

**The Relationship of Phosphatidase Activity to the Hypersensitive Reaction in Tobacco Induced by Bacteria**

Jeng-sheng Huang and Robert N. Goodman

Graduate Assistant and Professor, respectively, Department of Plant Pathology, University of Missouri, Columbia 65201.

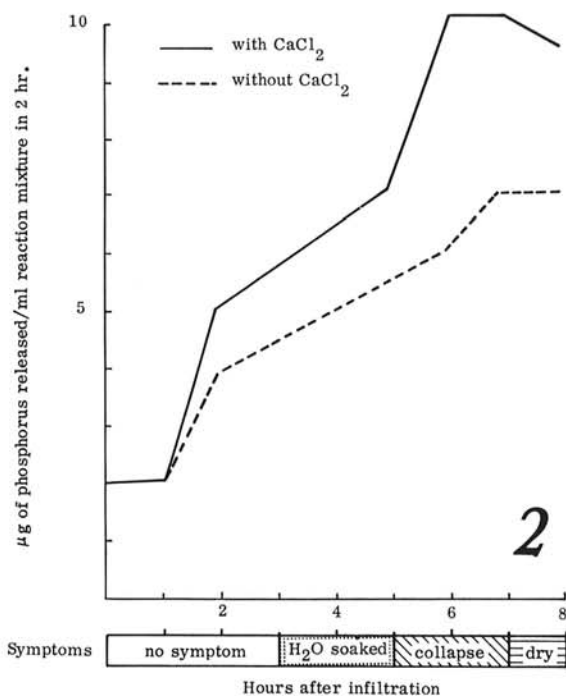
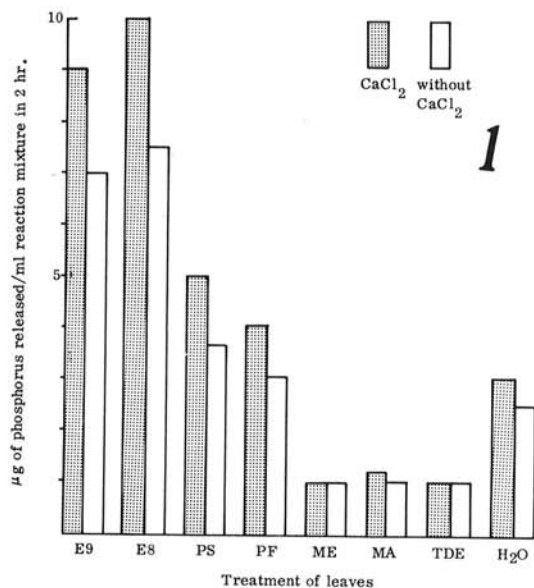
Missouri Agricultural Experiment Station Journal Series Paper No. 5626. Supported by a grant (GB 7472) from the National Science Foundation.

The authors wish to express their gratitude to D. F. Bateman for helpful suggestions made during the course of these experiments.

The predominant characteristics of the bacterially induced hypersensitive reaction (HR) in plants are that the cells of the tissues containing the bacteria lose their turgor; that is followed by desiccation and necrosis (2, 6). These symptoms suggest that an increase in the permeability of host cell membranes occurs during HR (2, 6). Phosphatides and proteins are the primary structural components in plant cell membranes. Alteration of either of these constituents should alter cell permeability. The ability of a number of phytopathogens to produce phosphatidases *in vitro* has been reported (9), and the enzymes have recently been found in some fungus-infected plants (7, 8). Five types of phosphatidases, e.g., phosphatidases A, B, C, D, and lysophosphatidase have been described (3), each of which is specific for the hydrolysis of one of the ester linkages in the phosphatide molecule. Phosphatidases A, B, C, and lysophosphatidase have been found in animal tissues and microorganisms; phosphatidase D has been reported only from higher plants (4). In this investigation we sought to determine the activity of phosphatidases in tissues undergoing HR, and their involvement in the cause of HR.

