CHARACTERIZATION OF *Schizaphis graminum* (RONDANI) (HOMOPTERA: APHIDIDAE) BIOTYPE EVOLUTION VIA VIRULENCE AND FITNESS ON *Sorghum bicolor* (L.) MOENCH and *Sorghum halepense* (L.) PERSOON

A Dissertation

by

ROBERTO LUIS GORENA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Entomology

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May 2004

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ABSTRACT

Characterization of *Schizaphis graminum* (Rondani)

(Homoptera: Aphididae) Biotype Evolution via Virulence and Fitness on

Sorghum bicolor (L.) Moench and *Sorghum halepense* (L.) Persoon.

(May 2004)

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Greenbug is one of two key insect pests of sorghum, and biotype evolution hinders the long-term usefulness of resistant sorghums. The current study sought to identify plant resistance mechanisms, plant damage characteristics, and greenbug fitness in sorghum/greenbug interactions. Choice tests were conducted to elucidate resistance mechanisms displayed by four sorghum genotypes towards several greenbug biotypes and isolates. Results indicated all three resistance modalities (antibiosis, antixenosis, tolerance) were identified in sorghums, with some genotypes displaying two or more modalities towards some biotypes. This suggests some sorghum genotypes do not select for greenbug biotypes, and the sorghum genotypes cultivated may have relatively long-term resistance.

Non-choice tests were used to determine plant damage associated with greenbug feeding. Four sorghum genotype, Johnson grass, and five greenbug biotype combinations were used to elucidate plant characteristics associated with visible plant

damage. Fluid loss and plant stunting were significantly associated with visible plant damage, and were also observed in some plants not incurring heavy visible damage. Additionally, some biotypes avirulent to cultivated sorghum caused significant damage to Johnson grass. These results suggest visible plant damage, routinely used in damage studies, reflects underlying causes that could lead to poor agronomic performance. Additionally, Johnson grass may harbor greenbug biotypes not commonly found in sorghum fields.

Greenbug colony and individual fitness were determined by reproduction rates of five biotypes on four sorghum genotypes and Johnson grass in non-choice tests. Generally, colony and individual fitness estimates were not different within genotype/biotype combinations. Also, biotypes did best on more susceptible and worst on more resistant sorghum genotypes. Colonies and individuals of all biotypes had lowest fitness on Johnson grass. These results suggest virulent biotypes may have a fitness advantage over avirulent ones, at least in the presence of the cultivated host.

The results presented herein reflect the diversity of sorghum/greenbug interactions, and underscore the need for further understanding of the nature of greenbug biotypes, and how they interact with cultivated and non-cultivated host plants.

This dissertation is respectfully dedicated to my parents, Rene Absalom and Maria Alicia Gorena, and to the rest of my family and friends who have kept faith in me over the years. Thanks for your support, and finally here is “It.”

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CHAPTER I

INTRODUCTION

Greenbug, *Schizaphis graminum* (Rondani) (Homoptera: Aphididae), has been a serious insect pest of cereal crops in the U.S. since at least 1882 when large numbers of the aphid were found destroying oats, *Avena sativa* (L.), around Culpeper, Virginia (Webster and Phillips 1912). Because this was a rather isolated incident and the genus *Toxoptera* (now *Schizaphis*) was not known in the U.S., early investigators erroneously assigned specimens of the aphid to *Siphonophora* (now *Sitobion*) *avenae* F. Despite the confusion surrounding these early accounts, it was clear that the insect had the capability of causing damage to oats, wheat (*Triticum* spp.), and rye (*Secale cereale* L.) (Webster and Phillips 1912).

The destructive nature of greenbug was not fully appreciated until a serious and devastating outbreak during late 1889/early 1890 on oats and wheat (Webster and Phillips 1912). During this outbreak, much of the oat and wheat crops grown in the eastern and southeastern U.S. were damaged to a greater or lesser extent, as in subsequent outbreaks in the late 19th and early 20th centuries (Webster and Phillips 1912). Investigations of these situations revealed some cases in which whole fields escaped the damaging effects of greenbug infestations:

> Some fields were not attacked by the louse, though it infested surrounding fields. From the fields not attacked by it there were splendid yield of oats; while, of course, the other fields yield scarcely anything. In every township there were a few fields that were not attacked by the green louse and that made good yield. The fact that those fields not

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This dissertation follows the style and format of the Journal of Economic Entomology.

> attacked by the green louse invariably made a good yield, while those that were attacked made a poor yield, is proof that in this part of the State, at least, the green louse was the prime cause of the failure (Webster and Phillips 1912, p. 23).

Webster and Phillips (1912) noted that this "immunity" was routinely observed in some fields, particularly those adjacent to others with acute destruction, and similar situations of plant susceptibility or resistance in small grains were observed in the outbreaks of 1901, 1903, and 1907. Thus, during the initial damaging outbreaks of greenbug on small grains in the U.S. there appear to have been resistance mechanisms at work within certain small grain cultivars. Although early breeding efforts in cereal crops, especially wheat, focused on winter hardiness (Fowler 1997), it is perhaps through these efforts that resistance to greenbug feeding was noted in some early cultivars.

Despite continuing efforts to breed for resistance, greenbug has continued to be a major pest of small grains since early reports (Starks et al. 1976), primarily due to its ability to overcome plant resistance. In one of the earliest reports, Dahms (1948) studied resistance of particular small grain varieties to two greenbug cultures obtained from Oklahoma and Mississippi. This study demonstrated that barley (*Hordeum vulgare* L.) plants had lower overall growth and were killed faster when infested with Oklahoma greenbugs than when infested with Mississippi greenbugs (Dahms 1948). This is perhaps the first documented evidence of differential damage to varieties by different greenbug populations. Populations that overcame plant resistance would later be referred to as "biotypes." Virulent greenbug biotypes are capable of damaging previously resistant varieties (Shufran et al. 1997), and are the reason that resistance in

small grains (and eventually sorghum) is short-lived and has not achieved the effectiveness initially envisaged (Starks and Merkle 1977).

Prior to the summer of 1968 greenbug only occasionally infested sorghum, *Sorghum bicolor* (L.) Moench, and caused no economic damage to it (Dickson and Laird 1969, Harvey and Hackerott 1969, Hamilton et al. 1982). Circumstantial evidence that greenbug was not an economic problem deserving resistance breeding efforts in sorghum before this time is its absence in Painter's (1951) in his seminal treatise on insect resistance in crops. During the 1968 growing season, greenbug densities on sorghum reached damaging numbers and, as a consequence, increased the cost of treatment up to 100-fold in subsequent years (Teetes and Johnson 1973). Greenbug populations capable of reproducing at high rates on, and damaging sorghum were classified as biotype C (Harvey and Hackerott 1969). Biotype C differed from biotypes A and B (identified on wheat by Wood 1961) by a greater tolerance to high temperatures and more efficient use of, and damage to sorghum (Dickson and Laird 1969, Harvey and Hackerott 1969). This led to concerted efforts towards identification of sorghum resistance traits effective against greenbug. By 1999, approximately 29,000 sorghum germplasm lines had been evaluated for greenbug resistance, with 28 lines exhibiting resistance (Teetes et al. 1999).

Greenbug has been considered a key insect pest (one that consistently is the focus of insecticidal treatment) of sorghum since 1968, and certain biotypes have the ability to substantially reduce yield potential in some sorghum varieties (Teetes 1980, Dharmaratne et al. 1986, Bush et al. 1987). Greenbug outbreaks do occur despite the use

of resistant cultivars. In these situations, insecticidal treatments are necessary, although the financial and environmental costs of these are high (Eikenberry and Rogers 1974, Teetes 1992, Hays et al. 1999). Numerous chemical treatments are generally not feasible in sorghum due to its relatively low market value. Sorghum does, however, exhibit extraordinary tolerance and compensatory re-growth after pest attack (Flattery 1982). Therefore, plant resistance in sorghum remains the most cost effective method of greenbug control (Porter et al. 1997, Hays et al. 1999). Resistance may also influence biotypes: evolution of virulent greenbug biotypes hinders the sustainability of resistant sorghum varieties (Shufran et al. 1991).

Adding to the potential for greenbug biotypic evolution and/or maintenance are a multitude of non-cultivated grasses upon which greenbug may feed (see Michels 1986). For example, experimental evidence suggests that cool season wild grasses were instrumental in the evolution of biotype F (Kindler and Hays 1999). One of the most notorious and ubiquitous potential greenbug hosts is Johnson grass, *Sorghum halepense* (L.) Persoon. Johnson grass has been regarded as a pest grass since the early 20th century and is listed as one of the world's ten worst weeds (Long 1930, Holm et al. 1977, Anderson 1999). Johnson grass occurs virtually everywhere that sorghum is grown (Arriola and Ellstrand 1996). Therefore, the influence of Johnson grass on the potential expression and maintenance on greenbug biotypes warrants study.

The primary goal of the research reported herein was to identify differences in fitness and virulence parameters among several greenbug biotypes on susceptible and resistant sorghum genotypes and the non-cultivated host Johnson grass. Data on fitness

and virulence were also analyzed for patterns in the interactions of specific biotype/genotype combinations in an attempt to identify interactions that may have been important in the evolution and/or maintenance of greenbug biotypes. The specific objectives, and the corresponding null hypotheses, were to:

1) Identify plant resistance mechanisms among sorghum genotypes towards greenbug biotypes using choice tests (Chapter IV)

H_o = sorghum genotypes do not display differential plant resistance to greenbug biotypes;

2) Compare differential virulence of greenbug biotypes on sorghum and Johnson grass genotypes based on subjective and objective damage measures using non-choice tests (Chapter V)

H_o = different biotypes do not exhibit differential virulence among sorghum varieties;

3) Compare intrinsic rates of increase (r_m) and generation times (T) as indices of biotype fitness of individual apterous females on sorghum and Johnson grass genotypes using non-choice tests (Chapter VI)

H_o = individual female fitness parameters do not vary among biotypes on plant genotypes;

4) Compare colony r_m and doubling time values as indices of colony fitness on sorghum and Johnson grass using non-choice tests (Chapter VI)

H_o = colony fitness parameters do not vary among biotypes on plant genotypes;

5) Attempt to identify differential gene expression in damaged versus undamaged sorghum plants using suppression subtractive hybridization (Chapter VII)

H_o = damaged plants do not display differentially expressed genes.

CHAPTER II

LITERATURE REVIEW

The biotype concept

The term "biotype" as used in current literature on plant resistance to insects is perhaps inconsistent with regard to its original meaning. Coined in 1905 by the Danish botanist Wilhelm Johannsen (Shull 1912), biotype meant a group of organisms with the same hereditary characteristics, i.e. the same genotype. The need for a specific term to identify such groups arose from confusion over usage of several other terms proposed by Johannsen, particularly "genotype" and "pure line" (Jennings 1911). It was apparent to researchers of the time that crops differed in many phenotypic respects, even within the same species. Combined with the burgeoning science of genetics, these terms grew out of a necessity to differentiate between the genetic constitution of an organism (genotype), groups of organisms with the same genotype (biotype), groups of individuals derived via self-fertilization of a single homozygous ancestor (pure line), and groups of individuals of similar genotype traceable through asexual reproduction to a single zygote (clone) (Shull 1912). Biotype notions in entomological literature usually combine the latter three concepts.

Biotypes of particular organisms are designated for a variety of reasons, including herbicide (Wagner et al. 2002) or insecticide (Teetes et al. 1975) resistance, overcoming plant resistance mechanisms (Eastop 1973, Porter et al. 1997), competitive ability (Solbrig and Simpson 1974), virus transmission ability (Eastop 1973) or other factors. The use of the term to distinguish between, for example, aphid populations

capable of overcoming host plant resistance is fraught with problems. Eastop (1973) noted that biotype is a taxonomic concept most used by non-taxonomists, which likely has resulted in its misuse. In the study of aphids the term has come to mean "behaving similarly as far as the researcher's immediate interests are concerned" (Eastop 1973, p. 40). Because most aphids reproduce parthenogenetically during part of the year, and fundatrices arise from different fertilized eggs, populations of aphids during the parthenogenetic stage are distinct clones (Eastop 1973). For this reason Eastop (1973) believes that there is no advantage in using "biotype" over "clone." However, the term "biotype" has a practical usefulness in agricultural research, particularly in regard to overcoming resistance mechanisms, and is used extensively in greenbug research.

Greenbug biotypes are identified by their ability to overcome resistance mechanisms in previously resistant crop varieties. In this sense, greenbug biotypes could perhaps fall under the "polymorphic or polygenic variation within populations" category of Diehl and Bush (1984), as it is unclear whether different biotypes represent distinct genotypes or simple polymorphism within a given genotype (e.g., Rider and Wilde 1998). This is particularly puzzling when examining variation within clones, which are presumably genetically identical. Greenbug biotypes have been hypothesized to develop through selective pressure from the use of resistant crop varieties (Eisenbach and Mittler 1987). This view was challenged by Porter et al. (1997) who claim that greenbug biotypes are preexisting opportunists only observable when the appropriate crop variety is developed and deployed.

While the debates as to what constitutes a biotype *per se*, and how greenbug biotypes come into being remain, the concept of greenbug biotypes is nonetheless useful in the search for crop varieties capable of minimizing their effects. Because greenbug is intimately associated with its host plants, the following discussion will focus primarily on the interaction between greenbug feeding and host plant reactions as a basis for what ultimately may be referred to as a "greenbug biotype."

Greenbug origin, morphology, and life history

Greenbug is of Mediterranean origin and was originally described from a large outbreak of the species in and around the city of Parma, Italy, during the summer of 1852 (Rondani 1852). Adults reach a length of about 2 mm and are deep green to yellow with a darker green longitudinal stripe along the dorsal abdomen. Antennae and eyes are completely black, as are the distal tarsi and distal tips of the cornicles (Rondani 1852).

As in most aphids, asexual females may be alate or apterous (Dixon 1977). Generally, the apterae are most common and alatae are produced mainly during periods of diminishing resources or crowding, as seen in other aphid species (e.g., Lees 1967, Watt and Dixon 1981, Williams et al. 2000). Apterous oviparae (sexually reproducing females) are distinguishable from apterous viviparae (asexuals) by blackened, enlarged metathoracic tibiae in the former (Puterka and Slosser 1983). Greenbug males are winged, have sclerotized genitalia, and are approximately half the size of winged viviparous females (Puterka and Slosser 1983).

Greenbug exhibits cyclic parthenogenesis (Fig. 1), also known as agamy (Sokal 1952), as its reproductive strategy, which it shares with the majority of temperate aphid species (Dixon 1998, Pietro and Caillaud 1998, Raymond et al. 2001). This strategy involves asexual production of only female nymphs from viviparous females (thelytoky) during spring through mid to late autumn. This is followed by production of at least one sexual generation (males and oviparous females) triggered by a variety of environmental cues, including temperature and photoperiod (Marcovitch 1923, Puterka and Slosser 1983, 1986; Margaritopoulos et al. 2002). Often, the sexual generation in aphids is associated with an alternate host, which has not been documented in greenbug, at least not outside of Poaceae (grasses). Fundatrices (founding mothers) hatch from eggs in early spring and initiate production of that year's parthenogenetic generations.

Subsequent to mating, females produce eggs which are the overwintering stage (Dixon 1998). Reportedly, greenbug does not exhibit a natural sexual cycle below the 35th parallel (Webster and Phillips 1912, Daniels 1956), or at least it is limited (Shufran et al. 1991). Several authors have noted greenbug eggs in greenhouses south of the 35th parallel (e.g. Puterka and Slosser 1983, 1986), yet eggs have not been found under field conditions. Daniels (1956) suggested that greenbugs in areas of the Texas Panhandle may come from eggs oversummering at high altitudes.

Puterka and Slosser (1983) determined that biotype C greenbug would produce

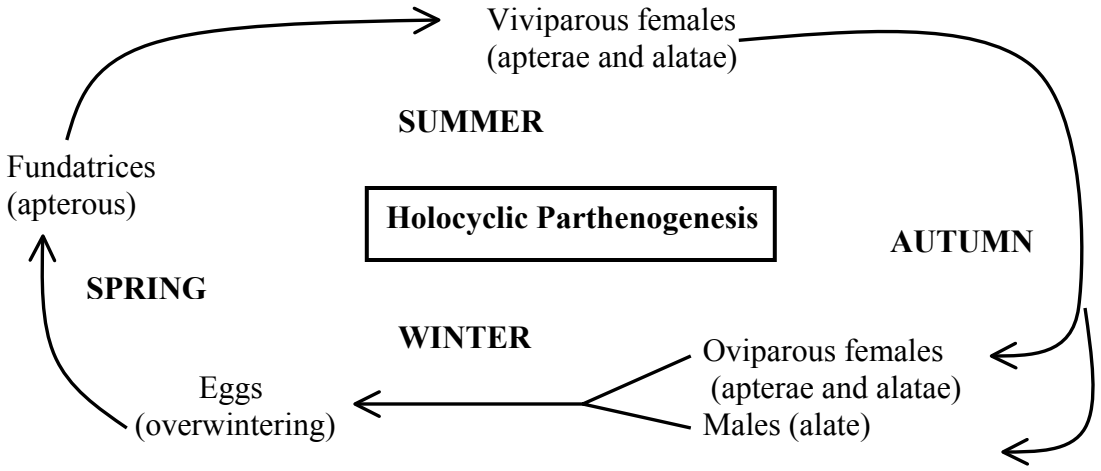

Fig. 1. *S. graminum* reproductive strategy, particularly north of the 35th parallel. Development of oviparae and males occurs with the onset of autumn conditions. Eggs overwinter and hatch to fundatrices in early spring. In warmer areas, greenbug may subsist in the viviparous stage for the majority of the year.

oviparae and males, provided the day length (photoperiod) was approximately 11 h. Cool temperatures (22^{o} or 18^{o} C) increased the proportion of oviparae and males: however, it was concluded that photoperiod was the major factor inducing bisexual morphs (Puterka and Slosser 1983). Additionally, Puterka and Slosser (1986) found that greenbug egg hatch was highly influenced by low temperatures ($\leq$ 1.7^{o} C) and time spent at those temperatures ($\geq$ 6 wk), yet percentage hatch was still quite low (13% and 11.5%) on sorghum and wheat, respectively. Although this experimental evidence supports the idea that greenbug eggs can overwinter in relatively cold temperatures, many authors believe that greenbug is incapable of withstanding winter conditions in the northern U.S., and argue that infestations in these states are from immigrants from the south (e.g., Irwin and Thresh 1988).

Viviparous greenbug females give birth to live young, all of which are female. Although this in itself may be sufficient to produce large numbers of offspring in a short period of time, it is perhaps the phenomenon of "telescoping of generations" that is responsible for the enormous numbers produced in very short time periods (Hughes 1963, Dixon 1998). Telescoping of generations refers to the development of two generations (daughter and granddaughter) within an individual female. This is possible because parthenogenetic eggs do not require fertilization, thus they begin to develop as soon as they are ovulated (Dixon 1998). This system has given aphids approximately a three-fold reproductive advantage over similarly sized insects (Dixon 1990), and population increase rates comparable to much smaller arthropods such as mites (Dixon 1998). In addition, population growth rates are influenced by host plant, cultivar, and/or

environmental parameters (e.g. Beregovoy and Peters 1994). Host plant quality also influences potential and achieved fecundity in many phytophagous insects (Dixon 1987, Awmack and Leather 2002). Further, the number and size of ovarioles, birth weight, adult weight, and myriad other physiological mechanisms may influence aphid fecundity (Dixon 1987).

Greenbug host plants, food, and endosymbionts

Recorded host plants of greenbug are limited to poaceous plants and include most, if not all, small grain and sorghum species widely cultivated today. Rondani (1852) listed 11 grass species, including maize (*Zea mays* L.) and rice (*Oryza sativa* L.), as hosts for this insect. He noted that the species with highest levels of infestation was "…the common wheat, *Triticum sativum* or *Vulgare hybernum*, or varieties of these" (Rondani 1852, p. 10; English translation). Patch (1938) provided one of the earliest extensive lists of greenbug hosts, listing 63 species in 28 genera, including maize and rice. In a study of host preference in greenbug biotype C, Dickson and Laird (1969) determined that average colony sizes derived from one adult female per plant after 10 days were approximately 42 and 36 times larger on small grains and sorghum than on rice, respectively: rice was described as a very poor host. In addition, they reported that greenbug biotype C generally did not establish well on maize, with colony sizes 157 and 133 times larger on small grains and sorghum than on maize, respectively (Dickson and Laird 1969). While these studies suggest maize and rice to be unsuitable hosts relative to small grains and sorghum, greenbug occasionally feeds on maize (e.g., Stoetzel and

Miller 2001). Future interactions between greenbug and maize may lead to the expression of a biotype capable of damaging it, as occurred in 1968 with sorghum.

Michels (1986) published an extensive list of greenbug hosts, which included 70 species in 44 genera of Poaceae: 24 species of grasses included in a previously published list (Patch 1938) were not positively confirmed as host plants of greenbug. Additionally, it is debatable whether orchardgrass (*Dactylis glomerata* L.) and switchgrass (*Panicum virgatum* L.) are hosts of greenbug (Michels 1986). The number of host species listed for greenbug has prompted some researchers to label them as polyphagous in their diet, although Painter (1936) suggests that such terms can be misleading as it is a classification of the apparent food and not indicative of foods of equal nutritive value. Nevertheless, greenbug seems capable of surviving upon and reproducing (at least to a limited extent) on a wide variety of grasses. Genetic evidence suggests that some greenbug biotypes are host-adapted races that developed an association with their poaceous hosts around 0.3 – 0.6 million years ago (Powers et al. 1989, Shufran et al. 2000). This is consistent with the hypothesis that modern aphid/host associations and life histories may, in a large part, be determined by evolutionary history (Moran 1989).

Aphids feed primarily on phloem tissues, particularly within the sieve cells where plant sap is found (Auclair 1963, Klingauf 1987a, Fujimaki et al. 2000). Chemoreceptors located within the greenbug food canal and epipharynx are responsible for monitoring the chemical composition of ingested fluids for suitability (Anderson and Bromley 1987, Klingauf 1987b). Stimuli that guide the stylets to the phloem include chemical gradients and pH differences around phloem tissue (Klingauf 1987b).

