ABSTRACT

EICKHOLT, DAVID PATRICK JOSEPH. Evaluating a Novel Method of Inbreeding in Tobacco and Investigating the Economic Value of an Introgressed *Nicotiana tomentosa* Chromosome Segment Influencing Leaf Number. (Under the direction of Dr. Ramsey Lewis.)

Tobacco (*Nicotiana tabacum* L.) is an integral part of the United States economy. In 2010, U.S. tobacco production had a total economic value of over $1.2 billion. As a result of tobacco's economic importance, plant breeding methods which increase the yield of tobacco varieties and increase the rate at which new varieties can be released are of great importance. In plant breeding, one of the rate limiting steps in the release of new cultivars is the time required to complete the inbreeding process. Inbred line development can be expedited through the use of off-season nurseries and all-season greenhouses. If the plant breeder wishes to perform selection during the inbreeding process, these approaches are of little value, however. Selection and line advancement for disease resistance in tobacco grown under artificial conditions is generally not possible, because the pathogen density overwhelms plants from even the most resistant cultivars before they can reach maturity.

In this research, constitutive transgenic expression of the *Arabidopsis thaliana* gene, *FT*, in tobacco, was used to dramatically reduce generation time from 160 days to 69 days on average. The *FT* gene encodes for a protein which originates in the leaves and travels to the shoot apex via the phloem, where it induces flowering. During the inbreeding process, tobacco populations were subjected to intense disease screening using artificial inoculation techniques with the pathogen *Phytophthora nicotianae* to select for favorable alleles associated with field resistance to the disease known as black shank. After three generations

of inbreeding coupled with selection for black shank resistance, selection against the *35S:FT* transgene resulted in the development of regular flowering populations of inbred lines. Results indicated that groups of inbred lines derived from the selection process exhibited significantly higher levels of field resistance compared to groups of random inbred lines not selected for resistance. Using the transgenic approach, the development of populations of inbred lines with an enhanced frequency of highly black shank resistant lines was achieved in approximately one half of the time that would have been required using traditional field breeding.

The portfolio of a successful plant breeding program is diversified in the sense that it focuses on the development of varieties that not only have high levels of disease resistance, but that also have a high yielding ability. Previous attempts to increase tobacco yields by delaying flowering using photoperiod sensitive genotypes have had limited success. Short-day mutants generally produced higher yields, but quality characteristics were adversely affected. In this research, an alternative genetic system of increasing leaf number was investigated for its potential value for increasing flue-cured tobacco yields. An introgressed chromosome segment derived from the species *Nicotiana tomentosa,* designated as *Many Leaves* (*Ml*), was backcrossed into three genetic backgrounds of flue-cured tobacco. Homozygous (*MlMl*) lines and heterozygous (*Mlml*) F_1 hybrids were evaluated for yield, flowering time, morphological characteristics, and physical and chemical quality of the cured leaf.

Days to flowering was increased in an additive to partially dominant fashion across genetic backgrounds, coinciding with significant increases in leaf number and yield. Delayed

flowering genotypes showed no significant reduction in physical quality of the cured leaf. Significant increases in hectare value were observed in the later flowering genotypes, but increases in percent reducing sugars and reductions in percent total alkaloids also were observed. The utility of increasing the nitrogen (N) rate to achieve higher yields in delayed flowering genotypes was also investigated. Results demonstrated that increasing the N rate had a significant effect on increasing yield and percent total alkaloids, while having no effect on leaf quality.

Evaluating a Novel Method of Inbreeding in Tobacco and Investigating the Economic Value of an Introgressed *Nicotiana tomentosa* Chromosome Segment Influencing Leaf Number

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Crop Science

Raleigh, North Carolina

2013

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DEDICATION

This thesis is dedicated to my grandparents, Kenneth and Florence Eickholt. Their altruistic actions regarding the adoption of my father afforded him, as well as me, the greatest opportunity possible. Without my grandparents, I certainly would not be where I am today.

BIOGRAPHY

David Patrick Joseph Eickholt was born in 1987 and raised on the outskirts of Chesaning, Michigan. Being raised on a multi-generational family farm, his gravitation towards agriculture was inevitable. Having a keen interest in the science behind farming, he pursued a degree in agriculture from the original land grant university, Michigan State University. He received his Bachelor of Science degree for the Advanced Study Option from the Department of Crop and Soil Science in 2009. Upon completion of his B.S., he decided to further his education and pursue a graduate degree in plant breeding from North Carolina State University. His Master of Science thesis work was performed in the tobacco breeding and genetics program under the supervision of Dr. Ramsey Lewis.

ACKNOWLEDGMENTS

In completion of the Master of Science degree, I would like to personally thank all of the individuals who have made this milestone in my life possible. I express my gratitude to the Crop Science Department, specifically the Plant Breeding Center, for allowing me to study plant breeding and genetics at North Carolina State University. I am especially grateful to Dr. Ramsey Lewis for accepting me into his program. His insight for my research, as well as my life, has been immeasurable. I also would like to thank Dr. Loren Fisher, Dr. Paul Murphy and Dr. David Shew not only for their academic support, but for serving on my graduate committee.

This thesis research would not have been possible without the technical assistance of our laboratory manager, Sheri Kernodle. I would also like to thank Mike Maher, Tomas Moreno, Jessica Nifong-Lott and fellow graduate students Katherine Drake, Wesley Hancock and Patrick McCachren. Their assistance on a daily basis made these ambitious research projects possible. In addition, I would like to thank fellow graduate students Charlie Zila and Dr. Jacob Delheimer for their statistical support of this experiment. I would also like to thank all of the personnel at the Kinston, Rocky Mount and Oxford research stations. Without their support, even the best planned research projects wouldn't be possible.

I am very grateful to Monsanto for providing me with a graduate research fellowship, Philip Morris International and Altria Client Services for their financial support, and Gold Leaf Seeds and Lorillard for awarding me graduate scholarships.

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INTRODUCTION

Nicotiana

The Solanaceae, or nightshade family, contains many agronomically important plants such as the potato, tomato, and pepper. Currently, over 100 recognized genera exist under the Solanaceae family, with the genus *Nicotiana* being the sixth largest member (Knapp et al., 2004). The genus *Nicotiana* was first described by Carl Linnaeus in 1753. Seventy-six species are classified under the genus *Nicotiana* (Knapp et al., 2004), while only one, *Nicotiana tabacum* L., is of significant economic importance today.

Tobacco (*Nicotiana tabacum* L.) is a true amphidiploid, meaning that its tetraploid somatic cells contain the diploid chromosome compliments of two parental species. *Nicotiana tabacum* (2n = 48) most likely arose by the union of unreduced gametes from two diploid progenitors (2n = 24), or the union of normal gametes (n = 12) from distinct diploid species, followed by chromosome doubling (Gerstel and Sisson, 1995). Chloroplast sequencing evidence strongly suggests that an ancestor of modern day *N. sylvestris* contributed the maternal genome and cytoplasm to *N. tabacum* (Yukawa et al., 2006). The donor species of the paternal genome is not as clear. However, research has shown that the most likely donor may have been an introgressive hybrid between *Nicotiana tomentosiformis* and *Nicotiana otophora* (Riechers and Timko, 1999; Kitamura et al., 2001).

General Characteristics

The tobacco plant, which is perennial in nature, is cultivated as an annual for production purposes around the world. In order for seed germination to occur, the seed

requires adequate moisture and light. Following the emergence of the di-cotyledons, true leaves develop spirally in a 2/9 phyllotaxy along the main stem (Avery, 1933). Once vegetative growth ceases, a terminal inflorescence develops. The inflorescence of tobacco is capable of producing hundreds of flowers and hundreds of thousands of seeds. Successful pollination of a single flower can produce between 2,500 to 3,500 seeds (Wernsman and Rufty, 1987). Reproductive growth can last 6-8 weeks and creates an energy sink which significantly reduces leaf yield. To limit yield loss, the inflorescence is removed at the button stage (Fisher et al., 2012a).

Tobacco Types

Before science based tobacco breeding efforts began in the early 20th century, eight distinct market types of tobacco existed. These types are known as burley, cigar filler, cigar wrapper, dark air-cured, dark fire-cured, flue-cured, Maryland and oriental (Lewis, 2011). These tobacco types emerged in response to consumer demand for specific tobacco uses. The classes can be easily separated based on the production practices they are associated with and the visual characteristics which describe them. Flue-cured tobacco is the most prominent form of tobacco grown world-wide, followed by burley, oriental and dark air-cured (Universal Leaf, 2012).

ECONOMIC IMPORTANCE

Production

Tobacco is grown for its cured leaf on all continents except Antarctica. The world's largest producer of tobacco is China, followed by Brazil, India and then the United States

(FAO, 2012). It is estimated that 4,498 million kg of flue-cured tobacco (green leaf weight) was harvested globally in 2011, with the United States producing 169 million kg (Universal Leaf, 2012). In the United States, tobacco had a total value of production over $1.2 billion in 2010. Of that $1.2 billion, $589 million came from North Carolina production, almost solely from flue-cured sales (NASS, 2012). Tobacco is the number one agricultural crop for farm cash receipts in North Carolina, comprising approximately 6.1% of sales (NCDA, 2012).

Tax Revenue

The sale of tobacco products generates a significant source of revenue for state governments as well as the federal government. In 2009, the federal excise tax on cigarettes was raised from $0.39 per pack to $1.01 per pack (Tiller et al., 2011). In 2011, the federal government collected over 16 billion dollars in revenue from tobacco excise taxes (TTB, 2012). On top of the federal excise tax, each state has an additional excise tax on cigarette sales. State excise taxes are quite variable and range from a high of $4.35 per pack in New York to a low of $0.17 in Missouri (CDC, 2012). On top of the standard excise tax rate for the state, individual counties and cities may impose an additional tax on cigarette sales.

TOBACCO BREEDING

Historical Perspective

Some of the earliest tobacco varieties grown were the result of visual farmer selections. The first genetic attempt at improving tobacco varieties was conducted at the Connecticut Agriculture Experiment Station at the turn of the 20th century. In the 1930's, the United States Department of Agriculture (USDA) began tobacco breeding efforts focused on

incorporating disease resistance into burley and flue-cured cultivars (Wernsman and Rufty, 1987). Following the USDA's tobacco breeding endeavor, agricultural research stations began breeding efforts of their own. Joint ventures between state research stations and the USDA continued until 1994, when the USDA exited from tobacco research.

In addition to the federal and state tobacco research programs, many private tobacco breeding companies emerged in the early 1940's (Wernsman and Rufty, 1987). Some of the most popular varieties to date have been released by private breeding companies (Fisher et al., 2012b).

Breeding Methods

The methods used to develop new tobacco cultivars have differed based on the time period and the characteristics being improved upon. The earliest breeding efforts focused on mass selection within heterogeneous cultivars (Wernsman and Rufty, 1987). Pedigree breeding followed mass selection and it is still the most widely used method for genetic improvement. Backcrossing is widely used to transfer specific resistance genes and more recently harm reduction traits into established cultivars. Doubled haploids have also been used, but due to the cost and effort required, they are generally developed for experiments with an academic focus.

Tobacco, being a self-pollinated species, would not be expected to suffer from significant inbreeding depression. Heterosis in F_1 hybrids developed from the hybridization of inbred lines is generally low (Aycock, 1980). As a result, the development of F_1 varieties wasn't the focus of early breeding programs. Instead, breeders initially focused on the development of pure-line varieties. Only recently have breeding programs began to release

hybrid varieties for varietal protection. Since tobacco is harvested for its vegetative tissue, the ability to produce seed is not a concern for growers. As a result, the majority of varieties released today use cytoplasmic male sterility for varietal protection. F_1 hybrids also facilitate the deployment of disease resistance genes introgressed from wild *Nicotiana* relatives. Significant linkage drag effects are often observed with introgressed disease resistance genes. In F_1 hybrids, these resistance genes can be deployed in a heterozygous condition with less of a deleterious effect on yield and/or cured leaf quality.

Haploids

The production and identification of haploid plants in tobacco occurs with relative ease. Tobacco haploid plants can be produced by several different methods and researchers today still dispute which method is best. In 1967, Bourgin and Nitsch first described the development of haploid plants from tobacco pollen, also known as anther culture. In 1979, Burk et al. described the production of haploid tobacco plants when *N. tabacum* was crossed with *N. africana*. The researchers found that although most seeds germinated, the majority of the seedlings died before they ever produced true leaves. Approximately 0.25-1.42 percent of the seedlings grew well and were either F_1 hybrids or maternal haploids. Subsequent visual evaluation allowed for rapid haploid identification. In 1985, Kumashiro and Oinuma reported on the production of maternal haploid plants by the in vitro pollination of *N. tabacum* ovules with irradiated *N. alata* pollen.

In 2010, Ravi and Chan described the production of haploid *Arabidopsis* plants through centromere-mediated genome elimination. The researchers targeted the highly conserved protein CENH3 and were able to produce haploid plants in frequencies ranging

from 25-45%. This method has yet to be attempted in tobacco, but may play a significant role in the future.

Doubled Haploids

Doubled haploids are produced by the doubling of the haploid chromosome set. Early methods for chromosome doubling utilized the application of colchicine to seeds or seedlings. Pressurized nitric oxide, growth regulators and midvein culture have also been used (Kato and Geiger, 2002). In tobacco, the most common method currently used to double the chromosome number is the leaf midvein tissue culture method first described by Kasperbauer and Collins (1972). The researchers demonstrated that haploid tobacco somatic cells spontaneously chromosome double to produce doubled haploid plants. The spontaneous chromosome doubling eliminates the need for the handling of hazardous chemicals and exposure to potentially dangerous pressurized chambers.

Research has shown that the yield of doubled haploid plants can be significantly lower than the parental line from which they were developed. In addition, the type of haploid gamete which was doubled, maternal versus paternal, has a significant effect on yield as well. Across several genetic backgrounds, doubled haploids produced from anther culture derived haploids yielded 10% lower than their maternal counterparts (Wernsman et al., 1989). Despite these findings, a few breeding programs within the United States and across the world still use anther culture. Doubled haploids are most often used today for QTL mapping studies and molecular marker research.

DISEASE

Economic Loss

Any threat to the health of the tobacco crop, whether biological or environmental, can have a significant impact in terms of dollars lost to the tobacco farmer, and the economy as a whole. In 2010, 13.3% of the North Carolina tobacco crop was lost due to disease, which represents an economic loss of 91 million dollars (Mila et al., 2010). From 2006 through 2010, the disease which caused the greatest economic loss was black shank, followed closely by bacterial wilt and tomato spotted wilt virus (TSWV). During that time period, black shank caused an estimated economic loss of over 103 million dollars (Mila et al., 2010).

Black Shank

Black shank of tobacco, a polycyclic disease, is caused by the oomycete *Phytophthora nicotianae* (Van Breda de Haan). Black shank was first reported on the Indonesian island of Java in 1896 by Van Breda de Haan. Since the initial reporting, black shank has spread to tobacco growing regions worldwide (Shoemaker and Shew, 1999). Black shank first appeared in the United States in southern Georgia in 1915, and by 1922 it had become a serious problem for the cigar tobacco growing regions of Florida and Georgia (Tisdale and Kelley, 1926). In 1931, black shank was first reported in Forsyth County of North Carolina and has subsequently spread to most of the tobacco growing counties in the state (Lucas, 1975).

Symptoms

A comprehensive account of symptoms associated with black shank is given by Lucas (1975). Black shank primarily affects the roots and lower portion of the tobacco stem. Tobacco stems and leaves can become infected through the splashing of zoospores from rainwater and irrigation, although this is much less common than root infection. Once the initial infection of the plant has occurred, the black shank pathogen continues to colonize and infect the roots, stem and leaves through hyphal growth. Infection of the plant results in wilting, chlorosis, root, stem and leaf necrosis, and usually plant death.

Infection of a tobacco plant by *P. nicotianae* can occur at any growth stage. Young seedlings are especially vulnerable to black shank. Near the soil, the stem of infected seedlings turns dark brown to black and the entire plant displays the symptom known as "damping off". In larger, actively growing plants, one of the first symptoms of infection is the wilting of leaves. Within a few days, the leaves will begin to display severe signs of chlorosis and a loss of turgor pressure will cause the leaves to severely droop. If an older plant which is no longer undergoing vigorous growth is infected, the stem may turn dark brown to black for nearly 30cm. The leaves will then turn brown, shrivel and the entire plant will be dead within a few days.

Infection

The occurrence of the disease is strongly influenced by environmental factors such as temperature and soil moisture. Black shank is considered a warm-weather disease and soil temperatures greater than 20°C are usually required for significant infection levels to occur, with lesion expansion being optimized at 22-28°C (Shew and Lucas, 1991). However,

infection can occur at temperature levels as low as 16°C. High soil moisture conditions enhance disease occurrence, since soil saturation is required for zoospore release and spread (Shew and Lucas, 1991).

The exact method by which zoospores identify and infect plant roots is quite complex, but research has shown that zoospores are attracted to some specific chemical signal, or combination of chemical signals. Zoospores of most *Phytophthora* species show attraction to certain amino acids, sugars, aldehydes, alcohols, isoflavones, calcium ions and electrical fields (Tyler, 2002). If a plant root is injured during transplanting, cultivation, by a feeding insect or a number of other methods, the plant root releases many of these compounds. A wound is not needed for infection to occur, however. Plant roots also create small electrical fields at their tips and branch points. Certain species of *Phytophthora* have demonstrated a strong affinity towards electrical fields (Morris and Gow, 1993).

PHYTOPHTHORA NICOTIANAE

Phytophthora pathogens are a diverse group of heterotrophic organisms that morphologically and physiologically resemble fungi, but are in-fact phylogenetically distant from them (Tyler, 2002). *Phytophthora nicotianae* is a heterotrophic hemibiotroph that grows primarily as coenocytic hyphae (Tyler, 2002). *Phytophthora nicotianae* and oomycetes in general commonly produce 3 types of asexual spores, sporangia, zoospores and chlamydospores. In addition to asexual reproduction, *Phytophthora nicotianae* also undergoes heterothallic sexual reproduction to form oospores (Apple, 1959).

Asexual Spores

A sporangium can germinate to produce hyphae through direct germination, or it can form zoospores through indirect germination. Under very wet conditions, such as a flood, zoospore formation is triggered. Zoospores are the most important means of plant root infection, especially when the soil is flooded (Tyler, 2002). Zoospores are motile due to their bi-flagellate morphology. Under wet conditions, zoospores are able to easily move throughout the soil water and locate plant roots, where they then form cysts on the roots through a process called encystment. For encystment to occur, the zoospore must initially attach to the plant root. The zoospore almost always attaches with its ventral side, which contains the flagella, against the plant root (Deacon and Donaldson, 1993). The zoospore's flagella are then resorbed and a hypha will develop from the original site of the flagella during germination of the cyst (Tyler, 2002).

Chlamydospores are the third type of asexual spore, which serve as the primary inoculum and initiator of epidemics. Chlamydospores are thick-walled spores that are thought to be produced as long-term survival structures (Zentmyer and Erwin, 1970). Chlamydospores can germinate directly to produce hyphae, or germinate indirectly to form sporangia. The sporangia can then germinate directly to form hyphae or indirectly to form zoospores.

Race Structure

There are currently four races of *P. nicotianae* that have been identified. These races have been numerically named in the order of their discovery (0, 1, 2 and 3). Apple (1962a) reported that a strain of *P. nicotianae* was virulent on *N. plumbaginifolia*. The new strain was

designated as race 1. The wild-type strain, which was non-virulent on *N. plumbaginifolia*, was designated as race 0. In North Carolina and other tobacco growing regions of the United States, race 0 has historically been the most prevalent strain of the pathogen (Lucas, 1975). Variation in aggressiveness within a race was first reported by Dukes and Apple (1961), and Sullivan et al., (2005a) found race 0 to be more aggressive than race 1.

Race 2 was first reported in South Africa based on a differential response to check varieties (Jaarsveld et al., 2002). However, race 2 has not been described elsewhere and is considered epidemiologically insignificant (Gallup and Shew, 2010). Race 3 was first reported in Connecticut by McIntyre and Taylor (1978). In 2010, Gallup and Shew identified race 3 in North Carolina. Race 3 is defined as a strain which can overcome the *Phl* resistance gene introgressed from *P. longiflora*, but cannot overcome the *Php* resistance gene from *N. plumbaginifolia*.

Although race 1 was identified in the early 1960's, race 0 was the most prevalent strain in North Carolina up until the mid-1990's (Sullivan et al., 2005b). Cultivars containing the *Php* gene were successful for some time in reducing losses due to black shank, but as race 0 resistant varieties were continuously planted, the disease began to appear more and more frequently. A shift from race 0 to race 1 occurred in all fields where continuous deployment of the *Php* resistance gene occured. Melton and Broadwell (2003) reported that race 1 of *P. nicotianae* was present in 58% of the flue-cured production areas of North Carolina.

PLANT DEFENSE

Constitutive Defenses

Since plants are immobile, they are sitting targets for a multitude of pathogen attacks. As a result, they must be able to naturally protect themselves from a number of different offenders. In order for a successful infection to occur, the pathogen must be able to first overcome the constitutive plant defenses. One of the first lines of defense against pathogens is the waxy cuticle layer that plants possess. Other common constitutive plant defenses are preformed peptides, proteins and non-proteinaceous secondary metabolites which have antimicrobial activity (Heath, 2000).

Inducible Defenses

If a pathogen can overcome these non-specific plant defenses, the pathogen must then either avoid detection by the plants natural inducible defense system or overcome whatever reactionary measures the plant employs. Common inducible plant defenses are the production of antimicrobial phytoalexins, localized necrosis of cell tissue as a result of hypersensitive response, production of activated oxygen species, production of proteins that inhibit degradative enzymes produced by the pathogen and the modification of plant cell walls (Freeman and Beattie, 2008).

Elicitors

In order for a plant's defenses to be induced, some chemical signal, or elicitor, must be present. An elicitor, as the name implies, is a compound that elicits a response. Elicitors are the basic structural components of a pathogens surface, or the result of the pathogens

metabolism (Ebel and Cosio, 1994). Elicitors can be grouped into two main categories, endogenous and exogenous. Exogenous elicitors, which are considered the primary signal in the plant-pathogen interaction, originate in the pathogen and evoke a plant response in the immediate vicinity of the pathogen (Ebel and Cosio, 1994). Endogenous elicitors are found in the plant and originate as a result of the plant-pathogen interaction. In order for the plant to detect the elicitor, a receptor is needed.

The simplest model which explains the plant-pathogen interaction is the gene-for-gene model, which was first proposed by Flor (1942). According to the model, the dominant avirulence (avr) gene in the pathogen is detected by the dominant resistance (R) gene in the host plant. It has been proposed that dominant R genes act as receptors for specific elicitors, which are produced by the pathogens avr genes (Bent and Mackey, 2007). Upon recognition of the elicitor, the plants defenses would be induced.

Phytophthora nicotianae Elicitins

One distinguishing feature of *Phytophthora* species is the production of 10-kDa proteins called elicitins. The function of elicitins is to uptake sterols from host membranes (Osman et al., 2001). Sterols are essential components of all eukaryotic membranes and are involved in maintaining membrane fluidity and permeability (Gaulin et al., 2010). *Phytophthora* species have lost the ability to synthesize sterols and instead acquire them from host tissues. The production of elicitins has been shown to induce hypersensitive responses in *Nicotiana* and *Brassica* species (Huitema et al., 2005).

One exception to the production of elicitins in species of *Phytophthora* is that of *P. nicotianae*. *Phytophthora nicotianae* cultures isolated from tobacco plants suffering from

black shank frequently do not produce elicitins. However, cultures of *P. nicotianae* that do produce elicitins can be found on black shank infected plants, but the severity of the disease is reduced (Kamoun et al., 1994). Elicitins are produced by several genes within a multi-gene family, however, one gene, parA1, is the main gene which is expressed (Colas et al., 2001). *Phytophthora nicotianae* strains which were able to infect tobacco while still producing elicitins were found to down regulate parA1 gene expression (Colas et al., 2001). The results indicate that elicitins may be involved in a quantitative decrease in aggressiveness of *P. nicotianae* strains. These strains may have evolved mutations that allow the suppression of elicitins as first described by Kamoun et al. (1994).

