

## Enzyme-linked Immunosorbent Assay for Pierce's Disease Bacteria in Plant Tissues

S. F. Nomé, B. C. Raju, A. C. Goheen, G. Nyland, and D. Docampo

First and last authors, professors, Facultad de Ciencias Agropecuarias, Universidad Nacional de Cordoba, Argentina. Second, third, and fourth authors, postgraduate research plant pathologist, research plant pathologist, United States Department of Agriculture, Science and Education Administration, and professor, respectively, Department of Plant Pathology, University of California, Davis 95616.

Work was done in the Department of Plant Pathology, University of California, Davis, where the first and fifth authors were supported through a fellowship from Consejo Nacional de Investigaciones Cientificas y Tecnicas de la Republica Argentina.

Mention of a trademark or proprietary product does not guarantee a warranty of the product by the U.S. Department of Agriculture and does not imply approval of it to the exclusion of other products that also may be suitable.

Accepted for publication 31 January 1980.

---

### ABSTRACT

NOMÉ, S. F., B. C. RAJU, A. C. GOHEEN, G. NYLAND, and D. DOCAMPO. 1980. Enzyme-linked immunosorbent assay for Pierce's disease bacteria in plant tissues. *Phytopathology* 70:746-749.

An antiserum with a reciprocal titer of 4096 was prepared in rabbits against cultures of an isolate of Pierce's disease bacterium from grapes. The immunoglobulin of the antiserum was purified and conjugated with alkaline phosphatase by standard procedures. The conjugate was standardized against an isolate of Pierce's disease bacterium and used in enzyme-linked

immunosorbent assay for detecting bacteria in host plant tissues. The conjugate reacted specifically to Pierce's disease bacterium either in pure culture or host tissue, but did not react with 15 isolates of other plant pathogenic bacteria, four saprophytes, or two symbionts.

---

The enzyme-linked immunosorbent assay (ELISA) technique has been used successfully since 1971 for the detection and study of several different pathogens causing diseases of humans and animals (13,14), and since 1976 in the detection of plant pathogens (2,12). In plants, the emphasis has been in virus detection, especially in circumstances in which either the virus is difficult to detect or in which it is necessary to work with a large number of samples (2,12). No work with the fastidious bacteria, which are limited to xylem tissues of their host, has yet been reported.

Pierce's disease (PD) and almond leaf scorch (ALS) are caused by the same bacterium (10). The experienced observer can readily identify each disease by symptoms during late summer when

expression is strong, but often at other times symptoms may be confused with those of other diseases or nutritional problems. Confirmation of diagnosis is usually done by electron microscopy, culture of bacteria, or, in almond, by a tissue staining procedure (9,10).

For other pathogens, ELISA is a reliable, sensitive and fast method, so we adapted it for the detection and study of the pathogen causing PD and ALS, having in mind that it might be useful in epidemiological studies. This paper reports the use of this method for the detection and quantitative assay of the PD bacteria, in pure culture and in diseased plants.

### MATERIALS AND METHODS

Two isolates of PD bacteria were used, one from almond and the other from grape. The grape isolate (G-NA-5) was obtained from Pierce's disease-infected grape leaves collected in the Napa Valley

---

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1980.

of California. The isolation was made on JD-3 agar medium (4). The almond isolate (AL-CC-8) was from almond leaf scorch-infected almond leaves collected in Contra Costa County, California. The isolates were maintained on JD-3 medium at 27 C by weekly subcultures.

The isolates from grape and almond were compared with 21 isolates of bacteria, most of them plant pathogens, belonging to the genera *Agrobacterium*, *Escherichia*, *Pseudomonas*, *Xanthomonas*, *Corynebacterium*, *Erwinia*, *Bacillus*, and *Rhizobium*. Each was tested by ELISA to determine the specificity of the PD bacterium antisera (Table 1). Cultures were provided from stock collections in the Department of Plant Pathology, University of California, Davis.

**Antiserum production.** An antiserum against the Pierce's disease bacterial isolate (G-NA-5) was produced in New Zealand white rabbits. The bacteria were harvested from broth cultures by centrifugation (23,000 g, 15 min) and washed five times in phosphate-buffered saline (PBS), 0.1 M, pH 7.0. The pellet was suspended in PBS (at a concentration of  $10^9$  bacteria per milliliter), sonicated with a Brownwill®-III sonicator (Brownwill Scientific, Rochester, NY) to break the bacterial cells, and the broken bacterial cells were used as an antigen. Each rabbit was intravenously injected with increasing dosages of 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 ml at 3-day intervals over a period of 3 wk. After one week this was followed by two intramuscular dosages of 4.0 and 4.5 ml of antigen emulsified with equal volume of Freund's incomplete adjuvant at 3-day intervals and the rabbits were bled 1 wk after the final injection. The antiserum had a reciprocal titer of 4,096 against homologous antigen.

