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**SOLUBLE AND CELL WALL BOUND  
PHENOLIC-MEDIATED INSECT RESISTANCE  
IN CORN AND SORGHUM**

by

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Thesis submitted to the  
Faculty of Graduate and Postdoctoral Studies  
University of Ottawa  
In partial fulfillment of the requirements for the  
Ph.D. degree in the

Ottawa-Carleton Institute of Biology

Thèse soumise à la  
Faculté des études supérieures et postdoctorales  
Université d'Ottawa  
En vue de l'obtention du Doctorat

L'Institut de Biologie d'Ottawa-Carleton



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**A la mémoire de ma grand-mère,**

**Hazra Ramjaun**

## Abstract

This thesis examined the role of phenolic substances in resistance to insect pests of maize and sorghum. Eight landraces of sorghum collected in Ethiopia were assessed for methanol soluble phenolic content (as catechin equivalents) by the Prussian blue procedure and storage insect resistance in standardized tests with *Sitophilus oryzae* and were found to show significant variation by genotype. Significant inverse linear relationships were also found between resistance parameters such as weight loss of grain, the Dobie index of susceptibility, number of eggs laid and progeny emerged and the phenolic content of the grain ( $r^2 = 0.85, 0.55, 0.46$  and  $0.52$  respectively). The results suggest that the soluble phenolic content, which has been shown to consist primarily of proanthocyanidins, can be used as a leading indicator of resistance.

Host plant resistance in maize, *Zea mays* L. to the southwestern corn borer (SWCB), *Diatraea grandiosella*, and the sugarcane borer (SCB), *Diatraea saccharalis*, was investigated in relation to bound phenolics in the leaf cell wall. A mapping population was developed from CML131 X CML 67 as the susceptible and resistant parents, respectively. Recombinant inbred lines were rated for leaf feeding damage after artificial infestation. Leaf tissue toughness, fiber content and gravimetric soluble metabolites (GSM) were quantified. A significant negative correlation was found between tissue toughness and leaf feeding damage ( $r = -0.37, P = 0.001$ ) with the SCB in 1997A. Fiber and GSM were not significantly correlated to leaf feeding damage. Cell wall bound phenolics were investigated as possible mechanisms for elevated levels of tissue toughness. Both cell wall bound p-coumaric acid and ferulic acid were significantly and negatively correlated to leaf feeding damage. Diferulic acid (DFA), which cross-links cell wall arabinoxylans was found to be significantly correlated to tissue toughness in 1997A. Also, significant negative correlations were found between DFA and leaf feeding damage for both insects in 1996B and 1997A. Leaf nitrogen levels were investigated as a nutritional basis for resistance. Nitrogen levels were positively correlated with leaf feeding damage, lower levels of nitrogen resulted in reduced feeding.

Approximately 40 % of the variation in leaf feeding damage for SCB could be accounted for by nitrogen, DFA and ferulic acid content in leaf tissue.

Putative phytochemical traits for insect resistance were further investigated with quantitative traits loci (QTL) mapping. Five out of eight QTLs for 5-5' DFA were located at the same position or close to QTLs for SWCB and SCB leaf feeding damage. Eight QTLs for 5-5' DFA could explain 44% of the phenotypic variation for this dimer. QTLs for p-coumaric acid and ferulic acid were also associated with QTLs for leaf feeding damage. QTLs for cell wall bound phenolics linked with QTLs for leaf tissue toughness in four chromosome locations.

A molecular transformation of maize was employed to increase cell wall hydrogen peroxide in order to enhance DFA formation and insect resistance. Corn transformed with a wheat oxalate oxidase gene under the control of the rice actin promoter elements (pActOXO) expressed the enzyme activity constitutively. With two independent transformation events, enzyme activity was stable after seven generations of outcrossing into three inbred lines. Enzyme activity remained associated with the cell wall debris fraction of water-extracted tissues. Leaf tissue of the OXO transgenics had elevated levels of H<sub>2</sub>O<sub>2</sub>. *In vitro* leaf-feeding bioassays demonstrated that ECB larval feeding was significantly reduced on all OXO transgenic lines. This was confirmed under field conditions on plants infested with ECB larvae. Most significantly, stalk tunneling damage, measured at plant harvest, was substantially reduced in all OXO transgenic lines. The reduction of tunneling by 50% in the OXO transgenic lines is indicative of lower levels of ECB survival and this should also reduce crop losses resulting from the effect of heavy infestations of ECB on plant lodging. The transformation did not increase DFA formation as predicted and resistance was associated with the direct effects of H<sub>2</sub>O<sub>2</sub> and oxalate oxidase on insect physiology.

## Résumé

La présente thèse porte sur l'étude du rôle des composés phénoliques dans la résistance du maïs et du sorgho aux insectes pathogènes. Le contenu phénolique soluble dans le méthanol (en équivalent catéchine) de huit populations naturelles de sorgho récoltées en Éthiopie a été évalué au moyen de la méthode du "bleu de Prusse" et une variation significative de la résistance aux ravageurs d'entrepôt a été observée lors de tests normalisés avec *Sitophilus oryzae*. Des relations linéaires négatives significatives ont également été observées entre des paramètres de résistance comme la réduction de la masse des grains, l'indice de susceptibilité Dobie, le nombre d'œufs pondus et de larves émergentes, et le contenu phénolique des grains ( $r^2=0,85; 0,55; 0,46$  et  $0,52$ , respectivement). Ces résultats suggèrent que le contenu phénolique soluble constitué, d'après les recherches, principalement de proanthocyanidines, peut être utilisé comme un bon indicateur de la résistance.

La résistance du maïs, *Zea mays* L., à la pyrale du maïs du sud-ouest (SWCB), *Diatraea grandiosella*, et au perceur de la canne à sucre (SCB), *Diatraea saccharalis*, a été étudiée. Une population cartographique a été développée à partir des lignées CML131 comme parent susceptible et CML67 comme parent résistant. Le dommage aux feuilles de lignées autofécondées recombinantes causé par la déprédation suite à une infestation artificielle a été évalué. La rigidité du tissu foliaire, le contenu fibreux et les métabolites solubles gravimétriques (GSM) ont été quantifiés. Une corrélation négative significative a été observée entre la rigidité du tissu foliaire et le dommage résultant de la déprédation ( $r = -0.37$ ,  $P = 0.001$ ) causée par SCB en 1997A. Ni les contenus en fibre ou en GSM n'étaient significativement corrélés au dommage causé par la déprédation. Les composés phénoliques liés à la paroi cellulaire ont été examinés comme mécanisme possible régissant l'augmentation de la rigidité du tissu foliaire. Une corrélation négative significative a été observée entre les concentrations d'acide p-coumarique et le dommage foliaire causé par la déprédation. Une corrélation significative a été observée en 1997A entre la rigidité des tissus et la production d'acide diférulique, un composé qui crée des liens entre les arabinoxylanes de la paroi cellulaire. Des corrélations négatives significatives ont également été observées entre le taux de production de DFA et les niveaux de dommage foliaire dans le cas des deux insectes, en 1996B et en 1997A. Les niveaux d'azote ont été évalués en tant que facteur nutritionnel possible contribuant à la résistance observée. Les niveaux d'azote étaient corrélés positivement au dommage foliaire. Ainsi, pour des

niveaux réduits d'azote, on a observé des taux réduits de déprédation. On a pu attribuer approximativement 40 % de la variation observée au niveau du dommage foliaire au contenu des tissus en azote, en DFA et en acide férulique.

Les traits phytochimiques putatifs de la résistance aux insectes ont été examinés ultérieurement en construisant des cartes génétiques par analyse de loci quantitatifs (QTL). Cinq des huit QTLs pour le DFA 5-5' étaient situés à la même position ou proche des QTLs pour le dommage foliaire causé par la déprédation par le SWCB et par le SCB. Huit QTLs pour le DFA 5-5' expliquaient 44 % de la variation phénotypique au niveau de ce dimère. De plus, les QTLs pour l'acide p-coumarique et l'acide férulique étaient associés aux QTLs pour le dommage foliaire. Les QTLs pour les composés phénoliques liés à la paroi cellulaire étaient reliés aux QTLs pour la rigidité du tissu foliaire sur quatre régions chromosomiques.

Le maïs a été génétiquement modifié de manière à augmenter la teneur de la paroi cellulaire en peroxyde d'hydrogène pour augmenter la production de DFA et la résistance aux insectes. Le maïs transformé avec un gène pour l'oxydase d'oxalate de blé sous le contrôle d'éléments promoteurs d'actine du riz (pActOXO) exprimait son activité enzymatique constitutivement. Avec deux événements de transformation indépendants, l'activité enzymatique était stable après sept générations de pollinisation croisée chez trois lignées autofécondées de maïs. Après une extraction par eau des tissus, il a été observé que l'activité enzymatique se retrouvait dans la fraction qui contenait les débris de la paroi cellulaire. Des niveaux élevés de  $H_2O_2$  ont été observés dans le tissu foliaire provenant des plantes transgéniques OXO. Des expériences de phyllophagie *in vitro* ont démontré que les quantités de feuilles consommées par les larves de pyrales du maïs européenne (ECB) étaient réduites chez toutes les lignées transgéniques OXO. Ces résultats ont été confirmés lors d'expériences sur terrain avec des plantes infestées artificiellement de larves d'ECB. En particulier, le dommage causé par le minage de la tige, mesuré lors de la récolte, était significativement réduit dans toutes les lignées transgéniques OXO. La réduction du minage de 50 % dans les lignées transgéniques OXO est indicatrice d'un taux de survie plus faible chez les ECB. Ceci devrait également réduire les pertes associées aux versements des plantes ayant lieu lors d'infestations majeures d'ECB. La transformation n'a pas entraîné l'augmentation prévue du taux de DFA et la résistance était associée aux effets directs de l' $H_2O_2$  et de l'oxydase d'oxalate sur la physiologie des insectes.

## **Acknowledgements**

Without any hesitation, I would like to thank my supervisor Dr. John Thor Arnason for his support, guidance and encouragement. Thank you Thor for showing me the softer side of science. To Dr. Bernard Philogène *mo dire mari meci* for keeping up the insect portion of the plant-insect interaction equation and for the PMRA preparations. I thank Dr. John Simmonds, Lesley Cass, and the staff at Agriculture Canada, Ottawa for their work on the transgenic corn. I also thank Dr. David Bergvinson, Silverio Garcia Lara, Valentin Perez and the staff at CIMMYT, Mexico for all their help in providing samples, maps and for your hospitality. I would also like to thank Dr. John Sinclair and Dr. Christiane Charest for their helpful inputs during my studies. To all the members of the lab, I say thank you for making the journey enjoyable. I acknowledge the help of John Livesey with the HPLC, Antoine Bily and Dave Johnston with lab and field-work, and France Duval for maintaining the ECB culture. The abstract in french was prepared by Alain Boucher and Anne-Sophie Belzile. To Dr. Semir Omar and Dr. Moussa Keita, I say thank you for giving me perspective.

To my parents Idris and Farida, I will never be able to replace even one tear. To my sisters Zinnat and Laila and their families, thank you for your support. To my kids Hadiya and Wasseem, thank you for the distraction, the joy and the comfort. To my wife Swadeka, thank you for everything especially your patience, Paradise is truly at your feet.

Funding for this project was provided by CIDA (Canadian International Development Agency) Linkage Fund and NSERC (National Science and Engineering Research Council).

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## Chapter 1

### 1.1 Introduction and Hypotheses

*In cultivating maize man also cultivated himself.*

Museum of the Popular Cultures of Mexico – *Maize, the basis of Mexican popular culture*

The Food and Agriculture Organization (FAO) of the United Nations estimates that 10 to 15 % of the world's maize production is lost every year to insects, diseases, and other pests. This number is much higher in many developing countries. The focus of this thesis is mostly concerned with small-scale farmers in developing countries who frequently do not have access to chemical means of controlling insects and who frequently cannot afford chemical controls even when they are available. Thus, for many farmers in the developing world, host plant resistance is the least expensive and perhaps the only means by which they can protect their maize crops under insect attack and stabilize their production over the long term. This thesis examined the putative role of phenolic substances as the biochemical basis for insect resistance.

An initial study examined resistance in sorghum to the storage pest *Sitophilus oryzae*. In this case, soluble phenolics were investigated as leading indicators of resistance. Subsequent studies focussed on maize because CIMMYT (International Centre for the Improvement of Maize and Wheat) requested our collaboration in this area.

It has been demonstrated previously that leaf feeding damage by the European corn borer (ECB), *Ostrinia nubilalis* (Pyralidae: Lepidoptera), is negatively correlated with the toxic hydroxamic acid DIMBOA in temperate grown corn (Klun et al., 1970).

But, maize resistance based on DIMBOA does not explain many situations in which host plant resistance is observed. Other resistance factors appear to operate in exotic accessions such as highly resistant Antigua germplasm (Sullivan et al., 1974). Cross-linking of matrix polymers by phenolic acid dimers, such as diferulic acid (DFA), in the plant cell wall was postulated to interfere with herbivore nutrition (Fry 1986). This hypothesis was supported by field and phytochemical studies by Bergvinson et al. (1994a) that showed ECB resistance was strongly correlated to DFA in maize. This present study is a continuation of research examining the role of DFA in host plant resistance to the important tropical maize borers which are tolerant to DIMBOA. It was hypothesized that these dimers would constitute a defense against the southwestern corn borer and the sugarcane borer in tropical and subtropical maize germplasm where the biochemical basis of resistance is unknown. A mapping population was developed to examine the correlations between insect feeding and DFA. It was also hypothesized that if DFA correlates with tropical borer resistance then quantitative trait loci (QTLs) for insect resistance would be closely associated with QTLs for DFA. This was an attempt to combine phytochemical analyses with molecular marker technology.

Molecular engineering offered the potential to specifically alter DFA levels and observe the effects on insect resistance. Corn plants were transformed with a wheat gene (germin) for oxalate oxidase, which converts oxalic acid to hydrogen peroxide and CO<sub>2</sub>. It was hypothesized that increasing hydrogen peroxide would also increase the peroxidase-mediated production of DFA. All three approaches: phytochemistry, mapping and transgenic work, were all used to provide a better understanding of DFA as one factor of host plant resistance.

## **1.2 Sorghum**

*Sorghum (Sorghum bicolor)* is an important human food plant in Africa, South-East Asia, India, Central America and China. Sorghum was domesticated over 5000 years ago in Africa and then spread through South-East Asia to China (Vaughan and Geissler, 1999). In general appearance sorghum is somewhat similar to maize and may grow into a large plant. The different varieties can be cultivated in varying degrees in arid and semi-arid zones, and in areas of unpredictable rainfall and poor soil, where larger-seeded cereals cannot be grown. The grains may be consumed like rice or the flour converted into gruel, porridge, or unleavened bread.

## **1.3 Maize**

*Zea mays* L. (*zeia*, Greek for cereal and *maís*, Mexican for corn) is an adaptable plant, its cultivars are able to grow in regions from the tropics to temperate areas and from sea level to 3500 m. Maize is the New World's gift to humanity, where it was domesticated more than 7,000 years ago by the people living in pre-Columbian Mexico. It was the basis for their ancient civilizations and it continues to play a major role in modern agriculture. It is remarkable that only four plant species out of 20 major crops provide 60% of all human food: wheat, rice, maize and potatoes. In 1999 the World produced 595 million metric tons (MMT) or 23.4 billion bushels of maize. In Ontario and Quebec, corn production is 9 MMT or 1.5% of the world total with a value of approximately \$900 million (OCPA, Ontario Corn Producers Association, 1999).

Corn (as it is called in Canada and the United States, mealies in South and central Africa, and maize in the rest of the world) is known to have over 1,000 nutritional and agro-industrial uses, which include animal feed, fertilizers, medicines, fuel, packaging, crafts, food, starches and oils. Although most of the world's corn is used for livestock feed, corn grown for human consumption is important in some regions, primarily in developing nations. In much of Africa, ground corn is a staple food. In Latin America, coarse flour from corn is formed into tortillas, a staple food for many people.

In 1940 the average yield of corn in the US mid-West was 35 bushels per acre (2.2 tons per hectare). Today that number has increased to 140 bushels per acre (8.7 tons per hectare). At the turn of the century, plant scientists demonstrated that self-fertilization for successive generations produced essentially homozygous lines and that these true breeding lines were not an end in themselves but could serve as parental stocks from which to produce superior performing hybrids. Early corn breeders showed that when two different inbred lines were crossed, their progeny usually performed better than either parent, a trait called hybrid vigor. These hybrid lines contain gene combinations which provide improved physiological functioning and anatomical and morphological organization, more resistance to disease and insect pests, greater uniformity, and better adaptation to specific environmental conditions. Today, virtually all corn produced in the US is grown from hybrid seed, conventional breeding efforts are now directed towards improvement of populations from which superior inbreds are selected to serve as hybrid parents.

Another important contribution to the high production of corn has been the massive use of pesticides and nitrogen fertilizers. Between 1945 and 1990, insecticide

use in corn production has increased 1000 fold, and despite this increase, total crop losses from insect damage has nearly doubled from 7% to 13% (Paoletti and Pimentel 1996). In order to maintain high yields, a vast array of pesticides (insecticides, fungicides and herbicides) are now used to control several corn pests. It is becoming increasingly apparent that this heavy reliance on chemical pesticides has a high economic, environmental and human cost.

#### **1.4 Integrated Pest Management**

The public concern over the use of pesticides came to the foreground with the publication of *Silent Spring* (Carson, 1962). Integrated Pest Management (IPM) was established during the 1960s to address these concerns. IPM as defined by the Entomological Society of America is “a sustainable approach to managing pests that combines biological, cultural, physical and chemical tools in a way that minimizes economic, health and environmental risks”. IPM is a systems approach that provides an ecologically-based solution to pest control problems. The term pest refers to insects, weeds, diseases, rodents and other organisms that compete with humans for food and shelter, or affect human health. Over the last 40 years IPM has broadened from single-tactic pest management largely with chemical pesticides to multiple pest management with integrated crop management.

An agronomy solution is only necessary when there is a problem. To determine if there is a problem, the initial step in any IPM strategy is to determine the Economic injury level (EIL). This is a determination of when an insect (or any other organism) becomes a pest, so that IPM needs to be implemented. EIL levels were pioneered by

Stern et al., (1959). Upon introduction to a favourable environment, any population will increase until, the combined effects of dwindling food supply, increased predation and parasitisation limit further increase. At this point a general equilibrium position is established, if this position exceeds the EIL, then the insect is considered a pest, and IPM is initiated. Hudon and Ogilvie (1984) has calculated that the EIL for the ECB is 2.3 larvae per plant in Ontario and Bigler (1983) suggested that an EIL of 5-10 ECB egg batches per 100 corn plants for Switzerland.

There are three main types of control that are critical to IPM: cultural practices, host plant resistance and biological control. Cultural practices include: crop rotation or intercropping; modifying the planting date is a long-known technique for altering the synchrony of host and pests, to allow the crop to avoid infestation; and tillage to physically bury or injure the pest, this is a particularly effective tool for managing numerous weed species and some pathogens and insects; and cleaning produce to remove weed seeds, insects, and seed-borne pathogens, this sanitation process helps remove residual infestation sources.

Biological control is the use of biological control agents including predators, parasites, pathogens, antagonists and competitors to reduce losses from pests (Cate and Hinkle, 1994). Diverse cropping systems and noncultivated land adjacent to cornfields may provide overwintering sites and additional food sources (nectar, pollen, alternative prey) for beneficial insects (Wright and Van Duyn, 1999). Research is ongoing to evaluate the use of *Trichogramma* species (wasps that parasitize insect eggs) for control of the ECB and corn earworm. A second insect, the parasitic fly *Lydella thompsoni*, is being considered as a potential natural enemy to the ECB.

Another type of biological control is the release of sterilized males against the insect pest. Sterile insect technique (SIT) was used to combat the screwworm fly attacking cattle in the southern US and Latin America. Ionizing radiation causes sterility of male insects by inducing dominant lethal mutations in the sperm (Knipling, 1979). The eradication program in northern Mexico required 500 million sterile flies per week.

### **1.5 Host Plant Resistance**

Painter (1951) defined host plant resistance as the heritable qualities possessed by the plant which influence the ultimate degree of damage caused by the pest. Host plant resistance forms the foundation of ecologically-based IPM systems. In such a systems, host plant resistance is the most effective component of IPM, also it is the cheapest technology, it is the easiest to introduce, and it is compatible with other control methods (Panda and Kush, 1995). The three types of genetic resistance are antixenosis, antibiosis and tolerance. Antixenosis describes the insect's response to unattractive or unsuitable plants to serve as hosts. Biophysical or biochemical factors or a combination of both influence the insect's behaviour in host selection. Antibiosis is a mechanism that operates after insects have colonized and started utilizing the plant. The natural products produced by the plant adversely affect the insect's biology, growth, development, reproduction and survival. Detrimental factors include: a decrease in weight, metabolic processes, an increase in restlessness, prolong periods of development in immature stages and an increase in larval mortality. The third form of resistance is tolerance, this is a plant genetic trait, which enables the host to withstand infestation or recover from damage caused by an insect that would severely damage susceptible plants of the same

species. Tolerance does not affect the rate of population increase, but raises the economic threshold level and is independent of the effect of the insect (Panda and Kush, 1995). These three forms of resistance are not mutually exclusive but rather form a continuum of host plant resistance.

Recently, the concept of host plant resistance has been expanded into crop plants via pyramided resistance. This approach combines multiple resistance, either through conventional breeding or through the incorporation of transgenes to combat multiple pests. Genetic engineering is rapidly providing new avenues for development and deployment of pest-resistant cultivars. Through this process, corn, cotton and other crops are genetically modified to produce biological products such as Bt (*Bacillus thuringiensis*). This technology provides new opportunities and challenges for the development and implementation of comprehensive IPM programs.

## **1.6 Co-evolution**

Host plant resistance has been made possible through 380 million years of plant/insect interaction. In the close ecological intimacy between a plant and an insect species, each becomes a major source of selection operating on the other, in such situations co-evolution occurs. Plants have evolved a vast array of static defenses to deal with insect predation. Chemical defenses (secondary metabolites or the preferred term natural products) are employed to provide resistance to the host plant. There are 30,000 described natural products out of an estimated 500,000 (Harborne and Baxter 1999). It is widely believed that many of these natural products have insecticidal activity. The idea of co-evolution was first proposed by Ehrlich and Raven (1964), which examined

butterflies, plants and chemical defenses. While plants were evolving chemical defenses, the insect herbivores were evolving ways to avoid or detoxify these chemicals. The long evolutionary experience of herbivores with plant natural products has provided the necessary mechanisms for the pest to evolve resistance to many synthetic pesticides. The insect pest has evolved enzyme systems such as P450 that detoxify natural products, with some modification these can detoxify synthetic pesticides. In addition, the natural enemies that keep herbivore populations within ecological levels are more susceptible to synthetic pesticides due to bioaccumulation and less adept at evolving detoxifying mechanisms.

Natural products can be grouped into three major categories: nitrogen compounds, terpenoids, and phenolics. Compounds that do not fit into one of these categories can be listed as "other". Within the nitrogen compounds there are alkaloids, amines, non-protein amino acids, cyanogenic glycosides and glucocinolates. Terpenoids include: monoterpenes, sesquiterpene lactones, diterpenes, saponins, limonoids, cucurbitacins, cardenolides and cardenoids. Within the phenolic category are simple phenols, flavonoids and quinines. Polyacetylenes are classified as "other".

With the declining use of insecticides and the increased importance of host plant resistance within the IPM framework, an understanding of plant defenses has become increasingly important. Corn hybrids vary in their resistance to insect pests. Corn breeding programs have incorporated insect resistance to a few of the major insects pest into commercial hybrids. Breeding for resistance to the ECB has largely focused on antibiosis targeted to the first generation or neonate larvae. First generation neonate larvae feed on leaf tissue within the whorl, and most larval mortality occurs during

feeding on resistant varieties (Guthrie et al., 1960). Klun and Brindley (1966) showed a positive correlation between the concentration of the hydroxamic acid MBOA (6-methoxybenzoazolinone) in whorl tissue and field resistance to the ECB. Leaf feeding resistance to corn borers is partially imparted by the presence of a plant aglucone DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), which is chemically labile and decomposes to MBOA (Fig. 1.1). DIMBOA is stored in the plant as a glucoside which is released on damage as the aglycone. It is most concentrated in whorl stage corn, it decreases with plant age, thus providing little protection for second generation ECB which feed primarily on sheath-collar tissue (Guthrie et al., 1986).

The role of DIMBOA has been extensively studied as a resistance factor to the ECB. Houseman et al., (1992) demonstrated that DIMBOA is toxic by inhibiting the activity of the gut proteolytic enzyme chymotrypsin. MBOA has been shown to inhibit the trypsin protease. These hydroxamic acids interfere with the insect's ability to obtain proper levels of nutrition since their digestive enzymes are inhibited.

DIMBOA though, cannot account for all of the resistance found in the corn plant, there are other studies, which offer alternative mechanisms of resistance. Manuwoto and Scriber (1985) found that plants grown under a reduced light regime and higher levels of ECB feeding, these plants also had higher levels of DIMBOA. The explanation being that under reduced light treatments the plant produces higher levels of nitrogen, and this in-turn leads to higher levels of leaf consumption. In leaf tissue, nitrogen is limiting and insects need nitrogen for normal growth and development. In another study, Hedin et al., (1984) showed that DIMBOA had little activity against the southwestern corn borer (*Diatraea grandiosella*). Scriber et al., (1975) demonstrated that tropical borer resistant

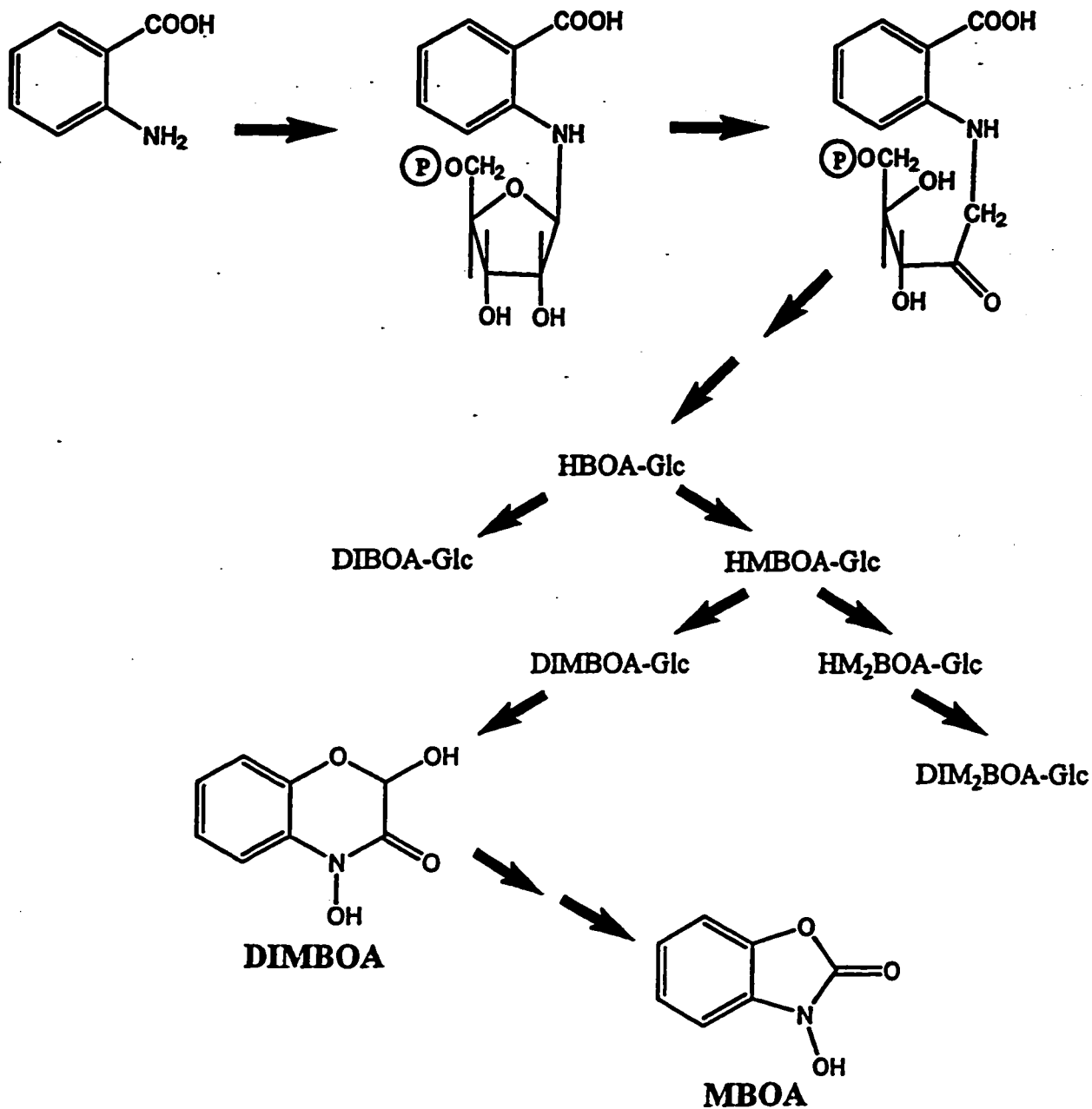


Fig. 1.1. The biosynthesis and degradation of hydroxamic acids (Niemeyer 1988).

lines that were derived from Antigua and other exotic material had low levels of leaf feeding damage despite having low levels of DIMBOA, lower levels than are found in susceptible North American inbreds. This suggests that in tropical and subtropical corn, DIMBOA is not effective against the tropical and subtropical corn borers, there must be other resistance mechanisms. The tropical and subtropical insects (sugarcane borer, southwestern corn borer and fall armyworm) have had longer to evolve on the tropical material and have overcome this chemical defense. DIMBOA is still moderately effective against the western corn rootworm (*Diabrotica vigifera vigifera*) in field trials in Ontario (Assabgui et al., 1995).

### **1.7 Phenolics**

Phenolic compounds or polyphenols are plant substances which possess in common an aromatic ring bearing one or more hydroxyl groups. Many phenolics arise from the shikimic acid pathway and subsequent reactions. Among these are cinnamic, p-coumaric, caffeic and ferulic acid, an important reaction in the formation of these phenolics is the conversion of phenylalanine to cinnamic acid. This is a deamination in which ammonia is split out of phenylalanine to form cinnamic acid, the reaction is catalyzed by phenylalanine ammonia lyase.

### **1.8 Plant Cell Wall**

The presence of a cell wall distinguishes plant cells from animal cells. The most characteristic component of the plant cell wall is cellulose (glucose units linked  $\beta$  1-4). A single molecule of cellulose may contain 3,000 to 10,000 glucose units in an unbranched

chain (Carpita, 1985). Several dozen linear cellulose chains form a microfibril, the plant cell wall consists of a rigid skeleton of cellulose microfibrils held together by a gel-like matrix. The matrix is built up of several noncellulosic polysaccharides and glycoproteins. The microfibril surfaces are coated with noncellulosic polysaccharides such as xyloglucans, glucomannans and arabinoxylans, these polysaccharides are cellulose-like in their conformations (Iiyama et al., 1994). Covalent cross-linking between wall polymers (Fig. 1.2) is a physiologically significant strategy contributing to the termination of wall extensibility, wall strengthening, and the blocking of the ingress of pathogens.

### **1.9 Diferulic Acid**

Diferulic acid was first reported in plants by Markwalder and Neukom (1976) in *Triticum aestivum*, followed by Hartley and Jones (1976) in *Lolium multiflorum*. The first proposed role for oxidative phenolic coupling was the lowering of cell wall extensibility (Fry, 1979). Secondly, Ride (1980) suggested that induction of resistance to fungal invasion was mediated by cell wall cross-linking. And thirdly, the initiation of lignification was due to peroxidase-catalysed coupling (Fry, 1983). The link between cell wall cross-linking and insect resistance was first suggested by Fry (1986). The view that cross-linking wall polymers renders the wall impenetrable to fungal hyphae (Friend, 1981) and restricts larval access (Bergvinson et al., 1997) is not in conflict with the proposed role in growth regulation, since it is quite plausible that a defence mechanism should evolve by adaptation of an existing wall reaction. In most cases, characters that confer resistance may have additional physiological or ecological functions (Rausher,



2001). Diferulic acid is an example of a secondary metabolite that functions in conferring structural reinforcement and hence resistance. The three main structural isomers of DFA are shown in Fig. 1.3. The cell wall cross-linking of adjacent arabinoxylan chains via ester-linked DFA is depicted in Fig. 1.4.

#### **1.10 Life cycle of the European corn borer**

The European corn borer is an introduced insect, it probably arrived in North America during the early 1900s in broom corn imported from Hungary and Italy for the manufacture of brooms (Smith, 1920). The European corn borer is a polyphagous insect utilizing over 200 host plant species although maize is its preferred host (Hudon and LeRoux, 1986). The life cycle of this insect is closely synchronous to the developmental stages of maize (Fig. 1.5), despite the polyphagous nature of the ECB. ECB populations in Ontario and Quebec have historically had one generation per season (the univoltine strain). Increasingly, corn producing regions of central Ontario are reporting two generations (bivoltine strain) each year. With increasing global warming, the range of the bivoltine strain may expand northwards. The ECB overwinters in the 5<sup>th</sup> larval instar in corn stalk and stubble. After adult emergence, the females will lay eggs for the first generation in cornfields or on other crops, typically on the undersides of leaves. Oviposition elicitation in ECB is influenced by the presence of several *n*-alkanes in the epicuticle of corn leaves (Udayagiri and Mason, 1997). Fatty acid methyl esters extracted from ECB egg masses act as oviposition deterrents in order to facilitate the spatial distribution of egg masses (Thiery and Le Quere, 1991). Hatching will occur after one week. Neonates immediately move into the whorl, larvae remain in these sites and

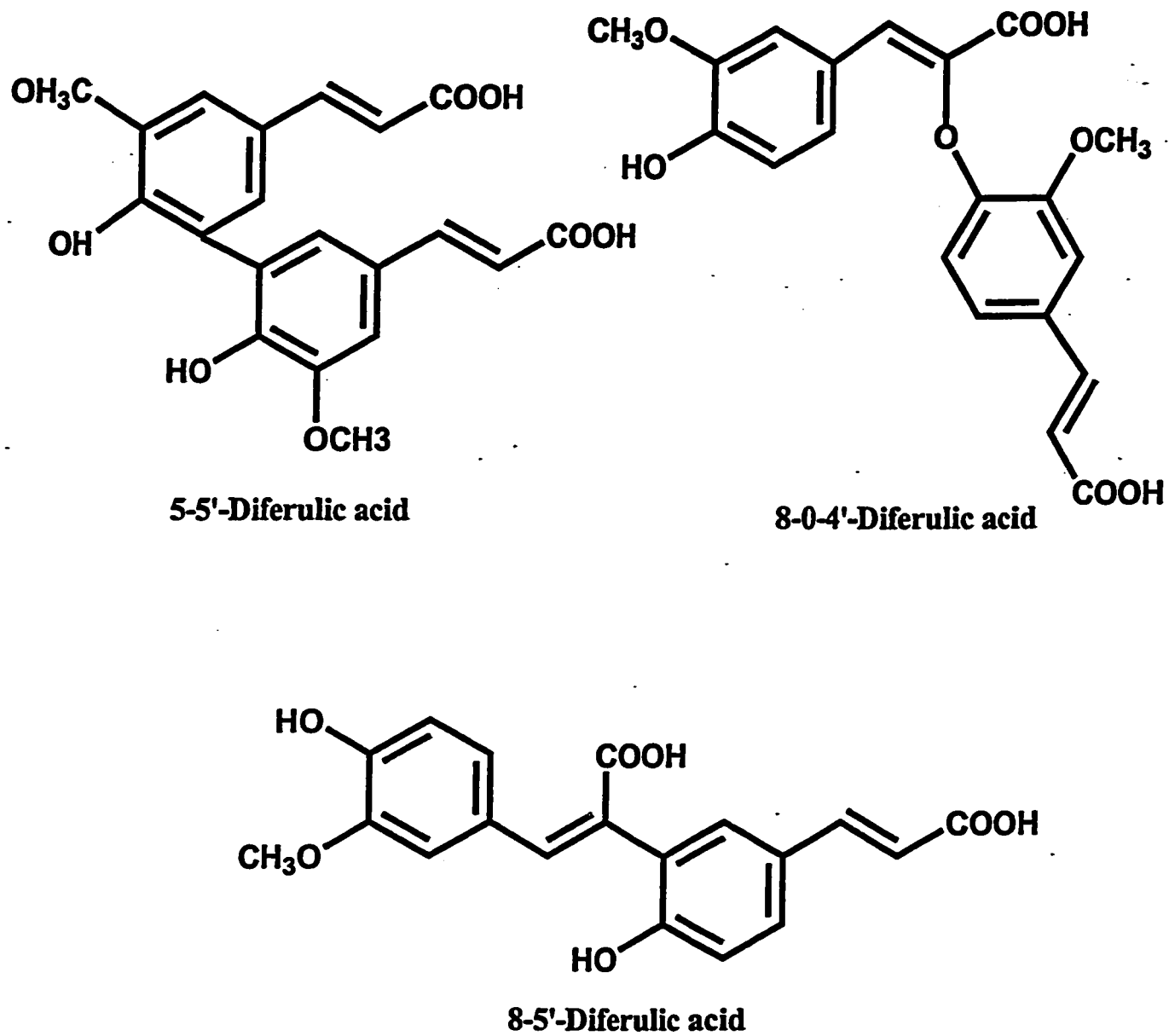


Fig. 1.3. Three main structural isomers of diferlic acid

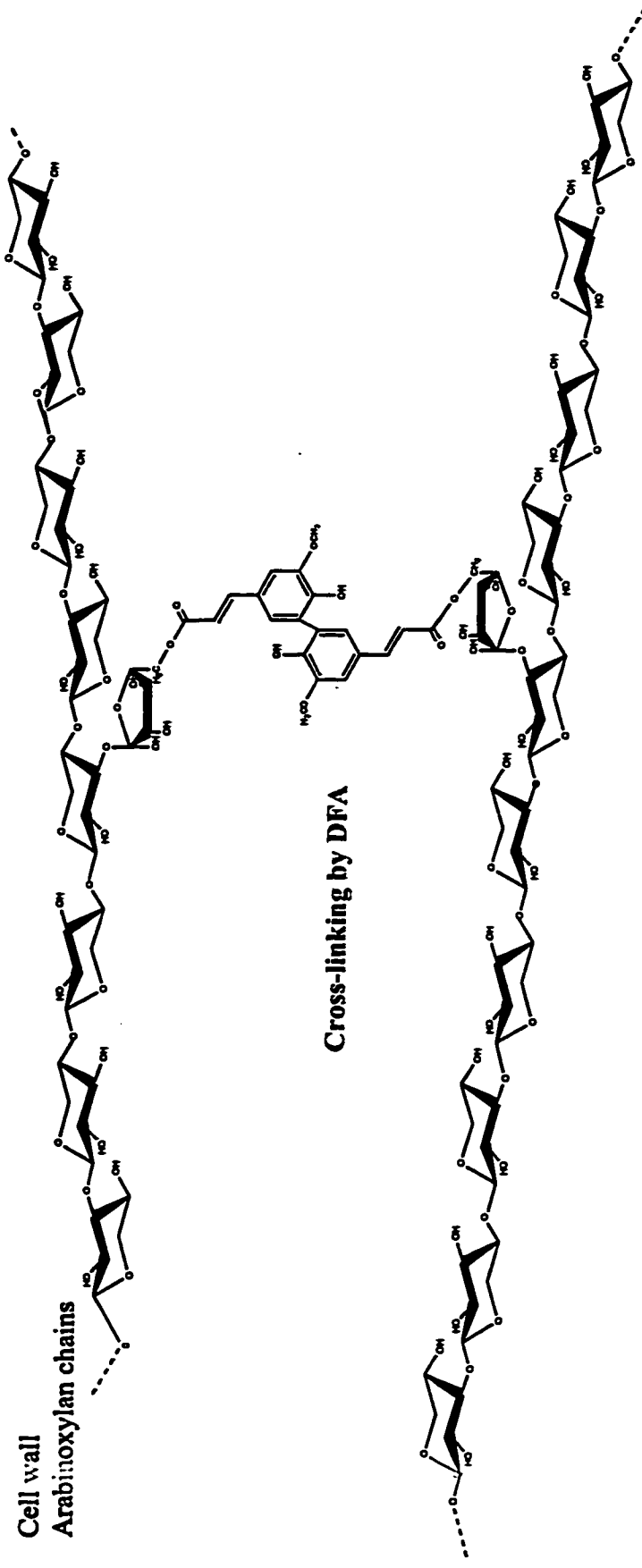


Fig. 1.4. Structure of a dimer of difenulic acid – carbohydrate complex (DFAXX).

