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ɜe control. The lack of yield differences probably due to the tolerance of South- ı Runner. Several factors have been plicated in that tolerance. The cultivar ıes fewer pods when peanut plants are ʹerted than does the susceptible cultivar ɔrunner (22). Southern Runner contin- s to produce new leaves throughout the ɔwing season to compensate for leaves ɜt due to infection and defoliation by ɪf spot pathogens (21); this allows ɪuthern Runner to maintain a higher leaf ɜa index during severe leaf spot epidem- ; than does Florunner (21). However, s continued leaf production reduces the otosynthate partitioning coefficient of ɪuthern Runner (22). Southern Runner rtitions 80% of its photosynthate to pods mpared with 92% for Florunner. An- ɪer factor possibly involved in the toler- ce of Southern Runner is that it develops ʍer stem lesions caused by *C. per- natum* than Florunner does under severe ɪf spot pressure (6).

The AU-Pnuts advisory is a rule-based stem. Beyond new and/or modified rules r new cultivars with disease resistance, ch as Southern Runner, the system al- ʍs for the addition of new rules for new ngicides as they become available. The J-Pnuts advisory could also be updated include rules for the control of other ɜeases such as southern stem rot (*Scle- tium rolfsii* Sacc.) and Rhizoctonia limb t (*Rhizoctonia solani* Kühn AG-4). Pea- ɪt growers are confronted with more than ɪe disease problem in a growing season; ɔorporating rules for other important ɜeases of peanut increases the useful- ss, acceptance, and adoption of the advi- ry system.

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Survival and Tumorigenicity of *Agrobacterium vitis* in Living and Decaying Grape Roots and Canes in Soil

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ABSTRACT

Burr, T. J., Reid, C. L., Yoshimura, M., Momol, E. A., and Bazzi, C. 1995. Survival and tumorigenicity of *Agrobacterium vitis* in living and decaying grape roots and canes in soil. Plant Dis. 79:677-682.

Agrobacterium vitis was recovered from living and decaying grape roots and canes 23 months after grapes were artificially inoculated with a mixture of six strains of the bacterium. Each strain contained a unique plasmid profile. Following inoculation, some plants were treated with the herbicide Roundup to speed up plant death and tissue decay. Roots and canes were assayed over time, and by comparing plasmid profiles of recovered strains it was determined that certain *A. vitis* strains used in the inoculum mixture were recovered more frequently than others. Profiles identical to those identified for each strain used in the inoculum mixture were observed at least twice in strains recovered during the experiment. Of 133 plasmid profiles that were observed, only 18 did not resemble any of the strains used in the inoculum mixture. Of 333 strains recovered from roots and canes, 321 were tumorigenic, indicating that this trait was stable throughout the experiment. A group of six strains having plasmid profiles identical to strain CG49 that were recovered over an 16-month period were further characterized using restriction fragment length polymorphic analysis of plasmid DNA, random amplified polymorphic DNA analysis of total genomic DNA, and ribofingerprinting of a chromosomal region including 1,479 bp (99.5%) of the 16S rDNA, the intergenic spacer between 16S and 23S rDNA genes, and 132 bp of the 23S rDNA gene. All six strains were shown to be identical to CG49.

Survival in soil of *Agrobacterium tumefaciens,* the cause of crown gall disease, has been implicated in the development of sporadic disease outbreaks (27). When field soils are assayed for the presence of *Agrobacterium* spp., nontumorigenic strains are often predominant (5); however, high proportions of tumorigenic *A. tumefaciens* strains may be detected infrequently (6). *Agrobacterium* spp. may also survive in association with symptomless plant material and in latent infections (9).

Agrobacterium vitis (24), previously *A. tumefaciens* biovar 3, is the major cause of crown gall of grape. The systemic survival of *A. vitis* in grape propagation material provides an important means of pathogen spread (8). Strategies that are being tested for producing *A. vitis*–free grapevines include the use of heat therapy (12) and propagation of vines from shoot tips (10). A potential source of inoculum for infecting "clean" plants is the presence of *A. vitis* in the soil. *Agrobacterium vitis* has been detected in vineyard soils but thus far not in other soils (5,6,9). When soils were artificially infested with *A. vitis*, and then left fallow, or planted to grape or a nonhost (*Avena sativa*), populations declined significantly over a 10-week period in the fallow soil and in oat rhizosphere soil but not in grape rhizosphere soil (3). Two important considerations related to the management of crown gall on grape are how long *A. vitis* will survive in vineyards after crown gall–infected vines are removed, and whether tumorigenicity is maintained by strains of *A. vitis* that survive in soil. In this paper we report on the survival and tumorigenicity of *A. vitis* in living and decaying grape roots and canes in soil over a 23-month period.

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MATERIALS AND METHODS

Bacterial strains. Tumorigenic strains of *A. vitis* that were isolated from grape in different states of the U.S. and in other countries were used. These included: CG49 (N.Y.), CG56 (Mich.), CG98 (Va.), CG106 (Miss.), NW-161 (Germany), and K306 (Australia). Plasmids of *Erwinia stewartii* strain SW2 (obtained from D. Coplin, Ohio State University) were used as size markers in gel electrophoresis. pTHE 17 (contains *Eco*RI fragments 16,32,28, 14,4, omega, and one of nopaline Ti plasmid pTiC58 cloned into the cosmid pLAFRI) carried in *Escherichia coli* strain DH5 was used as a DNA probe (11). All strains were maintained in cryogenic storage (–80°C) and were subcultured on potato-dextrose agar (PDA, Difco) or Luria-Bertani agar (LB) (20) with appropriate antibiotics. For DNA extractions, strains of *E. coli* were grown on L broth (20) and *A. vitis* on yeast mannitol (YM) broth (16).

