PHYTOCHEMICAL MEDIATION OF POST-HARVEST INSECT RESISTANCE IN TROPICAL MAIZE

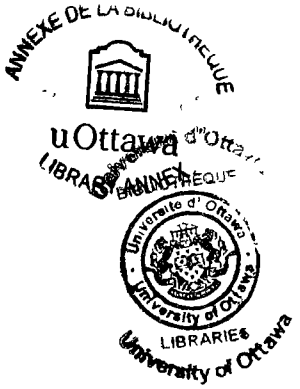
Andrew J. Burt

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University of Ottawa
in partial fulfillment of the requirements for the
M.Sc. degree in the

Ottawa-Carleton Institute of Biology

Thèse soumise à
Faculté des études supérieures et postdoctorales
Université d'Ottawa
en vue de l'obtention de la maîtrise ès sciences

L'Institut de biologie d'Ottawa-Carleton



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Abstract

The resistance of maize grain (*Zea mays* L.) to the stored insect pest, *Sitophilus zeamais*, was investigated in relation to the grain content of two groups of phytochemicals. The distribution of hydroxycinnamic acid amide conjugates (HAACs) in Mexican landraces was investigated and the toxicity of the HAACs in maize grain versus *S. zeamais* was examined by short-term feeding bioassays and a two-generation life cycle. Results suggest that the HAACs are not an effective defence compound against *S. zeamais*, but may be effective against generalist feeders.

The function of cell wall bound phenylpropanoids to *S. zeamais* resistance in a collection of maize grain with variable post-harvest resistance and in a Quantitive Trait Locus (QTL) mapping population was examined. Correlational data suggest that diferulic acid content is an important resistance factor. Evidence was found for nine chromosomal locations where QTLs for cell wall bound phenylpropanoid content correspond with QTLs for insect resistance. These results indicate a defence against grain attack by *S. zeamais* mediated by cell wall bound phenylpropanoids.

Résumé

La résistance du grain de maïs (*Zea mays* L.) contre l'insecte phytophage de post-récolte, *Sitophilus zeamais*, a été étudiée en se basant sur le contenu des grains en composés appartent à deux groupes phytochimiques. La distribution des conjugués acides hydroxycinnamiques d'amide (HAACs) dans les populations naturelles mexicaines a été étudiée et la toxicité des HAACs du grain sur *S. zeamais* a été élucidée par des tests biologiques d'alimentation de courte durée, ainsi que par un suivi du cycle de vie de deux générations. Les résultats suggèrent que les HAACs ne sont pas des composés efficaces dans la défense contre *S. zeamais*, mais peuvent être efficaces contre les insectes généralistes.

La fonction des phénylpropanoïdes de la paroi cellulaire a été aussi examinée dans une collection de grains de maïs avec différentes résistances post-récolte et dans une population utilisée pour une cartographie « Quantitative Trait Locus » QTL. L'analyse corrélationnelle des données suggère que le contenu du grain en acides diféruliques (DFA) est un facteur important de résistance. Cette évidence a été trouvée pour neuf emplacements sur les chromosomes du maïs où les QTLs pour le contenu en phénylpropanoïdes correspondent aux QTLs pour des mesures de résistance aux insectes. Ces résultats indiquent que la défense du grain contre l'attaque des *S. zeamais* implique les phénylpropanoïdes liés à la paroi cellulaire.

Acknowledgements

I would like to thank my supervisors, Dr. Thor Arnason and Dr. Bernard Philogène, for all of their input, time, attention, guidance, and support. Most of all I would thank them for showing such great care that each of their students should succeed. I would also like to thank Dr. David Bergvinson and the staff at CIMMYT, Mexico for their help and collaboration. Special thanks to Silverio García-Lara, for his close collaboration, all his hard work and help, and for the gracious hospitality that he and his wife Sofia showed me in Mexico. I would also like to thank Dr. Naomi Cappuccino and Dr. Doug Johnson for their insight during the course of my studies. I also thank Dr. Paul Fields for providing insects and helpful advice and Dr. Jeffrey Atkinson for donating pure compounds and lending recommendations. To all the members of the lab, I say thank you - extra thanks to John Livesey for HPLC counselling, to Antoine Bily for aid in MS analysis, and to Antoine and Virginie Treyvaud Amiguet for correcting my résumé. Special thanks goes to Zoë, my partner in everything, thank you for providing support, motivation, and inspiration throughout this process.

Table of Contents

Abstract i

Résumé ii

Acknowledgements iii

Table of Contents iv

List of Figures vi

List of Tables viii

List of Abbreviations x

1. Chapter 1. Introduction 1

    1.1 Biology of *Sitophilus zeamais* 2

    1.2 Phytochemical Basis for *Sitophilus zeamais* Resistance in *Zea mays* L. Grain 7

    1.3 Hypotheses and Objectives 9

2. Chapter 2. The Hydroxycinnamic Acid Amide Conjugates in Tropical Maize Grain

    2.1 Introduction 13

    2.2 Methods and Materials 16

        2.2.1 Separation of maize grain tissues and sample preparation for extraction 16

        2.2.2 Extraction of hydroxycinnamic acid amides from maize tissues 16

        2.2.3 HPLC analysis 18

        2.2.4 Recovery analysis 18

        2.2.5 Acute toxicity – Wheat disk feeding assay 19

        2.2.6 Chronic toxicity – Lifecycle assay 19

    2.3 Results and Discussion 21

3. Chapter 3. Cell Wall Bound Phenolic Compounds in Tropical Maize Grain and Correlations with Resistance to *Sitophilus zeamais*.

3.1 Introduction 54

3.2 Methods and Materials 59

3.2.1 Plant material 59

3.2.2 Insect bioassay trials 60

3.2.3 Agronomic and physical resistance factors 61

3.2.4 Separation of maize grain tissues and sample preparation for extraction 62

3.2.5 Extraction of cell wall ester-bound phenolic acids from maize tissue 63

3.2.6 HPLC & HPLC/MS analysis 63

3.2.7 QTL mapping 66

3.3 Results and Discussion 67

4. Chapter 4. General Discussion and Conclusions

4.1 Main conclusions 114

4.2 Discussion

4.2.1 Identification of phenolic X 116

4.2.2 Role of HAACs 118

4.2.3 A Comparison of QTL Maps for Cell-Wall Bound Phenylpropanoids 119

4.3 Future Work 120

References 121

Appendices 127

Appendix 1 128

Appendix 2 130

List of Figures

1.1 Four representative stages in the lifecycle of *Sitophilus oryzae* 6

2.1 Chemical structure of hydroxycinnamic acid amide conjugates examined in this thesis. 15

2.2 Representative chromatogram of HAACs in extract of outer tissues of maize grain. 22

2.3 Illustration of the wide variation in HAAC content in the Mexican landraces 30

2.4 a. Linear regression of maximum altitude range of landrace on diferuloyl putrescine content in outer grain tissues. 31

2.3 b. Mean diferuloyl putrescine content in outer grain tissue by major landrace groups. 32

2.3 c. Mean diferuloyl putrescine content of outer grain tissues for different collection sites. 33

2.4 Response of *Sitophilus zeamais* to diet treated with various concentrations of feruloyl putrescine and N8-coumaroyl spermidine. 36

2.5 Response of *Sitophilus* species to diet treated with various concentrations of A) feruloyl putrescine and B) Diferuloyl putrescine. 37

2.6 Effect of feruloyl putrescine treatment on the first generation of a *Sitophilus zeamais* lifecycle bioassay. 41

2.7 Effect of feruloyl putrescine treatment on the second generation of a *Sitophilus zeamais* lifecycle bioassay. 42

3.1 Structure of a dimer of phenolic acid-carbohydrate complexes (DFAXX) 55

3.2 Representative chromatogram of cell wall bound phenylpropanoids in extract of outer tissues of maize grain 65

3.3 Mass spectrum of the unknown soluble phenolic X 74

3.4 Absorbance spectrum and structure of Diferuloyl putrescine and absorbance spectrum and proposed structure of unknown soluble phenolic X 75

3.5 Simple regression models for main factors appearing in multiple regression model of the Dobie Index rating in season 2000B 89

3.6 Simple regression models for main factors appearing in multiple regression model of percentage of kernels damaged in season 2000B 90

3.7 Simple regression models for main factors appearing in multiple regression model of the percent grain weight loss in season 2000B 91

3.8 Simple regression models for main factors appearing in multiple regression model of the number of progeny emerged in season 2000B 92

3.9 Simple regression models for main factors appearing in multiple regression model of the Dobie Index rating in season 2001A 93

3.10 Simple regression models for main phytochemical factors appearing in multiple regression model of the percentage of damaged kernels in season 2001A 94

3.11 Simple regression models for main factors appearing in multiple regression model of the percent grain weight loss in season 2001A 95

3.12 Simple regression models for main phytochemical factors appearing in multiple regression model of the number of progeny emerged in season 2001A 97

3.13 Two models showing effect of total phenolic content on the percentage of damaged kernels in season 2000B 98

3.14 a QTL map (chromosome 1 and 2) 107

3.14 b QTL map (chromosome 3 and 4) 108

3.14 c QTL map (chromosome 5 and 6) 109

3.14 d QTL map (chromosome 7 and 8) 110

3.14 e QTL map (chromosome 9 and 10) 111

4.1 Mass spectra (electron impact) and proposed structures with fragmentation patterns of two unknown UV absorbing compounds found in corn bran extract From Moreau *et al.* (2001) 117

List of Tables

2.1 Description of germplasm used in Chapter II 16

2.2 Method precision analysis 22

2.3 Recovery analysis of HAACs in maize grain samples 23

2.4 Minimum detection levels of the HAACs in maize grain 24

2.5 HAACs in different tissues in maize landrace Yucatan 7 25

2.6 Summary of HAAC content of pericarp and aleurone tissues in the thirty Mexican landraces analyzed 28

2.7 Summary of reclassification of landraces into Wellhausen's Landrace groups using Model 1 45

2.8 Summary of misclassified landraces for Model 1. 46

2.9 Summary of reclassification of lancraces into Wellhausen's Landrace groups using Model 2 47

2.10 Summary of misclassified landraces for Model 2 48

2.11 Comparison of relative distances between centroids of Wellhausen groups as computed by Model 1 and Model 2 49

2.12 The Reclassification of the Poorly Defined group by Model 2 50

3.1 Characterization of the phytochemical resistance factors and insect ratings for the CIMMYT post-harvest resistance genotypes, season 98A 66

3.2 Characterization of the phytochemical resistance factors and insect ratings for the CIMMYT post-harvest resistance genotypes, season 99B 67

3.3 Correlations between phytochemical, physical and resistance traits in CIMMYT resistant populations 68

3.4 Equations and results of multiple regressions of phytochemical markers on resistance and physical traits in CIMMYT resistant populations in season 98A 69

3.5 Characterization of the parental lines of the QTL mapping population 74

3.6 Characterization of the phytochemical resistance factors and insect ratings for the mapping population, TL2000B 75

3.7 Characterization of the phytochemical resistance factors and insect ratings for the mapping population, TL2001A 76

3.8 Correlations between phytochemical, physical and resistance traits in QTL mapping population 78

3.9 Equations and results of multiple regressions of phytochemical markers on resistance and physical traits in the mapping population 79

3.10 Correlations between the physical and insect resistance parameters of season 2000B and 20001A 80

3.11 Correlations between the phytochemical parameters of season 2000B and 2001A 81

3.12 Correlations between phytochemical parameters within season 2000B 82

3.13 Correlations between phytochemical parameters within season 2001A 83

3.14 Weather statistics from Tlaltizapan for season 2000B and 2001A 98

3.15 Quantitative Trait Loci Interval Data for phytochemical markers 101

3.16 Quantitative Trait Loci Interval Data for Insect Resistance Markers 104

List of Abbreviations

APCI	Atmospheric Pressure Chemically-assisted Ionization
CIMMYT	Centro Internacional de Mejoramiento de Maíz Y Trigo International Centre for Maize and Wheat Improvement
CFP	p-Coumaroyl-feruloyl putrescine
cM	centi-Morgan
DFA	Diferulic Acid
DFAXX	Dimer of phenolic acid-carbohydrate complexes
DFP	Diferuloyl putrescine
HAAC	Hydroxycinnamic acid amide conjugate
HPLC	High Pressure Liquid Chromatography
HPLC-MS	High Pressure Liquid Chromatography – Mass Spectroscopy
LR	Likelihood Ratio
MAS	Marker Assisted Selection
MS	Mass Spectroscopy
QTL	Quantitative Trait Locus
RFLPs	Restriction Fragment Length Polymorphisms
RIL	Recombinant Inbred Line
SSRs	Short Sequence Repeats
TFA	Triflouroacetic acid

Chapter I: Introduction

Archaeological evidence shows that around 7000BP the residents of the Tehuacan valley in Central Mexico cultivated a primitive cereal crop, which would later develop into the Meso-American staff of life (Mangelsdorf *et al.*, 1964; MacNeish, 1964). This plant is now known as maize (*Zea mays* L.) or as corn in Canada and the United States. In the pre-colonial era, the maize crop was spread by indigenous people's north and south to what is now modern Canada and Argentina, exploiting every limit of its possible natural range in the Americas (Eubanks, 2001). The Spanish conquest of the Americas eventually brought maize to the rest of the world. Currently maize is one of the three most important cereal crops worldwide, and is arguably the most important plant indigenous to the Americas. Maize grain is an important staple food for human consumption in many Central and South American countries as well as parts of sub-Saharan Africa and is used around the world as a major source of animal feed.

A considerable research effort in recent years has been directed towards improving maize. The success of these efforts may be measured by the large numbers of maize varieties and hybrids that have been developed with improved productivity, grain quality and tolerance to biotic and abiotic stress. However, much of the accrued benefit of these inputs is often lost during grain storage. This is a particularly important issue in developing countries where the grain is grown, stored and used by small-scale, subsistence farmers. In these areas, post-harvest losses due to the maize weevil (*Sitophilus zeamais*), a stored grain pest, have been recognized as an increasing constraint (Vowotor *et al.*, 1995). Storage is often under adverse conditions: optimal grain drying is not possible, airtight storage facilities are not available, and chemical control is seldom available and/or prohibitively expensive. Improving innate resistance in the

stored grain is the least expensive and, in many cases, the only practical means by which many farmers in the developing world can improve the protection of their food supply.

1.1 Biology of *Sitophilus zeamais*

Distribution of the Sitophilus *spp.*

The genus *Sitophilus* is part of the sub-family Calandrinae and the family Curculionidae, the snouted beetles or weevils, of the order Coleoptera. There are three species of *Sitophilus* that are pests of economic importance: *S. granarius*, *S. oryzae*, and *S. zeamais*. The three species are all storage grain pests, but differ in distribution, size, and feeding and habitat preferences (Longstaff, 1981). *S. granarius*, the granary weevil, is most commonly found as a pest of wheat and barley in cool temperatures. In warmer parts of the world, it is restricted to higher altitudes. The range of *S. oryzae*, the rice weevil, is primarily restricted by the humidity of the grain; it is rarely found where the grain moisture is less than 10% or higher than 16%. While it is one of the most important pests of cereals, it tends not to be successful in areas that have a very high summer temperatures and is frequently displaced by *S. zeamais* in hot, moist areas. Birch (1953) demonstrated that, when utilizing the same resources, *S. zeamais* will out-compete *S. oryzae* in maize grain, but will be out-competed when in wheat. The moisture range of successful habitation for *S. zeamais* is much larger than that of *S. oryzae*. *S. zeamais* can thrive at relative humidities around 90% (Okelana and Osuji, 1985) and has even been found to attack stored apples (Longstaff, 1981). While the maize weevil flourishes in warm, moist areas, from the southern U.S.A. and throughout the tropical and sub-tropical areas, in cooler areas it is usually restricted to maize as a host.

Morphology and history of the Sitophilus *complex*

There is a history of confusion between the *oryzae* and *zeamais* species of *Sitophilus*. The biology of the two weevils is sufficiently similar that it was unclear if they were distinct species for many years. Both Richards (1944, from Longstaff, 1981) and Birch (1944) were unable to find any non-overlapping morphological differences between the two strains of what was then called *Calandra oryzae* (L.). Often the insects were referred to as either the large or the small strain of *C. oryzae.* Floyd and Newsom (1959) established the two species as distinct through differences in the genitalia: the large form was now called *Sitophilus oryza* (L.) and the small form, *Sitophilus sasakii* (Tak.). Finally, Kuschel (1961, from Longstaff, 1981) corrected the taxonomy by establishing that it was the small strain that Linnaeus had described in 1763 so it was now properly named *Sitophilus oryzae* (L.). The large form was named *Sitophilus zeamais* as described by Motschulsky in 1855.

Life cycle

The maize weevil will infest grain either in the field or in storage. When ovipositing onto loose or "shelled" grain, the egg may be laid at any spot of the grain. Few eggs are laid in the embryo, however, and the most frequent area chosen is the side opposite the embryo (Kossou *et al.*, 1992). When infesting unshelled grain, only the crown end is accessible. The female weevil excavates a small hole in the grain into which she oviposits a single egg. She secretes a mucilaginous plug to cover the egg and seal the hole. This plug is the only evidence of infestation. It is possible for more than one egg to be laid in a single grain; usually this will occur only when there is pressure from over-crowding (Longstaff, 1981). Due to cannibalism, it is, however, rare for more than one larva to grow to maturity in a single kernel (Longstaff, 1981).

The duration of the life cycle depends on the type of grain infested, temperature and humidity. In maize, the development time is longer in flint types and shorter in floury types; weevils also spend longer developing in grain on the cob, than in loose grain (Vowotor *et al.*, 1995). In maize, from mid-oviposition time to hatching takes approximately 7 to 9 days. The first instar, upon hatching, burrows into the tissues of the grain heading towards the radicle end, feeding as it goes. This instar lasts between 7 and 10 days. The second and third instar last for approximately 5 and 7 days, respectively. During these stages the larva continues to feed and tunnels first to the radicle and then turns to the plumule end. Toward the end of the fourth and final instar (4 to 6 days), the larva uses a mixture of larval secretion and frass to seal off the end of the tunnel to create a pupal chamber (Kirkpatrick and Wilbur, 1965). The majority of pupal chambers cross both the endosperm and the embryo, demonstrating that most larvae feed on the embryo. The pre-pupal and pupal stages last for a total of between 5 and 6 days. An adult emerges from the pupal casing, but does not emerge from the grain for several days. This period ranges from 5 to 12 days, or even longer in low temperatures. The lifespan of the adult weevil is temperature and humidity dependent: under normal conditions it averages about 10 weeks, but in lower temperatures it can average as long as 30 weeks. Approximately 400 eggs can be laid can be laid during the reproductive lifespan of the adult female (Birch, 1944). Selected stages from the *S. oryzae* lifecycle are shown in Figure 1.1.

Damage to stored maize

Post-harvest losses in maize usually amount to between 5 and 15% in developing countries; surveys in Mexico have shown a mean kernel damage of 30%, for which *S. zeamais* is largely responsible (Tigar *et al.*, 1994). Damage to stored maize is caused not only by the grain damage occurring from the feeding of both larval and adult *S. zeamais*, but also by a reduction of

grain quality as the insect produces frass, a mixture of grain dust and excrement. These factors decrease the viability of grain and the palatability of the grain for human or animal consumption.

In addition to the direct damage, the grain is heated as a result of insect respiration, and the temperature of the grain increases. This increase in temperature creates positive feedback by decreasing the development time of the insect and increasing its rate of egg laying (Longstaff, 1981). As the grain heats locally, the adult insects disperse from the site causing an enlargement of the heated area (Oxley & Howe, 1944). Related to this phenomenon is a migration of moisture to the periphery of the heated area; this is caused simply by the temperature differential that affects the capacity of air to carry moisture. This increases the grain moisture at the edges of the infected areas and creates conditions more suitable to insect development (Oxley & Howe, 1944) and invasion of fungi (Barney *et al.*, 1995).

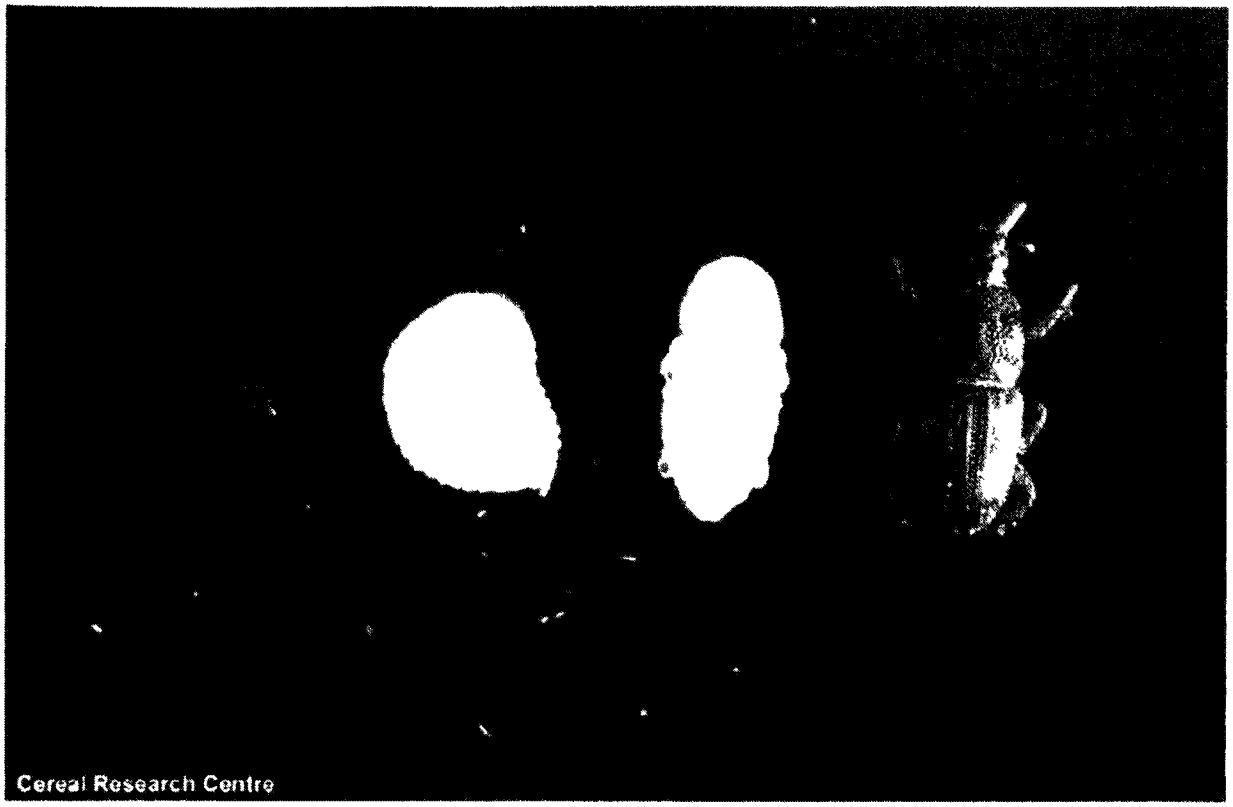


Figure 1 1 Four representative stages in the lifecycle of *Sitophilus oryzae* Stage 1, the egg is laid in the cereal grain Stage 2, a second instar larva, and 3, a pupa, grow inside the grain The insect does not emerge until after the adult stage, shown here as the fourth stage, is reached Photo courtesy of Cereal Research Centre, AAFC

Natural enemies and parasitization

There are several insects associated with grain storage that are enemies of storage pests. Some of these, such as many tenebrionid beetles, are not only predators to storage pests, but will, in the absence of insect prey, subsist on the grain itself (Savidan and Bergvinson, 2000). This is one reason why there hasn't been a serious effort to use these enemies as bio-control agents. Several species of pteromalid wasps are readily found in tropical maize stores, such as *Chaetospila elegans* (Westwood), *Lariophagus distinguendus* (Foerster), *Cerocephala dinoderi* Gahan, and *Anisopteromalus calandrae* (Howard) (Longstaff, 1981). In all these species, the female wasp punctures the pericarp with her ovipositor, paralyses the *Sitophilus* spp. larvae, and lays one egg. The wasp larva develops inside the host insect, killing the host larva in the process. These insects are not generally considered suitable candidates for bio-control because they have significantly shorter life spans and lower productivity than their hosts, so that effective control would require regular introduction of large numbers of the control vector (Longstaff, 1981). In the case of *A. calandrae*, the female adult lives six to seven days over which time it can produce between 30 and 40 progeny; the development time for the larvae is approximately fifteen days from oviposition to emergence (Smith, 1993).

1.2 Phytochemical Basis for *Sitophilus zeamais* Resistance in *Zea mays* L. Grain

Past work has examined sources of resistance in maize grain storage. Many studies have found that cultural practices such as the method used to dry and store the grain (e.g. shelled grain, or on the cob whether dehusked or undehusked) have an effect on the infestation rate or the damage inflicted by *S. zeamais* (Floyd and Powell, 1958; Kossou *et al.*, 1992; Kossou *et al.*, 1993; Tigar *et al.*, 1994; Vowotor *et al.*, 1995). As important as it is to determine what cultural

or technological changes could decrease grain storage losses, it is at least as important to determine a source of resistance that can be improved by selection and breeding. Such a source of resistance can be delivered to farmers directly, as seed, and does not need to break through traditions and cultural barriers.

Widstrom *et al.* (1975) determined that there is genetic variation in maize grain for resistance to storage pests, demonstrating that most of the additive variation in resistance originated maternally. This result was in accordance with Van Schoonhoven *et al.* (1975) who determined that factors for resistance to *S. zeamais* in the grain were located in the pericarp tissue, which is maternally derived.

Early research to clarify the source of *S. zeamais* resistance found that resistant grain varieties displayed intense fluorescence, associated with ferulic acid and related compounds, in the pericarp and aleurone tissues (Serratos *et al.*, 1987). Subsequent work used more direct measures to correlate cell wall bound trans-ferulic acid with grain resistance and with grain hardness (Classen *et al.*, 1990). Other work confirmed by HPLC of extracts of the various tissues that the grain phenolics are localized in the pericarp, aleurone and embryo tissues (Arnason *et al.*, 1992). Generation means analysis found that the genetics of the phenolic acids in maize grain correspond closely to the genetics of grain susceptibility (Serratos *et al.*, 1993).

Later work revealed the presence of dehydro-dimers of ferulic acid bound to heteroxylans of maize cell walls (Arnason *et al.*, 1994). It has been proposed that these cell wall bound diferulic acids (DFA) fortify the cell wall, thus clarifying the correlation between phenolics and grain hardness (Arnason *et al.*, 1994; Saulnier and Thibault, 1999). The presence of diferulates in maize cell walls has become very well established. Fry *et al.* (2000) have determined that the majority of the ferulate dehydro-dimers bound to the cell wall are secreted to the cell wall as

dimers, and that in mature tissues little additional dimerization occurs in the cell wall. An implication of this finding is that cell wall bound phenolic based resistance, unlike the DIMBOA-based resistance seen in maize roots, is not inducible.

Other researchers have proposed that another type of phenolic conjugate, the hydroxycinnamic acid amide conjugates (HAACs) may contribute to insect resistance by an antibiosis mechanism (Panagabko *et al.*, 2000). These compounds are also primarily located in the maternally derived outer tissues of the grain (Sen *et al*, 1994).

1.3 Hypotheses and Objectives:

The research presented in this thesis is focused on increasing the understanding of these phytochemical resistance factors to *S. zeamais* in stored maize grain. The work has been split into two main parts based on the role in post-harvest defence by two different phytochemical types: the HAACs and the cell wall bound phenylpropanoids.

The first experimental section investigates the potential role of the HAACs in maize post-harvest resistance to *S. zeamais*. Although the distribution of the HAACs has been well described throughout the angiosperms (Martin-Tanguy *et al.*, 1978), relatively little is known of their biological role, particularly in grain. The following hypotheses have been made in an attempt to frame the work in this section.

It was hypothesized (1a) that the HAACs are toxic compounds located in maize grain that have a negative effect on *S. zeamais* upon dietary exposure. The testable predictions that follow this hypothesis are:

1a.1. The HAACs will have an acute toxic or anti-feedant effect on the maize weevil *S. zeamais*. i.e. the *S. zeamais* adults will feed at a lesser rate on diet treated with maize HAACs as compared to a control diet.

1a.2. The HAACs will have a toxic effect on *S. zeamais* that are exposed to maize HAACs over their lifecycle. i.e. *S. zeamais* consuming a diet treated with maize HAACs throughout their lifecycle will produce fewer and/or inferior offspring when compared to a population reared on a control diet.

A corollary hypothesis was (1b) that the HAACs are toxic compounds located in maize grain to which *S. zeamais*, as a maize specialist, is adapted and which have no negative effect upon the maize weevil. The testable predictions that follow this hypothesis are:

1b.1. The HAACs will have no acute toxic or anti-feedant effect on the maize specialist *S. zeamais*, i.e. the *S. zeamais* adults will not feed at a lesser rate on diet treated with maize HAACs as compared to a control diet.

1b.2. The HAACs will have an acute toxic or anti-feedant effect on generalist grain feeding insects such as *Sitophilus oryzae*, the rice weevil, a closely related species to *S. zeamais*.

1b.3. The HAACs will have no toxic effect on *S. zeamais* that are exposed to maize HAACs over their lifecycle. i.e. *S. zeamais* consuming a diet treated with maize HAACs

throughout their lifecycle will not produce fewer and/or inferior offspring when compared to a population reared on a control diet.

The null hypothesis that corresponds to both the alternative hypotheses is that the HAACs are not toxic compounds to *S. zeamais* of *S. oryzae*. This would be supported if no negative effect were seen on any insects exposed to HAACs.

The second experimental section of the thesis seeks to increase our understanding of the role of the cell wall bound phenylpropanoids in *S. zeamais* resistance. The relationship between the concentration of total cell wall bound phenylpropanoids and resistance to *S. zeamais* is well defined, as previously described. A possible mode of action for these compounds has been determined in leaf tissue to be an increase in cell wall toughness mediated by the fortification of the plant cell wall by arabinose bound ferulic acid dehydro-dimers. These two ideas are combined to provide the hypothesis (2) that the cell wall bound diferulic acid content increases cell wall toughness and provides a major source of *S. zeamais* resistance in maize grain. The testable predictions that follow this hypothesis are:

2.1. The peak force needed to puncture maize grain should strongly correlate to the DFA concentration of the outer grain tissues.

2.2. The resistance to *S. zeamais* in maize grain should strongly correlate to the DFA concentration in the outer grain tissues.

2.3. The Quantitative Trait Loci (QTLs) for DFA content should occupy the same regions on the maize chromosome with the QTLs for *S. zeamais* resistance.

Objectives

The objectives of this study were 1) to verify the tissue location of the hydroxycinnamic acid amides (HAACs) in maize grain; 2) to establish a range of variation of the HAACs in maize grain by analysing the concentration in the external tissues of the grain of Mexican maize landraces; 3) to examine possible toxic effects of HAACs on maize grain feeding insects to determine if the HAACs are a possible source of resistance; 4) to elucidate the role of the cell wall bound phenylpropanoids in the defence of grain against *S. zeamais*; and 5) to obtain a QTL map for the putative phytochemical resistance traits, particularly the cell wall bound phenylpropanoids.

Chapter II: The Hydroxycinnamic Acid Amide Conjugates in Tropical Maize Grain

2.1 Introduction:

Hydroxycinnamic acid amide conjugates (HAACs) have been identified as the main phenolic constituents in the reproductive organs of many flowering plants. Martin-Tanguy *et al.* (1978) characterized 24 different hydroxycinnamic acid derivatives of the polyamines: putrescine, spermidine, tryptamine, tyramine, and spermine from plants representing 13 different families. These compounds are absent from the leaves, stems, and even the petals and sepals of these plants; they are found only in meristems, reproductive organs, and seeds (Martin-Tanguy *et al.*, 1978).

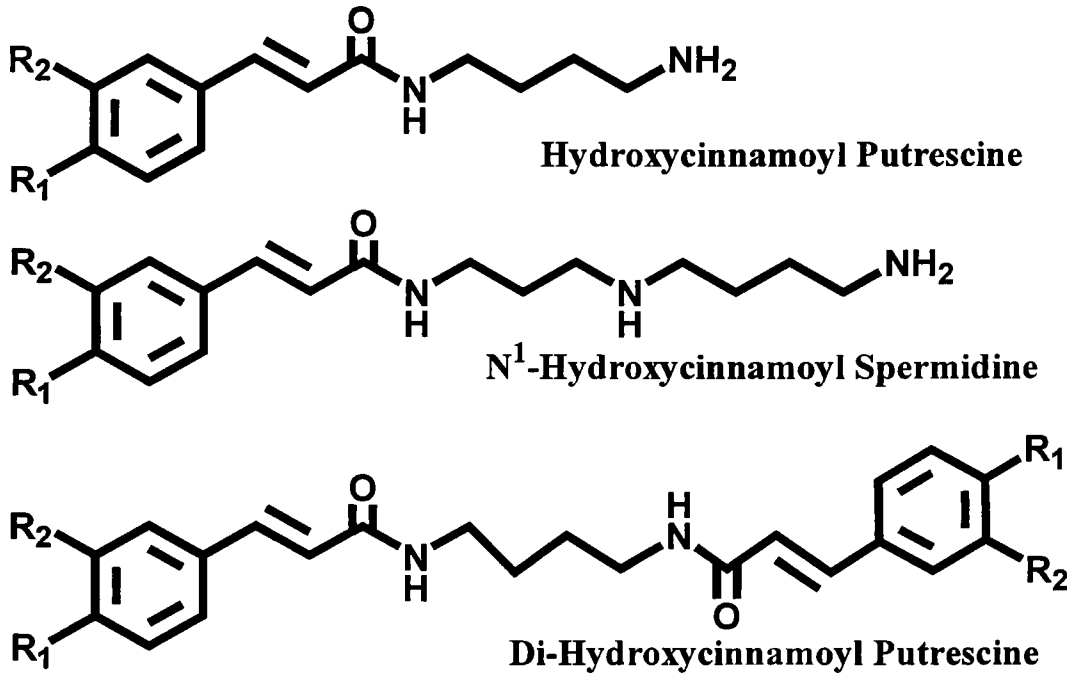
In maize, HAACs have been shown to be present in large quantities in the anthers of male fertile maize and absent in male sterile lines, and appear to be biochemical markers of male fertility (Martin-Tanguy *et al.*, 1982). After pollination, the HAACs accumulate in the seed as it develops; feruloyl putrescine increases rapidly following fertilization and then falls as diferuloyl putrescine increases. At maturity feruloyl putrescine is six times greater than on day four after fertilization and diferuloyl putrescine is 34 times greater (Martin-Tanguy *et al.*, 1982). Previous results have shown that most of the HAACs in the maize grain are located in the outer tissues, the pericarp and aleurone layers (Sen *et al.*, 1994).

In contrast to the extensive literature on the chemistry and distribution of HAACs in plants, relatively little is known about the biological function of these compounds. Considerable research has attempted to define the relationship between the accumulation of HAACs and floral development (Martin-Tanguy, 2001). The levels of HAACs in rice grain have been correlated with seed viability (Martin-Tanguy, 2001). Other work has correlated the accumulation of

HAACs in response to fungal pathogens, such as the development of smut infection in sugarcane buds (Legaz *et al.*, 1998). The HAACs have been hypothesized to be toxic to phytophagous insects. Panagabko *et al.* (2000) remarked on the structural similarity of the HAACs to the polyamine toxins isolated from spider and wasp venoms. If this were the case, the HAACs may be partially responsible for the ability of a plant to defend against insect pests.

The current work attempts to provide a foundation for future work describing patterns in HAACs in maize grain. An HPLC method was developed to separate and quantify the HAACs. Different grain tissues were analyzed to verify the tissue location of these compounds. Mexican maize landraces were analyzed to estimate the variability of HAAC content in maize grain. HAAC concentration in the Mexican landraces was analyzed to determine if any eco-geographical patterns explain the variation between landraces or groups of landraces. Finally, an attempt to use the HAACs as taxonomic marker compounds was explored; models were created to determine how well the variation in HAACs in the Mexican landraces supports the landrace groups established by Wellhasusen *et al.* (1952).

