Brown-Pigment Formation in Tobacco Leaves Infected with Alternaria

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ABSTRACT

Brown-spot leaf lesions from field-grown susceptible and resistant cultivars were separated mechanically into prehalo, halo, and necrotic tissue zones for comparative studies with adjacent healthy tissue. A gradient increase in total nitrogen, total protein, and oxidase activities was observed from prehalo, to halo, to necrotic tissues. The enzymatic oxidation of phenols was a primary reaction resulting in the formation of pigments. Enzymatically oxidized phenols were shown to complex with extracted proteins to form brown pigments. The results, in toto, support the hypothesis that complexing between certain oxidized phenols, proteins, and perhaps other constituents, results in brown-pigment formation and necrosis. This process seems basic to lesion development. The formation of brown pigments may be a nonspecific process in plants, activated by many types of infections or injuries. A notable difference in oxidase activity was observed between the susceptible and resistant cultivars studied using chlorogenic acid as a substrate. The resistant cultivar had a gradient increase in activity from prehalo to necrotic tissue, whereas all lesion tissue zones of the susceptible cultivar had similarly high activities.

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The brown-spot disease of tobacco is a foliar disease caused by the fungal pathogen *Alternaria alternata* (Fries) Keissl. (*A. longipes* (El. & Ev.) Mason or *A. tenuis* Nees). The leaf lesion was defined (5) as consisting of three zones of tissue: the brown central tissue is necrotic and contains the fungal pathogen; the necrotic zone is surrounded by a chlorotic halo which is not invaded by the fungus; and the halo is surrounded by a narrow zone named "prehalo". Biochemical and physiological studies of these and adjacent tissue revealed a gradient of changes during lesion development (5, 8, 11, 12). The changes in the noninvaded halo and prehalo tissues were attributed to the diffusion of toxic metabolites from the fungus (9, 10).

We have now examined phenoloxidase activities during lesion development attendant to changes in protein and phenols and interactions of these constituents to form the brown pigments of necrotic tissues in susceptible and resistant cultivars.

MATERIALS AND METHODS.—Naturally infected tobacco leaves were collected from field-grown, brown-spot-susceptible cultivar 'Coker 298' and the resistant breeding line NC 80383-5-9, at Oxford, N.C. (6, 7). Lesions measuring ca. 30-mm diam, including the halo, were mechanically separated into necrotic, halo, and prehalo tissues as described previously (5). Healthy tissue was obtained from noninfected areas adjacent to the prehalo zones. Tissues from more than 500 lesions were combined, freeze-dried, or processed into acetone powders, and stored over calcium chloride desiccant at 4 C in the dark. All analyses were repeated at least three times.

Enzymatic alteration of tyrosine and DOPA.—The capacity of tissue acetone powders to metabolize tyrosine or dihydroxyphenylalanine (DOPA) enzymatically was determined by following the disappearance of substrate from a reaction mixture. Acetone powder (500 mg) and 50 ml of buffer were agitated in a 250-ml Erlenmeyer flask on a rotary shaker for 15 min and centrifuged at 7,700 *g* for 10 min. The supernatant was adjusted to a 50-ml volume with buffer and transferred to a 250-ml Erlenmeyer flask, then 50 ml of buffered substrate was added to initiate a reaction. The mixture was incubated at 30 C or 40 C on a rotary shaker.

Tyrosine and DOPA were used as substrates at a final concn of 1.65×10^{-3} M. Two buffers were used: 0.067 M sodium-potassium phosphate adjusted to pH 6.0 (phosphate buffer) and 0.05 M sodium potassium borate (borate buffer) at pH 8.0. Enzymatic activity was followed by determining the residual substrate in the reaction mixture, using the Folin-phenol reagent (Folin-Ciocalteau reagent, Fisher Scientific Co.) (4). At various times, a 10-ml sample was withdrawn from the reaction flask, 0.05 ml 10% acetic acid was added, and the mixture heated in boiling water for 10 min. The solution was filtered and the precipitate washed with distilled water. The filtrate was diluted to 50 ml; 5 ml of freshly prepared 1 N Folin-phenol reagent

and 25 ml of saturated sodium carbonate were added; the mixture was incubated for 30 min at room temp and the volume brought to 100 ml. The absorbance of the solution was read at 420 nm with a spectrophotometer. At zero reaction time, the 10-ml sample of the reaction mixture contained about 3 mg of tyrosine and Folin-phenol-positive protein equivalent to 1 mg of tyrosine. DOPA was more Folin-phenol-positive than tyrosine; therefore, samples were decreased to 5 ml when DOPA was the substrate.

