MECHANISMS OF DISEASE RESISTANCE IN *GOSSYPIUM* SPECIES AND VARIATION IN *VERTICILLIUM DAHLIAE*

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Abstract

Studies on Verticillium wilt of cotton have concentrated on host resistance to the pathogen and on genetic variation and mechanisms of virulence in *Verticillium dahliae*. The resistance of cotton to *V. dahliae* depends on a number of anatomical and chemical characteristics that occur both constitutively and as active defense responses. Terpenoid phytoalexins and condensed tannins appear to be especially important in defense reactions. Resistance in cultivars correlates with the concentration of tannin in leaves, the rate of phytoalexin synthesis in xylem vessels in response to infection, and the toxicity of the phytoalexins and tannins to *V. dahliae*. Enzymes that appear to have critical roles in regulating terpenoid and tannin synthesis have been characterized, and partial genes coding for these enzymes have been cloned. Strategies for manipulation of these genes and for introduction of foreign genes from other malvaceous plants to improve cotton resistance to wilt are being developed. Vegetative compatibility (V-C) tests and isozyme analyses have been used to show that there are four genetically isolated populations (V-C groups) within the species *V. dahliae*, and that each V-C group has at least two subgroups. The V-C groups and subgroups vary in geographical occurrence, virulence to cotton and other crops, and sensitivity to fungicides. The severe defoliating form of Verticillium wilt found in the southwestern USA and in Peru is caused by isolates in the V-C 1A subgroup, whereas wilt in Europe and Asia is caused by isolates in the V-C 2 group. Wilt in Australia is incited by isolates in the V-C 4B subgroup. Isolates in the V-C 1A group show unique resistance to the antibiotic sanguinarine and have the ability to induce large accumulations of ammonium ions in cotton leaf tissues. Identifying the specific V-C groups responsible for wilts in a given field and geographical area should allow better recommendations for disease control measures.

Verticillium dahliae is the most important pathogen of cotton, causing worldwide losses of about 1.5 million bales from potential cotton production (Bell, 1992). These losses persist in spite of intense efforts to control the disease. Considerable progress has been made in improving resistance or tolerance to the pathogen, but even the most resistant cultivars can become severely diseased by defoliating strains of the fungus if mean soil temperatures fall below 25˚C (Bell and Presley, 1969). Consequently, an integrated management system, employing all possible control techniques, is necessary to control the disease in many states and countries (El-Zik, 1985).

Efforts to control Verticillium wilt of cotton have been frustrated by the absence of high levels of resistance in *Gossypium* species and by the spread of a highly virulent strain of the fungus, often referred to as the defoliating pathotype. This review summarizes current knowledge regarding mechanisms of resistance to wilts in *Gossypium* species and regarding genetic variation in *V. dahliae*. Future research needs and approaches to improving resistance of cotton to wilts are also discussed.

Nature of Resistance

The resistance of cotton to *V. dahliae* depends on both anatomical and chemical

characters that occur constitutively and as active defense responses. Both the spatial and chronological occurrences of these characters are important for their effectiveness. Defense mechanisms are discussed under the following topics: active defense, toxicity and mode of action of phytoalexins, constitutive defense, and molecular biology of defense.

Active defense

When xylem vessels of cotton roots and stems become infected with *V. dahliae*, the xylem tissues respond by synthesizing both antibiotic terpenoids that behave as phytoalexins and antibiotic flavanoids that behave as tannins (Bell *et al.*, 1992; Bell *et al.*, 1993a). The major terpenoid phytoalexins formed in xylem of Upland cotton (*Gossypium hirsutum* L.) are the terpenoid aldehyde hemigossypol (HG) and its naphthofuran precursor desoxyhemigossypol (dHG) (Fig. 1). In resistant cultivars of *Gossypium barbadense,* HG and dHG are accompanied by similar or larger percentages of their methyl ethers MHG and dMHG, respectively (Fig. 1). The phytoalexins dHG and HG are intermediates in the biosynthesis of gossypol, which occurs in lysigenous glands in seeds and in the bark of roots and stems of healthy cotton plants in concentrations that are toxic to many herbivores (Bell and Stipanovic, 1977; Bell *et al.*, 1987). However, gossypol and its methyl ethers occur only in low concentrations in stele tissue, probably due to low activity of a peroxidase that catalyzes the dimerization of HG to gossypol (Veech *et al.*, 1976).

The terpenoid phytoalexins are synthesized mostly in the paravascular parenchyma cells that adjoin the vessels (Mace *et al.*, 1976, 1989; Mace, 1978) and are secreted into the infected xylem vessels as part of an osmiophilic material that accumulates, forms aggregates, and plugs the vessels (Mueller and Morgham, 1993). Fungal structures in these vessels become coated with the phytoalexins. About 50% of the phytoalexin in infected stems can be rapidly eluted from xylem vessels with ethanol or acetone (Bell, 1969), indicating that most of the phytoalexin is exuded into the infected vessels.

