EVIDENCE THAT *AGROBACTERIUM* SPECIES CAUSE BRONZE WILT

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Abstract

Plants affected by bronze wilt in the field usually show extensive browning and necrosis of the feeder roots. The ability of *Agrobacterium* species to cause these symptoms was evaluated in these studies. Attempts to perform Koch's postulates as proof of pathogenicity were complicated by survival of *Agrobacterium* in pasteurized soils, ready dispersal of bacteria in environment chambers, and seed transmission of *Agrobacterium* species. Plants inoculated with isolate 25A of *Agrobacterium* biovar 1, compared to uninoculated controls, had marked increases in bacterial concentrations in roots and reduction of shoot growth at 36 ºC. However, the controls had mean bacterial concentrations greater than 30 M/gm, and necrosis occurred in all treatments. Under greenhouse conditions (38 º day and 30 º night), *Agrobacterium* biovar 1 isolates showed distinct differences in their ability to cause root necrosis. The ability to cause necrosis was correlated with the ability to colonize roots at 37 ºC. The patterns of specificity for necrosis induction by *Agrobacterium* were not changed by subsequent inoculations of 4-week-old plants with *Rhizoctonia solani*, although the frequency of plants with necrosis, when it occurred, was increased. When isolines of 'Acala 44' containing different bacterial blight resistance genes (*B* genes) were inoculated in the greenhouse with isolate 16B of *Agrobacterium* biovar 1, there were marked differences among isolines in frequency and intensity of root necrosis, which were reflected in reduced plant growth and seedcotton yield. Both B_2 and B_6 genes gave marked increases in necrosis, while B_4 and B_7 had no effect on necrosis. Progeny from 10 plants of 'Hartz 1215' were uniformly resistant to race 2 of *Xanthomonas campestris* pv. *malvacearum* indicating that this cultivar contains the B_2 gene. When seed from 'Hartz 1215' plants infected with isolate 34B of *Agrobacterium* biovar 1 were planted at 30 ºC days (14 hr) and 20 ºC nights (10 hr), some plants developed symptoms of bronze wilt. The severity of root necrosis, accompanied by stunting of the plant and chlorosis of terminal leaves, was correlated with concentrations of *Agrobacterium* biovar 1 in roots. Collectively, the observations indicate that certain *Agrobacterium* isolates can cause root necrosis especially in the presence of specific *B* genes and of a conductive microbial environment.

Introduction

The tap and secondary roots of plants affected by bronze wilt normally show numerous brown scars where feeder roots once emerged from the root. Core samples taken under the plants also reveal that most feeder roots are brown and necrotic. The necrotic scars on the live roots invariably contain high concentrations of *Agrobacterium*. Either or both *Agrobacterium* biovar 1 and *Agrobacterium* biovar 2 species are found, and various extracellular polysaccharide-deficient (-EPS) mutants accompany the wild type bacteria.

In this study, various *Agrobacterium* species and mutants were evaluated for their ability to cause root necrosis. Koch's postulates, the standard approach for proving that a microbe causes specific symptoms or disease, were attempted. When this failed, various isolates of *Agrobacterium* were established on 'Paymaster 1220 B/R' and used to obtain qualitative and quantitative differences in root necrosis under greenhouse conditions (cooling fans set at 38 ºC and heater set at 30 ºC). The contributions of temperature, other microbial infections, and bacterial blight resistance genes (*B* genes) to the frequency of necrosis were determined. Finally, seeds harvested from plants infected with isolate 34B, a necrosis-inducing strain, were planted at 30 ºC days (14 hr) and 20 ºC nights (10 hr), and relationships between development of root necrosis and *Agrobacterium* concentrations in roots were determined.

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Materials and Methods

Koch's Postulates

Plants were grown in environment chambers as described previously (Bell, 2000a,b). Soil mix (25% clay: 75% sand) was prepared as described by Bell (2000a), placed in solo cups, and wet to saturation. After 48 hr, the mix was pasteurized by heating with aerated steam at 165 ºF first for 6 hr and, 48 hours later, for 8 hr. Acid-delinted seed was treated with 70% acetone for 2 minutes followed by 3% hydrogen peroxide for 15 minutes and rinsed with sterile water. Seeds were planted directly in the pasteurized mix and 1ml of bacterial suspension (O. D. 0.5 at 600 nm) was applied directly over each seed for the inoculation treatment. At 5 wk after planting, plants were harvested, shoot, hypocotyl and root weights were determined, and bacterial concentrations in roots were determined as described by Bell (1999).

