The Relationship of Peroxidase and Ortho-Diphenol Oxidase to Resistance of Sugarbeets to Cercospora Leaf Spot

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ABSTRACT

The localization and fluctuation of peroxidase and *o*-diphenol oxidase was related to the mechanism of resistance of sugarbeets (*Beta vulgaris*). In all four varieties of sugarbeets representing a wide range of resistance, peroxidase was located mainly in the soluble fraction, whereas chloroplasts and mitochondria had only small amounts. On the other hand, *o*-diphenol oxidase was located mainly in the chloroplasts, but mitochondria also had an appreciable amount. The amount of *o*-diphenol oxidase associated with the soluble fraction was small. The response to infection was characterized by an immediate increase in the amounts of these enzymes, and this increase was consistently higher in resistant than in susceptible varieties. Toward the advanced stages of the disease, this pattern was reversed, and the increase in the amounts of enzymes was higher in susceptible varieties. The significance of localization and changes in the amounts of these enzymes in the resistance of sugarbeet leaves is discussed. Phytopathology 60:238-245.

The significance of peroxidase and phenolase in the disease resistance of many plants and the inhibitory effect of the oxidized phenolic compounds on the growth and certain enzymes of pathogenic microorganisms are well documented. This area has been the subject of many recent reports, books, and review articles, some of which are especially noteworthy (3, 6, 7, 10, 13, 15, 19, 20, 21, 22, 24). In addition, as was reported by Sizer (23), peroxidase and phenolase can inactivate certain enzymes directly by oxidizing their tyrosyl groups.

In studies of the biochemical nature of resistance in sugarbeet (*Beta vulgaris* L.) leaves against the leaf spot disease caused by *Cercospora beticola* Sacc., we found that an oxidation product of an *o*-dihydroxyphenol (13) isolated from sugarbeet leaves, later identified as 3-hydroxytyramine (8, 9), was highly toxic to cultures of *C. beticola*. Subsequent statistical studies using several resistant and susceptible varieties of sugarbeets revealed that 3-hydroxytyramine in disease-free plants was associated with high *Cercospora* leaf spot resistance (12, 17).

The present work deals with the localization of peroxidase (Donor: H_2O_2 oxidoreductase, E.C.1.11.1.7) and *o*-diphenol oxidase (*o*-diphenol:O_2 oxidoreductase, E.C.1.10.3.1) in subcellular organelles of sugarbeet leaves. The work also includes a study of changes in the amounts of these enzymes during the disease development. An attempt has been made to determine the possible significance of the enzymes in *Cercospora* leaf spot resistance.

MATERIALS AND METHODS.—*Leaves.*—Leaves from four sugarbeet varieties that vary in resistance to *C. beticola* were used. US 201, highly resistant, is a synthetic variety from several inbred lines, and is a heterogeneous population of heterozygous plants. GWI-29, resistant, is inbred and is a homogeneous population of homozygous plants. R&G Pioneer, lightly resistant, is a mass-selected variety and a heterogeneous population of heterozygous plants. 52-334, least resistant, inbred, is a product of several generations of selfing, and is a homogeneous population of homozygous plants.

All the leaves used in this work were supplied from field plots by Richard J. Hecker and John O. Gaskill, research geneticist and research pathologist, respectively, Crops Research Division, ARS, USDA, Fort Collins. One set of leaves was obtained from each of four varieties of sugarbeets growing in a field under normal conditions (Field A). The other set of leaves was obtained from a field (Field B) where the varieties were mass-inoculated with spores of *C. beticola*. Field B was maintained under high humidity conditions to favor the disease development. In both fields, A and B, each sugarbeet variety was growing in four replicates in separate plots. The leaves of a given variety were picked from each of the four plots and combined. The sampling procedure was similar to that described by Harrison et al. (12).

Sampling schedule.—The sugarbeet leaves at different disease development stages (Field B) and corresponding healthy stages (Field A) were sampled as given below. Stage 1: The first samples were collected on 5 July 1968. The plants were about 2 months old. Plants in both Field A and Field B were healthy, as the latter field was not yet inoculated. On 9 July 1968, 4 days after Stage 1, all the plants in Field B were inoculated. Stage 2: The second series of samples were collected on 15 July 1968, or 6 days after inoculation. At this stage, no leaf spots had appeared on leaves from either Field A or Field B. Stage 3: The third series of samples were collected on 31 July 1968, or 22 days after inoculation. At this stage, the disease

had advanced considerably. In Field B, the spots had covered almost the entire surface of the leaves of susceptible varieties (R&G Pioneer and 52-334). At the same time, there were very few spots (2-8 spots/leaf) on the leaves of resistant varieties (US 201 and GWI-29). In Field A, there were few spots (5-10/leaf) on the leaves of susceptible varieties, and practically none on the leaves of resistant varieties.

All the leaf samples were frozen immediately in the field, using dry ice, and held at −20°C in the laboratory.