Phenol oxidase activity.—Oxidase activity was measured with a GR-20 differential respirometer (Gilson Medical Electronics, Middleton, Wis.). Fifty mg of acetone powder, in 10 ml of cold (4 C) phosphate buffer was homogenized in a glass tissue-grinder. Substrate, 0.006 M tyrosine, 0.006 M DOPA, or 0.06 M chlorogenic acid, was dissolved in phosphate buffer and adjusted to pH 6.0. Substrate concn and pH were selected to maximize the reaction rate. One ml of homogenate was mixed with 0.5 ml of substrate in the respirometer flask and incubated at 26 C with 0.2 ml 20% KOH in the centerwell of each flask to absorb CO_2 from the system.

Total N and protein.—Total N in 100-mg samples of freeze-dried or acetone powders preparations was determined by the semimicro-Kjeldahl method. Quantitative and qualitative analyses of protein were made after molecular sieving. A 2- × 10-cm column was prepared according to the manufacturer's directions (Pharmacia Fine Chemicals, Inc.) using 12.5 g dry Sephadex G-25 to obtain a hydrated column with a total volume of 62.5 ml (V_t) and a void volume of 25 ml (V_o). Twenty-five mg of acetone powder was homogenized with 5 ml phosphate buffer in a glass tissue-grinder. One ml of homogenate was added to the top of the column and eluted with distilled water. Thirty-five fractions (each, 3 ml) were collected from the column, and their protein content determined with Folin-phenol reagent by the Lowry procedure (4). Results were expressed as mg total protein per gram acetone powder, computed as bovine serum albumin equivalents from a standard curve.

Soluble protein was determined by centrifuging acetone powder homogenates for 10 min at 7,700 *g* and adding 1 ml of the supernatant to a G-25 column for separation. The influence of 0.2 M urea on protein solubility was examined on duplicate samples by adding urea to the phosphate extraction buffer (12 mg/ml) before homogenization.

Polyacrylamide gel disk electrophoresis was used for qualitative assessment of proteins in the lesion tissues. The phosphate-buffered supernatant obtained from acetone powders described above was examined. In addition, all column fractions under peak I (Fig. 4) were combined, decreased in volume by membrane filtration, and examined by electrophoretic procedures. Protein content after membrane filtration was from 260 to 530 μg per ml. Sucrose was added to the protein solution to obtain a 0.2 M concn, and 0.1 ml was layered on the gel tubes. Three milliamperes current per tube was applied during stacking in the coarse gel, and 5 mA per gel during separation through the small gel at pH 8.9. The gels were fixed with acetic acid (1% v/v) and stained with amido-schwarz reagent (Buffalo Black NBR) (1%, w/v). Electrophoretic mobility values (E_f) were computed as the ratio of distance each band moved from the origin, compared to a fast-moving protein band common to all extracts. This protein band moved at a rate very similar to that of a dye marker (bromphenol blue) commonly used in gel electrophoresis. We did not use dye marker because it reacted with certain of the constituents in the samples.

Gels were tested for oxidase and peroxidase isozymes. Gels were removed from the tubes and incubated in phosphate buffer for 30 min, placed in small glass test tubes containing 4 ml of 0.2 M DOPA in buffer, and incubated at room temp for 30 min. Visible bands were recorded as zymograms. Two drops (0.05 ml) of 30% hydrogen peroxide were added to each tube, and new bands were recorded after 5 min.

Leaf protein –phenol complexing.—Tobacco-leaf proteins and phenols were incubated together under conditions favoring enzymatic oxidation, and then subjected to molecular sieving as follows. Fifty mg acetone powder from healthy greenhouse-grown, brown-spot-susceptible Coker 298 plants was extracted in phosphate buffer and centrifuged as before. A 2-ml sample of the supernatant (protein extract) was reacted with 0.4 ml of 0.01 M chlorogenic acid for 1 h at 26 C. Two ml of the mixture were layered on a Sephadex G-25 column and eluted with 0.02 N NaCl. Three-ml fractions were collected as before, analyzed by ultraviolet absorption at 260 and 280 nm and reacted with Folin-phenol reagent. The degree of complexing, if any, was observed in the elution patterns. Controls consisted of protein extract mixed with water, and water mixed with chlorogenic acid.

RESULTS.—*Enzymatic alteration of tyrosine and DOPA.*—Extracts from brown-spot lesions of both susceptible and resistant cultivars reacted enzymatically with tyrosine and DOPA. Healthy, prehalo, halo, and necrotic tissue-zones had similar reaction rates; however, reactions with tyrosine differed from those with DOPA (Fig. 1). A 20-h lag was observed before a decrease in free tyrosine was detected compared to an immediate and pronounced reaction with DOPA. Reactions were similar at pH 6.0 and 8.0. Black precipitates formed when samples of the reaction mixture were added to 10% acetic acid. This precipitate was detected 2 h after the start of the reaction with DOPA and after 4 h with tyrosine. More black precipitate formed initially (2-4 h) from halo and necrotic tissues than from either prehalo or healthy tissues. However, this distinction was not obvious after 4 h.

