The Stabilization of Artificial and Natural Cell Wall Membranes by Phenolic Infusion and its Relation to Wilt Disease Resistance

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ABSTRACT

Pectinase alone did not cause artificial calcium pectate-pectin membranes to swell. But these membranes did swell and eventually disintegrated when exposed to oxalate alone or, more rapidly, when exposed to a combination of oxalate and pectinase. They were also degraded, without apparent swelling, by *Fusarium oxysporum* f. sp. *cubense*. These membranes became highly resistant to swelling and disintegration when infused with the oxidation products of the phenolic, 3-hydroxytyramine, either before or, to a lesser extent, during treatment. Natural perforation plates in banana root vessels were unaffected by oxalate + pectinase, or by *F. oxysporum* f. sp. *cubense*. They were caused to swell and became very fragile, however, following three to four cycles of low pH/high pH + oxalate. Phenolic infusion prevented the swelling of these natural perforation plates by such treatment. It is postulated that the phenolic infusion of infection sites, commonly associated with vascular browning, is a final step in a localization process that prevents systemic infection in many wilt disease-resistant plants. It is further postulated that this infusion of infection sites with phenolics may finally serve to insulate the infection from healthy surrounding tissue, thereby turning off host responses. The timing of phenolic infusion may be critical to the success or failure of defense responses, since too early an infusion may inhibit the processes necessary for localization; whereas, too late an infusion could permit degradation of the sealing-off structures and systemic distribution of the parasite.

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Earlier evidence has shown that vascular infections by most microorganisms are regularly localized by occlusion of the affected vessels (2). Although other types of defense [i.e., lignitubers (25), endodermis (28), and phytoalexins (9, 10, 11, 15)] are certainly operative against many microorganisms, once entrance into vessels has been gained by vascular pathogens, this localization mechanism appears to be an essential factor in resistance to wilt in banana (6), tomato (5), cotton (10), elm (14), and several other plants (2). This defense system is dependent upon at least three factors: (i) initial trapping of the inoculum on perforation plates and end-walls; (ii) swelling of vessel wall materials to form a gel that temporarily seals off the infected vessels; and (iii) the initiation of growth of vascular parenchyma cells that crush the small protoxylem or invaginate the large metaxylem elements and wall off the infection permanently (6). A fourth possible factor (i.e., phenolic infusion) is responsible for the vascular discoloration that is a common internal symptom of vascular disease (7, 13, 18, 30, 31). Such phenolic infusion could play a role in making the occluding structures more resistant to chemical and physical breakdown.

The objective of this research was to test the hypothesis that infusion of wall structures and gels with a phenolic would make them more resistant to chemical degradation and physical rupture. Simple pectinaceous membranes and natural perforation plates were infused with oxidative products of 3-hydroxytyramine (HT), a phenolic known to be involved in vascular browning of banana roots (18), and then subjected to treatments known to cause swelling and degradation of noninfused membranes.

MATERIALS AND METHODS.—Artificial calcium pectate:pectin (3:1) membrane disks were prepared as described earlier (3). Membranes used in the first experiment were of low molecular-density; i.e., they were prepared by spraying 1% pectate-pectin solutions with $CaCl_2$, whereas those in the second experiment were of higher molecular-density, i.e., prepared from 3% pectate-pectin solutions. Low-molecular-density membranes were used in the first experiment to favor pectolytic enzyme action by providing greater hydration and dispersion of the pectinaceous polymers and making

cleavage points of the polymers spacially more accessible to the large molecules of the enzyme. The pectinase enzyme was supplied by Nutritional Biochemicals Corp., Cleveland, Ohio.

