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PRODUCTION OF OCHRATOXIN A AND CITRININ BY *Penicillium verrucosum* AND *P. viridicatum* FROM GRAIN AND HERBAL FODDER COLLECTED IN VARIOUS REGIONS OF RUSSIA

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Abstract

Over the past decades, significant progress has been made in elucidating the prevalence of fungi *Penicillium* subgenus *Penicillium* in the environment and their ability to produce a wide range of metabolites with toxic effects (T. Rundberget et al., 2004; J. O'Callaghanet al., 2013; M. Schmidt-Heydtet al., 2015). In a number of European countries, one of the species, the *P. verrucosum* Dierckx has been shown to predominate on cereals, and a direct relationship has been revealed between the grain infestation and the frequency of detection of nephrotoxins (F. Lund, J.C. Frisvad, 2003; M. Lindblad et al., 2004; S. Elmholt, P.H. Rasmussen, 2005). However, the available data on toxins produced by this fungus in grain products and feeds remain very limited (M.R. Bragulat et al., 2008; V. Koteswara Rao et al., 2011). In Russia, a series of publications has recently been devoted to the toxigenic potential of microscopic fungi affecting feed, including 11 species of *Penicillium* (A.A. Burkin et al., 2019; G.P. Kononenko et al., 2021). The whole set of cultural and morphological features previously accepted for the taxon *P. viridicatum* Westling (K.B. Raper et al., 1949), according to Russian researchers, turned out to be characteristic of isolates occurring with a frequency of 20 % in wheat, barley, oats, rye (E.A. Piryazeva, L.S. Malinovskaya, 2014) and with 1.3 % in dry grass feeds (hay, straw) (E.A. Piryazeva, 2017). Here, for the first time, it is shown that a set of isolates differing in terms of sites and years of collecting is mostly *P. verrucosum*, and its capability of toxigenic production in vitro is assessed. In addition, this work is the first in which *P. verrucosum* Dierckx and P. *viridicatum* Westling were discovered among isolates previously assigned to the taxon (according to K.B. Raper et al., 1949), and the characteristics of their toxin production in vitro were assessed. The aim of the study was to evaluate ochratoxin A (OA) and citrinin (CIT) production on grain substrates in a set of 33 isolates from grain and fodder sampled in various Russian regions in different years and deposited into a local collection as *Penicillium viridicatum* Westling. Prior to toxin production assessment, the modern morphological criteria were applied to check taxonomic assignment of the isolates tested. Species identification was performed in accordance with the guidelines (J.C. Frisvad, R.A. Samson, 2004). Cultures were grown on sucrose agar with yeast extract (YES), sucrose agar with creatine (CREA), Chapek agar with yeast autolysate extract (CYA) for 7 days at 25 °C and 30 °C. The acidity index was determined by the change in the CREA medium colour. Fungal inoculum was grown on Chapek-Dox agar for 7-10 days at 23-25 °C. After culturing fungi on moistened rice grain (7 days, 25 °C, without lighting), the OA and CIT content in biomass extracts was measured by indirect competitive enzyme immunoassay (ELISA) using certified commercial test systems (VNIIVSHE, Russia). According to the growth rate, colony pigmentation, acidity reaction, and the morphological traits when grown on a panel of 3 test media, 8 cultures were found to be *P. viridicatum* and 25 cultures were identified as *P. verrucosum*. None of *P. viridicatum* strains formed OA and/or CIT. All strains of *P. verrucosum* produced CIT and some of them OA. In 17 strains implementing the biosynthesis of both metabolites, the accumulation of CIT in the sample on average was about 15 times higher than OA. OA levels above 10 μg/g were detected in only 12 % of producers. The absolute majority (92 %) of *P. verrucosum* strains accumulated

CIT in amounts of more than 10 μg/g, of which 48 % of 100 μg/g or more, which allows us to classify them as highly active producers. The data obtained suggest the involvement of the *P. verrucosum* species in co-contamination of OA and CIT of domestic grain products and herbal feeds.