Reactions and black precipitate formation did not occur when the extracts were heated before adding substrate, or if extracts were incubated without substrate. When incubation temp were increased to 40 C, enzymatic activity decreased greatly.

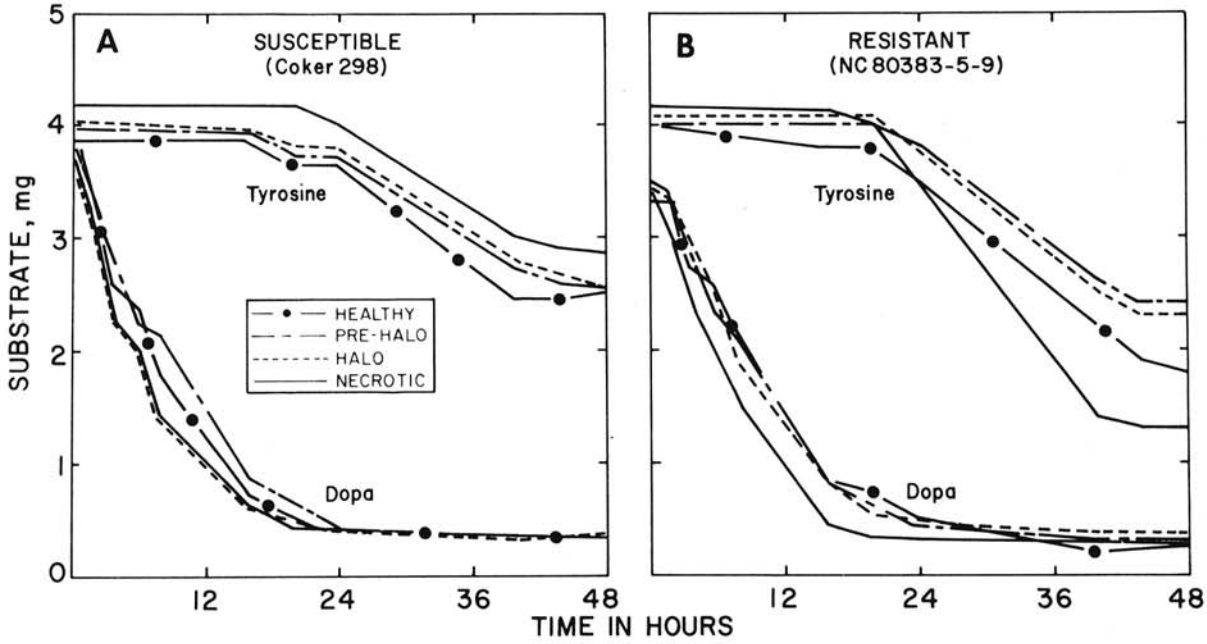

Fig. 1. Enzymatic alteration of tyrosine and DOPA by acetone-powder preparations from brown-spot lesion and adjacent healthy tissues.

Phenoloxidase activity.– Homogenized acetone powders enzymatically oxidized tyrosine, DOPA, and chlorogenic acid (Fig. 2). Tyrosine and DOPA were oxidized slowly. Measurements were made over a period of 8 h. The faster reactions with chlorogenic acid were measured during a 1-h period. Preparations from healthy and prehalo tissues were inactive with tyrosine, had low activities with DOPA, and were most active with chlorogenic acid. Preparations from halo tissue had higher activities on tyrosine and DOPA than healthy and prehalo tissues, and preparations from necrotic tissue had higher activities than their corresponding halo preparations. The halo and necrotic leaf tissue preparations from the resistant tobacco had considerably more oxidase activity on tyrosine and DOPA than their counterparts from susceptible tobacco.

With chlorogenic acid as a substrate, the preparations from the resistant tobacco increased progressively in oxidase activity from healthy and prehalo, to halo, to necrotic. Again, the activity of the preparation from necrotic tissue showed a pronounced increase in oxidase activity. This pattern was not observed in tissue preparations from the susceptible cultivar where all tissue preparations had high oxidase activity with chlorogenic acid.

As healthy and prehalo preparations oxidized DOPA, the reaction mixture became grey whereas halo and necrotic preparations turned the mixture black. The enzymatic oxidation of chlorogenic acid resulted in a browning of the reaction mixture.