The current work also attempts to illuminate the role of the HAACs in maize grain as an insect defence compound. The hypothesis that the HAACs are toxic compounds was investigated by performing acute feeding bioassay trials with various HAACs on *S. zeamais* and the close relative *S. oryzae*, and by performing a two-generation life-cycle bioassay with feruloyl putrescine using *S. zeamais*.



Coumaroyl if R_1 = HO- R_2 = H- Caffeoyl if R_1 & R_2 = HO-
Feruloyl if R_1 = HO- R_2 = CH_3O- Cinnamoyl if R_1 & R_2 = H-

Figure 2.1: Chemical structure of hydroxycinnamic acid amide conjugates examined in this thesis. Shown are the hydroxycinnamic acid mono- and di-conjugates of putrescine and spermidine.

2.2 Methods & Materials:

Representative accessions of Mexican landraces of maize were selected from the germplasm bank and propagated at CIMMYT (Centro Internacional de Mejoramiento de Maíz Y Trigo – the International Centre for Maize and Wheat Improvement) before shipment to Canada. A summary of the germplasm used in this study is shown in Table 2.1.

Separation of maize grain tissues and sample preparation for extraction

Dried maize grain was soaked in water overnight at 4°C. The grain was blotted dry and run through a Strong-Scott Pearler mill to coarsely crush the kernels. Tissues were then separated manually into three parts – the external portions, or the pericarp and aleurone; the endosperm; and the embryo. The separated tissues were then frozen, lyophilized, and ground to a fine powder with a UDI cyclone mill.

Extraction of hydroxycinnamic acid amides from maize tissue

The extraction procedure was modified from Panagabko *et al.*, 2000. One half gram of dried ground sample was extracted with ultrasound for 10 minutes in 10 mL 80% methanol (water) acidified with 0.25% concentrated hydrochloric acid by volume. The supernatant was collected and the extraction was repeated twice. The pooled supernatants (30ml) were dried by rotary evaporator and re-dissolved in 3ml methanol (100%).

Table 2.1: The Landraces and the altitude range at which they have been cultivated according to Wellhausen *et al.* (1952) and the CIMMYT accessions for the germplasm that was used in this study. N.A. indicates that the material or information were not available. Landraces 33 and above were defined after 1952.

	Cultivation Altitude Range (meters)	CIMMYT Accession Used in Study
A. Ancient Indigenous Races		
1. Palomero Toluqueño	2200 – 2800	Mexico-55
2 Arrocillo Amarillo	1600 – 2000	Puebla-463
3. Chapalote	100 – 600	Sinaloa-2, Sinaloa-35
4. Nal-Tel	100	Yucatán-7, Yucatán-16
B. Pre-Columbian Exotic Races		
5. Cacahuacintle	2200 – 2800	Mexico-212
6. Harinoso de Ocho	100	Nayarit-24
7. Olotón	2000 – 2400	Chiapas-124
8. Maíz Dulce	1000 – 1500	Guanajuato-93A
C. Prehistoric Mestizos		
9. Cónico	2200 – 2800	Mexico-182
10. Reventador	0 – 1500	Nayarit-39
11. Tabloncillo	0 – 1500	Jalisco-222
12. Tehua	600 – 1000	- N.A.
13. Tepicintle	0 – 600	Guanajuato-207
14. Comiteco	1100 – 1500	Chiapas-235
15. Jala	1000	Nayarit-72
16. Zapalote Chico	100	Oaxaca-179
17. Zapalote Grande	100 – 600	Chiapas-236
18. Pepitilla	1000 – 1700	Morelos-52
19. Olotillo	300 – 700	Chiapas-237, Chiapas-239
20. Tuxpeño	0 – 500	V-520-C
21. Vandeño	0 – 500	Oaxaca-4
D. Modern Incipient Races		
22. Chalqueño	1800 – 2300	- N.A. -
23. Celaya	1200 – 1800	Guanajuato-101
24. Cónico Norteño	1600 – 2100	Guanajuato-102
25. Bolita	900 – 1500	Oaxaca-130
E. Poorly Defined or New Races		
26. Conejo	200 – 350	Guerrero-168
27. Mushito	2400	N.A.
28. Complejo Serrano de Jalisco	1500 – 2700	Jalisco Grp 12
29. Zamorano Amarillo	1500 – 2200	Michoacan Grp 13
30. Maíz Blando de Sonora	0 – 500	Sonora-32
31. Onaveño	0 – 500	Sonora-139
32. Dulcillo del Noroeste	1500	Sonora-159
33. Bofo	N.A.	Nayarit-222
34. Tabilla de Ocho	- N.A. -	Nayarit-185
35. Gordo	N.A.	Chihuahua-140
36. Apachita	- N.A. -	Chihuahua-166
37. Azul	- N.A	Chihuahua-133

HPLC Analysis

HPLC Analysis was accomplished with a Hewlett-Packard LC Series 1100. The solvent system was comprised of acetonitrile (A), methanol (B), and an aqueous 25 mM sodium phosphate buffer with 0.5% acetic acid by volume (pH 3.18) (C). Starting conditions were 0% A, 5% B, 95% C. The gradient was as follows: 5% B throughout entire run; 0-10% A in 10 min; 10-70% A in 10 min; 70-0% A in 5 min; allow column to re-equilibrate for 10 min. A C4 Phenomenex Kromasil 3.5μ column, 150x2.0mm, fitted with Prosphere 300 5μ C4 guard column, 7.5x3.2mm, was used in a 50°C column oven to achieve compound separation. The flow rate was 0.200 mL/min, and detection was at 290 nm and 320 nm. An injection program was used to dilute the samples to 20% methanol in order to improve peak shape and resolution. Peak identification was determined by comparison of retention times and spectra with purified compounds obtained from Dr. J. Atkinson (Brock University). Details of the separation, relative standard deviation and minimum recovery can be found in the results section.

Recovery Analysis

A standard solution of a mix of the six hydroxycinnamic acid amide conjugates was added in incremental volumes to 500 mg of Sinaloa 35 maize external grain tissue samples. These samples were dried under nitrogen flux and extracted as detailed above. Three control replicates (with no added compounds) were used to determine the amount in the samples.

Acute Toxicity – Wheat disk feeding assay

Flour disks were prepared according to the method of Xie *et al.*, 1996. Treatment disks were prepared by mixing 800mg wheat flour with 3600µl distilled water and 400µl methanol containing different amounts of the dissolved test substance (feruloyl putrescine or diferuloyl putrescine). Control disks were prepared with flour, water, and methanol only.

The bioassay was conducted by placing five disks (approximately 80 mg) in a Petri plate with 25 unsexed weevils for seventy-two hours. Four replicates were performed for each treatment with each insect species. Amount eaten was determined by weighing the dried disks before the assay and at the end of the seventy-two hours. Tests for both insects were run concurrently using a single preparation of the wheat disks.

Chronic Toxicity – Lifecycle assay

Feruloyl putrescine was dissolved in methanol in order to create the highest treatment concentration. Serial dilutions were performed to obtain various concentrations of feruloyl putrescine in methanol. Six millilitres of each concentration were added to 120g of soft winter wheat equilibrated at 27°C and 80% relative humidity. The control consisted of 120g of wheat, equilibrated similarly to the treatments, with six millilitres of methanol added to it. The wheat from each treatment was rolled for 30 minutes in a round bottom flask to ensure a good distribution of the compounds throughout the sample. The grain samples were then put under vacuum over a warm water bath until the grain was very dry and the solvent was removed, or for no less than 30 minutes. The grain from each treatment was split equally into four vials and allowed to equilibrate in a growth chamber to 27°C and 80% relative humidity for 6 weeks. This method of coating the wheat with the compound, assuming minimal penetrance of the compound

into the grain, was used as the simplest method of mimicking the distribution of the compound i maize grain (see results section), with the majority located in the outer tissues. After this time the grain replicates were weighed and twenty *S. zeamais* adults (0-7 days old) were introduced into each vial. The insects were sexed according to Halstead (1963) and a 1:1 sex ratio was used in the trial. The insects were left on the grain in the growth chamber for one week, and then removed. The vials of grain were then left in the controlled conditions for five weeks at which point they were examined for emerged adults every two days for two weeks or until all the F1 generation had emerged. The grain was then re-weighed to determine the weight consumed by the weevils. A 5 mL sample of the grain was visually examined under white light to determine the proportion of the grain that was damaged.

An F2 generation test was performed using the progeny of the original trial. Ten males and ten females from each vial of the original trial were added to a fresh vial of the same treatment and kept as detailed above.

2.3 Results & Discussion:

HPLC Analysis

Separation, Retention Times and RSD

An example chromatogram can be seen in Figure 2.1. The elution times were as follows: N1-coumaroyl spermidine 8.27 min; N8-coumaroyl spermidine 9.27 min; caffeoyl putrescine 9.84 min; N1-feruloyl spermidine 11.13 min; coumaroyl putrescine 12.90 min; feruloyl putrescine 14.96 min; cinnamoyl putrescine 18.72 min; and diferuloyl putrescine 21.80 min. Feruloyl and diferuloyl putrescine were quantified at 320 nm, very close to the compounds peak absorption, as it gave better resolution and more accurate quantitation than 290 nm. All other compounds were quantified more accurately at 290 nm. Method precision analysis gave relative standard deviations of 1.02% to 2.38%; the relative standard deviation is obtained by dividing the standard deviation of the mean by the mean value, see Table 2.2 for details.

Recovery Analysis

The recovery analysis results are summarized in Table 2.3. Recovery of the hydroxycinnamic acid amide conjugates was very good, averaging, for each compound, between 93.6% for caffeoyl putrescine and 101.6% for coumaroyl putrescine. The mean recovery rate of all the compounds was 101%. In general, the recovery rate was most inaccurate when the amount added to the sample was much less than the amount already in the sample – indicating that it is an effect of sampling error. No recovery analysis was performed for the N1 and N8 coumaroyl spermidines because they could not be found in quantifiable levels in the maize tissue.

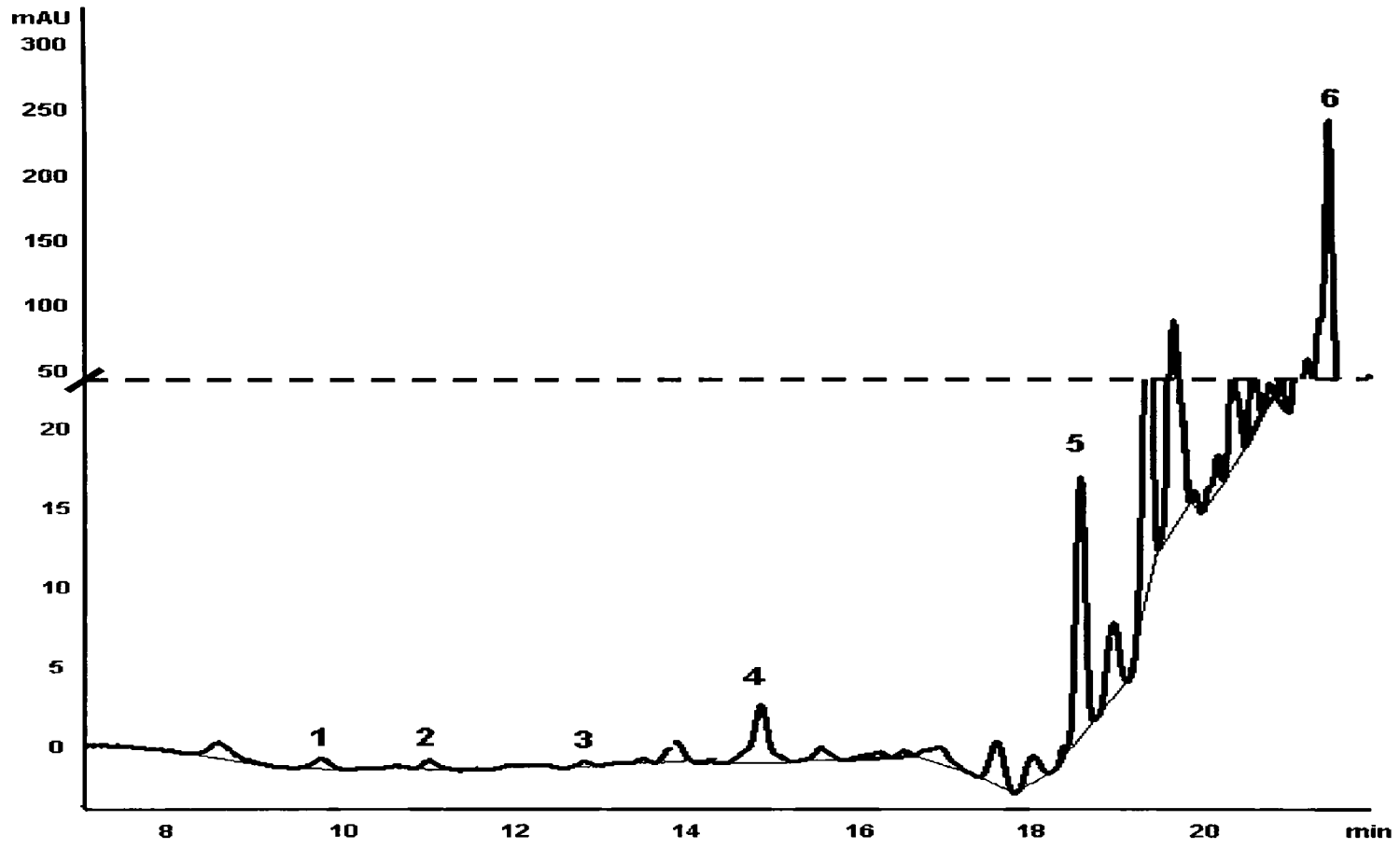


Figure 2.2: Chromatogram of the Mexican landrace Comiteco. The trace for only 320 nm is shown. Feruloyl putrescine and diferuloyl putrescine were quantified at 320 nm as it allowed for better resolution of the peaks; all other compounds were quantified at 290 nm. The peaks shown are: 1: caffeoyl putrescine 9.84 min; 2: N1-feruloyl putrescine 11.13 min; 3: coumaroyl putrescine 12.90 min; 4: feruloyl putrescine 14.96 min; 5: cinnamoyl putrescine 18.72 min; and 6: diferuloyl putrescine 21.80 min.

Table 2.2: Method precision analysis: Repeatability of quantification of HAACS in replicate maize grain samples of variety Chiapas 235.

Rep	Quantity in Extract (μg/ml)					
	Diferuloyl Putrescine	Cinnamoyl Putrescine	Feruloyl Putrescine	p-Coumaroyl Putrescine	N1-Feruloyl Spermidine	Caffeoyl Putrescine
1	612.7	36.62	20.39	0.982	0.7099	6.2654
2	585.1	34.24	18.52	1.160	0.8149	6.1708
3	565.4	31.65	23.26	0.920	0.6399	7.3673
4	580.6	33.55	22.18	0.988	0.6526	6.9805
5	600.3	31.98	20.99	1.035	0.7425	7.0356
Mean	588.8	33.61	21.07	1.017	0.7120	6.7639
St Dev	18.27	1.998	1.803	0.0899	0.0711	0.5208
Rel. St. Dev.	0.0310	0.0594	0.0856	0.0884	0.0999	0.0770

Table 2.3: Recovery analysis of HAACs from the external grain tissues of maize variety Sinaloa 35.

Diferuloyl putrescine			
Amount Added (µg)	Amount Measured (µg)	Amount Recovered (µg)	Recovery (%)
0.00	260.77	0.00	
57.73	324.28	63.49	110%
115.45	369.30	108.52	94%
230.90	475.51	214.74	93%

Cinnamoyl putrescine			
Amount Added (µg)	Amount Measured (µg)	Amount Recovered (µg)	Recovery (%)
0.00	32.39	0.00	-
8.32	39.96	7.57	91%
16.64	49.86	17.47	105%
33.28	63.67	31.28	94%

Feruloyl putrescine			
Amount Added (µg)	Amount Measured (µg)	Amount Recovered (µg)	Recovery (%)
0.00	8.53	0.00	-
2.72	11.33	2.80	103%
5.44	14.02	5.49	101%
10.88	19.08	10.55	97%

Coumaroyl putrescine			
Amount Added (µg)	Amount Measured (µg)	Amount Recovered (µg)	Recovery (%)
0.00	3.94	0.00	
2.02	6.06	2.12	105%
4.04	8.10	4.16	103%
8.08	11.78	7.84	97%

N1-Feruloyl spermidine			
Amount Added (µg)	Amount Measured (µg)	Amount Recovered (µg)	Recovery (%)
0.00	0.42	0.00	-
0.31	0.72	0.30	97%
0.61	0.99	0.57	94%
1.22	1.74	1.32	108%

Caffeoyl putrescine			
Amount Added (µg)	Amount Measured (µg)	Amount Recovered (µg)	Recovery (%)
0.00	0.43	0.00	-
0.35	0.75	0.32	91%
0.70	1.04	0.61	87%
1.41	1.74	1.31	93%

Table 2.4: Minimum detection levels, by HPLC-DAD, of the HAACs in maize grain as determined by serial dilutions of a standard stock solution.

Minimum Detectable Amount Loaded on Column (ng)							
Diferuloyl Putrescine	Cinnamoyl Putrescine	Feruloyl Putrescine	p-Coumaroyl Putrescine	N1-Feruloyl Spermidine	Caffeoyl Putrescine	N1-Coumaroyl Spermidine	N8-Coumaroyl Spermidine
4.22	3.24	3.46	3.19	2.75	2.76	2.49	2.52

Table 2.5: Hydroxycinnamic acid amide conjugates in different tissues in maize variety Yucatan 7. Values given are μg/g dry weight plus or minus the standard error. Means followed by the same letter in a column are not significantly different according to ANOVA and a post-hoc Bonferronni multiple comparison test ($p < 0.05$). n=4 sub-samples of a single harvest for each tissue type.

	Diferuloyl putrescine		Feruloyl putrescine		Cinnamoyl putrescine		Caffeoyl putrescine		Coumaroyl putrescine	
Endosperm	4.16 ± 0.70	d	0.00 ± 0	d	1.62 ± 0.43	d	0.00 ± 0	c	0.00 ± 0	c
Embryo	335 ± 8.58	b	6.96 ± 1.54	b	16.67 ± 2.77	b	6.30 ± 1.89	a	2.27 ± 0.87	a
Pericarp & Aleurone	1023 ± 152	a	37.9 ± 2.08	a	42.46 ± 19.67	a	0.42 ± 0.19	b	1.10 ± 0.14	b
Whole Grain	23.64 ± 0.11	c	0.84 ± 0.84	c	6.17 ± 0.47	c	0.92 ± 0.92	bc	0.00 ± 0	c

Minimum Detection Levels

The minimum amounts detectable by HPLC for the HAACs ranges from 2.49 ng and 4.22 ng for N1-coumaroyl spermidine and diferuloyl putrescine respectively, and averages at approximately 3.08 ng (see Table 2.4).

Tissue Distribution of HAACs in maize grain

The hydroxycinnamic acid amide conjugates are found in the largest quantity in the external tissues of the maize grain – the pericarp and aleurone fraction (Table 2.5). The major representative of these compounds in all maize grain tissues is diferuloyl putrescine, followed by the related monomer feruloyl putrescine and cinnamoyl putrescine. No N1-coumaroyl spermidine or N8-coumaroyl spermidine was found at quantifiable levels in any maize sample.

HAACs in Maize Landraces

For each landrace accession the tissue collection and extraction was performed in triplicate, except where insufficient tissue was available: the accessions Guanajuato-102, Jalisco Group 12, and México 182 were only analysed in duplicate. The maize landraces exhibited considerable variation in the levels of the hydroxycinnamic acid amide conjugates (see Figure 2.2 and Table 2.6). Mexico is the centre of origin for maize, and the indigenous Mexican landraces are thought to contain a large amount of the total genetic and phenotypic variation in the crop. The ranges provided in Table 2.6 should therefore provide a good estimate of the total natural range of these compounds in maize grain.

In order to get a larger scale view of a pattern of variation in the hydroxycinnamic acid amide conjugates, the grain tissue HAAC content was analysed according to eco-geographical

data available on the Mexican landraces. The maximum altitude of the growing range of the race and the historical origin group assigned to the landrace (both from Wellhausen *et al.*, 1952) as well as the collection site of the landraces were used as the independent variables in ANOVA and regression models; the simple relationships from the model for diferuloyl putrescine content are shown in Figures 2.3a, b and c.

A significant negative correlation is seen between the diferuloyl putrescine content and the maximum altitude of origin for the landraces (Figure 2.3a). A similar trend is seen with the other HAACs. A similar result was obtained by Reid (1988) in the concentration of DIMBOA in maize plants over differing altitudes. The implication of this negative relationship with the altitudinal gradient is that these compounds were selected for in lower altitude growing ranges, where the insect pest densities are greater, and therefore, selection pressure by insects is theoretically greater. Levin and York (1978) suggested a similar evolutionary basis for the differences found in toxicity of alkaloids within a genus across eco-geographical strata. If indeed, herbivore pressure did result in a selection for these compounds, that selection would be predicted by the hypothesis that the HAACs are toxic to insects as based on the structural similarity of the HAACs to spider and wasp venoms (Panagabko *et al.*, 2000).

No significant difference is seen in the HAACs between the ancestral landrace groups (Figure 2.3b) defined by Wellhausen *et al.* (1952). This indicates that the variation is not strongly linked to the pre-historical origin of the landrace and implies that the selection for these compounds occurred in their current ranges.

Table 2.6: Summary of hydroxycinnamic acid amide conjugate content of pericarp and aleurone tissues in the 30 Mexican landraces analyzed.

	Diferuloyl Putrescine	Cinnamoyl Putrescine	Feruloyl Putrescine	p-Coumaroyl Putrescine	N1-Feruloyl Spermidine	Caffeoyl Putrescine	N8-Coumaroyl Spermidine	N1-Coumaroyl Spermidine
Mean (μg/g)	1172	25	23	1.10	0.60	0.42	0.00	0.00
± S.E.	84.10	2.84	2.79	0.14	0.08	0.19	0.00	0.00
Min	428.97	5.91	6.56	0.00	0.00	0.00	0.00	0.00
Max	2336.17	85.20	85.00	2.85	1.68	6.60	0.00	0.00

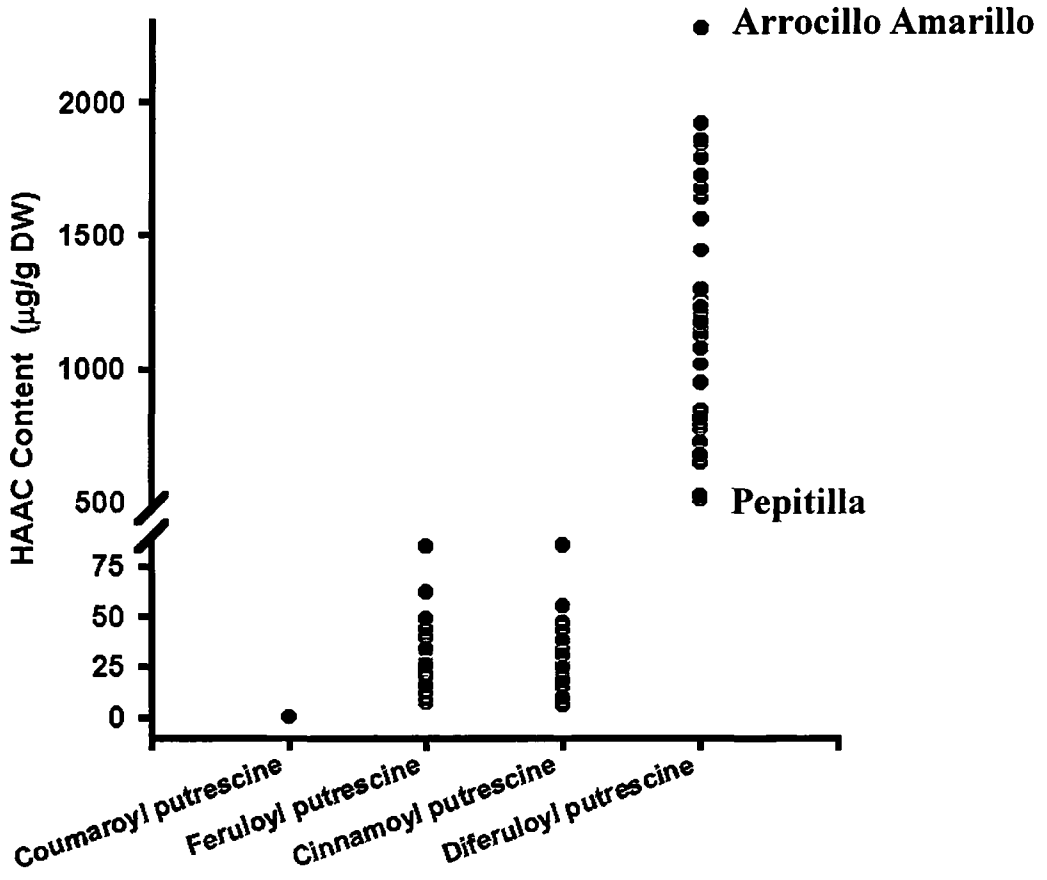


Figure 2.3: Illustration of the wide variation in hydroxycinnamic acid amide content in the Mexican landraces. Each point represents a single landrace; highest and lowest content of diferuloyl putrescine shown. Note log scale.

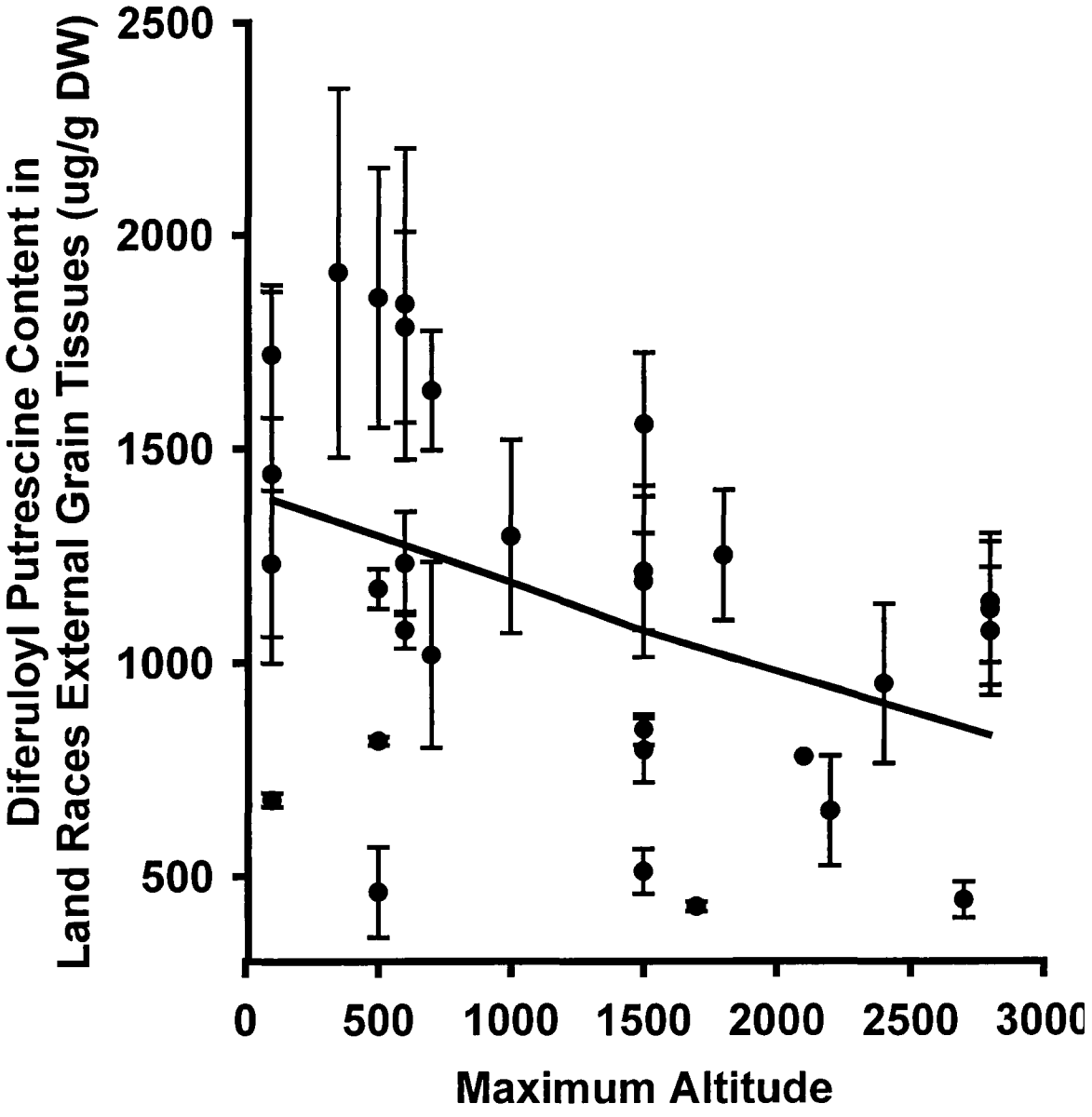


Figure 2.4-a: Linear regression of maximum altitude range of landrace on diferuloyl putrescine content in outer grain tissues. A significant negative relationship was found between maximum altitude and DFP content. A general linear model factored landrace group, collection location and maximum of altitude range of landrace against diferuloyl putrescine content in external grain tissues. Model $r^2 = 0.603$, $p=0.018$, partial $r^2 = 0.350$ for maximum altitude factor, n = 31 landraces, error bars show standard error based on 3 repetitions per point.

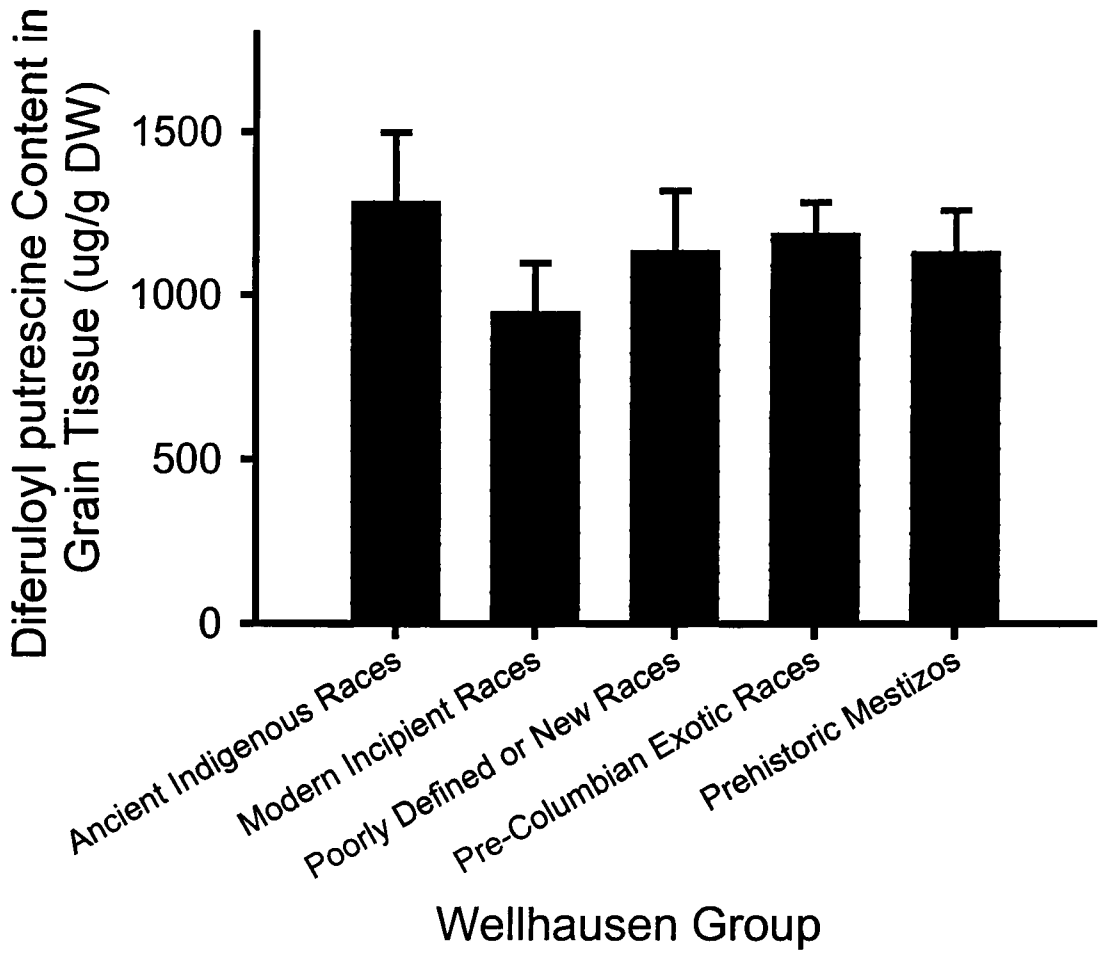


Figure 2.4-b: Mean diferuloyl putrescine content in outer grain tissue by major landrace groups. A general linear model factored landrace group, collection location and maximum of altitude range of landrace against diferuloyl putrescine content in external grain tissues. No significant effect of landrace group was seen on diferuloyl putrescine content, $p=0.963$, partial $r^2 = 0.073$. Model $r^2=0.603$.

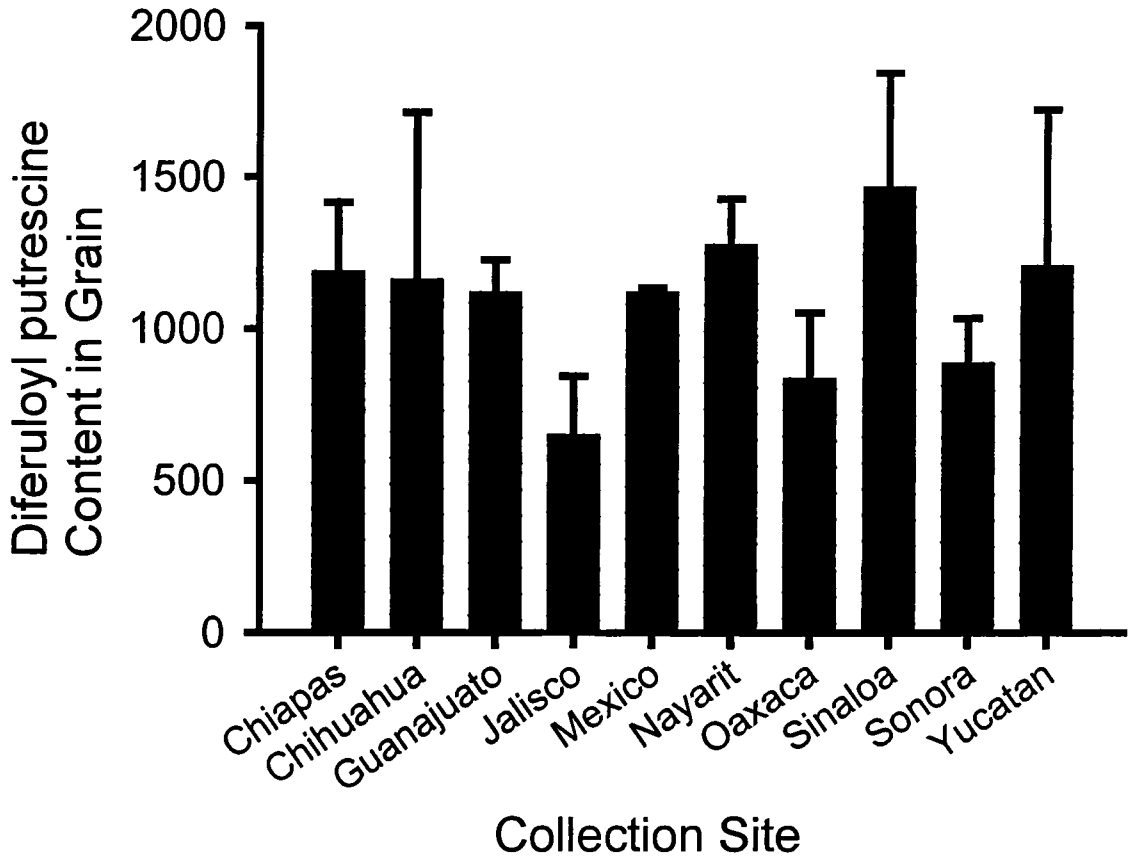


Figure 2.4-c: Mean diferuloyl putrescine content of outer grain tissues for different collection sites. A general linear model factored landrace group, collection location and maximum of altitude range of landrace against diferuloyl putrescine content in external grain tissues. No significant effect of collection site was seen on diferuloyl putrescine content, p=0.113, partial $r^2 = 0.4464$. Model $r^2=0.603$.

