Chlorophyll degradation in wheat lines elicited by cereal aphid infestation
by Tao Wang

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Entomology
Montana State University
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Abstract:
The Russian wheat aphid, Diuraphis noxia (Mordvilko) (Hemiptera: Aphididae), is a serious pest of cereal crops and causes chlorosis along with a multiple of other symptoms. Temporal changes of plant and aphid [i.e., D. noxia, Rhopalosiphum padi (L.), the bird cherry-oat aphid] biomass from ‘Tugela’ wheat and three near-isogenic lines (isolines) (i.e., Tugela-Dn1, Tugela-Dn2 and Tugela-Dn5) were tested to assess aphid resistance of the Tugela wheat lines. When infested by D. noxia, all D. u emu-resistant isolines sustained growth. Biomass of D. noxia collected from non-resistant Tugela was significantly higher than those plants with resistant Dn genes. Biomass of D. noxia collected from Tugela-Dn1 and Dn2 plants was not different from each other, but was lower than that from Tugela-Dn5 In contrast, there was no difference in R. padi biomass from the four wheat lines. Photosynthetic pigment (chlorophylls a and b, and carotenoids) concentrations, chlorophyll a/b ratio, and chlorophyll/carotenoid ratio among the four wheat lines were assayed. Concentrations of chlorophylls a and b, and carotenoids were significantly lower in D. noxia-infested plants when compared with R. padi-infested and the uninfested plants. However, no difference was detected in chlorophyll a/b or chlorophylls/carotenoids ratio among Tugela wheat lines with different aphid treatments. When infested by D. noxia, chlorophylls a and b contents were not different among the four wheat lines at day 3, but were lower in Tugela and Tugela-Dn1 plants than in Tugela-Dn2 and Dn5 plants at days 6 and 12. To elucidate the biochemical mechanisms of chlorosis formation, activities of three chlorophyll degradation enzymes (i.e., chlorophyllase, Mg-dechelatase and chlorophyll oxidase) extracted from the wheat lines were measured. Chlorophyllase activities were higher on R. padi-infested wheat lines and lower on D. noxia-infested plants. Mg-dechelatase activities were higher on D. noxia-infested plants and lower on R. padi-infested plants. Experimental results confirmed the previous reports that Tugela is a D. noxia susceptible variety while Tugela Du plants are resistant to D. noxia. Chlorosis on Tugela wheat lines induced by D. noxia infestation was most likely driven by unbalanced chlorophyll biosynthesis and catabolism processes.
CHLOROPHYLL DEGRADATION IN WHEAT LINES ELICITED BY CEREAL APHID INFESTATION

by

Tao Wang

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Entomology

MONTANA STATE UNIVERSITY
Bozeman, Montana

June 2003
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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date  June 27, 2003
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ABSTRACT

The Russian wheat aphid, *Diuraphis noxia* (Mordvilko) (Hemiptera: Aphididae), is a serious pest of cereal crops and causes chlorosis along with a multiple of other symptoms. Temporal changes of plant and aphid [i.e., *D. noxia*, *Rhopalosiphum padi* (L.), the bird cherry-oat aphid] biomass from ‘Tugela’ wheat and three near-isogenic lines (isolines) (i.e., Tugela-*Dn1*, Tugela-*Dn2* and Tugela-*Dn5*) were tested to assess aphid resistance of the Tugela wheat lines. When infested by *D. noxia*, all *D. noxia*-resistant isolines sustained growth. Biomass of *D. noxia* collected from non-resistant Tugela was significantly higher than those plants with resistant *Dn* genes. Biomass of *D. noxia* collected from Tugela-*Dn1* and *Dn2* plants was not different from each other, but was lower than that from Tugela-*Dn5*. In contrast, there was no difference in *R. padi* biomass from the four wheat lines. Photosynthetic pigment (chlorophylls *a* and *b*, and carotenoids) concentrations, chlorophyll *a/b* ratio, and chlorophyll/carotenoid ratio among the four wheat lines were assayed. Concentrations of chlorophylls *a* and *b*, and carotenoids were significantly lower in *D. noxia*-infested plants when compared with *R. padi*-infested and the uninfested plants. However, no difference was detected in chlorophyll *a/b* or chlorophylls/carotenoids ratio among Tugela wheat lines with different aphid treatments. When infested by *D. noxia*, chlorophylls *a* and *b* contents were not different among the four wheat lines at day 3, but were lower in Tugela and Tugela-*Dn1* plants than in Tugela-*Dn2* and *Dn5* plants at days 6 and 12. To elucidate the biochemical mechanisms of chlorosis formation, activities of three chlorophyll degradation enzymes (i.e., chlorophyllase, Mg-dechelatase and chlorophyll oxidase) extracted from the wheat lines were measured. Chlorophyllase activities were higher on *R. padi*-infested wheat lines and lower on *D. noxia*-infested plants. Mg-dechelatase activities were higher on *D. noxia*-infested plants and lower on *R. padi*-infested plants. Experimental results confirmed the previous reports that Tugela is a *D. noxia* susceptible variety while Tugela *Dn* plants are resistant to *D. noxia*. Chlorosis on Tugela wheat lines induced by *D. noxia* infestation was most likely driven by unbalanced chlorophyll biosynthesis and catabolism processes.
CHAPTER 1

INTRODUCTION

The Russian wheat aphid, *Diuraphis noxia* (Mordvilko) (Hemiptera: Aphididae), is a serious pest of cereal crops worldwide, except in Australia. This species completes its entire life cycle on a wide range of cereal grains and grasses. Estimated economic losses caused by *D. noxia* feeding to wheat, *Triticum aestivum* L., and barley, *Hordeum vulgare* L., in the western U. S. between 1987 and 1993 averaged over $127 million per year (Webster et al. 1994). In recent years, various management perspectives (e.g., biological control, chemical control, cultural control, and plant resistance) have been studied to reduce *D. noxia* populations and the economic impact of this pest on cereals.

*Diuraphis noxia* typically aggregates on the new growth of the host plants. The most obvious plant injury symptom is chlorosis, which can subsequently develop into longitudinal white streaks on leaves (Hewitt et al. 1984). Other injury symptoms include folding or convolute leaf rolling, blasted heads, and purple discoloration. These injury symptoms can lead to plant stunting, prostrate growth or even sterility, and finally loss of yield.

Von Wechmar and Rybicki (1981) showed that the typical injury symptoms of plants associated with *D. noxia* feeding were induced by a salivary toxin injected during feeding. Although the nature of the toxin was unknown, Kruger and Hewitt (1984) reported that *D. noxia* extracts adversely affect photosynthetic rates *in vitro* and proposed this could be secondary to the degradation of the chloroplast membrane or *vice versa*. Haile et al. (1999) found that *D. noxia* feeding caused less reduction of photosynthetic
rates in PI 262660 (*D. noxia* tolerant) when compared with PI 137739 (antibiotic) and 'Arapahoe' (susceptible) wheat. They suggested this phenomenon might be caused by varying resistance mechanisms that provided different photosynthetic compensation abilities to aphid feeding injury. Ni et al. (2000) studied the hydrolases and oxido-reductases in the homogenate of aphids and plant tissues. Pectinesterase, polygalacturonase, cellulose, and amylase activities were examined in the hydrolase group, while catalase, peroxidase, catechol oxidase, superoxide dismutase, and ascorbate oxidase activities were examined in the oxido-reductases group. They detected the same hydrolase activities from *D. noxia* and bird cherry-oat aphid, *Rhopalosiphum padi* (L.), but different oxido-reductase activities. Catalase activity was detected from *D. noxia* salivary tissue but not from *R. padi*, whereas peroxidase was detected from *R. padi* salivary tissue but not *D. noxia*. They suggested that this salivary enzyme difference between the two aphid species is important in the development of injury symptom formation on susceptible wheat plants. Ni et al. (2001a) detected a peroxidase increase in resistant ‘Halt’ wheat and suggested the increase may have contributed to *D. noxia* resistance. Ni et al. (2001b) demonstrated that herbivore-elicited chlorophyll loss differed from chlorophyll degradation in naturally senescing plants because neither chlorophyllase nor oxidative bleaching activities described by Janave (1997) and Matile et al. (1999) in the senescing plants was detected in aphid-infested wheat. *Diuraphis noxia*-infested susceptible wheat (Arapahoe) showed a significantly higher Mg-dechelatase activity than the uninfested wheat. The finding suggested chlorosis is the result of increased Mg-dechelatase activity and possibly other plant physiological changes.
The research reported in this thesis was a continuation of previous research by Ni et al. (2001b, 2002) to understand the biochemical mechanisms of aphid-elicited chlorosis using ‘Tugela’ and three wheat near-isogenic lines (isolines) (i.e., Tugela-Dn1, Tugela-Dn2 and Tugela-Dn5) and two species of cereal aphids (i.e., chlorosis-eliciting D. noxia and non-chlorosis-eliciting R. padi). The objectives were to assess the resistance mechanisms of Tugela and Tugela isolines through analysis of aphid and plant biomass; to quantify photosynthetic pigments (i.e., chlorophylls a and b, and carotenoids) in wheat plants with different aphid treatments (i.e., D. noxia, R. padi, and control); and to assay changes in chlorophyll degradation enzyme (chlorophyllase, Mg-dechelatase and chlorophyll oxidase) activities elicited by aphid infestation in wheat plants and their correlation with chlorosis formation. Chapter three focused on resistance assessment and pigment quantifications of aphid-infested wheat plants, while Chapter four reports measurement of chlorophyll degradation enzyme activities in wheat plants elicited by aphid feeding. The experimental results of chlorosis rating and quantifications of photosynthetic pigments of aphid-infested wheat plants in Chapter three were basal to the further discussion of enzymatic chlorosis formation in D. noxia-infested wheat plants in Chapter four.
Diuraphis noxia is native to the area between the Caucasus Mountains and the Tian Shan Mountain of Central Asia. It was first described in the early 1900s when outbreaks occurred in Moldova and Ukraine, followed by a period of relative obscurity (Halbert and Stoetzel 1998). It was introduced into the Republic of South Africa where it became a serious pest in 1978 (Halbert and Stoetzel 1998). Diuraphis noxia was detected in Mexico in 1980 and in the U. S. at Muleshoe, TX in March 1986 (Stoetzel 1987). The aphid soon spread to the seven Great Plains states (Colorado, Kansas, Nebraska, New Mexico, Oklahoma, Texas and Wyoming) and the following year spread north and west, increasing its range to include seven more western and northwestern states (Arizona, Idaho, Montana, South Dakota, Oregon, Utah and Washington). In North America, the estimated cumulative total economic losses to the U.S. cereal industry from D. noxia from 1987 through 1993 have been over $900 million (Webster et al. 1994). Morrison and Peairs (1998) estimated the associated cost (i.e., crop loss and use of pesticides) in the U. S. between 1986 and 1994 from D. noxia damage was 893.1 million dollars.

Diuraphis noxia has established itself as an important pest of small grains in the arid and semi-arid areas of the western U. S. The Canadian provinces of Alberta, British Columbia, and Saskatchewan have also reported the aphid’s presence and damage on
cereals (Jones et al. 1989). To date, D. noxia occurs in all major cereal production areas of the world, except Australia (Halbert and Stoetzel 1998).

Host Range

Hosts of D. noxia are restricted to the Graminae, including barley (Hordeum vulgare L.), wheat (Triticum aestivum L.), oats (Avena sativa L.), rye (Secale cereale L.), and triticale (X Triticosecale Wittmack). It also can survive and reproduce on several other cool-season and warm-season grasses (Kindler and Springer 1989). Wheat, barley, and some cool-season grasses are particularly favorable host plants.

It is clear that volunteer wheat is the most important oversummering host for this aphid (Hewitt et al. 1984). Suitability of some cool-season grasses appears to vary geographically. The most important grass hosts of D. noxia in the Great Plains are crested wheatgrass, Agropyron cristatum (L.) Gaertner; Canada wildrye, Elymus Canadensis (H.); jointed goatgrass, Triticum cylindricum (Host) Ces.; and downy brome, Bromus tectorum (L.) (Hammon et al. 1989, Armstrong et al. 1991, Montandon et al. 1993). Crested wheatgrass and Canada wildrye are reported to be the grass species most capable of supporting oversummering populations of D. noxia (Armstrong et al. 1991).