Subcellular fractionation.—The procedure given below was similar in principles to those described by Arnon et al. (2) and Mayer & Friend (18). All of the operations were carried out at 0-2°C. Ten g of sliced leaf blades were ground by hand in a precooled mortar with 25 ml of an "isotonic" medium and 10 g of precooled sand. This medium consisted of 550 ml 0.5 M sucrose, 80 ml Tris [tris (hydroxymethyl) amino methane] buffer (pH 7.8, $\mu = 0.05$), 30 ml 0.01M KCl, and ascorbate in above to give a concentration of 0.005 M. The grinding was continued for 2 min. The resulting slurry was squeezed through two layers of cheesecloth and centrifuged for 1 min at 200 *g* to sediment whole cells, debris, and sand. The supernatant was collected and centrifuged for 10 min at 1,100 *g*. The sediment consisted mainly of chloroplasts, and the supernatant contained mitochondria, soluble fraction, and some broken chloroplasts (X).

The chloroplast fraction was washed by suspending it in 15 ml of "isotonic" medium using a stirring glass rod, and was centrifuged again for 10 min at 1,000 *g*. The supernatant was discarded. The sediment (Y) was resuspended in 15 ml of the same medium and centrifuged for 2 min at 200 *g* to remove clumped chloroplasts. This suspension containing chloroplasts was abbreviated as C.

The fraction (X) was centrifuged for 30 min at 20,000 *g*. The supernatant containing soluble fraction (S) was decanted and collected. The sediment (Z) was resuspended in 15 ml of "isotonic" medium mentioned above by gentle stirring in a glass homogenizer. The homogenized preparation was centrifuged for 5 min at 1,000 *g* to remove any chloroplasts and clumps. The supernatant fraction (M) consisted of mitochondria.

For peroxidase assay (see below) it was not possible to use particulate fractions C and M; therefore, both these fractions were extracted. For obtaining chloroplast extract (CE), the sediment (fraction Y) was homogenized for 2 min in 15 ml of Tris-KCl medium (8 parts Tris buffer, pH 7.8, $\mu = 0.05$ and 3 parts 0.01 M KCl). The resulting homogenate was centrifuged for 10 min at 20,000 *g*. The residue was discarded, and supernatant (CE) was collected. For obtaining mitochondrial extract (ME), the fraction (Z) was processed similarly.

Enzyme assay.—Peroxidase units in CE, ME, and S fractions were determined. The spectrophotometric assay given in Worthington Manual (25) was followed. Accordingly, the rate of decomposition of hydrogen peroxide by an enzyme preparation with *o*-dianisidine as hydrogen donor was determined by measuring the change in absorbance at 460 mμ. One unit of peroxidase activity is that amount of enzyme catalyzing the decomposition of one μmole of hydrogen peroxide/min at 30°C. The various preparations were diluted so that the reaction rate followed a linear rate for at least 4 min, and the absorbance did not exceed 0.4 at the end of this reaction period.

Ortho-diphenol oxidase activity was determined by measuring the O_2 consumption at 35°C, using the conventional Warburg techniques. Using 3-hydroxytyramine as substrate, the enzymatic activity present in chloroplasts (C), mitochondria (M), and soluble (S) fractions was determined. The main compartment of flask contained 1.0 ml phosphate buffer (pH 7.0, $\mu = 0.2$), 1.0 ml 0.02 M ascorbic acid (20 μmole), and 0.5 ml 0.008 M 3-hydroxytyramine HCl (4 μmole). The center well contained 0.1 ml of 10% KOH. The side arm contained 0.5 ml of the given fraction. The reaction was carried for 12 min, and readings were taken at intervals of 1 min. From the initial velocities, μliter of O_2 consumption per min was calculated and converted to μmole of O_2/min. One unit of *o*-diphenol oxidase activity is that amount of enzyme catalyzing the consumption of 1 μmole of O_2/min.

It has been generally accepted (4, 5, 14) that in the presence of ascorbic acid, the over-all reaction rate depends only upon the velocity of *o*-diphenol oxidation. Ascorbate reduces quinone back to phenol instantaneously and for a duration of time sufficient to allow an accurate measure of initial velocity. In addition, ascorbic acid prevents inhibitions caused by the oxidation products of the substrate (Fig. 1).

Protein determination.—Protein was extracted from all the fractions (C, CE, M, ME, and S), following the procedure of Anderson (1). An equal volume of 20% trichloroacetic acid was added to the above-mentioned fractions (final TCA concentration 10%). The resulting precipitate was washed twice with acetone. The washed precipitate was extracted with 0.1 N NaOH for 2 hr at 50°C. The undissolved material was centrifuged off. The clear NaOH extract was used for determination of protein by the method of Lowry et al. (16).

