# Tissue Printing Immunoassayを用いたRalstonia solanacearumの検出


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# Detection of *Ralstonia solanacearum* Using Tissue Printing Immunoassay

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**Key words:** *Ralstonia solanacearum*, *Lycopersicon esculentum*, tissue printing immunoassay.

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most important diseases of solanaceous plants, affecting several hundred species[5]. This soil-borne bacterium invades root systems, causing rapid wilting of the host plants as a result of heavy colonization and multiplication in vascular tissues[6,13,16]. The soil must be sterilized by solarization or with chemicals such as methyl bromide or chlorpicrin, either independently or in combination to control the disease in Japan. However, soil sterilization does not completely control the disease, and chemical control is mostly ineffective. The use of resistant varieties of tomato is the most effective means of controlling the disease. Grafting susceptible tomato cultivars onto resistant root-stocks is also a way to control the disease[1,3,11,12,14,17]. However, the use of resistant cultivars is insufficient in heavily infested fields.

The bacteria have usually been detected by incubating disrupted host tissues on selective medium. Because the method is intricate and takes much time and labor, the method is difficult for detecting the bacteria in field plants. To overcome this problem, we applied a tissue printing immunoassay (TPI), which had been successfully used for virus detection in plants[10]. Suzuki *et al.*[15] has reported that the detection limit of TPI for cucumber mosaic virus from *Gentian* plants is equal to that of enzyme-linked immunosorbent assay (ELISA). Lazarovits *et al.*[9] have detected *Xanthomonas campestris* pv. *vesicatoria* from supernatants from homogenized, boiled tomato leaf tissue using dot-ELISA on nitrocellulose membranes. Few studies, however, have applied of TPI to detect phytopathogenic bacteria in fields. In this study, we show that TPI is applicable for detecting *R. solanacearum* from tomato plants in fields.

*R. solanacearum* OE1-1 was shake-cultured in YP medium (bacto-yeast extract, 5 g; bacto-peptone, 10 g; deionized water, 1 liter, pH 6.8) for 18 hr at 30°C, suspended in 0.01 M phosphate buffer, pH 7.2 containing 0.85% NaCl (PBS), and washed twice by centrifugation (5000×*g*, 10 min). The final precipitate was resuspended in 4 ml of PBS with 50% Freund's complete adjuvant (FCA) at $10^8$ cells/ml.

Antiserum against *R. solanacearum* was produced in a rabbit by administering four intramuscular injections at $10^8$ bacterial cells/ml/rabbit over two months. The rabbit was bled 10 days after the final injection. Antibody titer against *R. solanacearum* was 1/10,240 in a micro-precipitin test. Forty ml of PBS was added to 10 ml of antiserum followed by 50 ml of water-saturated ammonium sulfate. The mixture was incubated at 0°C for 10 min, then centrifuged at 8000×*g* for 15 min. The precipitate was dissolved in 10 ml of PBS and dialyzed overnight to obtain crude IgG. Leaves of healthy tomato, tobacco and eggplant 10 g each were ground in acetone at −20°C with a mortar and pestle. The powder was then washed in acetone and suspended in 80% ethanol. Then the solution was incubated at 80°C for 10 min and centrifuged at 8000×*g* for 10 min. The precipitate was suspended in PBS, centrifuged at 8000×*g* for 10 min, and then added to the crude IgG for incubating at room temperature overnight. After centrifuging the solution at 3000×*g* for 5 min, the supernatant was the antibody removed nonspecific antibody binding to antigen derived from healthy tomato, tobacco and eggplant[7,8].

Immobilon-P (Millipore, USA) membranes were cut to the required size with a razor blade and marked with a pencil into an 10×10 mm grid. *R. solanacearum* OE1-1 was a gift from Dr. H. Date, and HAIP010, HAIP101, HAIP106, HAIP107, 8101, 8109, 8202, 8216 and E7402 were gifts from Dr. K. Ozaki. *Clavibacter michiganense* subsp. *michiganense* 8910 and *Pseudomonas cichorii* SPC9001 were given by Dr. T. Shirakawa. *Pseudomonas putida* ICR 3460 was from Dr. S. Nagata. *Burkholderia glumae* IPG8908 and *Escherichia coli* HB101 were kept in Iwate Biotechnology Research Center. One microliter of each bacterial solution was applied separately at $10^3$–$10^7$ cfu/ml to the membranes. After air drying, the membranes were immersed in the blocking solution, PBST (PBS containing 2.7 mM KCl, 0.05% Tween 20 and 0.005% $NaN_3$) containing 3% skim milk (Snow