Life Cycle

Throughout the summer season, all populations of D. noxia reproduce parthenogenetically. The live forms (viviparae) can be winged or wingless. Winged morphs (alatae) appear in response to a decline of the host plant, an increase of population density, and possibly other environmental stimuli (Kawada 1987, Baugh and
Phillips 1991). Some populations that are holocyclic produce males and oviparae. They mate to produce overwintering eggs in the autumn. Other populations are anholocyclic and do not produce males or oviparae and overwinter as viviparae.

_Diuraphis noxia_ is holocyclic in its native range of Central Asia (Kiriac et al. 1990), Hungary (Basky 1993), and Spain (Fernandez et al. 1992). There are no reports of sexual forms occurring in the Middle East or in South America. Similarly, the South African population of _D. noxia_ is anholocyclic (Hewitt et al. 1984). In the U. S., oviparae of _D. noxia_ were found in Idaho in 1989, both in the southwest Treasure Valley and in the northern Idaho Palouse region (Kiriac et al. 1990). However, no males have been found to date in North America, and eggs are not viable without fertilization (Halbert and Stoetzel 1998).

Survival of _D. noxia_ during winter depends on the duration of cold temperatures, cold temperature extremes, and precipitation patterns (Butts 1989, 1992; Armstrong 1994). _Diuraphis noxia_ does not seem to suffer adverse effects from cold until temperatures are below 0°C. However, numbers decrease slowly and almost linearly throughout winter when soil surface temperatures remain at or below 0°C. As soil surface temperatures decrease below -5°C, mortality increases at a faster rate and, when temperature is below -25°C, mortality is rapid and extensive (Armstrong 1994).

### Economic Thresholds

The concept of plant tolerance to pest injury was discussed by scientists as early as 1934; however, the original concepts of the Economic Injury Level (EIL) and Economic Threshold (ET) were first proposed in a landmark paper by Stern et al. (1959). Economic
damage is defined as the amount of injury that will justify the cost of control. The EIL is proposed as the lowest population density of pests that will cause economic damage, while the ET is defined as the density of pests at which control measures should be taken to prevent the pest population from reaching the EIL (Peterson and Higley 2002). The ET is a basic component of integrated pest management (IPM) and determinations of the EIL and ET for an insect species are important to an IPM program (Legg and Archer 1998). Poston et al. (1983) listed four categories of thresholds: (1) nonthresholds, where a pest is so damaging and the threshold is too low to be employed; (2) nominal threshold, which is determined by field observation and/or experience rather than data; (3) simple threshold, a threshold developed by research using an average or nominal system for a single pest and is based on correlations of damage potential of the pest, crop market value, costs of control, and potential crop yield; and (4) comprehensive threshold, which incorporates multiple pest and stress factors and is regarded as the ideal model.

Determination of the ET for *D. noxia* was complicated because small grains are grown in several climate zones throughout the U. S. with various cultural practices. *Diuraphis noxia* also infests both winter and spring grains at any plant growth stage. Consequently, EILs and ETs must be applicable to a wide range of climatic zones, production practices, and plant stages. This requires knowledge of aphid injury and yield loss interactions, insect and plant developmental rates, and the effects of stress on the interaction between the aphid and plant (Legg and Archer 1998).

The first economic threshold for *D. noxia* was determined in South Africa by du Toit (1986). He calculated an EIL level of 14% infested plants at growth stage 59 (seed spike clear of the leaf sheath, Tottman and Makepeace 1979) using the formula proposed
by Stone and Pedigo (1972) and Ogunlana and Pedigo (1974); $EIL = \frac{ED}{b}$, where $ED$ is the economic damage and $b$ is slope of the crop yield on pest infestation level regression. The ET was calculated at 4-7% infested plants at growth stage 31 (first node) by using the formula $ET = EIL \left( C^{-x} \right)$, in which $C$ is the amount of increase in infestation between growth stages 31 and 59 and $x$ is the time in weeks between growth stages 31 and 59 (du Toit 1986). Archer and Bynum (1992) reported a threshold of $D. noxia$ on dryland winter wheat which predominates in the western United States and Canada. They concluded an estimate of 0.46% and 0.48% yield loss for each 1% increase in damaged and infested tillers respectively. Girma et al. (1993) calculated an economic injury level of 2.2 and 4.0 aphids per 7 plants for fall infestations in northeastern Kansas in 1988 and 1989, respectively. The spring infestation caused more damage than the fall infestation with economic injury levels of 0.9 and 2.4 aphids per tiller, respectively.

Recently, U. S. scientists realized that the thresholds reported previously were not dynamic because they did not include other factors such as chemical control costs and crop value. Therefore, in the U.S., a dynamic economic threshold was developed that factored in the changing cost of chemical control and crop value into the decision process. This enabled a comprehensive threshold that was sensitive to regional differences. The comprehensive threshold was equally useful in the northern states where emphasis is placed on fall infestations in winter grain, and in southern states where spring infestations are of the most concern. Pesticide use then evolved from being conservative policy against any $D. noxia$ population to an as-needed policy based on output from varying economic environment (Legg and Archer 1998). Based on this concept, Archer et al. (1998) concluded a comprehensive economic threshold by different infestation seasons
and regions. For spring infestation through all regions, they suggested the formula proposed by Peairs et al. (1991): $EIL = \frac{CC \times \text{loss per unit}}{EY \times MV}$, where $CC =$ cost of control, $EY =$ estimated yield, and $MV =$ market value of wheat. However, economic thresholds for fall infestation vary by state: in southwestern states, the loss in bulk seed weight will be 4% per 1% of infested or damage tillers and 0.3% per infestation or damage day. In the central Great Plains or Pacific Northwest states, the value of loss will be 0.7% per percentage of infested or damaged tiller and 0.6% per infestation or damage day. In the coldest states or Canada, the loss value will be 1% per percentage infested or damaged tillers and 1.1% for infestation or damage days.

Management Strategies

In recent years, various management tactics have been studied and applied to manage *D. noxia*. These include: biological control, chemical control, cultural control practices, and host plant resistance.

Biological Control

Even though endemic natural enemies do not seem to play a significant role in regulating *D. noxia* populations in the U. S., biological control is still recognized as a potential tactic for managing *D. noxia*. According to Prokrym et al. (1998), four exotic parasitoid species *Aphidius uzbekistanicus* (Luzhetzki) (Hymenoptera: Aphidiidae), *Aphidius colemani* (Viereck) (Hymenoptera: Aphidiidae), *Aphelinus albipodus* Hayat and Fatima (Hymenoptera: Aphelinidae), and *Aphelinus asychis* Walker (Hymenoptera: Aphelinidae) have been established in the U. S. to control *D. noxia*. *Aphelinus* spp. can be
found in most regions where *D. noxia* infestation occurs. They are solitary endoparasitoids of nymphs and adults of a variety of aphid species. They are regarded as promising candidates for *D. noxia* biological control because they are active during the season when *D. noxia* is active; they occur throughout most of *D. noxia*'s geographic distribution range, live longer, and can parasitize a large number species of aphids (Hopper et al. 1994). Syrphid flies (Diptera: Syrphidae) are another group of important natural enemies of *D. noxia*. According to Hopper et al. (1994), the most common species found in *D. noxia* colonies are *Episyrphus balteatus* (De Geer), *Eupeodes corollae* (F.), and *Sphaerophoria scripta* L. The adults feed on nectar, honeydew, and pollen, but their larvae prey on aphids. These three species of syrphids are promising predators for *D. noxia* biological control because of their frequent occurrence, large consumption rate, and vigor of their larvae. Gonzalez et al. (1990) summarized in their survey of natural enemies of *D. noxia* that aphelinids and chamaemyiids provided the best potential for rearing and colonizing for *D. noxia* management. Syrphids are next in order of importance, while *Aphidius colemani* (Viereck) and *Aphidius matricariae* (Haliday) are the third.

Other promising species reported by previous researchers include, predators [e.g., *Chrysopa* spp. (Neuroptera: Chrysopidae); *Aphidoletes aphidimyza* (Rondani); *Leucopis* spp. (Diptera: Chamaemyiidae); *Coccinella septempunctata* L.; *C. transversoguttata* Brown; *Propylea quatuordecimpunctata* (L.); *Scymnus frontalis* Fabricius; and *Adonia variegata* (Goeze), (Coleoptera: Coccinellidae)] (Bosque-Perez et al. 2002), parasitoids [e.g., *Lysiphlebus* spp.; *Aphidius picipes* (Nees); *Aphidius rhopalosiphi* DeStefani-Perez; *Ephedrus plagiator* (Nee); *Diaeretiella rapae* (McIntosh); and *Praon gallicum* Stary]
(Hymenoptera: Braconidae) (Brewer et al. 2001), parasites (e.g., mites), and fungal pathogens [e.g., *Pandora neoaphidis* (Remaudière and Hennebert)] (Feng et al. 1990, 1991).

**Chemical Control**

*D. noxia* colonization of the flag leaves of wheat can be inhibited and significant yield losses prevented by applying insecticides to plants when the first node is visible (du Toit and Walters 1984). Systematic insecticides have been recommended for *D. noxia* control. Contact insecticides are ineffective because *D. noxia* secludes itself within rolled leaves typically associated with the injury symptoms (Webster 1990). Mixing contact and systemic insecticides has reportedly resulted in effective control (Botha 1984, Valiulis 1986).

Numerous registered and unregistered insecticides in the U.S. have been tested against *D. noxia*. Materials most commonly used commercially include dimethoate, disulfoton, phorate, methyl parathion, and parathion. Other promising insecticides such as endosulfan, chlorpyrifos, esfenvalerate, and ethyl parathion also are effective (Pike and Suomi 1988).

Meyer et al. (1990) compared imidacloprid with other granular formulations of disulfoton, phorate terbufos, and carbofuran as seed treatments to control *D. noxia* and obtained effective control with imidacloprid. An evaluation of systemic insecticides applied in furrow at planting time for *D. noxia* control on winter wheat by Armstrong et al. (1989) showed that carbofuran plus liquid fertilizer were effective for aphid control. However, carbofuran was ineffective when applied with dry fertilizer. Hill and Butts
(1990) compared chlorpyrifos with dimethoate and malathion commonly used for aphid control in Canada. They found that chlorpyrifos was effective for *D. noxia* control because of its toxic vapor, efficient penetration through the plant epi-cuticular wax layer, and rapid diffusion to aphid feeding sites.

Chemical applications have limited success because temperature may affect the rate of insecticide absorption into insects and, in the case of a systemic insecticide, the rate of uptake by plants. Rainfall, occurring immediately after application, may dissolve and move active ingredients or mechanically dislodge residues. Volatility, which is directly related to temperature, can result in significant loss of insecticide from leaf surfaces after application (Johnson and Kammerzell 1990). The expense of insecticide application for managing *D. noxia* might be prohibitive and the cost to the environment is of concern (Burd et al. 1994). For instance, Flickinger et al. (1991) reported the poisoning of more than 200 Canada geese, *Branta canadensis* Lamark, in Texas as a direct result of parathion applications to winter wheat for control of *D. noxia*. Moreover, the use of insecticides for controlling *D. noxia* also has been reported to contribute significantly to the recent occurrence of insecticide-resistant greenbugs, *Schizaphis graminum* (Rondani) (Shotkoski et al. 1990, Shufran et al. 1993). Research programs directed toward the control and management of *D. noxia* populations in agroecosystems have emphasized the development of environmentally friendly alternative means of control such as plant resistance and biological and cultural controls (Burd et al. 1994).
Cultural Control

Cultural control can be defined as the alteration of crop environment through modification of normal farm practices for the purpose of either discouraging target pests or of encouraging biological control agents (Peairs 1998). Cultural controls are important management tactics especially for pests of extensively grown, low-return crops where profit margins leave little room for insecticide use (Holtzer et al. 1996). The production of dryland winter wheat typifies such a system. For instance, in 1992 in northeastern Colorado, the average return on winter wheat without federal price support payments on 1 ha of winter wheat was ≈$125 (Nitchie and Schaubert 1993). Cultural controls also help in postponing the development of insecticide resistance.