Acute Toxicity – Wheat disk feeding assay

The wheat disk assay was used to determine the acute effect of the HAACs on the feeding of *S. zeamais*. Several of the HAACs were tested: feruloyl putrescine, coumaroyl putrescine, diferuloyl putrescine, and N8-coumaroyl spermidine. These compounds were selected due to their availability and because they represent varying levels of prominence in the maize tissue, diferuloyl putrescine being the main representative of the HAACs in the outer grain tissues, and N8-coumaroyl spermidine not being present. Figure 2.4 shows the results of different trials. The results in this figure are shown as the percent of the control value so that comparisons between trials can be made. Of the HAACs tested, only N8-coumaroyl spermidine, which is not present in quantifiable amounts in the external tissues of maize, significantly decreased the feeding of *S. zeamais*, suggesting that the maize weevil, as a maize specialist, is well adapted to consuming the HAACs that are present in its host plant.

The results of the maize weevil acute feeding trials suggest that either it was well adapted to the HAACs present in maize tissue, or that these compounds are not toxic to the weevil family, Calandrae or to Coleopterans in general. Other researchers have found that these compounds are toxic to lepidopteran herbivores (Panagabko *et al.*, 2000). Simultaneous acute feeding trials with *S. zeamais* and the closely related *Sitophilus oryzae* were conducted to test the hypothesis that specifically the maize weevil is adapted to the HAACs present in maize grain. Feruloyl putrescine and diferuloyl putrescine were chosen for use in these bioassays because of their prominence in maize tissue and the availability of the pure compounds. Feruloyl putrescine significantly reduced the feeding of the rice weevil, *S. oryzae*, when presented at a dose of 2 mg/g of diet; at this same dose level there was no significant effect on the maize weevil, *S. zeamais*. Diferuloyl putrescine significantly reduced the feeding of the rice weevil at all doses present, as

low as 0.25 mg/g of diet. There was no significant reduction in the feeding of the maize weevil even at levels as high as 1 mg diferuloyl putrescine per gram of diet (Figure 2.5).

There are few visible differences between these two weevil species, as reported previously. Separation of the two insect species is based on, among other things, differences in feeding preferences (Hiddayat, 1994). These results clearly provide a phytochemical mechanism to explain the differences in feeding preferences of these two closely related species. The rice weevil, considered a maize generalist, is much more vulnerable to these maize compounds than the maize weevil, a maize specialist. Presumably, *S. zeamais* has evolved mechanisms to detoxify or tolerate these compounds. This pattern of deterrence of generalists, but tolerance by specialists, is consistent with the hypothesis that hydroxycinnamic acid amides are toxic plant defence compounds.

Previous research has indicated that maize is a less suitable and attractive substrate for *S. oryzae*, compared to wheat or rice (Birch, 1953, Kiritani, 1965; Ungsunantwiwat & Mills, 1979). This difference in preference may be attributed to the differences in grain size or other tactile differences. However, the results of Gomez *et al.* (1983), who coated various grains with paraffin, implied that mechanical and tactile senses do not play much role in oviposition choice – that more likely it is chemoreception that has the major role in decision making. It may be the case that the preference shown by *S. oryzae* for grain types other than maize is determined not by grain size or moisture but by the high hydroxycinnamic acid amide content in the external tissues of maize grain.

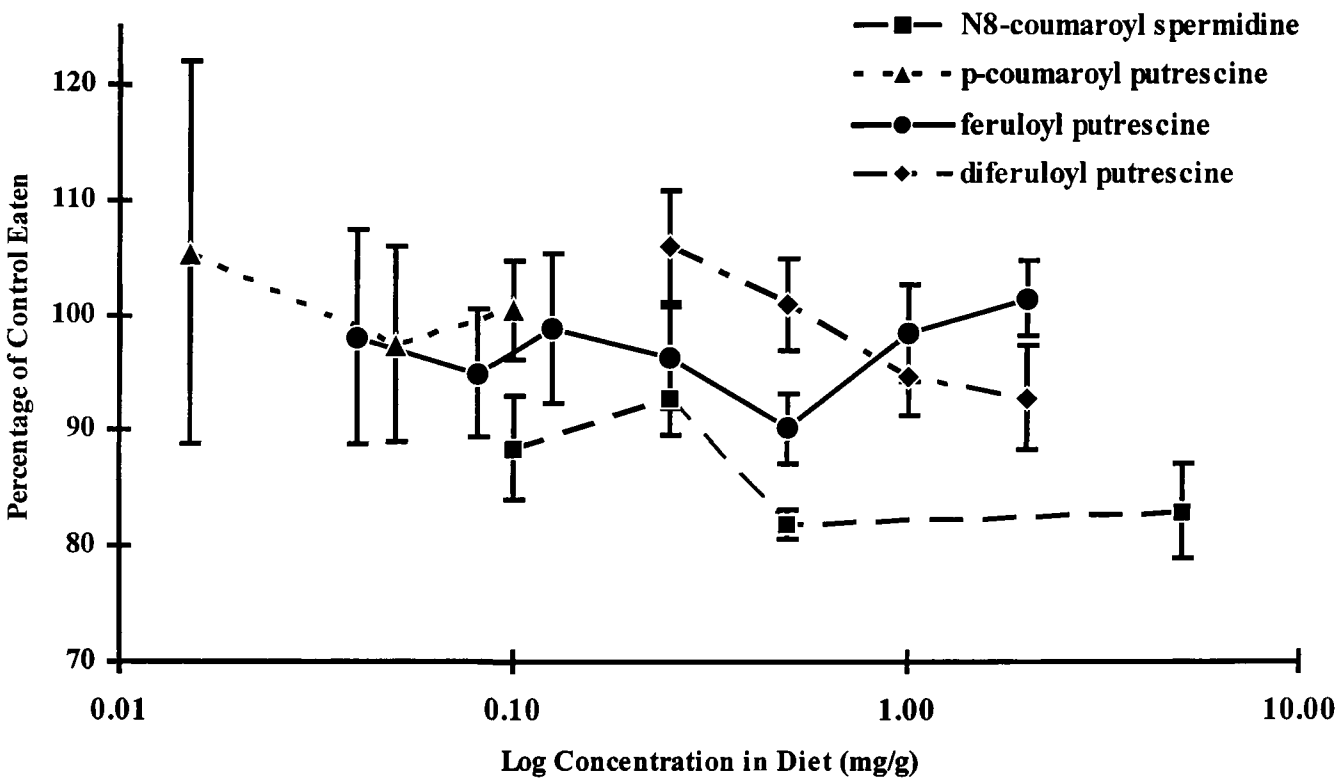


Figure 2.5: Response of *Sitophilus zeamais* to diet treated with various concentrations of diferuloyl putrescine, feruloyl putrescine, p-coumaroyl putrescine, and N8–coumaroyl spermidine. Feeding is significantly reduced, as compared to the control, only at the two highest N8-coumaroyl spermidine concentrations and at no level tested of the other compounds. Note that N8-coumaroyl spermidine is not found at measurable levels in any of the maize tissue examined. Each point represents the mean of four replicates as a percentage of the mean of four control replicates. Error bars represent standard error.

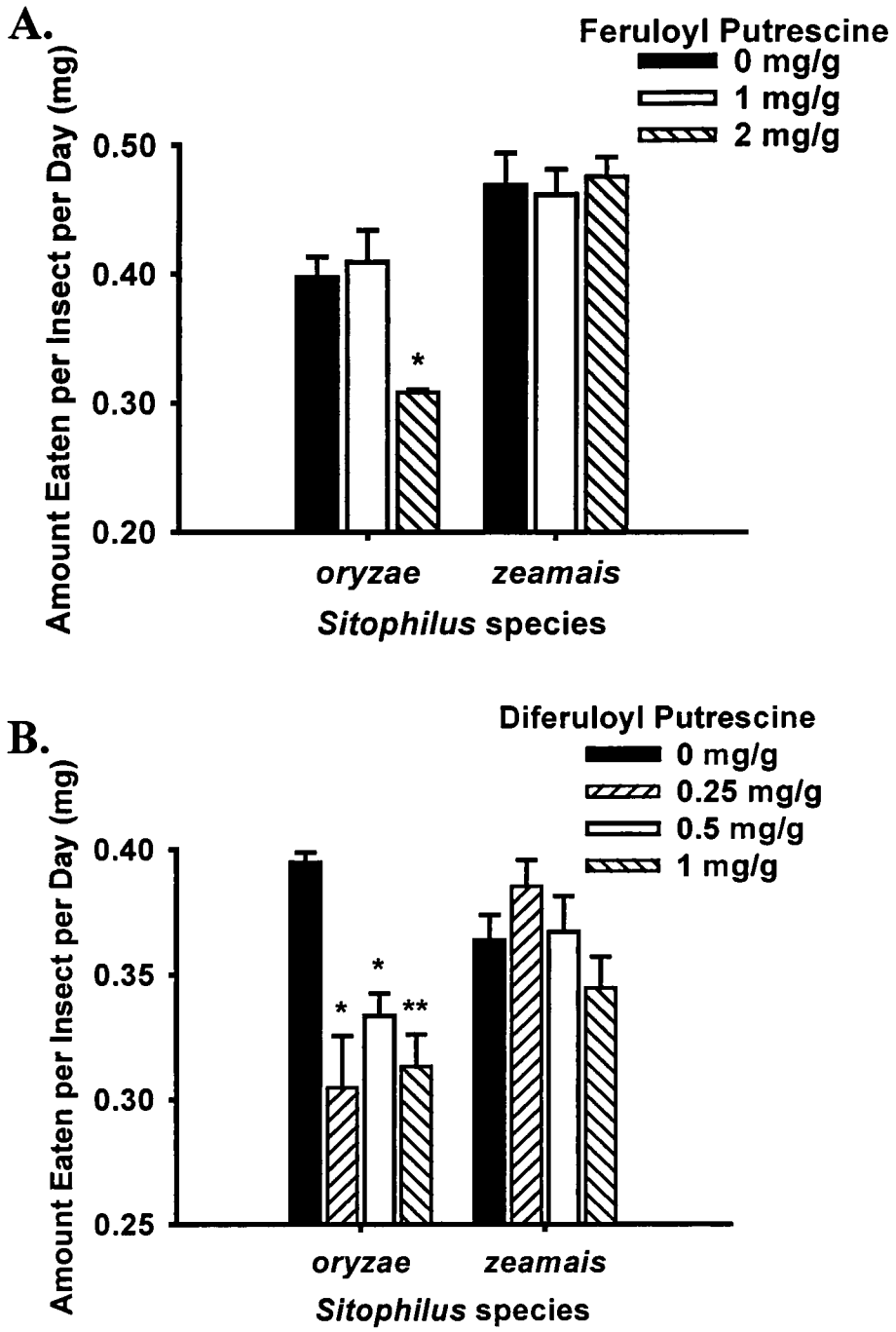


Figure 2.6: Response of *Sitophilus* species to diet treated with various concentrations of A) feruloyl putrescine and B) diferuloyl putrescine. No significant reduction of feeding in *S. zeamais* for any treatment. Feeding of *S. oryzae* is significantly reduced at high feruloyl putrescine concentrations and at biologically available levels of diferuloyl putrescine.

Chronic Toxicity – Lifecycle assay

The effect of chronic exposure to feruloyl putrescine on *S. zeamais* was tested with a two-generation lifecycle assay. The first generation results are summarized in Figure 2.6. In this trial, a control and four treatments were dosed at levels relative to the mean concentration of feruloyl putrescine in whole maize grain as seen in Table 2.5. The treatment [WG] is a dose of 0.72 μg/g of grain, approximately equivalent to the concentration of feruloyl putrescine in the whole grain. The [WG]/2 treatment is half the first dose, 0.36 ug/g grain. [WG] + 1SD is a dose of 2.17 ug/g, higher than [WG] by approximately one standard deviation. Finally, [WG]+2SD is approximately two standard deviations higher at 4.35 μg/g. No significant difference was seen in any of the three parameters examined: the number of F1 adults emerged, the percentage of damaged kernels, and the weight loss of the grain. The trend shown in the data was for an increase in the [WG] treatment as compared to the control, but this was not significant.

To further investigate the effect of long-term exposure to this feruloyl putrescine, the bioassay was extended to a second generation. These results are summarized in Figure 2.7. In this cycle the control and only the [WG] and [WG]+2SD treatments were used, in order to spare standard. In this cycle the [WG] treatment showed a significantly greater grain weight loss than the control. In addition, the percentage of kernels damaged in the [WG] treatment was significantly greater than either the control or the [WG]+2SD treatment. Although there was no statistical difference between treatments in the number of F2 adults emerged, the trend is also for an increase in the [WG] treatment as compared to the control. The treatments were examined for a difference in the weight loss and percent of damaged kernels per emerged adult. No significant difference was seen, however the mean weight loss per adult in the [WG] treatment was greater than the mean of the control. Therefore, it is difficult to determine whether the effects seen in

this generation is an increase in the fecundity of the F1 generation, an increase in survivorship to emergence in the F2 generation, or an increase in feeding in one or both of the F1 and F2 generations. Comparing the two trials, it appears that the control group in the second generation has decreased as compared to the first trial. It is thought that this is most likely the result of a decrease in the humidity of the environmental chamber during the second generation. A decrease in relative humidity would cause an increase in the hardness of the grain. This would confound comparisons between the two generations; the data analysis has been therefore limited to comparing only the variation within each generation.

Sub-lethal doses of other toxic compounds have been demonstrated to increase the feeding of the insect, such as in Stewart (1981) with *Manduca sexta* and in Tanton and Khan (1978) with *Parapsis atomaria*, in each study where the insects were exposed to sub-lethal or low mortality doses of fenitrothion. In Stewart's (1981) study, the reproductive potential of the treated groups was higher than the control group, in both the P generation and in some treatments of the F1 generation. This type of response is often indicative of an interference with the endocrine system. Both Tanton and Khan (1978) and Stewart (1981) observed debilitating effects of the treatment. Such effects were not seen in this case; however, it must be noted that the experimental design was not conducive to such an investigation.

An alternative explanation for the effect of the HAACs on *S. zeamais* involves a presumed evolved response of the maize weevil, as a highly adapted maize specialist, to the HAACs as prominent toxic compounds in the external tissues of the maize grain. In such a relationship, these compounds may be used by the adapted insect as an ovipositing stimulant and/or a phagostimulant. Further, although the weevil is well adapted to the compounds, when present levels higher than those that occur naturally, there may be both an attractant and a toxic

effect on the insect. What one would expect if this were the case is an increase in feeding and/or oviposition in the [WG] dose and a subsequent decrease in the higher doses, which approximates the results that were obtained. It is possible that if doses higher than [WG] +2SD were investigated, the toxicity of the compound would cause a decrease in the adult emergence, grain weight loss, and amount of damage as compared to the control.

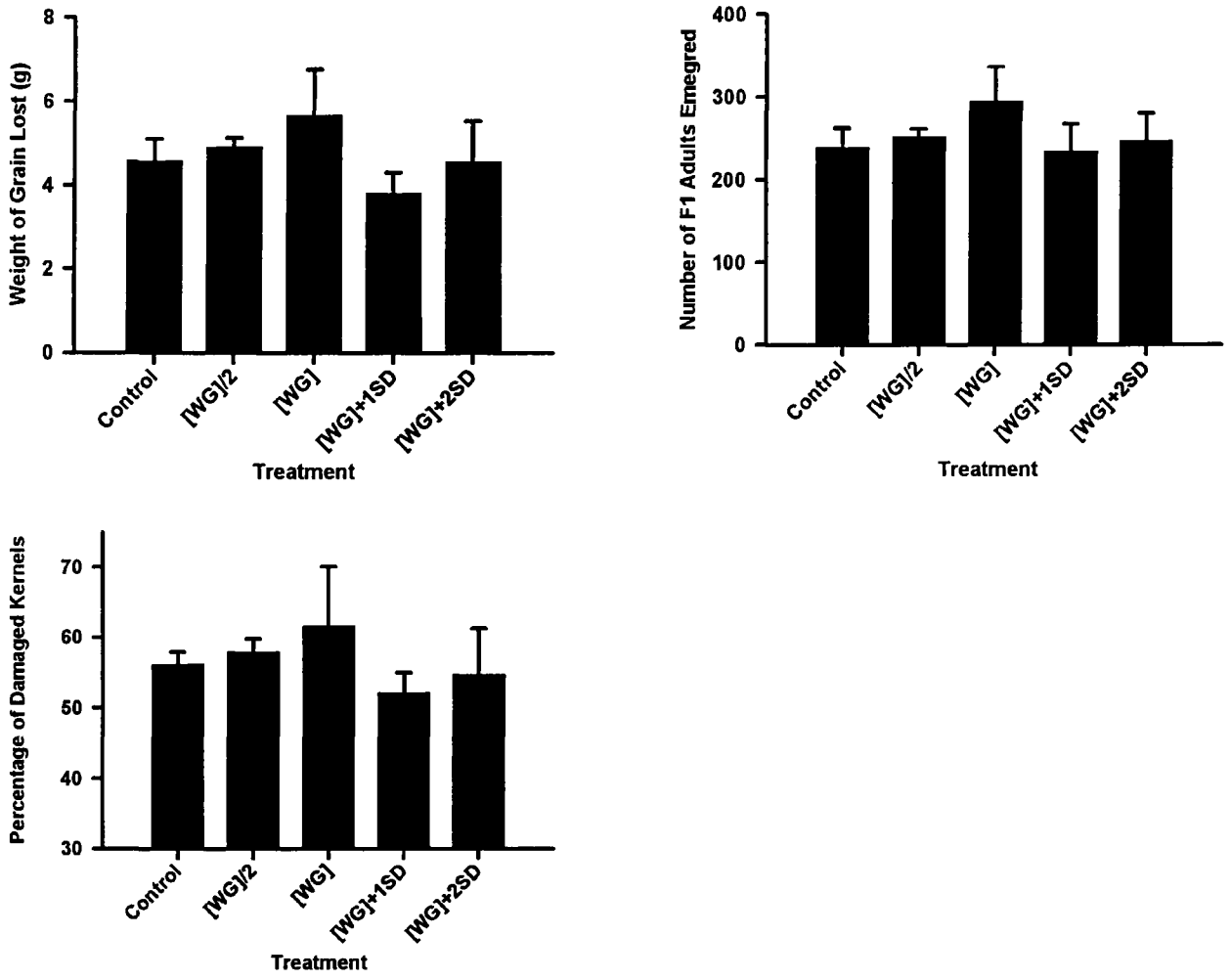


Figure 2.7: Effect of feruloyl putrescine treatment on the first generation of a *Sitophilus zeamais* lifecycle bioassay. The parameters measured were weight loss of grain, number of progeny emerged, and percentage of kernels damaged. The treatments represent different dose levels relative to the content in whole maize grain: [WG] denotes mean concentration of feruloyl putrescine in maize (0.72 μg/g grain), [WG]/2 is half this level (0.36 μg/g grain), [WG]+1SD is one standard deviation higher than [WG] (2.17 μg/g grain) , and [WG]+2SD is greater by two standard deviations (4.35 μg/g grain). No significant difference in any parameter was seen between any of these dose levels and the control. n=4 vials for each treatment.

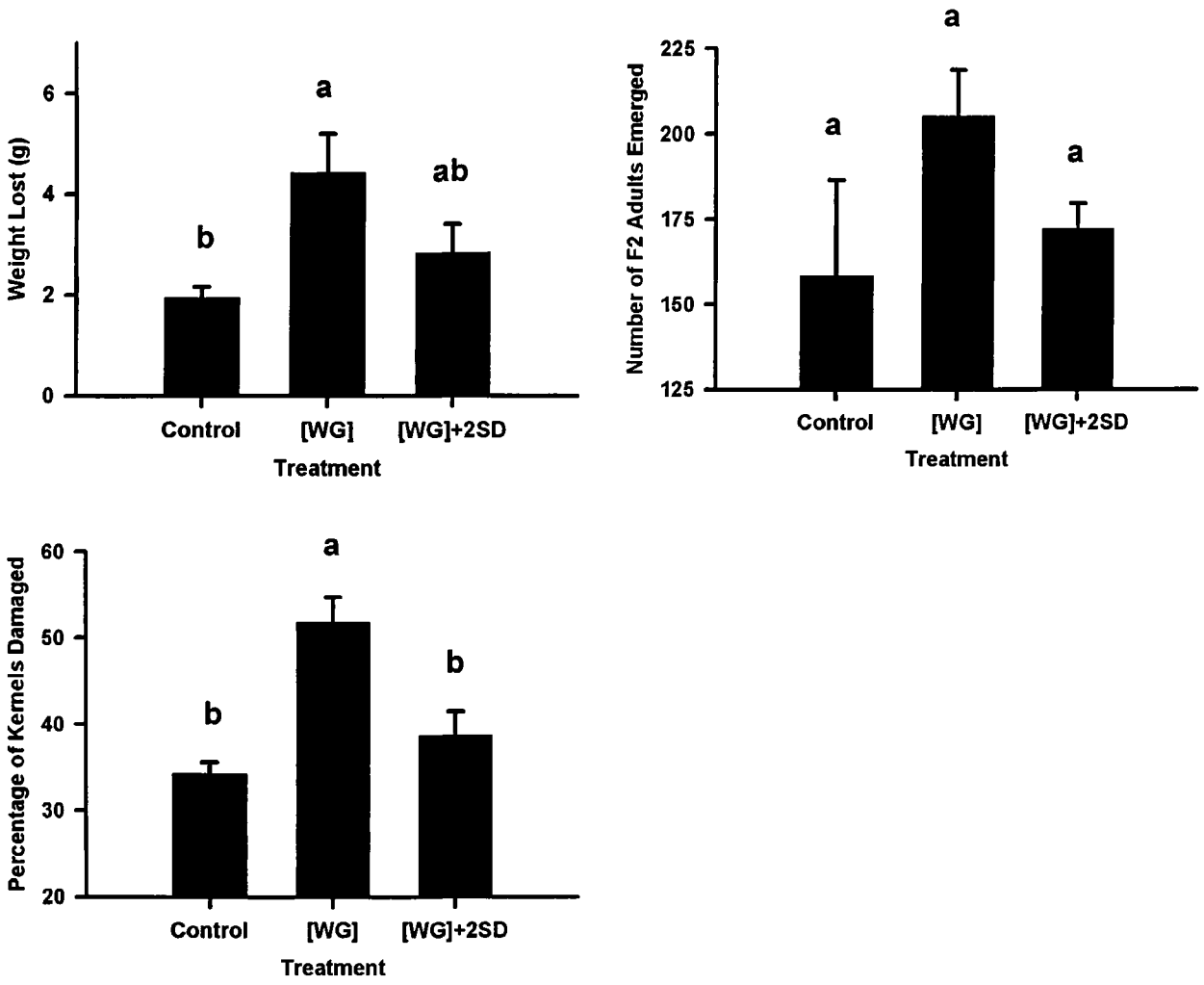


Figure 2.8: Effect of feruloyl putrescine treatment on the second generation of a *Sitophilus zeamais* lifecycle bioassay. The parameters measured were weight loss of grain, number of progeny emerged, and percentage of kernels damaged. The treatments represent different dose levels relative to the content in whole maize grain: [WG] denotes mean concentration of feruloyl putrescine in maize (0.72 μg/g grain) and [WG]+2SD is greater by two standard deviations (4.35 μg/g grain). A significant increase relative to the control is seen in the weight loss of the grain and in the percentage of damaged grain in the [WG] treatment. There is no significant difference between the dose level [WG]+2SD and the control for any of the parameters. n=4 vials for each treatment.

HAACs as Related to the Taxonomy of Maize Germplasm

To further examine the relationship between the hydroxycinnamic acid amides and the taxonomy of maize, phytochemical data were subjected to discriminant function analysis to see how well they fit into the existing classification of maize landraces by Wellhausen *et al.* (1952). Although the Wellhausen landrace groups were not a significant factor in a statistical analysis of any of the hydroxycinnamic acid amides, it is possible that there is a more complex, multi-factorial relationship. The six different compounds were used to generate discriminant functions that define the boundaries of the landrace groups. This analysis creates models that use either linear or quadratic functions to define the groups. The linear models use less complex functions and tend to be more robust; however, they require that all groups have equal covariance matrices. The quadratic models do not require this, but tend to be much less robust if the sample size (i.e. number of landraces) is relatively small.

Once the model is generated, the same factors that were used to create the model, in this case, the HAACs, are then used to re-classify the races into the previously defined groups. This is done to test the model for accuracy - to see which races are re-classified into their original group and which would be miss-classified by the model. The model can also be used to approximate the placement of new races into the established taxonomic groups.

The classification by Wellhausen *et al.* (1952) is the result of a collection process that spanned seven years and yielded 2000 varieties. A rigorous examination of the entire plant - the vegetative portions, the tassel, and the ear characters as well as physiological, genetic and cytological characters - was performed and the resultant classification is thought by many to be definitive. However, the taxonomy does have one area of weakness, where several landraces were grouped into a "Poorly Defined" group simply due to a lack of data. Also, since this

authoritative work, at least five new landraces have been defined in Mexico. A discriminant function analysis allows us to place these new landraces into Wellhausen's groups. Additionally, it enables us to test whether the "Poorly Defined" group is a viable group unto itself, or whether these landraces would be better classified into the other Wellhausen groups.

The first discriminant function models were built for all five of the landrace groups defined by Wellhausen et al. (1952). Stepwise discriminant analyses were used to determine which of the six compounds to use as factors in a linear model. Both forward and backward stepwise selection with a $p = 0.30$ to include or remove a factor selected four of the six compounds as the ones that provide the best separation between the landrace groups. These four factors were diferuloyl putrescine content, cinnamoyl putrescine content, feruloyl putrescine content, and N1-feruloyl spermidine content. These factors were then used, along with their pooled covariance matrix to generate linear discriminant functions that define the landrace groups. The model passed The Wilks' lambda test for the equality of the groups' covariance matrices, demonstrating that it met the criteria for a linear model. However, the successful reclassification rate using this model was only 57%. Of the 37 landraces used in the model, only 21 were reclassified into their correct Wellhausen landrace group. A linear model using all the factors did not increase the re-classification rate. The first model was deemed best as it contains fewer factors; it was named Model 1 (Tables 2.7 and 2.8).

A quadratic model was considered, but no significant improvement in the re-classification rate was seen. The number of factors cannot exceed the sample size of any group in a quadratic model, so any quadratic model using more than three phytochemical factors could not be used on the Pre-Columbian and the Modern Incipient groups. Additionally, the number of

races in each group is relatively small, so a quadratic model would not be sufficiently robust. In the face of these limitations, the quadratic model was rejected.

A second model was built including only four of the Wellhausen groups; the "Poorly Defined" group was omitted. The inclusion of this group may confound the model since the "Poorly Defined" group is a collection of the races for which sufficient data was lacking and therefore may contain races that should be in one of the other four groups. If the "Poorly Defined" group spans the boundaries of the other groups in Model 1, the possibility of misclassification will be increased. In such a case, removing the group would increase the re-classification rate of the other groups. This second model will also give an interesting opportunity to reclassify the "Poorly Defined" races into the other four landrace groups.

The factors for the second model were selected by forward and backward stepwise selection with a critical P to remove set at 0.20. The factors selected were diferuloyl putrescine content, cinnamoyl putrescine content, feruloyl putrescine content, p-coumaroyl putrescine content and N1-feruloyl spermidine content. The only factor left out of the model was caffeoyl putrescine content. The Wilks' lambda test showed that the linear model assumption of equality of the groups' covariance matrices was met. A linear model was generated using the selected factors and their pooled covariance matrix. The resultant model has a successful reclassification rate of 73%, or 19 of 26 races classified correctly. A model using all six compounds as factors resulted in the same reclassification rate, so the simpler model was chosen. The preferred model was dubbed Model 2 and is summarized in Tables 2.9 and 2.10.

By comparing Model 1 and Model 2 it can be seen that the removal of the "Poorly Defined" group improved the model fit - the rate of successful reclassification is improved in three of the four groups common to both models, with the fourth group having no change in the

rate of reclassification. This improvement is not a simple matter of the removal of one of five options. If this were the case then, based on the probability of reclassification in Table 2.8, of the cases misclassified in Model 1 only Palomero Toluqueno would be successfully reclassified in Model 2 giving Model 2 a successful reclassification rate of only 57%. However, the removal of the "Poorly Defined" group resulted in a successful reclassification rate of 73%. Furthermore, the exclusion of this group results in an overall increase in distance between the groups, in all cases except for between group A and C, as can be seen in Table 2.11. This indicates that the groups are more clearly resolved in Model 2. It may be concluded that the inclusion of the "Poorly Defined" group confounded Model 1 and that its removal allows a clearer definition of the boundaries between landrace groups.

Table 2.7: Summary of reclassification of landraces into Wellhausen's Landrace groups using Model 1 a linear model that used diferuloyl putrescine, cinnamoyl putrescine, feruloyl putrescine, and N1-feruloyl spermidine content to generate the discriminant functions.

Wellhausen Group	Number of Races Re-Classified into the Wellhausen Groups					
	A	B	C	D	E	% Correct
A – Ancient Indigenous	**4**	0	1	0	1	67
B – Pre-Columbian	1	**2**	1	0	0	50
C – Prehistoric Mestizos	3	0	**6**	2	2	46
D – Modern Incipient	0	0	0	**2**	1	67
E – Poorly Defined	0	1	3	0	**7**	64
					Total	57

Table 2.8: Summary of Misclassified Landraces for Model 1. The Wellhausen grouping is shown as well as the group that the model designated as most likely for the landrace to be in. Also shown are the probabilities of each landrace belonging to each landrace group.

Landrace	Classification		Probability of Membership in Group				
	Well.	Model	A	B	C	D	E
Nal, Tel Yucatán 16	A	C	0.17	0.28	0.31	0.02	0.22
Palomero Toluqueño Mexico 55	A	E	0.23	0.17	0.22	0.08	0.30
Cacahuacintle Mexico 212	B	C	0.10	0.20	0.44	0.06	0.19
Oloton Chiapas 124	B	A	0.37	0.24	0.12	0.01	0.26
Olotillo Chiapas 237	C	A	0.32	0.26	0.24	0.01	0.17
Pepitilla Morelos 52	C	E	0.16	0.27	0.25	0.03	0.29
Reventador Nayarit 39	C	E	0.12	0.28	0.19	0.07	0.34
Tepecintle Guanajuato 207	C	A	0.41	0.25	0.22	0.00	0.12
Vandeño Oaxaca 4	C	D	0.00	0.02	0.08	0.84	0.06
Zapalote Chico Oaxaca 179	C	A	0.33	0.14	0.27	0.05	0.22
Zapalote Grande Chiapas 236	C	D	0.08	0.04	0.29	0.45	0.14
Celaya Guanajuato 101	D	E	0.24	0.22	0.15	0.05	0.34
Bofo Nayarit 222	E	C	0.11	0.15	0.50	0.08	0.16
Maíz Blando de Sonora Sonora 32	E	C	0.05	0.09	0.40	0.26	0.19
Onaveño Sonora 139	E	C	0.16	0.15	0.34	0.11	0.24
Zamorano Michoacan Grp 13	E	B	0.18	0.59	0.18	0.00	0.06

Table 2.9: Summary of reclassification of landraces into Wellhausen's Landrace groups using Model 2 - a linear model that used diferuloyl putrescine, cinnamoyl putrescine, feruloyl putrescine, N1-feruloyl spermidine, and p-coumaroyl putrescine content to generate the discriminant functions.

Wellhausen Group	Number of Races Re-Classified into the Wellhausen Groups				
	A	B	C	D	% Correct
A – Ancient Indigenous	**4**	0	2	0	67
B – Pre-Columbian	0	**3**	1	0	75
C – Prehistoric Mestizos	1	2	**9**	1	69
D – Modern Incipient	0	0	0	**3**	100
				Total	73

Table 2.10: Summary of Misclassified Landraces for Model 2. The Wellhausen grouping is shown as well as the group that the model designated as most likely for the landrace to be in. Also shown are the probabilities of each landrace belonging to each landrace group.

Landrace	Classification		Probability of Membership in Group			
	Well.	Model	A	B	C	D
Nal, Tel Yucatan 16	A	C	0.12	0.23	0.65	0.00
Palomero Toluqueño Mexico 55	A	C	0.34	0.26	0.37	0.03
Cacahuacintle Mexico 212	B	C	0.10	0.11	0.78	0.00
Olotillo Chiapas 237	C	B	0.07	0.76	0.14	0.03
Reventador Nayarit 39	C	B	0.32	0.45	0.21	0.02
Vandeño Oaxaca 4	C	D	0.00	0.02	0.07	0.90
Zapalote Chico Oaxaca 179	C	A	0.50	0.03	0.47	0.00

Table 2.11: Comparison of Relative Distances between centroids of Wellhausen groups as computed by Model 1 (a) and Model 2 (b). Note that all distances increase in Model 2 except for the distance between group A and group C.

a)

	A	B	C	D	E
A	0				
B	1.128	0			
C	2.647	0.644	0		
D	3.672	2.101	1.862	0	
E	1.838	0.407	1.229	1.489	0

b)

	A	B	C	D
A	0			
B	1.310	0		
C	2.000	1.235	0	
D	4.656	2.175	3.870	0

Table 2.12: The Reclassification of the "Poorly Defined" group by Model 2. The group into which the landrace best fits is given, as well as p, the probability of the landrace fitting into the group, and M, the mahalanobis distance from the centroid of each group. No classification is given if all mahalanobis distances are 9.2 or greater.

Landrace	Classification	Mahalanobis Distance and Posterior Probability of Membership in Group							
		A		B		C		D	
		M	p	M	p	M	p	M	p
Apachita		38.1	0	29.9	0	41.8	0	**19.0**	**1.00**
Azul	D	13.2	0.01	7.2	0.24	12.9	0.01	5.0	**0.73**
Bofo	C	5.9	0.12	6.2	0.10	2.2	**0.77**	12.2	0.01
Complejo Serrano de Jalisco	C	6.8	0.10	5.3	0.22	3.2	**0.63**	8.2	0.05
Conejo		110	0	89.3	0	117	0	**74.9**	**1.00**
Dulcillo del Noroeste	B	4.6	0.40	4.3	**0.47**	7.0	0.12	12.4	0.01
Gordo	C	3.9	0.18	3.4	0.23	1.6	**0.56**	8.3	0.02
Maíz Blando de Sonora	D	20.7	0	12.3	0.07	12.7	0.05	7.2	**0.88**
Onaveño	C	5.2	0.11	3.1	0.29	2.1	**0.50**	5.2	0.10
Tabilla de Ocho	C	4.4	0.11	1.7	0.42	1.7	**0.43**	6.1	0.05
Zamorano Amarillo		**29.8**	**0.75**	40.3	0	32.0	0.25	75.1	0

Model 2 was used to reclassify the "Poorly Defined" races into one of the other four groups, as summarized in Table 2.12. Of the eleven "Poorly Defined" races, the model was able to place eight into the other landrace groups. Two are placed into the Modern Incipient group, five into the Prehistoric Mestizo group, and one into the Pre-Columbian group. The majority of the races are placed into the two landrace groups that might be considered the younger groups. Interestingly, the one race placed into the more ancient Pre-Columbian group is Dulcillo del Noroeste, a sweet corn. This is the group that contains the only other sweet corn landrace – Maíz Dulce. It is even considered in Wellhausen *et al.* (1952) that Dulcillo del Noroeste may have originated from the hybridization of Maíz Dulce with another landrace.