The flavanols synthesized in response to infection of the stele tissue are primarily heterogenous condensed proanthocyanidins (Fig. 2) that yield both cyanidin and delphinidin during acid hydrolysis in butanol. The condensed proanthocyanidins are always accompanied by smaller concentrations of catechins and gallocatechins (Fig. 2), which apparently are spurious monomeric flavanol products from the condensed proanthocyanidin biosynthetic pathway. The complete group of flavanols, often referred to as condensed tannins, have antibiotic and enzyme denaturing activities at the concentrations found in cotton tissues (Bell *et al.*, 1992). The oligomers generally have the greatest biological activity followed by the polymers and the monomers. In the remainder of this paper, I will refer to the flavanols simply as tannins.

Within tissues, tannins are synthesized only by a variable percentage of dedicated cells, so that certain cells have very high tannin concentrations while adjoining cells are completely free of tannin. The tannins first appear in small vacuoles within the dedicated cells. Later the vacuoles coalesce until most of the cell is filled with one or a few tannin containing vacuoles (Mueller and Beckman, 1978). Increases in tannin concentrations in tissues are the consequence of a greater percentage of cells being dedicated to tannin synthesis. Unlike the terpenoid phytoalexins, the tannins are not secreted but rather remain in cell vacuoles until the cell is ruptured or degenerates to release the tannins from the vacuoles. The enzyme-denaturing activity of catechin is enhanced by mixing it with peroxidase (Hunter, 1974), and similar enhancement of activity of oligomeric tannin by peroxidase probably occurs as well. Most of the tannin synthesized in response to infection of cotton stems by *V. dahliae* is located in the xylem ray cells (Mace *et al.*, 1978) and less than 5% of the tannin in stele can be eluted from xylem vessels (Bell and Stipanovic, 1978). This suggests that tannin contributes to resistance by confining the fungus to vessels rather than by killing it within

vessels.

The importance of the rate of active defense responses is indicated by studies of cultivar resistance (Bell, 1969; Bell and Stipanovic, 1978) and of the effects of temperature on disease severity (Bell and Presley, 1969). The cultivar `Seabrook Sea Island 12B2' (`SBSI') of *G. barbadense*, which is more resistant to Verticillium and Fusarium wilt than any other cultivar known (Wilhelm *et al.*, 1974), begins rapid synthesis of both terpenoid phytoalexins and tannins in infected stele tissues 24 to 48 h sooner than either susceptible or tolerant cultivars of *G. hirsutum* (Figs. 3, 4). As a consequence, the fungus is more readily contained in infected vessels before it can penetrate adjoining uninfected vessels and form new conidia to continue the systemic invasion of the xylem.

The quick response in `SBSI' apparently is due to its ability to respond to fungal conidia even before they germinate and begin growth. This cultivar responds strongly to heat-killed conidia introduced into xylem vessels, whereas cultivars of *G. hirsutum* show little or no active defense against heat-killed cells (Fig. 5; Bell and Stipanovic, 1978). Joost (1993) found evidence that `SBSI' has relatively high constitutive levels of beta-1,3-glucanase which might release elicitors from conidial walls. Glycoprotein elicitors that apparently originate from cell walls also have been purified from *V. dahliae* (Heinstein, 1980). Thus, `SBSI' may have greater ability to hydrolyze elicitors from fungal cell walls.

The resistance of cotton to Verticillium wilt decreases progressively as temperatures decrease from 30 to 22˚C. Bell and Presley (1969) showed that this change was probably due to the differential effects of temperature on secondary colonization of the pathogen and on active defense responses (Fig. 6). When the rate of terpenoid phytoalexin synthesis (a measure of active defense) is divided by the rate of conidial production (a measure of secondary colonization) and plotted as a function of temperature, a curve predicting the rapid decrease of resistance with declining temperatures is obtained (Fig. 7; Bell and Presley, 1969). Thus, the effects of temperature on disease resistance support the theory that the speed of active defense is critically important in resistance to Verticillium wilt.

Toxicity and mode of action of phytoalexins

More than 50% of the phytoalexins in `SBSI' and other cultivars of *G. barbadense* occur as the methyl ethers dMHG and MHG, whereas less than 10% of the phytoalexins in Upland cotton are methylated. Consequently, a series of experiments were designed to determine the effects of structural variations on the toxicity and efficacy of terpenoid phytoalexins in cotton and related plants. These studies included five areas: 1) surveys of structural variations in antibiotic terpenoids of *Gossypium* species, 2) effects of structural variations on toxicity to fungal pathogens, 3) genetic control of structural variations in Upland cotton, 4) effects of structural variations on resistance to wilt in Upland cotton, and 5) mode of action of phytoalexins. Each area is discussed in the following paragraphs.

