Phytochemistry, 1964, Vol. 3, pp. 269 to 272. Pergamon Press Ltd. Printed in England

BIOCHEMICAL CHANGES IN TOBACCO INFECTED WITH *COLLETOTRICHUM DESTRUCTIVUM*—I. FLUORESCENT COMPOUNDS, PHENOLS, AND SOME ASSOCIATED ENZYMES

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(*Received* 9 *September* 1963)

Abstract—Tobacco leaf tissue exhibited a 5- to 10-fold increase in peroxidase activity in the early stages of infection with *Colletotrichum destructivum*. Phenolase, ascorbic acid oxidizing ability, and quinone reductase increased only slightly or not at all. Chlorogenic acid content did not differ appreciably between infected and healthy tissue except that in some experiments chlorogenic acid was slightly reduced by the infection. Two u.v.-fluorescent compounds which were not detected in healthy tissue or in the pathogen were found in infected tissue.

INTRODUCTION

A NUMBER of biochemical changes are known to occur in plants following infection by various pathogens. These include alterations in the respiratory pattern, increased activity of oxidases such as phenolase (E.C. 1.10.3.1) and ascorbic acid oxidase (E.C.1.10.3.3), and qualitative and quantitative changes in several metabolites. Increased synthesis of phenolic compounds, synthesis of new phenolic compounds, and increased activity of phenolase are typical of a number of diseased plant tissues.[1]

Such metabolic alterations following exposure of a plant tissue to a pathogen may function as defense mechanisms, particularly the increased phenol synthesis and the increased activity of phenol oxidizing enzymes.[1,2]

The present investigation was undertaken to determine the effect of infection of tobacco leaf tissue by *Colletotrichum destructivum* O'Gara on the level of phenolic compounds in the host tissue and on the activity of enzyme systems which are thought to influence the oxidation level of these compounds.

RESULTS

Activity of Enzymes

The activities of peroxidase (E.C. 1.11.1.7), phenolase, quinone reductase (E.C. 1.6.51), and ascorbic acid oxidizing activity (possibly a coupled oxidation mediated by phenolase) were determined in buffered homogenates of healthy and infected tissue 5 days after inoculation. At this time, the only visible symptoms of the disease were small necrotic spots 2–3 mm

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[1] I. URITANI and T. AKAZAWA, in *Plant Pathology, an Advanced Treatise*, Eds. J. G. HORSFALL and A. E. DIAMOND, Vol. 1, p. 349, Academic Press, New York (1959).

[2] I. URITANI, *Symposium on Biochemistry of Plant Phenolic Substances*, p. 98, Colorado State University, Boulder (1961).

dia. The ratio of activity of each system in infected and healthy tissue (E_I/E_H) was determined using arbitrary units of activity (Δ 0.D./min or μl O_2/hr). Peroxidase increased 5- to 10-fold and phenolase up to 1·7-fold. Ascorbic acid oxidizing activity and quinone reductase were unchanged.

Phenolic and Other Fluorescent Compounds

Two-dimensional paper chromatograms of ethanolic tissue extracts revealed 6 fluorescent compounds in healthy tissue and 8 in infected tissue (Fig. 1). Compounds 1 and 2 were phenolic, and compound 1 was identified as chlorogenic acid on the basis of R_f values, u.v. absorption spectrum, and co-chromatography with known chlorogenic acid. In most experiments no difference in the quantities of these two compounds between healthy and

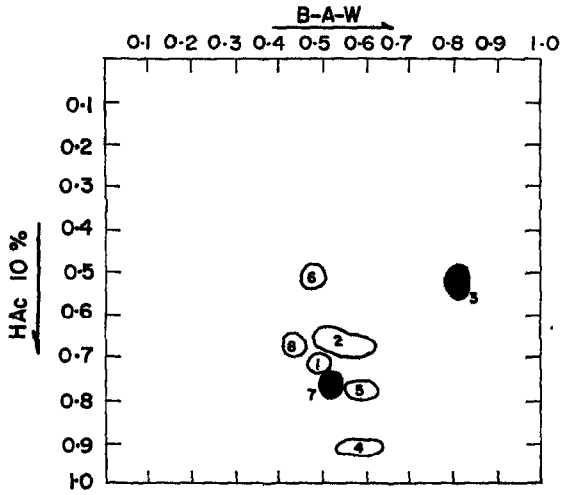

FIG. 1. FLUORESCENT COMPOUNDS IN INFECTED AND HEALTHY TISSUE.

Two-dimensional paper chromatogram developed with 10% acetic acid and n-butanol–acetic acid–water (4:1:5). Spots which are shaded were found only in infected tissue.

Spots 1, 2, 5 and 7 show purple fluorescence in long-wave u.v. light; 4, and 8 are yellow; 2, blue; and 6, red–brown. Spots 1 and 2 react with diazotized sulfanilic acid, $FeCl_3$ and Folin–Denis reagent. Spot 2 is acidic (bromcresol green).

infected tissue could be detected. However, in a few experiments, chlorogenic acid as judged chromatographically was reduced after infection. Compounds 3 and 7 were detected only in infected tissue. Both gave negative tests for phenols, quinones, amino acids, organic acids, and reducing groups (Fig. 1). No attempt was made to characterize the remaining compounds.