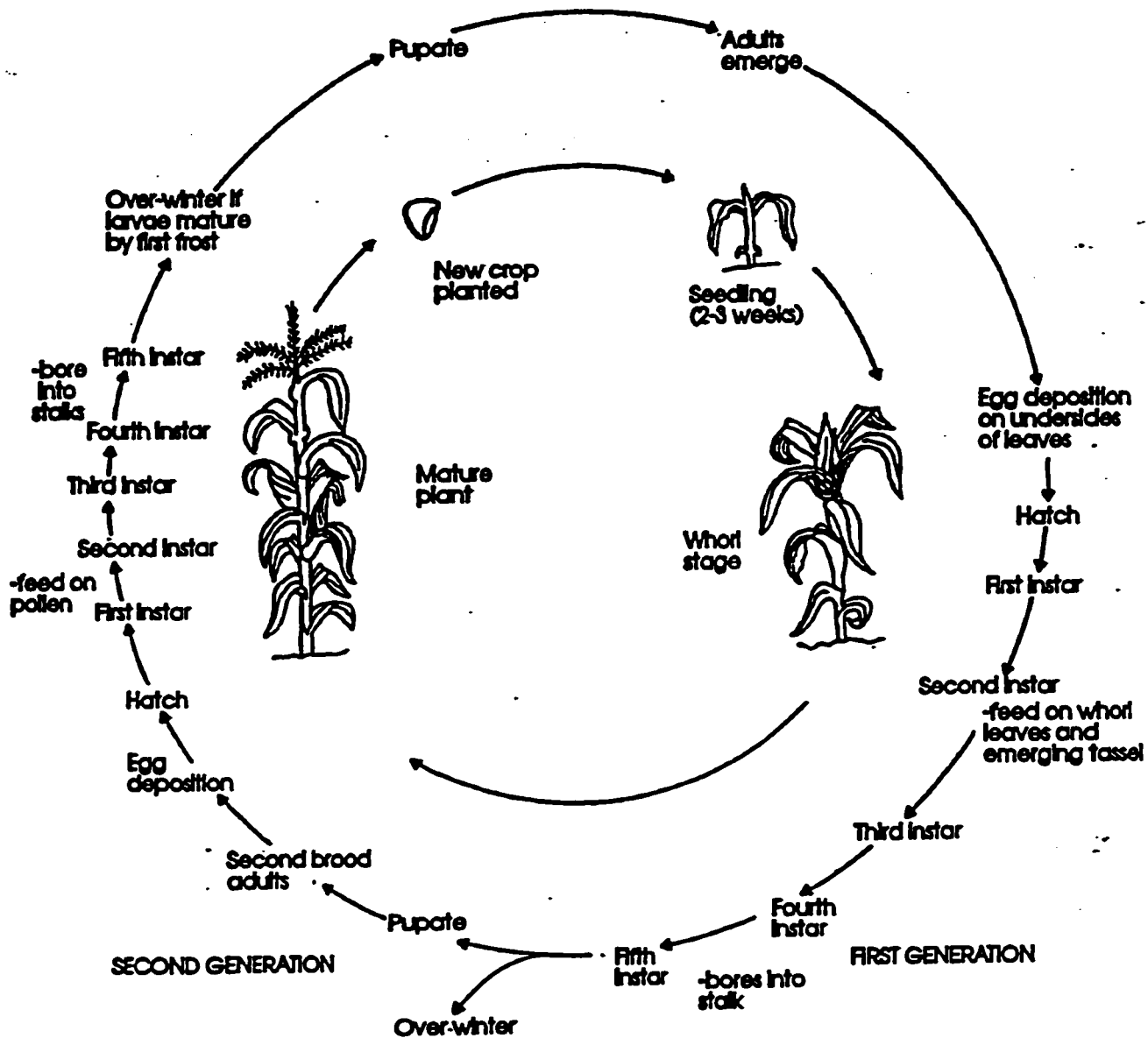


Fig. 1.5. The life cycle of the European corn borer (Reid 1989).

feed within the whorl. By the 3<sup>rd</sup> or 4<sup>th</sup> instar, larvae tunnel into the stalk where they develop and pupate. First generation adults emerge 7-10 days after pupation and congregate in grassy areas along field edges to mate and rest. Females lay second generation eggs mostly on the undersides of the ear leaf, the plant is now silking and shedding pollen. The second generation then passes through the same series of stages as the first generation feeding on sheath-collar tissue. Fifth instar larvae bore into the stalk to overwinter. Diapause is induced by a combination of decreasing day length, temperature and quality of food.

### **1.11 QTL Mapping**

Phenotypic variation such as insect resistance in plants is usually continuous instead of discrete and conditioned by allelic variation at several loci, each with a relatively small effect. Characters whose phenotypic variation is continuous and determined by the segregation of multiple loci are referred to as quantitative traits and the inheritance as polygenic. The mapping of quantitative trait loci (QTL) allows the detection and characterization of genetic factors contributing to the variation of a polygenically inherited trait (Groh et al., 1998). The idea of using single gene markers to systematically characterize and map individual polygenes controlling quantitative traits was first suggested by Thoday (1961). It was based on the idea that if the segregation of a single gene marker could be used to detect and estimate the effect of a linked polygene and if single gene markers were scattered throughout the genome, it should be possible to map and characterize all of the polygenes affecting a character. The lack of markers

made early mapping difficult. With the introduction of molecular markers like DNA-based restriction fragment length polymorphism (RFLP) (Botstein et al., 1980) the task became more feasible. Allelic variation for DNA-based markers usually has no detectable phenotypic effect, but the genetic variation is surveyed directly at the DNA level and thus can reveal the genetic polymorphism. Computer packages like MAPMAKER (Lander et al., 1987) have led to the construction of dense linkage maps. The subsequent identification of QTL has become routine. A large number of traits have been mapped in maize and other crops using RFLP markers (Lee, 1995).

Several biometrical methods have been developed for QTL detection. The recently introduced method of composite interval mapping (CIM) uses selected markers as cofactors in a multiple regression analysis (Zeng, 1994). It eliminates part of the genetic background noise and gives a better resolution of multiple linked QTL. The joint CIM as proposed by Jiang and Zeng (1995) uses phenotypic data of several environments or traits at a time and allows testing of the presence of QTL x environment (QTL x E) interactions and pleiotropy.

For insect resistance, QTLs have been mapped in populations of  $F_{2:3}$  lines in maize germplasm from the U.S. Cornbelt for resistance to the second generation ECB (Schön et al., 1993) and in tropical maize to the first generation SWCB and SCB (Khairallah et al., 1998; Bohn et al., 1997). Several QTLs have been found for corn borer resistance in tropical maize with mostly additive or partial dominant gene effects, confirming the assumptions of quantitative resistance with mainly additive gene action.

Most genetic studies employed populations of  $F_{2:3}$  lines or backcross progenies for the identification of QTL as the first step in marker-assisted selection (MAS).

Recombinant inbred lines (RIL) developed from  $F_2$  plants by single seed descent have been recommended as an alternative population type for QTL mapping (Burr et al., 1988). They are expected to have an increased power of QTL detection due to almost complete homozygosity at QTL and marker loci (Moreno-Gonzalez 1993). Furthermore, RILs allow a better resolution of linked QTL due to additional recombination during line development. RILs have been used for QTL mapping in maize for several traits, including yield and factors that affect yield (Austin and Lee, 1996). These authors found a greater number of QTLs for RIL than for  $F_{2:3}$  lines. Comparisons of QTL results from different generations are not only important for evaluating the efficiency of QTL mapping, but are also of great interest with respect to MAS. Only if QTL determined in early generations can largely be recovered in later generations, can they be used effectively for MAS during the process of line development.

As maps and markers progress from laboratory curiosities to practical and important tools, the appropriate use of markers should increase the precision and efficiency of plant breeding. Molecular marker technology and QTL mapping will expedite the acquisition of favourable genes from exotic populations or from wild species. These favourable genetic factors include insect resistance.

### **1.12 Transgenic Corn**

Genetically engineering inherent crop resistance to insect pests offers the potential of a user-friendly, environment-friendly and consumer-friendly method of crop protection to meet the demands of sustainable agriculture in the 21<sup>st</sup> century (Hilder and Boulter, 1999). Work to date has concentrated on the introduction of genes for expression of

modified *Bacillus thuringiensis* (Bt) toxins. This strategy has its limitations, the continuous exposure of insect pests to the Bt toxin can create a high selection pressure for the development of resistance against the toxin. Also, the Bt toxin could negatively affect non-target insects such as predators or parasites that feed on the pests. It is thus necessary to develop an optimum resistance management strategy with refuges (non-Bt plant) and high dose combination. An alternative to this approach has been the exploitation of plant-derived insect control genes. The challenge is to identify sources of resistance. Plants have evolved diverse mechanisms for protection against pathogens such as: fortification of cell walls, formation of antimicrobial phytoalexins, activation of defense related genes and localized cell death. Associated with these responses is the rapid and transient production of active oxygen species such as hydrogen peroxide.

### **1.13 Hypotheses and Objectives**

In a preliminary study with phenolics and insect resistance, it was hypothesized that there would be a negative correlation between soluble phenolic content in sorghum grain and feeding by *Sitophilus oryzae*. Subsequent studies focussed on the phytochemistry of maize resistance. The main hypothesis in these studies was that cell wall phenolic cross-linking, measured as total diferulic acid is negatively correlated to leaf feeding damage in maize to the SWCB and the SCB. Another hypothesis was that the QTLs for diferulic acid in the mapping population CML 131 X CML 67 would be closely associated with the QTLs for SWCB and SCB leaf feeding resistance in the same mapping population. The last hypothesis predicted that corn plants transformed with the wheat gene *germin* would have higher levels of oxalate oxidase activity, increased

hydrogen peroxide and increased DFA. This was predicted to translate into increased resistance to ECB feeding.

Objective 1. To determine the Soluble phenolic content of 8 sorghum landraces from Ethiopia and observe any correlations between soluble phenolic content and resistance to the rice weevil *Sitophilus oryzae*.

Objective 2. To determine any correlations between plant traits in the population CML131 X CML67 and leaf feeding damage by SWCB and SCB for 1996B and 1997A. The plant traits examined were cell wall bound phenolics, leaf toughness, leaf nitrogen, fiber and gravimetric soluble metabolites.

Objective 3. To obtain a QTL map of the putative phytochemical resistant traits in the mapping population.

Objective 4. To observe phytochemical and resistance changes in maize transformed with oxalate oxidase. Field evaluations of resistance in these transgenic plants was performed with the European corn borer. Changes in hydrogen peroxide and diferulic acid in the transformed lines were studied.

## CHAPTER 2

### **Soluble phenolic content as an indicator of sorghum grain resistance to the rice weevil, *Sitophilus oryzae***

*There is as much dignity in tilling the soil as in writing a poem.*  
Thomas Jefferson

#### **2.1 Introduction**

Post harvest cereal grain losses to insect pests in small-farm tropical agriculture can routinely lead to losses of more than 30% because of the lack of good storage facilities and the high humidity prevalent in the tropics. Although grain resistance has rarely been considered in conventional breeding programs as a selection criterion, in traditional tropical agriculture, farmers are acutely aware of the levels of resistance in various cultivars available to them and the need to develop management practices for grains of different storability. For example, Mayan farmers in Belize, Central America prefer resistant maize land races and CIMMYT synthetics for on-farm use and susceptible hybrids for quick sale (Fortier et al., 1982). Traditional knowledge of storability can be surprisingly accurate, perhaps because of its obvious importance to farmers without other means of protecting grain. We interviewed 300 farmers of the Welo district, Ethiopia and determined their mean consensus estimate of insect resistance for a group of 16 locally used sorghum varieties (Teshome et al., 1999). These varieties were then assessed for insect resistance in standardized laboratory tests with the granary weevil, *Sitophilus granarius*. The correlation between consensus estimates and laboratory estimates such as

grain consumption, number of eggs laid, and the Dobie index of susceptibility was high ( $r^2 = 0.85, 0.87$  and  $0.96, P < 0.05$ ).

If future varietal improvement for sustainable tropical agricultural systems is to include grain resistance as a possible criterion for some cultivars, mechanisms of resistance must be understood and simple selection criteria developed. Because of the need for controlled environmental conditions and the long duration of standardized insect testing procedures (2-3 months), more rapidly assessed indicators of resistance are required. In maize we have previously demonstrated the role of cell wall bound phenolics in resistance to *Sitophilus zeamais* (Arnason et al., 1992). In sorghum, the role of condensed tannins in resistance to birds and changes in digestibility is well known (Montgomery et al., 1986) but there is less information with respect to insects. In the present study, we examined the potential of determination of soluble phenolic content of grain as an indicator of resistance to the rice weevil, *Sitophilus oryzae*.

## **2.2 Materials and Methods**

### **2.2.1 Plant material**

Traditionally used landraces of sorghum (*Sorghum bicolor* L.) were collected in Ethiopia on farms in the Shewa and Wela districts in the 1993-1994 season at elevations between 1200-2400 m.

### **2.2.2 Insect resistance tests**

The method used is essentially the method of Dobie (1974). Grain was held at 25°C and 70% humidity for 7 weeks in a controlled environment chamber. Eight replicates of 25 g of grain were infested with 25 seven day old, unsexed weevils obtained from a laboratory culture and then returned to the chamber for one more week. The adult insects were then removed by sieving and four of the sieved replicates were stained with 20 ppm berberine chloride in water to visualize eggs laid. Oviposition counts were made using a magnifying glass and near UV illumination provided by a 20 W blacklamp to render the berberine stained eggs fluorescent. The four remaining unstained replicates were returned to the incubator and sieved for emergence every second day beginning three weeks after infestation until 7 weeks. The Dobie index (D.I.) of susceptibility was determined from the number of emerging insects and the time of their emergence:

$$\text{D.I.} = \ln (\text{progeny})/\text{median development period} \times 100\%$$

At the end of 7 weeks the grain was sieved to remove powder and the grain reweighed to determine weight loss from the original sample. The Farmers' index was measured as described in Teshome et al., (1999). Farmers rank the storability as short, medium, or

long, describing the harvested grain staying fresh for consumption and viable for planting for less than one growing season, less than two growing seasons, and for more than two growing seasons, respectively. Numerical values of 4, 2, and 1 were assigned accordingly for long, medium, and short duration of storability. The Farmers' Index (FI) for storability was determined using the formula:

$$FI = 4(L) + 2(M) + 1(S) / L + M + S$$

L, M, and S are the number of farmers who responded that the stored landrace has long, medium, or short duration, respectively.

### **2.2.3 Phenolic Tests**

The Prussian blue method of Price and Butler (1977) was used to quantify phenolics. Sorghum grain ground in a Wiley mill (60 mg, 60  $\mu$ m particle size) was vortexed for one minute with 3 mL of MeOH and filtered through a Whatman no. 1 filter paper using suction. The residue was washed with an additional 3 mL of MeOH and the combined filtrates were mixed with 50 mL of water. Three mL of 0.1 M FeCl<sub>3</sub> in 0.1 N HCl was added to 3 mL of 0.008 M K<sub>3</sub>Fe(CN)<sub>6</sub> to give a single colour reagent. Six mL of this solution was added in timed additions to the 50 mL of water. The absorbance was read at 720 nm after exactly 10 min in a 1 cm glass cuvette using a Beckman DU640 Spectrophotometer which was zeroed with water. A straight line calibration curve was obtained daily from standard solutions of D-catechin (0.02-0.40 mg/ml). The absorbance of a blank without catechin was subtracted from all readings. The results were expressed as catechin equivalents. All grain samples were analysed in four separately prepared replicates.

#### **2.2.4 Statistical analysis**

Statistical analysis of the data was undertaken using the statistical analysis system SAS (5.0.1) or SYSTAT for ANOVA's and multiple range tests. The Multiple linear regressions were determined with Cricket Graph III (1.0) software for MacIntosh computers.

## 2.3 Results and Discussion

The mean soluble phenolic content determined by the Prussian blue method of eight landraces of sorghum grain collected in the Welo and Shewa districts in Ethiopia showed significant variation in a one way ANOVA ( $F = 9.71$ ,  $df = 7$ ). There was an approximately 3-fold variation in the mean level of soluble phenolics measured as catechin equivalents between the highest and lowest genotypes (Table 2.1). The method was found to be sensitive (requiring little material), rapid, reliable and reproducible. We found the second method assessed, the vanillin-HCl method (Burns, 1963), was not easily reproducible as did Price and Butler (1977). The soluble phenolics that have been identified in sorghum grain include four proanthocyanidins having the basic formula epicatechin  $-(epicatechin)_n-$  catechin, glycosylated dimeric and trimeric flavonoids with eriodictyol and taxifolin units (Gujer et al., 1986).

The variation in several *Sitophilus oryzae* development parameters on the eight landraces collected showed significant variation by genotype ( $p < 0.05$ ). Genotypes used varied from highly susceptible in both lab and on-farm assessments to resistant with a broad coverage of intermediate susceptibilities (Table 2.2).  $F_1$  emergence from the grain showed the greatest variation of almost 40-fold while the oviposition and Dobie index of susceptibility varied by five fold or less. The development time of the progeny showed the least variation (1.3 x). These variations in insect performance led to considerable differences in grain damage as measured by weight loss in the tests, which varied from 0 to 3.95 g. On-farm interviews showed that these laboratory assessments agreed well with the mean Farmers' index of Ethiopian farmers (Teshome et al., 1999). In comparison to

**Table 2.1 Soluble phenolic content in Ethiopian landraces as catechin equivalents by the Prussian blue method.**

<b>Landrace</b>	<b>Mean <math>\pm</math> SE (mg/g)</b>
<b>Tuba</b>	<b>0.541 <math>\pm</math> 0.05 a</b>
<b>Abula Gorad</b>	<b>0.527 <math>\pm</math> 0.03 a</b>
<b>Key Jamuye</b>	<b>0.422 <math>\pm</math> 0.04 ab</b>
<b>Nech Jamuye</b>	<b>0.256 <math>\pm</math> 0.03 ab</b>
<b>Tenglaye</b>	<b>0.357 <math>\pm</math> 0.08 ab</b>
<b>Enat Gorad</b>	<b>0.197 <math>\pm</math> 0.04 b</b>
<b>Wogere</b>	<b>0.189 <math>\pm</math> 0.03 b</b>
<b>Merabete</b>	<b>0.233 <math>\pm</math> 0.04 b</b>

**Means followed by the same letter are not significantly different  
In Tukey's test (P<0.05, n=8)**

Table 2.2. Mean insect resistance parameters for landraces tested.

Landrace	Dobie Index	F1 emergence $\pm$ SE (insects/replicate)	Oviposition $\pm$ SE (eggs/replicate)	Weight loss (g/replicate)	Development time $\pm$ SE (days)
Tuba	3.30	1.00 $\pm$ 0.41	29 $\pm$ 1	0 $\pm$ 0	42.0 $\pm$ 2
Abula Gorad	7.30	4.38 $\pm$ 0.36	36 $\pm$ 4	0.21 $\pm$ .01	39.4 $\pm$ 4
Key Jamuye	7.42	5.81 $\pm$ 0.07	40 $\pm$ 4	1.52 $\pm$ .04	42.4 $\pm$ 2
Nech Jamuye	7.81	7.31 $\pm$ 1.33	48 $\pm$ 7	2.00 $\pm$ .07	43.0 $\pm$ 0.6
Tenglaye	8.84	12.0 $\pm$ 1.35	58 $\pm$ 2	2.21 $\pm$ .06	43.8 $\pm$ 2
Enat Gorad	10.1	17.1 $\pm$ 1.26	72 $\pm$ 4	2.79 $\pm$ .02	42.0 $\pm$ 1
Wogere	10.6	23.3 $\pm$ 1.94	98 $\pm$ 10	3.30 $\pm$ .03	43.0 $\pm$ 2
Merabete	15.3	39.5 $\pm$ 1.19	160 $\pm$ 11	3.95 $\pm$ .01	33.0 $\pm$ 0.4

landraces of maize that we have previously assessed for resistance to *Sitophilus* species these sorghum land races showed comparably large variation (Arnason et al., 1992).

The linear relationship between grain damage as assessed by weight loss and phenolic content of the genotypes were significant and inverse (Fig 2.1). Variation in phenolic content explained 85% of the genotypic variation in weight loss in the standardized tests with good coverage of the range between the extremes. Three of the insect development parameters also showed significant ( $P < 0.05$ ) inverse relationships with phenolic content (Figs 2.2-2.4). Variation in phenolic content explained 55% of the variation in the Dobie Index (Fig 2.2), 52 % of genotype variation in emergence (Fig 2.3) and 46% of variation in number of eggs laid (Fig 2.4). There was no significant relationship with development time (Table 2.2). The mean Farmers' index of the storability of the genotype also had a significant positive relationship with the phenolic content (Fig 2.5).

The significant relationship between grain damage (or insect development parameters or Farmers' index) and soluble phenolic content may be a cause and effect relationship. Phenolics are well known to be directly involved in insect resistance in many plants by antixenosis and antibiosis mechanisms. Sorghum phenolics may be involved in antibiosis to *Sitophilus oryzae* because of the negative relationship with  $F_1$  emergence, as well as in antixenosis because of the oviposition relationship. These mechanistic relationships require further investigation by feeding trials with purified sorghum proanthocyanidins, which are not available to us at this time. More precise relationships between individual sorghum proanthocyanidins and resistance should also be defined by HPLC. It would also be interesting to know whether farmers use any taste

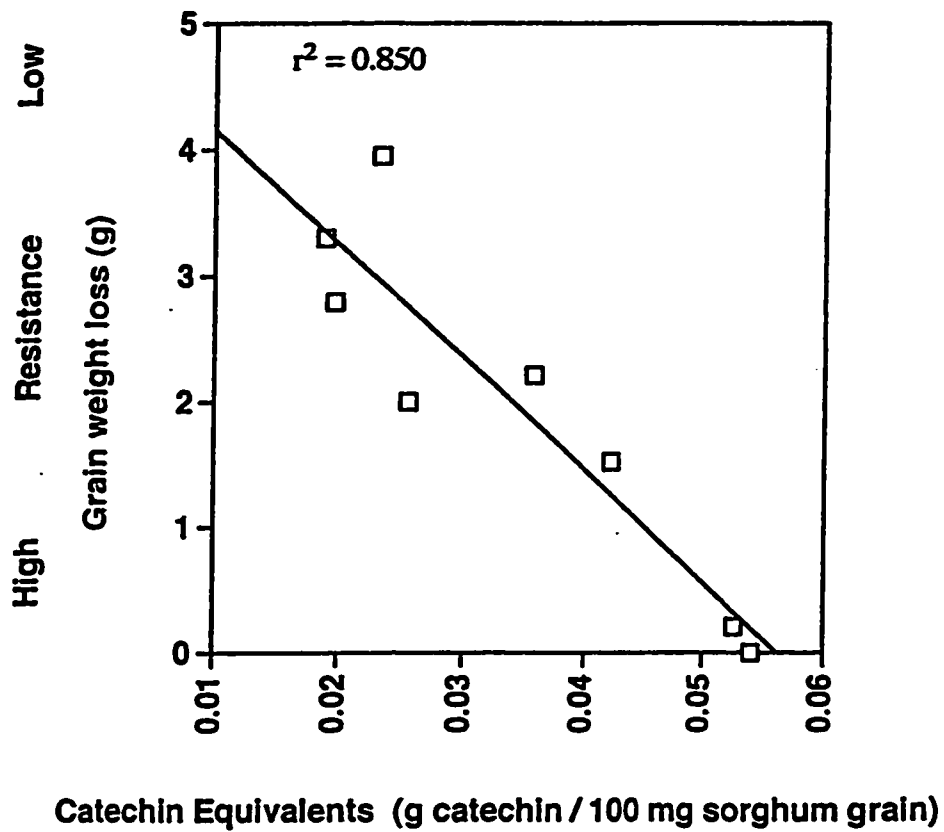


Figure 2.1. Mean consumption of grain (measured as grain weight loss) of eight sorghum genotypes exposed to the rice weevil, *Sitophilus oryzae* in standardized tests versus mean soluble phenolic content of grain ( $P < 0.05$ ,  $n = 8$ ).

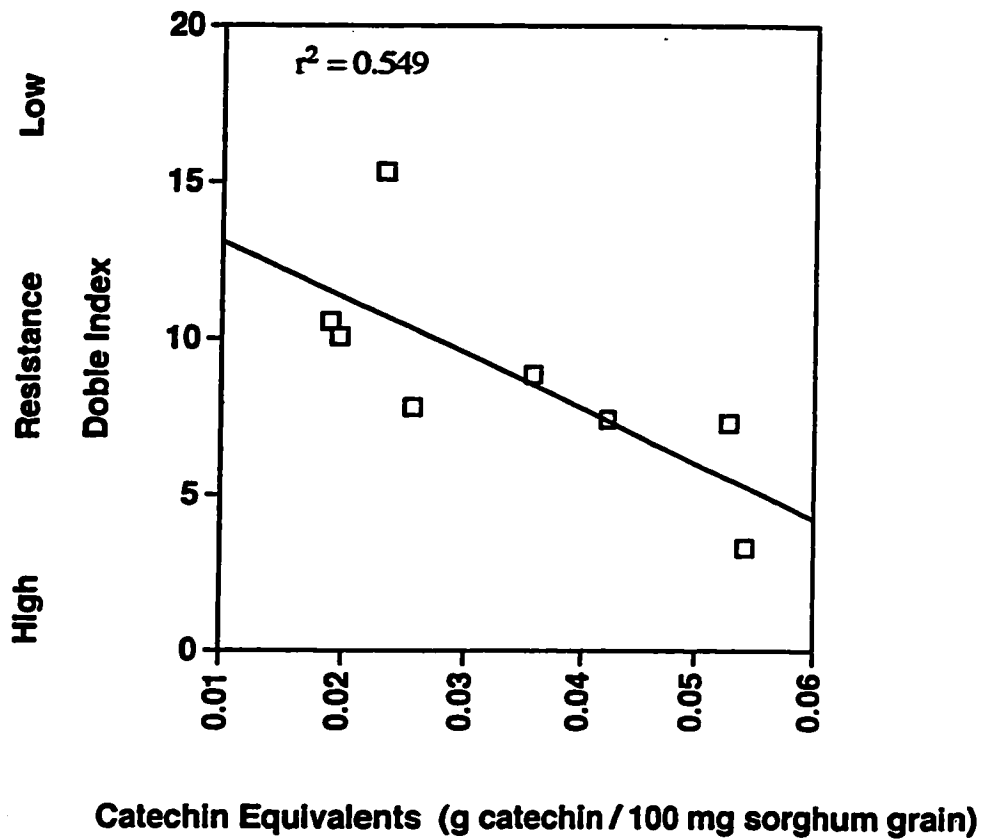


Figure 2.2. Mean Dobie index of susceptibility versus mean soluble phenolic content of grain ( $P < 0.05$ ,  $n = 8$ ).

and colour criteria in their assessment of grain resistance. Although similar inverse relationships between grain damage or insect development parameters and phenolic content in maize grain have been demonstrated, the phytochemical defences in sorghum and maize are different. In maize grain, the phenolics are largely cell wall bound phenolics that are released experimentally by base hydrolysis. They provide mainly mechanical resistance to *Sitophilus* spp. by the peroxidase-mediated production of diferulic acid cross-links in the arabinoxylan portion of the cell wall (Arnason et al., 1992). Cell wall bound phenolics in sorghum were not extracted in the present study.

Mechanistic considerations aside, the relationships demonstrated in the present study show that a simple phenolic test provides a rapid, reliable and easily determined prediction of the level of resistance to *Sitophilus oryzae* in sorghum grain. This methodology is suitable for rapid screening of germplasm in breeding programs for the purpose of reducing the number of accessions that will be submitted for a final insect screening procedure for verification of resistance.

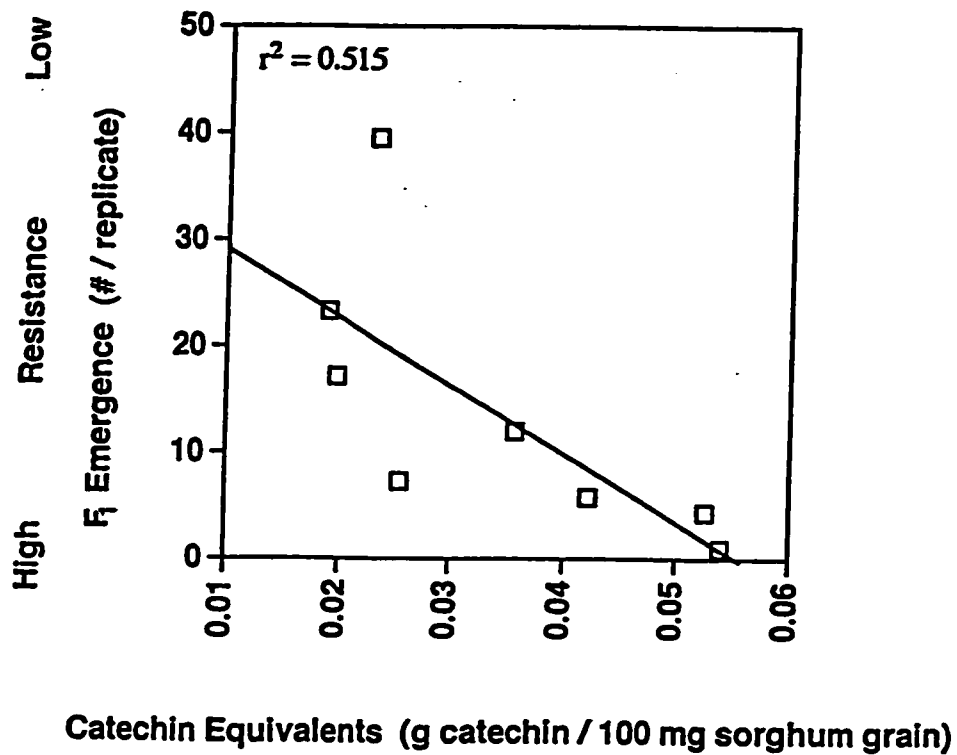


Figure 2.3. Mean F<sub>1</sub> progeny of rice weevil emerged from eight sorghum genotypes in standardized tests versus mean soluble phenolic content of grain ( $P < 0.05$ ,  $n = 8$ ).

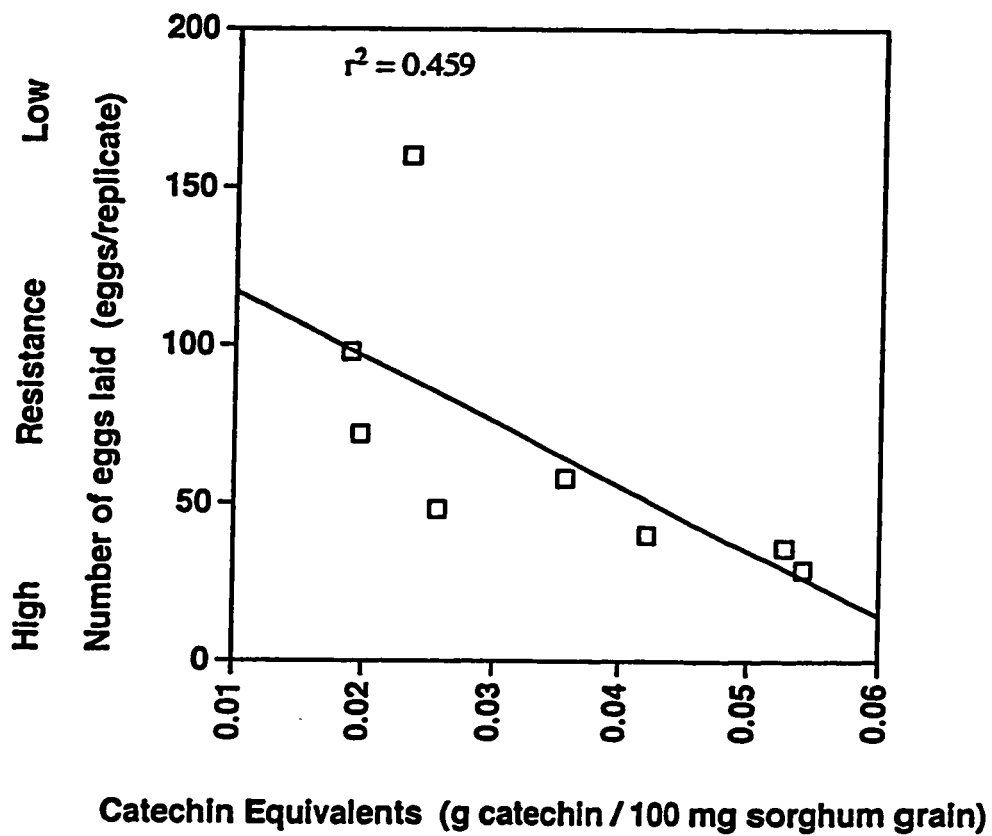


Figure 2.4. Mean number of rice weevil eggs laid on eight sorghum genotypes in standardized tests versus mean soluble phenolic content of grain ( $P < 0.05$ ,  $n = 8$ ).

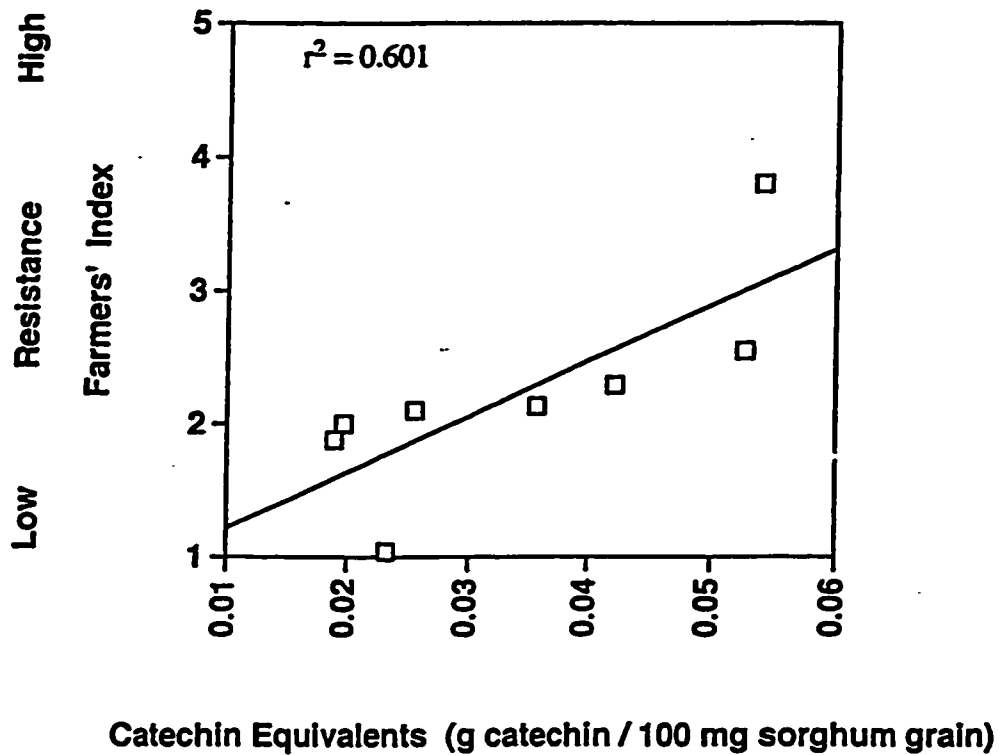


Figure 2.5. The farmers' consensus index (1 short duration, less than one growing season - 4 long duration, more than two growing seasons) on the storability of sorghum grain versus mean soluble phenolic content of grain ( $P < 0.05$ ,  $n = 8$ ).

## Chapter 3

### Correlations between quantitative phytochemical traits and resistance in maize to tropical and sub-tropical stem borers

*Only maize dough went into the meat of our first ancestors.  
The Popol Vuh, the Mayan tale of the creation and origin of man.*

#### 3.1 Introduction

The southwestern corn borer (SWCB), *Diatraea grandiosella* Dyar, and the sugarcane borer (SCB), *Diatraea saccharalis* Fabricius (Lepidoptera: Pyralidae) are important insect pests in tropical and subtropical areas of Central and South America. On many small-scale, resource-limited farms in these regions, low yield stability is partly due to the highly variable damage caused by the SWCB and the SCB. Yield losses measured in field studies under artificial infestation with larvae of both insect species ranged from 10 to 30%, reaching up to 70% in severe cases (Thome et al., 1992). Plants are damaged directly by feeding on various plant parts and indirectly by lodging and secondary pests that take advantage of entrance holes, such as *Fusarium moniliforme* and *Fusarium graminearum* (Dicke, 1975).

Host plant resistance to the SWCB and the SCB is a desired means of control, especially in developing countries, where insecticides are often not available or improperly applied to achieve maximum control. To achieve this goal, breeding for multiple borer resistance has been a major research objective at the International Maize

and Wheat Improvement Center (CIMMYT, Mexico) for 20 years. Mechanisms involved in resistance to insects include non-preference (antixenosis), antibiosis and tolerance (Painter, 1951). Agronomic research and maize breeding has largely focused on the antibiosis type of resistance to improve the levels of protection against SWCB and SCB feeding.