**ELISA.** Gamma-globulin was purified from the PD antiserum by precipitating it with ammonium sulphate and filtering it through DE52 cellulose (Whatman Ltd.) in a column preequilibrated with PBS. Unabsorbed fractions with the highest  $A_{278nm}$  were collected and diluted to an  $A_{278nm}$  value of 1.34. Conjugation with alkaline phosphatase (Type VII, Sigma Chemical Co., St. Louis, MO) was accomplished with glutaraldehyde at a final concentration of 0.06%. The preparation and handling of plates was done in a way

TABLE 1. Absorbance ( $A_{405nm}$ ) values obtained from enzyme-linked immunosorbent assay with different plant pathogenic bacteria including the Pierce's disease bacterium (PDB)

Bacteria tested <sup>a</sup>	Bacterial cells/ml		Reaction significance
	$10^8$	$10^6$	
<i>Agrobacterium tumefaciens</i>	.092	-.093	-
	.101	-.082	-
<i>Bacillus subtilis</i>	.093	.097	-
	.087	.103	-
<i>Corynebacterium michiganense</i>	.088	.087	-
<i>Escherichia coli</i>	.096	.090	-
	.104	.120	-
<i>Erwinia</i>			
<i>nigrifluens</i>	.092	.090	-
<i>amylovora</i>	.105	.100	-
<i>Pseudomonas</i>			
<i>syringae</i>	.110	.110	-
<i>lachrymans</i>	.112	.094	-
<i>phaseolicola</i>	.096	.100	-
<i>savastanoi</i>	.110	.101	-
<i>glycinea</i>	.105	.092	-
<i>morsprunorum</i>	.098	.082	-
<i>Rhizobium</i>			
<i>leguminosarum</i>	.102	.112	-
<i>japonicum</i>	.097	.139	-
<i>Xanthomonas</i>			
<i>malvacearum</i>	.101	.083	-
	.096	.090	-
<i>campestris</i>	.096	.101	-
<i>pruni</i>	.089	.097	-
PDB			
from almond	1.730	1.635	+
from grape	1.710	1.500	+
Phosphate-buffered saline (PBS) control	.149	.105	

<sup>a</sup>Bacterial isolates were obtained from J. E. DeVay and C. I. Kado, Department of Plant Pathology, University of California, Davis 95616.  
<sup>b</sup>-, not significantly different from PBS control; +, significantly different ( $P = 0.01$ ).

