Effect of Hot Water Treatment on Systemic *Agrobacterium tumefaciens* Biovar 3 in Dormant Grape Cuttings

T. J. BURR, Department of Plant Pathology, Cornell University, Geneva, NY 14456; K. OPHEL, Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064; B. H. KATZ, Department of Plant Pathology, Cornell University, Geneva, NY 14456; and A. KERR, Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064

ABSTRACT

Burr, T. J., Ophel, K., Katz, B. H., and Kerr, A. 1989. Effect of hot water treatment on systemic *Agrobacterium tumefaciens* biovar 3 in dormant grape cuttings. Plant Disease 73:242-245.

Agrobacterium tumefaciens biovar 3 (AT3) was eradicated or reduced to below the level of detection in dormant cuttings of grape rootstocks K5140 (*Vitis champinii* × *V. riparia*) and Ramsey (*V. champinii*) and in artificially infested Cayuga White (interspecific hybrid) by a hot water treatment (exposure to 50 C for 30 min). Strains of AT3 varied in their sensitivity to heat, but were generally more sensitive than strains of biovar 1 or biovar 2. Populations of about 10^3 colony-forming units per milliliter of AT3 in broth were killed by a 30-min treatment. Biovar 1 strains were apparently unaffected by 50 C, even when exposed for 30 min. Nontumorigenic biovar 1 strains were recovered from hot water treated cuttings. The hot water treatment may offer a simple, effective, economical, and environmentally safe means of eradicating AT3 from dormant cuttings.

The systemic nature of *Agrobacterium tumefaciens* (Smith & Townsend) Conn biovar 3 (AT3) in grapevines and the spread of the pathogen through propagation material are well documented (2,6, 15,16,20). Attempts to exclude the pathogen from grape by the use of shoot tip culture (8) and by repetitive propagation and indexing of green shoot cuttings (11) are effective, but time-consuming. A possible alternative for eradicating AT3 from grape cuttings is the use of heat therapy. Hot water treatment of dormant cuttings is a simple procedure that was reported in 1972 (10) as a means of freeing dormant grapevines of the Pierce's disease pathogen, now recognized as a bacterium (9). Treatments of 50 C for up to 150 min are not lethal to dormant vines (10,12), whereas only a 20-min treatment was required to kill the Pierce's disease pathogen. This treatment has been used effectively on imported grape propagation material in Australia since 1972 (1).

The purpose of this study was to determine the sensitivity of AT3 to heat and the potential of using hot water dips for eradicating the pathogen from dormant grape cuttings.

Accepted for publication 31 October 1988 (submitted for electronic processing).

MATERIALS AND METHODS

Thermosensitivity of *Agrobacterium*. The effect of exposure time at 50 C on survival of broth cultures of *Agrobacterium* strains was determined. This temperature was chosen because it is lethal to many bacteria and because dormant grapevines can survive exposures of up to 150 min. The identity of bacterial strains used in the first experiment and their sources are given in Table 1. Strains

Table 1. Time required to kill strains of *Agrobacterium* at 50 C in vitro

Strain	Biovar	Source[a]	No. colonies counted[b] according to treatment time (min)[c] 0	10	15	20	25
2437	1	NCPPB	+	+	+	+	+
CIRS	1	NCPPB	+	+	+	+	+
K188	1	Australia	+	+	+	+	+
K198	1	Australia	+	+	+	+	+
B1-4[d]	1	Australia	+	+	+	+	+
K84	2	Australia	+	+	50	5	0
K306	3	Australia	+	50	3	0	0
LK402	3	Australia	+	25	0	0	0
Schw 2	3	Australia	+	15	7	0	0
CG-49	3	United States	+	10	0	0	0
CG-484	3	United States	+	200	40	0	0

[a] Strains were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB), Harpenden, England, or were isolated by authors in Australia or the United States.
[b] Number of colonies counted on medium 523 after 72 hr at 28 C. + = Solid lawn of bacterial growth.
[c] About 10^4 cfu of bacteria in yeast extract broth were plated following heat exposures.
[d] Strain from hot water treated K5140 cutting.

were grown in yeast extract broth (YE) (18) overnight at 25 C on a shaker. Resulting bacterial suspensions were then diluted in YE to an optical density reading of 0.1 at 640 nm, which corresponds to approximately 10^7 colony-forming units (cfu) per milliliter. Samples (1 ml) of sterile YE were added to 1.5-ml Eppendorf microfuge tubes that were then incubated for 1 hr in a water bath adjusted to 50 C. Tubes were supported by a thin sheet of Styrofoam that allowed the basal end to be fully submersed in the water. A 30-μl sample of each prediluted suspension was then mixed with the preheated broth. Immediately, and at 5-min intervals for 25 min, 50-μl samples were removed and spread on plates of medium 523 (13). Plates were incubated at 28 C and final bacterial counts were recorded after 72 hr. This experiment was repeated once.