Dry wt determinations.—Healthy and diseased leaves of all four varieties from three stages were used. The whole set was done in duplicate. Ten g of sliced leaf blades were taken in aluminum weighing cups. The samples were first dried in an oven at 70°C for 12 hr. They were then transferred to a vacuum oven and were dried under vacuum over P_2O_5 for 24 hr. The dry wt was calculated by difference.

Expression of results.—The units of peroxidase and *o*-diphenol oxidase activities were expressed in two ways: units of enzyme activity/mg of protein and units of enzyme activity/g of leaf sample (dry wt).

All data on enzyme units were the averages of three separate measurements. Thus, the leaves of a given sugarbeet variety at a given stage were divided into three batches. Each batch was subjected to subcellular fractionation separately. Similarly, the enzyme activity

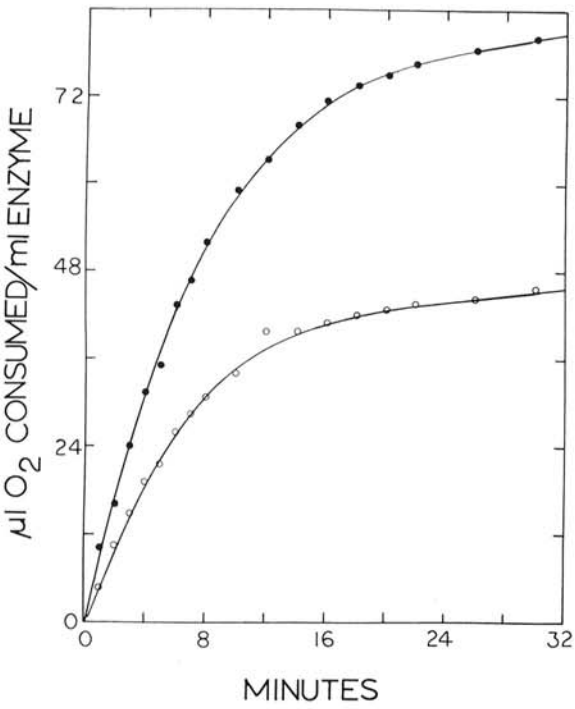

Fig. 1. Relationship between oxygen consumption and time during the oxidation of 3-hydroxytyramine catalyzed by *o*-diphenol oxidase in the presence (●) and the absence (○) of ascorbic acid. The upper curve (●) was obtained when the Warburg flask had all the reactants described in the text under *o*-diphenol oxidase assay. With the exclusion of ascorbic acid from the reaction mixture, the lower curve (○) was obtained.

and protein concentration were determined in each batch separately.

Enzymes of Cercospora beticola.—In order to assess the significance of peroxidase and *o*-diphenol oxidase present in sugarbeet leaves, it was necessary to determine whether the fungus produces these enzymes. Single spore cultures of *C. beticola* were used. The fungus was grown in the following media and the enzymes were extracted.

1) Beet leaf agar medium: Fifty g of finely sliced blades of sugarbeet leaves were boiled in 200 ml H_2O for 20 min. The resulting slurry was passed through two layers of cheesecloth. Three g Bacto-Agar was dissolved in the above extract, and final volume made to 200 ml. After sterilization, the medium was put into petri plates. Each plate was inoculated with a single spore of *C. beticola,* and incubated for 10 days at 28°C. The cultures were then sliced, homogenized with phosphate buffer (pH 7.0, $\mu = 0.1$), and centrifuged for 10 min at 10,000 *g*. The extract was brought to 0.8 saturation by the addition of solid ammonium sulfate. The resulting precipitate was separated by centrifugation, dissolved in one-tenth of the original extract volume in distilled H_2O, and assayed for peroxidase and *o*-diphenol oxidase.

2) Synthetic medium with beet leaf extract: The medium consisted of the following (per liter): Ammonium sulfate, 1.4 g; potassium dihydrogen phosphate, 2.0 g; magnesium sulfate heptahydrate, 0.3 g; calcium chloride, 0.2 g; ferrous sulfate heptahydrate, 5.0 mg; sucrose, 10.0 g; and beet leaf extract, 150.0 ml. It was dispersed, 100 ml/Roux bottle, and sterilized. Each Roux bottle was inoculated with 5.0 ml of *C. beticola* spore suspension in H_2O, laid flat, and incubated at 28°C. The cultures were harvested for enzyme extraction after 7 and 14 days of incubation. The culture filtrate was separated from the fungus by centrifuging for 10 min at 10,000 *g*. The procedure for extracting enzymes from the culture filtrate by ammonium sulfate fractionation was the same as given above.

3) Synthetic medium without beet leaf extract: The composition of this medium was similar to the one given above, except that no beet leaf extract was included. The procedure for growing the cultures and extracting enzymes was the same as above.

RESULTS.—*Enzymes of Cercospora beticola.*—The tests for peroxidase and *o*-diphenol oxidase in all three preparations were negative. Apparently, *C. beticola* does not produce either enzyme, and the increased enzyme activity observed in infected leaves (see below) can be attributed solely to the host response.