Based on the previous work of Bergvinson et al. (1994a), with temperate zone insect pest, the European corn borer, the leaf nitrogen concentration and leaf toughness were examined as putative components of resistance to tropical borers. These two traits were closely associated with leaf feeding resistance to the European corn borer in CIMMYT's MBR germplasm. Bergvinson et al. (1995) also found higher levels of crude fiber and cell wall bound phenolic acids in resistant maize genotypes. The mechanism of resistance proposed suggest that early cell wall fortification through phenolic cross linking of hemicellulose by diferulic acid increases the leaf tissue toughness. This increase in tissue toughness decreases neonate larvae penetration of leaf tissue. In addition, Bergvinson et al., (1997) demonstrated an increase in diferulic acid during recurrent selection for resistance to the European corn borer.

This present study extends the work of Bergvinson et al. (1994a) with the hypothesis that resistance to the important tropical and subtropical stem borers are sensitive to early cell wall fortification by phenolic cross-linking. As well, a second hypothesis was that low leaf nitrogen is critical for resistance to tropical borers. Insects need nitrogen to complete their life cycles and limiting the availability of nitrogen in the leaf would delay insect development. A SPAD meter was used as a quick field screening method for nitrogen determination. Once the leaf tissue was harvested and lyophilized,

an analytical nitrogen content determination was performed using the automatic micro-Kjeldahl.

### 3.1.1 Rationale for DFA analysis

Research on temperate maize resistance to the European corn borer (*Ostrinia nubilalis* Hübner) has largely focused on the hydroxamic acid DIMBOA [2,4-dihydroxy-7-methoxy 2H-1,4-benzoxazin-3-(4H)-one]. DIMBOA is considered the main resistance factor during leaf whorl tissue development to first generation ECB (Klun et al., 1970). However, other resistance factors appear to operate in exotic accessions (Sullivan et al., 1974). The toxic hydroxamic acids found in maize do not explain many situations where host plant resistance is observed (Scriber et al., 1975). DIMBOA may be effective against the Western corn rootworm (*Diabrotica virgifera virgifera* LeConte). Several studies have suggested that those maize inbreds that are high in DIMBOA exhibited increased resistance to the WCR (Xie et al., 1992; Assabgui et al., 1995). Yet, the prevailing notion regarding this classic resistance factor is that it is ineffective against the tropical borers (Scriber et al., 1975; Scriber and Slansky, 1981). It is within this context that the quantitative or structural defenses in maize may be employed against the tropical and subtropical borers.

Recent research has shown that cell wall bound phenolic acids can strengthen the cell wall through a peroxidase mediated dimerization that cross-links adjacent arabinoxylan molecules in the hemicellulose microfibrils with diferulic acid (Bergvinson et al., 1994a). The increase in DFA leads to an increase in the mechanical strength of the leaf tissue, which restricts access of neonate larvae of stem borers to nutrients and results

in larval mortality through desiccation. Other associated resistance factors include foliar nitrogen content, leaf toughness and fiber content. These components are based on leaf nutrition and suggest that increased resistance involves lower protein content, increased tissue toughness and higher fiber. This mechanism has now been verified with the ECB (Bergvinson et al., 1994a; Bergvinson et al., 1995) and can now be extended to the important stalk borers of the tropics and the subtropics, the SWCB and SCB.

## **3.2 Materials and Methods**

### **3.2.1 Plant Material**

Two homozygous inbred lines, CML 131 and CML 67, were used as parents (Table 3.1). CML 67 is a tropical, late maturity, yellow semi-dent line selected out of the Antigua group, a source of insect resistant germplasm. It has been selected to be highly resistant to leaf feeding by the SWCB and SCB. CML 131 is a subtropical, intermediate maturity white dent line selected out of CIMMYT Population 42. It is highly susceptible to leaf feeding by SWCB and SCB (Mihm et al., 1991). During the 1995 summer season, F<sub>2</sub> plant derived from two randomly chosen F<sub>1</sub> plants from the cross CML 131 X CML 67 were grown in Tlaltizapan, State of Morelos, Mexico. These F<sub>2</sub> plants were then selfed to produce 215 F<sub>3</sub> lines. These lines were subsequently selfed to produce 152 recombinant inbred lines (RIL) as described by Groh et al., (1998).

### **3.2.2 Field Trial**

Experiments with artificial infestations of SWCB larvae and SCB larvae were conducted for the population CML 131 X CML 67 at the CIMMYT experimental station in Tlaltizapan, Mexico (subtropical environment, 940 m elevation, 18° N lat). Trials were conducted during the summer season (June to October, wet season) of 1996 and the winter season (November to May, dry season) of 1997. Each experiment included 166 RILs (Recombinant inbred lines) and 6 parental inbred lines. Inbred parents were included (CML 67 and CML 131) to fill the 10 X 17 lattice design. The single row plots were 2.5 m long and 0.75 m apart. The trials were over planted and thinned to 10 plants

per row, corresponding to 60,000 plants per ha. Plots consisted of rows with 10 plants under infestation and an adjacent 10 plants with no infestation. This allowed for comparisons between infested and control plants, and provided leaf material from the control plants for inston measurements and phytochemical analysis. Thus, leaf ratings were conducted for the first 10 plants and leaf toughness and phenolic profiles for the second 10 plants for each of the 166 lines.

For evaluating the level of resistance (in terms of antibiosis) against the SWCB and SCB, plants were artificially infested with 30 to 45 neonate SWCB or SCB larvae at the six- to eight-leaf stage (mid-whorl development) three weeks after planting. This was conducted by mixing freshly hatched larvae with maize-cob grits and applying the mixture into the plant whorl using a mechanical dispenser, commonly called the bazooka (Mihm, 1983). Larvae were reared in CIMMYT's entomology laboratory. Leaf feeding damage (Table 3.2) was assessed two to three weeks after infestation using a rating scale from 1 (no visible leaf damage) to 9 (most leaves with long lesions) (Mihm 1989).

### **3.2.3 Leaf toughness**

The instron (force displacement meter) used was commercially available from Tricor Systems Inc., NJ (Model TM-M). The machine was calibrated and stationed in Tlaltizapan, Mexico. The leaf was placed on the test station with the lower epidermis facing upwards and is the first tissue the probe comes in contact. The probe itself is 1 mm in diameter with a rounded blunt tip (Bergvinson et al., 1994a). The lower (abaxial) epidermis was chosen for two reasons. First, it provided more consistent force measurements upon repetition and secondly, the borers feed from the outside inwards to

the center of the whorl, thus it more closely mimics insect feeding. The probe was placed between secondary and primary leaf veins to measure only the epidermal cell wall toughness.

Leaf toughness measurements were taken before tasseling from the second fully exposed leaf from the top of the plant. Leaves were harvested and immediately placed into ice water to prevent the loss of turgor. Toughness measurements were recorded within 12h of harvest. Two sections per leaf were sampled, the point of penetration for the inston probe was in the yellow-green transition area midway between the midrib and the edge of the leaf with no secondary leaf veins. Using the standardized inston technique, the peak force in grams required to puncture the lower epidermis of the leaves was determined.

#### **3.2.4 Leaf greenness**

Leaf greenness measurements were conducted with the SPAD-502 Chlorophyll Meter (Minolta Corp., Ramsey, NJ). The rationale behind utilizing this measurement is based on the strong correlations between SPAD readings and leaf nitrogen content. Agronomists extensively use this technology for monitoring leaf greenness to signal the approach of a potential nitrogen deficiency. Chlorophyll absorbs red light during photosynthesis, and the meter operation is based on the relationship between the amount of red light absorbed by chlorophyll and the amount transmitted through the leaf. The more red light absorbed indicates more chlorophyll present and a greener leaf.

### **3.2.5 Leaf nitrogen concentration**

Leaf nitrogen concentration was analysed in the recombinant inbred lines (RILs) of Population CML 131 X CML 67 from leaf tissue collected five weeks after planting. Two leaf sections from the middle part of the third fully expanded leaf from the top of each control plant were sampled, dried at 65°C for three days and ground into a fine powder. An automatic micro-Kjeldahl analyzer (Agriculture Canada, Ottawa) was used for nitrogen determination (Bremner, 1996).

### **3.2.6 Phenolic Quantification**

Cell wall bound phenolics were extracted from 0.5 g sample of lyophilized leaf tissue harvested as described above. Samples were extracted in 4 X 20 mL of 70 % methanol and mixed with a polytron mixer at high speed for 20 sec. After centrifugation the supernatants were discarded, the pellet that remained was washed in a Büchner funnel with 30 mL of water, 30 mL of methanol and 30 mL of ethyl acetate to remove residual chlorophyll and provide a crude cell wall preparation. Cell wall samples were dried in a desiccator for four days. This preparation was weighed and the weight loss was used as the gravimetric measure of soluble metabolites. Cell wall preparations were shaken in 20 mL of 2N NaOH for 4 h under N<sub>2</sub> and the beakers were wrapped in foil to hydrolyse phenolic ester linked to hemicellulose. Nitrogen was required to prevent oxidation of phenolics and a foil wrapping was required to minimize photoisomerization of phenolic acids. Samples were neutralized with 6 N HCl and the pH lowered to 2.0. After centrifugation the supernatant was extracted with 3 X 50 mL ethyl acetate. The pellet was resuspended in 10 mL water and centrifuged twice more with both supernatants

pooled and extracted with 3 X 50 mL ethyl acetate. The six ethyl acetate fractions were pooled and dried by rotary evaporator under darkness and stored at -20°C until HPLC analysis. The pellet that remained after extraction was dried and weighed to provide an estimate of the fiber content.

### 3.2.7 HPLC analysis

All analyses were performed with a Beckman HPLC system consisting of an Autosampler, Module 502, with 5 µL injection loop, a Solvent delivery system, Module 126, a Photo diode array detector, Module 168 and System Gold software, version 8.10. Separations were achieved using a C18 ODS reverse phase column (250 x 4.6 mm, 5 µm particle size, Beckman, Fullerton, CA) with a 5 µm LiChrospher RP-18, 4 x 4.6 mm guard cartridge (Merck/BDH, Toronto, ON). The solvent system consisted of methanol (A) and 10 mM H<sub>3</sub>PO<sub>4</sub>, pH 2.4 (B) at a flow rate of 1.5 mL/min. The gradient was as follows: 25 to 55 % A in 15 min, 55 to 80 % A in 5 min, 80 to 100 % A in 2 min, hold at 100 % A for 8 min, 100 to 25 % A in 2 min and 25 % A for 3 min. A detection wavelength of 325 nm was used. Cell wall bound phenolic acids were suspended in 10 mL methanol, 1.5 mL samples were filtered using a 0.22 µm PTFE membrane (Chromatographic Specialties, Brockville, ON) and injected onto the column. P-coumaric (Rt = 15.2 min) and ferulic acid (Rt = 15.6) were purchased from Sigma Chem. Co. and 5-5 DFA (Rt = 16.5 min) was prepared in the lab of G. H. Neil Towers (University of British Columbia) (Bergvinson et al., 1995). Peak identity of each phenolic was confirmed by on-line UV spectra and spiking of extracts with authentic standards.

**Table 3.1. Description of the parental inbred lines used for developing the Population CML 131 X CML 67.**

<b>Inbred</b>	<b>Texture</b>	<b>Colour</b>	<b>Adaptation</b>	<b>Maturity</b>	<b>Comments</b>
<b>CML 67</b>	<b>Semident</b>	<b>Yellow</b>	<b>tropical</b>	<b>late</b>	<b>Resistant to SWCB, SCB</b>
<b>CML 131</b>	<b>Dent</b>	<b>White</b>	<b>subtropical</b>	<b>intermediate</b>	<b>Susceptible to SWCB, SCB</b>

**Table 3.2. Rating scale applied for evaluating leaf damage caused by SWCB and SCB larvae feeding (Guthrie et al., 1960; Thome et al., 1992)**

<b>Rating scale</b>	<b>Level of resistance</b>	<b>Description</b>
1	Highly resistant	No damage or few pin holes
2	Resistant	Few shot holes on leaves
3		Shot holes on several leaves
4	Intermediately resistant	Several leaves with shot holes and few elongated lesions
5		Several leaves with elongated lesions
6		Several leaves with lesions $\geq 2.5$ cm
7	Susceptible	Long lesions on half of the leaves
8		Long lesions on $\frac{1}{2}$ to $\frac{1}{3}$ of the leaves
9		Most leaves with long lesions

### **3.3 Results and Discussion**

The insect ratings and phytochemical resistance factors for the two parental lines, CML 131 (susceptible) and CML 67 (resistant) are presented in Table 3.3. There was a significant difference (two sample T-test) between the two lines in leaf field ratings, GSM, ferulic acid, all forms of diferulic acid, and leaf toughness. In order to map these traits there must be a minimum 20% separation in values between parental lines (Chapter 4 of the thesis).

The mean and the range of leaf parameters associated with insect resistance in the mapping population CML131 X CML 67 are shown in Table 3.4 (1996B) and Table 3.5 (1997A). Temperatures, solar radiation and rainfall were lower during the winter season (1997A) than during the summer season (1996B). The two seasons were comparable with slightly lower infestations in the winter season. Leaf feeding damage after artificial infestation ranged from a low of 2 (resistant) to 8 (susceptible). Leaf feeding damage was higher for the SWCB, which is a more voracious insect compared to the SCB. The gravimetric soluble metabolites (GSM) washed from milled leaf tissue included sugars, soluble proteins, chlorophyll, phenolic conjugates such as flavonoids and hydroxycinnamic acids, and hydroxamides such as DIMBOA (Bergvinson et al., 1995). Both GSM and leaf fiber content were investigated as resistance factors and showed comparable levels in the two seasons. Five cell wall bound phenolic acids were quantified, p-coumaric acid, ferulic acid, and the three major structural isomers of diferulic acid (5-5', 8-5', and 8-0-4'). The DFA dimer levels were lower in the winter season. Leaf nitrogen, thickness, greenness (SPAD), and force were all comparable between the two seasons.

**Table 3.3. Characterization of phytochemical resistance factors in the parental lines CML 131 (susceptible) and CML 67 (resistant), Tlaltizapan, Mexico, 1996B, n=12.**

	CML 131	CML 67	T-test, P value
Rating SCB	9.5±1.0	4.0±0.5	0.002
Rating SWCB	9.7±1.2	4.3±0.6	0.004
GSM (mg/gDW)	249±4	334±5	0.04
Fiber (mg/gDW)	281±4	274±4	0.24
p-CA (µg/gDW)	952±78	1134±22	0.09
FA (µg/gDW)	2809±107	3456±104	0.02
5-5' DFA (µg/gDW)	24.4±2	37.7±2	0.04
8-5' DFA (µg/gDW)	89.3±3	122.9±6	0.02
8-0-4' DFA (µg/gDW)	92.5±3	123.5±4	0.01
Total DFA (µg/gDW)	206.2±5	284.1±11	0.008
Nitrogen (%)	2.09±0.03	1.94±0.12	0.22
Leaf thickness (µm)	216±5	221±6	0.94
SPAD	34.5±2	44.5±0.2	0.16
Force (g)	82.3±4	115.7±5	0.01

SCB (sugarcane borer), SWCB (southwestern corn borer), GSM (gravimetric soluble metabolite), p-CA (p-coumaric acid), FA (ferulic acid), DFA (diferulic acid), SPAD (leaf greenness).

**Table 3.4. Characterization of phytochemical resistance factors in the population CML 131 (susceptible) X CML 67 (resistant), Tlaltizapan, Mexico, 1996B, n=166.**

	Mean±SE	Range
Rating SCB	4.96±0.05	3.1-6.7
Rating SWCB	6.34±0.04	4.8-7.7
GSM (mg/gDW)	301±3	243-570
Fiber (mg/gDW)	335±3	240-433
p-CA (µg/gDW)	1443±21	818-2303
FA (µg/gDW)	3547±42	2369-5092
5-5' DFA (µg/gDW)	34.4±0.87	11.4-66.4
8-5' DFA (µg/gDW)	99.5±1.64	16.9-156.4
8-0-4' DFA (µg/gDW)	119.6±1.87	70.6-191.3
Total DFA (µg/gDW)	253.5±4.07	132.5-403.8
Nitrogen (%)	2.05±0.017	1.4-2.6
Leaf thickness (µm)	217.7±0.93	182.9-246.3
SPAD	38.6±0.41	28.7-48.7
Force (g)	91.4±0.63	67.6-114.1

SCB (sugarcane borer), SWCB (southwestern corn borer), GSM (gravimetric soluble metabolite), p-CA (p-coumaric acid), FA (ferulic acid), DFA (diferulic acid), SPAD (leaf greenness).

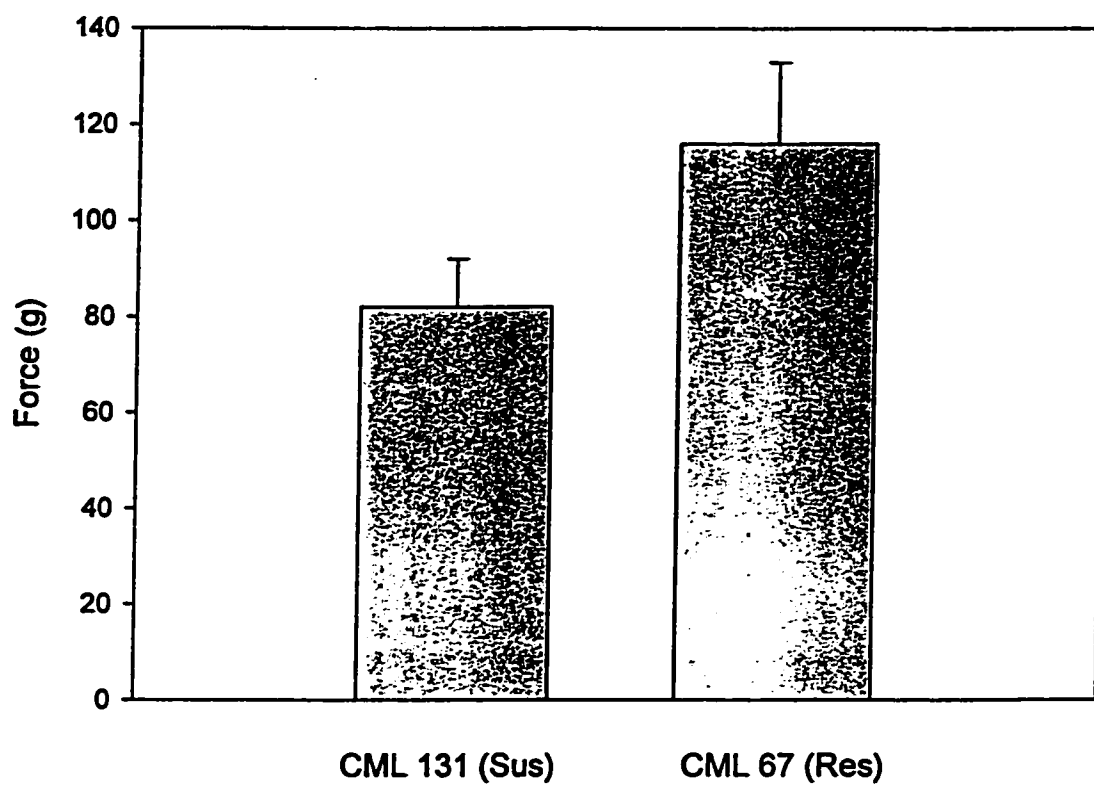
**Table 3.5. Characterization of phytochemical resistance factors in the population CML 131 (susceptible) X CML 67 (resistant), Tlaltizapan, Mexico, 1997A, n=166.**

	Mean±SE	Range
Rating SCB	4.82±0.07	2.21-7.05
Rating SWCB	5.25±0.08	2.70-7.68
GSM (mg/gDW)	359±2	283-440
Fiber (mg/gDW)	292±2	211-378
p-CA (µg/gDW)	1204±22	679-2340
FA (µg/gDW)	2524±38	1731-6289
5-5' DFA (µg/gDW)	15.4±0.44	4.55-33.41
8-5' DFA (µg/gDW)	61.81±1.04	33.20-94.97
8-0-4' DFA (µg/gDW)	62.75±1.42	36.19-186.22
Total DFA (µg/gDW)	150.9±2.75	88.23-298.25
Nitrogen (%)	2.06±0.02	1.4-2.5
Leaf thickness (µm)	215±1.4	167-262
SPAD	40.7±0.4	27.7-64.7
Force (g)	96.1±0.6	72.7-115.5

SCB (sugarcane borer), SWCB (southwestern corn borer), GSM (gravimetric soluble metabolite), p-CA (p-coumaric acid), FA (ferulic acid), DFA (diferulic acid), SPAD (leaf greenness).

The first goal of this project was to adapt the laboratory based leaf toughness technique used by Bergvinson et al., (1994a) at Agriculture Canada, Ottawa to the portable system described in the Materials and Methods section that could be used in Mexico. The first measurements recorded the force differences between the two parental lines CML 131 and CML 67. There was a 40% higher level of leaf tissue toughness in the resistant CML 67 line compared to the susceptible CML 131 line (Fig. 3.1). In terms of insect ratings, the resistant line had a field leaf rating of 4.5 compared to 9.0 for the susceptible genotype, a 50 % lower rating. Phytochemical parameters showed significantly higher levels of cell wall bound phenolics in the resistant parental line compared to the susceptible parent, with 19 % more p-coumaric acid, 23 % more ferulic acid, and 38 % more total diferulic acid. GSM showed higher levels in the resistant line, but surprisingly there was no difference in fiber content. No significant difference was observed for both leaf thickness and leaf greenness (SPAD) between the parental lines.

Tables 3.6 and 3.7 present a correlation matrix for the mapping population (CML 131 X CML 67) for both years, 1996B and 1997A for leaf field ratings (LFR) with all the phytochemical traits investigated. For both years and for both insects (SCB and SWCB), the 8-5' isomer of diferulic acid was highly significantly correlated with LFR. Overall, resistance to the insects tested was mainly correlated to tissue toughness and diferulic acid. The mapping population CML 131 X CML 67, 1997A infested with SCB showed a very strong correlation between LFR and tissue toughness ( $r = -0.379$ ,  $P = 0.001$ ) (Fig. 3.2), the higher ratings (more susceptible plants) were associated with low measurements for leaf toughness. The corollary is high leaf force corresponds to low field leaf ratings. This trend was previously demonstrated for the ECB (Bergvinson et al., 1994a) and now



**Fig 3.1 Mean force displacement measurements indicating Leaf tissue toughness in the parental lines CML131 and CML67, Tlaltizapan, 1996B (n=12).**

Table 3.6. Pearson correlation matrix with leaf field rating (LFR) and phytochemical resistance traits for mapping population CML 131 X CML 67 for 1996B, Tlaltizapan, Mexico.

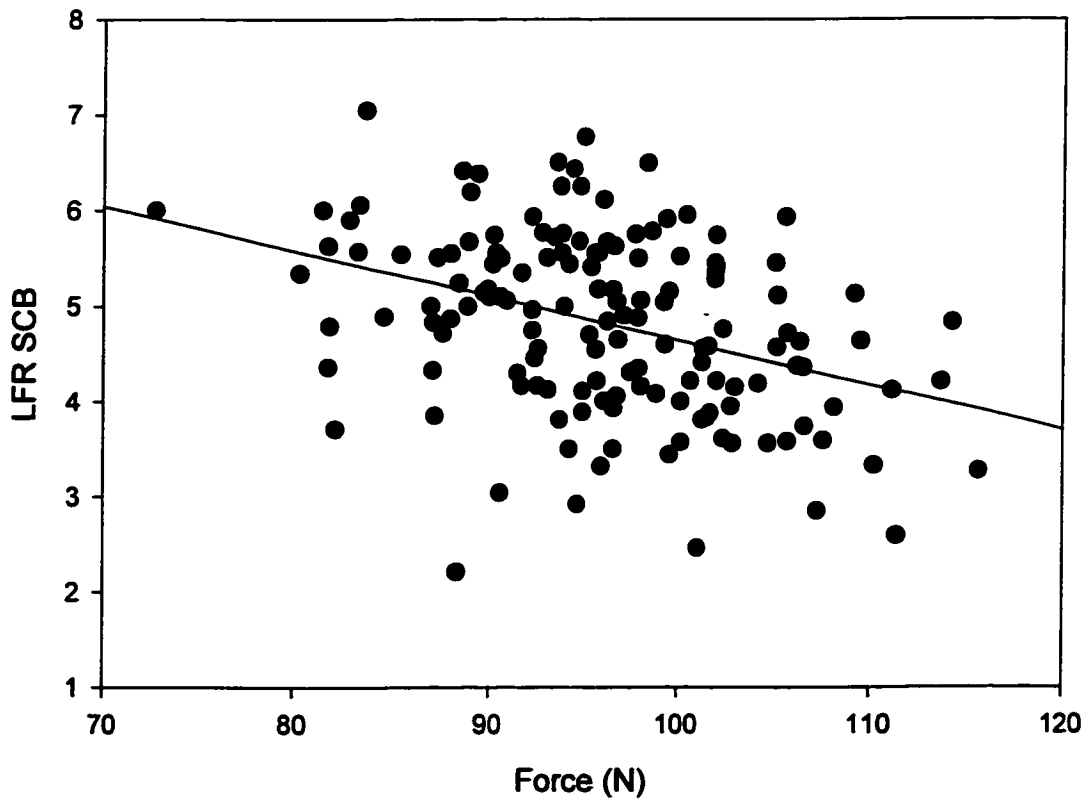
	SCB	SWCB	GSM	Fiber	p-CA	FA	8-5	5-5	8-0-4	DFA	N	Thick	SPAD
SCB	1.00												
SWCB	0.74**	1.00											
GSM	0.18	0.15	1.00										
Fiber	-0.10	-0.11	-0.05	1.00									
p-CA	-0.36**	-0.27**	-0.14	0.05	1.00								
FA	-0.40**	-0.24*	-0.24*	0.05	0.44**	1.00							
8-5	-0.38**	-0.31**	-0.11	0.07	0.32**	0.67**	1.00						
5-5	-0.36**	-0.27**	-0.12	-0.07	0.40**	0.74**	0.81**	1.00					
8-0-4	-0.29**	-0.24*	-0.05	-0.21	0.27**	0.53**	0.62**	0.82**	1.00				
DFA	-0.36**	-0.29**	-0.10	-0.10	0.36**	0.70**	0.84**	0.96**	0.92**	1.00			
N	0.39**	0.23*	0.08	0.10	-0.31**	-0.42**	-0.36**	-0.39**	-0.47**	-0.45**	1.00		
Thick	-0.14	-0.06	-0.12	0.06	-0.03	0.11	0.16	0.11	-0.02	0.07	0.06	1.00	
SPAD	-0.16	-0.15	-0.03	0.06	-0.02	0.10	0.14	0.07	0.02	0.07	-0.01	0.12	1.00
Force	-0.02	0.01	-0.03	-0.09	-0.09	-0.03	0.03	0.05	-0.05	0.01	0.08	0.41**	-0.03

Sugarcane borer (SCB), Southwestern corn borer (SWCB), gravimetric soluble metabolites (GSM), p-coumaric acid (p-CA), ferulic acid (FA), diferulic acid (DFA), nitrogen (N), \* P=0.05, \*\*P=0.001

Table 3.7. Pearson correlation matrix with leaf field rating (LFR) and phytochemical resistance traits for mapping population CML 131 X CML 67 for 1997A, Tlaltizapan, Mexico.

	SCB	SWCB	GSM	Fiber	p-CA	FA	8-5	5-5	8-0-4	DFA	N	Thick	SPAD
SCB	1.00												
SWCB	0.65**	1.00											
GSM	0.08	0.04	1.00										
Fiber	0.08	0.11	-0.34**	1.00									
p-CA	-0.26**	-0.13	-0.36**	0.22*	1.00								
FA	-0.19*	-0.01	-0.32**	0.18	0.45**	1.00							
8-5	-0.34**	-0.32**	-0.07	0.16	0.33**	0.43**	1.00						
5-5	-0.24*	-0.07	-0.16	0.20	0.54**	0.57**	0.68**	1.00					
8-0-4	-0.28**	-0.16	-0.16	0.13	0.43**	0.42**	0.44**	0.60**	1.00				
DFA	-0.31**	-0.18	-0.18	0.18	0.51**	0.55**	0.69**	0.88**	0.88**	1.00			
N	0.24*	0.22*	-0.04	0.05	0.03	0.02	-0.23*	-0.14	-0.03	-0.13	1.00		
Thick	-0.13	-0.07	-0.06	0.14	0.09	-0.01	0.11	-0.03	-0.01	0.01	-0.04	1.00	
SPAD	-0.12	-0.14	0.01	-0.01	-0.18	-0.14	-0.14	-0.25*	-0.16	-0.21*	-0.01	0.26*	1.00
Force	-0.37**	-0.30**	-0.13	-0.02	0.25*	0.21*	0.26*	0.19	0.31**	0.31**	-0.10	0.35**	0.30**

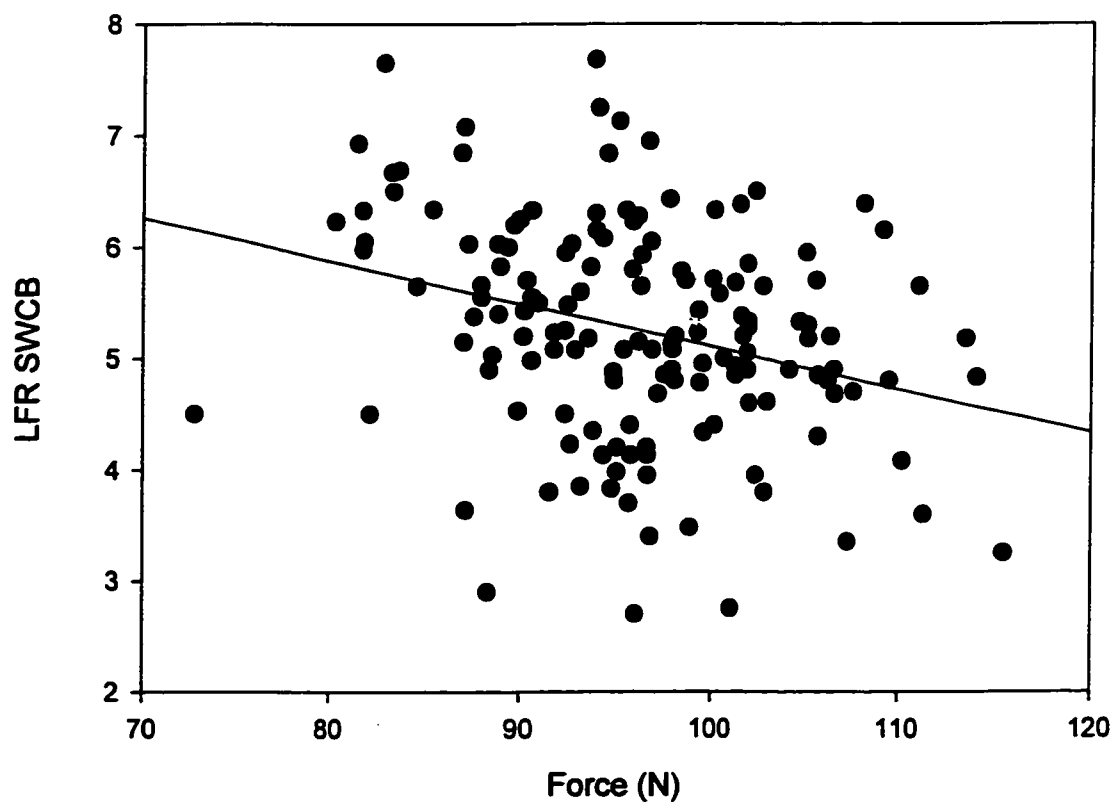
Sugarcane borer (SCB), Southwestern corn borer (SWCB), gravimetric soluble metabolites (GSM), p-coumaric acid (p-CA), ferulic acid (FA), diferulic acid (DFA), nitrogen (N), \* P=0.05, \*\*P=0.001



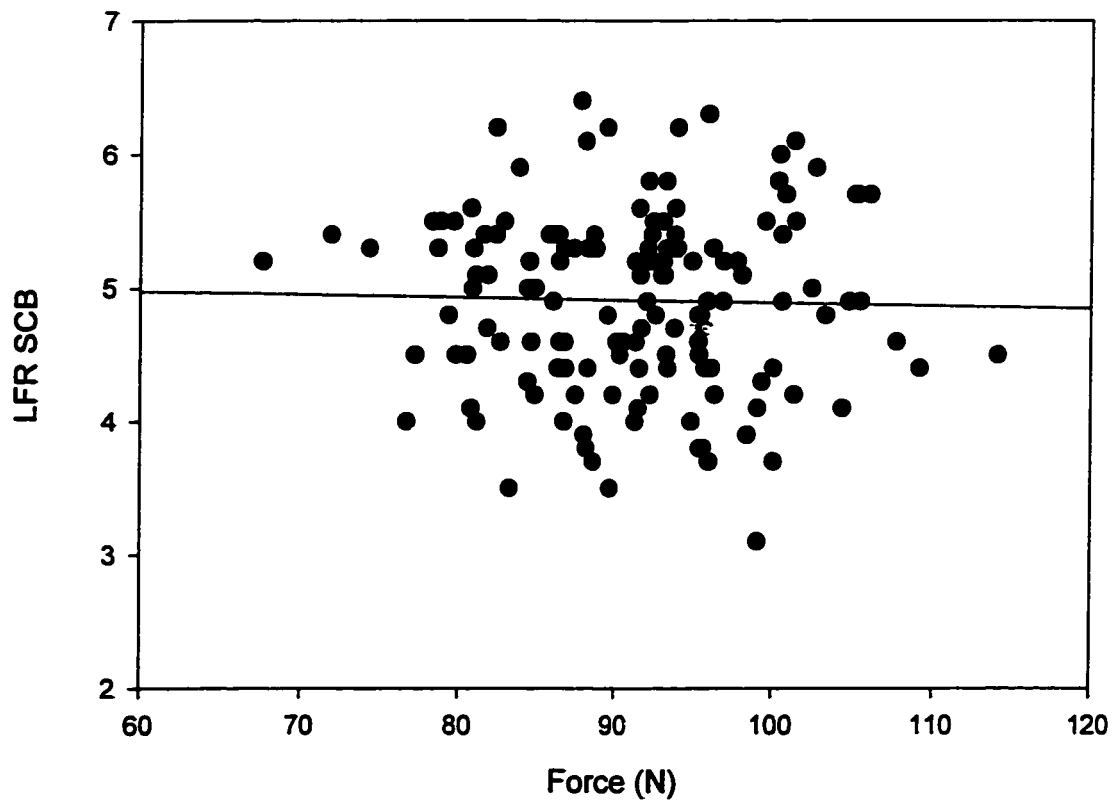
**Fig 3.2 Leaf field rating SCB and leaf toughness  
CML131 X CML67, 1997A, Tlaltizapan  
( $r = -0.379$ ,  $r^2 = 0.144$ ,  $P = 0.001$ ,  $n = 166$ )**

can be extended to two more stalk borers: SCB (Fig. 3.2), and SWCB mapping population 1997A (Fig. 3.3). There was considerable variation in the significance and the strength of the correlations. Although there were significant correlations between LFR and leaf toughness in 1997A, in the previous season 1996B there were no significant correlations between these traits (Figs. 3.4 and 3.5). The findings of this study are supported earlier the findings (Bergvinson et al., 1994a; Williams et al., 1998), in which corn leaves of susceptible hybrids were less tough than leaves of resistant hybrids. In a recent paper, Ossipov et al., (2001) showed that leaf toughness in mountain birch (*Betula pubescens*) correlated negatively with the relative growth rate (RGR) of larvae of the geometrid *Epirrita autumnata*. On leaves that had high toughness measurements, the RGR ranged from 0.196 to 0.296 and on leaves with low toughness the RGR ranged from 0.301 to 0.390. It is suggested that variations in the phenolic and phenolic-related traits such as gallotannins determine the suitability of birch leaves for *E. autumnata* larvae.