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Table 1. Substrate specificity of antibody against *Ralstonia solanacearum* in tissue printing immunoassay (TPI) and enzyme-linked immunosorbent assay (ELISA)

| Bacteria | TPI | | | | ELISA | | | |
|---|---|---|---|---|---|---|---|---|
| | $10^{4}$ a) | $10^{3}$ | $10^{2}$ | $10^{1}$ | $10^{5}$ | $10^{4}$ | $10^{3}$ | $10^{2}$ |
| *Ralstonia solanacearum* | | | | | | | | |
| OE1-1 | Y[b)] | Y | Y | N | Y | Y | Y | N |
| HAIP010 | Y | Y | Y | N | Y | Y | Y | N |
| HAIP101 | Y | Y | Y | N | Y | Y | Y | N |
| HAIP106 | Y | Y | Y | N | Y | Y | Y | N |
| HAIP107 | Y | Y | Y | N | Y | Y | Y | N |
| 8101 | Y | Y | Y | N | Y | Y | Y | N |
| 8109 | Y | Y | Y | N | Y | Y | Y | N |
| 8202 | Y | Y | Y | N | Y | Y | Y | N |
| 8216 | Y | Y | Y | N | Y | Y | Y | N |
| E7402 | Y | Y | Y | N | Y | Y | Y | N |
| *Clavibacter michiganensis* subsp. *michiganensis* 8901 | N | N | N | N | N | N | N | N |
| *Burkholderia glumae* | | | | | | | | |
| IPG8908 | Y/N | N | N | N | Y | N | N | N |
| *Pseudomonas cichorii* | | | | | | | | |
| SPC9001 | Y/N | N | N | N | Y | N | N | N |
| *Pseudomonas putida* | | | | | | | | |
| ICR 3460 | N | N | N | N | N | N | N | N |
| *Escherichia coli* | | | | | | | | |
| HB101 | N | N | N | N | N | N | N | N |

a) Bacterial population (cfu) blotted on Immobilon-P membranes (TPI) or ELISA plates (ELISA).

b) TPI : blue product (Y), faint one (Y/N) be observed, and blue one not observed (N) ; ELISA : Optical absorbance at 405 nm is more than 0.05 (Y) and others (N).

Brand, Japan) for 30 min. The membranes were washed twice with PBST for 10 min, then immersed in the blocking solution containing *R. solanacearum* antibody at a 1/5000 dilution for 1 hr. After washing twice with PBST, the membranes were immersed in the blocking solution containing alkaline phosphatase-linked anti-rabbit goat IgG for 1 hr. After washing with PBST, the membranes were then immersed in carbonate buffer ($Na_2CO_3$, 1.6 g ; $NaHCO_3$, 2.9 g ; deionized water, 1 liter ; pH 9.6) containing 0.34 mg/ml of nitro blue tetrazolium and 8.5 mg/ml of 5-bromo-4-chloro-3-indolylphosphate. The colorless substrate gave rise to a visible blue product.

Using this method, 10 isolates of *R. solanacearum* were detected to a limit of $10^{2}$ cfu/dot. *B. glumae* and *P. cochorii* was slightly detected at $10^{4}$ cfu/dot, and other bacteria were not detected (Table 1). These results show that *R. solanacearum* could be specifically detected at $10^{2}$ cfu/dot.

The detection limit of our TPI was compared with that of indirect ELISA as described by Clark and Adams[2)]. Wells of the ELISA plates (NUNCLON, Nunc, Denmark) were coated with 100 $\mu$l of the previous bacterial solutions at $10^{2}$–$10^{6}$ cfu/ml. All isolates of *R. solanacearum* were detected to a limit of $10^{3}$ cfu (Table 1). *B. glumae* and *P. cichorii* were weakly detected at $10^{5}$ cfu, and other bacteria were not detected. In comparison, the TPI method can detect a lower density ($10^{2}$ cfu/ml) of *R. solanacearum* than ELISA.