Induction of Root Necrosis by *Agrobacterium* Isolates

Bacterial stocks were maintained in 30 or 50% glycerol at –70 ºC or in sterile water at room temperature. Single colonies from diluted stocks spread on tryptic soy agar (TSA) were suspended in 2 ml of sterile water and 4 drops were spread on potato dextrose agar (PDA) plates containing 800 mg of fine $CaCO_3$ per liter. After 24 hr of incubation bacteria from a single plate were suspended in 1 L of water and 50 ml was applied to each of 20 solo cups. Similar concentrations were used to wet germination towels. Ninety isolates of *Agrobacterium* biovar 1, including the commonly studied isolates B6, C58, and A281, and 1 isolate each of *Pseudomonas aeruginosa* and *Burkholderia cepacia* were used along with 4 uninoculated groups of cups. Each group of 20 cups was isolated from all others on a greenhouse bench by splashguards. Cooling fans were set to come on at 38 ºC and heaters came on at 30 ºC. Seeds were germinated for 48 hr at 30 ºC in towels wet with bacterial suspension and transplanted to infested soil. Plants were fertilized weekly with Peters 15-5-25 soluble fertilizer (3 gm/liter; 50 ml/cup).

At 28 days after planting, 5 cups from each treatment were removed to a separate greenhouse and inoculated with *Rhizoctonia solani*, isolate AK-33 as described previously (Bell, 2000c).

At the time of initial flower, root balls were removed and scored for root necrosis (0=none; 3=most severe). At 3 wk after initial boll opening, all fruit were harvested and dried (40 ºC for 3 days), root necrosis was scored, plant parts were weighed, and bacterial concentrations in roots were determined.

Effects of Temperature on Isolates Differing in Necrosis Induction

Four isolates of *Agrobacterium* biovar 1 that induced the greatest frequency of necrosis in the greenhouse and 4 isolates that failed to induce necrosis, even with *R. solani*, were used to inoculate 'Paymaster 1220 B/R' plants in controlled environment chambers at continuous 33, 35 or 37 ºC. After 5 and 10 weeks, plant growth and bacterial concentrations in roots were determined.

Effects of Bacterial Blight Resistance Genes (*B* genes) on Necrosis

Isolines of 'Acala 44' that contain different *B* genes but otherwise are very similar were obtained from Dr. Margaret Essenberg at Oklahoma State University. These lines were inoculated with isolate 16B of *Agrobacterium*

biovar 1, grown in the greenhouse, and evaluated as described previously for the bacterial isolates.

Evaluation of Blight Resistance

Plants were grown in clay-sand mix in solo cups at 30 ºC days (14 hr) and 20 ºC nights (10 hr) with 90% relative humidity. *Xanthomonas malvacearum* race 1 and 2 were grown on PCDA (Bell, 1993) at 30 ºC. Single colonies were selected from suspensions spread on TSA and checked to ensure proper reactions on the host differentials, 'Acala 44', 'Stoneville 20', and 'Mebane B-1'. Cotyledons of 2-week-old plants were scratch-inoculated with bacterial suspension prepared from PCDA, or the 4th leaf of plants with 6 true leaves was infused with bacterial suspension using a hypodermic syringe without a needle. After 5-7 days, inoculation sites were scored as susceptible (water soaking visible) or resistant (only tan to brown necrosis of inoculated areas).

Correlation of Necrosis and *Agrobacterium* Concentrations in Roots at 30 ºC – 20 ºC

During evaluations of bacterial blight resistance, 1 'Mebane B-1' and 3 'Hartz 1215' plants began showing severe stunting and chlorosis of terminal leaves after about 4 weeks. After another 2 weeks, surrounding plants began to show the same symptoms. Diseased and healthy plants from these cultivars were scored for root necrosis, root weight, and *Agrobacterium* concentrations at 8 weeks.

