Characterization of Three Species of the Genus *Colletotrichum* with Aminopeptidase Profiles[1]

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Abstract

Aminopeptidase activity of *Colletotrichum coccodes* isolated from potato stems, tomato roots, and tomato fruits, *C. graminicola* isolated from sweet and dent corn, and *C. gloeosporioides* isolated from tomato fruit, was determined with a fluorometric aminopeptidase assay. Tubes containing 1.9 ml of each of 22 amino B-naphthylamide substrates (2.5 x 10^{-5}M in 0.05M Tris buffer pH 8.0) were inoculated with 0.1 ml inoculum. Inoculum was prepared by homogenizing fungal tissue harvested from 7 day PDA plate cultures in 0.05M Tris buffer pH 8.0 with a final concentration of 0.1 g fungal tissue per 10 ml buffer. Enzymatic activity, measured by the release of fluorescent B-naphthylamine was recorded with a fluoromicrophotometer (Corning 7-60 primary filter and a Wratten 2A secondary filter) after incubation for 6 hr at 37 C. Species differentiation of *Colletotrichum* on the basis of aminopeptidase activity was demonstrated. Differences in activity between isolates of the same species were not sufficiently large to permit the separation of isolates into groups which corresponded to isolate sources. The phenomenon of fluorescence quenching was observed and provided on additional parameter for isolate identification.

Introduction

The genus *Colletotrichum* contains a number of economically important fungal plant pathogens commonly observed on vegetable crops in Indiana. Three species of current interest include *Colletotrichum coccodes, C. graminicola* and *C. gloeosporioides.*

Colletotrichum coccodes (Berk. and Br.) Taub. frequently is involved in a fruit rot of tomatoes (2, 5) and was recently shown to be involved in a widespread disease of potato, black dot root rot, in Indiana (8). *C. coccodes* is also commonly associated with the brown root rot complex of greenhouse tomatoes (7). *C. graminicola* (Ces.) Wils., the causal agent of sweet and dent corn anthracnose, is a potential threat to corn production in Indiana. *C. graminicola* was involved in a severe epiphytotic of anthracnose on sweet corn in Benton County, Indiana, in mid-August 1972 (9) and reduced yields of several dent corn hybrids in 1975. *C. gloeosporioides* Denz., although of apparently minor importance in Indiana, is an additional incitant of tomato fruit anthracnose (5).

The fluorescent aminopeptidase assay technique was proposed in 1967 by Westley, *et al.* (10) for the identification of species of *Bacillus* and other bacteria. The assay has been shown to provide a rapid, reproducible means of identifying plant pathogenic fungi (4) and plant pathogenic bacteria (6). In studies on plant pathogenic microorganisms using the aminopeptidase assay technique, Huber *et al.* (3) separated isolates of *Pseudomonas pheseolicola* into two races and grouped isolates of *Pseudomonas syringae* into one of three profiles depending on the host of origin. The workers also noted an ability to differentiate between

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Xanthomonas phaseoli and *X. phaseoli* var. *fuscans*. Precise control of inoculum concentration, incubation time, prior growth media, and incubation temperature were shown to increase the accuracy of this technique (6).

The present study was undertaken to determine the potential value of the aminopeptidase assay in the separation of three plant pathogenic species of the genus *Colletotrichum* and to further determine whether distinct differences in aminopeptidase activity existed between isolates of a given species that could be directly related to the host of origin.

Materials and Methods

Stock cultures of *Colletotrichum* originated from the following sources: *Colletotrichum coccodes*—three isolates from Indiana grown potatoes (three separate locations) showing symptoms of black dot root rot, one isolate from infected certified potato seed pieces grown in a neighboring state, one isolate from an infested soil sample, one isolate from the root of an infected tomato plant grown in a greenhouse in England (provided by Dr. J. D. Farley, Ohio State University), two isolates from Ohio grown tomato fruit showing symptoms of tomato anthracnose (provided by Dr. J. D. Farley, Ohio State University), and one isolate from a Maryland grown tomato fruit (provided by Dr. T. Barksdale, USDA, Beltsville, Maryland); *Colletotrichum gloeosporioides*—one isolate from an Indiana grown tomato fruit showing symptoms of anthracnose; *Colletotrichum graminicola*—three isolates from anthracnose infected corn (provided by Dr. R. L. Nicholson, Purdue University). Stock cultures were maintained on potato dextrose agar (PDA) prior to and during the course of this study. The fungal isolates were prepared for assay by culture on a sterile 0.45μ Millipore filter (Millipore Corp., Bedford, Mass.) overlying the agar medium. Cultures were incubated at 24 C for 7 days with a 16 hr photoperiod light source of 300 foot candles. Fungal mycelium was harvested from filters after 7 days' growth, weighed, and homogenized in 0.05M Tris-HCl buffer pH 8.0 with a final concentration of 0.1 g fungal homogenate per 10 ml buffer. The inoculum homogenate (0.1 ml) was added to 1.9 ml of each of 22 amino-B-naphthylamides (2.5×10^{-5}M in 0.05M Tris-HCl buffer pH 8.0) (Table 1). A series of B-naphthylamines and Tris buffer

