An Antibacterial Compound from Solanum phureja and its Role in Resistance to Bacterial Wilt

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ABSTRACT

We reported previously that ethanolic extracts from tissues of wilt-resistant clones of *Solanum phureja* inhibited growth of *Pseudomonas solanacearum* to a greater degree than extracts from wilt-susceptible *Solanum tuberosum* cultivars. We now report on some of the chemical properties of one of the components of the main inhibitory fraction and on attempts to determine, by genetic means, the possible involvement of this fraction in the mechanism of disease resistance.

After partial purification by paper and column chromatography, the inhibitory fraction (IP) exhibited a strong ultraviolet absorption maximum at 264 nm, which shifted to 282 nm, accompanied by loss of biological activity, after treatment with 2 N HCl. Elution patterns from Sephadex G-10 columns showed nearly coincident peaks for bacterial growth inhibition and content of the 264-282 nm absorbing compound(s). However, a quantitative assay for this particular component did not yield a positive correlation between its presence and resistance of various potato clones to *P. solanacearum*.

Clones derived from segregating hybrid progeny from a cross between resistant (R) and susceptible (S) clones of *S. phureja* were used to test the relationship between IP content and resistance. Disease reaction segregated independently from total inhibitory content, and from content of the 282 nm-absorbing compound as well.

From our original data, based on a limited number of clones, a correlation between resistance and content of bacterial growth inhibitors appeared possible. The more critical evidence from segregating progenies of a R × S cross failed to support such a hypothesis.

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Studies on the mechanism of resistance of potato to bacterial diseases have failed to produce direct evidence for the involvement of any particular substance. Lyon (9) reported that rishitin and phytuberin accumulate in potato tubers inoculated with *Erwinia carotovora* var. *atroseptica,* but did not demonstrate antibacterial activity for these compounds. No correlation between levels of glycoalkaloids in 12 potato cultivars and resistance to bacterial ring rot could be established by Pacquin (10). Based on limited evidence, Livingston (7) suggested that the oxidation products of *o*-dihydric phenols are involved in resistance to bacterial soft rot. Work by Lovrekovich, Lovrekovich, and Stahmann (8) also suggested, but did not demonstrate, that oxidation of phenols by polyphenol oxidase blocks infection of potato tubers by *E. carotovora.*

In an earlier study, we established that ethanolic extracts of tuber, stem, and leaf tissues of *Solanum phureja* Juz. & Buk., and *S. tuberosum* L. 'Russet Burbank' inhibited growth of *Pseudomonas solanacearum* E. F. Sm., causal agent of bacterial wilt of potato (18). Inhibitory activity was consistently higher (three- to fourfold) in extracts from wilt-resistant clones of *S. phureja* than in those from wilt-susceptible *S. tuberosum.* In addition, inhibitory activity was reduced in extracts of *S. phureja* plants grown at low light intensity, a condition known to reduce resistance to the bacterium (13).

Because the inheritance of resistance in potato to race 1 and race 3 isolates of *P. solanacearum* is known (11, 12, 17), the potato-*P. solanacearum* system has definite advantages in the study of the nature of the resistance mechanism. For instance, segregating progeny from resistant (R) × susceptible (S) crosses have predictable R:S ratios and, because each individual plant can be propagated vegetatively, the content of any compound thought to be involved in resistance could be correlated with resistance. In such a system, the content of antimicrobial substances directly involved in resistance should parallel the disease reaction phenotypes. Unless there are linkage problems, correlation between the two factors should provide adequate proof for the mechanism of resistance.

Thus, the purposes of this work were (i) to establish some of the chemical properties of the inhibitory fraction from *S. phureja,* and (ii) to determine whether the content of this fraction in individual plants from segregating hybrid progenies was correlated with resistance to bacterial wilt.

MATERIALS AND METHODS.—*Bacterial culture.*—All cultures were obtained from the collection of phytopathogenic bacteria maintained at the Department of Plant Pathology, University of Wisconsin, Madison. Stock cultures of all *P. solanacearum* isolates were maintained in distilled water at room temperature (21-23 C) to reduce mutation (5). Fresh cultures were obtained by streaking a loopful of the stock suspension onto a medium containing triphenyl tetrazolium chloride (TZC) (4). After 48 hours of growth at 30 C, virulent colonies were selected on the basis of fluidity, color, and morphology.

