ABSTRACT

CHEEK, JOSEPH. Comparison of Transgenic and Non-Transgenic Tobacco Varieties for Low Alkaloid Flue-Cured Tobacco Production. (Under the direction of Dr. Loren Fisher)

Nicotine is a secondary plant metabolite produced by plants of the *Nicotiana tabacum* species, and is the compound primarily associated with addiction to tobacco products. The Family Smoking Prevention and Tobacco Control Act of 2009 places nicotine content of tobacco products under the control of the Food and Drug Administration (FDA), and it is speculated that the FDA may mandate the lowering of nicotine levels in cigarettes. As allowable nicotine decreases in tobacco products, it may be necessary to employ low-alkaloid varieties of tobacco to maintain smoke flavor characteristics. At present, low alkaloid cultivars have not been developed that are acceptable by grower and industry standards. Research was conducted in 2014 and 2015 to quantify the agronomic characteristics of three low alkaloid transgenic cultivars (DH16A, DH22A, and DH32), two non-transgenic standard cultivars (K326 and NC95), and one non-transgenic low alkaloid cultivar (LAFC53). Cultivars were managed under three N application programs: Recommended application rate (Rec.), 15% below recommended application rate (Rec. -15), and 30% below recommended application rate (Rec. -30).

As N application rate decreased, leaf length decreased and cured leaf reducing sugar concentration increased. The main effect of cultivar was significant for all parameters, with transgenic cultivars typically producing a narrower leaf with intermediate length that was darker in color and lower in cured leaf yield, quality, and value relative to K326. In general, the phenotypic characteristics of the three transgenic cultivars were similar to NC95 and LAFC53. Cured leaf alkaloid concentration (nicotine, nornicotine, anabasine, and anatabine)

was reduced as N application rate decreased in K326 and NC95; however, those same reductions were not typically observed in the low alkaloid cultivars. In addition, nornicotine concentration was increased in the transgenic cultivars relative to K326, NC95, and LAFC53. Ultimately, low alkaloid cultivars expressed certain phenotypic characteristics that may result in significant changes to agronomic management practices in order to ensure that leaf usability by manufacturers is maintained.

Comparison of Transgenic and Non-Transgenic Tobacco Varieties for Low Alkaloid Flue-Cured Tobacco Production

by
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DEDICATION

This thesis is dedicated to my parents, Nancy and Eric D. Cheek. Thank you for the unwavering support and encouragement you have provided throughout the years. Thank you for providing and environment that encouraged my curiosity in the natural world, and for entertaining the thousands of questions I had growing up. Most of all thank you for providing a hard-working, kind, Christian home for Daniel, Hannah and myself, your hard work and sacrifice has helped us all to achieve great things.

BIOGRAPHY

Joseph Aaron Cheek was born August 30, 1991 in the small town of Liberty, North Carolina to Nancy and Eric Cheek. Joseph's mother is a retired elementary school teacher, and his father was an engineer and farmer. Joseph has two older siblings, both alumni of NCSU. Growing up on a tobacco, row crop, and cattle farm, hard work was part of the everyday life for Joseph. He graduated from Southern Alamance High School in 2010 and started a Bachelor's Degree in Agronomic Science in the fall of 2010. Upon graduation from North Carolina State University in 2014 Joseph began a Master's degree in Crop Science under the direction of Dr. Loren Fisher. Joseph was able to work as an hourly technician for the Tobacco Agronomy project under Matthew Vann, as well as a technician for Dr. Lindsey Thiessen in Tobacco Pathology. Joseph married the love of his life Lauren Sander Cheek, on October 10th, 2015. They live in Raleigh, NC and eventually would like to relocate and start a family on the farm in Liberty.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

1.1 Introduction

Plants are capable of producing a broad spectrum of chemical compounds. These compounds can generally be separated into two fractions; primary metabolites, and secondary metabolites. Primary metabolites are compounds produced by plants that are directly involved in growth and metabolism, such as carbohydrates, lipids, and proteins. Secondary metabolites are produced by plants for many reasons such as being a deterrent to herbivory, a defense against pests and pathogens, or as an attractant to pollinators (Tso 1990). Although the term "secondary metabolites" might imply that plants do not heavy rely on the production of these compounds, in all actuality, for many species plant secondary metabolites are just as important to plant health and growth as primary metabolites (Bennet and Wallsgrove 1994).

Plant Secondary metabolites are generally divided into three major groups; flavonoids, terpenoids, and nitrogen containing metabolites (which include alkaloids) (Bennet and Wallsgrove 1994). Alkaloids are found in over 4,000 different plant species with over 12,000 different chemical forms, and have been used by mankind to create a wide range of products (Encyclopædia Britannica). By definition, alkaloids are a group of basic substances which contain a cyclic nitrogenous nucleus (Tso 1990). The pharmacological activity of some alkaloids has led to the large scale production of plant species which contain desirable amounts of the select alkaloids. Examples include *Papaver somniferum* for production of morphine and other opioids, *Nicotiana tabacum* for nicotine production, *Coffea arabica* for caffeine production, and *Erythroxylum sp*. for cocaine production. Alkaloids are not limited to plants, however, and can be found in a variety of animals, fungi and insects as

well (Tso 1990). Nicotine and related pyridine alkaloids such as nornicotine, anabasine, and anatabine are produced in varying amounts in many *Nicotiana* species, including commercial tobacco *Nicotiana tabacum*. The importance of nicotine rests in its role as a determinant of smoke flavor and primary cause of addiction in various combustible and non-combustible tobacco products (Bush 1999). The ability of tobacco to consistently produce high levels of nicotine in the leaf was the primary stimulus which lead to the global demand for tobacco after its discovery in the Americas (Salmon and Salmon 2013).