Results and Discussion

Koch's Postulates

As in previous studies (Bell, 1999; 2000b), it was not possible to perform a strict Koch's postulates experiment because most uninoculated controls were infected with *Agrobacterium* species and supported populations in excess of 1 million (M) colony forming units (cfu) per gram by 5 weeks after planting. Typical populations and variation for *Agrobacterium* biovar 1 in controls are shown in Figure 1 for 'Paymaster 1220 B/R'. The plant roots in which no biovar 1 was found contained in excess of 100 M cfu/gm of *Agrobacterium* biovar 2 which also occurred in three other plants. Thus, none of the controls were free *Agrobacterium* infections. In spite of strict pasteurization of soil mix and containment of control plants in a clean environment chamber separate from inoculated plants, 52 of the 53 control plants contained *Agrobacterium* concentrations in excess of 0.1 M cfu/gm by 5 wk after planting.

Plants inoculated with the aggressive 25A strain of biovar 1 contained higher and more uniform concentrations of bacteria in roots than did uninoculated control plants (Figure 1; Table 1). Bacterial concentrations were higher in the *Gossypium barbadense* cultivar 'Sakel' than in the *G. hirsutum* cultivars 'Paymaster 1220 B/R' and 'Stoneville 373', and were much higher with 27-15-12 fertilizer than with 15-5-25 fertilizer. Accordingly inhibition of shoot growth was greatest in inoculated plants, in the variety 'Sakel', and with 27-15-12 fertilizer. All plants under all conditions developed moderate to severe root necrosis by 5 wk after planting.

To confirm seed transmission and movement of *Agrobacterium* isolates in chambers, the experiment shown in Figure 2 and 3 was performed. Even though no wild type biovar 1 isolate was used as inoculum, roots in many pots contained wild type biovar 1 by 8 wk after planting. The ketolactose-deficient isolate 16B has never yielded a wild type revertant in more than 3 years of maintenance in the laboratory. Thus, seed transmission of the wild type is indicated. At 8 weeks, the variant 16B strain occurred in many pots apart from those inoculated. Some of this movement appeared to be due to water splash to adjoining cups, but movement by air or insects also is indicated. Similar results were obtained in three other chambers using other biovar 2 isolates. Any successful Koch's Postulate experiment must completely prevent seed transmission and may require disinfection of environment units and rigid insect control.

Specificity of Necrosis Induction by *Agrobacterium* Isolates

By 4 wk after planting, roots of all uninoculated control plants, plants inoculated with the laboratory strains of biovar 1 (B6, C58, and A281), and plants inoculated with *Pseudomonas aeruginosa* or *Burkholderia cepacia* also contained typical cotton isolates of biovar 1 and/or biovar 2, usually in excess of 1 M cfu/gm of root. The B6, C58, and A281 isolates could no longer be found and *P. aeruginosa* and *B. cepacia* usually occurred at populations less than 1 M cfu/gm which was considerably less than that of the invading *Agrobacterium* strains. Therefore, comparisons were made only among the 87 biovar 1 isolates from cotton. These included 14 marked isolates (7 deficient for ketolactose production, 5 that did make EPS on lactose only, 1 that did not make EPS on mannitol only, and 1 that overproduced EPS. Each of these still dominated at 4 wk after planting and was monitored as a measure of strain purity throughout the experiment.

Root necrosis on 5 to 75% of the 20 plants occurred with 58 of the 87 cotton isolates of biovar 1. The frequency of isolates causing different percentages of necrosis is shown in Figure 4. The specificity of necrosis was mostly unchanged by the additional infection with *R. solani* at 4 weeks after planting (Table 3). The 12 *Agrobacterium* strains that gave necrosis with but not without *R. solani* also had much higher concentrations of the bacteria in *R. solani*-infested roots. Thus, the necrosis still may have resulted from the *Agrobacterium.*

Roots of bronze wilt affected plants in the field often are infested with *R. solani* as well as *Agrobacterium*. The effects of root necrosis in the presence of both microbes are shown in Table 4. The first comparison (plants) is made between the 2 or 3 plants that had necrosis and the 2 or 3 that did not for each of 25 different biovar 1 isolates. This is a measure of the impact of susceptibility genes in the plant on plant damage. The second and third comparisons were made for all 5 plants between *Agrobacterium* isolates that caused 4 or 5 (or 2 or 3) plants to have necrotic roots compared to similar isolates that caused no necrosis. This is a measure of the impact of necrosis-inducing genes in the *Agrobacterium* isolate. In either comparison, the greatest impact of root necrosis is on fruit production due to delayed and diminished fruit set.

