# Characteristics of Colombian Isolates of *Pseudomonas solanacearum* from Tobacco

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ABSTRACT

Bacterial wilt of tobacco, caused by *Pseudomonas solanacearum*, is widespread in the tobacco-growing areas of Colombia. All local cultivars are susceptible, and resistant cultivars from the United States are apparently susceptible under field conditions. The susceptibility of these cultivars could be attributed to strains of the pathogen that are different from those in other tropical and subtropical countries where the same cultivars have been grown successfully for many years.

Under greenhouse conditions, however, five tobacco cultivars were resistant to *P. solanacearum* after stem inoculation with the most pathogenic of several isolates collected in Santander, Colombia. Resistance to these and other race 1 isolates could be overcome by increasing the number of stem inoculations per plant, or by inoculating plants at the seedling stage.

Resistance to the Colombian or other race 1 isolates was not expressed in callus tissues derived from the resistant tobacco cultivar NC 95. Rates of colonization of callus tissues derived from both resistant and susceptible cultivars were very similar.

Colombian isolates of *P. solanacearum* differed from a standard race 1 isolate from the United States (K60) only in relatively few physiological properties, among them: (i) their greater ability to utilize nitrate as the sole source of N, (ii) their lower tolerance to NaCl, and (iii) their ability to utilize malonate, tartrate, and L-phenylalanine as sole sources of C. In a wide variety of other physiological characteristics, such as utilization of many different carbohydrates and other organic compounds as sources of C, the production of lipase, catalase, oxidase, and tyrosinase, sensitivity to chemotherapeutants, etc., Colombian isolates were very similar to K60.

It was concluded that breakdown of resistance under field conditions in Colombia is probably not due to the presence of unusual strains of the pathogen with high pathogenic potential.

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Because of the increasing incidence and severe economic impact of bacterial wilt of tobacco (caused by *Pseudomonas solanacearum* E. F. Sm.) in Colombia, a breeding program was initiated at the Nataima Experimental Station in 1970 to incorporate resistance into acceptable local cultivars. The initial work involved screening potential parents, including the resistant cultivars NC 95, SC 66, Coker 254, and Coker 298. These cultivars were found to be susceptible under field conditions (Silvio Belalcazar, *personal communication*). Because of the significance of these findings to the breeding program, it became imperative to determine whether Colombian isolates of *P. solanacearum* from tobacco are substantially different in pathogenicity and physiological characteristics from other known isolates from tobacco. Previously, certain physiological traits (e.g., lack of acid production from lactose) have been associated with pathogenicity of certain strains to tobacco (19, 20).

All resistant tobacco cultivars from the United States have a common source of resistance, a seedling of *Nicotiana tabacum* L. identified as TI 448A (1). For more than three decades, tobacco cultivars with resistance originating from TI 448A have remained resistant to the many strains of the pathogen present throughout the tobacco-producing areas of the world. The reports from Colombia, however, indicated the possible presence of strains that could attack these cultivars.

Therefore, the objectives of this research were as follows: (i) to determine the reaction of different tobacco cultivars to isolates of *P. solanacearum* obtained from wilting tobacco in Colombia, and (ii) to compare the physiological characteristics of Colombian isolates with other race 1 isolates from tobacco (19, 20).

MATERIALS AND METHODS.—*Host plants.*—Seeds of tobacco cultivars NC 95, NC 17, NC 1007, Coker 254, Bottom Special, and Cuba 12, banana (*Musa balbisiana* Colla.), eggplant (*Solanum melongena* L. 'Black Beauty'), and tomato (*Lycopersicon esculentum* Mill. 'Bonny Best') were planted in "Jiffy Mix" or vermiculite. Seedlings were grown at 28 C under fluorescent lights with a 12-hour photoperiod. When seedlings were 2.0 cm tall, they were transplanted to individual pots containing a mixture of soil, sand, and peat (1:1:1, v/v) and grown either in a growth chamber at 28 C, 50% relative humidity, and $19.4 \times 10^3$ lux on a 12-hour photoperiod, or in the greenhouse at 28 C ($\pm$ 4 C) with $8.6 - 10.7 \times 10^3$ lux of supplemental lighting on a 12-hour photoperiod. Plants were watered daily with Hoagland's nutrient solution.