The *A. vitis* strains were tested for pathogenicity on sunflower (*Helianthus annuus* L.), tomato (*Lycopersicon esculentum* Mill.), *Kalanchoe diagremontiana* (Hamet. & Perrier) and grape (*Vitis vinifera* L.). Bacteria were grown for 48 h at 28°C on PDA. Plants other than grape were inoculated by placing bacterial growth on the stem of plants with a sterile toothpick and then wounding the plant by piercing a sterile needle through the bacterial cells into the plant. Grapevines, cv. Riesling, that were propagated from dormant cuttings, were inoculated by applying bacterial cell suspensions in holes that were bored with an electric drill in the woody cane portions of the plants. The drill bit diameter was about 6 mm and holes were drilled to the depth of the pith. Bacterial suspensions were made by suspending cells from 48-h-old cultures grown on PDA at 28°C in sterile distilled water and adjusting the concentration to about OD 0.1 at 600 nm using a spectrophotometer. This suspension contained about 10^8 cfu per ml as determined by dilution plating on PDA. Seventy-five microliters of bacterial suspensions was applied to three or four inoculation sites on each of three grape plants and sites were wrapped with Parafilm. The presence of galls was determined after 8 weeks.

Each *A. vitis* strain was examined to determine if it produced agrocins (19) that would inhibit growth of the other strains in vitro. A single streak of each strain was made across individual plates of MG agar medium (21). This medium is routinely used to bioassay for agrocins produced by *Agrobacterium.* After 48 h at 28°C, bacterial growth was killed by placing a filter paper saturated with chloroform in the lid of the glass petri dish. The plate was inverted for about 60 min to expose the bacterium to the vapor. Subsequently, plates were aired in a fume hood, and then the bacterial growth was scraped from the plates with a rubber spatula. Sensitivity of the other five *A. vitis* strains was determined by streaking them across the plate perpendicular to where the potential inhibiting strain had grown. Sensitivity to agrocin was then determined by the ap-

pearance of a zone of growth inhibition at the streak. A nontumorigenic strain of *A. vitis*, F2/5, that is known to produce an agrocin that inhibits growth of many *A. vitis* strains (13), was used as a positive control.

Inoculation with mixture of *A. vitis* strains Dormant Riesling grapes were collected from a vineyard that had less than 0.25% visible crown gall. Cuttings were rooted for about 1 month in moist perlite in the greenhouse. The *A. vitis* strains listed above were grown on PDA, and inoculum suspensions containing about 10^8 cfu per ml were made as described above. The grapevines were wounded by making a longitudinal cut (about 3 cm) in the basal end of the cuttings with pruning shears. The wounded areas and root systems of 34 plants were submersed for 10 min in an inoculum mixture that contained equal volumes (total 600 ml) of the suspensions of the six *A. vitis* strains. Vines were planted in 25-cm-diameter plastic pots in a soil mixture (pH 7.2) containing equal amounts of a sandy loam field soil and Cornell mix (4) and were maintained in the greenhouse. The field soil was collected from an apple nursery near Geneva, N.Y., and was determined to have no detectable levels of tumorigenic *Agrobacterium* spp. (data not shown). One month after planting, root samples from six of the vines were assayed for populations of *A. vitis* as described below.

Because we were interested in survival of *A. vitis* in living and decaying grape roots and canes, 25 vines were treated with the herbicide Roundup (41.0% glyphosate Monsanto, St. Louis, Mo.) 6 weeks after inoculation as a means of rapidly killing the vines. Foliage was sprayed to runoff with a 2% Roundup spray; 2 weeks after treatment, the foliage had completely turned necrotic. The aboveground portions of the vines, except for about 3 cm of woody cane, were cut off, and the remaining stem, crown, and roots were buried at least 5 cm below the soil surface. The remaining nine vines that were not sprayed with Roundup were maintained alive. Living vines were watered every other day and soils containing glyphosate-treated roots and canes were watered weekly.

To determine the possible bactericidal effect of glyphosate on *A. vitis*, 7-mm sterile filter paper disks were saturated with concentrations of Roundup (0, 10, 100, 1,000, and 10,000 mg per liter) and placed on the surface of PDA plates that were seeded with about 10^6 cfu per ml of *A. vitis* strains CG49 or CG56. Plates were observed for inhibition zones around the Roundup-saturated disks after 48 and 72 h.

Assays of root and cane tissue. Populations of *A. vitis* per gram of root tissue were initially determined for grapevines that were inoculated with the mixture of six *A. vitis* strains 1 month after inoculation. Ten grams of roots were collected from each of three inoculated vines that were treated or not treated with Roundup. Roots were placed in 250 ml of sterile distilled water in a blender and blended for about 1 min. Serial dilutions of the suspensions were plated on medium RS (9) that is semiselective for *A. vitis*. Colonies resembling *A. vitis* were subcultured on PDA and tested for reactivity to a species-specific monoclonal antibody using an immunoblot procedure (2). Strains were also tested for tumorigenicity by inoculation on sunflower, tomato, *K. diagremontiana,* and grape as previously described. After treatment with Roundup, dead and decaying tissues from glyphosate-treated vines and living tissues from nontreated vines were assayed for *A. vitis* every 1 to 3 months.

After 6 to 8 months, roots of the glyphosate-treated vines had decayed to the point that it was not possible to collect 10 g. Therefore, cane tissue that was buried with roots was collected and analyzed in the same way and the tissue sample size was reduced to 5 g. Assays were done every 2 or 3 months until all plant material had been utilized (19 to 23 months after the start of the experiments). For the first replication of this experiment, roots and canes were assayed from 29 May 1989 through 3 June 1991 and for the second replication from 3 January 1991 through 24 August 1992.

Plasmid analysis. Plasmids were isolated and visualized from *A. vitis* strains according to the method of Slota and Farrand (28). Undigested plasmids were electrophoresed in 0.7% agarose in Tris-borate-EDTA (TBE) (20).

To determine if certain strains in the inoculum mixture predominated in association with living and decaying grape roots and canes, plasmid profiles of strains isolated over time were compared with profiles of the known *A. vitis* strains that were used in the inoculum mixture. Plasmid profiles were run for 36 strains that were isolated from the first replication and for 97 strains collected during the second replication. These strains were isolated 3 to 23 months after the vines had been inoculated with *A. vitis*.