Four cultivated and 20 wild species of *Gossypium* were surveyed for the types of antibiotic terpenoids produced in stele tissue in response to infection by *V. dahliae* (Bell *et al.*, 1975) and in lysigenous glands of young healthy leaves (Bell *et al.*, 1978). These studies revealed two variations in the terpenoid biosynthetic pathway that yield qualitatively different terpenoids (Fig. 1). In one variation, 50-70% of the antibiotic terpenoids in leaves occur as the methyl ethers (dMHG, MHG, MHGQ). The ratios of dMHG/dHG, MHG/HG, and MHGQ/HGQ in a given species are nearly identical, indicating that the methyl group is introduced only at the point where dHG is methylated to form dMHG (Fig. 1). This recently has been confirmed in *G. barbadense* by using isotope-labeled methionine as the methyl donor (Benedict, Stipanovic, and Bell, unpublished data). In the second variation, dHG is oxidized to form 7-hydroxy dHG, which is subsequently methylated and oxidized to yield raimondal (R; Fig. 2). Raimondal makes up about 90% of the total antibiotic terpenoids in

leaves of *Gossypium raimondii* (Stipanovic *et al.*, 1980).

The effects of structural variations on the antibiotic potency of terpenoid phytoalexins were assessed by comparing the toxicity of dHG, dMHG, HG, and MHG against various fungi and by comparing the toxicity of HG and raimondal against *V. dahliae*. The naphthofuran phytoalexins (dHG and dMHG) are about twice as toxic as their terpenoid aldehyde counterparts (HG and MHG) and the methylated phytoalexins (dMHG and MHG) are only about half as toxic as their nonmethylated counterparts (dHG and HG) against *V. dahliae* (Mace *et al.*, 1985, 1990; Stipanovic *et al.*, 1988), *F. oxysporum* f. spp. *vasinfectum* (Zhang *et al.*, 1993), and zoopathogenic fungi (Mace *et al.*, 1993). Raimondal is only about half as toxic as HG against various strains of *V. dahliae* (Bell *et al.*, 1994). Thus, both of the variations in terpenoid biosynthesis that occur in *Gossypium* species other than *G. hirsutum* yield phytoalexins less toxic to fungi than those in *G. hirsutum*.

Genes controlling several structural variations in terpenoid phytoalexins were transferred into *G. hirsutum* by treating each chemical structure as a genetic character and backcrossing the character from the source *Gossypium* species into *G. hirsutum* four times. Plants obtained from the fourth backcross were selfed, and test crosses were used to select progenies that were homozygous for the presence or absence of specific terpenoid antibiotics. The homozygous BC_4S_1 progenies were used for genetic studies and for evaluating the effects of the characters on disease resistance. Various interspecific crosses showed that high levels of terpenoid methyl ethers transferred to *G. hirsutum* from all other *Gossypium* species, except *G. sturtianum*, were due to recessive genes (Bell *et al.*, 1987). Methylation was transferred from *G. sturtianum* to various other species as a completely dominant character, and raimondal synthesis was transferred as a partially dominant character. Detailed analyses showed that raimondal synthesis in the *G. hirsutum* lines is due to two dominant genes (Bell *et al.*, 1994). Presumably one gene is responsible for the oxidation at C-7 while the second controls the addition of a methyl group to the 7-hydroxyl group. High methyl ether concentrations appear to be due to a single recessive and a single dominant gene when transferred from *G. barbadense* and *G. sturtianum*, respectively (Bell and Stipanovic, 1977; Kohel and Bell, unpublished data). All of the genes transferred to date primarily affect the structure of terpenoids in green tissues, such as leaves, bracts, and carpel walls, and do not appreciably change the quality of terpenoids formed in roots and the stem stele tissue (Bell *et al.*, 1994). Thus, the high concentrations of terpenoid methyl ethers in stem stele of `SBSI' (Bell *et al.*, 1993) probably are due to genes that regulate concentrations of methionine or S-adenylmethionine (SAM) rather than those that regulate terpenoid biosynthesis.

The effects of qualitative changes in terpenoid phytoalexins on disease resistance has been determined by comparing the disease reactions of progeny from BC_4S_1 siblings homozygous for the presence and absence of the qualitative characters. The introduction into *G. hirsutum* `Tamcot CAMD-E' of either the recessive or dominant gene to increase formation of terpenoid methyl ethers or the two dominant genes to give raimondal synthesis increased the susceptibility of this cultivar to two isolates each of the defoliating and nondefoliating pathotypes of *V. dahliae*. Thus, partially substituting less toxic phytoalexins for those normally formed in foliage significantly increases susceptibility to Verticillium wilt. This suggests that increasing the toxicity of cotton phytoalexins should increase resistance to wilt diseases. Consequently, we have examined malvaceous genera for structurally similar but more potent terpenoid phytoalexins. Hibiscanone, a phytoalexin from kenaf, appears to have promise in this respect because it is four times more toxic than dHG to wilt fungi and apparently is made from the same precursor as cotton phytoalexins (Bell *et al.*, 1993b).

