Comparison Between a Culture Plate Method and an Immunoassay to Evaluate Vascular Colonization of Potato by *Verticillium dahliae*

Javier Plasencia, Former Graduate Research Assistant, and Ernest E. Banttari, Professor, Department of Plant Pathology, University of Minnesota, St. Paul 55108

ABSTRACT

Plasencia, J., and Banttari, E. E. 1997. Comparison between a culture plate method and an immunoassay to evaluate vascular colonization of potato by *Verticillium dahliae*. Plant Dis. 81:53-56.

Vascular colonization by *Verticillium dahliae* of 14 potato (*Solanum tuberosum*) cultivars was evaluated in field trials by a culture plate method and an indirect competitive enzyme-linked immunosorbent assay (IC-ELISA). Whereas the culture plate technique quantifies fungal propagules, the monoclonal antibody-based immunoassay detects the soluble antigen, providing an indicator of fungal biomass. Differences in vascular colonization were evident with both methods; thus, cultivars could be ranked as susceptible, intermediate, or resistant. Linear regression analysis of means of each cultivar showed correlation coefficients (r^2) ranging from -0.606 to -0.835. Spearman's rank correlation coefficient (r_s) ranged from -0.65 to -0.91. Estimation by IC-ELISA of vascular colonization by *V. dahliae* in potato is as accurate as that by the culture plate technique, and results can be obtained in 3 days with an approximately 50% reduction in costs of materials and reagents. This quantitative immunoassay can be applied in potato breeding programs to evaluate resistance to *Verticillium* wilt.

Host plant resistance is considered to be the most efficient, economical, and environmentally sound approach to control *Verticillium* wilt in potato (8,9). Although there are some cultivars with effective resistance to *Verticillium dahliae* Kleb., the most popular commercial varieties are susceptible, or tolerant at best. Tolerant cultivars sustain a high degree of vascular colonization by the fungus, but wilt symptoms are suppressed and yield reductions occur only under certain environmental conditions. Although tolerance is acceptable in some circumstances, it is not as desirable as resistance to infection because, under successive cropping, the pathogen will eventually build up in the soil to a level at which tolerance is no longer effective in preventing yield losses (11).

In practice, however, critical evaluation of potato germ plasm and clones for resistance to *V. dahliae* under field conditions is time consuming and expensive. Disease symptoms are not reliable indicators since the characteristic unilateral chlorosis and necrosis observed in *Verticillium* wilt are

often from natural senescence, and their expression is influenced by environmental variables (6). Further complications occur because of the wide range in genotype maturity existing in potato germ plasm. While early-maturing resistant clones may appear to be susceptible when they are simply senescing, late-maturing susceptible genotypes might show no symptoms at the time they are evaluated and would be classified as resistant (11).

Yield loss due to *Verticillium* wilt is the ultimate criterion for evaluating resistance to the disease, but because of the cost involved in yield trials, this test is reserved for only advanced breeding lines. Phyto-toxins produced in vitro by *V. dahliae* have been used in an excised leaf bioassay for rapid screening for resistance to disease. However, results with these bioassays did not correlate with the wilt reaction of clones tested in the greenhouse and the field (5).

Another approach for quantifying resistance of potato has been the use of a technique involving a selective medium and an assay to quantify *V. dahliae* in air-dried potato stems (2). Although this method showed high correlation with foliar wilt symptoms and yield, it is time consuming, thus limiting the number of samples that can be processed.

A simpler method has been developed that uses fresh, approximately 10-cm stem samples cut above the ground line (3). The stems are washed, surface disinfected, and squeezed to obtain sap from the basal part of the stem. A 0.1-ml subsample of sap is plated on a semiselective medium amended

with antibiotics and incubated for 7 to 10 days. Identity of *V. dahliae* in the cultures is based on morphology of microsclerotia. On this basis colony counts can be made. This assay method showed a high correlation with the foliar wilt rating of cultivars with varying degrees of susceptibility to *V. dahliae*. It has been proposed that the measurement of spore production of pathogens growing in infected host-plants provides a method to evaluate resistance of the host that is even more accurate than the use of assessment of visual symptoms (4). In practice, however, measurement of spore numbers is not as simple as symptom evaluation, thus limiting application of this method when large numbers of plants are evaluated, such as in a breeding program.