In order to detect the production of phosphatidases by bacteria *in vitro*, *Pseudomonas syringae* van Hall, *P. fluorescens* Migula, and virulent and avirulent bacteria of *Erwinia amylovora* (Burrill) Winslow et al. were grown in the following media adjusted to pH 6.8: Nutrient-yeast-glucose broth [NYGB (10)]; Czapek-Dox broth with or without 0.04% lecithin (vegetable lecithin, Mann Research Lab.); and a tobacco leaf decoction (10 g tobacco leaves in 100 ml water). Following incubation at 30 C for 48 hr, the culture media were assayed for phosphatidase activity on lecithin agar (pH 6.8) by the 'cup plate' method (8). Phosphatidase activity was not detected in cultures of either *P. syringae* or the virulent or avirulent strain of *E. amylovora*. It was detected, however, in nonautoclaved NYGB and Czapek-Dox broth (with or without lecithin) cultures of *P. fluorescens*.

*Nicotiana tabacum* L. 'Samsun NN' plants were grown in vermiculite, irrigated with Hoagland's solution in a growth chamber (day, 14 hr, 28 C; night, 10 hr, 18 C; light intensity 2,500 ft-c). Plants with 5 to 6 partially expanded leaves were used in the experi-



**Fig. 1-2.** 1) Phosphatidase activities of the extracts of tobacco leaves 8 hr after infiltrating with bacteria, sulfur-containing compounds, or distilled water. The reaction mixtures contained: 0.5 ml of 1.0% lecithin emulsion in 0.1 M Tris-HCl buffer (pH 8.5); 0.25 ml of water or 0.05 M CaCl<sub>2</sub>; and 0.25 ml of plant extract. The reaction mixtures were incubated for 2 hr at 30 C. E9 = Virulent strain of *Erwinia amylovora*; E8 = avirulent strain of *E. amylovora*; PS = *Pseudomonas syringae*; PF = *P. fluorescens*. ME = Mercaptoethanol; MA = mercaptoacetic acid. TDE = Thio-diethanol; H<sub>2</sub>O = sterile distilled water. 2) Phosphatidase activities in the extracts of tobacco leaves infiltrated with 10<sup>8</sup> cells/ml of the virulent strain of *E. amylovora*. The reaction mixtures contained the same constituents as Fig. 1. The reaction mixtures were incubated for 2 hr at 30 C.

ments. Phosphatidase activity was determined in leaves following infiltration of the intercellular spaces (5) with  $10^8$  cells/ml of bacterial suspensions, 0.025 M mercaptoethanol, mercaptoacetic acid, 0.1 M thiodiethanol (adjusted to pH 6.8), or distilled water. Eight hr after infiltration the leaves were detached, ground in water (1:1, w/v), and centrifuged. The supernatant was dialyzed against water for 36 hr at 5 C and assayed for phosphatidase activity by using the acid-soluble phosphorus procedure (8). Reaction mixtures (Fig. 1) supplemented with  $\text{CaCl}_2$  stimulated phosphatidase activity; release of acid-soluble phosphorus was maximal at pH 7.5 to 8.5. At pH 8.5, in the presence of  $\text{CaCl}_2$ , the extracts from leaves infiltrated with the virulent or avirulent strain of *E. amylovora* which cause HR released 9 and 10  $\mu\text{g}$ , respectively, of acid-soluble phosphorus/ml of reaction mixture at 30 C in 2 hr. The extracts from leaves injected with *P. syringae* or *P. fluorescens*, which showed no HR, had no significant increase in activity over the water control. Trace amounts of phosphorus were detected in reaction mixtures containing the extracts from leaves infiltrated with mercaptoethanol or mercaptoacetic acid which caused a hypersensitivelike reaction. The extract of the leaves infiltrated with thiodiethanol which showed no HR-like symptoms also exhibited trace amounts of phosphatidase activity (Fig. 1). In a similar experiment, a suspension containing  $10^6$  cells/ml of the virulent strain of *E. amylovora* caused no visible HR symptoms on tobacco leaves, whereas suspensions of  $10^7$  and  $10^8$  cells/ml induced HR. Acid-soluble phosphorus released from the  $10^6$ ,  $10^7$ , and  $10^8$  cells/ml treatments was 11, 10, and 9  $\mu\text{g}$ , respectively/ml of reaction mixture at pH 8.5, 30 C in 2 hr.