Roots of 4-week-old tomato (*Lycopersicon esculemtum*) seedlings of Oogata-Fukuju and LS-89, susceptible and resistant to bacterial wilt, respectively, were dipped into a solution of *R. solanacearum* OE1-1 at $10^{8}$ cfu/ml and incubated at 25°C in water culture with 5×-diluted Hoagland's solution. The stems were cut 10 mm below the cotyledon with a razor blade, and the cut surfaces were blotted onto Immobilon-P membranes. Then the stems were cut 5 mm above the cut surfaces to determine the population of *R. solanacearum* in the 5 mm section above the cut, blotted surface using Hara-Ono selective medium[4)].

Oogata-Fukuju seedlings started to wilt 3 days after inoculation and all seedlings wilted 8 days after inoculation. Using TPI at all samplings, bacteria were first detected 1 day after inoculation (Fig. 1A). At 5 days after inoculation, the bacteria were detected in all the Oogata-Fukuju seedlings. The population from the collars was estimated at more than $10^{6}$ cfu/g. By 13 days after inoculation, 26% of the LS-89 seedlings had wilted. The bacteria were detected from the collars of LS-89 seedlings when the population of bacteria was more than $2.0\times10^{5}$ cfu/g (Fig. 1B). From all wilting seedlings, the bacteria were detected using TPI. Non-specific blue products on the membranes were not observed from stems of non-inoculated Oogata-Fukuju and LS-89 seedlings.

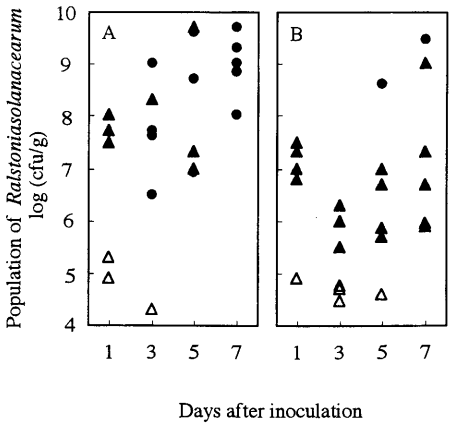


Fig. 1. Detection of *Ralstonia solanacearum* using the tissue printing immunoassay from tomato seedlings cultivars Oogata-Fukuju (A) and LS-89 (B) inoculated by the root dipping method and population of the bacteria in the seedlings by using Hara-Ono selective medium. Symbols △, ▲, and ● represent a non-wilting tomato seedling from which *R. solanacearum* is not detected, a non-wilting tomato plant from which the bacteria are detected, and a wilting tomato plant from which the bacteria are detected, respectively.

Table 2. Detection of *Ralstonia solanacearum* from infected tomato plants cultivar Oogata-Fukuju in a field using the tissue printing immunoassay

| Plant No. | Days after transplanting | | | | | | |
|---|---|---|---|---|---|---|---|
| | 32 | 36 | 39 | 43 | 46 | 50 | 53 |
| 1 | | | | | ●[a)] | ● | ● |
| 2 | | | | | ● | ● | ● |
| 3 | | | | | ● | ● | ● |
| 4 | | | | | | ● | ● |
| 5 | | | | | ● | ● | ● |
| 6 | | | | ▲ | ● | ● | ● |
| 7 | | | | | ▲ | ● | ● |
| 8 | ▲ | ▲ | ● | ● | ● | ● | ● |
| 9 | | ○ | ● | ● | ● | ● | ● |
| 10 | | | | | ● | ● | ● |
| 11 | | | | ● | ● | ● | ● |
| 12 | | | ● | ● | ● | ● | ● |
| 13 | | ● | ● | ● | ● | ● | ● |
| 14 | | | | | ● | ● | ● |
| 15 | | | ● | ● | ● | ● | ● |
| 16 | ▲ | ● | ● | ● | ● | ● | ● |

a) ▲, non-wilting tomato plant, bacteria detected; ●, wilting tomato plant, bacteria detected; ○, wilting tomato, bacteria not detected.

Sixteen seedlings each of Oogata-Fukuju and LS-89 were transplanted 44 days after sowing into a bacteria-infested field located at Nankoku in Kochi prefecture, Japan, on May 7, 1997. From 32 to 53 days after transplanting, the lateral branches of the lowest leaf were cut with a razor blade. Cut surfaces were then blotted onto the Immobilon-P membranes. Bacteria were first detected from two non-wilting Oogata-Fukuju plants 32 days after transplanting by TPI (Table 2). The two plants then wilted 36 days and 39 days after transplanting. The bacteria were detected from 15 plants (14 of them wilting) 46 days after transplanting. At 50 days after transplanting, the bacteria were detected from 16 wilting plants. LS-89 plants did not wilt at all, nor could the bacteria be detected.

