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A BIOCHEMICAL STUDY OF THE INTERVARIETAL RESISTANCE OF *PYRUS COMMUNIS* TO FIRE BLIGHT

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Abstract—The mechanism responsible for fire blight resistance in leaves of *Pyrus communis* appears to be wholly a host reaction, activated by mechanical damage to host tissues. The following factors were associated with leaves of highly resistant varieties: (1) presence of large amounts of arbutin and free hydroquinone in unaltered leaf homogenates; (2) accumulation and persistence of antibacterial concentrations of hydroquinone enzymatically released from arbutin during oxidation of leaf homogenates; and (3) disappearance, following cell disruption, of an unidentified antagonist of hydroquinone. The balance between the rapidity of the appearance and disappearance of antibacterial hydroquinone in mechanically damaged leaf tissue is a major determinant of relative blight resistance.

INTRODUCTION

DISEASE-RESISTANCE studies concerned with phenol metabolism in normal, mechanically injured, and deseased plant tissues have been primarily concerned with fungal pathogens.[1,2] Relatively little attention has been given the role of native resistance factors in the pathogenesis of bacterial diseases such as fire blight. Simple mechanical injury of pear trees caused by rain and hail storms presents the fire blight pathogen, *Erwinia amylovora* (Burrill) Winslow *et al.*, with penetration sites under conditions of high humidity and low temperature ideally suited for bacterial growth and fire blight development. Newly developed terminal shoots and leaves of rapidly growing trees represent potential infection courts, either for bacteria naturally present on the plant surface[3] or for those carried from diseased areas on the same or other trees. Following superficial injury of terminal shoots and leaves, infection of susceptible trees occurs with a high frequency as compared to that of resistant trees.

The antibacterial properties of hydroquinone,[4] and the widespread occurrence of its glucoside, arbutin, in *Pyrus* species has led to speculation that these compounds may play an important role in interspecific and intervarietal resistance of pear trees to fire blight.[5–7] Keil and Wilson[8], employing host plants maintained in a greenhouse, observed that leaf discs and stem cross-sections from a blight-resistant variety of *P. communis* were more inhibitory of *E. amylovora* than were comparable tissues from a susceptible variety.

[1] G. L. FARKAS and Z. KIRALY, *Phytopathol. Z.* **44**, 105 (1962).
[2] I. URITANI, In *Symposium of Plant Phenolic Substances* (Edited by G. JOHNSON and T. GEISMAN). Symposium of Plant Phenolic Substances, Colorado State University, Fort Collins, Colorado (1961).
[3] H. L. KEIL, B. C. SMALE and R. A. WILSON, *Phytopathology* (In press).
[4] W. B. GEIGER, *Arch. Biochem.* **11**, 23 (1946).
[5] E. M. OSBORN, *Brit. J. Exp. Pathol.* **24**, 227 (1943).
[6] G. RACZ, Z. HISGYORGY and I. FUZI, In *Lucrarile prenzentate conf. natl. farm.*, Bucharest (1959).
[7] A. H. WILLIAMS, In *Phenolics in Plants in Health and Disease* (Edited by J. B. PRIDHAM). Pergamon Press, Oxford (1960).
[8] H. L. KEIL and R. A. WILSON, *Phytopathology* **52**, 1218 (1962).

Hildebrand and Schroth[9] showed a relationship between hydroquinone (quinol) content and antibacterial activity of certain floral tissues of pear to *E. amylovora*. In subsequent studies with plants maintained in a greenhouse, they found that leaves initially high in antibacterial activity, when frozen and thawed, lost all antibiotic activity at the end of 2 hr of incubation at 22°.[10, 11] Neither arbutin nor quinol was detected in chromatograms of tissue macerates after 2 hr of incubation.

The present study was undertaken to determine whether the arbutin and hydroquinone levels in uninjured leaves and their metabolism, under conditions simulating mechanical injury of leaves among six varieties of *P. communis*, were correlated with observed fire blight resistance.