Prior to experimental treatment, the membranes (which had been stored in a dry state) were brought to aqueous equilibrium by submerging them in water or aqueous phenolic solutions for 4 h. The membranes were

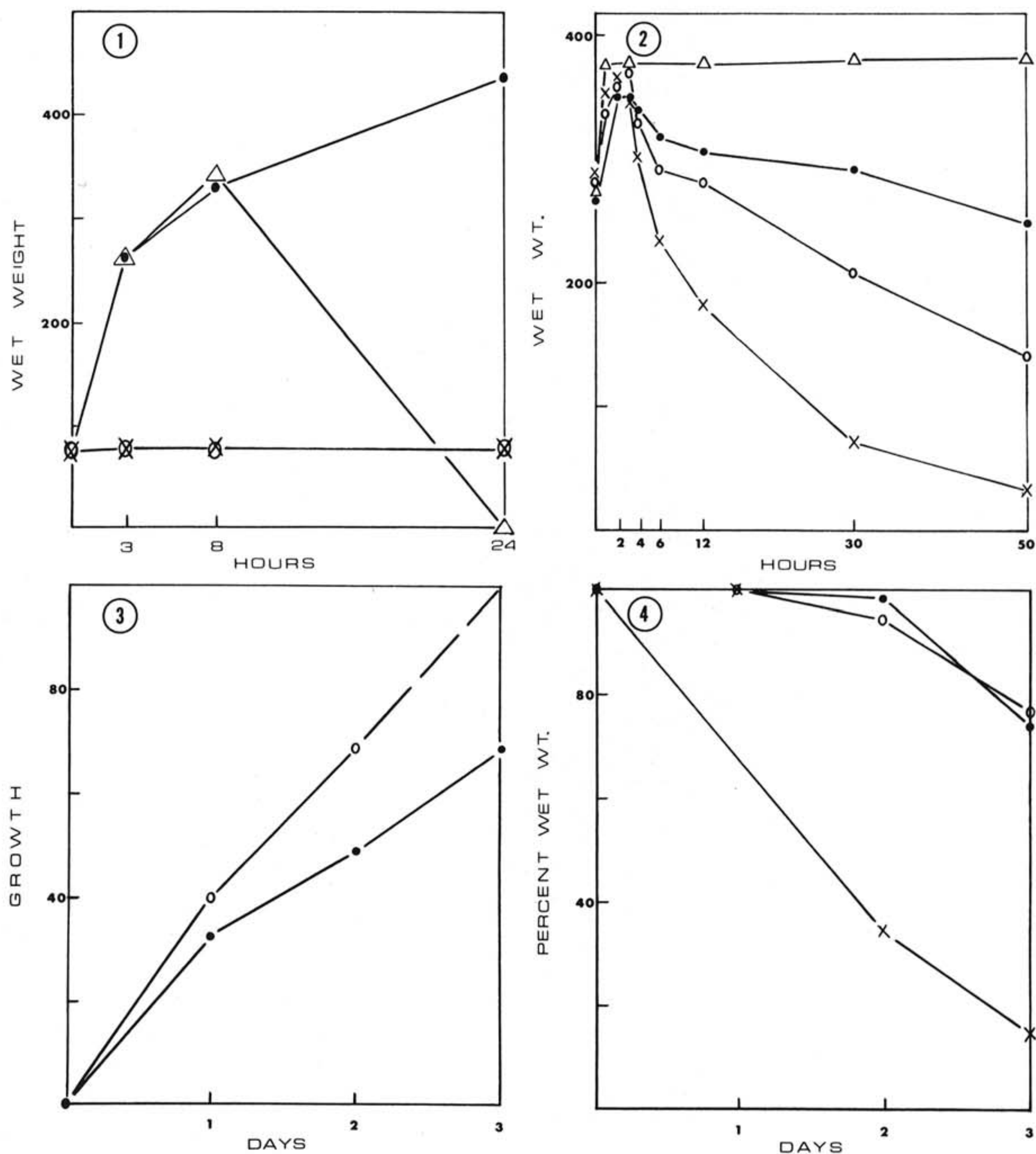

Fig. 1-4. 1) The wet wts (mg) of 3:1 calcium pectate:pectin membrane disks at various times after immersion in water (o), in pectinase solution (x), in 0.01M sodium oxalate solution (•), or in oxalate plus pectinase (△). **2)** The wet weights (mg) of 3:1 calcium pectate:pectin membrane disks treated with 0.01M sodium oxalate for various lengths of time after a preliminary 4-h aqueous equilibration period (before zero time) in which membranes were not infused with HT (x) or were infused with HT only during the 4-h aqueous equilibration period (•), continuously after aqueous equilibration (o), or continuously during and after aqueous equilibration (△). **3)** Growth (mm) of hyphae of *Fusarium oxysporum* f. sp. *cubense* from the margins of 3:1 calcium pectate:pectin disks that were (o) or were not (•) infused with phenol. **4)** Percent of the original wet wt of 3:1 calcium pectate:pectin disks that were seeded with spores of *F. oxysporum* f. sp. *cubense* and infused (o) or not infused (x) with HT, or held in water without fungal seeding or HT infusion (•) for 1, 2, and 3 days.

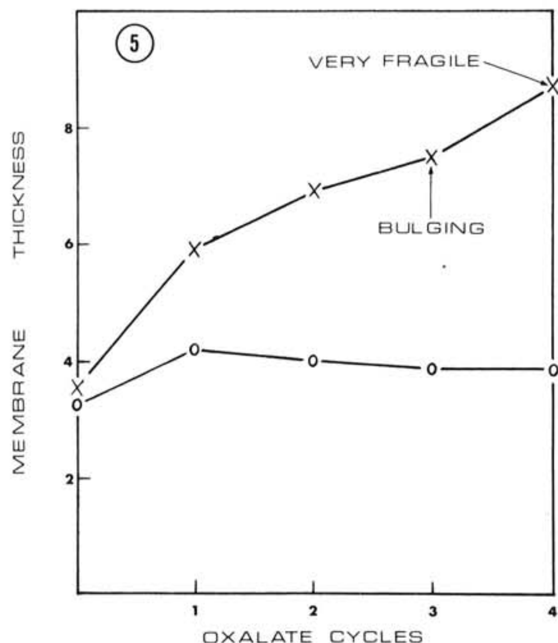

Fig. 5. The thickness (μm) of banana root perforation plates, infused (o) or not infused (x) with HT, when measured under the microscope after one to four cycles of low pH/high pH plus oxalate.

infused with oxidized phenolic products during, after, or both during and after aqueous equilibrium treatment by immersing them in a solution of 5 mg of 3-hydroxytyramine (HT) and 1 mg of polyphenoloxidase (PPO) in 30 ml of water. The PPO used was Tyrosinase having 3,690 units/mg solid and distributed by Sigma Chemical Co., St. Louis, Mo. This solution and the membranes immersed in it turned yellow to orange to red-brown within a few hours. Infused and noninfused membranes were then treated as described under Results and the degrees of swelling and eventual disintegration were measured by taking periodic wet wts. Weights increased as the membranes absorbed water and swelled, but as the membrane continued to swell, the surface substances dissolved, integrity was lost, and the weighable mass decreased.