Phloem fluids are notoriously low in both concentration of amino acids and other nitrogen sources, and proportion of essential amino acids (Wilkinson 1998, Sanström and Moran 2001). With the exception of xylem sap, phloem sap has the lowest concentration of nitrogen compared to other plant parts (Mattson 1980). Nitrogenous compounds may also only be available at very specific seasons or stages of plant or leaf phenology (Van Hook et al. 1980). To compensate for the generally low levels of available nitrogen in phloem sap, aphids have evolved at least two strategies to supplement their diet: endosymbiosis with amino acid-producing bacteria, and the ability to induce changes in host plants via feeding that influence the amino acid content in phloem, similar to the process of organ senescence.

Insects that feed on nutritionally poor diets are often associated with endosymbiotic organisms (bacteria, fungi, etc.) that enhance the nutritional content of their food (e.g., Kukor and Martin 1983, Boursaux-Eude and Gross 2000, Moran and Baumann 2000, Patricolo et al. 2001, Beard et al. 2002). Aphids harbor bacterial endosymbionts, particularly *Buchnera aphidicola* Buchner, a γ-Proteobacteria closely related to *Escherichia coli* Esch. (Douglas 1997, 1998, Rahbé et al. 2002) and *Haemophilus influenzae* Pfeiffer (Shigenobu et al. 2000). *Buchnera* is closely related to psyllid and whitefly endosymbionts (Charles et al. 2001, Darby et al. 2001).

Buchnera endosymbionts are present in almost all of the approximately 4500 extant species of Aphididae (Rouhbakhsh et al. 1997): *Buchnera* are not found in the Phylloxeridae, Adelgidae, and some species of Hormaphididae, which harbor other microbial endosymbionts (Douglas 1998). These bacteria are housed in special cells

called bacteriocytes that congregate into larger, bilobed structures called mycetocytes (Lai et al. 1994, Martinez-Torres et al. 2001), and are inherited maternally (Rouhbakhsh et al. 1997). The association is an obligate mutualism as the bacteria cannot be cultured outside of aphids, due to the loss of most of the genes essential for independent life (Ishikawa 2002). Similarly, aphids treated with antibiotics ("aposymbiotic aphids"), which kill the *Buchnera*, grow very slowly and cannot reproduce (Douglas 1996, 1998, Wixon 2001). Evidence suggests that the symbiosis originated early in aphid evolution, and speciation of aphid lineages has been paralleled by divergence of allied endosymbiont lineages (Baumann et al. 1995, Douglas 1997, Martinez-Torres et al. 2001). Funk et al. (2000) provided the first hard evidence of strict vertical transmission (maternal inheritance) and co-speciation between *Buchnera* and its aphid hosts.

Although completely dependent on hosts for survival, *Buchnera* retain characteristics of free-living ancestors, containing genes for DNA replication, transcription, and translation; chaperonins and other proteins for secretion; and, machinery for amino acid biosynthesis (Baumann et al. 1995, Wernegreen and Moran 2000). The latter function is perhaps the most important for the aphid host due to the limited supply of amino acids in phloem fluids, especially tryptophan (Rouhbakhsh 1997), cysteine (Baumann et al. 1995), leucine (Sandström and Moran 1999, 2001), and methionine (Douglas 1996, Sandström and Moran 1999). Rahbé et al. (2002) demonstrated that braconid parasitoids may enhance *Buchnera* performance, thus increasing a variety of amino acids in the larva's environment.

Shigenobu et al. (2000) report 54 genes in the *Buchnera* genome or in their plasmids that are responsible for amino acid biosynthesis, while Ishikawa (2002) lists 55 genes. Several of these genes have multiple copies (from 4 to 16), which seems unusual given the small size of the genome (640,681 bp). However, open reading frames (ORFs) average only 988 bp, and about 88% of the total genome is ORF (Shigenobu et al. 2000, Ishikawa 2002). Lai et al. (1994) determined that a plasmid of the greenbug *Buchnera* contained four tandem repeats of a 3.6-kilobase *trpEG* unit, the upstream origin for the genes *trpE* and *trpG*. *trpE* and *trpG* are responsible for production of anthranilate synthase, the rate-limiting enzyme in tryptophan biosynthesis (Rouhbakhsh et al. 1997). Lai et al. (1994) also report an average of four of these plasmids per cell of the greenbug endosymbiont, resulting in a 16-fold gene amplification and overproduction of tryptophan. Repeats of the *trpEG* origin and other genes involved in leucine synthesis (e.g., *leu*ABCD) have been found in the plasmids of *Buchnera* associated with several aphid species (Rouhbakhsh et al. 1997, Wernegreen et al. 2001).

In contrast to production of essential amino acids by *Buchnera*, aphid feeding may alter host plant physiology such that the availability of nitrogen sources in phloem fluids is enhanced. The influence of plant genotype on levels of aphid infestation and reproduction has long been recognized (e.g., Davidson 1922, 1925, Painter 1941, Maltais 1951), although the mechanisms involved were not fully understood. It is now established that part of the reason behind this is that some aphid species damage particular plant varieties in ways that are physiologically similar to senescence (e.g.,

Dorschner et al. 1987), which liberates nitrogenous compounds into the phloem, potentially enhancing the nutritional quality of the ingested material.

Senescence in plants is a highly controlled sequence of biochemical and physiological events from tissue maturation to death (Feller and Fisher 1994), somewhat similar to programmed cell death in animal tissue (Smart 1994). Senescing tissues have an overall proportion of soluble nitrogen higher than mature tissues and the nitrogen is less dilute (Awmack and Leather 2002): only young tissue has proportionately higher nitrogen concentrations (Awmack and Leather 2002). During senescence, photosynthetic tissues are dismantled and the nutrients are transported to young, growing tissues, storage organs, and/or fruit via phloem (Habibi et al. 1993, Feller and Fisher 1994, Smart 1994, Hörtensteiner and Feller 2002). The importance of nitrogen mobilization to seed from senescing tissues in wheat varieties is exemplified in attempts to exploit senescence to improve varietal performance in particular environments (Saulescu et al. 2001). However, since the nitrogen composition of phloem sap ingested by aphids influences their development (Maltais 1951, Dorschner et al. 1987), the effects and timing of senescence in wheat and other hosts on the development of aphids such as greenbug should be considered.

Physiological changes induced in host plants by aphid feeding are relatively well documented, particularly those leading to increased nitrogen availability for aphid consumption. The ability of some aphids to change the nutritional quality of ingested sap is, apparently, closely tied to the ability to cause plant damage (Telang et al. 1999), thus it will be addressed at the end of the following section.

Biochemistry of feeding and plant damage

Plant damage via homopteran feeding can occur in several ways: a) Directly from the biochemical interaction of saliva and plant tissues; b) by introduction of viral particles to the plant during probing or feeding (e.g. Blanc et al. 1996, Friess and Maillet 1997, Werker et al. 1998); c) directly from removal of plant fluids (Kantack and Dahms 1957, Gilreath and Smith 1988); d) through chlorophyll loss (Windle and Franz 1979, Deol et al. 2001); e) from mechanical disturbance of tissues arising from stylet penetration (Miles 1987); or f) from fungal growth due to honeydew excretion (Maxwell and Painter 1959, Freeman et al. 2001).

Aphids are primary vectors of a variety of plant viruses, that cause significant losses in certain crops (e.g., Power 1991, Kendall et al. 1992, Miller and Rasochová 1997, Stapleton and Summers 2002, Taylor et al. 2001). Additionally, aphid salivary fluids may act as toxins that cause plant damage (Paschke and Sylvester 1957, Nickel and Sylvester 1959, Dharmaratne et al. 1986). Several authors report that greenbug salivary secretions have toxic effects on host tissues, causing symptoms ranging from chlorosis to necrosis (e.g., Ryan et al. 1990, Puterka and Peters 1995, Flinn et al. 2001, Burd 2002). Cell death presumably results from cell wall degradation via enzymatic (e.g., pectinases; Ma et al. 1990, 1998) action, although the complete biochemistry of the saliva/cell interaction is not entirely understood (Puterka and Peters 1995).

At least two types of saliva are excreted during stylet penetration and active feeding by aphids, one type that gels as it is discharged, forming the salivary sheath, and another that remains watery (Miles 1965, 1987). The watery type is sucked up

periodically during probing to test the suitability of the ingested matter. Many sheath functions have been proposed (reviewed in Miles 1987) including stylet support, exclusion of material from accidental ingestion by the aphid, and sealing plant wounds. Lawson et al. (1954) concluded that salivary secretions were partly responsible for injury in tobacco plants. Some early reports (e.g., Nickle and Sylvester 1959) questioned whether the liberation of toxic compounds from aphid saliva was limited to sheath formation. Nickle and Sylvester (1959) showed that toxins were introduced slowly throughout feeding events, and toxic effects were "somewhat independent of the number of sheaths produced" (p. 254).

Aphid saliva often contains enzymes such as pectinases, which degrade pectin in the middle lamella (Adams and McAllen 1956, 1958). Pectin acts as the cementing material between plant primary cell walls (Willaman 1920, Davidson and Willaman 1927) and functions in regulating intercellular adhesion (Dreyer and Campbell 1984, Willats et al. 2001). Thus, pectin affects the rate of stylet penetration, and its degradation allows more-or-less unimpeded intercellular stylet movement (Saxena and Chada 1971, Dreyer and Campbell 1984). Degradation of pectin may also induce cell wall rupture, presumably allowing ingestion of cellular constituents, although enzymes other than/in addition to pectinases may be involved in this respect (Cole et al. 1998). Aphid species that utilize grasses as host plants may face further problems in that the chemical structure of primary cell walls, the nature of the pectin matrix, and the types of structural cross-linking molecules in grasses differ from those of all other flowering plants, including other monocots (Carpita 1996). Pectinase activity has been

demonstrated in a variety of plant feeding insects including *Deraeocoris nebulosus* (Uhler) (Boyd et al. 2002), *Sirex cyaneus* F. (Kukor and Martin 1983), *Sitophilus oryzae* (L.) (Shen et al. 1996, 1999), *Lygus rugulipennis* Poppius (Laurema et al. 1985), *Anthonomus grandis* Boheman (King 1972), and greenbug (Ma et al. 1990). Pectinase has also been used *in vitro* to isolate nucleic acids from cellular contaminants (e.g., Rogstad et al. 2001).

Dreyer and Campbell (1984) demonstrated that the degree of methylation of plant pectin is associated with resistance to pectinase activity of greenbug saliva. They reported that although levels of polygalacturonase (a common aphid pectinase) were identical in greenbug biotype C and E saliva, levels of pectin methylesterase were twice as high in E saliva as in C saliva. Additionally, saliva from biotype E greenbug hydrolyzed pectin from a biotype C resistant sorghum (high in methylated pectin) twice as rapidly as that of biotype C (Dreyer and Campbell 1984). The authors concludes that the interaction between biotype saliva and pectin is an important component of plant resistance to greenbug, and suggested that manipulation of such interactions through plant breeding or growth regulators would be beneficial. Thus, identification of wild sorghum germplasm exhibiting high methylated pectins potentially may be used in future sorghum breeding efforts (cf., Gurney et al. 2002).

The quality of ingested plant fluids may have profound influence on a variety of aspects of aphid development and reproduction (Klingauf 1987a, Srivastava 1987, Awmack and Leather 2002). Early research on factors associated with the development and timing of appearance of different morphs identified abiotic and biotic factors,

including plant characteristics, associated with alate production in aphids (e.g., Kelly 1913, Marcovitch 1923, 1924, Ewing 1925, Shull 1938). This research sparked a vast literature on aphid biology in relation to food quality, culminating in more recent interest in the effects of aphid feeding on host plant quality. Although resistance had been identified in pea (*Pisum sativum* L.) to pea aphid (*Macrosiphum pisi* Harris) during the 1930s (e.g., Searls 1932, Harrington and Searls 1940, Harrington 1941), the mechanisms behind it were unknown. Searls (1932), however, noted a correlation between the yellow coloration of certain varieties and resistance to pea aphid. Further, Harrington and Searls (1940) suggested that yield may not be a reliable measure of the plant resistance to insect attack, and "physiological maturity" had a large influence on the ability of a plant to recover from insect damage.

In 1951, Maltais reported that susceptible pea varieties had higher whole plant total nitrogen and amino acid content than resistant varieties. This study also revealed that pea aphid colonies grown on susceptible varieties were three times more numerous than colonies grown on resistant varieties over the nine years of the study. In a related study, Auclair and Maltais (1950) noted that the susceptible pea variety "Perfection" contained higher concentrations of amino acids than the resistant "Champion of England" variety, and pea aphid colonies in these experiments exhibited similar growth rates as reported by Maltais (1951). Contemporaneously, Kennedy et al. (1950) and Ibbotson and Kennedy (1950) reported increased development and reproduction, as a result of increased host nutritional quality, in *Myzus persicae* (Sulz.) and *Aphis fabae* Scop. colonies feeding on senescing tissue. These studies were among the first to

identify the relationship among plant nitrogen content, colony growth rates, and plant susceptibility to aphid feeding.

Not until the late 1980s, however, was it hypothesized that an aphid species' ability to cause plant damage improved the nutritional quality of the ingested plant matter. Dorschner et al. (1987) reasoned that greenbug damage to susceptible wheat varieties induced a condition similar to senescence that may increase the nitrogen content of damaged tissue. Indeed, analysis of greenbug damaged plants tissues revealed significantly higher free amino acid concentrations than tissues that did not incur damage. Plant damage occurred only when biotype E fed on wheat, but colonies of biotype C grown on previously damaged plants had significantly larger colony sizes and other developmental measures than C colonies not benefiting from damaged plants. These researchers concluded that the ability of some greenbug biotypes to induce plant damage ("virulence") may enhance the nutritional quality of the food. Other research has corroborated plant damage as a means of nutritional enhancement (e.g., Behle and Michels 1993, Burd et al. 1996, Telang et al. 1999, Burd 2002). Aphids apparently have a mechanism to enhance nutrition in addition to the action of symbiotic bacteria. For example, Telang et al. (1999) showed that wheat damaged by Russian wheat aphid (*Diuraphis noxia* Mordvilko) had higher levels of essential amino acids in phloem fluids relative to resistant plants. The authors concluded that nutritional enhancement via plant damage may have relaxed some constraints of amino acid production by *Buchnera*, and resulted in the lower number of certain genes for amino acid biosynthesis observed in *Buchnera* associated with Russian wheat aphid.

Constitutive and induced plant defense

Plants are continually exposed to pathogen and herbivore attack without the luxury of physical escape, yet each plant species is host to only a few of thousands of potentially harmful organisms (Heath 1981). Plants have evolved physical and chemical defenses that allow them to combat would-be attackers (Ouchi and Oku 1981). Characterization of these defenses began in the early 20th century, when plant breeders recognized the Mendelian inheritance of disease resistance in many plants (Staskawicz et al. 1995). Ward (1901, 1902) and Gibson (1904) provided some of the first evidence of plant resistance to fungal pathogens, especially the phenomenon that would later be termed the "hypersensitive reaction." The hypersensitive reaction is characterized by infection of a few cells of the plant tissue receiving the inocula, followed by the death of these cells and those immediately surrounding the infected cells. In this manner the growth and subsequent infection of other host cells is reduced or halted altogether (Ward 1902, Gibson 1904).

Gibson (1904) noted that Uridineae hyphae penetrating plant tissue were dead and shriveled, while hyphae remaining outside the plant were still living. This was attributed to "some poisonous substance emitted by the cells" (Gibson 1904, p. 186). It was clear, then, that the interaction was eliciting a response in the plant that, in some cases, was inhibitory to the pathogen. Stakman (1915), in the study of *Puccinia graminis* Pers. on cereal crops, reported a particularly distinctive reaction in certain combinations of pathogen and resistant hosts: the fungus was initially capable of infecting host tissue, however, infected cells died shortly thereafter, suppressing fungal

growth. The rapidity with which resistant host's cells died in response to pathogen invasion was indicative to the author of the host plant's level of resistance. Stakman (1915) called this phenomenon "hypersensitiveness," which is now referred to as the hypersensitive reaction.

Two broad types of defense systems are now known to exist in plants, constitutive and induced defenses (Staskawicz et al. 1995). Although each system is fundamentally different from the other, each plant cell is capable of defending itself through a combination of the two systems. The constitutive, or "background" defense system involves inhibition of pathogens and herbivores by pre-formed toxic compounds in or on the plant prior to infection/feeding, or inert chemicals that are rapidly made toxic following infection/feeding (Kelman and Sequeira 1972). These compounds include proteinase inhibitors (Green and Ryan 1972, Van Alstyne 1988, Gatehouse et al. 1993), phenolic (resorcinolic) lipids (Kozubek 2001), nicotine (Tomizawa and Casida 2003), terpenoids, indole, and other volatiles (Pichersky and Gershenzon 2002), leaf surface resins (Hare 2002), protocatechuic acid and other phenols (Wood 1972), lipopolysaccharide (Keyu 2002), and a wide variety of other compounds. Because constitutive compounds are constantly produced, plants relying on constitutive defense may be incurring higher physiological costs (Adler and Karban 1994). Mathematical models suggest that constitutive defenses are favored when herbivore pressures are relatively constant, or when multiple herbivores attack and defense cost is high (Adler and Karban 1994).

Constitutive defense compounds vary in their toxicity to herbivores, such that some compounds (e.g. alkaloids) are highly toxic at low concentrations to most herbivores while others (e.g. tannins) are "dosage-dependent," leading to detrimental effects only after ingestion of large concentrations. Dosage-dependent compounds are referred to as "quantitative," while highly-toxic, low-dosage compounds are "qualitative" (Feeny 1976). Further, some plant species rely primarily on quantitative defenses while others rely on qualitative. This dichotomy has lead to the theory of "plant apparency" (Feeny 1976). Plant species differ in their apparency to herbivores based on the probability that a plant will be encountered by all herbivores: those easily encountered are "apparent" and those not so are "unapparent" (Feeny 1976). Apparent plants allocate a larger proportion of their metabolic resources to defense and utilize primarily quantitative compounds whereas unapparent plants rely primarily on qualitative compounds. Quantitative compounds are associated with low nutritive value, serve to reduce the growth rate and fitness of herbivores, and are not readily susceptible to counteradaptation (Feeny 1976). In contrast, qualitative compounds are detrimental to most herbivores by being highly toxic, yet they are susceptible to counteradaptation by a small number of herbivorous species (Feeny 1976). Some empirical studies have supported plant apparency theory (e.g., Johnson and Bentley 1991).

Induced defense mechanisms are those changes in the plant in direct response to infection/feeding that require some time before the effects are observable (Karban and Myers 1989). Some inducible resistance mechanisms may increase resistance by reducing the preference of the plant to the pathogen or insect (Karban and Myers 1989).

Perhaps the most well known of the inducible mechanisms is the hypersensitive reaction, in which cellular death occurs due to infection or herbivore feeding. Although a plethora of literature exists on induced defenses against pathogens, relatively less attention has been given to those against insects. Further, much of the work directed at induced insect defense has focused on those species that cause extensive plant damage, such as chewers and miners (Stotz et al. 1999, Walling 2000, Moran et al. 2002). Recently, there has been an increasing interest on the effect of phloem feeding on induced defense (e.g., Moran et al. 2002). Phloem feeders (Aleyrodidae, Aphidoidea, Cicadellidae, Psuedococcidae, and Psylloidea) are "perceived" as pathogens by host plants because the mechanics of their feeding is relatively unobtrusive (e.g., compared to a chewing herbivore), and thus elicit plant signaling pathways characteristic of pathogen defense, such as the jasmonic acid/ethylene-dependent, the reactive oxygen species/nitric oxide-dependent, and the salicylic acid-dependent (Walling 2000). Of particular importance for plant defense appears to be the jasmonate-signaling pathway, as plants deficient in jasmonate have reduced direct (towards herbivores) and indirect (natural enemy attraction) defenses (Thaler et al. 2002).

The products of defense pathways direct the expression of pathogenesis-related genes (Walling 2000). Pathogenesis-related proteins may directly interact with intruding organisms, however their function(s) and those of related proteins involved in induced defense remain unknown (Walling 2000). Other compounds such as aliphitic polyamines (Torrigiani et al. 1997), hyperforins, and hypericins (Sirvent and Gibson 2002) have been shown to accumulate in response to pathogen stress and are important

components of the hypersensitive reaction. The hypersensitive reaction in plants is considered to provide an effective means of resistance to pathogens (Canter and Jaworski 1979), and may influence the biology and/or behavior of a variety of vertebrate and invertebrate herbivores (Freeland 1974, Freeland and Janzen 1974, Ernest 1994, Bolser and Hay 1998).

In addition to localized defense induction, pathogens and herbivores may elicit a systemic acquired resistance, which activates defense mechanisms in plant parts remotely located from the actual infection/feeding (Métraux et al. 2002). Recognition of systemic acquired resistance began in the early 1900s when workers identified the ability of plants to display resistance to subsequent attack after an initial viral infection (e.g., Chester 1933). Endogenous elicitors of systemic acquired resistance include those that induce the hypersensitive reaction, such as salicylic acid (Glazebrook 2001, Métraux et al. 2002), jasmonic acid (Walling 2000), ethylene (Glazebrook 2001), and others. Exogenously applied chemicals may also induce systemic acquired resistance, particularly isonicotinic acid (Ryals et al. 1992), which has been used in pesticide manufacture. The cascade of events leading to systemic acquired resistance involves a large number of elements, many of which remain unknown (Métraux et al. 2002). Further, the signal transduction associated with regulation of systemic acquired resistance is not likely to be a linear chain of events (Métraux et al. 2002). Investigations of systemic acquired resistance against pathogens suggest that plant resistance genes (*R*) capable of recognition of pathogens carrying avirulence genes (*avr*),

known as gene-for-gene resistance (see below), are involved in the response (Glazebrook 2001).

