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Production of Naphthoquinone Mycotoxins and Taxonomy of *Penicillium viridicatum*

A. CIEGLER,* L. S. LEE, AND J. J. DUNN

Southern Regional Research Center, U. S. Department of Agriculture, New Orleans, Louisiana 70179

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Groups I and II of *Penicillium viridicatum* were further differentiated on the basis of synthesis of two mycotoxins, xanthomegnin and viomellein. Strains previously classified as group II produced these pigments, whereas those in group I did not. These napthoquinone pigments were quantitated by thin-layer chromatography and high-pressure liquid chromatography. A new mobile phase of toluene and acetic acid effected a baseline separation of the two components. It is proposed that such biochemical distinctions be incorporated into an artificial taxonomic scheme of use to nontaxonomists.

Isolates of *Penicillium viridicatum* Westling, as identified by the taxonomic key of Raper et al. (4), have been reported to produce a number of potentially important nephrotoxins, including ochratoxin, citrinin, and xanthomegnin. Unfortunately, taxonomic identification of cultures belonging to this genus and in particular to members of the subsection *Fasciculata*, into which *P. viridicatum* falls, has proven difficult; some member species tend to taxonomically merge imperceptibly into one another. New classification schemes have been proposed by Samson et al. (6) and Pitt (3), but neither has as yet attained full acceptance. Both of the new schemes as well as the older scheme of Raper et al. (4) use the classical criteria of macroscopic and microscopic characters as well as growth patterns to separate the various species members of the genus *Penicillium*; biochemial criteria are not used.

Ciegler et al. (1) divided isolates of *P. viridicatum* into three subgroups based on overt growth characteristics and mycotoxin synthesis; morphological differences were essentially nonexistent (Fig. 1). Members of subgroups II and III produced ochratoxin and/or citrinin, compounds not produced by group I cultures. Since the initial investigation, Stack et al. (7, 8) reported that, of nine isolates of *P. viridicatum* examined, six corresponding to our group II produced ochratoxin and/or citrinin, and three belonging to our group I produced the newly identified mycotoxins, xanthomegnin and viomellein.

We have reexamined our original isolates with respect to production of the latter two mycotoxins to provide additional means for taxonomic clarification of this confusing and important fungal species.

MATERIALS AND METHODS

Cultures and fermentation. Cultures of *P. viridicatum* were obtained from the Science and Education Administration, U.S. Department of Agriculture collection of the Northern Laboratory, and from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, and were maintained on yeast malt agar slants (Difco Laboratories, Detroit, Mich.). Spores were suspended in 0.001% Triton, and each culture was inoculated both onto 200 g of rice in Fernback flasks and onto 50 g of rice in 500-ml Erlenmeyer flasks. Before sterilization, the moisture level of the rice was raised to 35%. Nineteen strains of *P. viridicatum* were used; inoculated samples were incubated 21 days at 27°C.

Metabolite extraction. Rice cultures were soaked for approximately 2 h in about 500 ml of methylene chloride, the solvent was removed, and the residual solids were ground in another 500 ml of methylene chloride in a Waring blender and filtered. The solvent extracts were combined, and the solvent was removed under vacuum. The solvent-free residue was dissolved in acetone, adjusted to 30% with water, and transferred to a separatory funnel, and an equal volume of hexane was added. Lipids were partitioned into the hexane layer, and the pigmented metabolites remained in the aqueous acetone. The aqueous layer was drawn off into a second separatory funnel, and the pigments were partitioned into methylene chloride which was then removed under vacuum. All extractions were done as rapidly as possible to insure minimum degradation of the pigments from contact with solvents (7). Extracts were stored in a freezer as dry films and then dissolved in appropriate solvents and quantitated immediately. Final extracts were made to volume in benzene-acetonitrile (98:2) for thin-layer chromatographic (TLC) quantitation.

