Some Physiological Effects of Agrobacterium Rhizogenes on Tomato

D. Huisingh and R. D. Durbin

Associate Professor, Department of Plant Pathology, North Carolina State University, Raleigh 27607; and Leader, Pioneering Research Laboratory, Crops Research Division, ARS, USDA, Department of Plant Pathology, University of Wisconsin, Madison 53706.

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ABSTRACT

Physiological studies using tomatoes infected with *Agrobacterium rhizogenes* revealed that wet wt., soluble protein, ascorbic acid oxidase activity, and indole-3-acetic acid (IAA) concn increased about 100% while peroxidase activity increased as much as 1,000 to 1,200% over that in wounded-control plants by 15 days after inoculation and wounding, respectively. The peroxidatic activity was electrophoretically separable into several discrete isozymes, with two new ones appearing only in extracts from diseased tissues. One of the new isozymes appeared to oxidize IAA. Susceptibility to inhibition by chlorogenic acid and the low pH optimum attest further to the presence of IAA oxidation by a peroxidase in the diseased tomatoes. Phytopathology 60:1101-1105.

Additional key words: peroxidase, disc electrophoresis, hairy root.

Until 1930, when Riker and his associates erected *Agrobacterium rhizogenes* (Riker et al.) Conn. as a separate species, there was considerable controversy about its taxonomic position and whether or not the disease it caused, hairy root, was different from crown gall (16). However, once the etiology had been established and control measures developed, very little attention was directed to it until recently. The disease might be a very useful model for studies on the regulation of organ differentiation, since the infected host is stimulated to produce only roots. As a preliminary to such model system studies, we report here on some of the physiological alterations induced in tomato after inoculation with *A. rhizogenes*.

Materials and Methods.—All tomato plants, *Lycopersicon esculentum* Mill. 'Bonny Best', were grown in a controlled environment chamber with a 14-hr day length at 25 C and a 20 C night temp, except for one series which was grown in a greenhouse. Light at an intensity of 1,800 ft-c at pot level was provided by a combination of cool-white fluorescent and incandescent bulbs. Inoculum was prepared by centrifuging a 48-hr culture grown in a synthetic medium (6) at 12,000 *g* for 30 min, washing, then diluting the cells in distilled water to ca. 10^{14}/ml. Using a pin lug inoculator (7), the inoculum was introduced into the stems of 2-month-old plants between the second and third true leaves from the bottom of the plant, just before the end of a light period. Control plants were wounded with a sterile inoculator. At various days after inoculation, 1.5-cm stem sections were taken from inoculated and control internodes, weighed, sliced into 1-mm segments, and ground in 0.078 M phosphate buffer, pH 5.8 (5 ml/g, wet wt) for two 1-min periods with a Sorvall Omni-Mixer at 0 C. In experiments where the supernatant fraction was to be utilized, the breis was centrifuged for 1 hr at 27,000 *g*.

In vivo population dynamics of the bacteria were determined by running dilution plate series of the breis. Numbers of bacterial colonies were determined after plates had been incubated 72 hr at 25 C. Similar tests were done with tissue from the internode above and the internode below the wounded or inoculated one.

For carbohydrate analyses, 5 g wet wt, stem samples were ground with boiling ethanol and extracted with three successive 20-ml aliquots of boiling ethanol. The total carbohydrate and reducing sugar contents of the combined extracts were then analyzed by the methods of Dubois (4) and Somogyi (20), respectively.

Five-hundred g wet wt samples were used for indole-3-acetic acid (IAA) analyses. After grinding, the acidic substances were extracted from the aqueous phase according to the procedure of Sequeira & Kelman (17). The ether extracts were dried and the residues taken up in 95% ethanol, spotted on thin-layer chromatographic plates of silica gel-carboxymethylcellulose (14), and developed with either a chloroform:methanol:96% acetic acid (75:20:5, v/v) or isopropanol:ammonia:water (100:10:5) solvent systems in which authentic IAA had R_F values of 0.77 and 0.26, respectively. Using Ehrlich's reagent, the limit of visual detection on the plates was 0.05 μg IAA.