The three races that were not placed into new groups have mahalanobis distances that are too great to allow them to be classified into any group. The mahalanobis distance function has a distribution that approximates a Chi-square distribution with the degrees of freedom equal to the number of variables in the model. In this case, with five degrees of freedom, anything with all mahalanobis distances greater than 9.2 should be considered an outlier and should not be classified into any group. These three races, however, do not cluster together to form a viable new group – they remain as outliers to the rest of the races.

This technique allows for an interesting theoretical reclassification of the "Poorly Defined" group and the results of this reclassification make a certain amount of intuitive sense. There is no model possible, made only from the hydroxycinnamic acid amide conjugate content in grain tissue, that defines the groups well enough to be considered a definitive taxonomical revision. It would prove interesting to perform this analysis for other phytochemical or physiological traits to see if the reclassification defined here might be confirmed.

Chapter III: Cell Wall Bound Phenolic Compounds in Tropical Maize Grain and Correlations with Resistance to *Sitophilus zeamais*.

3.1 Introduction:

Current general models of the plant cell wall show that cellulose microfibrils are bound in a matrix of interwoven polysaccharides and proteins (Talbott and Ray, 1992). These structures become permanently and irreversibly associated through the creation of covalent cross-links (Iiyama *et al.*, 1994). The presence of ferulic acid and coumaric acid in ester linkages to cell wall components in the primary cell wall of several plants has been established; these hydroxycinnamic acids have been shown to link to arabinoxylans in maize and other cereals (Shibuya, 1984; Grabber *et al.*, 1995; Saulnier & Thibault, 1999), to pectins in sugar beets (Micard *et al.*, 1997; Saulnier & Thibault, 1999), and to xyloglucans in bamboo (Iiyama *et al.*, 1994). These esterified ferulic acids on the cell wall polysaccharide fibrils have the demonstrated ability to undergo a dehydrogenative coupling to form dehydro-dimers of ferulic acid or dehydrodiferulic acid (DFA) (Iiyama *et al.*, 1994). An example of a dehydro-dimer of ferulic acid cross-linking the cell wall polysaccharides is shown in Figure 3.1. It has been proposed that these ester bridges fortify the cell wall, increasing the ability of the wall to withstand internal turgor pressure, and resist penetration from an external force.

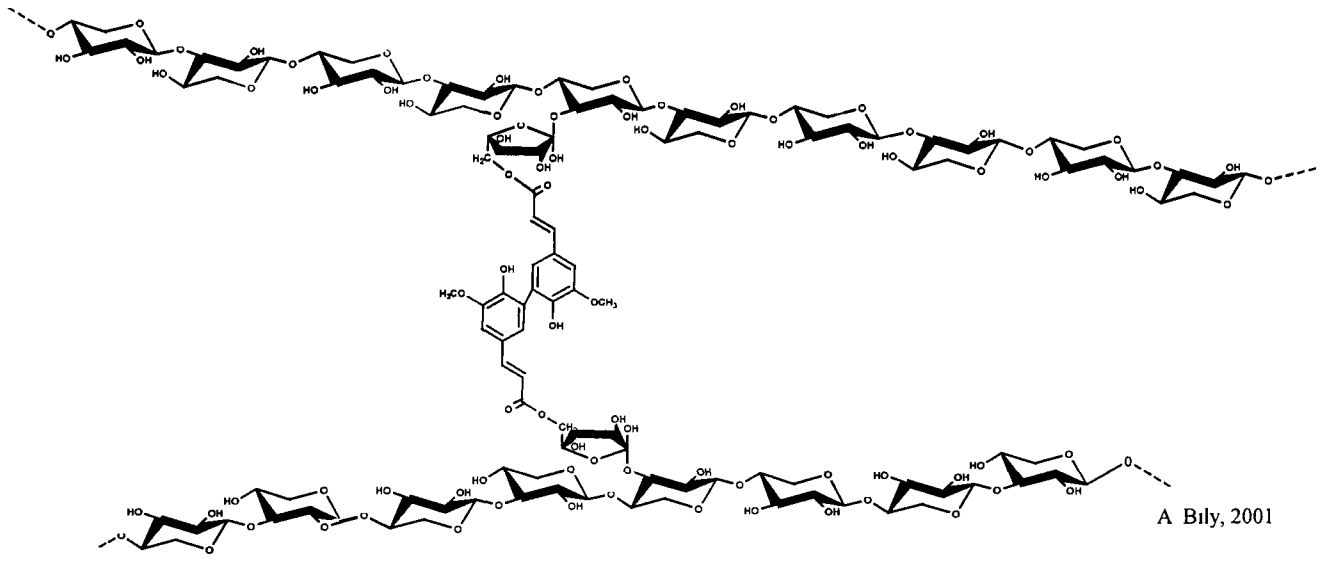


Figure 3.1: Structure of a dimer of phenolic acid-carbohydrate complex (DFAXX). O(5-O-feruloyl-α-L-arabinofuranosyl)-(1-3)O-β-D-xylopyranosyl-(1-4)-D-xylopyranose.

A strong correlation has been previously shown between *S. zeamais* resistance and the cell-wall bound phenolic content, particularly ferulic acid content, of the grain (Serratos *et al.*, 1987; Classen *et al.*, 1990; Arnason *et al.*, 1992; Serratos *et al.*, 1992; Arnason *et al.*, 1994). The different kernel tissues have been shown to have differing amounts of phenolic compounds (Arnason *et al.*, 1992): the endosperm, composing the bulk of the grain, has a relatively low phenolic content, whereas the embryo and the outer tissues of the grain are very rich in cell wall bound phenolics. Recent work by the author's group has shown that there is a much stronger correlation between *S. zeamais* resistance and phenolic content in the pericarp and outer tissues than between the resistance and the whole grain phenolic content (García-Lara, unpublished data).

QTL Mapping

The single locus theory that Mendel developed to explain hereditary events of traits with discrete variations in phenotype, or qualitative traits, is of limited use when studying traits showing a continuous pattern of variation, or quantitative traits. Quantitative traits do not have distinct or non-overlapping character states for different genotypes. The phenotypes expressed in quantitative traits are also often determined by the interaction of the genetic basis of the trait and environmental effects. The term polygenic trait is used to describe a single trait that is controlled by multiple genes. Statistical or biometrical methods have been developed to systematically characterize the trait by isolating and examining the effects of the polygenes controlling the quantitative trait (Kearsey & Pooni, 1996).

The advent of the molecular era allowed chromosome mapping of polygenic traits by the QTL technique. The QTL, or quantitative trait locus, is a chromosome region that has a

statistically derived high probability of contributing to the trait of interest. QTLs are determined relative to the known position of molecular markers. When allelic variation in the marker (i.e. presence or absence of that marker) coincides with variation in a quantitative trait, a gene responsible for some of the variation of that trait can be located on or near the site of the marker. A QTL is placed at this locus and is determined to be within a confidence interval around the marker or markers implicated in the variation of the trait. Complex algorithms increase the precision and accuracy of calculating QTLs. For example, composite interval mapping (Zeng, 1994) involves regressing quantitative traits on an unknown QTL genotype within an interval and on known marker loci outside the interval. This removes the effects of other QTLs outside the given interval so that higher power and more accurate estimates can be obtained. The introduction of dedicated computer packages such as MapMaker (Lander *et al.*, 1987) has made QTL detection routine.

QTL analysis done in the context of breeding programs and crop improvement projects is the first step towards Marker Assisted Selection (MAS). The purpose is to maximize productivity and efficiency of selection. If the progeny of a cross could be evaluated for desirable traits simply by looking for markers in young tissue rather than assessing mature plants for the desired traits, a breeding program could be accelerated greatly. The reality of MAS is, however, that it currently does not replace conventional evaluations; still, it has so much potential as an invaluable tool that work towards its development must be continued.

Previous efforts have used QTL mapping to identify loci associated with insect resistance, secondary metabolite production, and yield and other agronomic traits. For example, Yencho *et al.* (1996; 1998) found molecular markers in crosses of the domestic potato with the wild Bolivian potato that are associated with genes for resistance to the Colorado potato beetle and

with foliar glycoalkaloid aglycones. In cereals, several studies have been performed to map insect resistance, such as Alam & Cohen's (1998), who analyzed QTLs associated with resistance to a serious pest of high-yield rice varieties, the brown planthopper, *Nilaparvata lugens*.

There are many QTL studies specific to maize. Grain yield and grain weight were examined by Marsan *et al.* (2001) using an AFLP marker map. Insect resistance has been mapped in several instances: resistance to stalk borers was analyzed by Bohn *et al.* (1996; 2000), Groh *et al.* (1998), Cardinal *et al.* (2001), and Ramputh (2001). In an important series of studies, QTL analysis was used to identify regions associated with corn earworm antibiosis (Byrne *et al.*, 1997) and to determine the phytochemical basis for the antibiosis, maysin and apimaysin, and to clarify the genetic mechanism responsible for the synthesis of the glycosyl flavones (Byrne *et al.*, 1998; Lee *et al.*, 1998).

The current work attempts to refine the previously defined relationship between grain resistance to *S. zeamais* and cell wall bound phenylpropanoids in the pericarp. As a result of advances in chromatographic techniques, the individual species of ferulic acid dehydrodimers can be separated and quantified. The hypothesis that the diferulic acid content is highly correlated with kernel hardness and *S. zeamais* resistance was tested by analyzing a sample set showing a wide range of resistance levels. This relationship between resistance and cell wall bound phenylpropanoids was further examined by conducting a QTL mapping study of insect resistance and putative phytochemical resistance markers. The prediction that the loci for grain phytochemical traits will overlap with *S. zeamais* resistance loci on maize chromosomes was tested. This mapping study also provides a foundation for future use of molecular marker techniques in breeding programs. This is imperative given the delay in selection for resistance using standard bioassays.

3.2 Methods and Materials

Plant material

CIMMYT Post-Harvest Resistance trials

A combination of resistant landraces and CIMMYT breeding populations that were selected for variable resistance to *Sitophilus zeamais* and *Prostephanus truncatus* were used in this experiment. The material included grain of the landraces Sinaloa 35 and Yucatan 7, and the CIMMYT populations 1784 LGB, 1780 Ejura, and the crosses P84 x P47, P47 x P84, and the susceptible check cross of CML244 x CML349. The material was grown in Tlaltizapan, Morelos during 1998 (TL98A) and 1999 (TL99B). The "A" and "B" denote the winter and summer growing seasons, respectively. The seed were harvested and were dried and then dissected in three sections: pericarp and aleurone, endosperm, and embryo, by the staff at CIMMYT and was then sent to the University of Ottawa for analysis. The TL98A material was extracted and analyzed by Silverio García-Lara in the fall of 1999. These results will be presented along with the second set of material from the TL99B cycle analyzed by Andrew Burt in 2000. Nitrogen content, percent kernel damage, and peak grain puncture force were determined in Mexico, by Silverio García-Lara. The insect bioassay used is described later in this section.

QTL Population

The parental material for this population was selected by evaluating material in the CIMMYT maize improvement program. Several steps of selection of candidate material from the CIMMYT collection were made in order to obtain QTL parents of differing *S. zeamais* resistance levels and sufficiently homogenous genetic background to allow for the mapping techniques. The final parental material used resulted from CIMMYT's population 28, a yellow,

dent, late maturing plant. Thirty crosses of lines from this population were evaluated for agronomic characteristics. Five crosses were further evaluated for insect resistance. Finally, the cross between CML 290 and Muneng-8128-C0HC1-18-2-1-1 was selected for use as the mapping population due to an acceptable level of genetic polymorphisms. This mapping population was advanced to F4 and F5 seed for evaluation.

The breeding and selection of the parental material was done at CIMMYT, in Tlaltizapan, Mexico by David Bergvinson. The evaluation of the parents and mapping population for agronomic, physical and insect resistance factors was performed by Silverio García-Lara, in Batan, Mexico. The phytochemical evaluation of the parental stock and the mapping population was done at the University of Ottawa, by Andrew Burt.

Insect bioassay trials

A healthy colony of *S. zeamais* from Poza Rica, Veracruz was used in all the tests. This colony was maintained for two life cycles in the laboratory before being used. All maize grain being used in the trial was allowed to equilibrate at 27°C and 75% relative humidity.

For each family in the QTL mapping population (CML 290 x Muneng-8128-C0 HC1-18-2-1-1), four samples of 30 grams of maize kernels were placed into 100mL glass vials. Twenty newly emerged (0-7 days old), unsexed *S. zeamais* adults were introduced into each vial. After one week at the controlled environmental conditions the adult insects were removed. The sets of vials were maintained at control conditions for an additional five weeks. The samples were left undisturbed until the first adults of the F1 generation had emerged. The emerged adults were counted every two days until all of the F1 generation had emerged. The Dobie index, the percent of damaged kernels, and the percent weight loss of the grain were determined at the end of the

experiment. The Dobie index was calculated according to Dobie (1974) to obtain the index of susceptibility. The percent of damaged kernels is based on visual inspection under white light of every kernel used. The percent damage and percent loss were calculated as follows:

$$\% \text{ Damage} = 100 \times \frac{ND}{(NU+ND)}$$

$$\% \text{ Loss} = 100 \times \frac{[(WU/NU) - (WD/ND)] \times ND}{(NU + ND) \times (WU/WD)}$$

Where:

NU = number of undamaged kernels

ND = number of damaged kernels

WU = weight of undamaged kernels

WD = weight of damaged kernels

Agronomic and physical resistance factors

Grain Hardness

The grain hardness was measured using an Instron (Model 921A, Tricor Systems Inc, Elgin, Illinois 60123). This apparatus is comprised of a sensor (part no. 921-650-04) and a probe with a 0.8 mm diameter, blunt rounded tip. The kernel is placed embryo side down, on a metal surface and positioned so that the probe will test the area of the kernel opposite the embryo. The probe travels at a velocity of 1 cm/s until it reaches the kernel and measures the force necessary to puncture the grain surface. Fifty determinations were made for each family or genotype tested. Each repetition was performed at room temperature with grain that had equilibrated at 27°C and 70% relative humidity.

Kernel Density and Weight

The kernel density was determined by filling a beaker with a 120 mL capacity with grain and removing the excess with a flat edge. The kernels are then weighed and the density is calculated by dividing the weight in grams by 120 mL. Grain weight is determined by weighing out 30 grams of grain, counting the number of grains present and determining the average grain weight of this sample. These data were only available for the TL2001A samples.

Germination and Vigour

Healthy kernels remaining from the insect bioassay were used in the germination and vigour tests. At the end of the infestation the healthy kernels were separated and blotted dry for 1 hr. The kernels were then washed with 1% commercial chlorine solution for 5 min. 100 kernels were placed on a filter paper, which was then rolled into a tube. This tube was then wetted with distilled water and placed in a controlled environment of 20°C and 100% relative humidity for 5 days. After this time, the numbers of germinated kernels were counted and the length of the principal root was measured and recorded in cm. The percent germination was calculated and the vigour was defined as the mean length of the principal root. These data were only available for the TL2001A samples.

Separation of maize grain tissues and sample preparation for extraction

Dried maize grain was soaked in water overnight at 4°C. The grain was blotted dry and run through a Strong-Scott Pearler mill to coarsely crush the kernels. Tissues were then separated manually into three parts – the external portions, or the pericarp and aleurone; the endosperm; and the embryo. The separated tissues were then frozen and lyophilized.

Extraction of cell wall ester-bound phenolic acids from maize tissue

Cell wall bound phenolic acids were extracted from 0.5g samples of lyophilized pericarp and aleurone tissues prepared as described above. Samples were extracted in 20 mL of 2N NaOH under N_2 gas; this mixture was shaken for 4h in the dark to hydrolyze the ester linkages between the phenolics and the cell wall hemi-cellulose. Following this, the samples were mixed using a polytron mixer at high speed for 30s. The samples were then neutralized with concentrated HCl and the pH was equilibrated to 2.0. The supernatant was collected after centrifugation, the pellet was twice washed with 10 mL of distilled water, and the supernatants were collected and pooled. The pooled supernatant was then extracted with 4 x 50 mL ethyl acetate. The ethyl acetate fractions were pooled and dried by rotary evaporation under darkness. The dried extract was re-dissolved in 10 ml methanol and stored at -20°C until HPLC analysis.

HPLC & HPLC-MS analysis

All analyses were accomplished with an Agilent HP Chemstation LC 1100 system. The solvent system was comprised of methanol (A) and 0.05% TFA (aqueous) solution (B). Starting conditions were 30% A, 70% B. The gradient was as follows: 30-40% A in 3.5 min; 40-42% A in 6.5 min; 42-100% A in 4 min; hold at 100% A for 4 min; 100-30% A in 4 min; allow column to re-equilibrate for 5 min. A C18 YMC ODS-AM 3 μm column, 100mm x 2.0mm was used in a 50°C column oven to achieve compound separation. From each sample, 2 μl were taken and an injection program was used to dilute this with water to 50% methanol, in order to improve peak shape and resolution. The pump flow rate was 0.30 ml/min, and detection wavelength was at 280nm and 325nm. The elution times were as follows: p-coumaric acid 3.12 min; trans-ferulic acid 3.75 min; cis-ferulic acid 4.17 min; diferulic acid 8'-5' form 7.50 min; diferulic acid 5'-5'

form 8.09 min; diferulic acid 8-O-4 form 9.13 min; unknown soluble phenolic X 9.42 min; diferulic acid 8'-5' benzofuran form 10.08 min; and, diferuloyl putrescine 10.29 min; see Figure 3.2. Peak identification was determined by comparison of retention times and spectra with purified compounds. In cases where there was no standard, the absorption spectra were compared to spectral libraries and identification was verified by determining the mass spectra of the compounds in question.

An LC-MS method using an Agilent HP Chemstation LC 1100 system with an APCI MS detector was used on selected samples to obtain increased information on the unknown soluble phenolic X and on Diferuloyl putrescine. The column used was an YMC ODS-AM 100mm x 2mm. From each sample, 1 µl aliquot was injected onto the column. The column was maintained at 40°C and the flow rate was 0.30 ml/min. A binary gradient of Acetonitrile (A) and 0.05% triflouroacetic acid (TFA) in water, pH 2.4, was used to achieve separation. Starting conditions were 8% A, 92% B. The detection wavelengths were 280 and 325 nm. The gradient was as follows: 8-35% A in 12 min; 35-100% A in 3 min; hold at 100% A for 0.5 min; 100-8% A in 3 min; allow column to re-equilibrate for 5 min. Retention times for the compounds of interest were 8.28 min for the unknown soluble phenolic X and 8.54 min for Diferuloyl putrescine. MS detection was used.

APCI mass detector optimized parameters

Atmospheric Pressure Chemically-assisted Ionization (APCI) in positive ionization mode was used. Nitrogen was the nebulizing and drying gas. The spray chamber parameters were: gas temperature 300°C vaporizer temperature 400°C, fragmentor at 20 V, drying gas at 6.0 l/min, nebulizer pressure 40 psig, capillary voltage 3000 V, and corona current 3 uA. The mass detector was also used in positive SCAN mode from 100 to 800 (m/z).

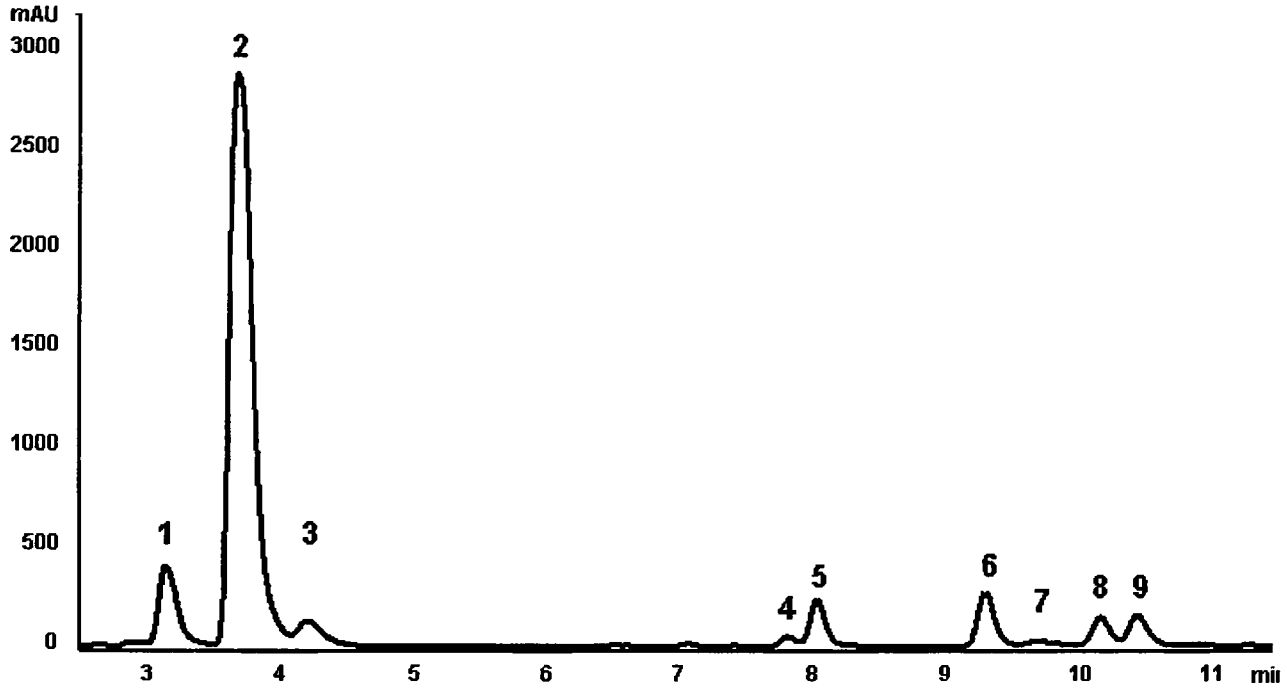


Figure 3.2: Chromatogram of the mapping population family number 3. The spectrum shown is at 325 nm. Trans-ferulic acid was quantified at 280 nm because the peak often exceeded the point of linear absorption on 325 nm. All other compounds were quantified at 325 nm. The peaks shown are: 1: p-coumaric acid - 3.12 min; 2: trans ferulic acid – 3.75 min; 3: cis-ferulic acid – 4.17 min; 4: DFA 8'-5' form - 7.50 min; 5: DFA 5'-5' form - 8.09 min; 6: DFA 8-0-4 form - 9.13 min; 7: Unknown soluble phenolic X - 9.42 min; 8: DFA 8'-5' benzofuran form - 10.08 min, and 9: diferuloyl putrescine - 10.29 min.

QTL Mapping

QTL analyses were performed in Mexico by Silverio García-Lara. The mapping was executed using a subset of 163 recombinant inbred lines (RIL) in the population CML 290 x Muneng 8128 for which molecular and phenotypic data were available. The map is composed of 61 polymorphic RFLPs and 90 polymorphic SSRs distributed over the ten maize chromosomes. In total 151 molecular markers were used to construct the map using the program MAPMAKER (Lander *et al.*, 1998). Genotypic segregation data were captured by HYPERMAPDATA developed by ABS, CIMMYT.

QTL Composite Interval Mapping Statistical Model

The model for the analysis was:

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

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} = t he number of alleles from the resistant parent at the selected marker k

e_{ij} = the residual variable of RIL j in environment I

3.3 Results and Discussion

Relationship of cell wall bound phenylpropanoid content in the external tissues of the grain and resistance to S. zeamais *infestation*

CIMMYT maize grain lines, selected for differing levels of resistance to *S. zeamais*, were investigated for cell wall bound phenylpropanoid content in the outer tissues. The data set is described in Table 3.1 for season TL98A and in Table 3.2 for season TL99B. The complete data set is presented in Appendix 1. The relationships between the amount of the phenolic compounds in the two seasons TL98A and TL99B and the resistance parameters in season TL98A were examined. Unfortunately, insect resistance bioassay data were available only for the TL98A season. The resulting correlations between the factors are presented in Table 3.3. In both TL98A and TL99B, the strongest correlation was a negative correlation between the total DFA content and the percent of damaged kernels in the insect trials. In both seasons the 8'-5' and the 8'-5' benzofuran forms of DFA were significantly, negatively correlated with percentage of kernels damaged. The 8'-5' benzofuran form content was also significantly, positively correlated with the peak puncture force in both seasons.

When performing multiple comparisons it is important to take into consideration the possibility of significant results occurring by chance. In the TL98A season of thirty statistical comparisons made, there were seventeen significant correlations. Of these, eight correlations have a p-value of less than 0.05 and nine have a p-value of less than 0.01. In the TL99B season, of twenty-seven comparisons, there were thirteen significant correlations, eight at the less than 0.05 level and five at the less than 0.01 level. Since there is a one in twenty chance of a $p<0.05$ result occurring arbitrarily, one or two of the correlations significant at the $p<0.05$ level in each set of comparisons might have been due to chance alone. The confidence in the correlations at

the $p<0.01$ level can be much higher, because the chance of such a relationship being a random result is only one in one hundred.

Interpretation of these results may be confounded by the lack of insect bioassay data in the TL99B season. Although the statistical comparison between phytochemical and bioassay data obtained in different seasons is not ideal, the strength of the correlations lends strong support to the accuracy of the patterns observed. The differences in the strength of the correlations between seasons may be accounted for primarily by the differences in the nature of the comparison, but also by the inclusion of the susceptible check, CML 244x349, in season TL98A. If this genotype is removed, the total range of variation in the parameters decreases and the r^2 values of the correlations decrease. For example, the relationship between total DFA and damage in TL98A decreases from 0.9042 (as shown in Table 3.3) to 0.7866 when CML 244x349 is removed from the calculation.

Multiple regression models were made for the TL98A season to provide a more in-depth understanding of the relationship between the *S. zeamais* resistance and the grain phytochemical traits. Peak puncture force and the percent of kernels damaged were set as dependent variables in two regression models (Table 3.4). In both models, the content of DFA 8'-5' form is a significant predictor. This form of the ferulic acid dimer has been shown to be highly correlated with leaf field resistance ratings against two lepidopteran stem borers, the sugarcane borer, *Diatraea grandiosella* Dyar, and the southwestern corn borer, *Diatraea saccharalis* Fabricius (Ramputh, 2001).

Table 3.1: Characterization of the phytochemical resistance factors and insect ratings for the CIMMYT post-harvest resistance genotypes, season TL98A. n = 7 genotypes. All phytochemical analysis was performed on the outer tissue layers of the grain.

	Mean ± S.E.	Range
Peak Puncture Force (N)	16.96 ± 2.32	5.95 – 21.54
% Kernels Damaged	18.83 ± 10.48	0.00 – 78.82
Nitrogen Content (%)	1.71 ± 0.08	1.36 – 1.97
p-Coumaric Acid (μg/g)	3060 ± 383	2068 – 5112
trans Ferulic Acid (μg/g)	30359 ± 1437	26101 – 35660
cis Ferulic Acid (μg/g)	823 ± 102	658 – 1423
DFA 8-5 (μg/g)	621 ± 55	333 – 786
DFA 5-5 (μg/g)	2149 ± 147	1492 – 2474
DFA 8-O-4 (μg/g)	2454 ± 204	1511 – 3165
DFA 8-5 benzofuran (μg/g)	1903 ± 192	1046 – 2666
Total DFAs (μg/g)	7127 ± 576	4382 – 9064
Total Phenolics (mg/g)	41.37 ± 1.71	36.14 – 46.82

Table 3.2: Characterization of the phytochemical resistance factors and insect ratings for the CIMMYT post-harvest resistance genotypes, season TL99B. n = 6 genotypes. All phytochemical analysis was performed on the outer tissue layers of the grain.

	Mean ± S.E.	Range
p-Coumaric Acid (μg/g)	3329 ± 671	1532 – 6274
trans Ferulic Acid (μg/g)	23953 ± 1470	18626 – 29804
cis Ferulic Acid (μg/g)	1206 ± 83	986 – 1452
DFA 8-5 (μg/g)	861 ± 182	234 – 1415
DFA 5-5 (μg/g)	1928 ± 66	1733 – 2195
DFA 8-O-4 (μg/g)	1958 ± 137	1797 – 2643
DFA 8-5 benzofuran (μg/g)	1080 ± 88	890 – 1502
Total DFAs (μg/g)	5826 ± 357	4654 – 7175
Total Phenolics (mg/g)	34.31 ± 2.14	25.80 – 40.88

Table 3.3: Correlations between phytochemical, physical and resistance traits in CIMMYT resistant populations. Numbers in bold show a significant correlation with * denoting $p<0.05$ and ** denoting $p<0.01$. n = 7 genotypes in season TL98A and 6 genotypes in season TL99B.

Season TL98A	Nitrogen Content	% Kernel Damage	Puncture Force
p-coumaric acid	0.357	-0.508	0.483
trans Ferulic acid	0.150	-0.025	-0.076
cis Ferulic acid	0.103	0.010	-0.203
DFA 5'-5' form	**0.906** **	**-0.907** **	**0.836** **
DFA 8-O-4 form	**0.791** *	**-0.876** **	**0.808** *
DFA 8'-5' form	0.478	**-0.857** **	**0.828** *
DFA 8'-5' benz	**0.762** *	**-0.851** **	**0.800** *
Total DFA	**0.808** *	**-0.904** **	**0.843** *
Total Phenolics	0.440	-0.381	0.259
Nitrogen Content		**-0.721** *	**0.666** *
Damage			**-0.973** **

Season TL99B	Nitrogen Content	% Kernel Damage	Puncture Force
p-coumaric acid	**0.699** *	**-0.862** **	**0.713** *
trans Ferulic acid	**0.683** *	-0.599	0.300
cis Ferulic acid	0.479	-0.306	-0.162
DFA 5'-5' form	0.539	-0.628	0.596
DFA 8-O-4 form	0.362	-0.593	**0.702** *
DFA 8'-5' form	**0.767** **	**-0.719** *	0.080
DFA 8'-5' benz	0.530	**-0.735** *	**0.801** **
Total DFA	**0.742** *	**-0.876** **	0.616
Total Phenolics	**0.662** *	**-0.778** **	0.467

Table 3.4: Equations and results of multiple regressions of phytochemical markers on resistance and physical traits in CIMMYT resistant populations in season TL98A. All regressions presented are significant and have been selected for maximized r^2 values.

	Equation	p-value	r^2
Season TL98A			
Peak Kernel Puncture Force	-4.35 – 0.005 cisFA – 0.050 DFA 8-O-4 + 0.012 DFA 8'-5' + 0.020 total DFA	0.0057	0.982
Percentage of Kernels Damaged	177.43 + 0.030 cisFA – 0.072 DFA 8'-5' + 0.131 DFA 8-5 benzofuran – 0.054 total DFA	0.0020	0.974

Putative Identification of Unknown Compound X as a Second Putrescine Di-conjugate

In all of the following analyses, an unknown compound, named unknown soluble phenolic X is included. Compound X, as shown in Figure 3.2, elutes at 9.42 minutes, and because of this position in the chromatogram, was first thought to be a fifth DFA form. The analysis of the mass spectrum of the compound concluded that this was not the case. Based on the mass spectrum (Figure 3.3), the molecular weight is 410 g/mole. Using the known rate of occurrence of natural isotopes, the major and related peaks of the mass spectrum were analyzed to determine that the chemical formula of the compound is C_{23} H_{26} O_5 N_2 (blow-up window Figure 3.3). The absorbance spectrum of the compound is very similar to that of diferuloyl putrescine (Figure 3.4). Furthermore, the concentration of this compound in the maize grain is highly correlated with diferuloyl putrescine content during both seasons (Tables 3.12 and 3.13). Based on all these considerations -- the compound mass, isotopic analysis of the mass spectrum, the similar absorbance spectrum, and on the correlation with diferuloyl putrescine -- it is hypothesized that this compound is coumaroyl-feruloyl putrescine – another putrescine di-conjugate.

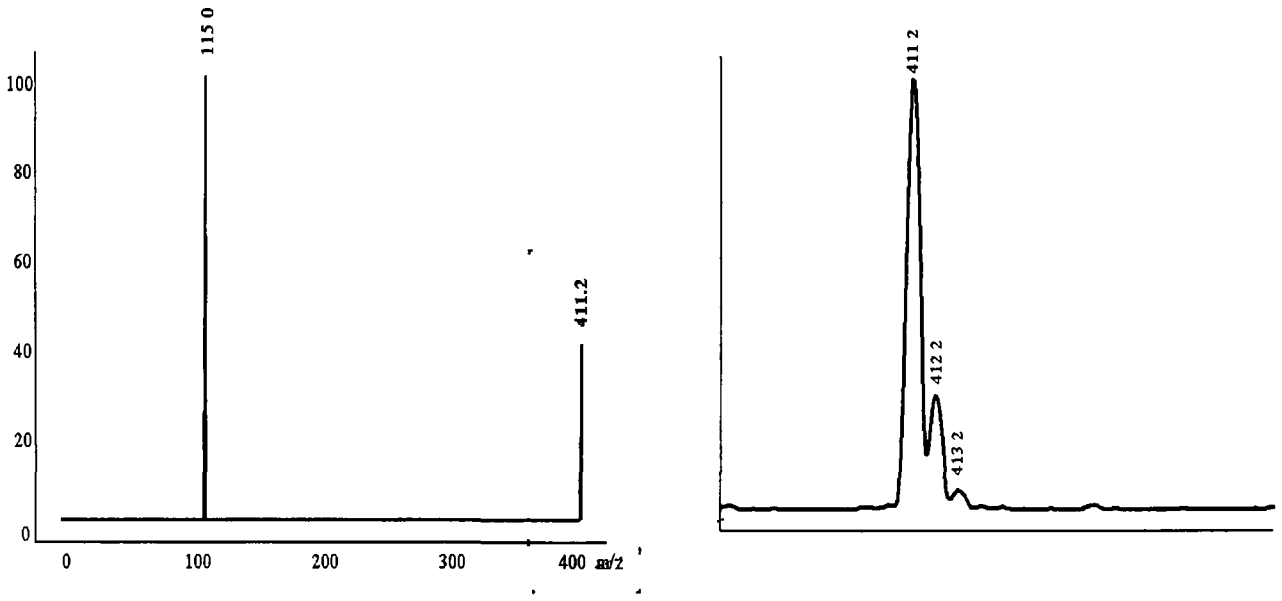


Figure 3.3: Mass spectrum of the unknown soluble phenolic X. Molecular weight is 410.2, spectrum shown is in positive ionization mode (MW+1). Analysis of the isotopic variation around the main peak, seen in magnified section, reveals that the chemical formula is $C_{23}H_{26}O_5N_2$.

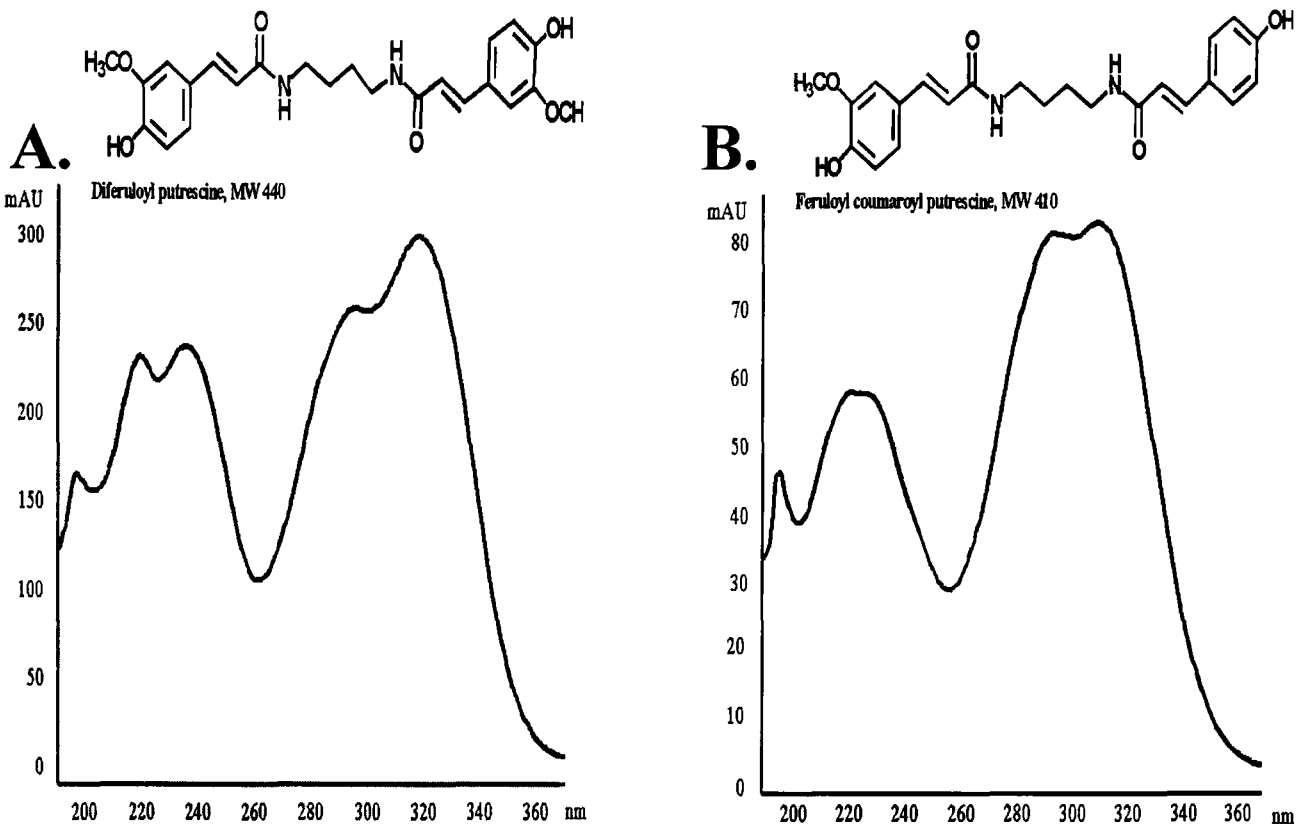


Figure 3.4: **A**: Absorbance spectrum and structure of diferuloyl putrescine and **B**: Absorbance spectrum and proposed structure of unknown soluble phenolic X.