A monoclonal antibody-based immunoassay developed to quantify *V. dahliae* biomass (7) has been tested with antigen produced in vitro and with infected greenhouse-grown potato plants. In this paper, we report the field testing of the immunoassay method to detect and quantify *V. dahliae* propagules in potato vascular tissue and a comparison between the immunoassay method and the culture plate method (3), using 14 potato cultivars with varying degrees of resistance to *Verticillium* wilt.

MATERIALS AND METHODS

Potato seed stocks. Potato (*Solanum tuberosum* L.) seed tubers of various cultivars were obtained either from plants grown as tuber-units in a disease-tested seed stocks nursery at Becker, MN, or from growers of certified seed. The cultivars chosen represented a wide array of varietal characteristics including maturity type, tuber and vine type, and levels of resistance to various diseases. All of the cultivars are adapted to the Minnesota region and represent about 40% of the acreage entered for seed certification. Plants were tested for potato virus Y (PVY), potato virus X (PVX), potato virus S (PVS), and potato leaf-roll virus (PLRV) by double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA). Plants that tested positive for PVS, PVX, PVY, or PLRV were rogued.

Inoculum production. For greenhouse inoculation, a mixture of *V. dahliae* isolates were grown in 200 g of rice medium (1) in 1-liter Erlenmeyer flasks. Flasks were incubated at room temperature for 14 to 20 days until all the grain was invaded and the

Corresponding author: Ernest E. Banttari;
Fax: (612) 625-9728; E-mail:
ernestb@puccini.crl.umn.edu

Current address of first author: Departamento de Bioquímica, Facultad de Química, UNAM 04510 México, D.F.

Accepted for publication 24 September 1996.

fungus formed microsclerotia. The contents of the flask were then used to inoculate trays containing sterile rice medium (700 g of rice, 85 g of vermiculite, and 500 ml of water). The trays were incubated at room temperature and after 12 days the contents were removed and allowed to dry in a hood. The contents of all trays were pooled in paper bags and kept in a cold room until used. The number of microsclerotia per gram of rice was estimated by grinding 1-g subsamples in 10 ml of sterile water and counting the microsclerotia in 20- μ l aliquots. An average concentration of 6.25×10^5 microsclerotia per gram of rice was estimated.

Verticillium wilt nursery. A *Verticillium* wilt nursery was established in 1992 at the Minnesota Agricultural Experiment Station, University of Minnesota, St. Paul campus. In 1992, 13 potato cultivars were tested and in 1993 one more entry (cv. Cascade) was added. Fields were planted on 1 May 1992 and 6 May 1993. A randomized complete block design was used in six-hill plots. Each seed piece was inoculated at planting with 40 cc of the rice grain/vermiculite mixture infested with *V. dahliae* before the furrow was closed. The

field spacing was 30 cm between plants in the same plot, 122 cm between plots in the same row, and 92 cm between rows. In 1992, there were six plots per row and the field was 13 rows wide. In 1993, seven plots per row were included in the 12-row-wide field. Sampling dates were 104 and 98 days after planting in 1992 and 1993, respectively. Samples consisted of a 10-cm-long piece of the main stem of each plant cut just above the soil line. Four plants at the center of each plot were sampled. Stems were washed, surface disinfected, and processed for the culture plate assay and immunoassay as described below. A field located 20 m south of the wilt nursery was planted with all 13 cultivars tested but without applying inoculum. In 1993, the experiment was replicated at a second location, at the Red River Valley Potato Growers Research Farm, Grand Forks, ND. This *Verticillium* wilt nursery has been in continuous potato cultivation since 1972, and it is where potato breeding lines from the University of Minnesota and North Dakota State University are evaluated for resistance to *Verticillium*. Seed tubers were not inoculated at planting (19 May 1993) at this site since this soil was infested with *V. dahliae* propagules (10 to 50 CFU per gram of soil). The same experimental design was followed as before and stems were sampled 97 days after planting.