To further study the importance of growth regulator inbalances on symptomatology, an auxin, naphthalene-acetic acid (NAA) and/or a compound with anti-auxin activity, 2,4-dichloroanisole, was applied to tomato plants as follows: The leaflets of the distal one-half of the 5th true leaf were removed with a sterile razor blade and the cut end of the petiole was submerged in the test solution contained in a shell vial taped to the main stem. Five-ml portions of the test solutions were absorbed by the plants within 8 hr. Distilled water (5 ml) was then added to each vial to prevent drying and to aid movement of the text compound. Both compounds were applied singly and in combinations to control and inoculated plants at 0.1 and 0.01 μmoles/plant at the time of inoculation.

Oxidation of ascorbic acid by the tissue breis was determined manometrically at 25 C. Twelve control or diseased stem sections were pooled to prepare each sample. For each sample, 2-ml aliquots of breis were pipetted into each of two Warburg flasks containing

1 ml 0.067 M phosphate buffer, pH 5.8, in the outer compartment. Six-tenths ml 0.13 M ascorbic acid (pH 6.0) was tipped in from the side arm after a 20-min equilibration period, and the rate of O_2 uptake measured at 15-min intervals for the next hr. The contents of the flasks were then rinsed into tared pans, oven-dried, and weighed. Ascorbic acid oxidase (AAO) activity was expressed as μliter O_2 uptake/hr per g wet wt. Duplicate analyses were run and averaged.

Analyses for peroxidase activity were done using the supernatant fraction after centrifugation. Twelve control or diseased stem sections were used to prepare each sample. The reaction mixture was composed of: 3.9 ml water; 0.5 ml of 0.1 M phosphate buffer, pH 6.0; 0.25 ml of 1.7 mM guaiacol; 0.1 ml of 0.44 mM hydrogen peroxide; and 0.1 ml of enzyme solution. The reaction rate was followed at 470 nanometer (nm) using a Bausch & Lomb recording spectronic 20 colorimeter. One unit of activity is defined as that amount of enzyme which causes a change in absorbancy of 0.3/min under the conditions of the assay.

The technique of Davis (2) and of Ornstein (15) for polyacrylamide gel disc electrophoresis was used to determine isozyme patterns. Samples containing 200 μg of protein, determined by the method of Lowry et al. (11), were layered on top of the spacer gel, and a current of 3 ma/tube was applied. When the marker dye band had migrated to within 0.5 cm of the anodic end of the gel, the gels were removed from the tubes, washed, and submerged in the peroxidase reaction mixture used above, except that no enzyme was added.

Visualization of IAA oxidase activity was done by first incubating the gels for 1 hr in a reaction mixture containing: 3.25 ml of 0.01 M phosphate buffer, pH 4.5; 0.25 ml of 1.0 mM 2,4-dichlorophenol; 0.2 ml of 4.8 mM H_2O_2; and 1.0 ml of a 1.0 mM IAA and 0.7 mM $MnCl_2$ followed by several successive rinses in buffer and, finally, incubation in Tang and Bonner's reagent for 1 hr. An absence of color development at certain locations in the acrylamide gel was considered indicative of IAA oxidation.

Since hairy root symptoms usually developed below, but never above, the point of inoculation on the stem, all physiological parameters studied in this work were measured 8 days after inoculation in the internode subjacent to the inoculated one.

RESULTS.—*Wet weight.*—The response of the inoculated plants was first detectable as a slight decrease, followed by a gradual increase in wt over that of the control plants. By 15 days after inoculation, the wet wt of diseased sections was about twice that of the controls (Table 1).

Soluble carbohydrates.—The plants used for the sugar analyses were greenhouse-grown; thus there was a high level of variability due to changing environmental conditions. The results indicate, however, that there were no consistent differences between healthy and diseased stem sections in the concentrations of either reducing sugars or total carbohydrates (Table 2).

Soluble protein.—The protein concentration of the supernatant fractions was 20% higher in diseased stems than in controls 2 days after inoculation (Table 1), and it continued to increase thereafter. By 15 days after inoculation, it was nearly 100% higher in the diseased stems than in the controls.