*Tissue cultures.*—Tobacco pith tissue cultures were prepared from resistant (cultivar NC 95) and susceptible (cultivar Bottom Special and Cuba 12) clonal plants following the methods described by Helgeson et al. (7). Parental plants were grown until approximately 1 m in height and then decapitated. Sprouts from basal buds of the decapitated plants were rooted to give clonal lines. Pith tissues isolated from these lines were induced to form callus on Linsmaier and Skoog's medium (15) containing 2 $\mu$M indole-3-acetic acid and either 1.0 $\mu$M or 0.2 $\mu$M kinetin. Callus tissue was incubated at 20 C or 28 C either under fluorescent lights ($19.4 \times 10^3$ lux and a 12-hour photoperiod) or in continuous darkness.

*Bacterial cultures.*—Eighteen isolates of *P. solanacearum* (race 1) from widely diverse geographical locations were used (Table 1). Most isolates from Colombia (G2 through G11) were isolated from wilting tobacco plants in 1972; other isolates were from the

TABLE 1. Isolates of race 1 of *Pseudomonas solanacearum* used for comparative studies of pathogenicity and physiological characteristics

| Isolate no. | Location | Host | Isolated by |
|---|---|---|---|
| G2 | Santander, Colombia | Tobacco | G. A. Granada |
| G3 | Santander, Colombia | Tobacco | G. A. Granada |
| G4 | Santander, Colombia | Tobacco | G. A. Granada |
| G7 | Santander, Colombia | Tobacco | G. A. Granada |
| G9 | Santander, Colombia | Tobacco | G. A. Granada |
| G10 | Santander, Colombia | Tobacco | G. A. Granada |
| G11 | Santander, Colombia | Tobacco | G. A. Granada |
| K60 | Wake Co., N.C., U.S. | Tomato | A. Kelman |
| K74 | Worth Co., Georgia, U.S. | Tomato | A. Kelman |
| K105 | Quincy, Florida, U.S. | Tobacco | A. Kelman |
| S123 | Coto, Costa Rica | *Eupatorium odoratum* | L. Sequeira |
| S247 | Santander, Colombia | Tobacco | H. D. Thurston |
| S213 | Paraiso, Costa Rica | Potato | L. Sequeira |
| S221 | Nairobi, Kenya | Potato | R. Robinson |
| S222 | Coast, Kenya | Eggplant | D. C. Harris |
| S225 | Lupuna, Peru | Tomato | L. Sequeira |
| S236 | Nambour, Australia | Tomato | A. C. Hayward |
| S240 | Ingham, Australia | Tobacco | A. C. Hayward |

TABLE 2. Reaction of resistant and susceptible tobacco seedlings to single-site stem inoculation[a] with two isolates of *Pseudomonas solanacearum*