Keywords: *Penicillium verrucosum*, *P. viridicatum*, grain products, herbal feeds, ochratoxin A, citrinin, ELISA

In recent decades, significant progress has been made in studying the prevalence of *Penicillium* species of the subgenus *Penicillium* in environment and their ability to produce a wide range of metabolites with toxic effects [1-3]. In a number of European countries *P. verrucosum* Dierckx predominates on cereals and a direct relationship occurs between the damage to grain and the detection of *P. verrucosum* metabolites with nephropathic effects [4-6]. However, the available information on the peculiarities of toxin formation in this fungus isolated from grain and feed remains very limited. Thus, in Spain, 0.02 to 213 μg/g of ochratoxin A (OA) was detected in 11 isolates of *P. verrucosum* from cereals and feed, and the citrinin (CIT) production was confirmed in 10 isolates [7]. In a feed survey in India, 22 out of 30 strains showed the ability to produce OA and CIT, but there was no explanation regarding their quantity, separate or joint detection [8].

For many years, the set of cultural and morphological characteristics introduced to describe the taxon *P. viridicatum* Westling [9] served as the basis for the search for this micromycete in environment [10, 11] and the study of toxin formation [12, 13]. However, several approaches were later proposed to distinguish groups within a species based on phenotypic, biosynthetic, and environmental criteria [14-16].

In Russia, a series of recent publications have been devoted to the toxigenic potential of microscopic fungi that cause feed contamination, including 11 *Penicillium* species [17, 18]. According to Russian researchers, the cultural and morphological traits considered specific to *P. viridicatum* Westling [9] turned out to be characteristic of 20% isolates from wheat, barley, oats, and rye grains [19], and of 1.3% isolates from dry grass feeds (hay, straw) [20].

In this work, we revealed for the first time that this group of isolates is mainly *P. verrucosum* and, less frequently, *P. viridicatum*. We assessed their toxin formation in vitro, and showed that *P. verrucosum* may cause grain and grass contamination with nephrotoxins.

This study aims to assess the toxigenicity of *P. viridicatum* Westling cultures [9] isolated from grain and grass feeds, describe their macro- and micromorphological properties and compare the identified species in ability to produce ochratoxin A and citrinin.

Materials ad methods. We studied 33 isolates tentatively identified by the key [9] as *P. viridicatum* Westling (the collection of the Laboratory of Mycotoxicology and Feed Sanitation, ARRIVSHE — branch of the Federal Scientific Center for Experimental Veterinary RAS). Fungi were isolated from grain after surface disinfection, from sunflower and pea meal after grinding and preparing suspensions by 10-fold dilutions in a 0.1% aqueous Tween 80, and from silage and hay by direct sowing of small crushed fragments. Pure cultures were stored in test tubes on Czapek-Dox agar (CDA) at 5 °C.

Micromorphological traits (tassel structure, sizes of conidiophores, branches, metulae, phialids and conidia) were assessed in isolates grown on CDA [9] using a microscope (Eclipse E200, Nikon, Japan) at 400× magnification. The macromorphological properties of fungi were studied as described [21]. The cultures were grown for 7 days on following media. YES medium contains yeast extract 20 g, sucrose 150 g, magnesium sulfate $\times 7H_2O$ 0.5 g, copper sulfate $\times 5H_2O$ 0.005 g, zinc sulfate $\times 7H_2O$ 0.01 g, agar 20 g, distilled water 1000 ml (incubation

temperature 25 °C). CREA medium contains creatine monohydrate 3 g, sucrose 30 g, potassium phosphate ×7H_2O 1.6 g, magnesium sulfate ×7H_2O 0.5 g, potassium chloride 0.5 g, iron sulfate (II) ×7H_2O 0.01 g, copper sulfate ×5H_2O 0.005 g, zinc sulfate ×7H_2O 0.01 g, bromocresol purple 0.05 g, agar 20 g, distilled water 1000 ml (incubation temperature 25 °C). CYA (Czapek agar with yeast autolysate extract) conteines sodium nitrate 3 g, yeast autolysate extract 5 g, sucrose 30 g, potassium phosphate disubstituted ×3H_2O 1.3 g, magnesium sulfate ×7H_2O 0.5 g, potassium chloride 0.5 g, iron sulfate (II) ×7H_2O 0.01 g, copper sulfate ×5H_2O 0.005 g, zinc sulfate ×7H_2O 0.01 g, agar 15 g, distilled water 1000 ml (incubation temperature 25 °C 30 °C). Acidification of the CREA medium during incubation was indicated by changes in its color [21].