Black Shank Resistance Breeding

Black shank has been a serious concern for tobacco growers since it was first reported in the United States in 1915, and specifically for North Carolina growers since it was first reported in 1931 (Lucas, 1975). The first breeding efforts for black shank resistance began in the 1920's and the first black shank resistant variety was released in 1931. The resistant variety, Florida 301, was released by W. B. Tisdale who crossed the black shank resistant cigar tobacco variety Big Cuba to the susceptible cigar tobacco variety Little Cuba.

At the time of his breeding efforts, the variety Big Cuba was no longer grown on any significant acreage in Florida. Instead, a newly released variety called Connecticut Round Tip proved to have superior leaf quality and yield, and was subsequently planted almost solely in just 4 years after release (Tisdale, 1931). Approximately the same time that almost all of the acreage was planted to Connecticut Round Tip, a major black shank outbreak occurred and it became apparent that the Connecticut Round Tip variety was highly

susceptible. The marketplace had shifted away from the leaf quality and characteristics of Big Cuba, and as a result, there was little incentive offered to the grower to switch back to Big Cuba (Tisdale, 1931).

Tisdale's release of Florida 301 is the first recorded tobacco variety to be released that employs quantitative resistance to *Phytophthora nicotianae*. Tobacco varieties which employ quantitative resistance have varying degrees of resistance, ranging from very low to very high. The Florida 301 style of resistance is the principal source of black shank resistance used in tobacco breeding programs since 1941 (Sullivan et al., 2005b).

The nature of inheritance of Florida 301 resistance has been debated since its initial deployment. Initially, the resistance was thought to be polygenic and to act in an additive fashion (Smith and Clayton, 1948). Clayton later reported (1958) that Florida 301 resistance was simply inherited and recessive in nature. Moore and Powell (1959) then reported that the resistance was partially dominant and controlled by modifying factors that vary based on the genetic background the resistance is placed in. Crews et al. (1964) and Chaplin (1966) both reported that the resistance was polygenic and additive in nature as Smith and Clayton (1948) first proposed. The most recent research (Xiao et al., 2012) demonstrates that the Florida 301 resistance is, in fact, polygenic and additive in nature, being controlled by genes at multiple loci with varying levels of effect.

The second major source of black shank resistance utilized in breeding programs is the result of interspecific crosses between *Nicotiana tabacum* to either *Nicotiana plumbaginifolia* (Apple, 1962b and Chaplin, 1962) or *Nicotiana longiflora* (Valleau and Stokes, 1960). Tobacco varieties which employ resistance derived from either *N.*

plumbaginifolia or *N. longiflora* do so as the result of a single gene. This single gene resistance is classified as *Php* if it originated from *N. plumbaginifolia* or *Phl* if it originated from *N. longiflora*. Both genes provide complete resistance against race 0 of *P. nicotianae* but no resistance against race 1. The *Php* gene has been used mainly in flue-cured varieties while the *Phl* gene has been used exclusively in burley varieties.

Screening for Black Shank Resistance

When using the pedigree method of inbreeding, some method of selecting for disease resistance is usually needed during generation advancement. Currently, the only practical way to simultaneously advance generations while selecting for black shank resistance is through the use of a soil-borne nursery. The need for field based testing only allows one round of selection and self-pollination to occur each year, due to tobacco's generation time (approximately 160 days). Field trials are labor intensive, expensive and time consuming (Jaarsveld et al., 2003). For some traits, generation advancement can occur in greenhouses, but space and money often become an issue. For black shank disease resistance testing, growing plants in the greenhouse and performing selection is not an option, however.

When inoculating young seedlings and selecting for resistance in the greenhouse, even the most resistant varieties typically die before flowering (Lewis, personal communication). Although this method works for testing resistance early on, it is of little use for selecting and advancing generations. Because greenhouse systems fail when selecting for black shank resistance, other strategies for identifying resistant genotypes have been investigated.

A number of different screening methods have been proposed using root, stem and leaf tissue (Apple, 1957; Hendrix and Apple, 1967; Litton et al., 1970; Rufty et al., 1987;

Tedford et al., 1990). Although these results are useful for identifying individuals which are resistant under artificial conditions, the results are often not correlated with field observations (Hendrix and Apple, 1967; Wills, 1971; Tedford et al., 1990).

GENETIC GAIN

A formula to express genetic gain per cycle, ($G_c = h^2D$), was first given by Lush in 1945, where h^2 is equal to the narrow sense heritability and D is equal to the selection differential. In order to calculate the genetic gain per year ($G_y = G_c/y$), the genetic gain per cycle is divided by the number of years required to complete one cycle (Eberhart, 1972). For black shank resistance breeding using field-based disease nurseries, the genetic gain per year is equal to the genetic gain per cycle.

Increasing Genetic Gain

In order to increase the genetic gain per year for tobacco breeding, the genetic gain per cycle would need to be increased, or the time required to complete a cycle would need to be reduced. In crop species such as soybean [*Glycine max* (L.) Merr.] and maize (*Zea mays* L.), off-season nurseries are heavily relied upon for inbreeding, backcrossing, and seed increase, allowing multiple generations of breeding to be completed each year. Due to differences in temperature, humidity, soil type, pest type and disease pressure, off-season nurseries are not used for pedigree-based breeding programs. Because tobacco breeding is mainly conducted using the pedigree method, off-season nurseries are of little use when selecting for quantitative disease resistance and yield.

Photoperiodism

Another method to increase the genetic gain per year is to reduce the generation time of the plant. One of the most common ways to reduce generation time is by altering the photoperiod. An organism's ability to measure the proportion of daylength in a 24-hour period is known photoperiodism (Hopkins and Hüner, 2004).

Photoperiodism was first reported by Garner and Allard (1920) when working with the mutant tobacco type called Maryland Mammoth. The researchers observed that during the normal summer growing conditions, the mutant tobacco type would not flower like all other tobacco types. The mutant tobacco type would only flower during the winter when the nights were long. Plants which display this growth habit are referred to as having a short-day photoperiodic response. Other than the Maryland Mammoth mutant tobacco type, normal tobacco is generally accepted to be day-length neutral, meaning that it doesn't respond to changes in photoperiod. As a consequence, altering the photoperiod through the use of artificial lighting is not useful in tobacco breeding.

FT System

In 2009, Lewis and Kernodle demonstrated the utility of a transgenic approach to significantly reduce the generation time of tobacco. The researchers demonstrated that by using an *Arabidopsis thaliana FT* cDNA clone, coupled with the cauliflower mosaic virus (CaMV) 35S promoter, generation time was reduced from 160 days to 69 days, on average. The *Arabidposis thaliana* gene, *FT,* produces a 20 kDa protein which originates in the leaf and moves to the shoot apex via the phloem, triggering flowering (Yoo et al., 2013). The researchers were successful in performing several rounds of backcrossing using the

transgenic plants and then selecting against the transgene at the end of the process to produce regular flowering plants. In addition to the significant reduction in generation time, the constitutive overexpression of the *FT* transgene produced plants that typically only reach 5.5-6 inches in height and produce 4-5 leaves which are between 2-3 inches in diameter (Lewis, unreported).

YIELD and QUALITY

Tobacco is different from most agronomic crops since its leaf tissue is harvested instead of its seed. Components of leaf yield include leaf number, leaf length, leaf width, leaf thickness and leaf density. Genes which directly or indirectly influence the yield components could be thought of as yield genes on which selection could be performed. Studies in burley (Legg and Collins, 1971) and flue-cured tobacco (Matzinger, 1968; Pandeya et al., 1983) have shown that in addition to the yield components listed above, days to flowering can also be added to that list because of its positive correlation with yield.

An equally important component to grower profitability other than yield is the quality of the cured leaf. Prior to the contract growing tobacco market of today, there were over 100 tobacco grades recognized by the USDA. The contract growing market has greatly simplified the number of grades recognized by tobacco companies to fewer than 50 (Fisher et al., 2012b). Characteristics such as leaf color, thickness, grain, and size are taken into account when determining grade and subsequent pricing.

Increasing Yield

If quality was not a concern for tobacco, the easiest way to increase the amount of harvested tissue per unit area is to increase the planting population. Increasing the number of plants per acre by decreasing intra-row and/or inter-row spacing has been shown to significantly increase yield per acre (Chaplin, 1968; Elliot, 1970; Collins and Hawks, 1993; Bukan et al., 2010). When increasing planting populations, the quality of the tobacco is adversely affected, however. At higher planting densities, reducing sugars are often increased while total alkaloids are decreased (Chaplin, 1968; Collins and Hawks 1993). Strong negative correlations exist between yield and total alkaloids (Matzinger, 1968; Legg and Collins, 1971), making the development of high yielding, high nicotine type tobaccos difficult.

Delayed Flowering

Since the number of days to flowering has a strong positive correlation with yield, methods to delay flowering should increase yield. The first breeding efforts focused on delaying flowering were with the Maryland Mammoth type tobacco. Determining the number of underlying genes which cause the mutant phenotype was an area of confusion for tobacco breeders for several decades. When Allard first reported on the Mammoth phenotype in 1919, he reported that, in the F_2 generation (derived from a cross between true breeding mammoth types and regular flowering types), the number of observed photoperiod sensitive plants neared the theoretical ratio for two contrasting unit characters that would be observed if the trait were controlled by two recessive genes. In 1957, Mann and Chaplin reported that the Mammoth phenotype was conditioned by a single gene difference. In 1980, Wernsman

and Matzinger reported that the delay in flowering was actually caused by recessive alleles at two loci.

Mammoth Tobacco

The first Mammoth type variety to be grown commercially was Bunn Special. Bunn Special was never grown on large acreage because of its small leaf size, average yield and susceptibility to black shank and bacterial wilt (Mann and Chaplin, 1957). Since Mammoth tobacco types do not flower during the summer in North Carolina, the possibility of increasing leaf number to harvest higher quality upper-stalk position tobacco was explored. Wernsman and Matzinger (1980) reported that when Mammoth tobacco types were topped 4 leaves higher than normal tobacco varieties, while removing and discarding the 4 lowest leaves, yield increases could be achieved. The increased leaf number caused reductions in nicotine levels and increases in reducing sugars, however. In 1986, King reported that Mammoth tobacco types could be topped 4 leaves higher than normal to aid in mechanical topping without significant reductions in yield, grade index and leaf chemistry. The additional leaves were left on the plant and were not harvested.

Although yield gains could be achieved without detrimental effects on quality, Mammoth tobacco types did not become widely popular. The development of Mammoth varieties is difficult for plant breeders due to the timing of pollen shed (winter) and the overall height of the plants (Lewis, Personal Communication). In addition, the lack of a visual cue (flower bud development) for hand topping added more complication for growers managing labor during the topping process.

Many Leaves

In 2007, Lewis et al. reported on the analysis of an introgressed chromosome segment from the species *Nicotiana tomentosa*. The researchers found that when this segment was evaluated across several genetic backgrounds, significant delays in flowering were observed. The chromosome segment, designated as Many Leaves (*Ml*), acted in a partially additive to dominant fashion and caused increases in leaf number, plant height and green leaf yield. The effect this chromosome segment has on the chemical and visual quality of tobacco has not yet been evaluated.

GERMPLASM INTROGRESSION

Nicotiana Resistance Sources

In tobacco, wild *Nicotiana* species have proved to be sources of commercially useful genetic variation. Resistance to diseases and pests such as tobacco mosaic virus (TMV), wildfire, angular leaf spot (ALS), black shank, blue mold, root-knot nematode, powdery mildew, tomato spotted wilt virus (TSWV), potato virus Y (PVY) and tobacco cyst nematode have been found in *Nicotiana* species (Lewis, 2011).

Introgressing Wild Germplasm

When attempting to incorporate new genes into *N. tabacum*, there are four steps which are conventionally followed. They are, (1) the identification of a species possessing the trait of interest, (2) hybridization with *N. tabacum*, (3) generation of fertile offspring and (4) backcrossing to the cultivated tobacco type to transfer the gene(s) of interest (Lewis, 2011).

If difficulties with hybridization and fertility can be overcome, there is often significant linkage drag associated with the alien chromosome segments. Linkage drag associated with tobacco mosaic virus resistance, black shank resistance and black root rot resistance have been shown to have adverse effects on yield and cured leaf quality (Lewis, 2011). One way to alleviate linkage drag effects associated with qualitative resistance genes has been the development and deployment of hybrid cultivars.

Markers and Backcrossing

During the backcrossing process, each subsequent round of backcrossing is expected to increase the percentage of the recurrent parent genome. When working with two inbred lines, by the 5^{th} backcross generation it is expected that over 98% of the recurrent parent genome will be restored. Recombination suppression can often distort the actual recovered genome percentages, however. Young and Tanksley (1989) reported that when backcrossing a resistance gene from wild tomato into modern tomato cultivars, significant portions of alien chromosome other than the resistance gene were still present after 20 backcrosses in some cases.

One method used to increase the percentage of the recovered parental genome is through marker assisted backcrossing. The identification of markers which flank the resistance gene and are in extreme linkage disequilibrium is an effective means to track the gene(s) through subsequent rounds of backcrossing (Tanksley, 1983). This method is commonly referred to as foreground selection. In addition to foreground selection, methods to increase the amount of recurrent parental genome through marker selection during backcrossing have also been demonstrated, commonly referred to as background selection

(Young and Tanksley, 1989; Frisch et al., 1999). Foreground selection has been used extensively in tobacco breeding but background selection has yet to be attempted by public programs.

REFERENCES

Alcohol and Tobacco Tax and Trade Bureau [TTB] (2012). TAX COLLECTIONS TTB S 5630-FY-2011 CUMULATIVE SUMMARY FISCAL YEAR 2011. http://www.ttb.gov/statistics/final11.pdf.

Allard, H.A. 1919. Gigantism in *Nicotiana tabacum* and its alternate inheritance. Am. Nat. 53:218-233.

Apple, J. L. 1957. Pathogenic, cultural, and physiological variation within *Phytophthora parasitica* var. *nicotianae*. Phytopathol. 47:733–740.

Apple, J.L. 1959. Sexuality of *Phytophthora parasitica* var. *nicotianae*. Phytopathol. 49:37-43.

Apple, J.L. 1962a. Physiological specialization within *Phytophthora parasitica* var. *nicotianae*. Phytopathol. 52:351-354.

Apple, J.L. 1962b. Transfer of resistance to black shank (*Phytophthora parastica* var *nicotianae*) from *Nicotiana plumbaginifolia* to *N. tabacum*. Phytopathol. (Abstr.) 52:1.

Avery, G.S.,Jr.. 1933. Structure and development of the tobacco leaf. Am. J. Bot. 20:565-592.

Aycock, M. K., Jr.. 1980. Hybridization among Maryland, burley and flue-cured tobaccos. Tob. Sci. 24:109-113.

Bent, A.F.,and D. Mackey. 2007. Elicitors, effectors, and R genes: The new paradigm and a life supply of questions. Annu. Rev. Phytopathol. 45:399–436.

Bourgin, J., and J. Nitsch. 1967. Production of haploids in *Nicotiana* from excised stamens. Annales de physiologie végétale 9:377-382.

Bukan, M., A. Budimir, M. Boic, H. Sarcevic, and V. Kozumplik. 2010. Effect of within-row spacing on agronomic and morphological characteristics of the flue-cured tobacco cultivars. Agric. Conspec. Sci. Vol. 75 No. 1:27-31.

Burk, L.G., D.U. Gerstel, and E.A. Wernsman. 1979. Maternal haploids of *Nicotiana tabacum* L. from seed. Science 206:585-585.

Centers for Disease Control and Prevention. 2012. State Cigarette Excise Taxes — United States, 2010–2011. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6112a1.htm?s_cid=mm6112a1.htm_w#tab.

Chaplin, J.F. 1962. Transfer of black shank resistance from *Nicotiana plumbaginifolia* to flue-cured *N. tabacum*. Tob. Sci. 6:184-189.

Chaplin, J.F. 1966. Comparison of tobacco black shank resistance from four sources. Tob. Sci. 10:55-58.

Chaplin, J.F., Z.T. Ford, J.B. Pitner, and R.E. Currin. 1968. Effect of row and within-row spacings on yield and quality of flue-cured tobacco. Agron. J. 60:314-316.

Clayton, E.E. 1958. The genetics and breeding progress in tobacco during the last 50 years. Agron. J. 50:352-356.

Colas, V., I. Lacourt, P. Ricci, F. Vanlerberghe-Masutti, A. Poupet, and F. Panabières. 1998. Diversity of virulence in *Phytophthora parasitica* on tobacco, as reflected by nuclear RFLPs. Phytopathol. 88:205-212.

Colas, V., S. Conrod, P. Venard, H. Keller, P. Ricci, and F. Panabières. 2001. Elicitin genes expressed in vitro by certain tobacco isolates of *Phytophthora parasitica* are down regulated during compatible interactions. Mol. Plant-Microbe Interact. 14:326-335.

Collins, W. K. and S. N. Hawks Jr.. 1993. Principles of Flue-Cured Tobacco Production. Raleigh, NC: W. K. Collins and S. N. Hawk Jr. p. 11-17.

Crews, J.W., W.H. Wills, and J.L. La Prade. 1964. Black shank disease reactions of six flue cured tobacco varieties and the F_1 hybrids among them. Tob. Sci. 8:128-132.

Deacon, J.W., and S.P. Donaldson. 1993. Molecular recognition in the homing response of zoosporic fungi, with special reference to *Pythium* and *Phytophthora*. Mycol. Res. 97:1153–71.

Dukes, P., and J.L. Apple. 1961. Influence of host passage on virulence of *Phytophthora parasitica* var. *nicotianae*. Plant Dis. Rept. 45:362.

Ebel, J., and, E.G. Cosio. 1994. Elicitors of plant defense responses. In: K. W. Jeon and J. Jarvik, editors, International Review of Cytology. A Survey of Cell Biology. Academic Press, San Diego. p. 1-36.

Eberhart, S.A. 1972. Techniques and methods for more efficient population improvement in sorghum. In N.G.P. Rao and L.R. House, editors, Sorghum in the Seventies. Oxford and IBH Publishing, New Delhi. p. 197-213.

Elliot, J.M. 1970. The effect of topping height and plant spacing on yield, grade and some physical characteristics of bright tobacco. Tob. Sci. 14:73-77.

Food and Agriculture Organization of the United Nations. 2012. Unmanufactured Tobacco Production Quantities by Country. http://faostat3.fao.org/home/index.html#VISUALIZE.

Fehr, W.R. 1993. Principles of cultivar development. Vol I. Theory and technique. Macmillian Publishing Company Inc, New York.

Fisher, L.R., S. Stewart, B. Collins, and J. Priest . 2012a. Topping, Managing Suckers, and Using Ethephon. In North Carolina State University: Flue-cured tobacco guide 2012 (AG-187 (Revised) ed., pp. 99-123). Raleigh, North Carolina: North Carolina Cooperative Extension.

Fisher, L.R., S. Stewart, W.D. Smith, G. Tart, and K. Barnes. 2012b. Selecting a Variety. In North Carolina State University: Flue-cured tobacco guide 2012 (AG-187 (Revised) ed., pp. 23-44). Raleigh, North Carolina: North Carolina Cooperative Extension.

Flor, H.H. 1942. Inheritance of pathogenicity in *Melampsora lini*. Phytopathol. 32:653-669.

Freeman, B.C., and G.A. Beattie. 2008. An Overview of Plant Defenses against Pathogens and Herbivores. The Plant Health Instructor. DOI: 10.1094/PHI-I-2008-0226-01.

Frisch, M., M. Bohn, and A.E. Melchinger. 1999. Comparison of selection strategies for marker-assisted backcrossing of a gene. Crop Sci. 39:1295-1301.

Gallup, C.A., and H.D. Shew. 2010. Occurrence of race 3 of *Phytophthora nicotianae* in North Carolina, the causal agent of black shank of tobacco. Plant Dis. 94:557-562.

Garner, W.W., and H.A. Allard.1920. Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. *J. Agric. Res.* 23: 871–920.

Gaulin, E., A. Bottin, and B. Dumas. 2010. Sterol biosynthesis in oomycete pathogens. Plant Signal Behav. 5:258–260.

Geiger, H.H. 2009. Doubled haploids. In J.L. Bennetzen, and S. Hake, editors, Handbook of maize. Springer, New York. p. 641-657.

Gerstel, D.U., and V.A. Sisson. 1995. Tobacco. In: Smartt J and Simmonds N.W., editors, Evolution of crop plants, 2nd ed. John Wiley & Sons, Inc., New York. P. 458–463.

Hardham, A.R., and L.M. Blackman. 2009. Molecular cytology of *Phytophthora*–plant interactions. Australas. Plant Pathol. 39:29-35.

Hendrix, J. W., and J. L. Apple. 1967. Stem resistance *to Phytophthora parasitica* var. nicotianae in tobacco derived from *Nicotianae longiflora* and *N. plumbaginifolia*. Tob. Sci. 11:148–150.

Heath, M.C. 2000. Nonhost resistance and nonspecific plant defenses. Curr. Opin. Plant Biol. 3:315–19.

Hopkins, W.G., and N.P.A. Hüner. 2004. Introduction to plant physiology. 3rd ed. Wiley, Hoboken, NJ.

Huitema, E., V.G.A.A. Vleeshouwers, C. Cakir, S. Kamoun, and F. Govers. 2005. Differences in intensity and specificity of hypersensitive response induction in *Nicotiana* spp. by INF1, INF2A, and INF2B of *Phytophthora infestans*. Mol. Plant-Microbe Interact. 18:183-193.

Jaarsveld, E., M. Wingfield., and A. Drenth. 2002. Evaluation of tobacco cultivars for resistance to races of *Phytophthora nicotianae* in South Africa. J. Phytopathol. 150:456-462.

Jaarsveld, E.M., M. Wingfield, and A. Drenth. 2003. A rapid seedling based screening technique to assay tobacco for resistance to *Phytophthora nicotianae*. J Phytopathol. 151:389-394.

Kamoun, S., M. Young, H. Förster, M.D. Coffey, and B.M. Tyler. 1994. Potential role of elicitins in the interaction between *Phytophthora* species and tobacco. Appl. and Environ. Micro. 60:1593-1598.

Kasperbauer, M.J., and G.B. Collins. Reconstitution of diploids from leaf tissue of anther-derived haploids in tobacco. Crop Sci. 12:98-101.

Kato, A., and H.H. Geiger. 2002. Chromosome doubling of haploid maize seedlings using nitrous oxide gas at the flower primordial stage. Plant Breed. 121:370-377.

King, M.J. 1986. Leaf number at topping and yield, grade index, and leaf chemistry of a mammoth-type tobacco. Agron. J. 78:913–915.

Kitamura, S., M. Inoue, N. Shikazono, and A. Tanaka. 2001. Relationships among *Nicotiana* species revealed by the 5S rDNA spacer sequence and fluorescence in situ hybridization. Theor. and Appl. Genet. 103:678-686.

Knapp, S., M.W. Chase, and J.J. Clarkson. 2004. Nomenclatural changes and a new sectional classification in *Nicotiana* (Solanaceae). Taxon. 53:73-82.

Kumashiro, T., and T. Oinuma. 1985. Comparison of genetic variability among anther-derived and ovule-derived doubled haploid lines of tobacco. Jap. J. Breed. 35:301-310.

Legg, P.D., and G.B. Collins. 1971. Genetic parameters in burley populations of *Nicotiana tabacum* L. I. 'Ky 10' × 'Burley 21'. Crop Sci. 11:365-367.

Lewis, R.S., and S.P. Kernodle. 2009. A method for accelerated trait conversion in plant breeding. Theor. Appl. Genet. 118:1499–1508.