Correlations between cell wall bound phenylpropanoid content and resistance to Sitophilus zeamais in QTL mapping population

To examine the characteristics of the mapping population, first the parental lines of the population must be examined to ensure that there is sufficient variation in the traits of interest. The phytochemical traits of the parental lines CML 290 and Muneng 8128 are summarized in Table 3.5.

The results of the analysis of the mapping population are summarized by season in the Tables 3.6 and 3.7, and the complete data set for both years is available in Appendix 2. There is a wider range of variation in almost all of the resistance factors in the TL2000B season than in the TL2001A season. The notable exceptions are the phytochemical traits, DFA 8'-5' benzofuran form content and diferuloyl putrescine content, which show somewhat greater variability in the second season.

Table 3.5: Parental lines of the QTL mapping population. Characterization of the phytochemical resistance factors in the outer layers of the grain of CML 290 and Muneng 8128. Means and standard errors are presented, n = 3 sub-samples of a single harvest for each line.

	CML 290	Muneng 8128	t-test, p-value (Dunn-Sidak Adjustment)
p-Coumaric Acid (μg/g)	1480 ± 40	1910 ± 144	0.40
trans Ferulic Acid (μg/g)	16743 ± 422	28113 ± 412	0.0004
cis Ferulic Acid (μg/g)	683.5 ± 34.9	733.2 ± 9.9	0.95
DFA 8-5 (μg/g)	253.2 ± 6.3	313.9 ± 10.4	0.080
DFA 5-5 (μg/g)	1452 ± 26	1798 ± 35	0.015
DFA 8-O-4 (μg/g)	759.4 ± 8.2	834.2 ± 4.7	0.015
Soluble Phenolic X (μg/g)	316.6 ± 12.6	196.9 ± 23.4	0.11
DFA 8-5 benzofuran (μg/g)	446.6 ± 21.6	628.5 ± 20.3	0.039
Diferuloyl putrescine (μg/g)	634.5 ± 76.6	188.9 ± 24.2	0.056
Total DFAs	2911 ± 18	3574 ± 27	0.0006
Total Phenolics	22535 ± 525	34422 ± 592	0.0013

Table 3.6: Characterization of the phytochemical resistance factors and insect ratings for the mapping population, TL2000B. n = 162. All phytochemical analysis was performed on the outer tissue layers of the grain.

	Mean ± S.E.	Range
Peak Puncture Force (N)	16.178 ± 0.109	11.754 – 19.318
% Kernels Damaged	75.19 ± 1.13	42.80 – 100.00
Dobie Index	12.69 ± 0.08	9.65 – 14.99
% Loss	13.56 ± 0.42	3.88 – 33.07
# Progeny Emerged	133.5 ± 3.3	67.0 – 276.2
p-Coumaric Acid (μg/g)	2079 ± 40	1065 – 3882
trans Ferulic Acid (μg/g)	23907 ± 269	15728 – 35197
cis Ferulic Acid (μg/g)	1344 ± 27	698 – 2362
DFA 8-5 (μg/g)	124.9 ± 7.5	33.0 – 597.7
DFA 5-5 (μg/g)	2014 ± 28	1350 – 3152
DFA 8-O-4 (μg/g)	937.4 ± 12.7	648 - 1483
Soluble Phenolic X (μg/g)	357.5 ± 14.0	0.00 – 1030
DFA 8-5 benzofuran (μg/g)	583.1 ± 7.0	257.3 – 810.4
Soluble Phenolic Y (μg/g)	670.1 ± 27.2	164.1 – 1864
Total DFAs	3659 ± 50	2468 – 5809
Total Phenolics	30991 ± 314	21854 - 44142

Table 3.7: Characterization of the phytochemical resistance factors and insect ratings for the mapping population, TL2001A. n = 162. All phytochemical analysis was performed on the outer tissue layers of the grain.

	Mean ± S.E.	Range
Peak Puncture Force (N)	14.33 ± 0.07	12.04 – 16.63
% Kernels Damaged	70.94 ± 0.54	53.70 – 89.13
Dobie Index	11.91 ± 0.04	10.39 – 13.58
% Loss	10.73 ± 0.18	6.09 – 18.30
# Progeny Emerged	113.2 ± 1.1	78.0 – 173.7
p-Coumaric Acid (µg/g)	2280 ± 38	1158 – 3769
trans Ferulic Acid (µg/g)	23403 ± 228	16386 – 31067
cis Ferulic Acid (µg/g)	1170 ± 16	714 – 1698
DFA 8-5 (µg/g)	310.5 ± 6.3	68.5 – 512.0
DFA 5-5 (µg/g)	1537 ± 19	907 - 2303
DFA 8-O-4 (µg/g)	850.6 ± 8.4	605.8 – 1161
Soluble Phenolic X (µg/g)	402.0 ± 14.1	0.00 – 948.9
DFA 8-5 benzofuran (µg/g)	543.8 ± 7.6	194.3 – 850.5
Diferuloyl putrescine (µg/g)	689.0 ± 31.7	86.4 – 2429
Total DFAs	3242 ± 37	2177 – 4585
Total Phenolics	30096 ± 265	21676 - 39552
Mean Grain Weight (g)	2.425 ± 0.022	1.474 – 3.362
Grain Width (cm)	0.8143 ± 0.0033	0.7000 – 0.9100
Density	0.7918 ± 0.0012	0.7600 – 0.8500
% Germination	9.06 ± 0.56	0.00 – 42.00
Vigour	1.760 ± 0.063	0.00 – 5.23

Correlations . . . cont'd

The correlations between the bound phenylpropanoid content and the *S. zeamais* resistance bioassay results for two consecutive seasons were examined (Table 3.8). In the first season, many of the phytochemical factors have significant, negative correlations with the insect bioassay factors. From this it can be inferred that the higher the grain outer-tissue content of these phytochemicals, the better the insect resistance of the grain. Total cell wall bound phenolics is the phytochemical factor that is most highly correlated with the bioassay results, with r values between -0.21, for the percent loss of the grain, and -0.43, for the Dobie index rating. Of the other phytochemical traits, total diferulic acids, the 5'-5' and 8-O-4 DFA forms, and trans-ferulic acid all have significant, negative correlations with all of the insect bioassay factors.

The correlations in the TL2000B season are all much higher than those seen in the TL2001A season. There are comparatively few strong correlations between the phytochemical traits and the bioassay factors in the second season. The best correlated phytochemical traits are total cell wall bound phenolics, the 8-O-4 form of DFA, and cis-ferulic acid; however, the strength of these correlations are all less than 20%. The strongest correlation in this season is that of the peak kernel puncture force with the Dobie index rating and with the number of insect progeny emerged, at 24% and 25% respectively.

Table 3.8: Correlations between phytochemical, physical and resistance traits in QTL mapping population. Numbers in bold show a significant correlation; bold alone indicates $p<0.10$, * denotes $p<0.05$ and ** denotes $p<0.01$

TL2000B	Puncture Force	Dobie Index	% Kernel Damage	% Loss	Progeny Emerged
Puncture Force	---	**-0.258** **	**-0.221** **	**-0.150**	-0.088
p-Coumaric Acid	0.016	**-0.152**	**-0.154**	**-0.151**	**-0.188** *
trans Ferulic Acid	**0.214** **	**-0.422** **	**-0.253** **	**-0.155**	**-0.281** **
cis Ferulic Acid	**0.179** *	**-0.160** *	**-0.271** **	**-0.237** **	-0.062
DFA 8'-5'	0.062	-0.129	**-0.243** **	**-0.274** **	-0.068
DFA 5'-5'	**0.180** *	**-0.245** **	**-0.222** **	**-0.173** *	**-0.165** *
DFA 8-O-4	**0.232** **	**-0.283** **	**-0.264** **	**-0.212** **	**-0.168** *
DFA 8'-5' benzo.	**0.136**	-0.103	**-0.204** **	**-0.139**	**-0.176** *
Soluble Phenolic X	-0.033	-0.117	-0.128	-0.058	**-0.142**
Diferuloyl putrescine	-0.073	-0.123	**-0.249** **	-0.063	**-0.130**
Total DFA	**0.188** *	**-0.242** **	**-0.256** **	**-0.212** **	**-0.169** *
Total Phenolics	**0.230** **	**-0.434** **	**-0.301** **	**-0.206** **	**-0.297** **

TL2001A	Puncture Force	Dobie Index	% Kernel Damage	% Loss	Progeny Emerged
Puncture Force	---	**-0.240** **	0.079	0.054	**-0.253** **
p-Coumaric Acid	0.077	-0.106	-0.093	-0.108	-0.113
trans Ferulic Acid	0.123	**-0.158** *	0.023	-0.019	**-0.137**
cis Ferulic Acid	-0.006	-0.096	**-0.154**	**-0.129**	**-0.138**
DFA 8'-5'	0.075	-0.027	-0.063	-0.004	-0.082
DFA 5'-5'	0.103	-0.102	-0.036	0.008	**-0.155** *
DFA 8-O-4	**0.145**	**-0.134**	-0.048	-0.031	**-0.192** **
DFA 8'-5' benzo.	0.088	0.021	0.022	-0.012	-0.035
Soluble Phenolic X	-0.068	-0.042	-0.091	-0.047	-0.066
Diferuloyl putrescine	-0.103	-0.078	-0.088	-0.057	-0.086
Total DFA	0.118	-0.084	-0.036	-0.006	**-0.145** *
Total Phenolics	**0.133**	**-0.169**	-0.008	-0.041	**-0.163** *

Table 3.9: Equations and results of multiple regressions of phytochemical markers on resistance and physical traits in the mapping population. All terms in the models are significant and were selected to maximize the r^2 value with the minimum number of terms.

	Equation	p-value	r^2
Season TL2000B			
Dobie Index Rating	17.06 – 0.120 Force + 0.004 DFA 85 benzo – 0.0004 total DFA – 0.0001 total Phenolics	<0.00001	0.242
Percentage of Kernels Damaged	138.81 – 2.08 Force – 0.040 DFA 85 + 0.053 Soluble Phenolic X – 0.032 Diferuloyl putrescine – 0.003 total DFA – 0.0004 total Phenolics	<0.00001	0.288
Percent Loss	26.69 – 0.472 Force – 0.0001 trans FA – 0.021 DFA 85 + 0.010 Soluble Phenolic X – 0.004 Diferuloyl putrescine	0.00037	0.135
Number of Progeny Emerged	261.11 + 0.028 cisFA – 0.089 DFA 85 benzo. – 0.014 Diferuloyl putrescine – 0.003 total Phenolics	0.00021	0.129
Season TL2001A			
Dobie Index Rating	17.84 – 0.494 Grain Weight – 5.239 Density – 0.0005 cisFA + 0.001 DFA 85 benzo. + 0.001 Soluble Phenolic X – 0.0003 Diferuloyl putrescine – 0.00002 Total Phenolics	0.00023	0.163
Percentage of Kernels Damaged	75.31 + 0.195 %Germination – 1.77 Vigour – 0.010 cis FA + 0.015 DFA 85 benzo.	0.00685	0.085
Percent Loss	9.943 – 1.65 Grain Weight + 0.092 %Germination – 0.457 Vigour – 0.443 Force – 0.0007 pCA	0.01517	0.092
Number of Progeny Emerged	316.69 – 12.51 Grain Weight – 195.06 Density – 0.014 cisFA + 0.030 DFA 85 benzo. + 0.031 Soluble Phenolic X – 0.014 Diferuloyl putrescine – 0.006 total DFA	0.00009	0.175

Table 3.10: Correlations between the physical and insect resistance parameters of season TL2000B and TL2001A. Numbers in bold show a significant correlation ($p < 0.05$).

2000B \ 2001A	Puncture Force	Dobie Index	% Kernel Damage	% Loss	Progeny Emerged
Puncture Force	0.1130	**-0.1794**	-0.1043	-0.1022	-0.1329
Dobie Index	-0.0713	**0.6178**	**0.3194**	**0.2509**	**0.5141**
% Kernel Damage	0.0288	**0.4663**	**0.5152**	**0.2899**	**0.4123**
% Loss	0.0987	**0.1934**	**0.2428**	**0.3951**	**0.1718**
Progeny Emerged	0.0124	**0.4514**	**0.4623**	**0.4291**	**0.4709**

Table 3.11: Correlations between the phytochemical parameters of season TL2000B and TL2001A. Numbers in bold show a significant correlation ($p < 0.05$).

2000B \ 2001A	p-Coumaric Acid	trans Ferulic Acid	cis Ferulic Acid	DFA 8'-5'	DFA 5'-5'	DFA 8-O-4	DFA 8'-5' benzo.	Soluble Phenolic X	Diferuloyl putrescine	Total DFA	Total Phenolics
p-Coumaric Acid	**0.6619**	-0.0574	0.0339	**0.1643**	0.0552	0.0815	0.0516	**0.1861**	**0.1644**	0.0858	0.0592
trans Ferulic Acid	-0.0126	**0.5221**	**0.2643**	0.0192	**0.1939**	**0.1575**	0.1377	-0.0076	-0.0452	**0.1682**	**0.48765**
cis Ferulic Acid	-0.0174	0.1106	0.0543	-0.0709	-0.0052	-0.0069	0.0784	-0.0879	-0.1305	-0.0001	0.0961
DFA 8'-5'	0.1086	-0.1143	-0.1245	-0.0659	-0.0883	-0.0503	-0.0467	-0.0196	-0.0311	-0.0791	-0.1014
DFA 5'-5'	**0.1657**	0.1284	0.0819	**0.1569**	**0.2530**	**0.2539**	**0.1864**	-0.0268	-0.0910	**0.2544**	**0.1748**
DFA 8-O-4	0.1323	0.0711	-0.0088	0.0895	**0.1430**	**0.2184**	0.1258	-0.0857	-0.1406	**0.1652**	0.1027
DFA 8'-5' benzo.	0.1005	0.0612	**0.1584**	0.1317	**0.1762**	**0.1940**	**0.2662**	-0.0484	-0.1248	**0.2131**	0.1064
Soluble Phenolic X	0.0933	**-0.2183**	-0.1122	-0.0000	-0.1300	-0.0805	-0.1333	**0.3574**	**0.3602**	-0.1134	**-0.1974**
Diferuloyl putrescine	0.0600	**-0.2090**	-0.1091	-0.0650	**-0.1908**	**-0.1875**	**-0.2174**	**0.3995**	**0.4817**	**-0.1978**	**-0.2058**
Total DFA	**0.1562**	0.0809	0.0467	0.1186	**0.1885**	**0.2164**	**0.1660**	-0.0465	-0.1086	**0.2016**	0.1230
Total Phenolics	0.0976	**0.4630**	**0.2433**	0.0502	**0.2029**	**0.1793**	**0.1579**	0.0024	-0.0462	**0.1873**	**0.4539**

Table 3.12: Correlations between phytochemical parameters within season TL2000B. Numbers in bold denotes a significant correlation ($p < 0.05$)

Season TL2000B	p-Coumaric Acid	trans Ferulic Acid	cis Ferulic Acid	DFA 8'-5'	DFA 5'-5'	DFA 8-O-4	DFA 8'-5' benzo.	Soluble Phenolic X	Diferuloyl putrescine	Total DFA	Total Phenolics
p-Coumaric Acid	**1**										
trans Ferulic Acid	0.1508	**1**									
cis Ferulic Acid	0.1962	**0.3056**	**1**								
DFA 8'-5'	**0.3678**	0.0489	**0.6833**	**1**							
DFA 5'-5'	**0.2696**	**0.3875**	**0.6104**	**0.6332**	**1**						
DFA 8-O-4	**0.3248**	**0.3943**	**0.6528**	**0.5950**	**0.8193**	**1**					
DFA 8'-5' benzo.	0.24361	**0.2916**	**0.6486**	**0.6136**	**0.8036**	**0.6936**	**1**				
Soluble Phenolic X	**0.3823**	0.0157	0.0472	**0.40344**	0.1807	0.1808	0.0824	**1**			
Diferuloyl putrescine	**0.2636**	0.0599	-0.0472	0.1470	-0.0470	-0.0035	-0.1379	**0.8414**	**1**		
Total DFA	**0.3220**	**0.3642**	**0.6992**	**0.7397**	**0.9723**	**0.8970**	**0.8557**	0.2186	-0.0242	**1**	
Total Phenolics	**0.3262**	**0.9617**	**0.4850**	**0.2658**	**0.5742**	**0.5789**	**0.4734**	0.1014	0.0774	**0.5730**	**1**

Table 3.13: Correlations between phytochemical parameters within season TL2001A. Numbers in bold denotes a significant correlation ($p < 0.05$)

Season TL2001A	p-Coumaric Acid	trans Ferulic Acid	cis Ferulic Acid	DFA 8'-5'	DFA 5'-5'	DFA 8-O-4	DFA 8'-5' benzo.	Soluble Phenolic X	Diferuloyl putrescine	Total DFA	Total Phenolics
p-Coumaric Acid	1										
trans Ferulic Acid	0.0712	1									
cis Ferulic Acid	**0.2740**	**0.4215**	1								
DFA 8'-5'	**0.4808**	0.1563	**0.6371**	1							
DFA 5'-5'	**0.3072**	**0.5147**	**0.5720**	**0.7288**	1						
DFA 8-O-4	**0.3544**	**0.5391**	**0.5579**	**0.6829**	**0.8392**	1					
DFA 8'-5' benzo.	**0.2627**	**0.4200**	**0.6550**	**0.5816**	**0.5894**	**0.6366**	1				
Soluble Phenolic X	**0.2822**	-0.0124	**0.2920**	**0.3705**	0.2068	-0.0197	0.1836	1			
Diferuloyl putrescine	0.1922	-0.0510	0.0395	0.0508	-0.0311	-0.2259	0.0018	**0.8646**	1		
Total DFA	**0.3762**	**0.5034**	**0.6676**	**0.8239**	**0.9558**	**0.9110**	**0.7567**	0.2036	-0.0586	1	
Total Phenolics	**0.2735**	**0.9678**	**0.5564**	**0.3571**	**0.6555**	**0.6762**	**0.5449**	0.0758	-0.0222	**0.6676**	1

Multiple regression models were built to explain the variation in the insect bioassay factors (Table 3.9). The TL2000B season data provided much more powerful models than those for the 20001A season data. The terms used in the models for the first season included any of the phytochemical data and the kernel puncture force. All regressions were highly significant ($p < 0.001$). Both the Dobie index rating and the percent of damaged kernels were quite well modeled by the phytochemical data, $r^2 = 0.24$ and 0.29 respectively. The grain percent weight loss and the number of progeny emerged fit the models less well, each having an $r^2 = 0.13$. The most important phytochemical traits in these models were total DFA content, total phenolic content and diferuloyl putrescine content, all of which, along with peak puncture force, were included as terms in three of the four models built. Both the 8'-5' DFA content and the 8'-5' benzofuran DFA form were included in two models each. The simple regressions of these important phytochemical factors on the insect bioassay results can be seen in Figures 3.5 through 3.8.

Additional agronomic data were available in the TL2001A season. These parameters were included in determining the best multiple regression models, to maximize the model fit. The multiple regression models for the TL2001A season were all significant ($p<0.01$ for all except percent weight loss where $p<0.05$). In all cases except for the number of progeny emerged, the second season models explained less of the variation in the insect bioassay results than in the first season's. The r^2 for the second season model of progeny emerged was 0.1754, an increase from the first season. The factors that were most important in the second season models were cis-Ferulic acid content and 8'-5' benzofuran DFA content, both of which were included in three of the four models. Diferuloyl putrescine content is included in two of the four models. Also prominent is "grain weight", a factor not available for inclusion in the first season. The

important phytochemical factors are regressed against the insect bioassay results in Figures 3.9 through 3.12.

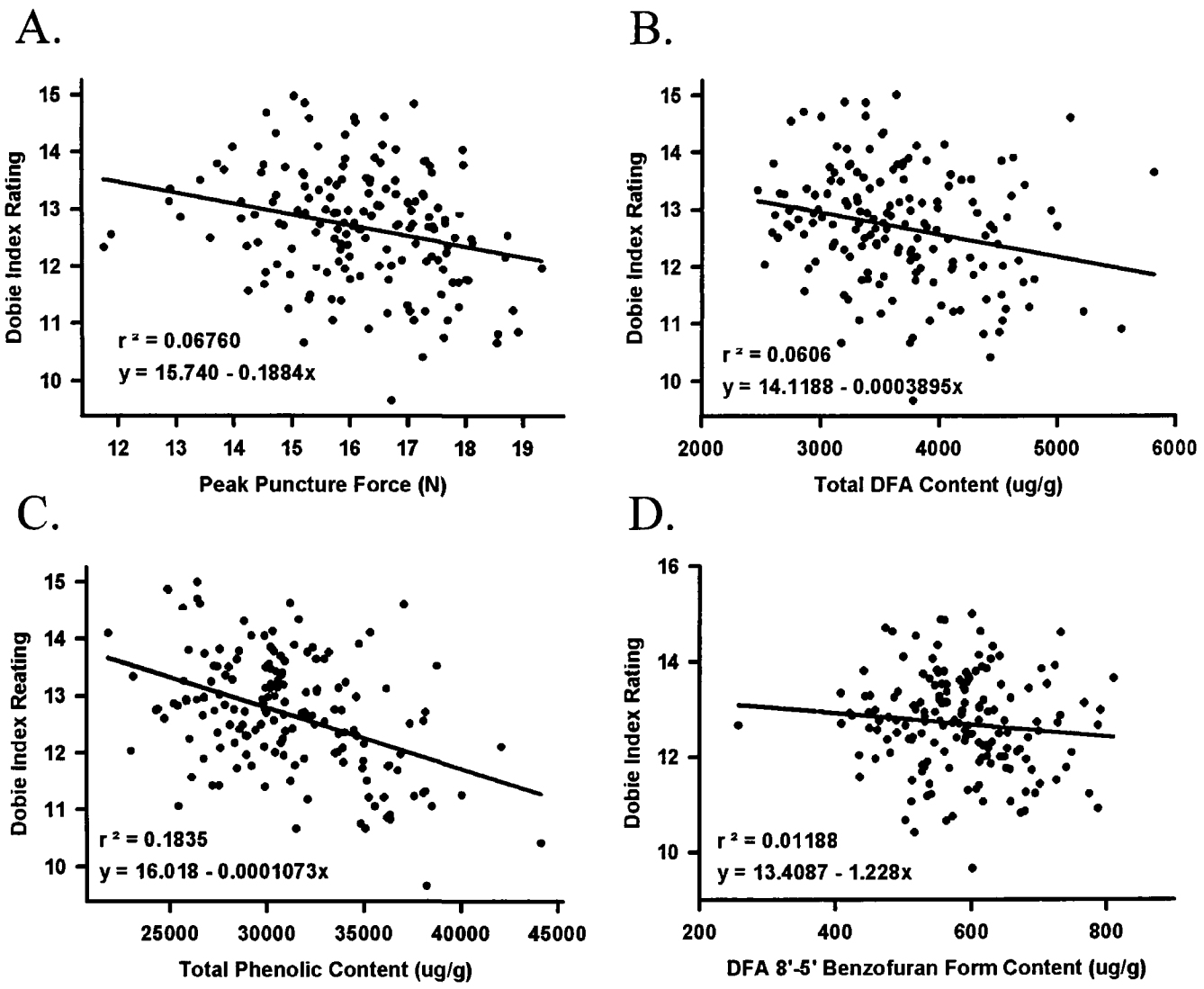


Figure 3.5: Simple regression models for main factors appearing in multiple regression model of the Dobie Index rating in season TL2000B. Shown are A: peak kernel puncture force; B: total DFA content; C: total phenolic content; and D: DFA 8'-5' benzofuran form content. See Table 7 for complete model: model $r^2 = 0.2424$, $p < 0.00001$. n = 163.

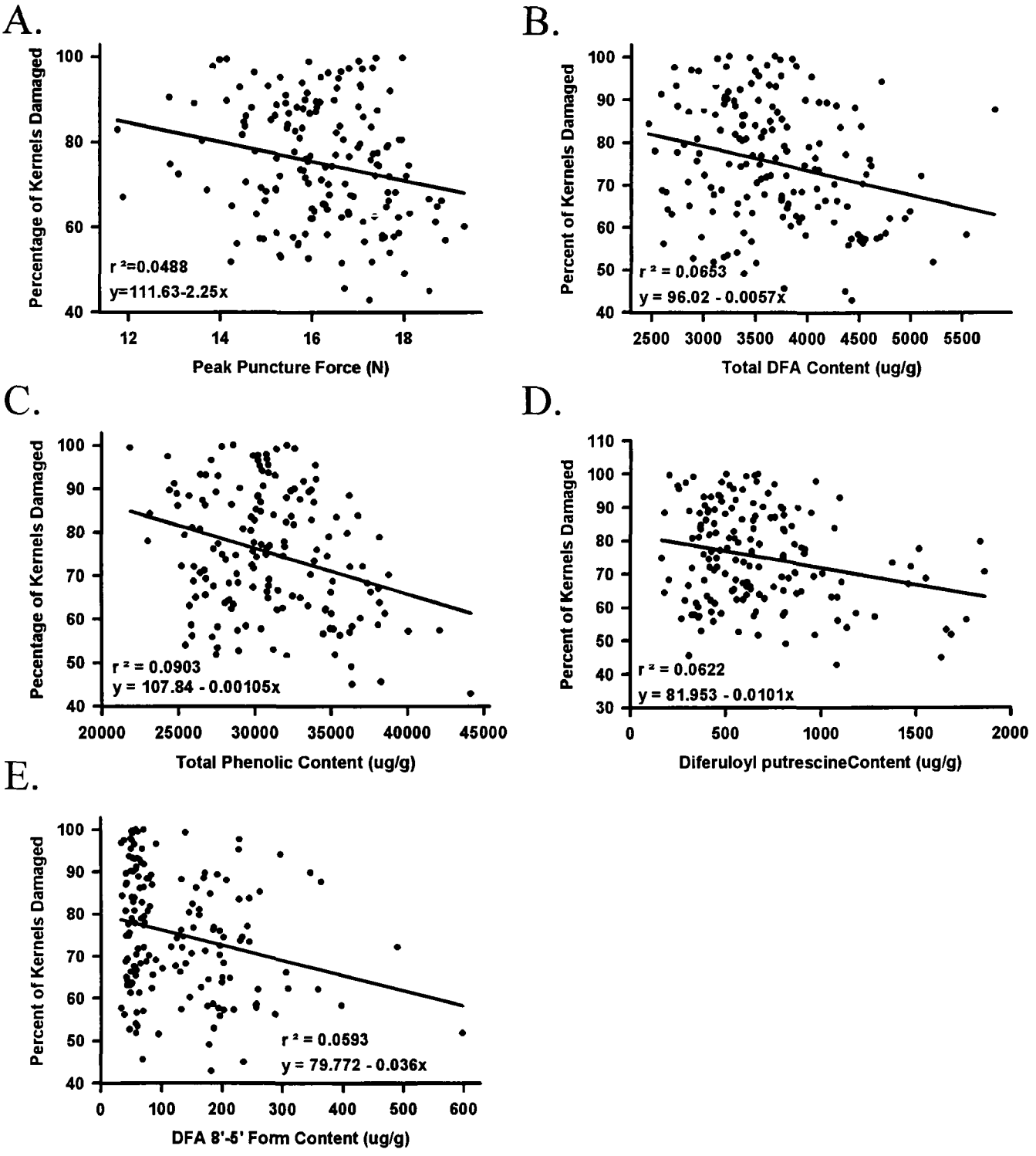


Figure 3.6: Simple regression models for main factors appearing in multiple regression model of percentage of kernels damaged in season TL2000B. Shown are A: peak kernel puncture force; B: total DFA content; C: total phenolic content; D: diferuloyl putrescine content; and E: DFA 8'-5' benzofuran form content. See Table 7 for complete model: model $r^2 = 0.2879$, $p < 0.00001$. n= 163.

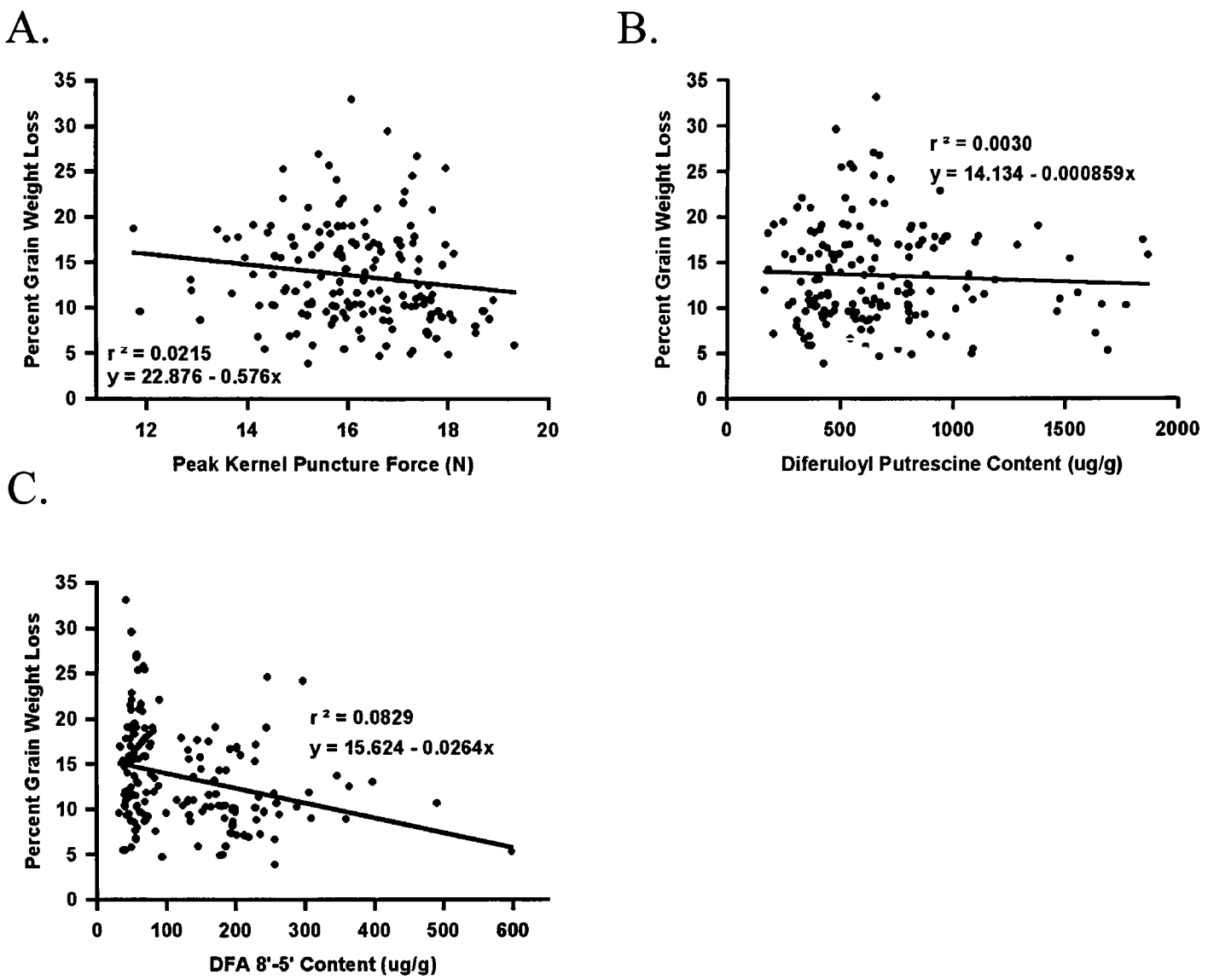


Figure 3.7: Simple regression models for main factors appearing in multiple regression model of the Percent Grain Weight Loss in season TL2000B. Shown are A: peak kernel puncture force; B: total diferuloyl putrescine content; and C: DFA 8'-5' benzofuran content. See Table 7 for complete model: model $r^2 = 0.1347$, $p = 0.00037$. $n = 163$.

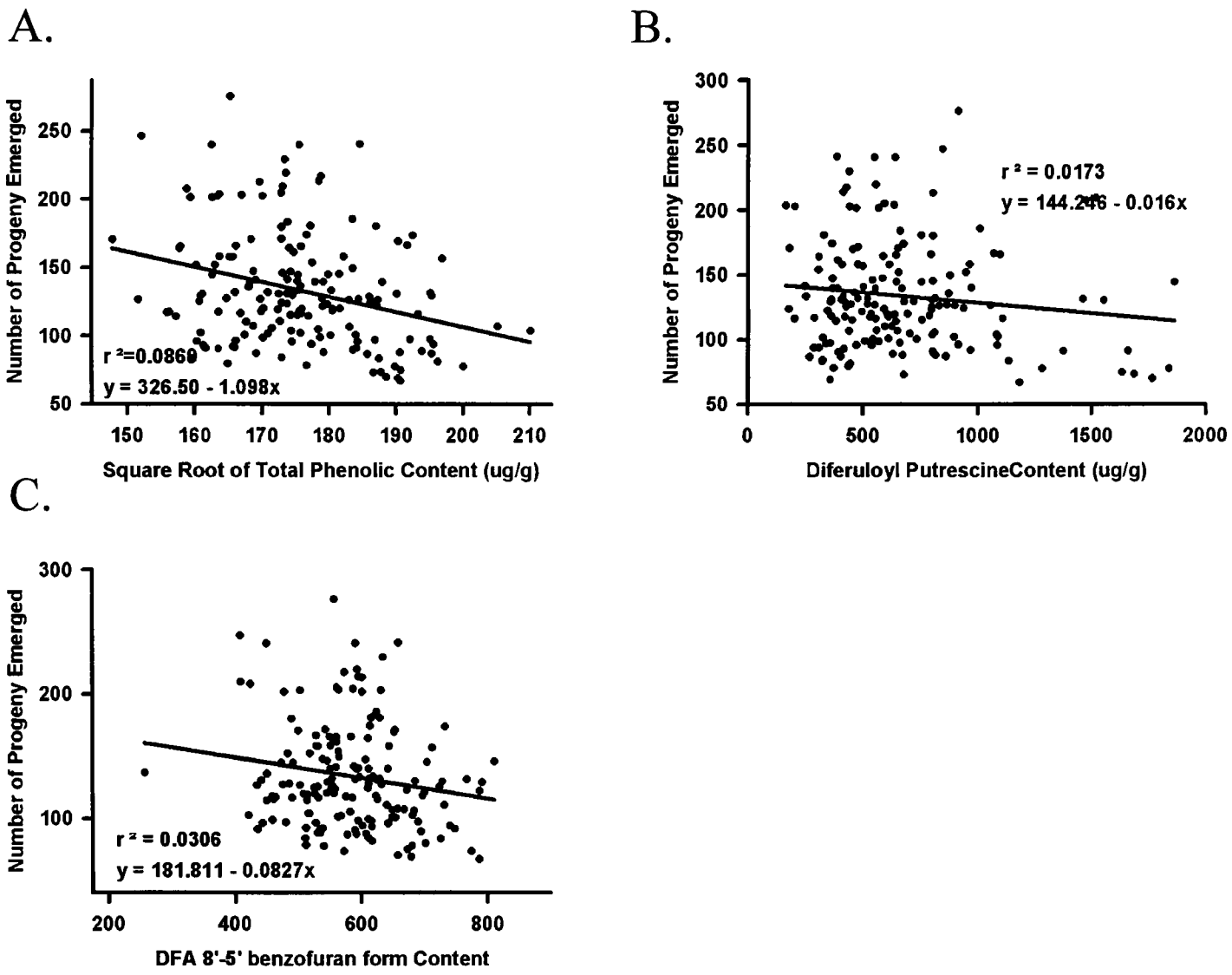


Figure 3.8: Simple regression models for main factors appearing in multiple regression model of the number of progeny emerged in season TL2000B. Shown are A: total phenolic content; B: diferuloyl putrescine content; and C: DFA 8'-5' benzofuran form content. See Table 7 for complete model: model $r^2 = 0.1287$, $p = 0.00021$. $n = 163$.

