APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1990, p. 3885–3887
0099-2240/90/123885-03$02.00/0

Vol. 56, No. 12

Variation in Polygalacturonase Production among *Aspergillus flavus* Isolates

PETER J. COTTY,* THOMAS E. CLEVELAND, ROBERT L. BROWN, AND JAY E. MELLON

Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, Louisiana 70179-0687

Received 27 July 1990/Accepted 22 September 1990

Pectinase production by *Aspergillus flavus* was determined by measuring clear zones formed around colonies stained with ruthenium red. Several isolates produced red zones instead of clear zones. Red zones were reproduced with pectinesterase and correlated with absence of specific polygalacturonases. Of 87 isolates tested, 15 produced red zones.

Aspergillus flavus Link ex Fries, a widely distributed filamentous fungus, infects and contaminates crops with aflatoxins (7, 15). Aflatoxins are potent carcinogenic and mutagenic agents whose presence in commodities is carefully regulated (7). Many plant pathogenic fungi produce enzymes which degrade pectin (2), and *A. flavus* produces several pectinases in culture and in infected cotton bolls (4). Pectinases may be important determinants of pathogenicity (5). It was found recently that several *A. flavus* strains with low virulence and low pectinase production lack the specific pectinase complex P2c (T. E. Cleveland and P. J. Cotty, Phytopathology 79:1208, 1989). In this report we describe a simple rapid method for detection of isolates of *A. flavus* lacking pectinase P2c. The incidence of isolates lacking P2c among 87 isolates of *A. flavus* is documented.

Eighty-seven isolates of *A. flavus* from cottonseed and soil collected in southwestern Arizona in 1986, 1987, and 1988 were tested for pectinase production. Representative isolates of both *A. flavus* strains from Arizona, strains L and S, described previously, were included (6). Methods of fungal isolation and culture were described previously (6). Pectinase production was assayed on a pectin medium (pH 5.5) containing the following, per liter: 5 g of pectin (grade 1 from citrus fruits; Sigma Chemical Co., St. Louis, Mo.), 3.0 g of $(NH_4)_2SO_4$, 10 g of KH_2PO_4, 2 g of $MgSO_4 \cdot H_2O$, 0.7 mg of $Na_2B_4O_7 \cdot 10H_2O$, 0.5 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10.0 mg of $Fe_2(SO_4)_3 \cdot 6H_2O$, 0.3 mg of $CuSO_4 \cdot 5H_2O$, 0.11 mg of $MnSO_4 \cdot H_2O$, 17.6 mg of $ZnSO_4 \cdot 7H_2O$, and 10 g of agarose. Culture plates containing 25 ml of pectin medium were inoculated in the center with 10 μl of a suspension containing about 50 spores per μl and incubated at 30°C. After 3 days, each plate was stained with 10 ml of a 0.05% ruthenium red aqueous solution for 1 h as in the pectinase cup plate assay (8) modified by Cleveland and McCormick (4). After 1 h, the plates were rinsed with deionized water and the clear zone around the fungal colony was measured.

Isolates varied widely in their reaction to this test (Fig. 1). Clear zones did not form around colonies of 15 of the 87 isolates assayed, indicating that if pectinases were produced they were at a level below the sensitivity of our assay (Fig. 1). However, red zones formed around each colony without a clear zone (Fig. 2). Three isolates exhibiting the red zone and three exhibiting pronounced clear zones were grown in stationary culture at 30°C for 5 days both in the pectin medium lacking agarose and on autoclaved cottonseed. Pectinases were eluted from the cottonseed into 0.1 M acetate buffer (pH 4.6). Cottonseed extracts and culture filtrates were dialyzed, and the protein component was concentrated as described previously (4). These test solutions (20 μl) were placed in 3-mm-diameter wells cut into the pectin medium, incubated at 30°C for 1 h, and stained as above. The solutions produced either clear or red zones around the well similar to the corresponding strain's specific reaction in the culture assay. Solutions of a given strain from culture and cottonseed behaved similarly. Pectinases in the concentrated test solutions were resolved with isoelectric focusing as described previously (11). Solutions producing clear zones in the cup plate assay contained all pectinase activities identified previously (4). However, solutions producing a red zone consistently lacked detectable levels of the major endopolygalacturonate hydrolase (EC 3.2.1.15) complex designated by Cleveland and McCormick (4) pectinase P2c (Fig. 3). The other previously described pectinase activities were present.