RFLP analysis. Predominant plasmid profiles were observed in strains that were isolated from grape roots and canes during the course of this study. A common profile that was observed in strains recovered during replication two was identical to that of CG49. A set of these strains recovered 3, 7, 12, 14, and 16 months after inoculation of vines were compared with CG49 using restriction fragment length polymorphic (RFLP) analysis. Ten microliters of plasmid DNA from each strain was suspended in TE8 buffer and digested with the restriction endonuclease *Eco*RI for 2 h at 37°C. Digested plasmid DNA was electophoresed in 1% agarose in TBE at 80V for 2 h. Restriction fragments were probed with digoxigenin-labeled pTHE17 that was labeled by random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate using a nonradioactive DNA labeling kit (Genius, Boehringer Mannheim, Indianapolis, Ind.). Following a 2-h prehybridization at 68°C, hybridizations were done for 20 h, 40 min at 68°C. A chemiluminescent detection system (Lumi-Phos 530, Boehringer Mannheim, Indianapolis) was used to detect the DNA fragments that hybridized with pTHE17.

RAPD analysis. The same set of six strains used above were compared by random amplified polymorphic DNA (RAPD) analysis using polymerase chain reaction (PCR). Chromosomal DNA was prepared from 5-ml overnight cultures incubated at room temperature in YM broth. The cells were centrifuged at 5,000 rpm and suspended in 0.2 ml of SET buffer (20% sucrose, 50 mM EDTA, and 50 mM Tris, pH 7.6) containing 5 mg per ml of lysozyme and 1 mg per ml of RNase, vortexed, transferred to a 1.5-ml Eppendorf tube, and incubated at 37°C for 20 min. To a suspension, 0.4 ml of 1% sodium dodecyl sulfate was added and mixed by inverting the tubes, and incubated at 65°C for 15 min. After incubation, an equal volume of 1:1 mixture containing Tris-buffered phenol/chloroform was added and the sample was vortexed and centrifuged at 13,000 rpm for 5 min. The aqueous layer was removed and reextracted with chloroform/isoamylalcohol twice. Nucleic acids were precipitated with 1/10 volume of 3 M NaAc and 2 volumes of ethanol at –20°C for 2 h and centrifuged for 20 minutes at 13,000 rpm. The pellet was washed with 70% ethanol, dried at room temperature for 30 minutes, and dissolved in 50 µl of sterile water. Quantification of DNA was done by using Gene Quant (Pharmacia, Piscataway, N.J.).

For RAPD analysis, PCR amplifications were carried out in 100-µl volumes and contained 50 ng of DNA, 2.5 mM $MgCl_2$, 75 pg primer, 0.1 mM (each) deoxynucleoside triphosphate (dNTP), 2.5 U *Taq* polymerase (Promega, Madison, Wis.), reaction buffer (10 mM TrisCl, 50 mM KCl and 0.1% Triton X-100) under 3 drops of mineral oil. Amplification was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., San Francisco) programmed for one cycle of 2 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 42°C, and 2 min at 72°C, and final extension for 5 min at 72°C. Ten-base pairs random oligonucleotides were purchased from Genosys Biotechnologies Inc. (Woodlands, Tex.). From kits Gen 1-RE and Gen 3-60, fourteen primers were tested. The three primers RE 5 (5′ GCGAATTCCG 3′), RE 2 (5′ GCGGTACCCG 3′), and 30-60 (5′ GAGCAGGCTG 3′) were selected for analyzing the variation among the strains.

DNA amplification products were separated by 1.5% agarose gel electrophoresis. Gels and running buffer were prepared in TBE buffer (100 mM TrisHCl, 83 mM boric acid, 1 mM $Na_2EDTA \cdot H_2O$. Electrophoresis was at 60 volts for 2 h. DNA was stained with ethidium bromide (EtBr) (0.5 µg per ml) and visualized under UV.

Ribofingerprinting. Oligonucleotides FGPS6 (5′GGAGAGTTAGATCTTGGCTCAG 3′) and FGPL 132 (5′CCGGGTTTCCCCATTCGG 3′) were used to amplify a ribosomal region containing 1,479 bp (99.5%) of the 16S rDNA, the intergenic spacer between 16S and 23S rDNA genes, and 132 bp of the 23S rDNA gene of the six strains described above. Sequence information of oligonucleotides was obtained from Normand et al. (23) and oligonucleotides were purchased from Genosys Biotechnologies Inc. The thermal controller was programmed for 2 min at 94°C and following the 35 cycles of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C and the final extension for 5 min at 72°C. Amplification products were purified using Milipore regenerated cellulose columns (Milipore Corp., Bedford, Mass.). Amplicons were digested with *Taq*I, *Rsa*I, and *Ava*I purchased from Promega and digests were analyzed by 2% agarose gel electrophoresis in TBE buffer. Gels were stained with 0.5 µg per ml of EtBr and photographed under the UV source.

RESULTS AND DISCUSSION

The wild-type strains of *A. vitis* that were used to inoculate grape were tumorigenic on sunflower, tomato, *K. diagremontiana,* and grape, except for strain CG98, which was nontumorigenic on *K. diagremontiana* and sunflower, and strain CG964, which was nontumorigenic on *K. diagremontiana.* Crown galls were observed at the basal ends of grape cuttings 1 month after they were inoculated with the mixture of six *A. vitis* strains. At that time, *A. vitis* populations averaged 2.2×10^6 cfu per gram of root tissue. All of the strains that were isolated from the galls resembled *A. vitis* on RS medium, reacted positively with the *A. vitis*–specific monoclonal antibody, and were tumorigenic. No other galls were observed on any of the plants for the duration of the experiment.

Populations of *A. vitis* ranged from 6.0 $\times 10^3$ to 1.2×10^7 cfu per gram of root or cane over the 23-month sampling periods for the two replications of the experiment (Table 1). Populations from the dead and decaying roots and canes were usually lower than those detected on living vines; however, differences over time were not statistically significant. The trend of lower populations being associated with dead and decaying tissues was observed at all but one sampling times at which comparisons could be made. One possible explanation for the trend is that Roundup, used to kill the vines, is inhibitory to *A. vitis.* This hypothesis is supported by the observation that a zone of growth inhibition against CG49 and CG56 formed around disks that were saturated with 10,000 mg per liter of Roundup but not around disks with lower concentrations.

From 116 strains of *A. vitis* that were isolated over the duration of replication one, only one was nontumorigenic. In replication two, 206 of 217 strains were tumorigenic. Therefore, *A. vitis* can stably maintain its Ti plasmid while surviving for extended periods in association with living or dead grape roots and canes. Tumorigenic strains are apparently not at a great competitive disadvantage even when surviving in association with dead roots and canes, i.e., a nontransformable substrate. However, as mentioned above, populations were generally lower in association with decaying and dead tissues. The experiment was ended when there was insufficient root and cane tissue to sample. From these data we conclude that *A. vitis* may persist for at least 23 months and possibly longer in decaying grape and cane tissue. Moisture and temperature conditions during this experiment were relatively stable; therefore, the effect of extreme conditions that may be encountered in the field, such as freezing and thawing, or the influence of a wide range of native plants or weed species, on the genetic stability and survival of strains is unknown.

