SYNERGISM BETWEEN *RHIZOCTONIA SOLANI* AND *AGROBACTERIUM TUMEFACIENS* IN CAUSING CROWN GALL OF COTTON

Alois A. Bell
USDA, Agriculture Research Service
College Station, TX

Abstract

When cotton seedlings were inoculated sequentially with three cotton isolates of *Agrobacterium* carrying Ti plasmids (i.e., *A. tumefaciens*) followed 4-14 days later by *R. solani*, a high frequency of crown gall tumors developed at the soil line. No tumors developed when plants were inoculated with the *A. tumefaciens* isolates alone using a variety of wounding techniques. The synergistic interactions were observed with each of the 8 isolates of *R. solani* from cotton and each of the10 different cotton cultivars. *Macrophomina phaseoli*, *Diplodia gossypii* or *Phoma exigua* used with *A. tumefaciens* failed to induce galls on cotton. The results indicate that *R. solani* or its infections may produce unique metabolites that activate virulence genes in infectious plasmids of *A. tumefaciens* strains obtained from cotton roots.

Introduction

Cotton roots in the field normally are parasitized by species of *Agrobacterium* biovar I (Bell, 1999). In 1998, all but three of more than 500 plants from across the USA contained concentrations of this bacterium in excess of 0.1 million per gram of fresh root bark. Additionally, more than 90% of the plants contained concentrations greater than 1 million per gram. The *Agrobacterium* invades and moves systemically in the xylem and eventually invades most of the seed formed by the plant. It is not unusual to find 100% of a 100 seed sample infested by *Agrobacterium* biovar 1 (Bell et al., 1997). The bacterium invades the young radical during the first 24 hours of germination, and concentrations of 1 million or more are reached within two weeks and maintained through the life of the plant.

A detailed study of 86 isolates of *Agrobacterium* biovar I (two each from 43 different cottonseed lots grown in various parts of the USA) showed that most *Agrobacterium* isolates apparently contained Ri or Ti plasmids (Cui et al., 1997). Forty-seven isolates contained 200 Kb plasmids that were indistinguishable in size from the standard *A. tumifaciens* isolates B6 and C58. An additional 13 isolates showed PCR amplification of a 730 bp section of the V*ir C* gene which is specific for Ri and Ti plasmids. A section of the *Vir C* gene from isolate 34B was cloned and had 97% homology with the TIPVIRC plasmid pTiA6NC (*A. tumifaciens*). The observations indicate that functional plasmids are probably present in many isolates.

The plasmids in cotton isolates of *Agrobacterium* biovar I generally have not shown activity in various bioassays. None of 86 isolates caused tumors when introduced into stem wounds of cotton, tomato, fava bean, sunflower, tobacco, kalanchoe, or snap bean. Likewise, inoculation of cotton seedlings in pasteurized soils failed to give tumors, even though the bacterium readily colonized roots and populations at natural wound sites normally exceeded 10 million per gram. These observations indicate that the 200 Kb plasmids in cotton isolates either are not functional or require unique conditions to activate the V*ir* gene complex in the plasmid.

Soreshin lesions caused by *Rhizoctonia solani* on cotton hypocotyls in the field invariably contained concentrations of *Agrobacterium* biovar 1 species more than 1000 times greater than that of adjoining healthy hypocotyl tissues. Evidence that *R.* solani can uniquely activate *Vir* genes in certain *A. tumefaciens* isolates is presented here.

Materials and Methods

Plants were grown in 16 oz white Solo cups containing 450 g of a soil prepared by mixing a fine sand mined from the Brazos River Valley with a clay soil from the Texas A&M Plantation (3:1). The soil was amended with 10g of gypsum and 20g of dolomitic limestone per 1 kg soil to insure adequate Ca, Mg, and S nutrition. Both soil components were passed through a 5-mm screen before blending. This mix contained about 25% clay (pH 8.0 – 8.5) and has consistently given *Agrobacterium* populations of 5-10 million per gram of root at 24-30 ˚C. Plants were fertilized weekly with 50 ml of a solution of 3 g of Peter's 15-16-17 soluble fertilizer containing chelated minor elements in 1 liter of water purified by reverse osmosis (RO). The Solo cups were drilled to provide three 6-mm drainage holes, which were covered with a vinyl-coated fiberglass screen cloth before the soil was added. The soil in cups was pasteurized with aerated steam (160 ˚F) for 6-8 hours immediately before planting.