TABLE 1. Wet wt and soluble protein content at various times after inoculation of control and diseased tomato stem sections. *Agrobacterium rhizogenes* was used as inoculum

Days after inoc-ulation	Wet wt (g/1.5-cm stem section)		Protein content (mg/g wet wt)	
	Control	Diseased	Control	Diseased
2	0.76[a]	0.71	1.88	2.25
4	0.99	1.23	2.45	3.40
6	1.01	1.25	3.45	4.35
8	1.01	1.60	2.40	3.85
11	1.13	1.79	2.15	3.90
13	1.11	2.35	3.10	4.60
15	1.03	1.84	2.60	4.85

[a] Each value is the average of two replicates run on extracts prepared from sections of 12 plants.

Auxin.—While wounded control stem sections contained approximately 0.05 μg IAA/100 g wet wt, diseased stems contained 0.10 μg IAA/100 g wet wt 8 days after inoculation. Thereafter, no significant change in concentration was found.

Exogenous growth regulators.—Anisole at both concentrations markedly reduced the number of roots initiated in inoculated plants. NAA, when applied alone, stimulated the initiation of roots in all internodes below the fifth node. Anisole at 0.1 and NAA at 0.01 μmole/plant caused no prominent changes in the initiation of roots in response to inoculation. When anisole was used at 0.01 and NAA at either 0.01 or 0.1 μmole/plant, however, the effect of NAA was dominant and more roots were initiated than with inoculation alone.

Ascorbic acid oxidation.—The rate of ascorbic acid oxidation was somewhat higher for all stem homogenates from the first series of experiments, using greenhouse grown plants, than in the second where the plants were grown in the controlled environment (Fig. 1). In homogenates from diseased stems, a peak at 4

TABLE 2. Total carbohydrate and reducing sugar content of homogenates prepared from control and diseased tomato stem sections. *Agrobacterium rhizogenes* was used as inoculum

Days after inoc-ulation[a]	Total carbohydrate (mg/g wet wt)		Reducing sugar (mg/g wet wt)	
	Control	Diseased	Control	Diseased
1	2.4[b]	2.8	2.0[c]	2.0
2	2.3	1.2	2.1	0.6
5	6.5	8.0	3.3	2.5
8	6.5	11.3	1.5	2.6
12	15.0	11.0	3.0	5.0
15	13.0	13.0	8.0	8.5
20	15.0	17.5	6.5	9.0

[a] Each sample was taken at 6 AM, and is the average of two replicates run on extracts prepared from sections of 12 plants.

[b] Each value is the average of triplicate analyses performed according to the methods of Dubois (4).

[c] Each value is the average of triplicate analyses performed according to the method of Somogyi (20).

