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Penicillium viridicatum, *Penicillium verrucosum*, and Production of Ochratoxin A

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The taxonomy of the important mycotoxigenic species *Penicillium viridicatum* and *P. verrucosum* was reviewed to clarify disagreements relating to the three *P. viridicatum* groups erected by Ciegler and coworkers (A. Ciegler, D. I. Fennell, G. A. Sansing, R. W. Detroy, and G. A. Bennett, Appl. Microbiol. 26:271–278, 1973) and the mycotoxins produced by them. Cultures derived from the types of these two species and authentic cultures from each group and from many other sources were examined culturally, microscopically, and for mycotoxin production. It was concluded that *P. viridicatum* group II has affinities with *P. verrucosum* and not with *P. viridicatum*, as indicated by J. I. Pitt in the 1979 monograph (*The Genus Penicillium and Its Teleomorphic States Eupenicillium and Talaromyces*). As a result of this study it can now be unequivocally stated that the mycotoxins ochratoxin A and citrinin are not produced by *P. viridicatum*. Of species in subgenus *Penicillium*, only *P. verrucosum* is known to produce ochratoxin A.

Ciegler et al. (3) drew attention to the fact that isolates of *Penicillium viridicatum* as defined at that time (16) could be divided into three groups on the basis of various properties, including growth rates, mycotoxin production, and source. Isolates allocated to *P. viridicatum* group I grew rapidly, were bright yellow green when young and forest green in age, were mostly isolated from moldy grain, and did not produce the mycotoxins ochratoxin A or citrinin. Isolates placed in group II grew slowly, were colored yellow green both at maturity and in age, came from various plant sources, and mostly produced both ochratoxin A and citrinin. Isolates placed in group III grew moderately quickly, became brown in age, and came from meats or meat packing plants in Europe. Such isolates produced ochratoxin A, at least when freshly isolated, but did not produce citrinin. Important features of these groups are given in Table 1.

In his revision of *Penicillium* spp., Pitt (12) examined representative isolates from among those cited by Ciegler et al. (3). He concluded that *P. viridicatum* group I isolates were correctly assigned to *P. viridicatum*. One isolate (NRRL 963), accepted as authentic for *P. viridicatum* by Raper and Thom (16) and placed in group I by Ciegler et al. (3), was designated as the neotype of *P. viridicatum* (12). This nomenclaturally fixed the name *P. viridicatum* to the concept described by Raper and Thom (16) and unequivocally attached group I to this species. Pitt (12) considered that group III isolates did not belong in *P. viridicatum* but in *P. verrucosum*, a species which predates *P. viridicatum* but which was placed in synonymy with the latter by Raper and Thom (16). The type isolate of *P. verrucosum* was observed to be similar in morphological characters and growth rates to the group III cultures examined. Group III isolates were confidently placed in that species.

Based on the cultures examined at that time, Pitt (12) assigned isolates in group II of Ciegler et al. (3) to *P. viridicatum*, concluding that although the isolates examined were not identical with those of group I, "overall characters lay entirely within the current circumscription of *P. viridicatum*."

Ciegler et al. (4) disagreed sharply with some of the conclusions of Pitt (12). In particular, they pointed out that more recent studies (21, 22) had shown that *P. viridicatum* group I cultures produced newly discovered naphthoquinone mycotoxins, xanthomegnin and viomellein, toxins quite different from those produced by groups II and III isolates. They concluded that "after even a casual comparison of isolates of groups I and II, . . . to infer that they belong to the same species strains credulity." Nevertheless, Ciegler et al. (4) retained both groups I and II isolates in *P. viridicatum*, as Ciegler et al. (3) had done previously. The same taxonomic decision, that all three groups belonged in a single species, *P. verrucosum* var. *verrucosum*, had been accepted by Samson et al. (18).

Such criticism prompted the present author to reexamine his earlier conclusions, using both the strains studied previously and also fresh cultures of the same isolates from the NRRL collection at USDA Northern Regional Research Center, Peoria, Ill. It was immediately apparent that the previous conclusion concerning the disposition of group II cultures was in error, caused by questionable interpretation of results or by incorrect cultures.