Rapid and accurate detection of the causal agents of diseases is essential for plant health certification. Using TPI, although we can detect *R. solanacearum* from wilting tomato plants in which the population of bacteria was more than $10^6$ cfu/g, we cannot always detect the bacteria from latently infected tomato plants. The sample volume for tissue blotting on the membranes was a few microliters and detection limit of the bacteria *in vitro* was $10^2$ cfu. Therefore, the detection limit from infected tomato plants using tissue blotting is theoretically $10^5$ cfu/g. After the bacteria proliferate to more than $10^7$–$10^8$ cfu/g in the roots and collars of tomato plants, they move into and proliferate in the upper stems[7,12]. Bacterial wilt then develops in the plants. By the time wilting is observed then, the bacterial population will be high enough to be measured by the TPI method.

In tomato fields, bacterial wilt caused by *R. solanacearum* is difficult to distinguish visually from fungal wilt by *Fusarium oxysporum* f. sp. *lycopersici* and *Phytophthora drechsleri*. The TPI technique will be sensitive enough for the early detection of *R. solanacearum* in wilting tomato plants and thus distinguishing bacterial wilt from fungal wilt.

Immunological procedures are commonly utilized in pathogen detection[10] with improved procedures being designed for specific diagnosis of plant diseases caused by various bacterial pathogens. Immunological detection of plant bacteria requires either preparation of tissue specimen sections or extraction of bacteria from infected tissues. Tissue sectioning or extraction is often cumbersome and time consuming. Methods that reduce the time required for sample preparation are desirable for the routine indexing of large numbers of field samples and the study of plant bacteria epidemiology.

One of the advantages of the tissue blotting technique for the detection of plant pathogens is that someone can prepare the blotting membrane in any laboratory, greenhouse or fields after a few instructions. *R. solanacearum* could also be detected from wilting and non-wilting eggplants surrounding wilting ones cultivated in a grower's greenhouse using the technique (Fig. 2). Furthermore, the tissue blot membranes could be stored at 4°C and/or transported and processed 3 weeks after the samples were applied without changing the detection limit. In addition, a large number of samples can be processed in a short time. These advantages should make this method useful for the diagnosis of other bacterial diseases as well as bacterial wilt by *R.*

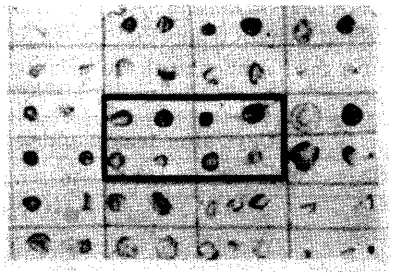

Fig. 2. Detection of *Ralstonia solanacearum* from eggplants in a grower's greenhouse using the tissue printing immunoassay. The eight blots in the black frame are from wilting eggplants. The others are from non-wilting eggplants surrounding the wilting plants in the greenhouse.

*solanacearum*.

