Differential Enzyme Synthesis by Haploid and Diploid Forms of Verticillium albo-atrum

C. R. Howell

Research Plant Pathologist, Crops Research Division, ARS, USDA, Lubbock, Texas 79401.
Cooperative investigation of Crops Research Division, ARS, USDA, and the Texas A&M University Agricultural Research and Extension Center at Lubbock.
Accepted for publication 17 October 1969.

ABSTRACT

Haploid and diploid forms of two strains of *Verticillium albo-atrum* were virulent and avirulent, respectively, to cotton. Both in agar cultures and in liquid cultures, haploid forms released approximately 3.5 times as much polygalacturonase, 5.5 times as much polyphenol oxidase, and 10 times as much β-glucosidase to the medium as did corresponding diploid forms during 5 days' incubation at 25 C. Wide differences in enzyme-synthesizing capacity, exhibited by haploid and diploid forms of *V. albo-atrum,* may account for differences in their virulence to cotton (*Gossypium hirsutum*). Phytopathology 60:488-490.

Physiological changes associated with vascular wilts caused by *Verticillium albo-atrum* Reinke & Berth. and *Fusarium oxysporum* Schlecht. ex Fr. exhibit a number of similarities (4, 8, 10, 13, 16). Many symptoms are attributed to polygalacturonase (1, 3, 12), polyphenol oxidase (7), and β-glucosidase (2).

Recently Tolmsoff (14) isolated diploid forms from a number of haploid *V. albo-atrum* isolates. When injected into stems of susceptible cotton hosts, diploid forms were consistently nonpathogenic; their haploid counterparts were highly pathogenic. Both forms were readily isolated from leaves and petioles of inoculated plants (Tolmsoff, *personal communication*).

In a preliminary investigation of haploid and diploid forms of isolates T9-50 and V-44 grown in cotton-seedling extract agar, the haploids induced a browning reaction similar to that observed in vascular systems of infected plants. Diploid colonies induced a lesser reaction later in their development.

This study reports the capacity of diploid and haploid forms of *V. albo-atrum* to produce enzymes associated with wilt-disease symptoms.

MATERIALS AND METHODS.—*Qualitative analyses for polygalacturonase (PG), polyphenol oxidase (PO),* and *β-glucosidase (BG).*—Single spore isolates of haploid and diploid forms of isolates V-44 and T9-50 of *V. albo-atrum* were grown on Eckert's medium (5), containing agar and specific enzyme substrates, modified with 1.36 g of KH_2PO_4, 1.68 g of K_2HPO_4, and 5 g of sucrose/liter. Cultures were incubated 5 to 6 days at 25 C before enzyme activities were determined.

PG activity was detected by adding 0.5% polygalacturonic acid (pH 5.0), in place of sucrose, to the modified Eckert's medium. After incubation for 6 days, the cultures were flooded with 1 N HCl for 2 hr to make the medium opaque, except where PG activity left a clear zone.

PO activity was detected by incorporating 0.05% tannic acid into the medium. The cultures were observed for formation of dark oxidation products indicative of PO activity (11). Hyphal tip isolates of haploid and diploid forms were also analyzed for PO, using 0.1% tannic acid medium.

BG activity was determined by adding 5×10^{-4} M *p*-nitrophenyl-β-D-glucopyranoside to the medium. Enzymatic hydrolysis of the colorless glucoside releases *p*-nitrophenol, a yellow compound.

Quantitative analyses for PG, BG, and PO.—Haploid and diploid forms were grown in a modified Wood's medium (15) for PG and BG production, and in modified Eckert's medium for PO. Cultures contained either 0.5% sodium polypectate, *p*-nitrophenyl-β-D-glucopyranoside (1×10^{-5} M), or tannic acid (1.5×10^{-4} M) as inducers of PG, BG, and PO production, respectively. Wood's medium was modified with 1.36 g of KH_2PO_4, 1.68 g K_2HPO_4, and by adding 5 instead of 10 g sodium polypectate/liter.

The media were inoculated with single germinated conidia of the fungal forms on agar discs, and were incubated for 5 days in shake culture. Cultures were centrifuged at 2,000 *g* for 15 min. The decanted supernatants were treated according to a modification of the method of Patil & Dimond (9). In the method used here, 50-ml aliquots of protein solution were concentrated and brought to 5 ml with deionized water. They were then stored in the freezer until used. Mycelial dry wt of the cultures were used in determining the efficiency of enzyme production by the haploid and diploid forms.