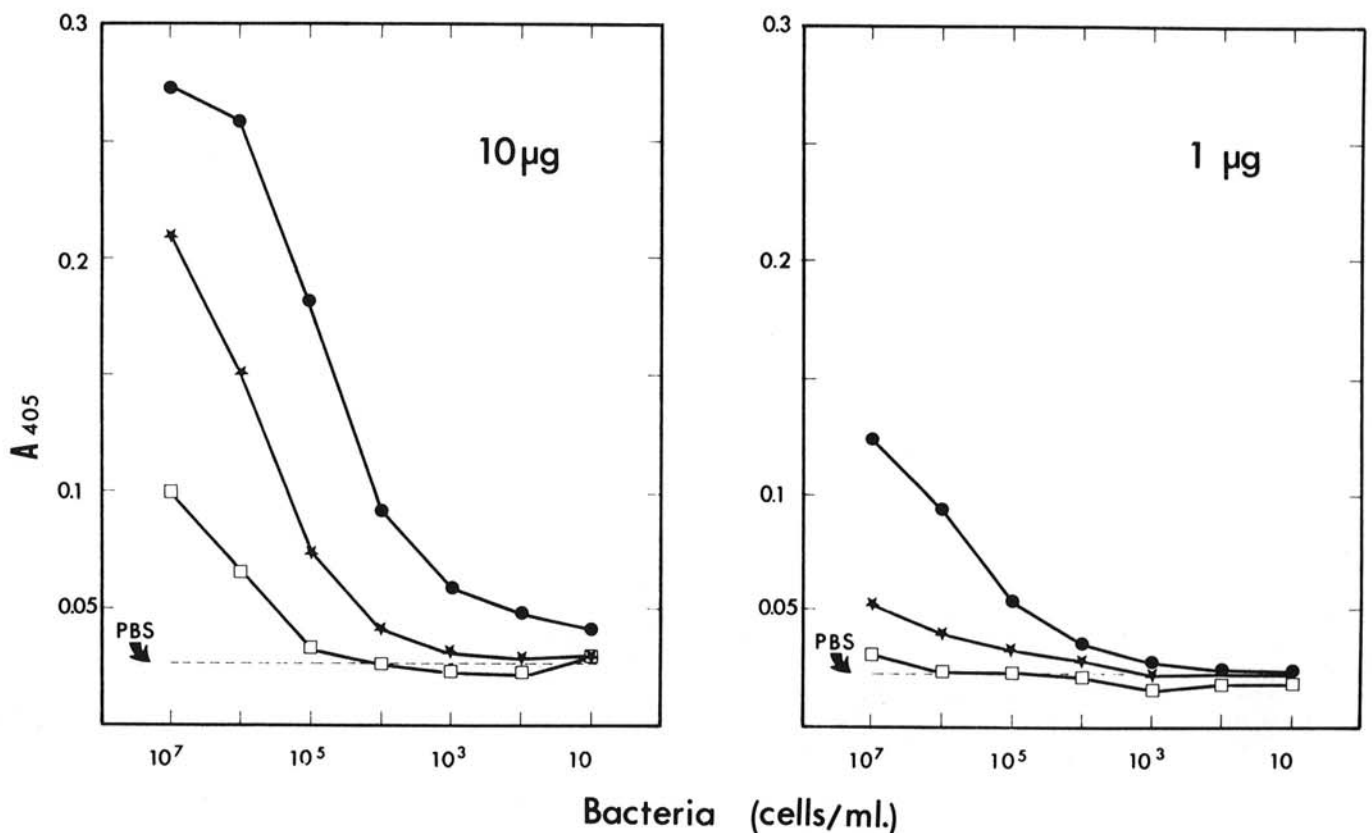


Fig. 1. Absorbance values ( $A_{405nm}$ ) for 10 and 1 µg coating  $\gamma$ -globulin of antiserum prepared against Pierce's disease bacterium (PDB) and various concentrations of PDB. Enzyme conjugate dilutions are: ●-●, 1/200; \*-\*, 1/800; and □-□, 1/3,200.

similar to that described by Clark and Adams (2) with some modifications. Flat-bottom Micro-ELISA® plates (Dynatech Laboratories) were coated with gamma globulin and incubated at 36 C for 3–4 hr with enzyme-labeled conjugates. Samples were incubated in the plate wells at 6 C for 16–18 hr. The plates were washed gently with a stream of PBS + 0.05% Tween-20. The enzyme substrate (*p*-nitrophenyl phosphate, Sigma 104-105) at a concentration between 0.6–0.9 mg/ml was allowed to act for 30–40 min after which the reaction was stopped with 3 M NaOH (50  $\mu$ l/well). We kept the plates over ice until the readings were finished because we noticed some increase in color after adding the NaOH if the plates were not kept on ice.

Tests results were scored visually and spectrophotometrically at 405 nm. For the latter, a spectrophotometer (Acta III; Beckman) was used and the readings were made after diluting the sample 1:1 with PBS. In the experiments with different bacterial species (Table 1) a Beckman 25 spectrophotometer was used without diluting samples.

**Preparation of samples.** Bacterial samples were prepared from agar slants to an initial concentration of  $1 \times 10^8$  cells per milliliter in PBS + Tween (0.05%) + PVP 2% (w/v) (polyvinylpyrrolidone, BDH Chemicals, MW 44,000). Each sample was sonicated for 45 sec in a Biosonik III to break up the tissue. Tenfold dilutions to 10 cells per milliliter were prepared for PD bacteria. For the other species, dilutions  $10^6$  to  $10^8$  were employed.

Plant samples were prepared separately from petioles, leaf veins,

TABLE 2. Enzyme-linked immunosorbent assay minimum and maximum absorbance ( $A_{405\text{nm}}$ ) values obtained with tissue of healthy and diseased grape and almond plants and with phosphate-buffered saline (PBS) as controls

Plant	$A_{405\text{nm}}^a$					
	Healthy		Diseased		PBS	
	min	max	min	max	min	max
Almond	.054	.065	.180	.265	...	...
Grape	.040	.068	.132	.283	.040	.065

<sup>a</sup> Each value represents the mean of three determinations. Values from diseased plants are significantly different ( $P=0.01$ ) from those of healthy plants and of PBS controls.