Artificially infused or noninfused natural perforation plates in longitudinal sections of banana (*Musa acuminata* L. 'Valery') roots were treated with low pH/high pH + oxalate cycles (3) to determine their resistance to swelling and degradation. The thickness of 25 to 30 perforation plates in both phenolic-infused and noninfused sections of three roots were measured microscopically with an occular micrometer after each low pH/high pH + oxalate cycle. The slide mounts were then repeatedly compressed and released manually to determine if the membranes had become flaccid or fragile.

In addition, small areas of cortical tissues on healthy, vigorous banana roots were injured by incision or compression, and harvested 7 days later at which time the natural phenolic infusion and browning of these tissues seemed to be complete. Cross-sections that included both darkened and apparently healthy tissue were then immersed in 1N NaOH or several cycles of low pH/high pH + oxalate, placed on microscope slides, compressed manually as above, and examined under the microscope to determine if these tissues had become softened and could be easily macerated.

Finally, tomato plants (*Lycopersicon esculentum* Mill.) that were susceptible (Improved Pearson) or resistant (Improved Pearson VF-11) to wilt were inoculated as reported earlier (5) with a suspension containing vinyl tracer particles and microspores of *Fusarium oxysporum* (Schlecht.) emend. Snyd. & Hans. f. sp. *lycopersici* (Sacc.) Snyd. & Hans. race 1. Plants were harvested at daily intervals through 7 days, and at 14 and 21 days after inoculation. Fresh, free-hand cross-sections of the lower hypocotyls were then examined microscopically for the earliest evidences of phenolic release and oxidation, for phenolic infusion of vascular structures, and for the presence of tyloses.

