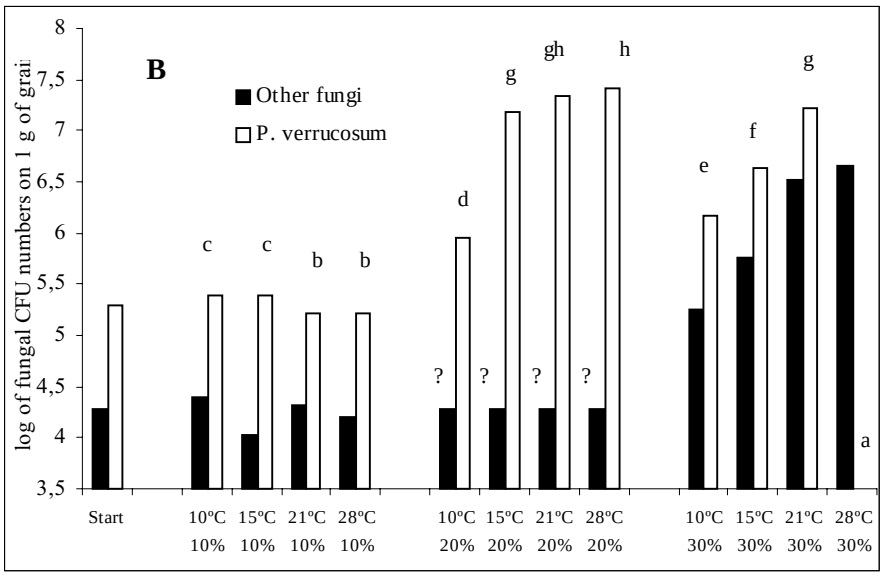
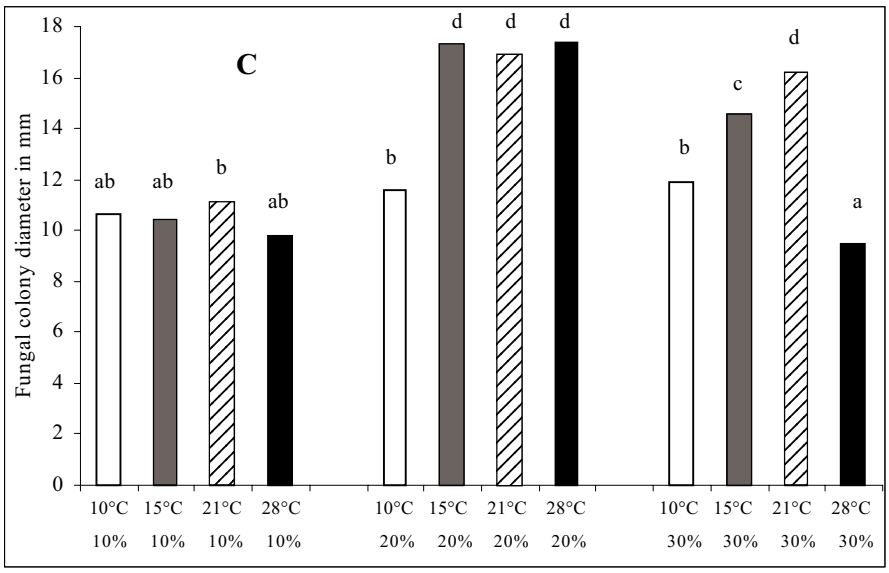
21ºC 20%
28ºC 20%
10ºC 30%
15ºC 30%
21ºC 30%
28ºC 30%


B
Other fungi
P. verrucosum
log of fungal CFU numbers on 1 g of grain
8
7,5
7
6,5
6
5,5
5
4,5
4
3,5
c
c
b
b
?
?
?
?
d
g
gh
h
e
f
g
a
Start
10ºC 10%
15ºC 10%
21ºC 10%
28ºC 10%
10ºC 20%
15ºC 20%
21ºC 20%
28ºC 20%
10ºC 30%
15ºC 30%
21ºC 30%
28ºC 30%