Understanding the mode of action of phytoalexins may allow the development of more toxic phytoalexins or manipulation of phytoalexin efficiency in tissues. Treatments such as reducing agents, strong chelators, and catalase decrease the rate of oxidation of dHG in water

solutions (Stipanovic *et al.*, 1992) and also decrease toxicity to *V. dahliae* (Stipanovic *et al.*, 1991). Adding a methyl group to dHG to form dMHG decreases both rate of oxidation and toxicity of the phytoalexin. Thus, the toxicity of dHG apparently involves free radicals and hydrogen peroxide generated during oxidative degradation. Because disruption of the plasmalemma is a primary effect of dHG in cells of *V. dahliae* (Mace *et al.*, 1992), the polarity of a phytoalexin probably is also important for determining its affinity for fungal membranes and its ability to cause membrane damage. Gossypol has a high affinity for lipid bilayers and disrupts these layers in water, and this is thought to contribute to its toxicity to animal cells (Reyes *et al.*, 1984).

Constitutive defense

Antibiotic terpenoids and tannins are limited to specific tissues in healthy plants, where they restrict the entry and spread of wilt fungi and contribute to effects of age and environment on cultivar resistance. Gossypol and its methyl ethers along with smaller concentrations of HG, dMHG, and MHG occur in the epidermis and root hairs of the developing seedling root (Mace *et al.*, 1974) and are infused later throughout the periderm of the root bark. Terpenoids are exuded by the epidermis of the root, and exudation can be increased by microbial infections of the plant (Hunter *et al.*, 1978; Hedin *et al.*, 1984). Only the root tip is devoid of terpenoids, which may explain why this is the only region of the root penetrated by *V. dahliae* and several other soilborne pathogens.

Tannins in young seedling roots occur in the root cap, the endodermis, and the hypodermis (Mace and Howell, 1974). The region of cell division and enlargement near the root tip is devoid of tannins as well as terpenoids. As the root matures, the endodermis progressively forms a continuous cylinder of tannin cells around the xylem tissues, and tannin cells are formed later in a scattered pattern throughout the root bark until tannin makes up 5 to 10% of the dry weight of the bark (Bell *et al.*, 1992). Only a very few cells of the stele, usually in the pith, form tannins in young or old healthy plants. These observations indicate that tannins restrict the movement of *V. dahliae* in cotton tissues. The fungus normally colonizes and penetrates the epidermis only near the root tip. It then grows directly to the outer wall of the endodermis where it spreads circumferentially and longitudinally. Very few hyphae, however, are able to penetrate the endodermis and reach xylem vessels. Moreover, the fungus is unable to penetrate the mature bark of roots and stems of live plants (Huisman and Gerik, 1989). In each case, the fungus fails to penetrate or invade tissues that contain lethal concentrations of tannin (Bell *et al.*, 1992).

Tannin also appears to restrict the development of *V. dahliae* in leaves. Fungal propagule numbers in leaves are inversely correlated with tannin concentrations (Howell *et al.*, 1976). Tannin concentrations in leaves depend on leaf age, leaf position on the plant, and light quality and quantity. Most of the tannin in leaves is synthesized while the leaves are in very early stages of development. Consequently, the young developing leaf has much higher tannin concentrations than the same leaf when mature. Tannin concentrations in mature leaves increase progressively with each leaf formed during growth until the plant has about 10 true leaves. Because of these dynamic changes, the lowest tannin concentrations are always found in the first true leaves above the cotyledonary scars and the highest concentrations are always found in the immature leaves near the shoot terminal. Progressive changes in hot water-extractable tannins as a function of leaf position in susceptible `Stardel' and resistant `SBSI' cultivars are shown in Figure 8, and the ability of these leaf extracts to support growth and conidia production of *V. dahliae* are shown in Figure 9. The occurrence of tannin concentrations low enough to allow fungal growth in the leaf extracts correlates very closely with the appearance of foliar symptoms at different leaf positions in these cultivars (Bell, 1969). Progressive increases in resistance to wilt diseases in the first cycles of the Acala and

MAR breeding programs also are correlated with progressive increases in tannin concentrations in leaves (Bell *et al.*, 1992). Further evidence supporting the importance of tannins is shown by the effects of light quality on tannins. Plants grown under glass (i.e., in greenhouses) contain only about half the concentrations of tannins in leaves as plants grown outdoors, and the greenhouse plants are more susceptible to wilt (Bell *et al.*, 1992). Collectively, these observations indicate that tannins probably have an important role in resistance to *V. dahliae*. However, it may be difficult to breed for more effective tannin concentrations because of the strong influence of development and environment on tannin concentration.