Plant resistance: definitions, mechanisms, and applications

The concepts and techniques of plant resistance to insect attack received considerable attention beginning in the late 19th century, and in particular more recently in concert with other tactical components of integrated pest management (IPM) strategies (e.g., Empson et al. 1968, Lewis et al. 1997, Deol et al. 2001). Current plant resistance tactics were undoubtedly heavily influenced by the dramatic early success against grape phylloxera, *Phylloxera vitifoliae* (Fitch), in European grapevines during the 1880s. Grape phylloxera, native to North America, was introduced into France sometime prior to 1863 (Essig 1929). By 1884 it infested nearly half (2.5 million acres) of the vineyard acreage in France (Essig 1929, Granett et al. 1985) and had the French wine industry on the brink of collapse (Kogan 1994). Subsequent introduction of North American rootstocks resistant to grape phylloxera was extremely effective in managing the pest, such that by 1891 those areas where resistant vines were used necessitated fewer chemical treatments than others (Riley 1891).

Because the last several decades have seen a rise in resistance of insect pests to chemical insecticides (e.g., Georghiou 1972, Brattsten et al. 1986, Gatehouse et al. 1993, Heckel et al. 1999), and increasing concern over environmental and other non-target impacts of insecticides (e.g., Keith 1966, Ratcliffe 1970, Powell 1984, Newton and Wyllie 1992, Cairns and Niederlehner 1996), plant resistance has gained favor as a beneficial and environmentally-safe alternative to pest management strategies based on

chemical control (Teetes 1992). Current thinking in crop pest management seeks to emphasize biological control and plant resistance tactics to keep pests within acceptable bounds and de-emphasizes therapeutic use of chemical agents (Lewis et al. 1997). This is especially true in low value crops such as sorghum where numerous insecticidal treatments are not economically sustainable (Flattery 1982). Resistance in certain sorghum varieties to greenbug remains the most cost effective control (Porter et al. 1997, Hays et al. 1999).

Resistance to insects in crops has been defined as "the relative amount of heritable qualities possessed by the plant which influence the ultimate degree of damage done by the insect" (Painter 1951, p. 15). A more inclusive definition was offered by Beck (1965, p. 208): "plant resistance is…the collective heritable characteristics by which a plant species, race, clone or individual may reduce the probability of successful utilization of that plant as a host by an insect species, race, biotype, or individual." Wood (1972, p. 213) provided a definition of disease resistance, which is useful in the current context particularly in regard to plant damage: "…resistance can be regarded as the properties of the plant that prevent the agent from growing on it, and which prevent the plant from becoming damaged by the agent, its metabolic products, or by both." In the practical sense plant resistance is measured by the ultimate output of a crop plant in relation to another variety: resistant plants are able to produce a larger crop than a more susceptible variety at the same infestation level of the insect pest (Painter 1936).

The most extreme case of plant resistance is immunity, which refers to a plant variety that an insect species will never consume or injure under any condition (Painter

1936, 1951). Painter (1936) claimed that such immune relationships do not exist, although there are cases in which the interaction between insect and plant approach immunity. There are, on the other hand, innumerable cases in which a plant is considered a "non-host" of some organism (Heath 1981). In this case the plant does not develop visible symptoms of damage. Resistance in crops, therefore, is relative in terms of the performance of one plant variety against that of the susceptible or commonly grown variety (Painter 1936). This qualification allows for the possibility of extensive injury to a normally resistant variety under heavy insect infestation or other extreme situations.

Painter (1941, 1951) identified three main mechanisms of plant resistance to insects: a) antibiosis, b) preference/non-preference, and c) tolerance. Kogan and Ortman (1978) proposed the term "antixenosis," meant to convey a property of the plant that makes it an unsuitable host and thus avoided by the pest, to replace Painter's non-preference modality of resistance. Since proposed, antixenosis seems to be the preferred term. Additionally, Beck (1965) recognized "pseudoresistance" as instances of "phenological evasion, induced resistance, and other examples of transitory or incidental resistance" (p. 208). Further, literature regarding plant resistance to disease is replete with definitions, many of which seem contradictory or incompatible (see Browning 1980). The work presented herein addresses antibiosis, antixenosis, and tolerance in sorghum varieties against greenbug.

Antibiosis. This modality of plant resistance is characterized by adverse effects directly on the insect's life history as a result of feeding on a resistant plant variety

(Painter 1941, Kogan 1994). These effects include reduced fecundity and fertility, decreased size of the adult insect, abnormal life span, extended pre-reproductive period, and increased mortality rates. Antibiosis towards insects may often be indistinguishable from severe antixenosis, as the inhibition of feeding may result in starvation (Kogan 1994).

Antixenosis. Antixenotic plant mechanisms are those that deter the insect pest from feeding, colonizing, or ovipositing on the host, or some other manipulation of the behavior of the insect, which keeps it from utilizing the plant (Painter 1951). The basis of antixenosis is the modification of insect behavior (Painter 1951), where an "allelopathic relationship" is established at the insect's sensorial level (Kogan and Ortman 1978). A variety of physical and chemical cues may be involved with this form of resistance, including color or intensity of light, contact surfaces of the plant, and plant chemical constituents (Painter 1951). Due to the nature of the plant characteristics involved, this may be the easiest modality to modify genetically in resistance breeding programs (Painter 1951).

Tolerance. Tolerance is displayed by a plant that is capable of compensatory re-growth and maintenance of fitness in the face of tissue loss and other stresses, relative to that of the undamaged state (Painter 1951, Stowe et al. 2000). While herbivore damage is generally detrimental to plant fitness, tolerant plants compensate for plant damage with little or no loss in fitness (Stowe et al. 2000, Pilson and Decker 2002). Only recently have evolutionary ecologists focused on the evolution of tolerance as a response to herbivore damage, and these studies typically focus on operational definitions of

tolerance rather than the underlying mechanisms, although a number of mechanisms have been proposed (Pilson and Decker 2002, and references therein).

Knowledge of the biogeographical history of insect/plant associations may help in the identification and utilization of plant resistance against insect pests (Harris 1975, Harris and Frederiksen 1984). It is generally assumed that sources of plant resistance against pests are most likely to be found in plant germplasm from the aboriginal home of a crop, as this is likely where the greatest genetic diversity is found (e.g., Singh 1986, Hoisington et al. 1999). This approach may assume, at least in part, an evolutionary history between the progenitors of the crop plant and the pest. Insects may also become pests upon plant species with which they share no evolutionary history. One well-documented example of a novel association resulting in pest status of native insects to an introduced crop is apple in the Eastern U.S. and its host of native insect pests (e.g., Harris 1975, Feder and Bush 1989). Resistance mechanisms identified in such systems would therefore obviously not be a result of shared evolutionary history, a situation which Harris (1975) has termed "allopatric resistance." Harris (1975) notes that allopatric resistance has major potential advantages over sympatric resistance, or resistance exhibited towards co-evolved pests: a) it may be polygenic and disrupt the insect's biology in numerous ways, b) it may benefit the plant's strategies for survival, and c) hybrid vigor should be expected in such plant species.

Overcoming host plant resistance

Plant resistance may influence insect herbivores in ways similar to insecticidal treatments (Gould 1984, Castillo-Chavez et al. 1988). Specifically, the use of plants

exhibiting a combination of antibiosis and antixenosis has effects on insect behavior analogous to the use of repellent insecticides (Gould 1984). As a result, insects may adapt to these resistance mechanisms via physiological or behavioral means (Castillo-Chavez et al. 1988). Therefore, deployment of antibiotic crop varieties based on expression of a single, major gene (e.g., Eisenbach and Mittler 1987, Wilhoit and Mittler 1991, Lazar et al. 1995) may drive the development of insect biotypes, including greenbug. Although empirical evidence supporting this view is not available, simulation models suggest that pure stands of a resistant variety can lead to evolution of pest insects adapted to a crop (e.g., Gould 1983). Alternatively, Porter et al. (1997) reviewed the history of greenbug biotype development and suggested that virulent biotypes evolve regardless of minimal selective pressure from resistant sorghums.

Interactions between insect herbivores and plants are exceedingly uncommon given the potential based on numbers of insect and plant species (Harris 1980). In nature, insect/plant interactions are regulated by a complex of genes in plants and insects that may optimize the fitness of one or both players via an evolutionary arms race (e.g., Dawkins and Krebs 1979). Insect herbivores may select for resistance in plants, given that phytophagous insects reduce the fitness of the plants they damage (Jermy 1984). In addition, genetic diversity in plants and their herbivores may be quite extensive in nature, allowing for a large number of possible interactions among species (e.g., Browning 1980). In agriculture, insect/plant interactions are often explained by gene-for-gene interactions. Gene-for-gene interactions, first espoused by Flor (1956; see below) involve selection of a resistance gene in a crop variety followed by the evolution

of a gene in the pest to overcome the resistance. Gene-for-gene interactions, best described in plant/pathogen systems (see De Wit 1997, Hulbert et al. 2001 for reviews), provide an explanation of how plant parasites can adapt to and overcome plant resistance in agricultural crops.

Flor (1956) developed the concept of the gene-for-gene relationship over several decades of research on flax, *Linum usitatissimum* L., and flax rust, *Melampsora lini* (Pers.) Léveillé. The initial impetus leading to the discovery of the gene-for-gene system was the identification, development, and deployment of flax varieties resistant to flax rust. It was quickly realized, however, that resistance was ephemeral as flax rust either evolved new races or changed the prevalence of existing races that were capable of overcoming the resistance mechanisms of the deployed flax varieties (Flor 1956). The resistance or susceptibility of a plant, the virulence or avirulence of the pathogen, and the interaction between the two was hypothesized to be a complex process involving the genetic and cytoplasmic complexes of the host and pathogen, plus the effects of the environment on each organism separately and in combination (Flor 1956). Since this early seminal work, a vast amount of literature has been devoted to identification of gene-for-gene systems in many crops.

The most basic tenet of a gene-for-gene interaction is that resistant plants carry a particular resistance (*R*) gene, the protein products of which are capable of recognizing parasite avirulence (*avr*) gene products (Hammond-Kosack et al. 1996, Glazebrook 2001). This recognition — genetic incompatibility (Hammond-Kosack et al. 1996) — triggers defensive responses in the plant (Glazebrook 2001). When a gene evolves

allowing some individuals to overcome plant resistance, the gene is virulent towards the otherwise resistant plant (e.g. Harris and Frederiksen 1984). Protein products of virulence genes presumably are not recognized by the host — genetic compatibility (Hammond-Kosack et al. 1996) — and disease or damage ensues. Monogenic (single gene) resistance is considered more vulnerable to parasite virulence than polygenic (multi-gene) resistance (Harris and Frederiksen 1984), as the probability of acquiring one mutation is higher than that of acquiring multiple mutations (Gould 1983).

Fitness costs associated with plant resistance and parasite virulence in concert determine allele frequencies and maintain genetic variation of the two traits (Simms and Triplett 1994). Individuals carrying genes capable of overcoming a stress (e.g., antibiotics, insecticides, plant resistance) typically incur a fitness cost, relative to the fitness of the wild-type individuals, in the absence of the stress (e.g., Morell 1997). Much of the literature on impacts of insecticide resistance in insects report fitness costs in the absence of the insecticide (e.g., Minkoff and Wilson 1992, Scott et al. 1997, Jinfu et al. 1998, Gazave et al. 2001, Berticat et al. 2002). Plant resistance may elicit similar effects. For example, the flea beetle *Phyllotreta nemorum* L. exhibits a similar fitness cost in the absence of plant resistance stress: individuals homozygous for genes conferring virulence towards *Barbarea vulgaris* (Opiz.) Simkovics (Brassicaceae) suffer a fitness reduction relative to their susceptible conspecifics when larvae are raised on the susceptible host *Raphanus sativus* L. (De Jong and Nielsen 2000). In contrast, populations of streptomycin-resistant *E. coli* have been reported to maintain fitness levels comparable to or exceeding that of the wild-type in the absence of streptomycin

(Morell 1997). The streptomycin-resistant populations carry a second gene, in addition to the gene conferring antibiotic resistance, which renders them competitive in the absence of the stress. Nonetheless, available evidence suggests that, with few exceptions, resistance to a stress comes at a fitness cost in the absence of the stress.

CHAPTER III

GENERAL MATERIALS AND METHODS:

GREENBUG STOCK CULTURES

Stock cultures of greenbug used in the present studies were initiated in April 1999 in a greenhouse at the Entomology Research Laboratory, Texas A&M University, College Station, Texas. Between 50 and 100 adults and nymphs of each of 8 biotypes, B, C, E, F, G, H, I, K, and 3 isolates, New York (NY), Canadian Wild Rye (CWR), and South Carolina (SC), were received in Petri dishes with small amounts of plant material and damp paper napkin from USDA-ARS, Stillwater, Oklahoma. Biotypes B, C, E, F, G, H, I, and K are reviewed in Porter et al. (1997). Biotypes C, E, I, and K are commonly associated with grass crops and have been referred to as the "agricultural biotypes" (Shufran et al. 2000). The NY isolate is biotype A-like in its host relationships (J. D. Burd, personal communication), and is phylogenetically similar to biotypes F and G in certain mitochondrial DNA sequences (Shufran et al. 2000). Biotypes F, G, and the NY isolate are reported to be extremely virulent of wheat and sorghum, but usually do not occur on these crops (Shufran et al. 2000). CWR isolate is described as a host adapted race on non-cultivated grasses (Shufran et al. 2000, Anstead et al. 2003). It is unclear whether the SC isolate represents a previously described biotype.

Aphid biotypes and isolates were enclosed separately in 22 cm diameter × 20 cm tall pots with approximately 20 – 30 sorghum plants (variety ATx399 × RTx430) between the 3rd and 4th growth stage (Vanderlip 1972, Vanderlip and Reeves 1972).

Fig. 2. Pot and enclosure used to maintain greenbug stock cultures. Each biotype was maintained separate from others. Plastic enclosure holes are covered with sheer mesh for ventilation. Small amounts of plant material from waning cultures were transferred to fresh pots when needed.

Each pot was covered with a cylindrical, nitrocellulose plastic cage to prevent contamination of the plant material by other greenbug biotypes or natural enemies (Fig. 2). The plastic cages had two large holes cut out of the sides and covered with sheer curtain liner material to provide ventilation. Additionally, curtain material was used to enclose the top, allowing for further ventilation and water evaporation. Pots were monitored daily to determine whether the insects had established on the plants and had begun reproduction. All biotypes had established and begun reproducing on sorghum within two weeks. Small amounts of plant material with a few aphids were removed from waning cultures and transferred to fresh pots as needed. In addition to this stock culturing method, additional cultures were initiated by placing one adult female of each biotype in a 22 cm diameter × 20 cm tall pot with 10 – 12 sorghum plants (ATx399 × RTx 430) in an attempt to develop clones. Most of these attempts failed, and initial cultures consisted of colonies started from at least 50 greenbugs/pot. Once initiated, clones were easily produced from individual females of all biotypes.

At least twice yearly, differential virulence tests were conducted on biotypes virulent on sorghum (C, E, and I) to ensure the purity of these cultures. Plant damage reactions allowed the identification of "unknown" biotypes based on plant resistance as follows: Tx7000 susceptible check (Teetes et al. 1974), Tx2737 resistant to C (Peterson et al. 1984), Tx2783 resistant to C and E (Peterson et al. 1984), and PI550607 resistant to C, E, and I (Andrews et al. 1993). Biotype K, virulent on sorghum, was not tested as PI550607 exhibits low levels of resistance (Katsar et al. 2002), and plant damage is highly variable (G. L. Teetes, personal communication). Twelve 22 cm diameter × 20

cm tall pots (i.e., four replications per biotype) were planted with 4 – 6 seeds of each genotype in a radial pattern and labeled with plastic markers to ensure the identity of plants following germination. When plants reached the three-leaf stage (three true leaves plus the cotyledon), each pot was thinned so only one plant of each genotype remained. At this point approximately 100 adult and nymphal greenbugs (the "unknown") were introduced to the pots directly on the soil in the middle of the circle of plants, and pots were covered with plastic enclosures. Each pot received equal numbers of insects. Replications were then maintained until the susceptible check (Tx7000) was rated at 7 or higher on a standard 9-point damage rating scale, modified from the greenhouse seedling evaluation technique (10 point scale) of Johnson et al. (1976), where 1 = 0 – 10, 2 = 11 – 20, to 9 = 81+% visible plant damage. At this time all plants within a pot were rated.

Since the resistance reactions of the sorghum genotypes were known, the differential tests were expected to yield results that would correctly identify the unknown biotype used in the test: 1) Tx7000 high damage, others low = biotype C; 2) Tx7000 and Tx2737 high damage, others low = biotype E; 3) Tx7000, Tx2737, and Tx2783 high damage, PI550607 low = biotype I. In all differential tests, the unknown biotypes were correctly identified by damage reactions of sorghum genotypes (data not shown) and the cultures were deemed pure. Differential tests of the other biotypes were not possible as the plant varieties used to identify them were not available. However, since the same practices were used to maintain all biotype stock cultures, those not subjected to differential tests were assumed to be pure.

During the summer of 2000, greenhouse temperatures exceeding 48^{o} C caused the loss of biotype H and the CWR isolate, which were not recovered. Subsequently, stock cultures were relocated to an area inside the Entomology Research lab along a windowsill, and were kept there throughout the remainder of the study period.

CHAPTER IV

PRELIMINARY IDENTIFICATION OF RESISTANCE MECHANISMS IN SORGHUM TO GREENBUG (HOMOPTERA: APHIDIDAE) BIOTYPES

Virulent aphid biotypes are generally regarded as comprised of numerous clones (populations derived from one parthenogenetic female) capable of feeding on and damaging plants resistant to other clones (e.g., Claridge and Den Hollander 1983). This is consistent with the view that biotypes are recognized by function, such as differing physiological effect on the host plant, rather than morphology (Eastop 1973). A widespread view of biotype evolution is that introduction of new forms of plant resistance causes the evolution of new virulent biotypes (e.g., Eastop 1973), particularly when resistance sources are single gene antibioses (Eisenbach and Mittler 1987, Wilhoit and Mittler 1991, Lazar et al. 1995). Porter et al. (1997), however, provided evidence suggesting that greenbug biotypes develop despite minimal selection pressure from resistant sorghum and wheat varieties. They concluded that biotypes are preexisting opportunists with substantial genetic variability, and future breeding efforts should not hesitate to deploy cultivars with single gene, antibiotic resistance sources (Porter et al. 1997).

The majority of greenbug biotypes that have been identified to date are based on differential damage incurred by wheat (Table 1; reviewed in Porter et al. 1997). These biotypes may have the ability to infest and damage other small grains, sorghum, or uncultivated grasses. Biotypes E, I, and K are currently the populations that

Table 1. Greenbug biotypes in the U.S., excluding biotype D[a], their hosts, and year they were first reported.

Biotype	Host	Author/Year
A	Wheat	Wood (1961)
B	Wheat	Wood (1961)
C	Sorghum	Harvey & Hackerott (1969)
E	Wheat	Porter et al. (1982)
F	Canadian Bluegrass	Kindler & Spomer (1986)
G	Wheat	Puterka et al. (1988)
H	Wheat	Puterka et al. (1988)
I	Sorghum	Harvey et al. (1991)
J	Wheat	Beregovoy & Peters (1994)
K	Sorghum	Harvey et al. (1997)

[a] Biotype D (Teetes et al. 1975) refers to a population resistant to organophosphates, thus is not considered in the scope of host plant resistance mechanisms as are the other greenbug biotypes.

predominate on wheat and sorghum (Webster and Porter 2000).

While it is likely that all greenbug biotypes are capable of utilizing most, if not all, recorded host plants (Michels 1986), certain biotypes may be better suited for certain hosts. This has led some researchers to suggest that these biotypes may be "host races" (e.g., Shufran et al. 2000). The terms "host race" and "biotype" are not clearly distinguished in most of the literature (however see Diehl and Bush 1984) and their use adds to the existing confusion surrounding what constitutes a biotype, host race, strain, etc. (Claridge and Den Hollander 1983, Diehl and Bush 1984). The use of the term "biotype" to represent greenbug populations capable of overcoming plant resistance mechanisms dates to the early 1960's when Wood (1961) named the first two greenbug biotypes (A and B) based on the ability to overcome resistance in wheat: biotype B caused damage to a wheat variety resistant to biotype A. Although the use of "biotype" in reference to insect populations with the ability to overcome certain stresses has been criticized (e.g., Eastop 1973), it is useful to the extent that it allows the identification of crop varieties that are resistant to greenbug damage (e.g., Katsar et al. 2002).

Research on identification of sorghum resistance to greenbug began after 1968 when biotype C, the original sorghum biotype, was identified (Harvey and Hackerott 1969, Teetes and Johnson 1973). Greenbug had rarely caused economic damage on sorghum prior to 1968, although it had the ability to utilize it (Harvey and Hackerott 1969). Biotype C was predominant in sorghum fields until the early 1980s when it was replaced by the newly recognized biotype E (Porter et al. 1982). Biotype I developed subsequently and has been the most prevalent biotype in sorghum since 1995 (Harvey et

al. 1999). The status of biotype K in crop fields is unclear; however, it was present in some areas in at least a small proportion of samples taken in the late 1990s (Harvey et al. 1999).

A substantial amount of work has been done towards sorghum resistance to greenbug biotypes, but most to date have dealt with biotypes C, E, I, and K because these are commonly associated with cultivated sorghum (Katsar et al. 2002) and other grass crops, and have been referred to as the "agricultural biotypes" (Shufran et al. 2000). Studies on all known biotypes suggest that some may be host-adapted races on non-cultivated grasses (e.g., Shufran et al. 2000), and their role in the evolution of new biotypes is questionable. The present study addressed sorghum resistance mechanisms towards most of the named greenbug biotypes and several isolates.

Materials and methods

Experimental design. Choice tests were initiated by planting 3 – 4 seeds each of four sorghum genotypes in a radial pattern in 25 cm pots, each pot receiving all genotypes. The sorghum genotypes used, and their resistance, were: Tx7000 susceptible check (Teetes et al. 1974), Tx2737 resistant to C (Peterson et al. 1984), Tx2783 resistant to C and E (Peterson et al. 1984), and PI550607 resistant to C, E, and I (Andrews et al. 1993). Greenbug biotypes B, C, E, F, G, H, I, K were used. Three isolates (populations acquired from specific hosts or geographic areas) were tested: Canadian Wild Rye (CWR), New York (NY), and South Carolina (SC). Small plastic stakes marked with the genotype designation were placed in the center of the pot so that as plants germinated their identity would be known. Pots were covered with plastic cages as described for the

stock culture procedure, watered as needed, and maintained in a greenhouse. Experiments were conducted during the summer and winter, during which temperature varied between 20 – 31^{o} and 29 – 41^{o} C, respectively. Four replications of each biotype/genotype combination were conducted for both winter and summer conditions. Each replication consisted of one plant of each genotype per pot.