1.2 Tobacco Alkaloids

The ability of *N. tabacum* to produce alkaloids and other plant secondary metabolites has been the subject of extensive research, with more than 2,500 compounds having been identified (Nugroho and Verpoorte 2001). Of the many alkaloids found in cultivated tobacco, nicotine is the most abundant, constituting roughly 90% of the total alkaloid pool. Nornicotine, anabasine, and anatabine can also be found in varying levels in cured tobacco leaf. For example, 50 to 60% of *Nicotiana* species produce nicotine as the predominant alkaloid, while 30 to 40% of *Nicotiana* species produce nornicotine as the primary alkaloid (Bush 1999). Nicotine is of particular interest due to the fact that it is primarily responsible for the pharmacological effect experienced by users of tobacco products and is credited with the addictive nature of combustible and non-combustible tobacco products. Although found in relatively small quantities compared to nicotine in flue cured tobacco, nornicotine, anabasine, and anatabine are important due to their role as precursors to carcinogenic tobacco specific N-nitrosamines (TSNAs).

Nicotine

The metabolic pathways that produce nicotine in tobacco have been the subject of a great deal of research. Nicotine is produced primarily in the roots of tobacco and then transported through the xylem to leaf tissue where it is stored in vacuoles to prevent cytotoxic concentrations (Bush et al. 1999). Specifically, primary nicotine production is located in endodermis and cortex regions, behind apical points of growing roots (Bush et al., 1999). Biosynthesis of nicotine is conducted through a series of metabolic pathways which independently produce the pyrrolidine and pyridine rings found in nicotine. Figure 1 illustrates the most current interpretation of the nicotine biosynthetic pathway as outlined by Lewis et al. (2015). Nicotine is produced by the condensation of a pyrrolidine ring, N-methylpyrrolinium, and a pyridine ring, 3,6-dihydronicotinic acid. The ring structures necessary for nicotine synthesis are derived from two distinct pathways. Orinithine decarboxylase (ODC) and arginine decarboxylase (ADC) create putrescine from the amino acids orinithine and arginine, respectively. Putrescine is a key metabolite in pyrrolidine ring construction, and is utilized for many cellular processes. Putrescine-*N*-methyltransferase (PMT) is considered the first dedicated enzyme in nicotine biosynthesis as it directs putrescine into alkaloid production, as opposed to other plant functions. *N*-methylputrescine is formed by PMT, which is converted to 4-methylaminobutanol by *N*-methyputrescine oxidase (MPO). 4-methylaminobutanol undergoes a spontaneous cyclization to form *N*-methylpyrrolinium, which provides the pyrrolidine ring in nicotine. Quinolinate phosphoribosyltransferase (QPT) initiates the pyridine ring synthesis by utilizing quinolinic acid to make nicotinic acid mononucleotide (NAMN) which enters the nicotinamide adenine dinucleotide (NAD) biosynthetic pathway, of which, nicotinic acid is an intermediate.

Nicotinic acid can be combined with Δ^1-piperidine to produce anabasine, a nicotinic acid derivative to form anatabine, and with *N*-methylpyrrolinium to form nicotine (Lewis et al. 2015).

Nornicotine

Nicotine and nornicotine are very similar in structure, and only differ by a single methyl group. Nicotine is converted to nornicotine by nicotine *N*-demethylase (NND). Typically nornicotine is a relatively minor alkaloid present in tobacco, representing about 3-5% of the total alkaloid content (Bush 1999). Some species are more prone to increased levels of nornicotine, these individuals are known as "converters" and can convert as much as 97% of the nicotine in senescing leaf to nornicotine. Increased rate of conversion is due to the amplified production of NND by the *CYP82E4* gene, which is typically silenced in traditional non-convertor lines (Siminszky et al. 2005). Tobacco lines that have high concentrations of nornicotine can yield a tobacco product that has undesirable smoke flavor and quality, and due to its role as the principal precursor for *N*'-nitrosnornicotine (NNN) efforts to regulate nornicotine levels have been extensively evaluated.

Anabasine

Anabasine is composed of pyridine and piperidine rings. As is the case with nicotine, nicotinic acid contributes the pyridine ring found in anabasine, however as indicated in Figure 1, the piperidine ring is formed through a separate pathway. The piperidine ring is synthesized from lysine, through decarboxylation by lysine decarboxylase (LDC) to form cadaverine. Cadaverine is oxidized by diamine oxidase (DAO) to produce 5-aminopetanol, which is followed by spontaneous cyclization creating Δ^1-piperidine. It is believed that these

rings are coupled in a similar fashion to nicotine, with common enzymes (potentially those encoded by *BBL* genes).

