# Some Chemical Aspects of Resistance to Cercospora Leaf Spot in Sugar Beets[1]

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Cercospora leaf spot, potentially a highly destructive disease of the sugar beet (*Beta vulgaris* L.) in many parts of the world, is being partially controlled by the use of resistant varieties. Leaf-spot resistant commercial varieties currently available in the United States, under conditions of moderate disease exposure, provide relatively satisfactory control; however, in seasons or localities in which conditions are especially favorable for development of the causal organism (*Cercospora beticola* Sacc.), such varieties may be seriously damaged by the disease. Consequently, varieties with improved resistance are urgently needed. The investigations reported in this article where undertaken in an attempt to explore the mechanism of leaf-spot resistance and to point the way toward improved selection techniques.

## Review of Literature

In order for an infection to be successfully completed there must be an interaction among the host, the pathogen, and the environment, which favors the pathogen and its parasitic establishment. It is only in the last few decades that a pathogen's preference for certain varieties of the same species has been investigated. In the majority of cases investigated, the factors responsible for disease resistance have fallen into one of the following categories: disease escape, mechanical exclusion, lack of an essential substance for development of the pathogen and presence or production of a substance toxic to the pathogen (6, 7, 21, 22).[3]

A possible mechanism of resistance of young leaves to Cercospora leaf spot was postulated regarding stomata size when it was observed that the pathogen had to gain its entrance through the open stomata (16), but there is adequate evidence that even seedlings with small stomates can be infected (20). In another investigation of resistance to Cercospora leaf spot no significant differences were found in regard to the size and number of stomates on resistant and susceptible varieties (8).

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[3] Numbers in parentheses refer to literature cited.

Kovacs (13) found that the low incidence of local lesions on the leaves of a resistant sugar beet variety was correlated with the presence of diffusable inhibitors on healthy leaves. Fewer spores of *C. beticola* germinated on the resistant leaves and a correspondingly smaller percentage of the spores germinated in dew or water washings collected from resistant leaves. No attempt was made to identify or characterize the inhibitor.

From growth studies of *C. beticola* in pure culture it seemed unlikely that the resistant leaves could be lacking in any essential nutrient for the growth of the organism (4).

As early as 1911 Cook et al. (3) pointed out that at the point of penetration of a pathogen the action of polyphenoloxidases might bring about reactions which could inhibit the progress of the pathogen. Since that time there has been a great deal of literature published regarding the role of phenolic compounds in relation to disease resistance. Thoroughly investigated examples are those of resistance to onion smudge (14, 15) and resistance of potatoes to common scab (9, 10). In the first instance the resistance was found to be due to concentrations of catechol and protocatechuic acid and in the latter to chlorogenic acid. In the case of potato scab resistance, certain polyphenolic compounds were found to promote the development of a thicker suberized layer (17).

The polyphenolase enzyme may function in the disease resistance mechanism and has been found capable of being activated (1, 11, 12). In sugar beet leaves most of the enzymes are in the stable adsorbed condition on the plastids and autolysis of the plastids causes the enzymes to go into solution (18, 19).

## Material

The eight inbred lines of sugar beets used in this investigation are listed in Table 1. The leaf-spot readings, furnished by the United States Department of Agriculture[1], were made in field plots in 1957 under severe, artificially induced leaf-spot exposure. On this scale 0.0 represents complete immunity while 10.0 represents complete defoliation.

The causal organism, *C. beticola*, was isolated from infected leaves, and single-spore cultures were used for toxicity studies. Potato-dextrose agar was used as the growth medium for the toxicity tests.

## Experimental Design

The eight inbred lines were planted in the United States Department of Agriculture's Sugar Beet Greenhouse in Fort

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Table 1.—Contributors of the inbreds and the relative leaf-spot readings.

| Contributor | Contributor's number | Leaf-spot reading | Experimental line number |
|---|---|---|---|
| United States Department of Agriculture | US 201 | 0.5 | 1 |
| | 52-437 | 7.5 | 7 |
| | 52-335 | 1.0 | 3 |
| The Great Western Sugar Company | GWI-29-55L | 1.0 | 2 |
| | GWI-20-55L | 7.0 | 8 |
| | GWI-21 | 8.0 | 6 |
| American Crystal Sugar Company | ACS-I | 1.3 | 4 |
| Holly Sugar Corporation | 5126-0 | 9.0 | 5 |

Collins, Colorado, September 22, 1959. Each line occurred in four plots. Each plot consisted of two eight-inch pots—each containing two plants—making a total of four plants of a single line per plot. A randomized-block experimental design was employed.