Pots were thinned to one plant per genotype per pot when plants reached the 3-leaf stage between 12 and 17 cm in height. Approximately 100 greenbug nymphs and adults from stock cultures were placed on the soil in the center of the circle of plants with a camel's-hair brush and were given 24 h to distribute themselves.

Initial aphid counts per plant were taken 1 d post infestation, and subsequently at 2, 3, and 7 d. Greenbugs were counted with the aid of a hand-held magnifying lens. Assessment of plant resistance was achieved using a standard 9 point damage scale where 1 = 0 – 10%, 2 = 11 – 20%, to 9 = 81+% red spotting (modified from Johnson et al. 1976). Damage ratings were recorded at 4, 5, and 6 d post infestation.

Plant resistance tests. Plant resistance mechanisms towards greenbug biotypes were identified on sorghum genotypes Tx2737, Tx2783, and PI550607 relative to the susceptible Tx7000. Antixenotic resistance was defined as a significant decrease in greenbug numbers between 1 and 2 d post infestation. Antibiosis was defined as a significant decrease in greenbug numbers on sorghum genotypes between 2 and 3 d post infestation relative to Tx7000, an estimate of reproductive reduction. Tolerance was determined by analysis of greenbug numbers 3 d post infestation and plant damage 4 d post infestation: genotypes exhibiting similar (non-significantly different) greenbugs but

significantly lower plant damage were identified as exhibiting tolerance relative to Tx7000. Oneway ANOVA for differences in greenbug number on sorghum genotypes within greenbug biotypes were used to test for resistance mechanisms: mean differences among genotypes were separated by Dunnet's pair-wise multiple comparison t-tests (SPSS, Inc. 1999) with Tx7000 as the comparison group.

Results and discussion

Significant differences in greenbug numbers were identified in five greenbug biotypes in at least one sorghum genotype relative to the susceptible (Table 2). PI550607 had significantly fewer aphids of biotypes C, F, H, and the NY isolate (Table 2). Tx2783 was antixenotic towards F, H, and the NY isolate; Tx2737 displayed antixenosis towards biotype F (Table 2). Tx2737 displayed antixenosis towards biotype F (Table 2). When faced with an early and large infestation of greenbug, seedling sorghum may benefit most from antixenosis as the reduction in insects colonizing the plant may result in reduction in plant damage. Reduction in early damage from antixenotic plant resistance could allow phenological escape, as sorghum may incur heavier yield losses when damaged early (developmentally) and for longer periods of time (Teetes and Johnson 1974).

PI550607 had fewer greenbugs of biotypes B, C, G, and the NY isolate between 2 and 3 d post infestation relative to Tx7000. Tx273 displayed antibiosis towards biotypes E and H; Tx2737 was antibiotic towards biotypes B and E

Table 2. Mean difference in greenbug numbers between 1 and 2 d post infestation as a measure of plant antixenosis relative to Tx7000[a].

Biotype	Tx2737	Tx2783	PI550607	*N*
C	–	–	31.3 (0.05)[b]	32
F	23.1 (0.01)	22.0 (0.01)	20.6 (0.03)	29
H	–	24.9 (0.02)	22.9 (0.04)	32
NY	–	25.1 (0.02)	25.2 (0.02)	32

[a] Entries with "–" represent no antixenosis identified.

[b] Numbers in parentheses are p values associated with significant differences in greenbug numbers, Dunnett's pairwise multiple comparison t-test.

(Table 3). While antibiotic plant resistance has been suggested to lead to insect biotype evolution (e.g., Gould 1984), some greenbug biotypes appear to be preexisting within the genetic variability of natural populations (Porter et al. 1997). Antibiotic resistance in sorghum, then, should not lead to biotype evolution *per se*, particularly if coupled with other resistance mechanisms in a genotype. Only biotype B was associated with antibiosis alone, in Tx2737 and PI550607 (Table 3).

Tolerance was identified towards five biotypes and two isolates in at least on sorghum genotype relative to the susceptible (Table 4). Tx2737 displayed tolerance towards four biotypes and the two isolates, followed by Tx2783 (four biotypes, one isolate), and PI550607 (three biotypes, one isolate). Plant damage was relatively low considering some mean colony sizes were rather large (e.g., Tx2737 and biotype C), suggesting plant damage may not be a simple function of colony size. Although tolerant plants may not show visible signs of damage, plants with relatively large greenbug colonies may be damaged through other means, such as fluid loss (see, e.g., Chapter V).

In summary, the sorghum genotypes used in the present study displayed varied resistance mechanisms towards greenbug, which may allow for increased usability of these genotypes, particularly in sorghum breeding programs. Of the greenbug biotypes

Table 3. Mean difference in greenbug numbers between 2 and 3 d post infestation as a measure of plant antibiosis relative to Tx7000[a].

Biotype	Tx2737	Tx2783	PI550607	*N*
B	15.4 (0.05)	–	22.1 (0.005)[b]	32
C	–	–	19.8 (0.04)	30
E	13.8 (0.05)	15.4 (0.04)	–	32
G	–	–	18.6 (0.04)	32
H	–	14.2 (0.04)	–	32
NY	–	–	20.1 (0.03)	32

[a] Entries with "–" represent no antibiosis identified.

[b] Numbers in parentheses are p values associated with significant differences in greenbug numbers, Dunnett's pairwise multiple comparison t-test.

Table 4. Mean plant damage and cumulative greenbug numbers on sorghum genotypes as a measure of tolerance relative to Tx7000[a].

Biotype	Tx2737	Tx2783	PI550607
C	2.1*	1.9*	–
	151.0	140.7	–
E	2.2*	1.0*	1.5*
	98.5	94.8	108.1
G	1.9*	–	–
	99.0	–	–
H	2.4*	1.2*	1.5*
	137.4	109.8	110.1
K	–	1.1*	1.4*
	–	97.6	80.1
NY	1.8*	–	–
	146.9	–	–
SC	2.4*	3.0*	1.5*
	72.2	155.4	71.4

[a] First row of numbers is mean plant damage at 4 d post infestation; second row is mean cumulative greenbug numbers at 3 d post infestation. Tolerance is identified by a significantly lower damage relative to the susceptible (Tx7000), but no difference in greenbug numbers. Entries with "–" represent no tolerance identified.

studied, biotype B and F were associated with only one plant resistance mechanism, antibiosis and antixenosis, respectively (Tables 2 and 3). Antibiosis alone may lead to selection for greenbugs capable of overcoming the resistance mechanism, while antixenosis may select for greenbugs capable of avoiding resistant plants. Future studies, therefore, should identify how particular resistance mechanisms would influence a particular biotype's physiology and/or behavior.

CHAPTER V

CORRELATES OF GREENBUG (HOMOPTERA: APHIDIDAE) BIOTYPIC INTERACTION WITH *Sorghum bicolor* (L.) MOENCH AND *Sorghum halepense* (L.) PERSOON. I: GREENBUG VIRULENCE

Greenbug, *Schizaphis graminum* (Rondani), biotypes can exhibit extreme variability in the damage they cause to certain sorghum, (*Sorghum bicolor* (L.) Moench), genotypes. Genetic evidence suggests substantial variability both between and within biotypes (e.g., Black 1993, Shufran et al. 2000), and such variability is likely involved in the unpredictable interactions between aphid clones and hosts (e.g., Wilhoit and Mittler 1991). Additionally, this variability may be a key factor in the evolution of biotypes capable of overcoming host plant resistance. One theory states that widespread use of pure stands of resistant cultivars, particularly those that influence pest fitness, fuels the evolution of new virulent biotypes (e.g., Wilhoit and Mittler 1991, Ratcliffe et al. 1994, Lazar et al. 1995, Deol et al. 2001, El Bouhssini et al. 2001). Plant resistance may influence insect herbivores in ways similar to insecticidal treatments (Gould 1984, Castillo-Chavez et al. 1988). Specifically, the use of plants exhibiting a combination of antibiosis and antixenosis has effects on insect behavior analogous to the use of repellent insecticides (Gould 1984). As a result, insects may adapt to these resistance mechanisms via physiological changes (Castillo-Chavez et al. 1988). While resistant plants undoubtedly impose at least minimal selection pressure on greenbug populations, some research suggests greenbug biotypes are merely expressions of their underlying genetic variability brought to light by resistant plant varieties (Porter et al. 1997). The

importance of aphid populations capable of overcoming plant resistance lies in the stability of resistant sorghum varieties and their long-term usefulness in the field (Starks and Merkle 1977, Shufran et al. 1997). It is therefore critical to understand how existing greenbug biotypes interact with available plant genotypes to elucidate factors that may be important in allowing biotypic expression, particularly relating to plant damage and greenbug fitness.

The study of parasite virulence places much emphasis upon fitness consequences incurred by the host organism given some parasite load (Ebert and Herre 1996, Poulin and Combes 1999). In agricultural systems the emphasis is placed on realized yield which, in a crop where the seed is harvested, is in direct relation to the fecundity of the plant. For example, in the interaction of greenbug and sorghum, this may ultimately be manifested by yield reduction of the plant variety in question. Certain greenbug biotypes are capable of significantly reducing yield potential of particular varieties relative to non-infested or resistant plants (Teetes 1980, 1992, Dharmaratne et al. 1986, Wilhoit and Mittler 1991). However, the onset of plant damage from greenbug feeding may initiate much earlier and lead to plant death prior to flowering (Burton 1986), thus effectively nullifying host fecundity.

Plant damage from greenbug is characterized by red or brown spotting, chlorosis, and/or necrotic lesions around the area of feeding caused by toxic elements in greenbug saliva (Burton 1986, Dorschner et al. 1987, Behle and Michels 1993, Deol et al. 2001). Greenbug populations capable of causing plant damage on previously resistant varieties are deemed virulent and are designated as new biotypes (Bush et al. 1987, Harvey et al.

1991, Lazar et al. 1995, Kindler et al. 2001). This method of naming greenbug biotypes has practical applications in agricultural systems but is criticized by researchers considering the evolution of parasite virulence, in part because the genetic basis for greenbug virulence is poorly understood. In particular, critics note that the use of terms such as "virulence genes" and "avirulence genes" in describing parasite genomes is faulty in most instances. Poulin and Combes (1999), for example, state that there are no genes in a parasite genome that code for a specific level of host damage. They argue that the only exceptions are cases in which harming the host organism directly facilitates parasite transmission, and these may be rare. In addition, Poulin and Combes (1999) claim it is illogical to speak of avirulence genes in the context of gene-for-gene theory (e.g., Flor 1956) when products of these same genes would be virulent in a host not capable of recognizing them. True genes for avirulence would rapidly be eliminated from the parasite genome (Poulin and Combes 1999).

Virulence is generally defined as the magnitude of the negative effect that a parasite inflicts on its host (e.g., Ewald 1994, Ebert and Herre 1996, Gandon et al. 2002). While this definition is widespread in both medical and agricultural literature, Poulin and Combes (1999) maintain that the study of virulence evolution must incorporate parasite fitness as well as host damage. Many studies on greenbug identify virulence solely as plant damage (e.g., Dorschner et al. 1987, Wilhoit and Mittler 1991, Shufran et al. 2000), although some may include aspects of greenbug fitness (Wilhoit and Mittler 1991). Poulin and Combes (1999) reserve the term "pathogenicity," which is a property of the host-parasite association, to describe damage incurred by a host due to parasite

infection. To avoid confusion with other published reports on greenbug, use of the term virulence in the present study will be used to describe the deleterious effect greenbug has on its host (cf., Gandon et al. 2002, Zhan 2002). The primary goal of the present study was to identify plant characteristics associated with damage incurred via feeding by virulent greenbug biotypes. In a companion study (Chapter VI), greenbug fitness associated with virulence is investigated.

Materials and methods

Experimental design. Virulence tests were conducted for five greenbug biotypes: NY, B, C, E, and I, on four sorghum genotypes and non-cultivated Johnson grass, *Sorghum halepense* (L.) Persoon. The sorghum genotypes were selected as representatives of varieties grown prior to greenbug virulence on sorghum (Tx7000), bred and deployed in response to the identification of biotype C (Tx2737), developed in response to biotype E (Tx2783), and genotypes exhibiting resistance to most greenbug biotypes (PI550607). Sorghum seed was received from Texas A&M Agricultural Research and Extension Center, Lubbock, and Texas Foundation Seed (some genotypes as needed), Texas A&M, College Station, Texas. Johnson grass seed was acquired from field margins and roadsides in and around College Station and Waco, Texas. *A priori* assumptions of sorghum resistance towards biotypes C, E, and I based on published reports were as follows: Tx7000 susceptible check (Teetes et al. 1974), Tx2737 resistant to C (Peterson et al. 1984), Tx2783 resistant to C and E (Peterson et al. 1984), and PI550607 resistant to C, E, and I (Andrews et al. 1993). All sorghum genotypes were assumed to be resistant to NY and B because these biotypes were expressed prior to the

first biotype capable of damaging sorghum, C (Harvey and Hackerott 1969). Since Johnson grass has essentially been wild for over a century, no *a priori* assumptions were made with regard to damage from specific biotypes. However, it was assumed that Johnson grass would incur less damage than sorghum, in general, due to greater genetic variability.

All experiments were conducted in ~52 m^3 environmental chambers housed at the Norman Bourlag Southern Crop Improvement Facility, Texas A&M University, College Station, Texas. Environmental conditions in the chambers were: 28 ± 1° C, 60 ± 2% R.H., and 13:11 L:D. These conditions were within the optimal range for greenbug as determined by a previous study (Thindwa and Teetes 1994). Light intensity averaged 700 lumens. To initiate non-choice experiments, individual 25 cm plastic pots were planted with 4 – 6 seeds of one sorghum genotype and plants were allowed to grow to the 3-leaf stage in a greenhouse. Pots were subsequently thinned to one plant/pot, representing one replication. Plants were measured for initial height, infested with approximately 30 greenbugs of one biotype, covered with nitrocellulose cages, and transported to the environmental chamber. All biotype/genotype combinations were tested in a full factorial design. Ten replications were conducted for all sorghum genotypes in combination with biotypes C, E, and I. Five replications were conducted for all other genotype/biotype combinations. Plants were watered every 2 – 3 d. Control plants were not infested and were grown under the conditions described above in a separate environmental chamber.

Plant damage was measured using a 9-point scale (modified from Johnson et al. 1976), and by measuring plant weight and growth. Plants remained in the environmental chamber until the susceptible check (Tx7000) was rated at a 7 on the damage scale. All plants of that replication were then rated for plant damage, measured for final height, removed from soil, weighed (fresh weight), dried at approximately 55^{o} C for 48 h, and weighed (dry weight). Weights were to the nearest 0.1 g. Plant growth was calculated as the difference between final and initial plant heights. The proportion of weight that was attributable to water (WTR) was determined by first subtracting dry from fresh weight ("weight difference") and dividing weight difference by fresh weight. The proportion of dry matter was calculated as 1 – WTR. The two proportions were arcsine transformed, i.e. arcsine $\sqrt{y}$ (Ott 1984), prior to use in statistical analyses.

Data analyses and statistical tests. Stepwise multiple regression analysis was used to identify significant predictors of the dependent variable, plant damage rating. Stepwise regression was used because it provides control for multicollinearity (Neter et al. 1996) observed in the plant parameters measured. Nine plant measures (beginning and final plant height, fresh and dry plant weight, weight difference, proportion of fresh and dry weight, plant growth, and days to kill susceptible) were used as independent variables in model building. Probabilities of F for the independent variables were used as the stepping criteria in regression model building as follows: F-to-enter = 0.05, F-to-remove = 0.10. The regression model was selected based on R^2 change with the criterion of acceptance arbitrarily set at ≥ 0.05 for the final variable entered in the equation. Experimental means of regression predictors were then compared to control

means with one-way ANOVA (biotypes C, E, and I) or one-way Kruskal-Wallis ANOVA (NY and B) to identify significant differences.

To further assess the differential virulence of greenbug biotypes, the mean number of days post infestation (DPI) to cause ≥ 7 damage ("kill") on the susceptible was determined. Differences in means attributable to biotypes were analyzed with one-way ANOVA. Post hoc comparisons of the range utilized Tukey's honestly significant difference because the standard errors of means were extremely low (SPSS, Inc. 1999). This allowed for the identification of mean differences that likely were biologically as well as statistically significant.

Results

In general, greenbug feeding reduced the water weight and growth of plants, with the exception of some combinations. These variables were identified by stepwise regression analysis as the two most significant predictors of plant damage rating (Table 5). The proportion of plant weight that was water (WTR) had the highest correlation with plant damage rating ($r = 0.67$), followed by mean plant growth (HDIFF; $r = 0.32$). These variables were highly significant and accounted for 55% of the variance in plant damage rating (Table 5).

Sorghum genotypes exhibited characteristic susceptibility or resistance to biotypes, e.g. biotype C was virulent on Tx7000 but not on other genotypes (Fig. 3). Significant differences between mean water weight and plant growth in experimental versus control plants were identified for the Tx7000/C (Figs. 3 and 4); the Tx7000/E and Tx2737/E (Figs. 5 and 6); and the Tx7000/I, Tx2737/I, and Tx2783/I combinations

Table 5. Significant predictors of plant damage rating identified from stepwise multiple regression analysis of 9 independent variables on plant damage rating.

Para[a]	β	R^2	R^2 Change[b]	t	p
WTR	-3.5	0.45	0.45	-7.4	< 0.001
HDIFF	-0.08	0.55	0.10	-6.2	< 0.001
Y-Intercept	9.7			23.4	< 0.001

[a] Parameter in the equation of the regression model: WTR = proportion of weight that was water; HDIFF = plant growth. [b] Contribution in R^2 from the new parameter in the equation; $F = 108.7$, $p < 0.001$, d.f. 2, 178 for whole model.

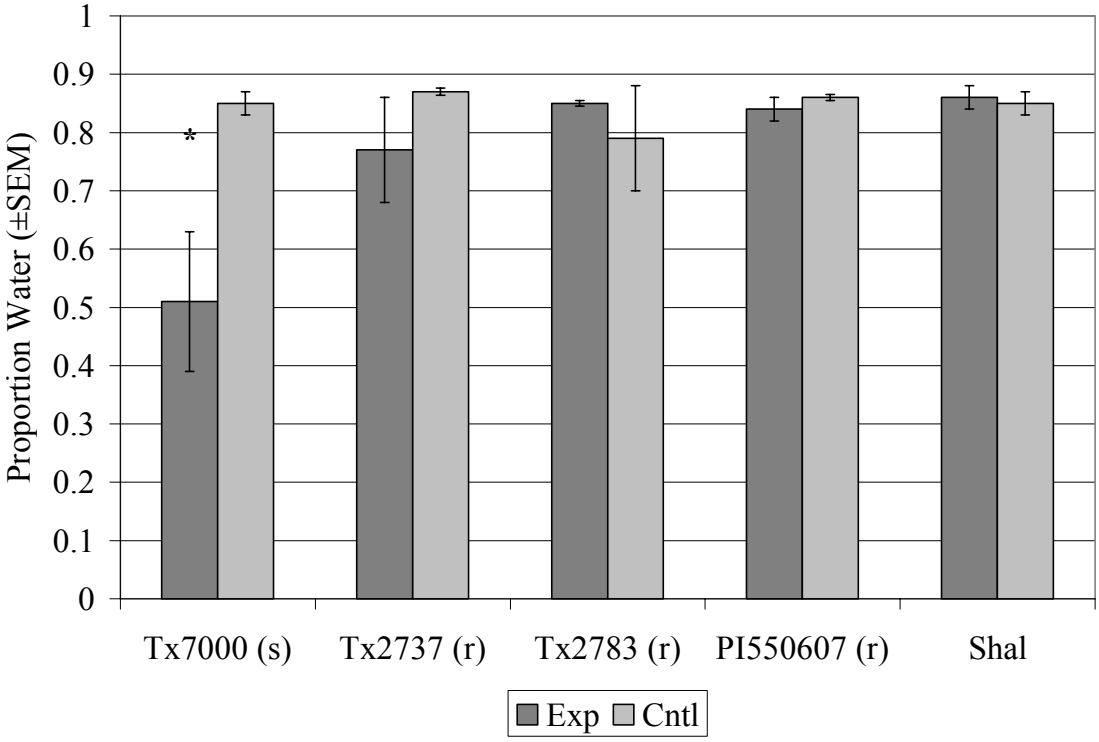

Fig. 3. Mean proportion of plant weight attributable to water of biotype C infested (Exp) and non-infested (Cntl) plants at mean DPI = 13.0. Letters following *S. bicolor* genotypes designate susceptibility (s) or resistance (r). Experimental mean topped by star significantly different from control, one-way ANOVA. * $p = 0.01$, d.f. = 1, 18.