C
Fungal colony diameter in mm
18
16
14
12
10
8
6
4
2
0
ab
ab
b
ab
b
d
d
d
b
c
d
a
10°C 10%
15°C 10%
21°C 10%
28°C 10%
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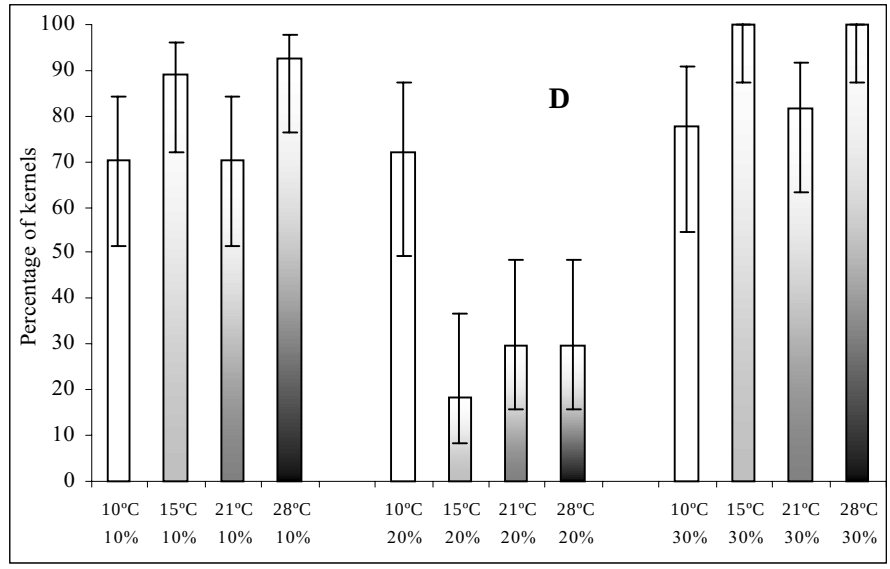
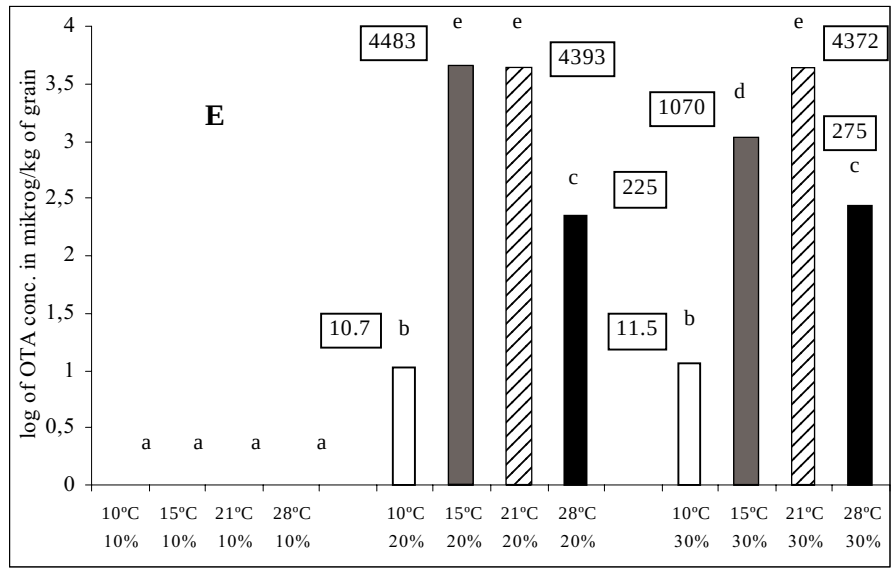
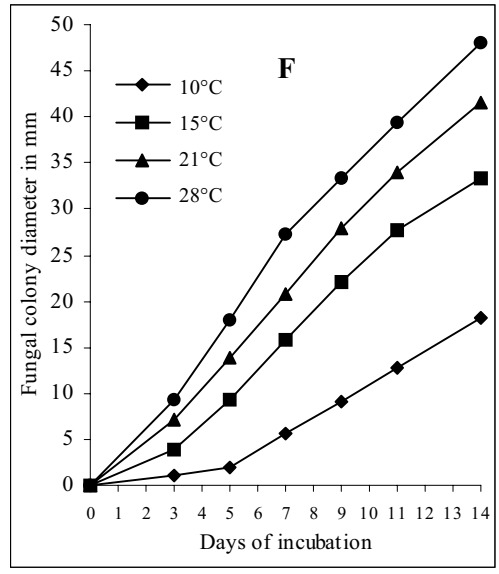
15°C 30%
21°C 30%
28°C 30%