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## Literature cited

1. Chellemi, D.O., Dankers, H.A. and Olson, S.M. (1994). Evaluating bacterial wilt-resistant tomato genotypes using a regional approach. J. Am. Soc. Hortic. Sci. 119: 325-329.
2. Clark, M.F. and Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34: 475-483.
3. Enfinger, J.M., MacCarter, S.M. and Jaworski, C.A. (1979). Evaluation of chemicals and application methods for control of bacterial wilt of tomato transplants. Phytopathology 69: 637-640.
4. Hara, H. and Ono, K. (1983). Ecological studies on the bacterial wilt of tobacco, caused by *Pseudomonas solanacearum* E. F. Smith. I. A selective medium for isolation and detection of *Pseudomonas solanacearum*. Bull. Okayama Tob. Exp. Stn. 42: 127-138.
5. Hayward, A.C. (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 29: 65-87.
6. Hikichi, Y., Nasu, Y., Toyoda, K., Suzuki, K., Horikoshi, M., Hirooka, T. and Okuno, T. (1998). Behavior of bioluminescent *Ralstonia solanacearum* YN5 containing the *lux*CDABE in tomatoes susceptible and resistant to bacterial wilt. *In* Bacterial Wilt Disease (Prior, P.H., Allen, C. and Elphinstone, J. eds.), Springer, Berlin, pp. 233-242.
7. Hikichi, Y., Okuno, T. and Furusawa, I. (1993). Immunofluorescent antibody technique for detecting *Pseudomonas glumae* on rice plants. Ann. Phytopathol. Soc. Jpn. 59: 477-480.
8. Hikichi, Y., Saito, A. and Suzuki, K. (1996). Infection sites of *Pseudomonas cichorii* into head leaf of lettuce. Ann. Phytopathol. Soc. Jpn. 62: 125-129.
9. Lazarovits, G., Zutra, D. and Bar-Joseph, M. (1986). Enzyme-linked immunosorbent assay on nitrocellulose membranes (dot-ELISA) in the serodiagnosis of plant pathogenic bacteria. Can. J. Microbiol. 33: 98-103.
10. Lin, N.S., Hsu, Y.H. and Hsu, H.T. (1990). Immunological detection of plant virus and a mycoplasma like organism by direct tissue blotting on nitrocellulose membranes. Phytopathology 80: 824- 828.
11. Monma, S., Narikawa, T., Sakata, Y. and Hida, K. (1993). Resistant to bacteria wilt in tomato varieties. Bull. Natl. Res. Inst. Veg. Ornamental. Plants Tea, A6: 1-12.
12. Monma, S., Sakata, Y. and Matsunaga, H. (1997). Inheritance and selection efficacy of bacterial wilt resistance in tomato. JARQ 31: 195-204.
13. Nakaho, K. (1997). Distribution and multiplication of *Ralstonia solanacearum* (synonym *Pseudomonas solanacearum*) in tomato plants of resistant rootstock cultivar LS-89 and susceptible Ponderosa. Ann. Phytopathol. Soc. Jpn. 63: 83-88.
14. Scott, J.W., Somodi, G.C. and Jones, J.B. (1992). Testing tomato genotypes and breeding for resistance to bacterial wilt in Florida. *In* Bacterial Wilt. Proceedings of an International Symposium (Hartman, G.L. and Hayward, A.C. eds.), Kaohsiung, Taiwan. ACIAR Proc. 45: 126-131.
15. Suzuki, K., Saito, A., Piyasak, C., Okumura, A. and Hikichi, Y. (1995). Infection route of cucumber mosaic virus in *Gentian*. 3) Detection of CMV from stems at cutting after harvest. Annu. Rept. Plant Prot. North Japan 46: 91-94.
16. Vasse, J., Frey, P. and Trigalet, A. (1995). Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 8: 241-251.
17. Wang, J-F., Hanson, P. and Barnes, J.A. (1998). Worldwide evaluation of an international set of resistance sources to bacterial wilt in tomato. *In* Bacterial Wilt Disease (Prior, P.H., Allen, C. and Elphinstone, J. eds.), Springer, Berlin, pp. 269-275.

## 和 文 摘 要

中澤（那須）佳子・北之園忍・長谷川久恵・奥野谷圭司・八重垣史彦・鈴木一実・曳地康史・奥野哲郎：Tissue Printing Immunoassay を用いた *Ralstonia solanacearum* の検出

*Ralstonia solanacearum*（RS）菌液をブロットした Immobilon-P(I-P) を，RS に対するウサギ IgG 液，続いて alkaline phosphatase 結合抗ウサギ IgG ヤギ IgG 液に浸漬後，5-bromo-4-chloro-3-indolylphosphate と nitro blue tetrazolium を含む炭酸緩衝液に浸漬し発色したところ，検出限界は $10^2$ cfu/dot であった。根に RS 菌液を浸漬接種したトマト苗（品種：大型福寿と LS-89）の地際部断面を I-P にブロットしたところ，青枯病発病苗ばかりか接種1日後の苗からも RS が検出され，検出限界は $10^5$-$10^6$ cfu/g であった。大型福寿と LS-89 を青枯病汚染圃場で栽培し，最下位基部を経時的に I-P にブロットした。LS-89 では RS は検出されず，青枯病の発病も認められなかった。大型福寿では発病株のみならず未発病株からも RS が検出された。本法を用いて，ハウス栽培の萎凋症状を呈すナスと周囲の未発病ナスから RS が検出できた。以上の結果から，本法は，圃場における RS の検出に有用であることが示された。

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