To assess the toxin formation, polished short-grain rice was used as a substrate. Additionally, for one of the strains (No. 74/2) we used oat flakes, wheat, barley, oats, millet and corn as groats. Inoculums were grown on Czapek-Dox agar slants for 7-10 days at 23-25 °C. Using, the inoculum was transferred with a mycological hook into 10 ml glass vials with a bottom diameter of 18 mm, containing1 g of grain substrate, which was moistened with 1 ml of water before autoclaving. Each treatment was performed in triplicate. After seeding, the vials closed with cotton-gauze stoppers and a layer of film (Parafilm M® PM-996, Pechiney Plastic Packing, USA) were kept in the dark at 25 °C for 7 days. Then, 3 ml of a mixture of acetonitrile and water in a ratio of 84:16 v/v was added to each vial. Vials were shaken vigorously at the beginning and end of the 14-hour stationary incubation. After diluting the extracts with a phosphate-buffered saline solution pH 7.4 with Tween 20, the concentrations of OA and CIT were measured by indirect competitive enzyme immunoassay with a certified commercial test systems in accordance with the manufacturer's recommendations (ARRIVSHE, Russia). The limits of measurrement for OA and CIT were 0.08 ng/ml and 0.4 ng/ml, respectively (a tablet photometer Stat Fax-2100, AWARENESS Technology, Inc., USA, state register No. 47063-11).

The results were expressed as arithmetic means (*M*) and standard errors of the means (±SEM).

Results. Grain and grass samples were collected in different years (Table 1):

1. The origin of the isolates earlier identified as *Penicillium viridicatum* Westling

Sample (*n*)	Place, year	*n* (No.)
Wheat grain (*n* = 5)	Tula Province, 2005	1 (56/3)
	Volgograd Province, 2005	1 (64/3)
	Primorsky Krai, 2008	1 (173/6)
	Republic of Sakha (Yakutia), 2009	2 (253/10, 253/11)
	Republic of Sakha (Yakutia), 2009	6 (254/3, 254/6-1, 254/6-2, 254/10, 254/13-1, 254/13-2)
Barley grain (*n* = 4)	Tula Province, 2005	1 (54/3)
	Altai Krai, 2011	1 (263/2)
	Krasnoyarsk Krai, 2011	1 (279/7)
	Tula Province, 2011	1 (287/4)
Oat grain (*n* = 2)	Lipetsk Province, 2005	1 (50/2)
	Tula Province, 2005	1 (61/2)
Rye grain (*n* = 1)	Orenburg Province, 2011	3 (292/3, 292/4, 292/5)
Pea grain (*n* = 2)	Tyumen Province, 2005	3 (45/3, 45/4, 45/5)
	Tyumen Province, 2005	4 (46/1, 46/4, 46/7, 46/ 8)
Sunflower meal (*n* = 2)	Krasnodar Krai, 2006	1 (74/2)
	Primorsky Krai, 2006	1 (101/11)
Silage (*n* = 1)	Moscow Province, 2013	1 (5/1)
Hay (*n* = 3)	Moscow Province, 2013	3 (181/3, 314/2, 462/8)

The initial isolates were characterized by common micromorphological features. Rough conidiophores measuring 200×3.4-4.0 μm formed coremia. There were spherical and hemispherical conidia (3.0-3.5 μm) with smooth or slightly

rough walls, asymmetrically branching brushes of several non-pressed branches (10-20×2.8-3.5 μm) with cylindrical panicles measuring 9-15×2.5-3.3 μm, 2-4 in a bunch, phialides of 7-9×2.5-3.5 μm in size, 3-5 in a bunch. More pronounced roughness of conidiophores was noted in eight of the studied isolates. In these isolates, the diameter of the colonies on YES medium was 25-40 mm (reverse side yellow), on CREA 17-24 mm, on CYA 19-35 mm at 25 °C and 6-18 mm at 30 °C. In cultures on CREA, the medium became acidic and its color changed. The data obtained allowed us to identify these strains as *P. viridicatum* Westling (series *Viridicata*). Two of them were isolated from a pea sample (Nos. 46/7, 46/8), one from barley grain (No. 56/3) and five from wheat grain (Nos. 254/6-1, 254/6-2, 254/10, 254/13-1, 254/13-2). The remaining 25 strains expressed lower growth rate, did not acidify the CREA medium, and in size and color of the colonies corresponded to the species *P. verrucosum* Dierckx (*Verrucosa* series). Their colonies on the YES medium were 20-32 mm in diameter with terracotta color of the reverse side of the colony, on the CREA 12-15 mm, on CYA at 25 °C 15-24 mm (the reverse side was creamy yellow, often with a brown center), at 30 °C no growth occurred. Based on the combination of micro- and macromorphological characters, both species belonged to the section *Viridicata* [21, 22].