## Methods

The four youngest fully expanded leaves on each plant were harvested January 15, 1960. The leaves, with petioles removed, were frozen immediately and stored in polyethylene bags in a freezer until analysis.

It was found that if the leaves were allowed to thaw at room temperature, the phenolic compounds were partially lost—apparently through oxidation by the polyphenolase enzyme. Hence, for extraction of phenolic compounds, the frozen leaves were placed in 95% ethanol and ground immediately in a Waring blender for three minutes. The concentration of the ethanol was kept above 80% to insure deactivation of the enzyme as soon as the cell was ruptured.

For the extraction, 15 grams of frozen leaves were ground in 75 ml of 95% ethanol and filtered through a Buchner funnel using vacuum. The residue was washed with an additional 25 ml of 95% ethanol. The ethanol solution was allowed to stand for one hour at room temperature and then refiltered through Whatman No. 50 filter paper. This solution was taken to dryness under partial vacuum with gentle heat and the residue taken up in 10 ml of distilled water. The aqueous solution was made to pH 2.5 using concentrated hydrochloric acid. The acidic solution was extracted three times with 10 ml portions of ether to remove some undesirable components, and the resulting solution was used for further experiments. For convenience this crude solution will be referred to as a phenolic extract.

The phenolic extracts and the oxidized phenolic extracts were tested for toxicity by placing a drop next to a 2 cm mat of *C. beticola* and observing the growth three days later.

The polyphenolase enzyme was isolated from the frozen leaves by allowing them to thaw and pressing to extract the juice. This juice was centrifuged, dialyzed at 4° C with a phosphate buffer at pH 6.7, lyophilized and separated electrophoretically in a phosphate buffer at pH 6.7 with 25 ml of glycerol added per liter. The polyphenolase fraction migrated toward the (–) electrode.

## Analyses and Results

The causal organism grew well on many simple media, including potato-dextrose agar, Czapek's agar etc.; however, the best growth and conidia formation were obtained using a beet-leaf agar from either the resistant or susceptible lines. Conidia production was slight on media such as Czapek's agar, but could be increased many times by the addition of a small amount of riboflavin.

When the phenolic extract from a resistant line was applied to the organism growing in culture, there was very little inhibition, but when the phenolic extract was oxidized prior to application there was severe inhibition. The substance responsible for the inhibition was heat stable, allowing the solutions to be heated to remove the oxidizing agents and also to increase the concentration.

Figure 1 shows the effect of adding two drops of phenolic extract from the most resistant line which had been heated in a water-bath until it was concentrated five times (about 2 to 3

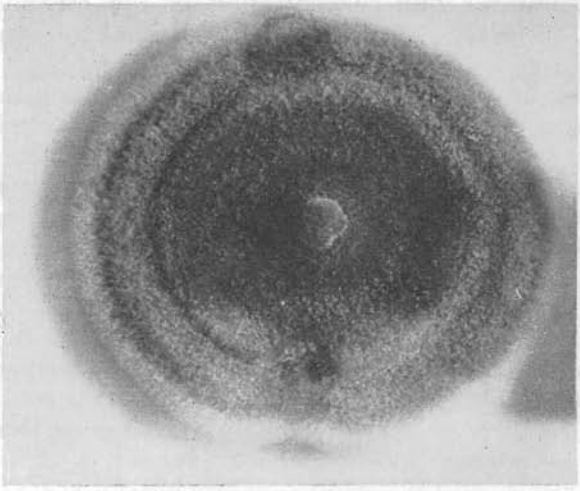

**Figure 1.—The effect of adding two drops of concentrated phenolic extract to a culture of C. *Beticola*.**

hours). The picture was taken three days after application and shows very little inhibition.