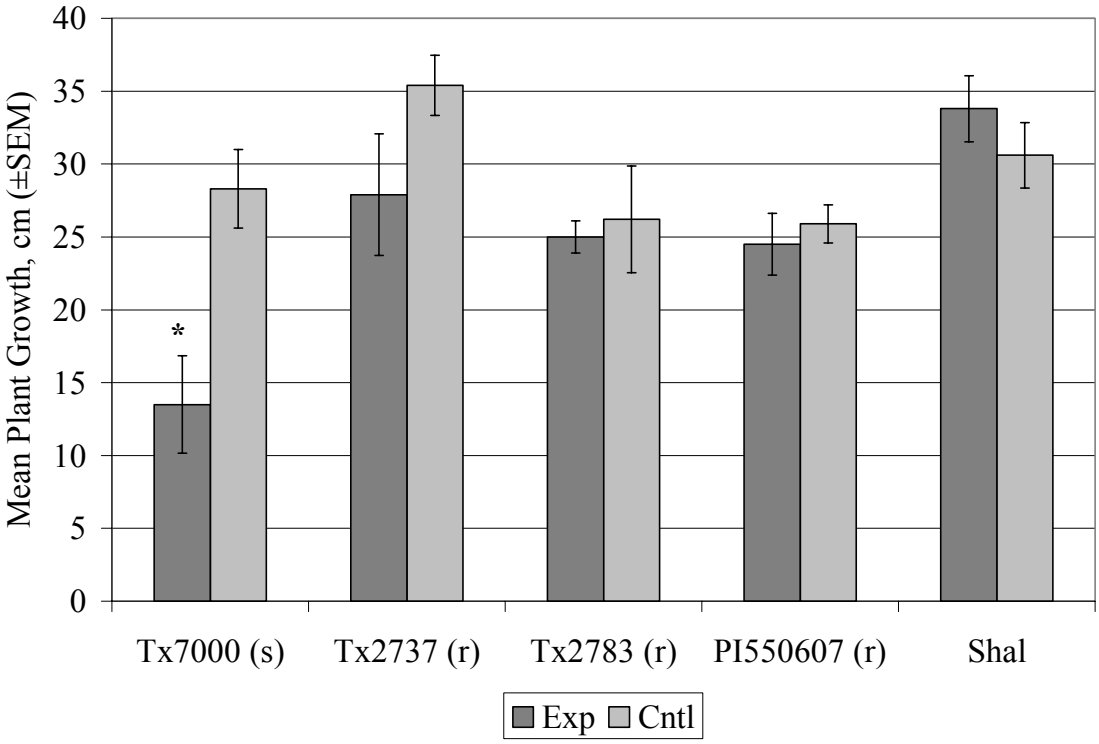

Fig. 4. Mean plant growth of biotype C infested (Exp) and non-infested (Cntl) at mean DPI = 13.0. Letters following *S. bicolor* genotypes designate susceptibility (s) or resistance (r). Experimental mean topped by star significantly different than control, one-way ANOVA. * $p = 0.003$, d.f. = 1, 18.

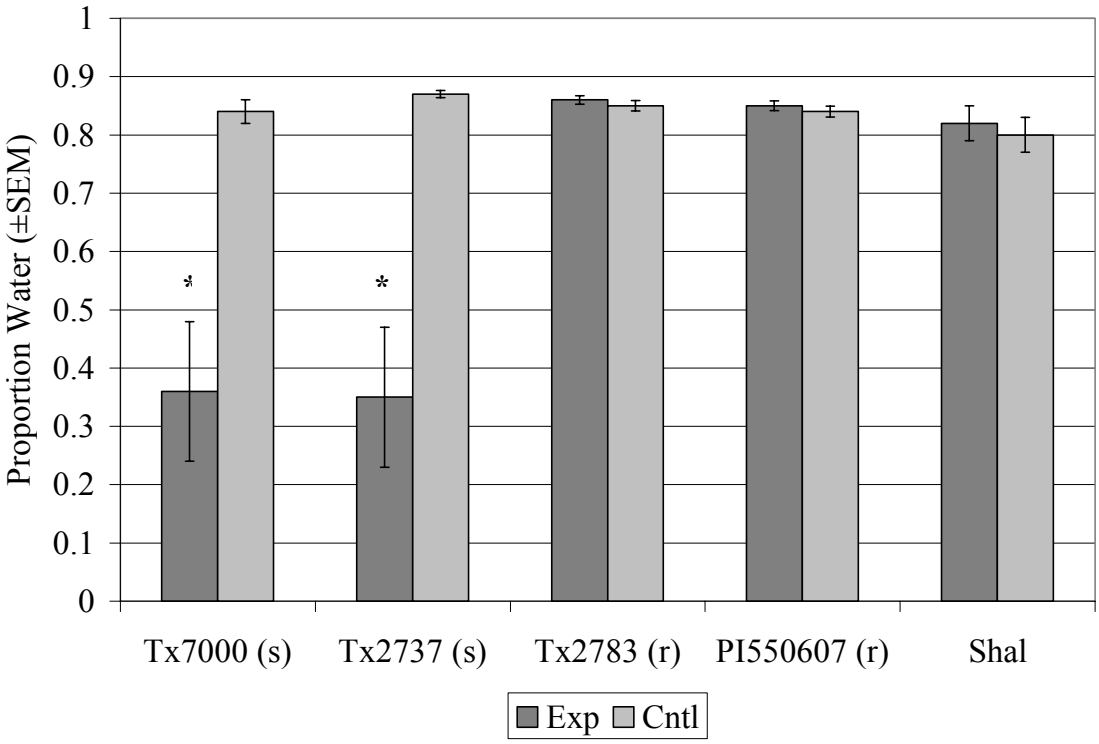

Fig. 5. Mean proportion of plant weight attributable to water of biotype E infested (Exp) and non-infested (Cntl) plants at mean DPI = 11.8. Letters following *S. bicolor* genotypes designate susceptibility (s) or resistance (r). Experimental means topped by a star significantly different than control within genotype, one-way ANOVA. * $p = 0.001$, d.f. = 1, 18.

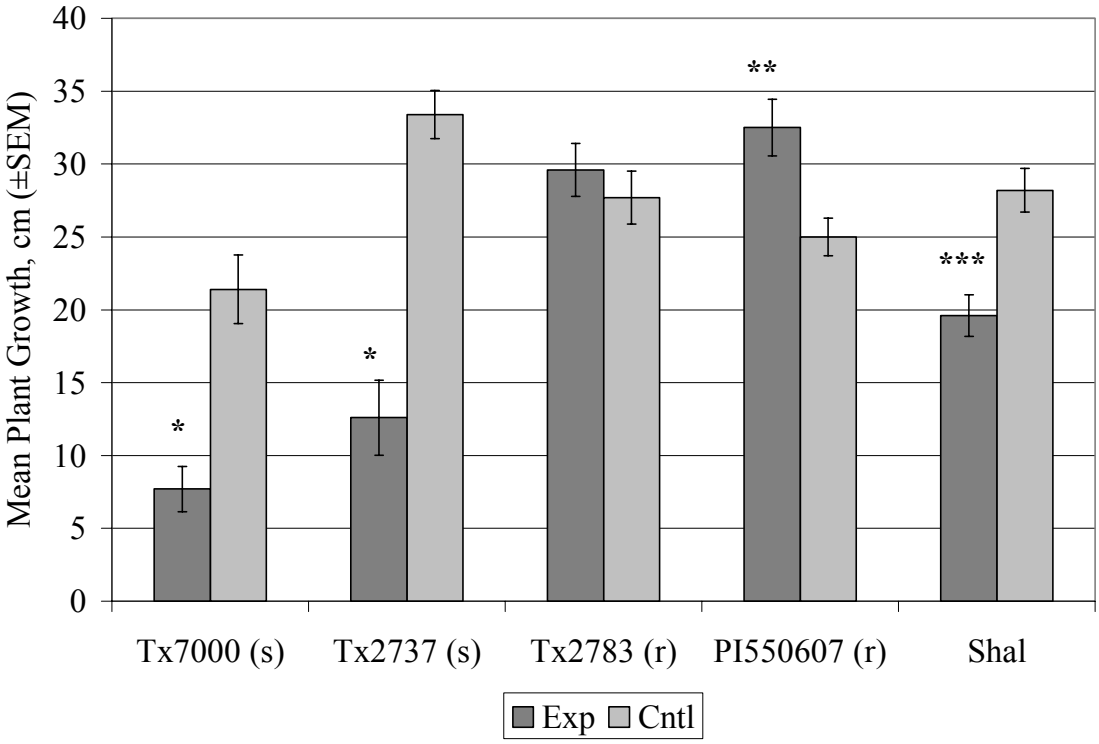

Fig. 6. Mean plant growth of biotype E infested (Exp) and non-infested (Cntl) plants at mean DPI = 11.8. Letters following *S. bicolor* genotypes designate susceptibility (s) or resistance (r). Experimental means topped by a star significantly different than control, one-way ANOVA. * $p < 0.001$, d.f. = 1, 18; ** $p = 0.005$, d.f. = 1, 18; *** $p = 0.01$, $N = 10$.

(Figs. 7 and 8). NY caused significant damage to some genotypes (e.g. Fig. 9). In one case, the susceptible exhibited greater mean plant growth relative to control plants when infested with NY (Fig. 10). No biotype caused significant loss of water weight or growth on PI550607. In fact, when combined with biotypes E and B, infested PI550607 plants showed significantly higher growth (Fig. 6) and water weight (Fig. 11) than controls, respectively. In contrast, Johnson grass showed significantly lower mean growth when combined with biotype E (Fig. 6), and significantly less water weight with NY (Fig. 9) and B (Fig. 11). This suggests some greenbug biotypes are virulent on the non-cultivated host. Biotype B also significantly reduced growth in Tx7000 and Tx2783 relative to the controls (Fig. 12).

Biotypes NY and B required an average of 24.0 and 25.7 days to kill Tx7000, respectively, which was significantly longer than the other three biotypes, but they did not differ from each other (Fig. 13). Biotypes C, E, and I required an average of 13.0, 11.8, and 11.9 days to kill the susceptible, respectively, but they were not significantly different from each other (Fig. 13).

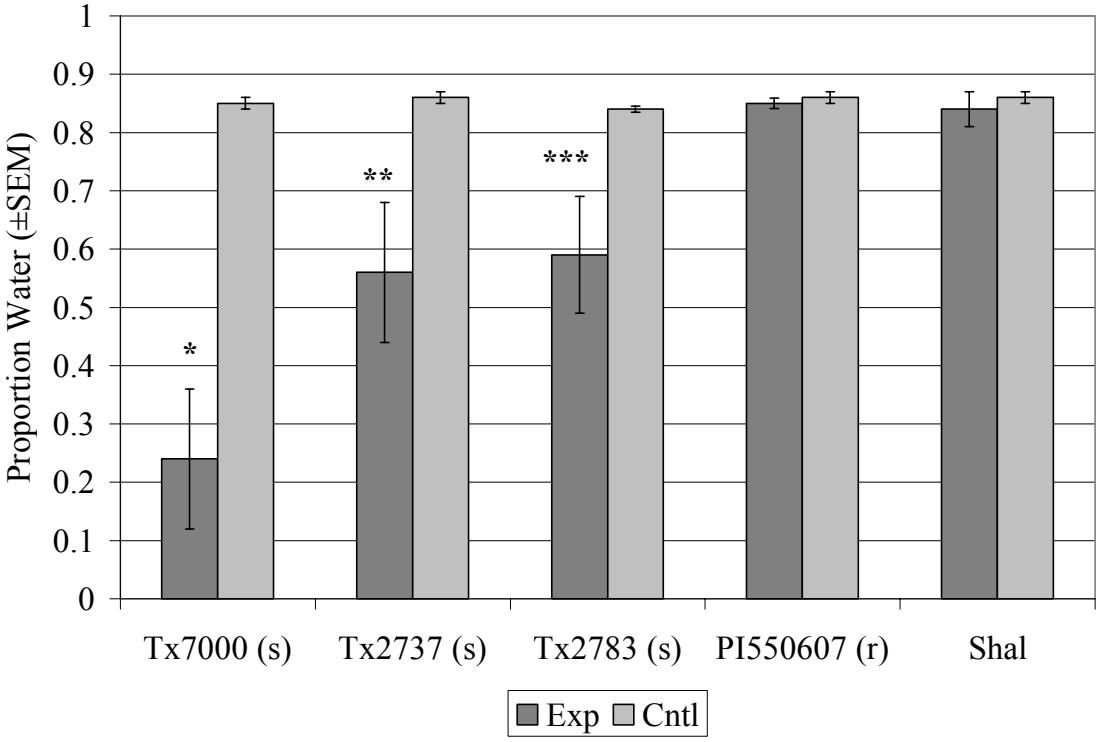

Fig. 7. Mean proportion of plant weight attributable to water of biotype I infested (Exp) and non-infested (Cntl) plants at mean DPI = 11.9. Letters following *S. bicolor* genotypes designate susceptibility (s) or resistance (r). Experimental means topped by a star significantly different than control, one-way ANOVA. * $p < 0.001$, d.f. = 1,18; ** $p = 0.03$, d.f. = 1, 18; *** $p = 0.04$, d.f. = 1, 18.

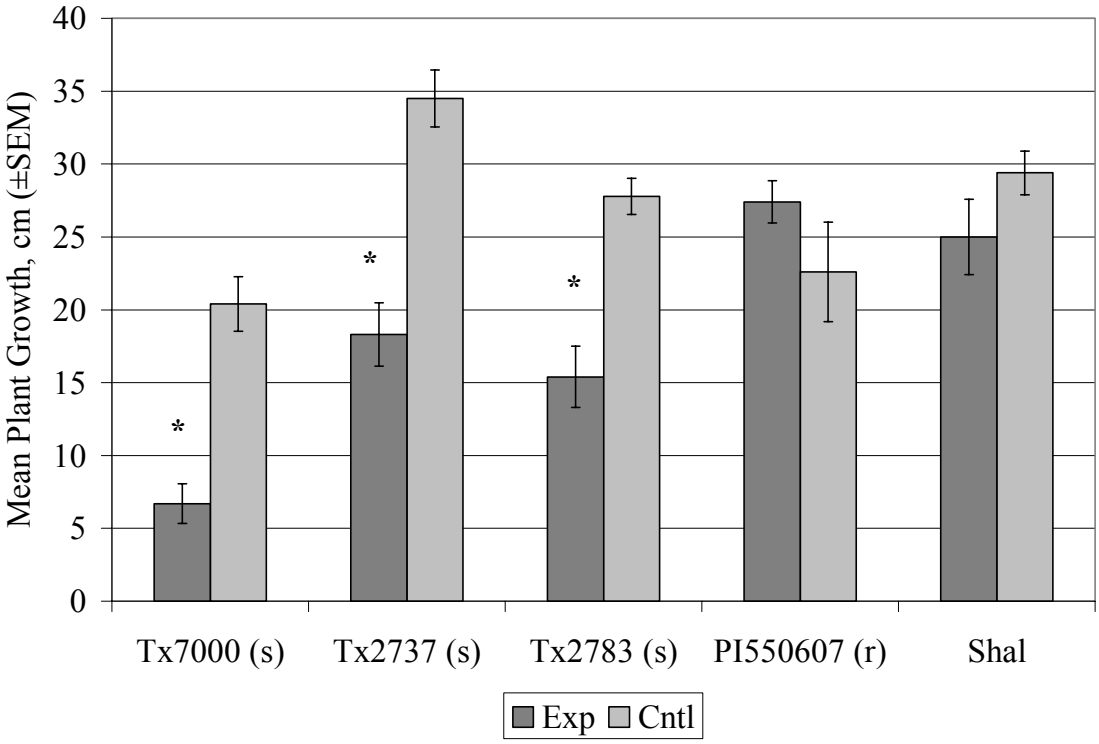

Fig. 8. Mean plant growth of biotype I infested (Exp) and non-infested (Cntl) plants at mean DPI = 11.9. Letters following *S. bicolor* genotypes designate susceptibility (s) or resistance (r). Experimental means topped by a star significantly different than control, one-way ANOVA. * $p < 0.001$, d.f. = 1,18.

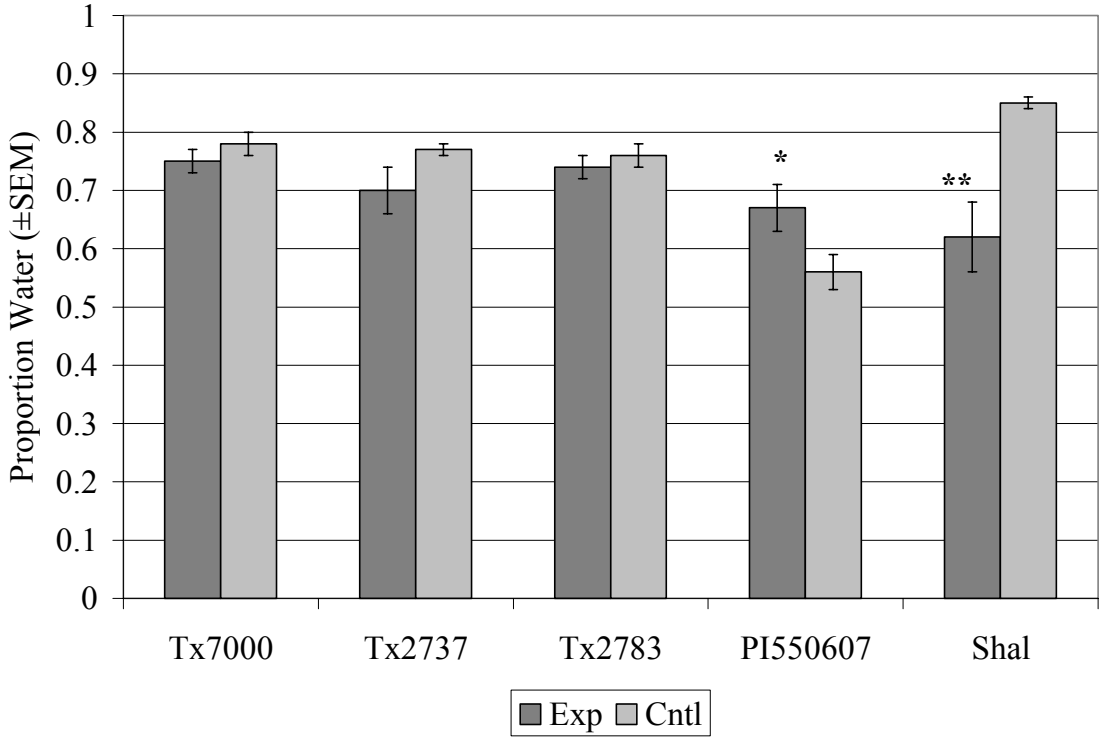

Fig. 9. Mean proportion of plant weight attributable to water of biotype NY infested (Exp) and non-infested (Cntl) plants at mean DPI = 24.0. Experimental mean topped by star significantly different than control, one-way Kruska-Wallis ANOVA. * $p = 0.05$, $N = 10$; ** $p = 0.03$, $N = 10$.

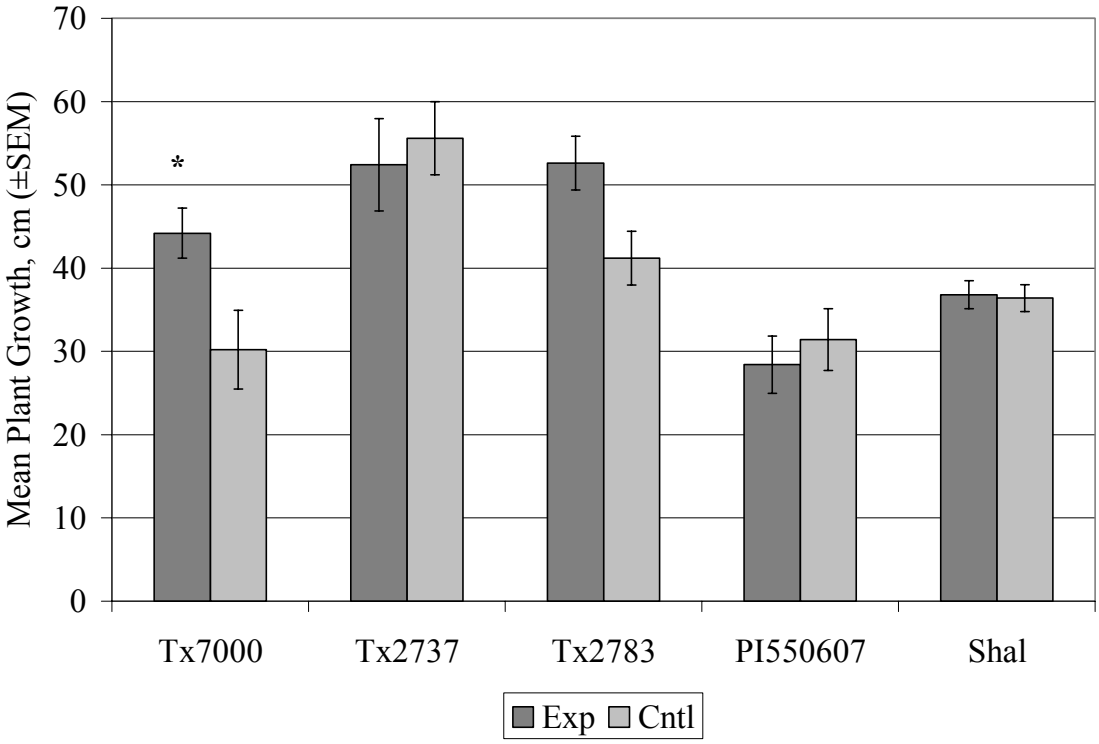

Fig. 10. Mean plant growth of biotype NY infested (Exp) and non-infested (Cntl) plants at mean DPI = 24.0. Experimental mean topped by star significantly different than control, one-way Kruskal-Wallis ANOVA.

* $p = 0.05$, $N = 10$.