The effectiveness of tannins *in situ* depends not only on concentration but also on mean polymer size, concentration of destructive enzymes (especially peroxidase), and sap pH. Tannins extracted from cotton stem bark with water or with 10-30% acetone in water are more toxic to *V. dahliae* per unit of tannin than are tannins extracted with 50-80% acetone (Bell *et al.*, 1992). Only monomers and small oligomers are extracted with the water or dilute acetone, whereas larger, less toxic oligomers and polymers as well as monomers and small oligomers are extracted with the higher acetone concentrations. Very large polymers and oxidized polymers can not be extracted even with the high acetone percentages, but can be measured by acid hydrolyses of finely ground tissue residues in butanol. Among Acala cultivars the percentage of tannin that is not extractable is correlated with susceptibility to Verticillium wilt (Bell *et al.*, 1992). The percentage of extractable and small tannin molecules in tissues may be a function of peroxidase activity since this enzyme oxidizes catechin moieties to melanoid pigments (Hunter, 1974). Peroxidase activity in leaves is inversely related to cultivar resistance to Verticillium wilt (Table 1). Lower peroxidase activity may allow the more toxic tannin molecules to persist at greater concentrations and for a greater length of time in cell sap. Lewis and Papavizas (1967) showed that various tannins were much more toxic to *V. dahliae* at pH 5 than at pH 6-7. Therefore the specific toxicity of tannin among cultivars probably varies considerably *in situ* because the pH of cotton leaf sap varies from 5.2 to 6.5 among cultivars (Bell, unpublished data). Ratios of catechin to gallocatechin and ratios of different optical isomers within the tannin polymer might also affect toxicity, but these have not been studied with regard to *V. dahliae*.

Molecular biology of defense

Joost (1993) recently studied the early genetic events in the interaction of *V. dahliae* with *Gossypium* species. The reaction of resistant *G. barbadense* `Seabrook Sea Island 12B2' (SBSI) was compared with that of susceptible *G. hirsutum* `Rowden' against a defoliating (D) and a nondefoliating (ND) strain of *V. dahliae*. Responses to live conidia were also compared with those to dead conidia. Key genes were studied for four groups of enzymes that possibly are involved in defense reactions: 1) beta-1,3-glucanase and chitinase enzymes that degrade fungal cell walls, 2) 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) that forms isoprenoid units used for synthesis of terpenoid phytoalexins, 3) phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), and dihydroflavonol reductase (DHF), which are involved sequentially in the synthesis of tannins, and 4) glutamine synthetase (GS), which incorporates ammonia into anabolic nitrogen metabolism in cotton and could act as an ammonia scavenger. Coding regions of the selected genes were amplified from the cotton genome by polymerase chain reaction using specifically designed oligonucleotide primers and were used as probes for messenger RNA of cotton plants.

All of the defense genes in the first three groups occurred as gene families with three to nine copies in the genome. Only two copies of the GS gene were found and no messenger RNA from these was detected in stele tissue. Relatively high levels of messenger RNA from glucanase, PAL, and DHF genes occurred in healthy tissues and there was either no change or

a decrease in messenger RNA in response to infection. In contrast, large increases in messenger RNA from chitinase, HMGR, and CHS genes occurred in response to infection. The increases in messenger RNA from HMGR and CHS genes occurred more rapidly and were more intense in the resistant than in the susceptible cultivar from 10-72 h after inoculation. Beyond 72 h both of these genes were downregulated more rapidly in the resistant than in the susceptible cultivar. This downregulation may have minimized damage to the host from its toxic products in the diseased tissues. Messenger RNA for chitinase could be detected in the resistant but not the susceptible cultivar at 10 h after inoculation, but beyond 24 h concentrations were greatest in the susceptible cultivar and were proportional to fungal propagules.

The behaviour of the HMGR and CHS genes are consistent with patterns of terpenoid phytoalexin and tannin accumulation in diseased cotton (Figs. 3, 4). When plants are stem-puncture inoculated with *V. dahliae*, both tannins and terpenoids accumulate 24-48 h sooner in resistant `SBSI' than in susceptible `Rowden'. In both cultivars, the accumulation of tannin lags about 24 h behind that of terpenoids (Bell, 1969; Mace, 1978; Bell and Stipanovic, 1978). Also, dead conidia strongly activate synthesis of terpenoids (Fig. 5) and HMGR in `SBSI' but not in `Rowden'. These observations indicate that HMGR and CHS genes may have key roles in regulating active defense responses in cotton.

The elicitor of HMGR and CHS has not been identified. However, the large increases in messenger RNA of HMGR by 10 h after inoculation, before conidia germinate and in response to dead cells in `SBSI' suggests that this cultivar recognizes elicitors from the conidial cell wall, whereas `Rowden' does not recognize the pathogen until after spore germination and possibly conidiation. The ND strain was more efficient in activating chitinase, HMGR and CHS genes in both cultivars than was the D strain (Joost, 1993). This might explain their differences in virulence. The beta-1,3-glucanase and chitinase enzymes of `SBSI' may more efficiently release elicitors from conidial walls than these same enzymes from `Rowden.' Likewise, the enzymes from either cultivar might be more effective against ND than D strains of *V. dahliae*. Future studies need to address these possibilities as well as the specific mechanisms of regulation of HMGR and CHS.