The six *A. vitis* strains that were used to inoculate grapes had different plasmid profiles that could be identified by agarose gel electrophoresis (Fig. 1). In addition to a Ti plasmid, each strain contained at least one cryptic plasmid that varied in size, making it possible to differentiate strains. By analyzing several strains that were recovered from the living and decaying grape roots over the period of the experiment, predominant profiles became apparent. In replication one, 24 of 36 strains that were examined had profiles identical to CG56 (Table 2). Fewer strains with pro-

Table 1. Populations of *Agrobacterium vitis* surviving in association with living and decaying grape roots and canes in soil

	Average population of *Agrobacterium vitis* per g of tissue[b]			
	Replication one		Replication two	
Months after inoculation[a]	Living[c]	Decaying	Living	Decaying
0	1.5×10^6	. . .	2.8×10^5	. . .
3	1.2×10^7	9.7×10^6	2.3×10^6	4.2×10^4
7	5.7×10^6	6.0×10^3	2.5×10^6	1.1×10^4
12	1.8×10^6	2.3×10^5	9.5×10^4	1.2×10^5
16	ND[d]	ND	1.5×10^5	2.7×10^4
19	ND	2.8×10^4	ND	ND
23	ND	5.8×10^5	1.1×10^5	2.8×10^4

[a] Vines were sampled on 16 different dates in replication one and on nine dates in replication two. Only data for seven sampling times are reported.
[b] The average population of *A. vitis* that was detected from samples of 5 g or 10 g of root or cane that were collected from each of three nontreated or Roundup-treated single-vine replicates. Populations are averages for the three replications of each dilution that were plated. Differences between populations over time or between treatments were not statistically significant ($P = 0.05$) as determined using the Waller-Duncan *k*-ratio *t* test contained in the procedure of SAS (Statistical Analysis Systems, SAS Institute Inc., Cary, N.C.).
[c] One month after inoculation vines were either sprayed with a 2% Roundup (glyphosate) solution (decaying) or left untreated (living). When foliage on decaying vines was dead, the above ground parts of plants (except about 3 cm of cane) were removed and the remaining root and cane tissue was buried at least 5 cm below the soil line.
[d] ND= not determined.

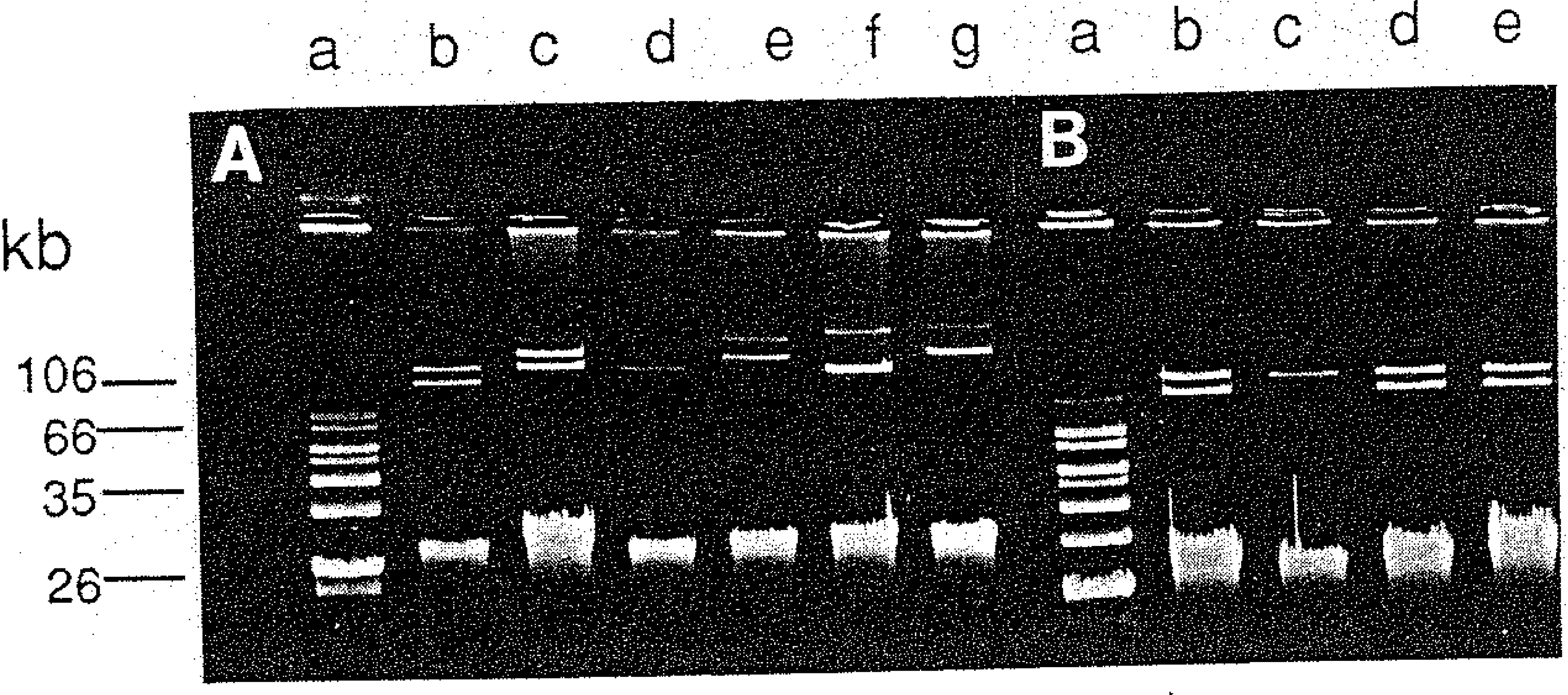

Fig. 1. (A) Plasmid profiles of *Agrobacterium vitis* strains that were used to inoculate cv. Riesling grapes. Lane a = SW2 (*E. stewartii* used as size marker), b = CG49, c = CG56, d = CG98, e = CG106, f = NW161 and g = K306. (B) Plasmid profiles of *A. vitis* strains that were recovered from grape roots and canes. Lane a = SW2; lanes b, d, and e are strains with plasmid profiles identical to CG49, c = *A. vitis* strain with plasmid profile unlike strains used in inoculum mixture.

files resembling CG49, CG98, CG106, and K306 were also detected. In replication two, a much more diverse array of profiles was observed and the predominant types resembled those of CG49 and K306. Profiles resembling those of CG98, CG106. and NW161 were rarely observed in both replications; however, profiles resembling each of the strains used in the inoculation mixture were observed at least twice during the experiment. Two of the recovered *A. vitis* strains had profiles unlike any of the six wild-type strains and two strains had no detectable plasmids.