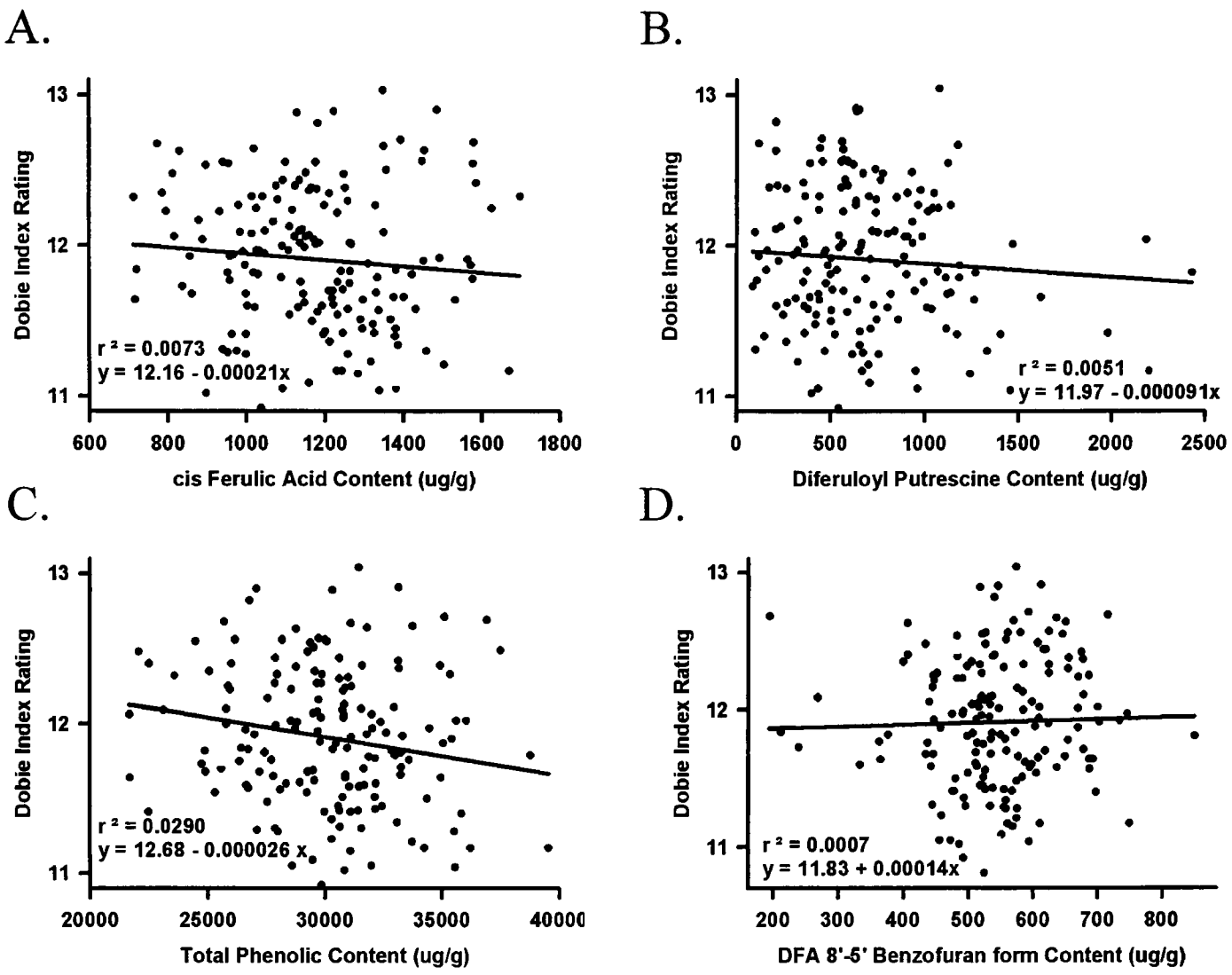


Figure 3.9: Simple regression models for main factors appearing in multiple regression model of the Dobie Index rating in season TL2001A. Shown are A: cis ferulic acid content; B: diferuloyl putrescine content; C: total phenolic content; and D: DFA 8'-5' benzofuran form content. See Table 7 for complete model: model $r^2 = 0.1631$, $p = 0.00023$. $n = 163$.

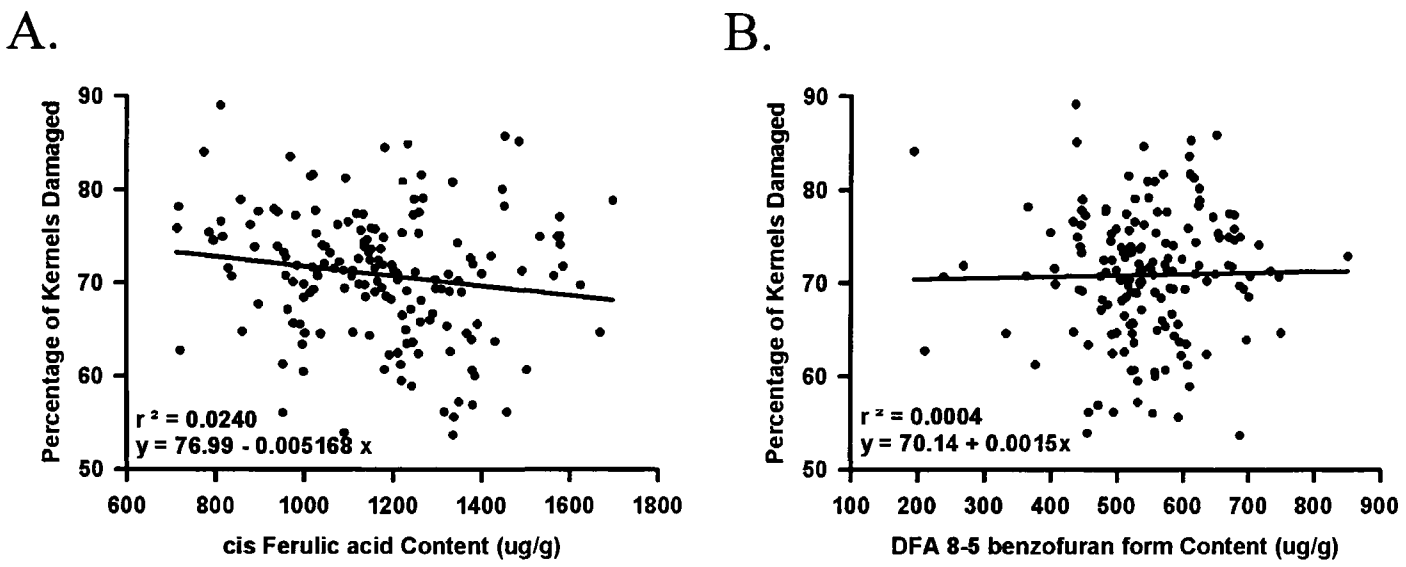


Figure 3.10: Simple regression models for main phytochemical factors appearing in multiple regression model of the percentage of damaged kernels in season TL2001A. Shown are A: cis ferulic acid content; and B: DFA 8'-5' benzofuran form content. See Table 7 for complete model: model $r^2 = 0.0851$, $p = 0.00685$. $n = 163$.

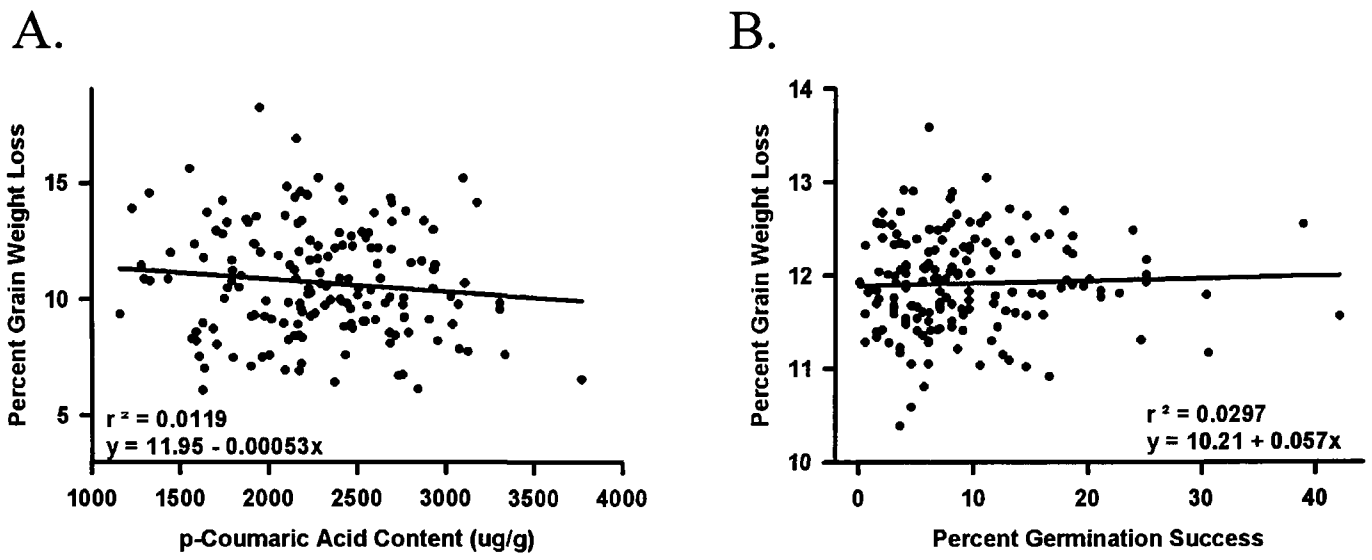


Figure 3.11: Simple regression models for main factors appearing in multiple regression model of the percent grain weight loss in season TL2001A. Shown are A: p-coumaric acid content; and B: percent germination success. See Table 7 for complete model: model $r^2 = 0.0922$, $p = 0.01517$. $n = 163$.

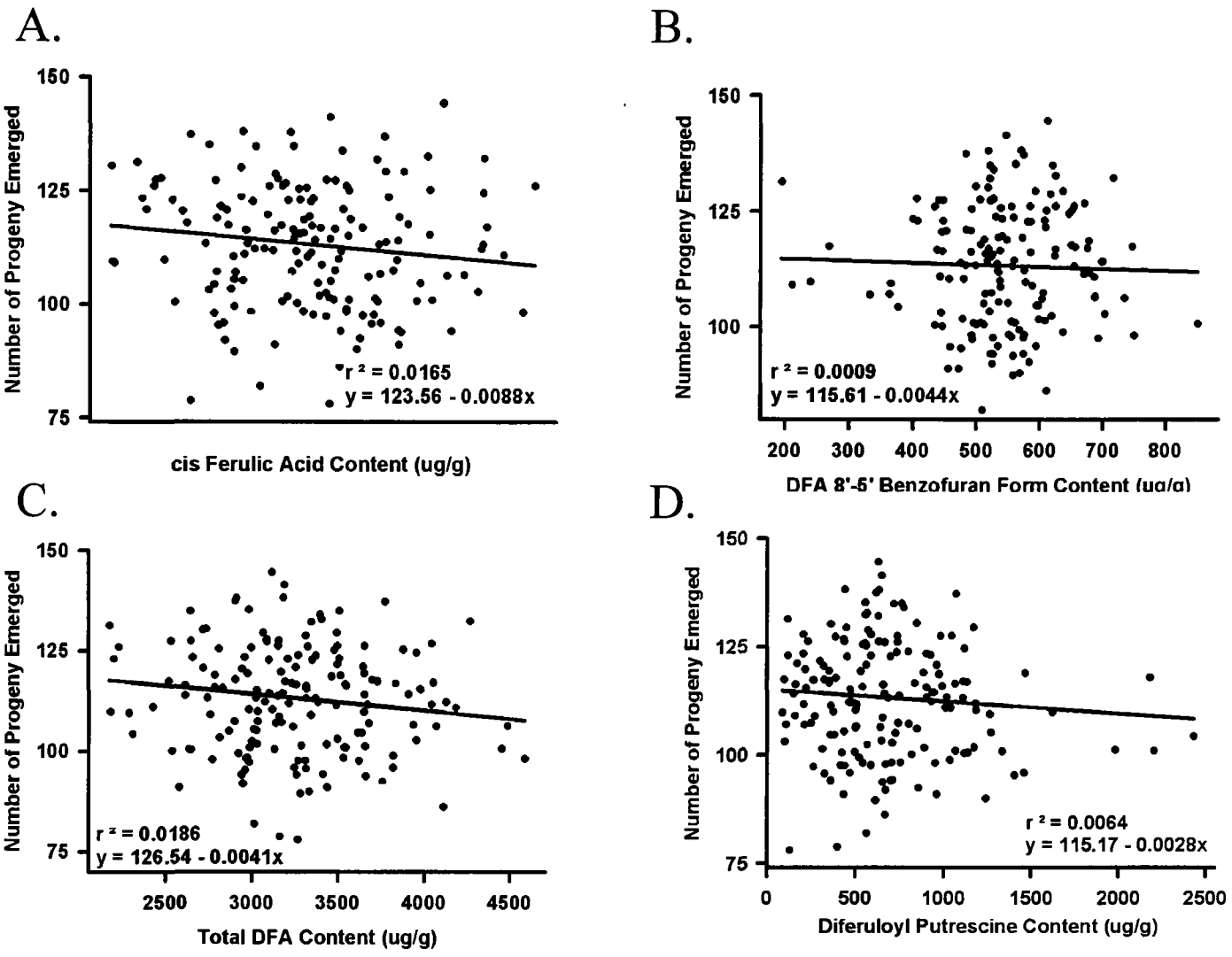


Figure 3.12: Simple regression models for main phytochemical factors appearing in multiple regression model of the number of progeny emerged in season TL2001A. Shown are A: cis ferulic acid content; B: DFA 8'-5' benzofuran form content; C: total DFA content; and D: diferuloyl putrescine content. See Table 7 for complete model: model $r^2 = 0.1754$, $p = 0.00009$. $n = 163$.

The major trend from the first to the second season is an overall decrease in goodness of fit both in the simple correlations and in the multiple regression models. This difference is also seen in the correlations of the insect bioassay parameters between the two seasons (Table 3.10) and in the correlations of the phytochemistry between the two seasons (Table 3.11). The auto-correlation between seasons is much lower than might be expected; for many of the parameters there is a similarity of less than 50%. This decrease in modelling power from one season to the next may be partially explained by the lower range of variation of the traits in the second season as compared to the first (refer to Tables 3.6 and 3.7). When analyzing large data sets, especially if the range of total variation present is relatively small, the spread of data decreases and the data cloud increases in density and become inherently less representable by a single line. This is, in fact, the problem inherent in QTL studies. The molecular work necessary for the mapping necessitates that the resistant, "high" and susceptible, "low" parents have a similar genetic background, which limits the range of variation possible in the trait of interest. This relatively low range of variation in the trait of interest means that if the entire QTL population is examined, the tightness of the relationship between the factors will decrease. Examining only the individuals within the population with extreme values may alleviate this problem, but doing so decreases the ability of the technique to detect additive QTLs or epistasis amongst the QTLs. Figure 3.13 shows an example of the improvement in the r^2 value of a regression, if only the extreme high and low individuals in the population are analyzed; in this example the top and bottom twenty-five ranked by Dobie index are used.

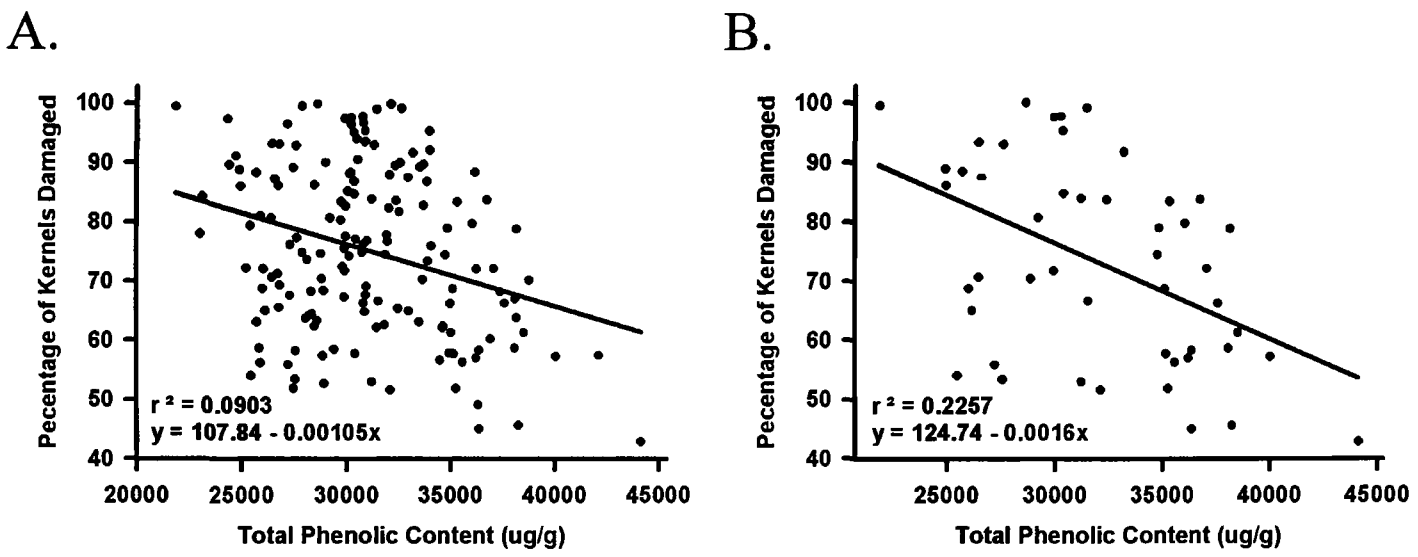


Figure 3.13: Two models showing effect of total phenolic content on the percentage of damaged kernels in season TL2000B. Contrast model fit of regression of the whole mapping population (A; n = 163) and regression using extreme values of the mapping population (B; n = 50). Model fit increases by more than a factor of two. Extreme values are chosen using the twenty-five families rated highest and the twenty-five rated lowest on the Dobie Index.

The root of this decrease in variation in the measured traits in the second season, and also the low auto-correlation between seasons may be attributed to an genotype x environment interaction. Table 3.14 summarizes the weather statistics during the two growing seasons. While there was no difference in the daily high temperature between the two seasons, the winter crop experiences significantly cooler nights, much drier weather and a much lower total number of growing degree days. This difference in the environmental conditions of the two seasons may have obscured the relationship between the phytochemistry and the insect resistance.

Despite this possible genotype x environment interaction, it is nevertheless clear that the content of cell wall bound phenylpropanoids show a strong positive correlation with the resistance to *S. zeamais*. This supports the findings of Serratos *et al.* (1987), Classen *et al.* (1990), and Arnason *et al.*(1992). The current work has attempted to refine this relationship by examining the different dehydrodiferulic acid forms as well as the total DFA content and the total bound phenolic content. There is no evidence to suggest that there is an overwhelming difference in the effect of the different DFA forms. It is the total DFA and total bound phenolic content that is the most important to the insect resistance.

This work does suggest that a second mode of resistance is present in this population. The content of diferuloyl putrescine may have a role in the resistance to *S. zeamais* feeding, shown in the correlational analysis (Table 3.8) and as demonstrated by the inclusion of diferuloyl putrescine content in many of the regressions (Table 3.9). Furthermore, this compound shows a relative lack of correlation with the cell-wall bound phenylpropanoids (Tables 3.12 and 3.13). These two trends combine to indicate that diferuloyl putrescine may be an alternate or additive source of resistance to *S. zeamais*, distinct in its mode of action from the fortification action of the ferulic acid dehydro-dimers.

Table 3.14: Weather statistics from Tlaltizapan for season TL2000B and TL2001A. Means and totals of the daily environmental readings are presented; means are presented with their standard deviation. The TL2000B season was planted on June 15, 2001; TL2001A was planted November 10, 2001.

	Mean Daily Tmax (°C)	Mean Daily Tmin (°C)	Length of Season (days)	Total Growing Degree Days	Total Rain (mm)
TL2000B	31.4 ± 1.7	16.9 ± 1.6	108	1528.7	339.0
TL2001A	30.8 ± 1.6	11.3 ± 2.9	111	1256.4	7.9

QTL Mapping

The results of the QTL mapping are summarized in Tables 3.15 and 3.16 as well as graphically in Figure 3.14. For each of the 126 QTLs found, the location on the maize chromosomes is described and the following data is given: the Likelihood ratio (LR); the constancy of the QTL across the two seasons or environments (QTL x E); and the amount of phenotypic variation explained by that QTL. Each of these measures provides information on the value the QTL for a breeding program.

The LR is related to the LOD score ($\log_{10}$ of the likelihood odds ratio) which is a standard measure for the likelihood that the QTL found is "real." The LOD score may be seen as analogous to a p-value. For example, an LOD score of 3.0 indicates that the probability of this locus of the chromosome being associated with the given trait is one thousand times greater than that of a locus chosen at random. In this study, the critical value of the LR for a LOD score of 3.0 is 13.81, and an LR of 11.51 indicates a LOD score of 2.5. Of the 126 QTLs found, 60 have an LOD score of 3.0 or greater. The LR was used as the critical value for identifying QTLs in this study and no loci with an LR of less than 11.51 was taken as a QTL.

The QTL x E measure indicates the stability of the QTLs over environments. A low QTL x E interaction indicates that the expression of the involved gene is largely independent of the environment. OF the 126 QTLs described in this study, 42 have a QTL x E value of 10 or greater. This indicates that there is a large environmental factor involved in the expression of the genes at these QTLs. This has a large implication for a potential breeding program; any resistance improvement based on these QTLs may not be stable over time or across different environments.

The phytochemical trait with the highest amount of phenotypic variation explained was the trans-Ferulic acid concentration, with just less than half the variation explained by the ten QTLs found (Table 3.15). The phytochemical trait with the least total phenotypic variation explained is the concentration of the 5'-5' form of DFA, with six QTLs describing just over one-quarter of the variation in the trait. The other phytochemical traits had between 30 and 45% of their total variation described. On chromosomes one, two, four, six, eight, and nine there are areas of overlapping or closely-neighbouring QTLs for different forms of DFA. These areas suggest a common genetic basis for the production of the different forms of the ferulic acid dehydro-dimers. Of especial interest are the regions on chromosomes six and eight where QTLs for the 8'-5' benzofuran, 8-O-4, and 5'-5' forms of DFA as well as for total DFA overlap completely. The regions on chromosome one, two and nine where the two types of the 8'-5' DFA co-occur imply a very close relationship between these two forms.

The insect resistance markers shown in Table 3.16 have less of their total variation explained by the QTLs found than the phytochemical traits. Both the total variation in the Dobie Index and in the grain hardness is over 25% described by eight and seven QTLs, respectively. Percent weight loss of the grain has just under one-quarter of its total variability explained by seven QTLs. The number of progeny and the percent of kernels damaged have only four and two significant QTLs, which explain about 12 and 17% of the phenotypic variation respectively.

Table 3.15: Quantitative Trait Loci Interval Data for phytochemical markers. From phenotypic data of 163 RIL in the 5x1 cross of CML 290 and Muneng 8128, TL2000B and TL2001A, Tlaltizapan, Mexico. * Denotes a LOD > 2.5, and ** denotes a LOD > 3.0.

Trait	QTL Location			Likelihood Ratio (LR)	QTL x E Interaction	Phenotypic Variance Explained (%)
	Chr	cM	Marker			
p-Coumaric Acid	1	197	13	16.32**	15.36	0.38
	1	234	16	13.44*	4.48	7.71
	3	205	12	14.60**	5.32	6.05
	4	85	4	12.70*	10.83	5.40
	6	66	5	11.60*	0.73	3.57
	7	19	3	14.00**	5.51	5.34
	8	58	4	11.87*	2.06	5.38
	9	177	14	11.96*	3.77	11.58
					Total	39.71
trans-Ferulic Acid	2	258	18	15.99**	14.27	0.87
	3	22	2	16.34**	0.54	4.46
	3	75	6	12.14*	10.88	0.51
	3	205	12	12.31*	4.03	8.89
	3	238	15	12.98*	8.98	5.74
	3	288	17	12.14*	4.24	3.43
	5	104	8	12.99*	4.93	2.29
	6	85	6	11.64*	3.59	9.34
	7	142	10	16.18**	2.04	6.85
	10	125	6	20.27**	5.90	13.76
					Total	47.25
cis-Ferulic Acid	1	44	3	13.15*	10.39	0.87
	1	232	16	20.68**	17.72	4.57
	2	60	3	13.06*	1.87	3.81
	3	204	12	13.49*	7.92	4.77
	4	97	4	11.99*	5.54	2.03
	6	140	9	16.40**	15.99	11.46
	8	27	2	12.48*	10.07	0.28
	8	72	5	15.84**	13.56	1.22
	9	28	3	15.35**	8.90	4.52
	10	41	2	13.93**	4.41	2.57
					Total	32.13
DFA 8'-5'	1	168	11	11.62*	8.47	2.72
	1	230	16	19.33**	14.72	6.49
	1	270	18	16.97**	8.99	3.71

DFA 8'5' cont'd	2	224	16	12.43*	10.02	4.96
	4	245	12	11.61*	9.99	3.38
	4	283	14	11.97*	8.04	7.84
	8	21	2	13.82**	10.45	0.30
	8	67	5	20.58**	6.18	5.52
	8	195	14	12.57*	10.10	3.16
	9	14	2	15.40**	10.20	4.12
					Total	33.87
DFA 5'-5'	2	1	1	12.34*	2.66	5.74
	2	139	11	18.74**	2.72	7.01
	3	259	16	11.66*	0.36	6.72
	6	33	3	11.53*	2.05	1.47
	6	146	9	14.52**	13.71	4.20
	8	72	6	14.38**	7.44	3.77
					Total	25.88
DFA 8-O-4	1	233	16	16.54**	9.66	4.30
	1	265	18	13.42*	7.13	1.80
	3	283	17	16.09**	0.89	7.20
	4	248	12	14.00*	13.22	1.98
	5	24	2	12.36*	3.14	1.90
	5	89	6	13.34*	7.68	0.78
	6	94	6	11.99*	1.57	6.73
	6	140	9	13.65*	12.62	2.57
	6	175	10	12.14*	11.80	2.26
	8	73	6	20.59**	11.78	5.02
					Total	32.41
DFA 8'-5' benzofuran	1	182	12	16.48**	0.88	1.41
	2	33	2	14.07**	0.66	5.85
	2	219	16	17.14**	9.51	8.12
	3	39	3	12.17*	11.81	2.89
	4	215	11	12.84*	0.40	2.98
	6	49	4	12.12*	1.25	8.67
	6	73	5	16.65**	4.30	11.00
	6	147	9	11.55*	9.74	5.21
	8	74	6	21.77**	6.03	4.34
	9	9	2	13.51*	5.50	3.44
					Total	40.83
Soluble Phenolic X	1	264	18	21.26**	18.27	8.80
	3	0	1	17.01**	7.17	0.95
	3	39	3	11.92*	9.25	2.35
	3	79	6	13.59*	10.97	1.86
	3	131	10	16.84**	14.30	1.53

Soluble Phenolic X cont'd	3	225	13	16.10**	15.67	2.12
	3	263	16	15.61**	11.28	2.62
	5	112	8	12.40*	8.58	7.72
	6	161	10	38.46**	36.86	4.31
	8	53	4	11.60*	7.38	4.20
	10	132	7	21.50**	21.80	10.16
					Total	43.49
Diferuloyl putrescine	1	254	17	14.21**	10.07	0.52
	2	95	6	13.41*	12.49	4.48
	4	283	14	14.92**	3.75	3.35
	6	20	3	16.38**	7.81	7.41
	7	141	10	21.20**	1.24	7.61
	7	179	14	15.54**	4.34	8.05
	9	120	9	12.80*	5.93	5.78
	10	150	9	27.43**	26.62	7.19
	10	140	8	23.20**	19.14	4.88
					Total	34.46
Total DFAs	1	233	16	13.37*	8.73	4.53
	2	18	2	14.36**	0.84	6.72
	2	221	16	11.98*	6.55	6.01
	3	260	16	12.40*	0.59	5.25
	6	89	6	11.57*	1.68	8.08
	6	144	9	15.06**	14.55	5.11
	8	73	6	21.78**	9.99	5.08
					Total	33.33
Total Phenolics	2	76	5	11.62*	0.27	1.88
	3	18	2	13.55*	1.23	5.69
	3	275	17	19.13**	2.21	9.62
	4	283	14	13.04*	3.12	2.84
	6	88	6	13.42*	3.12	10.96
	7	141	10	14.11**	2.50	5.78
	10	126	7	13.39*	3.18	10.33
					Total	41.82

Table 3.16: Quantitative Trait Loci Interval Data for Insect Resistance Markers. From phenotypic data of 163 RIL in the 5x1 cross of CML 290 and Muneng 8128, TL2000B and TL2001A, Tlaltizapan, Mexico. * Denotes a LOD > 2.5, and ** denotes a LOD > 3.0.

Trait	QTL Location			Likelihood Ratio (LR)	QTL x E Interaction	Phenotypic Variance Explained (%)
	Chr	cM	Marker			
Percent of Kernels Damaged	5	69	5	16.68**	11.87	7.30
	6	161	10	21.65**	12.95	9.98
					Total	17.17
Dobie Index	1	81	5	17.31**	0.87	2.74
	2	170	13	12.06*	3.86	3.33
	3	149	11	16.53**	5.35	6.25
	3	216	13	13.34*	12.20	6.20
	4	275	13	15.25**	12.81	7.91
	7	45	4	16.32**	16.20	4.85
	7	140	10	14.64**	8.34	3.76
	9	120	9	18.53**	15.07	2.74
					Total	26.77
Percent Weight Loss of Grain	2	236	17	12.47*	4.41	5.04
	4	57	3	13.35*	9.23	2.18
	6	36	3	12.52*	3.65	4.02
	6	174	10	12.74*	5.67	2.58
	7	46	4	20.33**	17.17	9.93
	9	79	5	13.01*	12.74	1.41
	9	178	14	11.86*	8.02	4.70
					Total	24.12
Number of Progeny Emerged	1	245	17	11.65*	8.85	5.61
	2	169	13	11.98*	5.65	6.60
	9	118	9	13.48*	7.88	4.40
	10	98	5	12.84*	9.14	7.76
					Total	11.84
Grain Hardness (Peak Puncture Force)	1	41	3	18.82**	11.14	7.61
	1	151	10	16.78**	5.88	7.40
	4	0	1	17.54**	13.35	1.64
	5	146	11	11.95*	3.88	3.50
	7	72	6	12.10*	9.68	2.75
	7	179	14	14.36**	9.74	7.53
	10	133	7	11.79*	7.88	4.90
					Total	28.65

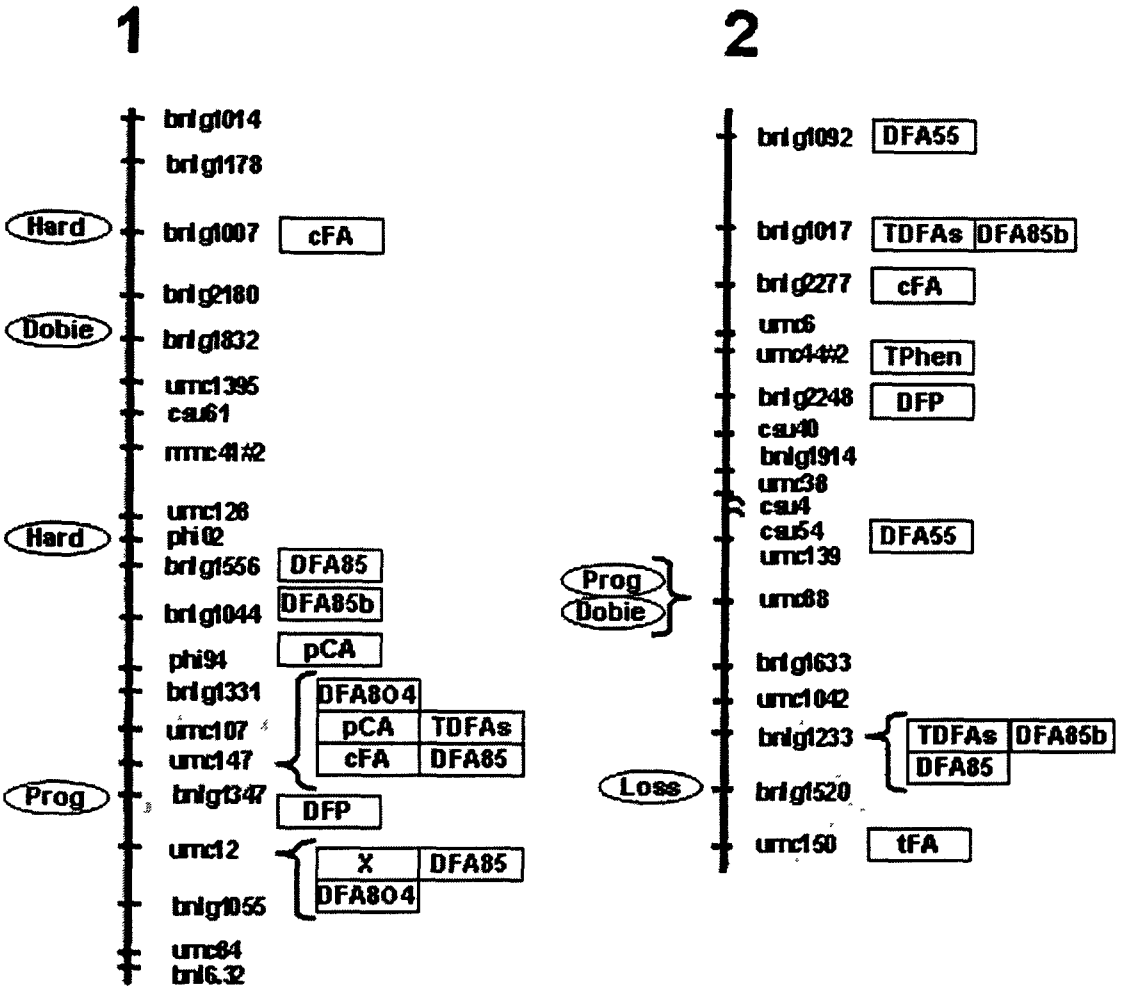


Figure 3.14a: QTL map (chromosome 1 and 2) showing *S. zeamais* bioassay results on the right of the chromosome and the phytochemical markers on the left. Shown are: Percent of kernels damaged (Dmg); Dobie index (Dobie); Number of progeny emerged (Prog), Peak kernel puncture force (Hard); p-coumaric acid (pCA); trans-ferulic acid (tFA); cis-ferulic acid (cFA); various DFA forms (DFA85, DFA55, DFA8O4, DFA85b); soluble phenolic X (X); diferuloyl putrescine (DFP); total DFA content (TDFAs); and, total cell wall bound phenylpropanoid content (TPhen). Areas where QTLs for insect resistance coincide with QTLs for phytochemical markers are highlighted.