Anatabine

Unlike anabasine, both ring structures of anatabine are formed via the nicotinic acid biosynthetic pathway, which implies direct dependency on QPT activity, the pyridine nucleotide cycle, and an "anatabine synthase" (Garrod and Jacob 1999). In tobacco varieties with reduced alkaloid content, anatabine levels tend to be reduced to a greater extent than nicotine, suggesting there is a lower K_m of nicotine for the substrate (Garrod and Jacob 1999).

1.3 Genetic Control of Nicotine Biosynthesis

Genetic regulation of nicotine production is largely influenced by the *Nic1/Nic2* loci, which are semi-dominant, unlinked loci that behave synergistically in regulating alkaloid content. Most genes involved with nicotine biosynthesis and transport such as *ODC, PMT, MPO, QPT, A622, BBL, MATE1/2* and *NUP1* are classified as *NIC*-controlled genes (Shoji and Hashimoto). *Nic1* and *Nic2* have been found to have varying levels of control over total alkaloid accumulation. *Nic1* has a 2.4 fold greater effect compared to *Nic2*, and their effects are additive (Legg and Collins 1971). Mutants expressing recessive *nic1/nic2* loci have been studied using whole plants and cell cultures extensively.

Transgenic cell cultures have provided great utility in studying the biosynthesis of nicotine due to their ability to be created and produced in a controlled environment, with relative limited space, and with a high degree of specificity as to which enzymes are targeted.

Enzymes that have been targeted to alter nicotine biosynthesis include *ODC/ADC, PMT, MPO, QPT2, A622, BBL*.

ODC/ADC

In most plants, ornithine decarboxylase and arginine decarboxylase synthesize putrescine from ornithine and arginine, respectively. Ornithine decarboxylase is capable of synthesizing putrescine directly from ornithine, while putrescine formed from arginine must undergo a three step metabolism initiated by arginine decarboxylase (Malmberg et al. 1998). Efforts to reduce *ADC* activity in transgenic tobacco plants has been successful in developing low *ADC* activity mutants, however, these reductions in activity were not accompanied by similar reductions in nicotine content (Chintapakorn and Hamill 2007). Although there was no notable decrease in nicotine production when *ADC* production was inhibited, there was a slight increase in anatabine concentration. Unlike *ADC*, similar efforts to reduce *ODC* expression in tobacco resulted in large decreases in nicotine content with substantial increases in anatabine production (DeBoer et al. 2011). The ratio of nicotine to anatabine ratio was much lower for *ODC* reduced transgenic plants, averaging 2:1, when compared to non-transformed checks which averaged a 20:1 ratio (DeBoer et al. 2011). Increases in anatabine production are likely due to the build-up of nicotinic acid derivatives formed through the pyridine nucleotide cycle. Anatabine requires the joining of two pyridine rings, while nicotine requires the condensation of a pyridine ring and pyrrolidine ring. Therefore in plants where putrescine synthesis is reduced, nicotinic acid self-condensation is favored. While nicotine production was reduced, it was not reduced to zero which is likely caused by *ADC* also supplying putrescine to feed into the pyrrolidine ring biosynthetic pathway. *ADC*

enzyme activity increased in *ODC* inhibited plants, indicating that the plant was able to partially compensate by increasing an alternate pathway.

PMT

Putrescine-*N*-methyltransferase plays an important role in alkaloid biosynthesis in that it is considered the first dedicated step in alkaloid biosynthesis (figure 1). While *ODC/ADC* provide putrescine, which can be used for both primary and secondary metabolic pathways, *PMT* acts as a gatekeeper, utilizing putrescine and shuttling it into the nicotine biosynthetic pathway. Transgenic plants with lowered *PMT* expression have been well studied with consistent results as summarized by Dewey and Xie 2013. When expression of *PMT* is reduced to varying degrees, nicotine concentration is also reduced in similar proportions which imply a large degree of control over nicotine biosynthesis. Reductions in nicotine production through reduced *PMT* expression result in increased anatabine production, similar to the increases seen in *ODC* suppression. Anatabine is typically found in relatively small quantities in commercially produced cultivars, however, when *PMT* expression is reduced; anatabine can become the primary alkaloid produced. It is possible that the buildup of anatabine is due to the excess production and self-condensation of the pyridine ring, since pyrrolidine ring production has been limited. Unlike suppressed *ODC* mutants, reduced *PMT* variants experienced a much larger reduction in nicotine production, likely due to the lack of an alternate pathway as was observed with *ADC.*

MPO

N-methylputrescine oxidase (MPO) catalyzes the second step in pyridine ring biosynthesis, forming 4-methylaminobutanol from N-methylputrescine. 4-Methylaminobutanol then undergoes spontaneous cylclization to form N-methylpyrrolinium,

which is the pyrrolidine ring necessary for nicotine production (Figure 1). Transgenic manipulation to lower expression of *MPO* in tobacco had similar effects on total alkaloid concentrations when compared to *PMT* suppression with lower nicotine and higher anatabine concentrations (Shoji and Hashimoto 2008).