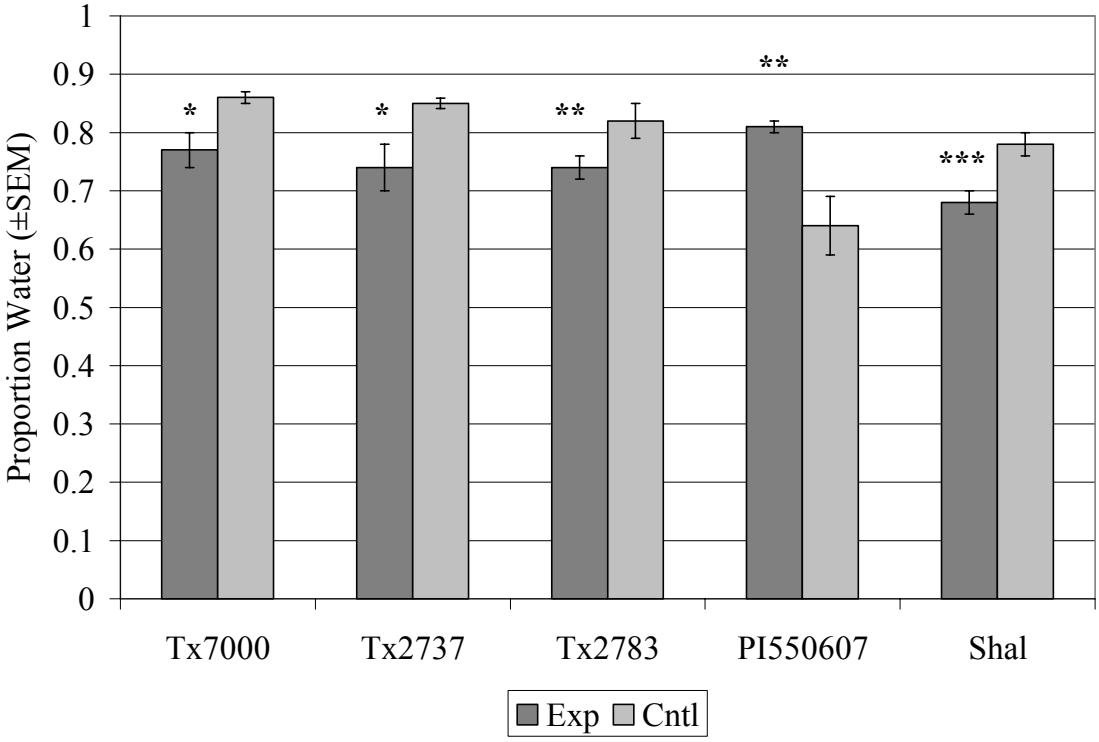

Fig. 11. Mean proportion of plant weight attributable to water of biotype B infested (Exp) and non-infested (Cntl) plants at mean DPI = 25.7. Experimental means topped by a star significantly different than control, one-way Kruskal-Wallis ANOVA. * $p = 0.01$, $N = 10$; ** $p = 0.05$, $N = 10$; *** $p = 0.03$, $N = 10$

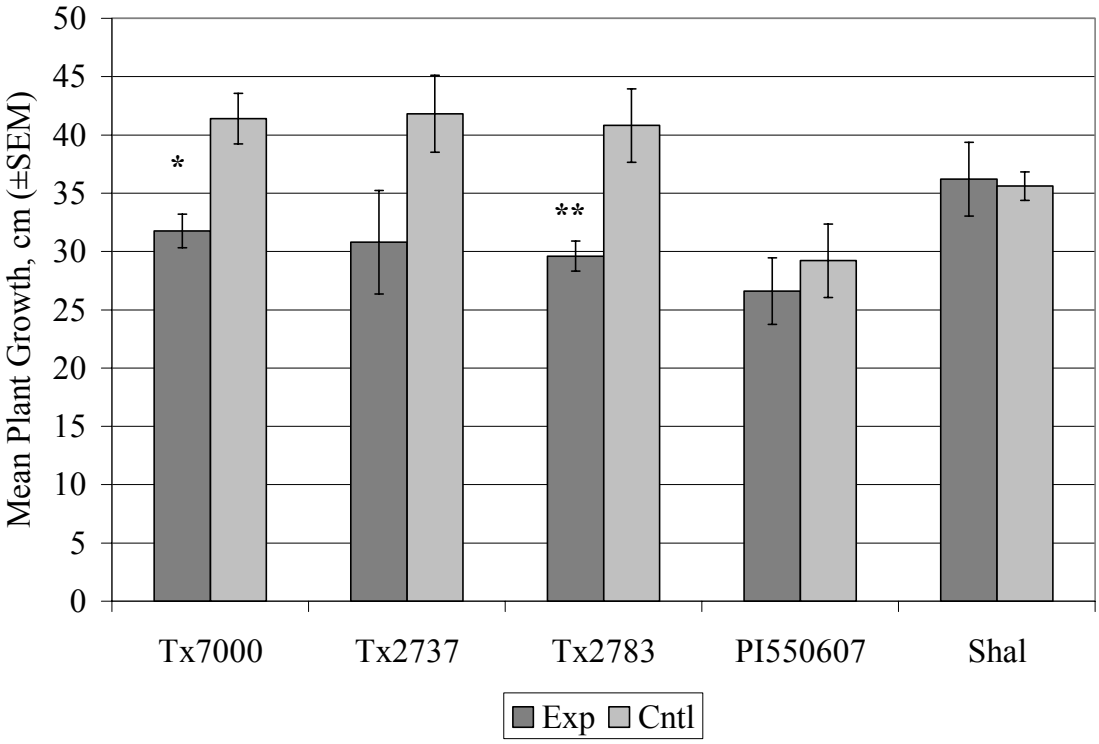

Fig. 12. Mean plant growth of biotype B infested (Exp) and non-infested (Cntl) plants at mean DPI = 25.7. Experimental means topped by a star significantly different than control, one-way Kruskal-Wallis ANOVA. * $p = 0.01$, $N = 9$; ** $p = 0.03$, $N = 10$.

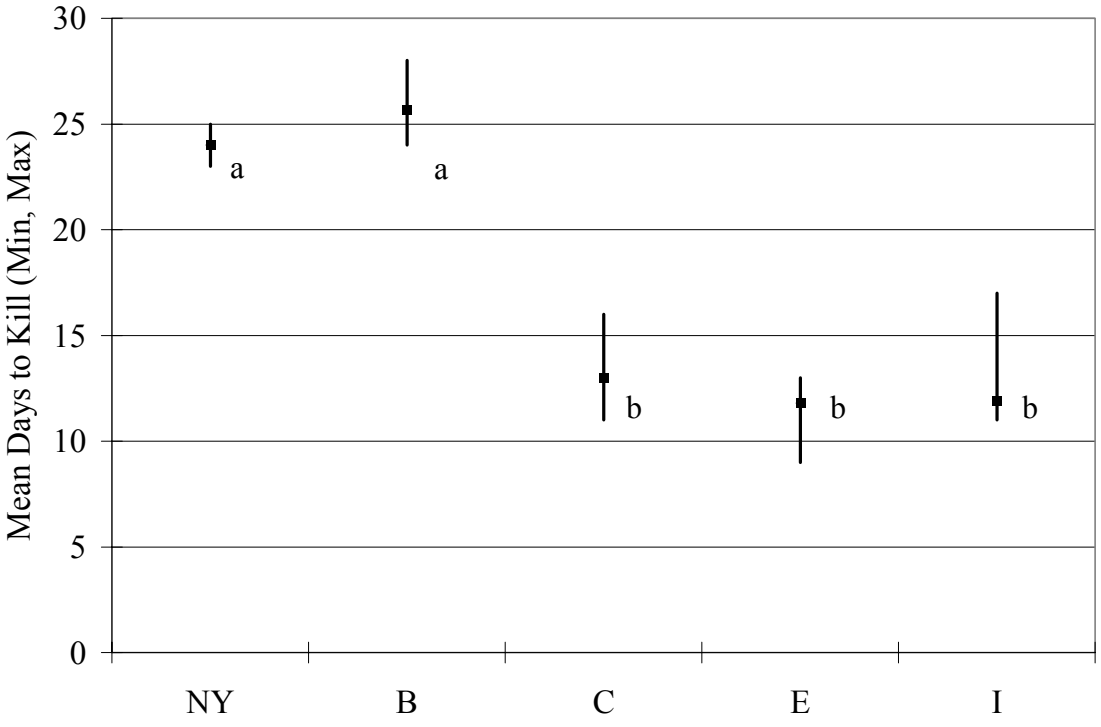

Fig. 13. Assessment of greenbug biotypic virulence via days to kill the susceptible. Boxes indicate mean days to kill Tx7000, and bars represent the range. Bars followed by the same letter not different. ANOVA with Tukey's HSD mean separation, $F = 166.7$, $p < 0.001$, d.f. = 4, 38.

Discussion

Because greenbug virulence is the criterion used to identify new biotypes it is critical to understand how virulence is manifested in plants. Mathematical models have shown insects to be capable of physiological adaptation to resistant plants in ways similar to adaptation to insecticides (Gould 1984, Castillo-Chavez et al. 1988). Greenbug biotypes are capable of developing physiological mechanisms, such as modified pectinases, allowing them to overcome antibiosis-based plant resistance (Dreyer and Campbell 1984). Here we demonstrate that visible plant damage is associated with important plant physiological characteristics, primarily water content and plant growth. The influence of homopteran feeding on plant damage via fluid loss has been explored in other studies (e.g., Gilreath and Smith 1988), however it is commonly assumed that aphid saliva contains toxins which may affect plant structural components (Adams and McAllen 1956, 1958, Saxena and Chada 1971). It therefore seemed reasonable that plant dry weight would be significantly lowered following feeding by virulent but not avirulent biotypes. For example, black bean aphid (*Aphis fabae* Scop.) has been demonstrated to reduce fava bean growth by reducing the amount of dry matter (Garsed et al. 1987). The results presented here suggest greenbug may not reduce significant amounts of dry matter as plant dry weight was not a significant predictor in the regression model. Perhaps the amount of dry weight loss in damaged plants was so miniscule when compared with fluid loss as to be trivial. If so, it may require an extremely large greenbug colony to significantly affect plant structural components, but this would also result in large amounts of fluid removal leading to

extensive plant damage. Additionally, since the regression model considered all biotypes and genotypes combined, it would only be sensitive to overall trends in these data. It would therefore be necessary in future studies to increase the number of replications per biotype/genotype combinations to analyze each separately and also to directly inspect host tissues to elucidate the amount of structural damage virulent biotypes impose on their hosts. It would not be surprising if greenbug were found to affect sorghum plant components as has been documented for other grass crops (e.g., Dreyer and Campbell 1984, Burd 2002). The influence of structural as opposed to fluid loss in the overall damage incurred by a plant is questionable at best.

One important factor in the development of plant biomass is the transpiration rate, such that the conversion of water to plant dry matter (e_w) increases as the saturation deficit (the difference between the maximum and actual vapor pressure) of the air decreases (Wallace and Batchelor 1997). Because a major limiting factor in crop production in many parts of the world is water availability, efforts to increase water use efficiency now include selection or manipulation of plants with higher e_w values (Wallace and Batchelor 1997). Considering the potential impact of greenbug on grass crops, significant loss of plant fluids may negate benefits gained from selection of cultivars that profit from higher transpiration rates simply due to the added stress of further water loss. Intense aphid feeding may also lead to an increased need for irrigation in some areas, as plant transpiration, not including loss to insects, accounts for approximately half of the total water loss through evaporation in crop systems (Wallace and Batchelor 1997). Water loss to fluid-feeding insects may therefore compound the

problem of water use and conservation in agriculture. However, it may explain why some sorghum genotypes (e.g., PI550607) seemed to benefit from greenbug infestation when compared to control plants (e.g., Fig. 17). The impact of greenbug and other fluid-feeders on the dynamics of plant transpiration and biomass anabolism should be a primary concern, in addition to abiotic conditions, when developing new crop cultivars. Host plant resistance to insects such as greenbug may, in this case, help alleviate two problems.

Stunting of plants from virulent biotype feeding is consistent with other studies showing aphids influence plant growth. For example, Burd et al. (1993) demonstrated that Russian wheat aphid (*Diuraphis noxia* Mordvilko) can significantly reduce the growth of susceptible hosts. In addition, Burton (1986) reported that greenbug feeding significantly influenced not only wheat plant above ground biomass but also roots, suggesting greenbug damage may be systemic in some cases. Since the present study identified reduction in growth of aerial plant parts only it cannot be stated unequivocally that roots were also affected. Further study in this area should include assessment of the relationship between damage and root biomass.

The genetic interactions between several insect pests and their host plants have been characterized by gene-for-gene mechanisms (e.g., Ratcliffe et al. 1994, Puterka and Peters 1995, El Bouhssini et al. 2001). Puterka and Peters (1995) reported on the gene-for-gene system in greenbug and sorghum. They identified a complex of several genes associated with virulence, modifier genes, and epistatic effects in certain biotype/plant variety combinations. They concluded virulence in greenbug may be recessively

inherited and sorghum resistance is dominantly inherited (Puterka and Peters 1995). The actual genes involved and their inheritance, however, appear to depend on the interacting greenbug biotypes and plant genotypes. Because greenbugs used in their experiments were crosses of several different biotypes, they developed unique gene combinations in greenbug that may better reflect genetic variability in natural conditions. The results of the present study suggest that genetic variability acquired through recombination in sexual generations may be maintained in clones for many generations, as well as the ability of "sub-clones" to express this variability. In particular, it appears as though greenbug is capable of plasticity in virulence within clones, a topic that should be addressed in the future.

The evolution of parasite virulence was commonly thought to lead to benign coexistence with host organisms, as high virulence was thought to lead to decreased parasite fitness (Ewald 1994). It is now believed that high virulence can be maintained in parasite populations, particularly if it is associated with increased transmission ability (Ewald 1994, Poulin and Combes 1999). In greenbug, development of winged progeny is the primary dispersal mechanism, and occurs as a result of waning food resources and crowding as in other aphid species (Lees 1967, Watt and Dixon 1981, Williams et al. 2000). If greenbug virulence is associated with increased dispersal, we would expect virulent biotypes to produce more alate individuals than avirulent biotypes. The present study has shown that virulent biotypes are capable of damaging host plants faster and to a greater extent than avirulent biotypes. This could be advantageous in the evolutionary maintenance of greenbug virulence if this damage is positively associated with alate

production and, therefore, parasite transmission. Additionally, if virulence is associated with higher greenbug fecundity (see Chapter VI) this may accelerate crowding and lead to alate production. These two mechanisms may be necessary in the evolution of greenbug virulence. Future studies should investigate the association between virulence and alate production.

The role of non-cultivated grasses in the maintenance of virulence levels in greenbug is an interesting area to be considered (e.g., Anstead et al. 2003). The present study demonstrated that biotypes typically avirulent to cultivated sorghums may reduce growth and increase fluid loss in wild sorghums. In particular, wild grass hosts may serve to maintain biotypes that may be outcompeted in agricultural fields (Anstead et al. 2003). Because sexual recombination can lead to unique greenbug genotypes with the ability to damage plants resistant to all known biotypes (Puterka and Peters 1995), the presence of avirulent biotypes such as NY and B around others may provide the genetic diversity necessary to produce new biotypes when interbred. It appears that Johnson grass can allow these avirulent biotypes to persist in such environments. Environmental heterogeneity plays an important role in the maintenance of genetic variability expressed in clonal organisms (Service and Lenski 1982). The fitness of greenbug biotypes on sorghums and Johnson grass will be determined in the companion study (Chapter VI). Ultimately, the interaction between greenbug and host plant genomes determines the virulence/avirulence reactions seen in grass crops and should therefore be studied further.

One of the most perplexing questions in the evolutionary biology of parasite-host relationships has been the issue of parasite virulence. Virulence is an unavoidable consequence of parasites evolving towards a higher rate of reproduction (Kirchner and Roy 2002, Zhan et al. 2002). Therefore, extremely virulent parasites may persist if they have a fitness advantage over less virulent conspecifics in certain situations. In addition, virulence can be maintained by genetic correlations with other traits of the parasite (Ratcliffe et al. 1994, Ebert and Herre 1996). In the greenbug/*Sorghum* system, virulence is a continuum with many possible advantages and disadvantages for both participants. Continued study of the interaction between these two organisms is warranted to further elucidate how biotypes maintain their levels of virulence, particularly the identification of genes associated with virulence and resistance (e.g., Katsar et al. 2002).

CHAPTER VI

CORRELATES OF GREENBUG (HOMOPTERA: APHIDIDAE) BIOTYPIC INTERACTION WITH *Sorghum bicolor* (L.) MOENCH AND *Sorghum halepense* (L.) PERSOON. II: GREENBUG FITNESS

The per capita intrinsic rate of increase, or instantaneous rate of increase, (r) has been an important tool in the study of population dynamics since its introduction in the early 1900's (e.g., Lotka 1907). The values of r are expressed as individuals/(individual * time period), and represent population exponential growth ($r > 0$), constant population size ($r = 0$), or population decline ($r < 0$) (Gotelli 2001). In a given species, the intrinsic rates of increase determined under different sets of conditions provide measures of the relative suitability of the respective conditions (Evans and Smith 1952). For example, the influence of biotic and abiotic factors on the intrinsic increase rates of a variety of aphid species has been examined (e.g., Dean 1974, Bowling et al. 1998, Fraser and Grime 1999, Stadler et al. 2002). Of particular usefulness in applied agriculture is the identification of plant varieties capable of decreasing pest insect fitness proxies such as r and increasing generation time to help manage pest populations. Food quality has been shown to influence aphid fitness, and may be quantified using estimates of r (Dixon 1987). Intrinsic increase rates are usually calculated via life table data, requiring tedious calculations (Dixon 1987). Several studies suggest aphid females produce most of their young in the first few reproductive days, particularly their contribution to the next generation (e.g., DeLoach 1974, Wyatt and White 1977, Dixon 1987). Also, the value of r is determined to a greater extent by the reproductive rate in early adult life than by the

total nymphs produced throughout a female aphid's lifetime (Dixon 1987). Therefore, Wyatt and White (1977) developed a simplified method of calculating *r* for aphids based on the production of offspring during a female's first few reproductive days, which has been satisfactorily utilized on a number of aphid species (e.g., Wyatt and White 1977, Dixon 1987, Bowling et al. 1998). Because the present study was concerned with maximum potential population growth, maximum intrinsic increase (r_m) rates for individual greenbug females were calculated using the method of Wyatt and White (1977).

Prior to the 1980's, statistical comparison of *r* values was generally not possible primarily due to the lack of closed-form algebraic equations for their calculation (Meyer et al. 1986). With the advent of more powerful, easily accessible computers, algorithms designed to resample existing data have been used to estimate error in a variety of situations (Efron and Tibshirani 1991, Young 1994). The "bootstrap" is a resampling method useful for estimating uncertainty in observed data, and is more widely applicable and dependable than a similar method, the jackknife (Efron 1979, 1982). Bootstrapping involves calculating statistics from some large number, *B*, of independent samples, each of size *N* (i.e., the size of the original sample), taken with replacement directly from the observed data. Ideally *B* would approach infinity, however the randomness due to a finite *B* is negligible for $B > 200$ (Efron and Tibshirani 1991). The standard error, confidence intervals, or a host of other error estimates may then be calculated from the *B* samples (Efron and Tibshirani 1991). Assuming full knowledge of the population variance (e.g., from hypothetical data), bootstrapping provides very close estimates

(Efron 1982), and thus is useful to calculate error of *r* coefficients. Resampling techniques are now widely used to estimate variance in *r* coefficients (e.g., Meyer et al. 1986, Lanciani 1995).

Aphid generation time may also be altered by food quality (Dixon 1987). In applied agriculture, increasing a pest insect's mean generation time may give host plants sufficient time for phenological escape (e.g., Beck 1965). In this case, identification of plant varieties that increase generation time would be desirable. Generation time is an estimate of the mean time it takes one cohort to mature and replace the previous cohort, and may be given as the average age of the parents of a particular cohort (Gotelli 2001). Because aphid populations rarely exhibit a stable age distribution and generations overlap considerably (Carter et al. 1978), generation times for aphid colonies are somewhat more nebulous than for individuals, particularly when lacking life table data. Doubling time has been offered as a surrogate for generation time for small organisms during exponential growth (e.g., Yarwood 1956, Dykhuizen and Hartl 1981). Generation and doubling times may, however, be very dissimilar; Mondedji et al. (2002), for example, reported generation times 5 times longer than doubling times in the parasitoid wasp *Dinarmus basalis* Rondani.

The focus of the present study was to determine the maximum potential intrinsic rates of increase (r_m), individual generation times, and colony doubling time for five greenbug, *Schizaphis graminum* (Rondani), biotypes on sorghum [*Sorghum bicolor* (L.) Moench] and Johnson grass [*Sorghum halepense* (L.) Persoon], as measures of relative biotype fitness. Colony doubling times were calculated as an estimate of colony

generation time during exponential growth to allow comparison between different biotype/genotype combinations. Results were examined in conjunction with prior results on biotype virulence (Chapter V) to identify possible fitness advantages gained by virulent biotypes.

Materials and methods

Experimental design. Both colony and individual fitness were determined in the present study. Experimental setup for plants used in colony fitness experiments were as described for the virulence tests (Chapter V). Colony sizes were determined by visually counting aphids on plants at three day intervals ("count days") beginning one day post infestation (DPI). Individual fitness tests were initiated by planting 4 – 6 seeds of one sorghum genotype in 15 cm pots, which were thinned to one plant when seedlings reached about 5 cm. Sorghum genotypes evaluated, and their resistance based on published reports, were: Tx7000 susceptible check (Teetes et al. 1974), Tx2737 resistant to C (Peterson et al. 1984), Tx2783 resistant to C and E (Peterson et al. 1984), and PI550607 resistant to C, E, and I (Andrews et al. 1993). All sorghum genotypes were assumed to be resistant to NY and B because these biotypes were expressed prior to the first biotype capable of damaging sorghum, C (Harvey and Hackerott 1969). Since Johnson grass has essentially been wild for over a century, no *a priori* assumptions were made with regard to damage from specific biotypes.

Individual apterous adult females were placed on seedlings at the three-leaf stage in a full factorial design. Plants were then covered with cages and transported to environmental chambers with conditions as described previously (Chapter V). Females

were checked daily until they produced nymphs, at which time all but one approximately one day old nymph were removed. The remaining aphid was allowed to mature and begin to produce nymphs. Nymphs were counted and removed from plants daily during the time period equal to the individual's pre-reproductive period (see below).

Fitness parameters and statistical tests. Greenbug colony intrinsic rates of increase (r_m) were determined by simple linear regression analysis of log colony size on the number of days post-infestation (DPI), with the slopes of the best fit regression used as the r_m for the biotype/genotype combination (cf., Jarošík et al. 1997, Gotelli 2001). Colony doubling time was calculated with the method of Yarwood (1956):

(1) (t * 0.301)/log population increase

where: t = time interval in DPI, and population increase is expressed as the fold increase in population size from the first to the last count day.

The r_m and generation time (T) for individual parthenogenetic females were calculated with (2) and (3), respectively:

(2) $r_m = 0.74 (\ln M_d)/d$

(3) $T = 4d/3$

where: d = time, in days, equal to the time from a female's birth to her first reproduction (Wyatt and White 1977). During the time period d, nymphs were counted and removed from the plant daily. M_d represents the total number of nymphs produced by a female in time d (Wyatt and White 1977). The r_m values were subjected to a bootstrap algorithm (Howell 2002), with $B = 10^3$ resamples, to determine the 95% confidence intervals (CI). CI not overlapping were considered significantly different.