2. Ochratoxin A and citrinin production in 25 isolates of *Penicillium verrucosum* Dierckx during growth on rice grain (7 days, 25 °C, *M*±SEM)

Strain, No.	Toxin, μg/g of substrate	
	ochratoxin A	citrinin
5/1	1,8±0,4	82±10
45/3	1,3±0,3	130±33
45/4	1,8±0,5	92±12
50/2	4,5±0,8	37±6
64/3	34,0±6,0	140±28
74/2	4,2±0,8	50±8
101/11	3,0±0,6	70±12
173/6	2,2±0,4	40±5
181/3	2,7±0,5	20±5
253/10	0,02±0	4±1
253/11	0,04±0,01	112±20
263/2	0,9±0,2	65±14
287/4	23,0±5,0	100±20
292/3	15,0±3,0	317±50
292/4	1,3±0,3	43±5
314/2	0,5±0,1	68±10
462/8	1,9±0,4	119±18
min-*M*-max	0,02-6-34	4-88-317
45/5	–	100±20
46/1	–	180±30
46/4	–	5±1
54/3	–	130±25
61/2	–	124±23
254/3	–	67±12
279/7	–	170±35
292/5	–	77±12
min-*M*-max	–	5-107-180

N o t e. Dashes mean that ochratoxin A was not detected.

At present, to determine and clarify the taxonomic position of fungi, their culturing on solid and in liquid media is followed by chromatographic analyze [23, 24]. To assess the mycotoxins production, we used a grain substrate which, according to our data, provides the highest toxin synthesis [17, 18], and enzyme-linked immunosorbent assay (ELISA test) for quantification. Our results are fully consistent with the chemotaxonomic markers reported for both species [25, 26]. In *P. viridicatum* samples OA and CIT were not detected, while *for P. verrucosum* the ability to biosynthesize nephrotoxins was confirmed. All *P. verrucosum* strains produced CIT, and some of them produced OA (Table 2).

In 17 strains producing both metabolites, the accumulation of CIT on

average was approximately 15 times higher than OA. One of the producers, No. 74/2 with the OA/CIT ratio of 4.2±0.8/50±8 μg/g on rice grain (see Table 2), on five other types of grain under the same conditions also predominantly produced CIT vs. OA, 2±0.4/37±5 μg/g on wheat, 0.9±0.2/21±2 μg/g on barley, 1.7±0. 4/38±6 μg/g on oats, 0.2±0/10±2 μg/g on millet and 0.1±0/55±8 μg/g on corn. Similar OA/CIT ratio was previously revealed for the collection strain *P. verrucosum* KKP 480 during long-term culture (40 days, 20 °C) on rice (30/75 μg/g), wheat (15/60 μg/g), barley (10/30 μg/g), triticale (8/10 rg/g) and corn (5/8 μg/g) [27]. Apparently, variation in the biochemical composition of these grain media is not significant for the general stage of the OA and CIT polyketide component synthesis and for the stages at which the completion of the OA molecule with the amino acid part and the introduction of a chlorine atom occurs [27].

In the other 8 strains of *P. verrucosum*, the detected amounts of CIT remained approximately in the same range as in the producers of the two mycotoxins, but OA was not found (see Table 2). While recognizing this species as phenotypically distinct, researchers draw attention to the high genetic diversity of its populations in cereals [28]. Indeed, all strains lacking the ability to produce OA were isolated from cereal grains — wheat, barley, oats, rye, and also from peas. Apparently, both chemotypes of the fungus can be sources of contamination of domestic grain products. According to generalized data for 2004-2009, in feed grain, feed mixtures, cakes, a general pattern was observed in the ratio of the content of these toxins, that is, the CIT accumulation was, as a rule, 1.1-10 times higher than OA [29, 30], but these studies did not consider the *P. verrucosum* infestation of contaminated samples.