The 4 isolates that caused the greatest percentages of root necrosis in the greenhouse experiment (2A, 25A, 27A, and 38A) were compared with four similar wild type isolates that did not cause necrosis (2B, 15B, 37A, and 41A). Comparisons of plant damage and bacterial concentrations in roots were made at continuous 33, 35, and 37 ºC. The only consistent and dramatic difference between the groups was in their ability to colonize roots at 37 ºC (Figure 5). In spite of this difference, root necrosis occurred with all isolates at constant 37 ºC in the environment chambers. However, under the diurnal changes of temperature in the greenhouse the differences in growth at 37 ºC might be a contributing factor to the differences in necrosis. Concentrations of all strains were lower under the greenhouse conditions, but those of necrosis-inducing strains were consistently the highest.

Effects of *B* Genes on Necrosis

In the 1960s, Dr. Brinkerhoff at Oklahoma State University began transferring various bacterial blight resistance genes (*B* genes) into the 'Acala 44' background. In recent years this program has been continued by Dr. Essenberg at the same institution. These isolines are essentially identical except for the *B* genes. Different isolines contain the individual B_2, B_4, B_{5a}, B_6, B_7, and B_{In} genes. The lines '101-102B' and 'S295' which contain B_2 combined with B_3 and B_{12}, respectively, were also included in the study. The lines inoculated with the *Agrobacterium* biovar 1 isolate 16B showed profound differences in root necrosis and root damage (Figure 6). Severe necrosis and root damage also resulted in decreased seedcotton yields (Figure 7). The B_2 and B_6 genes resulted in consistent severe necrosis

in all plants, while genes B_5 , B_{In} and B_{12} were associated with intermediate and more variable necrosis. Plants with B_4 and B_7 genes had slighter less necrosis than the 'Acala 44E' control which lacked *B* genes.

The results with B_7 are at variance with those obtained using isolate 34B and 'Stoneville 20' as the source of B_7 (Bell, 2000a). This might indicate that different races of *Agrobacterium* exist. Alternatively, the gene identified as B_7 in the 'Acala 44' isolines may not be the same as the B_7 in 'Stoneville 20'. Essenberg (personal communication) has found that the B_7 in 'Acala 44' is a dominant gene, whereas B_7 in 'Stoneville 20' is usually considered to be recessive (Brinkerhoff, 1970).

The specificity of necrosis induction with different *B* genes was not related to bacterial concentrations in 6-week-old plants (Figure 8). Thus, some sort of specific recognition, or differential response to high temperature, is indicated.

Five progeny from each of 10 'Hartz 1215' plants previously selected for uniform susceptibility to *Agrobacterium* isolate 34B, were tested for resistance to race 1 and 2 of *X.c.m*. All plants were uniformly resistant to both races (Table 5). This indicates that the B_2 gene probably occurs in a homozygous condition in this cultivar. The 'Tamcot SP37' parent used in the development of 'Hartz 1215' contained the B_2, B_3, B_7 gene combination. Thus the B_2 gene, at least, would need to be present to give resistance to race 2 of *X.c.m*.

Root Necrosis without Stress

In all of the previous experiments herein and published (Bell, 1999; 2000a; 2000b), high temperature or nutritional stresses were used in addition to *Agrobacterium* inoculations to trigger root necrosis. When seed were harvested from 'Hartz 1215' or 'Mebane B-1' plants inoculated with the necrosis inducing isolate 34B and were grown at 30 º days (14 hr) and 20 º nights (10 hr) with complete fertilizer (15-16-17 plus minor elements), a few plants developed severe root necrosis which was accompanied by loss in root mass, stunting, and severe chlorosis of terminal leaves. These symptoms soon spread to immediate adjoining plants and later to plants beyond.

Plants at various distances from one infection site were rated for severity of root necrosis which was related to *Agrobacterium* biovar 1 concentrations and root weight (Table 6). Foliar symptoms generally agreed with root necrosis scores, although the latter slightly preceded foliar symptoms. A very strong correlation was observed between *Agrobacterium* concentrations, root necrosis, and plant damage under these conditions. The *Agrobacterium* isolates obtained from these plants were indistinguishable from 34B in various biological tests, indicating that infections originated from infested seed.

No fungi other than *Penicillium* species were found on the roots. However, two other bacteria also occurred at concentrations in excess of 10 M/gm. Concentrations of these bacteria did not correlate with root necrosis. Their role, if any, in root necrosis is being evaluated.