Pectinase P2c was purified by isoelectric focusing, eluted from the gel into acetate buffer, and concentrated. When the concentrated P2c was added to filtrates which alone produced a red zone in the cup plate assay, a clear zone was produced. Lack of pectinase P2c in filtrates producing the red zone and elimination of the red zone by addition of pectinase P2c to those filtrates suggested that the red zone may result from some activity which was masked by the activity of P2c. Pectinesterase (EC 3.2.1.11) may be that masked activity. Pectinesterase may expose binding sites on pectin for ruthenium red through hydrolysis of the methyl ester groups of the uronic acid residues. Exposure of more binding sites should result in greater binding of the stain and a darker red pigmentation in areas exposed to pectinesterase.

Pectinesterase (Sigma) from orange peel was tested in the cup plate assay, and the red zone formed around treated wells upon staining with ruthenium red. Pectinesterase was detected in both red zone and clear zone filtrates by titrimetric assay (9). These results suggest that the red zone reaction might be caused by pectinesterase activity and indicates an absence of the P2c pectinase complex. The results further suggest that the culture assay can be used to assess the incidence of P2c in populations of *A. flavus*. We are purifying the pectinesterase of *A. flavus* to test the specific involvement of this enzyme in the red zone reaction.

The incidence and distribution of P2c within *A. flavus*

* Corresponding author.

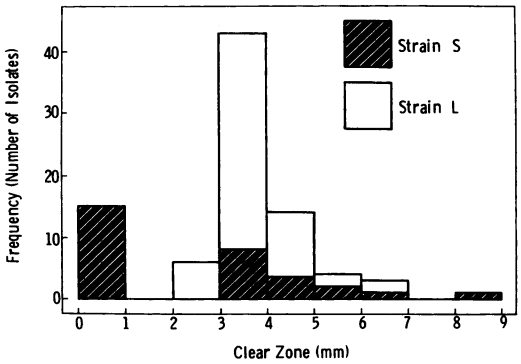

FIG. 1. Pectinase production by isolates of two strains of *A. flavus*. The width of the clear zone around fungal colonies in the pectinase culture assay indicates the relative level of pectinase produced.

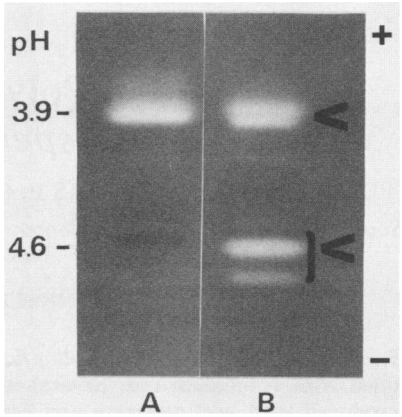

FIG. 3. Isoelectric focusing of pectinases produced by strains exhibiting red zone (A) and clear zone (B) reactions in the pectinase culture assay. Arrows indicate two pectinase complexes, P1 and P2c, described by Cleveland and McCormick (5). The bottom complex (pectinase P2c) was not detected in enzyme preparations from isolates which exhibited the red zone reaction in the culture assay.

populations of the desert valleys of Arizona were determined by using culture assay. Two physiologically distinct strains of *A. flavus*, strains S and L, occur in these valleys (6). Strain S is composed of isolates similar to the atypical isolates of Saito et al. (14). Strain S produces both abundant small sclerotia (<400 μm in diameter) and large quantities of aflatoxins. Strain L is composed of isolates which produce fewer but larger sclerotia (>400 μm in diameter) and less aflatoxins. Both strains fit the taxonomic description of *A. flavus* (13).

Thirty isolates of strain S and 57 isolates of strain L were tested in the culture assay. None of the L-strain isolates and 15 (50%) of the S-strain isolates exhibited the red zone reaction. Isolates lacking P2c were thus restricted to strain S. Strain S isolates which did not produce a red zone produced levels of pectinase similar to levels produced by strain L isolates (Fig. 1) and produced P2c detectable on isoelectric focusing gels. All strain L isolates produced a clear zone typical of isolates that produce P2c.

A. flavus infects diverse crops (7). Certain strains, however, exploit other ecological niches. Some *A. flavus* strains are insect pathogens (1, 12) and others are associated with mammals (16). The high frequency of S-strain isolates lacking P2c may indicate adaptation to an ecological niche where P2c is not required. *A. flavus* is highly variable and dichotomies among isolates exist for several important characters, including production of secondary metabolites (i.e., cyclopiazonic acid and aflatoxins) and sclerotia (3, 10). This population diversity may have developed over long periods of coevolution with specific resources. However, the diversity may not result from specific adaptation; the population may maintain a constant state of flux to exploit resources which temporarily become available.