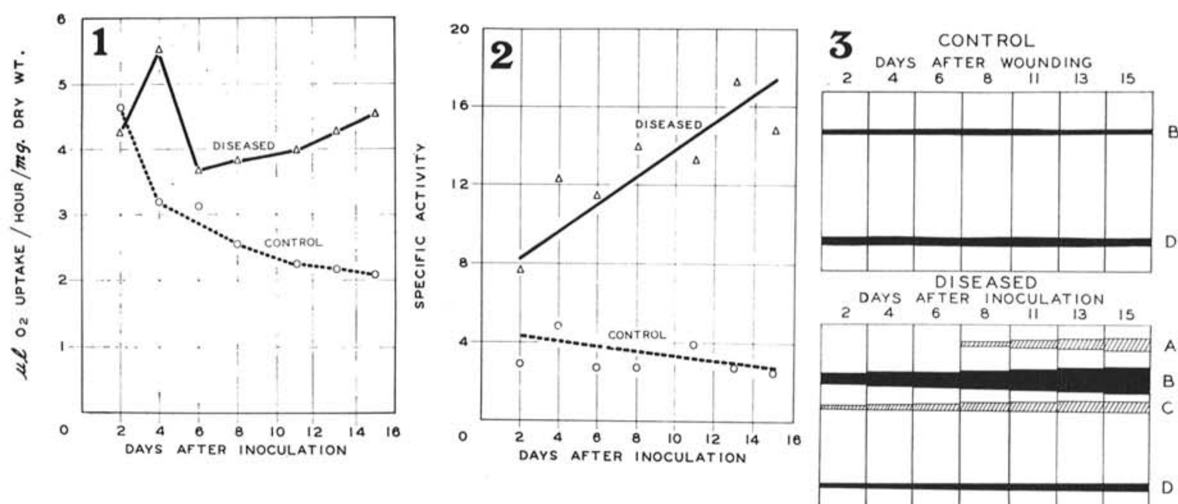

Fig. 1-3. **1)** Ascorbic acid oxidase activity in homogenates of control and diseased tomato stem sections at various times after wounding or inoculation. *Agrobacterium rhizogenes* was used as inoculum. **2)** Specific activity (units/mg protein) of peroxidase in extracts from control and diseased tomato stem sections at various times after wounding or inoculation. *A. rhizogenes* was used as inoculum. **3)** Zymograms showing the peroxidase activity of control and diseased tomato plant extracts at the indicated times after wounding and inoculation, respectively. *A. rhizogenes* was used as inoculum. The bars with diagonal lines indicate those bands which are present only in extracts from diseased tomato stem sections. The reaction mixture was composed of 3.90 ml water, 0.5 ml of 0.1 M phosphate buffer, pH 6.0, 0.25 ml of 1.7 mM guaiacol, and 0.1 ml of 0.44 mM hydrogen peroxide.

days after inoculation and a subsequent drop followed by a steady increase in a set pattern was obtained in both experiments. On the other hand, the rate of ascorbic acid oxidation in control stem homogenates continued to drop throughout both experiments. Sodium diethyldithiocarbamate (10^{-4} M) inhibited O_2 uptake of both healthy and diseased tissues (85-95%), whereas phenylthiourea (10^{-3} M) did not. Boiled homogenates had no AAO activity. Eighty-five to 90% of the ascorbic acid oxidation in all treatments was associated with the pellet after centrifugation.

Peroxidase.—A pronounced increase in the peroxidatic activity of extracts from diseased stems was evident 2 days after inoculation, and it continued to increase throughout the experimental period; in fact, it was 10 to 12-fold higher in diseased than in control plants by 15 days after inoculation (Fig. 2). Delaying the addition of guaiacol to the reaction mixture did not affect the subsequent rate of oxidation, but the addition of catalase (1-100 µg/ml final concn) halted the reaction immediately. From this it was concluded that there was little or no catalase activity in the tomato stem extracts. Guaiacol was not oxidized if H_2O_2 was omitted. The reaction was also cyanide-sensitive, since 50% inhibition was obtained with 2.7×10^{-6} M KCN. Diethyldithiocarbamate, azide, phenylthiourea, and fluoride gave the same level of inhibition at 1.5×10^{-4} M, 2.6×10^{-3} M, 4.4×10^{-3} M, and 1.1×10^{-2} M, respectively.

The percentage increase in peroxidase activity, in contrast with relatively smaller increases in wet wt, soluble protein, and ascorbic acid oxidation, suggested that there was preferential activation and/or synthesis of peroxidases. Following electrophoretic separation, zymograms were made ½ hr after beginning incubation of the gels in the peroxidase reaction mixture. The bands labeled B and D (Fig. 3) were present in extracts of both control and diseased stem sections. Band D showed no appreciable change in the infected tissue extracts, but band B increased about 6-fold in rate of appearance, and final density over those of controls, within 2 weeks after inoculation. Two bands, A and C, appeared only in extracts from diseased stem sections, and were not present in extracts made from healthy roots, apical meristems, flowers, or fruits. Band C became apparent 2 days after inoculation and continued to increase slowly throughout the remainder of the experiment. Band A did not appear until the 8th day after inoculation, but increased rapidly during the following 7 days.