Various cultural control techniques have been studied for the management of *D. noxia*: modifications in crop diversity, sanitation, grazing, fertilization, irrigation, row spacing, and planting dates (Peairs 1998). Increasing crop diversity is one of the practices that may possibly increase the abundance of the natural enemies of *D. noxia*, however, there are no studies of this technique.

Sanitation is the process of removing volunteer crop plants, crop residues, weeds, alternate hosts, and other potential pest refuges that can be sources of reinfestation (Peairs 1998). A number of alternate hosts of *D. noxia*, mostly cool-season grasses, as indicated above, have been identified (Kindler and Springer 1989, Armstrong et al. 1991). Volunteer wheat is generally considered to be the most important infestation source for the newly emerging crops. Management of volunteer wheat is very important because these plants compete with the crop for moisture, and act as alternative host for many
other pests including cereal aphids, the wheat curl mite (*Aceria tosichella* Keifer), and the Hessian fly [*Mayetiola destructor* (Say)] (Stuckey et al. 1989).

Grazing is a very common cultural practice in the southern Great Plains especially in southeastern Colorado where ≈ 50% of the wheat is grazed (Peairs 1998). The frequency of grazing decreases in north regions because of the severe winter conditions. Grazing can affect insects by trampling, ingestion, or competition for host plant tissues. Therefore, under proper utilization, grazing can be an effective cultural control.

Fertilization of wheat is another popular practice in the management of *D. noxia*. Appropriate fertilization will improve the plant’s vigor and enable the plant to better tolerate the stresses from *D. noxia* and other unfavorable conditions (Peairs 1998). Proper plant fertilization can also alleviate aphid injury and result in less yield loss. For instance, Riedell (1990) reported a ≈ 50% more grain produced in the presence of *D. noxia* in ‘Marshall’ wheat plants provided with 100% of normal nitrogen requirements in comparison with wheat plants given only 10% of the same nutrients. Changes in plant nutrition may also affect the reproductive potential of *D. noxia* (Peairs 1998). Soil moisture can be a limiting factor affecting fertilizer utilization. According to the research by Walker et al. (1990b), the application of varying levels of nitrogen has been beneficial under irrigated conditions when compared with dryland sites. *Diuraphis noxia* is more likely to cause economic losses in dryland wheat than in irrigated wheat, which is most likely attributed to the drought stress in plants (Peairs 1998). Therefore, modification of irrigation practices may provide an opportunity to improve *D. noxia* management. The direction in which a field is irrigated also correlates the severity of *D. noxia* infestation (Hammon and Peairs 1992). In western Colorado, the percentage of infested plants or
tillers and aphids per plant or tiller in early spring were \( \approx 10 \) times greater in fields irrigated from east to west than irrigated from north to south on furrow-irrigated ‘Schuyler’ barley. This was due to the higher temperature accumulations during the winter on the south-facing slopes of fields between the irrigation furrows.

Finally, modification of planting date is another common cultural tactic because crops can be planted within a range of dates that still have acceptable quality and yield. Planting date recommendations for *D. noxia* generally propose that spring wheat should be planted as early as possible to maximize crop maturity by the time *D. noxia* migrates from fall-sown, small-grain crops. This is based on the assumption that more mature plants will be less attractive to the aphids. Meanwhile, the plants will suffer less damage from *D. noxia* infestation when they are closer to maturity (Peairs 1998). On the other hand, decisions on fall seeding are more site-specific. In regions where spring infestation of *D. noxia* is of concern, early fall seeding is recommended because it enables the plants to mature early and be less attractive to aphids during spring *D. noxia* dispersal. Where fall infestations are severe, however, late fall seeding is suggested. During fall aphid dispersal, the crops are still small and presumably less attractive to aphids. Some crops may emerge after aphid dispersal (Peairs 1998). For instance, Butts (1992) in Alberta and Kammerzell and Johnson (1990) in Montana recommended late fall seeding in comparison with Walker et al. (1990a) in Colorado who support an early or intermediate fall seeding date.
Host Plant Resistance

Among the various pest management strategies, host plant resistance may be the most effective strategy, both from economical and environmentally sound perspectives, to manage *D. noxia* on cereal crops. Other management tactics, such as using biological control agents and chemical applications, have limited success because aphid feeding causes leaf rolling that protects aphids from natural enemies and direct contact of insecticides. Consequently, the major initial management tactics for this pest have been limited to systemic insecticides and cultural practices such as delayed plantings in late 1980’s (Pike 1988, Smith et al. 1991).

The phenomena of plant resistance to insects are usually based on heritable traits (Panda and Khush 1995). Nevertheless, some characteristics are quite plastic and fluctuate widely under the influence of environmental conditions. The environment may favor the plant or the insect unequally and may prevent or aggravate damage; therefore, it is likely to affect the expression of insect resistant traits. Accordingly, plant resistance to insects may be classified as “genetic,” implying characteristics are under the primary control of genetic factors, or “ecological,” implying the characteristics are under the primary control of environmental factors.

In terms of plant genetic resistance, Painter (1951) defined insect-resistance as nonpreference, antibiosis, and tolerance. Kogan and Ortman (1978) then proposed to replace the term “nonpreference” with “antixenosis” because the word “nonpreference” is mostly related to the insect feeding behavior instead of host plant’s intrinsic response to pest infestation. Plant resistance to insects can be categorized as antibiosis, antixenosis, tolerance or combination of the three categories (Painter 1951, Kogan and Ortman 1978).
Antixenosis is the resistance mechanism employed by the plant to deter or reduce colonization by the insects. Generally, insects orient themselves toward plants for food, oviposition sites, or for shelter. However, because of certain characteristics, the plant deters the insects. In certain situations, even though the insect may come in contact with the plants, the antixenotic characteristics of the plant do not allow the insect to colonize. Sometimes, the antixenosis mechanism is so effective that the insects starve and die (Painter 1968). Antibiosis is the resistance mechanism that functions after the insects have colonized and have started feeding. When an insect feeds on a plant exhibiting antibiotic resistance, its growth, development, reproduction, and survival will be affected. The antibiotic effects may result in a decline in insect size or weight, reduced metabolic processes, increased restlessness, and greater larval or preadult mortality. Indirectly, antibiosis may result in an increased exposure of the insect to its natural enemies. In certain cases antibiosis cannot be clearly separated from antixenosis because of the chemicals or physical factors in the plant may both negatively affect the insect's biological or physiological perspective and be deterrent to the pest. Tolerance is a genetic characteristic that enables the plant to support an insect population that will damage a susceptible host variety. There is no economic yield loss occurring when a plant is tolerant to insect damage. Tolerance does not affect the increase of the pest population but does raise the threshold level.

Not all host plant insect resistance phenomena can be distinguishingly assigned to a single category of resistance. These categories of resistance do not exclude each other, but are correlated along with other biotic and abiotic factors in the expression of resistance. Therefore, different cultivars may possess the same level of resistance but
with different resistant mechanisms (Panda and Khush 1995). This phenomenon has also been found in the wheat accessions examined by Souza (1998) for *D. noxia* resistance.

Ecological resistance has been categorized as pseudoresistance and induced resistance (Panda and Khush 1995). Pseudoresistance in the host plant does not result from the inherent characters, but from some temporary favorable environmental conditions (Painter 1951). As a consequence, certain crop varieties may pass through a susceptible stage rapidly and avoid pest damage. Induced resistance is either qualitative or quantitative enhancement of the plant's defense capability against the pests (Panda and Khush 1995).

In the U. S., the first source of significant level of resistance found in wheat to *D. noxia* was in PI 372129 (Turcikum 57 = T-57) in Colorado (Quick et al. 1991). PI 372129 is plant introduction line collected from Russia but generally unadapted to modern agriculture. In 1991, CORWA1, a hard, red winter wheat germplasm, was released for breeding and experimental purposes (Quick et al. 1991). The first North American Russian wheat aphid-resistant hard red winter wheat cultivar, known as 'Halt' (a sister line to CORWA1) was released by Colorado Agricultural Experiment Station in 1994 (Quick et al. 1996). The Western Regional Coordinating Committee No. 66 (WRCC-66) facilitated the identification of *D. noxia*-resistant genes and deployment of these genes in cultivated cereals. Systematic surveys were conducted of landraces and old cultivars from the former USSR, Central Asia, and the Near East. Research groups participating within WRCC-66 have evaluated >25,000 accessions, and identified at least 86 accessions to have reproducible resistance to *D. noxia*. Harvey and Martin (1990), Smith et al. (1991), Webster et al. (1991), and Porter et al. (1993) identified new sources
of *D. noxia* resistance in wheat, barley, and triticale accessions originated from these same areas (Souza 1998). These researchers also were able to confirm *D. noxia* resistance in PI 137739, PI 262660, and PI 294994 wheat that were initially identified in South Africa by du Toit (1987).

In recent years, scientists have identified a number of plant introduction lines that possessing at least one main category of resistance. Budak et al. (1999) reported PI 137739 and PI 294994 as antibiotic lines. Of particular interest are accessions that have been identified with strong sources of tolerance and limited or no antibiosis. These wheat accessions include PI 262660 (du Toit 1989, Budak et al. 1999), CI 15465 (Formusoh et al. 1992), Sando collection accession SS36 and SS385 (Formusoh et al. 1994), and PI 366447 (Webster et al. 1991). The sources of *D. noxia* resistance in current cereal breeding programs represent ten unique wheat genes (Table 1) (Quisenberry and Clement 2002).

The biochemical and physiological mechanisms associated with aphid resistance genes are not clearly understood. The resistance factors in PI 386148 triticale and PI 372129 wheat seem to minimize the loss of plant cell turgor pressure that normally result from *D. noxia* feeding (Burd and Todd 1992). *Diuraphis noxia* infestation induces the suppression of large and small subunits of Ribulose 1, 5-bisphosphate carboxylase on wheat and barley (Porter 1992, Rafi et al. 1993, 1996, Miller et al. 1994). Different protein biosynthesis between resistant and susceptible genotypes has also been documented in wheat and barley following *D. noxia* feeding (Miller et al. 1994, Rafi et al. 1996). Ni et al. (2001a) detected peroxidase increase in resistant ‘Halt’ wheat and suggested the increase may have contributed to *D. noxia* resistance. Preliminary evidence
indicates that some of the induced proteins (i.e., 32, 33, and 35k polypeptides) may be related to the resistance and early senescence in *D. noxia*-resistant wheat lines (e.g., PI 137739) (Rafi et al. 1996).