It was not surprising to detect strains with plasmid profiles that were different from those of wild-type strains. These may represent the natural population of *A. vitis* surviving in association with the cuttings. Considering that the cuttings were collected from a vineyard with a low level of crown gall, some resident *A. vitis* is expected. Nontumorigenic strains of *A. vitis* are also common in grape (7) and therefore the detection of strains with no detectable plasmids is also expected. It is possible that plasmids were conjugally transferred from the strains in the inoculum mixture to naturally residing nontumorigenic strains, thus generating strains with profiles different from any of the strains in the inoculum mixture. Such conjugal transfer of Ti plasmids in planta is well documented (18) and is dependent on the presence of specific conjugal opines that are produced by plant cells that are transformed by specific *Agrobacterium* strains (14). We have not determined the opine types for the strains used in the inoculum mixture except for CG49, which is a nopaline, and K306, an octopine type (T. J. Burr, *unpublished*). Because galls were induced at inoculation sites after 1 month, we can assume that opines were produced and present in the environment around which samples were collected. Depending on the strain or strains that caused the gall, the appropriate opines for inducing conjugal transfer may or may not have been present. Since plasmid profiles of recovered strains almost always resembled those of strains used in the initial inoculum mixture, there is little evidence for conjugal transfer of plasmids occurring between agrobacteria over the 23 months of our experiment.

Strains of *A. vitis* that were recovered more frequently than others (CG49, CG56, and K306) from the grape tissues were apparently better able to compete under the conditions of this experiment. None of the strains produced agrocins that were inhibitory to other *A. vitis* strains in vitro, suggesting that agrocins were not a factor affecting survival in roots and canes. Another factor that may affect competition and survival of strains is their relative virulence on Riesling grapes. It has recently been shown that *A. vitis* strains vary greatly with regard to virulence on grape genotypes (29) and that strain/genotype interactions exist. Therefore, if a genotype other than Riesling had been used in this experiment, other strains in the inoculum mixture may have predominated. In another study, the relative virulence of *A. vitis* strains was measured on Riesling (13): of 10 *A. vitis* strains tested, CG49 and K306 were two of the most virulent. Conversely, NW161 was less virulent on Riesling. The correlation between strain virulence in that study and predominant strain recovery in our present paper is not perfect, however, because strain CG106 had a relatively high virulence rating on Riesling but was infrequently recovered from roots and canes in this study. Factors such as bacterial growth rate and differences in response to nutritional or environmental conditions (15) may have affected the strains' survival.

Six of the strains from replication two that had plasmid profiles identical to CG49 were analyzed by RFLP, RAPD, and ribofingerprinting methods. When plasmid DNA was compared, no differences were noted for the strains when pTHE17 was used as a probe for RFLP analysis (Fig. 2). Further verification of strain identity was achieved by RAPD analysis of total genomic DNA (Fig. 3). Amplification patterns obtained with all three primers RE5, 60-30, and RE2 revealed identical products that were common to all six strains and to CG49 but were different for CG98, CG106, and K306.

Ribofingerprinting analysis has also proven to be a very useful technique for identification of fungi and bacteria (17,22, 25,31). Because the polymorphism of 16S rDNA is usually not sufficient to identify closely related bacterial species or strains within a species, we decided to amplify the 16S rDNA plus the more variable intergenic spacer region between 16S and 23S rDNA genes. Amplifications using the FGPS6 and FGPL132 primers yielded single amplification products of about 2,500 bp for the strains tested. Following digestion, identical patterns were observed for the six strains and CG49 (regardless of restriction endonuclease used), but different patterns for other *A. vitis* strains (Fig. 4). Therefore, our analysis of plasmid and total genomic DNA and specific chromosomal regions of strains indicates that these are subcultures of strain CG49 that persisted in grape tissues in soil for at least 16 months. The strains maintained tumorigenicity and did not appear to be altered genetically during this period.

Strategies being tested for producing grapevines free of *A. vitis* include the use of heat therapy (12) and propagation by shoot tip culture (10). In addition, grape-

Table 2. Comparison of plasmid profiles from *Agrobacterium vitis* strains isolated from living and decaying or dead grape tissues to profiles from the wild-type strains that were *used as inoculum*

			Wild-type strain						
Sampling period[a]	Replications[b]	No. dates sampled	CG49	CG56	CG98	CG106	NW161	K306	None
3 to 6	1	3	...[c]	...	...	...	...	...	...
	2	3	20/18[d]	0	0	0	1/0	0	3/1
7 to 12	1	6	0	5/7	0/2	0/1	0	0	0
	2	3	2/4	1/1	0	1/0	1/1	8/4	3/1
13 to 18	1	4	0/5	0/5	0	0	0	0	0
	2	2	1/4	0	0	0	0	5/0	0/8
19 to 23	1	3	0	0/7	0/1	0/1	0	0/1	0/1
	2	1	1/1	0	0	0	1/1	3/1	1/0

[a] Months after plants were inoculated with mixture of *A. vitis* strains.
[b] For the first replication of this experiment, roots and canes were assayed from 29 May 1989 through 3 June 1991. For the second, samplings were from 3 January 1991 through 24 August 1992.
[c] Plasmid profiles were not determined for strains recovered during this period.
[d] First number: total of isolated strains with profiles characteristic of wild-type strain. Second number: total of strains from living/and decaying or dead vines with plasmid profiles identical to the wild-type strain at top of column.