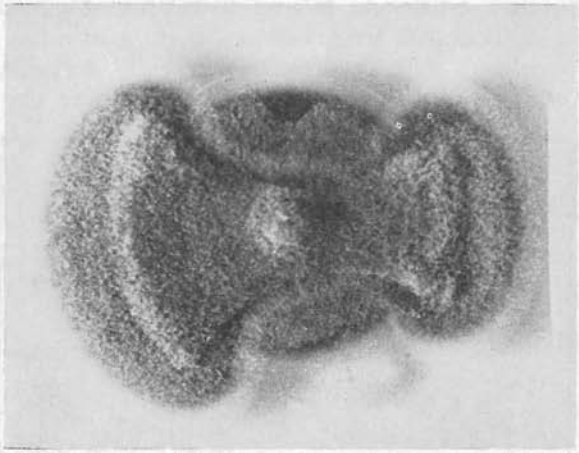

**Figure 2.—The effect of adding, to a culture of *C. beticola*, two drops of concentrated phenolic extract which has been ozidized with hydrogen peroxide.**

Figure 2 shows the effect of phenolic extract treated in a similar manner except that one drop of 30% hydrogen peroxide was added to 10 ml prior to heating. The water-bath treatment was sufficient to remove any excess hydrogen peroxide as indicated by testing with catalase enzyme. There was severe inhibition and even some existing growth was destroyed. This inhibition cannot be attributed to the interaction of the hydrogen peroxide and the hydrochloric acid because, when a water blank was treated with hydrochloric acid, hydrogen peroxide and heated, no inhibition was observed. When the extract from a susceptible line was oxidized and tested in a similar manner negligible inhibition was observed.

A similar inhibition to that in Figure 2 was observed when nitric acid was used instead of hydrochloric to bring the extract to pH 2.5, indicating that the inhibition must have been due to oxidation and not merely to hydrogen ion concentration. Other chemical oxidizing agents had similar effects and the extract could also be oxidized to a toxic substance using the polyphenolase enzyme.

Due to the low concentration of the phenolic extract, paper chromatography seemed to offer the best means of separation and characterization. Figure 3 shows a diagramatic composite of two dimensional chromatograms. The first dimension was run in a butanolacetic acid-water mixture[5], and the second in five

[5] 120 ml butanol, 30 ml acetic acid and 60 ml water.

percent acetic acid. Spot E represents ninhydrin positive compounds (mostly amino acids), spot D represents a sugar spot (mostly glucose), while A, B and C represent phenolic spots. When spots A, B and C were isolated from a number of chromatograms, oxidized with hydrogen peroxide, concentrated and tested for toxicity on the organism; spot A was the only one

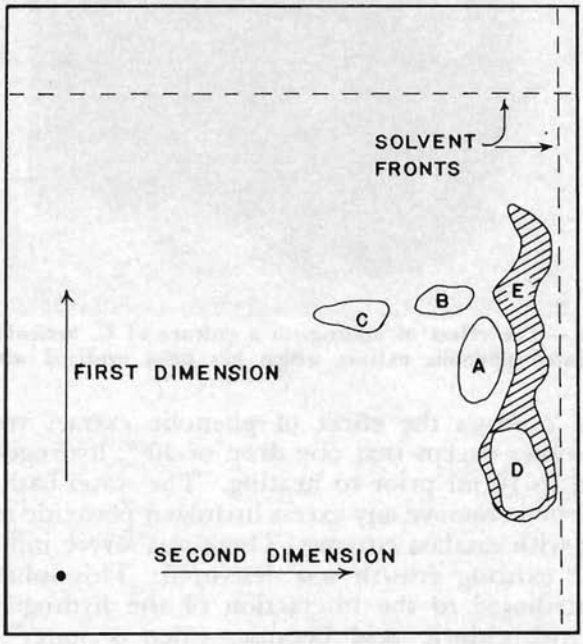


Figure 3.—A diagramatic composite of two dimensional chromatograms.

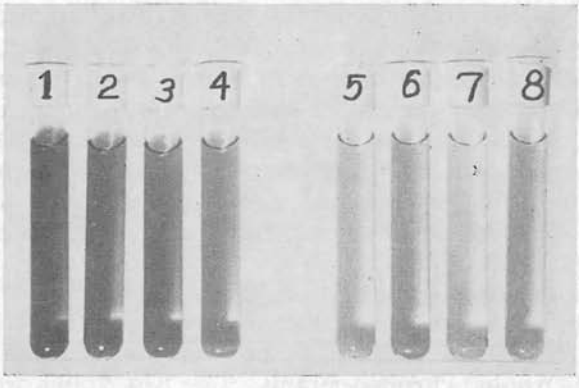


Figure 4.—The relative intensities of the violet color obtained by treating representative samples of sugar beet leaf extracts from lines 1 to 8 with Arnow's reagent.

exhibiting toxicity. It was found that Arnow's reagent (2) modified for paper chromatography[6] reacted selectively with spot A to produce an immediate reddish-brown color in the presence of ammonia vapors.