Table 1. Conserved cereal germplasm identified with *D. noxia* resistance (Quisenberry and Clement 2002)

<table>
<thead>
<tr>
<th>Source</th>
<th>Origin</th>
<th>Wheat type or class</th>
<th>Gene</th>
<th>Chromosome location</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI 137739</td>
<td>Iran</td>
<td>Hard white</td>
<td><em>Dn1</em></td>
<td>7DS</td>
</tr>
<tr>
<td>PI 262660</td>
<td>Russia</td>
<td>Hard white</td>
<td><em>Dn2</em></td>
<td>7DS</td>
</tr>
<tr>
<td>SQ 24</td>
<td>Unknown</td>
<td><em>Aegilops tauschii</em></td>
<td><em>dn3</em></td>
<td>Unspecified</td>
</tr>
<tr>
<td>PI 372129</td>
<td>Russia</td>
<td>Hard white</td>
<td><em>Dn4</em></td>
<td>1DS</td>
</tr>
<tr>
<td>PI 294994</td>
<td>Hungary(Bulgaria)</td>
<td>Hard red</td>
<td><em>Dn5</em></td>
<td>7DS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7DS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1DL</td>
</tr>
<tr>
<td>PI 243781</td>
<td>Iran</td>
<td>White</td>
<td><em>Dn6</em></td>
<td>Unspecified</td>
</tr>
<tr>
<td>Turkey 77</td>
<td>Turkey</td>
<td><em>Secale cereale</em></td>
<td><em>Dn7</em></td>
<td>1RS</td>
</tr>
<tr>
<td>PI 220127</td>
<td>Afghanistan</td>
<td>Red</td>
<td><em>Dnx</em></td>
<td>7DS</td>
</tr>
</tbody>
</table>

Etiology of Injury Symptoms

*Diuraphis noxia* typically aggregates and feeds on the new growth of susceptible host plants. *Diuraphis noxia* is a leaf-gall adapted aphid that induces “pseudogalling” by preventing newly formed leaves from unrolling (Burd and Burton 1992). *Diuraphis noxia* is well adapted to this niche because of its tubular body shape, waxy excretory products, and lack of well-developed cornicles (Nault and Phelan 1984, Wool 1984). Nonetheless, different aphid colonization behavior and plant response have been observed by Burd et al. (1993). Some aphids fail to aggregate on new growth and tend to be widely dispersed on the resistant wheat such as PI 386148. This suggested poor host suitability and an
inability of the aphids to alter the feeding site condition. Aphid aggregations may have an adaptive role in aphid performance through a conditioned improvement of the quality of host tissues as a food source (Hayamizu 1984, Dorschner et al. 1987, Dorschner 1990). A positive relationship between aggregation and aphid fecundity has also been shown for aphid species that, under natural conditions, form compact aggregations (Way and Cammell 1970, Hayamizu 1984).

The most obvious characteristics of *D. noxia* injury symptoms are leaf rolling and chlorosis (Walters et al. 1980, Hewitt et al. 1984) which can lead to plant stunting, prostrate growth or even sterility, and finally yield loss. The prevention of new leaf unfolding and reduction in leaf size are resulted from loss of leaf turgor below the threshold for elongation and cell-wall extensibility (Burd and Burton 1992, Burd et al. 1993, Bradford and Hsiao 1992).

Chlorosis indicates the loss of chlorophylls (i.e., chlorophylls *a* and *b*) caused by *D. noxia* feeding. Although *D. noxia*-elicited chlorophyll losses and their effects on plant’s photosynthetic efficiency have been studied extensively (Hewitt et al. 1984, Riedell 1989, Burd and Todd 1992, Miller et al. 1994, van der Westhuizen and Pretorius 1995, Burd and Elliott 1996, Ni et al. 2002, Burd 2002, Macedo et al. 2003, Heng-Moss et al. 2003), the biochemical mechanism of chlorosis induced by infestation of *D. noxia* is still unknown.

The toxic effect of *D. noxia* feeding on cereal crops resembles the symptoms of plant diseases. Particularly, the chlorotic streaks resemble plant viral infestation symptoms. Thus, it is not by accident that *D. noxia* was discovered for the first time in several areas by plant pathologists, and that the toxic effects of feeding have been thought
to be associated with transmission of plant pathogens (e.g., viruses). Hewitt et al. (1984) concluded that the injury symptoms caused by *D. noxia* infestation were not caused by phytopathogen infection. Fouche et al. (1984) also indicated that the typical symptoms associated with *D. noxia* feeding were induced by a toxin injected during aphid salivation and not by a virus or viruses. Kruger and Hewitt (1984) showed that *D. noxia* extracts adversely affected photosynthetic rates of spinach chloroplasts *in vitro*. They speculated that impact on photosynthetic rates could be secondary to the degradation of the chloroplast membrane or *vice versa*.

*Diuraphis noxia* is a phloem-feeding aphid whose stylets typically follow an intercellular route through the leaf mesophyll until phloem contact is achieved (Fouche et al. 1984, Girma et al. 1992, Ni and Quisenberry 1997). Early symptom development in susceptible barley indicates that localized changes in the ultrastructure and photosynthetic function of the chloroplast occur adjacent to the stylet paths (Belefant-Miller et al. 1994). Ultrastructural analysis of *D. noxia* injury to wheat by Fouche et al. (1984) described sequential events occurring at the cellular level and demonstrated the involvement of the chloroplast membrane and photosynthetic pigments as primary sites of action in the injury response. In their study, the initial response to *D. noxia* feeding was the retraction and convolution of the plasmalemma, followed closely by the distention of the chloroplast granal and stromal lamellae. The result was a substantial increase in the volume of plastoglobuli. Subsequent degeneration of the chloroplast envelope was followed by the disintegration of other cell organelle membranes that culminated in cell bleaching. Burd and Elliott (1996) compared the chlorophyll content kinetics between susceptible and resistant plants and found *D. noxia* feeding caused significant reductions
in chlorophyll $a$, chlorophyll $b$, and total chlorophyll content in susceptible wheat ('Pavon' and 'TAM W-101') and barley ('Wintermalt') but not in resistant plants. Likewise, $D. \text{noxia}$ infestation resulted in a significant increase of nonvariable fluorescence ($F_0$), a decrease of maximal fluorescence ($F_m$), and a variable fluorescence ($F_v$) in susceptible plants. The photochemical efficiency of photosystem II ($F_v/F_m$) and the half-rise time from $F_0$ to $F_m$ ($t_{1/2}$) were reduced significantly in the $D. \text{noxia}$ infested susceptible entries but remained relatively unchanged in the resistant cultivars. They concluded that $D. \text{noxia}$ injury went beyond the simple removal of photosynthates from the plant because of the substantial decrease in $F_v/F_m$ following aphid infestation for both susceptible wheat and barley showed a significant decrease in the capacity and efficiency of the primary photochemistry of photosystem II. Haile et al. (1999) compared the changes in CO$_2$ exchange rates from susceptible and resistant (i.e., antibiotic or tolerant) wheat lines elicited by $D. \text{noxia}$ infestation. There was significant permanent reduction of photosynthetic rates, especially in PI 137739. Nevertheless, the impact of $D. \text{noxia}$ feeding on the photosynthetic rates of PI 262660 (tolerance) was less than on PI 137739 ($D. \text{noxia}$-antibiotic) and 'Arapahoe' ($D. \text{noxia}$-susceptible) wheat. They suggested that this phenomenon was caused by different resistance mechanisms that led to different photosynthetic compensation abilities after aphid feeding.

Chlorophyll breakdown \textit{in vitro} results from the activities of a series of degradative enzymes (e.g., chlorophyllase, Mg-dechelatase, and chlorophyll oxidase) in two degradation pathways (i.e., pheophorbide $a$ and oxidative bleaching pathways) (Vicentini et al. 1995, Janave 1997, Matile et al. 1999, Takamiya et al. 2000, Dangl et al. 2000). Commonly, the hydrolysis of chlorophyll into chlorophyllide and phytol is regarded as
the initial step of degradation by chlorophyllase in the pheophorbide \textit{a} pathway (Matile et al. 1999). Without any inhibitor (i.e., ascorbate), enzymatic chlorophyll degradation may go through both pheophorbide and oxidative bleaching pathways (Janave 1997). Ni et al. (2001b, 2002) reported a significantly higher Mg-dechelatase activity in \textit{D. noxia}-infested susceptible wheat plant extracts when compared with the \textit{R. padi}-infested and the uninfested controls. However, neither chlorophyllase nor oxidative bleaching activity, described by Janave (1997) and Matile et al. (1999) in naturally senescing plants, was detected in their experiment. They suggested that the increase of Mg-dechelatase activity indicated that chlorosis formation on \textit{D. noxia}-infested plants was possibly an Mg-dechelatase-driven physiological phenomenon and the elicitor for leaf chlorosis might not be the direct result of aphid salivary secretion.
CHAPTER 3

ASSESSMENT OF APHID RESISTANCE IN WHEAT NEAR-ISOGENIC LINES

Introduction

Chlorophyll catabolism can be differentiated into two types. One is senescent chlorophyll catabolism associated with normal progressive senescence and the other is leaf chlorosis in growing plants elicited by herbivore feeding, nutritional deficiencies, or pathogen-infections (Ni et al. 2002). Although the chlorophyll degradation in vitro has been well documented (Janave 1997, Matile et al. 1999, Takamiya et al. 2000, Dangl et al. 2000), the mechanism of chlorosis caused by herbivore infestation remains unclear.

The Russian wheat aphid, *Diuraphis noxia* (Mordvilko) (Hemiptera: Aphididae), is an important pest of cereal crops that causes chlorosis, which indicates the loss of photosynthetic pigments (e.g., chlorophyll *a*, chlorophyll *b*, and carotenoids). Photosynthetic pigments are vital for plant growth and without them, light cannot be absorbed and therefore energy cannot be stored. All photosynthetic organisms contain one or more organic pigments capable of absorbing visible radiation that will initiate the photochemical reactions of photosynthesis (Blankenship 2002). Four major classes of pigments found in plants, bacteria, and algae are the chlorophylls (e.g., chlorophyll *a*, *b*, *c* and *d*), the bacteriochlorophylls (e.g., bacteriochlorophyll *a*, *b*, *c*, *d*, *e*, *f* and *g*), the carotenoids (e.g., β-carotene, α-carotene, luteol, violaxanthol and fucoxanthol) and the phycobilins (e.g., Phycoerythrins, phycocyanins and allophycocyanins) (Hall and Rao
1992, Biswal 1995). The pigments in higher plants mainly consist of chlorophyll \(a\), chlorophyll \(b\), and most of the carotenoids (Blankenship 2002).

Chlorophylls \(a\) and \(b\) have the absorption maximum at 663 and 645 nm respectively (in acetone) that gives them characteristic green color. They act as primary light harvesters in plant photosynthesis. Carotenoids, which are responsible for the orange-yellow colors observed in the leaves of plants, absorb light between 400 and 500 nm, a range in which absorption by chlorophylls is relatively weak. As such, carotenoids play a minor role as accessory light-harvesting pigments, absorbing and transferring light energy to chlorophyll molecules (Malkin and Niyogi 2000). Most importantly, carotenoids function in a process called photoprotection. Under the high light intensities often found in nature, plants may absorb more light energy than they can actually use for photosynthesis. This excessive excitation of chlorophylls can result in increased formation of the singlet oxygen that is detrimental to plant photosynthesis (Malkin and Niyogi 2000, Blankenship 2002). Carotenoids are able to accept excitation energy and prevent singlet oxygen formation (Malkin and Niyogi 2000, Blankenship 2002). Therefore, reduction of chlorophylls and/or carotenoids in plants induced by herbivore infestation will negatively affect the photosynthetic capacity of plants.

Many researchers have assessed the impact of sap-feeding herbivore-elicited photosynthetic pigment changes and their effects on plant photosynthetic efficiency (Kruger and Hewitt 1984, Riedell 1989, Burd and Todd 1992, Miller et al. 1994, van der Westhuizen and Pretorius 1995, Burd and Elliott 1996, Ni et al. 2002, Macedo et al. 2003, Heng-Moss et al. 2003). The objectives of this study were to assess the resistance of Tugela and three wheat near-isogenic lines (or isolines) (i.e., Tugela-\(Dn1\), Tugela-\(Dn2\),...
and Tugela-Dn5) to cereal aphids [i.e., *D. noxia* and the bird cherry-oat aphid, *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae)] through comparisons of aphid and plant biomass; to quantify changes of photosynthetic pigment concentrations (i.e., chlorophylls *a* and *b*, and carotenoids) in Tugela wheat lines with three aphid infestation treatments (i.e., *D. noxia*, *R. padi*, and control); to elucidate the effects of photosynthetic pigment changes on photosynthetic capacities among the aphid-infested plants; and to correlate photosynthetic pigment variations with the particular genes among the Tugela and Tugela *Dn* plants that confer aphid resistance.

**Materials and Methods**

**Insects and Plants**

*Diuraphis noxia*, a chlorosis-eliciting species, and *R. padi*, a non-chlorosis-eliciting species used in the experiment were from the colonies established from field-collected aphids. The colony of *D. noxia* was established originally using aphids collected near Scottsbluff, NE in 1994; *R. padi* colony was established using the aphids collected near Lincoln, NE in 1996 (Ni et al. 2001a). Aphids were maintained on ‘Stephens’ (*D. noxia* susceptible) wheat in Plexiglas cages (30 x 15 x 15 cm) in a Percival™ growth chambers (Percival Scientific, Boone, IA) at 21°C, with a photoperiod of 16:8 (L: D) h and 40-50% RH.

