Infection of Grapevines by Soilborne *Agrobacterium tumefaciens* Biovar 3 and Population Dynamics in Host and Nonhost Rhizospheres

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This work was supported in part by grants from the New York State Wine/Grape Foundation, the American Vineyard Foundation, and the U. S. Tobacco Company.

Accepted for publication 10 February 1988.

ABSTRACT

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.

Host (*Vitis vinifera* 'Chardonnay') and nonhost (*Avena sativa* 'Astro') rhizospheres enhanced survival of *Agrobacterium tumefaciens* biovar 3 (AT3) in artificially infested unpasteurized field soil in a greenhouse study; populations in host rhizospheres were greater than in nonhost rhizospheres. Populations declined over time in both fallow soil and oat rhizospheres but not in grapevine rhizospheres. AT3 survived 10 mo in association with 54% of grapevines assayed; 35% of these developed tumors, and systemic infections were detected in 10%. Populations of rifampicin-resistant AT3 did not change over 10 wk in Concord grapevine (*Vitis labrusca* L.) rhizosphere; 60% of Concord seedlings in soil infested with 10^6 cfu/g became systemically infected within 10 wk, but none became systemically infected at lower levels of soil infestation (10^4, 10^2, and 1 cfu/g).

Agrobacterium tumefaciens (E. F. Smith & Townsend) Conn biovar 3 (AT3) is associated with crown gall of grapevine (*Vitis* spp.) throughout the world (5,9,12,15,17–19). AT3 survives saprophytically as systemic infections in grapevine xylem and is transmitted to new plantings by vegetative propagation of grapevine cuttings (6,13,20). Tumor induction follows wounding of systemically infected grapevines by sudden drops in temperature or mechanical means (3,9,13,14).

Testing of grapevine propagation stocks and production of AT3-free grapevines have been proposed as approaches to disease control (1,6,8,20). Success of these control strategies will depend on continued freedom of grapevines from AT3 after planting in the field. AT3 appears to be restricted to soils where grapevines have grown (5,7), indicating that AT3 is introduced with diseased or systemically infected propagation material. Survival of AT3 in soil apart from grapevines and infection of grapevines by soilborne AT3 have not been described.

Our objectives were to compare the survival of AT3 in host and nonhost rhizospheres to that in fallow soil, to evaluate the ability of soilborne AT3 to initiate tumors and to systemically infect grapevines, and to determine if low initial populations of AT3 increase in the grapevine rhizosphere.

MATERIALS AND METHODS

Bacterial strains. Strains CG48 and CG49 of AT3 were isolated from galls on grapevine (*Vitis vinifera* L. 'Baco Noir' and 'Reisling,' respectively) in New York state. Rifampicin-resistant AT3 strain ABR15 was selected from a strain isolated from a Riesling gall. All strains were tumorigenic on *Nicotiana glauca* Graham. CG48 and CG49 inocula were cultured on potato-dextrose agar (PDA, Difco); ABR15 was grown on PDA plus 175 μg/ml of rifampicin (PDA-rif). All cultures were incubated at 28 C.

Host and nonhost rhizosphere effects on AT3. Current season grapevine galls used for soil infestation were collected from Riesling trunks in August immediately before use. *N. glauca* galls used to infest soil were collected 6 wk after inoculation of *N. glauca* stems in the greenhouse. *N. glauca* was inoculated by smearing internodes with a bacterial colony and inserting an insect mounting pin through the smear several times to wound the stem. Soil (sandy loam collected from an apple nursery) was infested with 1% (v/w) suspensions of 4-day-old PDA cultures of CG48 or CG49 in distilled water adjusted to $A_{600nm} = 0.1$, approximately 10^8 cfu (colony-forming units)/ml (estimated by dilution plating), or 1% (w/w) gall tissue (approximately 10^6 cfu/g, estimated by diluting plating) comminuted in an electric coffee grinder. Control soils were treated with distilled water (1%, v/w) in each experiment.

V. vinifera 'Chardonnay' plants, freed of AT3 by propagation of shoot tip cultures in vitro (8), were transplanted one per 8-cm pot in infested and control soils when several leaves had expanded and shoots were 3–5 cm in length. Nine oat (*Avena sativa* L. 'Astro') seeds per 8-cm pot were sown 1 cm deep. Plants were maintained in a greenhouse (approximately 24 C, 16-hr photoperiod) and were watered daily with approximately 9 ml of distilled water.