Plant growth and inoculation.—Botanical seed of potato was germinated in vermiculite and seedlings were grown at 21 C and a 14-hour photoperiod from a combination of Sylvania Gro-Lux and General Electric cool-white fluorescent and tungsten-filament incandescent lights providing 2×10^4 lux. After 3 weeks, individual seedlings were transplanted to 5-cm diameter peat pots and, 14-18 days later, to 10-cm diameter clay pots containing a mixture of sterilized muck soil and silica

sand (3:1, v/v). Plants were grown for a total of 4 weeks from date of transplanting. Plants propagated from tubers were planted directly in 10-cm diameter pots and grown for 1 month.

When required, cuttings were obtained by excising lateral shoots from 5-week-old plants. The cut end of the shoot was dipped in Rootone (American Chemical Products, Inc., Fremont, California 95202) and then the shoots were placed in moist silica sand in a wooden flat. The flat was covered with polyethylene plastic, and, after 10 days, rooted cuttings were transplanted into 10-cm diameter clay pots containing the silica sand-muck soil mixture and grown to the prebud stage.

Plants were inoculated by placing a drop of bacterial suspension containing 5×10^8 cells/ml ($O.D._{600\ nm} = 0.5$) on the axil of the third fully expanded leaf from the top and thrusting a sterile needle through the drop into the stem. After inoculation, plants were transferred to a growth chamber held at 28 C, 70 ± 3% relative humidity, and 2×10^4 lux light intensity supplied as described above. Disease indices were recorded at 3-day intervals for 15 days, according to the method of Sequeira and Rowe (13).

Preparation of crude extracts and purification procedure.—Stem, leaf, or root tissues from 4- to 6-week-old plants were cut into 3- to 4-cm pieces, weighed, frozen in liquid nitrogen, and stored in plastic bags at −30 C. Crude ethanolic extracts were obtained from these tissues by the procedure described previously (18).

Partial purification of the inhibitor of *P. solanacearum* present in these crude extracts was attempted by means of paper and column chromatography. For paper chromatography, 0.1-0.5 ml of crude extract was streaked on washed No. 3 MM chromatography paper and separated with butanol-acetic acid-water, (4:1:5, v/v) (BAW), by the descending method. After separation, chromatographs were dried and cut into 5.1-cm (2-inch) transverse sections beginning 1.3 cm (1/2 inch) below the origin. Each section was eluted overnight by capillary action with 70% ethanol. Eluates from equivalent sections of several chromatograms were pooled, evaporated to dryness, resuspended in 0.05 M phosphate buffer (pH 7) and assayed as described below. For further purification, the dried residue was dissolved in a small volume of water and applied to the top of a 3 × 27 cm column of Sephadex G-10 and eluted with distilled water. Sixteen 4-ml fractions were collected and assayed directly or stored at −15 C until used for further purification.

Bioassay.—The liquid culture assay involved the use of specially designed culture flasks (Fig. 1). Each culture flask consisted of a 25-ml Erlenmeyer flask connected to a 13 × 100 mm test tube by means of a glass T-joint. The side arm of the glass T was plugged with cotton and, after 1 ml casamino acids-peptone-glucose medium (4) and 1 ml phosphate buffer (0.02 M, pH 7.0) was added, the entire apparatus was autoclaved. After cooling, filter-sterilized extract (1 ml) and bacterial suspension (1 ml containing 2×10^8 cells) were added through the side arm. The assemblies were incubated in a water bath-shaker at 30 C. Optical densities (600 nm) were determined at 2-hour intervals by inverting the assembly so that the medium was transferred directly into the test tube, which could then be placed in a Bausch & Lomb Spectronic 20 colorimeter. Each test tube was marked to insure consistent alignment in the colorimeter for subsequent O.D. measurements. Control tubes contained buffer, medium, distilled water (instead of extract), and bacterial suspension. Inhibition was calculated from: (i) the difference in O.D. between tubes containing extracts and control tubes after a designated period of growth, or (ii) the difference in time required to reach an O.D. difference of 0.20 between tubes containing extracts and control tubes.