Tugela (susceptible), Tugela-*Dn1* (antibiosis), Tugela-*Dn2* (tolerance), and Tugela-*Dn5* (antibiosis and antixenosis) wheat lines were used in the experiment. Seeds of the four wheat lines were planted at the rate of three plants per Conetainer™ (3.81 cm diameter by 21 cm depth) (Stuewe and Sons, Inc., Corvallis, OR). Conetainers™ were
filled with Sunshine™ soil mix No.1 (SunGro Horticulture, Bellevue, WA) and placed in Conetainer™ racks (61 x 30 x 18 cm), leaving a space among Conetainers™ to provide adequate light. Plants were watered uniformly from the bottom by placing a rack over a plastic tray (54 x 28 x 6 cm) filled with water. Before aphid infestation, plants were thinned to two seedlings per Conetainer™. Experiments were maintained in a growth chamber at 21°C with a photoperiod of 16:8 (L: D) h and 40-50% RH.

Aphid Precondition and Infestation

Aphids were preconditioned on Stephens wheat caged with polyethylene tubes (30-cm length by 4-cm diameter) in a Percival™ growth chamber (Percival Scientific, Boone, IA) at 21°C with a photoperiod of 16:8 (L: D) h and 40-50% RH (Schotzko and Smith 1991). The adults were placed on Stephens wheat plants with three leaves at growth stage 13 of Zadoks scale (Zadoks et al. 1974) and removed after three days. Nymphs were maintained on the plants for approximately 10 days before infestation. Thus, the protocol provided us with age-specific aphids with a 3-day age variation.

There were three types of aphid infestations on plants: 0 aphid, 10 R. padi adults, and 10 D. noxia adults. The experiment was initiated when plants were at Zadoks stage 13. All plants were caged using polyethylene tubes and maintained in a Percival™ growth chamber under the conditions described previously.

Collection of Aphid and Plant Samples and Chlorosis Evaluation

Wheat leaves and aphids from the experimental plants were collected on the 3rd, 6th, 9th, and 12th days after initial aphid infestation. Aphids (i.e., D. noxia and R. padi) were
weighed to evaluate effects of Tugela wheat lines. Chlorotic injuries of wheat leaves caused by *D. noxia* infestation were qualitatively evaluated by rating the relative amount of chlorosis based on the report by Webster (1990) and Burd et al. (1993). Chlorosis was quantified using a nine point scale define the chlorosis area on leaves: 1, plants appear healthy and have scattered yellow spots; 2, isolated chlorotic spots obvious; 3, chlorosis ≤15% of total leaf area, chlorotic lesions coalesced; 4, chlorosis >15% but ≤25% of total leaf area, leaf streaks appear; 5, chlorosis >25% but ≤40% of total leaf area, obvious streaks; 6, chlorosis >40%, but ≤55% of total leaf area; 7, chlorosis >70%, but ≤85% of total leaf area; 9, plant appears dead or beyond recovery (Webster 1990).

After the evaluation of chlorosis, leaf samples were excised and weighed. A small part (0.3 g) of the entire leaf samples was randomly selected for pigment assay, the rest was analyzed later for chlorophyll degradation enzyme activities. All leaf samples were stored in a -20°C freezer until final analysis.

**Photosynthetic Pigment Measurement**

Control and aphid-infested plant leaves (about 0.3 g) were ground with liquid nitrogen in a mortar and pestle under low light condition. Acetone (3 ml of 80%) was added to extract photosynthetic pigments (i.e., chlorophyll *a*, chlorophyll *b*, and total carotenoids). About 1.5 ml of the mixture was aspirated by polyethylene pipette (Fisher Scientific, Pittsburgh, PA) and centrifuged at 6000 × g for 10 min to remove insoluble plant tissues. Final supernatant was diluted with 80% acetone to ensure the absorbance readings at 663 nm were lower than 1.5. The absorbance of pigment extracts were measured in a spectrophotometer respectively at wavelength of 470 nm, 646 nm, and 663
nm. Concentration of the three types of pigments was obtained following the equation described by Bertrand and Schoefs (1997):

\[
C_a = 12.21 A_{663} - 2.81 A_{646} \\
C_b = 20.13 A_{646} - 5.03 A_{663} \\
C_c = (1000 A_{470} - 3.27 C_a - 104 C_b)/198
\]

\( C_a, C_b, \) and \( C_c \) are the concentrations in \( \mu g / ml \) of chlorophyll \( a \), chlorophyll \( b \), and total carotenoids, respectively. \( A_x \) represents the absorbance at \( x \) nm. Final concentrations of the pigments were determined in microgram per gram of fresh wheat leaf tissue (\( \mu g/g \)). Thus, \( C_a, b, c \, (\text{final}) = (C_a, b, c \times r \times v)/w \) (\( r \) is the dilute ratio of pigment measurement, \( v \) and \( w \) are the volume of 80\% acetone to extract the pigments and the weight of sample plant leaves respectively). \( C_a / C_b \) and \( C_{a+b} / C_c \) also were calculated to determine the photosynthetic efficiency.

**Experimental Design and Data Analysis**

The experiment was a split-split plot design. The experiment was repeated six times. Four sampling dates (3\(^{rd} \), 6\(^{th} \), 9\(^{th} \), and 12\(^{th} \)) were the main plots within each trial. Three aphid treatments (control, \( D. \, noxia \), and \( R. \, padi \)) were the sub-plots within each main plot (sampling dates) and the four wheat lines (Tugela, Tugela-Dn1, Tugela-Dn2, and Tugela-Dn5) were the sub-sub-plots within each sub-plot (aphid treatments). Six plants were used for each treatment on each sampling date and therefore, 36 plants in total were used per treatment per sampling date. Data were analyzed using the PROC GLM procedure of the SAS software followed by TEST statements to ensure correct error terms were used.
in assessing main effects of experimental factors (Cochran and Cox 1957, SAS Institute 1989). The means were separated using the Fisher’s LSD test ($\alpha = 0.05$).

**Results**

**Plant Biomass**

Plant weight of the Tugela wheat lines was significantly affected by the three-way (isoline by date by aphid) interaction ($F = 2.41$, df = 18, 90, $P = 0.0034$). Also, plant weights were significantly affected by aphid-date interaction ($F = 94.15$, df = 6, 30, $P < 0.0001$), and isoline-date interaction ($F = 2.96$, df = 9, 45, $P = 0.0075$), but not by isoline-aphid interaction ($F = 1.59$, df = 6, 30, $P = 0.1835$). Significantly different plant weight was detected in the four wheat lines ($F = 3.88$, df = 3, 15, $P < 0.0310$), the four sampling dates ($F = 254.74$, df = 3, 15, $P < 0.0001$), and the three aphid infestation treatments ($F = 179.34$, df = 2, 10, $P < 0.0001$). Because the three-way interaction significantly affected plant weight, plant weights were further analyzed within each aphid treatment.

Plant weight of the uninfested plants was higher than aphid-infested plants (Fig. 1). Although injury symptoms (e.g., chlorosis and leaf rolling) appeared only on *D. noxia*-infested plants, plant weights of all *D. noxia*-infested wheat lines were greater than those of *R. padi*-infested plants in all sampling dates (Fig 1). Therefore, Tugela isolines that had been reported to be resistant to *D. noxia* were not resistant to *R. padi*. We found that when infested by *D. noxia*, all Tugela *Dn* plants sustained growth throughout the infestation durations (Fig. 1C). However, plant weight of Tugela decreased on day 12.
Figure 1. Temporal changes of plant biomass of the four wheat lines with three aphid treatments A: Uninfested Tugela plants; B: *R. padi*-infested Tugela plants; C: *D. noxia*-infested Tugela plants (*n* = 18). Each data point represents the mean on each sampling date. Error bar indicates the standard error of the mean.
This finding confirmed that Tugela is susceptible to *D. noxia* infestation, while Tugela-
*Dn1*, Tugela-*Dn2*, and Tugela-*Dn5* are resistant. No statistical difference in plant weight:
was detected among *R. padi*-infested and uninfested Tugela wheat lines on all sampling
dates (Figs. 1A and 1B).

**Aphid Biomass**

Biomass of aphids collected from Tugela wheat lines was significantly affected by
the three-way (isoline by date by aphid) interaction (*F* = 4.39, df = 9, 45, *P* = 0.0004).
Aphid biomass also was significantly affected by all the two-way interactions (*P*-values
< 0.0001). Significantly different aphid biomass was detected in the four isolines (*F* =
72.92, df = 3, 15, *P* < 0.0001), the four sampling dates (*F* = 650.20, df = 3, 15, *P* <
0.0001), and between the two type of aphids (*F* = 504.32, df = 1, 5, *P* < 0.0001). Because
the three-way interaction was significant, temporal developments of *R. padi* and *D. noxia*
biomass were analyzed.

*Rhopalosiphum padi* biomass was not affected by date-isoline interaction (*F* = 0.63,
df = 9, 45, *P* = 0.7686). No significant difference was detected in aphid biomass of *R.
padi* collected from different wheat lines (*F* = 2.51, df = 3, 15, *P* = 0.0983) (Fig. 2A).
However, significant difference was found in biomass of *R. padi* collected at different
dates (*F* = 178.82, df = 3, 15, *P* < 0.0001).

Biomass of *D. noxia* was significantly affected by date-isoline interaction (*F* =
10.05, df = 9, 45, *P* < 0.0001) (Fig. 2B), which indicated different impacts on *D. noxia*
growth rate among the four wheat lines. Significantly different aphid biomass was
detected in *D. noxia* collected from different wheat lines (*F* = 202.09, df = 3, 15, *P* <
The biomass of *D. noxia* collected from Tugela plants was higher than from the other Tugela *Dn* plants (Fig. 2B). Biomass of *D. noxia* collected from Tugela-*Dn5* was higher than that from Tugela-*Dn1* and Tugela-*Dn2*. No statistical difference was detected in the comparison of aphid biomass collected from Tugela-*Dn1* and Tugela-*Dn2*.

Figure 2. Temporal changes of aphid biomass. A: Biomass of *D. noxia*; B: Biomass of *R. padi*. Each data point represents the mean (n = 18) on each sampling date. Error bar indicates the standard error of the mean.

Chlorosis Rating

Because *R. padi* was a non-chlorosis-eliciting species and did not cause any injury symptoms after its colonization on wheat, chlorosis rating was only conducted on *D. noxia*-infested plants. Chlorosis ratings of *D. noxia*-infested plants were affected by the two-way (isoline by date) interaction (*F* = 3.63, df = 9, 45, *P* = 0.0018). Significant differences of chlorosis ratings were detected among wheat lines (*F* = 75.11, df = 3, 15, *P* < 0.0001), and in different sampling dates (*F* = 11.12, df = 3, 15, *P* = 0.0004). According to our observations, Tugela (susceptible) plants had the most severe chlorosis and Tugela-*Dn2* (tolerance) plants had the least. Tugela-*Dn1* (antibiosis) plants had more
chlorosis than Tugela-\textit{Dn5} (antixenosis and antibiosis) plants. Therefore, chlorosis rating was highest in Tugela plant and lowest in Tugela-\textit{Dn2} plants (Fig. 3). Chlorosis rating was higher in Tugela-\textit{Dn1} plants when compared with Tugela-\textit{Dn2} plants. We also noticed that chlorotic injury increased constantly on \textit{D. noxia}-infested Tugela plants within the sampling period. Tugela \textit{Dn} plants, however, showed less chlorosis on the late sampling dates (i.e., 12\textsuperscript{th} day) (Fig. 3). This indicated that Tugela \textit{Dn} plants were able to adapt to the injury caused by \textit{D. noxia} feeding as their development.