Because the biovar 3 strains used in the above tests appeared to vary in their sensitivity to heat, an additional four strains (Table 2) were tested. They were grown overnight in 523 broth, diluted with 523 to about 10^7 cfu/ml as described above, and then serially diluted in sterile tubes containing 2.5 ml of 523. Four sets of 10-fold dilution tubes (10^{-2} to 10^{-7}) were prepared for each strain and were placed in a shaking water bath (50 C) for 10, 20, or 30 min. One dilution set for each strain was immediately plated on 523 agar to determine initial populations.

After exposure to the hot water, the tubes were removed from the bath and incubated on a shaker at room temperature (25–30 C) for 6 days. A loopful of broth from tubes that appeared free of bacterial growth (no turbidity) was streaked on a plate of medium (RS) that is semiselective for AT3 (7). We then determined the number of colony-forming units per milliliter in the lowest dilution where total kill of each strain was detected. Subcultures of strains that survived the treatments of 50 C were tested for pathogenicity on sunflower (*Helianthus annuus* L.) and tobacco (*Nicotiana glauca* Graham) and for reactivity to an antibody that is specific for biovar 3 (4).

The temperature of broth suspensions before placement in the water bath was about 25 C. By inserting a thermometer in a noninoculated broth tube and placing the tube in the water bath, we determined that it took about 2.5 min for the broth to reach 50 C. This measurement was repeated three times.

Heating of cuttings. The time required for internal tissues of dormant 1-yr-old grape cuttings to reach 50 C when submersed in a water bath (50 C) was determined using a thermocouple recorder. Single-node cuttings (10 cm long) of rootstock K5140 (*Vitis champinii* × *V. riparia*) were used. Nodes and internodes of cuttings with cross-sectional areas of 33–297 mm^2 were tested. Cross sections of cuttings were considered oval in shape. Therefore, areas were calculated based on measurements of the shortest and longest radii at the points where the thermocouple was inserted. Temperature measurements were made by drilling a 1.5-mm-diameter hole with an electric drill to the pith of the cutting and inserting a 0.5-mm thermocouple probe (copper, constantan wire) so that the thermocouple measured the temperature of the pith and innermost vascular tissue. The area surrounding the point of entry of the wire was covered with a thermoinsulating sealant. Cuttings were fully submersed in a hot water bath (50 C) to a depth of about 5 cm. The temperature of the water bath was monitored throughout the experiments with a thermocouple recorder. The water bath for these and subsequent experiments contained about 20 L of water, and no detectable change in temperature was observed when cuttings were submersed in it. Measurements were taken from a total of 18 K5140 cuttings: nine from nodal and nine from internodal areas. In addition, six cuttings each from the cultivars Sauvignon Blanc and Munier and from rootstock Ramsey (*V. champinii*) were tested. Cross-sectional areas of these cuttings ranged from 94 to 132, 102 to 138, and 121 to 153 mm^2, respectively.

Hot water treatments. Two rootstocks that are commercially important in Australia, K5140 and Ramsey, were used because they contained a high level of systemic AT3 (16). In all cases, single-node, 1-yr-old dormant cuttings, 10 cm in length, were used. A test was also conducted using artificially infested cuttings of an important interspecific hybrid, Cayuga White. Two-node cuttings, about 20 cm in length, were artificially infested by vacuum-infiltrating them with 500 μl of an aqueous cell suspension (about 10^7 cfu/ml) of a rifampicin-resistant biovar 3 strain, ABR-15 (3) and then incubating the infested cuttings for 24 hr at room temperature. Eight hot water experiments were conducted over a 6-mo period. Cuttings were treated by completely submersing groups of them in a hot water bath that was adjusted to 50 C. No change in temperature was detected when groups of 10 cuttings were placed in the bath. However, when 100 cuttings (two groups of 50) were submersed in the bath at the same time, a two-degree decrease in temperature was observed for about 3 min. The number of cuttings that were treated for each experiment and the duration of each treatment are given in Table 3. The range of cross-sectional