RESULTS

Antibacterial activity of leaf discs and leaf extracts. The antibacterial activity of leaf discs from *P. communis* varieties did not accurately reflect the observed resistance to fire blight (Table 1). For example, inhibition zones associated with leaf discs from the highly resistant Kieffer variety were the same as those associated with leaf discs from the susceptible Dawn

TABLE 1. AREA OF INHIBITION ZONES ASSOCIATED WITH 10 MM LEAF DISCS EXCISED FROM FIRE BLIGHT-RESISTANT AND SUSCEPTIBLE VARIETIES OF *P. communis*

	Resistant			Susceptible		
	Kieffer	Magness	Moonglow	Dawn	Bartlett	DeVoe
Area (mm^2)*	176	217	187	176	89	82

* Inhibition annulus in mm^2 excluding the area covered by leaf disc, represents the average of four discs, one per leaf, excised from several plants of each variety.

variety. Anomalous results were also obtained in the bioassay of leaf discs from Kieffer and Moonglow varieties, in that third-ranked Moonglow consistently produced inhibition zones greater than those produced by first-ranked Kieffer.

Extracts of unaltered tissue, essentially representing the biochemical condition of the uninjured leaf, exhibited low antibacterial activities and apparently were not related to fire blight resistance (Fig. 1). Antibacterial activities of extracts of oxidized homogenates, however, were correlated with blight resistance. The biochemical alterations in leaf homogenates, occurring during 6 and 18 min oxidation, resulted in enhanced bioactivities that paralleled relative resistance of the six varieties used.

The antibacterial activity of Kieffer homogenates reached a maximum of 326 mm^2 with 6 min oxidation, and represented the most striking injury-induced enhancement of antibiotic activity. This high activity, however, was short-lived and with 12 min additional oxidation decreased to the level of the extract of the unaltered homogenate. The transitory nature of the Kieffer antibiotic activity explains, in part, the inability to relate resistance to tissue activity by leaf disc assay.

[9] D. C. HILDEBRAND and M. N. SCHROTH, *Nature* **197**, 513 (1963).
[10] D. C. HILDEBRAND and M. N. SCHROTH, *Phytopathology* **54**, 59 (1964).
[11] D. C. HILDEBRAND and M. N. SCHROTH, *Phytopathology* **54**, 640 (1964).

In contrast to Kieffer, neither Magness nor Moonglow homogenates reached maximum levels of antibacterial activity with 6 min oxidation. Bioactivities of extracts of these homogenates, while much greater at the end of 18 min than at the end of 6 min oxidation, still had not attained maximum levels. It was necessary to extend oxidation intervals beyond 18 min in order to establish the time required for maximum antibiotic development in Magness and Moonglow homogenates. We were able to prepare, with a supplementary sample of leaves, Magness homogenates oxidized for intervals as long as 90 min. Extracts of these Magness homogenates oxidized for 6 and 18 min were equivalent in bioactivity to those obtained with

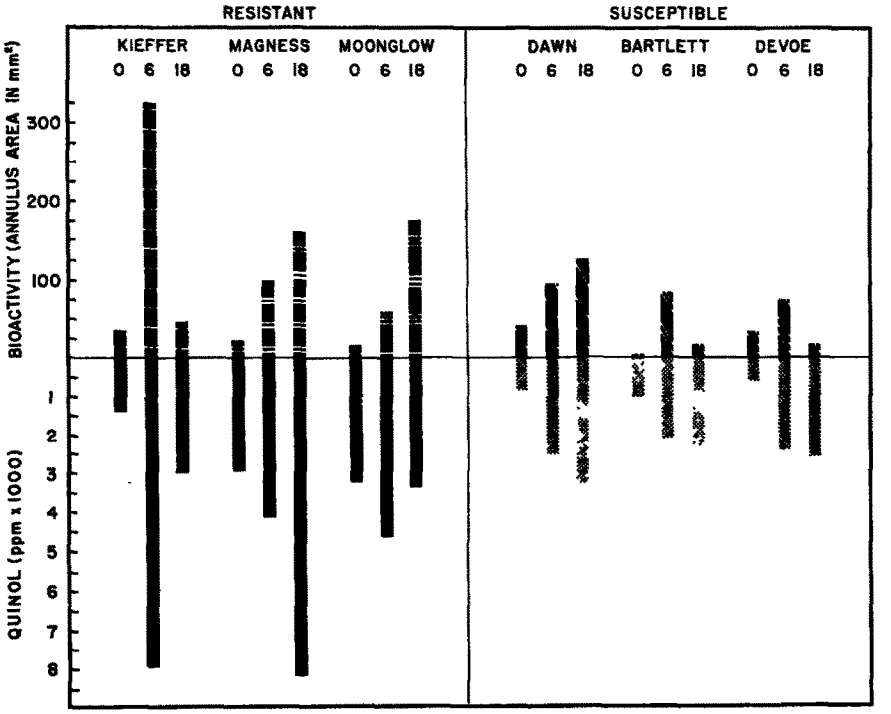

FIG. 1. HYDROQUINONE (QUINOL) CONCENTRATIONS, AND ANTIBACTERIAL ACTIVITIES (BIOACTIVITY) OF UNALTERED AND OXIDIZED LEAF HOMOGENATES FROM FIRE BLIGHT RESISTANT AND SUSCEPTIBLE *P. communis* VARIETIES.