RESULTS.—*Factors that cause membrane degradation.*—Experiments were conducted initially to establish the kinds of chemical actions to which membranes might be susceptible; i.e., cleavage of polymers and breaking of interpolymer bridges, or both. Artificial, low molecular-density, calcium pectate-pectin membrane disks that had not been infused with HT were treated with pectinase, with sodium oxalate, with pectinase + oxalate, or kept in water. Water or pectinase alone had no effect on these membranes (Fig. 1). Oxalate alone caused a marked swelling (gain in wt) of the membranes during the first 24 h. Complete dissolution of the membranes occurred after 48 h, but that is not shown in Fig. 1. Pectinase with oxalate caused an initial swelling comparable to that in oxalate alone, but that was followed by a very rapid and complete dissolution of the gel, as indicated by the complete loss in weight within 24 h. Polymer cleavage by pectinase thus enhanced the dissolution of membranes, but did not affect the swelling process nor cause dissolution without a preparatory swelling treatment that removed calcium from the pectic substances. Removal of the calcium was therefore essential to membrane swelling and eventual dissolution, whereas pectinase action merely enhanced the dissolution process.

Effect of HT infusion on the degradation of artificial membranes.—In an experiment to determine the effect of HT, membranes of high molecular-density were infused with HT continuously, infused only during the period (4 h) required for the dry membranes to reach aqueous equilibrium, or infused continuously only after aqueous equilibrium had been reached. A comparable degree of membrane swelling had occurred by the time of aqueous equilibrium with or without HT infusion (0 h in Fig. 2). Therefore, HT infusion did not affect the capacity of membranes to absorb water to the point of aqueous equilibrium. The aqueous equilibrium wt of these high-density membranes was considerably greater than that of the preceding low-density membranes, but their innate capacity to swell was less. The membranes were then treated with 0.01 M sodium oxalate (at 0 h) and the wet wts determined at various time intervals thereafter (Fig. 2). HT infusion did protect membranes from dissolution by oxalate, and the time and duration of infusion had a considerable effect on the relative resistance of membranes to dissolution.

Calcium pectate-pectin disks were then immersed in the standard solution of HT and PPO, whereas controls were immersed in PPO alone to provide a comparable level of protein. These solutions were then seeded with 1.0×10^6 spores in 1.0 ml of a conidial suspension of *Fusarium oxysporum* Schlecht. f. sp. *cubense* (E. F. Sm.) Snyd. & Hans. Growth of fungal hyphae was at least as great or greater in the treatment containing oxidized phenolic as in the phenolic-free treatments during 72 h of incubation (Fig. 3), but the HT-infused membranes were far more resistant to fungal action than were noninfused membranes (Fig. 4).

Effect of HT infusion on the degradation of natural cell walls of banana.—Natural cell walls are far more complex than the artificial membranes used in the preceding experiments, and are not affected by a simple oxalate or oxalate + pectinase treatment or even by the growth of *Fusarium* (3). Therefore, repeated low pH (2.8)/high pH (5.0) + oxalate cycles were employed to swell the perforation plates in longitudinal sections of