Lewis, R.S., S.R. Milla, and S.P. Kernodle. 2007. Analysis of an introgressed *Nicotiana tomentosa* genomic regions affecting leaf number and correlated traits in *Nicotiana tabacum*. Theor. Appl. Genet. 114:841–854.

Lewis, R.S. 2011. *Nicotiana*: In C. Kole, editor, Wild Crop Relatives: Genomic and Breeding resources – Plantation and Ornamental Crops. Springer, Berlin Heidelberg. p. 185-208.

Litton, C., P. Legg, and G. Collins. 1970. A greenhouse technique for screening tobacco seedlings for black shank resistance. Tob. Sci. 14:124-125.

Lucas, G.B. 1975. Disease of Tobacco: 3rd Edition, Raleigh, NC: Biological Consulting Associates. p. 115-141.

Lush, J. L. 1945. Animal Breeding Plans. Iowa State University Press, Ames.

Mann, T. J., and J. F. Chaplin. 1957. The effect of the mammoth gene on certain quantitatively inherited characters of flue-cured tobacco. Agron. J. 49:230-233.

Matzinger, D.F. 1968. Genetic variability in flue-cured varieties of *Nicotiana tabacum* L. III. SC58 x Dixie Bright 244. Crop Sci. 8:732-735.

McIntyre, J., and G. Taylor. 1978. Race-3 of *Phytophthora parasitica* var. *nicotianae*. Phytopathol. 68:35-38.

Melton, T. A., and A. Broadwell. 2003. Disease Management. In North Carolina State University: Flue-cured tobacco guide 2012 (AG-187 (Revised) ed., pp. 142-166). Raleigh, North Carolina: North Carolina Cooperative Extension.

Mila, A., and J. Radcliff. (2010) Flue-Cured Tobacco Disease Report. http://www.ncsu.edu/project/tobaccoportal/wp-content/uploads/2010/12/2010-Disease-Reports.pdf.

Moore, E.L., and N.T. Powell. 1959. Dominant and modifying genes for resistance to black shank of tobacco. Phytopathol. 49:318-319.

Morris, B.M., and N.A.R Gow. 1993. Mechanism of electrotaxis of zoospores of phytopathogenic fungi. Phytopathol. 83:877–82.

National Agricultural Statistics Service (NASS). 2012. Crop Values 2010 Summary. http://usda01.library.cornell.edu/usda/nass/CropValuSu//2010s/2011/CropValuSu-02-16-2011.pdf.

North Carolina Department of Agriculture and Consumer Services (NCDA). 2012. CASH RECEIPTS from Farm Marketing by Commodity. http://www.ncagr.gov/stats/economic/IncomePercent.pdf.

Osman, H., S. Vauthrin, V. Mikes, M. Milat, F. Panabières, A. Marais, S. Brunie, B. Maume, M. Ponchet, and J. Blein. 2001. Mediation of elicitin activity on tobacco is assumed by elicitin-sterol complexes. Mol. Biol. Cell 12:2825-2834.

Pandeya, R.S., V.A. Dirks, and G. Poushinsky. 1983. Quantitative genetic studies in flue-cured tobacco (*Nicotiana tabacum*). I. Agronomic characters. Can. J. Genet. Cytol. 25:336-345.

Ravi, M., and S.W.L. Chan. 2010. Haploid plants produced by centromere-mediated genome elimination. Nat. 464:615-618.

Riechers, D.E., and M.P. Timko. 1999. Structure and expression of the gene family encoding putrescine N-methyltransferase in *Nicotiana tabacum*: New clues to the evolutionary origin of cultivated tobacco. Plant Mol. Biol. 41:387-401.

Rufty, R. C., E. A. Wernsman, G. V. Gooding Jr.. 1987. Use of detached leaves to evaluate tobacco haploids and double haploids for resistance to tobacco mosaic virus, *Meloidogyne incognita*, and *Pseudomonas syringae* pv. *tabaci*. Phytopathol. 77:60–62.

Shew, H.D. and G.B. Lucas. 1991. Compendium of Tobacco Diseases, APS Press, St. Paul, MN.

Shoemaker, P. B., and H. D Shew. 1999. Major tobacco diseases: Fungal and bacterial diseases. In: D. L. Davis and M. T. Nielsen, editors, Tobacco: Production, Chemistry and Technology. Blackwell Science, Oxford. p.183-197.

Smith, T.E., and E.E. Clayton. 1948. Resistance to bacterial wilt and black shank in flue-cured tobacco. Phytopathol. 38:227-229.

Sullivan, M.J., T.A. Melton, and H.D. Shew. 2005a. Fitness of races 0 and 1 of *Phytophthora parasitica* var. *nicotianae*. Plant Dis. 89:1220-1228.

Sullivan, M.J., T.A. Melton, and H.D. Shew. 2005b. Managing the race structure of *Phytophthora parasitica* var. *nicotianae* with cultivar rotation. Plant Dis. 89:1285-1294.

Tanksley, S.D. 1983. Molecular markers in plant breeding. - Plant Molecular Biology Reporter 1:3-8.

Tedford, E. C., T. L. Miller, and M. T. Nielsen. 1990. A detached-leaf technique for detecting resistance to *Phytophthora parasitica* var. *nicotianae* in tobacco. Plant. Dis. 74:313–316.

Tiller, K., S. Feleke, and J. Starnes. Federal excise tax increase and its effects on U.S. tobacco production. Empir. Econ.1-17.

Tisdale, W.B. 1931. Development of strains of cigar wrapper tobacco resistant to black shank *(Phytophthora nicotianae* Breda de Haan). Fla. Agr. Exp. Stn. Bull. 226.

Tisdale, W. B., and J. G. Kelley 1926. A *Phytophthora* disease of tobacco. Florida Agric. Exp. Sta. Bull. 179, 159–219.

Tyler, B. M. 2002. Molecular basis of recognition between *Phytophthora* pathogens and their hosts. Annu. Rev. Phytopathol. 40:137-167.

Universal Leaf Tobacco Company, Incorporated. 2012. Estimated Leaf Production by Crop Year. http://www.universalcorp.com/PubReports/World%20Leaf%20Production%20as%20of%2018%20May%202012.pdf.

Valleau, W.D., G.W. Stokes, and E.M. Johnson. 1960. Nine years experience with the *Nicotiana longiflora* factor for resistance to *Phytophthora parasitica* var. *nicotianae* in the control of black shank. Tob. Sci. 4:92-94.

Van Jaarsveld, E., M.J. Wingfield, and A. Drenth. 2002. Evaluation of tobacco cultivars for resistance to races of *Phytophthora nicotianae* in South Africa. J. Phytopathol. 150:456-462.

Wernsman, E.A., and D.F. Matzinger.1980. Mammoth genotypes and tobacco management regimes for reduced production of downstalk tobaccos. Agron. J. 72:1047–1050.

Wernsman, E.A., D.F. Matzinger, and R.C. Rufty. 1989. Androgenetic vs. gynogenetic doubled haploids of tobacco. Crop Sci. 29:1151-1155.

Wernsman, E. and R. C. Rufty. 1987. Tobacco. In: W. Fehr, editor, Principles of Cultivar Development, Vol. 2. Macmillan Publishing Company Inc, New York. p. 669-698.

Wills, W. 1971. A comparison of black shank resistance of tobacco measured in inoculated whole plants and excised leaf tissue. Tob. Sci. 15:47-50.

Xiao, B., K. Drake, V. Vontimitta, Z. Tong, X. Zhang, M. Li, X. Leng, and R.S. Lewis. 2012. Location of genomic regions contributing to *Phytophthora nicotianae* resistance in tobacco cultivar 'Florida 301'. Crop Sci. (In Review)

Yoo, S. J., Hong, S. M., Jung, H. S., and J. H. Ahn. 2013. The Cotyledons Produce Sufficient FT Protein to Induce Flowering: Evidence from Cotyledon Micrografting in Arabidopsis. Plant and Cell Phys. 54(1):119-128.

Young, N.D., and S.D. Tanksley. 1989. RFLP analysis of the size of chromosomal segments retained around the tm-2 locus of tomato during backcross breeding. Theor. and Appl. Genet. 77:353-359.

Yu, L.M. 1995. Elicitins from *Phytophthora* and basic resistance in tobacco. Proc. Natl. Acad. Sci. USA 92:4088-4094.

Yukawa, M., T. Tsudzuki, and M. Sugiura. 2006. The chloroplast genome of *Nicotiana sylvestris* and *Nicotiana tomentosiformis*: Complete sequencing confirms that the *Nicotiana sylvestris* progenitor is the maternal genome donor of *Nicotiana tabacum*. Mol. Genet. and Genom. 275:367-373.

Zentmyer, G.A., and D.C. Erwin. 1970. Development and reproduction of *Phytophthora*. Phytopathol. 60:1120-1127.

Chapter 2: A Novel *FT*-Based Breeding Method for Developing Tobacco Inbred Populations with Enhanced Disease Resistance in an Expedited Manner

ABSTRACT

In order to release cultivars fixed for favorable alleles affecting disease resistance, up to four generations of selection and inbreeding in disease nurseries may be required. This selection period, in turn, may last four years. Methods to accelerate the inbreeding process while still allowing for phenotypic selection would be of great value. In this study, the efficacy of a novel, *FT*-based, modified single-seed descent method of inbreeding was investigated. The purpose of this experiment was to develop tobacco (*Nicotiana tabacum* L.) inbred lines resistant to the disease causing oomycete, *Phytophthora nicotianae*, in an expedited manner. Using a climate controlled growth chamber and oat grain inoculation procedures, two sets of $F_{4:5}$ inbred lines were developed within a single year using the new method. Selection against the *FT* transgene in the $F_{4:5}$ generation permitted development of two regular-flowering populations of $F_{5:6}$ lines. On average, inbred lines developed using this selection method exhibited significantly higher levels of field resistance to *P. nicotianae* relative to randomly derived inbred line populations. The average area under the disease progress curve (AUDPC) value for the selected population (1371.0) was significantly lower than that for the population of randomly derived inbred lines (1883.4). The average end percent survival (EPS) value for the selected population (56.8%) was significantly higher than that for the group of randomly derived lines (44.7%). The *FT*-based breeding approach allowed for the development of inbred lines in one-half of the time required using traditional, field-based, disease nursery screening and resulted in an increased frequency of lines exhibiting elite levels of resistance. In the future, *FT*-based breeding technology might be of

value for a number of different crop species to expedite the development of inbred lines resistant to a multitude of pathogens.

INTRODUCTION

One of the largest obstacles to profitable crop production on a global scale is the presence of disease causing microorganisms. Billions of dollars in crop value are lost annually due to diseases in the United States alone (Pinstrup-Andersen, 2001). In addition to reductions in crop value, monetary losses also result from the use of lower value rotation crops and costly pesticide applications. From a production standpoint, the most cost-effective strategy to control disease is through the use of disease-resistant cultivars.

The production of tobacco may be severely limited by plant diseases and the impacts of disease are compounded by the relatively high value of the crop. Over the last five growing seasons, diseases of tobacco have resulted in an estimated loss nearing 400 million dollars in North Carolina (Mila et al., 2010). Of that 400 million, an estimated 25% is due to the disease called black shank, caused by the oomycete *Phytophthora nicotianae*.

Black shank has been a problem in certain tobacco growing regions of the United States for nearly a century. The disease was first reported in North Carolina in 1931 and subsequently spread across the state (Lucas, 1975). Due to the large economic value of tobacco in North Carolina, breeders have had as a major objective the development of high-yielding varieties with high levels of black shank resistance.

Resistance to black shank was first identified in the 1920's by W.B. Tisdale, who was working with cigar type tobaccos in Florida (Tisdale, 1931). The type of resistance he

identified is not simply inherited, and is referred to as quantitative. Breeding efforts using quantitative resistance have produced varieties with varying levels of resistance, but never complete control. In the 1960's, research with the wild *Nicotiana* species *N. plumbaginifolia* and *N. longiflora* resulted in the introgression of two new genes which provided complete resistance against race 0 of *P. nicotianae*, designated as *Php* and *Phl* (Valleau and Stokes, 1960, Apple, 1962, Chaplin, 1962). Flue-cured tobacco varieties carrying this form of resistance began to occupy very significant acreage around the year 2000. Large-scale planting of varieties carrying the *Ph* forms of resistance resulted in race shifts, however. Many fields now have pathogen populations that predominantly consist of a race other than the race 0 wild type. This new race is able to overcome the resistance conferred by the *Ph* genes (Sullivan et al., 2005). With increased prevalence of race 1, the focus of breeding efforts has reemphasized incorporation of quantitative resistance into new varieties, since the quantitative source of resistance provides moderate levels of control against all known races.

The use of quantitative resistance is much more complex than the use of single gene resistance. Due to complexities associated with harvesting, curing, and storage of tobacco, uniform varieties are a necessity for growers. In order to generate genetically uniform and stable varieties, several generations of inbreeding and selection are required. When attempting to incorporate quantitative disease resistance into new, higher yielding genetic backgrounds, large population sizes are generally needed to identify individuals with high levels of resistance. The size of the population required is a function of the number of genes which contribute to the resistance and their expression patterns.

In order to screen large segregating populations, expensive and time consuming field disease nurseries are required (Jaarsveld et al., 2003). Due to tobacco's generation time (160 days, on average), only one cycle of field-based inbreeding and selection can typically be performed each year. The use of greenhouses or off-season nurseries can be used to advance generations, but are typically not of use when selection for soil-borne disease resistance is required. When selecting for black shank resistance under greenhouse growing conditions, even the most resistant cultivars frequently die before they reach maturity (Dr. David Shew, personal communication). As a result, 3-5 years are often spent during the field inbreeding and selection process when black shank resistance is concerned.

Methods which significantly reduce generation time may expedite development of inbred lines. Manipulation of temperature and lighting conditions has proven to be an effective means to reduce generation time in plant species which are photoperiod sensitive. These methods have produced erratic results for tobacco, however (Kasperbauer 1966, 1969; Kasperbauer and Lowe 1966). The use of grafting in tobacco has also allowed for reductions in generation time (Lang et al., 1977), but this is not feasible for large scale breeding efforts. One possible method to reduce the generation time of tobacco is through constitutive overexpression of the *Arabidopsis thaliana*-derived gene *FT*. The results of Lewis and Kernodle (2009) showed that expression of a *35S*:*FT* transgene system in tobacco reduced average generation time from approximately 160 days to 69 days on average, while significantly decreasing overall plant stature. Lewis and Kernodle (2009) proposed the use of an *FT*-based system to facilitate rapid backcrossing, but did not investigate the potential utility of an *FT*-based system to expedite inbreeding with selection for disease resistance.

In this report, we demonstrate a breeding system based upon constitutive expression of *FT* to rapidly advance through inbreeding generations coupled with selection for quantitative levels of resistance to black shank. In this system, selection for *35S*:*FT* and favorable alleles affecting black shank resistance is performed at each generation, except the final generation. In the final generation, selection is performed against the *FT* transgene to generate regular-flowering individuals. Data demonstrated that $F_{5:6}$ lines with increased levels of black shank resistance could be produced in nearly one-half the time required using traditional methods.

MATERIALS AND METHODS

Development of Lines Selected for Black Shank Resistance

Flue-cured tobacco cultivar 'K 346' was selected as a source of a very high level of polygenic black shank resistance for this investigation. Using procedures described by Lewis and Kernodle (2009), the *Arabidopsis thaliana* gene *FT*, under the control of the CaMV 35S promoter, was transferred to K 346 to develop a BC_7F_1 family segregating for *FT* and hereafter referred to as K 346 *FT*. K 346 *FT* plants were hybridized with the standard burley tobacco variety, TN 90LC, and the burley parental line, NC1209-23, to generate two separate F_1 hybrids that were expected to segregate for *35S*:*FT*. NC1209-23 and TN 90LC are non-*Ph* genotypes that exhibit low and low-intermediate levels of black shank resistance, respectively. Three *35S*:*FT* individuals from each cross were self-pollinated to produce two F_2 populations segregating for both the *FT* transgene and for alleles affecting black shank resistance. Seed from each F_2 population were uniformly dispersed across 16 cm x 22 cm

plastic pans (Republic Molding Corporation, Chicago, IL). The F_2 populations were germinated and maintained in a laboratory growth room at 24-26°C with a photoperiod of 16 h light/ 8 h dark for 30 days. Individual seedlings carrying *35S*:*FT* were identified approximately 20 days after germination due to the presence of a lower trichome density on the uppermost leaves and visible signs of flower bud initiation. Thirty days after germination, 864 *35S*:*FT* F_2 plants from each population were transplanted into plastic trays (Landmark Plastic 50 Corporation, Akron, OH) containing eight sectors of six cells (6 cm x 4 cm x 5 cm) each. Cells were filled with a 2:1 mix of Fafard 2 mix (Conrad Fafard Inc., Agawam, MA) and river bottom sand (Figure 2.4).

The multiple-seed method of single seed descent (SSD) inbreeding (sometimes also known as modified SSD (Fehr 1987)) was carried out to ultimately develop $F_{5:6}$ lines derived from each cross. In this system, multiple seeds from each plant are planted the following generation. In our situation, this was necessary as a form of 'progeny testing' needed to maintain *35S*:*FT* in a hemizygous condition throughout the inbreeding process and to avoid fixation of *FT* in resulting lines. At the end of the process, and consistent with SSD, each derived line could be traced back to a unique F_2 individual, however.

Transplanted *35S*:*FT* F_2 plants were grown in a climate controlled growth chamber at the North Carolina State University Phytotron (Raleigh, NC). Growing conditions were set at 16 h light at 30°C / 8 h dark at 25°C and soil moisture was held constant using sub-irrigation. Approximately 7 days after transplanting, each individual plant cell was inoculated with two oat grains infested with *P. nicotianae*. Oat grains were placed approximately 1-1.5 cm into the soil at opposite corners of each cell (Figure 2.5, Figure 2.6). $F_{2:3}$ seed was harvested from

individual F_2 plants lacking symptoms attributed to black shank (stem lesions, wilting, or plant death) (Figure 2.7).

Approximately 20 seeds from each selected $F_{2:3}$ family were then seeded into plastic pots (Kord Products, Toronto, Ca) (6.35 cm x 6.35 cm) containing Fafard 2 mix potting soil. Plants were grown in the laboratory growth room using the same conditions as previously described. Each $F_{2:3}$ family was visually screened to identify those segregating for *35S*:*FT*. One hundred and eighty segregating $F_{2:3}$ families were randomly selected from each cross. Six *35S*:*FT* plants per $F_{2:3}$ family were then transplanted and grown in the Phytotron using the same protocol as before. Each $F_{2:3}$ family corresponded to one 6 cell sector and one tray housed 8 families (Figure 2.8). Approximately 7 days post-transplanting, individual plant cells were inoculated using the same protocol as before. $F_{3:4}$ seed from plants without visible black shank symptoms was harvested.

Approximately 20 seeds from each selected $F_{3:4}$ family were germinated and families were examined to identify those segregating for *35S*:*FT*. Segregating $F_{3:4}$ families were selected so that each family could be traced back to a single F_2 individual. Six *35S*:*FT* plants from each $F_{3:4}$ line were then transplanted and grown in the Phytotron using the same protocol as before. Seven days after transplanting, individual cells were inoculated using the same protocol as previously described. $F_{4:5}$ seed was harvested from plants without visible black shank symptoms.

Selected $F_{4:5}$ families were germinated and screened for segregation of *35S*:*FT* as before. Following the modified SSD method, a single segregating $F_{4:5}$ family was selected per original F_2 individual. A single normal-flowering (non-*35S*:*FT*) $F_{4:5}$ individual was

identified for each selected family, grown in the greenhouse, and self-pollinated to produce $F_{5:6}$ seed that was not expected to segregate for *35S*:*FT*. For evaluation of field black shank resistance, 35 $F_{5:6}$ inbred lines were randomly selected from each cross (70 lines total).

Development of Random Lines

In order to evaluate the effectiveness of the system described above for selecting for black shank resistance, a random set of lines also was developed from each of the two initial crosses. Two-hundred *35S*:*FT* individuals from each of the two original F_2 populations were selected. The same procedures were followed during the inbreeding process, except that inoculations with the black shank pathogen were not performed. Furthermore, instead of using the Phytotron growth chamber, plants were grown in a greenhouse since the need for highly regulated conditions used to promote disease growth was not necessary. Thirty-five random $F_{5:6}$ inbred lines were selected from each cross for field testing of disease resistance (70 lines total).

Development of Inoculation Materials

Thirty days prior to the transplanting of seedlings, cultures of *P. nicotianae* were initiated on Petri dishes containing approximately 50 ml of carrot agar. Carrot agar was generated by autoclaving 50 ml of carrot juice (Bolthouse Juice Products LLC, Bakersfield, CA) with 20 grams of agar (Sigma Chemical Co. St Louis, MO) in 950 ml of distilled H_2O. An agar plug containing a race 0 isolate of *P. nicotianae* (provided by Dr. David Shew, NCSU, Raleigh, NC) was placed in the center of the carrot agar and allowed to grow until the agar was completely covered with hyphae (7-10 days). Inoculated Petri dishes were grown at

room temperature in complete darkness. Autoclaved oat grains were then spread across the agar in a single layer. Inoculated oat grains were grown at room temperature in complete darkness. Oat grains were considered ready for use once they were covered with *P. nicotianae* hyphae (15-21 days).

DNA-Based Verification of *35S*:*FT* Null Status in Derived Lines

In order to verify that field tested lines did not carry a silenced *35S*:*FT* transgene insertion, selected 'normal-flowering' $F_{4:5}$ individuals were genotyped for the presence of the *FT* coding sequence, the CaMV 35S promoter, and the *npt*II selectable marker gene. DNA isolation, PCR conditions, and primer sequences were previously outlined by Lewis and Kernodle (2009).

Evaluation for Field Black Shank Resistance

Thirty-five selected $F_{5:6}$ lines from each of the two crosses were evaluated for field black shank resistance. They were compared for resistance to 35 random $F_{5:6}$ lines from each of the two pedigrees. Also included in the field experiments were each of the parental lines (TN 90LC, NC1209-23, K 346) and a non-*35S*:*FT* line derived by self-pollinating a normal-flowering plant of K 346*FT*. Field black shank resistance was evaluated in black shank nurseries at three locations (the Lower Coastal Plain Tobacco Research Station, Kinston, NC; the Upper Coastal Plain Research Station, Rocky Mount, NC; and the Oxford Tobacco Research Station, Oxford, NC) in North Carolina during 2012. A randomized complete block design (RCBD) was used with three replications per environment. An experimental unit consisted of a single 12 plant row. Tobacco plants were transplanted and grown based on

common agricultural practices for flue-cured tobacco production in North Carolina (North Carolina Cooperative Extension, 2012).

Every 21 days post-transplanting, the number of plants killed by black shank in each plot was recorded. Four separate disease ratings were taken at the Lower Coastal Plain Research Station and the Oxford Tobacco Research Station. Five disease ratings were taken at the Upper Coastal Plain Research Station.

Statistical Analysis

Once the final disease ratings were taken, end percent survival (EPS) and area under the disease progress curve (AUDPC) were calculated for each plot. EPS was calculated by dividing the final number of surviving plants by the initial stand count. AUDPC was calculated using the method described by Madden et al. (2007).

After excluding the parental lines and checks, an analysis of variance was performed over locations using PROC GLM of SAS 9.2 (SAS Institute, Cary, NC) considering the nested treatment structure (pedigree, group within pedigree, and genotypes within group within pedigree). All factors except group (selected lines versus random lines) were considered as random factors. A square root transformation was performed on AUDPC data to reduce heterogeneous error variances. Transformed AUDPC values were used as the response variable in the ANOVA. Entry means were calculated using the LSMEANS statement. Dunnett's procedure (Dunnett, 1964) was used to compare the resistance of each genotype to the highly resistant control variety, K 346, using the ADJUST= DUNNETT statement. Transformed AUDPC values were used for mean comparisons.