Genetic Variation and Virulence of the Pathogen

Vegetative compatibility groups

Isolates of *V. dahliae* frequently show variation in morphology and virulence (Bell, 1992). Schnathorst and Mathre (1966) first showed that different levels of virulence to cotton and tomato occur among isolates of *V. dahliae* from cotton in California, USA. They designated a strain that completely defoliated `Acala 4-42' plants in the greenhouse as a defoliating strain (or pathotype). A strain that caused severe wilt of tomato but only mild wilt and no defoliation of `Acala 4-42' cotton was designated as a nondefoliating strain. Puhalla (1979) subsequently showed that defoliating and nondefoliating strains were vegetatively incompatible when either auxotrophic or melanin-deficient mutants were paired in genetic complementation tests. Using these techniques, he identified four subgroups within *V. dahliae* and reasoned that because heterokaryosis and the parasexual cycle are the only known means of gene exchange in the fungus, the subgroups may be viewed as genetically isolated populations. Each of the eight defoliating isolates in his study belonged to the P1 subgroup. In a later study, Puhalla and Hummel (1983) used only melanin-deficient mutants to distinguish 16 vegetative compatibility (V-C) groups among 96 strains from 38 plant species and 15 countries. More recently, Joaquim and Rowe (1990) and Strausbaugh *et al.* (1992) reinvestigated vegetative compatibility relationships among isolates of *V. dahliae* using nitrate-nonutilizing (*nit*) mutants. Both studies showed that the melanin-deficient mutants are

probably overly discriminating with respect to vegetative compatibility and that as few as four vegetative compatibility groups (VCG) may exist.

The recent studies based on *nit* mutants are not in agreement or did not both include assignments of the isolates RI, CU, TO and PCW that were assigned to VCGs 9, 10, 15 and 16, respectively, by Puhalla and Hummel (1983). The recent studies also used methods developed for *Fusarium oxysporum* (Correll *et al.*, 1987) without modification for the differing requirements of *V. dahliae*. Because of these discrepancies I reinvestigated VCGs in *V. dahliae* using *nit* mutants and Puhalla's original isolates (placed in storage before his experiments). The reliability of tests was increased by making several modifications in test media: 1) 0.5 g L^{-1} dibasic potassium phosphate was added to the nitrate medium to bring the pH up to about 6.0 and increase the vigor of complementation, 2) niacin and riboflavin were added to all media at 5 mg/L^{-1} because *V. dahliae* isolates sometimes develop partial or complete requirements for these vitamins in stock culture, and 3) 20 mg L^{-1} of uric acid was added to hypoxanthine media because purine dehydrogenase is strictly activated by the product in some isolates of *V. dahliae*. Hyaline variants were avoided because some had a diminished capacity for complementation. Isolate PCW had a natural mutation for a pathway specific regulator gene of the purine catabolic pathway and therefore could not use hypoxanthine or uric acid as nitrogen source. A revertant for this mutation was obtained from PCW and used to obtain *nit-1* and *nit-M* mutants.

One study included 35 isolates from cotton and 54 isolates from 31 other hosts; these were collected in 15 states of the USA and 16 other countries. All of the isolates, except five from Russia, could be readily assigned to one of three VCGs. These are given in Table 2 along with the original assignments of Puhalla and Hummel (1983). The Russian isolates were self-incompatible and, therefore, could not be assigned. Except for two isolates (CIH and MG) all of the isolates within a VCG as identified by Puhalla and Hummel (1983) fit into a single VCG in the new classification. I obtained CIH and MG from several additional sources and confirmed that both belong to VCG 2. In my genetic complementation tests isolates TO and PCW clearly belonged to VCG 4 as proposed by Strausbaugh *et al.* (1992) and not to VCG 3 as proposed by Joaquim and Rowe (1990). Isolates RI and CU, which were assigned to VCG 2 and 5, respectively, by Strausbaugh *et al.* (1992), complemented several isolates that were clearly assigned to VCG 4 in my tests.

Isolates within each VCG could be further divided into two (VCG 1 and 2) or three (VCG 4) subgroups based on the vigor and specificity of complementations. Complementations between A and B subgroups of VCG 1 and 2 occur slower and with less vigor than those within the subgroups. Isolates in the A and B subgroups of VCG 4 do not complement each other but will complement those in the A/B subgroup.

The virulence of isolates from the various VCGs and subgroups is shown in Table 3. All isolates in VCG 1A caused defoliation of cotton regardless of their host or geographical origin. Isolates in VCG 1B were not defoliating pathotypes, although in all other respects except complementation vigor they were indistinguishable from isolates in VCG 1A. Isolates in VCG 1B and 2A generally were more virulent than those in 2B or in any of the VCG 4 subgroups. Russian isolates were the least virulent of any group. There was a wide range of virulence among isolates within any subgroup except 1A. Thus, generalities about relationships between V-C assignment and virulence to cotton could be misleading.