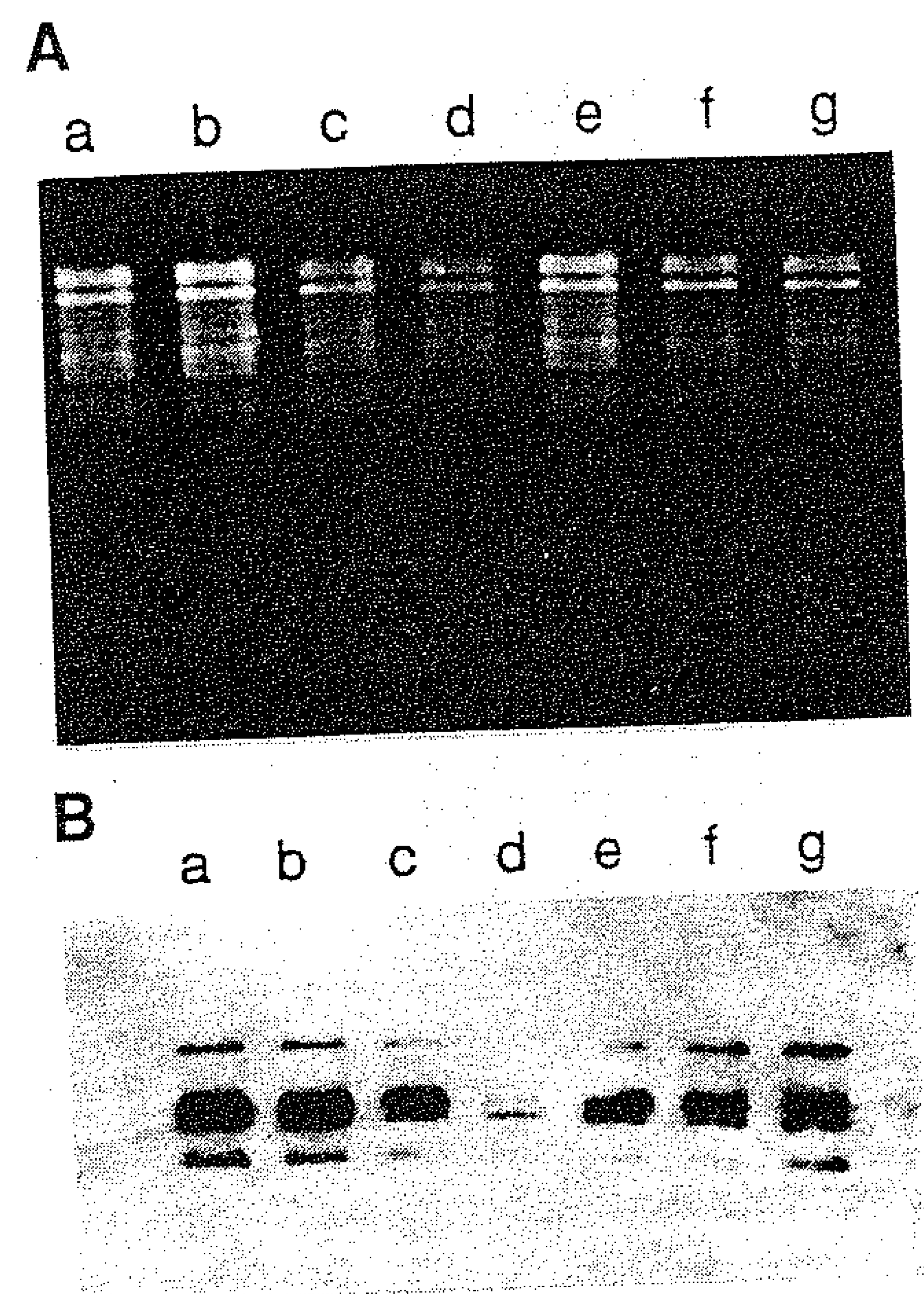

Fig. 2. **(A)** *Eco*RI restriction digest of plasmid DNA from strain CG49 (lane a) and strains with plasmid profiles identical to CG49 that were recovered from living and dead grape roots and canes over a 16-month period. **(B)** Southern hybridization of restriction digests with pTHE17 (described in text).