An additional ANOVA was conducted to determine if loci conditioning the yellow burley phenotype were linked to black shank resistance loci. The analysis was carried out in the same manner as described above, except PROC MIXED was used so that the correct error term would be assigned for additional contrasts. Sixteen inbred lines displaying the burley phenotype were compared to sixteen inbred lines displaying the flue-cured phenotype. An equal number of lines (burley and flue-cured) were chosen from each pedigree and from each selection method within pedigree to prevent bias. A single degree of freedom contrast was performed between burley and flue-cured lines.

RESULTS

Field Evaluation of $F_{5:6}$ Inbred Lines for AUDPC

In the evaluation of the genetic materials over three locations, significant differences were observed among locations, replications, and genotypes, and between the selected and random groups (Table 2.1). Although a significant genotype x location interaction was observed, location was considered a random factor and genotype means were averaged over locations. The selected $F_{5:6}$ inbred population had an average untransformed AUDPC value that was 513.4 lower than that for the randomly-selected population (Figure 2.1, Figure 2.9). The untransformed AUDPC least square means for the selected population, the random population, and the parental lines were 1370.66, 1883.41 and 1789.23, respectively (Figure 2.1). No statistically significant difference for AUDPC was observed between K 346 and the K 346 BC_7F_3 null segregant line (Table 2.6). Twenty-six lines from the selected group of $F_{5:6}$ lines and nine lines from the random group had an AUDPC value that was numerically

superior to that of resistant check cultivar, K 346 (Table 2.6). Only three lines from the selected population and one line from the randomly-selected population had an AUDPC value that was significantly lower (better) than that for K 346, however (Table 2.6). Thirteen lines from the selected population and twenty-two lines from the random group had an AUDPC value that was significantly higher (worse) than that for check cultivar K 346 (Table 2.6).

Field Evaluation of $F_{5:6}$ Lines for EPS

In the evaluation of the genetic materials over three locations, significant differences were observed between replications, genotypes, and between the selected and random groups of lines (Table 2.2). A significant genotype x location interaction also was observed for EPS, but data were averaged over locations as was done for AUDPC. The EPS least square means for the selected population, the randomly-selected population and the parental lines were 56.8%, 44.7%, and 46.4%, respectively (Figure 2.2). No statistically significant difference was observed for EPS between K 346 and the K 346 BC_7F_3 null segregant line (Table 2.7). Twenty-three lines from the selected population and ten lines from the random population had an EPS value that was numerically superior to that of resistant check cultivar K 346 (Table 2.7). Zero lines from the selected or random groups of lines had a statistically significant EPS value greater than that for K 346, however (Table 2.7). Seventeen lines from the selected population and 29 lines from the random-selected population had EPS values that were significantly lower than that for the check cultivar, K 346 (Table 2.7).

Linkage Between Black Shank Resistance Alleles and Alleles Affecting the *Yellow Burley* Phenotype

The flue-cured tobacco cultivar K 346 differs from the burley tobacco parental lines TN 90LC and NC1209-23 by having dominant alleles at the *Yellow Burley 1* and *Yellow Burley 2* loci. The double recessive genotype $yb_1yb_1yb_2yb_2$ results in the chlorophyll-deficient genotype exhibited by burley tobacco cultivars. We were interested in the possibility that alleles affecting black shank resistance/susceptibility may be genetically linked to these two loci. Results from a Chi-square analysis (Table 2.5) showed no significant difference between the number of observed $F_{5:6}$ inbred lines displaying the burley phenotype in the group of lines selected for black shank resistance and the number of lines expected to display the burley phenotype in the absence of selection. Results from the single degree of freedom contrast between the $yb_1yb_1yb_2yb_2$ (burley phenotype) and $Yb_1Yb_1Yb_2Yb_2$ (normal phenotype) groups for AUDPC and EPS showed no significant differences (Table 2.3, Table 2.4).

DISCUSSION

Using the *FT*-based inbreeding approach, four generations of inbreeding were performed in less than 300 days (three with selection for black shank resistance). Visual selection against *35S*:*FT* resulted in the successful generation of normal-flowering $F_{5:6}$ inbred lines of tobacco in nearly one-half the time required using conventional inbreeding methods. PCR screening of normal-flowering individuals confirmed the absence of any lingering transgene components.

In order to maintain genetic variability throughout the inbreeding process, each F_2 individual was allowed to contribute only one $F_{5:6}$ line for field evaluation. Significant differences were found between random $F_{5:6}$ lines and lines selected for black shank resistance for both AUDPC and EPS. These results demonstrated that the method was effective in shifting the population mean in the desired direction for black shank resistance.

Selection during the inbreeding process was performed using a race 0 isolate of *P. nicotianae*. The isolate used was chosen based on its highly aggressive nature, being much more aggressive than most race 1 isolates that were available. Since selection was performed for alleles affecting partial resistance (which is usually race non-specific), it was predicted that derived lines would also exhibit resistance to isolates other than race 0. Two of the three field disease nurseries used to evaluate the derived lines had a prevalence of race 1. As the shift in the population mean illustrated, selection using a race 0 isolate was effective in producing lines with a high level of field resistance to race 1.

Within the selected population, there were individual lines that had relatively poor EPS and AUDPC values. The selection system might be improved by performing inoculations with pathogen isolates with a greater level of aggressiveness or by increasing inoculum density. The system of inoculating segregating populations of *35S*:*FT* tobacco plants with *Phytophthora nicotianae* offers an advantage over field selection because selection for soil-borne disease resistance in the field can be conducted only one time per year.

There was some question about whether or not loci associated with the 'yellow burley' phenotype might be genetically linked with loci affecting black shank resistance or

susceptibility. If this was the case, it was expected that in the population where selection was imposed, significant deviations from the expected number of lines displaying the burley phenotype would exist. Results from the Chi-square analysis did not support this hypothesis, however. In addition, the results from the ANOVA between inbred lines which had the burley phenotype and inbred lines which did not, no significant differences for disease resistance were found between the groups.

As this experiment has shown, the modified system of inbreeding based on expression of *35S*:*FT*, coupled with selection for disease resistance, had an impact on genetic gain per year. In its simplest form, gain from selection for a plant breeder is explained by the equation $R = h^2S$, where R is the response from selection, h^2 is the narrow sense heritability, and S is the selection differential. Expanding the formula to estimate genetic gain per year, the number of generations of selection which can be performed within a single year is added to the equation. Reducing time expended on inbreeding reduced the duration required to complete a breeding cycle and consequently increased genetic gain per year. This *35S*:*FT*-method not only drastically reduced generation time (allowing for as many as five generations per year), but it also allowed for selection to be performed during each generation of inbreeding. As a result, the modified method was used to affect significant genetic gain in the short term and long term.

Although this modified method was successful in reducing the time required to complete a breeding cycle, there are some inconveniences that must be mentioned. First, in order for this method to work, a plant breeder must have access to the transgene in a suitable

parental line. If the method were used extensively over time, however, a breeding program would likely possess many potential parental lines segregating for *35S*:*FT*.

Second, performing visual selection in each generation for families segregating for *35S*:*FT* was an inconvenience. Identifying segregating families was only accomplished by careful visual selection for morphological traits which help distinguish normal flowering individuals from transgenic individuals. Screening in this manner only confirmed that the individual that was self-pollinated to create the family was heterozygous, however. It did not allow for the differentiation of heterozygous and homozygous individuals in the same generation. Although differentiation could be performed using genetic markers, a faster visual selection scheme would be ideal. We are currently unaware of a suitable method for visibly distinguishing *35S*:*FT* heterozygotes from homozygotes.

Finally, although selection can be performed for disease resistance (and maybe some other simply inherited characteristics) in each generation, phenotypic selection for some of the most economically important traits (such as yield) is not possible. Single seed descent (SSD) and doubled haploid (DH) breeding methods are also subjected to the same limitations. A multitude of visible qualitative characters such as simply-inherited disease resistance traits or characters for which markers are available could be easily selected for during the modified system of inbreeding described here, however. The system is also amenable to marker-based F_2 enrichment of desirable alleles or potentially additional methods designed to enhance the frequency of desirable alleles in segregating populations.

CONCLUSIONS

The development and execution of this novel *FT*-based breeding method and the evaluation of the inbred populations developed as a result showed that accelerated inbreeding and phenotypic selection for black shank resistance was an effective strategy. In addition to the success displayed for black shank resistance, this method may be used for the development of resistant inbred lines for other diseases of tobacco, and equally as important, for other diseases in different crop species in the future.

REFERENCES

Afanador, L.K., Haley, S.D. and J.D. Kelly. 1993 Adoption of a "mini-prep" DNA extraction method for RAPD marker analysis in common bean (*Phaseolus vulgaris* L.). Annual Report: Bean Improvement Cooperative 36:10-11.

Apple, J.L. 1962. Transfer of resistance to black shank (*Phytophthora parasitica* var *nicotianae*) from *Nicotiana plumbaginifolia* to *N. tabacum*. Phytopathol. (Abstr.) 52:1.

Dunnett, C.W. 1964. New tables for multiple comparisons with a control. Biometrics 20, 482-491.

Jaarsveld, E.M., M. Wingfield, and A. Drenth. 2003. A rapid seedling based screening technique to assay tobacco for resistance to *Phytophthora nicotianae*. J. Phytopathol. 151:389-394.

Johnson, E., Miklas, P.N., Stavely, J.R. and J.C Martinez-Cruzado. 1995. Coupling- and repulsion-phase RAPDs for marker-assisted selection of PI 181996 rust resistance in common bean. Theor. Appl. Gen. 90:659-664.

Kasperbauer, M.J. 1966. Interaction of photoperiod and temperature on flowering of burley tobacco. Tob. Sci. 10:119–120.

Kasperbauer, M.J. 1969. Photo- and thermo-control of pretransplant floral induction in burley tobacco. Agron. J. 61:898–902.

Kasperbauer, M.J., and R.H. Lowe. 1966. Flowering of three types of *Nicotiana tabacum* under controlled-environments. Tob. Sci. 10:107–108.

Lang, A., Chailakhyan, MKh,, and I.A. Frolova. 1977. Promotion and inhibition of flower formation in a day neutral plant in grafts with short-day and a long-day plant. Proc. Natl. Acad. Sci. USA 74:2412–2416.

Lewis, R.S., and S.P. Kernodle. 2009. A method for accelerated trait conversion in plant breeding. Theor. Appl. Genet. 118:1499–1508.

Lucas, G.B. 1975. Disease of Tobacco: 3rd Edition, Raleigh, NC: Biological Consulting Associates. p. 115-141.

Madden, L.V., Hughes, G. and F. van der Bosch. 2007. *The Study of Plant Disease Epidemics*, St. Paul: APS Press.

Mila, A., and J. Radcliff. 2010. Flue-Cured Tobacco Disease Report. http://www.ncsu.edu/project/tobaccoportal/wp-content/uploads/2010/12/2010-Disease-Reports.pdf.

North Carolina Cooperative Extension. 2012. Flue-Cured Tobacco Guide, Raleigh, NC: North Carolina State University.

Pinstrup-Andersen, P. 2001. The future world food situation and the role of plant diseases. The Plant Health Instructor. DOI: 10.1094/PHI-I-2001-0425-01.

Sullivan, M.J., T.A. Melton, and H.D. Shew. 2005. Managing the race structure of *Phytophthora parasitica* var. *nicotianae* with cultivar rotation. Plant Dis. 89:1285-1294.

Tisdale, W.B. 1931. Development of strains of cigar wrapper tobacco resistant to black shank *(Phytophthora nicotianae* Breda de Haan). Fla. Agr. Exp. Stn. Bull. 226.

Tisdale, W. B., and J. G. Kelley 1926. A *Phytophthora* disease of tobacco. Florida Agric. Exp. Sta. Bull. 179, 159–219.

Valleau, W.D., G.W. Stokes, and E.M. Johnson. 1960. Nine years experience with the *Nicotiana longiflora* factor for resistance to *Phytophthora parasitica* var. *nicotianae* in the control of black shank. Tob. Sci. 4:92-94.

Table 2.1. AUDPC ANOVA for field evaluation of selected and randomly derived $F_{5:6}$ lines.

Source	DF[a]	SS[b]	MS[c]	F-Value	P-Value
Location	2	66984	33492	7.5	0.021
Replication(Location)	6	25669	4278	44.4	< 0.0001
Pedigree	1	3004	3004	0.4	0.612
Group(Pedigree)	2	17038	8519	4.6	0.016
Genotype(Group(Pedigree))	136	203630	1497	7.7	< 0.0001
Location x Pedigree	2	623	311	0.6	0.596
Location x Group(Pedigree)	4	2178	545	2.7	0.051
Location x Genotype(Group(Pedigree))	272	53103	195	1.6	< 0.0001
Replication(Location) x Pedigree	6	578	96	0.8	0.611
Replication(Location) x Group (Pedigree)	12	1510	126	1.1	0.399
Error	816	97674	120	-	-

[a]DF, Degrees of Freedom; [b]SS, Sum of Squares, [c]MS, Mean Squares;

Table 2.2. EPS ANOVA for field evaluation of selected and randomly derived $F_{5:6}$ lines.

Source	DF[a]	SS[b]	MS[c]	F-Value	P-Value
Location	2	3.23	1.61	0.8	0.501
Replication(Location)	6	11.94	1.99	54.1	< 0.0001
Pedigree	1	1.47	1.47	0.6	0.514
Group(Pedigree)	2	4.73	2.36	5.0	0.009
Genotype(Group(Pedigree))	136	61.02	0.45	7.2	< 0.0001
Location x Pedigree	2	0.28	0.14	1.4	0.334
Location x Group(Pedigree)	4	0.34	0.08	2.0	0.107
Location x Genotype(Group(Pedigree))	272	16.91	0.06	1.6	< 0.0001
Replication(Location) x Pedigree	6	0.22	0.04	2.0	0.139
Replication(Location) x Group (Pedigree)	12	0.22	0.02	0.5	0.937
Error	816	31.95	0.04	-	-

[a]DF, Degrees of Freedom; [b]SS, Sum of Squares, [c]MS, Mean Squares;

Table 2.3. AUDPC ANOVA for field evaluation of burley and flue-cured phenotypes and single degree of freedom contrast between phenotypes across pedigrees.

Source	DF[a]	SS[b]	MS[c]	F-Value	P-Value
Location	2	12766	6382.96	6.0	0.0441
Replication(Location)	6	4975.06	829.18	4.2	0.053
Pedigree	1	2728.24	2728.24	0.4	0.6134
Group(Pedigree)	2	15600	7800.10	4.5	0.0239
Genotype(Group(Pedigree))	28	36259	1294.97	8.0	<.0001
Location x Pedigree	2	870.69	435.35	0.7	0.5599
Location x Group(Pedigree)	4	2368.84	592.21	3.5	0.0346
Location x Genotype(Group(Pedigree))	56	9076.79	162.09	1.3	0.1173
Replication(Location) x Pedigree	6	1192.94	198.82	1.5	0.2584
Replication(Location) x Group (Pedigree)	12	1589.91	132.49	1.1	0.4089
Error	168	21268	126.60	.	.
Burley vs. Flue-Cured Contrast	1			0.71	0.4006

[a]DF, Degrees of Freedom; [b]SS, Sum of Squares, [c]MS, Mean Squares;

Table 2.4. AUDPC ANOVA for field evaluation of burley and flue-cured phenotypes and single degree of freedom contrast between phenotypes across pedigrees.

Source	DF[a]	SS[b]	MS[c]	F-Value	P-Value
Location	2	0.44	0.22	0.5	0.6255
Replication(Location)	6	1.95	0.32	7.2	0.0151
Pedigree	1	0.98	0.98	0.4	0.5719
Group(Pedigree)	2	4.30	2.15	4.9	0.0163
Genotype(Group(Pedigree))	28	11.25	0.40	6.6	<.0001
Location x Pedigree	2	0.31	0.16	1.3	0.3396
Location x Group(Pedigree)	4	0.38	0.10	2.3	0.1108
Location x Genotype(Group(Pedigree))	56	3.43	0.06	1.4	0.0401
Replication(Location) x Pedigree	6	0.27	0.05	2.0	0.1499
Replication(Location) x Group (Pedigree)	12	0.28	0.02	0.5	0.8864
Error	168	7.15	0.04	.	.
Burley vs. Flue-Cured Contrast	1			0.54	0.4652

[a]DF, Degrees of Freedom; [b]SS, Sum of Squares, [c]MS, Mean Squares;

Table 2.5. Chi-square test for the number of observed vs. expected $F_{5:6}$ inbred lines from the selected group that were non-segregating for the burley phenotype.

Category	**Observed**	**Expected**	**|O -E|**	**$(O — E)^2$**	**$(O — E)^2 / E$**
Burley Phenotype	9	15.38	-6.38	40.71	2.64
Other	61	54.61	6.38	40.71	0.74
					$\chi^2 = 3.39$ Critical Value = 3.84

Table 2.6. Transformed AUDPC least square means for each experimental entry and adjusted *P*-values for comparison with the mean for K346 using Dunnett's procedure. Lines from the random group are indicated in red, while those presented in black are from the selected group. Lines with blue text are parental or control lines.

Genotype	Sqrt. AUDPC LSMEAN	Adjusted P-Value
TN90/K346 FT Selected Line 22, F5:6 Seed	3.23	0.000
TN90/K346 FT Selected Line 11, F5:6 Seed	6.49	0.006
NC1209-23/K346 FT Random Line 6, F5:6 Seed	9.38	0.039
TN90/K346 FT Selected Line 23, F5:6 Seed	9.40	0.040
TN90/K346 FT Random Line 24, F5:6 Seed	10.06	0.058
TN90/K346 FT Random Line 23, F5:6 Seed	10.16	0.061
TN90/K346 FT Selected Line 21, F5:6 Seed	11.13	0.102
TN90/K346 FT Selected Line 12, F5:6 Seed	11.15	0.103
TN90/K346 FT Selected Line 3, F5:6 Seed	11.65	0.132
NC1209-23/K346 FT Selected Line 19, F5:6 Seed	12.46	0.191
TN90/K346 FT Random Line 7, F5:6 Seed	13.10	0.250
NC1209-23/K346 FT Selected Line 6, F5:6 Seed	13.69	0.315
TN90/K346 FT Selected Line 19, F5:6 Seed	16.29	0.709
TN90/K346 FT Selected Line 27, F5:6 Seed	16.51	0.744
TN90/K346 FT Selected Line 5, F5:6 Seed	17.48	0.887
NC1209-23/K346 FT Selected Line 3, F5:6 Seed	18.19	0.956
NC1209-23/K346 FT Selected Line 5, F5:6 Seed	18.30	0.963
TN90/K346 FT Random Line 20, F5:6 Seed	18.38	0.968
TN90/K346 FT Selected Line 14, F5:6 Seed	18.68	0.982
NC1209-23/K346 FT Selected Line 7, F5:6 Seed	18.70	0.983
TN90/K346 FT Selected Line 8, F5:6 Seed	19.26	0.996
NC1209-23/K346 FT Random Line 14, F5:6 Seed	20.67	1.000
TN90/K346 FT Selected Line 15, F5:6 Seed	21.06	1.000
TN90/K346 FT Selected Line 28, F5:6 Seed	21.68	1.000
TN90/K346 FT Selected Line 16, F5:6 Seed	21.87	1.000
TN90/K346 FT Random Line 22, F5:6 Seed	21.90	1.000
NC1209-23/K346 FT Random Line 8, F5:6 Seed	23.06	1.000
NC1209-23/K346 FT Selected Line 20, F5:6 Seed	23.60	1.000
NC1209-23/K346 FT Selected Line 12, F5:6 Seed	24.22	1.000
TN90/K346 FT Selected Line 35, F5:6 Seed	25.40	1.000
NC1209-23/K346 FT Selected Line 13, F5:6 Seed	25.53	1.000
NC1209-23/K346 FT Selected Line 14, F5:6 Seed	26.04	1.000
TN90/K346 FT Random Line 9, F5:6 Seed	26.19	1.000

Table 2.6 Continued.

Genotype	Sqrt. AUDPC LSMEAN	Adjusted P-Value
NC1209-23/K346 FT Selected Line 22, F5:6 Seed	27.07	1.000
NC1209-23/K346 FT Selected Line 31, F5:6 Seed	27.13	1.000
K346	27.32	-
NC1209-23/K346 FT Selected Line 33, F5:6 Seed	27.59	1.000
TN90/K346 FT Random Line 12, F5:6 Seed	27.82	1.000
NC1209-23/K346 FT Selected Line 11, F5:6 Seed	27.95	1.000
NC1209-23/K346 FT Selected Line 8, F5:6 Seed	28.05	1.000
NC1209-23/K346 FT Selected Line 1, F5:6 Seed	28.21	1.000
NC1209-23/K346 FT Random Line 11, F5:6 Seed	29.05	1.000
NC1209-23/K346 FT Random Line 16, F5:6 Seed	29.20	1.000
NC1209-23/K346 FT Selected Line 27, F5:6 Seed	29.22	1.000
TN90/K346 FT Random Line 34, F5:6 Seed	29.32	1.000
NC1209-23/K346 FT Selected Line 24, F5:6 Seed	29.50	1.000
NC1209-23/K346 FT Random Line 27, F5:6 Seed	29.90	1.000
NC1209-23/K346 FT Random Line 9, F5:6 Seed	29.91	1.000
NC1209-23/K346 FT Random Line 2, F5:6 Seed	30.10	1.000
NC1209-23/K346 FT Selected Line 21, F5:6 Seed	30.14	1.000
TN90/K346 FT Random Line 5, F5:6 Seed	30.32	1.000
NC1209-23/K346 FT Random Line 31, F5:6 Seed	30.38	1.000
NC1209-23/K346 FT Random Line 24, F5:6 Seed	31.03	1.000
TN90/K346 FT Random Line 1, F5:6 Seed	31.22	1.000
TN90/K346 FT Random Line 2, F5:6 Seed	31.77	1.000
K346 non-FT; BC7F3 Line	32.14	1.000
TN90/K346 FT Selected Line 10, F5:6 Seed	32.22	1.000
TN90/K346 FT Selected Line 32, F5:6 Seed	32.48	1.000
NC1209-23/K346 FT Random Line 34, F5:6 Seed	32.69	1.000
TN90/K346 FT Selected Line 9, F5:6 Seed	32.98	1.000
NC1209-23/K346 FT Selected Line 23, F5:6 Seed	33.14	1.000
TN90/K346 FT Selected Line 13, F5:6 Seed	33.28	1.000
NC1209-23/K346 FT Selected Line 32, F5:6 Seed	33.29	1.000
TN90/K346 FT Random Line 8, F5:6 Seed	33.73	1.000
TN90/K346 FT Selected Line 25, F5:6 Seed	33.75	1.000
NC1209-23/K346 FT Selected Line 35, F5:6 Seed	33.93	1.000
NC1209-23/K346 FT Random Line 10, F5:6 Seed	34.00	1.000

Table 2.6 Continued.