Ascorbic acid (0.06 M) was also tested as an oxidase substrate. Ascorbic oxidase activities of the preparations from the two cultivars were similar to the pattern shown by the reaction of the susceptible cultivar tissues with DOPA (Fig. 2). The maximum oxygen uptake observed was 55 μl in 90 min. Ascorbic acid oxidase activity appeared intermediate between activities with chlorogenic acid and DOPA. Both cultivars had similar activities on ascorbic acid.

Total N and protein.–Total N in the acetone powders was half that of freeze-dried tissue (Table 1). This indicates that acetone extraction removed nonprotein, nitrogenous compounds. Total N in acetone-powder preparations from both cultivars increased in the following order: prehalo < halo < necrotic.

Fig. 3 summarizes the protein content of the tissue acetone powders after molecular sieving on Sephadex G-25 columns. Proteins eluted in two peaks (Fig. 4). Peak I was mainly proteins because it had a strong UV absorption at 260 and 280 nm. Peak II was mainly nucleoproteins because it absorbed strongly at 260 nm and weakly at 280 nm. Protein increased from prehalo < halo < necrotic tissue. This agrees with total N determinations. Necrotic tissue contained nearly twice as much protein as halo tissue. The presence of the pathogen itself in necrotic tissue probably adds to the protein level. However, the fact that an increase occurred in the fungus-free halo tissue, and the large magnitude of the increase, indicate that the proportion of the increase resulting from the fungus was small. Buffer extraction did not remove all protein from the acetone powders (Fig. 4). The addition of 0.2 M urea to the buffer increased extraction efficiency of peak I protein, but had little effect on peak II nucleoproteins. Differences in total protein between susceptible and resistant cultivars were small.