Conclusions

The reported experiments further indicate that infections of cotton roots by *Agrobacterium* species may contribute to root necrosis and subsequently to bronze wilt symptoms and yield losses. Unequivocal proof that *Agrobacterium* causes bronze wilt has not been possible because of the difficulty of performing Koch's postulates. The possibilities that certain *B* genes may enhance sensitivity of cotton plants to high temperatures and other stresses alone should be recognized. Likewise, a different bacterial pathogen might allow greater *Agrobacterium* populations by providing nutrients or suppressing competitors. Under field conditions, *Agrobacterium* may be only one of a complex of organisms that contribute to bronzing and wilt symptoms.

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Table 1. Effects of cultivar, fertilizer, and inoculation on *Agrobacterium* biovar 1 concentrations in roots at 5 weeks after planting[a].

	15 - 5 - 25		27 - 15 - 12	
Cultivar	**CK**	**Inoc[b]**	**CK**	**Inoc[b]**
	(M cfu / gm root)[c]			
Sakel	35	141	251	3398
PM 1220 B/R	56	150	48	2595
St 373	30	146	144	2563

[a]Plants were incubated 1 wk at 30 ºC followed by 4 wk at 36 ºC.
[b]Inoculated with isolate 25A.
[c]Data are means of 8-10 replications.

Table 2. Effects of cultivar, fertilizer, and inoculation on shoot weight at 5 weeks after planting[a].

	15 - 5 - 25		27 - 15 - 12	
Cultivar	**CK**	**Inoc[b]**	**CK**	**Inoc[b]**
	(grams / plant)[c]			
Sakel	1.32	0.92	0.34	0.33
PM 1220 B/R	2.62	2.04	1.15	0.96
St 373	2.24	2.26	1.97	1.73

[a]Plants were incubated 1 wk at 30 ºC followed by 4 wk at 36 ºC.
[b]Inoculated with isolate 25A.
[c]Data are means of 8-10 replications.

Table 3. Effects of *Rhizoctonia solani* (*R. s.*) on necrosis specificity.*

Result	No. of Isolates
No necrosis (*A. t.* only or with *R. s.*)	29
Necrosis (*A. t.* only or with *R. s.*)	33
Necrosis (with but not without *R. s.*)	12
Necrosis (without but not with *R. s.*)	13

*5 plants with and 15 without *R .s.* were observed.

Table 4. Effects of root necrosis when plants are inoculated with both *Agrobacterium* biovar 1 and *Rhizoctonia*.

Comparison	No. of Comparisons	Fruit Wt	Root Wt	Days to Fruit Set
Plants (Nec/Not)	25	-39%	-5%	+6 days
Strains (Nec(4,5)/Not)	7	-55%	-12%	+11 days
Strains (Nec(2,3)/Not)	8	-27%	-10%	+3 days

Table 5. Reaction of cultivars to race-1 and race-2 of *Xanthomonas campestris* pv. *malvacearum* with 30 ºC days (14 hr) and 20 ºC nights (10 hr).

	Reaction to Races*	
Cultivar	R-1	R-2
Acala 44 (CK)	S	S
Stoneville 20 (B_7)	R	S
Mebane B-1 (B_2)	R	R
Hartz 1215	R	R

*Reactions were consistent for 5 progeny from each of 10 parent plants.

Table 6. Relationships of necrosis, bacterial concentration, and root weight in 'Hartz 1215' plants at 30/20 ºC.

Plant No.	Necrosis Score	*Agrobacterium* Concn. (M / gm)	Root Weight (gm)
1	0	16.0	11.5
2	0	2.4	10.5
3	0	0.4	10.6
4	0	5.8	8.0
5	1	2.5	10.9
6	1	4.9	7.8
7	2	68.6	9.5
8	3	197.3	9.6
9	3	77.8	8.3
10	4	140.6	6.7
11	5	227.0	5.9
12	5	576.0	3.3

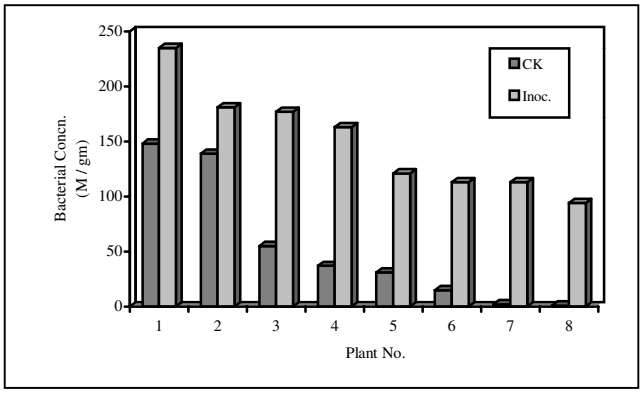

Figure 1. Effects of inoculation with isolate 25A on *Agrobacterium* biovar 1 concentrations in root of 'Paymaster 1220 B/R' treated with 15-5-25 fertilizer.