Genotype	Sqrt. AUDPC LSMEAN	Adjusted P-Value
NC1209-23/K346 FT Selected Line 10, F5:6 Seed	34.00	1.000
TN90/K346 FT Selected Line 4, F5:6 Seed	34.13	1.000
NC1209-23/K346 FT Random Line 12, F5:6 Seed	34.25	1.000
NC1209-23/K346 FT Random Line 3, F5:6 Seed	34.58	1.000
TN90/K346 FT Random Line 27, F5:6 Seed	34.61	1.000
TN90/K346 FT Selected Line 29, F5:6 Seed	34.95	0.999
TN90/K346 FT Random Line 15, F5:6 Seed	35.14	0.998
NC1209-23/K346 FT Selected Line 26, F5:6 Seed	35.53	0.994
TN90/K346 FT Selected Line 7, F5:6 Seed	35.53	0.994
NC1209-23/K346 FT Selected Line 17, F5:6 Seed	35.91	0.984
TN90/K346 FT Random Line 16, F5:6 Seed	36.00	0.981
TN90/K346 FT Random Line 19, F5:6 Seed	36.28	0.966
TN90/K346 FT Random Line 10, F5:6 Seed	36.38	0.960
TN90/K346 FT Random Line 17, F5:6 Seed	36.46	0.955
TN90/K346 FT Selected Line 31, F5:6 Seed	36.60	0.944
TN90/K346 FT Random Line 6, F5:6 Seed	36.72	0.933
TN90/K346 FT Selected Line 33, F5:6 Seed	37.30	0.868
NC1209-23/K346 FT Selected Line 4, F5:6 Seed	37.93	0.776
NC1209-23/K346 FT Random Line 17, F5:6 Seed	40.86	0.325
TN90/K346 FT Selected Line 26, F5:6 Seed	41.04	0.304
NC1209-23/K346 FT Random Line 18, F5:6 Seed	41.17	0.289
TN90/K346 FT Random Line 28, F5:6 Seed	41.45	0.259
TN90/K346 FT Random Line 18, F5:6 Seed	41.62	0.242
TN90/K346 FT Random Line 26, F5:6 Seed	41.76	0.227
TN90	41.91	0.214
NC1209-23/K346 FT Random Line 29, F5:6 Seed	42.02	0.204
NC1209-23/K346 FT Selected Line 9, F5:6 Seed	42.64	0.155
TN90/K346 FT Selected Line 30, F5:6 Seed	42.73	0.148
TN90/K346 FT Selected Line 18, F5:6 Seed	42.73	0.148
NC1209-23/K346 FT Selected Line 25, F5:6 Seed	42.91	0.137
NC1209-23/K346 FT Random Line 21, F5:6 Seed	43.03	0.129
TN90/K346 FT Random Line 21, F5:6 Seed	43.19	0.119
TN90/K346 FT Random Line 35, F5:6 Seed	43.36	0.110
TN90/K346 FT Random Line 14, F5:6 Seed	43.62	0.096

Table 2.6 Continued.

Genotype	Sqrt. AUDPC LSMEAN	Adjusted P-Value
NC1209-23/K346 FT Random Line 5, F5:6 Seed	43.66	0.095
TN90/K346 FT Random Line 13, F5:6 Seed	43.92	0.083
NC1209-23/K346 FT Selected Line 2, F5:6 Seed	44.25	0.069
NC1209-23/K346 FT Random Line 4, F5:6 Seed	44.58	0.058
TN90/K346 FT Random Line 33, F5:6 Seed	44.61	0.057
TN90/K346 FT Random Line 30, F5:6 Seed	44.64	0.056
NC1209-23/K346 FT Selected Line 18, F5:6 Seed	44.71	0.054
TN90/K346 FT Selected Line 20, F5:6 Seed	45.53	0.033
NC1209-23/K346 FT Random Line 33, F5:6 Seed	45.91	0.027
NC1209-23/K346 FT Selected Line 15, F5:6 Seed	46.04	0.025
TN90/K346 FT Random Line 11, F5:6 Seed	46.33	0.020
TN90/K346 FT Random Line 31, F5:6 Seed	47.01	0.013
NC1209-23/K346 FT Selected Line 16, F5:6 Seed	47.46	0.010
NC1209-23/K346 FT Random Line 23, F5:6 Seed	48.12	0.006
NC1209-23/K346 FT Random Line 15, F5:6 Seed	48.27	0.005
NC1209-23/K346 FT Random Line 35, F5:6 Seed	48.30	0.005
NC1209-23/K346 FT Selected Line 29, F5:6 Seed	48.36	0.005
NC1209-23/K346 FT Random Line 25, F5:6 Seed	48.51	0.005
NC1209-23/K346 FT Random Line 13, F5:6 Seed	48.52	0.005
NC1209-23/K346 FT Selected Line 28, F5:6 Seed	48.81	0.004
NC1209-23/K346 FT Selected Line 30, F5:6 Seed	48.98	0.003
NC1209-23/K346 FT Random Line 26, F5:6 Seed	50.34	0.001
TN90/K346 FT Selected Line 24, F5:6 Seed	51.57	0.000
TN90/K346 FT Selected Line 2, F5:6 Seed	51.72	0.000
TN90/K346 FT Selected Line 17, F5:6 Seed	52.02	0.000
NC1209-23/K346 FT Random Line 30, F5:6 Seed	52.43	0.000
NC1209-23/K346 FT Random Line 32, F5:6 Seed	52.84	0.000
NC1209-23	52.85	0.000
NC1209-23/K346 FT Random Line 22, F5:6 Seed	52.89	0.000
TN90/K346 FT Selected Line 1, F5:6 Seed	53.15	<.0001
TN90/K346 FT Random Line 32, F5:6 Seed	53.70	<.0001
TN90/K346 FT Random Line 4, F5:6 Seed	53.73	<.0001
NC1209-23/K346 FT Random Line 19, F5:6 Seed	53.81	<.0001
NC1209-23/K346 FT Random Line 28, F5:6 Seed	53.83	<.0001

Table 2.6 Continued.

Genotype	Sqrt. AUDPC LSMEAN	Adjusted P-Value
TN90/K346 FT Selected Line 34, F5:6 Seed	53.98	<.0001
NC1209-23/K346 FT Random Line 20, F5:6 Seed	55.08	<.0001
NC1209-23/K346 FT Random Line 1, F5:6 Seed	56.82	<.0001
TN90/K346 FT Selected Line 6, F5:6 Seed	57.35	<.0001
TN90/K346 FT Random Line 3, F5:6 Seed	57.73	<.0001
TN90/K346 FT Random Line 25, F5:6 Seed	58.09	<.0001
NC1209-23/K346 FT Selected Line 34, F5:6 Seed	60.61	<.0001
NC1209-23/K346 FT Random Line 7, F5:6 Seed	61.81	<.0001
TN90/K346 FT Random Line 29, F5:6 Seed	62.24	<.0001

Table 2.7. Least square means for EPS for each experimental entry and adjusted *P*-values for comparison with the mean for K346 using Dunnett's procedure. Lines from the random group are indicated in red, while those presented in black are from the selected group. Lines with blue text are parental or control lines.

Genotype	EPS LSMEAN	Adjusted P-Value
TN90/K346 FT Random Line 23, F5:6 Seed	0.954	0.269
TN90/K346 FT Selected Line 22, F5:6 Seed	0.954	0.269
TN90/K346 FT Selected Line 11, F5:6 Seed	0.951	0.284
TN90/K346 FT Selected Line 23, F5:6 Seed	0.944	0.333
NC1209-23/K346 FT Random Line 6, F5:6 Seed	0.923	0.490
TN90/K346 FT Selected Line 21, F5:6 Seed	0.916	0.555
TN90/K346 FT Random Line 24, F5:6 Seed	0.905	0.655
TN90/K346 FT Random Line 7, F5:6 Seed	0.886	0.828
TN90/K346 FT Selected Line 12, F5:6 Seed	0.880	0.873
TN90/K346 FT Selected Line 27, F5:6 Seed	0.878	0.885
NC1209-23/K346 FT Selected Line 19, F5:6 Seed	0.875	0.901
TN90/K346 FT Selected Line 19, F5:6 Seed	0.850	0.992
TN90/K346 FT Selected Line 3, F5:6 Seed	0.841	0.998
TN90/K346 FT Selected Line 16, F5:6 Seed	0.838	0.999
TN90/K346 FT Selected Line 8, F5:6 Seed	0.830	1.000
TN90/K346 FT Selected Line 5, F5:6 Seed	0.829	1.000
NC1209-23/K346 FT Selected Line 3, F5:6 Seed	0.822	1.000
TN90/K346 FT Random Line 20, F5:6 Seed	0.818	1.000
TN90/K346 FT Selected Line 14, F5:6 Seed	0.816	1.000
NC1209-23/K346 FT Random Line 14, F5:6 Seed	0.796	1.000
TN90/K346 FT Selected Line 28, F5:6 Seed	0.793	1.000
TN90/K346 FT Random Line 22, F5:6 Seed	0.774	1.000
NC1209-23/K346 FT Selected Line 20, F5:6 Seed	0.769	1.000
TN90/K346 FT Selected Line 15, F5:6 Seed	0.765	1.000
NC1209-23/K346 FT Selected Line 6, F5:6 Seed	0.759	1.000
NC1209-23/K346 FT Selected Line 14, F5:6 Seed	0.756	1.000
NC1209-23/K346 FT Selected Line 33, F5:6 Seed	0.747	1.000
NC1209-23/K346 FT Random Line 8, F5:6 Seed	0.746	1.000
NC1209-23/K346 FT Selected Line 13, F5:6 Seed	0.737	1.000
TN90/K346 FT Random Line 12, F5:6 Seed	0.737	1.000
NC1209-23/K346 FT Selected Line 5, F5:6 Seed	0.731	1.000
TN90/K346 FT Selected Line 35, F5:6 Seed	0.719	1.000
NC1209-23/K346 FT Random Line 31, F5:6 Seed	0.709	1.000

Table 2.7 Continued.

Genotype	EPS LSMEAN	Adjusted P-Value
K346	0.699	_
TN90/K346 FT Random Line 9, F5:6 Seed	0.688	1.000
NC1209-23/K346 FT Selected Line 31, F5:6 Seed	0.683	1.000
TN90/K346 FT Random Line 34, F5:6 Seed	0.672	1.000
NC1209-23/K346 FT Selected Line 12, F5:6 Seed	0.669	1.000
NC1209-23/K346 FT Selected Line 11, F5:6 Seed	0.657	1.000
NC1209-23/K346 FT Selected Line 7, F5:6 Seed	0.648	1.000
K346 non-FT; BC7F3 Line	0.639	1.000
NC1209-23/K346 FT Selected Line 27, F5:6 Seed	0.636	1.000
NC1209-23/K346 FT Random Line 2, F5:6 Seed	0.626	1.000
NC1209-23/K346 FT Selected Line 8, F5:6 Seed	0.622	1.000
NC1209-23/K346 FT Random Line 16, F5:6 Seed	0.616	1.000
NC1209-23/K346 FT Random Line 9, F5:6 Seed	0.600	1.000
NC1209-23/K346 FT Selected Line 22, F5:6 Seed	0.598	1.000
NC1209-23/K346 FT Random Line 34, F5:6 Seed	0.598	1.000
NC1209-23/K346 FT Selected Line 1, F5:6 Seed	0.596	1.000
NC1209-23/K346 FT Selected Line 21, F5:6 Seed	0.593	1.000
NC1209-23/K346 FT Random Line 12, F5:6 Seed	0.586	1.000
TN90/K346 FT Random Line 1, F5:6 Seed	0.582	1.000
NC1209-23/K346 FT Selected Line 10, F5:6 Seed	0.581	1.000
TN90/K346 FT Random Line 5, F5:6 Seed	0.581	1.000
NC1209-23/K346 FT Selected Line 35, F5:6 Seed	0.580	1.000
TN90/K346 FT Selected Line 32, F5:6 Seed	0.579	1.000
NC1209-23/K346 FT Random Line 27, F5:6 Seed	0.579	1.000
TN90/K346 FT Selected Line 13, F5:6 Seed	0.577	1.000
NC1209-23/K346 FT Selected Line 23, F5:6 Seed	0.577	1.000
NC1209-23/K346 FT Selected Line 24, F5:6 Seed	0.574	1.000
TN90/K346 FT Random Line 2, F5:6 Seed	0.561	0.999
NC1209-23/K346 FT Random Line 10, F5:6 Seed	0.557	0.998
TN90/K346 FT Selected Line 9, F5:6 Seed	0.557	0.998
NC1209-23/K346 FT Random Line 11, F5:6 Seed	0.556	0.997
TN90/K346 FT Selected Line 7, F5:6 Seed	0.546	0.988
NC1209-23/K346 FT Random Line 24, F5:6 Seed	0.535	0.960
TN90/K346 FT Selected Line 10, F5:6 Seed	0.532	0.947

Table 2.7 Continued.

Genotype	EPS LSMEAN	Adjusted P-Value
TN90/K346 FT Selected Line 33, F5:6 Seed	0.531	0.944
TN90/K346 FT Random Line 27, F5:6 Seed	0.526	0.917
TN90/K346 FT Selected Line 29, F5:6 Seed	0.526	0.916
TN90/K346 FT Random Line 8, F5:6 Seed	0.524	0.905
TN90/K346 FT Random Line 15, F5:6 Seed	0.524	0.901
NC1209-23/K346 FT Selected Line 17, F5:6 Seed	0.517	0.858
TN90/K346 FT Selected Line 4, F5:6 Seed	0.517	0.854
TN90/K346 FT Selected Line 25, F5:6 Seed	0.517	0.853
TN90/K346 FT Random Line 10, F5:6 Seed	0.513	0.828
TN90/K346 FT Random Line 17, F5:6 Seed	0.504	0.744
NC1209-23/K346 FT Selected Line 32, F5:6 Seed	0.495	0.663
TN90/K346 FT Random Line 16, F5:6 Seed	0.479	0.515
NC1209-23/K346 FT Random Line 3, F5:6 Seed	0.478	0.510
NC1209-23/K346 FT Random Line 18, F5:6 Seed	0.476	0.491
TN90/K346 FT Random Line 6, F5:6 Seed	0.471	0.453
TN90/K346 FT Selected Line 31, F5:6 Seed	0.469	0.437
NC1209-23/K346 FT Selected Line 26, F5:6 Seed	0.468	0.426
TN90/K346 FT Random Line 19, F5:6 Seed	0.464	0.394
TN90/K346 FT Random Line 14, F5:6 Seed	0.436	0.215
NC1209-23/K346 FT Selected Line 2, F5:6 Seed	0.431	0.193
TN90/K346 FT Random Line 30, F5:6 Seed	0.431	0.192
TN90/K346 FT Selected Line 18, F5:6 Seed	0.417	0.137
TN90/K346 FT Random Line 26, F5:6 Seed	0.409	0.110
NC1209-23/K346 FT Selected Line 4, F5:6 Seed	0.407	0.105
NC1209-23/K346 FT Random Line 17, F5:6 Seed	0.405	0.098
TN90/K346 FT Selected Line 26, F5:6 Seed	0.405	0.098
NC1209-23/K346 FT Random Line 29, F5:6 Seed	0.392	0.067
TN90/K346 FT Random Line 13, F5:6 Seed	0.389	0.062
TN90/K346 FT Random Line 28, F5:6 Seed	0.383	0.051
TN90/K346 FT Random Line 33, F5:6 Seed	0.378	0.044
TN90/K346 FT Random Line 18, F5:6 Seed	0.365	0.029
TN90/K346 FT Selected Line 30, F5:6 Seed	0.365	0.029
NC1209-23/K346 FT Selected Line 25, F5:6 Seed	0.365	0.028
TN90/K346 FT Random Line 21, F5:6 Seed	0.352	0.018

Table 2.7 Continued.

Genotype	EPS LSMEAN	Adjusted P-Value
TN90/K346 FT Random Line 31, F5:6 Seed	0.343	0.013
NC1209-23/K346 FT Selected Line 29, F5:6 Seed	0.337	0.010
TN90/K346 FT Random Line 11, F5:6 Seed	0.334	0.009
NC1209-23/K346 FT Random Line 21, F5:6 Seed	0.331	0.008
TN90/K346 FT Random Line 35, F5:6 Seed	0.330	0.008
NC1209-23/K346 FT Random Line 5, F5:6 Seed	0.328	0.008
TN90/K346 FT Selected Line 20, F5:6 Seed	0.327	0.007
NC1209-23/K346 FT Selected Line 9, F5:6 Seed	0.311	0.004
NC1209-23/K346 FT Random Line 13, F5:6 Seed	0.308	0.003
TN90	0.303	0.003
NC1209-23/K346 FT Random Line 33, F5:6 Seed	0.296	0.002
NC1209-23/K346 FT Random Line 4, F5:6 Seed	0.289	0.001
NC1209-23/K346 FT Selected Line 16, F5:6 Seed	0.287	0.001
NC1209-23/K346 FT Selected Line 18, F5:6 Seed	0.285	0.001
NC1209-23/K346 FT Selected Line 30, F5:6 Seed	0.266	0.001
NC1209-23/K346 FT Random Line 23, F5:6 Seed	0.260	0.000
NC1209-23/K346 FT Random Line 35, F5:6 Seed	0.256	0.000
NC1209-23/K346 FT Random Line 30, F5:6 Seed	0.247	0.000
TN90/K346 FT Random Line 4, F5:6 Seed	0.246	0.000
NC1209-23/K346 FT Selected Line 28, F5:6 Seed	0.241	0.000
TN90/K346 FT Selected Line 17, F5:6 Seed	0.232	<.0001
NC1209-23/K346 FT Selected Line 15, F5:6 Seed	0.222	<.0001
TN90/K346 FT Selected Line 24, F5:6 Seed	0.219	<.0001
NC1209-23	0.216	<.0001
NC1209-23/K346 FT Random Line 32, F5:6 Seed	0.214	<.0001
NC1209-23/K346 FT Random Line 22, F5:6 Seed	0.209	<.0001
TN90/K346 FT Selected Line 2, F5:6 Seed	0.208	<.0001
TN90/K346 FT Random Line 3, F5:6 Seed	0.205	<.0001
NC1209-23/K346 FT Random Line 15, F5:6 Seed	0.200	<.0001
TN90/K346 FT Selected Line 1, F5:6 Seed	0.188	<.0001
NC1209-23/K346 FT Random Line 25, F5:6 Seed	0.176	<.0001
NC1209-23/K346 FT Random Line 26, F5:6 Seed	0.176	<.0001
NC1209-23/K346 FT Random Line 20, F5:6 Seed	0.168	<.0001
TN90/K346 FT Random Line 32, F5:6 Seed	0.167	<.0001

Table 2.7 Continued.

Genotype	EPS LSMEAN	Adjusted P-Value
TN90/K346 FT Selected Line 34, F5:6 Seed	0.160	<.0001
NC1209-23/K346 FT Random Line 28, F5:6 Seed	0.149	<.0001
TN90/K346 FT Selected Line 6, F5:6 Seed	0.148	<.0001
NC1209-23/K346 FT Random Line 19, F5:6 Seed	0.136	<.0001
NC1209-23/K346 FT Random Line 1, F5:6 Seed	0.135	<.0001
NC1209-23/K346 FT Selected Line 34, F5:6 Seed	0.135	<.0001
TN90/K346 FT Random Line 25, F5:6 Seed	0.122	<.0001
NC1209-23/K346 FT Random Line 7, F5:6 Seed	0.121	<.0001
TN90/K346 FT Random Line 29, F5:6 Seed	0.066	<.0001

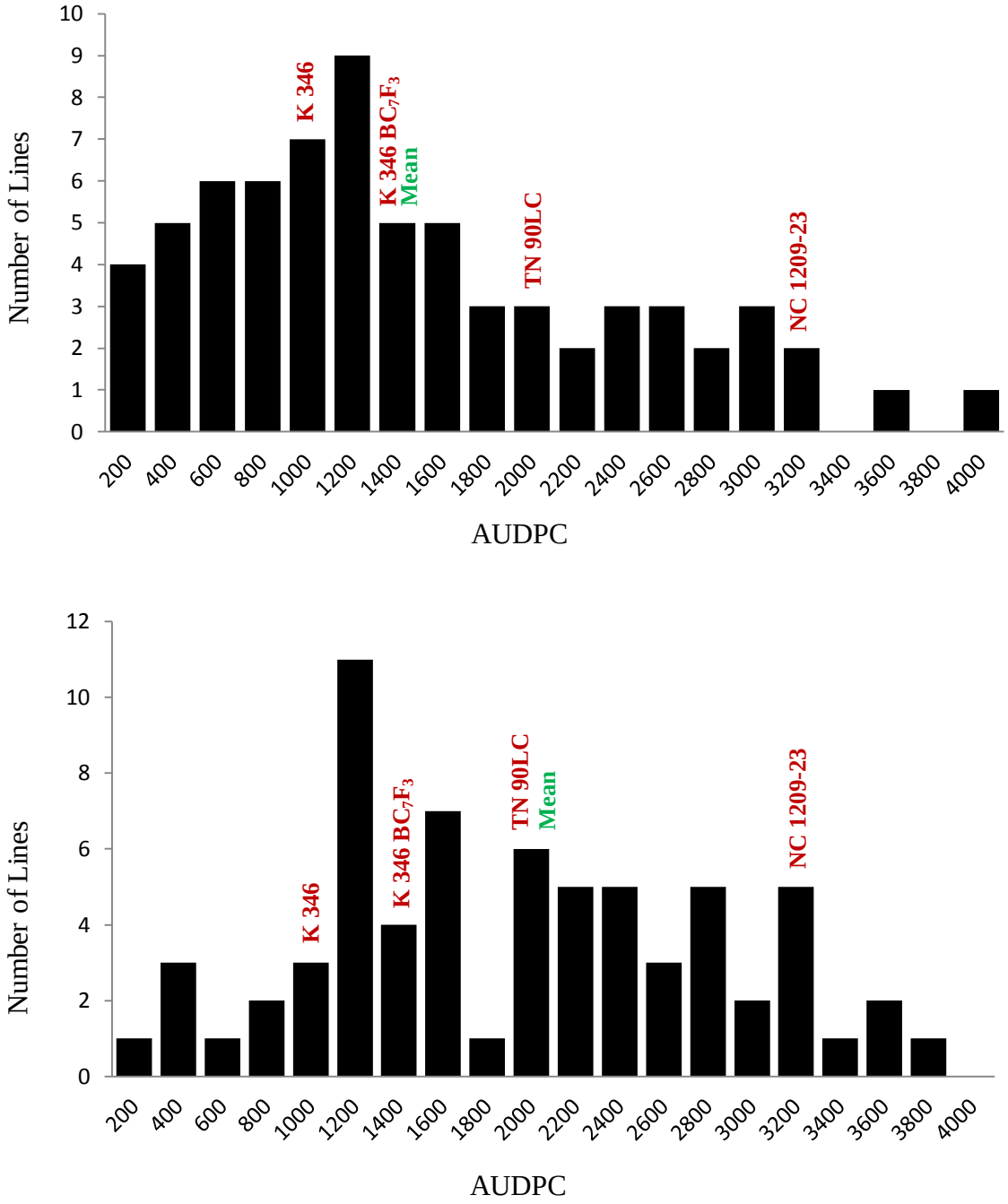


Figure 2.1. Frequency histogram for AUDPC of selected (top) and random groups (bottom) of $F_{5:6}$ inbred lines and parental checks evaluated over three disease nursery locations during 2012.