Table 2. Lethal effect of water treatment (50 C) on dilutions of *Agrobacterium tumefaciens* biovar 3 in medium 523 broth

Strain	Source	Highest population of bacteria killed (cfu/ml)[a]	Treatment time (min)[b]
CG-102	Virginia	1.0×10^3	30
CG-483	Washington	4.7×10^3	10
CG-485	New York	2.9×10^3	20
IPV-P02152	Italy	2.0×10^3	10

[a] In medium 523 (13), 10-fold dilutions (10^{-2} to 10^{-7}) were made from cell suspensions of each strain (initially about 10^7 cfu/ml) and were exposed for 0, 10, 20, or 30 min in a water bath of 50 C. The colony-forming units per milliliter of the lowest dilution that was killed is given.
[b] It required about 2.5 min for the broth in tubes to reach 50 C after being placed in a water bath.

Table 3. Effect of water treatment (50 C) on survival of *Agrobacterium tumefaciens* biovar 3 (AT3) in dormant grape cuttings[a]

Experiment	Rootstock or cultivar[b]	No. cuttings with AT3/no. cuttings tested according to treatment time (min)			
		0	10	20	30
1	Ramsey	6/10	···	···	0/10
2	K5140	8/12	···	0/10	0/10
3	K5140	6/10	···	0/10	0/10
	Ramsey	7/10	···	0/10	0/10
4	K5140[c]	8/10	6/10	0/10	0/10
	Ramsey	7/10	0/10	0/10	0/10
5	K5140	5/10	1/10	0/10	0/10
6	K5140	4/10	0/10	0/10	0/10
7	K5140[c]	8/20	···	0/50	0/50
8	Cayuga White	20/20	···	···	0/20

[a] All cuttings were assayed by flushing 500 μl of sterile distilled water through them with vacuum pressure and plating 10 μl of the vascular extract on RS medium. Presumptive biovar 3 colonies were tested with a monoclonal antibody.
[b] Ramsey and K5140 were naturally infested. Cayuga White was artificially infested.
[c] In addition to assaying by the vascular flush method, cuttings were callused for 1 mo in the greenhouse and isolations from the callus tissue were attempted as described in Materials and Methods. No AT3 were isolated from the callus of treated cuttings.

areas of different cuttings in each treatment was approximately 40–250 mm^2. For experiments 5 and 6, the cross-sectional areas were recorded for individual cuttings to determine if the diameter of the cuttings influenced pathogen survival in treated and nontreated cuttings. Cuttings for these two experiments had cross-sectional areas of 33–132 mm^2.

Assay of cuttings for *Agrobacterium*. All treated and nontreated cuttings were assayed for AT3 using a method that was prevously published by Bazzi et al (2). Sterile distilled water (500 μl) was forced through each cutting using vacuum pressure and then 10 μl of the vascular extract was spread on RS medium. Extracts from Cayuga White were plated on 523 agar amended with 100 μg of rifampicin per milliliter. Cuttings from experiments 4 and 7 were further indexed by planting them in moist sand to stimulate callus formation at their basal ends. Isolations from the callus tissue were attempted on RS medium after 1 mo, as previously reported (6,16). About 200–500 mg of callus from each cutting was triturated in 500 μl of sterile distilled water in a microfuge tube. The solid phase was allowed to settle for about 15 min and 10 μl of the liquid phase was spread on one-half of an RS plate. Plates were incubated at 28 C for 1 wk and inspected for the presence of typical biovar 3 colonies (6). Where presumptive AT3 colonies were apparent on isolation plates from assays of nonheated cuttings, at least one colony per cutting was selected for further characterization. For cuttings treated with hot water, all presumptive AT3 colonies were further characterized. Colonies were purified on medium 523 and verified as AT3 by a previously described solid-phase ELISA technique (14) using a monoclonal antibody that is specific for biovar 3 (4).

Twenty Cayuga White cuttings that were infiltrated or not infiltrated and treated or not treated with hot water were planted in the greenhouse after indexing to determine the effect of treatments on bud survival.