Peroxidase in sugarbeet leaves.—In all varieties from both fields, the soluble fraction (S) had higher specific activity than the chloroplasts or mitochondria. Compared to the soluble fraction, the chloroplasts (CE) and mitochondria (ME) had lower specific activities; however, between these two fractions, CE had more.

At Stage 1, with the exception of variety 52-334, the soluble fractions of the resistant varieties had more peroxidase than the soluble fractions of the susceptible varieties. This was true for varieties from both fields A and B. In variety 52-334, the peroxidase content of the soluble fraction was about equal to that of GWI-29. The increase in peroxidase content in the soluble fraction following inoculation distinguished resistant from susceptible varieties. Thus, between Stage 1 and Stage 2 this increase was greatest in US 201 (10 units) followed by GWI-29 (5 units). R&G Pioneer showed an increase of only 1.9 units, whereas 52-334, a most susceptible variety, had a negligible increase of 0.6 units.

Between Stage 1 and Stage 2 in US 201, which is the most resistant variety, the increase in specific peroxidase activity in CE and ME fractions was also highest as compared to the increase in the corresponding fractions in the other three varieties (Fig. 2-A). In varieties GWI-29, R&G Pioneer, and 52-334, the observed increments in specific peroxidase activity in CE and ME fractions were not clearly related to their resistance (Fig. 2).

Between Stage 2 and Stage 3, the increase in the peroxidase content in the soluble fraction was negatively correlated to the resistance. This pattern was just the reverse of that we observed between Stage 1 and Stage 2. Thus, variety 52-334, which showed the highest leaf spot incidence, also had the highest increment of peroxidase, and in US 201, which showed hardly any leaf spot development, the increase in peroxidase was negligible.

