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A Simple, Direct Plating Method, Alternative to Dilution Plating, for Estimation of the Abundance of *Penicillium verrucosum* on Incubated Cereal Grain

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Abstract

Because dilution plating is more labor intensive than direct plating, we would like to propose the use of a direct plating technique for estimation of *Penicillium verrucosum* abundance in cereal grain in incubation studies, with use of very selective and indicative for the fungus agar DYSG medium. The proposed method is based on the measurement of the diameter of *P. verrucosum* colonies grown around cereal kernels placed on DYSG medium. In three different experiments wheat grain contained a great range of *P. verrucosum* CFU number (from <25 to 77×10^6 per 1 g). When *P. verrucosum* CFU number was at least as high as 10^2 per 1 g of the grain, 100% of the wheat kernels, placed on the surface of DYSG medium, were surrounded by colonies of *P. verrucosum*. The diameter (x, mm) of *P. verrucosum* colonies surrounding wheat kernels on DYSG medium was correlated with the fungal CFU number (y) on the wheat grain. The relationship is described by the exponential regression equation ($y = 0.1258\ e^{0.9309x}$, $R^2 = 0.96$). The relationship became linear ($y = 0.404\ x - 0.901$, $R^2 = 0.96$) after transformation of *P. verrucosum* CFU numbers to logarithms to base 10.

K e y w o r d s: direct *versus* dilution plating method, *Penicillium verrucosum* abundance, wheat grain

Introduction

Penicillium verrucosum Dierckx is the major, if not the only, ochratoxigenic fungus in cereals in temperate climatic regions (Lund and Frisvad, 2003; Park *et al.*, 2005).

Estimation of the abundance of *P. verrucosum* on cereal grain is in most cases necessary in ecological studies on ochratoxin (OTA) synthesis by the fungus. In incubation studies of grain inoculated with *P. verrucosum* (when the fungal propagule number is relatively high) dilution plating has been commonly used (Abramson *et al.*, 1990; Lindblad *et al.*, 2004; Ramakrishna *et al.*, 1996). For evaluation of contamination of natural grain by the fungus (when the fungal propagule number is relatively low) direct plating is considered to be a more effective technique (Elmholt and Rasmussen, 2005; Lund and Frisvad, 2003; Park *et al.*, 2005). Development of the very efficient selective and indicative medium DYSG for specific detection of *P. verrucosum* in cereals (Frisvad *et al.*, 1992) offers excellent possibilities for estimating the abundance of the fungus in mixed populations of fungi, using both the dilution plating and the direct plating techniques (Elmholt *et al.*, 1999; Lund and Frisvad, 2003).

Dilution plating is a more labor consuming method than the direct plating. So, we would like to propose the use of direct plating instead of dilution plating for estimation of *P. verrucosum* abundance in grain in incubation studies, with the use of agar DYSG medium.

Experimental

Materials and Methods

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

The fungus growth medium. The fungus was isolated and grown on DYSG medium (final pH 5.6) containing: glycerol (anhydrous) – 220 g; sucrose – 150 g; yeast extract (Difco) – 20 g; $MgSO_4 \times 7H_2O$ – 0.5 g; $ZnSO_4 \times 7H_2O$ – 0.01 g; $CuSO_4 \times H_2O$ – 0.005 g; dichloran – 0.002 g; chloramphenicol – 0.05 g; agar – 20 g; dist. H_2O – 1000 ml (Lund and Frisvad, 2003).

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Nonsterile grain of spring wheat cultivar Histra, harvested in 2004, was used in the studies. On the basis of studies with methods described by Lund and Frisvad (2003), the grain was not contaminated with *P. verrucosum*. The grain used in our studies contained 12.1% water.