Isolates in VCG 1 and 4 generally occurred in distinct geographical areas, whereas isolates in VCG 2 occurred universally. All but 1 of the 12 isolates of VCG 1A were from the southern and southwestern U.S.A. (Arkansas, Arizona, California, Mississippi, Missouri, New Mexico, Oklahoma and Texas) and northern Mexico, while the 3 isolates from VCG 1B were from the north central U.S.A. (Illinois and Indiana) and Canada. Isolates of VCG 4 generally are found in the northern U.S.A. (California, Idaho, Minnesota, Oregon, Washington and Wisconsin) and Canada and isolates of VCG 4B have also been found in

New South Wales and South Australia. Isolates of VCG 2 have been identified from the Americas (U.S.A., Canada, Peru, Argentina), Europe (France, Greece, Italy, Netherlands), Central Asia (Syria, Iran, Jordan), Africa, (Congo, Swaziland) and South Australia. The geographical occurrences suggest that VCG 1 and 4 are specialized subgroups that originally evolved from isolates in VCG 2. This conclusion is supported by the fact that some VCG 2 isolates show weak complementation with VCG 1 or 4 isolates after several weeks, whereas isolates in VCG 1 and 4 are completely incompatible.

Verticillium wilt in the USA generally is caused by isolates in VCG 1A with some fields in California and Texas also infested with isolates of VCG 2A. Isolates in the VCG 4A/B group have been obtained only from four fields in Texas (Bell, Mertely and Kaufman, unpublished data). Verticillium wilt in South America, Europe, Asia and Africa is generally caused by isolates in VCG 2A or 2B. A single isolate from cotton in Australia belonged to VCG 4B. Localized occurrences of VCG 1A isolates in Peru, Spain and China probably have resulted from introductions from North America. Verticillium wilt of certain crops is caused mostly by isolates from a specific subgroup. For example, 7 of 8 isolates from tomato belonged to VCG 2A, 3 of 4 isolates from potato belonged to VCG 4A and 3 from sunflower belonged to VCG 4B. This indicates that a group of closely related isolates often are responsible for the most severe wilt of a specific plant. In these cases, VCG typing may give a quick indication of whether the most virulent forms are present.

Virulence Mechanisms

Symptom production in Verticillium wilt of cotton is due to water deficits caused by plugging of xylem vessels, especially in leaves and to premature senescence of leaves leading to defoliation and loss of photosynthetic area (DeVay, 1989). Damage from nondefoliating isolates in VCG 2A is due primarily to the water deficits, whereas both factors contribute to damage caused by defoliating isolates in VCG 1A. Defoliating isolates cause much larger increases in ethylene and abscisic acid concentrations in leaves than do nondefoliating isolates (Wiese and DeVay, 1970) and these changes apparently contribute to both increased plugging of vessels and defoliation (Misaghi *et al.*, 1972). The defoliating isolates also cause as much as 10-fold increases in ammonium ions in leaves compared to less than 50% increases caused by nondefoliating isolates (Bell, 1991). When concentrations of ammonia similar to those in leaves affected by defoliating strains were supplied either as ammonium nitrate or urea, many of the symptoms typical of Verticillium wilt, including defoliation, were reproduced.

The severity of symptoms shown by the plant is proportional to the amount of colonization by either defoliating or nondefoliating isolates in the vascular system. Isolates from VCG 1A are better adapted to grow in the yeast form at temperatures of 28 to 32˚C than are isolates from VCG 2A (Wyllie and DeVay, 1970; Bell, unpublished data). Isolates from VCG 1A also are much less sensitive to sanguinarine, an alkaloid phytoalexin from poppy, than are isolates from VCG 2A (Puhalla and Hummel, 1983). The decreased sensitivity is due to their ability to detoxify sanguinarine by reducing it to dihydrosanguinarine (Howell *et al.*, 1972), indicating that VCG 1A isolates possess unique detoxifying enzymes. However, isolates from VCG 1A and VCG 2A do not vary significantly in their sensitivity to cotton phytoalexins (Mace *et al.*, 1985) or tannins (Bell *et al.*, 1992). Also, isolates from VCG 1B show the same adaptations to high temperature and sanguinarine as do those in VCG 1A, but isolates from VCG 1B do not cause a defoliating syndrome (Bell, unpublished data). Thus, adaptations to temperature or to terpenoid or flavonoid antibiotics of the cotton plant probably are not a primary cause of the increased virulence of the defoliating isolates.

Understanding the basis of increased virulence to cotton in defoliating strains is extremely important for future progress in breeding for resistance. Several sources of resistance that almost completely control nondefoliating isolates are available but these are

much less effective against the defoliating strains, particularly when soil temperatures drop below 28˚C. If the biochemical basis of defoliation were completely understood, it might be possible to introduce an additional gene(s) that directly counteracts the mechanism of enhanced virulence in VCG 1A isolates. For example, if these strains produced a unique toxin, it might be possible to introduce a detoxification system from a foreign species into cotton. Currently, we are studying the mechanism of ammonia accumulation in leaves in hope that this may give us a clue to the mechanism of enhanced virulence.