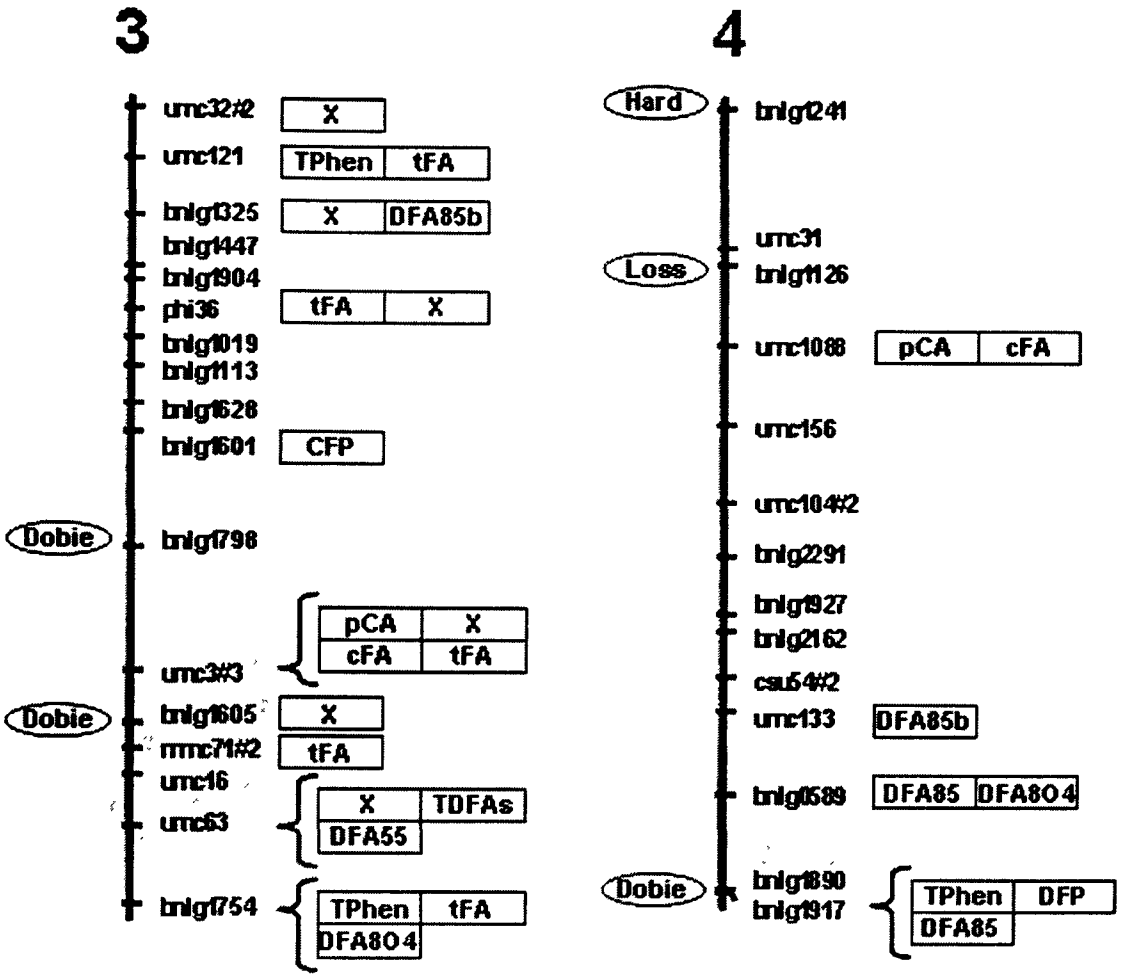


Figure 3.14b: QTL map (chromosome 3 and 4) showing *S. zeamais* bioassay results on the right of the chromosome and the phytochemical markers on the left. Shown are: Percent of kernels damaged (Dmg); Dobie index (Dobie); Number of progeny emerged (Prog), Peak kernel puncture force (Hard); p-coumaric acid (pCA); trans-ferulic acid (tFA); cis-ferulic acid (cFA); various DFA forms (DFA85, DFA55, DFA8O4, DFA85b); soluble phenolic X (X); diferuloyl putrescine (DFP); total DFA content (TDFAs); and, total cell wall bound phenylpropanoid content (TPhen). Areas where QTLs for insect resistance coincide with QTLs for phytochemical markers are highlighted.

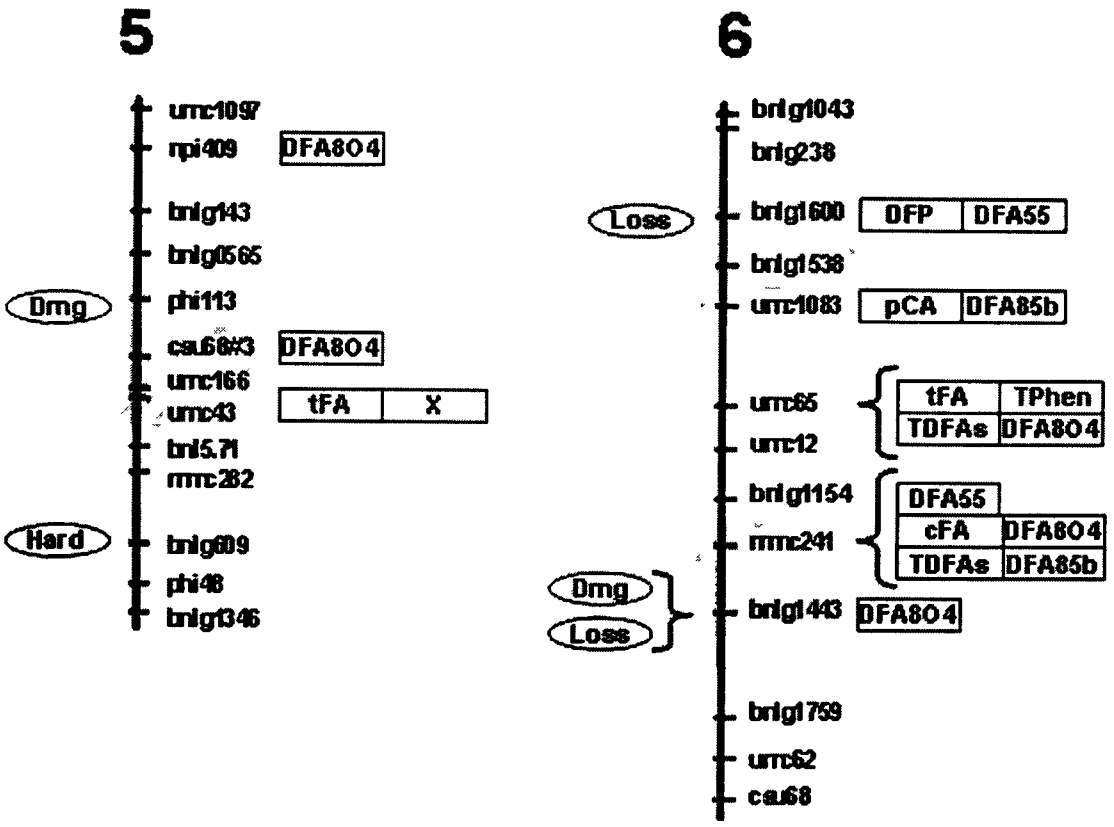


Figure 3.14c: QTL map (chromosome 5 and 6) showing *S. zeamais* bioassay results on the right of the chromosome and the phytochemical markers on the left. Shown are: Percent of kernels damaged (Dmg); Dobie index (Dobie); Number of progeny emerged (Prog), Peak kernel puncture force (Hard); p-coumaric acid (pCA); trans-ferulic acid (tFA); cis-ferulic acid (cFA); various DFA forms (DFA85, DFA55, DFA8O4, DFA85b); soluble phenolic X (X); diferuloyl putrescine (DFP); total DFA content (TDFAs); and, total cell wall bound phenylpropanoid content (TPhen). Areas where QTLs for insect resistance coincide with QTLs for phytochemical markers are highlighted.

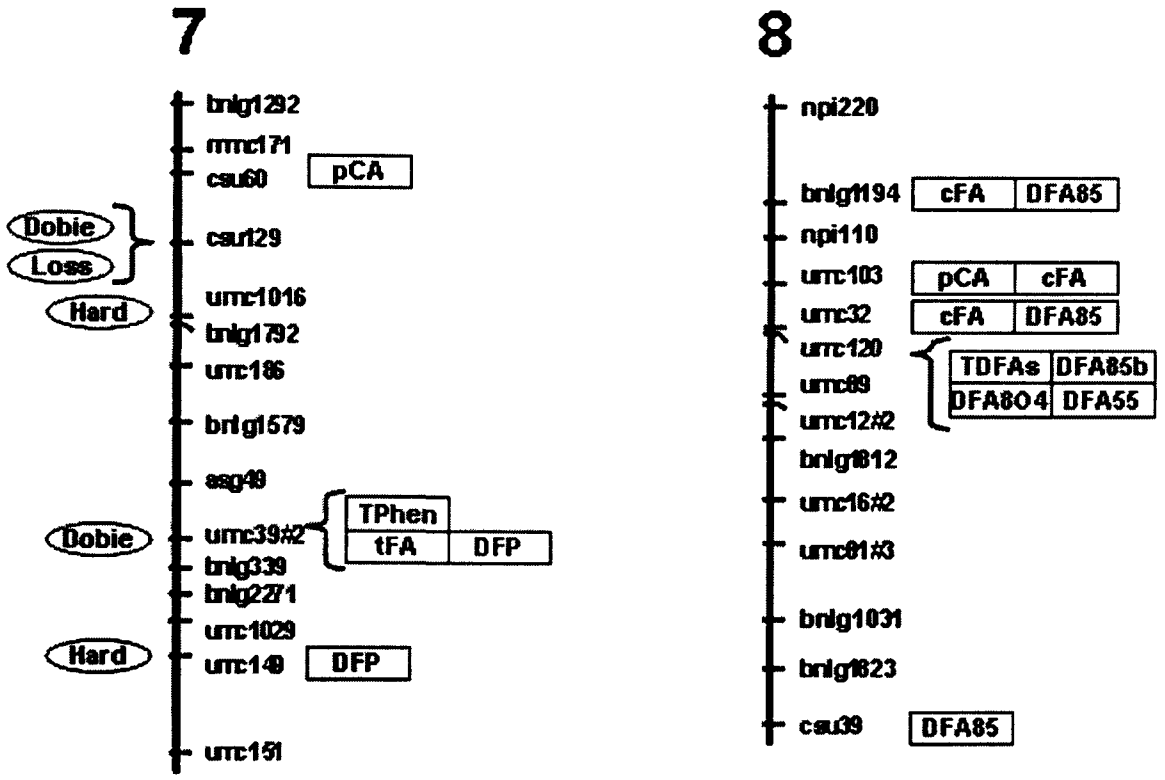


Figure 3.14d: QTL map (chromosome 7 and 8) showing *S. zeamais* bioassay results on the right of the chromosome and the phytochemical markers on the left. Shown are: Percent of kernels damaged (Dmg); Dobie index (Dobie); Number of progeny emerged (Prog), Peak kernel puncture force (Hard); p-coumaric acid (pCA); trans-ferulic acid (tFA); cis-ferulic acid (cFA); various DFA forms (DFA85, DFA55, DFA8O4, DFA85b); soluble phenolic X (X); diferuloyl putrescine (DFP); total DFA content (TDFAs); and, total cell wall bound phenylpropanoid content (TPhen). Areas where QTLs for insect resistance coincide with QTLs for phytochemical markers are highlighted.

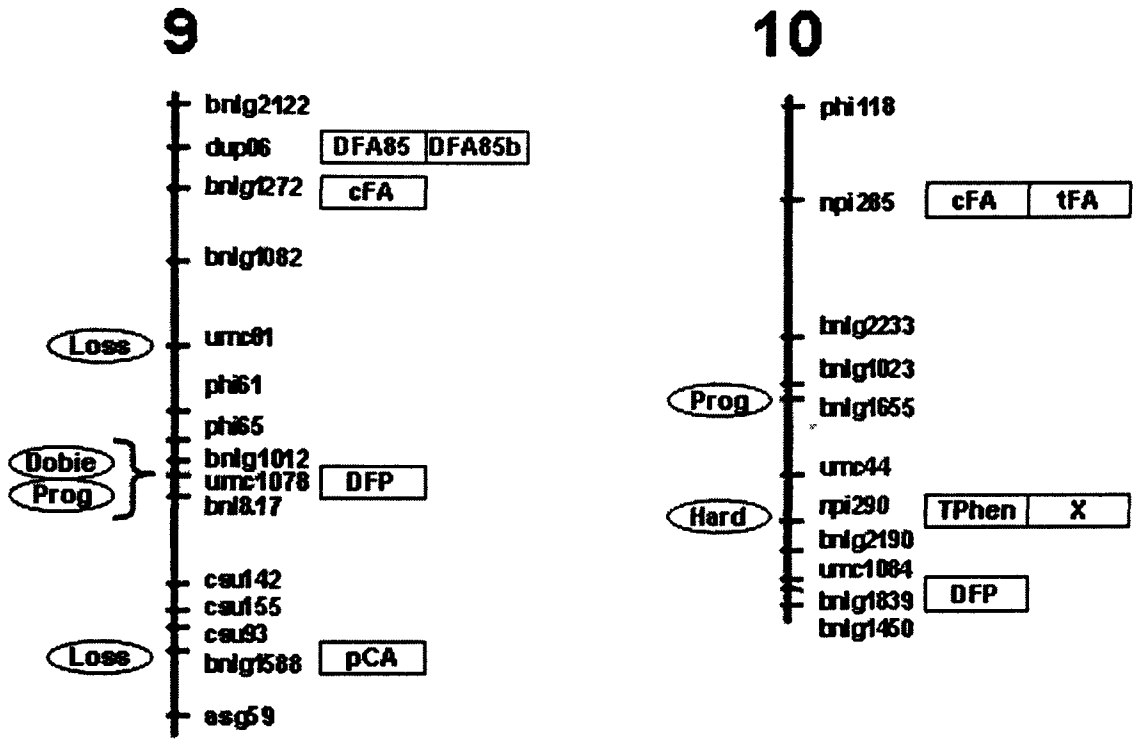


Figure 3.14e: QTL map (chromosome 9 and 10) showing *S zeamais* bioassay results on the right of the chromosome and the phytochemical markers on the left. Shown are: Percent of kernels damaged (Dmg); Dobie index (Dobie); Number of progeny emerged (Prog), Peak kernel puncture force (Hard); p-coumaric acid (pCA); trans-ferulic acid (tFA); cis-ferulic acid (cFA); various DFA forms (DFA85, DFA55, DFA8O4, DFA85b); soluble phenolic X (X); diferuloyl putrescine (DFP); total DFA content (TDFAs); and, total cell wall bound phenylpropanoid content (TPhen). Areas where QTLs for insect resistance coincide with QTLs for phytochemical markers are highlighted.

The fact that the phenotypic variation in the chemical traits is more completely described by the QTLs than that of the insect resistance traits is not completely unexpected. The production of phytochemicals is more directly under direct genetic control than the ability of the grain to resist insect attack. There is more random variation in the actual resistance of the grain to insects than there is in the abundance of phytochemicals simply because there are many more uncontrollable variables in insect bioassay testing, even under controlled laboratory conditions, than there are in extracting and measuring the phytochemical content of plant tissue.

The graphic representation of the QTL map (Figure 3.14) provides an essential visualization of these data. There are ten regions, one on each chromosome save number eight, and two on chromosome six, where there is a co-occurrence or overlap of the QTLs for insect resistance markers and for phytochemical markers. These regions show an overlap in the QTLs for the concentration in the cell wall bound phenylpropanoids and the insect resistance markers. This is evidence of a physical overlap of the regions of genetic control for both phytochemical content and insect resistance. This, in combination with the correlational evidence provided earlier in this chapter, provides sufficient justification to presume a causal relationship between the cell wall bound phenylpropanoids and resistance to *S. zeamais*. That is, sufficient evidence has been provided to conclude that the cell wall bound phenylpropanoids are mediating at least a portion of the post-harvest resistance.

There is no single phenylpropanoid form that was predominantly linked to the insect resistance markers over all others. Most of the different phenylpropanoid species occur three times in the ten areas of interest; trans-ferulic acid and total DFAs each occur four times, indicating that these two measures may be good phytochemical markers of resistance to *S. zeamais*. Diferuloyl putrescine also occurs four times in the regions of interest, and soluble

phenolic X, the putative coumaroyl-feruloyl putrescine occurs five times; this supports the correlative findings earlier in this chapter that these hydroxycinnamic acid amide di-conjugates may mediate some resistance against the maize weevil.

Although all of the ten regions are of interest and further fine mapping of these areas may be useful in finding more information on the phytochemical mediation of post harvest resistance, there are some regions that may be classified as the most interesting for future work. The regions that are responsible for relatively large amounts of variation in the insect resistance measures can be rated highly important: the regions on chromosomes three and four explain 6.2 and 7.9% of the variation in the Dobie index respectively; the area on chromosome five and the second area on chromosome six are responsible for 7.3 and almost 10% of the variation in the percentage of kernels damaged; and the region on chromosome ten is responsible for 7.7% of the variation in the number of progeny emerged. Regions may also be rated as more important when more than one QTL for an insect resistance marker occurs in the area. These regions are: on chromosome six, where percent of kernels damaged and weight loss co-occur; on chromosome nine, where QTLs for the Dobie index and for the amount of progeny co-occur; and, on chromosome ten, where QTLs for number of progeny and grain hardness neighbour. Finally, the region on chromosome nine where the QTLs for the Dobie index rating and the amount of progeny overlap with the QTL for diferuloyl putrescine content is of great interest essentially because of the absence of QTLs for cell wall bound phenylpropanoids. Further investigation into this area may provide information on the role of this HAAC in post-harvest insect resistance.

Chapter IV: General Discussion and Conclusions

4.1 Main Conclusions

This thesis has contributed new information to the understanding of post harvest resistance of tropical maize grain to the maize weevil. In particular, the roles of the hydroxycinnamic acid amide conjugates and cell wall bound phenylpropanoids in post harvest resistance were elucidated.

The investigations on the HAACs confirmed that the majority of these compounds are located in the external tissues of the grain. Also, a range of variation of HAAC content in the Mexican landraces was established as a useful guide for the natural range in maize germplasm in general. Wheat disk feeding assays determined that the HAACs deterred feeding by *Sitophilus oryzae*, but not by *S. zeamais*. This indicated that the HAACs are effective defence compounds against generalist grain feeders, but not against the maize specialist, *S. zeamais*. Further lifecycle trials determined that feruloyl putrescine, at the levels found in the maize grain, increased the weight loss of the grain and the percentage of damaged grain in the second generation of weevils. These results indicate that the maize weevil is adapted to the presence of feruloyl putrescine at the levels that are naturally occurring in maize.

Work with the CIMMYT material selected for variable levels of post-harvest resistance refined our understanding of the relationship between *S. zeamais* resistance and the grain outer tissue content of cell wall bound phenylpropanoids. The results of the analysis of material from two growing seasons implicated the dehydro-dimers of ferulic acid in both the grain hardness and the insect resistance. In both seasons the 8'-5'form and the 8'-5' benzofuran form of DFA and the total DFA content were significantly correlated with the percentage of kernels damaged by *S.*

zeamais. Multiple regression models were constructed to explain 97% and 82% of the variation (based on the r^2 values) in the percentage of kernels damaged in the TL98A and the TL99B seasons respectively.

In addition, the QTL analysis showed that the QTLs for the insect resistance mapped to chromosome locations corresponding to QTLs for phytochemical traits. This result provides support for a causal relationship between phytochemical traits and resistance to *S. zeamais*; it indicates, at a given locus, the contribution to the control of the expression of the phytochemical is also contributing to resistance to *S. zeamais*.

The work with the QTL mapping population also provided additional information on the role of the di-hydroxycinnamic acid amide conjugates. An unknown compound in the samples was identified as coumaroyl-feruloyl putrescine, based on the mass of the compound, the isotopic variation around the major mass spectrum peak, and the similarity of the UV absorbance spectrum to that of diferuloyl putrescine. Contrary to the earlier bioassay findings in chapter 2, the content of diferuloyl putrescine and the putative coumaroyl-feruloyl putrescine is positively correlated with maize weevil resistance in the QTL mapping population, indicating that the HAACs may indeed have a role as defence compounds in maize grain against *Sitophilus zeamais*.

4.2 Discussion

Identification of Phenolic X

The tentative structure deduced for the unknown phenolic X was the same as that suggested by Moreau *et al.* (2001). This group proposed the same structure, calling it *p*-coumaroyl-feruloyl putrescine (CFP), this was inferred by the mass of the compound and a mass spectrum with a fragmentation pattern consistent with the identification. They produced fragments with masses corresponding to the cleavage of a ferulic acid group and a coumaric acid group, as shown in Figure 4.1. Moreau *et al.* found that the highest yield of these compounds were 3.28 mg/g of maize bran for diferuloyl putrescine and 1.02 mg/g of maize bran for coumaroyl-feruloyl putrescine. They found that the maize bran content of coumaroyl-feruloyl putrescine was reliably one third that of diferuloyl putrescine. The highest amounts of these two compounds found in this study were 2.43 mg/g of outer grain tissue for diferuloyl putrescine and 1.03 mg/g of outer grain tissue for coumaroyl-feruloyl putrescine. The ratio of coumaroyl-feruloyl putrescine to diferuloyl putrescine in these analyses ranged from 0 to 1.47, averaging at 0.54 in TL2000B season and 0.64 in the TL2001A season. This indicates that the relative amounts of these two compounds found in maize are more variable than reported by Moreau *et al* (2001).

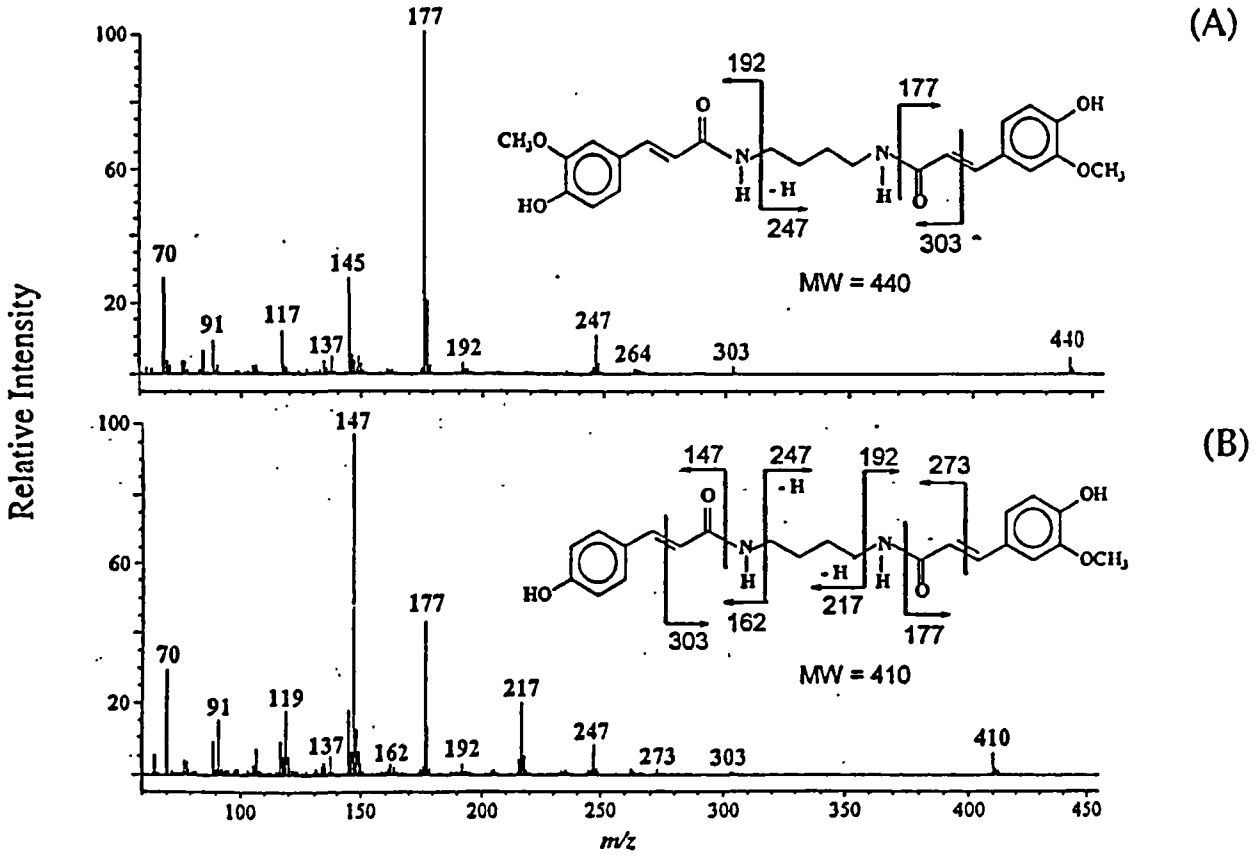


Figure 4.1: Mass spectra (electron impact) and proposed structures with fragmentation patterns of two unknown UV absorbing compounds found in corn bran extracts. (A) DFP (diferuloyl putrescine) and (B) CFP (coumaroyl-feruloyl putrescine). From Moreau *et al.* (2001).

Role of HAACs

The roles defined for the HAACs in chapter 2 and chapter 3 are seemingly at odds with one another. As previously stated, the experiments reported in chapter 2 found that the HAACs did not deter feeding by *S. zeamais* and lifecycle evidence demonstrated that low doses of feruloyl putrescine increased the damage done to the grain by second-generation weevils. The results of the third chapter show a strong correlation between the insect resistance and the presence of diferuloyl putrescine and coumaroyl-feruloyl putrescine, independent of the presence of elevated cell wall bound phenylpropanoid levels, as well as strong evidence from the QTL map to suggest a role in *S. zeamais* resistance by diferuloyl putrescine. There are three possible explanations to reconcile these opposite trends. The first is the fact that the di-hydroxycinnamic acid putrescine conjugates are more abundant than feruloyl putrescine: the difference shown may either simply be the effect of a higher dose of HAACs, or the putrescine di-conjugates may also be more toxic to *S. zeamais* than feruloyl putrescine. Secondly, rather than acting alone as in the bioassays, in maize the HAACs are present in combination: the effect seen may be an additive effect or may be a demonstration of the synergistic effect of the presence of two (or more) HAACs. Thirdly, the HAACs are not the only defence found in the maize grain, despite the lack of statistical correlation with the cell wall bound phenylpropanoids, the two defence mechanisms are by no means mutually exclusive: the effect seen may be due to the coupling of the mechanical resistance provided by the fortification by the phenylpropanoids and the toxic action of the HAACs – a naturally occurring example of pyramidal resistance.

A Comparison of QTL Maps for Cell-Wall Bound Phenylpropanoids

A similar QTL mapping project concerning defensive traits in leaves rather than grain was performed by Groh *et al.* (1998) in which resistance to Southwestern corn borer (*Diatraea grandiosella* Dyar) and Sugarcane borer (*Diatraea saccharalis* Fabricius) in tropical maize was mapped using two RIL mapping populations containing Ri3 x CML 139 and CML131 x CML67. Using the same mapping populations, Ramputh (2001) investigated the role of cell wall bound phenylpropanoids in resistance in young stem and leaf tissue. That QTL map may be directly compared to that of this work. Fewer QTLs were detected in Ramputh's work. This is most likely due to the fact that only RFLP markers were used to create the map by Ramputh (2001), which results in a less even distribution of markers across the chromosomes making it more difficult to obtain strong QTLs. Despite this difference and the difference in tissue examined, several similarities may be seen. There are some QTLs that occur in both maps: a QTL p-coumaric acid on chromosome six between 50 and 70 cM; one for ferulic acid occurring on chromosome five between 100 and 130 cM; and, a QTL for 8'-5' DFA on chromosome one between 200 and 230 cM.

In another study, Méchin *et al* (2001) found QTLs for in vitro cell wall digestibility on chromosome one at 174 and 250 cM, and on chromosome five at 90 cM. These QTLs correspond closely to several found for different DFA forms in this work and by Ramputh (2001). Cell wall bound phenylpropanoids, especially dehydro-dimers of ferulic acid have long been thought to decrease cell wall digestibility in maize (Saulnier and Thibault, 1999).

Even more interesting are areas that occur on both maps that are associated with phytochemically mediated resistance to both the post-harvest insects and to the stem borers of Ramputh (2001). These regions occur on chromosome five between 70 and 100 cM, on

chromosome six between 130 and 160 cM, and on chromosome nine between 90 and 120 cM. The similarities between these two maps may indicate that a breeding program for increased borer resistance may also increase post-harvest resistance, and vice-versa.

4.3 Future Work

A rationale has been presented to reconcile the lack of efficacy in bioassay shown in chapter 2 for the HAACS and the evidence for the association with *S. zeamais* resistance seen in chapter 3. More exhaustive bioassay testing could be performed, specifically a lifecycle assay with diferuloyl putrescine and short-term feeding assays with binary combinations of HAACs could improve the understanding of the role of these compounds in weevil resistance. Maize germplasm could be assessed to determine if breeding for diferuloyl putrescine and coumaroyl-feruloyl putrescine would provide a valuable second source of weevil resistance in grain storage.

Molecular markers from the ten chromosome regions of interest may be valuable for use in marker-assisted selection. Fine mapping would have to be performed before this would be a possibility. It may be possible for future work to determine the identity of genes responsible for the insect resistance seen at these chromosome regions. Such an investigation may yield genes controlling phenolic biosynthesis or cell wall development. Of the ten areas, the ones that are especially interesting for future work were explicitly defined in chapter three. These areas may be a basis for future marker assisted selection for accelerated crop improvement programs. The region on chromosome nine may also provide new information on a putative role of diferuloyl putrescine, and possibly other HAACs, as a new source of post-harvest resistance to *S. zeamais*.

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Appendices

Presented in the appendices are the complete data sets used in the analyses in Chapter III.

Appendix 1 contains the complete phytochemistry and insect bioassay data for season 1998A, seen summarised in Table 3.1, and the phytochemistry data for season 1999B, seen summarised in Table 3.2.

Appendix 2 contains the phytochemical and insect bioassay data for the QTL mapping population for seasons TL2000B and TL2001A, which was summarised in Tables 3.6 and 3.7.

Appendix 1. Complete data set of CIMMYT Resistant populations.

1998A

DESCRIPTION	Fiber	p-CA	Trans FA	Cis FA	5-5 DFA	8-0-4 DFA	8-5 DFA	8-5 DFA Be	Total DFA	Total Phenol	% Damage	% N	FORCE
Sinaloa 35	419.40	5188.8	30697.6	786.4	2552.0	3325.6	813.6	2827.2	9518.4	46.1912	0.0	1.82	21.54
Yucatan 7	347.00	3323.2	33305.6	658.4	2081.6	2585.6	703.2	1976.0	7346.4	44.6336	1.9	1.97	
1780 Ejura	409.80	3033.6	33607.2	1423.2	2456.8	2770.4	690.4	2107.2	8024.8	46.0888	8.9	1.77	16.99
1784 LGB	363.00	2617.6	35660.8	673.6	2473.6	2704.8	560.8	2128.0	7867.2	46.8192	6.6	1.78	19.68
P84 x P47	372.20	2234.4	33948.0	794.4	2339.2	2436.0	588.8	1926.4	7290.4	44.2672	10.0	1.78	20.44
P47 x P84	434.80	3040.8	26378.4	708.8	1764.8	2037.6	665.6	1545.6	6013.6	36.1416	25.6	1.36	17.17
CML 244 x 349	405.40	2068.0	29003.2	822.4	1492.0	1511.2	332.8	1046.4	4382.4	36.2760	78.8	1.48	5.95

Appendix 1. Complete data set of CIMMYT Resistant populations (cont'd)

1999B

DESCRIPTION	Trans p-CA	Trans FA	Cis FA	5-5 DFA	8-0-4 DFA	8-5 DFA	8-5 DFA Be	Total DFA	Total Phenolic
Sinaloa 35	6274.7	23419.7	1021.6	2034.0	2643.2	996.6	1501.6	7175.4	37.9
St. Error	718.7	1334.6	33.7	9.5	59.3	9.1	24.1	83.8	2.2
Yucatan 7	2741.1	23486.4	1150.5	2195.2	1850.4	991.2	1068.6	6105.4	33.4834
St. Error	314.0	1338.4	38.0	10.3	41.5	9.0	17.1	71.3	1.9
1780 Ejura	3397.0	25101.1	1448.0	1838.3	1809.6	1414.8	963.7	6026.3	36.0
St. Error	104.8	1150.8	29.6	83.2	61.6	23.7	38.1	206.6	1.0
1784 LGB	3739.4	29804.4	1452.1	1886.3	1828.3	1111.6	1060.1	5886.2	40.9
St. Error	20.0	1630.6	153.3	129.7	50.2	28.4	37.0	245.3	1.7
P84 x P47	2288.7	23280.4	1178.1	1878.7	1817.8	418.8	995.5	5110.8	31.8580
St. Error	262.1	1326.7	38.9	8.8	40.8	3.8	16.0	59.7	1.8
P47 x P84	1532.1	18625.7	986.0	1733.3	1797.0	233.6	890.2	4654.2	25.8
St. Error	26.6	283.5	26.0	76.4	60.0	8.2	80.7	225.4	0.6

Appendix 2. Complete data set of QTL mapping population TL2000B and TL2001A from Tlaltizapan, Mexico.

TL2000B.