Inoculation of the grain with *P. verrucosum*. The 10 g samples of the grain were placed in beakers. The separate grain samples were inoculated with 1 ml of *P. verrucosum* spore suspensions in solution containing 0.1% peptone, 0.85% NaCl and 1 drop of Tween 80 (Frisvad, 1986) in Experiments I and II, and, in Experiment III, with 0.5 ml of suspension of the fungus spores in water. In Experiment I, 1 ml of *P. verrucosum* spore suspensions contained approximately 3.5×10^7, 3.5×10^6, 3.5×10^5, 3.5×10^4, 3.5×10^3, 3.5×10^2, 3.5×10^1 or 3.5×10^0 spores to obtain from 0.35 to 3.5×10^6 spores of the fungus per 1 g of the grain. In Experiment II, 1 ml of spore suspension contained approximately 1.7×10^8, 1.7×10^5 or 1.7×10^2 spores to obtain about 17×10^6, 17×10^3 and 17×10^0 per 1 g of the grain. In Experiment III, 0.5 ml amounts of spore suspension, contained approximately 1.5×10^6 spores were added to 10 g samples of the wheat grain containing 15% and 17% of water to obtain 150×10^3 spores per 1 g of the grain.

Incubation of the wheat grain before microbial analyses: Experiment I – the grain, wetted by the fungal inoculum, was equilibrated in a refrigerator at 1°C for 24 hours, with periodical shaking during this time. Experiment II – the wetted grain was incubated: (1) for 48 hours at 1°C; (2) for 24 hours at 1°C and then for 24 hours at 22.5°C; (3) for 48 hours at 22.5°C. Experiment III – the wetted grain was equilibrated in a refrigerator at 1°C for 24 hours, and after mixing, it was incubated for 2 weeks at 10, 15 or 20°C and at 20% or 22% initial water content.

Determination of fungal number 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, 1986). The numbers of colony forming units (CFU) of all fungi and *P. verrucosum* in the obtained suspensions were determined by dilution plating on DYSG medium. Inocula (0.1 ml per plate) were spread on the surface of the agar medium. The Petri dishes with DYSG medium were incubated in the dark at 22.5°C for 7 days. After this time *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 in four replicates.

Determination of diameter of *P. verrucosum* colonies around the wheat kernels placed on agar DYSG medium. Thirty six wheat kernels were placed on DYSG medium (9 kernels per 1 Petri dish). The diameters of the fungal colonies were measured in the crosswise direction of the kernels after 4, 5 and 6 days of incubation in the dark at 22.5°C (Experiment I) or after 5 days (Experiments II and III).

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

Statistical evaluations. The data were subjected to one-way analysis of variance and the means were separated with Student's t-test. For statistical evaluation of significant differences between the percentages of wheat grains with colonies of *P. verrucosum* or with fungi different from *P. verrucosum* on DYSG medium, confidence intervals were calculated according to the equation (Oktaba, 1966):

$$\frac{2Y+u_\alpha^2-K}{2(n-u_\alpha^2)} < p < \frac{2Y+u_\alpha^2+K}{2(n-u_\alpha^2)}$$

where: p is a confidence interval; $K=u_\alpha\sqrt{x}$ and $x=u_\alpha^2+4Y\,(1-Y/n)$; Y is the number of kernels with growing colonies of fungi different from *P. verrucosum*; n is the total number of kernels; u_α is the Student's t value obtained from tables for an infinite number of freedom (1.96 for 95% confidence intervals). The confidence limits are presented as percentages ($p\times100$).

For estimation of the relationships between the diameter of *P. verrucosum* colonies and the fungus CFU numbers or $\log_{10}$ of the CFU numbers, linear correlation analysis as well as linear and exponential regression analyses were applied. Together with correlation coefficients (r), probability (P) and the number of kernels (n) are presented. Determination coefficients (R^2) are presented with linear and exponential regression equations.

Results and Discussion

In these studies, a temperature of 22.5°C (the arithmetic mean of 20°C and 25°C) was chosen for incubation of DYSG Petri plates to determine both *P. verrucosum* CFU number and the diameter of the fungal colonies, because in many studies temperatures of 20°C or 25°C were used for incubation of Petri dishes with DYSG medium in both dilution plating and/or direct plating methods for enumeration of *P. verrucosum* CFU in grain or soils and for the number of cereal kernels colonized by the fungus (Elmholt, 2003; Elmholt and Rasmussen, 2005, Elmholt *et al.*, 1999; Lund and Frisvad, 2003; Kristensen *et al.*, 2005).