The figures indicate time of oxidation in minutes.

extracts prepared from the original Magness leaf samples. Antibacterial activities of the extracts of homogenates oxidized for 36, 72, and 90 min were essentially equal to that of the extract of the 18-min oxidized homogenate. This plateau of antibacterial activity, developed in oxidized homogenates of Magness leaves, partially accounts for the high antibacterial activity associated with Magness leaf discs.

Antibacterial activity of the Dawn homogenate increased with oxidation in a manner similar to that of the Magness homogenate during the 0–6 min oxidation interval. The increment in the bioactivity of Dawn, however, was only one-third that of Magness during the 6–18 min oxidation interval.

The antibacterial activities of extracts of Bartlett and DeVoe leaf homogenates were greater with 6 min oxidation than they were with 18 min oxidation. Homogenates of these

two susceptible varieties and resistant Kieffer responded to oxidation in a similar way, in that maximum antibacterial activities were exhibited in extracts of homogenates of the three varieties 6 min after tissue disruption. There was no similarity, however, between the antibacterial activities of leaf discs from the resistant Kieffer and those of the susceptible Bartlett and DeVoe varieties. Antibiotic activities of Bartlett and DeVoe leaf discs and extracts of leaf homogenates oxidized for 6 min, although low and producing inhibition zones of less than 100 mm^2, were nearly equal. Antibiotic activity of Kieffer leaf discs, however, was only one-half that of the very high activity of the Kieffer leaf homogenate oxidized for 6 min.

Hydroquinone metabolism in unaltered and oxidized homogenates. Extracts of unaltered homogenates from the six varieties contained free or non-glucosidic hydroquinone, which occurred in the largest amounts in resistant Kieffer, Magness and Moonglow varieties (Fig. 1). Concentrations of authentic hydroquinone equal to those in unaltered extracts of Magness and Moonglow were two to three times more inhibitory of *E. amylovora* than the unaltered tissue extracts. Hydroquinone recovered from chromatograms of unaltered Magness and Moonglow homogenates was also more antimicrobial than that not separated from other extract constituents. The antimicrobial activities of hydroquinone in unaltered Kieffer extracts and of authentic hydroquinone at an equivalent concentration were essentially the same.

Extracts of homogenates, oxidized for 6 min, contained more hydroquinone than did extracts of unaltered homogenates (Fig. 1). Kieffer and Moonglow homogenates reached maximum levels with 6 min oxidation, while maxima in homogenates of Magness and the susceptible Dawn, Bartlett, and DeVoe were delayed and may not have been reached by the end of 18 min oxidation. Hydroquinone maxima or high levels, detected in oxidized homogenates of the resistant Kieffer, Magness, and Moonglow, were 7980, 8160, and 4680 ppm, respectively; and those detected in the susceptible Dawn, Bartlett, and DeVoe were 3220, 2320, and 2590 ppm, respectively.

The difference between the high level of hydroquinone developed during homogenate oxidation of Moonglow (4680 ppm) and Dawn (3220 ppm) is not of sufficient magnitude to account for the superior resistance of Moonglow. The higher degree of resistance of Moonglow, however, may involve the four-fold greater hydroquinone concentration of the unaltered Moonglow homogenate. This uncombined hydroquinone, although not antibacterial in uninjured tissue, could play a major role in resistance when injury-induced metabolism allows expression of its antibacterial potential.

Arbutin metabolism in unaltered and oxidized homogenates. Extracts[12] of unaltered leaf homogenates of blight-resistant pear varieties contained more arbutin than did comparable extracts of susceptible varieties (Fig. 2). The arbutin concentrations of the unaltered extracts of resistant Kieffer and Moonglow exceed those of the susceptible varieties by several thousand ppm and correlated with fire blight resistance. However, the 1500 ppm difference in the arbutin levels of unaltered Magness and DeVoe extracts was not sufficient alone to account for the superior resistance of Magness.