| Tobacco cultivar | Fungus isolate | Age[b] of seedlings (weeks) | Days after inoculation | | | | | Reaction |
|---|---|---|---|---|---|---|---|---|
| | | | 3 | 6 | 9 | 12 | 15 | |
| NC 95 | K60 | 1 | 2.1[c] | 3.3 | 3.7 | 3.8 | 4.0 | S[d] |
| | | 2 | 1.6 | 2.6 | 3.0 | 3.5 | 3.5 | S |
| | | 3 | 1.7 | 2.7 | 3.5 | 3.8 | 3.8 | S |
| | | 4 | 1.2 | 2.0 | 2.6 | 2.5 | 2.6 | R |
| | G11 | 1 | 1.4 | 2.0 | 2.7 | 3.4 | 3.7 | S |
| | | 2 | 1.9 | 2.5 | 3.4 | 3.5 | 3.7 | S |
| | | 3 | 1.8 | 2.7 | 3.8 | 3.6 | 4.6 | S |
| | | 4 | 1.3 | 1.6 | 2.3 | 2.6 | 2.9 | R |
| Bottom Special | K60 | 1 | 1.6 | 2.7 | 3.1 | 3.5 | 3.8 | S |
| | | 2 | 2.1 | 3.5 | 4.0 | 4.3 | 4.6 | S |
| | | 3 | 1.7 | 2.8 | 3.8 | 4.1 | 4.1 | S |
| | | 4 | 1.0 | 1.8 | 2.8 | 3.0 | 3.3 | S |
| | G11 | 1 | 2.0 | 3.4 | 3.9 | 4.4 | 4.6 | S |
| | | 2 | 2.0 | 2.6 | 4.0 | 4.5 | 4.5 | S |
| | | 3 | 1.7 | 3.5 | 4.5 | 4.8 | 5.0 | S |
| | | 4 | 1.2 | 1.9 | 2.8 | 3.1 | 3.2 | S |

[a]Plants inoculated with a bacterial suspension containing approximately $7.6 \times 10^8$ cells per ml at one location on the stem.
[b]Weeks after transplanting.
[c]Average disease indices of ten plants. Index ranged from: 1 = no symptoms, to 5 = complete wilting. (see text for details).
[d]S = susceptible; R = resistant.

collection of phytopathogenic bacteria maintained in the Department of Plant Pathology, University of Wisconsin, Madison. Stock cultures of each isolate were maintained in sterile distilled water (13).

Isolates were grown on tetrazolium agar medium (TZC) for 48 hours at 30-32 C (12). Wild-type colonies were selected on the basis of their fluidity, color, and morphology; water suspensions prepared from them were used for inoculation.

*Inoculation.*—Tobacco plants were inoculated 3 and 4 weeks after transplanting following the stem-puncture method of Winstead and Kelman (24). A drop of bacterial suspension containing $7.6 \times 10^8$ cells/ml was placed on the axil of the third fully expanded leaf from the top, and the stem was pierced by thrusting a hypodermic needle downward through the inoculum drop. Five to ten plants of each tobacco cultivar, or of other host plants, were inoculated with each isolate. Disease indices were recorded at 3-day intervals for 15 days on the following scale: 1 = no symptoms, 2 = wilting of the inoculated leaf, 3 = 1/3 of the leaves wilted, 4 = 2/3 of the leaves wilted, and 5 = all leaves wilted. Plants of varieties with final indices of 3.0 or below usually recovered and were considered resistant; those with indices above 3.0 usually died and were considered susceptible.

Tobacco callus tissues were inoculated when 4 weeks

TABLE 3. Reaction of resistant and susceptible tobacco seedlings to multiple site stem inoculation[a] with two isolates of *Pseudomonas solanacearum*

| Cultivar | Isolate | Days after inoculation 3 | 6 | 9 | 12 | 15 | Reaction |
|---|---|---|---|---|---|---|---|
| Coker 254 | K60 | 1.7[b] | 2.9 | 3.5 | 3.8 | 4.1 | S |
| | G11 | 1.6 | 2.4 | 3.3 | 3.6 | 4.0 | S |
| Coker 298 | K60 | 1.7 | 2.5 | 2.8 | 3.3 | 3.7 | S |
| | G11 | 1.8 | 1.9 | 2.2 | 2.3 | 2.8 | R |
| NC 17 | K60 | 1.8 | 2.2 | 2.8 | 2.9 | 3.0 | R |
| | G11 | 1.8 | 2.3 | 2.8 | 2.7 | 3.0 | R |
| NC 95 | K60 | 1.8 | 2.8 | 3.3 | 3.6 | 3.9 | S |
| | G11 | 1.9 | 2.7 | 3.3 | 3.6 | 4.1 | S |
| NC 1007 | K60 | 1.9 | 2.3 | 3.1 | 3.3 | 4.1 | S |
| | G11 | 1.9 | 2.6 | 3.2 | 3.4 | 4.1 | S |
| Cuba 12 (susceptible) | K60 | 2.0 | 2.4 | 3.6 | 4.0 | 4.8 | S |
| | G11 | 2.0 | 2.8 | 3.3 | 4.3 | 4.3 | S |

[a]Plants inoculated with a bacterial suspension containing $7.6 \times 10^8$ cells per ml at three locations on the stem, 4-5 weeks from transplanting.