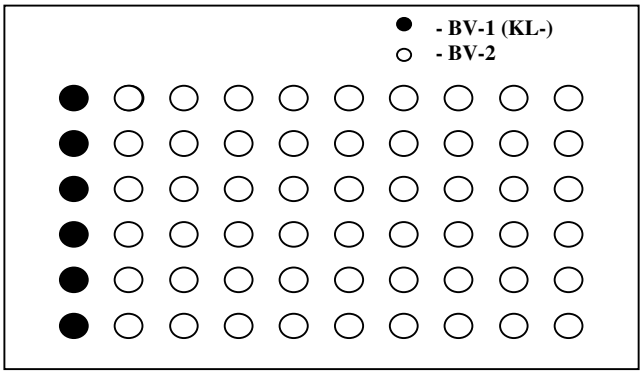

Figure 2. Inoculation pattern and distribution of *Agrobacterium* biovars and variants in the environment chambers. Both soil and germinating seed were inoculated, and different biovar 2 (BV-2) isolates were used in each row of pots. BV-1 is the ketolactose-deficient isolate 16B.

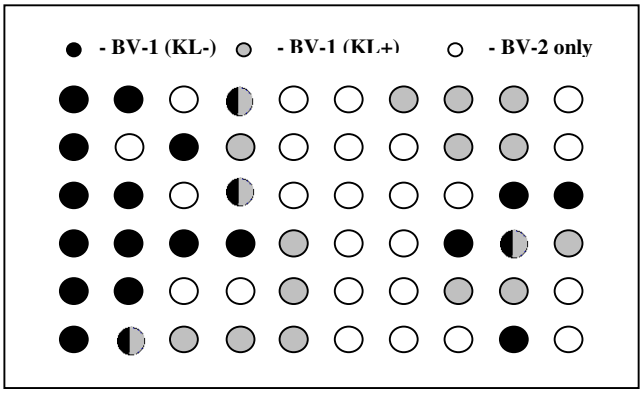

Figure 3. Distribution pattern of *Agrobacterium* biovars and variants in environment chambers. Conditions were same as in Fig. 2. Plants were incubated 4 wk at 33 ºC followed by 4 wk at 36 ºC. BV-1 (KL+) are wild type strains of biovar 1 that apparently originated from seed.

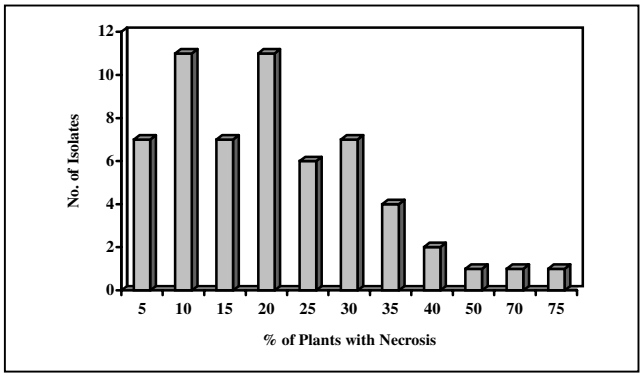

Figure 4. Number of isolates of Agrobacterium biovar 1 that induced different percentages of cotton root necrosis.

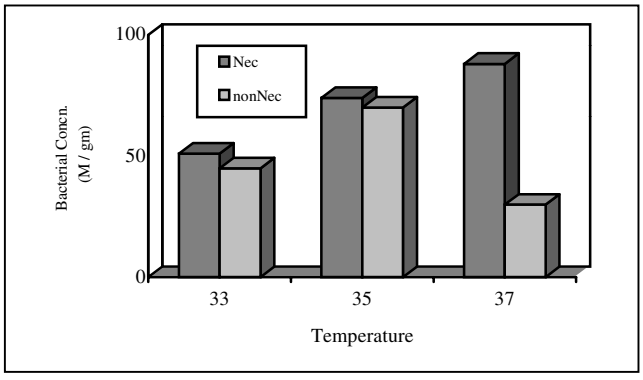

Figure 5. Comparison of bacterial concentrations in 'Paymaster 1220 B/R' plants infected with necrogenic and non-necrogenic isolates at 33, 35, and 37 ºC. Data are means of 4 isolates, 6 replications per isolate for each group.