The solvent used for the mobile phase of the high-

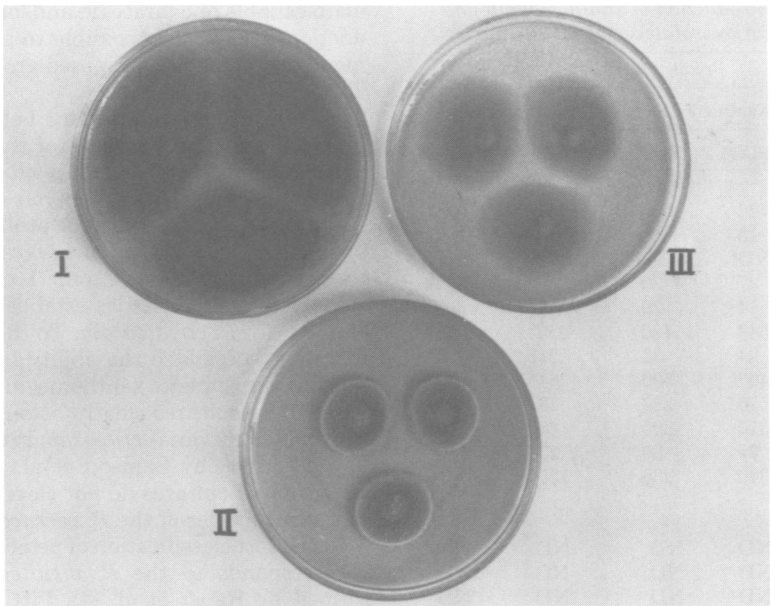

FIG. 1. *Representative strains of P. viridicatum groups I, II, and III grown on Blakeslee malt agar for 12 days at 24°C.*

pressure liquid chromatographic (HPLC) quantitation was also used to dissolve extracts quantitated by this technique. Reference standards of xanthomegnin and viomellein were purified by preparative TLC using silica gel GHR plates (Brinkmann Instruments, Inc., Westbury, N.Y.) developed in benzene-methanol-acetic acid (90:5:5). Structures of these compounds are given in reference 8. Pigments were eluted from the gel with acidic chloroform, and the chloroform eluant was washed immediately with water in a separatory funnel. Upon solvent evaporation, the pigments separated from the acid-free chloroform layer as an amorphous mass. Purity of standards was judged by HPLC and TLC comparison with authentic standards (7, 8). Chromatographically pure pigments were dissolved in benzene-acetonitrile (98:2) to obtain a concentration of 100 μg/ml. One-milliliter volumes were pipetted into separate 4-dram (ca. 16-ml) vials fitted with Teflon liners. The solvent was evaporated under a stream of nitrogen, and the standards were stored as thin films in a freezer. Standards were reconstituted with the appropriate solvent immediately before quantitation.

TLC. For TLC, samples were spotted on precoated silica gel 60 plates (E. Merck AG, Darmstadt, Germany), as were samples of the TLC standard, and developed in benzene-methanol-acetic acid. Developed plates were air dried and then exposed to ammonia fumes (both pigments change from yellow-brown to violet) (7) (Table 1). The serial dilution technique described by Robbers et al. (5) was used to compare the intensities of samples with those of standards.

HPLC. The HPLC detection was a modification of the system described by Stack et al. (7). We used the same column (μporasil; Waters Associates) and ultraviolet detection at 405 nm, but substituted toluene with 1% acetic acid-methanol (493:7) for their chloroform-acetic acid mobile phase. A mixture of standards containing 0.2 μg of xanthomegnin per ml and 0.5 μg of viomellein per ml was injected after each sample extract. The amount of each pigment in the extracts was determined by comparing peak heights of sample components with those of the authentic standards.

TABLE 1. *Pigments of P. viridicatum*[a]

Pigment	R_f	Visualization after exposure to ammonia	
		Daylight	UV[b]
Xanthoviridicatum	0.74	Tan	Black
Viomellein	0.58	Brown-violet	Brown
Xanthomegnin	0.48	Violet	Black
Brevianamide	0.37	Yellow	Yellow
Rubrosulphin	0.55	Black	Black
Viopurpurin	0.0	Violet	Brown

[a] Adsorbisil 1 plates developed in benzene-acetic acid-methanol (95:5:5).

[b] UV, Ultraviolet.

RESULTS

Results of quantitation by HPLC and TLC (Table 2) show that all strains classified as group I (1) synthesize both xanthomegnin and viomellein, which are the major pigments produced by these molds. Amounts of pigments varied from about 2,700 μg of xanthomegnin per g of rice (strain 963) to just a trace (strain 15105). There

TABLE 2. *Production of xanthomegnin and viomellein by isolates of P. viridicatum*