To identify general trends in r_m, individual generation, and colony doubling times, data were pooled within biotypes and genotypes for separate analyses. Further, Kruskall-Wallis oneway ANOVA (Ott 1984) was used to identify significant differences in r_m and generation times among the specific biotype/genotype combinations for individual females, and also for colony doubling time. Colony r_m were compared with analysis of covariance (ANCOVA) to test for parallel slopes (Neter et al. 1996); combinations exhibiting significantly different slopes had different population growth rates.

Because of the extreme variability observed in some colony doubling times, the regression diagnostic *DFFITS* was used *post hoc* to identify extreme outliers in colony doubling time. *DFFITS* calculates the difference between the fitted value $\hat{Y}_i$ for the *i*th case when all *N* cases are fitted to the regression function and $\hat{Y}_{i(i)}$ when the *i*th case is omitted (Neter et al. 1996). This allows for the identification of "influential outliers", defined as those whose omission cause major changes in the fitted regression function (Neter et al. 1996). In general, a case with a *DFFITS* value ≥ 1 is considered an influential outlier.

Individual r_m and generation times for biotypes E and I were compared to those determined in a previous study (Bowling et al. 1998). The environmental conditions under which the greenbugs were raised in Bowling et al. (1998) were the same as the present study with the exception that the light regime was 16:8.

Results

In general, colony and individual r_m values were very similar when pooled within biotypes (Fig. 14) or plant genotypes (Fig. 15). No significant differences were identified in pooled colony and individual values within biotypes or genotypes (Figs. 14 and 15). There were, however, significant differences among some biotypes or genotypes. Biotype B, for example, had significantly lower colony r_m than biotypes E and I, and biotype E colony r_m was significantly higher than the average for all pooled data (Tot; Fig. 14). No apparent differences were identified among individual r_m pooled within biotypes. Biotype B exhibited a lower individual intrinsic increase rate than NY, I, and the total pooled value (Tot). Analysis of r_m values pooled within plant genotypes provides an estimate of the host plant suitability, at least to the extent greenbug biotypes can reproduce upon genotypes. The susceptible check (Tx7000) supported the highest r_m for both colonies and individuals (Fig. 15). Tx7000 had significantly higher colony r_m values than PI550607, Johnson grass, and the total pooled value (Fig. 15). Johnson grass suppressed individual reproduction to significantly lower levels than all other plant

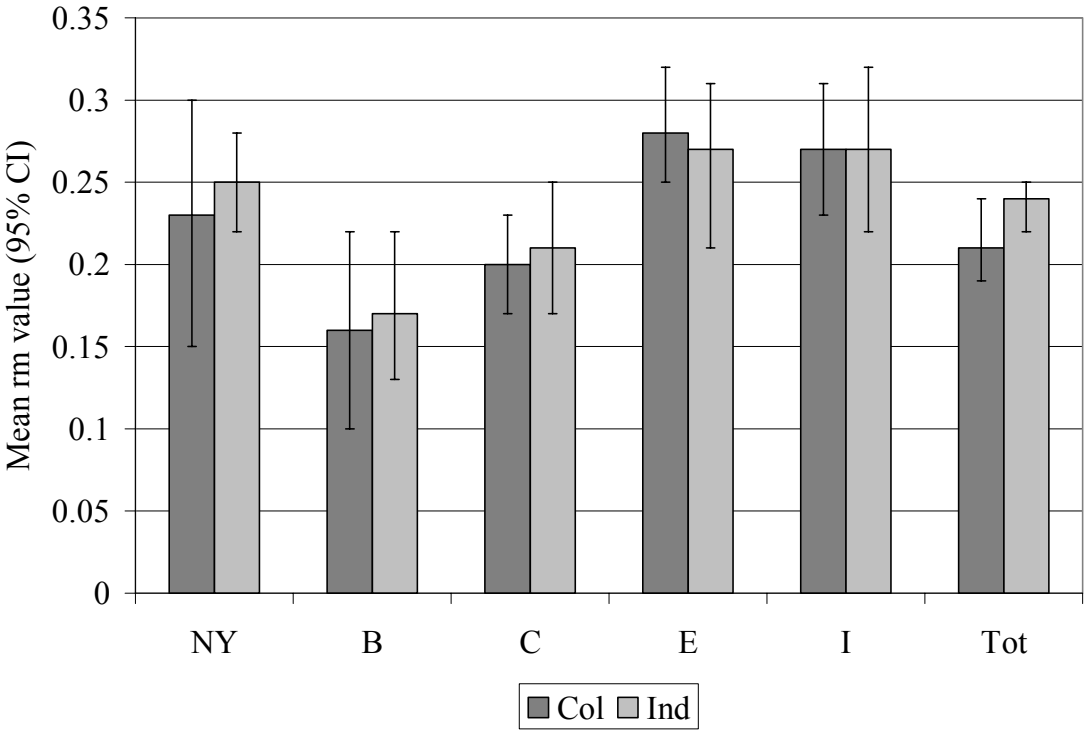

Fig. 14. Mean r_m values and 95% confidence intervals (CI) for colony (Col) and individual females (Ind) pooled within greenbug biotypes, and for all biotypes combined (Tot). Overlap of CI between any bars represents non-significant differences. $N = 28$ for each biotype.

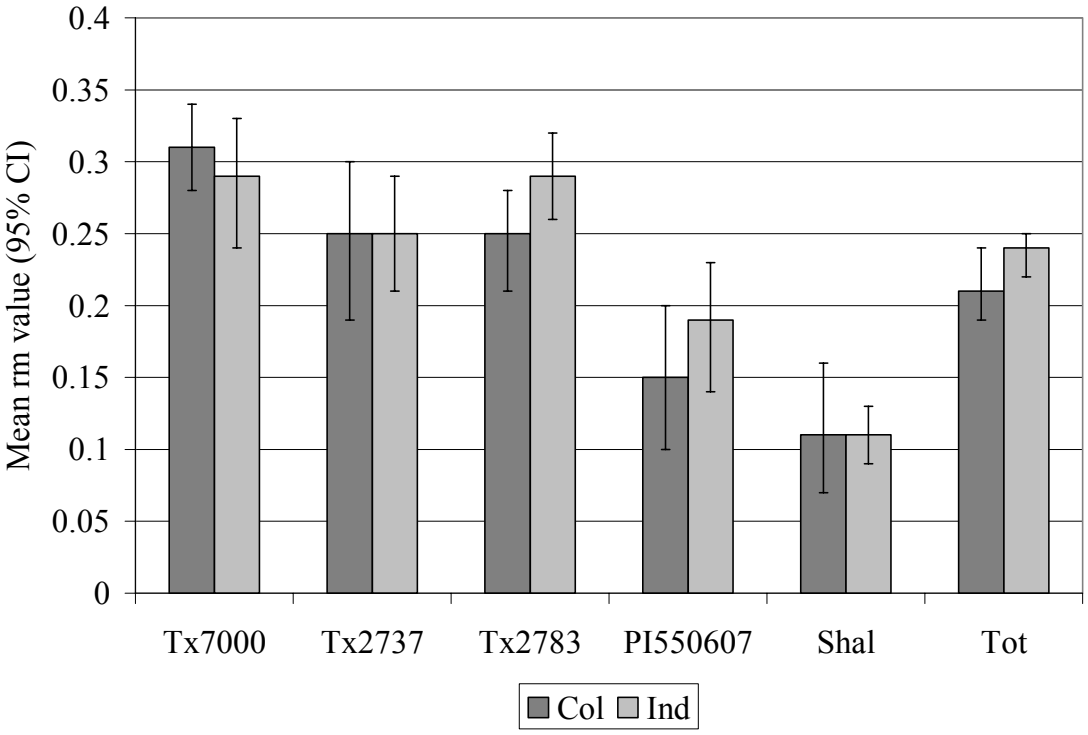

Fig. 15. Mean r_m values and 95% confidence intervals (CI) for colony (Col) and individual females (Ind) pooled within sorghum genotypes, and all genotypes combined (Tot). Overlap of CI between any bars represents non-significant differences.

genotypes (Fig. 15). In addition, Tx2783 supported significantly higher r_m than PI550607, Johnson grass, and total pooled data.

Mean colony doubling times were generally less than 12 days with the exception of biotype B which exhibited a mean time of 29 days with extreme variability (Fig. 16). *DFFITS* identified three influential outliers in colony doubling time, two in the biotype B/Johnson grass combination (87.9 days and 207.8 days) and a third in the biotype C/PI550607 combination (114.8 days). Mean times were then determined with the influential cases removed, which brought the means to more comparable levels with the other biotype/genotype combinations and provided tighter 95% CI's (Figs. 16 and 17; DF). Colony generation times were significantly shorter in biotype E than all other biotypes and the total pooled data (Fig. 16). No significant differences in generation time were identified for individual females between biotypes. Johnson grass increased the mean generation time of greenbug colonies, which was significantly longer than the susceptible (Tx7000) but not for other plant genotypes (Fig. 17). Johnson grass also increased the mean individual generation time, which was significantly longer than all other genotypes (Fig. 17).

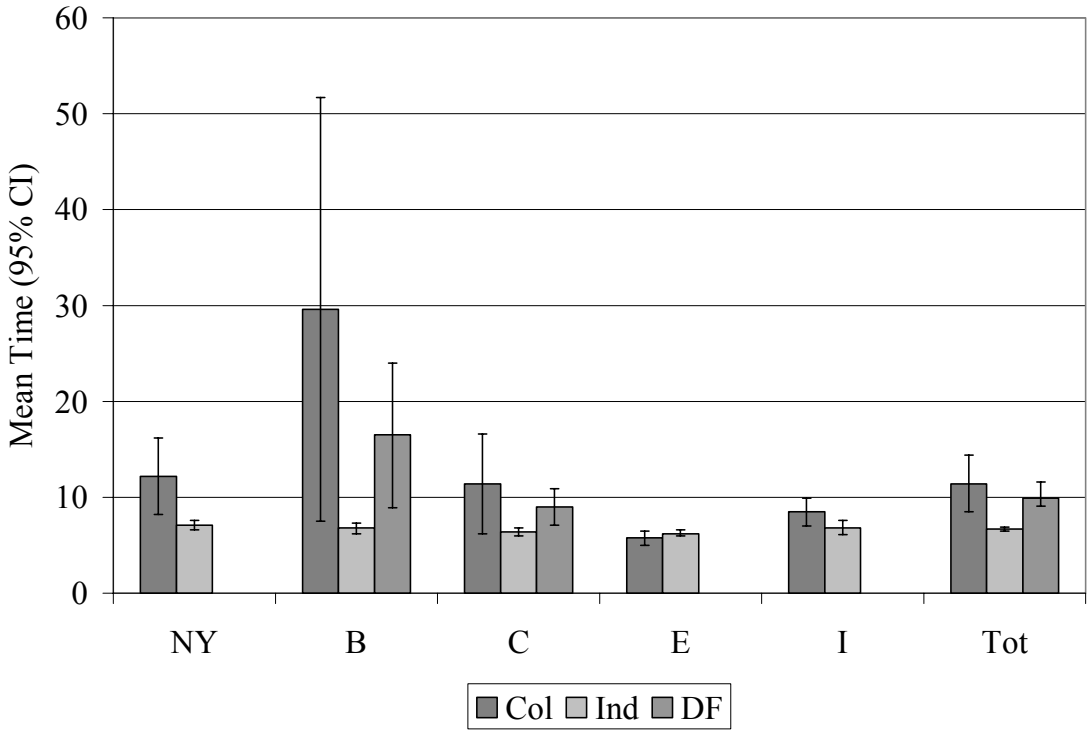

Fig. 16. Mean colony (Col) and individual female generation (Ind) time and 95% confidence intervals (CI) of greenbug biotypes, all sorghums pooled. DF is the mean colony doubling time in biotypes after the removal of the influential cases identified by *DFFITS*. Overlap of CI between any bars represents non-significant differences.

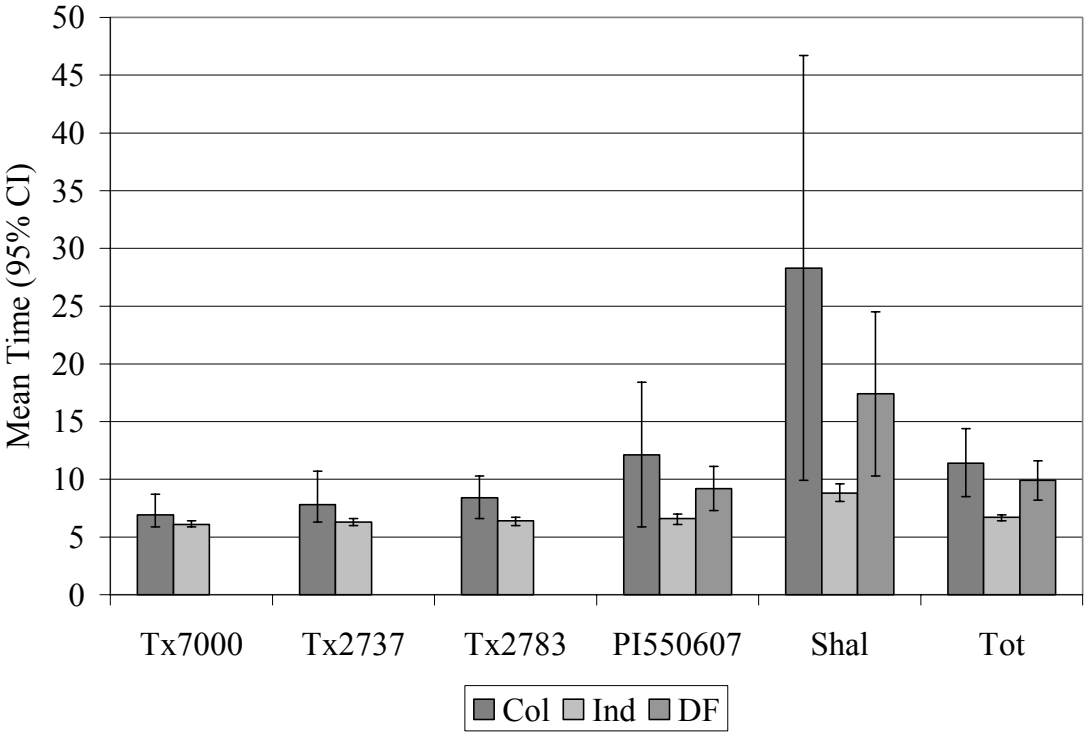

Fig. 17. Mean colony (Col) and individual female (Ind) generation time and 95% confidence intervals (CI) on sorghum genotypes, all biotypes pooled. DF is the mean colony doubling time on genotypes after the removal of the influential cases identified by *DFFITS*. Overlap of CI between any bars represents non-significant differences.

Table 6 lists the mean individual and colony r_m values for each biotype/genotype combination. Individual female r_m differed significantly among biotypes within Tx2783 and Johnson grass, but not within the remaining genotypes. Far more significant differences were identified among sorghum genotypes when data were pooled within greenbug biotypes. The susceptible check (Tx7000) generally supported the highest reproduction, significantly higher than other genotypes in many cases (Table 6). Biotype E exhibited significantly higher reproduction on Tx2783, a genotype upon which it is avirulent. In general, individual greenbugs exhibited higher reproduction on more susceptible genotypes.

Colony r_m values followed a similar trend as individual values, but were more variable among biotype/genotype combinations. The trend was toward higher reproduction in more susceptible sorghum genotypes, particularly within biotypes C, E, and I (Table 6). Within the "sorghum" biotypes (C, E, and I), virulence was consistently associated with increased r_m. Also, biotype E consistently exhibited higher r_m than C and biotype I had consistently higher r_m than E (Table 6).

Within biotype C, r_m did not differ between Tx7000 and Tx2737. Tx2737 suffered significantly less damage than Tx7000 from biotype C feeding (see Chapter V). In conjunction, these results suggest tolerance in Tx2737 towards biotype C. Antibiosis was observed in biotype E/Tx2783 in relation to Tx7000 and Tx2737, as the latter two sorghums incurred heavy damage from biotype E and supported significantly higher r_m (Table 6). All greenbug biotypes exhibited significantly lowered reproduction on PI550607 (except B) and Johnson grass relative to the susceptible check, suggesting

Table 6. Intrinsic rates of increase in biotype/genotype combinations for individual females and colonies.

	Tx7000[a]	Tx2737	Tx2783	PI550607	Shal
NY[b]	0.32Aa	0.27Aab	0.23Aab	0.22Aab	0.15Ab
B	0.27Aa	0.17Aab	0.25ABab	0.09Ab	0.06Bb
C	0.24Aa	0.24Aa	0.26ABa	0.16Aa	0.14ABa
E	0.26Aab	0.33Aab	0.35Bb	0.27Aab	0.12ABa
I	0.37Aa	0.25Aab	0.35Ba	0.23Aab	0.10ABb
	Tx7000	Tx2737	Tx2783	PI550607	Shal
NY[c]	0.36ACa	0.31ACa	0.26ABab	0.11Ac	0.14Abc
B	0.26Bac	0.11Bb	0.27ABc	0.12Aab	0.03Bb
C	0.31Ca	0.27Aa	0.18Bb	0.11Ac	0.08Cc
E	0.40Aa	0.41CDa	0.30ACb	0.23Bc	0.15Ad
I	0.42Aa	0.48Da	0.38Cab	0.32Bbc	0.19Ac

[a] Mean values followed by the same uppercase letter are not significantly different within columns, same lowercase letter are not significantly different within rows, Kruskal-Wallis oneway ANOVA.

[b] Mean r_m for individual females.

[c] Mean r_m for colonies.

antibiosis. However, in combination with biotypes E and I, PI550607 and Johnson grass supported moderately high r_m. Johnson grass also supported rather high reproduction in combination with biotype NY. Additionally, biotypes E and NY caused significantly lowered plant growth and water loss, respectively, on Johnson grass, suggesting virulence is positively associated with greenbug fitness in these two cases. In combination with biotype B, however, Johnson grass incurred significant water loss yet it severely restricted biotype B's reproduction (Table 6).

Mean individual generation and colony doubling times for combinations are listed in Table 7. Individual generation times were quite consistent across all combinations. The shortest mean individual generation time (5.6 days, biotype I/Tx7000) was significantly different than the longest (10.3, biotype I/Johnson grass). As with the individual r_m values, few significant differences were found among biotypes pooled within genotypes. In Tx2783, biotype E had significantly shorter generation time than biotype NY (Table 7). Biotype E also had significantly shorter generation time compared to biotype I when reared on Johnson grass.

Colony doubling times were much more variable, with a low of 4.6 (biotype C/Tx7000) and high of 54.0 days (biotype B/Johnson grass, influential cases removed). Within biotype B no significant differences were found despite the large difference in doubling time between Johnson grass and all other biotypes (Table 7). This is due to small *N* within biotype B. In contrast, within biotype C mean doubling times were

Table 7. Individual generation and colony doubling times in biotype/genotype combinations for individual females and colonies.

	Tx7000[a]	Tx2737	Tx2783	PI550607	Shal
NY[b]	6.0Aa	6.7Aab	7.5Aab	6.7Aab	9.3ABb
B	6.2Aab	6.7Aab	6.0ABa	6.4Aab	9.3ABb
C	6.4Aa	6.2Aa	6.0ABa	6.2Aa	7.7ABa
E	6.2Aa	6.2Aa	5.8Ba	6.2Aa	7.0Aa
I	5.6Aa	5.8Aa	6.4ABab	7.3Aab	10.3Bb
	Tx7000	Tx2737	Tx2783	PI550607	Shal
NY[c]	6.9ABa	9.4ABab	10.2Aab	10.8ABab	23.1ABb
B	10.8Aa	15.3Aa	11.0Aa	11.1ABa	54.0Ba
C	4.6Ba	5.4Ba	8.4ABab	14.0Ab	16.4ABb
E	5.1Ba	6.0ABa	4.7Ba	5.8Ba	8.2ABa
I	8.8Aa	8.3ABa	10.1ABa	7.1Ba	7.4Aa

[a] Mean values followed by the same uppercase letter are not significantly different within columns, same lowercase letter are not significantly different within rows, Kruskal-Wallis oneway ANOVA.

[b] Mean generation time, in days, for individual females.

[c] Mean colony doubling time in days.

significantly longer in the most resistant genotype, PI550607, compared to the susceptible check (Table 7). Doubling time was longest in Johnson grass for most biotypes. No significant differences in doubling time were observed among plant genotypes when combined with biotypes E and I (Table 7).

Table 8 lists individual female r_m values and development times for biotypes E and I from the present study in comparison to those from Bowling et al. (1998). Intrinsic increase rates were quite similar for biotype E with the exception of the pairings with I resistant plants (Table 8). Intrinsic increase values were rather dissimilar in all groupings involving biotype I. In general r_m values reported in Bowling et al. (1998) were higher (in six of the eight pairings) than those for the present study. Similarly, mean individual generation times were somewhat disparate between the two studies; the biggest difference was 2.0 days in the biotype I/E-resistant pairings (Table 8).

Discussion

Insect life history strategies may be altered by a variety of biotic and abiotic factors (e.g., Dean 1974, Jarošík et al. 1997, Gonzales et al. 2002, Stadler et al. 2002). For example, the influence of food quality on aphid fitness has received considerable attention (e.g., Dixon 1987), yet food quality remains difficult to measure. The present study has demonstrated that the most resistant sorghum genotype, PI550607, and non-cultivated Johnson grass significantly decreased greenbug r_m and increased colony doubling time relative to the susceptible check, Tx7000. Anstead et al. (2003) suggest

Table 8. Comparison of biotype E and I individual r_m values and generation times of the present study with those from a previous study on sorghum[*].

Data set[a]	E sus[b]	E res	I sus	I res
Individual r_m				
E_G	0.30	0.25	0.31	0.20
E_B	0.27	0.28	0.28	0.26
I_G	0.31	0.23	0.32	0.16
I_B	0.38	0.36	0.36	0.29
Generation time				
E_G	6.2	6.3	6.1	6.6
E_B	6.8	7.8	7.5	8.0
I_G	5.7	8.0	5.9	8.8
I_B	5.9	6.0	6.0	7.2

[a] Capital letter represents greenbug biotype, subscript represent data set: G = present study, B = Bowling et al. (1998).

[b] Susceptibility (sus) or resistance (res) exhibited by sorghum genotypes (see text).