[b]Average disease indices of ten plants

| | | $P = 0.05$ | $P = 0.01$ |
|---|---|---|---|
| LSD between | Cultivars | 0.393 | 0.518 |
| | Isolates | 0.238 | 0.313 |
| | Isolates × Cultivar | 0.533 | 0.701 |

old by placing 0.1 ml of bacterial suspension containing $2.3 \times 10^5$ or $9.2 \times 10^7$ cells inside a small ring of Tygon tubing affixed on top of the callus (7). Of the six pieces of tissue grown in each plate, five were inoculated as above and one received distilled water instead of inoculum.

*Bacterial populations.*—Populations of *P. solanacearum* in inocula were estimated by determining the absorbance at 600 nm of the bacterial suspension by means of a Bausch & Lomb Spectronic 20 colorimeter. The number of cells/ml was estimated by reference to a standard curve calculated from serial dilution on TZC of cell suspensions at various absorbance values.

To estimate bacterial populations in callus tissues, each piece of callus was weighed and then ground with a Waring Blendor in 200 ml of sterile distilled water for one minute. A logarithmic dilution series was prepared from this suspension, and aliquants were spread on TZC plates; typical colonies were counted after 48 hours incubation at 30 C.

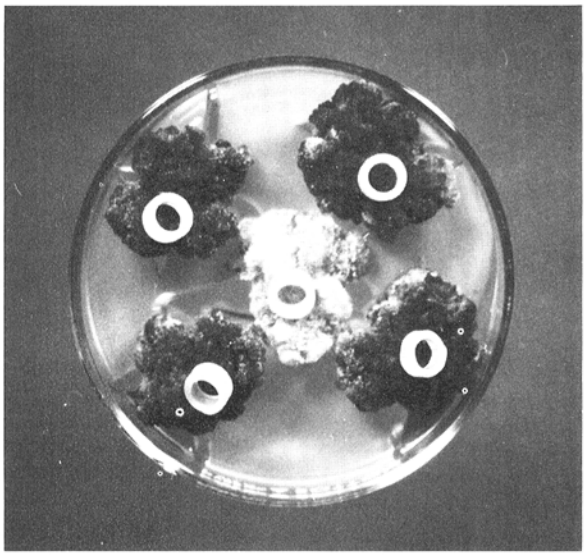

**Fig. 1.** Browning of tobacco (cultivar NC 95) callus tissues 4 days after inoculation with *Pseudomonas solanacearum* K60. Small Tygon tubing pieces were placed on top of the callus to maintain the inoculum in contact with the tissue. Callus in the center was not inoculated.

*Physiological tests.*—Media for carbohydrate oxidation studies were prepared as indicated by Hayward (6). The utilization of other carbon sources was tested by adding them (0.1%, w/v) to Husain and Kelman's (HK) basal medium (9). For growth rate studies, a medium (CPG) containing 1.0 g casamino acids, 10.0 g peptone, and 5.0 g glucose in 1,000 ml distilled water was used. These media were dispensed in either 25-ml Erlenmeyer flasks or test tubes and, after inoculation with approximately $1 \times 10^8$ cells, cultures were incubated at 30-32 C with or without shaking. The references for the specific methods used for these standard physiological tests are given in the results section.

The sensitivity of each isolate to antibiotics and sulfa drugs was tested with standard sensitivity disks (Difco Laboratories, Detroit, Mich.) following the technique recommended by the manufacturer. All experiments were repeated two or three times.