Conductivity measurements.—The concentration of electrolytes in plant extracts was measured with a model

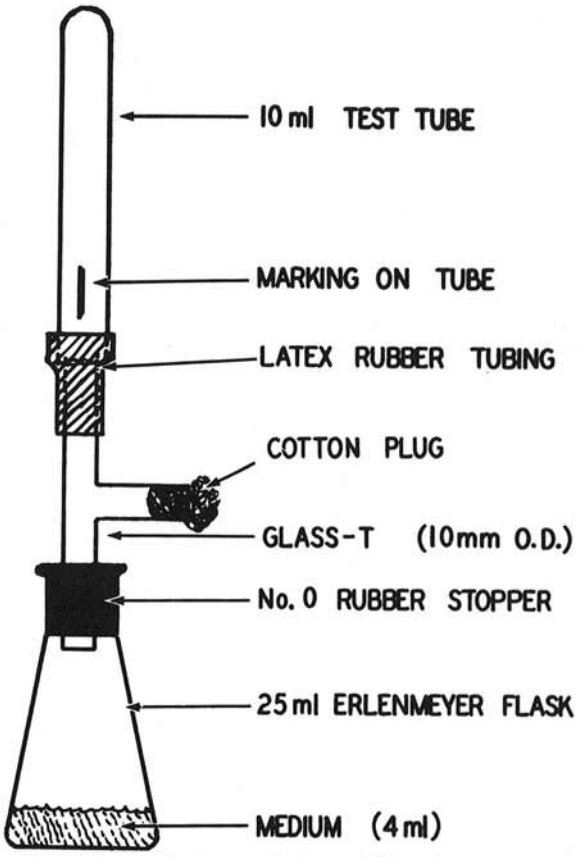

Fig. 1. Culture flask assembly for turbidimetric measurement of bacterial growth.

TABLE 1. Sensitivity of various isolates of *Pseudomonas solanacearum* to crude potato extracts[a]

Isolate number	Original host	Location	Race	Percent inhibition[b]
S-123	*Eupatorium*	Coto, Costa Rica	1	17
K-60	Tomato	Wake Co., N. Carolina	1	38
S-225	Tomato	Lupuna, Peru	1	66
S-115	*Heliconia*	Coto, Costa Rica	2	37
S-118	*Heliconia*	Coto, Costa Rica	2	39
S-222	Plantain	Timicuro, Peru	2	52
S-207	Potato	Popayan, Colombia	3	65
S-206	Potato	Las Palmas, Colombia	3	74
K-56	Potato	Israel	3	76

[a]Determined by the standard liquid culture method in casamino acids, peptone, glucose medium containing 1.0 g fresh weight equivalent/ml of potato extract.

[b]Determined by comparison with yield of the same isolate after 8-hours of incubation in medium containing no extract.