RESULTS

In the initial experiment, all strains of AT3 were killed when suspended in YE broth for 20 min at 50 C. However, some variability between strains was apparent because some cells survived exposures of 10 and 15 min (Table 1). No detectable reduction in survival of any biovar 1 strain was observed even when treated for 30 min. In later experiments, where four strains were diluted in 523 broth, populations of AT3 at 10^3 cfu/ml or less were killed at 50 C within 10–30 min, depending on strain (Table 2). Some cells of each strain survived 30 min when initial suspensions contained greater than 10^3 cfu/ml. Cells that survived exposures of 50 C produced typical colonies on RS, remained tumorigenic, and reacted positively to the biovar-specific antibody.

The time required for internal tissues of dormant cuttings to reach 50 C was affected by the diameter of the cutting (Figs. 1 and 2). Internodal areas of thin cuttings required only about 2.5 min to reach 50 C, whereas very thick nodal areas required up to 9 min. All rootstocks and scion cultivars reacted similarly to the rootstock K5140. The time required for different-sized cuttings of Sauvignon Blanc, Munier, and Ramsey to reach 50 C ranged from 3.5 to 4.5, 3.3 to 4.2, and 4.5 to 7.0 min, respectively.

AT3 could not be isolated from vascular extracts or callus of cuttings treated for 20 or 30 min at 50 C (Table 3). In two experiments, the pathogen was detected following the 10-min treatment. The percentage of the control cuttings that were contaminated with the pathogen ranged from 40 to 100. Thickness of cuttings did not appear to influence infestation by AT3 because the bacterium was detected in cuttings of various sizes that were not given the hot water treatment. Frequently, cuttings that were treated yielded relatively large and mucoid *Agrobacterium*-type colonies. Five of these colonies were purified and characterized as nontumorigenic biovar 1 strains, using methods previously published (17,19).

Exposure of dormant cuttings to 50 C for 30 min affected survival of primary buds. However, the cuttings used were not fully dormant and bud swell or initial shoot growth was apparent. The primary buds or shoots were often killed by the treatment. However, shoots developed from secondary buds from an equal number of nodes, as with nontreated cuttings.

DISCUSSION

Hot water treatment has great promise as a simple, effective, economical, and environmentally safe method for eradicating AT3 from dormant grape cuttings. The diameter of the cuttings affected the time required for internal tissues to reach 50 C. However, even cuttings with a cross-sectional area of 297 mm, which is larger than that desired for propagation material, were heated to 50 C in 9 min. Although primary buds were often killed by this exposure, there was excellent shoot development from secondary buds. This is not surprising because we were using a temperature and treatment times well within the limits for survival of dormant grapevines (10,12). Dormant cuttings imported into Australia are routinely treated at 50 C for 20 min and no harmful growth effects have been

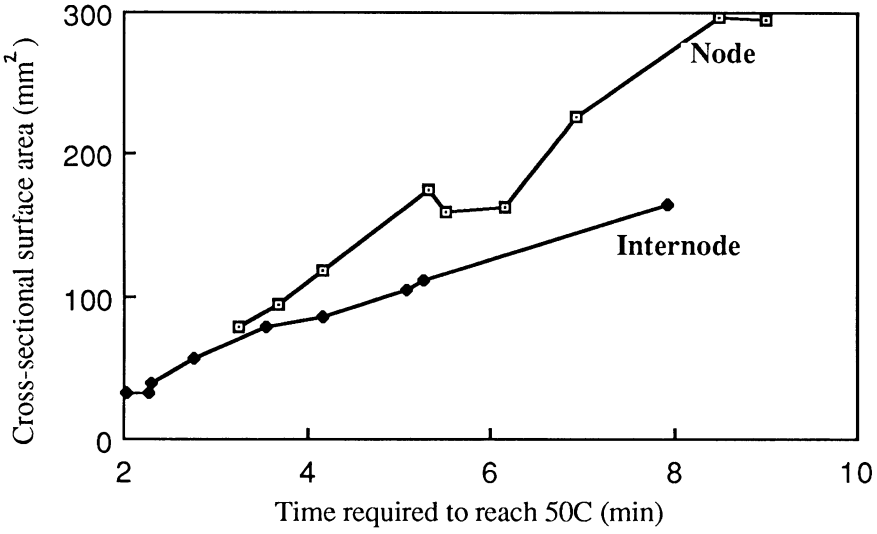

Fig. 1. Effect of cross-sectional area of nodes and internodes of dormant K5140 grape cuttings on the time required for pith temperatures to reach 50 C when submersed in a water bath of 50 C. Temperatures were measured with a thermocouple placed in the pith region of nodes and internodes of different sized cuttings.