When analyzing two samples (wheat and pea), along with *P. verrucosum*, *P. viridicatum* was found, which did not produce OA and CIT. We identified this same species as the only one in one of the barley samples. There is no doubt that it is necessary to study in more detail the prevalence of *P. viridicatum* due to its ability to produce xanthomegnins known for their high hepato- and nephrotoxicity, and viridic acid which is a poorly studied tetrapeptide mycotoxin [25, 31]. Note that to assess the toxigenicity of *Penicillium* isolates contaminating grain products, modern morphological methods of species identification should be supplemented by molecular genotyping and assessing genes associated with toxigenesis [32, 33].

Despite the small sample of *P. verrucosum* strains from grass feeds, some comparisons can be made based on our data. The same type of toxin formation as in crops from grains, with a higher accumulation of CIT than OA (see Table 2), is quite consistent with the general situation with the contamination of meadow grasses and hay [34]. None of the grass feed samples was a source of atypical cultures that do not form OA, and the reason for this, in addition to the rare occurrence of the species, may well be the homogeneity of its populations in these objects. Unfortunately, a targeted study of the toxigenicity of the species *P. verrucosum* associated with meadow forbs has not yet been carried out.

Noteworthy is the tendency of *P. verrucosum* to adapt the biosynthesis of secondary metabolites when changing external conditions [35, 36]. A sharp shift towards the accumulation of OA has been described under shock influences, for example, with excessive salinity of the substrate, oxidative stress in the presence of copper ions [3], or upon the use of glycerol or galactose as carbon sources [2]. Apparently, this is precisely what is associated with the unusual result obtained in studying collection *P. verrucosum* strains (IBT, BioCentrum-DTU, Denmark) on bread analogues medium made from wheat flour added with margarine, baking powder, yeast extract, glycerin and salt. Three strains formed CIT and OA in equal amounts or with a predominance of OA, and two strains only OA [37]. This

metabolic plasticity of the fungus certainly requires accurate investigation in lab tests. It cannot be excluded that factors leading to a shift in the equilibrium in the biosynthesis pathways of these metabolites may also operate in natural populations. Thereof, many vegetative meadow grasses, like grain products, are characterized by joint contamination of OA and CIT, and, as a rule, with a noticeable quantitative predominance of CIT [34]. However, in rare cases, a fold increase in OA content occurred and both metabolites were found in comparable quantities, for example in licorice *Glycyrrhiza glabra* and in flowers of clover *T. hybridum* and *T. medium* [38].

In our study, 92% of *P. verrucosum* strains accumulated more than 10 μg/g CIT, of which 48% had a CIT content of ≥ 100 μg/g which allows us to consider these strains as highly active producers [39]. On the contrary, >10 μg/g OA was detected in only 12% of the producing strains, and the rest of them were completely devoid of this ability. All this gives reason to doubt the validity of the very widespread indications in the literature that *P. verrucosum* is a producer of OA [14, 40, 41] with a clear underestimation of its ability to accumulate CIT [25]. As is known, both metabolites are synthetized via a common pathway. For OA. significant progress has already been made in identifying the genes responsible for its biosynthesis [42], and the study of factors that influence the transcription levels of the *otapks*PV gene and other components of the cluster is actively continuing. However, for CITs, only analogies have so far been traced with other fungi, in particular with representatives of the genus *Monascus* [2, 43, 44]. Identification of mechanisms regulating toxin formation in fungi from natural populations is becoming one of the priority. The development of alternative typing methods based on proteome analysis and genetic markers [45] allows us to hope for their use in the near future in investigating toxigenic micromycetes important for hygienic and sanitary control.

Thus, in the studied *Penicillium* isolates from grain and grass samples collected in different years in the Russian Federation, the species *P. verrucosum* producing citrinin (from 5 to 180 μg/g grain substrate) and ochratoxin A (from 0.02 to 34 *r*g/g) dominates, while *P. viridicatum* not capable of biosynthesizing these metabolites is rare. Our findings indicate the possible involvement of *P. verrucosum* in the combined contamination of domestic grain products and grass feeds with citrinin and ochratoxin A. Considering the pronounced responses of the *P. verrucosum* species to environment, mycotoxicological studies of its populations should be continued.

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