Since the phenolic compound A in Figure 3 was the only substance in the extract that reacted appreciably with Arnow's reagent, the relative concentration of the compound responsible

Table 2.—Optical density measurements for 32 greenhouse plots of sugar beets.

| Line number | Leaf-spot reading (field) | Plot number (greenhouse) | Optical density | Average of 4 plots |
|---|---|---|---|---|
| 1 | 0.5 | 8 | 2.30 | 1.480 |
| | | 11 | 1.60 | |
| | | 20 | 0.80 | |
| | | 29 | 1.22 | |
| 2 | 1.0 | 1 | 1.60 | 0.885 |
| | | 15 | 0.40 | |
| | | 19 | 0.71 | |
| | | 28 | 0.83 | |
| 3 | 1.0 | 7 | 1.60 | 0.925 |
| | | 14 | 1.50 | |
| | | 21 | 0.50 | |
| | | 30 | 0.10 | |
| 4 | 1.3 | 4 | 0.77 | 0.530 |
| | | 10 | 0.50 | |
| | | 22 | 0.48 | |
| | | 32 | 0.37 | |
| 5 | 9.0 | 6 | 0.22 | 0.2775 |
| | | 9 | 0.61 | |
| | | 17 | 0.15 | |
| | | 27 | 0.13 | |
| 6 | 8.0 | 5 | 0.50 | 0.475 |
| | | 16 | 0.38 | |
| | | 18 | 0.44 | |
| | | 26 | 0.31 | |
| 7 | 7.5 | 2 | 0.24 | 0.200 |
| | | 12 | 0.15 | |
| | | 23 | 0.20 | |
| | | 31 | 0.21 | |
| 8 | 7.0 | 3 | 0.38 | 0.225 |
| | | 13 | 0.22 | |
| | | 24 | 0.11 | |
| | | 25 | 0.19 | |

[6] 1 part water, 2 parts 95% ethanol, and 1 part Arnow's reagent (10 g sodium nitrite and 10 g sodium molybdate in 100 ml water).

for the potential inhibition of the pathogen could be determined in solution. For this analysis 0.5 ml of the phenolic extract, 1.0 ml Arnow's reagent (2), and 25 ml water were added to an Evelyn tube. One ml of 10% sodium hydroxide was added and the resulting violet color read at 515 mμ on a Beckman Model B spectrophotometer. Figure 4 shows a representative sample from each of the eight lines. The violet color is much more intense in the resistant than in the susceptible lines.

The results of this method of analysis are given in Table 2, and Table 3 gives the analysis of variance for those data. From these tables it can be seen that: 1) there is a significant difference at the 1% level between resistant and susceptible lines in the amount of phenolic compound present, 2) the resistant lines contain varying concentrations of the phenolic compound, 3) line number 1 contained the highest concentration of the phenolic compound, and 4) there are significant differences at the 1% level among replications.

**Table 3.—Analysis of variance for optical density measurements presented in Table 2.**

| Source of variation | Mean square | Degrees of freedom |
|---|---|---|
| Resistant vs. susceptible lines | 3.6700** | 1 |
| Within resistant lines | 0.6161** | 3 |
| Within susceptible lines | 0.0342 | 3 |
| Replications | 0.5085** | 3 |
| Lines vs. replications | 0.1152 | 21 |

**Significant at the 1% level.

In an attempt to characterize compound A from Figure 3, identical chromatograms were run and dyed with various reagents. These results are presented in Table 4.

**Table 4.—Color reactions of compound A in Figure 3.**

| Reagent | Color reaction |
|---|---|
| Folin-Denis | blue |
| Sulfanilic acid | red |
| P-nitraniline | purple |
| Arnow's | reddish-brown |
| Ferric chloride | green-blue which fades rapidly |
| Ultra-violet light | very faint purple |