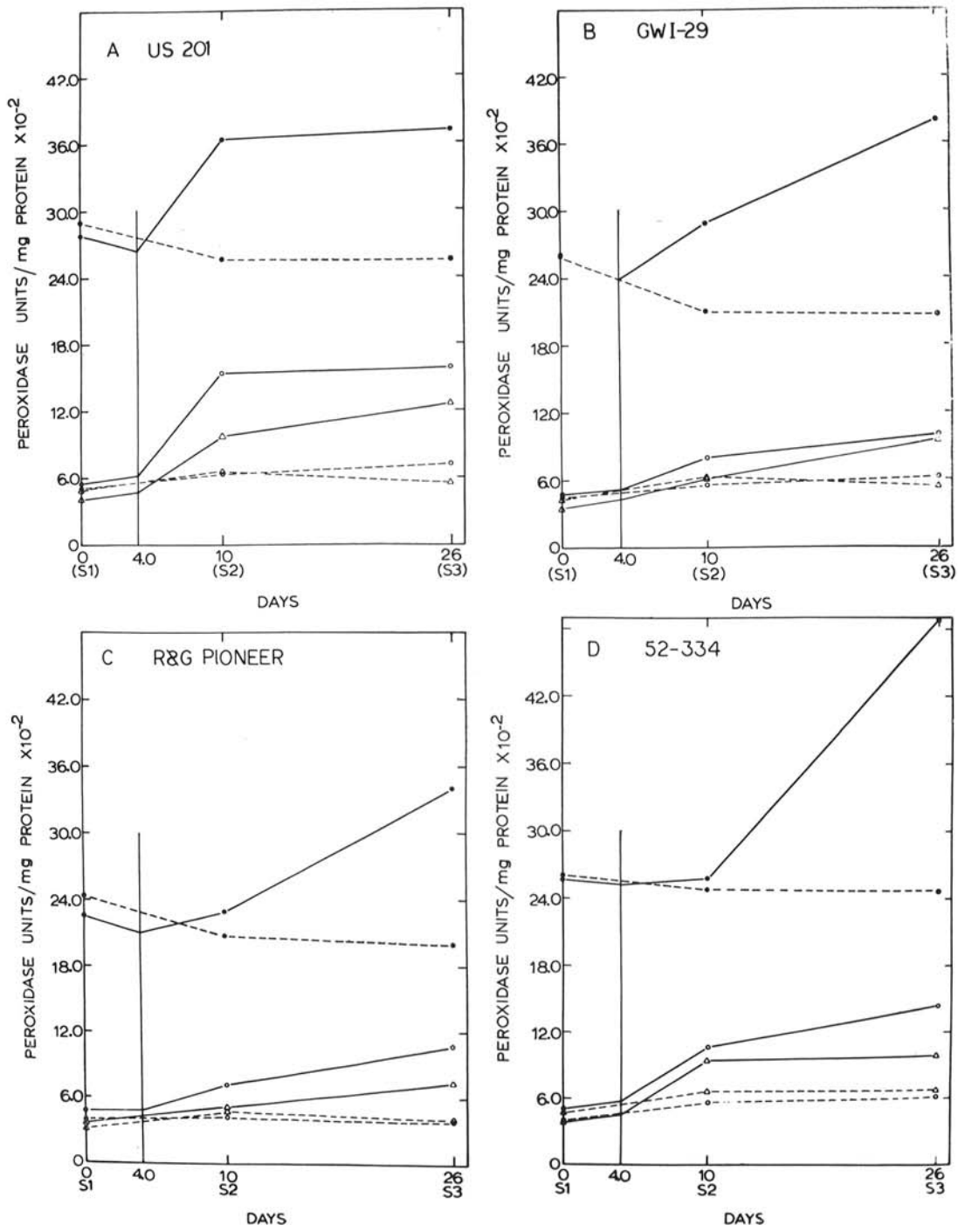

Fig. 2. The effect of infection by *Cercospora beticola* on peroxidase concentration in three subcellular fractions of the leaves of four varieties of sugarbeets: US 201 (A); GWI-29 (B); R&G Pioneer (C); and 52-334 (D). The three subcellular fractions assayed were: chloroplast extract (○); mitochondria extract (△); and soluble fraction (●). Peroxidase units/mg protein were plotted as function of Stage 1 (S_1), Stage 2 (S_2), and Stage 3 (S_3). At S_1, leaves of all the varieties in both fields, A and B, were free of infection. After 4 days (shown by vertical line), all varieties in Field B were inoculated, while Field A remained under normal conditions to provide healthy controls. S_2 and S_3 represent 6 and 22 days, respectively, after inoculation. The specific peroxidase activity in three subcellular fractions during the disease development (solid lines) and in the healthy controls (broken lines) are shown.

TABLE 1. Total peroxidase and *o*-diphenol oxidase units/g dry wt of leaves in four varieties of sugarbeet during *Cercospora* leaf spot development. Total peroxidase and *o*-diphenol oxidase units represent the sum of units present in chloroplast, mitochondria, and soluble fractions

Var.	Enzyme units (E.U.)			Increase in E.U.		Increase in E.U. corrected[b]	
	S_1[a]	S_2	S_3	From S_1-S_2	From S_2-S_3	From S_1-S_2	From S_2-S_3
			Peroxidase units				
US 201 (HR)[c]	26.27	32.22	41.11	5.95	8.89	5.35	4.00
GWI-29 (R)	18.15	23.97	38.10	5.82	14.13	5.20	10.32
R&G Pioneer (S)	15.86	20.72	34.98	4.86	14.26	4.40	10.37
52-334 (HS)	17.19	20.11	38.30	2.92	18.19	2.28	14.34
			Ortho-diphenol oxidase units				
US 201	12.25	14.85	20.87	2.60	6.02	3.55	4.54
GWI-29	9.65	12.65	19.51	3.00	6.86	2.87	4.60
R&G Pioneer	10.11	11.88	24.34	1.77	12.46	0.80	6.71
52-334	9.48	11.40	43.53	1.92	32.13	0.96	25.52

[a] S_1, S_2, and S_3 = Stage 1, Stage 2, and Stage 3, respectively.
[b] Corrected by using the values for changes in enzyme units in healthy controls.
[c] HR, R, S, and HS represent highly resistant, resistant, susceptible, and highly susceptible varieties.

Between Stage 1 and Stage 2, the response (measured in terms of change in peroxidase) to infection by different subcellular fractions of a given variety was more or less the same. Variety 52-334 was an exception in this regard, and here the increase in peroxidase in CE and ME fractions was greater than the increase in the soluble fraction. Between Stage 2 and Stage 3, the increase in the specific peroxidase activity was greatest in the soluble fractions of all four varieties (Fig. 2).

Specific peroxidase activity decreased in the soluble fractions of healthy leaves in the four varieties between Stages 1 and 2. Between Stage 2 and Stage 3, the activity was relatively constant. The peroxidase content in CE and ME fractions of all four varieties remained relatively constant throughout the three growth stages (Fig. 2).

The total peroxidase units (sum of enzyme units present in CE, ME, and S fractions) per g of dry wt of leaves from Field B are shown in Table 1. At Stage 1, the resistant varieties had more peroxidase units than the susceptible varieties. At Stage 2, 6 days after inoculation, the increase in peroxidase units was higher in resistant than in susceptible varieties. This increment was greatest in US 201, and was lowered in proportion to the resistance of the varieties. Between Stage 2 and Stage 3, the pattern was reversed. Thus, toward the advanced stages of disease development the increase in peroxidase units was higher in the susceptible varieties. The data on patterns of fluctuation in total enzyme units (Table 1) were consistent with the specific activity data for the soluble fractions (Fig. 2).

Ortho-diphenol oxidase in sugarbeet leaves.—The highest content of *o*-diphenol oxidase was located in the chloroplasts (C) in all varieties from both fields (Fig. 3). The mitochondrial fraction (M) was next in this respect. The soluble fraction (S) had very low *o*-diphenol oxidase content.