PG activity was determined with a size 200 modified Ostwald, Cannon-Fenske viscosimeter at 32 C. Reaction mixtures consisted of 9 ml of 0.5% sodium polypectate in 0.15 M phosphate buffer (pH 6.35) and 1 ml enzyme solution. Results are expressed in relative enzyme activity units obtained from 1,000/t, where t equals the time in min for 50% reduction in viscosity.

Assay for PO was made using 1.5×10^{-5} M chlorogenic acid in 0.15 M phosphate buffer (pH 5.7) as the substrate. Two ml of substrate were mixed with 1 ml of enzyme solution, and the mixture was incubated for 90 min at 35 C. Optical-density readings were taken with a Bausch and Lomb Spectronic 600 at 326 nm to determine the amount of chlorogenic acid oxidized. The readings were then converted to arbitrary enzyme units. An enzyme unit is that amount of enzyme necessary to change the optical density by 0.001/min during the incubation period.

BG activity was determined by adding 1 ml of en-

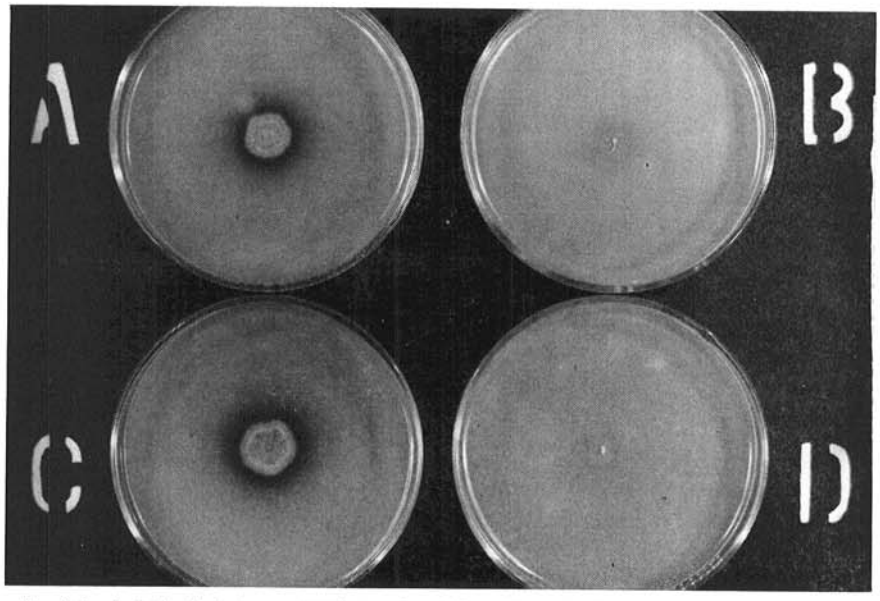

Fig. 1. Growth of hyphal tip isolates on 0.1% tannic acid medium. Dark zones represent polyphenol oxidase activity. **A, B)** T9-50 haploid and diploid forms; **C, D)** V-44 haploid and diploid forms, respectively.

zyme concentrate to 2 ml of 1.5×10^{-3} M *p*-nitrophenyl-β-D-glucopyranoside in 0.015 M phosphate buffer (pH 6.5). The amount of *p*-nitrophenol released was measured with a Bausch and Lomb Spectronic 600 at 400 nm. Readings were taken at the end of a 40-min incubation period at 35 C, and the data were converted to arbitrary enzyme units. An enzyme unit is that amount of enzyme necessary to change the optical density by 0.001/min during the incubation period.

Heat-inactivated enzyme controls were used in the assays of all three enzymes to correct for nonenzymatic changes occurring during the incubation periods.

RESULTS.—Agar cultures of haploid forms of T9-50 and V-44, compared with diploid forms, had wider zones of enzyme activity and exhibited superior growth. The greatest contrast was observed on 0.1% tannic acid medium. Single spore isolates of both forms failed to grow on this medium. However, hyphal tip isolates from established colonies did grow. Haploids showed greater enzyme activity on the 0.1% medium than on lower concentrations of tannic acid. They grew well and readily oxidized tannic acid, whereas diploids appeared to be inhibited by the substance (Fig. 1).

TABLE 1. Enzyme activities in culture filtrates of haploid and diploid forms of *Verticillium albo-atrum*

Fungus		Production of		
Strain	Form	Polygalacturonase	Polyphenol oxidase	β-glucosidase
		enzyme units/100 cc[a]		
V44	Haploid	344[b]	1.74[c]	11.4[c]
T9	Haploid	334	1.22	6.3
V44	Diploid	93	0.22	1.0
T9	Diploid	100	0.40	0.7

[a] Enzyme unit values represent an average of three replications.