[*] Part of this chapter is reprinted with permission from "Relative fitness of greenbug (Homoptera: Aphididae) biotypes E and I on sorghum, wheat, rye, and barley" by Bowling et al. 1998. J. Econ. Entomol. 91: 1219 – 1223. Copyright 1998 by the Entomological Society of America.

that the absence of greenbugs on certain non-cultivated grasses, including Johnson grass, may reflect poor host quality. Hunter and McNeil (1997) demonstrated that low quality host plants favored diapause, resulting in decreased voltinism via increased generation time, in natural populations of *Choristoneura rosaceana* (Harris). Results presented herein suggest that some sorghum genotypes (e.g., PI550607) and Johnson grass are poor hosts relative to others (e.g., Tx7000) as reflected by decreased reproduction and increased doubling times. While it is difficult to compare results from an agricultural system, with low genetic diversity, to a natural system with much higher genetic diversity, the current study underscores the value to examining agricultural systems to identify and formulate broader ecological questions. Agricultural systems provide some measure of control, as a result of low genetic diversity, that may be difficult to attain or manipulate in natural systems.

Results show that virulent biotypes routinely exhibited higher r_m than avirulent biotypes, however since virulence is determined via plant reaction (i.e., resistance/susceptibility) it is difficult to separate the influence of virulence from plant resistance on greenbug reproduction. For example, tolerance is exhibited by Tx2737 towards biotype C when compared to the biotype C/Tx7000 combination. In this case, virulence does not appear to be associated with greenbug fitness. If plant damage leads to increased nutrition (e.g., Dorschner et al. 1987, Burd 2002), it may then be predicted that virulence increases fitness; in the case of tolerance this prediction is not supported. In contrast, other resistant genotypes exhibited antibiosis which decreased greenbug fitness. Because few genes for greenbug resistance have been identified (Katsar et al.

2002), and pyramiding of resistance genes does not provide plant protection over that of single-gene resistance (Porter et al. 2000), single-gene antibiosis may become more important in future breeding efforts despite the possibility of increased selection of new virulent biotypes.

Damage to Johnson grass was associated with high r_m in combination with NY and E, but not B. This suggests the relationship between virulence and fitness may be variable in relation to the genetic constitution of wild hosts. Because Johnson grass is non-cultivated, it was assumed that genetic variability among plants would be greater than in sorghum. Thus, the interactions between Johnson grass and greenbug biotypes would better reflect a natural condition. Since Johnson grass is damaged by NY and B, this could allow for these otherwise avirulent biotypes (towards sorghum) to persist in areas and interbreed with other biotypes (e.g., Anstead et al. 2003). However, since reproduction was severely reduced in the B/Johnson grass combination, biotype B may accrue little or no reproductive benefit from its ability to damage Johnson grass. This underscores the importance of identifying how host damage and parasite fitness interact to lead to "virulence" as defined by Poulin and Combes (1999), particularly the interactions under natural conditions. While recognized greenbug biotype designations rely on the insect overcoming plant resistance, presumably through a gene-for-gene system, the interactions occurring on non-cultivated grasses would be genetically more complex. Therefore, identification of plant damage and greenbug fitness on wild grasses is necessary to further elucidate how wild hosts could potentially impact greenbug biotype evolution and maintenance, particularly grasses commonly associated with

sorghum and small grains. If selection pressure from host plants, particularly wild hosts, is an important determinant in the development of virulent insect biotypes (Gould 1984, Ratcliffe et al. 1994, Deol 2001), Johnson grass appears to be one of the prime candidates for selection and/or maintenance of greenbug biotypes.

The superior performance of biotypes E and I on Johnson grass relative to C may help explain the predominance of E and I on both cultivated (Porter et al. 2000, Anstead 2003) and non-cultivated (Anstead et al. 2003) hosts. Anstead et al. (2003) report that biotype I accounted for most of the greenbugs collected, followed by biotype E, on non-cultivated hosts. The ability of biotypes E and I to reproduce well on Johnson grass may confer a competitive advantage for these biotypes, at least in those areas where Johnson grass predominates. Johnson grass is ubiquitous, particularly around cultivated grasses (e.g., Holm et al. 1977), and this may benefit greenbug biotypes capable of utilizing it effectively. In the absence of cultivated sorghum, biotypes E and I may find a reproductive refuge on non-cultivated grasses in close proximity to fields. Study of the reproductive potential of greenbug biotypes on other non-cultivated grasses would help to better understand the maintenance and evolution of biotypes.

Differences in fitness parameters of biotype E and I between the present study and Bowling et al. (1998) may be attributable to several factors. Initial plant heights likely differed between the two studies, as Bowling et al. (1998) infested plants after two weeks, whereas plants were approximately 10 days old when infested in the present study. Additionally, Bowling et al. (1998) provided three hours more light than the present study. The differences in light regime between the two studies may also have

influenced r_m values. Insects other than greenbug have been shown to cease reproduction at night (e.g., Tessmer et al. 1995).

The two studies utilized different varieties of susceptible and resistant plants. This is the most obvious and perhaps most influential difference between the studies, as food quality influences aphid development and reproduction. Presumably, resistant plants are resistant because they are of lower quality than susceptible plants. Therefore a rough measure of the plant quality could be determined by comparing greenbug fitness index values for different plant varieties. In this case, plant varieties utilized by Bowling et al. (1998) were of higher quality than those in the present study, as r_m values were consistently higher than those reported here. This is reasonable as Johnson grass was included as resistant to both E and I. Although Johnson grass may be damaged by greenbug feeding (Chapter V), this does not necessarily reflect its quality, which ultimately should be manifested by reproduction. Future studies in this area should incorporate energy assimilation assays in conjunction with fitness studies to better reflect plant quality influences on fitness.

The present study demonstrated that greenbug virulence is positively associated with increased fitness on most sorghum genotypes. Further research on host quality is necessary to elucidate how virulence influences greenbug fitness. The relation between longevity and reproduction should be explored in future studies.

CHAPTER VII

AN ATTEMPT TOWARDS AN INVESTIGATION OF DIFFERENTIAL GENE EXPRESSION IN GREENBUG DAMAGED VERSUS UNDAMAGED *Sorghum bicolor* (L.) MOENCH

To date, research on the genetic basis of greenbug biotype expression and their interaction with grass crops has been limited to identification of genetic diversity among natural greenbug populations (e.g., Jensen 2001, Anstead et al. 2003), or plant genes conferring resistance to particular biotypes (e.g., Porter et al. 2000, Katsar et al. 2002). In contrast, no known effort has been made towards identifying differential expression of genes in plants incurring greenbug damage relative to undamaged plants; i.e. what, if any, genes are differentially regulated among damaged versus undamaged plants? This question is more than academic as damage caused by greenbug biotypes may vary considerably among sorghum genotypes (see Chapters IV and V). Therefore it may be expected that certain plant genes would be differentially expressed when damaged by greenbug feeding. Because messenger RNA (mRNA) results from gene transcription in cells, inspection of the mRNA species common to and, more importantly, differing among individuals is instrumental in determining differential gene expression. Inspection of mRNA species for such purpose has been employed since the early 1990s (e.g., Peng and Pardee 1992). A relatively new technique, called suppression subtractive hybridization (SSH), has been used to identify tissue-specific gene expression (Diatchenko et al. 1996) as well as differential gene expression in organisms under environmental stress (e.g., Zhang et al. 2002).

SSH is a PCR-based technique in which differentially expressed complimentary DNA (cDNA) fragments (target fragments) are amplified, and commonly expressed non-target cDNA fragments are simultaneously suppressed between two samples of cDNA's derived from source material representing different treatment groups or tissues. For example, Zhang et al. (2002) recently identified several differentially expressed genes between two populations of the green algae *Dunaliella salina*, one population of which had been raised under hyperosmotic shock. SSH is ideal for the amplification of rare mRNA gene species, which are often the primary differentially expressed species, because it incorporates a hybridization step that normalizes the mRNA sequence abundance via standard hybridization kinetics (Diatchenko et al. 1996). In addition, SSH is superior to other methods because it is less labor intensive and is capable of achieving greater than 10^3-fold enrichment of differentially expressed cDNA fragments (Diatchenko et al. 1996).

The control of mRNA stability is a key component of gene regulation, and a multitude of cellular components have been identified in eukaryotic organisms that function to control stability in mRNA species (reviewed in Gutiérrez et al. 1999). In addition, mRNA displays inherent chemical instability and susceptibility to degradation by RNases (Voet et al. 1999), making it more difficult to isolate and use than DNA. The present study attempted to identify differentially expressed genes between greenbug damaged and undamaged sorghum plants via SSH.

Materials and methods

Two source populations, ATx399 × RTx430 sorghum plants damaged by greenbug biotype I and control plants not infested with greenbug, were used in the present study. Experimental treatments were designed as previously described (Chapter IV), with the exception that pots contained approximately 6 plants, all of one genotype, which were infested after 3 weeks (≥ 60 cm) with between 500 and 700 biotype I greenbugs. Plants were maintained in a greenhouse at the Entomology Research Lab, Texas A&M University, College Station, Texas.

When infested plants had incurred damage ratings of 7, they were harvested by first removing greenbugs (if infested) under running tap water, cutting the plant at the base, flash-freezing in liquid N_2, and storing at -80^o C for future RNA extraction. Approximately 3.5 g of total plant tissue were used per treatment. Total RNA was extracted by crushing frozen sorghum plants under liquid N_2 in a mortar until reduced to a fine powder. Extraction was accomplished with the Qiagen RNeasy® Plant Mini Kit. The kit protocol was modified as follows: (a) 18 ml of the guanidine isothiocyanate-containing buffer was poured directly onto the entirety of frozen, crushed sorghum tissue in the mortar, (b) the material (between 15 and 20 ml) was transferred to a 50 ml tube and allowed to thaw, (c) once thawed the material was homogenized with a handheld tissue tearer for approximately 30 minutes, and (d) 450 µl aliquots (samples) of the homogenate were used downstream. It was determined that this method resulted in total RNA concentrations equal to or better than the method suggested by the manufacturer,

and also allowed for more rapid collection of numerous samples. Any material that was not immediately processed was stored at -80^{o} C until processing could begin.

Because high purity, more concentrated total RNA results in better mRNA isolation, an essential step is to determine the purity and concentration of samples to be used downstream. Sample RNA quality and concentration were determined by measuring the 260 (A_{260}) and 280 (A_{280}) nm absorbance values. For pure RNA, the $A_{260/280}$ ratio is routinely reported as between 1.9 and 2.1, and degraded samples tend to lie outside this range. However, good quality RNA of $A_{260/280} \geq 1.8$ is suitable for downstream applications (e.g., Lal et al. 2001). Therefore, samples with $A_{260/280}$ between 1.8 and 2.1 were used in the present study. RNA concentrations were determined as follows:

(4) $40 * A_{260} *$ dilution factor

where 40 μg/ml of RNA is equivalent to 1 unit of absorbance at 260 nm, and the dilution factor was 20 for the present study. Total RNA content of all samples selected for downstream use was visualized using a denaturing agarose gel electrophoresis to ensure 18S and 28S bands were present (e.g., Fig. 18), confirming the isolation of total RNA. Isolation of mRNA from total RNA was attempted with the Oligotex^{TM} mRNA Mini Kit.

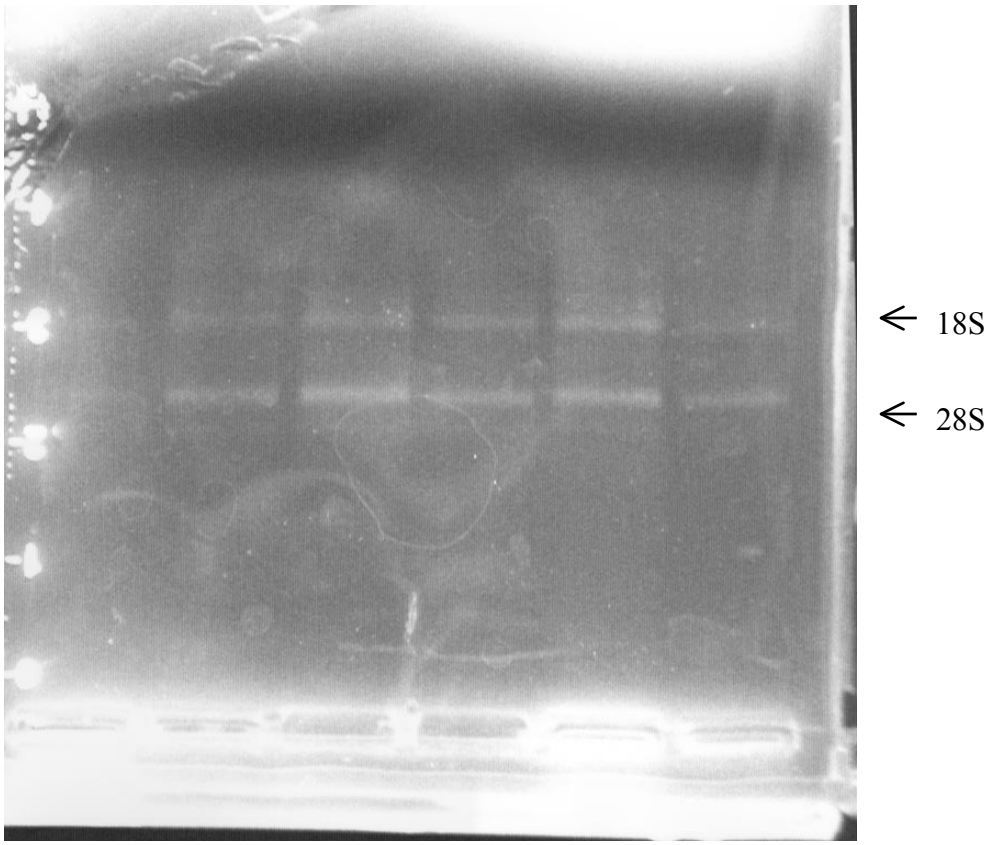

Fig. 18. Gel electrophoresis of total RNA from damaged plants. Sample numbers are below lanes. Bands are of low intensity due to the low concentration of total RNA in samples. Absence of bands in sample 3D suggests degraded RNA.

Results

Total RNA extraction resulted in marginal $A_{260/280}$ ratios and relatively low concentrations in undamaged plants, and very pure but extremely low RNA concentrations in damaged plants (Table 9). Four of 11 samples from undamaged plants and five of 20 samples of damaged plants displayed adequate $A_{260/280}$ ratios to attempt mRNA extraction (Table 9). Isolation of mRNA was unsuccessful for all samples: absorbance was not detected at either 260 or 280 nm for any mRNA samples.

Discussion

The analysis of differential gene expression was not attempted in the present study due to an inability to isolate messenger RNA, the molecule necessary for cDNA synthesis. Degradation of RNA in samples was probable, as the elution/storage material was RNase-free water, which may not have provided adequate protection against RNase action. A buffer with RNase inactivation capacities may have been a more appropriate storage material, however elution with RNase-free water was given in the protocol. Further, the very low concentrations of total RNA are likely to be more susceptible to degradation, or at least the effect was more pronounced due to low concentration. Minimal cell wall disruption is likely to have limited the amount of RNA extracted from plant tissues: therefore techniques to help degrade cell wall constituents may be beneficial to future research efforts.

Research has shown that pathogens capable of causing plant disease use extracellular enzymes that degrade cell walls and these enzymes are associated with

Table 9. $A_{260/280}$ ratios and total RNA concentrations extracted from sorghums.

Sample[a]	$A_{260/280}$	Total RNA (μg/μl)
6D	2.0	0.103
8D	1.9	0.106
9D	2.0	0.104
12D	1.9	0.121
14D	1.9	0.121
1C	1.8	0.31
7C	1.8	0.33
10C	1.8	0.38
11C	1.8	0.39

[a] D = damaged plants, C = undamaged plants.

pathogen virulence (e.g., Tonukari et al. 2000). A class of cell wall-degrading enzymes (i.e., pectinases) is present in greenbug saliva and may help confer virulence in this insect to some of its hosts (e.g., Ma et al. 1998). Therefore, it seemed reasonable to assume that greenbug feeding would break down cell walls and this, in turn, would increase the amount of RNA available to be isolated from damaged tissues. This was not the case however, as control plants consistently had higher RNA concentrations (on the order of 3X more) than damaged plants. It has been reported that greenbug saliva contains enzymes capable of degrading plant macromolecules to enhance the nutrition of the ingested material, as has been shown in other studies (e.g., Burd 2002), and these enzymes may have lowered the overall RNA content in damaged tissues (had high levels of RNase activity).

Identification of differentially expressed genes in sorghum related to greenbug feeding may have important implications in future sorghum resistance management. Since plant resistance remains the most viable option in sorghum production for protection against greenbug infestation, future work in this area should include research on differentially expressed genes in addition to other more conventional approaches such as QTL analysis (e.g., Katsar et al. 2002).

CHAPTER VIII

SUMMARY AND CONCLUSIONS

"It has fallen on me, an Entomologist, to hear strange and absurd words about insects..."

- Camillo Rondani, 1852 (English Translation)

Greenbug has been a serious insect pest of sorghum since the late 1960s when biotype C developed which was capable of damaging sorghum. Since then, a struggle has ensued to develop and sustain resistant sorghum varieties under the constant threat of evolution of new virulent greenbug biotypes. Because of this, research on the interactions among existing greenbug biotypes and sorghum genotypes is essential to elucidate the insect and plant parameters that may be important in the evolution of new virulent greenbug biotypes. The present study was conducted to characterize the interactions among greenbug biotype/*Sorghum* genotype combinations with regard to plant damage, and greenbug virulence and fitness.

Choice tests were conducted on four cultivated sorghum genotypes and eight published greenbug biotypes plus three clones isolated from particular geographic areas or host plants. These tests identified resistance mechanisms in some plant genotypes towards certain greenbug biotypes. Some biotypes not commonly associated with sorghum and relatively rare in field collections, such as G, H, and SC, were shown to reproduce as well as more common biotypes (e.g., C, E, and I) but did not cause substantial damage on sorghum genotypes. This may allow uncommon biotypes to persist and enter the gene pool during the reproductive period. Previous research has suggested volunteer grass crops and non-cultivated grasses such as Johnson grass are

important in the maintenance and evolution of some greenbug biotypes (e.g., Shufran et al. 2000, Anstead et al. 2003). The choice tests results suggest a mechanism by which these uncommon biotypes may persist, at least in cultivated sorghum: lacking noticeable damage, sorghum varieties infested with avirulent biotypes may not receive the attention from the plant breeder perspective as would varieties exhibiting greenbug damage. Continued research in population dynamics and virulence of all known biotypes on susceptible and resistant sorghum varieties is warranted to further elucidate insect and plant parameters important in the interaction of the two organisms. Additionally, molecular genetic analysis would be instrumental in identifying genes that are directly involved with the outcome of a particular biotype/genotype interaction.

Non-choice tests on five greenbug biotypes (NY, B, C, E, and I) were conducted on four sorghum varieties and the non-cultivated host Johnson grass to identify more specific variables involved in the virulence/avirulence of biotypes, the susceptibility/resistance of plants, and the population dynamics of biotypes. The five greenbug biotypes were chosen because they represented the chronological development of greenbug biotypes prior to cultivated sorghum damage (i.e., NY, which is A-like, and B) and those for which resistant sorghum varieties have been developed (i.e., C, E, and I). Tests were conducted in environmental chambers with constant conditions to minimize the impact of temperature, humidity, and light availability. Virulence tests revealed plant damage rating to be negatively associated with both plant water weight and plant growth. Therefore, a significant amount of damage that is visualized by the plant damage rating scheme is due to water loss and plant stunting, which corroborates

other findings (e.g., Garsed et al. 1987). However, the present study did not identify plant dry matter as significantly influenced by greenbug feeding, as has been demonstrated by other researchers (e.g., Burd 2002). This is likely due to the limitation of the measurements in the present study, therefore necessitating more precise measurement of dry matter loss from greenbug feeding. Specific biotype/genotype combinations exhibited characteristic virulence patterns (e.g., Tx7000 damage by biotype C, but all other genotypes resistant) with regard to water loss and plant stunting. Surprisingly, NY and B caused significant damage relative to the control plants, although no visible signs of damage were observed and these biotypes took significantly longer to kill the susceptible variety. This stresses the importance of non-cultivated grasses, particularly those invariably associated with cultivated sorghum, in the maintenance and evolution of new greenbug biotypes.

Non-choice tests on biotype reproduction rates were conducted as for virulence tests by calculating intrinsic rates of increase, r_m, for colonies and individuals. Generally, colony and individual r_m were very similar within biotypes and genotypes. Values did vary between biotype/genotype combinations; some differences were significant. The most resistant sorghum genotype (PI550607) and Johnson grass restricted the reproduction of many biotypes, particularly in colonies. In general, resistant varieties restricted the reproduction of greenbug biotypes relative to susceptible ones, however the differences were not all significant. While this could suggest increased nutrition via greenbug virulence, the plant genotypes restricting greenbug reproduction could be displaying antibiosis. The interaction is dependent upon both

organisms, and particular outcomes in a biotype/genotype combination involved multiple factors of which greenbug virulence and sorghum resistance are but two. For example, antibiotic effects on greenbug reproduction could be the result of poor host quality from whatever source, whether or not there is truly a "resistance mechanism." In the present study, the observation of reduced reproduction is argued to reflect poor host quality, regardless of how it comes about. Further examination of other greenbug biotypes, sorghum varieties, and non-cultivated grasses would help illuminate the relationship between greenbug virulence and fitness, and the role of host quality and resistance mechanisms.

Because plant resistance remains the most economically and environmentally sound option towards protection against greenbug damage in sorghum and small grains, research on the interaction between greenbug and its hosts continues to be an important focus in grass crops. Additionally, sorghum cultivation may benefit other crops, such as cotton, that share or have very similar insect pest composition (e.g., Prasifka et al. 2001). The importance of sorghum to humanity, as a primary food source, feed for livestock, industrial product, etc., will likely continue to grow as it is suited to grow in the face of many of the stresses associated with modern agriculture. It is therefore necessary to improve as much as possible our understanding of its relationship with one of its primary insect pests, greenbug.

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1998 – 2003	Graduate Assistant Teaching Assistant	Texas A&M University College Station, Texas
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1996 – 1997	Instructor Biology Dept.	University of Texas-Pan American Edinburg, Texas

Organizational Memberships:

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