In Experiment I, where wheat grain was inoculated with a great range of number of *P. verrucosum* spores, the number of *P. verrucosum* CFU varied from less than 25 (under the detection limit) to more than 4×10^6 per gram of the grain (Table I). From 10^2 CFU number upward, 100% of the wheat kernels, placed on the surface of DYSG medium, were surrounded by colonies of *P. verrucosum*. However, *P. verrucosum* grown only beside 3–11% of these wheat kernels, inoculated with approximately 0.35–35 spores per 1g (about 23 kernels) of the grain (Table I). Simultaneously with a progressive decrease of *P. verrucosum* CFU number on the grain, a progressive increase in the percentage of the kernels (placed on DYSG medium) with growing fungi different from *P. verrucosum* was observed (Table I). In the case of *P. verrucosum* CFU number lower than 100 per 1 g of the grain, all wheat kernels, placed on DYSG medium, were surrounded by colonies of other fungi (Table I).

Diameter of *P. verrucosum* colonies surrounding wheat kernels on DYSG medium was correlated with the fungal CFU number on the wheat grain (Table I). The visible initial growth of *P. verrucosum* around wheat kernels placed on the surface of DYSG medium was observed earlier, when CFU number of the fungus was higher (results not shown), but later radial growth rates (only in experimental series with *P. verrucosum* CFU number higher than 100 per 1 g of the grain) were faster in the cases of series with lower CFU number, so the differences between the series gradually decreased with increasing time (Table I).

Experiment II was conducted to evaluate the significance of the physiological state of *P. verrucosum* on the diameter of the fungal colony around the wheat kernels placed on DYSG medium. In the series incubated at 1°C for 48 h *P. verrucosum* existed only in the form of spores; in the series incubated at 1°C for 24 h and at 22.5°C for 24 h the fungus predominantly existed in the form of germinated spores; and in series incubated at 22.5°C for 48 h it existed mostly in the form of short hyphae. Results of this experiment, presented in Table II, show that the physiological state of *P. verrucosum* was less important than CFU number of the fungus for its colony diameter around wheat kernels placed on the surface of DYSG medium. Nevertheless, the results of percentage of kernels with *P. verrucosum* colonies grown beside the kernels on DYSG medium show that the physiological state is important in competition with other microorganisms, because higher percentage of the kernels with growing *P. verrucosum* was observed in the case of the grain inoculated with the short fungal hyphae than with fungal spores (Table II).

Table I
P. verrucosum abundance on wheat grain and growth rate of the fungus around wheat kernels placed on DYSG medium as well as the percentage of kernels with *P. verrucosum* and other fungi in Experiment I

P. verrucosum CFU numbers per 1 g of grain	*P. verrucosum* colony diameter (mm)					Percentage of wheat kernels with *P. verrucosum* colony		Percentage of wheat kernels with colony of fungi different from *P. verrucosum*
	Days of incubation of Petri dishes with wheat kernels					Days of incubation		
	4	5	6	14	6–4 ◊	6	14	
4165500 f □	13.06 e	17.31 e	21.44 f	–	8.39	100 (90.4–100) ‡	–	0 (0–9.6) ‡
368000 e	11.50 d	15.86 d	20.19 e	–	8.69	100 (90.4–100)	–	2.8 (0.5–14.2)
32372 d	9.53 c	13.97 c	18.33 d	–	8.81	100 (90.4–100)	–	11.1 (4.4–25.3)
3150 c	6.85 b	11.31 b	15.86 c	–	9.01	100 (90.4–100)	–	19.4 (9.8–35.0)
250 b	3.19 a	7.33 a	12.28 b	–	9.08	100 (90.4–100)	–	63.9 (47.6–77.5)
50 a	n.m.○	n.m.	5.00 a	7.00 b	–	11.1 (4.4–25.3)	11.1	100 (90.4–100)
<25	n.m.	n.m.	2.50 a	2.67 a	–	5.6 (1.5–18.1)	8.3	100 (90.4–100)
<25	n.m.	n.m.	0	1.00	–	0 (0–9.6)	2.8	100 (90.4–100)