In the chloroplast fractions at Stage 1, the resistant varieties had more *o*-diphenol oxidase than the susceptible ones. This was true for all the varieties from Field B, and all but variety 52-334 from Field A.

Following infection, at Stage 2, there was a rise in specific *o*-diphenol oxidase activity in different subcellular fractions of all four varieties. Unlike the peroxidase, the increment in *o*-diphenol oxidase content was more or less the same in C, M, and S fractions in the resistant and susceptible varieties. Toward the advanced stage (Stage 3) of the disease development, the increment in the enzyme content in all three fractions was much higher in the susceptible varieties. The more susceptible the variety, the higher was the increase (Fig. 3).

Between Stage 1 and Stage 2, the response, as indicated by an increase in specific *o*-diphenol oxidase activity, was most pronounced in the chloroplast fraction (C) of all four varieties. Between Stage 2 and Stage 3, however, this increase was greatest in the mitochondrial fraction (M) of all varieties.

At Stage 3, the increased specific activity observed in the soluble fraction may have resulted from leakage of the enzyme from chloroplasts and mitochondria. The disintegrated chloroplasts were evident in and around the central necrotic zone of spots on sugarbeet leaves (Fig. 4). The increment at Stage 3 was greatest in the soluble fraction of variety 52-334, which would be expected, as the damage by leaf spots was most extensive in this variety.

The data on the leaves of healthy controls showed that the specific *o*-diphenol activity of all fractions remained relatively constant throughout the three growth stages.

The total *o*-diphenol oxidase data presented in Table 1 are for the leaves from Field B. At Stage 1, with the exception of R&G Pioneer, the resistant varieties had more enzyme units. At Stage 2, 6 days after the inoculation, the susceptible varieties had a higher increase in *o*-diphenol oxidase units than the resistant varieties. Comparison of data in Table 1 with those in Fig. 3 shows that between Stage 1 and Stage 2, the data on