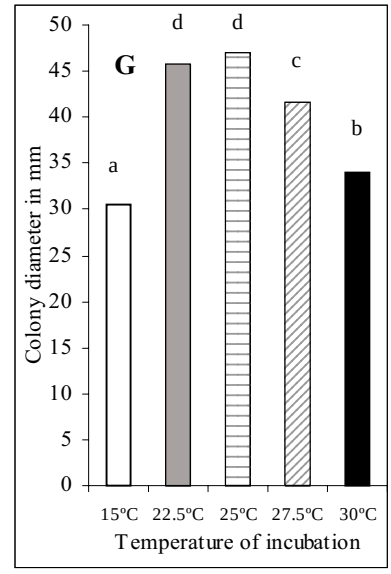


Fig 1. Ochratoxin A production and growth of *P. verrucosum* and other fungi on wheat grain at different temperatures and water contents in Experiment I (Fig. 1**A** – 1**E**) as well as radial growth of *P. verrucosum* on DYSG medium at different temperatures in Experiment A (Fig. 1**F**) and Experiment B (Fig. 1**G**).

(**A**) CFU numbers of *P. verrucosum* on DYSG medium (the numbers in squares are the CFU × 10^6 per 1 g of grain); (**B**) CFU numbers of *P. verrucosum* and other fungi on Martin's medium. (In experimental series with 20% grain water content the number of other fungi could not be determined, because it was masked by high number of *P. verrucosum*); (**C**) Diameters of *P. verrucosum* colonies growing around the wheat kernels placed on DYSG medium; (**D**) Percentage of wheat kernels placed on DYSG medium with colonies of fungi different from *P. verrucosum* growing by the kernels (The vertical lines represent 95% confidence intervals of the proportions); (**E**) OTA production by *P. verrucosum* (the numbers in squares are OTA concentration in ng per 1g of grain). The values of any columns with different letters are significantly different at $P<0.05$ (Fig. 1**A** and 1**B**), $P<0.0001$ (Fig. 1**C** and 1**E**) and $P<0.01$ (Fig. 1**G**). Although concentrations of OTA and CFU numbers are presented on a logarithmical scale, only basic values were used in the statistical analyses.

a temperature of 28°C could be too high for proper metabolism of the fungus, including OTA formation. Probably, OTA synthesis is important for competition of *P. verrucosum* against other microorganisms. Ramakrishna *et al.* (1996) obtained similar results. They reported that at 30°C, but not at 20°C, *P. verrucosum* CFU numbers in barley grain coinoculated with *Aspergillus flavus*, *Fusarium sporotrichioides* or *Hyphopichia burtonii* were much lower, in comparison to a series, in which *P. verrucosum* was growing alone. Especially, marked decreases in *P. verrucosum* CFU were noted in competition with *A. flavus*.

The highest level of IMC (30%) can also be too high for metabolism of *P. verrucosum*. Cairns-Fuller *et al.* (2005) reported that Scandinavian strains of the fungus could grow better and produce OTA on gamma irradiated wheat grain at 0.95 a_w (25% MC) than at 0.995 a_w (~30% MC). Similarly, Arroyo *et al.* (2005) found that *P. verrucosum* strains synthesized more OTA on autoclaved bread at 0.93 and 0.95 a_w than at 0.97 a_w.

It should be added that degradation of OTA by other microorganisms should be taken into consideration. For example, Abrunhosa *et al.* (2002), Varga *et al.* (2000) and Varga *et al.* (2005) reported an ability of some isolates of *Aspergillus*, *Alternaria*, *Cladosporium*, *Mucor*, *Penicillium*, *Rhizopus* and some other fungal genera to degrade OTA. Similar conclusion can be drawn from results of Ramakrishna *et al.* (1996), who reported that OTA produced by *P. verrucosum* during a 2-week incubation disappeared after three weeks when barley grain was coinoculated with *A. flavus*. Bacteria are also able to decompose OTA (Škrinjar *et al.*, 1996; Piotrowska and Żakowska, 2005).