QPT

Unlike enzymes involved in pyrrolidine ring biosynthesis such as *PMT*, quinolate acid phosphoribosyltransferase (*QPT*) is involved in both primary and secondary metabolism. *QPT* utilizes quinolinic acid as a substrate, acting as the entry point for the pyridine nucleotide cycle, of which, nicotinic acid is an intermediate used for nicotine biosynthesis. The pyridine nucleotide cycle also produces nicotinamide adenine dinucleotide (NAD) which is an important co-factor for primary cell metabolism. Research suggests that there are two members of the *QPT* gene familiy, one of which is responsible for NAD synthesis and primary cell metabolism that is found in varying amounts in all plant tissues (*QPT1*), the other found almost exclusively in the plant roots (*QPT2*), which helps provide a dedicated source of nicotinic acid for nicotine biosynthesis (Shoji and Hashimoto 2011). Efforts to decrease nicotine content by reducing expression of *QPT2* were successful. Xie et al. (2004) created transgenic low alkaloid line Vector 21-41 which produced only 8% of the nicotine found in parent line Burley 21 by targeting cells where nicotine production was taking place for *QPT* suppression, (Xie et al. 2004). It is likely however that increased levels of suppression would have negative impacts on overall plant health due to the role *QPT* plays throughout the plant.

A622

One of the enzymes which plays a role late in nicotine biosynthesis is *A622*. *A622* is a member of the PIP-family of NADPH-dependent reductases, which is believed to potentially form a coupling component in which nicotinic acid is a substrate (Kajikawa et al. 2009). Similar to many other enzymes with dedicated nicotine biosynthesis roles, *A622* is exclusively expressed in the roots and its inhibition has been shown to drastically reduced nicotine content in hairy root systems. The exact enzymatic activity encoded by *A622* is not fully known, however a buildup of nicotinic acid β-N-glucoside (NaNG) has been observed in genetically modified hairy root and cell cultures. It is preposed that NaNG is a detoxification product which is produced in response to the buildup of phytotoxic nicotinic acid, a substrate utilized by *A622* (Kajikawa et al. 2009).

BBL

Another downstream enzyme that seems to play a key role in nicotine biosynthesis is the berberine bridge enzyme-like (*BBL*) gene family. The *BBL* enzyme is a Flavin containing oxidase, which is suspected to play a role in the final oxidation process to form nicotine, anatabine and anabasine (Kajikawa et al. 2011). The *BBL* gene family is regulated by the NIC loci, and was first elucidated using homozygous recessive genotypes (Kajikawa et al. 2011). Transgenic reduction in *BBL* expression has been shown to reduce cured leaf nicotine content by roughly 75% compared to the untransformed control (Lewis et al. 2015).

There have been several successful attempts to lower nicotine by silencing or down regulating various enzymes in both the pyridine and pyrrolidine ring biosynthesis pathways. Nicotine reduction strategies that interrupt the nicotine biosynthetic pathway early within the pyridine or pyrrolidine ring sub-pathways tend to have deleterious effects on leaf texture and

physiology which negatively affect yield and grade index, leading to unmarketable leaf when produced in the field. It is probable that interruptions in the nicotine biosynthetic pathway create buildups of potentially toxic constituents, and/or prevent primary metabolism from taking place.

1.4 Plant hormones regulating nicotine biosynthesis

Auxin

Traditionally, the flowering parts of commercial *Nicotiana tabacum* are removed as a cultural method to increase yield and ensure appropriate leaf chemistry. By removing the apical meristem, auxin production is decreased, thus ceasing apical dominance. As a result, axillary buds begin to proliferate and leaf mass and nicotine production begin to increase. The increase in alkaloid production can largely be credited to auxins' role in suppressing alkaloid production in the roots of *Nicotiana tobacum*. Application of *N*-1-naphthylphthalamic acid (NPA), which is an auxin transport inhibitor to the area of the tobacco stalk just below the apical meristem has been shown to have similar effects to topping, increasing nicotine concentration in the entire plant, without any physical wounding (Li 2007). Unlike treatments where the apical meristem was removed, plants to which NPA was applied exhibited no increase in jasmonic acid as shown in Table 1, yet still had increased nicotine production compared to the control, implying that auxin likely provides an inhibitory regulation on nicotine production, independent of the stimulant effect of jasmonic acid (Li 2007). Auxin has also been shown to effectively suppress nicotine production in tobacco cultured callus, and suppress enzymatic activities in the nicotine biosynthetic pathway (Takahashi and Yamada 1973, Hashimoto et. al 1995).

Jasmonic Acids

Jasmonic acid and methyl jasmonate (MeJA) collectively known as jasmonates, are essential plant hormones that regulate plant responses to environmental stresses such as drought, UV, ozone, wounding, and attacks by pathogens and insects (Farmer et al. 2003). Increases in jasmonate concentration in tobacco can be influenced by mechanical topping, herbivory and mechanical injury to leaf tissue. Leaf lamina injury activates the wound response pathway, mediated primarily by jasmonic acids (JAs) (Baldwin et al. 1994). Jasmonic acid is derived from linolenic acid via the octadecanoid pathway (Shoji et al. 2000). JAs produced in the leaf tissue of *Nicotiana* are transported to the roots through the phloem, where they are credited with activating most of the genes necessary for nicotine production and accumulation: *ODC, PMT, MPO, AO, QS, QPT, A622, BBL, MATE1/2, JAT1, and NUP1* (Shoji and Hashimoto 2013).