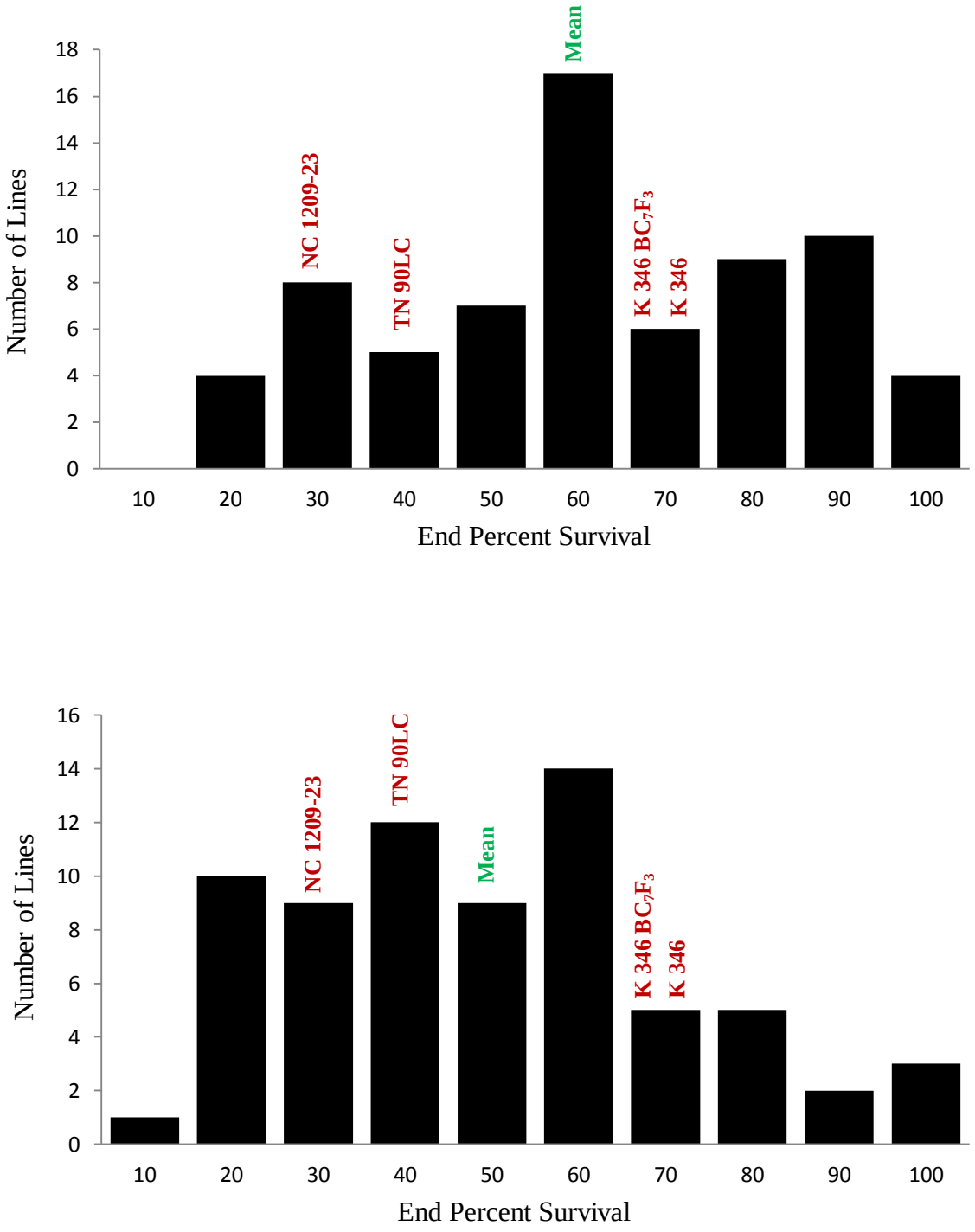
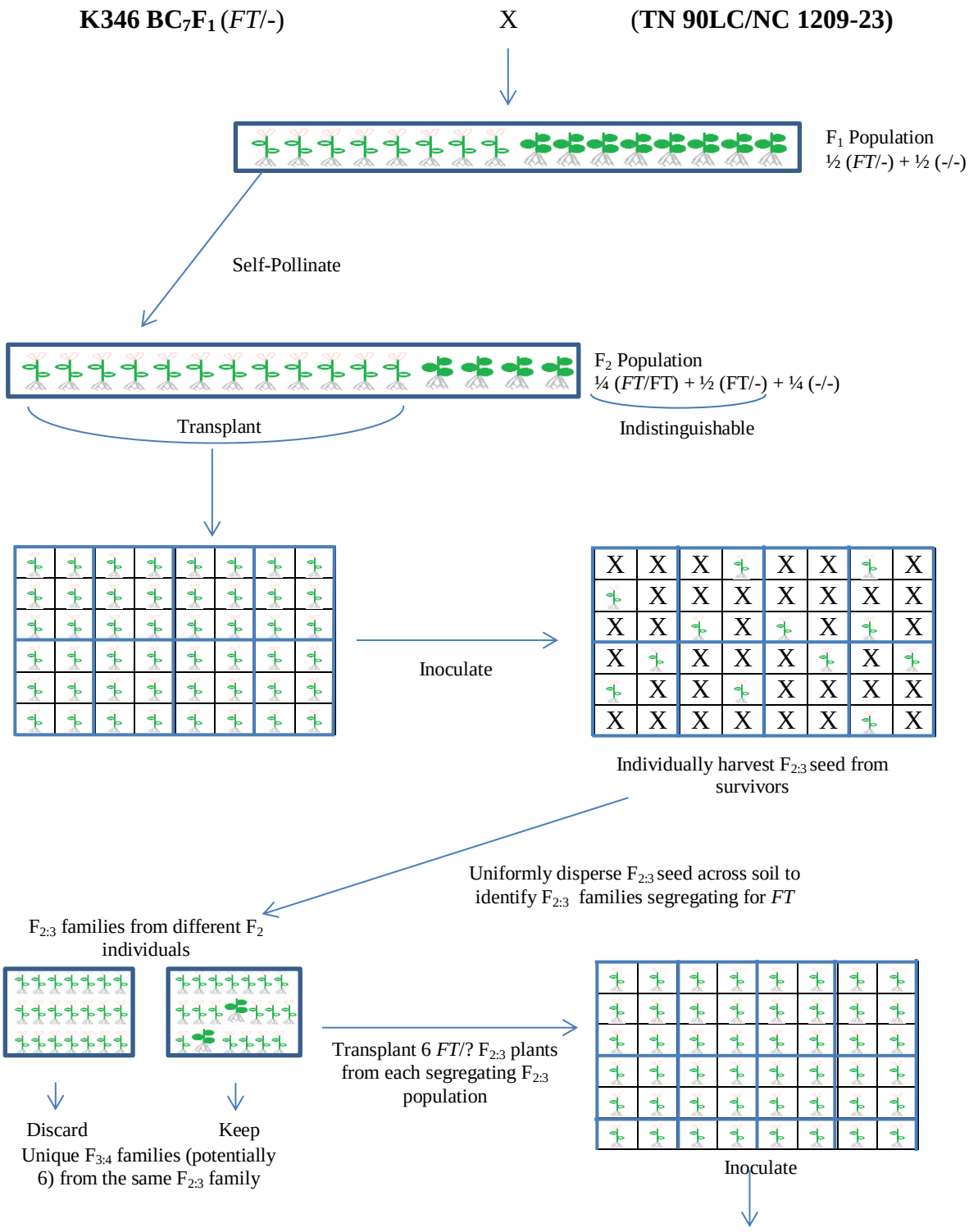
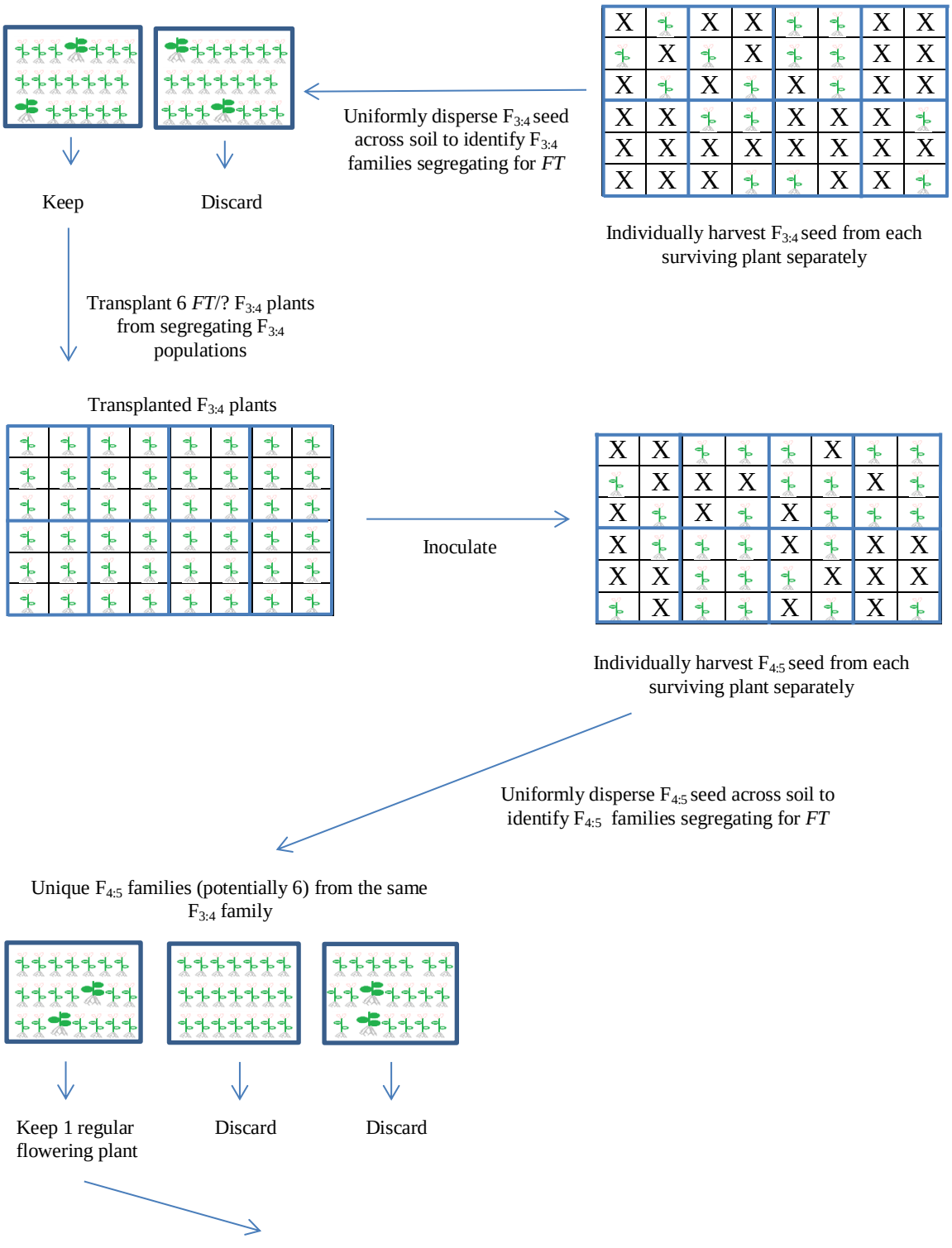


Figure 2.2. Frequency histogram for EPS of selected (top) and random groups of (bottom) $F_{5:6}$ inbred lines and parental checks evaluated over three disease nursery locations during 2012.

Figure 2.3. Inbreeding and selection method utilized during the development of $F_{5:6}$ lines for field evaluation.


K346 BC7F1 (FT/-)
X
(TN 90LC/NC 1209-23)
F1 Population
½ (FT/-) + ½ (-/-)
Self-Pollinate
F2 Population
¼ (FT/FT) + ½ (FT/-) + ¼ (-/-)
Transplant
Indistinguishable
Inoculate
Individually harvest F2:3 seed from survivors
Uniformly disperse F2:3 seed across soil to identify F2:3 families segregating for FT
F2:3 families from different F2 individuals
Transplant 6 FT/? F2:3 plants from each segregating F2:3 population
Discard
Keep
Unique F3:4 families (potentially 6) from the same F2:3 family
Inoculate


Uniformly disperse $F_{3:4}$ seed across soil to identify $F_{3:4}$ families segregating for *FT*
Keep
Discard
Individually harvest $F_{3:4}$ seed from each surviving plant separately
Transplant 6 *FT*/? $F_{3:4}$ plants from segregating $F_{3:4}$ populations
Transplanted $F_{3:4}$ plants
Inoculate
Individually harvest $F_{4:5}$ seed from each surviving plant separately
Uniformly disperse $F_{4:5}$ seed across soil to identify $F_{4:5}$ families segregating for *FT*
Unique $F_{4:5}$ families (potentially 6) from the same $F_{3:4}$ family
Keep 1 regular flowering plant
Discard
Discard
PCR screen for presence of *FT* and kanamycin

Self-pollinate $F_{4:5}$ plant and harvest $F_{5:6}$ seed

Field evaluation of $F_{5:6}$ lines in black shank disease nurseries

Figure 2.4. Phytotron growth chamber with two *35S:FT* F_2 populations (NC-120923 and TN-90) pre-inoculation.

Figure 2.5. Phytotron growth chamber with two *35S:FT* F_2 populations (NC-120923 and TN-90) post-inoculation, pre-selection.

Figure 2.6. *35S:FT* F_2 plant post-inoculation, showing symptoms of black shank (blackened stem).

Figure 2.7. Surviving *35S:FT* F_2 plants at time of harvest and collection of $F_{2:3}$ seed.

Figure 2.8. NC1209-23 *35S:FT* $F_{2:3}$ plants at time of inoculation, with 8 unique $F_{2:3}$ families per tray.

Figure 2.9. TN 90 $F_{5:6}$ lines (randomly derived left, selected right) being evaluated in a black shank disease nursery at the Lower Coastal Plain Tobacco Research Station, Kinston, NC.

Chapter 3: Field Evaluation of an Introgressed QTL Affecting Days to Flowering and its Relation to Yield and Quality in Flue-Cured Tobacco

ABSTRACT

Annual yield gains in flue-cured tobacco (*Nicotiana tabacum* L.) have tapered off since the early 1980's. Cultural practices designed to maximize yield generally focus on decreasing plant stresses to allow the maximum genetic potential of the variety to be expressed. The only way to truly increase the maximum genetic potential of a variety is through focused breeding efforts. Previous attempts to increase tobacco leaf number and yield by altering photoperiodic response have been successful. Cured leaf quality was often reduced in these materials, however. An introgressed QTL from the species *N. tomentosa*, designated as *Many Leaves* (*Ml*), significantly increased green leaf yield in tobacco, but, to date, the effects this QTL has on flue-cured tobacco yields, cured leaf quality, and leaf chemistry have not been investigated. The purpose of this research was to determine if the introgressed *Ml* region could be used to achieve yield gains while maintaining acceptable cured-leaf quality and chemistry.

Ml was backcrossed into three genetic backgrounds (K 326, Speight 168, and NCTG-61) for field evaluation. Homozygous lines (*MlMl*) and F_1 hybrids (*Mlml)* were evaluated in six field environments and at two nitrogen (N) fertilization rates for agronomic, morphological, and quality characteristics. Results demonstrated that *Ml* acts in a partially additive to dominant fashion to increase days to flowering, plant height, leaf number, leaf density, and percent reducing sugars. *Ml* acted to decrease mid-leaf length, mid-leaf width, and percent total alkaloids, while having minimal effects on physical quality. In addition, an increased nitrogen rate of 28 kg N ha^{-1} increased yield while have no effect on visual leaf

quality in the delayed flowering genotypes. The increased N rate also increased total alkaloids in later flowering genotypes.

Across all genetic backgrounds and nitrogen rates, one copy of *Ml* increased yield by approximately 378 kg ha^{-1}, thus increasing revenue by approximately \$1,289 ha^{-1}. Development of new cultivars with *Ml* could result in significant increases in harvestable yield potential, allowing for significant increases in grower revenue.

INTRODUCTION

The main objective for an applied plant breeding program is to develop and release new varieties having a superior yielding ability. Although yield is measured and quantified as a single trait, it actually results from the interaction of many genetic and environmental factors that contribute to the final measurement (Sebastian et al., 2010). Despite the genetic complexities associated with yield, varying levels of heterogeneity exist within breeding populations for breeding efforts to capitalize on. If quality (visual and chemical) is not a constraint for the release of new varieties, mass selection and advanced cycle breeding are an effective means to increase yield.

Tobacco is different from most other agronomic crops in that its vegetative tissue is harvested for sale, rather than its seed. In addition, the quality of the cured leaf is as important, if not more important, than the overall mass of the harvested leaf. Tobacco leaves are strongly affected by physiological and environmental factors at many stages of development that can affect their quality after curing. Because of this, increasing tobacco yields while maintaining acceptable quality is a major obstacle for breeders.

Over the last three decades, average flue-cured tobacco yields in North Carolina have been relatively consistent, with fluctuations mainly attributed to environmental variation. The general trend for tobacco varieties enetered into the North Carolina Officical Variety Trial (NCOVT) shows fairly consistent increases in yield for approximately the first 30 years (pre-1982) of the program's existence. There has been an obvious plateau in yields since the early 1980's, however.

Several factors may have contributed to this apparent yield plateau. First, this plateau corresponded with the 1982 release of cultivar, 'K 326.' Although K 326 is an extremely high yielding variety, it is severely lacking in disease resistance. As a result, significant breeding efforts have been devoted to incorporating disease resistance into the K 326 background. Most cultivars in the last 20 years are highly related to K 326, thus contributing to reduced genetic variation amongst modern varieties (Moon et al., 2009).

Another contributing factor to the plateau in average NCOVT yields are the leaf chemistry tolerances set forth by the United States Regional Minimum Standards Program. This program sets the acceptable ranges for cured leaf chemistry, particularly percent reducing sugars and percent total alkaloids. In general, as the yield of a variety is increased, the nicotine content decreases (Matzinger, 1968; Legg and Collins, 1971). Higher-yielding varieties consequently often fall outside the limits of acceptability for percent total alkaloids.

Agronomic studies in tobacco (Chaplin, 1968; Elliot, 1970; Collins and Hawks, 1993; Bukan, 2010) have investigated alternative (non-genetic) methods to increase the amount of harvestable leaf material per unit area of production. Significant yield gains can be achieved by altering the intra-row and inter-row spacings. These yield gains often come at the expense

of favorable leaf chemistry characteristics, however. At higher planting densities, reducing sugars are often increased while total alkaloids are decreased (Chaplin, 1968; Collins and Hawks 1993), adversely affecting the important sugar/alkaloid ratio. Alternative approaches to increase yield without increasing competition between plants have also been investigated, mainly through the altering of leaf number through a photoperiodic response (Wernsman and Matzinger, 1980; King, 1986)

Tobacco, generally being a day-neutral species, does not typically respond to changes in photoperiod. Short-day mutants do exist as first demonstrated by Allard (1919), however. The first effort to utilize photoperiodic response in an attempt to increase yield was with the trait referred to as "gigantism" in the variety Bunn Special (Mann and Chaplin, 1957). Gigantism, or short-day photoperiodic response, produces varieties that will not flower during North Carolina's tobacco growing season. The extended vegetative growth period caused production of very high leaf numbers, which resulted in the reduction of nicotine levels and increases in percent reducing sugars, however. In addition to the changes in leaf chemistry, non-flowering varieties were difficult to manage for field workers. Without the visual cue (flower bud emergence) for topping within a field, significant variation in plant height and leaf number would result. Finally, because photoperiod sensitive cultivars would only flower during the winter, they proved to be very difficult to manage from a breeding standpoint.

Because the number of days to flowering is positively associated with yield (Matzinger, 1968; Legg and Collins, 1971: Pandeya et al., 1983), genetic mechanisms other than photoperiodic response which affect the length of the vegetative growth period might

prove to be useful. Lewis et al. (2007) reported on an introgressed chromosome segment from the species *N. tomentosa* which affected flowering time. They found that when this segment was evaluated across several genetic backgrounds, significant delays in flowering time were observed. The chromosome segment, designated as *Many Leaves* (*Ml*), acted in a partially additive to dominant fashion to cause increases in leaf number, plant height and green leaf yield. The effect this chromosome segment has on cured leaf yield, cured leaf quality, and leaf chemistry in a flue-cured genetic background under normal production situations has not yet been evaluated.

The objective of this research was to determine if *Ml* might be used to increase yields without adversely affecting leaf quality and chemistry across several flue-cured genetic backgrounds. If a delay in flowering proves to be beneficial, this QTL may be introgressed into already existing cultivars, or used in the development of new cultivars to significantly increase yield and profitability for growers.

MATERIALS AND METHODS

Development of Genetic Materials

Seed from the genetic stock Red Russian *MlMl* was obtained from the North Carolina State University (NCSU) tobacco breeding program. This stock was produced by backcrossing *Ml* into the line 'Red Russian' using an unknown number of backcrosses. Using pollen from the Red Russian *MlMl* genetic stock, the *Ml* QTL was transferred into three flue-cured varieties, 'Speight 168,' 'K 326,' and 'NCTG-61' to develop F_1 hybrids. After the initial hybridization, six cycles of backcrossing were performed to develop BC_6F_1

individuals. At each backcross generation, selection was made for the presence of four amplified fragment length polymorphism (AFLP) markers ((M4, M5) and (M1, M8)) flanking the *Ml* QTL (Lewis et al., 2007). The only phenotypic selection conducted during backcrossing was against red flower color and for a normal leaf attachment. AFLP genotyping was conducted by first extracting DNA using a modified cetyltrimethylammonium bromide (cTAB) procedure (Afanador, Haley and Kelly, 1993; Johnson et al., 1995), with the exception being that a BIO 101 FastPrep machine (BIO 101, Holbrook, N.Y.) was used to grind tissue samples. AFLP reaction conditions were conducted according to Lewis et al. (2007).

BC_6F_1 individuals that tested positive for any combination of the flanking *Ml* AFLP markers were self-pollinated to generate three segregating BC_6F_2 populations (one for each recurrent parent). Nine BC_6F_2 individuals for each genetic background that tested positive for a combination of the flanking dominant AFLP markers were selected for self-pollination and also for testcrossing with their respective parents to determine their zygosity for the *Ml*-associated markers. Twenty testcross progeny from each BC_6F_2 individual were genotyped for the presence of the *Ml*-associated markers to identify BC_6F_2 plants homozygous for *Ml*. Homozygous (*MlMl*) BC_6F_2 plants were self-pollinated to produce BC_6F_3 seedlots, and also hybridized with their respective recurrent parents to produce seed of heterozygous (*Mlml*) F_1 hybrids. For field evaluation, one homozygous line and one heterozygous hybrid was selected for each of the three genetic backgrounds. In addition, one BC_6F_3 null-segregant line (*mlml*) was selected for each of the genetic backgrounds.

Field Evaluation

Twelve genotypes were selected for field evaluation during the 2011 and 2012 growing seasons. This included the homozygous (*MlMl),* heterozygous *(Mlml*), and null segregants (*mlml*) for each of the three genetic backgrounds, in addition to commercial seedlots of K 326, Speight 168 and a foundation seedlot of NCTG-61 (Table 3.1). A split-plot design was used to evaluate genotypes in each environment, with six replications per environment. Nitrogen rate was assigned to whole plots and genotype was assigned to sub-plots. Nitrogen fertilization rates of normal and normal + 28 kg N ha^{-1} were chosen under the hypothesis that additional nitrogen may be needed to realize yield gains in higher leaf number tobaccos.

Research was conducted at three locations (the Lower Coastal Plain Tobacco Research Station, Kinston, NC; the Upper Coastal Plain Research Station, Rocky Mount, NC; and the Oxford Tobacco Research Station, Oxford, NC). Plots consisted of single rows of 20 plants per row and were cultivated according to standard flue-cured production practices for North Carolina (North Carolina Cooperative Extension, 2012). Intra-row spacing was 56 cm at the three research stations. Inter-row spacing was 122 cm at the Oxford and Rocky Mount research stations and 112 cm at the Kinston research station. 'Normal' N rates were applied based upon NCSU fertilization recommendations for the soil type at each research station (North Carolina Cooperative Extension, 2012). Normal N rates were 67.3, 85.2 and 79.6 kg N ha^{-1}, respectively.

Phenotypic data were collected for days to flowering, plant height (cm), leaf number, leaf length (cm) and width (cm) of the leaf at mid-stalk position, leaf mass per unit area

(mg/cm^2) of the uppermost leaf, and yield ($kg\ ha^{-1}$). A plot was considered to have flowered when greater than 50% of the row exhibited pink coloration in the corolla. Flowering time observations were made at all six environments. Once an individual plant showed pink coloration in its corolla, it was subsequently decapitated to remove leaves that were less than 20 cm in length and 10 cm in width (approximately 1-2 leaves below the lowest flowering branch). Immediately following decapitation, each individual plant was treated with Prime + EC (Syngenta Crop Protection, Greensboro, N.C.) according to label recommendations to suppress future lateral meristem development (suckering). Once all plants within a location had been decapitated, plant height was recorded by measuring the distance from the ground to the top of the stalk for 10 plants per plot. Plant height measurements were taken at all six environments. Leaf number was also measured at this time for 10 plants per plot at all six environments. Prior to the third priming, the length and width of a mid-stalk-position leaf (approximately the 13^{th} leaf) was taken on 10 plants per plot at five environments. Prior to the final harvest, two leaf punches were made on the upper-most leaves for 10 plants per plot, avoiding major veins, at three environments. Leaf punches were taken using precision machined stainless steel punches with a diameter of 1.91 cm. Leaf samples were dried and mass per unit area was then calculated. Yield data were collected from only five environments due to hurricane damage at the 2011 Kinston location.