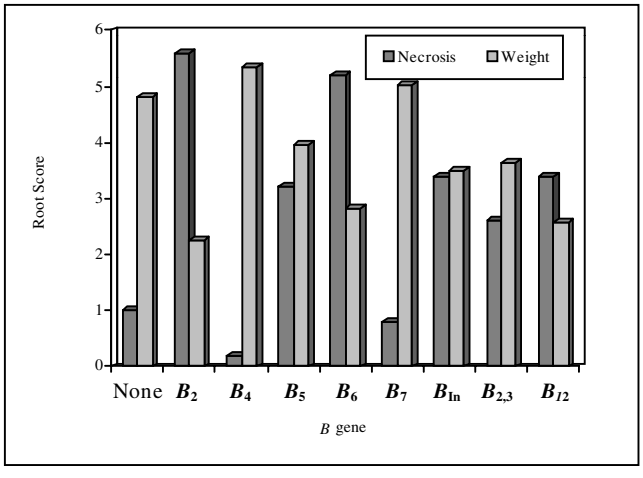

Figure 6. Effects of B genes of root necrosis and weight of plants inoculated with Agrobacterium

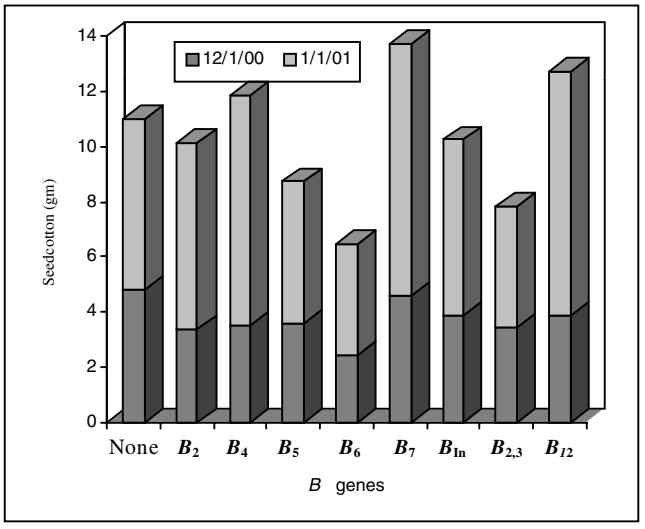

Figure 7. Effects of *B* genes on seedcotton yield of plants inoculated with *Agrobacterium* biovar 1, isolate 16B. Conditions were the same as Fig. 6.

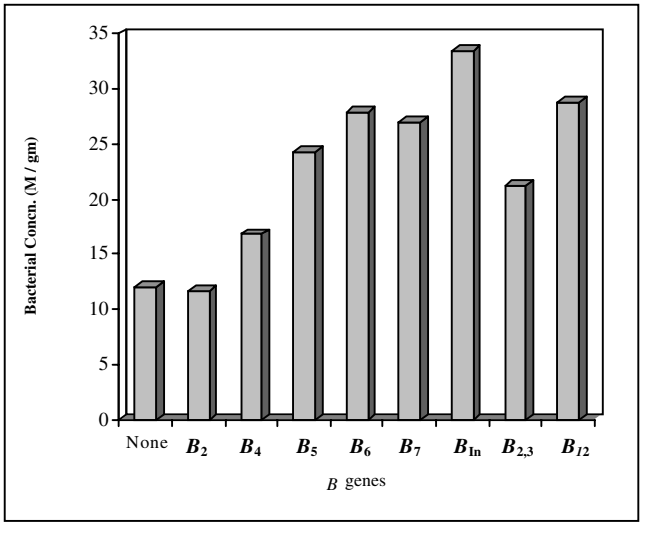

Figure 8. Effects of *B* genes on bacterial concentrations of 6-week-old plants inoculated with *Agrobacterium* biovar 1, isolate 16B.