Figure 3. Temporal chlorosis ratings in \textit{D. noxia}-infested Tugela wheat lines using scale of Webster (1990). Each data point represents the mean ($n = 18$) of chlorosis rating on each sampling date ($n = 3$). Error bar indicates the standard error of the mean.
Chlorophyll a Concentration

Chlorophyll a concentration was not affected by the three-way (isoline by date by aphid) interaction ($F = 1.06$, df = 18, 88, $P = 0.4051$). None of the three two-way interactions significantly affected chlorophyll a concentration ($P$-values $> 0.05$). Chlorophyll a concentrations were significantly different among the aphid infestation treatments ($F = 7.83$, df = 2, 10, $P = 0.0090$), and among the wheat lines ($F = 4.04$, df = 3, 15, $P = 0.0272$), but not among the sampling dates ($F = 0.28$, df = 3, 15, $P = 0.8405$). Injury symptoms (e.g., chlorosis and leaf rolling) were observed on $D. noxia$-infested plants but not on $R. padi$-infested or the uninfested plants. Chlorophyll a concentration was not statistically different between $R. padi$-infested and the uninfested plants, but was lower on $D. noxia$-infested ones (Fig. 4).

Chlorophyll b Concentration

Chlorophyll b concentration was significantly affected by the three-way (isoline by date by aphid) interaction ($F = 2.06$, df = 18, 88, $P = 0.0141$). However, none of the three two-way interactions affected chlorophyll b concentration ($P$-values $> 0.05$).

Chlorophyll b concentration was significantly different among the aphid infestation treatments ($F = 12.50$, df = 2, 10, $P = 0.0019$), but not among the sampling dates ($F = 1.01$, df = 3, 15, $P = 0.4177$), or among the wheat lines ($F = 3.02$, df = 3, 15, $P = 0.0627$). Similar to the analysis of chlorophyll a content, chlorophyll b was significantly lower on $D. noxia$-infested wheat lines in comparison with $R. padi$-infested and the uninfested plants (Figs. 5). Further analysis was conducted within each aphid treatment.
Figure 4. Chlorophyll $a$ concentration in four wheat lines (Tugela, Tugela-$Dn1$, Tugela-$Dn2$, and Tugela-$Dn5$) with three aphid treatments (RWA=$D.\ noxia$; BCO=$R.\ padi$; CK=Control).
There was no difference in chlorophyll \( b \) concentration among \( D.\) noxia-infested wheat lines on day 3 (Fig. 5C). However, significantly lower chlorophyll \( b \) concentrations were found in plant samples collected from Tugela with more severe injury symptoms on days 6, 9, and 12. \( Diuraphis\) noxia-elicited injuries also were obvious on Tugela-\( Dn1 \) plant. Consistently, chlorophyll \( b \) concentration was lower in Tugela-\( Dn1 \) on days 6 and 12 but not on day 9 when compared with Tugela-\( Dn2 \) and Tugela-\( Dn5 \). There were no differences of chlorophyll \( b \) concentrations between \( R.\) padi-infested and the uninfested Tugela wheat lines. Chlorophyll \( b \) concentrations were not statistically different among \( R.\) padi-infested or uninfested plants on all sampling dates.

**Carotenoids Concentration**

Carotenoid concentration was not affected by the three-way (isoline by date by aphid) interaction \( (F = 1.28, \text{df} = 18, 88, P = 0.2235) \). None of the three two-way interactions affected carotenoid concentration \( (P\)-values > 0.05). Carotenoid concentrations were significantly different among the aphid infestation treatments \( (F = 7.90, \text{df} = 2, 10, P = 0.0088) \), but not among the wheat lines \( (F = 2.6, \text{df} = 3, 15, P = 0.0906) \), or among the sampling dates \( (F = 1.06, \text{df} = 3, 15, P = 0.3965) \). Further analysis of carotenoid concentration was carried out within each aphid infestation treatment.

Carotenoid concentration was lower \(<200 \mu g/g\) on \( D.\) noxia-infested wheat lines when compared with that of \( R.\) padi-infested and the uninfested plants \( >220 \mu g/g\) (Fig 6). There were no significant differences of carotenoid concentrations between \( R.\) padi-infested plants and the uninfested plants. Unlike the analysis of chlorophylls \( a \) and \( b \), carotenoids were not significantly different among the Tugela wheat lines when infested.
Figure 5. Temporal changes of chlorophyll \( b \) concentration (\( \mu g/\text{gram leaf} \)). A: Uninfested Tugela plants; B: \( R. \ padi \)-infested Tugela plants; C: \( D. \ noxia \)-infested Tugela plants (\( n = 6 \)). Each data point represents the mean (\( n = 6 \)) on each sampling date. Error bar indicates the standard error of the mean.
by *D. noxia*. Also, carotenoid concentrations were not significantly different among the four wheat lines under *R. padi*-infested or the uninfested condition.

**Chlorophyll a/b Ratio**

The chlorophyll a/b ratios on Tugela wheat lines were not significantly affected by the three-way (isoline by date by aphid) interaction (*F* = 1.01, df = 18, 88, *P* = 0.4564). None of the three two-way interactions had any significant effect on the chlorophyll a/b ratio (*P*-values > 0.05). Chlorophyll a/b ratio was not significantly different among the aphid infestation treatments (*F* = 0.66, df = 2, 10, *P* = 0.5391), among the wheat lines (*F* = 0.21, df = 3, 15, *P* = 0.8883), or among the sampling dates (*F* = 1.29, df = 3, 15, *P* = 0.3141).

**Chlorophylls/carotenoids Ratio**

The chlorophylls/carotenoids ratios were not affected by the three-way (isoline by date by aphid) interaction (*F* = 0.89, df = 18, 88, *P* = 0.5908), or by any one of the three two-way interactions (*P*-values > 0.05). Furthermore, chlorophylls/carotenoids ratios did not differ among the sampling dates, the wheat lines, or the aphid infestation treatments (*P*-values > 0.05).

**Discussion**

Both plant and aphid biomass analysis in this experiment supported the previous reports by du Toit (1987, 1989) that Tugela is susceptible to *D. noxia* infestation but Tugela *Dn* plants are resistant. When infested by *D. noxia*, Tugela plants showed much more severe chlorotic symptoms and decreased plant weight on day 12 when compared
Figure 6. Carotenoid concentration in four wheat lines (Tugela, Tugela-Dn1, Tugela-Dn2, and Tugela-Dn5) with three aphid treatments (RWA=D. noxia; BCO=R. padi; CK=Control).
with the Tugela Dn plants (Figs 1C, 3). Although chlorotic symptoms were only observed on D. noxia-infested wheat plants, plant weight of R. padi-infested wheat was lower than D. noxia-infested plants (Fig 1B, 1C). Therefore, Tugela wheat lines are resistant to D. noxia but not to R. paid infestation. We found that D. noxia biomass from Tugela-Dn5 (antixenotic and antibiotic resistance) was higher when compared with Tugela-Dn1 (antibiosis) and Tugela-Dn2 (tolerance) (Fig 2B). Budak et al. (1999) observed similar results when comparing the biomass of D. noxia collected from different 'Betta' wheat lines with Dn genes. They concluded Betta-Dn5 did not show the same level of resistance to D. noxia as the donor line PI 294994 and suggested that resistance inherited from PI 294994 might be controlled by more than one gene. Zhang et al. (1998) in their genetic study of PI 294994 study indicated that there might be three resistance genes in PI 294994, but in each PI 294994 plant from the original accession there may be only one or two D. noxia resistant genes. Therefore, it is possible that Tugela-Dn5 did not fully inherit the resistance from PI 294994. Biomass of D. noxia collected from Tugela-Dn1 was lower than that from Tugela and Tugela-Dn5 (Fig 2B). The results support that Tugela-Dn1 is antibiotic and has negative impact on the biology and/or physiology (e.g., growth rate, reproduction) of D. noxia during its colonization. It is worth noting that we observed less injury symptoms on Tugela-Dn2 (tolerance) plants after their being infested by D. noxia, while the biomass of D. noxia collected from Tugela-Dn2 was similar to that from Tugela-Dn1 but less than Tugela and Tugela-Dn5. Therefore, Tugela-Dn2 appears to be both tolerant and antibiotic to D. noxia. Similar results were reported by Haile et al. (1999) and Heng-Moss et al. (2003) of D. noxia on Betta-Dn2 (tolerance) wheat.
Chlorophylls $a$ and $b$ are primary pigments in plants to harvest light energy for photosynthesis. $D. noxia$ feeding causes chlorosis in plants and could potentially affect the photosynthetic capacity. Chlorophyll concentrations in $D. noxia$-infested Tugela-$Dn2$ and Tugela-$Dn5$ plants were significantly higher than in Tugela and Tugela-$Dn1$ plants. Tugela-$Dn2$ and $Dn5$ plants were better able to sustain $D. noxia$ damage and therefore maintain the chlorophyll contents and photosynthetic potential than Tugela-$Dn1$ and Tugela (Figs. 4 and 5). Ni et al. (2001b, 2002) detected significantly higher Mg-dechelatase activities in $D. noxia$-infested wheat leaves in comparison with those of $R. pad$-infested and the uninfested plants. We also detected higher chlorophyllase activities in asymptomatic $R. pad$-infested plants and higher Mg-dechelatase activities in symptomatic $D. noxia$-infested plants. When infested by $D. noxia$, Tugela showed more plant chlorosis than the other three isolines with $D. noxia$-resistant genes and Mg-dechelatase activity was lower than the other $Dn$ plants. Therefore, chlorophyll loss in $D. noxia$-infested wheat plants is most likely correlated with chlorophyll degradative enzyme activities.

Carotenoids in higher plants are important in photosynthesis and act as accessory light harvesters and harmful quanta quencher (Blankenship 2002). $D. noxia$ feeding caused reduction in carotenoids and, thus, was detrimental to wheat photosynthesis. Although carotenoid biosynthesis and its correlated enzymes are well characterized (Dangl et al. 2000, Hundle and Hearst 1991, Hundle et al. 1991), there is no clear mechanism of carotenoid degradation. The intermediate steps and nature of the catabolites in the degradation pathway of carotenoids remain largely unclear (Biswal 1995). As a consequence, the biochemical mechanism of carotenoid degradation on
Tugela isolines caused by *D. noxia* infestation and its correlation with chlorophyll content changes are not clearly understood. What can be delineated is that the lower carotenoid level among the Tugela wheat lines imposed by *D. noxia* feeding would cause higher potential of oxidative damage to plant, which is detrimental to plant physiology (Bi and Felton 1995, Blokhina et al. 2003) and possibly correlated to the loss of chlorophylls (Burd and Burton 1992).

Decrease of the chlorophyll *a/b* ratio has been widely reported in the natural plant senescing process (Wolf 1956; Sanger 1971; Watts and Eley 1981; Bricker and Newman 1982; Adams et al. 1990). Burd and Todd (1992) detected a significant reduction of chlorophyll *a/b* ratio in *D. noxia*-infested wheat (TAM W-101). Ni et al. (2002) also reported a significantly lower chlorophyll *a/b* ratio on *D. noxia*-infested ‘Arapahoe’ (susceptible) wheat. The chlorophyll *a/b* ratio of Tugela isolines did not accompany the chlorosis process caused by *D. noxia* feeding, but maintained a 3:1 ratio observed in natural growing plants, unlike the reports noted in either natural senescent or *D. noxia*-infested wheat (Adams et al. 1990, Ni et al. 2002). Burd and Elliott (1996) and Heng-Moss et al. (2003) have reported similar results.
ENZYMATIC CHLOROPHYLL DEGRADATION IN WHEAT NEAR-ISOGENIC LINES ELICITED BY CEREAL APHID INFESTATION

Chapter 4

Introduction

The Russian wheat aphid, *Diuraphis noxia* (Mordvilko) (Hemiptera: Aphididae), is a serious pest of cereal crops worldwide, except in Australia. This aphid species has a wide range of cereal grains and grasses as host plants. The most obvious plant injury symptoms on cereal plants after *D. noxia* feeding include chlorosis, which indicates the loss of chlorophylls (Burd and Elliott 1996, Rafi et al. 1996, Heng-Moss et al. 2003).