RESULTS.—*Pathogenicity.*—Preliminary pathogenicity tests of all isolates (Table 1) on the susceptible cultivar Cuba 12 indicated that the two most pathogenic isolates were G11 from Colombia and K60 from the

United States. Therefore, these isolates were selected to test the performance of resistant tobacco cultivars.

Plants of resistant NC 95 and of susceptible Bottom Special cultivars were inoculated at different stages of development with isolates G11 or K60 at $7.6 \times 10^8$ cells/ml and one stem puncture. Both cultivars were killed when very young plants were stem-inoculated, but by the fourth week after transplanting, NC 95 plants were highly resistant to both isolates. In contrast, the cultivar Bottom Special was highly susceptible, even at 4 weeks after transplanting (Table 2). The other resistant cultivars were similar to NC 95 in their response to a single stem inoculation at the 4-week stage.

When tested under extremely severe conditions; i.e., high inoculum level ($7.6 \times 10^8$ cells/ml) and multiple (3) stem punctures, only one of the five resistant cultivars (NC 17) had a disease index of less than 3.0 by 15 days after inoculation with either of the two isolates (Table 3). One cultivar (Coker 298) was resistant to one isolate (G11), but susceptible to the other (K60). Although there were significant ($P = 0.01$) differences in the reaction of cultivars to the bacterium, there were no significant differences between isolates or in the interaction cultivars × isolates (with the exception of Coker 298).

There were no significant differences in the rates of colonization of callus tissues from susceptible or resistant plants by either G11 or K60 isolates at the two inoculum concentrations used. Complete browning, presumably due to invasion of the tissue, was obtained by the third or fourth day after inoculation (Fig. 1). No browning of control tissues was observed during this period. Bacterial multiplication, as determined by population counts at 0, 1, 2, 3, and 4 days after inoculation of either loose (0.2 $\mu$M kinetin) or tight (1.0 $\mu$M kinetin) callus, was rapid during the first two days after inoculation, reaching a maximum after 3 days (approximately $10^{10}$ cells per g). Bacterial growth rates were very similar in tissues derived from either resistant or susceptible plants. When clonal 4-week-old plants, derived from the same plants used as a source of callus, were stem-inoculated with isolates K60 or G11, severe wilting was observed only in plants of the Bottom Special cultivar by 2 weeks after inoculation.

All Colombian isolates of *P. solanacearum*, as well as isolate K60, were highly pathogenic to tomato and eggplant inoculated at 15 days after transplanting with $7.6 \times 10^8$ cells/ml and one stem puncture. All isolates were nonpathogenic to *Musa balbisiana*. There were no significant differences in the pathogenicity of different isolates to these various hosts.

*Colony morphology.*—After 48 hours incubation on TZC at 30 C, colonies of all isolates of *P. solanacearum* were round-to-elliptical, entire, slightly raised or flat (isolates K60 and S123), or convex in the case of the Colombian isolates. Colonies of K60 and S123 were slightly larger than those of the Colombian isolates. Since all isolates had similar growth rates (doubling time approximately 100 minutes in CPG medium), differences in colony size were probably due to differences in amount of slime produced. All isolates reduced triphenyltetrazolium chloride; when grown on TZC medium, red pigmentation developed at the center of the colonies after 48 hours incubation at 30 C.

*Physiological characteristics.*—All isolates were tested for their ability to utilize 18 different carbohydrates. All