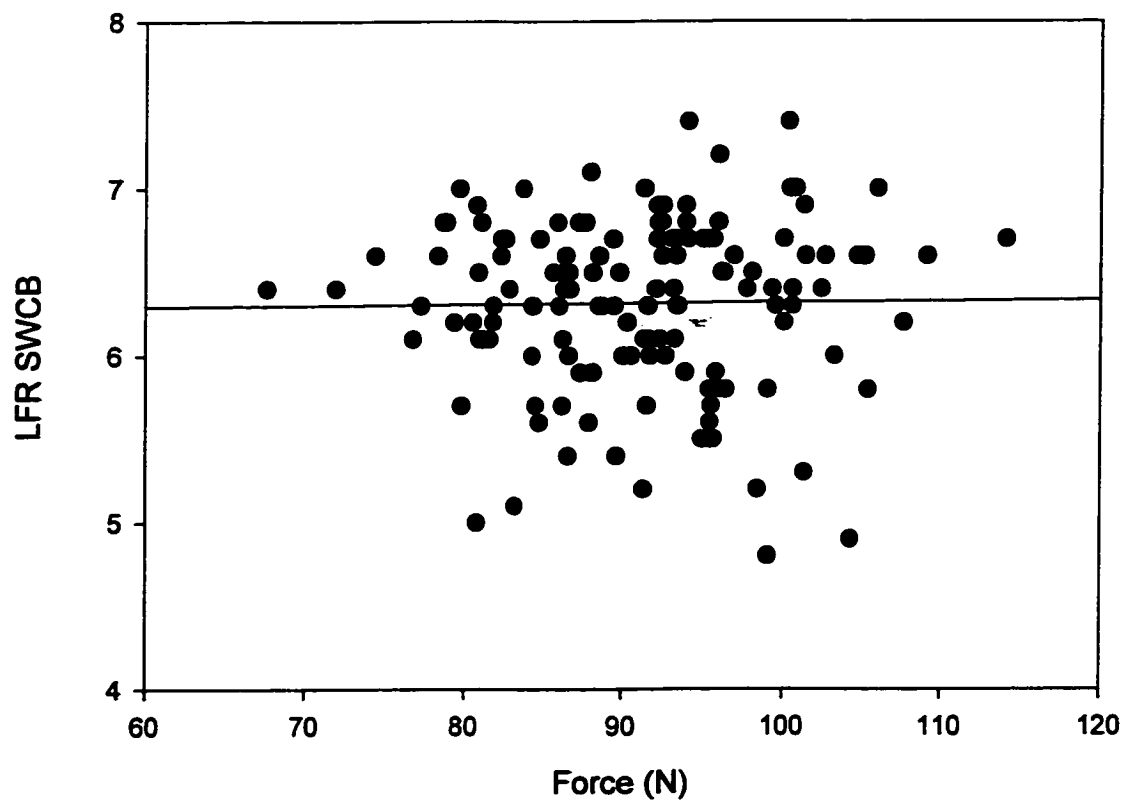
The other field traits investigated included leaf thickness and leaf chlorophyll content measured as leaf greenness with the SPAD meter. Leaf thickness (measured using a digital micrometer) did not correlate significantly with leaf field ratings, but there was a significant correlation between leaf thickness and leaf toughness. A thicker leaf does not imply a more resistant leaf. One possible explanation may be that a thicker mesophyll layer provides greater nutrition quantity leading to a more susceptible plant. In this study the correlations between leaf field ratings and SPAD measurements were not significant. However, the correlations between leaf field ratings and leaf nitrogen content were significant. In the mapping population infested with SCB 1996B,  $r = 0.393$ ,  $P = 0.001$  (Fig. 3.6). The same trend was noticed for SWCB in 1996B (Fig. 3.7) and 1997A



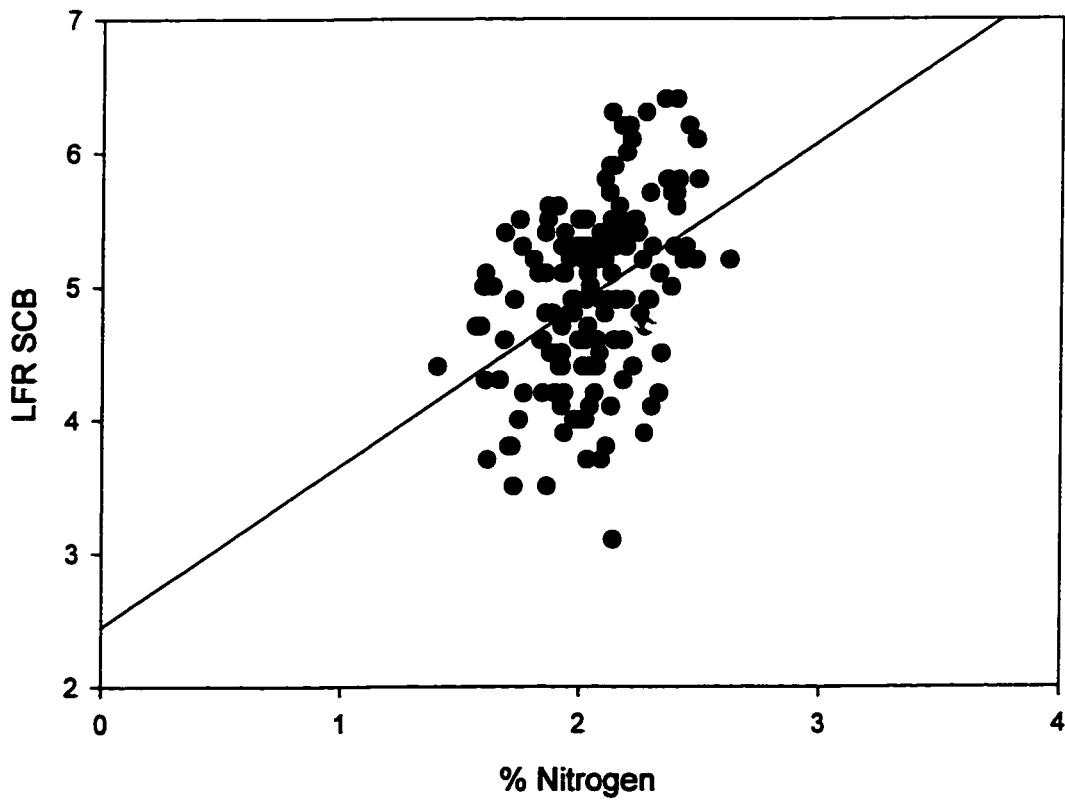
**Fig 3.3 Leaf field rating SWCB and leaf toughness**  
**CML131 X CML67, 1997A, Tlaltizapan**  
**( $r = -0.301$ ,  $r^2 = 0.091$ ,  $P = 0.005$ ,  $n = 166$ )**



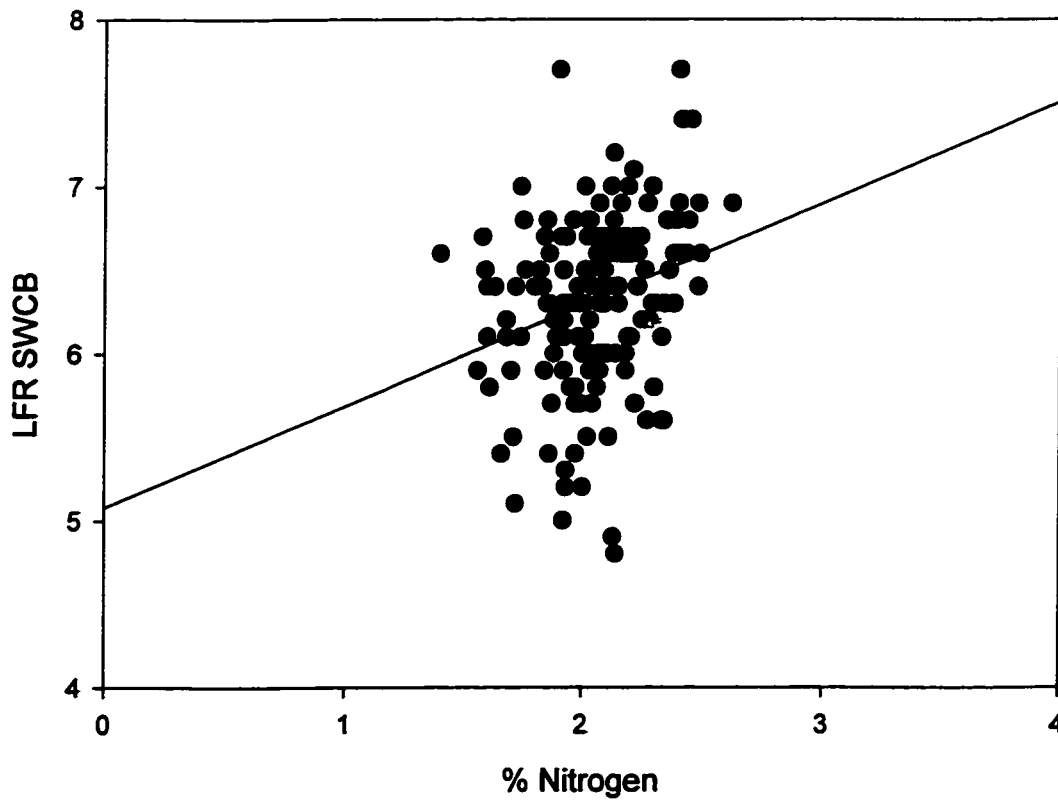
**Fig 3.4 Leaf field rating SCB and leaf toughness,  
CML131 X CML67, 1996B, Tlaltizapan  
(P=ns)**



**Fig 3.5 Leaf field rating SWCB and leaf toughness,  
CML131 X CML67, 1996B, Tlaltizapan  
(P=ns)**



**Fig 3.6 Leaf field rating SCB and leaf nitrogen  
CML131 X CML67, 1996B, Tlaltizapan  
( $r = 0.393$ ,  $r^2 = 0.166$ ,  $P = 0.001$ ,  $n = 166$ )**



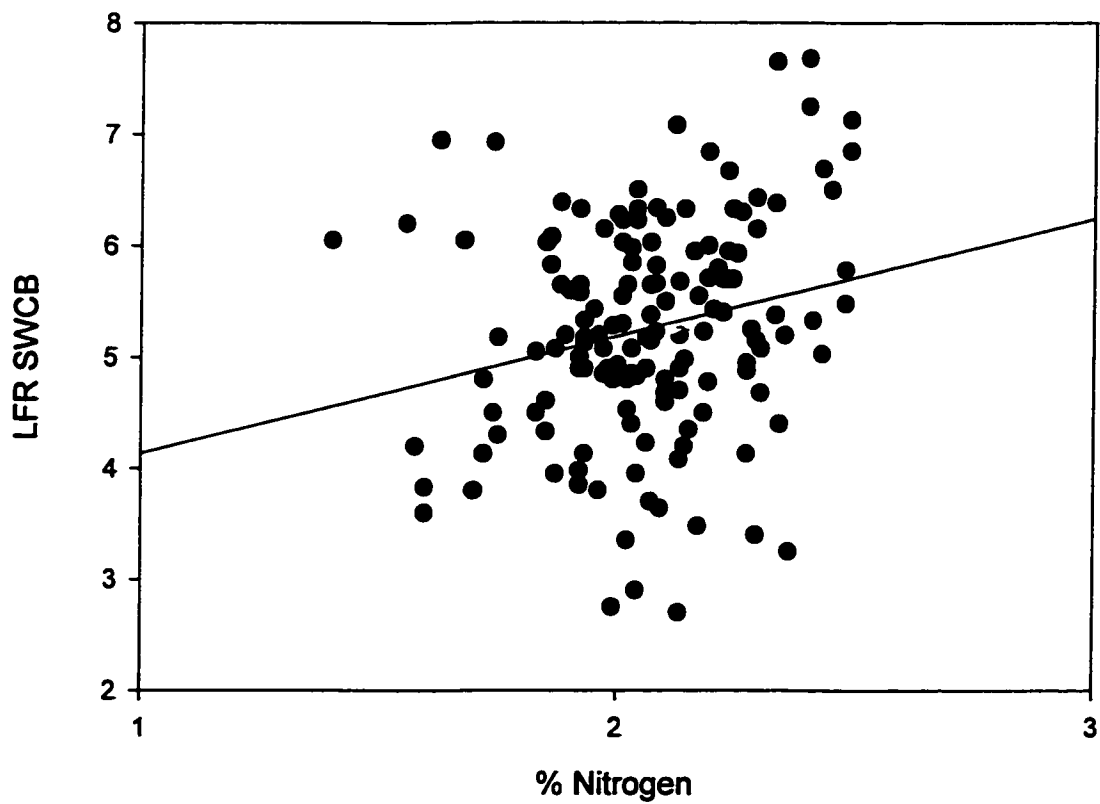
**Fig 3.7 Leaf field rating SWCB and leaf nitrogen  
CML131 X CML67, 1996B, Tlaltizapan  
( $r= 0.234$ ,  $r^2= 0.069$ ,  $P= 0.05$ ,  $n= 166$ )**

(Fig. 3.8). Correlations between field rating and leaf nitrogen were positive, an increase in nitrogen led to a more susceptible plant, as indicated by a higher rating.

Investigating leaf nitrogen, leaf toughness and insect resistance, Wheeler and Center (1996) examined the influence of leaf nutritional quality of the submersed aquatic weed, *Hydrilla verticillata* on the larval growth and development of the biological control agent *Hydrellia pakistanae* (Diptera: Ephydriidae). Leaf nitrogen content and leaf toughness varied among different sites, *H. pakistanae* individuals reared from neonate larvae to the adult stage on poorer quality hydrilla (low nitrogen, higher toughness) had higher mortality, longer developmental periods, and decreased female biomass compared with larvae fed on the more nutritious hydrilla (high nitrogen, lower toughness).

Leaf toughness can be used as an important trait in the screening of elite maize lines. The development of a rapid and portable instron could find applications in breeding programs which do not have access to artificial insect infestations, yet still want to select for insect resistance in their germplasm.

Diferulic acids can serve as a cross-linking agent within the cell wall to increase the leaf toughness and render the tissue less accessible to the herbivore. In a comparison between the parental genotypes, CML 131 (susceptible) and CML 67 (resistant), there was a 38 % difference in the levels of total diferulic acid in the resistant line compared to the susceptible line (Fig. 3.9). There was also a significant correlation between the levels of diferulic acid and leaf field ratings ( $r = -0.378$  for the mapping population infested with SCB 1996B (Fig. 3.10) and  $r = -0.344$  in 1997B (Fig. 3.11). Similar results were obtained for the SWCB with  $r = -0.312$  for the mapping population 1996B (Fig. 3.12) and  $r = -0.325$  1997A (Fig. 3.13). In 1997A in the mapping population there was a significant



**Fig 3.8 Leaf field rating SWCB and leaf nitrogen  
CML131 X CML67, 1997A, Tlaltizapan  
( $r = 0.222$ ,  $r^2 = 0.05$ ,  $P = 0.05$ ,  $n = 166$ )**

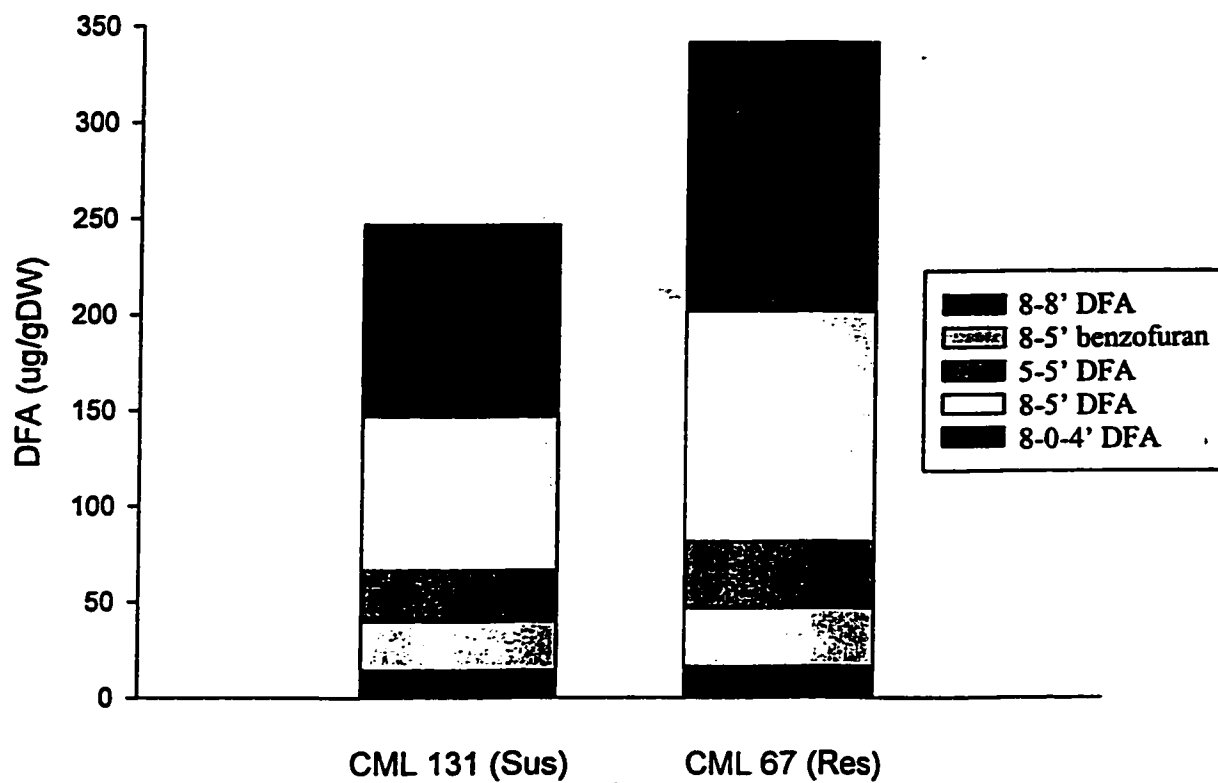
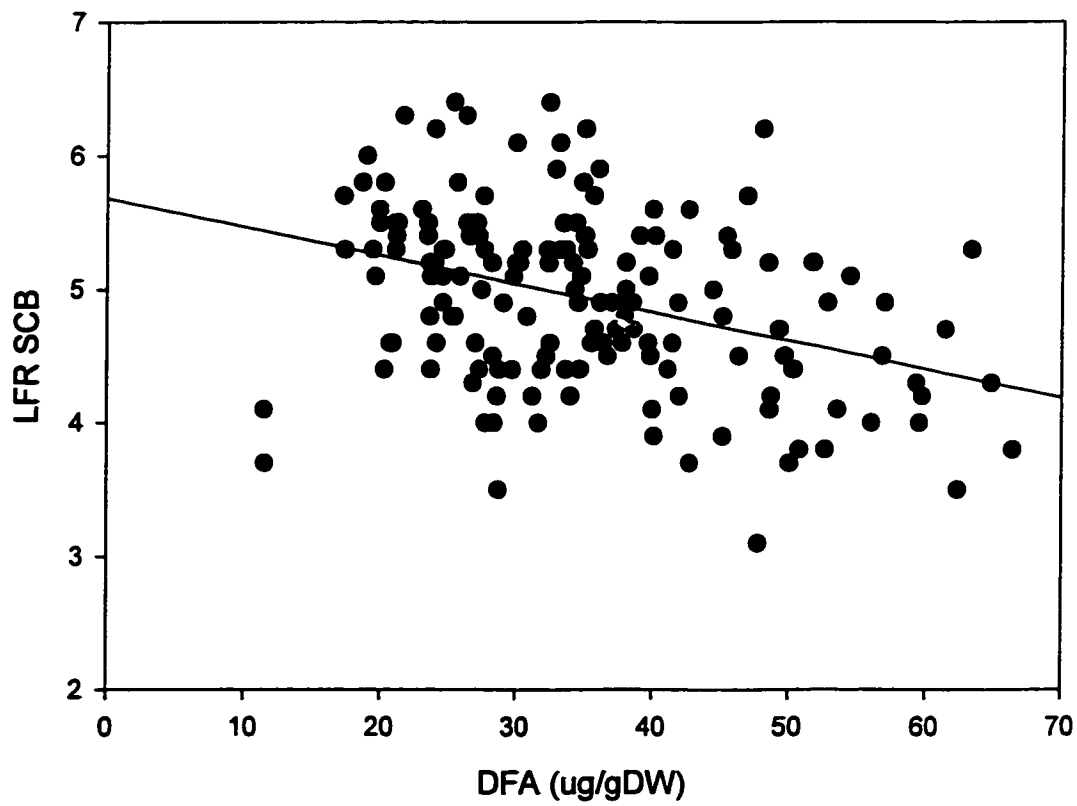


Fig 3.9 Mean levels of DFA isomers in the parental lines CML131 and CML67, Tlaltizapan, 1996B (n=12).



**Fig 3.10 Leaf field rating for SCB and DFA  
CML131 X CML67, 1996B, Tlaltizapan  
( $r = -0.378$ ,  $r^2 = 0.140$ ,  $P = 0.001$ ,  $n = 166$ )**

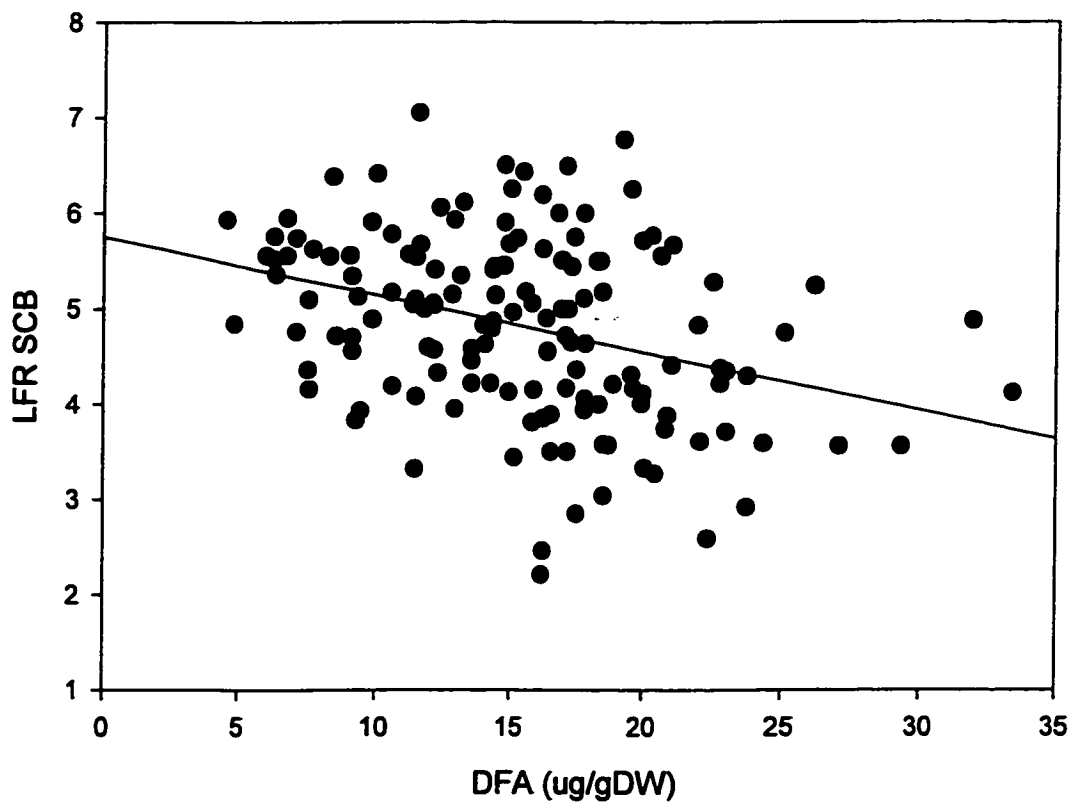


Fig 3.11 Leaf field rating SCB and leaf DFA  
CML131 X CML67, 1997A, Tlaltizapan  
( $r = -0.344$ ,  $r^2 = 0.118$ ,  $P = 0.005$ ,  $n = 166$ )

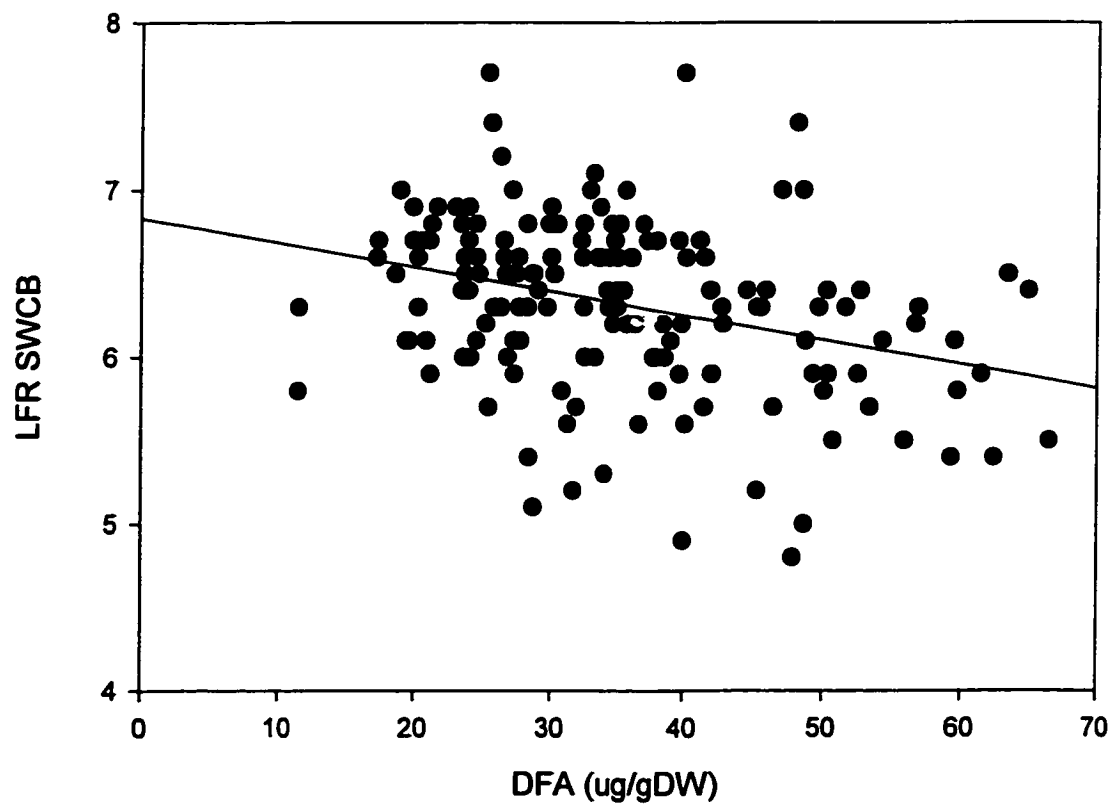
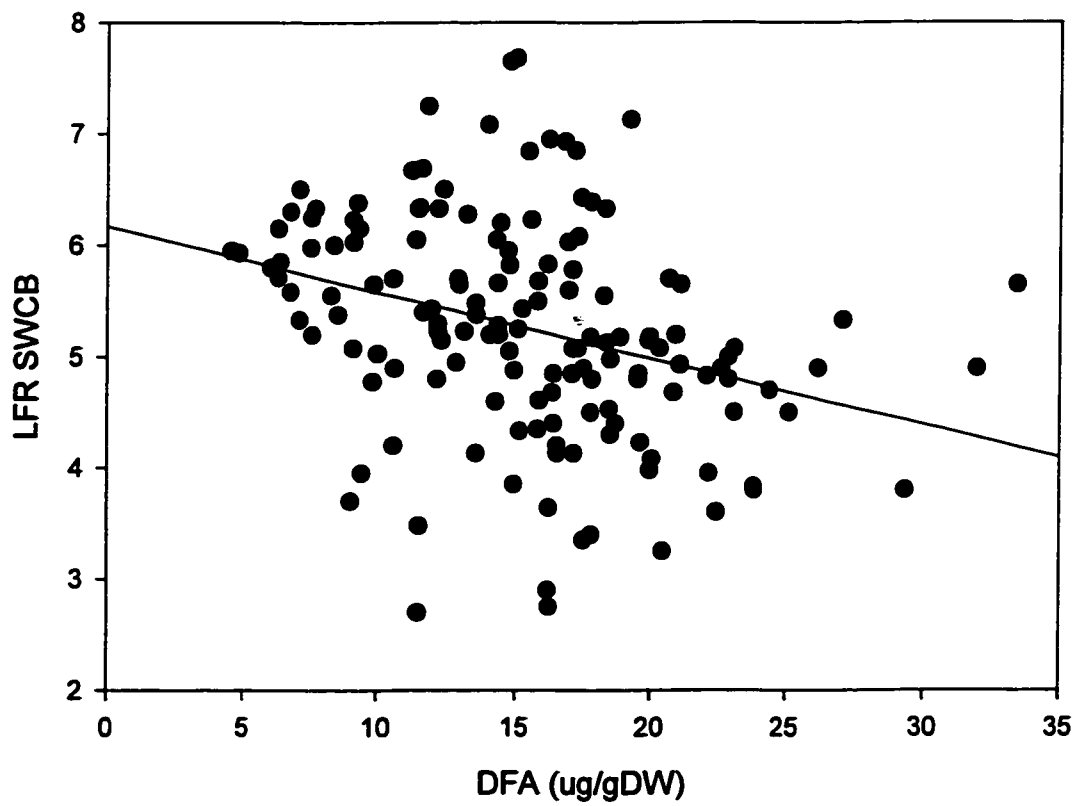


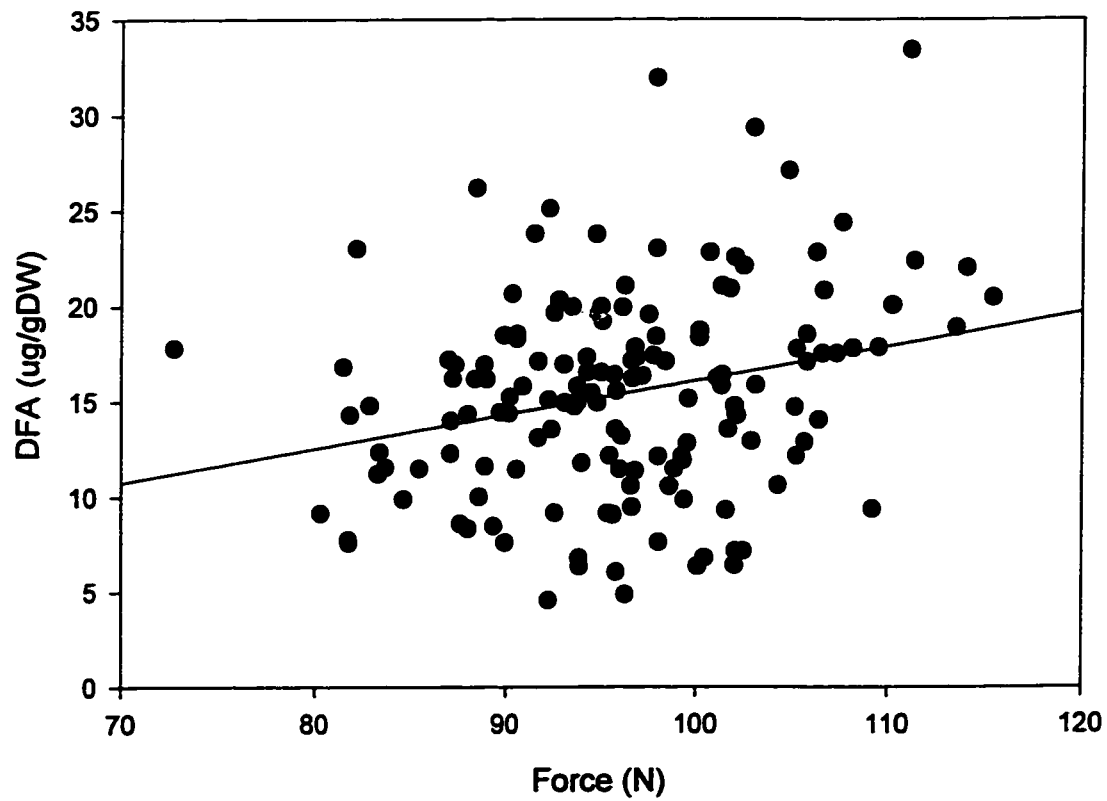
Fig 3.12 Leaf field rating for SWCB and DFA  
CML131 X CML 67, 1996B, Tlaltizapan  
( $r = -0.312$ ,  $r^2 = 0.107$ ,  $P = 0.001$ ,  $n = 166$ )

correlation between leaf DFA and tissue toughness (Fig. 3.14). This was not observed in 1996B (Fig. 3.15). This may be attributable to lower levels of leaf toughness in the summer season.

Host plant resistance in tropical maize to the lepidopteran pests appears to be polygenically controlled and involves primarily additive variation (Hinderliter, 1983). Host plant resistance involves multiple defenses against the invading herbivore. To explore these relationships, multiple regression analysis using biochemical parameters as independent variables and plant resistance parameters as dependent variables was performed (Table 3.8). In the various regression models examined the most common independent variables within all the traits investigated included diferulic acid content and leaf nitrogen content. Thirty percent of the variation in rating could be explained by variations in these two plant traits (Fig. 3.16). Adding the amount of cell wall bound ferulic acid to the forward regression equation provided the most significant model (Multiple R = 0.625, Multiple R<sup>2</sup> = 0.391, P = 0.0001) (Table 3.8). These regressions are consistent with the hypothesis that MBR (Multiple borer resistant) varieties employ a nutritional resistance mechanism whereby lower protein content acts in concert with increased cell wall mechanical strength through higher fiber content and higher levels of cell wall phenolics, to reduce the nutrient availability to early instar larval feeding (Bergvinson et al., 1997). This model supports the earlier reports of FAW showing reduced digestion of Bermuda grass (*Cynodon dactylon*) with high cell wall content (Quisenberry and Wilson, 1985). This model may also explain the reduced growth rate of SWCB larvae feeding on MBR cultivars compared to those feeding on susceptible cultivars (Davis et al., 1988). The main advantages of polygenic resistance are the



**Fig 3.13 Leaf field rating SWCB and leaf DFA**  
**CML131 X CML67, 1997A, Tlaltizapan**  
**( $r = -0.325$ ,  $r^2 = 0.105$ ,  $P = 0.005$ ,  $n = 166$ )**



**Fig 3.14 Leaf DFA and leaf toughness  
CML131 X CML67, 1997A, Tlaltizapan  
( $r = 0.311$ ,  $r^2 = 0.065$ ,  $P = 0.001$ ,  $n = 166$ )**

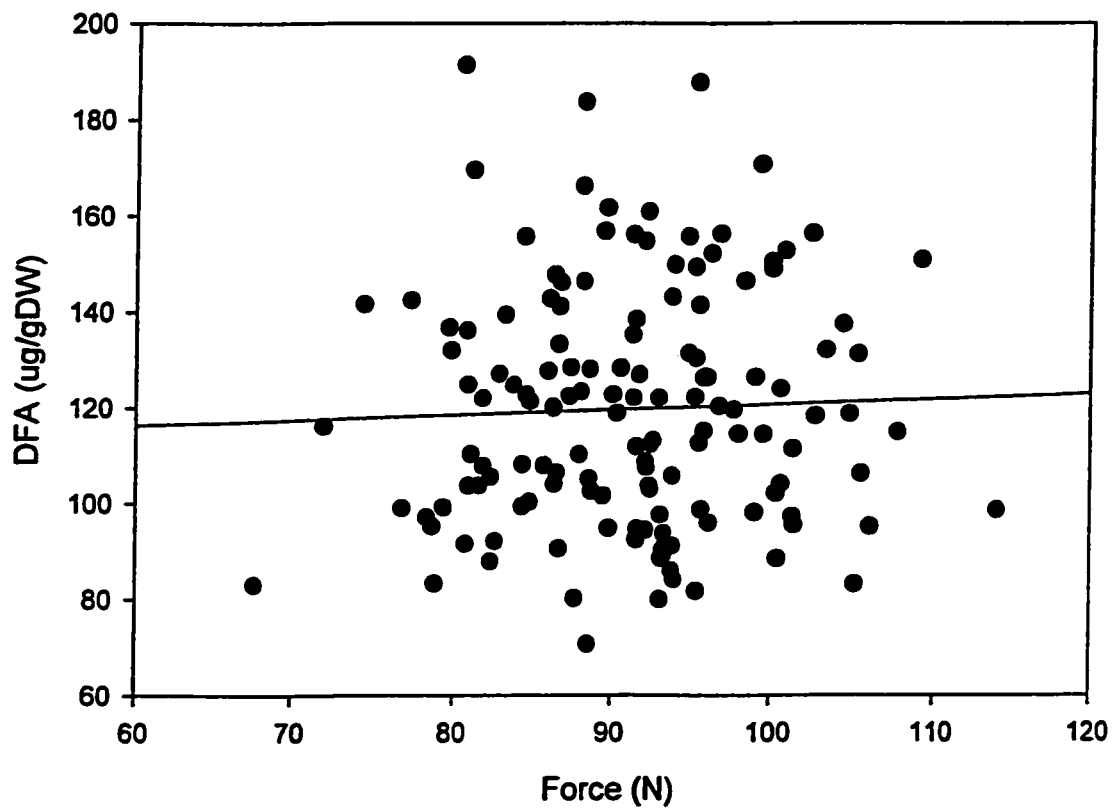


Fig 3.15 Leaf DFA and leaf toughness CML131 X CML67, Tlaltizapan, 1996B (P=ns).

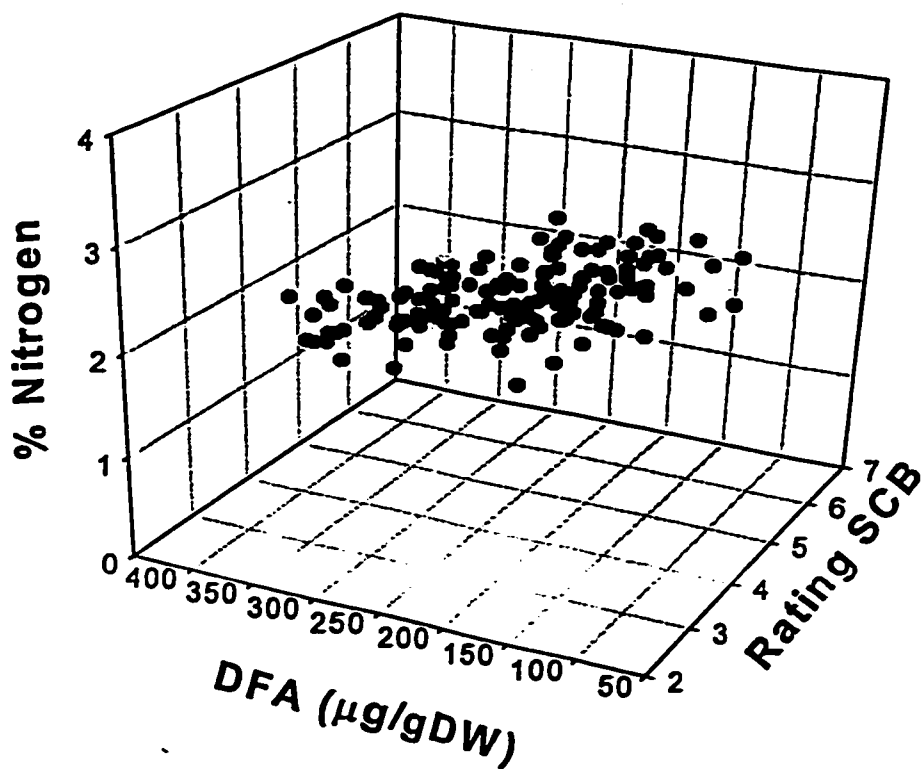


Fig 3.16 Leaf field rating for SCB, leaf nitrogen, and DFA in CML131 X CML67, Tlaltizapan, 1996B (multiple R = 0.613, P= 0.0001, n=166).

Table 3.8. Forward multiple regression analysis of plant traits with plant resistance parameters for the Mapping population CML131 X CML67, Tlaltizapan, Mexico, 1996B and 1997A.

Year	Dependent Variable	Regression Equation	multiple $r^2$	P
1996B	Leaf Rating (SCB)	LR=4.481+0.778(N)-0.012(DFA)-0.001(tFA)	0.390	0.0001
	Leaf Rating (SWCB)	LR=6.561+0.278(N)-0.010(DFA)-0.001(pCA)	0.376	0.0001
	Leaf Toughness	LT=31.256+0.293(TH)-0.011(cFA)+0.009(DFA)	0.197	0.0001
1997A	Leaf Rating (SCB)	LR=2.184+1.811(N)-0.028(DFA)-0.001(tFA)	0.326	0.0001
	Leaf Rating (SWCB)	LR=2.491+1.496(N)-0.051(DFA)-0.001(tFA)	0.286	0.0001
	Leaf Toughness	LT=46.086+0.163(TH)+0.098(DFA)+0.004(tFA)	0.245	0.0001

SCB (Sugarcane borer), SWCB (Southwestern corn borer), N (leaf nitrogen content), DFA (diferulic acid), pCA (p coumaric acid), tFA (trans ferulic acid), TH (leaf thickness), (n=150 for both years).

reduced likelihood of resistance developing in the pest population coupled with effective resistance over a broader spectrum of pest organisms.

There has been significant increase in the area of research directed towards the hydroxycinnamic acids in plants. The hydroxycinnamic acids, such as ferulic, sinapic, caffeic and coumaric, are found covalently attached to the plant cell wall and as soluble forms in the cytoplasm. Ferulic acid is the most abundant member of this class of phytochemicals (Faulds and Williamson, 1999). A portion of the ferulic acid in the cell wall is present as dehydrodimers (diferulic acids), the function of which is to cross-link and strengthen the cell wall (Iiyama et al., 1994). In wheat straw it has been estimated that the dimmers comprise 25% of the total ferulic acid complement, indicating a high degree of cell wall cross-linking (Waldron et al., 1996). Arabinoxylan hemicelluloses are the main polymers that are cross-linked.