Ethylene

Ethylene is a gaseous plant hormone that commonly plays a role in germination, senescence, and fruit ripening. Ethylene acts as a suppressor of JA activated structural genes such as *ODC, PMT,* and *A622* (Shoji et al. 2000). By suppressing response to JA, ethylene can effectively reduce production of nicotine as a response to wounding. The antagonistic relationship between jasmonate and ethylene signals is relatively unique to the nicotine biosynthetic pathway when compared to synergistic relationships found in many other plant species. It is possible this relationship exists to prevent plant from producing excess nicotine when under herbivory stress, due to the heavy nitrogen requirement for nicotine synthesis. Utilization of nitrogen for increased nicotine production can lead to a decrease in seed production, therefore ethylene is required to regulate the jasmonate induced response.

Nicotine tolerant larvae of *Manducta sexta* utilize this response by inducing ethylene production, preventing a subsequent increase in nicotine as a result of herbivory (Kahl et al. 2000).

1.5 Cultural practices effecting nicotine biosynthesis

The production of flue cured tobacco has been manipulated throughout the years to optimize the nicotine to reducing sugar ratio so that high quality, full flavored cigarettes can be produced (Collins and Hawks 1994). The physical and chemical properties of flue cured tobacco leaves are influenced by genetics, agricultural practices, soil type, nutrients, weather conditions, plant disease, herbivory, stalk position, harvesting and curing procedures (Collins and Hawks 1994). The primary alkaloids produced and the inherent production capacity of each is determined by the genetic makeup of the plant (Mann et al. 1975). Altering any number of cultural practices and environmental influences can have a large effect on leaf alkaloid content. Generally speaking, any factor that would enhance the growth of the tobacco plant, will increase alkaloid production and accumulation. Increasing nitrogen fertility, decreasing topping height, decreased soil temperature, increased length of photoperiod, increased time between topping and harvest, and increased sucker control all increased nicotine production, likely due to increases in leaf yield (Bush 1999). Inversely, wet conditions, root inhibiting layers, under-fertilization, and anything which hinders the development of a healthy root system will lead to decreased nicotine production. Since nicotine is synthesized in growing root tips, nicotine concentrations are highest there, however, when those areas are combined with the entire root system, total concentration falls below that of leaf lamina (Bush 1999). Alkaloid concentrations can be 200 to 300% higher

near the top of the stalk compared to lower stalk positions at harvest (Bush 1999). Alkaloid concentrations in *Nicotiana* germplasm maintained by the U.S. Department of Agriculture can range from 0.2 to 7.8% (Tso 1990).

Nitrogen (N) fertility has long been recognized as one of the primary influences on nicotine content in flue cured tobacco. Although most necessary plant nutrients (phosphorus, potassium, sulphur, manganese, etc) have little to no effect on nicotine content, N has been repeatedly studied for its relationship with tobacco nicotine content. As N rate increases, nicotine content in cured leaf also increases as shown in table 1.2 (Woltz and Hutcheson 1953). This correlation between increased N rate and increased nicotine content is largely due to the role N plays as a primary constituent in the synthesis of nicotine and other alkaloids. While N rate has a direct influence on alkaloid content of tobacco, when comparing urea ammonium nitrate, calcium nitrate, and ammonium nitrate, N source has not been shown to have any influence on total alkaloid content (Parker 2008).

1.6 Tobacco Regulation

Domestic tobacco consumption has been decreasing for a several decades, beginning with the Surgeon General's report: *Smoking and Health* in 1964, which identified cigarettes as a public health threat. From 1965 to 2012 adult smoking rates have fallen from 43% to 18% (U.S. Department of Health and Human Services. 2014). Despite lower rates of consumption, tobacco use continues to be the leading cause of preventable death in the United States, resulting in more than 480,000 deaths annually and an associated healthcare cost of nearly $US 300 billion each year (FDA 2017). Former US president Barack Obama signed into law the Family Smoking Prevention and Tobacco Control Act (Tobacco Control

Act) of 2009 which placed tobacco products under the regulatory jurisdiction of the Food and Drug Administration (FDA). The Tobacco Control Act authorizes the FDA to restrict marketing and sales to youth, require large warning labels on smokeless tobacco products, and request the disclosure of ingredients in tobacco products from manufacturers (H.R. 2009). Most importantly, the FDA now has the authority to regulate the level of nicotine in commercial tobacco products (H.R. 2009). Current domestically produced cigarettes range from 10-15 mg nicotine per stick (Benowitz and Henningfield 2013). Although the Tobacco Control Act prevents the FDA from eliminating nicotine in tobacco products, the agency has taken actions to potentially decrease total nicotine content of cigarettes to "achievable market standards" which translate into near-zero levels of nicotine as the end goal.

Nicotine is the primary constituent in tobacco smoke that results in consumer addiction. Many harm reduction efforts have focused on reducing the addictive nature of tobacco products by reducing the nicotine content of those products. Reducing levels of nicotine in combustible products has been shown to increase consumption in some instances, as more cigarettes are subsequently required to achieve the same level of nicotine intake (Nakazawa et al. 2004). American blend cigarettes have, on average, 8-9 mg of nicotine per stick, with a maximum bioavailability of roughly 40% (Benowitz et al. 1983). Research suggests, however, that if bioavailable nicotine levels are low enough (0.17 mg per cigarette) addiction cannot be sustained; therefore, the desire to over-consume cigarettes would not exist (Benowitz and Henningfield 1994). A reduction that large would constitute a 95% reduction in nicotine content for American blend cigarettes. An additional public health benefit to utilizing sub-addictive levels of nicotine in cigarettes is that the propensity of first time smokers to become addicted is greatly decreased (Benowitz and Henningfield 2013).