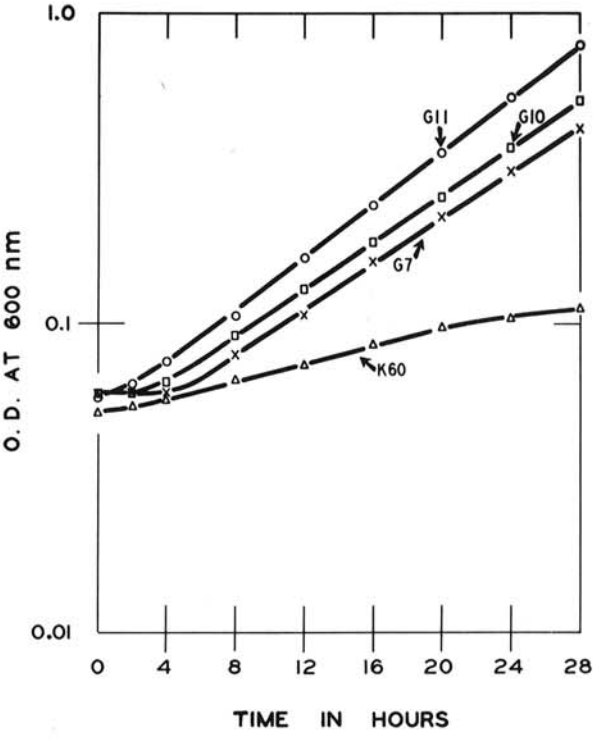


**Fig. 2.** Growth of *Pseudomonas solanacearum* isolates G7, G10, G11 (Colombian) and K60 (United States) in HK basal medium containing $NaNO_3$ (0.075%, w/v) as the sole N source at 32 C.

isolates grew and produced acid with D-glucose, glycerol, sucrose, trehalose, and D-fructose; none grew or produced acid with dulcitol, D-sorbitol, D-mannitol, cellobiose, lactose, maltose, L(−)rhamnose, D(+)galactose, D(−)ribose, D(+)arabinose, or raffinose. All isolates, except G7 and G11, grew and produced acid with inositol in the medium. Acid production, but no growth, was obtained with D(+)galactose.

All isolates utilized a wide variety of amino acids and other organic acids as sole sources of C including: L-tyrosine, DL-tryptophan, $\beta$-alanine, L-phenylalanine, L-serine, D-gluconate, tartrate, malonate, citrate, and acetate, but none utilized glycollate, lactate, caproate, L-arginine, ornithine, or glycine. However, K60 differed from the Colombian isolates in that it could not utilize tartrate, malonate, or L-phenylalanine as sole C sources.

Isolate K60 grew very slowly when either $NaNO_3$ or $KNO_3$ was added to HK basal medium at 0.075% (w/v), but all Colombian isolates grew rapidly with these sources of N (Fig. 2). This was a consistent, significant difference between K60 and the Colombian isolates.

Colombian isolates also differed from K60 in their tolerance to NaCl. With 1.6% NaCl in HK medium, all Colombian isolates had a longer lag phase than K60 (35 and 20 hours, respectively), and grew more slowly (55.6 and 12.7 hours doubling time, respectively). None of the isolates grew at salt concentrations of 2% or higher.

All isolates accumulated poly-$\beta$-hydroxybutyrate (6) and were positive for catalase (2), oxidase (22), lipase (21), tryosinase (3), and pitting of polypectate gel (8). All isolates were negative for 2-ketogluconate production

from gluconate (5), starch hydrolysis (22), and gas production with nitrate (23), and produced only slight liquefaction of gelatin after 2 weeks. All isolates produced an alkaline reaction when grown in litmus milk for 2 weeks at 30 C.

All isolates were very sensitive to kanamycin (5 μg/ml), aureomycin (5 μg/ml), streptomycin (2 μg/ml), and tetracycline (5 μg/ml), but resistant to bacitracin (10 μg/ml), chloromycetin (30 μg/ml), penicillin (10 μg/ml), and vancomycin (30 μg/ml). Although resistant reactions were obtained at low concentrations (50 μg/ml) of furadantin, elkosin, novobiocin, and sulfathiazole, slightly sensitive or sensitive reactions were obtained at high concentrations (300 μg/ml) of these chemotherapeutants.

All Colombian isolates, as well as K60, caused a slow, compatible response (16) when infiltrated at $10^8$ cells/ml into the intercellular spaces of leaves from resistant (NC 95) or susceptible (Cuba 12) tobacco plants.