Until the early 1990s, only one diferulate was identified, the 5-5' coupled dehydrodiferulate. Waldron et al., (1996) demonstrated that the levels of dehydrodimers were much higher than previously estimated. The total quantity of dehydrodiferulates was up to 20 times the level of the 5-5' dimer alone. Using reverse phase HPLC to separate the phenolics and diode array detector for identification, up to six dehydrodiferulic acids could be identified in extracts of graminaceous monocot cell walls. In this present study, five out of six isomers could be positively identified (Fig. 3.9). The relative proportion of each dimer is similar to that found for wheat straw (Waldron et al., 1996) and in Cocksfoot and Switchgrass, two other cereals (Ralph et al., 1994). The overall quantity of diferulic acid appears to be more variable than the individual content of each isomer. In a recent paper, Renger and Steinhart (2000)

suggested that only 40 % of the DFA is ester linked, the other 60 % is ether linked in mature leaf of corn. The method used in this present study cleaves only the ester link (2 N NaOH, 4 h at room temp). In order to cleave the ether link a teflon pressure bomb was used with 4 N NaOH at 170° for one hour (Renger and Steinhart, 2000). However, the ether-linked DFA is far more important in mature leaf tissue than young tissue studied here. The absolute DFA content may still be underestimated in this investigation.

There have been a number of roles proposed for diferulic acid. Ferulic acid and diferulates have been implicated to act as nucleation sites for lignification in grasses (Ralph et al., 1997). In another study, Ikegawa et al. (1996) suggests that the accumulation of diferulic acid may play a role in resistance during the hypersensitive response of oat leaves to the invasion of the pathogen *Puccinia coronata*. The cell wall cross-links may decrease cell wall extensibility during cell maturation (Sanchez et al., 1996). There is an induced increase in diferulic acid content in etiolated dark grown *Avena* coleoptiles when exposed to light (Miyamoto et al., 1994). Ng et al. (1998) suggest that diferulic cross-links may promote cell-cell adhesion in beet root (*Beta vulgaris*). Diferulic acid may play a role in cell separation, maintaining fruit and vegetable tissue texture during cooking (Waldron et al., 1997). Grabber et al., (1998) reports that diferulic acid limits cell wall enzymatic degradation and digestibility in forage grasses. Using <sup>13</sup>C NMR data, Hedin et al., (1996) demonstrated that the intensity of the carbonyl carbon in resistant corn hemicellulose was stronger, indicating a greater degree of cross-linking. Resistant hemicellulose is both structurally different from susceptible hemicellulose and present in greater quantities, thus resistance to the FAW appears to be correlated with both a greater amount and a higher degree of cross-linking

of the hemicellulose (Hedin et al., 1996). This finding is consistent with the idea that cell wall thickness is of vital importance in plant resistance (Bergvinson et al., 1994b). The thicker the cell wall, the more hemicellulose microfibrils, the more cell wall bound phenolics, the more diferulic acid and ultimately the increased tissue toughness. Cell wall bound phenolic acids have previously been correlated with maize kernel toughness and maize resistance to the storage pest *Sitophilus zeamais* (Classen et al., 1990). This present study has demonstrated the role of leaf DFA in resistance to SCB and SWCB. Future investigations in this area should include cell wall thickness and the developmental regulation of the cell wall as a plant resistant trait.

### **Conclusions**

Maize leaf tissue toughness represents an important component of leaf feeding resistance and reduced plant damage observed with two important tropical borers in this study. Larval feeding by SCB and SWCB on leaf whorl tissue was reduced in plants with elevated levels of cell wall phenolic dimers. Diferulic acid within the immature leaf tissue may serve to increase the toughness of this tender tissue. Fortification of epidermal cell wall tissue is probably more important as a physical barrier than as a means of reducing digestibility. The advantage of this type of resistance factor is that a structural defense is more difficult to overcome than a toxic defense for these tropical and subtropical borers.

## Chapter 4

### **QTL Mapping in Tropical Maize: Phytochemical Resistance to Southwestern corn borer and Sugarcane borer**

*If all the ways I have been along were marked on a map and joined up with a line, it might represent a minotaur.*

Pablo Picasso

#### **4.1 Introduction**

The southwestern corn borer (*Diatraea grandiosella* Dyar) and the sugarcane borer (*Diatraea saccharalis* Fabricius) are important insect pests in tropical and subtropical areas of maize production in Central America and the southern US. Larvae of both insect species can cause extensive damage due to leaf feeding at the whorl stage and stalk tunneling. Total yield loss due to maize borer infestation range from 14 to 50 % (Hinderliter, 1983). In Central America, the low yield stability on small-scale, low-input farms is partly due to the highly variable damage caused by these borers. In tropical environments, up to seven generations can develop annually.

The overlap in the distribution of these pests has led to the development of maize germplasm that is resistant to leaf feeding and stalk tunneling generations of both insect species. Resistance to only one of these insect species would be insufficient (Bohn et al., 1997). Therefore, research at CIMMYT (International Maize and Wheat Improvement Centre) has focused on a breeding scheme to combine different germplasm sources conferring resistance to a number of maize stem borers, including both *Diatraea* species into a multiple borer-resistant (MBR) population (Smith et al., 1989). Breeding for

resistance to insects is laborious and time consuming. Large scale rearing facilities and repeated and effective artificial infestations are required at each cycle of selection (Smith et al., 1989). Owing to these difficulties, marker assisted selection (MAS) is being viewed increasingly as both feasible and necessary. A resistant parent is selected to carry a RFLP marker in a breeding program. The progress of the resistant gene through the generations is followed simply by RFLP screening of DNA from seedlings. This is a far less time-, space- and resource-demanding process than carrying out full-scale insect resistance trials.

Resistance to insects is often continuously variable, polygenic, additive (Hinderliter, 1983) and controlled by the segregation of quantitative trait loci (QTL). In the present experiment, a segregating population was derived from the cross of two lines clearly contrasting in the expression of insect resistance (CML 131 susceptible x CML 67 resistant). Previous studies (Khairallah et al., 1998; Bohn et al., 1997; Groh et al., 1998) have obtained RFLP genetic linkage maps in order to identify genomic segments responsible for the expression of insect resistance in maize to SWCB and SCB.

Other studies have focused on the biochemical mechanisms of insect resistance in corn. Resistant maize lines were found to have higher physical strength of the cell wall because of higher concentrations of phenolics, phenolic dimers and fiber (Bergvinson et al., 1995). Diferulic acid, a cell wall cross-linking phenolic has been implicated in antibiosis to the ECB (Bergvinson et al., 1997). Diferulic acid also has a significant role in resistance to the SWCB and SCB (Chapter 3 of this thesis). For these reasons, these putative phytochemical traits conferring insect resistance were mapped in the present

study in order to gain some insight into the genetic basis of the underlying mechanisms of insect resistance.

Lee et al., (1998) have mapped QTL controlling resistance to the corn earworm (*Helicoverpa zea*) in maize, as well as levels of two C-glycosyl flavones, maysin and apimaysin, compounds in the silk tissue that confer resistance to the earworm. They identified maysin and apimaysin QTLs tightly linked to insect resistance and one QTL for insect antibiosis that was unrelated to flavonoid levels. This combined approach to mapping insect resistance QTLs and their phytochemical components is one of the fundamental tools that can elucidate the genetic basis for resistance to insects.

In this report we present results of a QTL study that extends the understanding of cell wall phenolic cross-linking and resistance to leaf feeding by SWCB and SCB in maize.

## **4.2 Materials and Methods**

### **4.2.1 Plant Material**

Two maize inbred lines were selected as parents to produce one recombinant inbred line (RIL) population. CML131, a highly susceptible, subtropical white dent line out of CIMMYT's population 42, was crossed to CML67, a highly resistant, tropical red-yellow semi-dent line from Antigua Group 2. RIL was developed by selfing a random subsample of F<sub>2</sub> plants by single seed descent until the generation of F<sub>6:7</sub> lines in the mapping population. Each line was the sib-mated for seed increase. Population size was 166 RIL for CML131 X CML67.

The phytochemical analyses of these lines are described in Chapter 3.

### **4.2.2 QTL Mapping**

QTL analyses were performed on a subset of 166 RIL in Population CML 131 X CML 67 for which both phenotypic and molecular data were available. The composite interval mapping (CIM) procedure of Zeng (1994) and Jiang and Zeng (1995) were used to combine data for both years 1996B and 1997A in a joint analysis of multiple environments and multiple traits. CIM was employed to map phytochemical QTLs and to estimate their genetic effects. All computations were performed using software developed by C. Jiang at CIMMYT. The QTL mapping was performed by M. Vargas at CIMMYT.

### 4.2.3 Model

The Statistical model for the QTL analysis was:

$$y_{ij} = b_i + b_i^* x_j^* + \sum_k b_{ik} x_{jk} + e_{ij}$$

for the RILs, where

$y_{ij}$  = the phenotypic value of RIL  $j$  in environment  $i$

$b_i$  = the mean phenotypic value of RIL with genotype qq at the putative QTL and mm at the markers used as cofactors in environment  $i$

$b_i^*$  = the additive effect of a putative QTL in environment  $i$

$x_j^*$  = the number of alleles from the resistant parent at the putative QTL, taking values 0, 1, and 2 with probabilities depending on the genotype at the flanking markers in the interval under search (0 = QQ, 1 = Qq, 2 = qq)

$b_{ik}$  = the partial regression coefficient of the phenotype on the marker  $k$

$x_{jk}$  = the number of alleles from the resistant parent at the selected marker  $k$

$e_{ij}$  = the residual variable of RIL  $j$  in environment  $i$ .

The threshold used for QTL detection with data from one environment was set to a likelihood ratio (LR) of 11.5 (equivalent to a LOD 2.5). This critical value is equivalent to a significance level  $P = 0.0032$  in a distribution with  $df = 2$  (Groh et al., 1998).

### 4.3 Results and Discussion

The primary phytochemical data set used to map QTLs from the population CML131 X CML67 for 1996B and 1997A is described in Chapter 3 of the thesis. The complete data set for all RILs for both years is presented in the Appendix. The results of the QTL analyses are presented here (Table 4.1). Field data was obtained for the mapping population over two seasons to examine genotype by environment interactions. Stability of genotypes over environments is a major goal in plant breeding. Most QTLs found for phytochemical components were consistent across environments (low QTL X E interaction, Table 4.1), indicating that expression of genes conferring resistance to SWCB and SCB was mainly independent of the environment.

For any individual plant trait, a LOD score ( $\log_{10}$  of the likelihood odds ratio) of 3.0 indicates that there is a one thousand times greater probability that this portion of the chromosome is associated with this particular trait. In this study a LOD of 3.0 indicated a Likelihood Ratio (LR) of 13.81 and a LOD of 2.5 indicated a LR of 11.51. These LRs were used as critical values to identify QTLs. QTLs were identified for p-coumaric acid, ferulic acid, 5-5' DFA, 8-5' DFA, and GSM. The sole QTL for 8-0-5' DFA linked with the 5-5' DFA QTL on chromosome 5 but the likelihood ratio for this QTL was 11.41 and the QTL identity threshold for significance ( $\text{LOD} > 2.5$ ) is 11.51. The overlap in the QTLs for 8-5', 5-5', and 8-0-4' DFAs on chromosomes 1, 4, and 5 suggests a common genetic basis for the production of these cell wall dimmers. A total eight QTLs can explain 44% of the genetic difference between parental lines CML 131 and CML 67 for the production of 5-5' DFA (Table 4.1).

Table 4.1. Parameters associated with QTL for cell wall bound phenolic acids, gravimetric soluble metabolites (GSM), and fiber estimated from phenotypic data of 166 RIL for Population CML131 X CML67, 1996B and 1997A, Tlaltizapan, Mexico.

Trait	Chromosome	QTL Position	Marker	Likelihood Ratio (LR)	QTL X E interaction	Phenotypic Variance Explained
		cM				%
p-coumaric acid	3	101	10	19.30**	0.08	2.53
	3	148	15	17.26**	2.47	5.47
	4	135	8	11.84*	7.11	0.24
	6	49	5	23.99**	1.56	8.66
	9	93	6	17.04**	8.93	8.99
	Total					
ferulic acid	1	172	15	19.96**	1.69	11.06
	1	197	17	14.38**	0.28	8.90
	5	129	11	30.57	13.64	17.97
	Total					
8-5' DFA	1	203	17	14.98**	0.32	11.84
	4	83	4	13.31*	8.16	4.12
	Total					
5-5' DFA	1	83	7	24.32**	0.04	9.78
	1	203	17	11.67*	0.01	12.58
	2	64	5	11.56*	1.46	5.05
	3	94	9	27.82**	0.81	9.67
	4	88	5	11.75*	9.54	3.09
	5	153	13	12.79*	9.03	2.05
	6	91	8	15.83**	0.02	7.17
	10	116	10	12.43*	6.29	1.90
	Total					
8-0-4' DFA	5	154	13	11.41	1.35	4.84
GSM	2	62	5	10.04	3.78	1.65
	5	55	5	9.78	9.10	1.39
		130	11	11.58*	5.14	10.35
	Total					
Fiber	6	85	7	10.36	2.70	6.96

\*\* LOD>3.0, \* LOD>2.5

QTLs for phytochemical traits and insect feeding damage are compared in Figs. 4.1a to 4.1e. In the present study five out of eight QTLs for 5-5' diferulic acid were located at the same position or close to QTLs for SCB leaf feeding damage and SWCB leaf feeding damage on chromosomes 1 (2 QTLs), 3, 5, and 6 that were detected by Groh et al., (1998). One QTL for 8-5' DFA also matched with a QTL for leaf feeding damage on chromosomes 1 for both insects. The other 8-5' QTL matched with a QTL for leaf toughness on chromosome 4. This QTL on chromosome 4 for leaf toughness was also linked to a QTL for 5-5' DFA. Four QTLs for p-coumaric acid linked with QTLs for leaf feeding damage on chromosomes 1 (2 QTLs), 6, and 9. Two QTLs for trans-ferulic acid on chromosome 1 were associated with leaf feeding damage. The only significant QTL for gravimetric soluble metabolites (GSM) was also linked to leaf feeding damage and leaf toughness on chromosome 5. There were five QTLs for leaf nitrogen, all linked to leaf feeding damage: two on chromosome 1, and one each on chromosomes 5, 8, and 9. Two QTLs were identified for leaf greenness (SPAD) on chromosomes 3 and 4, these were not linked to leaf feeding damage, suggesting that leaf greenness is not a good indicator of leaf resistance. No QTL for crude fiber content was identified in the mapping population (Table 4.1). In addition, three QTLs for 5-5' DFA also matched QTLs for leaf toughness on chromosomes 1, 4, and 5. Leaf toughness could be used as a quick method for screening germplasm. Both of these traits, DFA and leaf toughness are associated with insect resistance, these plant traits could be used for indirect selection. The advantage of such a selection process is the elimination of the need for costly mass rearing of insects. The identification of QTLs for these traits also can be used to develop

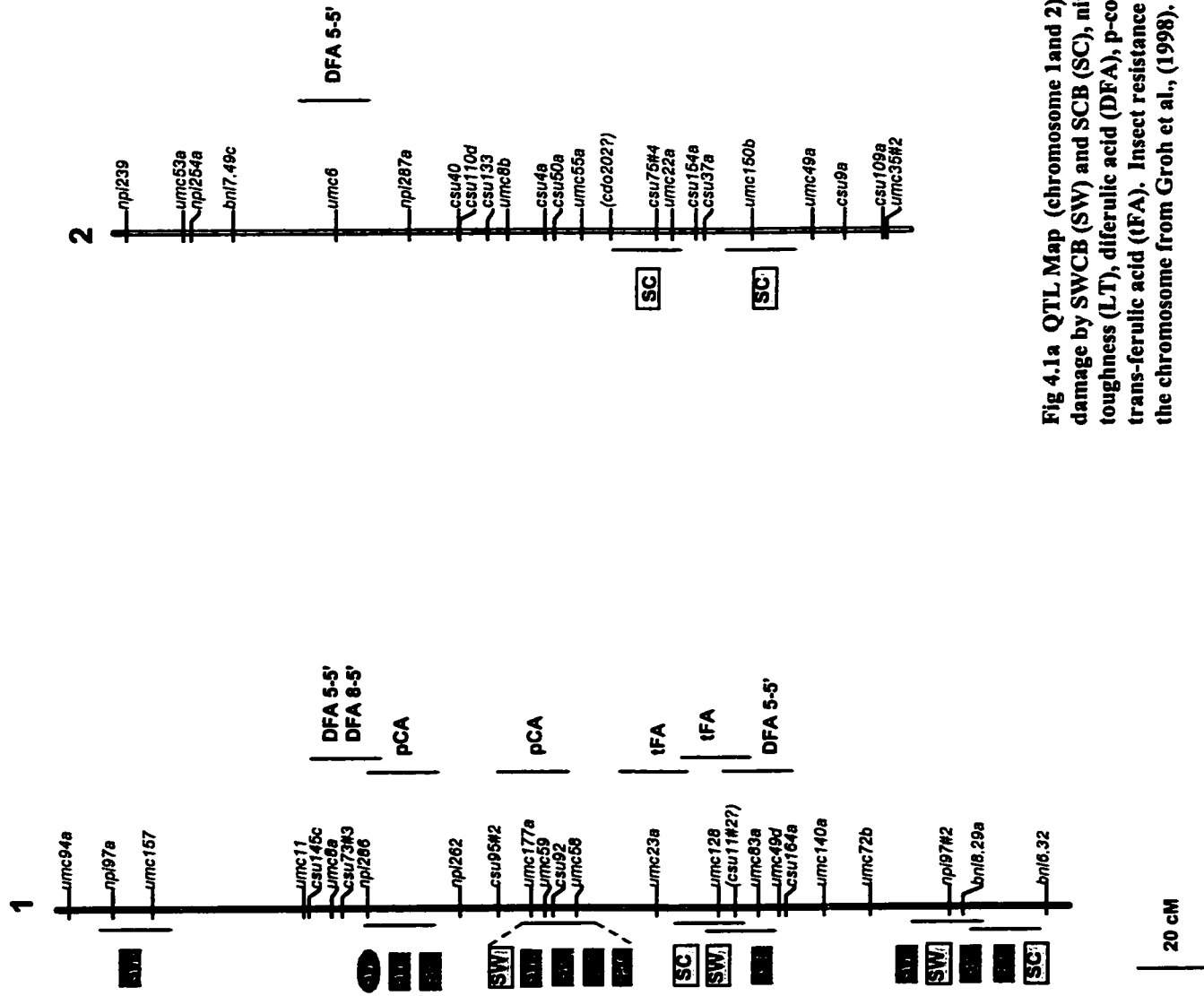


Fig 4.1a QTL Map (chromosome 1 and 2) for leaf feeding damage by SWCB (SW) and SCB (SC), nitrogen (PC), leaf toughness (LT), diferulic acid (DFA), p-coumaric acid (pCA), trans-ferulic acid (tFA). Insect resistance QTLs on the left of the chromosome from Groh et al., (1998).

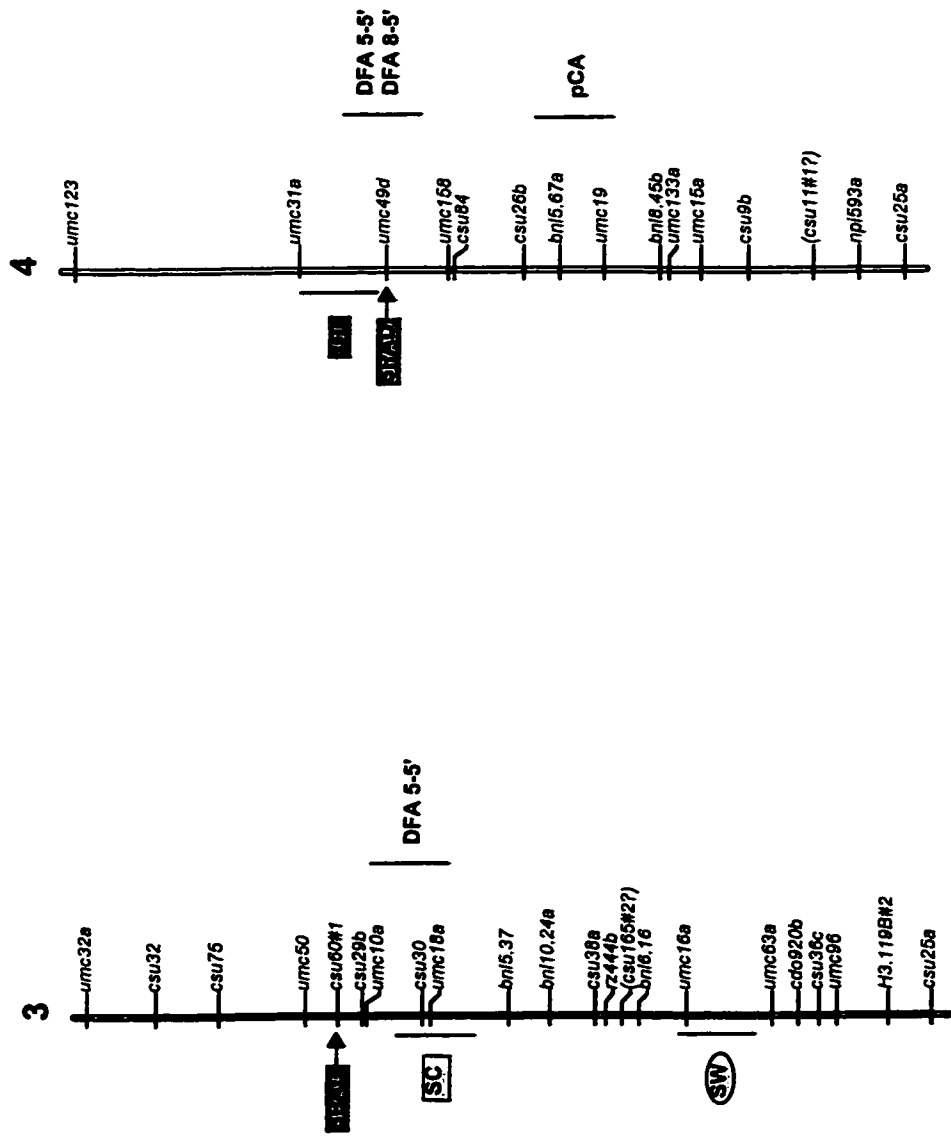


Fig 4.1b QTL Map (chromosome 3 and 4) for leaf feeding damage by SWCB (SW) and SCB (SC), leaf toughness (LT), leaf greenness (SPAD), diferulic acid (DFA), p-coumaric acid (pCA). Insect resistance QTLs on the left of the chromosome from Groh et al., (1998).

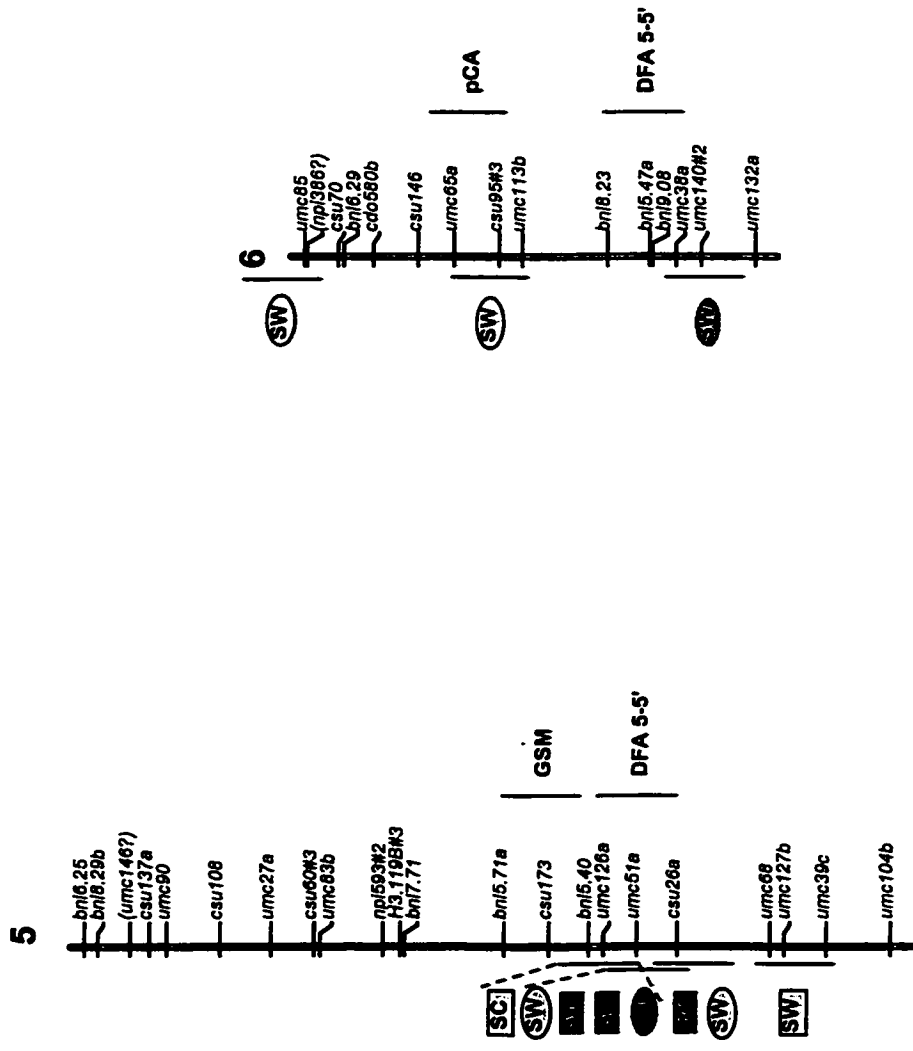


Fig 4.1c QTL Map (chromosome 5 and 6) for leaf feeding damage by SWCB (SW) and SCB (SC), nitrogen (PC), leaf toughness (LT), diferulic acid (DFA), p-coumaric acid (pCA), and gravimetric soluble metabolite (GSM). Insect resistance QTLs on the left of the chromosome from Groh et al., (1998).

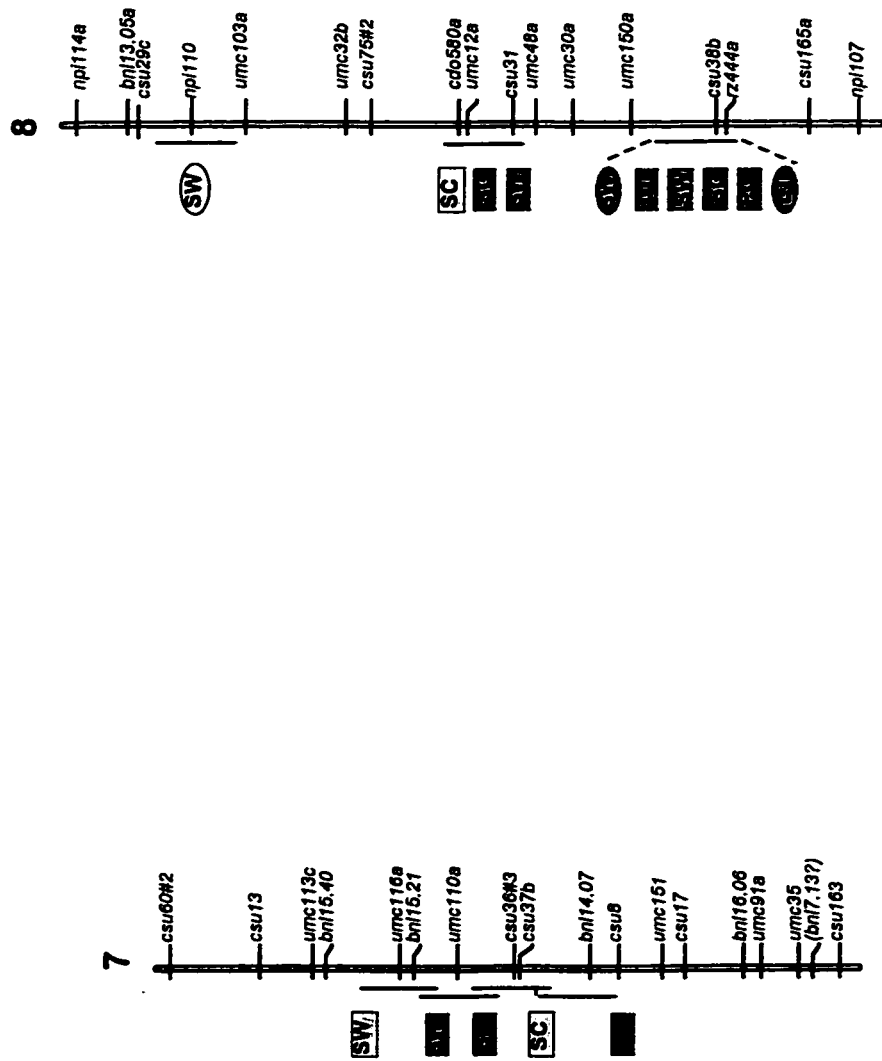


Fig 4.1d QTL Map (chromosome 7 and 8) for leaf feeding damage by SWCB (SW) and SCB (SC), nitrogen (PC), leaf toughness (LT). Insect resistance QTLs on the left of the chromosome from Groh et al., (1998).

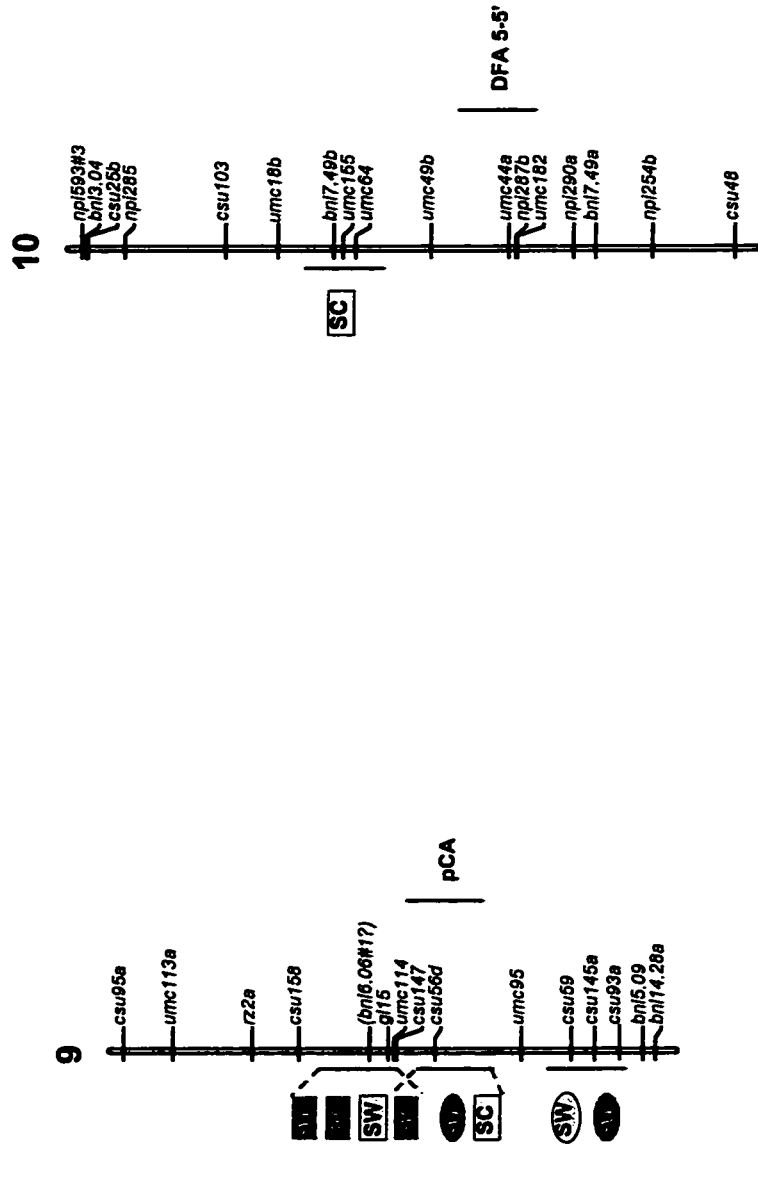


Fig 4.1e QTL Map (chromosome 9 and 10) for leaf feeding damage by SWCB (SW) and SCB (SC), nitrogen (PC), diferulic acid (DFA), p-coumaric acid (pCA). Insect resistance QTLs on the left of the chromosome from Groh et al., (1998).

marker assisted selection (MAS) techniques that avoid the costly infrastructure needed for direct phytochemical analysis.

The proposed mechanism of insect resistance is that leaf toughness in early stages of development in the whorl is mediated by cell wall fortification through the cross linking of hemicellulose microfibrils with diferulic acid. Stalk borers feed on immature leaf tissue progressively towards the center of the whorl. The cell walls in this tissue are still expanding and diferulic acid may act as a cell wall toughening agent before the deposition of lignin when cell wall expansion is halted. This increase in DFA contributes to antibiosis by inhibiting neonate larval penetration into the leaf tissue. In this study we have identified DFA QTLs tightly linked to insect resistance. Since insect resistance is polygenic, there are other compounds not measured that contribute to plant resistance. A greater understanding of secondary metabolism, cell wall biochemistry and plant development is needed. This combined approach to mapping insect resistant QTLs and putative phytochemical components is one of the fundamental tools that can elucidate the genetic basis of insect resistance (Stotz et al., 1999). Insect resistance in tropical and subtropical maize germplasm could be further improved by marker assisted selection through the accumulation of more desirable alleles.

## Chapter 5

### **Reduced feeding of the European corn borer (*Ostrinia nubilalis*) on corn transformed with germin, a wheat oxalate oxidase gene.**

*We cannot turn back the clock on agriculture and only use methods that were developed to feed a much smaller population.*

Norman Borlaug

#### **5.1 Introduction**

Lepidopteran stalk boring larvae cause significant economic losses to corn (*Zea mays* L.) production throughout the world. One of the major insect pests of corn is the European corn borer (ECB) (*Ostrinia nubilalis* Hübner). Damage from this pest occurs not only from direct feeding which causes loss of productivity but stalk tunneling damage from heavy ECB predation causes plant lodging resulting in further losses at harvest. European corn borer also acts as a vector for several *Fusarium* stalk-rot fungi which lead to a further destruction of plant material. Most corn producers do not use chemical treatments for ECB control in grain and silage crops but rely upon host plant resistance as the centerpiece of integrated pest management.

Plants have evolved diverse mechanisms for protection against pathogens eg fortification of cell walls, production of antimicrobial phytoalexins and activation of defense-related genes (Kombrink and Somssich, 1995). In response to herbivory, direct defense mechanisms are also activated that interfere with feeding, growth and development, fecundity and fertility of the herbivore. In addition, the induction of an array of volatiles creates indirect mechanisms of defense (Wallin, 2000).

A rapid production of reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ) is often associated with plant defense responses to induced genes that are involved in cellular protection (Levine et al., 1994). Also, the oxidative potential of  $H_2O_2$  to contribute to cell wall strengthening could retard insect predation, through the oxidative cross linking of specific cell wall structural proteins (Brisson et al., 1994), polymerization of coniferyl alcohol affecting lignin formation (Richardson et al., 1997) and cell wall peroxidase mediated cross linking of hemicellulose microfibrils with diferulic acid (Fry, 1986). These observations, together with the demonstration that physiological levels of  $H_2O_2$  could inhibit the growth of pathogens (Peng and Kuc, 1992), and  $H_2O_2$ -induced lipid peroxidation harms insect development and reproduction (Downer, 1986) led to the strategy of over-expressing a cell wall localized  $H_2O_2$ -generating enzyme in corn to reduce insect predation.

The wheat germin gene, isolated from genomic clone gf 2.8 (Lane et al 1991) codes for an apoplastic (Lane et al., 1986), protease-resistant (Grzelczak and Lane 1984) protein with peroxide-generating oxalate oxidase (OXO) activity (Lane et al., 1993), which catalyzes the oxidation of oxalate to carbon dioxide and  $H_2O_2$ . Germin is tenaciously and selectively associated with the highly substituted glucuronogalactoarabinoxylans (HS-GGAX) (Jaikaran et al., 1990) whose synthesis is closely allied with increased cell wall extensibility in cereals (Gibeaut and Carpita, 1991). Monocot cell walls are rich in glucuronoarabinoxylans which would favour a strong association of germin-like oxalate oxidases with the cell wall. This would include those generated from ectopically expressed transgenes. The role of germin-like oxalate oxidase in host plant resistance to pathogens is supported in studies of powdery mildew (*Erysiphe graminis* f. sp. *tritici*) infection of barley. Early phases of infection were associated with localized induction of oxalate oxidase and production of  $H_2O_2$  (Zhang et al.,

1995). This localized production of  $H_2O_2$  was associated with protein cross linking in papillae to reinforce the apposition and arrest pathogen invasion (Thordal-Christensen et al., 1997).

In order to augment  $H_2O_2$  production in the extracellular matrix of corn to affect insect predation, the wheat germin gene encoding mature protein, including the transient peptide, was expressed constitutively under the control of rice actin promoter in transgenic corn. This potential to increase ROS exploits the broad spectrum of physiological effects of  $H_2O_2$  on the interaction between plant and insect. We report here the effects of two independent germin transgenic events on ECB feeding in three corn inbred lines.

## **5.2 Materials and Methods**

### **5.2.1 Plant Material and Transformation**

The transformed plant material was provided by John Simmonds (Agriculture Canada, Ottawa). Immature embryos (1.5 – 2.0 mm long) of an A188 X B73 derivative were isolated and cultured on medium consisting of N6 salts and vitamins (Chu et al., 1975), 2 % sucrose, 1 mg/L 2,4-D, 25 mM proline, 100 mg/L vitamin-free casamino acids, 10  $\mu$ M silver nitrate and 0.6 % Phytagar (GibcoBRL). Embryonic cultures were maintained at 25°C in the dark and transferred to fresh medium every 2 weeks.

**Vector Construction.** **Pact-OXO:** The wheat oxalate oxidase (germin) gene was isolated from genomic clone gf-2.8 in pGermin (Lane et al., 1991) as a 0.9 kb Sph I fragment which was then blunt ended and ligated into the Sma I site of pCOR113 (McElroy et al., 1991). This construct was digested with HindIII to remove approximately 200 bp of germin 5' UTR, and then re-ligated to create pCOR113-ger. The nos 3' region was obtained from pAct1-D as a SacI/XbaI fragment, and this approximately 300 bp fragment was inserted downstream of the germin coding sequence in pCOR113-ger to create pact-OXO.

### **5.2.2 Particle Bombardment**

Particle acceleration (Klein et al., 1988) was performed with a PDS 1000/He gun (BioRad). Five micrograms of circular plasmid DNA, 2.5  $\mu$ g each of pAct-OXO and pAHC25 (Ubi-GUS-Ubi-BAR) (Christensen and Quail 1996) were precipitated onto 1.0  $\mu$  gold particles.

Embryonic maize cultures were bombarded 5-7 days after subculturing. Cultures were transferred to medium supplemented with 0.6 M mannitol for 2-4 hrs prior to bombardment and were removed to culture medium 14 hrs post-bombardment. Seven days post-bombardment, cultures were transferred to medium containing 2 mg/L bialaphos (Meiji Kaisha Ltd, Japan) and then subcultured onto fresh herbicide medium containing 0.5 mg/L 2,4-D and 10 mg/L benzyl adenine and cultured for one week in the dark. They were then transferred to 0.5 X salts, hormone-free, sucrose-free medium and cultured at 25°C in a 16 h photoperiod ( $50 \mu\text{Em}^{-2}\text{s}^{-1}$ ), from CW fluorescent tubes, for plant development. Rooted plants were established in soil and grown to maturity in greenhouses. Transgenic lines were confirmed by Southern analysis and transgenic function by OXO activity assays. Primary transgenic lines were backcrossed to a range of corn inbred lines.