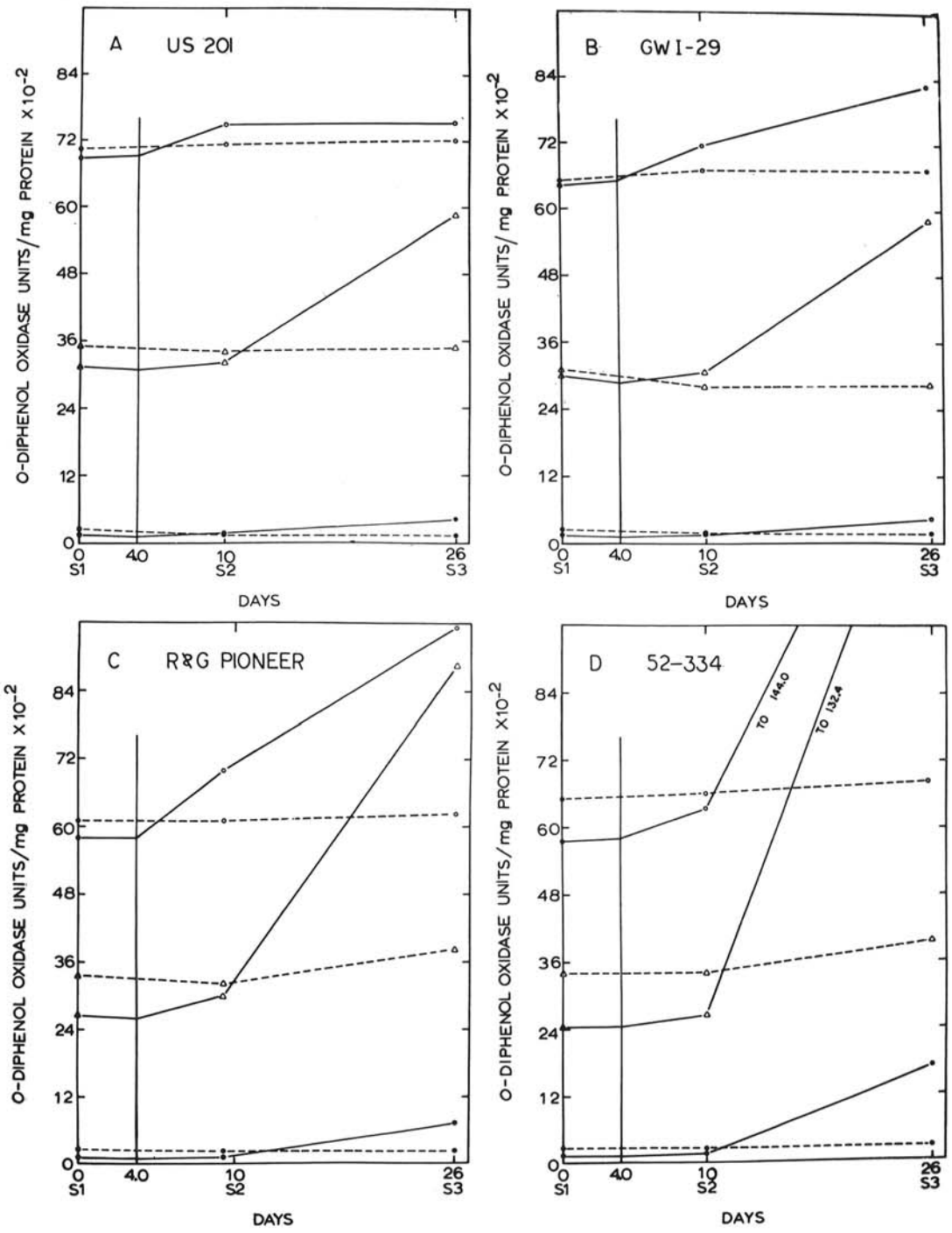

Fig. 3. The effect of infection by *Cercospora beticola* on *o*-diphenol oxidase concentration in three subcellular fractions of the leaves of four varieties of sugarbeets: US 201 (A), GWI-29 (B), R&G Pioneer (C), and 52-334 (D). The three subcellular fractions assayed were: chloroplasts (○); mitochondria (△); and soluble fraction (●). Units of *o*-diphenol oxidase/mg protein were plotted as function of Stage 1 (S_1), Stage 2 (S_2) and Stage 3 (S_3). At S_1, leaves of all the varieties in both fields, A and B, were free of infection. After 4 days (shown by vertical line), all varieties in Field B were inoculated, while Field A remained under normal conditions to provide healthy controls. S_2 and S_3 represent 6 and 22 days, respectively, after infection. The specific *o*-diphenol oxidase activity in three subcellular fractions during the disease development (solid lines) and in the healthy controls (broken lines) are shown.

enzyme units/g dry wt of leaves were not consistent with the data on specific activities. In the latter case, no distinction between the resistant and susceptible varieties could be shown. This situation may result if in the resistant varieties, following infection, some proteins (other than *o*-diphenol oxidase) were produced at a rate faster than that in the susceptible varieties.

Between Stage 2 and Stage 3, the pattern of change in *o*-diphenol oxidase units was just the reverse of that between Stage 1 and Stage 2. Thus, toward the advance stages (Stage 3) of disease development, the increment in total *o*-diphenol oxidase units was more pronounced in the susceptible varieties. This pattern was consistent with the specific *o*-diphenolase activity pattern shown by all three subcellular fractions between Stage 2 and Stage 3 (Fig. 3). This pattern was also similar to that exhibited by peroxidase (Table 1).

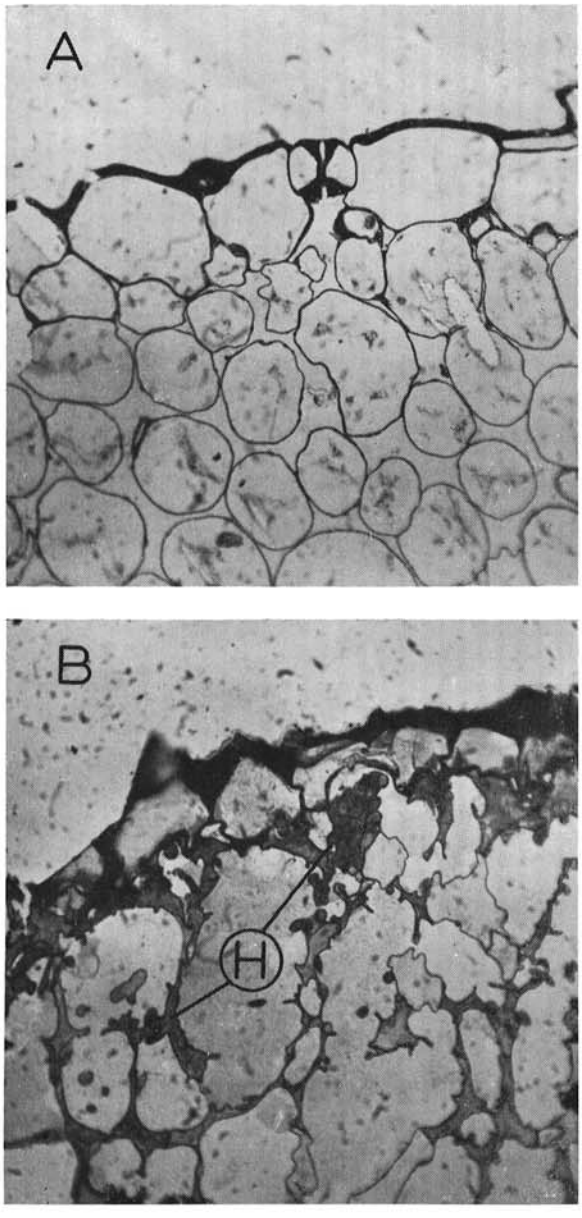

Fig. 4. Photomicrographs of sections through a spot on the leaf of sugarbeet (R&G Pioneer). **A)** Section at zone surrounding the central necrotic zone of the leaf spot. Note the disintegrated chloroplasts. The cells in this zone are not disorganized. **B)** Section through the dark necrotic center zone. Note that the cells are extensively damaged and the chloroplasts are disintegrated. The hyphae (H) of *Cercospora beticola* were present throughout this zone, both inter- and intracellularly.