This paper clarifies the status of the *P. viridicatum* groups of Ciegler et al. (3), by using morphology, cultural techniques (12), and mycotoxin production.

MATERIALS AND METHODS

Cultures. A large number and wide variety of isolates were examined in this study, particularly FRR 963 (NRRL 963) from the neotype of *P. viridicatum* and FRR 965 (NRRL 965) from the type of *P. verrucosum*. FRR denotes the culture collection of the CSIRO Division of Food Research, North Ryde, New South Wales. Isolates representative of each Ciegler group were studied, including those mentioned by Pitt (12): from group I, FRR 1636 and FRR 1637 (and their immediate parents, NRRL 5569 and NRRL 5570); from group II, FRR 1638 and FRR 1639 (and parents NRRL 5570 and NRRL 5571); and from group III, FRR 1641 and FRR 1642 (plus parents NRRL 5573 and 5574). A selection of the isolates listed as representative of *P. viridicatum* and *P. verrucosum* (12) was also examined, together with 60 iso-

TABLE 1. Important features of *P. viridicatum* groups[a]

Group no.	Colony diam (mm)[b]	Conidial color		Source	Reverse color	Mycotoxin produced
		When young	In age			
I	60–65	Bright yellow green	Forest green	Cereals	Orange	Xanthomegnin, viomellein
II	20–25	Duller yellow green	Yellow green	Cereals, other foods	Red brown or uncolored	Ochratoxin A, citrinin
III	30–35	Fairly bright yellow green	Brown	Processed meats	Uncolored	Ochratoxin A (mostly)

[a] From Ciegler et al. (3), except mycotoxins in group I (from references 8 and 21).
[b] When grown on Czapek agar for 12 to 14 days at 25°C.

lates of *P. viridicatum* and 68 isolates of *P. verrucosum* that were identified personally from the large culture collection at the Bundesanstalt für Fleischforschung, Kulmbach, Federal Republic of Germany. Culture morphology was examined both macro- and microscopically, and colony diameters were measured as described previously (12).

Mycotoxin production. Except for the Kulmbach cultures, for which the types of mycotoxins produced had already been established at that laboratory, cultures were examined after growth for 7 to 14 days on Czapek yeast extract agar (CYA) or malt extract agar (MEA) (12), or both, by the thin-layer chromatographic methods of Filtenborg et al. (6). Plugs of mycelia were taken from the colonies with a 3-mm-diameter cork borer, moistened with chloroform-methanol (2:1) and pressed onto thin-layer chromatography plates (no. 5748; E. Merck, Darmstadt, Federal Republic of Germany). The solvent systems used were benzene-methanol-acetic acid (90:5:5) and toluene-ethyl acetate-formic acid (5:4:1) (6).

In a further check on the stability of mycotoxin production under various cultural conditions, representative isolates of *P. viridicatum* and *P. verrucosum* were grown on a wide variety of culture media, with different carbon sources, water activities, and major solutes.

RESULTS

Taxonomy. The species *P. viridicatum* and *P. verrucosum*, which include groups I and III of Ciegler et al. (3), respectively, possess several characteristics considered to distinguish them in culture (Table 1). The simplest and most effective distinguishing characteristic, however, is the difference in colony diameters of the two species on CYA and MEA after incubation for 7 days at 25°C (Table 2). The most important point to note in Table 2 is that the ranges for both species quoted earlier (12) are too narrow. Many more isolates have been examined since that publication, from a much wider range of sources. The working ranges for colony diameters are now as follows: for *P. viridicatum*, 28 to 35 and 27 to 34 mm and for *P. verrucosum*, 18 to 24 and 13 to 18 mm on CYA and MEA, respectively (Table 2).

Group II isolates. Data on colony diameters for some isolates designated as belonging to *P. viridicatum* groups are shown in Table 3. In hindsight, it is apparent that apart from relatively fast growth by FRR 1638 (perhaps a factor in the earlier erroneous placement) the growth rates of the group II isolates are consistent with allocation to *P. verrucosum*. This is especially obvious on MEA, in which colony diameters are totally inconsistent with placement in *P. viridicatum*. Colonies of group II were reported previously (3), after 12 days of incubation, to be smaller than those of group III; my data do not bear this out (Table 3). In my experience, the differences between groups II and III isolates in colony diameters or in other morphological or gross physiological characters are insignificant. All these features indicate placement of the two groups within a single species, *P. verrucosum*.