Strain	Production of pigment (μg/g)			
	Xanthomegnin		Viomellein	
	HPLC[a]	TLC[b]	HPLC	TLC
Group I				
963	2722	1000	909	1000
15059	15	40	28	13
15105	ND[c]	trace	ND	trace
15402	27	20	trace	6
18343	14	20	13	46
18563	543	420	253	200
18565	24	33	21	20
18567	406	500	264	300
18616	70	233	47	100
18876	310	200	143	140
19118	35	20	27	14
19121	340	280	125	71
Group II				
3710	ND	ND	ND	ND
17732	ND	ND	ND	ND
18686	ND	ND	ND	ND
20216	ND	ND	ND	ND
20217	ND	ND	ND	ND
20218	ND	ND	ND	ND
20219	ND	ND	ND	ND

[a] Extracts quantitated from 200 g of cultured rice.
[b] Extracts quantitated from 50 g of cultured rice.
[c] ND, None detected.

was a reasonable correlation between results by TLC and HPLC. TLC was a slightly more sensitive quantitative technique than HPLC; TLC detected trace amounts of both pigments in strain 15105, whereas HPLC detected none. The HPLC developed in this study is an improvement over that proposed by Stack et al. (7). Our mobile phase effected baseline separation of xanthomegnin and viomellein, whereas we were unable to separate xanthomegnin from viomellein with the mobile phase (chloroform-acetic acid) proposed by Stack et al. (7). Viomellein, with a retention time of 1.5 min, eluted before xanthomegnin (retention time, 1.9 min).

DISCUSSION

A reexamination of 19 of the original 38 strains of *P. viridicatum* still available to us reconfirmed our original data separating groups I and II on the basis of mycotoxin synthesis and overt morphological and colony growth characteristics. Group I cultures synthesized no ochratoxin or citrinin, but all 12 strains produced both xanthomegnin and viomellein, ranging from a trace to over 1 g/kg of rice substrate. None of the cultures belonging to group II synthesized xanthomegnin and viomellein, although they remain capable of ochratoxin and/or critrinin production. Hence, it is possible to easily separate members of the two proposed groups by simple TLC.

We did not analyze strains belonging to our proposed group III because of the extreme genetic instability of this group which makes any taxonomic identification tenuous at best.

We are again faced with the problem of correct taxonomic classification of *P. viridicatum*. Cultures falling into our group I clearly fit the composite characteristics established by Raper et al. (4) for *P. viridicatum*. To these characteristics may be added the ability to produce the mycotoxin pigments xanthomegnin and viomellein. These cultures clearly belong to *P. verrucosum* Durckx var. *verrucosum* in the taxonomic key described by Samson et al. (6). However, our group II cultures do not clearly fit into any of the subspecies of the *P. verrucosum* scheme.

In the Pitt classification of penicillia (3), group I corresponds to the *P. viridicatum* circumscribed by Raper et al. (4). Pitt acknowedged that group II cultures, when grown on his Czapek yeast autolysate agar, grew more slowly than group I cultures, had more obvious fascicles, and produced a copious exudate with concomitant reduced conidiogenesis. Although Pitt used growth data and colony characters as major factors in his classification scheme, he nevertheless concluded that, with respect to group II isolates, overall characters lay entirely within the current circumscription of *P. viridicatum*. We do not dispute this conclusion with respect to his own classification, but after even a casual comparison of isolates of groups I and II (Fig. 1), to infer that they belong to the same species strains credulity. Existence of considerable biochemical and cultural differences further emphasizes the disparities between the two groups. We currently feel that our use of subgroupings for *P. viridicatum* within the "group" system devised by Raper et al. for *P. citrinum* is an unsatisfactory ploy that merely serves to defer an ultimate resolution of this classification problem.

Among the fusaria where similar classification problems exist, operational difficulties have been partially resolved by the existence of multiple taxonomic schemes that each inadvertantly serves a specific need. For example, the simple classification of Snyder and Hansen (9), in which only nine species of *Fusarium* are recognized, is used by many plant pathologists, whereas more sophisticated schemes are preferred by classical taxonomists (2, 10). Using this line of reasoning, we suggest that an arbitrary, perhaps a broader computer-assisted approach encompassing sev-

eral taxonomic schemes is required by mycotoxicologists and other nontaxonomic specialists for classification of the genus *Penicillium*. Such a scheme could utilize microscopic, macroscopic, and biochemical criteria which are either assigned equal weight (Adansonian principle) or reasonable gradations of weight. Current wide availability of modern instrumentation and simple chromatographic techniques permit ready measurement of biochemical characters. We acknowledge that the result would be artificial or arbitrary, but it should be reproducible and hence useful. It should permit all those in a given field to speak and understand the same language. We recommend that the responsibility of developing such a scheme be placed upon fungal taxonomists, perhaps under the aegis of some international scientific body.

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