Ultimately, it is difficult to provide any certainty about establishing minimum nicotine thresholds as this varies a great degree between individuals and also is influenced by previous exposure to nicotine (Benowitz et al. 2010).

Reducing the total nicotine content in cigarettes is a much different concept than creating low nicotine delivery cigarettes that have been developed within the industry. Low delivery cigarettes such as those labeled "low tar and nicotine" and "light" cigarettes deliver lower nicotine yields when evaluated on smoking machines; however, the actual amount of nicotine consumed by a human smoker can vary greatly based on how the individual smokes the cigarette (Benowitz et al. 1983). For example, Nakazawa et al. (2004) demonstrated that compensatory smoking can be observed when smokers are asked to switch from regular to light cigarettes, so that they can maintain an equivalent level of nicotine consumption (Nakazawa et al. 2004). The primary danger associated with the consumption of cigarettes is not exposure to nicotine specifically, but is instead the prolonged exposure to toxic combustion products that occur with nicotine delivery. Since it is smoke, and not necessarily nicotine, that is the primary danger, any strategies that seek to limit nicotine consumption must do so in a method that limits the risks of compensatory smoking (Benowitz and Henningfield 2013). Therefore, from a regulatory perspective, the only "safe" way to reduce nicotine consumption is to lower the total amount of nicotine in the cigarette so as to preclude and reduce dependency on nicotine and not just alter the construction of the cigarette to lessen the amount conveyed (Benowitz and Henningfield 2013). In order to produce tobacco which can be used to manufacture reduced nicotine cigarettes, cultivars with inherently low levels of nicotine must be developed and commercialized.

1.7 Conclusion

If the FDA mandates lower nicotine levels in combustible tobacco products, there will be a need for low alkaloid varieties and potentially altered cultural practices to produce tobacco that can provide similar flavor and leaf characteristics to traditional varieties so that smoke characteristics are preserved, while nicotine levels are decreased. Strategies to reduce nicotine levels in flue cured tobacco might include shifting current agricultural practices to influence leaf chemistry. Modification of agronomic practices, alone, will not be capable of reducing nicotine content of tobacco to levels deemed necessary by the FDA. Many attempts to alter the alkaloid content of flue cured tobacco through genetic manipulation have resulted in undesirable leaf quality, yield and smoke flavor characteristics when compared to traditional commercial varieties (references). Resent research has been conducted however that may provide a reduced nicotine variety of tobacco that avoids some of the common issues associated with low alkaloid varieties. By using RNAi constructs to down regulate the *BBL* gene family in K326, viable low alkaloid varieties have been developed. The interruption of the nicotine biosynthetic pathway at a very late stage decreases the likelihood of disrupting aspects of primary metabolism.

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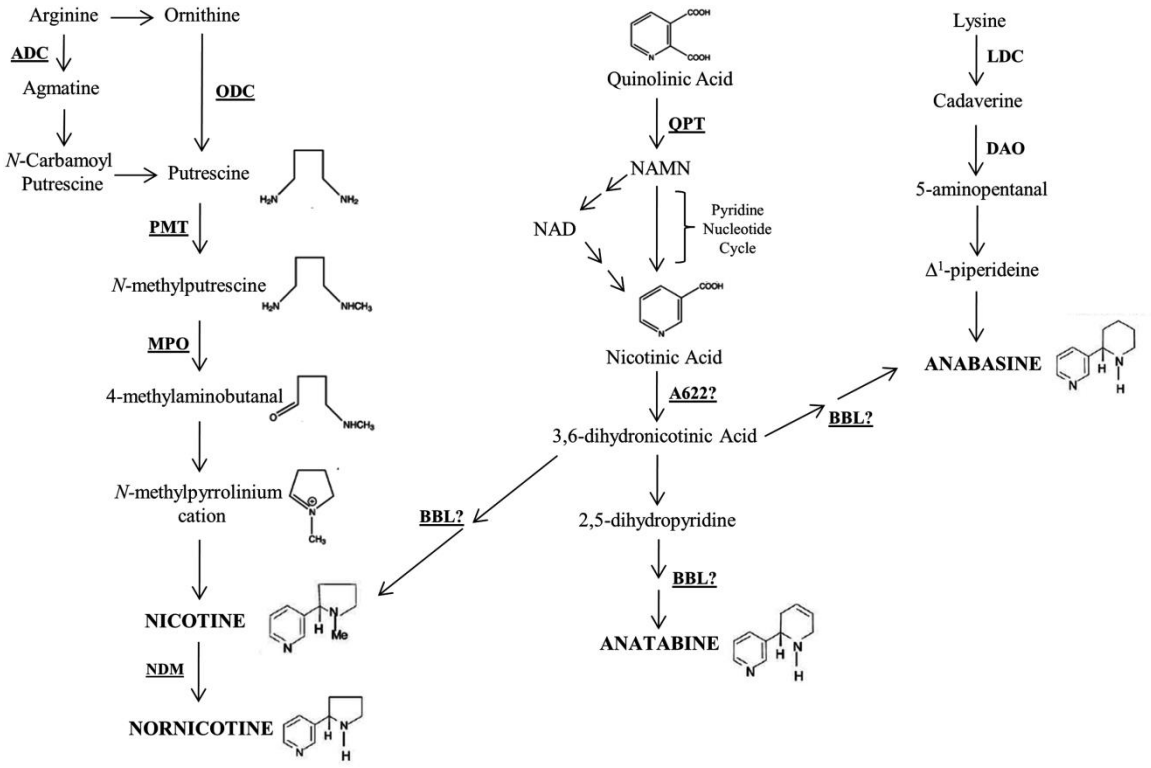