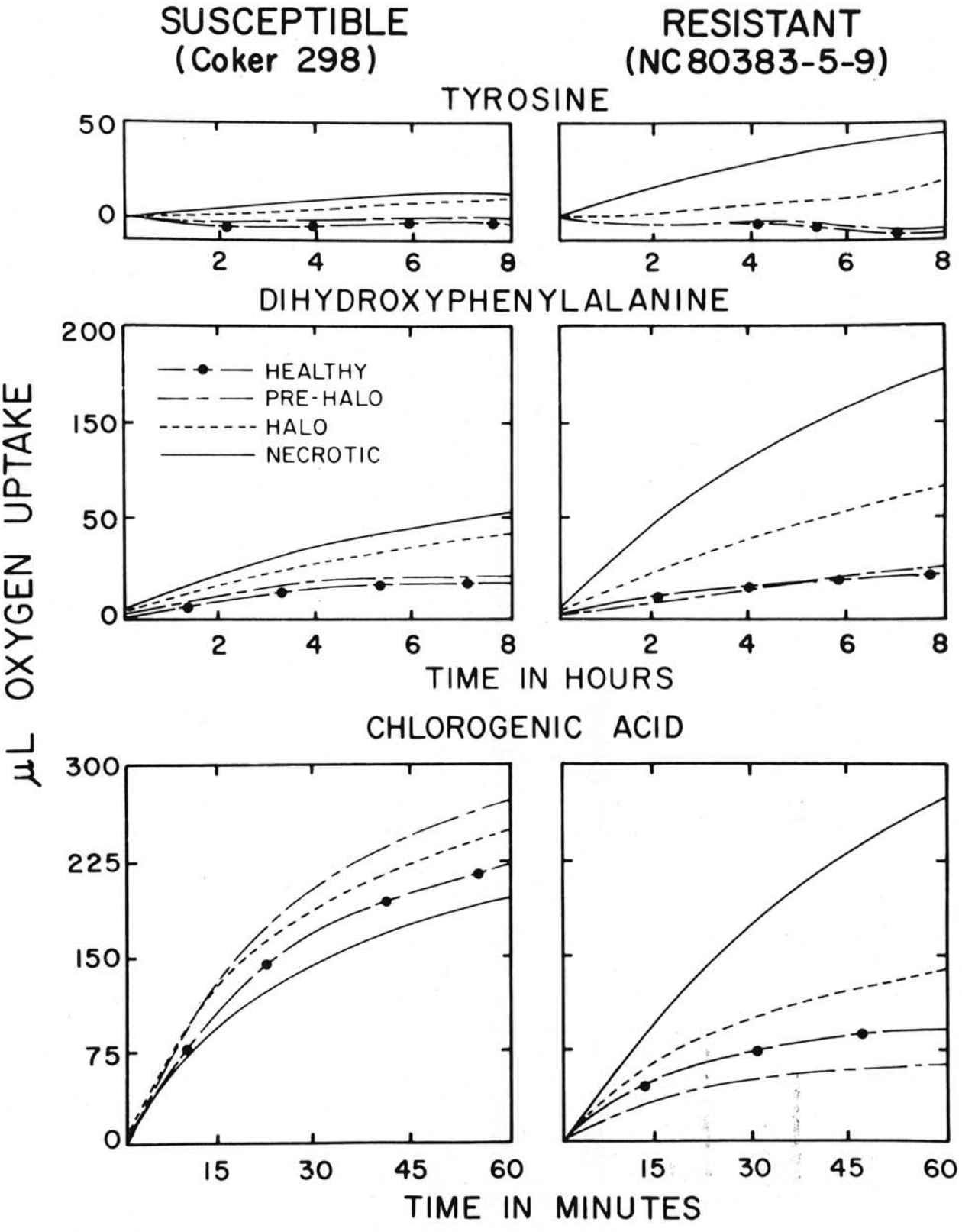

Fig. 2. Oxidase activities of acetone-powder preparations from brown-spot lesion and adjacent healthy tissues on tyrosine, DOPA, and chlorogenic acid.

TABLE 1. Total nitrogen in freeze-dried and acetone-powder preparations of brown-spot lesion and healthy tissue[a]

Tissue	Susceptible (C 298)		Resistant (NC 80383-5-9)	
	Freeze-dried	Acetone powder	Freeze-dried	Acetone powder
Healthy	3.2	1.9	3.2	1.1
Prehalo	3.1	1.2	2.9	1.0
Halo	2.7	1.4	2.7	1.3
Necrotic	3.1	1.5	4.1	1.7
LSD ($P = 0.05$)	0.1	0.1	0.2	0.1

[a]Data expressed as percent, dry-wt basis.

Disk gel electrophoresis.– Electrophoretic separations revealed qualitative changes in proteins associated with brown-spot formation (Fig. 5). The yellow reference band stained blue with the amido-Schwarz reagent, as did the other protein bands, although some bands were more grey than blue. Bands from the buffer extract differed somewhat from peak I bands. Therefore, results were influenced by the method used to prepare the sample. One consistent and prominent difference was a decrease in protein bands from halo to necrotic tissue. The progressive decrease in protein bands from healthy to necrotic lesion tissue in the susceptible cultivar was reported previously (5). The decrease in protein bands may result from the complexing of proteins into brown pigments. However, the number of oxidase and peroxidase isozyme bands did not appear to decrease in the necrotic tissues.