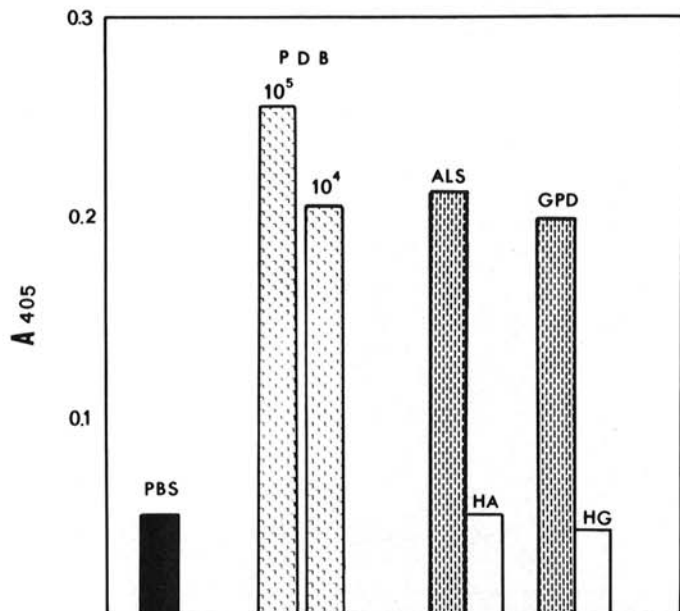


Fig. 2. Absorbance values ( $A_{405\text{nm}}$ ) of a pure culture of Pierce's disease bacterium (PDB) at two concentrations ( $10^4$  and  $10^5$  bacteria per milliliter) of affected almond leaf scorch (ALS) and healthy (HA) almond, and of affected (GPD) and healthy (HG) grapevines compared with those of phosphate-buffered saline (PBS).

or new shoots by grinding the tissue (1.0–2.5 g) in a mortar or by blending it in a high speed micro-blender (Sorvall Inc., Newtown, CT 06470) with extraction buffer (PBS-Tween-PVP). The homogenates were filtered through cheesecloth and centrifuged at 23,000 g for 15 min. The pellets were resuspended in additional extraction buffer containing 2% ovalbumin equivalent to one-half the tissue weight and sonicated in the manner described previously. If the resulting samples were too thick to pipette readily, several dilutions were employed.

## RESULTS

The sensitivity of ELISA varied greatly with the dilutions of gamma globulin and enzyme conjugate tested (Fig. 1). If 0.1  $\mu$ g/ml of coating gamma globulin was used, bacteria were not detected. With 1.0  $\mu$ g/ml, positive readings were obtained at a concentration of  $10^4$ – $10^5$  bacteria per milliliter. With 10  $\mu$ g/ml, positive readings were obtained at a concentration of 100–1,000 bacteria per milliliter when using the minimum enzyme conjugate dilution (1/200) (Fig. 1). At a dilution of enzyme conjugate of 1/200, the reaction was very fast and within 15 min it was possible to detect, visually, a color reaction representing a concentration of  $10^5$  bacteria per milliliter. The spectrophotometric values at 405 nm of this test were obtained by diluting the sample with buffer (240  $\mu$ l sample + 300  $\mu$ l PBS) and in general the readings were low. A visual color reading could be made with values close to  $A_{405\text{nm}} = 0.09$ .

The different bacterial species which were tested to check specificity did not react with PDB antiserum although PD bacterial isolates reacted strongly. The three dilutions employed for each of these species ( $10^6$ ,  $10^7$ , and  $10^8$ ) had similar  $A_{405\text{nm}}$  values ranging 0.087–0.120 and were always below the average values of the PBS control (0.123). PD bacteria had a strong reaction,  $A_{405\text{nm}} = 1.710$  at  $10^8$  cells per milliliter and 1.500 with  $10^6$  cells per milliliter (Table 1).

The ELISA worked well with plant samples. The sap constituents had no effect on the test because the absorption values for healthy plants were similar to or lower than values of the PBS control (Fig. 2). In blind tests, ELISA could readily discriminate between healthy and diseased plants. The extinction values for diseased samples were significantly greater than those of PBS controls, while values for healthy samples were the same as the controls (Table 2). Only one almond of 12 which showed symptoms, tested negative. All of the 15 grape plants, which were known to be infected with PD, tested positive.