Figure 1.1 Model for alkaloid biosynthesis in *N. tabacum.* (Lewis et al. 2015)

Table 1.1

IAA and JA concentration in stem tissue 90 min after removing the shoot apex or 210 min after application of NPA (Li et al. 2007)

	Control	Treatments Removing apex	NPA
IAA concentration (ng g^{-1} FW)	82 ± 19	59 ± 13	37 ± 12
JA concentration (ng g^{-1} FW)	5.6 ± 0.5	133.2 ± 25.2	3.7 ± 0.4

Samples of different treatments were taken at the same position, either below the cut-surface after excision of the shoot apex, or below the position of NPA application. GC-MS (Trace 2000-Voyager, Finnigan, Thermo-Quest, USA) was employed to analyze IAA and JA concentration. Means ± SE, n = 3.

Table 1.2.

Average nicotine contents of Oxford 26 tobacco differentially fertilized with nitrogen and potash. (Sandy loam soils at Oxford and Rocky Mount, 1946-1948) (Weybrew et al. 1953)

Nitrogen Fertilization	Potash Fertilization (lb/A)			Nitrogen
(lb/A)	30	90	150	Average
0	1.41	1.31	1.28	1.33
30	------	1.89	------	1.89
60	2.98	2.80	2.79	2.86
Potash Means	2.20	2.00	2.04	
				LSD 5% .16
				1% .21

Chapter Two

Comparison of Transgenic and Non-Transgenic Tobacco Varieties for Low Alkaloid Flue-Cured Tobacco Production.

Abstract

Nicotine is a secondary plant metabolite produced by plants of the *Nicotiana tabacum* species, and is the compound primarily associated with addiction to tobacco products. The Family Smoking Prevention and Tobacco Control Act of 2009 places nicotine content of tobacco products under the control of the Food and Drug Administration (FDA), and it is speculated that the FDA may mandate the lowering of nicotine levels in cigarettes. As allowable nicotine decreases in tobacco products, it may be necessary to employ low-alkaloid varieties of tobacco to maintain smoke flavor characteristics. At present, low alkaloid cultivars have not been developed that are acceptable by grower and industry standards. Research was conducted in 2014 and 2015 to quantify the agronomic characteristics of three low alkaloid transgenic cultivars (DH16A, DH22A, and DH32), two non-transgenic standard cultivars (K326 and NC95), and one non-transgenic low alkaloid cultivar (LAFC53). Cultivars were managed under three N application programs: Recommended application rate (Rec.), 15% below recommended application rate (Rec. -15), and 30% below recommended application rate (Rec. -30).

As N application rate decreased, leaf length decreased and cured leaf reducing sugar concentration increased. The main effect of cultivar was significant for all parameters, with transgenic cultivars typically producing a narrower leaf with intermediate length that was darker in color and lower in cured leaf yield, quality, and value relative to K326. In general, the phenotypic characteristics of the three transgenic cultivars were similar to NC95 and

LAFC53. Cured leaf alkaloid concentration (nicotine, nornicotine, anabasine, and anatabine) was reduced as N application rate decreased in K326 and NC95; however, those same reductions were not typically observed in the low alkaloid cultivars. In addition, nornicotine concentration was increased in the transgenic cultivars relative to K326, NC95, and LAFC53. Ultimately, low alkaloid cultivars expressed certain phenotypic characteristics that may result in significant changes to agronomic management practices to ensure that leaf usability by manufacturers is maintained.

2.1 Introduction

The ability of tobacco (*Nicotiana* sp.) to produce alkaloids and other secondary plant metabolites has been subject to an extensive amount of research, with more than 2,500 compounds having been identified (Nugroho and Verpoorte, 2001). Of the many alkaloids produced by the commercially cultivated species *Nicotiana tabacum*, nicotine is the most abundant, constituting roughly 90% of the total alkaloid pool (Tso, 1990). In addition, nornicotine, anabasine, and anatabine are also found at varying concentrations in cured tobacco leaf. Nicotine is of particular interest due to the fact that it is primarily responsible for the pharmacological effects associated with tobacco product consumption, and is credited with the addictive nature of combustible and non-combustible tobacco products. Nornicotine, anabasine, and anatabine, although found in relatively small quantities compared to nicotine, are important due to their role as precursors to carcinogenic tobacco specific N-nitrosamines (TSNAs).