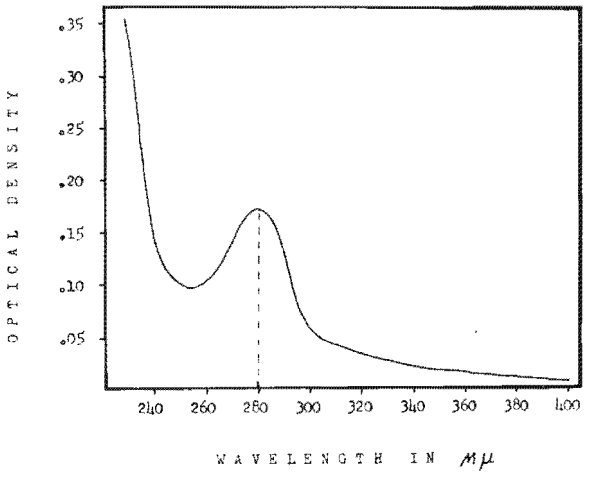


**Figure 5.—The ultraviolet absorption spectrum of the potentially toxic phenolic compound.**

Figure 5 gives the ultraviolet absorption curve obtained by means of a DU Beckman spectrophotometer using substance isolated from spot A Figure 3. For this isolation many chromatograms were run, one chromatogram was dyed, and spot A isolated from the other chromatograms. These spots were eluted with water to give a solution for the analysis. There is a maximum absorption at 280 mμ indicating a phenolic substance which has no conjugation with the benzoid structure.

The phenolic substance responsible for the inhibition of *C. beticola* is water-soluble and heat-stable. When the extract was extracted with ether, ethyl acetate and amyl alcohol and oxidized there was no inhibition to the organism in the ether or ethyl acetate layers, only slight inhibition in the amyl alcohol layer, with most of the toxicity remaining in the aqueous portion.

No differences could be found in the electrophorograms from resistant and susceptible leaves. The portion containing the polyphenolase activity moved farther toward the cathode than any other zone of protein. This portion accounted for a considerable percentage of the entire cytoplasmic protein in the leaves. The activities of the enzymes obtained from resistant and susceptible lines were very nearly the same, although activation could have taken place during extraction. Because of the relative abundance and activity of the polyphenolase enzyme in the susceptible as well as the resistant lines, it appeared unlikely that the enzyme could be the limiting factor in any reaction controlling resistance.

In another experiment washings were taken from line number 1 and a susceptible line (not included in the greenhouse series) by washing the leaves with a washbottle filled with distilled water and collected in a polyethylene bag. The washings were taken from 50 leaves (both upper and lower surfaces) and were about 150 ml each. These were concentrated to 10 ml in a water bath and examined chromatographically. In the washings from the resistant line there was a faint spot corresponding to spot A in Figure 3. It gave the same color reactions as spot A and was entirely lacking in the washings from the susceptible line.

## Discussion

From the various color reactions with reagents on chromatograms and from the ultraviolet absorption data the compound responsible for the toxicity to *C. beticola* appears to be a single ortho-dihydroxy phenolic compound which has no conjugation in the side groups. This compound cannot be a simple dihydroxy compound as evidenced by its partition with water and ether. The simple phenolic compounds are soluble in ether. However, since the potentially toxic compound is water soluble it must not contain any long aliphatic side-chains.

A possible mechanism of disease resistance is as follows: The organism entering the cell (or for that matter any general injury) might cause the polyphenolase enzyme to act on the phenolic compound producing toxic substances. Since there is evidence that this enzyme can be activated by various agents: that in the sugar beet the enzymes are adsorbed on the plastids: and that autolysis of the plastids causes the enzymes to go into solution, injury could release, activate or merely mix the enzymes with the cell contents. The oxidized phenolic compound then would inhibit the pathogen directly aand perhaps exhibit a secondary inhibitory effect by stimulating the walling-off of the wound. Cunnngham (5) reported that the zone between the dead cells of the leaf-spot and the healthy cells is characterized by mature cells which have become meristematic and solidly packed. The fungus hyphae are never present in this zone or beyond. Furthermore, if the phenolic compound is presentt on the surfaces of the leaves of resistant lines it may inhibit the germination of the conidia of the pathogen.

Although it is risky to isolate an artifact from plant material and reason that it was the same and underwent the same reactions in the living cell, the results of this investigation strongly indicate that selection of individual plants for leaf-spot resistance can be performed effectively by chemical means. The most

promising technique appears to be that involving optical density determinations for solution samples prepared with Arnow's reagent.

## Summary

A phenolic compound was isolated from leaves of sugar beets which, when oxidized, is extremely toxic to *C. beticola* growing in pure culture. This compound was found to be more concentrated in the leaves from resistant inbred lines than in the leaves from susceptible inbred lines.

This compound appears, from chromatographic, solubility, and ultra-violet absorption data, to be an ortho-dihydroxy phenol with one or more side groups. These side groups probably contain no conjugation with the benzoid structure.

The polyphenolase enzyme isolated from the leaves does not appear to be the controlling factor in producing resistance.

The compound responsible for the toxicity was found in the washings from leaves of a resistant line growing in field tests, but was lacking in washings obtained from a susceptible line.