Chlorophyll degradation *in vitro* has been studied in recent years (Janave 1997, Matile et al. 1999, Takamiya et al. 2000, Dangl et al. 2000). Two chlorophyll degradation pathways and correlated enzymes are recorded (Figs. 7 and 8). In pheophorbide *a* pathway (Fig. 7), chlorophyllase catalyzes the dephytylation in the first step of chlorophyll degradation and transforms the chlorophyll into chlorophyllide. Magnesium dechelation takes place after dephytylation in which the Mg-dechelatase remove the magnesium from the tetrapyrrole macrocycle and yields pheophorbide *a*. After that is the additional modifications of the tetrapyrrole macrocycle: e.g., oxygenolytic cleavage of the macrocyclic ring of tetrapyrrole by pheophorbide *a* oxygenase; conversion of the cleavage product to colorless fluorescent compounds by red chlorophyll catabolite reductase; and conversion of the colorless fluorescent compounds to nonfluorescent compounds (Ginsburg and Matile 1993, Hörtensteiner et al. 1995, Mühlecker and
Figure 7. Chlorophyll pheophorbide $a$ pathway described by Takamiya et al. (2000). (a) Chlorophyllase (b) Magnesium dechelatase (c) Pheophorbidase (d) Pheophorbide $a$ oxygenase (e) Red chlorophyll catabolite reductase. Abbreviations: NCCs, nonfluorescent chlorophyll catabolites; pFCC, primary fluorescent chlorophyll catabolite; RCC, red chlorophyll catabolite.

Kräutler 1996, Hörtensteiner et al. 1998, Malkin and Niyogi 2000). Janave (1997) examined the chlorophyll degradation initiated by enzymes extracted from Cavendish bananas ($Musa cavendishi$ L.) and found chlorophyll catabolic activity was inhibited under anoxygenic conditions or by adding ascorbate. Therefore, an oxidative bleaching pathway was suggested based on his findings (Fig. 8).
Although chlorophyll degradation in plants has been extensively studied in terms of normal progressive senescence, little is known about chlorosis formation in plants caused by herbivore feeding. It is important though to monitor chlorophyll degradation enzyme activities in herbivore-infested plants and determine whether the chlorosis formation is the same as chlorophyll degradation in natural senescent plants. Ni et al. (2001b, 2002) did not detect either chlorophyllase or oxidative bleaching activity described by Matile et al. (1999) and Janave (1997) in *D. noxia*-infested ‘Arapahoe’ wheat (susceptible). They proposed that herbivore-elicited chlorophyll loss was different from the process in naturally senescing plants. They also reported that *D. noxia*-infested susceptible wheat showed a significantly higher Mg-dechelatase activity than *R. padi*-infested than the uninfested Arapahoe wheat. They suggested aphid-elicited chlorosis is most likely to be related to the increase of Mg-dechelatase activity and other plant physiological and/or biochemical changes.
The research proposed is a continuation of previous research by Ni et al. (2001b, 2002) to understand the biochemical mechanisms of aphid (i.e., *D. noxia* and *R. padi*) elicited chlorosis using Tugela and three *D. noxia*-resistant wheat isolines (i.e., Tugela-Dn1, Tugela-Dn2, and Tugela-Dn5). The main objective was to determine changes in chlorophyll degradation enzyme (chlorophyllase, Mg-dechelatase and chlorophyll oxidase) activities elicited by aphid infestation in wheat lines and their correlation with chlorosis formation.

**Materials and Methods**

**Plants and Insects**

*Diuraphis noxia*, the chlorosis-eliciting species, and *R. padi*, the non-chlorosis-eliciting species, were used in the experiment. Both aphids were from colony established from field-collected aphids. The colony of *D. noxia* was established originally using aphids collected near Scottsbluff, NE in 1994; the colony of *R. padi* was established using the aphids collected near Lincoln, NE in 1996 (Ni et al. 2001a). Aphids were maintained on Stephens wheat in Plexiglas cages (30 x 15 x 15 cm) in a Percival™ growth chambers (Percival Scientific, Boone, IA) at 21°C, with a photoperiod of 16:8 (L: D) h and 40-50% RH.

Tugela (susceptible), Tugela-Dn1 (antibiosis), Tugela-Dn2 (tolerance), and Tugela-Dn5 (antixenosis and antibiosis) wheat lines were used in the experiment. Seeds of the four wheat lines were planted at the rate of two plants per Conetainer™ (3.81 cm diameter by 21 cm depth) (Stuewe and Sons, Inc., Corvallis, OR). Conetainers™ were filled with Sunshine™ soil mix No.1 (SunGro Horticulture, Bellevue, WA) and placed in
Conetainers™ racks (61 x 30 x 18 cm), leaving a space between Conetainers™ to provide adequate light. Plants were watered uniformly from the bottom by placing a conetainer rack over a plastic tray (54 x 28 x 6 cm) filled with water. The Tugela isolines were maintained in a growth chamber at 21°C with a 16:8 (L: D) h photoperiod and 40-50% RH for 13 days until they were infested with aphids.

**Aphid Infestation and Plant Sample Collection**

Aphids were preconditioned on Stephens wheat for one generation following procedures of Schotzko and Smith (1991). The apterous adults of *D. noxia* and *R. padi* were placed on Stephens wheat plants with three leaves (Zadoks stage 13) (Zadoks et al. 1974) and then caged with polyethylene tube-cages (30-cm length by 4-cm diameter). After three days of infestation, adults were removed and nymphs were maintained on the plants for 7 or 8 days before infestation. Thus, the variation of aphid age was within 3 days. This protocol provided relatively age-specific aphids for our experiment.

There were three levels of aphid infestations on plants: 0 aphid; 10 apterous *R. padi* adults, and 10 apterous *D. noxia* adults. The experiment was initiated when plants were at the 3-leaf stage. After being infested with aphids, all plants were caged using polyethylene tube-cages (30-cm length by 4-cm diameter) and maintained in a Percival™ growth chamber under the conditions described previously. Wheat leaves from the experimental plants were collected and weighed on the 3\textsuperscript{rd}, 6\textsuperscript{th}, 9\textsuperscript{th}, 12\textsuperscript{th} days after the initial aphid infestation. Because the wheat leaf-blades are relatively small, leaves of three replications within a treatment were combined and processed as one sample.
Enzyme Extraction of Plant Samples

The enzyme extraction from wheat leaf samples was conducted according to methods reported by Mihailović et al. (1997) and modified based on the reports by Ellsworth (1971) and Janave (1997). Wheat leaves (3.0 to 10.0 g) were ground with liquid nitrogen in a mortar and pestle. Chilled extraction buffer (20 ml) containing 0.1 M potassium phosphate buffer (pH 6.2), 1% NaCl, 1% Triton X-100, and 1% polyvinylpyrrolidone (PVP) was used for all plant samples. Plant homogenates were filtered through one layer of miracloth. Chlorophylls were separated and removed once using one volume of n-butanol (Aldrich Chemical, Milwaukee, WI) and centrifuged at 3000 x g for 3 min.

The protein in the lower (aqueous) layer was collected and precipitated with three volume of cold (4°C) acetone. The mixture was swirled briefly and allowed to stand in ice for 10 min. The samples were then centrifuged at 10,000 x g for 10 min. The precipitate was re-suspended in 1 ml of 0.1 M potassium phosphate buffer (pH 7.0), and held at 4 °C for 2 h before initiation of enzymes assays. Only fresh enzyme samples were used for the assays.

Preparation of Enzyme Assay Substrates

Quantified substrates were prepared to assay chlorophyll degradation enzyme activities from wheat lines. Fresh spinach leaves were used for chlorophyll extraction according to Janave (1997). After the spinach leaves were ground in chilled acetone, the extracts were filtered through Whatman No. 2 filter paper and then centrifuged at 6000 x g for 10 min to remove insoluble plant tissues. The supernatant was purified twice by
dioxane precipitation (Iriyama et al. 1974, Janave 1997). The ratio of dioxane: acetone was 1:7 (v/v). The distilled water was then added drop-wise, swirling the mixture until a precipitate was formed. The precipitated chlorophylls were centrifuged at 3000 x g for 3 min and re-suspended in absolute acetone. The concentration of chlorophyll \(a\) was determined by diluting the original chlorophyll solution with 80% acetone and concentration was calculated according to the formula described by Bertrand and Schoefs (1997) using absorbance of 646 and 663 nm. The chlorophyll solution was placed in the microcentrifuge tubes wrapped with aluminum foil and stored in dark at -20°C. Prepared chlorophyll solution was used as the substrate for total chlorophyll degradation and chlorophyllase activity assays.

Chlorophyllin was prepared from chlorophyll by Molisch conversion as described by Vicentini et al. (1995). Spinach leaves (approximately 10 g) were ground in 80% acetone. The chlorophyll was partitioned in petroleum ether phase in a ratio of 1:1 (v/v). The petroleum ether phase was washed twice with distilled water. One hundred \(\mu l\) of 30% KOH in methanol was added to 12 ml chlorophyll solution in petroleum ether. The precipitated chlorophyllin was centrifuged at 3000 x g for 5 min and dissolved in 10 ml of distilled water. The pH of the chlorophyllin solution was adjusted to pH 9 by adding tricine. Chlorophyllin solution is stable at pH 9 when stored in the dark at -20°C. Chlorophyllin was used as the substrate for the Mg-dechelatase assay.

**Protein Determination**

Protein concentration of all enzyme samples was determined according to the dye-binding assay (Bollag and Edelstein 1991) using bovine serum albumin (Sigma chemicals).
as a standard. The enzyme extracts used in protein determination were treated with butanol (to remove pigments) and acetone (to concentrate protein) for assays of chlorophyll catabolic enzyme.

Total Chlorophyll Degradation Assay

The disappearance of chlorophyll $a$ was measured according to the method used by Janave (1997). The reaction mixture (1.0 ml) contained 0.36 ml 0.1 M potassium phosphate buffer (pH 7.0), 0.288 ml of acetone (to make 30% in final reaction mixture), 12 $\mu$l of chlorophyll in acetone (to make 10 $\mu$M of chlorophyll $a$ in final reaction), and 0.34 ml of enzyme extract. The control mixture did not contain enzyme extract (0.7 ml potassium phosphate buffer instead). The mixtures were incubated at 30°C in a water bath under dark condition for 30 min. The reaction was stopped by adding 0.1 ml of 1 N NaOH and followed by 3 ml acetone/hexane mixture (1/2, v/v). The reaction mixture was vortexed vigorously until emulsion formation, allowed to stand for 10 min, and centrifuged at 3000 $\times$ g for 5 min. The absorbance of the hexane layer at 663 nm was recorded. Chlorophyll $a$ concentration was determined by employing the specific absorption coefficient of 94.5 M$^{-1}$cm$^{-1}$. Activity was expressed by $\mu$ mol of chlorophyll $a$ degraded/30min/gram fresh leaf weight. Chlorophyll degradation in this protocol was the result of both chlorophyllase and chlorophyll oxidase activities (also known as chlorophyll oxidative bleaching activities) (Janave 1997).
Chlorophyllase Activities

Chlorophyllase activity was determined using a modified procedure based on the reports on Cavendish banana (Janave 1997) and rye (*Secale cereale* L.) seedlings (Tanaka et al. 1982). The reaction mixture containing 0.35 ml of 0.1 M potassium phosphate buffer (pH 7.0), 0.288 ml of acetone (to make 30% in final concentration), 0.01 ml of 0.1 M ascorbate (to inhibit the oxidative bleaching pathway or chlorophyllase oxidase activities), and 12 µl chlorophyll (for final concentration of 10 mM). The reaction was initiated by adding 0.34 ml enzyme extract. After 30 min at 30°C, 0.1 ml of 1 N NaOH was added to stop the reaction. Then, 3 ml of acetone/n-hexane (1/2, v/v) were added to the reaction mixture. The mixture was vigorously shaken in order to allow the chlorophyllide formed by the enzymatic reaction to be partitioned into the lower aqueous layer. The mixture was centrifuged at 3000 x g for 5 min. The enzyme activity was determined according to the decrease of chlorophyll *a* using the absorbance changes at 663 nm. The reduction of chlorophyll *a* only indicated chlorophyllase activities.