Sample processing. The culture plate method was performed as described by Hoyos et al. (3). Briefly, 0.1 ml of sap squeezed from approximately 10 cm of the basal section of the stem was placed in the center of a 100-mm petri dish and 12 to 15 ml of *Verticillium* isolation medium (45°C) was poured over the expressed sap. The sap and medium were then swirled to mix them. Plates were incubated at room temperature (22 to 25°C) for 10 to 14 days and the colonization index was visually estimated and converted to CFU per 0.1 ml of

sap according to a scale developed by Hoyos et al. (3).

For the immunoassay, a 0.5-ml aliquot of the same sap sample was diluted with 0.5 ml of ELISA buffer (phosphate buffered saline, pH 7.4 + 0.05% Tween 20 + 0.5% bovine serum albumin + 2% polyvinyl-pyrrolidone-10 + 0.2% sodium sulfite). The immunoassay format used was indirect competitive ELISA (IC-ELISA) as described by Plasencia et al. (7). Twenty microliters of diluted sap was mixed in each well of a Serocluster plate (Costar, Cambridge, MA) with 100 μ l of monoclonal antibody (mAb 7C2.A11 or mAb 5E7.G9) diluted 1:40,000 in ELISA buffer. Wells A2, A3, and A4 of each plate were used as blanks and 20 μ l of ELISA buffer was mixed with the antibody solution. The contents of each well were mixed and 90 μ l of the mixture was transferred to the replicated well of the antigen-coated plate (ELISA plate), which was incubated for 1 h at room temperature. The ELISA plate was washed three times with phosphate buffered saline + 0.05% Tween 20 (PBS-T20) and 50 μ l of rabbit anti-mouse IgG alkaline phosphatase conjugate (1:2,000 dilution) was added to each well. The plate was incubated overnight at 4°C or for 3 h at room temperature. The plate was washed three times with PBS-T20, and 200 μ l of substrate (0.75 mg *p*-nitrophenyl phosphate per ml of 10% diethanolamine, pH 9.8) was added to every well. The absorbance was read at 405 nm until wells A2, A3, and A4 reached a value of about 2.000.

Data analysis. For the colonization and absorbance values, the general linear model analysis of variance was employed to determine the statistical significance among cultivar means and block effects. Means were compared by the least significance difference method at 0.05 probability. Comparison between the culture plate method and the immunoassay was done with scatter plots, Pearson's correlation coefficient (*r*), and Spearman's rank correlation coefficient (*r_s*), and by simple linear regression analysis.

RESULTS AND DISCUSSION

In 1992, vascular colonization of 13 potato cultivars by *V. dahliae* was evaluated by both the culture plate technique and the immunoassay in the IC-ELISA format. The field inoculation procedure proved to be effective since high levels of *V. dahliae* were recorded from susceptible cultivars, and the plants showed symptoms about 70 days after planting. With both methods, differences in vascular colonization levels were evident ($P < 0.0001$) and ranking of cultivars was possible (Fig. 1). Grouping of cultivars into susceptible, intermediate and resistant categories was based on vascular colonization values, which placed cvs. Superior, Kennebec, Russet Norkotah, Krantz, and Shepody as susceptible with both techniques. Cultivars Reddale and

Fig. 1. Vascular colonization by *Verticillium dahliae* in 13 potato cultivars evaluated by (A) the culture plate technique and (B) the immunoassay with mAb 5E7.G9. Plants were grown in the *Verticillium* wilt nursery in St. Paul, MN, in 1992. Means with the same letter do not differ significantly with the least significant difference procedure at $P = 0.05$.