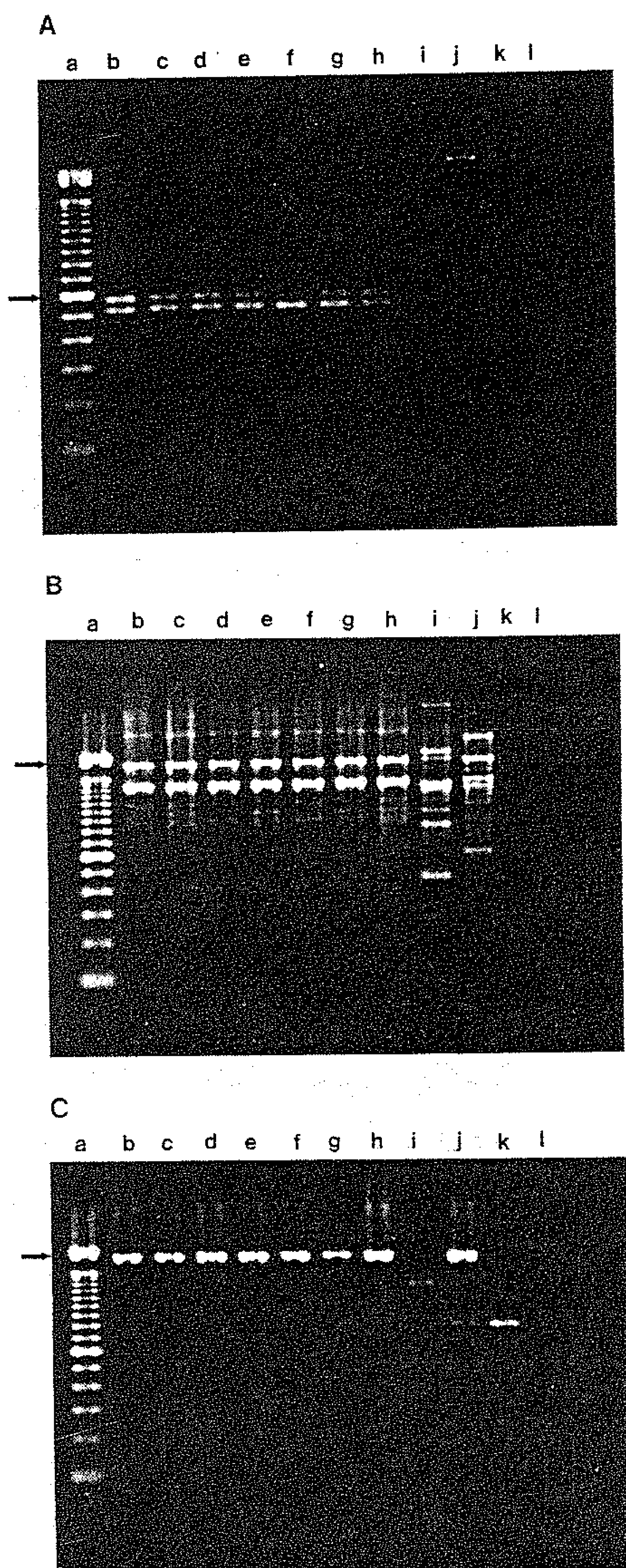

Fig. 3. Comparison of RAPD patterns of *A. vitis* strains using primers (**A**) RE5, (**B**) 30-60, and (**C**) RE2. Lanes a=DNA size marker (arrow points to about 600 bp in A and about 1,900 bp in B and C), b through g = the six strains recovered from roots and crowns that have identical plasmid profiles as CG49, h = CG49, i = CG98, j = CG106, k = K306, l = control reaction without DNA.

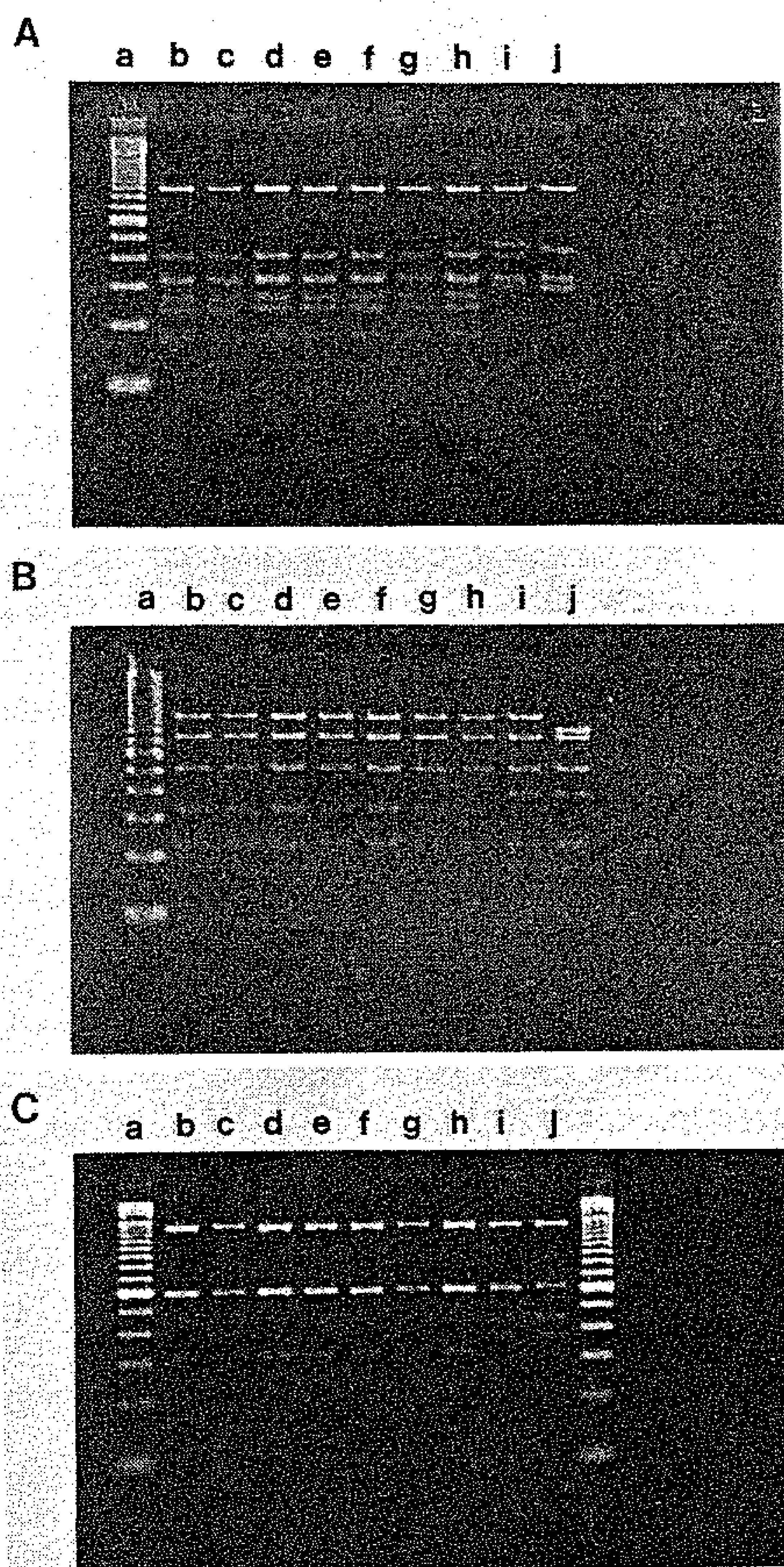

Fig. 4. Restriction patterns of the polymerase chain reaction–amplified chromosomal regions (16S rDNA, intergenic spacer, and 132 bp of the 23S rDNA gene) that were digested with (**A**) *Taq*I, (**B**) *Rsa*I, and (**C**) *Ava*I. Lanes a = DNA size marker, b through g = the six strains recovered from roots and crowns that have identical plasmid profiles as CG49, h = CG49, i = CG98, and j = CG106.

vines can be indexed for *A. vitis* using several approaches (1,30). However, the value of planting *A. vitis*–free vines in pathogen-infested soil has not been determined. From this study it appears that *A. vitis* surviving in association with grape tissues in soil may constitute an important source of inoculum for crown gall. Strategies being studied that may be of benefit for preventing infection from soil inoculum include the use of biological control (13) and the use of crown gall–resistant rootstocks (29).