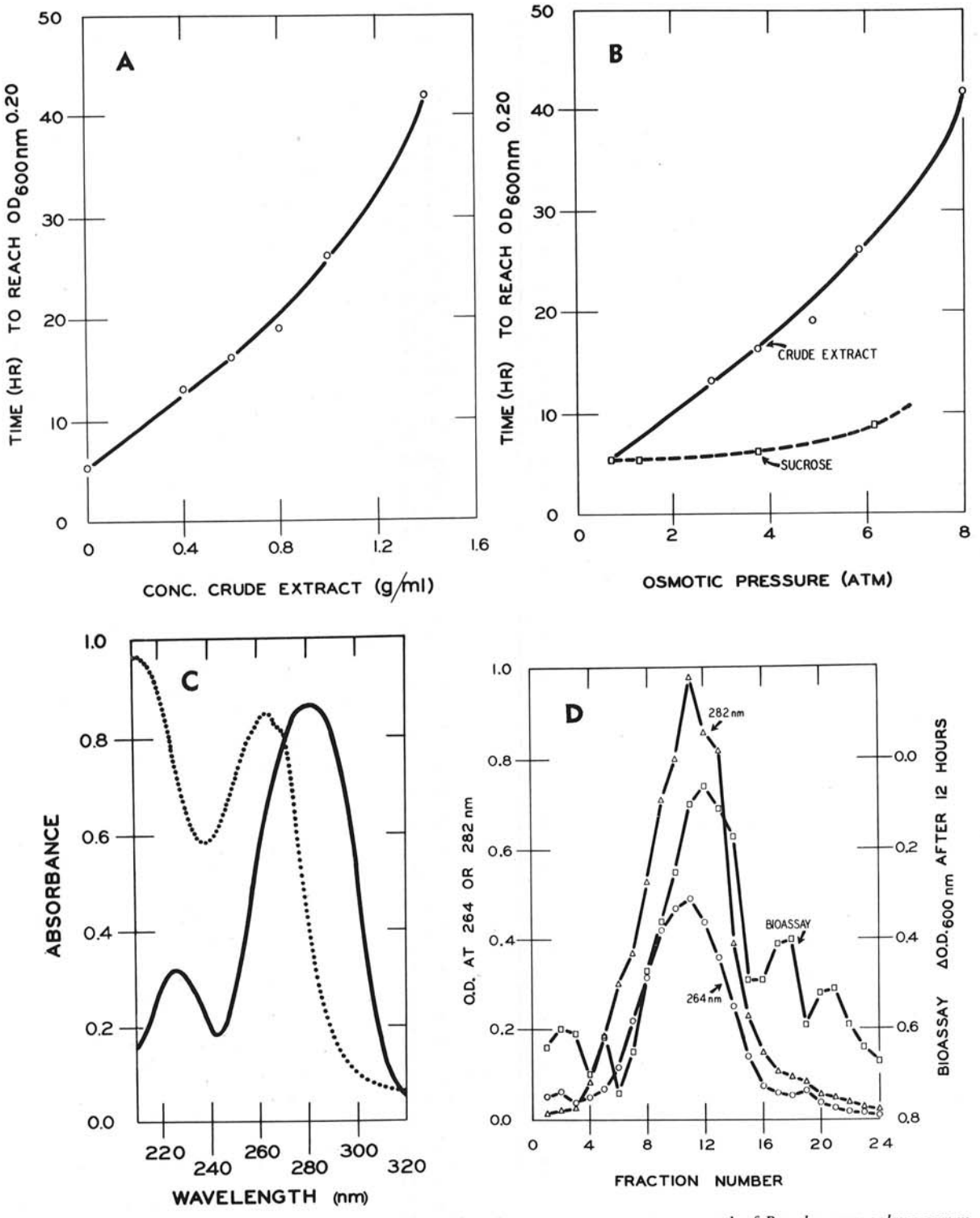

Fig. 2.-(A to D). **A)** Effect of increasing concentrations of crude potato stem extract on growth of *Pseudomonas solanacearum* (isolate S-206) in casamino acids-peptone-glucose liquid medium. Concentrations based on fresh weight of tissue. **B)** Comparisons of growth inhibition of *Pseudomonas solanacearum* (S-206) by crude potato extracts and sucrose solutions of increasing osmotic pressure. **C)** Ultraviolet absorption spectra of (a) active inhibitory fraction (dotted line) in 70% EtOH and (b) the same fraction diluted 1:10, treated for 30 minutes with 3 N HCl at 95 C, and extracted with ethyl acetate. The ethyl acetate was evaporated and the residue was resuspended in 70% EtOH. **D)** Chromatography on Sephadex G-10 of partially purified potato stem extracts before (264 nm) and after (282 nm) hydrolysis with 3 N HCl, and inhibitory activity of unhydrolyzed extract on growth of *Pseudomonas solanacearum* (K-60).

TABLE 2. Amounts of the 282-nm absorbing compound in stem extracts from selected resistant and susceptible *Solanum phureja* clones and a susceptible *S. tuberosum* clone

Clone	Reaction to isolate K-60[a]	mmoles per gram fresh weight tissue[b]
S. phureja - 1386.26	R	1.26×10^{-3}
S. phureja - P.I. 225709	S	1.32×10^{-4}
S. phureja - P.I. 225681	S	3.52×10^{-4}
S. phureja - WRF 354	S	1.01×10^{-3}
S. tuberosum - R. Burbank	S	6.40×10^{-3}

[a]R = resistant; S = susceptible.
[b]Based on $\epsilon = 8.45 \times 10^3$; each figure represents the average of two replicates.

RC 16-2B conductivity bridge (Industrial Instruments, Inc., Cedar Grove, N.J. 07009) equipped with a dip-type conductivity electrode with a range of 5×10^{-1} to 5×10^5 μmhos and a cell constant of 1.0. With this instrument, the conductivity of a solution of NaCl was linear between 100 and 10,000 ppm (2×10^2 to 1.5×10^4 μmhos).