Evaluation of Physical and Chemical Cured Leaf Quality

Official USDA grades were assigned to each priming of each plot by a former USDA tobacco grader for four environments. One environment in 2011 was lost due to hurricane damage and quality data could not be collected from the 2012 Rocky Mount location because

of loss of power to the curing barn. Fifty-gram cured leaf samples were prepared for each plot by compositing cured leaf from each priming on a weighted-mean basis. Oven-dried samples were ground and analyzed for percent total alkaloids and percent reducing sugars according to the method of Davis (1976). Percent reducing sugar estimates were obtained for four environments while percent total alkaloids were obtained for five environments. Based on the 2012 flue-cured pricing structure (North Carolina Cooperative Extension, 2013), average grade index, average value per kg, and value per hectare were assigned to each plot using data from the four environments which had corresponding quality and yield measurements. Grade index was calculated by first multiplying the associated grade of each priming by the percentage of yield resulting from that priming, and then summing over the primings. Value per kg was calculated in a similar weighted manner, except that grade index was replaced by the price per kg of the priming, which was based on the visual grade and associated price.

Statistical Analysis

An analysis of variance was performed over locations using the statement method=type3 in PROC MIXED SAS 9.2 (SAS Institute, Cary, NC), giving consideration to the nested treatment structure (genotype within genetic background). N rate, genetic background, and genotype within genetic background were considered as fixed effects, while environment and replication were treated as random effects. Linear contrasts were performed for genotypic comparisons of interest using ESTIMATE statements.

RESULTS

A QTL from *Nicotiana tomentosa* was evaluated to determine its effect on cured-leaf yields, physical quality, and cured leaf chemistry in flue-cured tobacco. Using marker assisted backcrossing, the QTL was successfully transferred into three important genetic backgrounds of flue-cured tobacco, resulting in the development of BC_6F_1 individuals. AFLP marker screening of BC_6F_2 individuals, coupled with progeny testing, led to the identification of BC_6F_2 individuals that were homozygous for the *Ml* introgression. This permitted the successful development of homozygous, heterozygous and null segregant lines for field evaluation.

Analysis of Variance

Significant environmental effects were observed for all measured traits except grade index, value per kg, and percent reducing sugars (Tables 3.2 Table 3.3 and Table 3.4). Significant differences between N rates were only observed for length and width of the mid-stalk leaf, yield, and percent total alkaloids (Table 3.2, Table 3.3 and Table 3.4). A significant N rate x environment interaction was observed only for length of the mid-stalk leaf (Table 3.2). Significant differences between genetic backgrounds were detected for plant height, leaf number, leaf mass per unit area, length and width of the mid-stalk leaf, yield and percent total alkaloids (Table 3.2, Table 3.3 and Table 3.4). Significant differences between genotypes within pedigree were found for all phenotypic traits measured except length of the mid-stalk position leaf, grade index, and value per kg (Table 3.2, Table 3.3 and Table 3.4). A significant environment x genetic background interaction was observed for length of the mid-

stalk position leaf, hectare value and percent total alkaloids (Table 3.2, Table 3.3 and Table 3.4). Significant genotype within genetic background x environment interactions were found for flowering time, plant height, leaf number, length and width of the mid-stalk leaf, hectare value, percent reducing sugars and percent total alkaloids (Table 3.2, Table 3.3 and Table 3.4). A significant nitrogen rate x genotype within genetic background interaction was found for length of the mid-stalk position leaf, grade index, value per kg and hectare value (Table 3.2, Table 3.3 and Table 3.4). Finally, a significant environment x N rate x genotype within genetic background interaction was found for flowering time (Table 3.2).

Linear Contrasts

Linear contrast estimates are reported for comparisons between genetic groups (*MlMl*, *Mlml*, and *mlml*) and standard varieties when averaged over genetic backgrounds and N rates. The results demonstrated that *Ml* significantly delayed flowering by 8.8 days when in a heterozygous condition and significantly delayed flowering by 14.1 days when in a homozygous condition (Table 3.5 and Figure 3.1). Similarly, plant height was significantly increased by 14.0 cm and 21.2 cm for *Mlml* and *MlMl* genotypes, respectively (Table 3.5, Figure 3.1, Figure 3.5 and Figure 3.6). Leaf number increased significantly by 5.0 leaves for *Mlml* genotypes and by 7.4 leaves for *MlMl* genotypes (Table 3.5 and Figure 3.1). Mass per unit area for the uppermost leaf increased significantly by 0.94 mg/cm^2 and 0.77 mg/cm^2 for *Mlml* and *MlMl* genotypes, respectively (Table 3.5 and Figure 3.1). Length of the leaf at the mid-stalk position was reduced by 1.1 cm for *Mlml* genotypes relative to the standard varieties, but no significant difference was observed for *MlMl* genotypes (Table 3.5 and Figure 3.2). Width of the leaf at mid-stalk position decreased significantly by 1.6 cm and 2.5

cm for heterozygous and homozygous genotypes (Table 3.5 and Figure 3.2). No significant differences were found to be associated with *Ml* for grade index or value per kg (Table 3.6 and Figure 3.2). Yield was significantly increased by 379 kg/ha and 443 kg/ha for *Mlml* and *MlMl* genotypes, respectively (Table 3.6). Percent reducing sugars were significantly increased by 2.2% and 2.4% for heterozygous and homozygous genotypes (Table 3.6 and Figure 3.4). Percent total alkaloids were significantly reduced by 0.8% and 1.1% for *Mlml* and *MlMl* genotypes, respectively (Table 3.6). Hectare value was significantly increased by $1,290 for *Mlml* genotypes and by $1,274 for *MlMl* genotypes (Table 3.6).

Relative to the standard varieties, the null segregant lines (*mlml*) displayed significantly reduced mass per unit area measurements, lower widths for the mid-stalk leaves, reduced yield, and reduced hectare value (Table 3.5 and Table 3.6). No significant differences were observed for this comparison for days to flowering, plant height, leaf number, lower lengths of mid-stalk leaves, grade index, value kg^{-1}, percent reducing sugars, or percent total alkaloids (Table 3.5 and Table 3.6).

Because ANOVA results indicated significant differences between N rates for yield and percent total alkaloids, (Table 3.7), separate ANOVA's were carried out at each N rate for increased insight. Linear contrast estimates are reported for comparisons between the genetic groups and the standard varieties. At the standard N rate, significant yield increases of 406kg/ha and 450 kg/ha were observed for *Mlml* and *MlMl* genotypes, respectively (Table 3.7). Null segregants were significantly lower yielding (189 kg/ha) than the standard varieties at the normal N rate (Table 3.7). At the higher N rate, significant yield increases of 348 kg/ha and 437 kg/ha were observed for heterozygous and homozygous genotypes, while a

significant yield decrease of 349.91 kg/ha was observed for null segregant lines (Table 3.7). At the standard N rate, percent total alkaloids were significantly reduced by 0.8% and 1.1% for *MlMl* and *Mlml* genotypes, while no significant difference was observed for null segregant lines (Table 3.7). At the higher N rate, significant reductions of 0.85% and 1.22% were observed for heterozygous and homozygous lines, while no significant difference was observed for null segregant lines (Table 3.7).

Least Square Means

A general trend for increased yield was observed for all genetic backgrounds as the zygosity for *Ml* increased (Table 3.8 and Figure 3.3). A general trend for decreased value per hectare was observed for the comparison between all null segregant lines and their corresponding standard varieties when the N rate was increased (Table 3.9 and Figure 3.3). A general trend of increased value per hectare was observed for all *Mlml* and *MlMl* genotypes, with the exception that the NCTG-61 *MlMl* genotype was reduced when compared to the standard variety (Table 3.9 and Figure 3.3). Finally, a general trend for increased percent total alkaloids was observed as N rate was increased (Table 3.10 and 3.4).

DISCUSSION

We found the marker assisted backcrossing method was effective at transferring the *Ml* introgression into the genetic backgrounds of three important flue-cured tobacco genetic backgrounds. The use of markers throughout the backcrossing procedure eliminated the need for field confirmation of the QTL's presence at each backcrossing generation, reducing the

total time required for backcrossing by 50%. The field results for flowering time verify the validity of the chosen markers and the backcrossing approach.

In agreement with Lewis et al. (2007), *Ml* was found to act in a partially additive to dominant fashion, significantly increasing days to flowering. As a consequence of delayed flowering, significant increases in plant height and leaf number were also observed. Although the increases in plant height were statistically significant, when evaluating average internode spacing, average internode length was comparable to the standard varieties (recurrent parents). As a result, increased leaf number and plant height should not impact the logistics of harvesting (hand or mechanical).

As hypothesized, the formation of additional leaves increased cured leaf yields. The results show that regardless of nitrogen rate, significant increases in yield were observed for heterozygous (*Mlml*) and homozygous (*MlMl*) lines when compared to the standard varieties and also the null segregants (*mlml*). The increased N rate increased the yield difference between homozygous lines and the standard variety counterparts, while it decreased the yield difference between heterozygous lines and the standard varieties. These results are slightly confusing and a better interpretation is made by evaluating the least square means. The least square means for all genotype entries (except null segregants) show that as nitrogen rate is increased, yield is increased. The response to fertilization is higher in the homozygous lines than it is in the heterozygous lines, explaining the differences when compared to the standard varieties.

Previous attempts to increase yield through altering photoperiodic response and changing cultural practices have resulted in only mild success. Cured leaf quality and leaf

chemistry were generally adversely affected by using this system, however (Chaplin, 1968; Elliot, 1970; Wernsman and Matzinger, 1980; King, 1986; Collins and Hawks, 1993; Bukan, 2010). As the results for grade index have shown in the current study, no statistically significant differences in visual quality were observed for heterozygous or homozygous lines when compared to the standard varieties. In addition, no significant differences were observed for value kg^{-1} for these comparisons.

Because grade index and value kg^{-1} were not reduced with the observed yield increase, hectare value estimates were significantly increased. Hectare value estimates for heterozygous and homozygous lines show almost identical values, although homozygous lines yielded roughly 65 kg more, on average. This discrepancy most likely arises because the numerical differences that exist for grade index between the homozygous lines and the standard varieties were greater than the differences between the heterozygous lines and the recurrent parents. Although the differences in grade index are not statistically significant, they are still factored into hectare value calculations.

Although significant increases in hectare value were observed for heterozygous and homozygous lines, candidate varieties must also meet the chemistry requirements of the Regional Minimum Standards Program. In agreement with previous attempts to increase yield by delaying flowering, percent reducing sugars was significantly increased while percent total alkaloids was significantly decreased in higher leaf number genotypes. Reducing sugars are a product of the curing process, where starch stored in the vacuole is broken down into glucose. One could hypothesize that the increased leaf density measurements observed for higher leaf number tobaccos were the result of increased starch

accumulation. This increased starch accumulation would allow for increased reducing sugar formation during the curing process.

The percentage of reducing sugars in the tobacco leaf is important as it contributes to the reducing sugar/total alkaloid ratio. Because percent total alkaloids for the *Ml* genotypes are significantly below the standard variety K 326, which is used as a check in the Minimum Standards Program, there is a combined effect to affect the total alkaloid: reducing sugar ratio in an unfavorable manner. This is a concern for higher leaf number tobaccos. Although there is a strong negative correlation between yield and percent total alkaloids (Matzinger, 1968; Legg and Collins, 1971), tobacco varieties can be produced in which the total alkaloid level is significantly greater than the acceptable tolerances set forth by the Minimum Standards Program. Because modern tobacco varieties are released as cytoplasmic male sterile hybrids, opportunity exists during the hybridization process to increase the total alkaloid percentage so that higher leaf number tobaccos may meet the minimum standards requirements. As the hectare value estimates have shown, heterozygous lines were almost identical to homozygous lines, supporting the potential utility of this approach.

Although this research has demonstrated that delays in flowering can increase yield and hectare value, a few inconveniences associated with the use of *Ml* should be mentioned. First, the time required to backcross the QTL from a relatively unadapted line into modern flue-cured tobacco varieties takes several years, depending on the level of residual heterozygosity that is accepted. Delays in time associated with backcrossing can be alleviated if a plant breeder chooses to use the same breeding line for hybrid development once it has been fixed for *Ml*. If this approach is taken, consequences regarding specific combining

ability and reductions in genetic variation need to be evaluated. Alternatively, if a plant breeder chooses to reduce generation time through transgenic means as described by Lewis et al. (2009), the time required to develop unique lines fixed for *Ml* to be used in the hybridization process is much less of a concern.

Second, throughout the backcrossing procedure, the QTL was tracked using flanking AFLP markers. Being that AFLP markers are dominant, there is no way to distinguish between homozygous and heterozygous individuals. The inability to distinguish between heterozygous and homozygous individuals was somewhat of an issue when generating field testing materials. Time consuming progeny testing was required to assure that lines were truly homozygous for *Ml*. The development and use of co-dominant markers such as SNP's would eliminate this problem.

Finally, because markers flanking the QTL were used during the backcrossing procedure, a large fraction of the entire introgressed region may have been present in the field tested materials. Fine mapping of this QTL may allow for the identification of markers in much closer proximity to the actual gene(s) of interest, eliminating unwanted introgressed genetic materials. The possibility that undesired genetic material from the Red Russian donor line could have been transferred to derived lines even after six generations of backcrossing is illustrated by the fact that the BC_6F_3 null segregants performed significantly worse than the standard varieties. Future efforts to eliminate undesirable alleles from the donor material may serve to increase the yield and quality of materials containing *Ml*. However, regardless of any inconveniences associated with this method, *Ml,* coupled with an increased nitrogen rate has proven to be an effective means to increase yield and acre value for tobacco growers

REFERENCES

Afanador, L.K., Haley, S.D. and J.D. Kelly. 1993 Adoption of a "mini-prep" DNA extraction method for RAPD marker analysis in common bean (*Phaseolus vulgaris* L.). Annual Report: Bean Improvement Cooperative 36:10-11.

Allard, H.A. 1919. Gigantism in *Nicotiana tabacum* and its alternate inheritance. Am. Nat. 53:218-233.

Bukan, M., A. Budimir, M. Boic, H. Sarcevic, and V. Kozumplik. 2010. Effect of within-row spacing on agronomic and morphological characteristics of the flue-cured tobacco cultivars. Agric. Conspec. Sci. Vol. 75 No. 1:27-31.

Chaplin, J.F., Z.T. Ford, J.B. Pitner, and R.E. Currin. 1968. Effect of row and within-row spacings on yield and quality of flue-cured tobacco. Agron. J. 60:314-316.

Collins, W.K. and S.N. Hawks Jr.. 1993. Principles of Flue-Cured Tobacco Production. Raleigh, NC: W. K. Collins and S. N. Hawk Jr. p. 11-17.

Davis, R.E. 1976. A combined automated procedure for the determination of reducing sugars and nicotine alkaloids in tobacco products using a new reducing sugar method. Tob. Sci. 20:139-144.

Elliot, J.M. 1970. The effect of topping height and plant spacing on yield, grade and some physical characteristics of bright tobacco. Tob. Sci. 14:73-77.

Fisher, L.R., S. Stewart, W.D. Smith, G. Tart, and K. Barnes. 2012. Selecting a Variety. In North Carolina State University: Flue-cured tobacco guide 2012 (AG-187 (Revised) ed., pp. 23-44). Raleigh, North Carolina: North Carolina Cooperative Extension.

King, M.J. 1986. Leaf number at topping and yield, grade index, and leaf chemistry of a mammoth-type tobacco. Agron. J. 78:913–915.

Legg, P.D., and G.B. Collins. 1971. Genetic parameters in burley populations of *Nicotiana tabacum* L. I. 'Ky 10' × 'Burley 21'. Crop Sci. 11:365-367.

Lewis, R.S., S.R. Milla, and S.P. Kernodle. 2007. Analysis of an introgressed *Nicotiana tomentosa* genomic regions affecting leaf number and correlated traits in *Nicotiana tabacum*. Theor. Appl. Genet. 114:841–854.

Lewis, R.S., and S.P. Kernodle. 2009. A method for accelerated trait conversion in plant breeding. Theor. Appl. Genet. 118:1499–1508.

Mann, T. J., and J. F. Chaplin. 1957. The effect of the mammoth gene on certain quantitatively inherited characters of flue-cured tobacco. Agron. J. 49:230-233.

Matzinger, D.F. 1968. Genetic variability in flue-cured varieties of *Nicotiana tabacum* L. III. SC58 x Dixie Bright 244. Crop Sci. 8:732-735.

Moon, H.S., J.S. Nicholson, A. Heineman, K. Lion, R. van der Hoeven, A.J. Hayes, and R.S. Lewis. 2009. Changes in genetic diversity of U.S. flue-cured tobacco germplasm over seven decades of cultivar development. Crop Sci. 49:498–508.

North Carolina Cooperative Extension. 2012. Flue-Cured Tobacco Guide, Raleigh, NC: North Carolina State University.

Pandeya, R.S., V.A. Dirks, and G. Poushinsky. 1983. Quantitative genetic studies in flue-cured tobacco (*Nicotiana tabacum*). I. Agronomic characters. Can. J. Genet. Cytol. 25:336-345.

Sebastian, S., L. Streit, P. Stephens, J. Thompson, B. Hedges, M. Fabrizius, J. Soper, D. Schmidt, R. Kallem, and M. Hinds. 2010. Contextspecific marker-assisted selection for improved grain yield in elite soybean populations. Crop Sci. 50:1196.

Wernsman, E.A., and D.F. Matzinger.1980. Mammoth genotypes and tobacco management regimes for reduced production of downstalk tobaccos. Agron. J. 72:1047–1050.

Table 3.1 Genetic materials tested in field evaluations

Entry	Genotype
K 326 (certified seed)	*mlml*
K 326 MlMl BC6F3	*MlMl*
K 326 Mlml F1 hybrid	*Mlml*
K 326 null segregant	*mlml*
NCTG-61 (foundation seed)	*mlml*
NCTG-61 MlMl BC6F3	*MlMl*
NCTG-61 Mlml F1 hybrid	*Mlml*
NCTG-61 null segregant	*mlml*
Speight 168 (certified seed)	*mlml*
Speight 168 MlMl BC6F3	*MlMl*
Speight 168 Mlml F1 hybrid	*Mlml*
Speight 168 null segregant	*mlml*

Table 3.2. ANOVA results displaying mean squares and significance levels for flowering time, plant height, leaf number, leaf mass per unit area, and length of mid-stalk analyzed over nitrogen rates and genetic backgrounds.

Source	Days to Flowering (days)		Plant Height (cm)		Leaf Number		Mass per Unit Area (mg/cm^2)		Length of Mid- Leaf (cm)	
Environment	11451.00	***	2181.35	**	266.60	***	290.08	**	4487.70	***
Rep(Environment)	105.17	***	256.18	**	7.25	**	14.33	*	44.82	**
N Rate	1.50	-	58.84	-	0.04	-	4.40	-	741.36	*
N Rate*Environment	23.09	-	20.41		0.49	-	6.96	-	54.07	*
N Rate*Rep(Environment)	28.77	***	101.33	***	2.28	*	5.60	***	12.20	*
Genetic Background	493.14	-	8302.29	***	174.36	**	66.41	*	680.44	**
Genotype(Genetic Background)	3657.86	***	9020.83	***	1133.75	***	36.30	***	30.10	-
Environment*Genetic Background	122.28	-	234.89	-	18.32	-	5.50	-	40.89	*
Environment*Genotype(Genetic Background)	83.45	***	364.16	***	15.39	***	2.38	-	15.39	***
Rep(Environment)*Genetic Background	9.21	-	21.09	-	1.25	-	1.40	-	5.21	-
Rep(Environment)*Genotype(Genetic Background)	8.03	*	29.74	**	1.65	*	0.99	-	5.08	-
N Rate*Genetic Background	1.85	-	20.52	-	0.31	-	1.50	-	3.16	-
N Rate*Genotype(Genetic Background)	3.36	-	14.37	-	1.50	-	0.74	-	13.52	**
N Rate*Environment*Genetic Background	8.73	-	48.93	-	1.96	-	0.21	-	4.48	-
N Rate*Environment*Genotype(Genetic Background)	9.90	*	29.49	-	1.55	-	1.07	-	4.44	-
N Rate*Rep(Environment)*Genetic Background	6.86	-	26.61	-	1.34	-	0.73	-	5.89	-

- = Not Significant at $P < 0.05$; * = Significant at $P < 0.05$; ** = Significant at $P < 0.01$; *** = Significant at $P < 0.001$.

Table 3.3. ANOVA results displaying mean squares and levels of significance for width of the mid-stalk leaf, yield, grade index and value per kg analyzed over nitrogen rates and pedigrees.

Source	Width of Mid-Stalk leaf (cm)		Yield (kg ha^{-1})		Grade Index (0-100)		Value per kg ($ kg)	
Environment	531.18	***	50,617,102	***	4670.32	-	13.17	-
Rep (Environment)	12.39	*	925,942	-	250.60	-	0.70	-
N Rate	137.46	*	651,531	***	81.57	-	0.58	-
N Rate*Environment	15.96	-	9271.78	-	65.00	-	0.14	-
N Rate*Rep (Environment)	4.84	**	443,445	**	202.66	***	0.57	***
Genetic Background	100.73	***	2,878,087	*	259.40	-	0.52	-
Genotype (Genetic Background)	167.40	***	6,317,051	***	48.45	-	0.15	-
Environment*Genetic Background	4.81	-	452,655	-	282.95	-	0.71	-
Environment*Genotype (Genetic Background)	5.63	**	202,707	-	102.71	-	0.30	-
Rep(Environment)*Genetic Background	2.71	-	125,654	-	36.02	-	0.10	-
Rep(Environment)*Genotype (Genetic Background)	2.27	**	113,076	-	82.30	-	0.25	-
N Rate*Genetic Background	2.01	-	163,651	-	18.76	-	0.03	-
N Rate*Genotype (Genetic Background)	3.47	-	211,655	-	272.03	*	0.86	*
N Rate*Environment*Genetic Background	2.49	-	55,247	-	264.69	-	0.86	-
N Rate*Environment*Genotype (Genetic Background)	1.78	-	152,855	-	101.42	-	0.31	-
N Rate*Rep (Environment)*Genetic Background	1.84	-	120,323	-	55.31	-	0.15	-

- = Not Significant at $P < 0.05$; * = Significant at $P < 0.05$; ** = Significant at $P < 0.01$; *** = Significant at $P < 0.001$.

Table 3.4. ANOVA results displaying mean squares and levels of significance for reducing sugars, total alkaloids and value per hectare analyzed over nitrogen rates and pedigrees.