### 5.2.3 OXO Assay

Histological: The assay buffer, comprised of 25 mM succinic acid, 3.5 mM EDTA, and 2.5 mM oxalic acid, pH 4.0; 0.6 mg/mL 4-chloro-1-naphthol, was added and dissolved by heating at 55°C just before use (Dumas et al., 1995). Oxalate oxidase activity resulted in the formation of a black precipitate after a few minutes at room temperature. Negative control reactions were transgenic tissue incubated without oxalic acid (Sigma) and non-transformed tissue incubated with oxalic acid.

**Quantitative OXO Assay:** Oxalate oxidase was determined spectrophotometrically as described in Sugiura et al., (1979) and Zhang et al., (1996) with some modifications. Corn leaf tissue was ground in liquid N<sub>2</sub>, approximately 0.05 g of this material was placed in a cold microfuge tube. Tubes and leaf tissue were kept on dry ice. Tubes with leaf tissue were then maintained at room temperature and 500 µL of dH<sub>2</sub>O was added and the tubes were vortexed for a few seconds. The tubes were then centrifuged at 1400 rpm for 10 minutes. The supernatant was discarded. To the pellet, 1 mL of dH<sub>2</sub>O was added and vortexed for a few seconds. The samples were then heated for 5 mins in an 80°C water bath to inactivate the endogenous catalases. The slurry was centrifuged at 1400 rpm for 10 mins, and the supernatant was discarded. The oxalate oxidase assay was carried out on the remaining pellet. For the OXO activity enzyme assay, the developing solution (100 mL) was prepared as follows: 40 mL of 0.1 M succinate buffer (pH 3.5), 60 mL of 100% ethanol, 8 mg of 4-aminoantipyrine, 8 mg of soybean peroxidase (60 U/mg) and 20 µL of N,N-dimethylaniline, plus 13 mg of oxalic acid. The oxalic acid was omitted from the control developing solution with transgenic tissue and was included in assays on non-transgenic tissue. One mL of developing solution (with oxalic acid) was added to the tissue pellet and 1 mL of control developing solution was added to the tissue pellet, and 1 mL of control solution to control tissue pellet, these were then vortexed for a few seconds. A standard curve for oxalate oxidase activity was generated using barley oxalate oxidase (Sigma). Standards, samples and control tubes were placed on a shaker at room temperature for 2 hrs. The reaction was stopped with the addition of 20 µL of 1 N NaOH. Tubes were then centrifuged at 1400 rpm for 5 min and the supernatant was transferred to a cuvette. Absorbance was read at 555 nm using a spectrophotometer. One

unit of enzyme activity was defined as the amount of the enzyme which formed 1  $\mu\text{mol}$  of  $\text{H}_2\text{O}_2$  per min in the standard assay system.

#### **5.2.4 $\text{H}_2\text{O}_2$ Assay**

The measurement of leaf  $\text{H}_2\text{O}_2$  was determined by luminol-dependent chemiluminescence based on the method described in Dat et al., (1998). Corn leaf tissue was harvested at noon from plants 6-7 weeks old grown under controlled conditions (25°C, 16h light: 8 h dark). The middle portion of a fully expanded V6 leaf (without the mid-rib) was used. Approximately 0.5 g was ground in liquid  $\text{N}_2$  and extracted in 3 mL of ice-cold 5 % trichloroacetic acid. (TCA). The crude extracts were centrifuged at 1400 g for 20 min. An aliquot of supernatant (0.5 mL) was passed through a 0.5 mL column (BioRad) containing 0.5 g of Dowex resin (1X1-100, chloride form, 1 % cross-linked). The column was equilibrated with 20 mL of cold 5 % TCA. After 0.5 ml of supernatant was added, the column was washed with 7 X 0.5 mL of cold 5 % TCA. The eluate from the column was collected on ice. All eluates were collected together. A mixture was prepared using 0.5 ml eluate, 0.5 ml 0.5 mM luminol (Aldrich) and 4.5 ml 0.2 M  $\text{NH}_4\text{OH}$  (pH 9.0) on ice. All solutions were kept cold. To a cuvette 0.5 ml of this mixture was added and analysed using a chemiluminescence meter (LKB1251 Luminometer). The chemiluminescence reaction was initiated by injecting 50  $\mu\text{L}$  of 0.5 mM potassium ferricyanide (Sigma) in 0.2 M  $\text{NH}_4\text{OH}$ . The emitted photons were counted for 5 s. A standard curve was generated using known amounts of  $\text{H}_2\text{O}_2$ .

### **5.2.5 *In vitro* insect feeding and greenhouse infestation assays**

*In vitro* insect leaf feeding bioassays were performed on mid-whorl leaf tissue from greenhouse grown plants using a modified Ascher et al., (1981) apparatus to measure the amount of consumption of a 1 cm<sup>2</sup> leaf disk by one third-instar ECB larva in a 24 h period. Larvae were reared and tested under a 16:8 (light:dark) photoperiod at 85% RH and 25°C/19°C (day/night) temperature regime. Forty bioassays were performed for each corn line.

Greenhouse infestation assays were initiated at mid-whorl stage (40 cm in height). Plants were manually infested with 2 egg masses (25 eggs/egg mass) at black head stage. Infestations were repeated after 2 days to simulate first generation corn borer egg laying. Egg masses were placed deep in the whorl of the plant using long forceps. Three weeks after infestation each plant was rated based on a 1 (no damage) to 9 (most leaves with long lesions) scale developed by Guthrie et al., (1960) to evaluate damage to whorl leaves from borer feeding. At maturity the plants were harvested, leaves removed and the stalk was split down the center. The cumulative lengths of borer tunnels were measured to determine the extent of internal ECB damage in a 90 cm section of stalk 45 cm above and below the primary ear node. Statistical analysis included an analysis of variance of each of the resistance parameters by genotype. These greenhouse trials were repeated twice.

### **5.2.6 Field Testing**

Transgenic maize plants in the field were evaluated under artificial ECB infestation. Null and Homozygous lines were planted in a randomized block design with 3 blocks of one row per rep with 15 plants per row. Each row was 3.8 m long with 76 cm between rows. Heterozygous lines were planted similarly, except two rows per block. Field plots were fertilized according to soil requirements and weeds were controlled using standard herbicides and hand weeding as necessary. Null and segregating transgenics were identified by OXO assay. Artificial infestation and effects of ECB feeding assays were performed as described in the greenhouse trials above.

### **5.2.7 Insect rearing and experimental diets**

A bivoltine strain of the European corn borer, obtained from French Agricultural Research (Lamberton, MN) was maintained in the laboratory at 80% RH and photoperiod of 18 h (26°C): 6 h (19°C) [L:D]. Insects were reared as described by Guthrie et al., (1985), except that corn cob grits were added to the culture diet but not to the experimental diets. The diets used in the experiments were prepared by mixing ultrapure 30% H<sub>2</sub>O<sub>2</sub> (Fisher) to unsolidified meridic diet at concentrations of 2 and 20 μmoles/g diet. These concentrations are realistic with respect to concentrations in transformed plants. Neonate ECB larvae were placed on the appropriate control or treated diets in glass scintillation vials plugged with cotton wool with 30 individuals per treatment. Larvae were weighed on days 7, 11, 14, 19, 26 and 29. Fresh diet was placed in the vial on day 15. In the oxalate oxidase experimental diets, concentrations of 2, 5, 10 and 20 X 10<sup>-4</sup> U/ml were prepared using barley oxalate oxidase (Sigma). Third instar larvae were first weighed and then placed on control or treated diets as described

previously and weighed 4 days later. The RGR (Relative growth rate =  $\ln$  weight gain/time) was calculated for each treatment.

### **5.3 Results and Discussion**

Leaf tissues of all three germin transgenic lines had significantly different oxalate oxidase activities whereas no activity could be detected in the corresponding germin null lines (Table 5.1). The highest level of expression was recorded for homozygous transgenic event (a) in CK44 (++), which was 3-fold greater than that obtained for the same event in the heterozygous state in CO286 (+-). This greater than additive response for the homozygous compared to the heterozygous transgenic state has also been observed in transgenic lines of the same genetic background (data not shown). The germin transformed lines and the null lines were morphologically indistinguishable.

Hydrogen peroxide levels in leaves were also significantly higher in the germin transgenic lines than in the null lines (Table 5.1) and these levels correlated with OXO activities. Germin homozygous (a) line had the highest level of hydrogen peroxide, followed by the higher heterozygous (b) event in CO328 and the level in the lowest heterozygous event (a) in CO286 was the lowest. Levels of  $H_2O_2$  were not significantly different in any of the null lines.

Oxalate oxidase activity was associated primarily with the cell wall of the transgenics. Histological assays clearly marked the cell walls (Fig 5.1A) but no activity was associated with cell walls of non-transformed tissue even after prolonged overnight staining in oxalate containing assay buffer (Fig 5.1B). Quantitative estimations of transient OXO activity in maize callus tissue (Table 5.2), were obtained from low speed tissue pellets, whereas the supernatant fractions were without activity. Similarly, OXO activity was only present in the pellet fractions of leaves of germin transformed lines (Table 5.1) and the

Table 5.1. Oxalate oxidase (OXO) activity, H<sub>2</sub>O<sub>2</sub> production and diferulic acid (DFA) in leaf tissue of corn transformed with the wheat germin gene.

Corn Line	Oxalate oxidase (X 10 <sup>-4</sup> / mg)*	H <sub>2</sub> O <sub>2</sub> (μmol/gFW)	DFA (μg/gDW)
<sup>a</sup> CK44++	5.74±0.20 a	1.85 ± 0.17 a	524.7 ± 167 ab
CK44--	0	0.87 ± 0.12 c	598.4 ± 184 a
<sup>a</sup> CO286+-	1.84±0.08 c	1.27 ± 0.27 b	525.6 ± 154 ab
CO286--	0	0.56 ± 0.06 cd	613.6 ± 127 a
<sup>b</sup> CO328+-	3.92±0.30 b	1.77 ± 0.18 a	436.7 ± 128 c
CO328--	0.	0.60 ± 0.05 cd	345.3 ± 130 cd

Corn plants are designated (++) homozygous for the transgene, (+-) heterozygous for the transgene, and (--) null for the transgene. <sup>ab</sup> designate unique transformation events. Plants were grown at 25°C day and 20°C night in a 16h photoperiod. OXO, H<sub>2</sub>O<sub>2</sub> and DFA were measured at mid-day on fully expanded V6 leaf. Means ± SEM within the same column followed by the same letter are not significantly different, SNL (P< 0.05), n=18 for each corn line.

\* One unit (U) of OXO activity forms 1 nmole H<sub>2</sub>O<sub>2</sub> per min from oxalate at pH 3 at 37°C. Activity was measured on low speed pellet fraction; the supernatant had no activity.

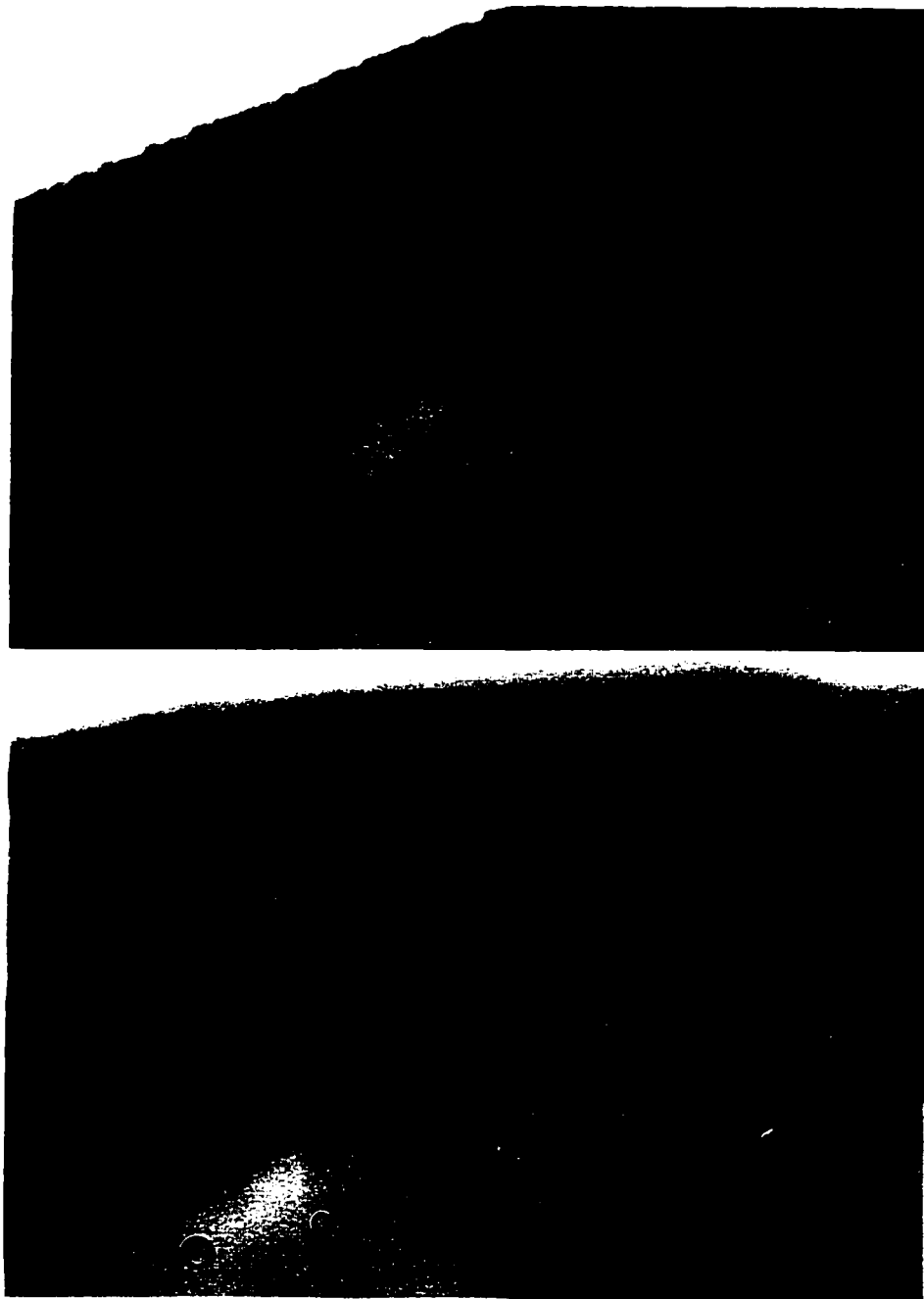


Fig 5.1A. Surface view of anther of pAct-OXO transformed corn. Oxidation of 4-chloro-1-naphthol produced a dark precipitate in the cell wall to locate activity of OXO. Viewed after 10 mins in histochemical stain; longer staining covers the anther surface to obscure wall architecture. Pollen viewed through tear in anther wall also shows OXO activity with longer staining.

Fig 5.1B. Surface view of anther of non-transformed corn. Viewed after 12 h in 4-chloro-1-naphthol histochemical stain.

Table 5.2. Localization of oxalate oxidase (OXO) activity in tissue pellets of water-extracted corn callus 48h after bombardment with pAct-OXO.

	<b>Oxalate Oxidase</b>	
	<b><u>Activity (U X 10<sup>-4</sup> / mg FW)*</u></b>	
	<b>Pellet</b>	<b>Supernatant</b>
<b>Non-Bombarded</b>	<b>0</b>	<b>0</b>
<b>Bombarded</b>	<b>0.32</b>	<b>0</b>

\* One unit (U) of OXO activity forms 1nmole H<sub>2</sub>O<sub>2</sub> per min from oxalate at pH 3 at 37°C

supernatant fraction was devoid of activity. This would suggest that the elevated  $H_2O_2$  levels were produced in the apoplast and could therefore influence chemistry in the extracellular matrix.

One aspect of cell wall chemistry investigated showed that diferulic acid (DFA), an indicator of cross linking of cell wall FAXX [O-(5-O-feruloyl- $\alpha$ -L-arabinofuranosyl)-(1 $\rightarrow$ 3)-O- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose] components was not influenced by the elevated levels of  $H_2O_2$ .

*In vitro* insect feeding assays, with third instar European corn borer larvae, on leaves from greenhouse grown plants showed that leaf consumption was similar on CK44 and CO286 germin null lines but was significantly less on CO328. However, in all cases the presence of the germin transgene significantly reduced ECB leaf consumption by 21-34 % (Table 5.3). Stalk tunneling activity in greenhouse grown plants was also significantly reduced by 40% in duplicated experiments (data not shown).

These greenhouse results were confirmed in a confined field trial. Field data is considered to be more reliable for such insect feeding assays because of the relatively low vigour of potted greenhouse plants and because greenhouse environments favour insect development. Field data confirmed the greenhouse results with the exception that the reduced feeding on the CO328 null line was not observed under field conditions. Visual leaf damage ratings, determined eight weeks after ECB infestation, were significantly reduced on all the germin transgene lines by 35-42% (Table 5.3). Genetic variation in stalk tunneling resistance was observed among the lines tested. CK44 had significantly more resistance than CO328, and CO286 was significantly the least resistant line. However, stalk tunneling damage, measured at plant harvest, was substantially reduced in all germin transgene lines

Table 5.3 European corn borer feeding on germin transformed maize.

Corn Line	Leaf Consumption (mm <sup>2</sup> )	Leaf Rating Field (1-9)	Stalk Tunneling Field (cm)
<sup>a</sup> CK44+	45.8 ± 5.43 b	2.17 ± 0.12 c	13.94 ± 1.54 d
CK44-	65.6 ± 8.46 a	3.75 ± 0.21 b	25.95 ± 2.11 c
<sup>a</sup> CO286+	41.3 ± 6.73 c	2.81 ± 0.23 bc	27.67 ± 1.52 c
CO286-	62.7 ± 9.45 a	4.38 ± 0.45 a	50.71 ± 2.17 a
<sup>b</sup> CO328+	37.9 ± 5.41 c	2.53 ± 0.09 bc	19.95 ± 1.23 cd
CO328-	48.1 ± 7.75 b	4.14 ± 0.54 a	33.35 ± 1.89 b

Corn plants are designated (++) homozygous for the transgene, (+-) heterozygous for the transgene, and (--) null for the transgene. <sup>ab</sup> designate unique transformation events. Leaf tissue bioassays ran for 24 hrs with one third-instar ECB larva per apparatus (n=40 for each corn line). Visual leaf ratings based on the Guthrie 1-9 scale were determined eight weeks after infestation (n=45 plants for each line). Stalk tunneling was measured at maturity, ECB tunneling damage was measured in a 90 cm section of stalk, 45 cm above plus 45cm below the primary ear node (n=45 plants for each line). Means±SEM within the same column followed by the same letter are not significantly different, SNK test (P<0.05).

(Table 5.3 and Fig 5.2). Reductions in stalk tunneling ranged from 40 % in CO286 (+-) to 46 % in CK44 (++) and CO286 (+-).

To test whether there was a direct effect of  $H_2O_2$  on insect feeding, artificial feeding trials were conducted with ECB larvae on diets supplemented with  $H_2O_2$  at a level ( $2 \mu\text{mol/g}$  diet) similar to that generated in the germin transgenic leaves. At this level of  $H_2O_2$ , growth and development of the ECB larvae were significantly reduced. On control diets larvae reached maximum weight in 20 days, whereas weight gain was reduced by 50% on the  $2 \mu\text{mol/g}$   $H_2O_2$  diet (Fig 5.3). The time interval to reach pupation was also substantially increased from 24 to 39 days. The diet containing  $20 \mu\text{mol/g}$   $H_2O_2$  was toxic to the larvae.

The relative growth of ECB larvae was not affected by the inclusion of barley oxidase (Sigma), at rates  $2 \times 10^{-4}$  -  $2 \times 10^{-3}$  U/mL, in the insect diet (Table 5.4). The highest dose,  $2 \times 10^{-3}$  U/mL, was more than 6-fold greater than the highest OXO activity recorded in the transgenic lines used in this study.

Plants react to pathogens with a broad range of defense responses including the generation of reactive oxygen species (ROS) constitutively in significant amounts, mainly  $H_2O_2$ , which is associated predominantly with the extracellular matrix (Bolwell and Wojtaszek, 1997). The strategy of targeting expression of a constitutively regulated germin gene to generate  $H_2O_2$  in the extracellular matrix, at the initial site of contact with the insect, augments natural ROS defense systems. Temporal and spatial correlation of germin (oxalate oxidase) activity in germinating wheat (Lane 1994) and immunocytological evidence (Lane 2000) proved that germin was associated with the cell wall. Oxalate oxidase activity was demonstrated in purified cell walls of germinating wheat (Lane 2000) and in cell walls isolated from maize seedlings (Vuletic and Sukalovic 2000). Ectopic localization of

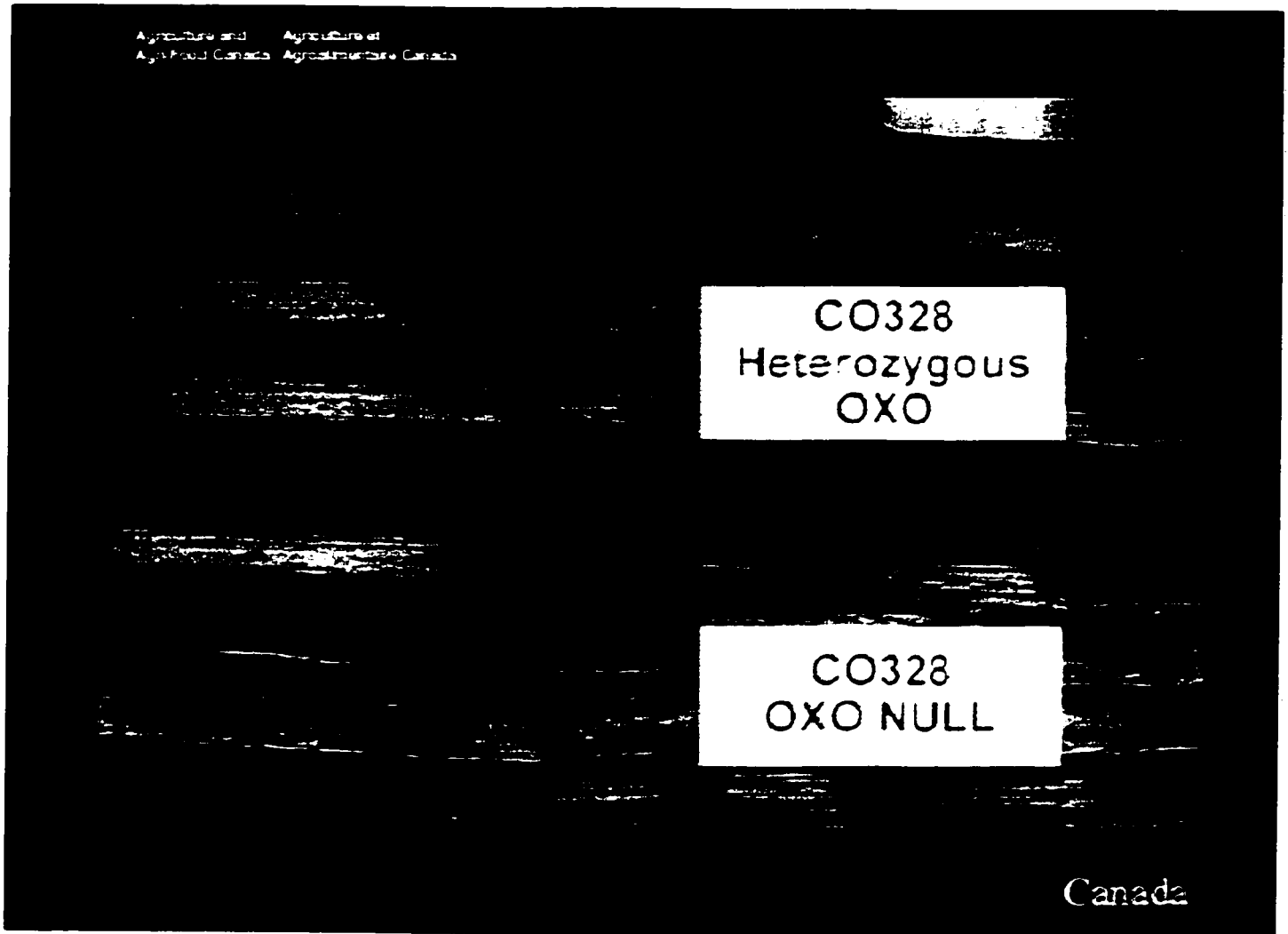


Fig 5.2. Split stalks of corn at harvest showing tunneling damage from European corn borer (ECB) infestation. Inbred line CO328, null for the germin transgene had severe damage. The (hemizygous) heterozygous germin line had significantly less ECB damage.

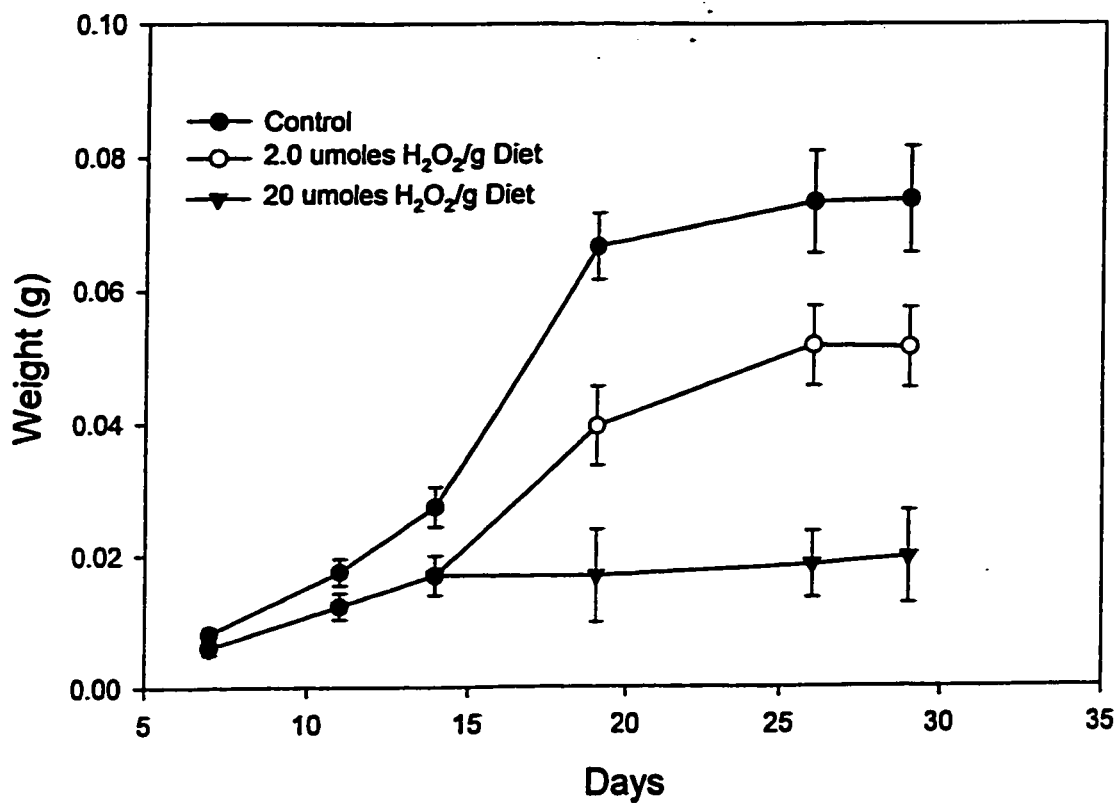


Fig 5.3. the effect of H<sub>2</sub>O<sub>2</sub> – supplemented diet on larval growth of the European corn borer.

Table 5.4. Effect of barley oxalate oxidase (Sigma) on relative growth rate *in vitro* of the European corn borer larvae.

	Oxalate Oxidase (U X 10 <sup>-4</sup> ) *				
	0	2	5	10	20
Initial wt (mg)	8.5	7.2	8.4	8.3	8.9
Final wt (mg)	31.5	26.6	31.9	29.1	31.9
Relative Growth Rate **	0.32	0.32	0.33	0.31	0.34
(Standard Error)	(0.022)	(0.011)	(0.016)	(0.012)	(0.015)

\* One unit (U) of OXO activity forms 1 nmole H<sub>2</sub>O<sub>2</sub> per min from oxalate at pH 3 at 37°C.

\*\* Relative Growth Rate = (ln Final Wt – ln Initial wt) / time ; n = 20.

OXO activity in transgenic corn was shown to be associated with the cell wall (Fig 5.1, Table 5.1 and 5.2).

Transgenic corn lines with stable expression of cell wall associated OXO activity for seven generations (Simmonds et al., 2001) were used for ECB predation studies. Germin homozygous and heterozygous lines were generated. OXO activity could not be detected in leaf tissue of the segregating transgene null lines. Native oxalate oxidase activity was associated only with the germination phase in corn. It has been well documented that germin is selectively expressed during germination and is an unambiguous molecular marker of wheat embryo germination (Grzelszak et al., 1985). Ectopic expression of germin produced significant OXO activity in corn leaf tissue. Activity in the germin homozygous line (CK44 ++ ) was three times greater than in the germin heterozygous line (CO286 +-). A similar greater than additive effect of the transgene in the homozygous state has been observed for the event in the same genetic background. This was unexpected, but encouraging, in view of reports that increased transgene copy number can initiate gene silencing (Matzke et al., 1994).

H<sub>2</sub>O<sub>2</sub> levels in leaves were measured at mid-day to eliminate any diurnal effects of native production. The levels in all null lines were not significantly different but all the transgenic lines contained significantly more H<sub>2</sub>O<sub>2</sub> (2-3 fold) than the null lines. The generation of this extra H<sub>2</sub>O<sub>2</sub> from oxalate is problematic because the level of oxalate in corn leaves is relatively low, 0.2 % d. wt (Wadleigh and Shive, 1939) and the neutral pH of the extracellular matrix would not favour OXO activity with a pH optimum of 3-4. Recent evidence that germin acts as OXO at pH 3.8 but has superoxide dismutase (SOD) activity at pH 7.5 (Woo et al., 2000) suggests that the elevated production of extracellular H<sub>2</sub>O<sub>2</sub> in the

maize germin transgenics is more probably the result of SOD activity. These high  $H_2O_2$  producing lines were maintained through seven generations of selection with no discernable phenotypic or morphological differences compared to the null lines.

Generation of hydrogen peroxide in the extracellular matrix is required for the key peroxidase cross-linking reactions of cell wall biochemistry including the cross-linking of ferulates, coumarates and/or extensins to the pectins and the hemicelluloses in plant cell walls (Fry 1986).

The content of isomers of dehydrodiferulic acid (DFA) in corn leaves is highly negatively correlated across genotypes of maize field leaf damage ratings by the European corn borer (Bergvinson et al., 1994a) and increased levels of ferulic acid have been localized in the epidermal cell wall of resistant maize lines (Bergvinson et al., 1994b). Additional evidence for the role DFA was provided by the observation that DFA content of maize increased in cycles of selection for borer resistance in the Iowa stiff stalk synthetic BS9 (Bergvinson et al., 1997). However, recent findings that DFA is produced intracellularly in the Golgi cisternea and then exported into the developing cell wall (Fry et al., 2000) suggests that cell wall bound peroxidase activity would not affect DFA production. This is supported by evidence of no significant correlation between the levels of extracellular peroxidase activity and the levels of DFA in corn leaves (MacIssaac 1999) and evidence (Table 5.1) that elevated  $H_2O_2$  generated from cell wall associated enzyme activity had no effect on DFA content of the OXO transgenic corn lines. Reduced predation of ECB on germin transgenics is the result of a novel, non-DFA source of resistance. It is possible that the elevated extracellular  $H_2O_2$  effects some other aspect of cell wall chemistry or leaf nutrient availability (Felton et al., 1989) to restrict insect feeding.

A central role has been proposed for H<sub>2</sub>O<sub>2</sub> as a signaling mechanism to activate plant defense genes (Brisson et al., 1994), thus elevated levels of H<sub>2</sub>O<sub>2</sub> could attenuate native insect predation resistance mechanisms. The induction of a germin-like protein (GLP) in *Nicotiana attenuata* leaves a few hours following exposure to larvae of the tobacco hornworm (*Manduca sexta*) (Hermsmeier et al., 2001) suggests that GLP-generated H<sub>2</sub>O<sub>2</sub> may be part of the plant defense response to insect predation. Enhanced resistance to ECB predation in the germin transgenics provides evidence that GLP induction has causality in induced resistance to insect feeding. Maize germplasm used for germin transformation exhibited diverse predation resistance, CK44 having most and CO286 having the least resistance to insect tunneling. Thus these germin transgenics provides a unique germplasm to identify and to explore the effects of H<sub>2</sub>O<sub>2</sub> on native resistant mechanisms.

The possibility that H<sub>2</sub>O<sub>2</sub> or germin could act directly on insect physiology to retard insect growth and development was investigated in *in vitro* feeding assays.

Lipid peroxidation is potentially very harmful to insects, not only because of general effects on membrane disfunction, but also because of affects on specific aspects of insect physiology such as cuticular dessication, function of juvenile hormones and on the production of pheromones (Downer et al., 1986). Oxidative damage from super oxide can be alleviated by superoxide dismutase, which generates H<sub>2</sub>O<sub>2</sub>. In turn, this can be converted to the hydroxyl radical which is more lipophilic than superoxide and therefore more capable of lipid peroxidation. Management of H<sub>2</sub>O<sub>2</sub> levels is therefore critical for successful insect predation. The constitutive levels of catalase (CAT) in larvae of the cabbage looper (*Trichplusia ni*), southern armyworm (*Spodoptera eridania*) and black swallowtail (*Papilio polyxenes*) all correlate with the natural feeding habits of these insects subjected to

endogenous sources of oxidative stress from H<sub>2</sub>O<sub>2</sub> (Ahmad and Pardini, 1990). This CAT activity, with higher and broader subcellular distribution than observed in most mammalian species, is critical for the prevention of excessive accumulation of cytotoxic H<sub>2</sub>O<sub>2</sub>.

When ECB larvae were fed an artificial diet supplemented with H<sub>2</sub>O<sub>2</sub>, at levels comparable to that in germin transformed leaf tissue, insect growth and development were significantly reduced (Fig 5.3) and the time interval to pupation was substantially increased from 24 to 39 days. A diet containing 20 µmol/g H<sub>2</sub>O<sub>2</sub> was toxic to the larvae. Similarly, the corn leafhopper (*Dalbulus maidis*) showed a reduction in feeding on H<sub>2</sub>O<sub>2</sub> containing diets (Dowd and Vega, 1996). The data demonstrate that H<sub>2</sub>O<sub>2</sub> has potential for providing resistance to herbivory through a direct affect on insect physiology.

Germin could affect insect physiology directly through its capacity to bind sugars, possibly affecting chitin production, or through an indirect affect of generating H<sub>2</sub>O<sub>2</sub> either from ingested or metabolic oxalate or from SOD activity. The failure of barley oxalate oxidase, at levels 6-10 fold higher than the content of the transgenic leaves, to impede insect growth (Table 5.4) suggests that reduced ECB predation was not due to an effect of germin on insect physiology.

Reduced ECB predation due to H<sub>2</sub>O<sub>2</sub> affects on cell wall biochemistry or activation of native resistance mechanisms remain to be explored. However, it has been demonstrated that the level of H<sub>2</sub>O<sub>2</sub> generated in germin transformed leaf tissue was sufficient to significantly retard the growth and development of ECB larvae and is therefore an important component of plant insect resistance mechanisms.

*In vitro* leaf feeding bioassays demonstrated that ECB larvae feeding was significantly reduced on all germin transgenic lines. This was confirmed under field conditions on plants

infested with ECB larvae. Most significantly, stalk tunneling damage, measured at plant harvest, was substantially reduced in all transgenic lines. The reduction of tunneling by 50% in the transgenic lines should also reduce further crop losses resulting from plant lodging caused by heavy infestations of ECB. This substantial reduction in tunneling is indicative of lower levels of ECB survival which may have significant effects on ecological ECB epidemiology.

Novel genes, such as Bt (insect toxins produced by *Bacillus thuringiensis*) have been introduced into corn hybrids to provide resistance against the ECB. Given the high selection pressure of Bt corn, it is inevitable that ECB resistance to Bt will develop unless effective resistance management practices are developed (de Maagd et al., 1999; Bourguet et al., 2000). Thus, it is important to develop alternative resistance mechanisms, based preferably strategies of natural plant resistance mechanisms, that slow insect growth and development and reduce fecundity rather than cause massive population depletion.

We have shown that the ectopic expression of a wheat gene, germin, conferred resistance to the European corn borer in transgenic corn plants. These transgenic plants with constitutively higher levels of H<sub>2</sub>O<sub>2</sub> afford a unique opportunity with which to study the physiological functions of elevated levels of extracellular H<sub>2</sub>O<sub>2</sub> on cell wall chemistry and insect herbivory.

## Chapter 6

### General Conclusions and Discussion

*Farming looks mighty easy when your plow is a pencil and you're a thousand miles from a corn field.*

Dwight D. Eisenhower

#### 6.1 Main Conclusions

The present thesis has contributed significantly to our understanding of corn resistance to insects, and in particular the role of phenolics, leaf toughness, segregation of QTLs, and cell wall oxalate oxidase.

The work with soluble phenolics in sorghum demonstrated the importance of phenolics as resistance factors in cereals to insect pests (Chapter 2). A simple, rapid and reliable test is a good indicator of resistance to *Sitophilus oryzae*, an important stored products pest. This study also confirmed the essential role that traditional farmers play in the development and maintenance of landraces.

The studies in corn presented here elucidated for the first time a phenolic based mechanisms of natural resistance to major tropical pests, southwestern corn borer and sugarcane borer. Plant toughness mediated through phenolic cell wall cross-linking represents an important correlate with reduced leaf feeding and plant damage. This has been observed in two years of field data. Reduced larval feeding by the southwestern corn borer and the sugarcane borer on leaf tissue is strongly correlated to elevated levels

of cell wall bound phenolics. Leaf toughness is inversely related to leaf damage ratings, with diferulic acid content being the best regression variable to account for toughness and leaf feeding (Chapter 3). Diferulic acid acts as a cross-linking agent within the immature leaf tissue and correlates the toughness of this tender tissue. Leaf nitrogen content is also a good indicator of resistance. The results of the study suggest a nutrition-based resistance in which lower levels of nitrogen act in concert with elevated levels of cell wall phenolic dimmers to reduce the accessibility of nitrogen and hinder larval development.