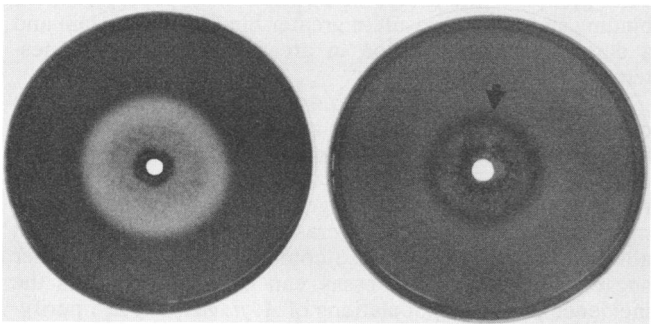

FIG. 2. Red zone (arrow) and clear zone reactions in the pectinase culture assay. The red zone correlates with absence of the major pectinase complex P2c.

We thank L. G. Fortuné and E. Bowers for technical assistance.

LITERATURE CITED

1. **Amonkar, S. V., and K. K. Nair.** 1965. Pathogenicity of *Aspergillus flavus* Link to *Musca domestica* nebulo Fabricius. J. Invertebr. Pathol. **7**:513–514.
2. **Bateman, D. G., and H. G. Basham.** 1976. Degradation of plant cell walls and membranes by microbial enzymes, p. 316–355. *In* R. Heitefuss and P. H. Williams (ed.), Encyclopedia of plant physiology, vol. 4. Springer-Verlag, New York.
3. **Blaney, B. J., M. A. Kelly, A. L. Tyler, and M. D. Connole.** 1989. Aflatoxin and cyclopiazonic acid production by Queensland isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. Aust. J. Agric. Res. **40**:395–400.
4. **Cleveland, T. E., and S. P. McCormick.** 1987. Identification of pectinases produced in cotton bolls infected with *Aspergillus flavus*. Phytopathology **77**:1498–1503.
5. **Cooper, R. M.** 1983. The mechanisms and significance of enzymatic breakdown of host cell walls by parasites, p. 101–135. *In* J. A. Callow (ed.), Biochemical plant pathology. John Wiley & Sons, Inc., New York.
6. **Cotty, P. J.** 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology **79**:808–814.
7. **Diener, U. L., R. J. Cole, T. H. Sanders, G. A. Payne, L. S. Lee, and M. A. Klich.** 1987. Epidemiology of aflatoxin formation by *Aspergillus flavus*. Annu. Rev. Phytopathol. **25**:249–270.
8. **Dingle, J., W. W. Reid, and G. L. Solomons.** 1953. The enzymatic degradation of pectin and other polysaccharides. II. Application of the cup-plate assay to the estimation of enzymes. J. Sci. Food Agric. **4**:149–155.
9. **Kertesz, Z. I.** 1951. The pectic substances. Interscience Publishers, New York.
10. **Maggon, K. K., L. Viswanathan, T. A. Venkitasubramanian, and K. G. Mukerji.** 1969. Aflatoxin production by some Indian strains of *Aspergillus flavus* Link ex Fries. J. Gen. Microbiol. **59**:119–124.
11. **Mellon, J. E., and L. S. Lee.** 1985. Elicitation of cotton isoperoxidases by *Aspergillus flavus* and other fungi pathogenic to cotton. Physiol. Plant Pathol. **27**:281–288.

12. **Ohtomo, T., S. Murakoshi, J. Sugiyama, and H. Kurata.** 1975. Detection of aflatoxin B_1 in silkworm larvae attacked by an *Aspergillus flavus* isolate from a sericultural farm. Appl. Microbiol. **30:**1034–1035.
13. **Raper, K. B., and D. I. Fennell.** 1965. The genus *Aspergillus*. The Williams & Wilkins Co., Baltimore.
14. **Saito, M., O. Tsuruta, P. Siriacha, S. Kawasugi, M. Manabe, and D. Buangsuwon.** 1986. Distribution and aflatoxin productivity of the atypical strains of *Aspergillus flavus* isolated from soils in Thailand. Proc. Jpn. Assoc. Mycotoxicol. **24:**41–46.
15. **Stephenson, L. W., and T. E. Russell.** 1974. The association of *Aspergillus flavus* with Hemipterous and other insects infesting cotton bracts and foliage. Phytopathology **64:**1502–1506.
16. **Wicklow, D. T., and C. Rebar.** 1988. Mold inoculum from cheek pouches of a granivorous desert rodent, *Dipodomys spectabilis*. Mycologia **80:**750–753.