DISCUSSION.—The main objective of this work was to determine whether available bacterial wilt-resistant tobacco cultivars would survive after stem inoculation with Colombian strains of *P. solanacearum*. Our results indicate that tobacco cultivars (with resistance originating from TI 448A) were fully resistant at 4 weeks after transplanting. Younger seedlings were susceptible, however. Ohashi and Kunisawa (18) reported earlier that resistance in Dixie Bright 101 plants was higher at 4 weeks after transplanting than in younger plants. Similarly, Winstead and Kelman (24) reported that the susceptibility of Marglobe tomato plants decreased as the age of the plants increased from 4 to 8 weeks.

With the stem inoculation procedure used, both inoculum concentration and the number of stem punctures affected the expression of resistance in tobacco to *P. solanacearum*. A combination of high inoculum concentration and more than one stem puncture per plant could overcome resistance. These results, of course, do not explain why resistant seedlings wilt when transplanted into infested soil in the field in Colombia. Resistance, however, can be overcome when roots are damaged by wounding, and by certain nematodes in soils heavily infested by *P. solanacearum* (10, 17). The severity of wilt symptoms on resistant tobacco plants is generally higher in the presence of *Meloidogyne incognita* and *P. solanacearum* than in the presence of the bacterium alone. Whether nematodes, or some other environmental factor interacted with *P. solanacearum* to reduce resistance in tobacco in Colombia is not known, however.

Colombian isolates of *P. solanacearum* from tobacco differed from a standard race 1 isolate from the United States, K60, only in relatively few physiological properties. They are as follows: (i) The Colombian isolates grew equally well with $NaNO_3$, $KNO_3$ or $(NH_4)_2SO_4$ in HK medium, whereas K60, as is typical of most *P. solanacearum* isolates (11), utilized ammonium salts much more effectively than nitrates as sources of N. (ii) K60 was much more tolerant to salt than the Colombian strains; concentrations of NaCl (1.6%) that inhibited growth of the Colombian isolates allowed substantial growth of K60. (iii) The Colombian isolates utilized malonate, tartrate, and L-phenylalanine as sole sources of C, whereas K60 did not grow under the same conditions.

Biochemically, all Colombian isolates belonged to biotype 1 in Hayward's (6) scheme; most tobacco isolates belong to this group. However, biotype 1 is a very heterogeneous group in which at least nine biochemical sub-biotypes have been proposed by Harris (4). It is clear that the biochemical differences between K60 and the Colombian isolates, as listed above, are within the variability expected in this group. It can be concluded, therefore, that Colombian isolates are not significantly different from K60 either in biochemical features or in pathogenicity.

Both Colombian (G11) and United States isolates (K60) of *P. solanacearum* multiplied rapidly in callus tissues from either resistant or susceptible tobacco plants. Modification in the amounts of cytokinin added to the medium, resulting in differences in tightness of the tissues, did not appear to alter the rate of colonization by the bacterium. Our results were different from those of Helgeson et al. (7), who found that modifications of the cytokinin regime of tobacco callus drastically changed colonization by *Phytophthora parasitica* var. *nicotianae*. Our results are similar to those of Kennedy et al. (14), who reported that the reaction of soybean callus tissue to inoculation with various bacteria was similar regardless of concentration of inoculum, species of bacteria, or genotype of the callus. Because bacteria remain in the intercellular spaces in the initial stages of infection, it might be expected that modification of the callus by different hormonal regimes would not have so direct an effect on bacteria as on a fungus that penetrates the host cells.

The results of the callus inoculation experiments provided additional evidence that the Colombian isolates of *P. solanacearum* are very similar to other race 1 isolates, such as K60. The total evidence, therefore, does not support the hypothesis that breakdown of resistance to bacterial wilt in tobacco under field conditions in Colombia is due to the presence of widely different, or more virulent strains of the bacterium. Although only a relatively small number of Colombian cultures isolated from susceptible tobacco cultivars was tested, these isolates appeared to be very uniform physiologically and constituted the predominant strain in the field. The results do not preclude the possibility that other, more highly pathogenic strains are present in small numbers in Colombian soils and become predominant only when resistant tobacco cultivars are planted.

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