Experiment II was done to determine the best conditions for growth of the isolated strain of *P. verrucosum* and OTA formation by the fungus in the range of the grain moisture suitable for xerophilic fungi. In this experiment, no symptoms of *P. verrucosum* growth and activity on wheat grain containing 14 and 16% water, at all temperatures studied, were noted (Fig. 2). At 10°C, in the cases of the two highest IMC (20 and 22%), the fungus produced small amounts of OTA (Fig. 2C) and revealed small "growth activity", which was expressed by higher (than those at 14% and 16% IMC) diameter of its colonies around the wheat kernels placed on agar DYSG medium (Fig. 2B), but its CFU number did not increase significantly. Czaban and Wróblewska (2006) found that the physiological activity of *P. verrucosum* on the wheat grain could increase slightly the diameter of the fungal colony growing around wheat kernels placed on DYSG medium. Similar (to that at 10°C and 22% IMC) weak growth activity of *P. verrucosum* were observed at temperature of 15°C and 18% IMC (Fig. 2). However, very distinct growth and OTA production by the fungus were observed only in the cases of the two highest temperatures (15 and 21°C) and two highest grain moistures (20 and 22% IMC). In general, the parameters of growth and activity of *P. verrucosum* were higher at higher temperature and higher grain moisture content (Fig. 2).

These results are consistent with data obtained by Harwig and Chen (1974), Abramson *et al.* (1990), Abramson *et al.* (1992) and Cairns-Fuller *et al.* (2005). Harwig and Chen (1974) studied the number of moulds and OTA synthesis by *P. viridicatum* (*P. verrucosum* at present) on ground wheat grain inoculated with the fungus and incubated at 12°C and 25°C and IMC 18% and 22% for four weeks. Similarly to the results of the present study (conditions 10°C and 18% IMC), Harwig and Chen (1974) did not observe any symptoms of growth of *P. viridicatum* and OTA synthesis by the fungus after two weeks of incubation at 12°C and 18% IMC, but the fungus produced OTA at 12°C and 22% IMC as well as at 25°C and 18% IMC. This strain of *P. viridicatum* produced the highest amounts of OTA at 25°C and 22% IMC, but these amounts were about 9 times smaller than those obtained in the present study at 20°C and 22% IMC (Fig. 1E and Fig. 2C). Studies of Abramson *et al.* (1990) on Canadian wheat grain and Abramson *et al.* (1992) on Bavarian cereal grain stored for 60 and 20 weeks, respectively, at 15 and 19% IMC, have shown that at 15% IMC neither OTA production nor symptoms of active fungal development were observed. Contrary to that, at 19% IMC both OTA production and fungal proliferation, especially of penicillia and aspergilli were distinct. Similarly, Cairns-Fuller *et al.* (2005) found that Scandinavian strains of *P. verrucosum* grew and produced OTA on milled wheat agar or wheat grain at water activities equal to or greater than 0.85 a_w (19% MC). The best conditions for fungal growth and OTA formation in their studies were 0.95 a_w (25% MC) and temperature 25°C. Our radial growth Experiment B also showed that a temperature of 25°C was the best for growth of the *P. verrucosum* strain isolated (Fig. 1G). Results of the present study are also consistent with results obtained by Elmholt and Rasmussen (2005). They found that moisture contents of stored cereal grain above 17% are conducive to OTA production. In their opinion, moisture contents above 17% constitute a serious risk of OTA formation in cereal grain.

Table I presents linear correlation coefficients between determined parameters of *P. verrucosum* growth and activity. All of the relationships are statistically very significant. Only low OTA production by the fungus at 28°C and IMC 20% in Experiment I, when the CFU number and the colony diameter of *P. verrucosum* were very substantial, reduced the correlation between OTA concentration and the other parameters.