Determination of osmotic pressure.—The osmotic pressure (OP) of extracts and culture media used in bioassays was estimated by the freezing point depression method (2). Freezing point depressions were determined by means of a Model 63-30 Milk Cryoscope (Advanced Instrument, Inc., Newton Highlands, Massachusetts 02161).

RESULTS.—To determine the amounts of extracted tissue required to yield detectable inhibition of isolate S-206, the time required to reach O.D. 0.20 in culture medium containing decreasing amounts of crude extract was recorded. The lowest concentration sampled (0.4 g fresh weight/ml) was inhibitory. It was apparent that extracts from even smaller quantities of tissue could inhibit growth of isolate S-206 significantly (Fig. 2-A). Seven isolates of *P. solanacearum* were grown in casamino acids-peptone-glucose medium with and without 1 g fresh weight equivalent per milliliter of crude extract from clone 1386.15. Individual isolates varied greatly in their sensitivity to substances in the extract. Race 3 isolates from potato (S-207, S-206, and K-56) were the most sensitive; isolates from other hosts (S-123, K-60) were the least sensitive (Table 1).

Crude extracts contain high amounts of glucose and other sugars, salts, amino acids and many other substances that contribute to the total osmotic pressure (OP) of crude extracts. Since *P. solanacearum* is particularly sensitive to high osmotic potential in the medium (3), OP values of crude extracts were determined by the freezing point depression method.

A linear relationship existed between OP and concentrations of crude extract between 0 and 2.0 g fresh weight equivalent per milliliter. Because Kelman (3) reported that OP values above 4.36 atm inhibit growth of *P. solanacearum* and high concentrations of crude extracts exceeded this value, it was necessary to determine if any portion of the total inhibition obtained with crude extracts was due to osmotic effects. A comparison of the inhibitory activity of crude extracts and of sucrose at

TABLE 3. Summary of various properties of crude extracts prepared from segregating progeny of a resistant (R) × susceptible (S) *Solanum phureja* cross[a]

Plant no.	Disease reaction	Inhibition[b]	Osmotic pressure[c] (atm)	Conductivity (μmhos)[c]	282-nm compound (mmoles per gram fresh weight)[d]
1	(R)	102	3.05	5,000	0.59
25	(R)	270	3.02	5,300	6.09
36	(R)	252	3.31	5,100	3.12
39	(R)	120	3.31	5,200	2.06
45	(R)	336	2.88	5,000	3.82
61	(R)	180	3.40	5,800	1.35
78	(R)	120	3.15	6,100	0.91
93	(R)	90	2.80	5,000	3.35
96	(R)	252	2.79	5,100	5.44
$\bar{X}_R$		191	3.08	5,289	2.97
6	(S)	126	3.08	4,600	0.94
7	(S)	180	3.57	5,700	...
11	(S)	96	3.19	4,900	2.50
18	(S)	318	2.96	4,300	5.18
32	(S)	120	2.91	5,200	4.76
51	(S)	198	3.39	6,900	0.56
72	(S)	144	3.91	6,000	0.56
89	(S)	216	3.34	6,050	1.12
95	(S)	162	3.34	6,600	2.35
$\bar{X}_S$		173	3.30	5,025	2.25

[a]Containing 0.5 grams fresh weight per milliliter.
[b]Increase in time (minutes) to reach O.D. = 0.20 as compared with growth of isolate S-206 in medium containing no extract.
[c]Determined prior to addition of culture medium.
[d]Based on $\epsilon = 8.45 \times 10^3$.

increasing OP values indicated clearly that within the range of crude extract concentrations normally assayed (0-1.2 g fresh weight per milliliter), the corresponding OP values (0.7-6.0 atm) do not contribute significantly to inhibition of bacterial growth (Fig. 2-B). Thus, it appeared that the inhibitory properties of crude extracts were due to the presence of toxic compounds, rather than to osmotic effects on bacterial growth.

When crude extract was filtered through a UM-2-membrane (MW retention = 1,000) in a Dia-flo pressure dialyzer (Amicon Corporation, Lexington, Massachusetts 02173), the portion of the extract that was retained had no inhibitory activity, but the portion that passed through the membrane was strongly inhibitory.