Discussion.—G. Minz and Z. Solel of Israel (*personal communication*) found that the percent of germination of *C. beticola* spores on the leaves of the resistant and susceptible varieties of sugarbeets is essentially the same. *Cercospora* spores, upon germination, form appressoria which give rise to infection hyphae. The latter penetrate the stomatal openings and infect the adjacent parenchyma cells. Branching of hyphae occurs either before or after the penetration of parenchyma cells. Penetration by the hyphae immediately induces necrotic reactions characterized by the formation of dark-colored compounds.

We observed that the sugarbeet leaves of susceptible varieties in Field B had necrotic spots 7-10 days after inoculation. As the infection progressed, more spots appeared on the leaves of susceptible varieties, and by Stage 3, practically the whole leaf surface was covered with leaf spots. Occasional spots appeared on the leaves of resistant varieties 13 days after the inoculation, and by Stage 3 there were 1-5 spots/leaf in US 201 and 2-8 spots/leaf in GWI-29. In resistant varieties, the number of leaf spots may not correspond to the number of necrotic zones. Here, the infection hypha may be killed soon after it penetrates a cell, and thus the necrosis will be limited to a very small number of cells, not enough to make a spot visible on the outside of the leaf. If this is true, the defensive reactions will be operative in the very early stages of infection, long before the appearance of leaf spots.

The host response measured in terms of peroxidase and *o*-diphenol oxidase suggested that the defense reactions occurred during the early stages of infection. There was a marked increase in the amounts of these enzymes in all four varieties immediately following infection. Although the response of all the varieties was basically similar, there were quantitative differences. Thus, at Stage 2 the increase in peroxidase and *o*-diphenol oxidase was closely correlated to the resistance of a given variety. The higher the concentration of these enzymes, the faster the rate of oxidation reactions that cause necrosis of host cells as well as that of fungal hyphae. Disruption of cell compartmentation and disintegration of subcellular organelles resulting from infection will also increase the availability of various substrates for oxidation by these enzymes.

With regard to imparting resistance, the amounts of enzymes present in disease-free plants probably were not as significant as the changes in their amounts in response to infection. Thus, even though US 201 had the highest amounts of peroxidase and *o*-diphenol oxidase before infection, in the other three varieties the amounts of these enzymes were not always correlated

with their resistance. On the other hand, as has been mentioned above, the increment in these enzymes in response to infection was always correlated with the resistance.

Toward the late stages of the disease development (between Stage 2 and Stage 3), the increase in peroxidase and *o*-diphenol oxidase levels became greater in susceptible varieties. This pattern was just the opposite of that we found between Stage 1 and Stage 2. This behavior suggested that in the susceptible varieties, the increase in the enzymes soon after infection was not large enough (and fast enough) to inhibit the pathogen. By Stage 3, numerous cells in the leaves of the susceptible varieties were infected. Thus, the net increase of enzyme levels in all these cells became greater than the increase shown by the resistant varieties, where only a few cells became infected by Stage 3. Also, a variety of other microorganisms infecting the leaf tissue in and around the necrotic zone in the late stages of the disease may have contributed to the increase of enzyme levels in the susceptible varieties.

Although our data indicated that both peroxidase and *o*-diphenol oxidase were significant in resistance, peroxidase may play a more important role in the initial defense reactions. Peroxidase in the presence of hydrogen peroxide or certain other inorganic peroxides is capable of oxidizing a wide variety of hydrogen donor compounds (11). Compared to peroxidase, *o*-diphenol oxidase has a limited range of substrates. Also, the localization of peroxidase mainly in the soluble fraction could be significant, as the fungus is more likely to come in contact first with the cytoplasm of host cells. The occurrence of *o*-diphenol oxidase in the chloroplasts and mitochondria suggested that this enzyme was involved in the defense reactions of these organelles rather than those of the cytoplasm. During the infection process, the chloroplasts and mitochondria are likely to be affected after the cytoplasm. Therefore, *o*-diphenol oxidase may operate after the peroxidase in the sequences of defense reactions.

The mechanism of *Cercospora* leaf spot resistance in sugarbeets probably involves several factors. On the basis of the data presented in this paper and the reports (12, 17) on the positive correlation between the amounts of 3-hydroxytyramine and resistance, we suggest that peroxidase, *o*-diphenol oxidase, and their substrates are some of the factors contributing significantly to the resistance of sugarbeets.

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