Family	para CA	Trans FA	Cis FA	DFA 8-5	DFA 5-5	DFA 8-O-4	Soluble Phenolic X
1	1881	25653	2120	358	2454	1331	323
2	2419	22708	1654	213	2026	1176	592
3	2561	20309	1662	309	1867	1109	347
4	1790	26647	1774	256	2597	1157	299
5	2465	23025	1581	130	1914	1079	362
6	2408	28832	1927	199	2856	1202	305
7	2164	27037	1545	62	2406	1109	135
8	2211	20230	1295	43	1569	930	502
9	2405	21938	1251	43	1379	780	301
10	2100	20799	1312	54	1640	871	307
11	2450	24199	1945	200	2400	1077	247
12	1384	27654	1825	83	2098	966	229
13	2598	23743	1592	165	2190	950	534
14	1732	31356	1566	101	1915	806	595
15	1892	34049	1490	132	2733	1130	349
16	3424	30464	1592	202	2444	1228	632
17	2172	21310	1459	256	2462	1080	590
18	1952	21083	1429	219	2444	1058	252
19	1685	19306	1476	256	1796	800	234
20	1742	21344	1375	139	2146	965	184
21	2481	20693	1211	57	1692	832	303
22	1326	20813	1456	176	2272	919	212
23	2177	31121	1417	65	1374	708	182
24	2672	26407	1093	47	1861	853	421
25	1553	23420	1088	46	1643	748	230
26	3080	26510	2363	490	2606	1265	376
27	1456	23786	1601	90	2239	1174	363
28	3422	28756	2317	79	2290	1173	184
29	1400	23995	1506	75	1758	914	0
30	1721	24771	1525	186	1668	826	160
31	1500	19650	1189	162	2022	818	384
32	2447	22538	1371	145	1907	787	258
33	2626	22161	2351	363	3152	1483	756
34	2158	26835	1633	194	2559	1036	191
35	2231	29796	1388	49	2353	1074	252
36	1674	25555	1370	94	1823	1050	314
37	2393	27947	1655	236	2316	1140	756
38	2070	21139	1113	59	1740	889	637
39	2872	17988	1289	57	1816	935	515
40	2256	22759	1648	259	2668	1125	0
41	2104	23510	1884	186	1940	832	256
42	2364	29069	1622	146	2115	955	207
43	1913	19377	1531	196	2457	1035	261
44	1661	24504	1319	45	1741	829	214
45	3666	24241	2115	598	2545	1296	1031
46	1643	29157	2114	178	1870	810	307
47	1171	32017	1396	48	2212	1037	249
48	2045	21722	1505	196	2567	1068	321
49	2211	23793	1332	122	2032	842	504
50	1518	31176	1442	66	2274	1060	377

51	1753	28487	1454	69	2615	1137	244
52	1564	19617	1209	134	1971	919	326
53	1716	26407	1668	245	2074	1036	652
54	2889	26232	1909	288	2363	1220	930
55	1998	20516	763	41	1603	780	464
56	2438	29359	1507	185	2575	1397	420
57	1322	29166	1423	75	1702	890	436
58	2234	29585	1428	55	1957	950	431
59	1501	18416	742	34	1378	648	348
60	2171	21768	1295	136	1903	894	454
61	1578	21076	819	41	1731	712	249
62	1395	21035	1187	132	2112	887	600
63	1670	23167	1469	263	1865	987	293
64	1515	23729	1489	185	2273	1020	338
65	1274	25143	807	45	1561	730	530
66	3146	24458	1527	196	2637	1070	619
67	2321	25388	1746	196	2593	1147	149
68	2041	20525	1989	177	1999	971	0
69	2441	28588	1391	70	2043	1028	279
70	2138	23913	1826	233	2131	1007	264
71	1875	24721	1167	58	2126	999	216
72	2713	30305	1446	69	2070	1036	134
73	1494	19497	1231	116	1681	783	556
74	1686	23194	1256	56	2192	940	284
75	1764	20071	1336	172	2071	801	331
76	2600	26528	1560	306	2286	1059	531
77	1938	23528	1307	125	1766	868	354
78	3195	35197	1325	182	2482	1246	600
79	1385	22019	1074	41	1886	914	0
80	1669	21368	1936	202	2138	971	0
81	2146	24661	1589	152	1949	877	355
82	2436	23600	935	46	1661	734	320
83	2477	19166	962	70	1545	716	327
84	1911	20511	1042	85	1814	841	352
85	2073	20425	1556	230	2265	928	345
86	1825	26897	2084	397	2957	1395	708
87	1995	25010	1123	56	2157	971	309
88	3008	26131	1030	51	1759	863	353
89	2151	27907	1005	50	2123	1023	333
90	1887	18689	1019	38	1503	709	206
91	1798	21852	1012	70	1680	724	440
92	2448	22965	902	60	2001	926	266
93	2012	22842	1023	55	1792	926	266
94	1303	25449	1442	55	1744	800	139
95	1367	21766	906	60	1517	698	257
96	1608	24921	980	41	1602	751	349
97	1065	28921	1521	54	2188	911	145
98	1809	27594	1252	66	1918	882	309
99	2943	27950	1024	162	2415	991	945
100	2426	21695	1644	296	2553	1172	663
101	1189	22553	1605	70	1701	911	202
102	2318	26626	1095	53	2066	944	453
103	1882	25339	1185	57	2078	951	412
104	2402	21010	998	50	1978	876	155
105	2557	23509	1452	45	1557	897	405
106	2307	23494	815	51	1858	872	419
107	2135	27203	1082	49	2095	854	254

108	2566	24816	1367	139	2236	913	424
109	2774	21154	1532	228	2475	980	675
110	2336	25769	1247	53	1853	801	361
111	1904	21401	896	91	1594	775	227
112	1633	25184	1305	60	1976	932	309
113	2233	26477	1429	84	2026	943	406
114	2035	22714	1535	228	2126	1065	452
115	2231	22487	1089	49	1817	783	358
116	2579	24011	837	47	1754	1395	230
117	1794	20003	1490	82	2338	1112	150
118	2057	26302	1584	71	1695	914	169
119	1943	22470	2272	51	1691	928	129
120	1279	25253	1149	52	2097	965	131
121	2182	22648	1499	229	2009	1022	569
122	3882	24175	1657	346	2150	911	584
123	2452	23277	990	55	1927	921	218
124	1596	26346	1096	41	1831	856	406
125	2251	15728	748	61	1717	850	342
126	1905	23355	1258	133	2031	901	314
127	1987	22488	1594	241	2472	1007	447
128	1365	21367	1179	63	2078	995	429
129	1805	24081	1250	49	2059	898	466
130	2453	24542	1451	151	1933	878	416
131	1872	25102	908	34	1645	708	319
132	1941	20077	1343	157	1887	810	330
133	2181	23778	1155	68	2144	909	199
134	2133	17446	1315	172	1954	819	472
135	2592	23635	1385	207	2370	1084	320
136	2490	27960	1420	170	2317	1018	420
137	1153	20235	1435	51	1996	977	325
138	2576	23830	923	58	1751	834	276
139	2879	25019	1501	192	2315	948	374
140	1452	19719	975	58	1422	656	371
141	1551	18006	932	72	1350	670	505
142	2527	20008	856	41	1460	658	678
143	1579	24897	963	33	1636	789	421
144	2583	19890	1125	149	1550	677	735
145	2235	20218	825	38	1486	668	503
146	1515	25820	1330	56	2096	1002	350
147	1944	22050	973	51	1614	854	347
148	2166	19838	939	79	1434	715	549
149	2006	23564	1160	180	1955	893	249
150	1703	24667	1478	245	2577	985	521
151	1928	22057	1167	50	1968	854	130
152	1635	22686	1047	70	1730	771	307
153	1319	24547	927	68	1767	778	294
154	2089	27710	1223	58	1943	878	0
155	1807	20527	698	43	1535	670	438
156	2541	25541	1132	80	1792	832	222
157	2398	24507	928	51	1885	819	169
158	1478	19018	1210	61	1753	825	109
159	1525	21157	903	43	1715	757	351
160	2517	26375	1238	202	2643	1040	506
161	2482	22245	1079	77	1864	833	343
162	1972	18306	1301	64	1799	944	253
163	2766	21844	706	58	1935	884	412

TL2000B cont'd

Family	DFA 8-5 benzo	Diferuloyl putrescine	Total DFAs	Total Phenolics	Progeny	Dobie	Damage	Losses
1	791	422	4935	34589	133.670	13.735	81.284	14.008
2	681	896	4096	30876	105.670	12.560	84.790	10.152
3	657	559	3941	28473	110.000	12.298	81.517	13.186
4	689	339	4699	34911	99.000	12.324	75.569	9.913
5	622	610	3746	30817	119.000	12.950	86.656	16.217
6	727	676	4984	38151	135.330	13.329	83.477	13.861
7	671	345	4249	34995	118.330	12.929	80.205	15.114
8	528	961	3070	26806	168.670	14.401	90.642	27.407
9	450	871	2653	28246	143.000	14.013	83.976	15.006
10	531	613	3096	27307	158.000	13.426	88.409	10.764
11	699	534	4376	32969	123.330	13.234	84.957	14.336
12	610	591	3757	34620	125.670	12.974	81.546	18.103
13	581	704	3887	31819	113.330	13.276	81.896	15.144
14	590	1461	3412	38066	133.000	13.303	87.632	13.977
15	667	648	4661	42092	110.330	12.771	75.042	13.820
16	680	1282	4555	40034	81.000	11.796	74.863	20.086
17	683	810	4481	29423	111.670	13.010	76.426	16.603
18	696	361	4418	28882	123.670	13.359	75.039	10.206
19	530	425	3383	25849	135.670	13.501	76.729	6.630
20	610	388	3861	28322	141.670	13.463	89.198	15.696
21	512	968	3092	27477	99.667	12.880	67.770	7.616
22	611	476	3978	27573	138.670	13.696	76.137	14.062
23	490	554	2636	37351	122.330	13.124	89.260	16.118
24	553	1054	3315	33486	136.670	13.301	82.471	11.701
25	459	569	2896	28957	102.330	12.611	68.848	15.062
26	732	674	5093	37045	190.330	15.231	94.322	15.162
27	582	794	4085	30929	174.330	14.305	90.392	18.122
28	711	498	4252	38748	160.330	14.337	91.720	20.867
29	552	178	3300	30200	138.670	13.765	79.604	13.626
30	512	373	3192	31209	86.667	12.011	47.705	4.851
31	546	358	3548	25887	139.330	13.591	73.089	9.456
32	526	632	3365	29721	130.330	13.048	72.405	14.080
33	810	802	5809	32948	162.330	14.269	78.940	9.983
34	725	323	4514	35140	91.000	12.007	52.008	6.005
35	694	627	4169	37583	89.667	11.773	59.723	7.026
36	535	670	3502	32102	94.667	11.620	46.516	3.771
37	672	1634	4365	36360	80.333	11.249	40.508	5.539
38	538	1660	3226	27548	98.000	11.939	48.095	8.430
39	511	1136	3319	25469	87.000	11.542	48.626	9.218
40	740	287	4791	31455	94.667	12.409	55.972	8.496
41	508	636	3466	30964	126.330	12.990	69.239	12.444
42	615	357	3832	36887	103.670	12.538	54.273	5.354
43	701	437	4389	27210	85.333	11.979	50.297	6.476
44	625	451	3239	30723	122.330	12.784	67.415	11.096
45	774	1689	5212	35236	82.333	11.670	46.666	4.064
46	530	814	3389	36303	96.667	12.294	44.223	3.769
47	617	443	3914	38498	84.000	11.570	55.219	9.070
48	731	625	4561	29833	123.000	13.419	65.238	6.809
49	585	1107	3582	30917	130.670	13.423	60.971	14.037
50	607	860	4007	38143	91.333	11.838	71.070	13.808
51	679	357	4500	36195	72.333	11.362	51.378	8.466
52	616	416	3640	26030	106.000	12.685	64.972	7.518
53	748	1376	4103	33894	100.670	12.633	66.124	15.358
54	657	1767	4528	35558	74.333	11.582	50.732	8.221

55	436	803	2861	26137	96.333	12.102	58.573	8.101
56	594	659	4751	38055	101.330	11.798	52.876	6.943
57	502	830	3169	35080	59.000	10.725	74.083	12.468
58	527	1067	3489	36735	87.333	11.679	90.347	18.019
59	408	843	2469	23128	129.330	13.424	90.917	22.013
60	600	803	3533	28767	110.330	12.538	80.460	9.378
61	449	547	2933	26406	125.000	12.966	86.975	19.641
62	556	911	3687	27304	146.330	13.571	82.100	19.241
63	633	438	3747	30053	117.670	13.105	91.870	13.766
64	590	637	4069	30802	128.670	13.438	82.233	12.706
65	408	1516	2744	29968	108.670	12.768	83.653	20.150
66	623	1008	4525	33656	95.667	12.064	75.733	12.925
67	657	384	4594	34050	127.330	13.279	81.899	13.102
68	652	181	3800	28355	87.333	12.252	69.495	16.999
69	651	454	3792	36211	85.333	11.821	77.727	12.540
70	594	413	3965	31842	110.000	12.766	80.350	14.749
71	563	305	3746	31510	72.333	10.653	71.821	10.414
72	601	307	3775	38238	48.333	9.626	49.086	8.381
73	424	1473	3004	25226	111.330	12.855	77.845	13.987
74	560	591	3748	29884	102.000	12.223	72.506	10.126
75	502	440	3546	26717	107.000	12.707	76.768	13.496
76	628	752	4278	34967	92.333	11.886	71.340	15.138
77	593	554	3351	30125	120.330	13.519	80.004	14.445
78	516	1083	4426	44143	54.000	10.386	46.137	6.635
79	563	164	3404	27882	112.330	13.288	80.703	15.590
80	630	202	3941	28913	103.000	12.407	73.669	9.787
81	572	426	3551	31947	110.000	12.891	82.743	14.276
82	489	802	2930	29901	99.333	12.681	81.427	19.170
83	478	566	2809	25414	110.670	12.760	85.617	11.542
84	586	633	3326	26790	106.330	13.075	70.636	9.845
85	640	594	4063	28118	120.000	13.163	62.036	6.423
86	787	1183	5536	36341	73.667	11.345	49.112	8.810
87	611	507	3794	31922	106.330	12.497	65.551	12.338
88	583	514	3256	33425				
89	572	677	3768	34831	75.333	11.234	66.548	8.194
90	464	288	2715	24310	131.000	13.306	82.113	12.290
91	480	915	2953	27615	108.670	12.574	65.201	11.965
92	611	324	3598	29913	90.000	11.955	60.432	9.644
93	567	325	3340	29217	107.000	12.387	68.067	11.309
94	513	382	3113	31307	122.330	13.104	78.423	13.992
95	458	421	2733	26772	131.330	13.566	78.491	12.036
96	461	755	2854	30363	125.330	13.193	73.279	5.978
97	641	367	3794	35301	162.330	14.662	70.358	12.393
98	476	549	3342	33997	133.330	12.984	77.690	14.131
99	540	1842	4108	36025	80.667	11.796	67.188	10.736
100	684	719	4706	30470	148.330	13.994	79.348	16.362
101	559	500	3242	28589	162.330	14.349	85.996	17.478
102	590	806	3652	33691	104.000	12.825	69.830	11.636
103	592	667	3678	32084	158.000	14.443	84.668	17.994
104	531	203	3436	27846	128.000	13.457	83.983	12.982
105	552	812	3051	30569	150.670	13.811	93.809	11.846
106	524	937	3305	29921	142.000	13.670	88.493	15.109
107	527	537	3525	33945	102.330	12.418	80.461	11.736
108	556	605	3845	32594	135.330	13.703	83.672	9.733
109	624	799	4307	29767	146.000	13.543	70.328	8.874
110	514	788	3221	32573	135.000	13.655	75.926	6.262
111	485	518	2945	27147	144.670	13.819	81.390	15.663

112	550	553	3517	31639	161.000	14.983	91.582	14.406
113	648	731	3701	33840	117.670	13.201	73.234	8.955
114	610	585	4029	30313	154.330	14.402	89.341	18.157
115	545	692	3193	29001	105.000	12.588	84.514	15.754
116	257	460	3453	30879	122.670	12.937	87.800	15.153
117	643	408	4175	27463	144.330	13.818	83.637	20.519
118	550	477	3230	33173	150.330	13.913	85.994	10.684
119	542	476	3212	29897	160.000	14.314	91.472	29.177
120	615	328	3729	31410	167.670	14.147	92.946	20.181
121	622	659	3882	30211	172.000	14.055	91.603	23.437
122	564	876	3971	33685	140.670	13.279	84.248	6.814
123	587	246	3490	30209	134.670	13.626	90.506	20.021
124	558	796	3286	32324	111.000	12.931	83.971	2.447
125	499	651	3127	21855	154.330	14.455	93.279	21.990
126	590	362	3654	30173	123.670	13.272	82.698	15.022
127	642	470	4361	30430	89.667	12.123	72.305	7.124
128	549	1096	3686	27597	154.000	14.083	87.149	22.804
129	595	970	3601	30736	134.670	13.836	91.765	12.286
130	611	448	3574	32019	120.330	13.205	77.236	15.667
131	491	753	2878	30760	134.000	13.574	90.801	12.391
132	534	394	3389	26749	85.333	11.987	80.857	10.124
133	617	253	3738	30852	124.330	13.392	89.530	13.085
134	575	528	3519	24413	110.330	12.922	84.114	20.259
135	787	494	4449	32061	111.670	12.847	82.522	10.699
136	766	405	4272	36142	120.670	13.343	82.975	14.499
137	600	469	3624	26447	194.670	15.138	87.458	18.299
138	560	388	3202	30531	143.670	13.397	84.950	9.117
139	649	439	4104	33504	94.667	12.264	83.744	12.353
140	450	641	2587	24734	106.000	12.785	85.524	25.726
141	435	897	2528	23017	110.330	12.235	73.111	11.965
142	441	1553	2600	25991	112.670	12.893	64.128	9.826
143	517	800	2975	30414	90.000	12.140	53.779	6.498
144	473	1864	2849	26446	127.670	13.736	65.879	14.440
145	421	1087	2613	25891	89.667	11.973	52.329	3.639
146	653	543	3808	32472	83.667	11.704	60.962	2.466
147	563	610	3081	28048	89.667	12.544	59.425	6.802
148	517	947	2745	25688	135.000	13.502	82.463	0.805
149	606	366	3633	30364	124.670	12.876	79.128	18.387
150	703	643	4510	32358	122.000	12.952	78.056	10.439
151	591	403	3464	28615	75.667	11.531	59.090	13.209
152	538	584	3109	28478	120.330	12.832	80.533	17.147
153	545	539	3158	29951	119.670	12.710	77.224	17.067
154	577	268	3457	34479	73.333	11.567	52.803	7.928
155	443	1084	2691	25723	84.333	12.341	58.835	11.141
156	596	414	3300	32514	115.670	12.731	76.339	8.779
157	613	365	3368	31200	148.000	13.748	78.341	20.042
158	554	306	3193	24899	138.670	14.061	82.934	20.148
159	483	652	2998	26582	134.670	13.615	81.509	13.171
160	723	798	4608	34738	108.670	13.006	69.518	13.991
161	628	519	3402	29208	114.330	13.172	75.336	18.471
162	560	642	3367	24946	144.000	13.890	80.303	8.623
163	631	863	3507	28822	115.670	13.247	65.702	21.660

TL2001A.

Family	para CA	Trans FA	Cis FA	DFA 8-5	DFA 5-5	DFA 8-O-4	Soluble Phenolic X
1	1976	23117	1031	234	1184	720	275
2	2142	16553	717	219	1077	628	492
3	2599	22649	977	269	1333	821	316
4	1687	23575	999	69	1641	770	327
5	1829	19355	1220	212	1628	780	620
6	2396	23450	1376	237	1995	840	761
7	2762	22013	1160	403	1728	849	515
8	1940	16386	817	252	1169	673	484
9	2573	21086	1030	260	1234	749	358
10	2108	19996	1047	243	1159	723	405
11	2218	24931	1135	290	1588	846	306
12	1735	23838	897	241	1413	769	382
13	2858	25930	1019	272	1401	803	735
14	1432	26354	1201	261	1553	804	408
15	2755	24404	999	257	1281	817	317
16	2685	23775	1284	402	1543	818	648
17	2933	22646	1068	306	1379	817	512
18	2542	21771	1137	357	1454	774	558
19	2235	19759	1099	346	1413	800	426
20	2168	22065	1014	241	1172	786	218
21	2613	16420	813	206	973	619	281
22	1790	25390	1107	321	1635	1027	141
23	2520	24398	1577	362	1506	846	535
24	2903	23127	889	269	1370	761	846
25	1925	21281	1022	208	1136	641	387
26	2275	19233	810	177	1160	606	361
27	1324	18817	714	233	1278	728	409
28	2394	26132	1484	323	1352	830	426
29	2088	28037	1698	353	1552	968	330
30	2785	24857	1380	368	1622	900	441
31	2458	20007	1240	374	1361	763	367
32	2609	23426	1295	378	1535	801	643
33	2014	21876	1176	303	1307	911	399
34	2754	26195	1503	324	1534	830	452
35	2001	24350	1367	261	1473	777	364
36	1706	23391	1232	253	1204	835	0
37	1961	22978	1092	211	1216	699	532
38	2429	21012	1459	280	1400	812	533
39	3304	18759	1262	310	1356	842	331
40	3035	20253	1219	350	1491	838	136
41	1913	22037	1148	257	1315	748	322
42	2470	23622	1212	254	1408	830	220
43	2257	19135	1091	298	1584	850	383
44	2323	21480	1139	257	1287	730	485
45	2372	21015	953	220	1234	764	361
46	1897	24340	1350	326	1514	776	475
47	1590	29203	1378	276	1713	963	0
48	2411	23833	1348	352	1625	880	643
49	3067	18400	1337	445	1818	985	473
50	2228	31067	1671	463	2303	1070	567
51	2187	21332	1193	423	1734	881	313
52	2083	17109	1123	235	907	656	307
53	1841	19739	1110	264	1095	777	225

54	3072	24088	1381	396	1625	943	441
55	1797	21166	721	205	1484	859	163
56	2711	24771	1002	379	1869	1096	259
57	1651	29641	1259	224	1327	846	488
58	2395	26406	1232	386	1775	945	431
59	2214	22390	932	260	1457	825	492
60	2345	20591	856	322	1548	894	121
61	1761	21979	878	257	1460	785	213
62	1737	23113	829	275	1525	882	229
63	2173	27698	1027	282	1740	1034	162
64	1588	18027	984	268	1280	703	143
65	1446	20288	837	164	1112	667	93
66	3334	22308	1317	382	1562	911	380
67	2922	27083	1250	315	1807	1040	0
68	2175	25862	958	244	1639	893	119
69	2684	27292	1452	406	1909	1039	287
70	1549	21204	774	151	1207	624	88
71	2231	23961	940	300	1726	1024	0
72	2761	25910	1220	275	1476	984	170
73	2160	22370	1577	427	1891	991	727
74	2730	28726	1229	302	1706	965	871
75	2544	22079	1247	407	1609	854	547
76	2414	26266	1089	270	1575	834	494
77	2926	22311	1310	298	1481	858	429
78	3123	27278	1339	446	1861	917	914
79	2769	23498	1127	231	1391	773	309
80	1631	24915	1109	249	1512	808	192
81	2098	28752	1264	262	1669	937	349
82	3091	31038	1334	249	1619	890	424
83	3171	23269	1453	427	1819	982	532
84	2330	22981	1625	481	1984	1032	709
85	3103	21692	1355	379	1603	832	596
86	1282	22441	999	284	1553	887	308
87	2558	26776	1573	416	1880	1161	400
88	2549	24598	1059	273	1437	795	375
89	2626	24749	1132	294	1531	832	394
90	2150	20504	1181	280	1375	765	39
91	1789	23055	1222	258	1336	761	397
92	2234	22822	1148	313	1532	908	258
93	2141	23848	1431	400	1654	964	387
94	1297	25336	1172	226	1432	785	218
95	1794	25621	1165	234	1406	821	364
96	1606	25283	1232	212	1250	739	352
97	1159	29460	1391	223	1475	778	291
98	1900	28169	1168	246	1545	848	289
99	3025	23392	1326	478	1734	890	949
100	2222	23259	1041	300	1628	838	393
101	1702	24504	1092	286	1582	829	362
102	2288	25157	1122	286	1639	909	493
103	2273	23423	1258	395	1727	877	557
104	2314	19452	1075	291	1512	828	73
105	2758	22107	1447	353	1567	861	508
106	2369	21666	1267	433	1664	871	687
107	2152	25178	975	261	1627	824	257
108	2498	24082	1079	296	1598	877	409
109	2841	22545	1014	289	1593	860	387
110	2170	23338	1159	273	1295	739	305

111	2184	23885	940	247	1381	795	281
112	2503	24970	1586	431	1912	1035	344
113	2187	25543	1493	377	1959	1000	224
114	1881	20787	1221	360	1466	819	449
115	2460	25885	1246	340	1699	949	275
116	2797	25353	967	316	1615	956	210
117	2688	23005	1117	470	1916	988	391
118	2412	30405	1151	297	1711	892	525
119	2592	28332	1185	356	1907	943	351
120	1869	27661	1018	244	1541	830	282
121	2144	21095	1134	434	1601	853	599
122	3769	24260	958	255	1254	768	394
123	2872	24885	1142	435	1864	1063	185
124	1626	25809	1211	237	1292	856	346
125	2468	17979	955	287	1462	818	299
126	1981	25728	1177	313	1572	933	279
127	2089	23860	1297	367	1632	928	369
128	2272	22338	1198	441	1814	955	648
129	2286	26227	1159	309	1584	892	524
130	2447	23981	1245	244	1359	813	264
131	1767	24376	980	207	1357	706	379
132	2109	24662	897	281	1578	819	247
133	2161	22232	1179	334	1337	870	274
134	2690	22897	1565	425	1806	1021	540
135	2681	24694	1422	500	1962	1138	472
136	2951	28088	1579	443	2073	1037	486
137	2116	24201	1346	436	1855	910	732
138	2680	25438	1401	397	1692	959	327
139	2654	26268	1533	512	2219	1066	488
140	1229	20014	1145	208	1215	670	440
141	1627	16628	1196	356	1459	737	669
142	2573	23479	963	274	1448	763	627
143	1565	23468	1331	334	1528	757	663
144	2463	19156	952	199	1047	688	841
145	2183	21510	861	233	1272	710	452
146	1636	21322	1322	259	1532	904	255
147	1917	20902	992	241	1253	801	345
148	2178	20065	1025	244	1206	720	529
149	1950	23309	1046	285	1671	893	262
150	1748	24588	1151	285	1628	888	467
151	2682	24394	1290	428	1871	875	520
152	1920	23605	1264	410	1764	862	576
153	1329	24523	1038	248	1425	802	330
154	2169	25860	1386	371	1872	858	518
155	2422	24074	997	251	1488	787	501
156	2528	23395	1381	432	1742	907	497
157	2924	20268	1328	433	1610	783	481
158	2150	23042	1211	382	1499	772	609
159	1579	20058	786	227	1314	718	416
160	2440	26437	1243	379	2096	1026	385
161	2400	21201	1182	306	1649	828	413
162	2052	19862	796	334	1569	863	399
163	3304	23063	1259	388	1815	981	580

TL2001A cont'd.

Family	DFA 8-5 benzo	Diferuloyl putrescine	Total DFAs	Total Phenolics	TLWEEVIL	DOBTL	DAMAGETL	LOSSESTL
1	475	473	2613	28736	133.670	13.735	81.284	14.008
2	365	1262	2289	21702	105.670	12.560	84.790	10.152
3	525	672	2949	29173	110.000	12.298	81.517	13.186
4	534	435	3014	29274	99.000	12.324	75.569	9.913
5	558	944	3178	25582	119.000	12.950	86.656	16.217
6	577	1618	3649	30872	135.330	13.329	83.477	13.861
7	551	707	3530	29465	118.330	12.929	80.205	15.114
8	440	984	2533	21676	168.670	14.401	90.642	27.407
9	498	903	2742	27431	143.000	14.013	83.976	15.006
10	520	718	2645	25795	158.000	13.426	88.409	10.764
11	536	838	3260	31543	123.330	13.234	84.957	14.336
12	482	617	2905	29375	125.670	12.974	81.546	18.103
13	549	1269	3025	32832	113.330	13.276	81.896	15.144
14	538	655	3156	32142	133.000	13.303	87.632	13.977
15	567	523	2922	31080	110.330	12.771	75.042	13.820
16	569	1241	3332	31076	81.000	11.796	74.863	20.086
17	575	931	3077	29725	111.670	13.010	76.426	16.603
18	516	934	3102	28552	123.670	13.359	75.039	10.206
19	526	590	3086	26177	135.670	13.501	76.729	6.630
20	517	363	2717	27964	141.670	13.463	89.198	15.696
21	433	670	2231	22076	99.667	12.880	67.770	7.616
22	746	325	3728	32015	138.670	13.696	76.137	14.062
23	654	685	3368	31864	122.330	13.124	89.260	16.118
24	506	2182	2906	29825	136.670	13.301	82.471	11.701
25	443	1010	2428	26656	102.330	12.611	68.848	15.062
26	436	827	2379	24696	190.330	15.231	94.322	15.162
27	498	849	2737	23592	174.330	14.305	90.392	18.122
28	611	630	3117	33126	160.330	14.337	91.720	20.867
29	624	439	3497	35321	138.670	13.765	79.604	13.626
30	522	710	3413	32436	86.667	12.011	47.705	4.851
31	537	374	3034	26740	139.330	13.591	73.089	9.456
32	518	1113	3233	30563	130.330	13.048	72.405	14.080
33	580	454	3100	28166	162.330	14.269	78.940	9.983
34	574	701	3261	33714	91.000	12.007	52.008	6.005
35	523	743	3034	30752	89.667	11.773	59.723	7.026
36	605	248	2898	29226	94.667	11.620	46.516	3.771
37	456	961	2581	28612	80.333	11.249	40.508	5.539
38	495	1333	2988	27887	98.000	11.939	48.095	8.430
39	523	474	3031	26356	87.000	11.542	48.626	9.218
40	608	314	3286	27794	94.667	12.409	55.972	8.496
41	492	544	2811	27910	126.330	12.990	69.239	12.444
42	493	264	2985	30289	103.670	12.538	54.273	5.354
43	585	660	3316	25798	85.333	11.979	50.297	6.476
44	514	994	2788	27729	122.330	12.784	67.415	11.096
45	555	672	2773	27113	82.333	11.670	46.666	4.064
46	532	748	3148	30735	96.667	12.294	44.223	3.769
47	697	149	3650	35822	84.000	11.570	55.219	9.070
48	636	1171	3493	31086	123.000	13.419	65.238	6.809
49	687	503	3935	26740	130.670	13.423	60.971	14.037
50	750	951	4585	39552	91.333	11.838	71.070	13.808
51	598	359	3636	28348	72.333	11.362	51.378	8.466
52	406	590	2203	22518	106.000	12.685	64.972	7.518
53	499	423	2636	25326	100.670	12.633	66.124	15.358
54	472	435	3436	31978	74.333	11.582	50.732	8.221

55	212	158	2759	26443	96.333	12.102	58.573	8.101
56	333	211	3678	32162	101.330	11.798	52.876	6.943
57	575	755	2972	35522	59.000	10.725	74.083	12.468
58	437	354	3543	33577	87.333	11.679	90.347	18.019
59	482	1016	3024	28560	129.330	13.424	90.917	22.013
60	447	119	3210	27003	110.330	12.538	80.460	9.378
61	445	323	2946	27564	125.000	12.966	86.975	19.641
62	406	207	3088	28767	146.330	13.571	82.100	19.241
63	491	163	3548	34445	117.670	13.105	91.870	13.766
64	269	97	2520	23120	128.670	13.438	82.233	12.706
65	239	86	2182	24752	108.670	12.768	83.653	20.150
66	458	326	3314	30272	95.667	12.064	75.733	12.925
67	483	172	3645	34901	127.330	13.279	81.899	13.102
68	363	108	3138	32134	87.333	12.252	69.495	16.999
69	623	222	3977	35404	85.333	11.821	77.727	12.540
70	194	117	2177	25703	110.000	12.766	80.350	14.749
71	444	100	3495	30627	72.333	10.653	71.821	10.414
72	532	130	3268	33158	48.333	9.626	49.086	8.381
73	645	1120	3954	30061	111.330	12.855	77.845	13.987
74	561	2201	3534	36220	102.000	12.223	72.506	10.126
75	526	774	3396	29267	107.000	12.707	76.768	13.496
76	534	1181	3213	32983	92.333	11.886	71.340	15.138
77	603	850	3240	29786	120.330	13.519	80.004	14.445
78	594	1457	3817	35557	54.000	10.386	46.137	6.635
79	517	634	2911	30306	112.330	13.288	80.703	15.590
80	584	233	3153	30809	103.000	12.407	73.669	9.787
81	610	564	3478	35592	110.000	12.891	82.743	14.276
82	556	1110	3314	38776	99.333	12.681	81.427	19.170
83	650	564	3879	31772	110.670	12.760	85.617	11.542
84	686	1039	4183	31119	106.330	13.075	70.636	9.845
85	557	735	3371	29521	120.000	13.163	62.036	6.423
86	557	615	3281	28003	73.667	11.345	49.112	8.810
87	669	485	4126	35033	106.330	12.497	65.551	12.338
88	509	563	3014	31220				
89	513	803	3171	31678	75.333	11.234	66.548	8.194
90	539	207	2960	26795	131.000	13.306	82.113	12.290
91	511	726	2866	28933	108.670	12.574	65.201	11.965
92	587	270	3340	29545	90.000	11.955	60.432	9.644
93	595	380	3613	31033	107.000	12.387	68.067	11.309
94	533	354	2977	30782	122.330	13.104	78.423	13.992
95	537	556	2999	31579	131.330	13.566	78.491	12.036
96	447	739	2648	30769	125.330	13.193	73.279	5.978
97	592	450	3067	35077	162.330	14.662	70.358	12.393
98	480	502	3119	34356	133.330	12.984	77.690	14.131
99	555	1979	3657	31399	80.667	11.796	67.188	10.736
100	585	701	3350	29871	148.330	13.994	79.348	16.362
101	616	575	3314	30612	162.330	14.349	85.996	17.478
102	598	891	3432	32000	104.000	12.825	69.830	11.636
103	654	633	3654	30607	158.000	14.443	84.668	17.994
104	540	217	3171	26013	128.000	13.457	83.983	12.982
105	624	568	3405	29717	150.670	13.811	93.809	11.846
106	547	1467	3514	28817	142.000	13.670	88.493	15.109
107	534	470	3245	31550	102.330	12.418	80.461	11.736
108	554	740	3326	30984	135.330	13.703	83.672	9.733
109	530	801	3272	29672	146.000	13.543	70.328	8.874
110	525	543	2832	29500	135.000	13.655	75.926	6.262
111	561	556	2984	29993	144.670	13.819	81.390	15.663

112	675	354	4053	33111	161.000	14.983	91.582	14.406
113	734	501	4069	33292	117.670	13.201	73.234	8.955
114	545	650	3190	27078	154.330	14.402	89.341	18.157
115	678	507	3665	33256	105.000	12.588	84.514	15.754
116	608	301	3496	32614	122.670	12.937	87.800	15.153
117	670	436	4043	30854	144.330	13.818	83.637	20.519
118	607	928	3507	37475	150.330	13.913	85.994	10.684
119	700	663	3906	36015	160.000	14.314	91.472	29.177
120	569	441	3184	33733	167.670	14.147	92.946	20.181
121	619	763	3507	27881	172.000	14.055	91.603	23.437
122	512	888	2789	31776	140.670	13.279	84.248	6.814
123	678	207	4040	32939	134.670	13.626	90.506	20.021
124	619	567	3003	31649	111.000	12.931	83.971	2.447
125	521	390	3088	24489	154.330	14.455	93.279	21.990
126	669	360	3488	32373	123.670	13.272	82.698	15.022
127	692	440	3620	30865	89.667	12.123	72.305	7.124
128	624	1135	3834	29642	154.000	14.083	87.149	22.804
129	677	976	3463	33134	134.670	13.836	91.765	12.286
130	526	361	2941	30614	120.330	13.205	77.236	15.667
131	452	962	2720	29843	134.000	13.574	90.801	12.391
132	486	399	3163	30831	85.333	11.987	80.857	10.124
133	655	265	3195	28766	124.330	13.392	89.530	13.085
134	703	709	3954	31106	110.330	12.922	84.114	20.259
135	851	497	4450	33247	111.670	12.847	82.522	10.699
136	716	558	4268	36886	120.670	13.343	82.975	14.499
137	573	1071	3773	31436	194.670	15.138	87.458	18.299
138	649	390	3697	33215	143.670	13.397	84.950	9.117
139	687	640	4484	34940	94.667	12.264	83.744	12.353
140	446	819	2539	24927	106.000	12.785	85.524	25.726
141	478	1173	3030	22480	110.330	12.235	73.111	11.965
142	476	1404	2961	29975	112.670	12.893	64.128	9.826
143	512	1137	3131	29495	90.000	12.140	53.779	6.498
144	376	2429	2310	24882	127.670	13.736	65.879	14.440
145	434	1118	2650	27205	89.667	11.973	52.329	3.639
146	574	417	3269	27549	83.667	11.704	60.962	2.466
147	520	648	2815	26626	89.667	12.544	59.425	6.802
148	446	1068	2617	25885	135.000	13.502	82.463	0.805
149	533	464	3382	29687	124.670	12.876	79.128	18.387
150	516	905	3317	30804	122.000	12.952	78.056	10.439
151	583	858	3757	32122	75.667	11.531	59.090	13.209
152	507	1068	3543	30332	120.330	12.832	80.533	17.147
153	492	543	2968	29857	119.670	12.710	77.224	17.067
154	558	654	3659	33074	73.333	11.567	52.803	7.928
155	457	1183	2982	30475	84.333	12.341	58.835	11.141
156	572	532	3653	30957	115.670	12.731	76.339	8.779
157	520	631	3346	27866	148.000	13.748	78.341	20.042
158	505	1047	3158	29561	138.670	14.061	82.934	20.148
159	399	905	2658	25081	134.670	13.615	81.509	13.171
160	611	669	4112	34232	108.670	13.006	69.518	13.991
161	526	589	3309	28092	114.330	13.172	75.336	18.471
162	490	564	3255	25965	144.000	13.890	80.303	8.623
163	636	1036	3820	31446	115.670	13.247	65.702	21.660