When crude extract was separated by paper chromatography with BAW solvent, the major peak of inhibition was consistently located at R_f .10-.25. Chromatograms of partially purified extracts (see following sections) contained a pale-blue fluorescent band at R_f .03-.24 under ultraviolet (UV) light which gave a positive reaction with diazotized sulfanilic acid, Folin-phenol reagent (1), bisdiazotized benzidine (6), and 2% ferric chloride (14). Only minor amounts of inhibition were detected in eluates from areas at other R_f values.

Twenty milliliters of extract which had been partially purified by paper chromatography were concentrated under vacuum to 1 ml, and this concentrate was layered on a G-10 Sephadex column and eluted with distilled water. Twenty-four fractions (2 ml each) were collected and assayed by the liquid culture technique. Fractions 8 through 15, corresponding to .50-.83 of the first elution volume after void volume contained the highest inhibitory activity (Fig. 2-D). Although minor peaks were detected before and after fractions 8-15, virtually all biological activity was associated with these fractions.

Active fractions had a UV absorbance maximum at 264 nm; the products obtained by treatment with 2 N HCl at 95 C for 1 hour and recovered with ethyl acetate, gave a strong absorbance peak at 282 nm (Fig. 2-C). Because of the relatively simple hydrolysis procedure and the sharp spectrum of the 282-nm product, the possibility that quantification of this compound might allow determination of inhibitor levels in crude extracts from various clones was explored. First, it was necessary to determine whether inhibitory activity and absorption at 264 and 282 nm were due to the same compound.

Samples from biologically active fractions obtained by chromatography on Sephadex G-10 were checked for absorbance at 264 nm, hydrolyzed with 2 N HCl, extracted with ethyl acetate, and then monitored for absorbance at 282 nm. Elution patterns for the inhibitor, the 264-nm and 282-nm absorbing compounds were nearly coincident. Highest absorbance at both 264 nm and 282 nm occurred in fraction 11, but the highest biological activity was detected in fraction 12 (Fig. 2-D). Variability in the bioassay was thought to account for this slight difference in elution patterns.

Because the results of chromatographic separation indicated that the 282-nm peak of the acid product was nearly coincident with biological activity of the nonhydrolyzed fractions, attempts were made to estimate the amounts of 282-nm absorbing compound in resistant and susceptible *S. phureja* and *S. tuberosum*. Crude extracts prepared from 25 g fresh weight each of stem tissues from potato clones 1386.26, P.I. 225709, P.I. 225681, WRF 254, and Russet Burbank were purified by chromatography as described previously. Active fractions were pooled, evaporated to dryness, resuspended in 2 ml distilled water, hydrolyzed with 2 ml 6 N HCl for 30 minutes and extracted three times with 10 ml of ethyl acetate. The ethyl acetate extract of each clone was evaporated to dryness in vacuo, redissolved in 4 ml 70% ethanol and the absorbance at 282 nm was determined. The amount of 282-nm absorbing compound in each clone, in millimoles per gram fresh weight tissue, was calculated on the basis of a molecular weight of 206 and an extinction coefficient of 8.45×10^3 (Sequeira and Harborne, *unpublished*). Contrary to expectation from past results based on total inhibitor content, Russet Burbank extracts contained the highest amount of the 282-nm compound, followed by clones 1386.26, WRF 354, P.I. 225681, and P.I. 225709 in that order (Table 2).

To examine the possible role of inhibitors of bacterial growth in the mechanism of resistance to *P. solanacearum* in potato, segregating progeny from a cross of susceptible (S) × resistant (R) *S. phureja* clones (1339.28 × 1386.22) were tested both for resistance and inhibitory activity. For this purpose, clones were developed from 100 hybrid plants and a single plant of each clone was inoculated with isolate S-206 of *P. solanacearum* at the prebud stage; the remaining plants of each clone were harvested and extracted individually. The inhibitor content in extracts from nine highly resistant and nine highly susceptible segregants was determined by the standard liquid culture bioassay against isolate S-206. The content of the 282-nm absorbing compound, osmotic pressure, and conductivity were determined as described previously.