Isolates 1A, 14A and 34B of *Agrobacterium* biovar I were kept in sterile distilled water at room temperature or in 30% glycerol at -70˚C. Isolate 1A has a unique fatty acid profile, 14A is a natural ketolactose-deficient variant, and 34B has a characterized Ti plasmid that can be detected by PCR amplification. These markers allow the strains to be traced in biological experiments. Bacteria were spread on potato dextrose agar medium containing 0.8g calcium carbonate (powder) light (Mallinckrodt U.S.P. Food Grade, LOT 4052 KPTY). The bacteria from 24-hr-old cultures were harvested by adding sterile water and agitating with a glass-spreading rod. Suspensions were stirred and adjusted to an absorbance of ca. 0.5 at 600 nm with sterile water.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 1:175-177 (2000)
National Cotton Council, Memphis TN

Seed of the cultivars were obtained from commercial companies or breeders that have developed them. Cottonseed were washed for about 5 min in 70% acetone and then thoroughly with tap water. Germination towels were wet with the bacterial suspension. Seeds were then placed on towels which were rolled and incubated for 24 hours at 30 ˚C in closed containers. The 24-hr-old seedlings were selected for uniformity and one was transplanted into each cup. Twenty replications per treatment were used.

The fungal isolates were all obtained from diseased cotton stems or roots. The fungi were stored as dry cultures on sorghum seed. Red sorghum seed was mixed with an equal volume of water and sterilized (15 p.s.i. for 20 min) before being inoculated with mycelial plugs of *R. solani* or *Macrophomina phaseoli* or pycniospores of *Diplodia gossypina* or *Phoma exigua*. The pycniospores were produced on PDA cultures. Infested sorghum seed from 7-day-old cultures were used as inoculum. Seed were buried 1 cm deep and 2 cm to the side of emerged seedlings. Two or four seeds per cup were used in different experiments.

After cotton bolls were mature, the root ball was carefully removed and submerged in tap water where most of the soil was washed away. The root was then placed in a tea strainer and washed thoroughly under running tap water. Roots were blotted with absorbent towels and allowed to air-dry for ca. 20 min. before the fresh weights were determined. The whole root or a 5-g sample was then placed in a dry plastic bag over cracked ice until roots were analyzed for bacterial content (always within four hours).

The bacterial concentrations were determined from roots ground and diluted in sterile water. The root was placed in sterile water (19 ml/gm root) and homogenized with a 20-mm standard saw teeth polytron generator for 30 – 60 seconds until a uniform homogenate was obtained. Three sequential 1/10 dilutions were normally prepared from the homogenate and four drops (1/6 ml) of the final dilution (1/20,000) was spread on modified D-1 medium (15.0 g mannitol; 5.0 g NaNO3; 6.0 g LiCl; 0.002 g Ca(NO3)2 ! 4H20; 1.7 g K2HPO4; 0.3 g KH2PO4; 0.36 g MgSO4 ! 7H20; 0.1 g bromothymol blue; 15 g agar; 1 liter water --- sterilized 20 minutes at 15 p.s.i.). The D-1 plates were generally allowed to dry for 3 days before they were used. The inoculated plates were incubated for 72 hours at 28 ˚C before the colonies were counted. Agrobacterium biovar 1 appeared as butyrous, blue-gray, convex colonies and was easily distinguishable from other bacteria.

Results and Discussion

Typical concentrations of *Agrobacterium* biovar 1 in soreshin lesions are shown in Table 1. Some *Agrobacterium* cells apparently remained with the embryos even though they were surgically removed and washed with sterile water. In fact, lesions on plants started from embryos had higher and more uniform numbers of the bacterium in lesions.

Only sequential inoculations with seedborne *Agrobacterium* biovar I isolates containing 200 Kb plasmids and *R. solani* gave rise to crown gall tumor formation in cotton (Table 2). Neither *Agrobacterium* alone nor combined infections of *Agrobacterium* and *Macrophomina phaseoli*, *Diplodia gossypina* or *Phoma exigua* resulted in any galls.

Crown gall tumors were induced at the soil line by the sequential inoculation of *Agrobacterium* and *R. solani* on 50% or more of the plants of each of 10 cultivars (Table 3). Debilitated plants that failed to successfully fruit during the experiment had a higher frequency of tumors and in most cases larger galls. Galls were often coated by the pigmented hyphae of *R. solani* giving them a dark brown color. The cultivars Paymaster 1215 B/R and Paymaster 1220 B/R which have had two bacterial genes introduced for herbicide and insect resistance via *A. tumifaciens* also had the largest tumors in fruited plants and a high frequency of tumors. More studies are needed to determine whether the use of *A. tumifaciens* in cultivar development may cause these cultivars to become more vulnerable to the wild seedborne isolates of *Agrobacterium*, such as those found in cotton.

Eight different isolates of *R. solani* from separate plants in three different fields in Arkansas gave a high frequency of galls in sequential inoculations with *Agrobacterium* in the cultivar Stoneville 132 (Table 4). The results suggest that certain *R. solani* isolates produce compounds that activate virulence genes in the plasmids of the seedborne isolates of *A. tumifaciens* that infect crop roots. This may be a widespread phenomenon since similar seedborne isolates of *Agrobacterium* biovar I also are prevalent in other major field crops such as soybeans, peanuts, field beans and sorghum (Bell et al., 1997; Bell, 1999).