A possible mechanism of leaf-spot resistance in sugar beets is described.

The results indicate the feasibility of selecting individual sugar beet plants for leaf-spot resistance on the basis of chemical determinations on leaf samples.

### Literature Cited

(1) ALLEN, T. H., RAY, O. M. and BODINE, J. H. 1938. Enzymes in orthopteran ontogensis. VI. Autocatalytic nature of *in vivo* formation of protyrosinase. Soc. for Expt. Biol. and Med. Proc. 39: 549-552.

(2) ARNOW, L. E. 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. 118: 531-537.

(3) COOK, M. T., BASSETT, H. P., THOMPSON, F. and TAUBENHAUS, J. J. 1911. Protective enzymes. Science 33: 624-629.

(4) COONS, G. H. and LARMER, G. F. 1929. The physiology and variations of *Cercospora beticola* in pure culture. Mich. Acad. of Sci., Arts, and Letters 11: 75-104.

(5) CUNNINGHAM, H. S. 1928. A study of the histological changes induced in leaves by certain leaf spotting fungi. Phytopathology 18: 717-751.

(6) GAUMANN, E. A. 1950. Principles of plant infection. p. 245-439. Hafner Publishing Company, New York.

(7) HORSFALL, J. G. and DIMOND, A. E. 1959. Plant pathology, an advanced treatise. Vol. I. p. 435-467, Academic Press, New York.

(8) HOSOKOWA, S. 1958. Annual report of sugar beet experiments in 1958. p. 46-60. Hokkaido National Agricultural Experiment Station. Division of Field Crops, Japan.

(9) JOHNSON, G. and SCHAAL, L. A. 1952. Relation of chlorogenic acid to scab resistance in potatoes. Science 115: 627-629.

(10) JOHNSON, G. and SCHAAL, L. A. 1957. Chlorogenic acid and other orthodihydricphenols in scab-resistant russet Burbank and scab susceptible triumph potato tubers of different maturities. Phytopathology 47: 253-255.

(11) KENTEN, R. H. 1957. Latent phenolase in extracts of broad-bean (*Vicia faba* L.) leaves. 1. Activation by acid and alkali. Biochem. J. 67: 300-307.

(12) KENTON, R. H. 1958. Latent phenolase in extracts of broad-bean (*Vicia faba* L.) leaves. 2. Activation by anionic wetting agents. Biochem. J. 68: 244-251.

(13) KOVACS, A. 1955. Uber die Ursachen der unterschiedlichen Resistenz der Zuckerrubensorten gegen *Cercospera beticola* Sacc. Phytopathol. Zeitschrift 24: 283-298.

(14) LINK, K. P., ANGELL, H. R. and WALKER, J. C. 1929. The isolation of protocatechuic acid from pigmented onion scales and its significance in relation to disease resistance in onions. J. of Biol. Chem. 81: 369-375.

(15) LINK, K. P. and WALKER, J. C. 1933. The isolation of catechol from pigmented onion scales and its significance in relation to disease resistance in onions. J. of Biol. Chem. 100: 379-383.

(16) POOL, V. W. and MCKAY, M. B. 1916. Relation of stomatal movement to infection by *Cercospora beticola*. U. S. Department of Agriculture, J. Agr. Research 5: 1011-1038.

(17) SIMONDS, A. O., JOHNSON, G. and SCHAAL, L. A. 1953. Comparative effects of catechol, some related compounds, and other chemicals on suberization of cut potato tubers. Botan. Gaz. 115: 190-195.

(18) SISAKYAN, N. M. and KOBYAKOVA, A. M. 1948. Activity and condition of enzymes in plastids. Biokhimiya 13: 88-94.

(19) SISAKYAN, N. M. and KUVAEVA, E. B. 1949. Polyphenoloxidase and peroxidase activity of isolated plastids. Doklady Akad. Nauk. S.S.S.R. 62. 121-124 (1948) in Chemical Abstracts v. 43 (1), col. 1083.

(20) VESTAL, E. F. 1933. Pathogenicity, host response and control of Cercospora leaf-spot of sugar beets. Iowa Agr. Expt. Sta. Research Bull. 168: 45-70.

(21) WALKER, J. C. 1957. Plant pathology. p. 584-585. McGraw-Hill Book Co., Inc., New York.

(22) WALKER, J. C. and STAHMANN, M. A. 1955. The chemical nature of disease resistance in plants. Annual Rev. of Plant Physiol. 6: 351-366.