○ – not measurable;
□ – the means in separate columns marked with different letters are statistically different at $P < 0.01$;
◊ – the differences between diameters after 6 days and diameters after 4 days;
‡ – the values in the parentheses are 95% confidence intervals. The percentages with confidence intervals overlapped each other did not differ statistically at $P = 0.05$;

Table II
The effect of introduction of different amounts of *P. verrucosum* propagules to the wheat grain and physiological state of the fungus in the inoculum on its growth rate around the wheat kernels placed on DYSG medium in Experiment II

P. verrucosum spore numbers added to 1 g of wheat grain	*P. verrucosum* colony diameters on DYSG medium (mm)		
	Incubation conditions of *P. verrucosum* spores		
	2 days at 1°C	1 day at 1°C and 1 day at 22.5°C	2 days at 22.5°C
17×10^6	18.50 c**	19.39 d**	19.53 d**
17×10^3	12.28 b	12.86 b	12.86 b
17×10^0	3.50 a (5.6%)*	3.69 a (36.1%)*	3.58 a (52.8%)*

* – the values in parentheses are the percentage of wheat kernels with colonies of *P. verrucosum* (all kernels from the remaining experimental series are surrounded by colonies of *P. verrucosum*)
** – the means marked with different letters are statistically different at $P<0.01$

Table III
P. verrucosum CFU number on wheat grain incubated under different conditions for 2 weeks and diameters of the fungus colonies surrounding the wheat kernels placed on DYSG medium in Experiment III

Conditions of incubation: temperature/initial grain moisture	*P. verrucosum* colony diameters on DYSG medium (mm)	*P. verrucosum* CFU numbers on DYSG medium
20°C/22%	20.54 c *	77.5×10^6 d
15°C/22%	20.13 bc	13.0×10^6 c
20°C/20%	19.47 b	78.1×10^5 c
15°C/20%	18.93 b	17.9×10^5 b
10°C/22%	16.26 a	18.8×10^4 a
10°C/20%	15.16 a	15.1×10^4 a

* – the means in separate columns marked with different letters are statistically different at $P<0.01$

Table IV
Linear correlation coefficients between diameter of *P. verrucosum* colonies surrounding the wheat kernels and CFU number (normal and presented as $\log_{10}$) of the fungus on wheat grain

	P. verrucosum colony diameters on DYSG medium	*P. verrucosum* CFU numbers on DYSG medium	Log_{10} of CFU numbers of *P. verrucosum* on DYSG medium
Exp. I (n = 5) after 4 days	1	0.653	0.996*
Exp. I (n = 5) after 5 days	1	0.635	0.981*
Exp. I (n = 5) after 6 days	1	0.633	0.982*
Experiment II (n = 3)	1	0.814	0.995*
Experiment III (n = 6)	1	0.590	0.955*

* – The correlation coefficients are significant at $P<0.01$

Results of Experiment III, where the nonsterilized wheat grain, inoculated with *P. verrucosum*, was incubated for 2 weeks at different temperatures and different initial moisture contents, show, similarly to the previous experiments, a distinct relationship between *P. verrucosum* CFU number on the grain and the diameter of colonies of the fungus surrounding the wheat kernels on DYSG medium (Table III). However,