DISCUSSION

The data presented above are of interest in several respects. Uritani[2] has recently pointed out that increased activity of phenolase, peroxidase, and frequently of ascorbic acid oxidase; and accumulation of phenols are characteristic of a number of plant diseases. The present case differs from this in that only peroxidase increases markedly and chlorogenic acid remains constant or decreases after infection. If a rough correction is applied to the data to compensate for the 10–20 per cent necrosis in the infected tissue, the enzyme activities would be

somewhat higher than noted above. However, this would still not represent the 2- to 3-fold increase in phenolase and ascorbic acid oxidase noted in some diseased plant tissues. The very large increase in peroxidase activity in infected tissue and the fact that quinone reductase does not increase correspondingly might result in the accumulation of quinones in the infected tissue. This in turn might lead to tissue necrosis, as suggested by Farkas and co-workers in the case of tobacco mosaic virus in hypersensitive hosts.[3] Since peroxidase is apparently capable of oxidizing phenols using either peroxides or molecular oxygen as an electron acceptor,[4] increased peroxidase activity would be expected to have the same effect as increased phenolase activity.

The two fluorescent compounds which were found only in infected tissue appeared to be identical to those found in tobacco infected with *Pseudomonas tabaci*,[5] a bacterial pathogen which also induces local necrosis in infected leaves. The appearance of two apparently identical compounds in tobacco after infection by either a fungal or bacterial pathogen suggests the possibility that these compounds might be associated with other tobacco diseases as well. Romanowski *et al.*[6] detected fluorescent compounds in bean seedlings infected with *Colletotrichum lindemuthianum* which induced tissue necrosis when introduced locally into healthy tissue. However, these compounds were acidic, whereas those from tobacco are not. An attempt is presently being made to obtain sufficient quantities of these compounds to characterize them and to determine their effects on the pathogen and host. The possibility that they accumulate in tobacco infected with other pathogens is also being investigated.

EXPERIMENTAL

Inoculation

Seedlings of *Nicotiana tabacum* (var. Ky 26) grown in 3-in. clay pots were inoculated in the 4- to 5-leaf stage with an aqueous suspension of spores and mycellium of *C. destructivum*. Immediately after inoculation, the plants were covered with individual polyethylene bags to maintain high humidity and placed in a fluorescent lighted incubator at 24° for 48 hr. The plastic bags were then removed and the plants were returned to the greenhouse. Tissue was harvested by punching disks from the leaves with an 8-mm cork-borer, using the lesions as the centers of the disks. Control tissue was obtained from plants which had been treated in the manner described above except that they were not inoculated.

Enzyme Assays

Tissue homogenates for enzyme assays were prepared by homogenizing frozen tissue in cold 0·05 M phosphate buffer, pH 6·5 (3 ml/g fresh wt.), in a Servall omnimixer for 3 min and centrifuging at 3600 rev/min for 15 min. The supernatants were decanted and placed in an ice-bath until used. Peroxidase using pyrogallol as substrate, phenolase with chlorogenic acid, and ascorbic acid oxidasing activity were assayed as previously described,[7] and quinone reductase was assayed by the method of Wosilait and Nason[8] using NADH and hydroquinone. Spectrophotometric assays were made using a Zeiss PMQ II spectrophotometer

[3] G. L. Farkas, Z. Kiraly and F. Solymosy, *Virology* **12**, 408 (1960)
[4] B. A. Ruben and T. M. Ivanova, *Life Sciences*, No. 4, 219 (1963).
[5] P. R. Fisher and R. E. Hampton, unpublished data.
[6] R. D. Romanowski, J. Kuc and F. W. Quackenbuch, *Phytopathology* **53**, 1259 (1963).
[7] R. E. Hampton, *Phytopathology* **53**, 497 (1963).
[8] W. D. Wosilait and A. Nason, *J. Biol. Chem.* **206**, 255 (1954).

adapted to record absorbance versus time. All assays were made using at least two concentrations of tissue homogenate to insure that the reaction rates were proportional to enzyme concentration.

Paper Chromatography

Ethanolic extracts of infected and healthy tissue were made by homogenizing tissue in 95% ethanol (3 ml/g fresh wt.) and centrifuging as described above. Supernatants were spotted (200–400 μl) on Whatman No. 1 paper and developed with 10% acetic acid and the upper phase of n-BuOH–acetic acid–H_2O (4:1:5) in the second direction, after 24 hr equilibration with the lower phase.

Acknowledgements—This investigation was supported in part by P.H.S. research grant AI-04632-BM from the National Institute of Allergy and Infectious Diseases, United States Public Health Service. We would like to express our appreciation to Mrs. Marjorie Eason for her technical assistance. Published with the approval of the director of the Kentucky Agricultural Experiment Station.