Arbutin disappeared rapidly in resistant and susceptible varieties during homogenate oxidation, with only 10–20 per cent remaining unaltered at the end of 18 min. Injury-induced metabolism of arbutin resulting from the hydrolytic action of β-glucosidase is associated with the temporary accumulation of hydroquinone and glucose. The significance of β-glucosidase

[12] Only partial extraction (about 50 per cent) of arbutin from homogenates is obtained with ethyl acetate. The data on arbutin levels is presented to show relative concentration and metabolism of the glucoside among blight-resistant and susceptible varieties.

activity in the metabolism of arbutin in mechanically damaged leaf tissue was established with Magness and Bartlett varieties. Initial arbutin concentrations of Magness and Bartlett homogenates, prepared in the presence of 1% D-glucono-*delta*-lactone, a glucosidase inhibitor, remained unchanged throughout the 18 min oxidation interval. Furthermore, these gluconolactone-containing homogenates did not exhibit the enhanced antibacterial activity associated with comparable homogenates prepared without gluconolactone.

The rate of arbutin disappearance or hydrolysis during homogenate oxidation apparently was not related to host resistance. Arbutin disappeared from homogenates of the most resistant and the most susceptible varieties at nearly identical rates during the initial 6-min oxidation interval with 70 per cent of the glucoside metabolized.

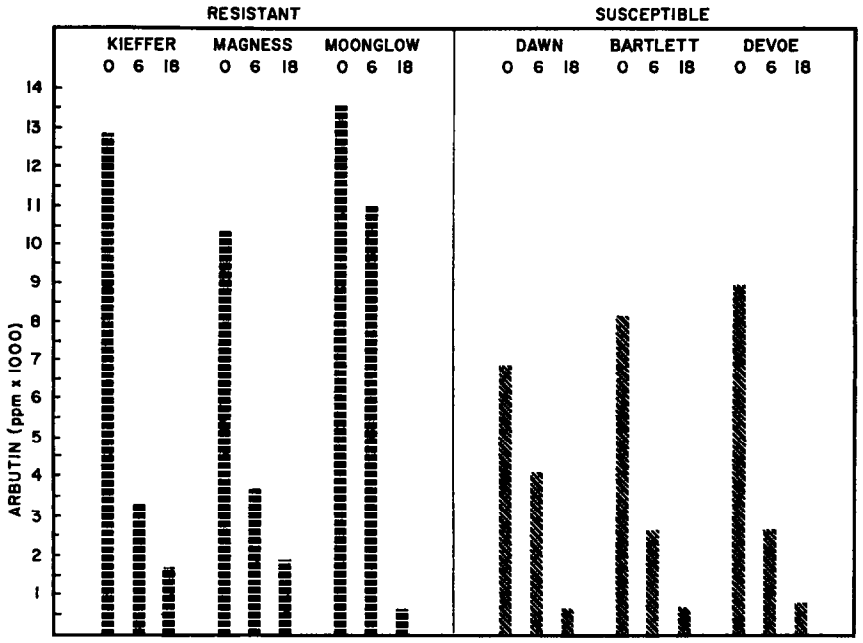

FIG. 2. ARBUTIN CONCENTRATIONS IN UNALTERED AND OXIDIZED LEAF HOMOGENATES OF FIRE BLIGHT RESISTANT AND SUSCEPTIBLE *P. communis* VARIETIES.
Figures indicate time of oxidation in minutes.

DISCUSSION

The defense mechanism in pear leaves responsible for resistance to fire blight appears to be wholly a host reaction that is activated by mechanical damage to host tissues. Little or no indication of a resistance mechanism, in the form of an antibacterial compound, is expressed by the unaltered extracts that represent the biochemical condition of leaf tissue prior to injury. With tissue damage, however, cell disruption allows contact between β-glucosidase and arbutin, which results in hydrolysis of the glucoside and liberation of antibacterial hydroquinone. The balance between the rapidity of the appearance and disappearance of antibacterial hydroquinone is a major factor in the resistance mechanism.

Widespread occurrence of hydroquinone and its glucoside in *P. communis* and other *Pyrus* species and the antibacterial properties of hydroquinone account, in part, for the presence

of a degree of blight resistance in most pear species. Although immune varieties of *P. communis* are unknown, the degree of resistance in Kieffer, Magness, Moonglow and others is sufficient to justify commercial exploitation.

The superior resistance of Kieffer, Magness, and Moonglow varieties over Dawn, Bartlett, and DeVoe cannot be attributed to a single factor but was associated with the following observations: (1) the presence of large amounts of arbutin and free hydroquinone in unaltered leaf homogenates; (2) accumulation and persistence of antibacterial concentrations of hydroquinone enzymatically released from arbutin during oxidation of leaf homogenates; and (3) disappearance, following injury-induced metabolism, of an unidentified antagonist and/or the appearance of a synergist of hydroquinone.