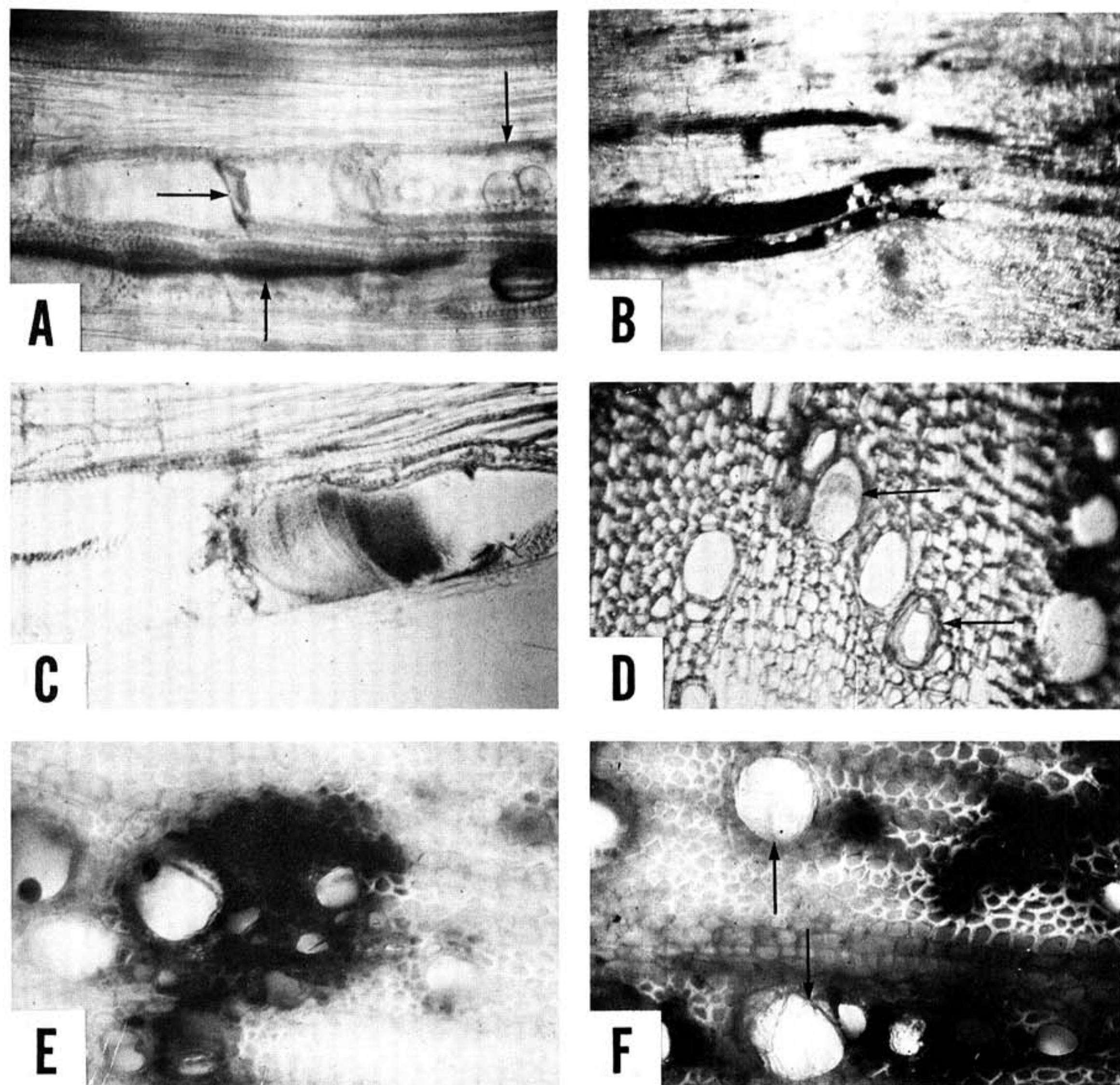

Fig. 6-(A to F). Vascular browning, phenolic-infusion of gels, and the presence or absence of tyloses as associated with the absence or presence of vascular browning in the hypocotyls of tomato plants that had been inoculated with *F. oxysporum* f. sp. *lycopersici*. **A)** early browning of randomly-occurring, phenol-storing parenchyma cells (vertical arrows) and discolored perforation plate (horizontal arrow) 3 days after inoculation, **B)** dark browning of vessels 4 days after inoculation, **C)** phenolic infusion of a vascular gel plug 4 days after inoculation, **D)** the persistence of phenolic-infused gels (arrows) 21 days after inoculation, **E)** the general absence of tyloses where strong vascular browning was apparent, and **F)** the frequent presence of tyloses (arrows) where browning was limited or absent 21 days after inoculation.