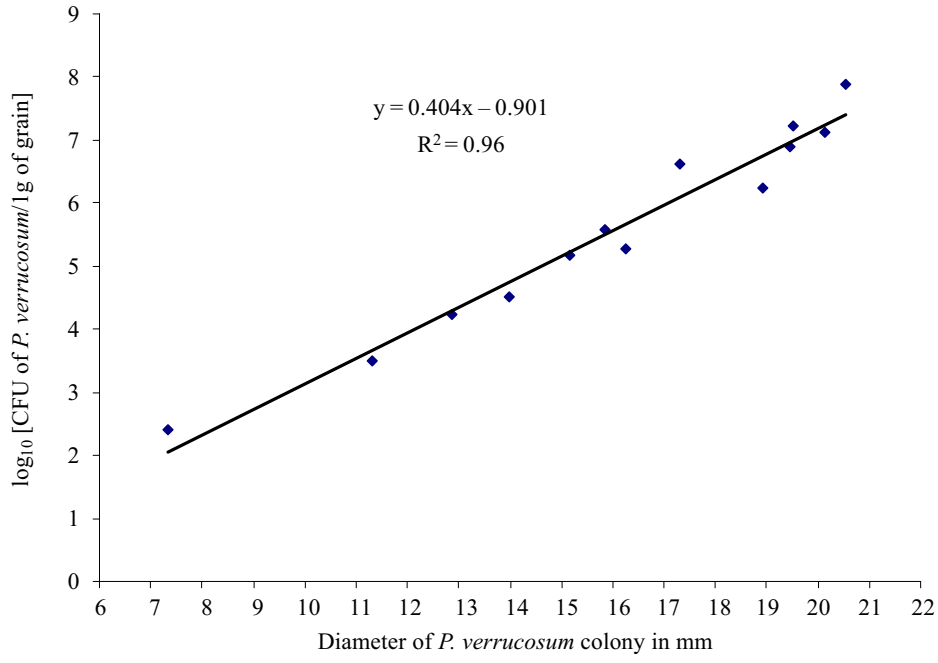

Fig. 1. Relationship between log CFU number of *Pencillium verrucosum* on the wheat grain and colony diameter of the fungus around the wheat kernels, placed on agar DYSG medium

the relationships are not linear in the case of all experiments. They become linear after transformation of *P. verrucosum* CFU numbers to logarithmic values. Then, linear correlation coefficients between diameter of *P. verrucosum* colonies and logarithms of CFU number are very high and statistically significant (Table IV).

Fig. 1 presents a linear regression curve and linear regression equation between diameter of *P. verrucosum* colonies surrounding the wheat kernels on DYSG medium (x) and $\log_{10}$ CFU numbers of the fungus on the wheat grain (y) in all performed experiments. When the CFU numbers are not transformed to logarithmical values, the relationship is described by the exponential regression equation:

$$y = 0.126\ e^{0.931x},\ R^2 = 0.96.$$

Some authors have used other methods than CFU counting for quantification of moulds in cereal grain *e.g.* ergosterol measurements or ELISA readings (Saxena *et al.*, 2001; Yong and Cousin, 2001). These methods are sensitive and rapid, but ergosterol measurement needs expensive equipment and it is not specific at the genus level. The ELISA test is based on the detection of fungal antigens by antibodies that are specific at least at the genus level, but some cross-reactions are possible (Yong and Cousin, 2001). It is interesting that readings of ELISA test using antibodies against antigens of *Aspergillus parasiticus* in the studies of Yong and Cousing (2001) are similarly related to *A. parasiticus* CFU number as the diameter of *P. verrucosum* colony to *P. verrucosum* CFU number in the present study. The present calculations on the basis of results of Yong and Cousin (2001) show that linear correlation coefficients between ELISA readings and *A. parasiticus* CFU numbers ($r = 0.504$ and 0.426 for naturally contaminated maize $< n = 20 >$ and inoculated maize and peanuts $< n = 37 >$, respectively) are much higher and statistically significant, when the fungal CFU numbers are transformed to logarithmical values ($r = 0.929$ and 0.945).

The proposed, very simple method (which is a derivative of direct plating using DYSG medium, which enables to be distinguished *P. verrucosum* from other fungi) can be useful in the determination of the relative abundance of this fungus on cereal grain in incubation experiments.

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