## DISCUSSION

Bacterial plant pathogens generally produce distinct disease reactions in affected plants within a short time, permitting easy diagnosis of disease by observing symptoms. This is not the case with the PDB in grapes and almonds. Often during the growing season, PD symptoms are masked, requiring either a period of waiting until distinct symptoms occur in late summer or an indexing procedure based either on culturing the bacterium (4), transmitting the causal bacterium from a suspected plant to a healthy one by insect vectors (6), or by examining suspected materials with a light- (9) or electron microscope (7). Similarly, several plant species do not show symptoms of PD, even though they may be infected (5). The ELISA test offers a rapid method for detecting PD bacteria in plant materials that do not yet show disease symptoms, as well as for bacteria in alternate host plants that may be reservoirs and sources of inoculum available to the vectors.

The ELISA test was specific for the PD pathogen. Tests against 22 isolates of other plant pathogenic and nonpathogenic bacteria showed no reactions.

The test was effective for detecting bacteria at low concentration and the pathogen could be detected in the presence of host plant tissues; this permits us to use the ELISA test for identification of the pathogen before symptoms develop or in the tissues of alternate host species, making the test very useful for epidemiological studies of Pierce's disease.

The early results of the ELISA test indicate that bacteria might

not be uniformly distributed throughout all tissues of affected almond trees. We did not get a positive ELISA test from one tree of 12 that were known to be affected. This bacterium produces a powerful toxin (8) that may kill tissue some distance from the location of the organism. A positive test with the ELISA method would not be expected unless the bacteria were present in the tissue sample. We can use ELISA as a tool to map distribution of the pathogen within the host. Mapping such distribution has been very difficult with the older detection methods.

Although ELISA has been used for detecting viruses (12), spiroplasmas (3), fungi (11), and bacteria (1,15), this is apparently the first full paper of its use for detecting bacterial plant pathogens that are transmitted by leafhoppers.

#### LITERATURE CITED

1. CLAFLIN, L. E., and J. K. UYEMOTO. 1978. Serodiagnosis of *Corynebacterium sepedonicum* by enzyme-linked immunosorbent assay. (Abstr.) *Phytopathology News* 12:156.
2. CLARK, M. F., and A. N. ADAMS. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34:475-483.
3. CLARK, M. F., C. L. FLEGG, M. BAR-JOSEPH, and S. ROTTEM. 1978. The detection of *Spiroplasma citri* by enzyme-linked immunosorbent assay (ELISA). *Phytopathol. Z.* 92:322-337.
4. DAVIS, M. J., A. H. PURCELL, and S. V. THOMSON. 1978. Pierce's disease of grapevines: Isolation of the causal organism. *Science* 199:75-77.
5. FREITAG, J. H. 1951. Host range of the Pierce's disease virus of grapes as determined by insect transmission. *Phytopathology* 41:920-934.
6. FREITAG, J. H., and N. W. FRAZIER. 1954. Natural infectivity of leafhopper vectors of Pierce's disease of grapes in California. *Phytopathology* 44:7-11.
7. GOHEEN, A. C., and S. K. LOWE. 1973. Use of electron microscopy for indexing grapevines for Pierce's disease. *Rev. Pathol. Veg. Entomol. Agric. Fr., (Sér IV)* 279-280.
8. LEE, R. F., B. C. RAJU, and G. NYLAND. 1978. Phytotoxin(s) produced by the Pierce's disease organism grown in culture. *Phytopathology News* 12:218.
9. LOWE, S. K., G. NYLAND, and S. M. MIRCETICH. 1977. A simple and rapid staining procedure for the in situ detection of almond leaf scorch bacterium. *Proc. Am. Phytopathol. Soc.* 4:208.
10. MIRCETICH, S. M., S. K. LOWE, W. J. MOLLER, and G. NYLAND. 1976. Etiology of almond leaf scorch disease and transmission of the causal agent. *Phytopathology* 66:17-24.
11. NACHMIAS, A., M. BAR-JOSEPH, Z. SOLEL, and I. BARASH. 1979. Diagnosis of mal secco disease in lemon by enzyme-linked immunosorbent assay. *Phytopathology* 69:559-561.
12. VOLLER, A., A. BARTLETT, D. E. BIDWELL, M. F. CLARK, and A. N. ADAMS. 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). *J. Virology* 33:165-167.
13. VOLLER, A., D. E. BIDWELL, and A. BARTLETT. 1976. Enzyme immunoassays in diagnostic medicine. *Bull. W. H. O.* 53:55-65.
14. VOLLER, A., D. E. BIDWELL, G. HULDT, and E. ENGWALL. 1976. A microplate method of enzyme-linked immunosorbent assay and its application to malaria. *Bull. W. H. O.* 51:209-211.
15. WEAVER, W. M., and J. M. GUTHRIE. 1978. Enzyme-linked immunospecific assay: application to the detection of seedborne bacteria. (Abstr.) *Phytopathology News* 12:156-157.