Inoculation tests of the progeny from this cross gave a R:S ratio (22:78) which was very close to the 1:3 ratio predicted by the four-gene hypothesis (17). However, disease reaction segregated independently from total inhibition and content of the 282-nm compound in tissue extracts (Table 3). Salt concentration (as estimated by conductivity) and osmotic pressure did not appear to contribute significantly to the inhibition caused by the crude extracts. Correlation coefficients for inhibition versus osmotic pressure, conductivity, and content of the 282-nm absorbing compound were −0.186, −0.141, and +0.542 ($P = 0.05$), respectively. Statistical analysis by the analysis of variance method failed to detect any significant difference between inhibition means for extracts from resistant and susceptible plants.

DISCUSSION.—It can be estimated that the inhibitor of *P. solanacearum* (IP) is present in potato tissues at biologically significant concentrations. The dry weight of crude ethanolic extracts represents approximately 1% of the fresh weight of tissue extracted. In addition, about one-tenth of the dry weight of crude extracts accounts for the inhibition from active fractions eluted from a G-10 Sephadex column. Thus, 0.2 g fresh weight of tissue could contain approximately 200 μg of IP. If it is assumed from Fig. 2-A that extracts containing 0.2 g fresh weight of tissue per milliliter inhibit bacterial growth, then pure IP must be active at concentrations below 200 μg/ml. Phytoalexins suppress growth of microorganisms at average concentrations of 100 μg/ml (16). Thus, it appears that IP is active at concentrations within the same

general range at which phytoalexins are biologically active.

The IP appears to have differential inhibitory effects on plant-pathogenic bacteria. Concentrations of crude extract which inhibited *P. solanacearum* (K-60) markedly stimulated growth of *E. carotovora* and *E. atroseptica* (18). Potato extracts have been reported previously to have differential inhibitory effects on bacteria. *Erwinia amylovora* was inhibited more strongly than *E. carotovora* by an extract from potato stems and leaves (15).

The initial comparison of *S. phureja* and *S. tuberosum* clones indicated that there was a close correlation between resistance to bacterial wilt and content of IP (18). Crude extracts from clones 1386.26, 1386.9, were consistently much more inhibitory to isolate K-60 than those from Russet Burbank. The apparent biological activity of IP, its differential inhibitory properties, and its presumed involvement in the mechanism of resistance to bacterial wilt in potato, encouraged attempts to determine the nature of this compound.

Purification of the IP was an especially difficult problem. Basic solvents, such as isopropyl-ammonium hydroxide-water (10:1:1) caused an immediate loss of biological activity. Similarly, prolonged exposure to acidic conditions resulted in a slow loss of activity. Column chromatography on Sephadex G-10 resulted in an active (A_{max} 264 nm) fraction which, however, gave positive reactions for sugars and amino acids when examined by thin layer chromatography. Although the 264-nm absorbance maximum did not shift with changes in pH, as is characteristic of phenolics, BAW paper chromatograms of these column fractions gave positive reactions with several phenolic reagents.

Because it was not possible to purify the active inhibitor completely, there was some question as to whether the inhibitor actually possesses the spectral properties depicted in Fig. 2-C. On Sephadex G-10, the peak of inhibitory activity consistently eluted one fraction later than the peaks for the 264- and 282-nm absorbing compound (Fig. 2-D). This slight difference in elution indicated the possibility that the IP was different from the 264-282-nm absorbing compound. In addition, assays of different *S. phureja* and *S. tuberosum* clones indicated that the content of this compound had no relation to resistance to *P. solanacearum,* although total inhibitory activity of crude extracts was correlated with resistance.

The presence of more than one inhibitory substance in the crude extracts could explain the discrepancy between levels of the 282-nm absorbing compound and inhibitory activity of extracts from different *S. phureja* and *S. tuberosum* clones. Paper chromatogram eluates obtained at R_f values greater than that of the IP exhibited some inhibitory activity. Thus, it seemed critical to examine the total inhibitor content, rather than individual components, in studying the mechanism of resistance to *P. solanacearum*. However, when the total inhibitory content of individual clones in a segregating progeny of a cross between resistant and susceptible *S. phureja* parents was examined, this factor was not correlated with resistance. It is clear that total inhibitor content and disease resistance are under independent control.

These results demonstrate the value of examining a segregating progeny for factors thought to be involved in resistance. In our original data, based on a limited number of clones, a correlation between resistance and total inhibitory activity appeared possible. The more critical evidence from segregating progenies of a R × S cross failed to support such a hypothesis.

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