Source	Value per Hectare ($ ha^{-1})		Reducing Sugars (%)		Total Alkaloids (%)	
Environment	908,693,362	**	424.35	-	21.96	**
Rep(Environment)	12,370,157	-	25.99	-	0.20	-
N Rate	20,220,297	-	349.97	-	10.28	*
N Rate*Environment	2,515,271	-	58.58	-	0.84	-
N Rate*Rep(Environment)	6,641,901	-	23.03	***	0.53	***
Genetic Background	23,153,996	-	80.64	-	17.95	***
Genotype(Genetic Background)	62,982,555	***	127.74	***	22.80	***
Environment*Genetic Background	41,387,305	*	23.11	-	0.67	*
Environment*Genotype(Genetic Background)	9,049,576	*	11.50	**	0.27	**
Rep(Environment)*Genetic Background	3,506,815	-	3.34	-	0.11	-
Rep(Environment)*Genotype(Genetic Background)	4,129,045	-	2.64	-	0.07	-
N Rate*Genetic Background	2,662,412	-	2.26	-	0.10	-
N Rate*Genotype(Genetic Background)	11,073,918	*	4.96	-	0.06	-
N Rate*Environment*Genetic Background	8,026,150	-	3.30	-	0.05	-
N Rate*Environment*Genotype(Genetic Background)	4,053,408	-	2.77	-	0.08	-
N Rate*Rep(Environment)*Genetic Background	3,874,301	-	4.91	*	0.11	-

- = Not Significant at $P < 0.05$; * = Significant at $P < 0.05$; ** = Significant at $P < 0.01$; *** = Significant at $P < 0.001$.

Table 3.5. Results from linear contrasts between selected genotypic groups for days to flowering, plant height, leaf number, leaf mass per unit area, length of the mid-stalk leaf, and width of the mid-stalk leaf analyzed over nitrogen rates and pedigrees.

Contrast	**Days to Flowering** (days)		**Plant Height** (cm)		**Leaf Number**		**Mass per Unit Area** (mg/cm^2)		**Length of Mid-Stalk Leaf** (cm)		**Width of Mid-Stalk Leaf** (cm)	
Homozygotes (*MlMl*)	-14.1574[a]	***	-21.215	***	-7.438	***	-0.78	**	0.6583	-	2.5172	***
Heterozygotes (*Mlml)*	-8.8843	***	-14.0793	***	-5.032	***	-0.95	***	1.1439	**	1.6344	***
Null segregant (*mlml*)	-0.1991	-	1.5721	-	0.6841	-	0.86	***	0.3389	-	-0.8228	**

[a] Comparisons made by subtracting the mean of the genotypic class indicated from the mean for the standard varieties.

- = Not Significant at $P < 0.05$; * = Significant at $P < 0.05$; ** = Significant at $P < 0.01$; *** = Significant at $P < 0.001$.

Table 3.6. Results from linear contrasts between selected genotypic groups for yield, grade index, value per kg, value per hectare, percent reducing sugars and percent total alkaloids analyzed over nitrogen rates and pedigrees.

Contrast	Yield (kg ha^{-1})		Grade Index (1-100)		Value per kg ($ kg^{-1})		Value per Hectare ($ ha^{-1})		Reducing Sugars (%)		Total Alkaloids (%)	
Homozygotes (*MlMl*)	-443.07[a]	***	0.9877	-	0.049	-	-1274.25	**	-2.42	***	1.14	***
Heterozygotes (*Mlml*)	-378.73	***	0.05228	-	-0.003	-	-1289.96	**	-2.20	***	0.83	***
Null segregant (*mlml*)	264.94	***	0.525	-	0.017	-	975.72	*	0.69	-	-0.06	-

[a] Comparisons made by subtracting the mean of the genotypic class indicated from the mean for the standard varieties.

- = Not Significant at $P < 0.05$; * = Significant at $P < 0.05$; ** = Significant at $P < 0.01$; *** = Significant at $P < 0.001$.

Table 3.7. Results from linear contrasts between selected genotypic groups for yield analyzed between nitrogen rates and over pedigrees.

Genotype	Yield Normal N Rate (kg ha^{-1})		Yield High N Rate (kg ha^{-1})		Total Alkaloids (%) Normal N		Total Alkaloids (%) High N	
Homozygotes (*MlMl*)	-449.85[a]	***	-437.64	***	1.10	***	1.22	***
Heterozygotes (*Mlml*)	-406.43	***	-347.89	***	0.82	***	0.85	***
Null segregant (*mlml*)	188.51	**	349.91	***	-0.12	-	-0.02	-

[a] Comparisons made by subtracting the mean of the genotypic class indicated from the mean for the standard varieties.

- = Not Significant at $P < 0.05$; * = Significant at $P < 0.05$; ** = Significant at $P < 0.01$; *** = Significant at $P < 0.001$.

Table 3.8. Least square mean estimates for days to flowering, leaf number, plant height, yield, mass per unit area, and length of mid- stalk position leaf analyzed over environments, replications and nitrogen rate in some cases.

Genotype	Days to Flowering	Leaf Number	Plant Height (cm)	Yield (Kg ha^{-1})		Mass per Unit Area (mg/cm^2)	Mid-Stalk Leaf Length (cm)
				Normal N	High N		
K 326 (certified)	133.19	22.42	92.96	3316.59	3380.33	16.54	64.96
K 326 *Mlml*	143.42	27.82	109.12	3392.85	3528.12	16.69	63.11
K 326 *MlMl*	150.10	30.59	115.83	3401.68	3678.73	16.84	63.19
K 326 Null Segregant	133.88	21.85	92.85	3065.49	2870.54	15.82	65.03
NCTG-61 (foundation seed)	133.17	21.75	83.42	2920.25	2996.95	15.29	61.75
NCTG-61*Mlml*	142.51	26.85	97.78	3459.76	3467.07	17.30	60.63
NCTG-61 *MlMl*	149.13	29.64	108.05	3771.60	3746.88	17.43	61.19
NCTG-61 Null Segregant	132.36	20.41	81.04	2700.67	2703.86	14.23	60.77
Speight 168 (certified)	133.22	21.46	94.34	2805.48	3029.55	15.21	61.28
Speight 168 *Mlml*	140.31	26.06	106.06	3408.99	3455.32	15.88	60.81
Speight 168 *MlMl*	142.83	27.71	110.48	3218.59	3294.15	15.09	61.64
Speight 168 Null Segregant	133.94	21.32	92.11	2710.62	2782.71	14.41	61.18

Table 3.9. Least square mean estimates for width of mid-stalk leaf, grade index, value per kg, and value per hectare analyzed over environments, replications and nitrogen rate in some cases.

Genotype	Mid-Stalk Leaf Width (cm)	Grade Index (1-100)	Value per kg ($ kg^{-1})	Value per Hectare ($ ha^{-1})	
				Normal N	High N
K 326 (certified)	28.01	76.82	3.51	12623.00	11565.00
K 326 *Mlml*	26.74	76.32	3.50	11903.00	13339.00
K 326 *MlMl*	25.74	77.61	3.57	12407.00	13594.00
K 326 Null Segregant	27.72	76.91	3.51	11061.00	10806.00
NCTG-61 (foundation seed)	27.93	77.78	3.55	10504.00	11610.00
NCTG-61*Mlml*	26.37	77.58	3.56	12497.00	12735.00
NCTG-61 *MlMl*	25.56	74.27	3.36	13173.00	12583.00
NCTG-61 Null Segregant	30.70	76.12	3.50	9888.00	9799.00
Speight 168 (certified)	29.60	78.58	3.60	11098.00	10796.00
Speight 168 *Mlml*	27.52	79.12	3.61	12003.00	13459.00
Speight 168 *MlMl*	26.68	78.33	3.58	11347.00	12737.00
Speight 168 Null Segregant	29.58	78.56	3.59	10405.00	10383.00

Table 3.10. Least square mean estimates for percent reducing sugars and percent total alkaloids analyzed over environments, replications and nitrogen rate in some cases.

Genotype	Reducing Sugars (%)	Total Alkaloids (%)	
		Normal N	High N
K 326 (certified)	15.21	2.74	3.03
K 326 *Mlml*	17.01	1.94	2.18
K 326 *MlMl*	16.53	1.56	1.68
K 326 Null Segregant	14.67	2.82	3.00
NCTG-61 (foundation seed)	15.94	3.36	3.62
NCTG-61*Mlml*	18.30	2.37	2.62
NCTG-61 *MlMl*	19.28	2.03	2.18
NCTG-61 Null Segregant	14.98	3.42	3.67
Speight 168 (certified)	15.23	2.91	3.22
Speight 168 *Mlml*	17.69	2.22	2.51
Speight 168 *MlMl*	17.86	2.11	2.46
Speight 168 Null Segregant	14.66	3.09	3.28

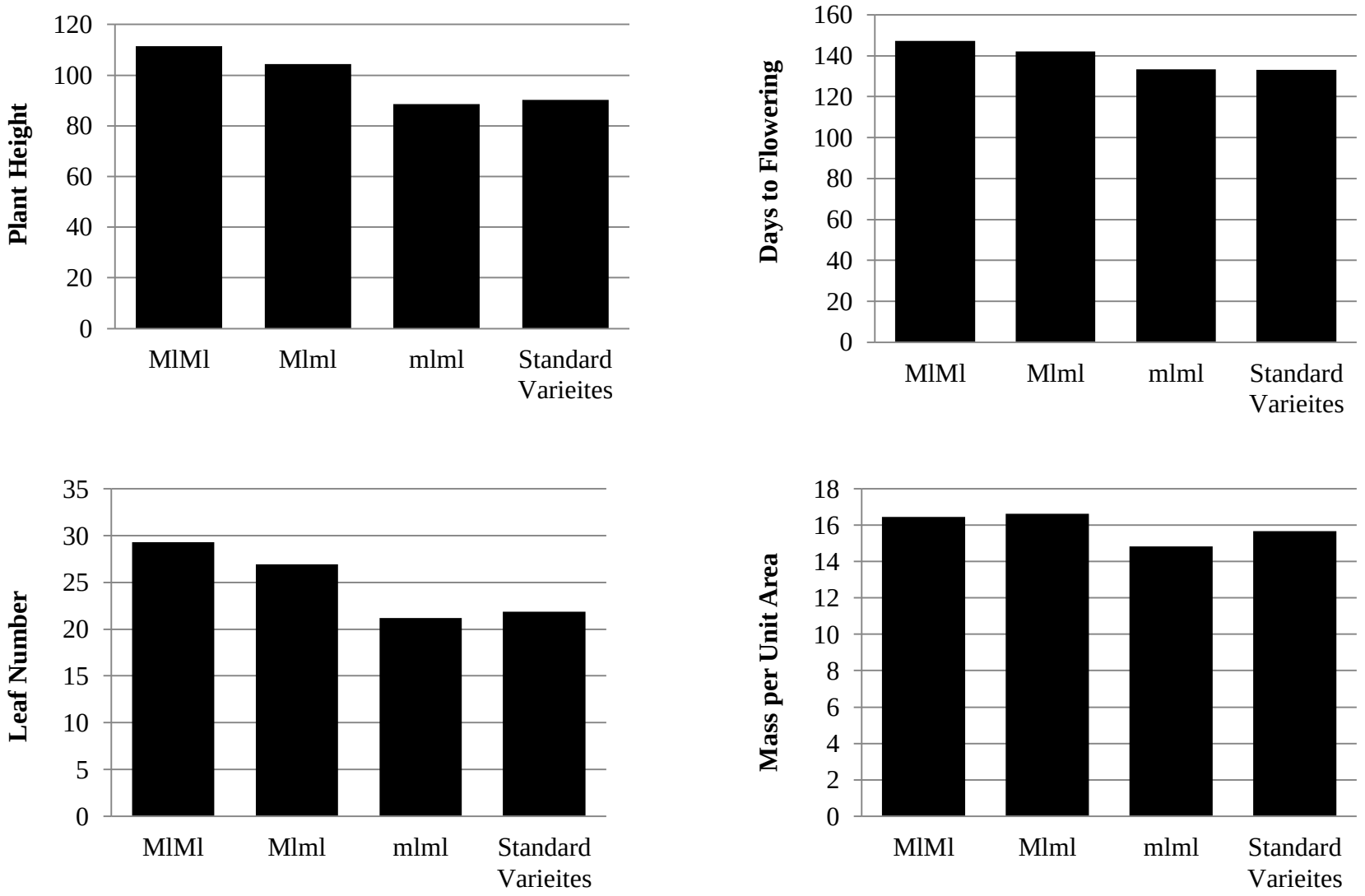


Figure 3.1. Least square mean estimates for plant height, days to flowering, leaf number and mass per unit area analyzed over environments, replications and nitrogen rate.

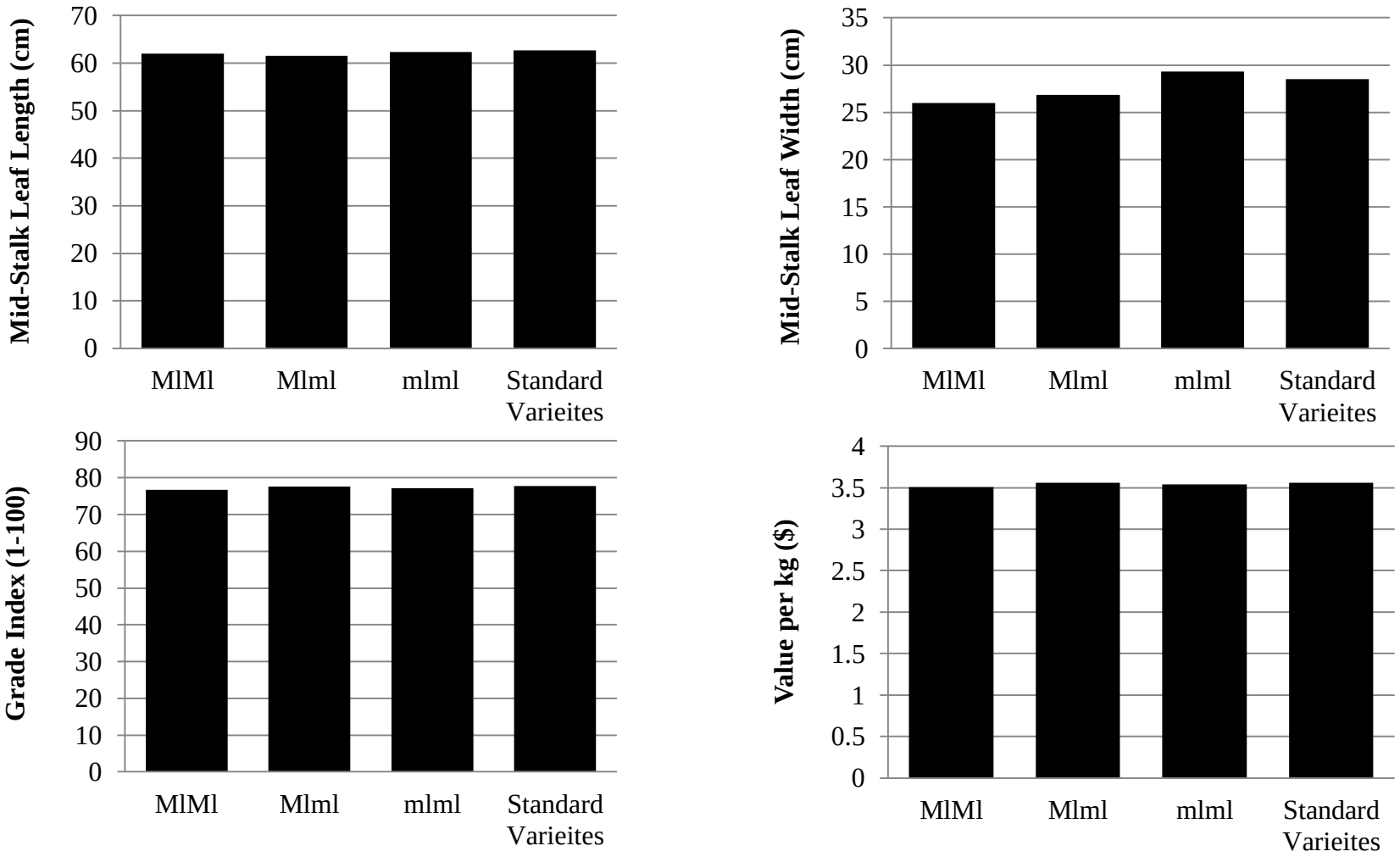


Figure 3.2. Least square mean estimates for mid-stalk leaf length and width, grade index and value per kg analyzed over environments, replications and nitrogen rate.

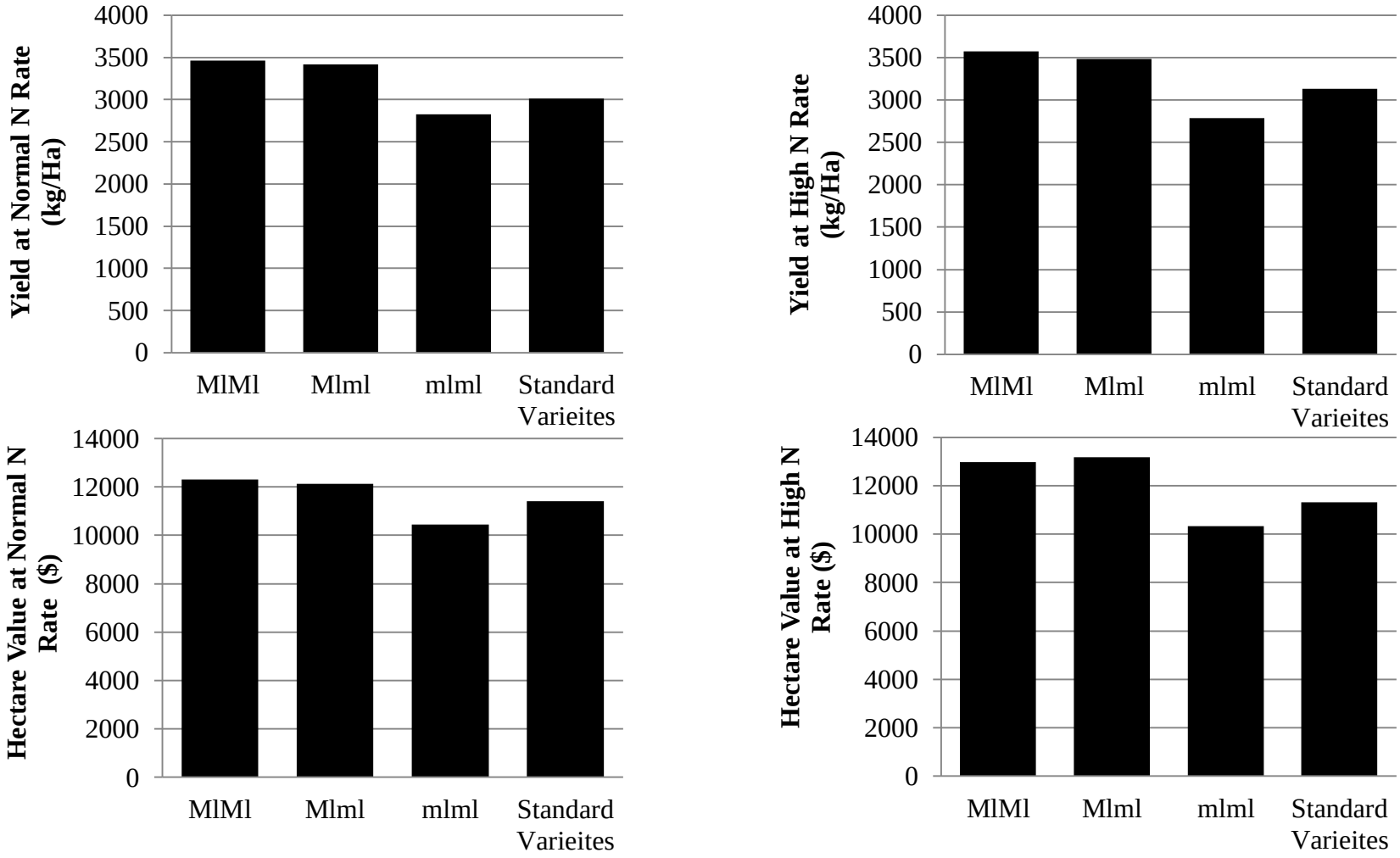
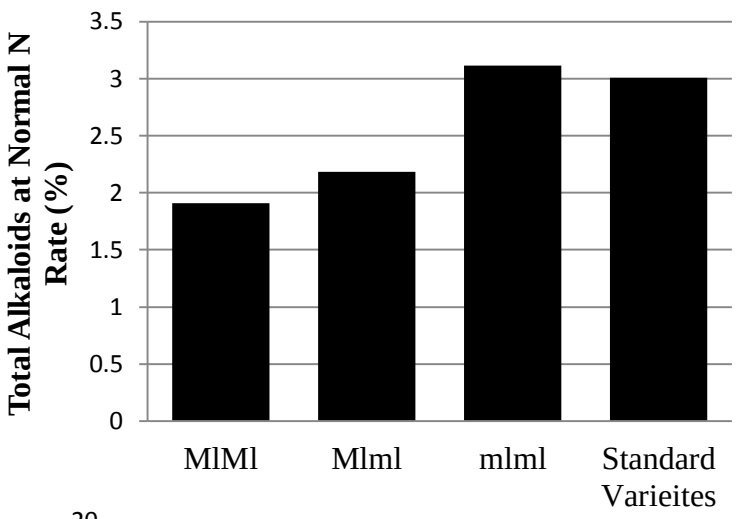
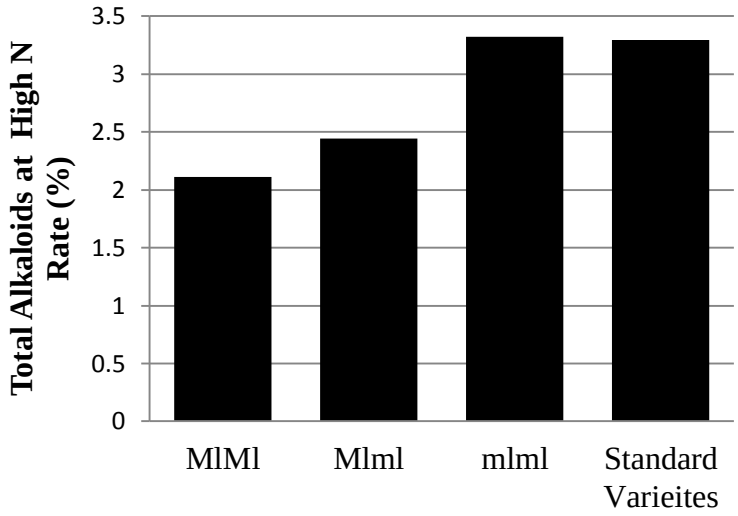


Figure 3.3. Least square mean estimates for yield and hectare value analyzed over environments, replications and at each nitrogen rate.





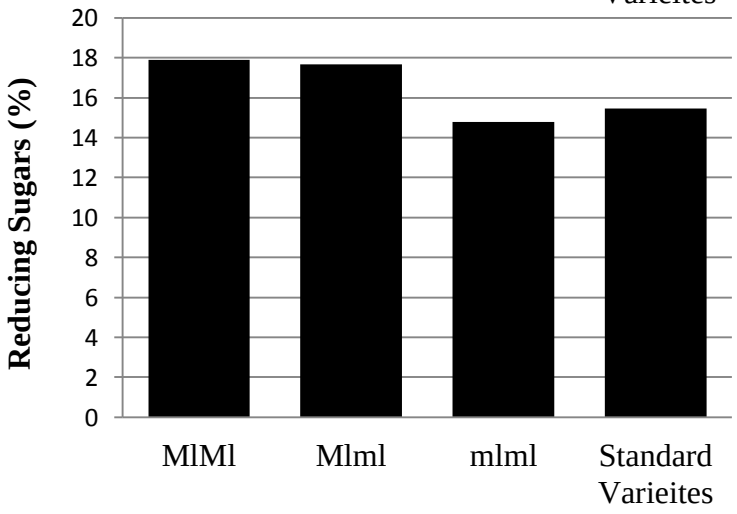


Figure 3.4. Least square mean estimates for percent total alkaloids and percent reducing sugars analyzed over environments, replications and at each nitrogen rate in some cases.

Figure 3.5. Yield testing of Speight 168 genotype entries (*Mlml*, *mlml* and *MlMl*) at the Oxford Tobacco Research Station, Oxford, NC, 2012.

Figure 3.6. Yield testing of K 326 *MlMl* and NCTG-61*MlMl* at the Upper Coastal Plain Research Station, Rocky Mount, NC, 2011.