Three replicates each of infested and control treatments were sampled, as described below, at the time of infestation and 3, 14, 28, and 70 days after infestation and planting. Populations of AT3 in soil amendments and fallow soil were estimated by suspending 10–15 g of soil or amendment in distilled water, diluting in sterile distilled water (SDW), plating appropriate dilutions on a modification of Roy and Sasser's medium (RS) selective for AT3 (7).

The entire contents of each pot of oats (including roots), approximately 100 g, less a 3–5-g subsample for estimation of water content, were weighed, suspended in 300 ml of distilled water, and shaken on an orbital shaker (250 rpm, 10 min), followed by dilution in SDW and plating on RS. Grape plants were uprooted and shaken to remove loose soil. Roots and adhering soil were washed by stirring vigorously for 10 min in 50 ml of distilled water; loose soil was suspended separately and shaken in 250 ml of distilled water. Root washes and suspended soil were subsequently combined, diluted, and plated on RS. The intact grape plants were then repotted in Cornell soil mix (2) and watered with tap water in the greenhouse.

Approximately 10 mo after the initial planting, xylem sap was collected by defoliating grape plants, then swabbing an internode 5–10 cm above soil level with 70% ethanol, cutting the stem at this internode, saturating the soil with water, and collecting the sap that exuded overnight in a sterile microfuge tube. Sap (0.1–1.5 ml) was spread on RS medium. A sample of roots and soil (3–5 g) was removed from each pot with a sterile cork borer, suspended in

SDW, and then plated on RS to determine if AT3 was still detectable in grapevine rhizospheres. Subsequently, plants were removed from pots, washed free of soil, and inspected for galls. Galls, or bark (5–20 mg) in cases where galls were not present, were scraped from the surface of plant crowns with a sterile razor blade, triturated in 0.1 ml of SDW, and spread on RS to determine the presence of AT3.

Strains were tentatively identified by growth rate, colony color, and morphology on RS. A scheme for identification of AT3 described previously (7) was applied to a subsample of colonies (30/replicate or 10/replicate, respectively, in the two repetitions).

Effect of grapevine rhizosphere on low-level populations of AT3. Two weeks after emergence, *V. labrusca* L. 'Concord' seedlings germinated in moist Cornell soil mix were transplanted one per 8-cm pot into soil infested with ABR15 as described below. Plants were watered with distilled water in the greenhouse.

A 4-day ABR15 culture from PDA-rif was suspended in distilled water (A_{600nm} = 0.1), diluted serially to yield four cell concentrations (approximately 10^8, 10^6, 10^4, and 10^2 cfu/ml, estimated by dilution plating), and added to Cornell soil mix:perlite:sand (2:1:1), at 1%, v/w, to give soil populations of approximately 10^6, 10^4, 10^2, and 1 cfu/g of soil.

Roots of Concord seedlings planted in ABR15 infested soil (five replicates per treatment) were sampled, 0, 5, and 10 wk after infestation and planting. Plants were removed from pots and loose soil was removed from the roots. Shoots were excised at soil level. Roots and adhering soil were shaken in 50 ml of distilled water for 10 min at room temperature. Aliquots of appropriate dilutions of these suspensions were spread on RS plus 175 μg of rifampicin per milliliter (RS-rif).

Shoots from plants sampled at 10 wk were surface sterilized (10 min, 0.5% sodium hypochlorite plus 0.01% Tergitol) and rinsed in running tap water for 10 min. Samples (0.5 g) were triturated in 1.5 ml of SDW with a mortar and pestle. After incubation for 30 min at room temperature, suspensions were mixed thoroughly, and two 0.3-ml aliquots from each sample were spread on RS-rif. ABR15 was identified on RS-rif by colony morphology.

RESULTS

Host and nonhost rhizosphere effects on AT3. Rhizosphere effects on AT3 populations were significant, $P < 0.05$, by 28 days after infestation and planting regardless of infestation technique. Seventy days after infestation, populations of AT3 in grapevine rhizosphere were significantly higher than those in oat rhizosphere, and populations in oat rhizosphere were generally higher than those in fallow soil, with one exception (70-day sample, pure culture-infested soil, Fig. 1D). AT3 was not isolated from the control (no artificial infestation), regardless of the crop planted. Regression of population level against time in soil infested with grapevine gall (Fig. 1A) indicated that AT3 did not change significantly in grapevine rhizospheres ($P > 0.25$) and declined in fallow soil and oat rhizospheres ($P < 0.001$). The decline was significantly greater in fallow soil than in oat rhizosphere ($P < 0.05$). Decline of AT3 in grapevine rhizosphere was insignificant ($P > 0.50$) in soil infested with pure culture of CG49, whereas declines of AT3 in fallow soil and oat rhizospheres were significant ($P < 0.01$) but not different from one another ($P > 0.50$) (Fig. 1D). All populations declined significantly in soil infested with *N. glauca* gall, with unique slopes for each treatment ($P < 0.001$) (Fig. 1C).