Mycotoxin production. As is the case with other fungi, mycotoxin production (Table 4) is not always consistently expressed in these species. However, once group II isolates have been reassigned to *P. verrucosum*, unpublished mycotoxin data obtained at the Federal Centre for Meat

TABLE 2. Colony diameters of *P. viridicatum* and *P. verrucosum*[a]

Source	Colony diam (mm) of:			
	P. viridicatum on:		*P. verrucosum* on:	
	CYA	MEA	CYA	MEA
Pitt (12)	28–32	25–30	15–25	12–15
Frisvad and Filtenborg (8)	24–34		8–22	
Culture ex type[b]	29–34	33–34	23–25	13–16
Recent data, Australia[c]	24–37	23–36	15–26	12–25
Recent data, Federal Republic of Germany[d]	23–38	23–36	13–27	8–24
All sources, 90% of readings[e]	27–36	26–35	17–24	10–20
All sources, 80% of readings[e]	28–35	27–34	18–24	13–18

[a] After growth for 7 days at 25°C.
[b] Measurements on *P. viridicatum* neotype isolate FRR 963, *P. verrucosum* type isolate FRR 965, and other strains derived from the types.
[c] Observations on 43 isolates of *P. viridicatum* and 23 isolates of *P. verrucosum*.
[d] Observations on 60 isolates of *P. viridicatum* and 68 isolates of *P. verrucosum*.
[e] Observations on 103 isolates of *P. viridicatum* and 91 isolates of *P. verrucosum*.

TABLE 3. Colony diameters of representative isolates assigned to *P. viridicatum* groups[a]

Group and isolate no.	Colony diam (mm) on:	
	CYA	MEA
I		
FRR 1636	35	33–34
FRR 1637	28–29	29–30
NRRL 5569	30–34	33–34
NRRL 5570	29–30	34–35
II		
FRR 1638	26	13–14
FRR 1639	22–23	13–15
NRRL 5571	20–22	13
NRRL 5572	19–20	17
III		
FRR 1641	23	17–18
FRR 1642	16–17	15
NRRL 5573	21–22	17–18
NRRL 5574	17–18	13–15

[a] Ranges of measurements from three colonies after 7 days of incubation at 25°C.

TABLE 4. Mycotoxins produced by *P. viridicatum* and *P. verrucosum*

Species and source	No. of isolates examined	No. producing indicated toxin				No. not producing toxins
		Xanthomegnin	Brevianamide A	Ochratoxin A	Citrinin	
P. viridicatum groups[a]						
I	21	NT[b]	NT	0	0	21
II	17	NT	NT	15	13	0
III	13	NT	NT	9	0	4
As now classified						
Neotype	1	1	1	0	0	0
Australia	17	13	11	0	0	4
Federal Republic of Germany	60	41	41	0	0	13
P. verrucosum						
Type	1	0	0	1	0	0
Australia	15	0	0	7	2	7
Federal Republic of Germany	68	0	0	39	4	27

[a] Data from Ciegler et al. (3).
[b] NT, Not tested.

Research, Kulmbach, and in my laboratory and data published by Frisvad and Filtenborg (8) provide entirely consistent information. It can be confidently stated that isolates of *P. viridicatum* (including group I isolates of Ciegler et al. [3] and Frisvad and coworkers [6, 7]) normally produce xanthomegnin, viomellein, brevianamide A, or all three, but never ochratoxin A or citrinin. Brevianamide A is not a mycotoxin but a very useful marker metabolite for this species. On the other hand, *P. verrucosum* isolates, including groups II and III of Ciegler and Frisvad, usually produce ochratoxin A and sometimes citrinin but never produce xanthomegnin, viomellein, or brevianamide A.

The influence of media on the quantities of mycotoxins produced was marked, as was to be expected. However, quite radical changes in medium formulation had no influence over the kinds of identifiable toxins and pigments produced.