[b] Data are expressed in relative enzyme activity units obtained from 1,000/t, where t equals the time in min for 50% reduction in viscosity.

[c] An enzyme unit is defined as that amount of enzyme necessary to change the optical density 0.001/min during the incubation period.

Quantitative analyses of haploid and diploid culture filtrates for PG, PO, and BG confirmed the results obtained from agar media. Haploid forms released approximately 3.5 times as much PG, 5.5 times as much PO, and 10 times as much BG to the medium as the corresponding diploid forms (Table 1). Calculation of mycelial dry wt showed haploids to be five times as productive as diploids in liquid culture.

DISCUSSION.—On a per-mg dry wt basis, haploids are twice as efficient as diploids in the production of BG. With respect to PG and PO, however, differences in efficiency of production between haploid and diploid forms do not appear to be significant. Therefore, major differences in enzyme production between the two forms must be attributed to differences in growth rate. Haploids are much more vigorous growers than diploids.

Whether or not a host plant becomes diseased often depends on the balance between production of microbial metabolites and those metabolites conferring resistance on the host (6). In the study reported here, the haploids are highly pathogenic, while the diploids produce no discernible symptoms, although their presence in the upper portions of inoculated plants can be easily demonstrated. The results of this study suggest that the wide differences in enzyme-producing capacity exhibited by these strains may account for differences in their virulence to cotton. It remains to be shown whether these differences in enzyme synthesis occur within the infected plant.

LITERATURE CITED

1. BLACKHURST, F. M., & WOOD, R. K. S. 1963. *Verticillium* wilt of tomatoes: Further experiments on the

role of pectic and cellulytic enzymes. Ann. Appl. Biol. 52(1):89-96.
2. Davis, D., P. E. Waggoner, & A. E. Dimond. 1953. Conjugated phenols in the *Fusarium* wilt syndrome. Nature 172:959.
3. Deese, D. C., & M. A. Stahmann. 1962. Formation of pectic enzymes by *Verticillium albo-atrum* on susceptible and resistant tomato stem tissues and on wheat bran. Phytopathol. Z. 46:53-70.
4. Dimond, A. E. 1967. Physiology of wilt disease. p. 100-120. *In* C. J. Mirocha & I. Uritani [ed.] The dynamic role of molecular constituents in plant-parasite interaction. Amer. Phytopathol. Soc., St. Paul, Minn.
5. Eckert, J. W. 1962. Fungistatic and phytotoxic properties of some derivatives of nitrobenzene. Phytopathology 52:642-649.
6. Kuć, O. 1967. Shifts in oxidative metabolism during pathogenesis. p. 183-202. *In* C. J. Mirocha & I. Uritani [ed.] The dynamic role of molecular constituents in plant-parasite interaction. Amer. Phytopathol. Soc., St. Paul, Minn.
7. Matta, A., & A. E. Dimond. 1963. Symptoms of *Fusarium* wilt in relation to quantity of fungus and enzyme activity in tomato stems. Phytopathology 53:574-578.
8. Page, O. T. 1959. Observations on the water economy of *Fusarium* infected banana plants. Phytopathology 49:61-65.
9. Patil, S. S., & A. E. Dimond. 1967. Inhibition of *Verticillium* polygalacturonase by oxidation products of polyphenols. Phytopathology 57:492-496.
10. Scheffer, R. P., & J. C. Walker. 1953. The physiology of *Fusarium* wilt of tomato. Phytopathology 43: 116-125.
11. Singh, P. 1968. Amylase and polyphenol oxidase production by germinating conidia of *Colletotrichum falcatum* Went. Experimentia 24(2) 117-118.
12. Stoddart, J. L., & A. J. H. Carr. 1966. Properties of wilt-toxins produced by *Verticillium albo-atrum*. Ann. Appl. Biol. 58:81-92.
13. Threlfall, R. J. 1959. Physiological studies on the *Verticillium* wilt disease of tomato. Ann. Appl. Biol. 47:57-77.
14. Tolmsoff, W. J. 1968. The microsclerotial cell as a major source for genetic variability in *Verticillium albo-atrum*, p. 231-232. Beltwide Cotton Prod. Res. Conf. Proc., 1968, Hot Springs, Ark. (Abstr.).
15. Wood, R. K. S. 1961. *Verticillium* wilt of tomatoes—The role of pectic and cellulytic enzymes. Ann. Appl. Biol. 49:120-139.
16. Wood, R. K. S. 1967. Physiological plant pathology, p. 560. Oxford & Edinburg.