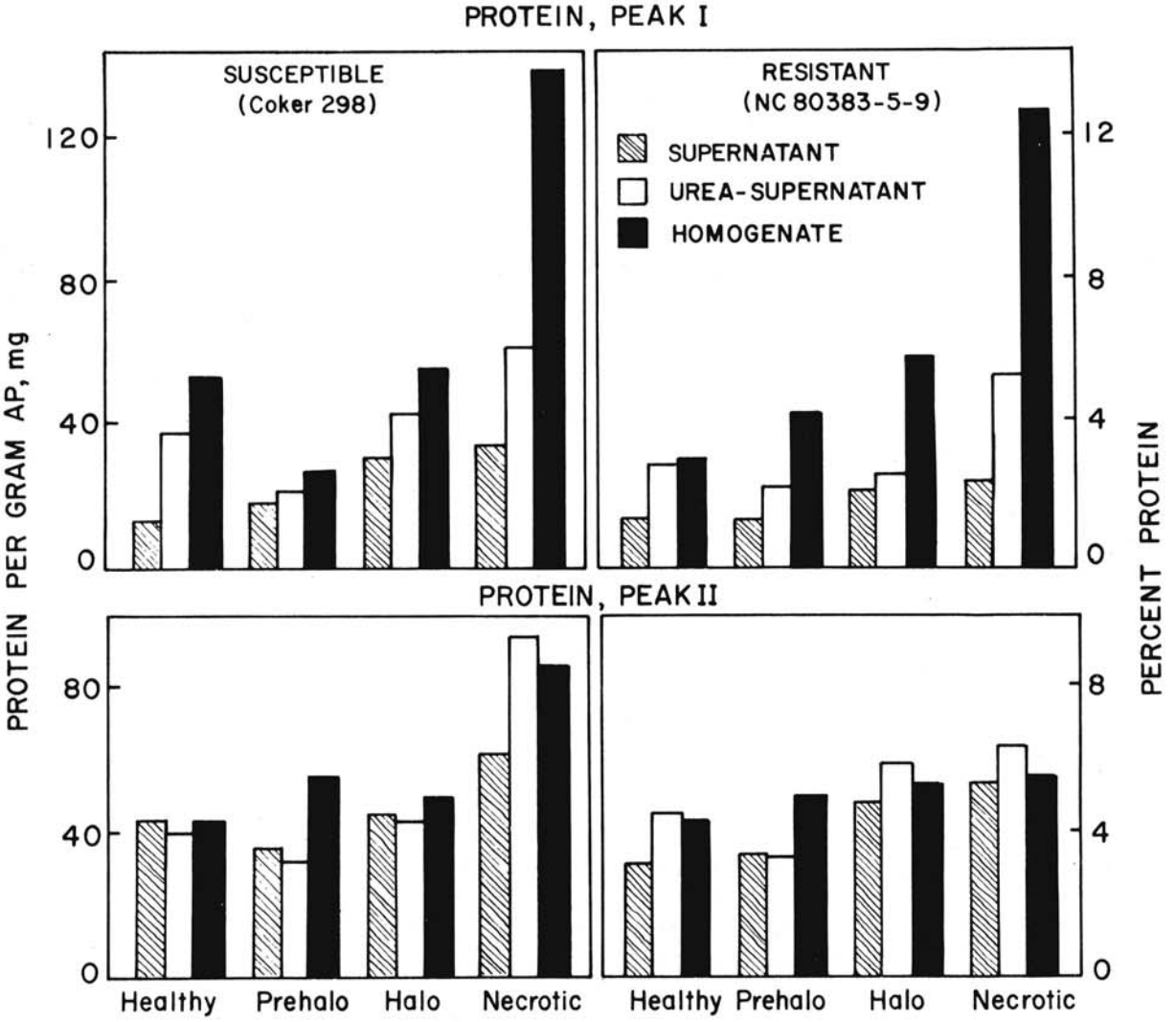

Fig. 3. Protein in acetone-powder preparations of brown-spot lesion and adjacent healthy tissues after separation on Sephadex G-25 columns. Protein was determined with Folin-phenol reagent and expressed as bovine serum albumin equivalents.

Leaf protein – phenol complexing.—The protein-extract/water-control mixture eluted from the Sephadex G-25 column revealed two peaks (I, II) described above (Fig. 4). The water – chlorogenic acid control mixture eluted with a single large peak. The single peak was composed of the unreacted chlorogenic acid. The protein-extract/chlorogenic acid mixture browned during the 1-h incubation and eluted with a large increase in protein peaks I and II and a correspondingly large decrease in the chlorogenic acid peak.

When protein extract – chlorogenic acid mixtures were incubated for 1 h at 0 C and eluted, smaller increases appeared in peaks I and II, and the chlorogenic acid peak decreased less. Heating the protein extract at 60 C for 10 min before adding chlorogenic acid, completely prevented increases in the protein peaks.

When DOPA (0.006 M) was substituted for chlorogenic acid, and the incubation period was extended to 8 h (Fig. 1, 2), a fine, black, filterable precipitate formed. The elution pattern changed also (Fig. 4). A decrease in peak I and an increase in peak II occurred.

We concluded that the enzymatic oxidations by the extracts resulted in the formation of pigments (protein-phenol complexes).

DISCUSSION.—We hypothesize that the progressive complexing between certain oxidized phenols, proteins, and perhaps other constituents results in pigment formation and necrosis and is basic to brown-spot lesion development. Others have reported that brown pigments in leaves caused by viral infection, mechanical injury, and high-temp curing processes were composed primarily of phenols, amino acids, metals, and proteins (1, 2, 14, 15). Thus, brown-pigment formation may be a nonspecific process in plants, activated by many types of stimuli.

Our results support this hypothesis when viewed in toto: total N, protein, and oxidase activities increased from prehalo to necrotic tissue; the number of protein bands decreased, particularly in necrotic tissues; enhanced oxidase activity was a primary enzymatic reaction characteristic of lesion tissue; enzymatic oxidation of phenolic substrates resulted in the formation of pigments; enzymatically oxidized phenols (quinones) complexed with proteins; chlorogenic acid, and several other phenols and certain amino acids decreased considerably from the yellow halo to the brown necrotic tissue (5); tissue permeability and structural integrity within lesion tissues decreased significantly from halo to necrotic tissue (13; and H. W. Spurr and C. E. Main, *unpublished*).

An increase in protein could result from the synthesis of existing protein or new protein in response to infection, or some of the increase in the necrotic tissue probably was due to the presence of the fungal pathogen. The Folin-phenol reagent used to determine protein did not differentiate between protein and protein-phenol complexes as evidenced by molecular sieving experiments. Thus, protein-phenol complexes could account for a part of the increase observed in halo and necrotic tissues. If chlorogenic acid, which does not contain nitrogen, is considered to be the principal phenol in the pigment of the brown, necrotic tissue, then the increase in total N probably resulted from an increase in protein. In addition to this research, previous research by others supports the contention that chlorogenic acid is the principal phenol in brown pigments (1, 2, 14, 15).