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Table 1. Peroxidase activity in comminutions from various cotton tissues. (Adapted from Bell and Stipanovic, 1978)

Tissue tested	Peroxidase per variety†		
	Stardel	Acala 4-42	SBSI
	(mg purpurogallin/min/g fresh tissue)		
Leaf	85.0	62.1	40.8‡
Stem bark	36.6	35.3	16.5‡
Stem stele	5.4	7.9‡	5.4

† Reactions to Verticillium wilt: Stardel, susceptible; Acala, tolerant; SBSI, resistant.
‡ Significantly different from other varieties (LSD.05).

Table 2. Reclassification of vegetative compatibility groupings (VCGs) of strains of *Verticillium dahliae*.

Reassigned VCG	No. of strains	Tester strains	Original VCG assignments by Puhalla and Hummel (1983)
1	21	V76,RN	VCG 1†
2	40	PH,OC	VCG 2,3,5,7,8,13,14
3	23	BB,TA,277,CU	VCG,4‡,6,9,10,11,12,13,15,16
Unassigned	5	RU, PR	Not assigned; all from Russia

† except isolate CIH assigned to VCG 2
‡ except isolate MG assigned to VCG 2

Table 3. Virulence of VCG groups of *Verticillium dahliae* to Rowden cotton plants with 10-12 true leaves.

VCG group	No. of strains tested	Growth reduction†	Defoliation Index†‡
1A	12	74-87 (79)	99-100 (100)
1B	3	34-65 (50)	34- 71 (52)
2A	22	11-77 (56)	1- 92 (59)
2B	5	16-61 (35)	6- 62 (42)
4A	8	13-83 (39)	0- 71 (30)
4A/B	7	9-61 (44)	0- 74 (43)
4B	4	0-80 (34)	0-100 (38)
Russian	5	25-30 (28)	13- 41 (27)

† Range (and means) for all isolates.
‡ Percentage reduction of fresh shoot weight/root weight ratio.

Figure 1. Structures and probable biosynthetic relationships among the major terpenoid phytoalexins formed by *Gossypium* species. Key to compounds: HG = hemigossypol; MHG = hemigossypol 3-methyl ether; 7-OH dHG = 7-hydroxydesoxyhemigossypol; dHG = desoxyhemigossypol; dMHG = desoxyhemigossypol 3-methyl ether; dR = desoxyraimondal; 5-OH dHG = 5-hydroxydesoxyhemigossypol; 5-OH dMHG = 5-hydroxydesoxyhemigossypol 3-methyl ether; R = raimondal; HGQ = hemigossypolone; and MHGQ = hemigossypolone 3-methyl ether.

Figure 2. Structures of catechins and condensed proanthocyanidins (tannins) formed by *Gossypium* species.

Figure 3. Accumulation of terpenoid aldehyde phytoalexins (hemigossypol equivalents) at intervals after inoculation of resistant *Gossypium barbadense* (G.b.) or susceptible *Gossypium hirsutum* (G.h.) cultivars with a defoliating (D) or non-defoliating (M) strain of *Verticillium dahliae*. (Adapted from Bell, 1969).

Figure 4. Accumulation of ethanol-extractable (soluble) and ethanol-nonextractable (insoluble) flavanols (tannins) at intervals after inoculation of resistant *Gossypium barbadense* `Seabrook Sea island 12B2' (SBSI) or susceptible *Gossypium hirsutum* `Stardel' with a defoliating strain of *Verticillium dahliae*. (Adapted from Bell and Stipanovic, 1978).

Figure 5. Phytoalexin concentrations (hemigossypol equivalents) in stem stele of resistant *Gossypium barbadense* `Seabrook Sea Island 12B2' (SBSI) and susceptible *Gossypium hirsutum* `Stardel' at 72 h following infiltration of heat-killed conidia (10^7/ml) from defoliating (103, 105) and non-defoliating (106, 116, 277) isolates of *Verticillium dahliae*. (Adapted from Bell and Stipanovic, 1978).

Figure 6. Effects of temperature on mean rates of conidial production by isolates of *Verticillium dahliae* and of phytoalexin production by cotton cultivars. (Adapted from Bell and Presley, 1969).

Figure 7. Effects of temperature on rate of conidia production of*Verticillium dahliae* and phytoalexin response of cotton. (Adapted from Bell and Presley, 1969).

Figure 8. Tannin concentrations (% fresh weight) of leaves located at various positions above the cotyledons in plants with nine true leaves. Susceptible *Gossypium hirsutum* `Stardel' is compared with resistant Gossypium barbadense `Seabrook Sea Island 12B2' (SBSI). (Adapted from Bell and Stipanovic, 1978).

Figure 9. Conidial production during 72 hours incubation in water extracts from leaves located at various positions above the cotyledon. Susceptible *Gossypium hirsutum* `Stardel' is compared with resistant `Seabrook Sea Island 12B2' (SBSI). (Adapted from Bell and Stipanovic, 1978).