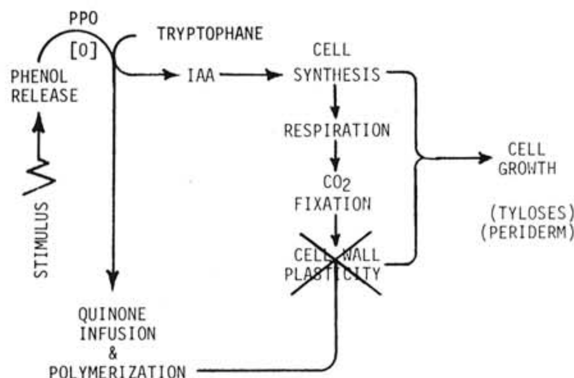

Fig. 7. A hypothetical scheme depicting the central role of phenolics in regulating growth responses to injury or infection. See discussion.

banana roots that had been artificially infused with HT or kept noninfused in a large volume of water to minimize infusion that would otherwise have occurred naturally. The thickness of perforation plates was then measured. Infusion with HT caused a marked stabilization of these membranes that were otherwise susceptible to swelling (Fig. 5). Whereas noninfused membranes swelled and eventually became fragile and disintegrated when mild pressure was alternately applied and released on the covering slide, membranes that were artificially infused with HT did not swell as a result of this treatment and never became fragile.

Observations concerning natural phenolic infusion in plant tissues.—Cortical tissues of banana roots were injured by needle punctures or by incisions with a razor blade. Tissues within or closely adjacent to the sites of injury became infused with phenolic naturally. Cross-sections of these roots were cut to include both infused and noninfused tissue and were treated with low pH/high pH + oxalate cycles or with 1.0 N NaOH. After four low pH/high pH + oxalate cycles, or four days in NaOH, the noninfused tissues had become very soft and were easily macerated on microscope slides when a little manual pressure was applied to covering slides. The NaOH treatment presumably softened tissues because the high level of sodium displaced calcium and broke the calcium bridges between pectate polymers. HT-infused tissues, on the other hand, remained intact even after pressure was applied vigorously and repeatedly.

Cross-sections of the lower portion of hypocotyls of tomato plants were examined at various intervals after inoculation (5) with *Fusarium*. Slight yellowing and release of phenolic compounds were associated with randomly scattered vascular parenchyma cells as early as 2 days after inoculation. Vascular discoloration and gel infusion were clearly apparent 3 and 4 days after inoculation [Fig. 6-(A to C)]. The process appeared to be essentially comparable, but possibly more rapid in the susceptible, than in the resistant, tomato isoline. After 21 days, when host responses were complete, phenolic-infused gels were still clearly visible (Fig. 6-D,E). At this time, tyloses were generally absent where phenolic infusion and discoloration was extensive (Fig. 6-E), but were generally abundant above the zone of phenolic infusion (Fig. 6-F).

DISCUSSION.—The capacity to alter the plastic-rigid state of cell walls is a determining factor in the everyday life of plants. During growth of cells, walls must be plastic to permit enlargement or elongation. Once differentiated, however, tissues depend upon the rigidity of cell walls for form and function. But cells do not always remain differentiated, nor do walls always remain rigid, because tissues must be able to respond to wounding or to new requirements dictated by development or the environment. Thus, thin-walled parenchyma, and even collenchyma cells can dedifferentiate, renew growth, and redifferentiate to form new tissues; e.g., adventitious roots and wound callus; and cell walls can be made plastic or rigid as need dictates. These walls are also susceptible to degradation by many microorganisms, especially those that produce calcium-binding organic acids as well as hydrolytic enzymes (1).

In some instances, however, cell walls or parts of walls are made irreversibly rigid by secondary thickening and lignification as in the formation of endodermis and sclerenchyma cells. In the process, cell walls may be made highly impervious and death may ensue so that the tissues form an inert protective barrier; e.g., in cork, periderm, abscission layers, or xylem sclerenchyma. These structures are highly resistant to microbial penetration and degradation (28).