This and previous studies of *A. vitis* in vineyard soils have concentrated on the grape rhizosphere (9) and therefore the ability of *A. vitis* to survive in vineyard soil devoid of grape debris is still unknown. This may be difficult to determine in a natural soil since we were able to detect *A. vitis* even in small pieces of dead grape debris. Researchers studying the biology of grapevine fanleaf virus demonstrated that grape roots can remain viable in soil after being removed from vines for at least 5 years (26) and would thus constitute a substrate favorable for survival of *A. vitis*. Vineyardists must now consider that as long as grape root and cane residues are present in soil, the pathogen may survive and provide an inoculum source for crown gall on subsequent plantings. Therefore, all possible precautions should be taken to prevent infestation of vineyard sites with *A. vitis*.

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The Influence of European Red Mites on Intensity of Alternaria Blotch of Apple and Fruit Quality and Yield

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ABSTRACT

Filajdić, N., Sutton, T. B., Walgenbach, J. F., and Unrath, C. R. 1995. The influence of European red mites on intensity of Alternaria blotch of apple and fruit quality and yield. Plant Dis. 79:683-690.

Two levels of Alternaria blotch (*Alternaria mali*) intensity and three levels of European red mite (*Panonychus ulmi*) populations were established to study the possible effect of an interaction between mite feeding and fungal infection on disease intensity and fruit quality and yield of apples (*Malus* × *domestica* cv. Delicious). The effect of mite feeding on disease and yield was most apparent in 1991, when disease severity and levels of mite infestations were higher than in 1992 and 1993. Disease severity was increased with increased mite densities in more instances than it was with defoliation and fruit drop. Fruit quality characteristics such as diameter, weight, firmness, commercial color, and soluble solids content were not affected to a great extent by increased mite densities although soluble solids content was reduced in about one-half of the tests with a high intensity of Alternaria blotch.

Alternaria blotch has become a serious disease of strains of Delicious apples (*Malus* × *domestica* Borkh.) in the southeastern United States. The disease is caused by *Alternaria mali* Roberts, which was first identified in 1924 in the United States (11) but was not considered to be a pathogen at that time. Currently, Alternaria blotch is the most important disease of apple in Japan and other Asian countries (6). After the disease was first confirmed in North Carolina in 1988, a survey of major apple-growing regions in the western part of the state was conducted to determine the distribution, incidence, and severity of the disease (6). High populations of European red mites, *Panonychus ulmi* (Koch), also were observed in many orchards severely affected by Alternaria blotch. Based on this observation, and a recent study that found that feeding of leaf miners (*Lyriomyza trifolii* Burgess, Diptera: Agromyzidae) increased infection of muskmelon by *Alternaria cucumerina* (Ellis & Everh.) J. A. Elliot (5), we hypothesized that mite populations may be affecting the intensity (severity and defoliation) of Alternaria blotch on apple leaves.

The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service nor criticism of similar ones not mentioned.

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Other studies have investigated the effect of European red mite on apple leaves, vegetative growth, flowering, and yield (2,3,4,7,9). Beers and Hull (3) reported that shoot length, leaf number, and trunk girth were not affected greatly by mite damage but defoliation was increased in Golden Delicious. Flowering was reduced in Golden Delicious and Stayman but not in Delicious. According to Avery and Briggs (2), mites feed on both upper and lower leaf surfaces and damage mesophyll and bundle sheath cells, resulting in bronzing of leaves.

The objective of this study was to determine if there is an interaction between *A. mali* and *P. ulmi*, and, if so, to quantify its influence on disease intensity, fruit quality, and yield parameters. A preliminary report has been published (7).

MATERIALS AND METHODS

Locations of experimental plots. Experimental plots were established in two orchards in Henderson County in western North Carolina (McKay and Staton orchards). The McKay orchard had a 5-year history (1989 to 1993) of severe Alternaria blotch, with defoliation up to 60% (N. Filajdić and T. B. Sutton, *unpublished*). The European red mite population was very high during the same period, averaging more than 3,000 cumulative mite days (CMD), (J. F. Walgenbach, *unpublished*). Alternaria blotch intensity in the Staton orchard was moderate from 1990 to 1992 and severe in 1993 (N. Filajdić and T. B. Sutton, *unpublished*), and the European red mite population was moderate from 1990 to 1993 (<2,000 CMD, J. W. Walgenbach, *unpublished*).

In the summer of 1991, 24 trees in a single row were used in the McKay orchard to establish two levels of Alternaria blotch intensity and three levels of European red mite density with four replications. Trees were of the cultivar Oregon Spur Delicious and were 12 years old at the initiation of the study. In 1992, the same trees were used in McKay (block 1), and the study was expanded to 24 additional trees in the same orchard (block 2). The same number of trees was used in the Staton orchard. Trees at the Staton orchard were of the cultivar Oregon Spur Delicious and were 8 years old.

1991 experiment. High and low levels of Alternaria blotch were established in the McKay orchard (block 1) with iprodione sprays (Rovral 4F, Rhone-Poulence Ag Company, Research Triangle Park, N.C.). One half of all assigned trees were sprayed with a high pressure handgun sprayer at 0.6 g a.i. per liter on a 2-week schedule beginning 28 May to 15 August. Three levels of European red mite populations were achieved using propargite (Omite 30WP, Uniroyal Chemical Company, Inc., Middlebury, Conn.) at 0.72 g a.i. per liter. The eight trees where a low mite density was intended were sprayed at 2-week intervals beginning on 28 May. Trees where a moderate mite density was desired were sprayed only when the motile mite population exceeded 25 per leaf (in weekly counts). If propargite was needed, it was applied on the same date as the propargite in the low mite density treatment. The last eight-tree group (high mite density) was not treated with propargite. Propargite was applied with a handgun sprayer in the same manner as iprodione. The experimental design was a randomized complete block with six treatments and four single-tree replications receiving the same sprays. Ten arbitrarily selected leaves per tree were taken to the laboratory, brushed with a mite-brushing machine onto the surface of greased circular glass plates and mites were counted with a dissecting scope. Mite numbers were expressed as cumulative mite days (CMD) = $\Sigma\ (((a + b) / 2) \times c) + d$ (equation 1), in which a = number of mites on a count date 1, b = number of mites on count date 2, c = the number of days between count days, and d = CMD on the previous sampling date.