The significance of a high level of arbutin in unaltered tissue as a factor associated with resistance is indicated by the reciprocal relationship of glucoside disappearance and hydroquinone appearance during homogenate oxidation.

The role in resistance of the high concentrations of the non-glucosidic hydroquinone in unaltered homogenates of the resistant varieties cannot be established with the present data. Williams[13] reported that the occurrence of small amounts of free hydroquinone was always accompanied by very much larger amounts of arbutin. The amounts of free hydroquinone detected in unaltered Kieffer, Magness, and Moonglow leaf extracts, however, cannot be considered low levels. The hydroquinone in the unaltered extracts of Magness and Moonglow, 3000 and 3300 ppm respectively, is of particular interest since it exhibited essentially no antibacterial activity. The hydroquinone in these same extracts of Magness and Moonglow, however, when separated from other extract constituents by chromatography was equivalent to authentic hydroquinone. These findings suggest the presence of an antagonist, but direct proof and identification will require further investigation.

The second factor associated with blight-resistant varieties concerns the appearance, accumulation, and disappearance of the hydroquinone during homogenate oxidation. Resistant and susceptible varieties are readily distinguished from one another by the magnitude of hydroquinone levels attained during homogenate oxidation.

The relationship between antibacterial activity and hydroquinone concentration in Kieffer homogenates indicates a resistance mechanism based upon the hydrolysis of a major portion of arbutin immediately after injury, and the transitory accumulation of hydroquinone at a time during the oxidation interval which permits maximum expression of antibacterial activity. With 18 min oxidation, a repression of bioactivity of the residual or unmetabolized hydroquinone occurred.

Magness homogenates reflect the presence of an inhibitor or antagonist of hydroquinone that is slowly and not completely metabolized during the 18-min oxidation interval. Homogenates, oxidized for 18 min, contain sufficient antagonist to repress hydroquinone bioactivity by 50 per cent.

Hydroquinone content and antibacterial activity of unaltered and oxidized homogenates of Moonglow represent another type of resistance mechanism. The effect of an antagonist of hydroquinone is marked in homogenates oxidized 6 min but is essentially absent in homogenates oxidized for 18 min.

Varieties Dawn, Bartlett, and DeVoe are not resistant to blight primarily because antibacterial levels of hydroquinone are not developed. In Dawn, this insufficiency appears to result from the rapid oxidation of hydroquinone, thus preventing accumulation. With

[13] A. H. WILLIAMS, *J. Sci. Food Agr.* **8**, 385 (1957).

Bartlett and DeVoe, the delayed appearance of an antagonist of the hydroquinone in oxidized homogenates could also reduce blight resistance.

The relationship between fire blight resistance and antibacterial activity of Magness and Bartlett leaf discs reported by Keil and Wilson[8] was confirmed in the present study. Evaluation of other varieties by the leaf disc assay, however, demonstrated the limitations of direct tissue assay and the questionable nature of conclusions based on tissue bioactivity. These findings are in apparent disagreement with those of Hildebrand and Schroth[9–11] who concluded that hydroquinone formation (antibacterial activity) in pear tissues was limited by inadequate amounts of the β-glucosidase enzyme, rather than by an insufficient amount of arbutin. Their glucosidase assay, basically a direct tissue assay established with the model species, may not necessarily be reliable when used with other species and varieties.

EXPERIMENTAL

Plant material. Newly formed leaves approximately 50 per cent expanded were randomly collected from early season, succulent, terminal shoots of three resistant and three susceptible *P. communis* varieties. One- to two-gram lots of leaves from blight-resistant Kieffer, Magness, and Moonglow varieties, and susceptible Dawn, Bartlett, and DeVoe varieties, were quick-frozen in polyethylene containers as collected in a mixture of dry ice and ethanol and maintained at dry ice temperatures until processed.

Preparation of extracts. Frozen leaf samples, used to prepare unaltered extracts, were killed and biochemical transformations minimized by boiling tissue, under reflux for 3 min, in 80% methanol. The tissue and methanol were blended for 2 min, filtered, and the filtrate was washed three times with 80% methanol. Combined extract and washes were reduced to near dryness at room temperature in a rotary evaporator, taken up in 50 ml distilled water and partitioned three times against ethyl acetate. Combined ethyl acetate extracts were reduced to a small volume and concentration adjusted to 0·5 g fresh weight per ml with a 4:1 ethyl acetate–ethanol diluent which provided a homogeneous extract.