Regression analysis was not appropriate to the data from infestation of soil with the pure culture of CG48, due to the anomalous collapse of AT3 populations in the first two samplings (Fig. 1B). The lower limit of detection by soil dilution and plating on RS was approximately 10^2 cfu/g. All AT3 isolated from soils infested with pure cultures of with *N. glauca* galls were

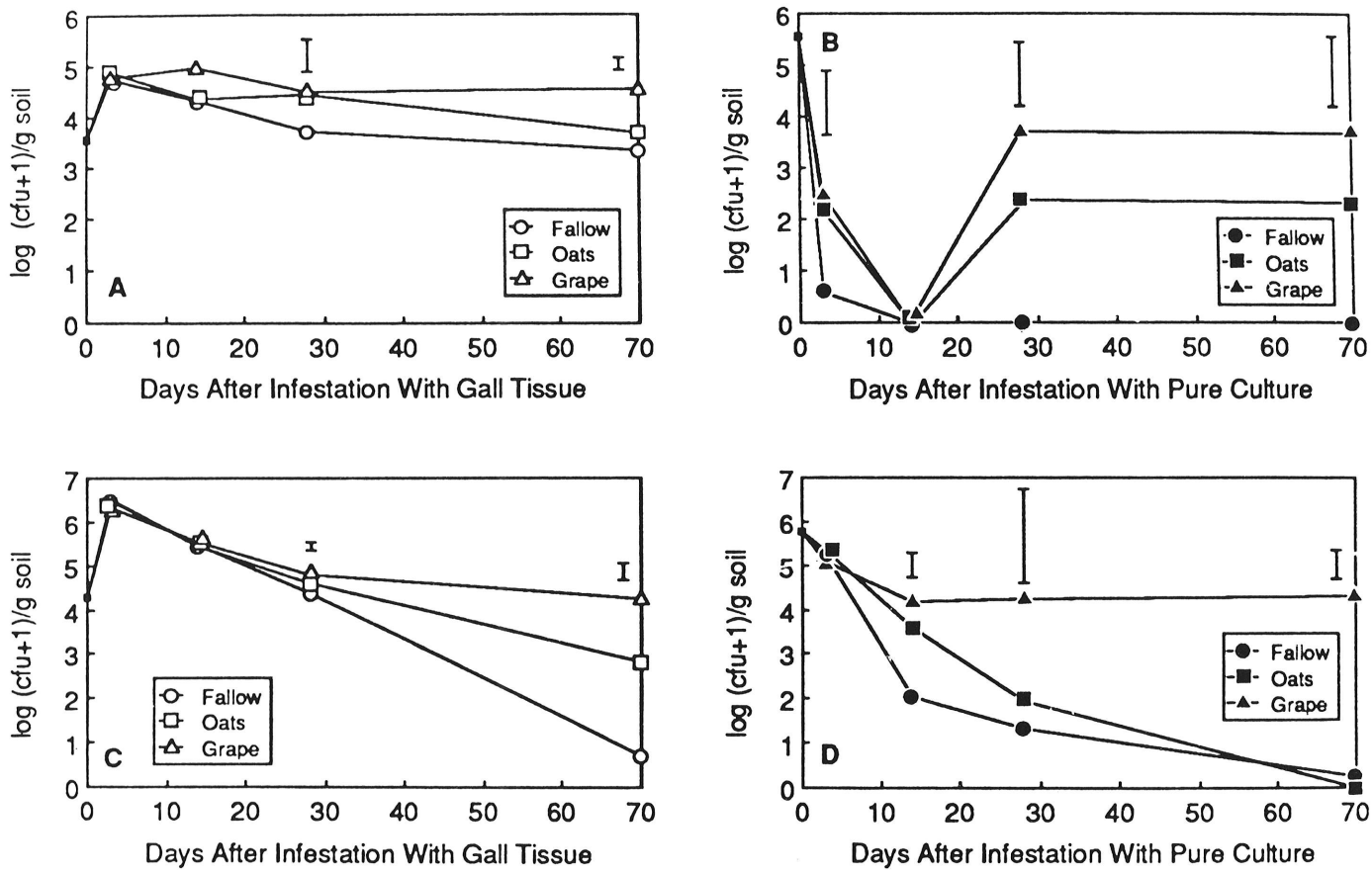

Fig. 1. Populations of *Agrobacterium tumefaciens* biovar 3 (AT3) in artificially infested soil and rhizosphere of grapevine (*Vitis vinifera* 'Chardonnay') and oat (*Avena sativa* 'Astro'). Calculations were based on oven dry weight of soil. Soil was infested with **A,** 1% (w/w) grapevine gall tissue; **B,** 1% (v/w) of a suspension of CG48 in distilled water, A_{600nm} = 0.1; **C,** 1% (w/w) *Nicotiana glauca* gall tissue; or **D,** 1% (v/w) of a suspension of CG49, A_{600nm} = 0.1. Vertical bars represent values of Fisher's protected LSD (P = 0.05) for sampling dates when differences among treatments were significant.

tumorigenic. The proportion of tumorigenic strains from soil infested with Riesling galls was the same as that detected in isolations from the galls themselves before soil amendment (approximately 90%).

Populations of AT3 in gall-infested soil were less variable than those in pure culture-infested soil, $P(\text{Fmax}) < 0.01$, violating the assumption of homogenous variance required for statistical comparison of infestation techniques. Population means in grapevine rhizospheres were generally similar regardless of infestation technique, while populations in oat rhizospheres or fallow soil were higher in gall-infested soil than in pure culture-infested soil. Oat plants in the soil infested with pure culture of CG49 did not survive 70 days. AT3 was undetectable after 70 days in this treatment (Fig. 1D).

More plants in soil infested with gall tissue developed tumors than those in soil infested with pure culture (Table 1). Longer periods of exposure to infested soil were associated with increased infection (Table 1). Soil infested with gall tissue was a more effective source of inoculum than soil infested with pure culture. AT3 was not detected in root and soil samples assayed after 10 mo of tap water irrigation, nor was AT3 isolated from plants exposed only to uninfested control soils.

Effect of grapevine rhizosphere on low levels of AT3. ABR15 remained undetectable in soil amended with 1 cfu/g of soil and uninfested controls (Fig. 2). Regression analysis showed no change over 10 wk in populations of ABR15 in grapevine rhizosphere amended with 10^2, 10^4, and 10^6 cfu ABR15/g of soil ($P > 0.50$). ABR15 was reisolated from shoots of three of five plants sampled at 10 wk from soil infested with 10^6 cfu ABR15/g of soil. ABR15 was not isolated from shoots of plants from lower levels of soil infestation.