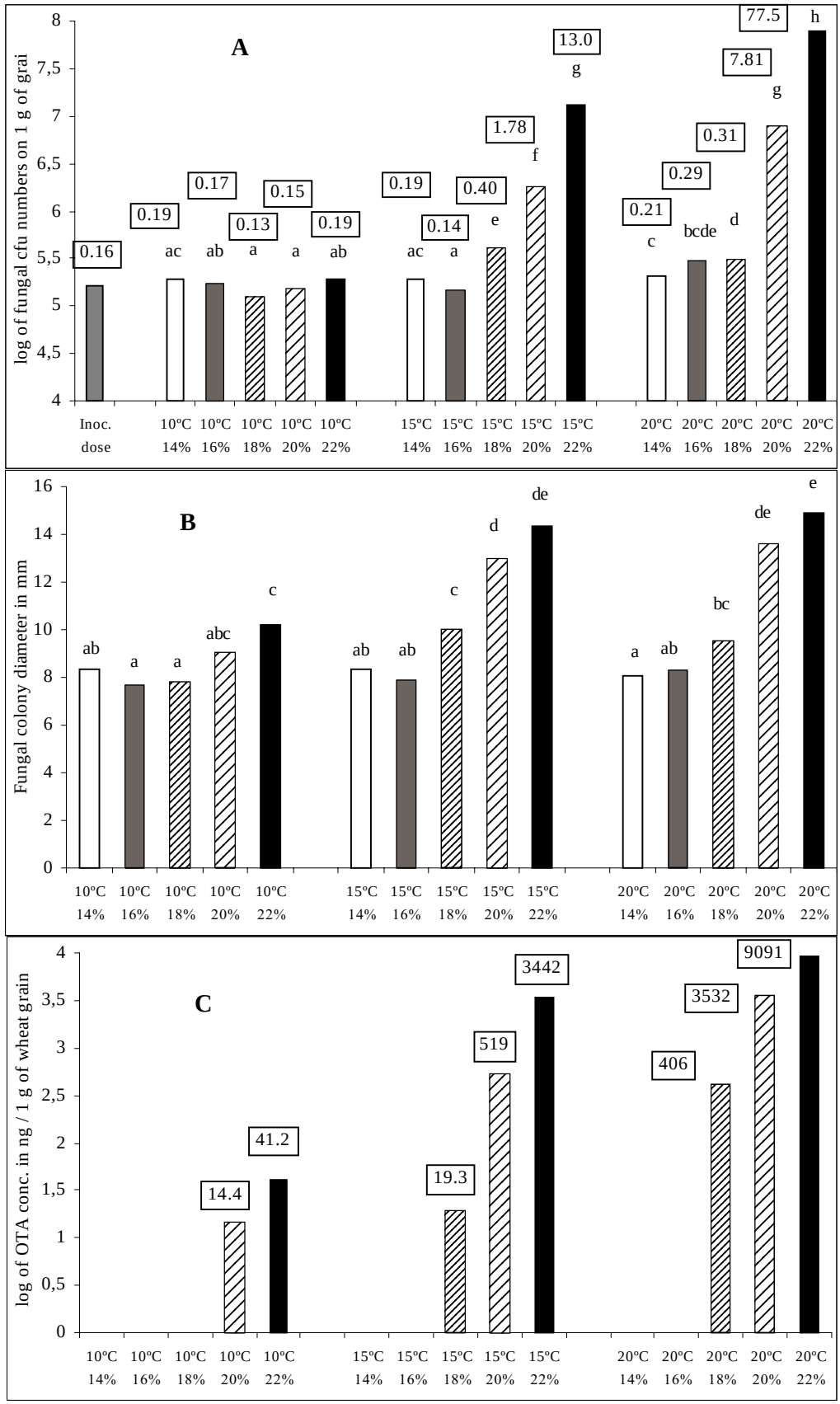


Fig 2. Ochratoxin A production and growth of *P. verrucosum* on wheat grain at different temperatures and water contents in Experiment II. (A) CFU numbers of *P. verrucosum* on DYSG medium (the numbers in squares are the CFU×10^6 per 1g of grain); (B) Diameters of *P. verrucosum* colonies growing around the wheat kernels placed on DYSG medium; (C) OTA production by *P. verrucosum* (the numbers in squares are OTA concentrations in ng/g of grain); The values of any columns with different letters are significantly different at $P<0.05$ (Fig. 2A) and $P<0.0001$ (Fig. 2B and 2C). Although concentration of OTA and CFU numbers are presented on a logarithmical scale only basic values were used in the statistical analyses.