Mg-dechelatase Activities

The dechelation of magnesium from chlorophyllin (or chlorophyllide) to form pheophorbide was determined by monitoring the change in absorbance with time at 686 nm according to Vicentini et al. (1995) and Janave (1997). The assay mixture was comprised of 800 µl 50 mM Tris-Tricine (pH 8.0), 95 µl chlorophyllin (*A*₆₈₆ nm=0.1), 100 µl 1% Triton X-100, and 5 µl of enzyme extract. The control mixture did not contain enzyme extract. The reaction was carried out at 25°C and activity expressed as Δ*A*₆₈₆/min/gram leaf. The decrease of substrate chlorophyllide and the increase of
pheophorbide in two minutes were monitored on a spectrophotometer (Model Genesys 5, Spectronic Instruments, Rochester, NY) based on the protocol described by Janave (1997).

**Chlorophyll Oxidase Activities**

Total degradation of chlorophyll without the inhibitor resulted from both pheophorbide a and oxidative bleaching pathways. When 2 mM ascorbate was added, only the pheophorbide a pathway occurred because the ascorbate in the reaction mixture completely inhibited the oxidative bleaching pathway (Janave 1997). The contribution of the oxidative bleaching pathway to overall chlorophyll degradation was calculated by subtracting the absorbance change with inhibitor from the absorbance change without the inhibitor.

**Experimental Design and Data Analysis**

The experiment was a split-split plot design. The experiment was repeated six times. The four sampling dates (3rd, 6th, 9th, and 12th) were the main plots within each trial. The three aphid treatments (control, *D. noxia*, and *R. padi*) were the sub-plots within each main plot (sampling dates) and the four wheat lines (Tugela, Tugela-Dn1, Tugela-Dn2, and Tugela-Dn5) were the sub-sub-plots within each sub-plot (aphid treatments). Six plants were used in each treatment on each sampling date (in total 36 plants were used per treatment per sampling date) for the analysis of chlorophyll degradation enzyme activities, plant and aphid biomass, and total protein contents. Data were analyzed using the PROC GLM procedure of the SAS software followed by TEST statements to assure correct error terms used in assessing main effect of experimental factors (Cochran and
Results

Total Protein Determination

Total protein content of wheat leaves collected from the four wheat lines was not significantly affected by the three-way (isoline by date by aphid) interaction ($F = 0.57$, df = 18, 70, $P = 0.9125$). Among the three two-way interactions, only aphid-isoline interaction significantly affected total protein content ($F = 2.66$, df = 6, 30, $P = 0.0343$). None of the three main effects (e.g., aphid treatment, sampling date, and wheat line) was significant on protein content ($P$-values > 0.05).

Chlorophyllase Activities

Chlorophyllase activity of Tugela wheat lines was not significantly affected by the three-way (isoline by date by aphid) interaction ($F = 0.89$, df = 18, 65, $P = 0.5975$), nor by the three two-way interactions ($P$-values > 0.05). Chlorophyllase activities were significantly different among aphid treatments ($F = 10.13$, df = 2, 8, $P = 0.0064$), but not among sampling dates ($F = 3.46$, df = 3, 11, $P = 0.0548$), or wheat lines ($F = 1.76$, df = 3, 12, $P = 0.2081$). Although chlorophyllase activities were not statistically different among sampling dates in the overall analysis above, chlorophyllase activity of $D. noxia$-infested and the uninfested wheat lines decreased over the sampling period (Fig. 9).

Chlorophyllase activity was lower in $D. noxia$-infested plants than $R. padi$-infested plants (Fig. 9). When infested with $D. noxia$, chlorophyllase activity in Tugela plants was
Figure 9. Chlorophyllase activity in four wheat lines (Tugela, Tugela-Dn1, Tugela-Dn2, and Tugela-Dn5) with three aphid treatments (RWA=D. noxia; BCO=R. padi; CK=Control).
statistically the same as in Tugela-Dn2 plants, but was higher than in Tugela-Dn1 and Tugela-Dn5 plants (Fig. 9).

Mg-dechelatase Activities

Mg-dechelatase activities were significantly affected by the three-way (isoline by date by aphid) interaction ($F = 2.53$, df = 18, 71, $P = 0.0029$), but not affected by either isoline-date interaction or aphid-date interaction ($P > 0.05$). Mg-dechelatase activity was significantly affected by isoline-aphid interaction ($F = 7.69$, df = 6, 24, $P = 0.0001$). Significantly different Mg-dechelatase activities were recorded in four wheat lines ($F = 34.68$, df = 3, 12, $P < 0.0001$), three aphid infestation treatments ($F = 16.87$, df = 2, 8, $P = 0.0013$), but not in four sampling dates ($F = 0.98$, df = 3, 12, $P = 0.4365$).

Mg-dechelatase activities were lower in R. padi-infested plants than in D. noxia-infested plants (Fig. 10). Mg-dechelatase activities were not different among the uninfested or R. padi-infested wheat lines, but were different among D. noxia-infested plants. Mg-dechelatase activity was lower in D. noxia-infested Tugela when compared with other Tugela plants with Dn genes on all sampling days except day 12. Mg-dechelatase activity was similar in Tugela and Tugela-Dn5 plants on day 12 (Fig. 10C).

Chlorophyll Oxidase Activities

Chlorophyll oxidase activity was not affected by any of the main effects or interactions ($P$-values > 0.05). All values of oxidase activity obtained by subtracting absorbance change with inhibitor from absorbance change without inhibitor were negative. This means that the oxidative bleaching pathway was not involved in chlorosis formation in aphid-infested wheat seedlings.
Figure 10. Temporal changes of Mg-dechelatase activity in four wheat lines were shown respectively by three aphid infestation treatments. A: Uninfested Tugela plants; B: *R. padi*-infested Tugela plants; C: *D. noxia*-infested Tugela plants. Each data point represents the mean \((n = 6)\) on each sampling date. Error bar indicates the standard error of the mean.
Discussion

The chlorotic injury symptoms on *D. noxia*-infested Tugela wheat lines indicated the degradation of chlorophylls. Chlorophyllase and Mg-dechelatase play critical roles in the first two steps of pheophorbide *a* pathway in chlorophyll catabolism (Matile et al. 1999, Dangl et al. 2000, Takamiya et al. 2000) (Fig. 7). In the very late stage of chlorophyll biosynthesis, protoporphyrin IX is metallated by insertion of Mg from Mg-chelatase which is a two-step reaction activated by ATP (Walker and Wenstein 1994, Blankenship 2002). In the following step, attachment of phytol chain to chlorophyllide *a* is catalyzed by chlorophyll synthase (Blankenship 2002). The Tugela wheat lines were young seedlings (3-leaf stage) and still in early development. Unlike the natural senescing plants, chlorophyll biosynthesis was, therefore, the main biological event occurring within the green tissue. We suggest that chlorophyll degradation and chlorophyll biosynthesis were balanced in *R. padi*-infested Tugela isolines, while chlorophyll degradation was the main event in *D. noxia*-infested plants and normal chlorophyll biosynthesis was interrupted.

In *R. padi*-infested wheat lines, chlorophyllase activities were significantly higher than in *D. noxia*-infested plants. Based on the chlorophyll degradation model (Fig. 7), more chlorophyllide *a* would be produced in the first step of chlorophyll degradation in *R. padi*-infested plants than in *D. noxia*-infested plants (Figs. 11A, 11B).

In contrast, Mg-dechelatase activities were significantly higher in *D. noxia*-infested plants than in *R. padi*-infested plants. This means more chlorophyllide *a* would have been degraded into pheophorbide *a* in *D. noxia*-infested plants than in *R. padi*-infested plants.
Figure 11. Hypothesis of chlorophyll loss in D. noxia and R. padi-infested wheat lines. Bold characters indicate significantly higher enzyme activities when compared with characters without bolding. Faint arrows indicate weak activity caused by lower enzyme (Mg-chelatase and chlorophyll synthase) activities.

We hypothesize that in R. padi-infested Tugela wheat lines, Mg-chelatase and chlorophyll synthase activities were not affected. Excess amount of chlorophyllide \( a \) produced by higher chlorophyllase activities would provide substrates for both chlorophyll catabolism (i.e., magnesium dechelation by Mg-dechelatase) and chlorophyll anabolism (i.e., chlorophyll formation by chlorophyll synthase) (Fig. 11A). Thus, both chlorophyll degradation and chlorophyll biosynthesis were in balance on R. padi-infested plants. Because low Mg-dechelatase activity, chlorophyll biosynthesis catalyzed by chlorophyll synthase recycled the chlorophyllide \( a \) into chlorophylls and no chlorosis was observed (Fig. 11A). In contrast, less chlorophyllide \( a \) was produced in D. noxia-infested Tugela isolines (Fig. 11B) because of lower chlorophyllase activities. Mg-dechelatase
activities in *D. noxia*-infested plants were higher and caused a higher demand of chlorophyllide \( a \) that contributed to chlorophyll degradation. Chlorophyll synthase activity most likely was limited because there were lower levels of chlorophyllide. These events would block chlorophyll \( a \) biosynthesis and contribute to chlorosis formation in *D. noxia*-infested plants.

Within *D. noxia* infestation, Tugela plants showed more chlorotic injury symptoms than the three *D. noxia*-resistant isolines. However, Mg-dechelatase activity in Tugela was significantly lower than in Tugela *Dn* plants (Fig. 10C). We speculate that chlorophyll biosynthesis enzyme (i.e., Mg-chelatase and chlorophyll synthase) activities in *D. noxia*-infested plants are less affected in Tugela isolines with *D. noxia*-resistant *Dn* genes but strongly inhibited in Tugela plants (Fig. 12). Both the chlorophyll biosynthesis process and chlorophyll degradation occur in Tugela *Dn* plants which would decrease the formation of chlorotic symptoms. Inhibition of the chlorophyll biosynthesis enzymes (i.e., Mg-chelatase and chlorophyll synthase) in *D. noxia*-susceptible Tugela plants would limit the plant's capacity to synthesize chlorophylls, which would account for severe chlorosis.

We assayed only the chlorophyll degradation enzyme activities. To fully understand the mechanism of aphid-induced chlorosis formation in wheat plants, measurements of chlorophyll biosynthesis enzyme (i.e., chlorophyll synthase and Mg-chelatase) activities should be considered in future investigations.
Figure 12. Hypothesis of chlorophyll loss in *D. noxia*-infested Tugela and Tugela plants with *Dn* genes. Bold characters indicate significantly higher enzyme activities when compared with characters without bolding. Faint arrows indicate weak activity caused by lower enzyme (Mg-chelatase and chlorophyll synthase) activities.
CHAPTER 5

CONCLUSION

The experiments conducted and reported in this thesis assess the resistance of Tugela and three wheat isolines (i.e., Tugela-Dn1, Tugela-Dn2, and Tugela-Dn5) by examination of aphid and plant biomass. The results supported previous reports concerning the mechanisms among \( D. noxia \)-resistant \( Dn \) genes. Tugela-Dn2 is not only \( D. noxia \)-tolerant as reported by du Toit (1987, 1989), but is also antibiotic. Although research has been conducted on enzymatic chlorophyll degradation in senescing plants and a few plant-herbivore systems, this is the first research to examine chlorophyll degradation enzyme (i.e., chlorophyllase, Mg-dechelatase, and chlorophyll oxidase) activities among wheat lines that have particular genes conferring aphid resistance. By comparing chlorophyll degradation enzyme activities in Tugela and Tugela isolines with varying chlorotic symptoms, we were able to hypothesize mechanisms of aphid-elicited chlorosis formation, which was most likely induced by unbalanced chlorophyll degradation and chlorophyll biosynthesis. The enzymatic analysis among wheat lines also affords baseline knowledge concerning gene function in wheat lines with varying resistant genes and will contribute to post-genomic studies. To validate the inference about the chlorosis formation in wheat lines after aphid infestation, further research is needed that measures chlorophyll biosynthesis enzyme (i.e., chlorophyll synthase and Mg-chelatase) activities.
REFERENCES CITED


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