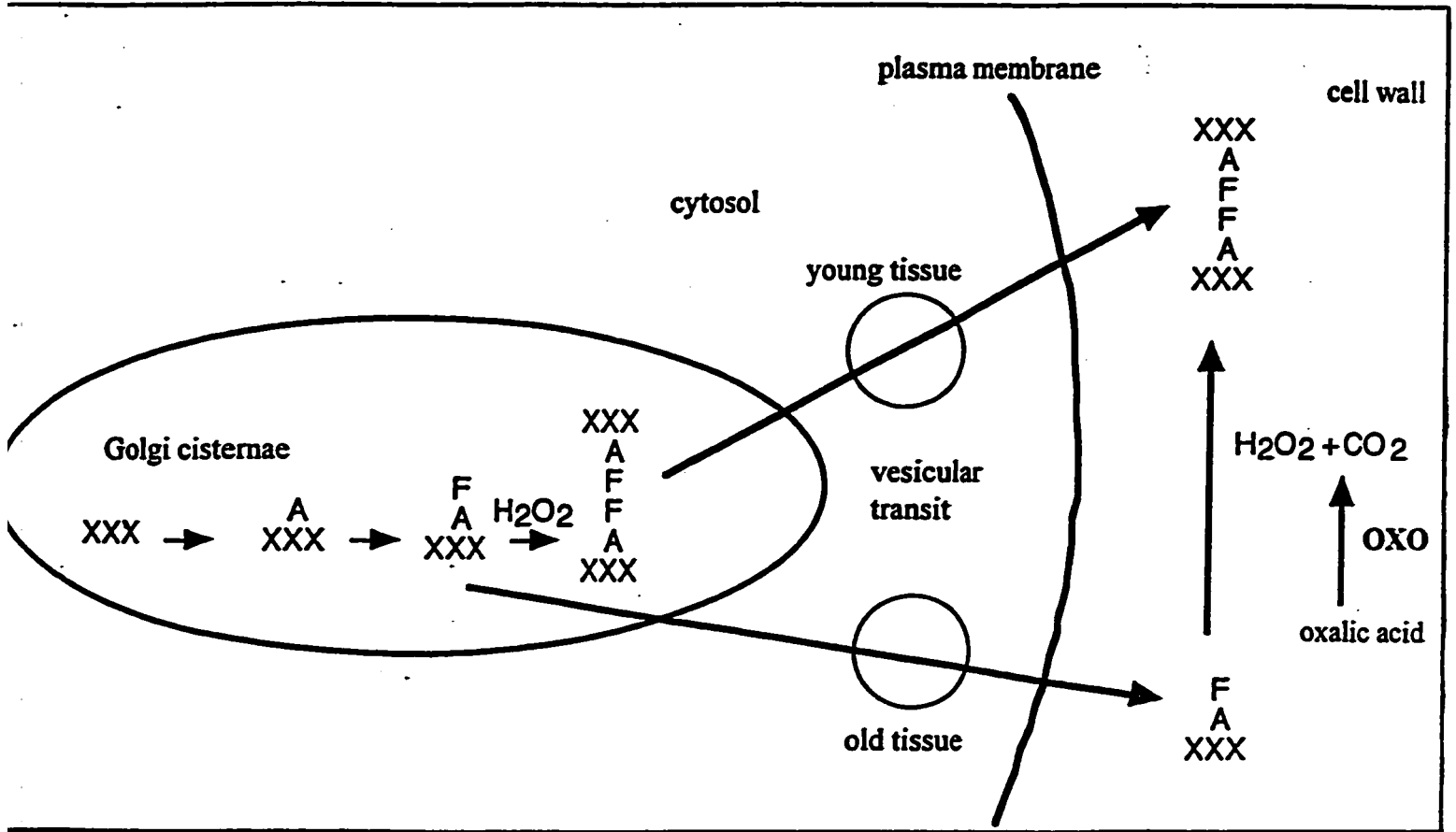
Resistance to insects is often continuously variable across genotypes and controlled by segregation of quantitative trait loci (QTLs). The QTL map for resistance to the SWCB and SCB detected eight QTLs for leaf feeding damage within common genomic regions for both insects (Groh et al., 1998). In this present study, eight QTLs were identified for 5-5' DFA and two for 8-5' DFA. Five QTLs for 5-5' DFA and one QTL for 8-5' DFA were closely associated with SWCB and SCB leaf feeding resistance (Chapter 4). Five QTLs for leaf toughness were linked to leaf feeding damage. This is the first study to demonstrate a QTL mapping link between the secondary metabolite DFA (5-5' and 8-5') and insect resistance to a tropical and a subtropical stem borer. This association of insect resistance QTLs and phytochemical QTLs provides evidence of a genetic basis for association of insect resistance and phytochemistry.

The transformation of maize cells with wheat oxalate oxidase resulted in higher levels of cell wall oxalate oxidase activity and hydrogen peroxide. The transformation was designed as a direct test of the DFA mechanism in which transformation was expected to increase DFA levels and insect resistance. Under field conditions with plants

infested with ECB larvae, feeding was significantly reduced on all OXO transgenic lines (Chapter 5). Most significantly, stalk tunneling damage, measured at plant harvest, was substantially reduced in all OXO transgenic lines. The reduction of tunneling by 50% in the OXO transgenic lines is indicative of lower levels of ECB survival and this should also reduce crop losses resulting from the effect of heavy infestations of ECB and subsequent plant lodging. Surprisingly, the levels of diferulic acid in the OXO transformed lines remained similar to non-transformed corn lines, despite 2-fold increase in hydrogen peroxide in the transgenic lines. This finding is consistent with the very recent results of Fry et al., (2000), in which the intraprotoplasmic rather than the extracellular formation of diferulic acid was demonstrated (Fig. 6.1). One possible route to manipulating DFA would be to alter the cytosolic concentration of hydrogen peroxide. Altering the extracellular levels of hydrogen peroxide in the transformed lines had no effect on the levels of DFA.

## **6.2 Implications**

The rising costs of pesticides, the increasing resistance of insects to them and their undesirable effects on the environment coupled with their negative public perception, has led to renewed efforts to understand, describe and exploit host plant resistance to pests. The challenge facing breeders, entomologists and phytochemists is finding non-anthropotoxic natural products that enhance the innate pest-fighting powers in cultivated plants. Higher plants use the products of the Shikimate pathway as precursors for a large number of secondary metabolites, among them plant pigments, compounds that defend



► Fig. 6.1. Model for the metabolic pathway for the synthesis of diferulic acid (XXX - xylan backbone, A - arabinose, F - ferulic acid, OXO - oxalate oxidase; based on Fry et al., 2000)

against insects and other herbivores (such as phenolics), UV light protectants and lignin (Herrmann, 1995). Under normal growth conditions, 20% of the carbon fixed by plants flow through the shikimate pathway (Haslam, 1993). The present study has demonstrated the importance of phenolic cell wall cross-linking as a mechanism of insect resistance. One recent approach for augmenting host plant resistance within the IPM framework has been pyramided resistance. This strategy develops crop plants (either through conventional breeding or genetic engineering) that produce two or more unrelated but compatible defense compounds simultaneously. The advantage of this system is a significant delay in pest adaptation to the multiple defenses. An example of this approach could be Bt corn with another resistance factor, insuring against massive crop failure once insects adapt to Bt.

Insect control in agriculture carries a heavy economic and environmental cost. The annual worldwide expenditure on insecticides is \$ 8.1 billion US. Worldwide crop losses caused by insect pests, in spite of the use of chemical insecticides is \$ 98 billion US (Krattiger, 1997). It is widely held that crop protection primarily based on chemical pesticides is unsustainable. It is therefore not surprising that insect-resistant transgenic plants were among the first products of plant biotechnology to reach the marketplace. We are now in an interesting phase in agriculture and plant biotechnology; some have called this era the third wave. Traditional farmers represent the first wave, modern plant breeders represent the second wave and genetic engineers are the third wave. We are actually entering a second green revolution. The current scientific and public controversies surrounding genetically modified organisms is particularly relevant to pest control. Public acceptability of a transgene, such as germin, from a related grass (wheat)

into corn may be more favourable than CryA bacterial gene producing the toxic Bt protein, which is currently in use. Ultimately though, the entry of any transgenic into the public domain should be through a combination of peer-reviewed scientific processes, regulation and public awareness.

### **6.3 Future Work**

The QTLs for diferulic acid can be used as a molecular marker for insect resistance. Fine mapping is now needed to determine the genes in these QTLs. Genes involved in the regulation of plant cell wall development or phenolic biosynthesis may be found in these QTLs. Yield trials would be the next step with the transgenic material. Incorporating germin into elite agronomic lines and repeating the ECB infestations would demonstrate its efficacy. The regulation of the synthesis of diferulic acid is still unknown. Diferulic acid is the product of a complex, multi-enzyme pathway. Whether cellular compartmentalization plays a major role in DFA synthesis remains to be determined. Manipulating the metabolic pathway to DFA is a challenge that was not achieved with OXO transformation, but may be achievable with other transformation strategies. Direct transformation of elite maize for higher expression of natural resistance based of DFA would eliminate tedious and costly recurrent selection for insect resistance.

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**Appendix Insect Ratings and Phytochemical Data for all RILs 1996B, Tlaltizapan.**

<b>PLANT RIL</b>	<b>SCB</b>	<b>SWCB</b>	<b>GSM (mg/gDW)</b>	<b>FIBER (mg/gDW)</b>	<b>P - CA (ug/gDW)</b>	<b>Trans FA (ug/gDW)</b>	<b>Cis FA (ug/gDW)</b>	<b>Total FA (ug/gDW)</b>
199	5.4	6.3	0.2543	0.364	1175.515	3135.091	507.7675	3642.858
	5.3	6.8	0.2839	0.322	1101.558	2779.178	492.0075	3271.185
292	5.9	6.6	0.2668	0.363	1126.224	2993.784	462.8269	3456.611
276	5.4	5.9	0.2841	0.356	1087.806	2437.98	414.6886	2852.669
319	4.4	6.3	0.3198	0.352	818.0425	2164.702	446.4513	2611.153
234	4.4	6.2	0.3418	0.366	951.7016	2503.096	460.5373	2963.633
213	5.2	6.3	0.3098	0.3222	1304.259	3994.982	439.6377	4434.62
181	3.8	5.9	0.2585	0.2794	1536.463	4026.593	734.3909	4760.984
328	4	5.4	0.2887	0.332533	2046.993	3517.695	583.7591	4101.454
331	4.1	4.9	0.2696	0.3562	1681.472	3161.507	432.1054	3593.612
251	4.4	6.7	0.2695	0.3134	1579.198	3613.147	398.0311	4011.178
178	4.2	5.8	0.2721	0.358133	1391.353	4179.765	577.1971	4756.962
211	4.6	6.7	0.2764	0.4119	1720.418	3229.843	348.5135	3578.356
305	4.6	6.2	0.2688	0.3691	1127.754	2784.632	478.8096	3263.442
284	6.7	6.8						
320	3.1	4.8	0.3248	0.3404	1650.785	2846.935	453.824	3300.759
	5.1	6.1	0.3266	0.3267	1863.786	3484.752	390.9903	3875.742
241	5.6	6.9	0.3546	0.2768	1404.078	2894.714	508.624	3403.338
209	6.1	6.9	0.2896	0.365533	1151.077	2428.696	398.1012	2826.797
340	4.9	6.6	0.2886	0.327	1689.983	2871.426	361.2744	3232.701
223	5.4	6.8	0.3010	0.3048	1542.852	2562.684	419.7611	2982.445
244	4.2	5.9	0.2898	0.394667	1353.43	3527.919	536.7203	4064.639
262	5.4	6.5	0.3282	0.3884	1247.814	2836.986	531.3743	3368.36
323	4.9	5.8	0.3580	0.2954	1256.12	2625.696	376.0256	3001.722
238	5	6.7	0.2906	0.3329	1547.092	3617.315	620.3485	4237.663
204	5	6.5	0.2981	0.3286	1247.009	3359.617	357.811	3717.428
278	4.9	6.4	0.2807	0.3132	1632.637	3919.799	456.6937	4376.492
338	4.7	6						
	6.4	7.7	0.3085	0.3415	1106.984	2410.701	296.3243	2707.025
220	5.3	6.9	0.5498	0.329667	1570.285	3337.544	368.3356	3705.88
308	4.3	5.4	0.2829	0.3116	1463.433	3727.144	535.2	4262.344
226	4.4	6.5	0.2836	0.285	1621.898	3043.577	419.8183	3463.396
246	5.4	6.3	0.2625	0.345333	1483.515	3692.214	467.05	4159.264
229	3.9	5.2	0.2937	0.3206	1939.446	3926.87	517.5605	4444.431
237	5.3	6.7	0.3191	0.299467	1159.417	2477.111	535.2719	3012.383
295	4.5	5.7	0.2821	0.338867	1664.897	3250.814	504.2123	3755.026
271	4.5	6.2	0.3186	0.296333	1569.13	3086.399	431.4173	3517.816
197	4.9	6.3	0.2802	0.3468	1949.62	3712.527	417.386	4129.913
317	4.3	6	0.2818	0.3896	1746.188	2911.475	383.0795	3294.555
217	4.6	6	0.2652	0.3402	1112.305	2996.327	389.6676	3385.994
264	5.5	6.6	0.3278	0.4008	1402.123	2373.153	405.9406	2779.093
180	4.8	6	0.2957	0.359467	1605.148	3240.95	489.0046	3729.954
	5.2	6.3	0.3023	0.299867	1429.273	2685.586	405.6228	3091.209
303	4.7	6	0.3073	0.305133	1765.091	3434.164	520.5551	3954.719
	4.7	5.9	0.3127	0.3772	1492.066	3688.959	479.2827	4168.241
245	4.7	6.2	0.2658	0.3375	1434.719	2878.495	509.0725	3387.568
265	5.5	6.6	0.2990	0.2924	1038.131	2343.986	396.7068	2740.693
286	4.4	5.9	0.2665	0.4116	1417.601	2947.531	370.9269	3318.458
232	4.5	6.3	0.2943	0.281933	1623.565	3575.959	555.1139	4131.073

PLANT RIL	SCB	SWCB	GSM (mg/gDW)	FIBER (mg/gDW)	P - CA (ug/gDW)	Trans FA (ug/gDW)	Cis FA (ug/gDW)	Total FA (ug/gDW)
310	4.7	6.7	0.3066	0.333	1149.958	2893.938	505.6896	3399.628
190	3.7	5.8	0.2874	0.3294	1909.756	3592.24	482.4345	4074.674
208	4.6	6	0.3302	0.284133	1401.45	2860.217	335.207	3195.424
187	5.2	6.4	0.3498	0.337267	1047.194	2468.785	379.6254	2848.411
289	5.8	6.7	0.2911	0.3791	1120.912	2904	397.88	3301.88
332	3.8	5.5	0.3246	0.311	1675.274	3751.747	410.6862	4162.433
321	4.1	5.7	0.2876	0.3102	1425.301	3630.863	551.7693	4182.632
215	5.6	6.9	0.3116	0.3106	1017.692	2478.949	414.8867	2893.835
186	5.1	6.1	0.2832	0.3118	1226.849	2347.26	414.1388	2761.399
231	4.6	6.1	0.3140	0.262	1223.307	2802.166	487.6641	3289.83
313	4.2	5.6	0.3496	0.365	1535.884	2689.01	380.0014	3069.012
255	3.9	5.6	0.2767	0.385867	1625.439	3257.7	430.5542	3688.254
198	5.5	6.7	0.2950	0.321267	1612.235	2428.404	307.0244	2735.429
298	4	6.1	0.2511	0.3777	1761.629	3498.691	515.0788	4013.77
270	4.5	5.6	0.2714	0.3563	1356.664	3522.142	652.0024	4174.145
293	5.2	6.9	0.3702	0.375	1089.816	2364.861	415.9664	2780.827
207	5.3	6.5	0.2743	0.3284	1753.891	3841.49	372.3783	4213.868
203	4.5	6.2	0.2479	0.345333	1514.573	3896.448	495.2262	4391.675
253	4.8	6.2	0.2713	0.3536	1271.419	2889.72	427.9065	3317.626
316	4.1	5	0.3224	0.4186	1574.607	3157.318	559.176	3716.494
327	4.2	6.1	0.2973	0.3786	1186.758	4120.013	558.8406	4678.853
263	4.4	5.7	0.2802	0.315867	1609.17	2714.111	465.3397	3179.45
195	4.6	6.4	0.3057	0.2996	1674.081	3693.662	458.4015	4152.064
267	4.6	6.5	0.2731	0.368	1953.569	2706.939	382.7916	3089.731
221	4.9	6.6	0.2763	0.335667	1718.028	3080.272	397.2793	3477.551
214	4.2	6.5	0.2701	0.285867	1139.225	3009.812	481.7652	3491.578
300	4.5	6.7	0.2829	0.349133	1233.066	2872.113	441.0863	3313.2
282	5.2	6.8	0.2780	0.3168	1198.02	2743.122	474.2284	3217.35
302	5.3	6.3	0.2885	0.3108	1216.539	2617.291	585.1064	3202.397
335	4.9	6.4	0.2741	0.337267	1475.157	2874.9	409.8047	3284.705
260	5.3	6.7	0.3258	0.3381	1079.438	3089.913	434.5187	3524.432
205	4.4	6.6	0.2636	0.3012	1689.809	3759.195	497.5239	4256.719
218	5.4	6.6	0.2814	0.3469	1334.402	3418.565	384.6156	3803.18
0	4.6	6	0.2992	0.3317	1546.608	3050.059	367.9697	3418.029
283	5.7	6.6	0.3370	0.321	887.158	2405.444	310.6773	2716.121
325	5	6.3	0.3087	0.3361	1441.365	2941.079	416.3704	3357.45
224	5.7	7	0.2470	0.3311	1477.712	4114.622	535.4069	4650.029
268	5.1	6.5	0.3098	0.3471	1682.463	3423.36	711.019	4134.379
247	5.3	6.1	0.3063	0.276333	1539.848	2337.781	306.5201	2644.302
309	4	5.5	0.3109	0.3275	1518.091	3780.621	550.0566	4330.677
274	5	6.4	0.2812	0.3272	1814.319	3488.583	517.4217	4006.005
336	5.7	7	0.2733	0.342533	1347.26	2881.042	397.2841	3278.326
			0.3714		1440.74	3111.362	297.1119	3408.474
301	5.8	7.4	0.3235	0.3258	1374.649	2607.373	410.8045	3018.178
182	3.7	6.2	0.2890	0.3158	1722.408	3674.12	431.8841	4106.004
258	5.8	6.5	0.3288	0.4004	1025.407	2643.683	387.1452	3030.828
233	5.7	6.6	0.2995	0.3035	1148.965	3045.556	406.9099	3452.466
296	5.1	5.9	0.2991	0.335733	1592.396	3197.153	438.6277	3635.781
228	4.8	6	0.2964	0.333867	1252.431	3395.994	447.125	3843.119

PLANT RIL	SCB	SWCB	GSM (umg/gDW)	FIBER (mg/gDW)	P - CA (ug/gDW)	Trans FA (ug/gDW)	Cis FA (ug/gDW)	Total FA (ug/gDW)
194	4.9	6.8	0.2932	0.3548	1597.15	3502.228	409.2951	3911.523
269	5.2	6.4	0.2780	0.346	1512.347	2420.998	428.944	2849.942
219	5.1	6.3	0.2571	0.3564	1680.37	3635.666	530.7197	4166.386
227	3.5	5.1	0.2890	0.3313	2115.613	4177.71	495.6425	4673.352
254	5.3	6.6	0.3161	0.321867	1349.389	3552.928	489.7961	4042.724
206	4	5.2	0.2863	0.375533	1365.4	3625.774	440.7815	4066.555
249	5.3	6.8	0.3020	0.3667	1516.547	3497.984	436.3518	3934.336
307	4.5	6.3	0.2866	0.3246	1022.218	2387.131	475.0081	2862.139
288	4.7	5.9	0.2542	0.3609	2147.809	3740.511	353.5591	4094.07
326	3.8	5.5	0.2984	0.378733	1744.135	4007.138	446.8729	4454.011
196	5.5	7	0.3119	0.2457	1371.393	3276.883	551.4094	3828.293
311	5.3	6.8	0.2600	0.327467	1079.115	2488.624	1613.607	4102.232
281			0.2937	0.385667	1456.96	2955.544	381.7308	3337.275
250	5.4	6.1	0.3142	0.3616	1315.734	2772.156	380.7553	3152.911
230	5.5	6.4	0.2761	0.2844	1530.118	3246.443	714.2076	3960.651
324	4.9	6.2	0.3885	0.240733	1104.417	2764.8	388.7164	3153.516
273	5.5	6.3	0.3113	0.4252	1522.411	2930.896	378.3942	3309.29
257			0.2940	0.3359	1602.379	2929.505	412.6248	3342.129
193	5.4	6.7	0.2578	0.338933	1111.685	2252.305	397.0554	2649.36
216	6.1	7.1	0.2752	0.3527	1169.477	3208.302	479.8667	3688.169
333	5.2	6.5	0.3124	0.3203	1387.308	2883.372	528.9201	3412.292
299	5.1	6.1	0.2928	0.2644	1473.015	3155.22	429.5558	3584.776
200	5.4	6.4	0.2945	0.334467	1339.729	2910.805	399.9444	3310.749
240	5.3	6	0.2867	0.294133	1952.05	3136.204	445.3789	3581.583
259	5.2	7	0.2940	0.3516	1594.77	3924.912	638.9589	4563.871
279	6	7	0.2899	0.3239	1142.644	1927.957	441.2654	2369.223
266	4.4	6.6	0.3487	0.3222	1579.341	2740.577	487.542	3228.119
287	5.3	6.5	0.3187	0.3504	1406.311	2730.181	308.0155	3038.196
188	4.4	5.9	0.3151	0.4024	1565.85	3498.058	409.9861	3908.044
0	4.9	6.4	0.3364	0.2824	1600.059	3331.795	437.6242	3769.419
315	5.2	6.6	0.3097	0.327467	1454.62	2500.341	351.8221	2852.163
183	5.5	6.6	0.2949	0.3154	1495.084	3281.226	498.3731	3779.599
0	0	0	0.3554	0.3026	1174.37	2496.221	400.0864	2896.308
243	4.8	6.3	0.2858	0.4334	1298.624	3619.133	507.3938	4126.527
0	6.3	6.9	0.2953	0.341333	1613.582	2424.025	302.3056	2726.33
318	4.8	5.7	0.3266	0.3383	965.0564	2301.394	443.9288	2745.323
256	6.3	7.2	0.2774	0.3113	1486.63	3368.621	454.6242	3823.246
277	5.4	6.3	0.2645	0.3403	1320.733	2923.696	644.1091	3567.805
239	5.3	6.8						
337	5.9	6.6	0.3064	0.292667	1375.907	3173.658	359.807	3533.465
184	5.4	5.9	0.2897	0.3904	950.5776	2295.541	378.5019	2674.043
272	4.4	6.3	0.3011	0.302	1509.83	2728.093	427.4206	3155.514
191	4.4	6.2	0.2911	0.351667	1546.93	3198.723	370.4445	3569.167
297	5.5	6.8	0.2829	0.404	1989.22	3270.798	371.96	3642.758
222	5.8	6.6	0.2872	0.309	1292.556	2575.515	397.7179	2973.233
261			0.2819	0.332	1481.486	2861.873	460.6233	3322.496
189	4.9	6.8	0.2958	0.327133	2136.098	2940.354	361.2982	3301.652
275	6.2	6.7	0.3165	0.2752	1283.478	2886.169	377.5532	3263.723
225	4.8	5.8	0.3077	0.324	1723.135	3173.482	365.6683	3539.15

<b>PLANT RIL</b>	<b>SCB</b>	<b>SWCB</b>	<b>GSM (mg/gDW)</b>	<b>FIBER (mg/gDW)</b>	<b>P - CA (ug/gDW)</b>	<b>Trans FA (ug/gDW)</b>	<b>Cis FA (ug/gDW)</b>	<b>Total FA (ug/gDW)</b>
314			0.3048	0.390867	1297.42	2757.16	360.7686	3117.929
210	6.2	7.4	0.2898	0.369067	1784.135	3589.883	316.5288	3906.412
329	5.1	6.8	0.3290	0.293333	1207.546	2955.001	389.1054	3344.106
242	4.1	5.8	0.2975	0.3236	1594.869	3217.116	620.9119	3838.028
306	4.6	6.7	0.3072	0.3928	1643.543	3406.433	444.0214	3850.454
290	5.6	7.7	0.3047	0.2802	1489.107	3433.9	748.1452	4182.045
177	3.5	5.4	0.3189	0.3572	2019.291	3841.578	455.5718	4297.15
	5.5	6.7	0.2957	0.319067	1357.281	2491.332	442.6956	2934.028
201	5.9	7	0.5707	0.3694	1313.076	2881.7	290.2545	3171.954
185	6	7.5						
280	4.2	5.3	0.3024	0.2732	1316.071	3137.499	411.3635	3548.862
304	6.4	6.8	0.3470	0.3808	978.9632	2422.286	551.072	2973.358
235	4	6.1	0.2686	0.356933	1415.102	4089.809	605.8154	4695.625
212	4.6	5.7	0.2789	0.324133	1581.417	3324.574	360.2347	3684.809
322	4.4	6.4	0.3055	0.339733	1830.433	3072.297	433.7691	3506.066
252	5.2	6.7	0.2766	0.2837	1433.617	3436.212	557.2551	3993.467
339	5.3	6.8	0.3110	0.3914	1808.526	4075.12	506.326	4581.446
291	5.1	6.7	0.4213	0.2776	1032.405	2767.273	376.5931	3143.866
294	5.2	6.4	0.3140	0.32	1481.032	2289.013	313.7712	2602.784
330	5.3	6.6	0.3194	0.317133	1266.697	2821.13	319.6715	3140.802
192	5.4	6.1	0.2912	0.3523	1478.419	3423.86	420.9542	3844.814
312	4.4	6.3	0.2849	0.3518	1108.219	2522.176	393.4862	2915.663
179	4.3	6.4	0.3074	0.3078	1426.637	4498.258	594.2954	5092.553
202			0.3576	0.36	1026.672	2782.023	355.7138	3137.737
	5.3	6.4	0.3135	0.3244	1248.528	3032.932	338.0195	3370.952
334	5.6	6.3	0.3262	0.292	2303.762	2871.94	309.7038	3181.644
248	6.2	6.6	0.2966	0.3326	1159.974	2835.604	428.74	3264.344
285	3.7	6.3	0.2987	0.395	1213.68	2446.946	307.4093	2754.355
236			0.2434	0.3662	1523.127	3124.403	458.1917	3582.594

PLANT RIL	8-5 DFA (ug/gDW)	5-5 DFA (ug/gDW)	8-0-4 DFA (ug/gDW)	Total DFA (ug/gDW)	%N	THICK (um)	SPAD	INSTRON (g)
199	39.72833	132.1542	131.4154	303.29792	1.86	227.40	34.3	96.8
	34.475	111.6333	117.5433	263.65167	2.17			
292	39.03063	124.9719	121.4013	285.40375	1.80	215.53	34.6	96.61
276	28.12752	89.03385	94.61551	211.77688	2.10	216.67	40.9	103.43
319	24.54292	84.95625	86.84417	196.34333	2.15	224.27	45.7	98.76
234	20.53288	94.0309	95.99737	210.56115	2.04	207.20	46.9	90.78
213	51.65645	155.6986	139.8755	347.23055	1.95	0.00	36.4	84.465
181	52.52965	166.2608	127.5495	346.33993	1.70	229.07	38	88.115
328	28.35533	133.2992	102.1216	263.77617	1.97	225.07	30.8	86.655
331	39.9586	137.4976	110.6385	288.0946	2.13	239.27	34.1	104.36
251	41.11833	150.4947	121.3851	312.99813	1.91	232.87	36.9	100.055
178	59.71442	152.2191	111.3693	323.30285	2.06	215.13	33.6	96.37
211	20.6473	92.0576	73.2051	185.91	1.84	206.07	45.1	82.625
305	36.3612	114.876	110.7314	261.9686	1.68	227.13	33.6	107.71
284						220.47	38.3	90.30
320	47.8136	126.4224	102.9208	277.1568	2.14	225.87	43.7	99.07
	54.34405	137.5058	95.6909	287.54075	1.60			
241	22.98	105.708	91.92	220.608	2.40	241.60	40.1	93.89
209	29.9796	97.2594	70.0686	197.3076	2.48	212.60	33	101.33
340	24.5147	118.6864	94.592	237.7931	2.11	224.93	42.6	104.77
223	23.4168	103.6688	86.21303	213.2986	1.85	213.93	33.3	92.39
244	41.98565	128.4206	122.6847	293.09085	1.84	221.53	38.9	87.405
262	26.64715	107.9415	98.1392	232.72785	2.09	188.47	43.8	85.72
323	38.0888	106.1624	111.8352	256.0864	1.97	216.20	43.9	105.465
238	37.9826	121.3152	112.445	271.7428	2.04	215.93	39.9	84.85
204	27.4068	124.8532	129.421	281.681	1.59	208.33	35.5	80.855
278	52.69325	154.746	118.5258	325.96505	1.72	243.67	37.2	92.075
338						209.53	31.7	90.4
	25.3464	101.8672	81.18955	208.40315	2.40			
220	33.6533	108.7137	90.37127	232.7383	2.07	192.73	33	92.155
308	59.26467	161.6843	126.2895	347.2384	1.66			
226	28.70375	126.4608	101.1315	256.296	1.92	204.13	38.7	96.135
246	45.5084	142.8563	100.5417	288.90643	1.93	204.27	39	86.085
229	45.24315	146.5096	141.9645	333.7172	1.93	216.67	35.1	98.41
237	17.3574	84.1009	70.2109	171.6692	2.14	217.13	39.5	94.03
295	46.40453	132.0145	108.4072	286.82627	1.87	223.20	43.5	79.81
271	39.8219	118.8775	92.34513	251.0445	1.89	203.67	35.9	90.315
197	56.8897	147.3515	116.6747	320.91597	2.29			
317	26.7896	99.35307	72.09947	198.24213	2.18	237.47	29.4	84.36
217	32.4716	122.6861	102.8721	258.0298	2.02	203.80	43.8	90.06
264	26.5434	97.04775	87.07945	210.6706	2.23	196.00	33.6	78.315
180	37.91448	132.1021	110.4889	280.50545	2.10	246.33	47.8	103.31
	32.36953	102.9723	88.98687	224.32867	2.07			
303	38.5716	126.9285	107.7246	273.22467	2.03	203.00	34.9	91.715
	61.4465	164.8212	123.6159	349.8836	1.92			
245	35.71085	122.0608	115.5692	273.34075	2.27	199.13	41.6	81.78
265	33.4656	95.5164	48.804	177.786	2.16	221.47	42.3	101.425
286	27.26067	98.7308	71.8819	197.87337	2.06	216.00	33.4	95.77
232	49.69607	142.413	123.2263	315.33533	1.92	209.93	34.8	77.255

PLANT RIL	8-5 DFA (ug/gDW)	5-5 DFA (ug/gDW)	8-0-4 DFA (ug/gDW)	Total DFA (ug/gDW)	%N	THICK (um)	SPAD	INSTRON (g)
310	37.2784	112.6456	113.456	263.38	1.58	232.80	40	95.63
190	50.05293	126.5379	113.2171	289.80795	1.61	215.53	48.7	96.005
208	24.0555	128.2861	115.1776	267.51917	2.14	223.20	35.1	90.55
187	34.1628	119.5698	97.2594	250.992	2.10	229.00	46	97.74375
289	34.843	94.3634	73.498	202.7044	2.10	220.87	35.6	92.18
332	66.4146	187.7229	132.8292	386.9667	1.71	229.67	37.7	95.46
321	53.43613	138.408	109.8533	301.69747	2.04	201.07	35.8	91.505
215	19.88473	91.48717	85.80003	197.17193	2.16	207.93	34.4	80.745
186	19.57898	88.52797	85.427	193.53395	2.33	222.73	38.5	93.2
231	20.8443	122.0604	95.22067	238.12537	2.01	196.73	37.8	91.355
313	31.1888	100.2554	87.3096	218.7538	2.33	238.20	46.6	84.82
255	40.1056	110.1742	79.763	230.0428	2.27	211.47	44.3	87.96
198	21.0046	97.55763	97.3166	215.87883	2.02	219.07	30.5	93.14
298	27.701	99.0166	73.1288	199.8464	1.98	212.13	44.1	76.755
270	36.6777	130.4262	110.5477	277.6516	2.34	212.33	48.4	95.42222
293	23.952	102.9936	106.9856	233.9312	2.62	219.73	37.9	92.46
207	63.3678	183.6865	156.4399	403.4942	1.92	216.33	30.3	88.2
203	56.70267	191.3337	155.7926	403.8289	1.88	210.53	40	80.49
253	25.1739	99.102	91.0344	215.3103	2.25	222.07	44.4	79.34
316	48.624	136.1472	123.1808	307.952	1.92	216.07	31.3	80.755
327	48.72443	160.9009	118.8698	328.4952	1.89	234.33	41.9	92.265
263	31.85407	120.2321	104.1733	256.25947	2.22	192.80	42.1	86.27
195	35.5109	147.7933	130.9484	314.25255	1.83	216.60	36.8	86.365
267	26.96667	90.5423	64.44027	181.94923	2.01	207.67	31.3	86.705
221	36.11537	120.3425	100.8183	257.27613	2.19	215.27	39.3	96.87
214	28.5852	94.8192	79.4808	202.8852	1.76	223.13	40.1	89.835
300	32.19973	98.56853	78.04527	208.81353	2.08	233.07	35.2	114.11
282	28.2159	107.4772	92.95983	228.6529	2.03	212.87	30.5	92.21
302	27.628	102.5903	84.90907	215.12733	2.30	217.20	44.5	88.74
335	29.02163	104.0422	88.77483	221.83863	2.15	237.33	44.1	100.605
260	21.0603	89.0753	80.2907	190.4263	2.07	209.27	35.2	93.33
205	33.62645	150.9184	132.8869	317.4317	2.06	213.73	31.8	109.17
218	40.15385	128.1119	120.5321	288.79785	2.08			88.60714
0	37.7226	103.8472	72.8363	214.4061	2.07			
283	17.25155	83.09545	76.86215	177.20915	2.40	232.40	31.4	105.12
325	34.2772	108.1093	79.04853	221.43507	2.38	209.53	43.4	84.36
224	46.976	152.7508	100.8094	300.5362	2.29	232.60	45.9	100.81
268	23.65125	114.5082	94.1128	232.2722	1.82	223.47	40.7	98.075
247	19.40047	103.653	100.4317	223.48517	2.19	203.40	35.1	80.925
309	55.9034	155.7356	123.483	335.122	2.02	219.53	38.7	94.91
274	44.47943	156.3469	116.2542	317.0805	1.63	232.20	39.2	102.45
336	35.6385	95.1216	69.0619	199.822	2.12	234.00	36.3	106.05
	31.0847	122.1701	109.8808	263.1356				
301	25.5817	102.1052	93.5337	221.2206	2.41	211.07	37.3	100.34
182	42.78927	149.0086	91.79172	283.58957	2.03	237.33	41.5	100.065
258	18.6252	89.0424	64.0926	171.7602	2.36			
233	27.5795	131.2361	113.3599	272.17545	2.38	230.13	38.5	105.29
296	39.67227	141.9629	124.4672	306.10233	2.03			
228	23.55677	113.1557	81.4887	218.2012	1.88	229.07	45.6	92.62

PLANT RIL	8-5 DFA (ug/gDW)	5-5 DFA (ug/gDW)	8-0-4 DFA (ug/gDW)	Total DFA (ug/gDW)	%N	THICK (um)	SPAD	INSTRON (g)
194	36.99647	126.3075	105.2424	268.5464	1.96	221.93	39.1	95.91
269	23.56785	79.91655	68.63685	172.12125	2.48	214.60	46	93.13
219	25.81127	107.8166	93.26747	226.89537	1.85	182.93	33.4	81.825
227	28.7012	139.4518	135.7376	303.89055	1.72	217.27	39.7	83.225
254	41.47767	141.6465	122.5965	305.72073	2.13	205.80	33.4	74.385
206	31.64627	135.2628	93.5328	260.4419	2.00	213.20	37	91.31
249	30.409	122.3427	107.3142	260.0659	1.75	201.20	35.1	87.29
307	28.26373	93.71853	85.48707	207.46933	2.34	228.20	45.7	93.335
288	49.30475	143.1961	115.0801	307.5809	1.56	209.20	37.2	93.86
326	50.73013	141.5043	95.29267	287.52707	2.11	228.87	48.5	95.635
196	27.1178	136.7498	119.8578	283.72535	1.74	211.20	42.9	79.665
311	35.19533	95.18373	78.84027	209.21933	2.39	237.43	32.4	78.67
281	44.02597	134.5485	107.9	286.4745	2.45	215.00	35.5	95.79
250	27.2194	103.6914	79.43273	210.34353	2.20	211.80	43.6	81.58
230	23.41605	127.0948	109.299	259.80985	2.13	224.60	47.9	82.86
324	38.4914	127.0346	104.638	270.164	2.28			
273	26.2944	114.4791	82.1866	222.96013	1.99	216.47	29.2	99.56
257	31.9941	113.478	102.6999	248.172	1.47	207.00	45.7	88.8
193	26.4936	87.8472	75.6462	189.987	2.24	227.20	35.2	82.34
216	33.16485	123.3675	115.2856	271.81795	2.21	197.13	39.9	88.04
333	30.1985	104.0433	83.19733	217.43913	2.26	223.13	32.6	86.385
299	24.4951	94.5648	87.6345	206.6944	1.92	208.60	39.8	91.63
200	34.9746	116.1134	95.452	246.54	2.23	210.07	42.6	71.905
240	33.23717	141.1953	115.5341	289.96657	2.00	219.20	38.2	86.7
259	48.5301	156.0981	126.0687	330.6969	2.01	227.36	42.2	91.32143
279	18.9323	88.47385	71.5709	178.97705	2.19	212.00	35.5	100.435
266	23.6384	106.3728	107.1115	237.1227	1.40	204.93	46.1	86.495
287	24.69043	96.01293	76.5714	197.27477	2.03	225.67	46.9	96.25
188	50.33598	146.36	106.2302	302.92615	2.07	216.60	41.2	88.195
0	41.8909	135.4842	106.2819	283.65703	2.08			
315	29.9808	156.2896	140.1479	326.41827	2.43	222.60	32.1	96.915
183	34.35788	112.2476	91.49353	238.09898	1.86	228.47	29.7	92.455
0	25.8815	95.8039	84.9973	206.6827	1.97			
243	45.238	156.8068	113.1322	315.17693	1.85	224.87	46.3	89.495
0	21.62797	102.4114	90.591	214.63033	2.27			
318	25.3778	81.7452	80.2132	187.3362	1.97	228.87	31.4	95.49
256	26.2446	115.1376	115.9842	257.3664	2.13	227.00	38.1	95.935
277	34.9928	123.9813	97.5167	256.49075	2.15	211.00	36.3	100.59
239						225.40	44.1	82.885
337	35.9985	118.2488	93.9759	248.22315	2.14	220.00	42.7	102.695
184	21.1533	85.98	71.1294	178.2627	2.18	224.87	41.4	93.87368
272	20.24677	92.41757	80.18447	192.8488	2.01	209.53	38.8	91.56
191	34.62895	114.5637	79.79515	228.98777	1.92			
297	21.2364	83.27	61.252	165.7584	2.13	221.20	32.5	78.845
222	20.2512	90.2755	65.07445	175.60115	2.49	220.13	39.6	93.305
261	24.79003	103.028	82.76493	210.58293	2.01	215.00	39.7	91.155
189	34.52137	127.6933	115.3268	277.54145	2.02	216.20	36.7	85.99
275	23.9379	101.631	89.5638	215.13273	2.17	215.27	35.6	89.43
225	30.77195	149.4185	128.7442	308.9346	1.95	203.33	29.1	95.375