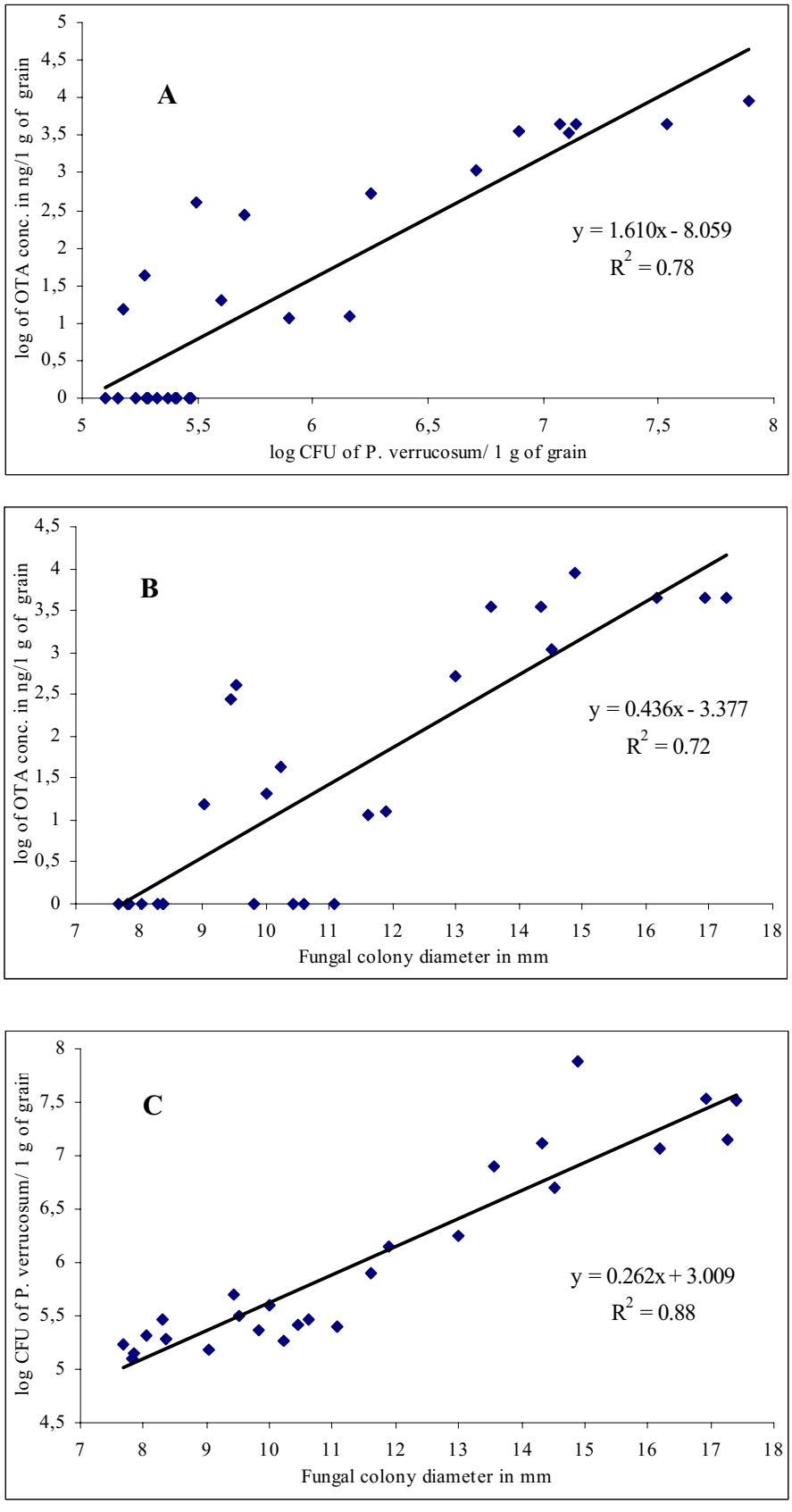


Fig. 3. Relationships between: log OTA concentration in wheat grain and log CFU number of *P. verrucosum* on the grain (A); log OTA concentration in wheat grain and *P. verrucosum* colony diameter growing around the kernels placed on DYSG medium (B) and log CFU number of *P. verrucosum* on the grain and *P. verrucosum* colony diameter growing around the wheat kernels placed on DYSG medium (C).

The data of determined parameters of *P. verrucosum* growth and activity are obtained from both Experiments (I and II).