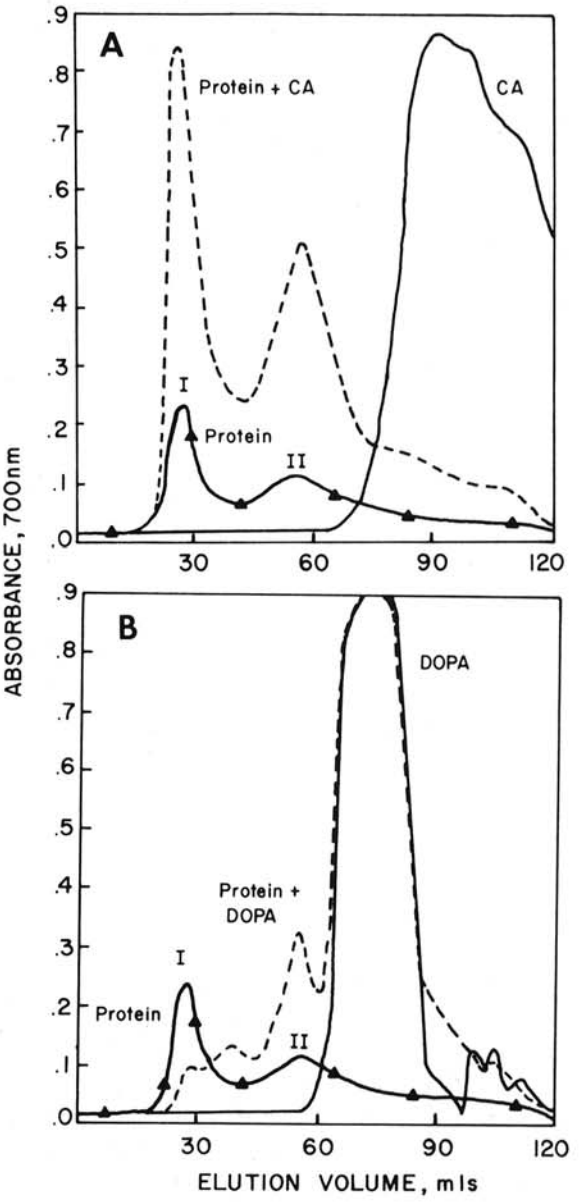

Fig. 4- (A, B). Reaction of protein extract from healthy tobacco-leaf tissue, Coker 298, with phenols to form protein-phenol complexes, as identified after separation on Sephadex G-25 columns. Two phenols were used: **A)** chlorogenic acid, **B)** DOPA. The constituents were determined with Folin-phenol reagent and ultraviolet absorption.