TABLE 1. *Beta-Naphthylamide substrates used for analysis of* Colletotrichum *isolates.*

L-Alanyl (ALA)	L-Lysyl (LYS)
L-Arginyl (ARG)	L-Methionyl (MET)
Benzyl-Arginyl (BANA)	4-Methoxy-L-Leucyl (4M-LEU)
L-alpha-Aspartyl (ASP)	L-Phenylalanyl (PHE)
L-gamma-Glutamyl (GLU)	L-Prolyl (PRO)
Glutamyl-L-Phenylalanyl (GLU-PHE)	L-Pyrolidonyl (PYR)
Glycyl (GLY)	L-Seryl (SER)
L-Histidyl (HIS)	L-Threonyl (THR)
L-Hydroxy-Prolyl (H-PRO)	L-Tryptophyl (TRY)
L-Leucyl (LEU)	L-Tyrosyl (TYR)
L-Isoleucyl (ILEU)	L-Valyl (VAL)

were also inoculated to determine the maximum fluorescence obtainable with complete hydrolysis and background fluorescence, respectively. After incubation for 6 hr at 37 C, aminopeptidase activity was determined fluorometrically by measuring the hydrolyzed B-naphthylamines in an Aminco fluoromicrophotometer equipped with a Corning 7-60 narrow band pass primary filter and a Wratten 47-B narrow band pass secondary filter. Fluorometric readings were corrected for background fluorescence and calculated as a percentage of the 100% relative fluorescence for each substrate. All assays were repeated a minimum of 6 times. Polar graph and bar graph profiles of average percent substrate hydrolysis were constructed for each fungal isolate.

Results

The greatest differences in aminopeptidase activity were generally observed between species of *Colletotrichum*. Small overall differences in activity were generally observed between isolates of the same species. Polar and bar graph profiles of selected isolates are shown in Figs. 1 and 2.

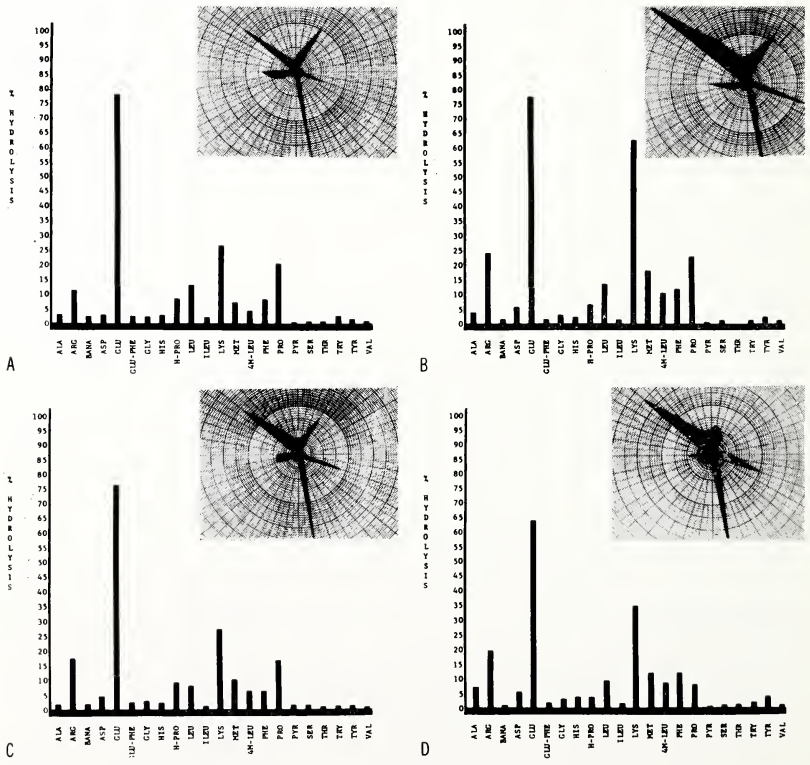

FIGURE 1. *Bar and polar graph profiles of aminopeptidase activity of four isolates of* Colletotrichum coccodes *A) 74-1S, Indiana potato stem; B) 75-5S, Certified potato seed piece; C) CA-8, Tomato root; D) T-7, Tomato fruit.*