References

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Table 1. Concentrations of *Agrobacterium* biovar 1 in soreshin lesions.[a]

Treatment	Bacterial Concentration (M/gm)		
	No. of Lesions	Mean (sd)	Range
Controls:			
Direct Seeded	9	48 (69)	2—194
Surgical [b]	7	79 (70)	11—166
Inoculated:			
Strain 38A	1	46	—
Strain 41A	1	5	—
Strain 43B	2	57	9—106
Strain R1B	2	2	1—2

[a] 19-day-old plants inoculated with two sorghum seeds infested with *Rhizoctonia solani* strain AK-33 at 30˚C. Lesions harvested after 7 days.

[b] Embryos were removed surgically, washed 3 x with sterile water, and germinated for 48 hrs at 30˚C in towels wetted with sterile water before planting.

Table 2. Frequency (%) of cotton plants with crown gall tumors when inoculated sequentially with *Agrobacterium tumefaciens* and various root-infecting fungi at different temperatures. [a]

FungalSpecies	Temperature (˚C)			
	24	27	30	33
Rhizoctonia solani	29	17	29	43
Diplodia gossypina	0	0	0	0
Macrophomina phaseoli	0	0	0	0
Phoma exigua	0	0	0	0
Control (No Fungus)	0	0	0	0

[a] Cottonseed were germinated in towels wetted with a suspension (10^8/ml) of *Agrobacterium tumefaciens*, isolates 1A, 14A, and 34B. After 24 hrs at 30 ˚C, the seedlings were transplanted into a pasteurized sandy soil and after an additional five days, sorghum seeds infested with the fungi were placed 1 cm deep and 2 cm away from each plant (two per plant placed at opposite sides).

Table 3. Development of crown galls in fruited and debilitated cotton plants of 10 cultivars inoculated sequentially with *Agrobacterium tumefaciens* and *Rhizoctonia solani*, isolate AK33, at 32 ˚C. [a]

Cultivar	Frequency of Galls		Mean Gall Score [b]	
	Fruited Plants	Debilitated Plants [c]	Fruited Plants	Debilitated Plants
Stoneville 132	4/12	5/6	2.3	4.2
Stoneville 373	8/13	4/4	2.8	3.8
Paymaster 1215 B/R	9/12	6/6	4.2	3.3
Paymaster 1220 B/R	12/16	2/2	3.6	2.5
PR-80	11/15	1/1	2.4	4.0
Deltapine 20	9/10	7/7	2.0	2.3
Deltapine 50	10/14	5/6	2.5	3.6
Suregrow 125	8/13	4/5	2.3	2.0
Stoneville 474	8/11	8/8	2.3	3.8
Stoneville LA 887	4/10	7/7	1.8	3.7

[a] 20 plants per cultivar inoculated as in Table 1, except four sorghum seeds used per plant.

[b] Rated on scale of 1-5 with largest galls scored as 5.

[c] Debilitated plants failed to set fruit for 100 days, when most bolls on fruited plants were mature.

Table 4. Crown gall development, root weight, *Agrobacterium* concentrations in root, and seedcotton yield of 'Stoneville 132' cotton plants inoculated sequentially with *Agrobacterium tumefaciens* and different isolates of *Rhizoctonia solani.* [a]

R. solani isolate	Freq. of galled plants	Mean Gall score [b]	Root Wt (gm)	Bacterial Concn (M/gm)	Seedcotton yield (gm)
AK-5	9/14	3.4 (1.3)	15.5 (3.0)	12.8 (9.8)	4.6 (1.8)
AK-6	8/15	2.0 (0.9)	15.2 (3.3)	7.5 (4.1)	4.7 (1.0)
AK-7	6/14	2.5 (1.2)	14.3 (3.2)	5.8 (3.8)	5.0 (1.8)
AK-8	11/14	3.3 (1.4)	14.0 (3.3)	8.8 (7.9)	4.9 (2.2)
AK-9	13/15	3.8 (1.1)	16.1 (2.4)	6.2 (3.5)	5.8 (0.9)
AK-10	9/15	3.2 (1.2)	13.1 (2.7)	5.5 (5.3)	4.3 (3.0)
AK-11	10/15	2.4 (0.8)	15.3 (3.5)	5.9 (4.2)	3.7 (2.3)
AK-13	8/15	2.1 (1.1)	15.4 (4.3)	6.3 (4.7)	4.6 (1.3)
Control [c]	0/150	0	13.3 (2.4)	4.8 (3.7)	4.9 (1.5)

[a] 15 plants per *R. solani* isolate inoculated as in Table 2, except fungus applied ten days after bacteria under greenhouse temperatures (25-35 ˚C).

[b] Rated on scale of 1-5 with largest galls scored as 5. Standard deviation in parentheses.

[c] Plants inoculated only with *Agrobacterium.*