DISCUSSION

Understanding the dynamics of AT3 populations in soil and their relationship to grapevines is essential to the development and evaluation of a control strategy for grapevine crown gall based on the use of *Agrobacterium*-free grapevines. AT3 has not been detected in nonvineyard soils (7), but its presence in association with grapevine roots and persistence in grapevine rhizosphere have been reported (16). Several edaphic factors influence the survival of *A. tumefaciens* in soil (10). We have demonstrated effects of rhizospheres on AT3 populations and that these effects vary between plant species. The observation that populations of AT3 in grapevine rhizospheres were sustained at higher levels than those in rhizospheres of oats should not be taken alone as evidence for a specific effect of grapevine rhizospheres on AT3. Simpler explanations (e.g., that the greater root mass of grapevines supports larger populations) are not excluded by our data. Results showing that AT3 causes a host and tissue specific decay of grapevine roots (4), however, suggest the possibility of host specific rhizosphere effects. AT3 does not decay oat roots (Bishop, *unpublished*).

Our results indicate that soilborne AT3 can function as inoculum in establishment of tumors and systemic infections of grape plants in the greenhouse. Initial populations in some treatments may have been artificially high initially. Nonetheless, these levels were sustained for up to 70 days in grapevine rhizosphere. Soilborne populations of AT3 could thus be a significant source of inoculum for grapevine crown gall where initial pathogen populations are high and grapevine culture is continuous. Such circumstances may prevail in established vineyards where populations of AT3 can be 10^3–10^7/g of soil or roots (7) or where grapevines are continuously replanted in nurseries.

Decline of AT3 in fallow soil and rhizosphere of oats indicated that populations in soil might be reduced by fallowing, though verification of this observation in field experiments is necessary before fallowing can be recommended as a measure to reduce soilborne AT3. Oat rhizosphere effects on populations of AT3 demonstrate an association with this nonhost plant that is beneficial to the pathogen. Other cultivated and weedy species not commonly infected by AT3 could have a similar effect, influencing survival of AT3 in soil. Systemic populations of AT3 in grapevine roots (14) may lead to greater persistence in the field, since roots are frequently left in soil after vine removal and could serve as a reservoir of inoculum (11).