Fig. 2. Relationship between the culture plate method and the immunoassay (mAb 5E7.G9) to quantify vascular colonization by *Verticillium dahliae* in 13 potato cultivars grown in the *Verticillium* wilt nursery in St. Paul, MN, in 1992.

Gemchip were the most resistant by both methods and cvs. Norchip, Alpha, Russet Burbank and Norland fell into the intermediate category. Only cvs. Monona and Norking were ranked more resistant by the immunoassay than by the culture plate technique. Linear regression analysis of the means of every cultivar (Fig. 2) gave a coefficient of determination (r^2) = 0.606 for both methods. Pearson's correlation coefficient was $r = -0.7783$ and Spearman's rank correlation coefficient was $r_s = -0.7308$.

For the 1993 tests, two mAbs were available and both were tested in the immunoassay. When the inoculation procedure was repeated at planting, good wilt symptoms were observed in the plants and high colonization values were recorded. Cultivars could be distinguished by both the culture plate technique (Fig. 3; $F = 6.04$, $P < 0.0001$) and the immunoassay ($F = 12.15$, $P < 0.0001$). A close correlation was obtained with both methods, giving a coefficient of determination of $r^2 = 0.835$, and grouping in each category was the same with both methods. For the immunoassay, both mAbs (5E7.G9 and 7C2.A11) were employed separately and in a 1:1 mixture in order to compare their performances in relation to the culture plate test. With mAb 7C2.A11 (Fig. 3A), the same

Fig. 3. Relationship between the culture plate method and the immunoassay tested with (A) mAb 5E7.G9 and (B) mAb 7C2.A11, to evaluate vascular colonization by *Verticillium dahliae* in 14 potato cultivars grown in the Verticillium wilt nursery in St. Paul, MN, 1993.

rankings were obtained ($F = 12.20$, $P < 0.0001$) as with mAb 5E7.G9 (Fig. 3B) and with the mixture of the two antibodies ($F = 13.19$, $P < 0.0001$, data not shown). Pearson's correlation coefficient and Spearman's rank ν correlation coefficient were very similar for either antibody or for the mixture.

Vascular colonization in 13 potato cultivars grown in the Verticillium wilt nursery at Grand Forks, ND, in 1993 was evaluated with the culture plate technique and with the immunoassay, using a 1:1 mixture of the two mAbs. The cultivar Norland was eliminated from this test because, at the time of sampling, plants were mature and dry. There was a good correlation between both methods in ranking the vascular colonization of the various cultivars (Fig. 4).

Since the level of a fungal pathogen in host plant tissue is an important parameter affecting the outcome of the host-pathogen relationship, this study was aimed at development of quantitative techniques for rapid and specific estimation of the extent of colonization of potato by *V. dahliae*. In contrast to viruses and bacteria that form homogeneous suspensions upon homogenization of the plant tissue, filamentous fungi are fragmented during homogenization. Therefore, a plate-count method would reflect the efficiency of the grinding procedure and the resistance of the organism to this treatment (10). This could be a limitation of the dry-sample method developed by Davis et al. (2) to quantify *V. dahliae* in potato stems. The use of fresh sap as sample (3) provides a homogeneous suspension to analyze, and the culture plate method detects both viable conidia and hyphae present in the sap. The immunoassay evaluated here detects soluble protein as indicator of fungal biomass. During this 2-year study, it was shown that the biomass as quantified by the immunoassay was a reliable indicator of vascular colonization by *V. dahliae* in potato. Correlation coefficients

Fig. 4. Relationship between the culture plate method and the immunoassay (mAbs 5E7.G9 + 7C2.A11 in a 1:1 mixture) to evaluate vascular colonization by *Verticillium dahliae* in 13 potato cultivars grown in the Verticillium wilt nursery in Grand Forks, ND, in 1993.

values ranged from -0.65 to -0.91 and both the Pearson's sample correlation coefficient and Spearman's rank correlation coefficient showed similar values. However, Spearman's rank correlation coefficient might be the best choice when ranking resistance levels of cultivars is the primary objective.