DISCUSSION

The results presented here provide unequivocal evidence that *P. viridicatum* and *P. verrucosum* are distinct species. For keys to, and descriptions of, these species, see Pitt (12, 13) or Pitt and Hocking (15). The most useful characteristics for distinguishing between these two species are the differences in growth rates on CYA and MEA. Appropriate colony diameters under standardized conditions are given in Table 2. The 80%-of-readings figures are considered to be the most useful range for specifying colony diameters of fresh isolates. The use of the 80% range can be justified on two grounds. First, for many species, only about 80% of isolates will be readily classifiable by the nonexpert (12); and second, some isolates studied in this work have been in culture for 20 years or more, so variation from normal is to be expected, especially a decrease in growth rate. Other morphological, physiological, and biochemical properties support the growth rate distinction but are less obvious or less readily used in determinative taxonomy. Such features include colony texture, reverse colors, fine details of penicillus structure, and isoenzyme patterns (R. H. Cruickshank and J. I. Pitt, Microbiol. Sci., in press; R. H. Cruickshank and J. I. Pitt, submitted for publication). The classification of *P. viridicatum* group I of Ciegler et al. (3) as *P. viridicatum* and of group III as *P. verrucosum* (12) has been confirmed.

The classification of Ciegler's group II has now been clarified also. The disposition of *P. viridicatum* group II in *P. viridicatum* (12) is demonstrably erroneous. The conclusion that group II lies within the circumscription of *P. verrucosum*, arrived at here on morphological and broadly physiological grounds, is in agreement with the mycotoxin data of Ciegler et al. (3, 4), Frisvad and Filtenborg (8), and my own studies in the Federal Republic of Germany and Australia. As noted above, it is now clear that these two species produce quite separate ranges of mycotoxins. In consequence, thin-layer chromatography techniques (6), which readily detect ochratoxin A or brevianamide, can be used as confirmatory tests for distinguishing *P. verrucosum* from *P. viridicatum*.

It should be noted that the First International Workshop on *Penicillium* and *Aspergillus* Taxonomy (17) rejected the nomenclaturally incorrect use of the term group as a subdivision of a species. The Workshop recommended that if secondary metabolism is to be used as a taxonomic criterion, it should be at a clearly defined subspecific level. The term chemotype was recommended for such subspecies (14), with the use of either Roman numerals (e.g., *P. viridicatum* chemotype I) or names derived from some property of the particular chemotype (e.g., *P. chrysogenum* chemotype citrinin).

In the present context, a case exists for distinguishing the isolates of *P. verrucosum* which produce citrinin from those that do not. It is therefore proposed here that *Penicillium verrucosum* chemotype *citrinin* is the new and nomenclaturally acceptable name for *Penicillium viridicatum* group II of Ciegler et al. (3) and Frisvad and Filtenborg (8).

The results presented above have important practical implications. The production of ochratoxin A by *P. viridicatum* was first reported (2, 20, 23) before *P. verrucosum* was established as a separate species (12). In consequence of this and of the confusion over the disposition of group II isolates, the notion that *P. viridicatum* produces ochratoxin A has become widely accepted both in literature reports and reviews (5, 11, 19). It can now be stated unequivocally that *P. viridicatum* does not produce ochratoxins or citrinin, but rather it produces xanthomegnin, viomellein, or brevianamide A, as originally reported by Stack et al. (21). Among the common grain, food, and feed-related species in subgenus *Penicillium*, only *P. verrucosum* is able to produce ochratoxin A.

The role of *P. viridicatum* in the disorders of pigs described by Carlton et al. (1) is clear, because one of their

isolates, Purdue 66-68-2, was used in the original study on xanthomegnin production (21). The production of this toxin by this isolate confirms its identity as a *P. viridicatum*. However, it is unlikely that *P. viridicatum* is involved in the production of ochratoxin A in Danish barley (9, 10), which is believed to be the cause of nephropathy in pigs and perhaps humans. The reported presence of both ochratoxin A and citrinin in Danish barley, with citrinin being encountered less frequently than ochratoxin A (9), indicates that the causative fungus is probably *P. verrucosum*.

Isolates of certain other *Penicillium* species, classified in subgenera other than subgenus *Penicillium*, have been reported to produce ochratoxin A on occasion. This topic is currently under investigation.

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