Results of planting grapevines in soils with low-level infestations of AT3 indicated that small populations did not infect grapevines systemically within 10 wk and that soilborne AT3 will not necessarily increase to problem levels in grapevine rhizosphere.

Thus, soil populations of AT3 might be managed by exclusion of the pathogen from new viticultural areas by planting only uncontaminated propagation material, or by meticulous removal of old vine roots followed by fallowing before replacement of galled grapevines. This suggestion can be evaluated in field experiments where grapevines free of AT3 are planted in areas not previously used for viticulture or as replacements for galled grapevines in established vineyards.

The tap water in our laboratory is toxic to pure cultures of AT3 in suspension (unpublished results). This may explain the failure to

TABLE 1. Incidence of galls, systemic infection, and persistent populations of *Agrobacterium tumefaciens* biovar 3 associated with crowns of *Vitis vinifera* 'Chardonnay' planted in artificially infested soil

Infestation technique	Days of exposure[a]	Galled (%)	Systemically infected (%)	Isolation from crown (%)
Pure culture[b]	3	0	17	33
	14	0	0	0
	28	50	17	17
	70	33	17	50
Mean		21	13	25
Gall tissue[c]	3	17	0	83
	14	50	0	100
	28	83	17	67
	70	83	17	83
Mean		58	9	83

[a] Plants transplanted at specified times following washing of roots for rhizosphere samples. Figures represent percent incidence in samples of six plants from two experiments. Assays conducted 10 mo after initiation of experiments.
[b] Suspension of *A. tumefaciens* biovar 3 added to soil (1%, v/w).
[c] Comminuted crown gall tissue added to soil (1%, w/w).

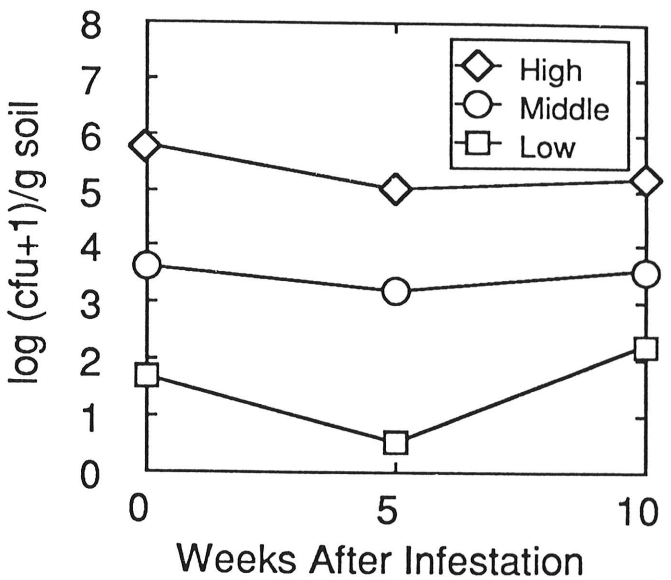

Fig. 2. Populations of *Agrobacterium tumefaciens* biovar 3 strain ABR15 in rhizosphere of grapevine (*Vitis labrusca* 'Concord') seedlings. Soil was amended with approximately 10^6 (high), 10^4 (middle), 10^2 (low), and 1 (undetectable, not shown) cfu/g soil. Slopes are significant ($P > 0.50$); intercepts (differences among treatments) are significantly different ($P < 0.001$).

detect AT3 in grapevine rhizosphere after transplantation and irrigation with tap water in the greenhouse for 10 mo subsequent to quantitative isolations from the rhizosphere. AT3 was still associated with crowns of a significant number of grape plants following this period, indicating an intimate association with these tissues. The effect of tap water may also explain the anomalous drop of AT3 populations in soil infested with pure culture of CG48 (Fig. 1B, 3–14 days); these plants were unintentionally watered with tap water at least once in the first few days of this experiment. AT3 introduced to soil as comminuted gall tissue was apparently protected from this effect.

The observation that grapevines free of AT3 were rapidly recontaminated by soil infested with 10^6 or more cfu/g is a caution against the use of clean planting stock to replace galled grapevines in established vineyards. However, the failure to detect AT3 in soils where grapevines have not been grown (7), the decline of soilborne AT3 in the absence of grapevines, and the failure of low levels of AT3 to increase in grapevine rhizospheres suggest that the use of clean planting stock in conjunction with management of soil populations of AT3 may provide effective control of grapevine crown gall. Long-term studies of reinfection of grapevines by AT3 in the field are currently under way to evaluate the significance of soilborne inoculum in the field.

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