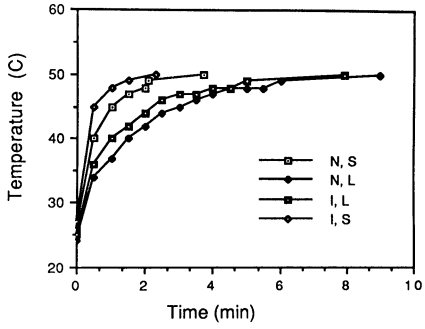

Fig. 2. Effect of thickness of nodal and internodal regions on pith temperatures over time of dormant K5140 cuttings submersed in a water bath of 50 C. Comparisons are made of relatively large (N, L) and small (N, S) nodal (cross-sectional areas of 94 and 294 mm) and large (I, L) and small (I, S) internodal (33 and 165 mm) regions.

noted (S. J. Navaratnam, Australian Quarantine and Inspection Service, *personal communication*). Goheen et al (10) reported that dormant vines survive hot water treatments of 50 C for up to 150 min. In South Africa, treatment of dormant Cabernet Sauvignon cuttings for 30 min at 50 C hastened callus formation and bud burst (12). In our study, water treatments of 50 C for 30 min were conducted with only a few cultivars. It is possible that some cultivars may be more sensitive to heat. Therefore, further testing on a range of cultivars and rootstocks is currently being done and is necessary before hot water treatment is applied on a large commercial scale. Also, the timing of the treatment may be important with regard to bud survival; cuttings treated before winter storage may sustain less bud injury. Regardless of the incidence of bud mortality, many cuttings survived the hot water treatment and could be useful as sources of AT3-free propagation wood.

The assay methods used in this study will detect as few as 50 cfu of AT3 in vascular extracts and from triturated callus tissues. Therefore, we cannot conclude that the treatments totally eliminated all of the pathogen in cuttings, but rather reduced them to a nondetectable level. In vitro, strains of AT3 varied in their sensitivity to heat. If high levels of relatively heat-tolerant strains are present in cuttings they may not be eradicated with a 30-min treatment. It is difficult to extrapolate from the results of laboratory tests to the potential effectiveness of the treatment on naturally infested cuttings because the population of AT3 within cuttings is difficult to measure and it is not known if cells residing within grape vascular tissue will respond identically to cells in culture. It is also possible that heating of cuttings may solubilize or alter plant metabolites that are toxic to the pathogen. Although hot water treatment looks very promising as a means of eradicating the pathogen, it can only be verified over time by continued indexing of treated plants in the field.

The variability of thermosensitivity of *Agrobacterium* biovars was apparent in vitro and also from assays of heat-treated cuttings. Previously, variability in sensitivity of strains of *Agrobacterium* to 37 and 38 C were reported (17,19), but sensitivity to 50 C was not measured. In the tests reported here, biovar 1 is relatively heat-tolerant and survived exposures of 50 C for 30 min in culture media and in naturally infested, heat-treated cuttings, as did other relatively heat-tolerant microorganisms. Although the strains of biovar 1 isolated from dormant cuttings were nontumorigenic, tumorigenic strains of biovar 1 have been detected infrequently in grape galls and systemically in symptomless cuttings (5,6). Biovar 1 is not considered a significant factor in grapevine crown gall. However, it is possible that tumorigenic strains may be selected in heat-treated cuttings. In general, AT3 appeared to be more heat-sensitive in experiments with 523 medium than with YE. This may reflect the differences between the strains tested or be related to the different broth media.

Once propagation material is freed of biovar 3, mother blocks of clean propagation material can be established. Previously, recontamination of clean stocks from high populations of soil inoculum has been reported (3). However, the pathogen has not been detected in nonvineyard soils (7). Periodic indexing of mother blocks will be necessary to verify that plants remain free of AT3.

ACKNOWLEDGMENTS

We thank the Riverland Vine Improvement Committee and Phylloxera Board of South Australia, the Australian Grape and Wine Research Council, and the University of Adelaide for financial assistance.