It appears that these same basic capabilities and processes of cells and tissues may be employed in a system of defense by which infections are walled-off from surrounding, healthy tissues. It has been shown that phenolics are stored (compartmented) and maintained in a reduced state in specialized cells that are randomly scattered throughout tissues of many higher plants (7, 8, 18). When mechanical injury or infection occurs at a particular site, the phenolics at that site are released (7, 20), become mixed with glucosidases and/or oxidases (13, 20), and are thus converted into quinones (24). The synthesis of phenolics de novo (13, 20, 22, 23) may also augment the supply. With the oxidation of these phenolics, two opposing processes apparently begin. The first turns-on dedifferentiation, growth and redifferentiation in adjacent tissues, whereas the second insulates the region and source of excitation from adjacent tissues to turn-off these processes (Fig. 7).

The processes of dedifferentiation and growth apparently are turned-on because the oxidation of ortho-dihydroxy phenolics mediates the enzymatic conversion of tryptophane to 3-indoleacetic acid (IAA) (16, 19, 21). IAA, being a hormone, is then transported for some distance into adjacent tissues and turns-on the processes of dedifferentiation and growth, including the synthesis of cellular constituents and the plasticizing of rigid walls that permits cell enlargement (4). Such a sequence of events seems to have been reasonably demonstrated in the case of vascular diseases where the conversion of phenolics to quinones (13, 20), the build-up of IAA (26, 27), the induction of tyloses by IAA (19), and the walling-off of infections by tyloses in wilt-resistant plants (5, 6, 10, 14) have been found (upper sequence in Fig. 7).

The oxidation of these same stored phenolics (and possibly phenolics synthesized de novo) could also set in motion events that turn-off these host response processes. It has been shown (3) that different cell wall polymers

incorporated into a single membrane structure interact to make such membranes highly resistant to any single chemical action. The results herein indicate that incorporation of yet another polymer by the infusion, and apparent oxidation and polymerization of phenolics (24), makes them all but immutable. The results of Corden and Chambers (12) have shown that naturally- and artificially infused tissues are also highly impervious to water and dyes.

It is well established that when phenolic derivatives are incorporated into cell walls during lignification and "suberization" (17, 23, 29), the cell walls are permanently fixed in a rigid state that prevents dedifferentiation and growth and makes them highly resistant to fungal penetration and degradation (17, 28). The results reported herein indicate that the infusion of small phenolic molecules into preformed cell walls and their incorporation into the polymer lattice of those walls, while not the same as lignification, provides a rapid alternative means by which cell walls can be made permanently rigid where and when that reaction occurs. As a result, infections and the stimulus they provide are contained and isolated from surrounding healthy tissues and the processes of host responses are eventually turned off (lower sequence in Fig. 7).

Since both the turning-on and turning-off of host responses seem to be regulated by the release, oxidation, and accumulation of phenolic compounds in affected tissues, the rate at which these processes occur could determine whether or not infections are sealed off, and thus whether a plant is resistant or susceptible to systemic infection and disease. Sequeira and Kelman (27) have demonstrated that a growth promoter, as well as growth inhibitors, are present in the vascular tissues of plants infected with *Pseudomonas solanacearum*. The growth promoter was apparently IAA, whereas the growth inhibitors were phenolic in nature. In addition, it has been found that although tylose initiation was comparable in both resistant and susceptible isolines of tomato infected with *Fusarium*, an inhibiting factor came into play in the susceptible isoline which delayed the occlusion of vessels and permitted systemic distribution of the pathogen (5). As reported herein, there is an inverse relationship between the occurrence of tyloses and the occurrence of visible vascular browning. These results suggest that the phenolic infusion responsible for vascular browning can prevent the normal plasticizing of walls and inhibit the growth of tyloses at infection sites. It seems, then, that a delicate balance and timing must be maintained between the mediation of IAA by phenolics, and the infusion of phenolics into wall structures, if the defensive system is to be effective. Too slow a release of phenolics could delay the turning-on of the localization response, but too rapid an accumulation of oxidized phenolics could turn-off the response before the walling off of infections becomes completed. In either case, systemic infection and disease could result. It appears, then, that phenolics may play a central role in regulating host responses to injury and infection and, thereby, relative resistance and susceptibility of the host plant to disease.

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