Accurate evaluation of vascular colonization plants growing in the field requires several samples to characterize a genotype or cultivar. Typically, variations are observed between samples evaluated either by the culture plate technique or the immunoassay. Block effects were detected in some cases and might explain to some extent the variability observed when individual experimental unit values of absorbance were plotted against log (CFU + 1)/0.1 ml of sap.

This study presents a novel approach to evaluate resistance to Verticillium wilt in potato and has been corroborated with the culture plate technique. In contrast to that technique, the immunoassay results are readily evaluated since quantitation is done by an instrument that also reduces subjectivity. The immunoassay is also faster, since results can be obtained in 2 to 4 days, and allows for retesting of the samples as well as transportation of samples to a specialized laboratory for analysis. With the available antibodies (7), costs of materials and reagents were estimated to be half of those for the culture plate technique. This capacity to process a large number of samples widens the potential application of the immunoassay to epidemiological studies for evaluating disease incidence and/or severity. The immunoassay does not offer an improvement in sensitivity or reliability over the culture plate method but it offers the possibility of increasing sample size at no increase in cost.

ACKNOWLEDGMENTS

Published as paper No. 22,034 of the contribution series of the Minnesota Agricultural Experiment Station based on research conducted under Project 22-77, supported by RRVGA and Area II Potato Council funds.

LITERATURE CITED

1. Abbas, H. K., Mirocha, C. J., and Gunther, R. 1989. Mycotoxins produced by toxic *Fusarium* isolates obtained from agricultural and non-agricultural areas (Arctic) of Norway. *Mycopathologia* 105:143-151.
2. Davis, J. R., Pavsek, J. J., and Corsini, D. L. 1983. A sensitive method for quantifying *Verticillium dahliae* colonization in plant tissue and evaluating resistance among potato genotypes. *Phytopathology* 73:1009-1014.
3. Hoyos, G. P., Zambino, P. J., and Anderson, N. A. 1991. An assay to quantify vascular colonization of potato by *Verticillium dahliae*. *Am. Potato J.* 68:727-742.
4. Johnson, R., and Taylor, A. J. 1976. Spore yield of pathogens in investigations of the race-specificity of host resistance. *Annu. Rev. Phytopathol.* 14:97-119.
5. Mohan, S. K., Davis, J. R., Corsini, D. L., Sorensen, L. H., and Pavsek, J. J. 1990. Reactions of potato clones and accessions of *Solanum* spp. to *Verticillium dahliae* Kleb. and

- its toxin. *Potato Res.* 33:449-458.
6. Pegg, G. F. 1981. Biochemistry and physiology of pathogenesis. Pages 193-253 in: *Fungal Wilt Diseases of Plants*. M. E. Mace, A. A. Bell, and C. H. Beckman, eds. Academic Press, New York.
 7. Plasencia, J., Jemmerson, R., and Bantari, E. E. 1996. Production and characterization of monoclonal antibodies to *Verticillium dahliae* and development of a quantitative immunoassay for fungal biomass. *Phytopathology* 86: 170-176.
 8. Powelson, M. L., and Rowe, R. C. 1993. Biology and management of early dying of potatoes. *Annu. Rev. Phytopathol.* 31:111-126.
 9. Rowe, R. C., Davis, J. R., Powelson, M. L., and Rouse, D. I. 1987. Potato early dying: Causal agents and management strategies. *Plant Dis.* 71:482-489.
 10. Strange, R. N. 1982. Pathogenic interactions of microbes with plants. Pages 472-489 in: *Experimental Microbial Ecology*. R. G. Burns, and J. H. Slater, eds. Blackwell Scientific Publ., Oxford, UK.
 11. Treadwell, F. J. 1991. Breeding for resistance to *Verticillium* wilt in potato. Ph.D. thesis. University of Minnesota, St. Paul.