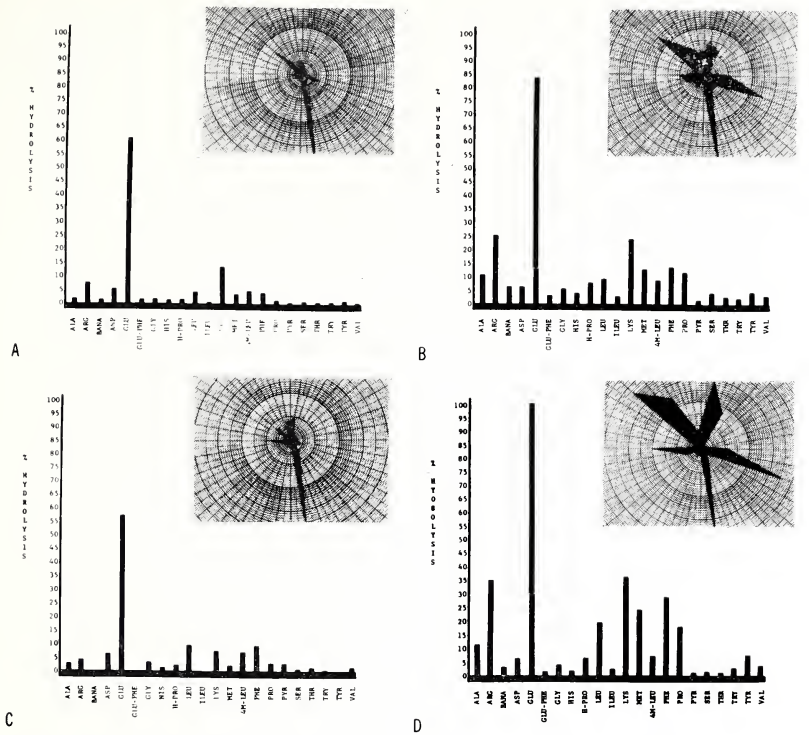

FIGURE 2. *Bar and polar graph profiles of aminopeptidase activity of selected isolates of* Colletotrichum *A) CG-103, C.* graminicola *from Indiana dent corn; B) CG-104,* C. graminicola *from Indiana sweet corn; C) CG-105,* C. graminicola *from North Carolina sweet corn; D) CGL-1,* C. gloeosporioides *from Indiana tomato fruit.*

C. gloeosporioides appeared to be the most enzymatically active species tested. The percent B-naphthylamine cleaved from amino acids by this species was greater than all isolates tested for the following substrates: alanine, arginine, gamma-glutamine, leucine, methionine, phenylalanine and tyrosine. *C. graminicola*, on the other hand, was generally characterized by low enzyme activity, except for a single Indiana sweet corn isolate which generally showed a higher activity than the other *C. graminicola* isolates for most of the substrates. *C. coccodes* isolates as a group were generally more active than the activity of *C. graminicola*, but less than the activity of *C. gloeosporioides*. A single isolate of *C. coccodes* from certified potato seed stock differed markedly from other isolates of *C. coccodes* by a two-fold increase in the hydrolysis of L-lysyl-beta-naphthylamide.

The phenomenon of fluorescent quenching with incubation was observed with all isolates studied (Table 2) and appeared to provide another parameter for comparison of isolates. The highest quenching was associated with an isolate of *C. coccodes*.

TABLE 2. *Origin and percent fluorescence quenching of* Colletotrichum *isolates.*

Isolate Code	*Colletotrichum sp.*	Origin	% Quenching
74-1S	*C. coccodes*	Indiana — potato stem	67.8
74-2S	*C. coccodes*	Indiana — potato stem	82.9
74-5S	*C. coccodes*	Indiana — potato stem	77.0
75-5S	*C. coccodes*	Certified potato seed piece	82.2
75-16	*C. coccodes*	Indiana — soil sample	83.9
CA-8	*C. coccodes*	England — tomato root	61.3
C-9	*C. coccodes*	Ohio — tomato fruit	95.4
C-13	*C. coccodes*	Maryland — tomato fruit	83.6
T-7	*C. coccodes*	Ohio — tomato fruit	90.1
CG-103	*C. graminicola*	Indiana — dent corn	82.8
CG-104	*C. graminicola*	Indiana — sweet corn	28.1
CG-105	*C. graminicola*	North Carolina — sweet corn	84.6
CGL-1	*C. gloeosporioides*	Indiana — tomato fruit	55.9

Discussion

The fluorometric aminopeptidase assay provides a rapid reproducible method of separating at least three species of the genus *Colletotrichum.* Although single isolates of a given species of *Colletotrichum* were quantitatively different from other isolates of the same species in their hydrolysis of specific naphthylamides, it was not possible to separate the isolates into distinct groups which correspond to their host of origin. Since several isolates were subcultured repeatedly on PDA medium after initial isolation from host tissue, it is conceivable that variability between isolates of the same species may have been affected. Isolate differences observed were comparable to those attributed to prior growth conditions by Westley, et al. (10) and Krawczyk and Huber (6). The relationship of peptidase activity to pathogenesis or host tissue preference would be an interesting extension of this study. Continued natural occurrence of these pathogens should provide an opportunity to evaluate isolate and environmental influences relative to peptidase relationships.

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