PLANT RIL	8-5 DFA (ug/gDW)	5-5 DFA (ug/gDW)	8-0-4 DFA (ug/gDW)	Total DFA (ug/gDW)	%N	THICK (um)	SPAD	INSTRON (g)
314	29.5573	108.8873	96.0287	234.4733	2.11	208.87	40	89.64
210	48.1068	149.898	78.0864	276.0912	2.45	216.20	40.1	94.005
329	29.83357	110.273	92.33613	232.4427	2.13	199.60	29.9	81.065
242	11.4661	98.1442	80.8853	190.4956	2.30	207.87	33.2	99.05
306	39.64447	122.2378	90.0658	251.94807	2.18	241.13	36.9	95.375
290	40.0056	177.5536	138.196	355.7552	1.90			
177	62.3846	161.7075	132.9777	357.06975	1.86	225.93	47.1	89.66
	19.89817	103.5591	84.91387	208.3711	2.22			
201	32.8855	124.8764	100.2942	258.05607	2.12	211.67	43.3	83.78
185								
280	33.9641	111.4061	97.5671	242.9373	1.93	228.33	44.2	101.35
304	32.416	80.2296	88.3336	200.9792	2.35	215.87	43.2	87.715
235	59.52233	169.572	142.5574	371.65167	1.74	227.13	41.2	81.12
212	41.45383	122.7293	88.50143	252.6846	1.99	206.53	42.5	84.57
322	50.27613	146.1279	113.2304	309.6344	2.04	224.73	39.1	86.77
252	37.997	131.4675	117.0747	286.5392	2.04	221.33	31.8	94.965
339	24.512	91.154	16.852	132.518	2.44	229.13	35.7	93.915
291	34.72293	122.0766	99.93667	256.7362	1.93	220.67	28.7	93
294	23.952	83.0336	89.4208	196.4064	1.80	202.47	43.7	67.615
330	32.3129	105.0946	75.03453	212.44203	2.19	208.67	37	88.575
192	39.023	119.9338	112.2365	271.1932	1.68	218.53	35.3	86.31
312	29.6722	90.6734	79.998	200.3436	2.07	226.13	35.8	93.4
179	64.84715	170.7368	155.1407	390.7246	1.60	213.40	45.1	99.335
202	24.12935	99.31015	89.3891	212.8286	1.89	210.47	42.5	94.9
	45.8552	134.7745	101.1594	281.7891	1.98			
334	42.691	111.7728	97.025	251.4888	1.86	211.27	34.4	91.56
248	35.0269	105.5128	104.2853	244.8249	2.20	204.07	35.8	82.31
285	11.52683	70.65153	55.8378	138.01617	2.09	216.67	36.7	88.565
236	16.8763	106.8908	86.15495	209.92205	1.92	239.67	37	101.49

**Insect Ratings and Phytochemical Data for all RILs 1997A, Tlaltizapan.**

<b>PLANT RIL</b>	<b>SCB</b>	<b>SWCB</b>	<b>GSM (mg/gDW)</b>	<b>Fiber (mg/gDW)</b>	<b>pCA (ug/gDW)</b>	<b>Trans FA (ug/gDW)</b>
RIL-152	3.71	4.50	0.366	0.2708	1470.55	2257.28
RIL-154	3.56	3.80	0.3704	0.2882	1363.91	2776.69
RIL-155	4.35	5.08	0.3772	0.2896	1277.59	2037.91
RIL-156	3.59	4.70	0.3714	0.3028	1268.79	2203.11
RIL-158	5.50	5.55	0.3734	0.2868	1005.42	2031.14
RIL-159	4.17	4.23	0.3958	0.2574	679.76	2146.24
RIL-160	2.92	3.83	0.364	0.2686	1166.03	2064.31
RIL-161	4.79	6.05	0.3536	0.3124	816.68	1921.86
RIL-162	5.77	5.08	0.3248	0.3018	1314.29	2217.29
RIL-163	2.59	3.60	0.3538	0.2724	1471.51	2193.64
RIL-164	4.21	5.18	0.3854	0.2518	1240.13	1987.42
RIL-165	4.46	5.48	0.3788	0.2804	1273.48	1966.94
RIL-166	6.49	5.78	0.4088	0.2762	983.26	1846.81
RIL-167	5.51	6.03	0.3724	0.3036	1169.25	2023.27
RIL-168	5.00	6.03	0.3684	0.285	1354.02	2205.22
RIL-170	3.85	3.64	0.3808	0.2756	808.18	1849.08
RIL-171	4.65	5.08	0.3876	0.2756	872.93	1859.40
RIL-172	4.00	6.33	0.3546	0.3334	1370.24	2425.95
RIL-173	4.31	4.85	0.3384	0.3016	1133.83	2258.65
RIL-175	6.25	4.80	0.3502	0.2726	905.67	2154.08
RIL-177	5.56	5.70	0.3416	0.3124	1451.76	2465.65
RIL-178	4.58	5.38	0.3704	0.2938	912.78	1980.06
RIL-179	4.41	4.93	0.3298	0.2566	1293.50	2320.93
RIL-180	5.28	4.90	0.3294	0.3144	1594.14	2614.05
RIL-181	3.94	6.39	0.3444	0.309	1038.80	2272.69
RIL-182	3.56	5.33	0.3648	0.3088	1727.63	2696.61
RIL-183	5.06	5.50	0.3436	0.3454	1192.32	2024.84
RIL-184	4.17	5.08	0.3746	0.2566	1495.19	2223.89
RIL-185	4.11	3.98	0.339	0.3332	1366.13	2353.65
RIL-187	4.06	3.40	0.335	0.3318	1411.76	2093.40
RIL-188	4.57	5.30	0.3402	0.2592	1637.79	2257.40
RIL-189	5.18	4.53	0.3614	0.3244	1057.90	2057.55
RIL-190	5.24	4.90	0.3438	0.3702	1366.34	1993.61
RIL-191	4.90	4.68	0.4104	0.2768	1003.20	1815.28
RIL-193	5.04	5.23	0.354	0.3598	1270.52	2195.46
RIL-194	5.36	5.85	0.3402	0.3616	1094.22	1859.47
RIL-195	4.88	4.90	0.3324	0.3146	1460.88	2675.82
RIL-196	5.15	4.95	0.3882	0.3056	1188.34	1975.84
RIL-197	4.64	4.80	0.3354	0.2918	1103.04	2268.16
RIL-198	5.11	5.18	0.3326	0.3042	1120.82	2120.82
RIL-199	5.68	4.88	0.4062	0.2748	1010.66	1771.86
RIL-200	5.17	4.20	0.3762	0.2984	881.77	1512.55
RIL-201	4.13	3.85	0.3906	0.2802	1225.30	1971.57
RIL-203	3.04	4.98	0.3812	0.316	1363.73	2415.39
RIL-204	3.93	3.95	0.35	0.2632	908.23	1608.64
RIL-205	4.22	4.60	0.4002	0.2724	1056.64	1983.61
RIL-206	3.58	4.30	0.3676	0.2892	1543.28	2399.71
RIL-207	3.61	3.95	0.3176	0.3336	1385.11	2604.92
RIL-208	4.22	4.13	0.386	0.304	895.62	1832.56

<b>PLANT RIL</b>	<b>SCB</b>	<b>SWCB</b>	<b>GSM (mg/gDW)</b>	<b>Fiber (mg/gDW)</b>	<b>pCA (ug/gDW)</b>	<b>Trans FA (ug/gDW)</b>
RIL-212	3.83	6.38	0.3424	0.3374	1330.48	2094.32
RIL-213	4.30	3.80	0.3158	0.3478	1319.06	2490.66
RIL-214	4.72	5.38	0.3432	0.3086	1598.43	1854.95
RIL-215	3.50	4.13	0.3688	0.2874	1206.86	2022.15
RIL-216	5.67	5.65	0.3338	0.378	1191.98	2491.34
RIL-218	6.41	5.03	0.3902	0.2918	953.91	1841.37
RIL-219	4.55	4.40	0.4028	0.2836	1049.52	2073.39
RIL-220	4.56	4.85	0.3812	0.3036	1126.47	2190.24
RIL-221	4.22	5.00	0.3756	0.3128	1254.72	2528.68
RIL-223	4.38	4.80	0.3648	0.3316	1120.77	2618.45
RIL-224	4.84	5.93	0.3544	0.2778	1181.60	1793.15
RIL-225	4.72	4.85	0.3866	0.2622	1010.68	2000.00
RIL-226	4.16	5.20	0.3354	0.2742	1418.19	1692.15
RIL-228	5.06	4.80	0.3652	0.2954	1245.55	1916.73
RIL-229	5.45	5.05	0.3812	0.2412	943.78	1982.43
RIL-230	6.77	7.13	0.3448	0.3216	1152.31	2303.91
RIL-231	6.43	6.84	0.3556	0.3064	1026.70	2204.50
RIL-232	5.52	5.71	0.3412	0.2632	916.37	1959.94
RIL-233	6.50	5.82	0.3356	0.2222	1094.17	2079.41
RIL-237	5.76	6.15	0.3662	0.2648	976.11	1953.62
RIL-238	4.70	5.08	0.3184	0.2504	1758.26	2914.97
RIL-239	5.41	6.33	0.387	0.2666	1017.14	1857.14
RIL-240	5.56	3.70	0.3388	0.3116	1280.06	2462.12
RIL-242	6.25	7.68	0.3914	0.2654	1150.00	2140.00
RIL-243	4.87	5.66	0.3592	0.3036	2122.88	2360.35
RIL-247	4.12	5.65	0.3442	0.3178	1724.29	2850.90
RIL-248	5.00	7.25	0.3434	0.2786	1028.71	2093.39
RIL-250	5.90	7.65	0.327	0.326	1594.79	2685.19
RIL-251	4.01	5.15	0.3646	0.3122	1479.43	2748.07
RIL-252	5.63	6.33	0.3618	0.318	849.10	1764.33
RIL-253	6.38	6.00	0.3726	0.3026	1295.11	2322.19
RIL-256	4.56	6.03	0.3414	0.3052	1156.52	1898.70
RIL-257	5.63	6.95	0.3752	0.261	930.61	2053.18
RIL-258	4.76	6.50	0.3874	0.2472	1020.75	1685.47
RIL-259	5.74	5.33	0.383	0.2792	992.87	1556.42
RIL-260	4.19	4.90	0.3464	0.3056	1232.40	2037.09
RIL-261	3.27	3.25	0.3184	0.2984	1915.22	2915.97
RIL-262	5.45	5.95	0.3602	0.327	919.84	1977.96
RIL-263	3.57	4.40	0.3964	0.3024	1452.24	2497.66
RIL-264	5.75	6.43	0.3214	0.3248	1438.30	2373.96
RIL-265	4.63	5.20	0.3738	0.3012	1154.31	1934.54
RIL-266	4.83	4.83	0.3426	0.3094	2340.04	2747.63
RIL-268	3.44	4.33	0.397	0.2756	1339.30	2294.56
RIL-270	4.89	5.65	0.345	0.3164	1022.39	1886.91
RIL-272	3.81	5.68	0.347	0.3028	1148.66	2205.09
RIL-274	3.95	5.65	0.3402	0.27	1218.98	2173.06
RIL-275	5.72	5.18	0.438	0.2724	1008.26	2202.34
RIL-276	4.36	4.90	0.3428	0.2876	983.19	1916.67
RIL-277	5.14	6.20	0.3496	0.3018	1668.94	2783.60

<b>PLANT RIL</b>	<b>SCB</b>	<b>SWCB</b>	<b>GSM (mg/gDW)</b>	<b>Fiber (mg/gDW)</b>	<b>pCA (ug/gDW)</b>	<b>Trans FA (ug/gDW)</b>
RIL-278	5.57	6.67	0.4046	0.2506	1362.75	1756.30
RIL-280	6.00	6.93	0.3886	0.3098	1186.97	2267.51
RIL-281	5.93	5.95	0.3702	0.2924	874.72	1667.43
RIL-282	4.60	5.43	0.3384	0.3032	1429.97	2166.67
RIL-283	5.10	6.25	0.3484	0.288	1271.83	2018.22
RIL-286	4.96	5.25	0.3706	0.3	1080.69	2005.28
RIL-287	5.41	5.28	0.3904	0.2808	1531.67	2046.00
RIL-288	5.78	5.70	0.3204	0.307	876.32	1863.50
RIL-289	5.56	5.80	0.3462	0.2984	960.03	1544.49
RIL-290	5.56	6.30	0.3432	0.3108	1520.36	1881.60
RIL-292	5.95	5.58	0.3552	0.2756	1093.51	1720.97
RIL-293	5.93	5.70	0.2992	0.2942	1254.63	2123.34
RIL-294	6.11	6.28	0.3802	0.2884	896.22	1841.72
RIL-296	5.55	5.55	0.3798	0.3236	967.84	1679.15
RIL-298	5.91	4.78	0.3416	0.2516	1050.32	1636.78
RIL-299	5.05	6.05	0.307	0.2956	998.86	1922.81
RIL-300	4.36	5.98	0.3698	0.3184	1023.84	1432.46
RIL-301	4.33	5.15	0.432	0.2642	918.86	1689.87
RIL-304	5.54	6.34	0.3302	0.2914	1075.28	1669.09
RIL-305	7.05	6.69	0.3678	0.2894	1474.94	1898.31
RIL-307	5.44	6.08	0.3882	0.25	1006.85	2195.46
RIL-308	4.15	4.61	0.3414	0.3272	1223.95	2073.57
RIL-310	3.50	4.13	0.3678	0.2928	1312.97	2102.05
RIL-311	5.13	6.15	0.3588	0.283	1526.41	2168.16
RIL-312	5.44	5.20	0.3782	0.2728	891.84	1748.47
RIL-313	3.81	4.35	0.3852	0.2498	1039.89	1920.95
RIL-314	3.89	4.20	0.3668	0.2682	1244.70	2080.49
RIL-317	4.75	4.50	0.3014	0.2598	1451.29	2521.31
RIL-318	5.34	6.23	0.3664	0.2766	896.50	1487.82
RIL-319	5.10	6.33	0.3186	0.2642	1059.41	1804.27
RIL-a10	3.32	2.70	0.3534	0.2186	971.06	1570.45
RIL-a12	5.00	6.85	0.392	0.2376	1002.87	2101.00
RIL-a14	2.85	3.35	0.3246	0.2526	1552.17	1964.20
RIL-a16	4.08	3.48	0.3712	0.2632	1075.93	1931.95
RIL-a17	5.74	5.43	0.3504	0.2824	1335.87	2179.74
RIL-a18	6.06	6.50	0.3512	0.3096	999.61	1989.21
RIL-a19	3.33	4.08	0.3218	0.3058	1188.44	2364.55
RIL-a23	3.74	4.68	0.3202	0.2994	1141.43	2289.02
RIL-a26	5.35	5.23	0.3684	0.2812	1218.50	2018.50
RIL-a27	5.51	5.60	0.345	0.2892	1200.00	2248.94
RIL-a28	6.19	5.83	0.3406	0.3294	1055.15	1981.62
RIL-a30	5.67	5.40	0.3196	0.3246	1300.08	1847.64
RIL-a31	4.83	7.08	0.3378	0.3152	916.18	1766.91
RIL-a32	5.50	5.13	0.3832	0.2956	1160.29	2112.50
RIL-a33	3.88	5.20	0.2826	0.3202	1674.40	2689.87
RIL-a34	6.00	4.50	0.3388	0.296	1064.84	2282.33
RIL-a36	2.46	2.75	0.358	0.2822	1390.56	2512.76
RIL-a39	5.18	6.23	0.44	0.2106	707.67	1888.85
RIL-a40	2.21	2.90	0.3142	0.238	1267.54	2361.60

PLANT RIL	Cis FA (ug/gDW)	Total FA (ug/gDW)	8-5 DFA (ug/gDW)	5-5 DFA (ug/gDW)	8-0-4 DFA (ug/gDW)	Total DFA (ug/gDW)
RIL-152	367.637102	2624.91537	23.0196344	77.18348	77.18348	190.250508
RIL-154	420.300752	3196.99248	29.3233083	81.2030075	61.6541353	178.947368
RIL-155	381.178064	2419.09276	23.0196344	66.3507109	68.3818551	170.616114
RIL-156	387.271496	2590.38592	24.3737305	79.2146242	81.9228165	209.207854
RIL-158	387.271496	2418.41571	18.2802979	68.3818551	68.3818551	162.491537
RIL-159	410.968179	2557.21056	19.634394	66.3507109	69.7359513	169.262018
RIL-160	382.383782	2446.69696	23.7679133	68.5075149	72.7018525	179.657462
RIL-161	355.371901	2277.23516	14.2749812	46.5815177	46.5815177	114.19985
RIL-162	379.699248	2596.99248	20.3007519	54.1353383	54.1353383	135.338346
RIL-163	377.490388	2571.12898	22.3698008	73.4009088	69.9056274	174.065012
RIL-164	321.565886	2308.98287	18.8745194	58.720727	58.720727	143.306536
RIL-165	315.552216	2282.49437	13.5236664	54.0946657	49.5867769	121.712998
RIL-166	438.902743	2285.71429	17.1001069	57.7128607	64.1254008	152.475953
RIL-167	359.661495	2382.93371	16.9252468	57.8279267	60.6488011	143.159379
RIL-168	366.713681	2571.9323	16.9252468	66.9957687	64.1748942	154.442877
RIL-170	366.713681	2215.7969	16.2200282	50.0705219	50.0705219	123.413258
RIL-171	336.842105	2196.2406	17.2932331	58.6466165	56.3909774	139.097744
RIL-172	394.217207	2820.16925	18.3356841	66.9957687	62.0592384	154.442877
RIL-173	355.639098	2614.28571	19.5488722	66.1654135	66.9172932	160.150376
RIL-175	369.034198	2523.11161	19.5415257	63.1341601	57.1213829	146.561443
RIL-177	405.833377	2871.48451	20.6479174	74.759701	71.1997152	175.151299
RIL-178	336.774653	2316.83873	13.5279459	56.247775	59.8077608	137.41545
RIL-179	366.779406	2687.71139	21.04472	75.1597144	72.904923	176.625329
RIL-180	365.276212	2979.33108	22.5479143	78.166103	69.1469372	175.873732
RIL-181	474.190103	2746.88501	17.7999288	75.4716981	75.4716981	183.695265
RIL-182	453.475936	3150.08913	27.0944742	92.6916221	89.8395722	243.137255
RIL-183	327.436959	2352.277	15.8073015	44.4109898	49.6800903	115.920211
RIL-184	378.609626	2602.49554	17.1122995	68.4491979	66.3101604	163.99287
RIL-185	380.748663	2734.40285	19.9643494	79.144385	77.7183601	191.800357
RIL-187	369.340463	2462.7451	17.8253119	64.8841355	67.0231729	158.28877
RIL-188	451.336898	2708.7344	12.1212121	72.7272727	77.0053476	177.540107
RIL-189	466.78508	2524.33393	18.4724689	76.731794	80.2841918	191.829485
RIL-190	361.545832	2355.1581	26.1897157	55.5732993	51.7406579	148.195465
RIL-191	453.285968	2268.56128	16.3410302	58.9698046	64.6536412	154.884547
RIL-193	321.303098	2516.76781	12.1366975	53.018205	53.6569786	131.587352
RIL-194	312.999042	2172.46886	6.38773555	44.7141488	47.2692431	106.03641
RIL-195	387.735548	3063.55797	31.9386777	77.2916001	80.4854679	212.711594
RIL-196	351.812367	2327.64748	12.793177	58.9907605	61.8336887	141.435679
RIL-197	426.023778	2694.18758	17.8335535	71.994716	74.6367239	215.984148
RIL-198	397.299218	2518.12367	17.7683014	63.2551528	63.9658849	152.09666
RIL-199	297.085999	2068.94101	14.9253731	51.8834399	50.4619758	124.378109
RIL-200	536.327609	2048.87715	10.5680317	52.8401585	50.8586526	155.217966
RIL-201	334.044065	2305.61478	14.9253731	51.1727079	52.594172	127.221038
RIL-203	427.502672	2842.89277	18.5251158	84.0755255	78.3754898	196.651229
RIL-204	579.622132	2188.25911	9.44669366	45.8839406	45.8839406	114.709852
RIL-205	386.889918	2370.50232	14.2500891	51.3003206	52.0128251	125.400784
RIL-206	426.077663	2825.79266	18.5251158	76.9504809	78.3754898	189.526185
RIL-207	503.028144	3107.94442	22.087638	74.8129676	67.687923	172.426078
RIL-208	384.752405	2217.31386	13.5375846	50.5878162	56.2878518	128.250802

PLANT RIL	Cis FA (ug/gDW)	Total FA (ug/gDW)	8-5 DFA (ug/gDW)	5-5 DFA (ug/gDW)	8-0-4 DFA (ug/gDW)	Total DFA (ug/gDW)
RIL-212	423.722758	2518.04216	9.2890318	52.8760272	52.8760272	122.186495
RIL-213	494.733265	2985.38906	23.7852531	67.9578661	67.2782875	173.972137
RIL-214	315.827081	2170.77528	8.57449089	56.4487317	57.8778135	130.760986
RIL-215	419.435513	2441.58628	17.1489818	57.1632726	55.7341908	139.335477
RIL-216	468.229698	2959.56507	21.0669385	70.6761808	67.9578661	167.176351
RIL-218	328.688817	2170.06074	10.0035727	45.0160772	48.5887817	110.753841
RIL-219	460.990381	2534.37834	16.3876024	68.4004275	75.525472	175.9886
RIL-220	354.827218	2545.06591	16.3876024	65.5504097	63.4128963	153.188457
RIL-221	403.27752	2931.95582	22.8001425	75.525472	66.9754186	173.851087
RIL-223	538.653367	3157.10723	22.8001425	87.6380477	76.9504809	204.488778
RIL-224	406.087859	2199.23902	4.84261501	44.9671394	43.5835351	98.9277067
RIL-225	340.213523	2340.21352	17.0818505	59.0747331	73.3096085	166.548043
RIL-226	405.396057	2097.5441	7.60982359	33.2065029	45.6589415	93.3932895
RIL-228	349.466192	2266.19217	12.0996441	55.5160142	56.9395018	131.672598
RIL-229	303.583978	2286.01546	14.7575545	43.5699227	43.5699227	107.519325
RIL-230	381.494662	2685.40925	19.2170819	59.7864769	57.6512456	144.483986
RIL-231	327.477161	2531.9747	15.4602952	52.7055517	56.9219958	134.223472
RIL-232	282.501757	2242.44554	6.3246662	44.2726634	46.3808855	102.600141
RIL-233	314.827829	2394.23753	14.7575545	50.5973296	58.3274772	133.520731
RIL-237	311.314125	2264.93324	6.3246662	44.2726634	47.7863668	104.708363
RIL-238	382.993675	3297.96205	9.13562895	59.0302178	63.246662	143.3591
RIL-239	313.571429	2170.71429	12.1428571	45.7142857	47.8571429	111.428571
RIL-240	439.50245	2901.62081	9.04636261	51.2627214	50.5088579	116.84885
RIL-242	327.142857	2467.14286	15	63.5714286	62.8571429	148.571429
RIL-243	320.392009	2680.73879	14.3234075	76.1402186	65.5861289	162.834527
RIL-247	471.079692	3321.97943	33.4190231	89.3316195	84.8329049	217.22365
RIL-248	4195.78001	6289.1733	11.7606365	59.4949844	63.6457973	148.045659
RIL-250	505.803728	3190.99543	14.773127	94.970102	88.6387619	215.969047
RIL-251	437.660668	3185.73265	19.9228792	87.403599	83.5475578	208.226221
RIL-252	428.420682	2192.75413	7.73830461	61.9064369	57.6855434	139.289483
RIL-253	528.31516	2850.51002	8.44178684	87.2317974	73.8656349	183.608864
RIL-256	500.175871	2398.87443	9.14526908	76.6795638	71.0517059	173.05663
RIL-257	389.753567	2442.93126	16.2127108	66.7963684	70.6874189	165.36965
RIL-258	315.823606	2001.29702	7.13359274	47.9896239	53.1776913	116.083009
RIL-259	315.175097	1871.59533	7.13359274	41.5045396	48.6381323	105.058366
RIL-260	377.744133	2414.83724	10.5980318	43.1491294	158.970477	224.072672
RIL-261	441.332324	3357.30507	20.4390613	78.7282362	186.222559	298.258895
RIL-262	384.101536	2362.05745	14.6960588	56.1122244	62.7922512	142.284569
RIL-263	358.717435	2856.37943	18.7040748	75.4843019	76.1523046	179.692719
RIL-264	381.529145	2755.48827	17.4110522	74.9432248	64.345193	164.269493
RIL-265	366.733467	2301.26921	14.0280561	59.4522378	67.4682699	151.636607
RIL-266	658.823529	3406.45161	22.0113852	94.1176471	81.2144213	219.354839
RIL-268	399.174123	2693.7371	15.1410874	66.0701996	66.0701996	158.293187
RIL-270	517.647059	2404.55408	9.86717268	57.6850095	55.4079696	138.140417
RIL-272	370.956641	2576.04955	15.8293187	66.0701996	63.3172746	150.722643
RIL-274	508.538899	2681.59393	12.9032258	69.0702087	65.2751423	164.705882
RIL-275	293.874742	2496.21473	19.9587061	61.9408121	59.1878871	145.905024
RIL-276	370.448179	2287.11485	17.5070028	58.8235294	62.32493	149.859944
RIL-277	469.248292	3252.84738	14.4267274	78.2080486	72.1336371	182.991648

PLANT RIL	Cis FA (ug/gDW)	Total FA (ug/gDW)	8-5 DFA (ug/gDW)	5-5 DFA (ug/gDW)	8-0-4 DFA (ug/gDW)	Total DFA (ug/gDW)
RIL-278	310.22409	2066.52661	11.2044818	55.3221289	60.9243697	139.355742
RIL-280	369.047619	2636.55462	16.8067227	77.0308123	74.2296919	179.971989
RIL-281	410.022779	2077.44875	4.55580866	44.7987851	41.0022779	97.1905847
RIL-282	341.736695	2508.40336	11.9047619	65.8263305	72.8291317	160.364146
RIL-283	481.397115	2499.62035	7.59301443	43.2801822	40.2429765	97.9498861
RIL-286	479.638009	2484.91704	15.0829563	58.0693816	52.7903469	139.517345
RIL-287	489.441931	2535.44495	14.3288084	70.8898944	65.6108597	169.683258
RIL-288	513.574661	2377.07391	10.5580694	49.0196078	49.7737557	122.926094
RIL-289	372.54902	1917.04374	6.0331825	39.2156863	36.199095	88.2352941
RIL-290	461.538462	2343.13725	6.78733032	47.5113122	42.2322775	104.072398
RIL-292	429.110106	2150.07541	6.78733032	47.5113122	42.9864253	104.072398
RIL-293	520.620507	2643.96519	12.8641695	59.7805524	56.753689	144.532728
RIL-294	322.804582	2164.52621	13.1898646	58.3130857	68.7261368	149.947935
RIL-296	495.648884	2174.80136	8.32387439	44.6462353	41.6193719	102.913356
RIL-298	441.922058	2078.69845	9.83730609	59.0238365	55.2402573	132.425274
RIL-299	518.350359	2441.16534	11.3507378	58.2671207	54.4835414	133.18199
RIL-300	298.902762	1731.36587	7.56715853	44.6462353	51.456678	115.777526
RIL-301	258.204111	1948.07068	12.2610891	43.9956726	43.9956726	105.301118
RIL-304	360.71838	2029.80512	11.4635078	56.5533053	47.382499	123.041651
RIL-305	258.204111	2156.5092	11.5398485	60.5842048	61.3054454	139.920664
RIL-307	327.443202	2522.89939	17.3097728	64.911648	63.469167	152.181753
RIL-308	360.620267	2434.1868	15.8672917	58.4204832	56.2567616	136.314461
RIL-310	303.988502	2406.03665	16.5289256	57.4919152	56.0546173	135.82465
RIL-311	341.358246	2509.5221	9.34243622	47.43083	48.149479	110.671937
RIL-312	275.242544	2023.71542	14.3729788	49.5867769	50.3054258	120.014373
RIL-313	293.927416	2214.87603	15.8102767	56.0546173	55.3359684	133.668703
RIL-314	303.269853	2383.75853	16.5289256	56.7732663	55.3359684	134.387352
RIL-317	703.196347	3224.50533	25.1141553	82.1917808	85.2359209	213.089802
RIL-318	509.13242	1996.95586	9.13242009	47.9452055	51.7503805	123.287671
RIL-319	584.158416	2388.42346	11.4242193	67.0220868	59.4059406	156.892612
RIL-a10	417.364813	1987.81417	11.4242193	47.2201066	47.2201066	120.33511
RIL-a12	345.988539	2446.9914	17.1919771	70.2005731	65.9025788	163.323782
RIL-a14	494.28789	2458.492	17.5171363	67.0220868	60.9291698	162.985529
RIL-a16	260.028653	2191.97708	11.4613181	53.008596	48.7106017	118.194842
RIL-a17	543.031226	2722.77228	15.2322925	77.6846915	69.3069307	181.26428
RIL-a18	543.352601	2532.56262	12.3314066	59.344894	63.9691715	151.830443
RIL-a19	490.17341	2854.72062	20.0385356	67.822736	83.2369942	188.05395
RIL-a23	507.899807	2796.91715	20.8092486	70.1348748	64.7398844	173.410405
RIL-a26	282.080925	2300.57803	13.1021195	66.2813102	63.9691715	152.601156
RIL-a27	319.075145	2568.01541	16.955684	61.6570328	60.1156069	147.206166
RIL-a28	294.117647	2275.73529	16.1764706	55.1470588	55.8823529	133.088235
RIL-a30	283.83604	2131.47718	11.6009281	47.1771075	51.0440835	116.782676
RIL-a31	246.323529	2013.23529	13.9705882	58.8235294	58.0882353	137.5
RIL-a32	323.529412	2436.02941	18.3823529	66.9117647	63.2352941	155.882353
RIL-a33	388.244393	3078.11292	20.8816705	66.5119876	64.9651972	159.319412
RIL-a34	346.795109	2629.1219	17.7843646	62.245276	54.0941089	142.274917
RIL-a36	375.87007	2888.63109	16.2412993	57.2312452	57.2312452	136.890951
RIL-a39	266.765469	2155.61319	15.561319	52.6120785	51.1300482	125.972582
RIL-a40	370.084811	2731.68851	16.1912105	60.1387818	61.6808019	144.178874

<b>PLANT RIL</b>	<b>Force (g)</b>	<b>Thickness (um)</b>	<b>Spad</b>	<b>% N</b>
RIL-152	82.12	195.50	37.95	1.83
RIL-154	102.88	243.00	36.10	1.7
RIL-155	97.90	262.90	41.50	1.97
RIL-156	107.61	243.70	39.40	2.13
RIL-158	90.57	211.70	36.80	2.17
RIL-159	92.55	232.00	48.90	2.06
RIL-160	94.74	202.40	40.35	1.6
RIL-161	81.81	232.40	41.45	1.68
RIL-162	92.80	225.50	37.30	2.3
RIL-163	111.35	240.40	47.10	1.6
RIL-164	113.57	227.90	45.55	1.93
RIL-165	92.41	253.80	35.55	2.48
RIL-166	98.36	211.60	45.55	2.48
RIL-167	87.29	232.50	40.15	1.85
RIL-168	88.86	228.60	39.15	2.07
RIL-170	87.17	232.30	39.90	2.09
RIL-171	96.86	245.90	40.40	2.03
RIL-172	100.12	230.30	40.95	2.04
RIL-173	97.51	218.40	39.95	1.97
RIL-175	94.85	204.50	38.00	1.72
RIL-177	90.31	189.90	37.75	2.22
RIL-178	101.61	211.60	41.55	2.07
RIL-179	101.26	221.50	44.00	2
RIL-180	101.93	222.40	37.20	1.92
RIL-181	108.16	214.60	34.05	1.88
RIL-182	104.75	242.80	42.30	1.93
RIL-183	90.87	206.00	47.85	2.1
RIL-184	91.71	215.20	43.90	1.87
RIL-185	95.02	232.50	41.20	1.92
RIL-187	96.80	226.20	42.90	2.29
RIL-188	105.18	216.30	40.10	2.01
RIL-189	89.89	232.60	39.65	2.02
RIL-190	88.40	217.80	37.80	2.13
RIL-191	97.17	225.50	37.60	2.1
RIL-193	99.24	219.30	44.55	2.08
RIL-194	101.97	233.40	46.85	2.03
RIL-195	97.87	230.30	41.10	2.06
RIL-196	99.51	213.20	43.15	2.27
RIL-197	109.51	227.40	42.85	2.02
RIL-198	105.21	236.20	38.20	2.06
RIL-199	94.80	212.90	40.65	2.27
RIL-200	96.60	223.30	44.90	1.58
RIL-201	93.09	223.10	39.05	1.92
RIL-203	90.57	230.60	33.20	2.14
RIL-204	96.64	215.30	38.90	1.87
RIL-205	102.03	222.60	36.25	2.1
RIL-206	105.75	215.80	37.30	1.75
RIL-207	102.39	224.50	45.65	2.04
RIL-208	95.77	223.10	51.50	1.93

<b>PLANT RIL</b>	<b>Force (g)</b>	<b>Thickness (um)</b>	<b>Spad</b>	<b>% N</b>
RIL-212	101.50	223.90	39.95	2.33
RIL-213	91.49	212.30	34.50	1.96
RIL-214	87.58	217.30	38.55	2.33
RIL-215	96.63	215.20	42.45	1.72
RIL-216	96.27	210.00	44.00	2.02
RIL-218	88.56	211.20	41.35	2.43
RIL-219	95.70	194.30	39.55	2.34
RIL-220	101.28	200.20	37.25	2.01
RIL-221	100.64	216.60	40.50	1.92
RIL-223	106.23	222.00	46.95	1.99
RIL-224	96.32	208.10	45.65	2.25
RIL-225	105.74	217.00	45.50	2.03
RIL-226	98.05	203.90	44.25	1.89
RIL-228	98.02	206.60	39.30	2.1
RIL-229	101.93	215.40	35.70	1.83
RIL-230	95.08	206.70	33.40	2.49
RIL-231	94.48	221.00	42.85	2.19
RIL-232	100.07	223.00	42.55	2.19
RIL-233	93.59	215.20	35.80	2.08
RIL-237	93.87	196.60	44.75	2.29
RIL-238	95.36	227.30	41.00	2.3
RIL-239	95.47	227.50	64.65	2.14
RIL-240	95.65	193.00	43.50	2.07
RIL-242	93.78	221.10	38.75	2.4
RIL-243	87.97	230.00	39.55	2.08
RIL-247	111.10	213.10	39.95	1.92
RIL-248	93.98	220.70	39.90	2.4
RIL-250	82.83	186.50	27.90	2.33
RIL-251	96.14	224.30	36.20	2.29
RIL-252	81.73	221.60	42.60	2.24
RIL-253	89.36	203.30	40.80	2.19
RIL-256	92.58	211.60	39.50	2.01
RIL-257	96.67	209.80	37.85	1.63
RIL-258	102.37	224.30	36.65	2.04
RIL-259	102.02	223.20	50.90	2.41
RIL-260	104.20	215.80	38.70	1.93
RIL-261	115.49	219.30	38.75	2.36
RIL-262	105.11	202.10	45.20	2.16
RIL-263	100.14	172.60	38.90	2.03
RIL-264	97.74	221.80	35.05	2.29
RIL-265	106.38	227.30	44.35	1.96
RIL-266	114.11	233.50	40.55	2.04
RIL-268	99.57	204.80	42.05	1.85
RIL-270	84.61	197.80	43.75	2.07
RIL-272	101.27	214.90	39.50	2.13
RIL-274	102.78	198.50	41.55	1.88
RIL-275	93.49	192.50	27.65	1.75
RIL-276	106.55	238.90	46.55	1.98
RIL-277	89.69	204.10	36.10	1.56

PLANT RIL	Force (g)	Thickness (um)	Spad	% N
RIL-278	83.27	228.50	31.25	2.23
RIL-280	81.46	194.50	33.60	1.74
RIL-281	92.27	213.40	38.50	2.23
RIL-282	99.30	219.10	46.70	2.2
RIL-283	89.96	194.90	41.20	2.1
RIL-286	92.25	189.50	37.00	2.28
RIL-287	102.01	208.50	37.40	1.99
RIL-288	98.60	240.10	43.80	2.24
RIL-289	95.83	242.30	43.60	2.21
RIL-290	93.85	221.90	39.55	2.26
RIL-292	100.40	241.70	44.40	1.92
RIL-293	105.63	230.10	46.05	2.23
RIL-294	96.10	221.00	46.75	2
RIL-296	87.99	237.40	44.80	2.01
RIL-298	99.36	219.90	38.35	2.19
RIL-299	96.80	219.70	41.10	1.4
RIL-300	81.75	199.90	36.00	2.03
RIL-301	87.06	208.90	39.95	2.07
RIL-304	85.44	221.30	39.15	2.08
RIL-305	83.65	193.90	32.50	2.43
RIL-307	94.25	177.00	35.50	1.86
RIL-308	103.02	195.70	41.80	1.85
RIL-310	94.28	215.10	37.90	2.27
RIL-311	109.18	229.90	41.25	1.97
RIL-312	90.16	216.70	39.00	2.13
RIL-313	93.75	220.80	39.40	2.15
RIL-314	95.01	211.30	34.50	2.14
RIL-317	92.27	200.20	29.95	2.18
RIL-318	80.28	181.10	38.45	2.01
RIL-319	90.55	198.20	35.00	1.92
RIL-a10	96.01	183.70	41.75	2.13
RIL-a12	86.95	167.80	39.60	2.49
RIL-a14	107.30	217.40	46.80	2.02
RIL-a16	98.85	199.80	41.35	2.17
RIL-a17	90.21	184.70	37.95	1.95
RIL-a18	83.34	167.70	35.20	2.45
RIL-a19	110.20	215.20	44.15	2.13
RIL-a23	106.63	204.20	42.85	2.3
RIL-a26	91.71	201.40	47.30	2.18
RIL-a27	93.05	226.90	46.00	1.9
RIL-a28	88.97	212.30	34.00	1.86
RIL-a30	88.87	238.00	37.80	2.22
RIL-a31	87.08	216.30	46.70	2.12
RIL-a32	97.87	229.20	48.00	1.93
RIL-a33	101.68	221.40	48.20	2.35
RIL-a34	72.68	168.33	36.95	1.74
RIL-a36	101.03	214.10	49.25	1.99
RIL-a39	95.83	206.70	52.60	2.04
RIL-a40	88.35	199.00	40.00	2.04