Table I
Linear correlation coefficients between the determined parameters of *P. verrucosum* growth and activity on wheat grain incubated at various temperature and moisture

	OTA concentration in wheat grain	Log_{10} OTA concentration in wheat grain	*P. verrucosum* CFU numbers on DYSG medium	Log_{10} *P. verrucosum* CFU numbers on DYSG medium	*P. verrucosum* colony diameters on DYSG medium	Percentage of kernels surrounded with colony of fungi different from *P. verrucosum*
Experiment I (n = 12)	1	–	0.565* (0.846***)	0.729** (0.853***)	0.756*** (0.932***)	–0.524* (–0.675**)
		1	–	0.873*** (0.907***)	0.808*** (0.848***)	–
			1	–	0.845***	–0.772***
				1	0.970***	–
					1	–0.735***
Experiment II (n = 15)	1	–	0.947***	0.926***	0.801***	–
		1	–	0.869***	0.947***	–
			1	–	0.641***	–
				1	0.944***	–

***, **, * – The correlation coefficients are significant at P < 0.01, < 0.05 and < 0.1, respectively.
(The values in parentheses are obtained after data deletion of the series incubated at 28°C and 20% IMC; n = 11)

Fig. 3 presents linear regression equations between logarithmical values of OTA concentration in the wheat grain in both (I and II) experiments, logarithmical values of CFU counts of *P. verrucosum* on the wheat grain in both experiments and the fungal colony diameters in both experiments. The data from Table I and Fig. 3 clearly show that OTA formation was distinctly related to *P. verrucosum* abundance, expressed both as the fungal CFU counts and the fungal colony diameter. Similarly, Lindblad *et al.* (2004) reported that the probability of high OTA concentration in wheat grain was significantly related to *P. verrucosum* colony counts at a range of grain moisture content from 19 to 24%.

OTA formation and abundance of *P. verrucosum* were negatively correlated with "competition activity" of other fungi, expressed as percentage of the wheat kernels, placed on DYSG medium, with growing colonies of fungi different from *P. verrucosum* (Table I). Czaban and Wróblewska (2006) also found that the percentage of wheat kernels placed on DYSG medium with growing fungi different from *P. verrucosum* was strictly inversely dependent on the abundance of *P. verrucosum* on the wheat grain.

Similarly to our earlier results (Czaban and Wróblewska, 2006), CFU counts of *P. verrucosum* on the wheat grain were related to the diameter of the fungal colonies around the wheat kernels placed on DYSG medium, but not in a linear manner. Only when CFU values are transformed to logarithmical values a very strong linear relationships is noted (Table I and Fig. 3C). When the CFU numbers are not transformed to logarithmical values, the relationship is described by equation of exponential regression:

$$y = 1021\ e^{0.603x}, \qquad R^2 = 0.88$$

Results of the present study clearly show that both moisture and temperature affect *P. verrucosum* growth on wheat grain and formation of OTA by the fungus. At initial moisture content of the wheat grain up to 16% (~0.76 a_w) neither *P. verrucosum* growth nor OTA formation were observed. In a range of IMC from 18% (~0.83 a_w) to 22% (~0.90 a_w) both fungal growth and OTA synthesis were distinct, and the parameters were higher at higher moistures and temperatures in a temperature range of 10–21°C.

Lindblad *et al.* (2004) concluded, on the basis of their own results and the literature cited, that OTA production occurred within a more restricted range of water activities than growth of *P. verrucosum*. Results of Lindblad *et al.* (2004) showed that at low water activity (0.8 a_w which corresponds to 17% MC) the fungus was able to reach high levels of CFU per gram of grain without forming significant amounts of OTA. At higher moistures (0.85–0.95 a_w which correspond to 19–24% MC) the risk of OTA formation increased with increasing CFU level. Such a shift was not observed in the present study, probably because of the lack of the experimental series with 17% IMC of the wheat grain. So, as concluded by Elmholt (2005), further studies within the critical range from 16 to 19% IMC are needed.

The present experiments were of only two weeks duration. To obtain measurable symptoms of *P. verrucosum* growth and OTA formation after such a short time, 10°C was chosen as the lowest temperature. But

the fungus can grow at lower temperatures when the moisture is sufficiently high. Elmholt (2005) reported that *P. verrucosum* grew well and formed OTA in rye grain after 100 days at a temperature as low as 2°C and 22% MC. So, further studies with longer incubation periods and low temperatures are needed.

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