The relationship of the development of HR symptoms to phosphatidase activity in tobacco leaves following infiltration of  $10^8$  cells/ml of virulent strain of *E. amylovora* is shown in Fig. 2.

The extracts prepared from leaves undergoing HR induced by *E. amylovora* and water-treated control released 8.5 and 3  $\mu\text{g}$ , respectively, of free choline/ml of reaction mixture of pH 8.5, 30 C in 2 hr when lecithin was used as substrate and assayed with potassium triiodide (1). No free choline was detected when either glycerylphosphorylcholine or phosphorylcholine (Sigma Chemical Co.) was used as substrate instead of lecithin. Thus, the enzyme presented in these two extracts was phosphatidase D.

When 10 mg/ml aqueous solution of phospholipase C (1-2 EU/ml, Mann Research Lab.) or phospholipase D (0.7 EU/ml, Calbiochem Co.) was infiltrated into tobacco leaves, HR symptoms were not induced.

The data we have presented may be summarized as follows: (i) *E. amylovora* did not produce phosphatidase in vitro but did stimulate the synthesis of host phosphatidase D; (ii) changes in plant cell permeability caused by SH-containing compounds (2, 6) resulting in an HR-like symptom do not potentiate phosphatidase activity; (iii) host phosphatidase activity was stimulated to the greatest extent by  $10^6$  cells/ml of *E. amylovora*, a cell concentration that did not induce HR; (iv) commercial preparations of phosphatidases C and D did not induce HR. Hence, it would appear that although bacteria and particularly *E. amylovora* are able to potentiate phosphatidase activity in tobacco leaf tissue, such potentiated activity does not cause HR.

## LITERATURE CITED

- APPLETON, H. D., B. N. LA DU, JR., B. B. LEVY, J. M. STEELE, & B. B. BRODIE. 1953. A chemical method for the determination of free choline in plasma. *J. Biol. Chem.* 105:803-813.
- GOODMAN, R. N. 1968. The hypersensitive reaction in tobacco: a reflection of changes in host cell permeability. *Phytopathology* 58:872-873.
- INTERNATIONAL UNION OF BIOCHEMISTRY, COMMISSION ON ENZYMES. 1965. Enzyme nomenclature. Elsevier Pub. Co., Amsterdam. 219 p.
- KATES, M. 1960. Lipolytic enzymes, p. 165-237. *In* K. Block [ed.] *Lipid metabolism*. John Wiley & Sons, N.Y.
- KLEMENT, Z. 1963. Rapid detection of the pathogenicity of phytopathogenic pseudomonads. *Nature* 199:299-300.
- KLEMENT, Z., & R. N. GOODMAN. 1967. The hypersensitive reaction to infection by bacterial plant pathogens. *Annu. Rev. Phytopathol.* 5:17-44.
- LUMSDEN, R. D. 1968. Phosphatidase production by *Sclerotinia sclerotiorum* in culture and in association with infected bean hypocotyls. *Phytopathology* 58:1058 (Abstr.).
- LUMSDEN, R. D., & D. F. BATEMAN. 1968. Phosphatidase degrading enzymes associated with pathogenesis in *Phaseolus vulgaris* infected with *Thielaviopsis basicola*. *Phytopathology* 58:219-227.
- TSENG, T. C., & D. F. BATEMAN. 1968. Production of phosphatidases by phytopathogens. *Phytopathology* 58:1437-1438.
- VÖRÖS, J., & R. N. GOODMAN. 1965. Filamentous forms of *Erwinia amylovora*. *Phytopathology* 55:876-879.