LITERATURE CITED

1. Barlass, M., Newman, H. P., and Possingham, J. V. 1987. Grapevine quarantine at Merbein, Victoria. Pages 15-17 in: Aust. Grapevine Winemaker.
2. Bazzi, C., Piazza, C., and Burr, T. J. 1987. Detection of *Agrobacterium tumefaciens* in grapevine cuttings. EPPO Bull. 17:105-112.
3. Bishop, A. L., Katz, B. H., and Burr, T. J. 1988. Infection of grapevines by soilborne *Agrobacterium tumefaciens* biovar 3 and population dynamics in host and nonhost rhizospheres. Phytopathology 78:945-948.
4. Burr, T. J., Bishop, A. L., Mittak, V. L., and Katz, B. H. 1988. Production and use of a monoclonal antibody specific to *Agrobacterium tumefaciens* biovar 3. (Abstr.) Phytopathology 78:1506.
5. Burr, T. J., and Katz, B. H. 1983. Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and sap, and from vineyard soil. Phytopathology 73:163-165.
6. Burr, T. J., and Katz, B. H. 1984. Grapevine cuttings as potential sites of survival and means of dissemination of *Agrobacterium tumefaciens*. Plant Dis. 68:976-978.
7. Burr, T. J., Katz, B. H., and Bishop, A. L. 1987. Populations of *Agrobacterium* in vineyard and nonvineyard soils and grape roots in vineyards and nurseries. Plant Dis. 71:617-620.
8. Burr, T. J., Katz, B. H., Bishop, A. L., Meyers, C. A., and Mittak, V. L. 1987. Effect of shoot age on systemic infestations of *Agrobacterium tumefaciens* biovar 3 and production of pathogen free vines. Am. J. Enol. Vitic. 39:67-70.
9. Davis, M. J., Purcell, A. H., and Thomson, S. V. 1978. Pierce's disease of grapevines: Isolation of the causal organism. Science 199:75-77.
10. Goheen, A. C., Nyland, G., and Lowe, S. K. 1973. Association of a rickettsialike organism with Pierce's disease of grapevines and alfalfa dwarf and heat therapy of the disease in grapevines. Phytopathology 63:341-345.
11. Goodman, R. N., Butrov, D., and Tarbah, F. 1987. The occurrence of *Agrobacterium tumefaciens* in grapevine-propagated material and a simplified indexing system. Am. J. Enol. Vitic. 38:189-193.
12. Goussard, P. G. 1977. Effect of hot-water treatments on vine cuttings and one-year-old grafts. Vitis 16:272-278.
13. Kado, C. I., Heskett, M. G., and Langley, R. A. 1972. Studies on *Agrobacterium tumefaciens*: Characterization of strains 1D135 and B6, and analysis of the bacterial chromosome, transfer RNA and ribosomes for tumor inducing ability. Physiol. Plant Pathol. 2:47-57.
14. Leach, J. E., Ramundo, B. A., Pearson, D. L., and Claflin, L. E. 1987. Dot-immunobinding assay for detecting *Xanthomonas campestris* pv. *holcicola* in sorghum. Plant Dis. 71:30-33.
15. Lehoczky, J. 1968. Spread of *Agrobacterium tumefaciens* in the vessels of the grapevine after natural infection. Phytopathol. Z. 63:239-246.
16. Ophel, K., Burr, T. J., Magarey, P. A., and Kerr, A. 1988. Detection of *Agrobacterium tumefaciens* biovar 3 in South Australian grapevine propagation material. Australas. Plant Pathol. 17:61-66.
17. Panagopoulos, C. G., Psallidas, P. G., and Alivizatos, A. S. 1978. Pages 221-228 in: Studies on Biotype 3 of *Agrobacterium radiobacter* var. *tumefaciens*. Proc. Int. Conf. Plant Pathog. Bact. 4th. Angers.
18. Rodriguez, R. L., and Tait, R. C. 1983. Recombinant DNA techniques: An introduction. Addison-Wesley, Reading, MA.
19. Sule, S. 1978. Biotypes of *Agrobacterium tumefaciens* in Hungary. J. Appl. Bacteriol. 44:207-213.
20. Tarbah, F. A., and Goodman, R. N. 1986. Rapid detection of *Agrobacterium tumefaciens* in grapevine propagating material and the basis for an efficient indexing system. Plant Dis. 70:566-568.