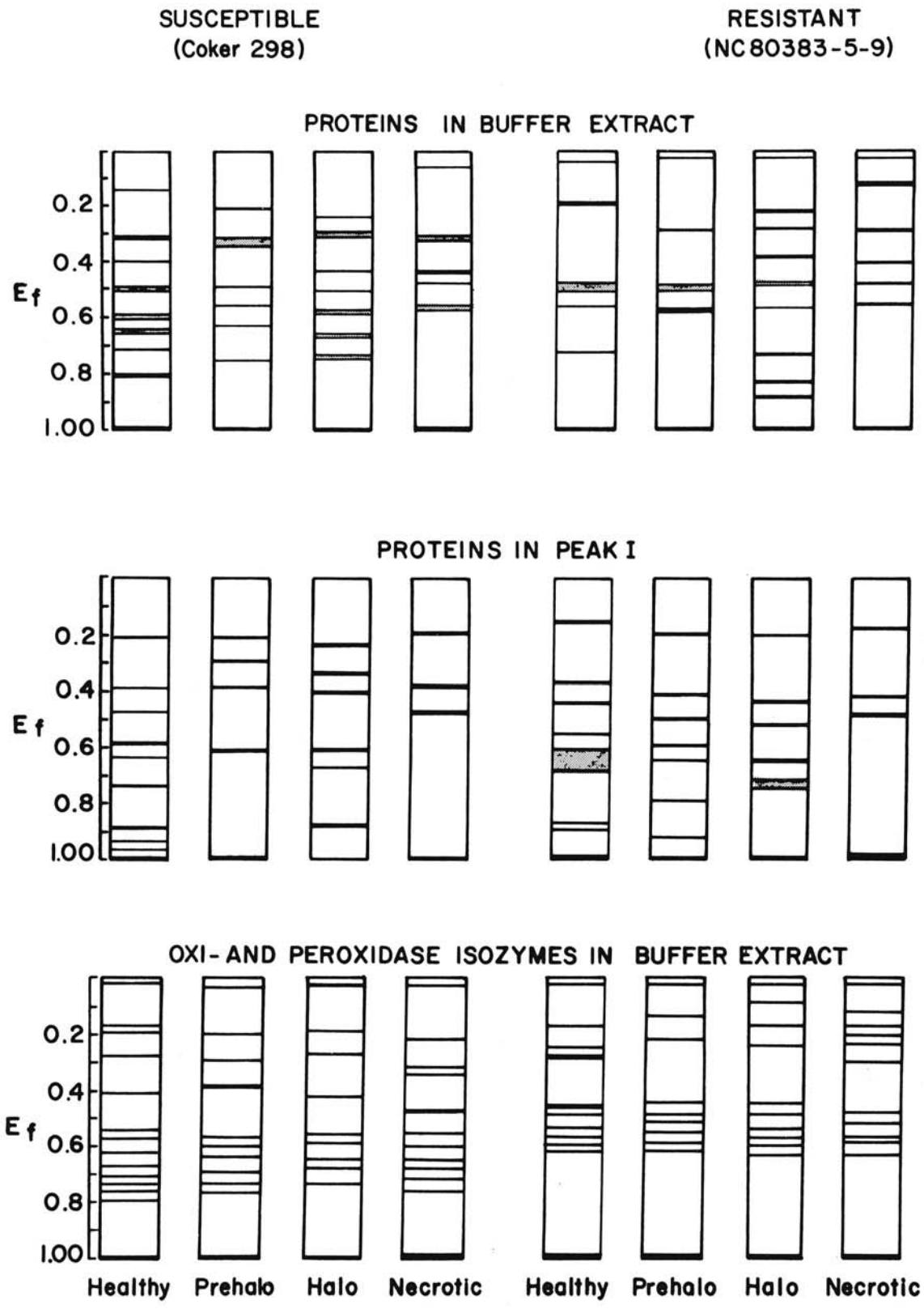

Fig. 5. Electrophoretic, polyacrylamide-gel separation of proteins and oxidase and peroxidase isozymes in acetone-powder preparations of brown-spot lesion and adjacent healthy tissues.

It is interesting to compare the oxidation of tyrosine and DOPA to the disappearance of these substrates from solution. The oxidized forms of these, and other, substrates are chemically reduced in the reaction with Folin-phenol reagent; thus, until these substrates or their oxidation products become bound (complexed), degraded, or altered structurally, no change in substrate concn would be detected. The "alteration" and "oxidase" assays (Fig. 1, 2) indicate that substrate oxidation is the primary enzymatic reaction under these conditions, because the presence of other enzymes that could compete for these substrates was not evident. Pigment formation by the complexing of oxidation products with available constituents, is a reaction that follows oxidation. The variation in oxidase activity with the three phenolic substrates may reflect isozyme-substrate specificity.

These studies do not clarify the mechanism of the resistance shown by cultivar NC 80383-5-9. This is quantitative, or horizontal, resistance and is characterized by fewer lesions per unit leaf area, coupled with less chlorotic halo formation and necrosis (3, 6, 7). Therefore, some level of constituent gradation rather than specific qualitative differences between cultivars, might be involved in the resistance mechanism. The gradational increase in the enzymatic oxidation rates of tyrosine, DOPA, and chlorogenic acid from prehalo to necrotic tissues (especially as seen in the resistant cultivar) may reflect horizontal resistance. The rapid oxidation of phenols and pigment formation provides a possible explanation of the mechanism by which fungal pathogen is contained and may explain the corresponding decrease in lesion numbers and size.

Observing the biochemistry of brown-spot lesion development leads to an understanding of the epidemiological significance of the changes. Since the growth and sporulation of the pathogen is confined to necrotic tissue, the rate of lesion enlargement regulates secondary inoculum production. The rate of increase in necrotic tissue was linear in both susceptible and resistant leaves, but twice as fast in susceptible leaves (8). In the field, sporulation was observed on 50% of a population of lesions when necrotic area approached 23 mm^2. Thus, susceptible tobacco is subjected to more extensive damage from rapidly expanding lesions and increased inoculum.

Leaves of all ages are susceptible to infection by *A. alternata* (13). However, in young leaves with a potential for meristematic activity, a cicatrix of host cells quickly forms which stops fungal advance and restricts the size of the lesion (1-mm diam). This links the physiological age of the leaf at the time of infection to the development and spread of brown spot.

Thus, two factors, susceptibility and physiological age, are important determinants to brown-spot lesion development. Our research has demonstrated an association between several biochemical parameters involved in brown-pigment formation and lesion development. We believe that by understanding the mechanism of complexing and brown-pigment formation we can explain the variables which affect lesion development in tobacco brown spot.

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