IAA oxidation was present at the same position as band C in diseased, but not in healthy, stem extracts. Chlorogenic acid (2.8×10^{-5} M) inhibited the appearance of this band; likewise, the oxidation of IAA by diseased tissue extracts, determined spectrophotometrically (18) at 247 nm, was reduced 41% by that concn of chlorogenic acid. The pH optimum for this IAA oxidase activity, using .02 M citrate-phosphate and phosphate buffer systems, was found to be 3.2 with a rapid decline above 5.0. In contrast, the pH optimum for the peroxidatic activity, using guaiacol as a substrate, was 6.3. Neither peroxidase nor IAA-oxidase could be demonstrated in electrophoretograms of bacterial extracts.

Subjacent internode.—The same parameters were measured for the internode subjacent to the inoculated and wounded ones. In all cases, extracts from the subjacent inoculated internode were similar but quantitatively smaller than those of the inoculated inter-

node. Extracts from the internode subjacent to the wounded internode showed no changes.

DISCUSSION.—Infection of tomato by *A. rhizogenes* results in the activation of a developmental pathway leading to root formation. It appears from our results that changes in oxidative enzymes may be involved in this development. The appearance of oxidative isozymes peculiar to diseased tissue has been previously reported, and their possible significance in regulating growth and differentiation stressed (5). Although our data do not differentiate among de novo synthesis, solubilization, and conversion from inactive forms, the new peroxidatic isozymes found in diseased tissue extracts do not appear to be formed at the expense of other soluble forms since increases, not decreases, were seen in all bands. It may be that the enzymes are particle-bound in healthy plants. These peroxidase isozymes are not found in extracts prepared from *A. rhizogenes* run at a wide range of protein concentrations, whereas in extracts from the plants, these bands were present over a 50-fold range in protein concn applied/gel.

Histological examination of disease-induced roots has shown that xylem elements differentiate in much closer proximity to the apex than they do in normal roots (8). In light of Torrey's finding (22) that IAA application stimulates xylem element development in pea roots, this accelerated maturation may be related to the increased IAA level of diseased tissue. But how this increased level is to be reconciled at the cellular level with the presumed higher rates of IAA oxidation in diseased tissue and the appearance of a soluble enzyme having IAA oxidase activity is unclear.

Peroxidases increase in tissues treated with IAA (21) are localized within the protoxylary regions of developing roots (9) and are involved in lignin synthesis (10, 19). However, De Jong (3) recently presented data which do not support the current hypothesis that in some manner peroxidatic enzymes are directly involved in lignification, but rather suggest that they function in ion transport across membranes by maintaining anisotropic redox gradients. Thus, although their precise function in vivo remains obscure, they may still play an important role in the vascular development of roots produced in diseased tissue.

An important role in cell elongation in developing maize roots has also been ascribed to ascorbic acid oxidase (12), and, as in the case of peroxidase, IAA-induced growth results in a marked increase in AAO activity (13). We did not study this increase by electrophoretic techniques, as practically all the activity was associated with the particulate fraction. Our attempts to solubilize the enzymes responsible for ascorbic acid oxidation using sonification, surfactants, and cellulolytic and pectic enzymes were unsuccessful. Other investigators have encountered similar problems, although in corn roots up to 30% of the AAO enzyme activity has been obtained in a soluble form (12).

The finding that similar physiological changes occur in the internode subjacent to the inoculated one suggests that bacterially synthesized substances which "trigger" the host response or products resulting from primary host-parasite interactions are translocated, and that they in turn exert a controlling influence on protein synthesis. The nature of such substances is at present unknown. The ability of NAA to partially mimic the effect of the bacterium and of 2,4-dichloroanisole to abolish symptom expression argues for an intimate role, as might be expected a priori, for auxins in such a triggering mechanism. However, natural and synthetic auxins alone are not active in inducing root formation on fleshy root slices from turnip, potato, carrot, or beet, whereas the bacterium induces root formation in all these cases (1).

The bacterium, *A. rhizogenes*, has the capability of redirecting host metabolism so that roots are invariably produced on all infected tissue. That an auxin-peroxidase system is involved appears likely, but other bacterial-host components are probably involved in selective depression that precedes the morphogenetic responses. Work now in progress is designed to elucidate these factors.

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