Samples of frozen leaves used in preparing oxidized tissue extracts were blended for 2 min with 50 ml distilled water. Homogenates were incubated with intermittent agitation for 6 or 18 min including blending interval. Enzymatic activity was terminated by boiling the samples for 3 min under reflux. Cooled homogenates were partitioned three times against ethyl acetate and the extracts were handled as previously described.

Chromatography. Separation and purification of hydroquinone and arbutin in extracts was accomplished by one-dimensional thin-layer chromatography on 250 μ layers of 0·3 M sodium acetate-buffered high purity silica gel H (Brinkman Silica Gel HR[14]). Extracts were applied as 3 cm bands to 20 × 20 cm silica gel coated plates divided into vertical lanes. Hydroquinone was well separated at R_f 0·77 with acetonitrile/chloroform/methanol/propyl acetate (1:1:1:1). Arbutin was resolved, R_f 0·40, on the buffered silica gel utilizing multiple development (twice) with ethyl acetate/pyridine/water (50:15:3).

Chromatograms were sprayed with 0·002% methanolic dichlorofluorescein after development and arbutin and hydroquinone located by their absorption of u.v. light. The band of silica gel (1·5 × 3 cm) containing arbutin or hydroquinone, quantitatively transferred to a test tube, was eluted by continuously stirring for 10 min with 5 ml 50% methanol at 80°. A test tube, magnetic stirring unit, with controlled heating block, simplified the elution procedure and aided reproducibility. Following centrifugation, and adjustment of volume to 5 ml, the

[14] Mention of this absorbent does not constitute an endorsement by the U.S. Department of Agriculture of this product over similar products or a guaranty or warranty of the standard of the product.

concentration was determined by reading absorbance at 295 mμ (hydroquinone [quinol]) or 283 mμ (arbutin). Data presented herein represent the averages of triplicate determinations.

Bioassay. Antimicrobial activities of leaf extracts and hydroquinone against *E. amylovora* were determined by paper-disc assay. One-tenth milliliter of an extract was applied to each of four 12·7 mm diameter assay discs and the solvent was evaporated. The air-dried discs were placed on nutrient agar inoculated with bacteria from a washed-cell suspension of *E. amylovora*. Diameters of inhibition zones were measured after 24 hr of incubation at 28°. These diameters were averaged, and the area of inhibition annulus was calculated by the equation:

$$A_a = \Pi(R_1 - R_2)(R_1 + R_2)$$

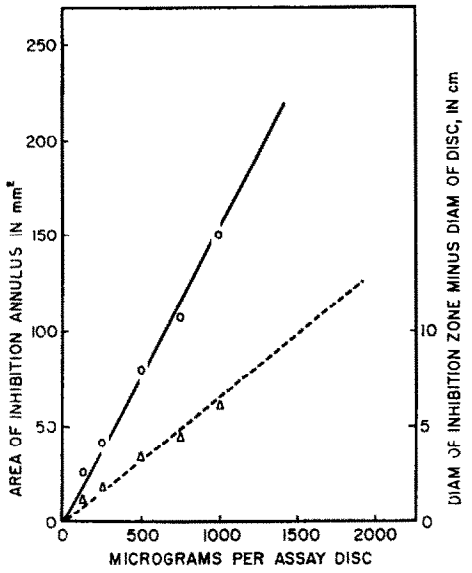

FIG. 3. THE RELATIONSHIP BETWEEN HYDROQUINONE DOSAGE RESPONSE CURVES OF *E. amylovora* BASED ON AREA OF INHIBITION ZONE AND ON DIAMETER OF INHIBITION ZONE.

Open circles, area of annulus associated with paper disc; open triangles, diameter of inhibition zone minus diameter of assay disc.

where R_1 is the radius of the zone of inhibition plus the radius of the assay disc, and R_2 is the radius of the assay disc. Dosage response curves, based on area of inhibition annulus and diameter of inhibition zone, are shown in Fig. 3. The greater differences in areas of inhibition resulting from altered hydroquinone concentrations favored use of inhibition areas in bioassays.

Antimicrobial activity of leaf tissue prior to extraction was obtained by direct assay of 10 mm discs excised from tissue adjacent to midrib. Quadruplicate discs from several plants of each variety were frozen at dry ice temperature and, while frozen, placed with adaxial surface toward inoculated nutrient agar. Diameter of inhibition zone including disc was measured after incubation and area of annulus calculated as previously described.

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