2.2 Tobacco Regulation

Domestic tobacco consumption has been decreasing for a several decades, beginning with the Surgeon General's report: *Smoking and Health* in 1964, which identified cigarettes as a public health threat. From 1965 to 2012 adult smoking rates have fallen from 43% to 18% (U.S. Department of Health and Human Services. 2014). Despite lower rates of consumption, tobacco use continues to be the leading cause of preventable death in the United States, resulting in more than 480,000 deaths annually and an associated healthcare cost of nearly $US 300 billion each year (FDA 2017). Former US president Barack Obama signed into law the Family Smoking Prevention and Tobacco Control Act (Tobacco Control

Act) of 2009 which placed tobacco products under the regulatory jurisdiction of the Food and Drug Administration (FDA). The Tobacco Control Act authorizes the FDA to restrict marketing and sales to youth, require large warning labels on smokeless tobacco products, and request the disclosure of ingredients in tobacco products from manufacturers (H.R. 2009). Most importantly, the FDA now has the authority to regulate the level of nicotine in commercial tobacco products (H.R. 2009). Current domestically produced cigarettes range from 10-15 mg nicotine per stick (Benowitz and Henningfield 2013). Although the Tobacco Control Act prevents the FDA from eliminating nicotine in tobacco products, the agency has taken actions designed to potentially decrease total nicotine content of cigarettes to "achievable market standards" which translate into near-zero levels of nicotine as the end goal.

Nicotine is the primary constituent in tobacco smoke that results in consumer addiction. Many harm reduction efforts have focused on lowering the addictive nature of tobacco products by reducing the nicotine content of those products. Reducing levels of nicotine in combustible products has been shown to increase consumption in some instances, as more cigarettes are subsequently required to achieve the same level of nicotine intake (Nakazawa et al., 2004). American blend cigarettes have, on average, 8-9 mg of nicotine per stick, with a maximum bioavailability of roughly 40% (Benowitz et al. 1983). Research suggests, however, that if bioavailable nicotine levels are low enough (0.17 mg per cigarette) addiction cannot be sustained; therefore, the desire to over-consume cigarettes would not exist (Benowitz and Henningfield, 1994). A reduction that large would constitute a 95% reduction in nicotine content for American blend cigarettes. An additional public health benefit to utilizing sub-addictive levels of nicotine in cigarettes is that the propensity of first

time smokers to become addicted is greatly decreased (Benowitz and Henningfield 2013). Ultimately, it is difficult to provide any certainty about establishing minimum nicotine thresholds as this varies a great degree between individuals and also is influenced by previous exposure to nicotine (Benowitz et al. 2010).

Reducing the total nicotine content in cigarettes is a much different concept than creating low nicotine delivery cigarettes that have been developed within the industry. Low delivery cigarettes such as those labeled "low tar and nicotine" and "light" cigarettes deliver lower nicotine yields when evaluated on smoking machines; however, the actual amount of nicotine consumed by a human smoker can vary greatly based on how the individual smokes the cigarette (Benowitz et al. 1983). For example, Nakazawa et al. (2004) demonstrated that compensatory smoking can be observed when smokers are asked to switch from regular to light cigarettes, so that they can maintain an equivalent level of nicotine consumption (Nakazawa et al 2004). The primary danger associated with the consumption of cigarettes is not exposure to nicotine specifically, but is instead the prolonged exposure to combustion products that occur with nicotine delivery. Since it is smoke, and not necessarily nicotine, that is the primary danger, any strategies that seek to limit nicotine consumption must do so in a method that limits the risks of compensatory smoking (Benowitz and Henningfield 2013). Therefore, from a regulatory perspective, the only "safe" way to reduce nicotine consumption is to lower the total amount of nicotine in the cigarette so as to preclude and reduce dependency on nicotine and not just alter the construction of the cigarette to lessen the amount conveyed (Benowitz and Henningfield 2013). In order to produce tobacco which can be used to manufacture reduced nicotine cigarettes, cultivars with inherently low levels of nicotine must be developed and commercialized.

2.3 Factors influencing alkaloid production

Flue-cured tobacco has been bred to optimize the reducing sugar:nicotine ratio so that high quality, full-flavored cigarettes may be produced (Collins and Hawks 2013). The physical and chemical properties of flue-cured tobacco leaves are influenced by genetics, agronomic practices, soil type, nutrient availability, climatic conditions, plant disease, herbivory, stalk position, and harvesting and curing procedures (Collins and Hawks 2013). The primary alkaloids produced and the inherent production capacity of each is determined by the genetic makeup of the plant (Mann et al. 1975). Altering any number of cultural practices and environmental influences can have a large effect on leaf alkaloid content. Generally speaking, any factor that may enhance the growth of the tobacco plant, will increase alkaloid production and accumulation. Increasing nitrogen fertility, decreasing topping height, decreased soil temperature, increased length of photoperiod, increased time between topping and harvest, and increased sucker control typically increase nicotine production (Bush 1999). Stalk position can also have a major effect, in that alkaloid concentrations can be 200 to 300% higher near the top of the stalk compared to lower stalk positions at harvest (Bush 1999). Two of the variables with the most influence over nicotine production are often viewed as nitrogen fertility and varietal selection (Tso 1990).

2.4 Role of Nitrogen

Nitrogen (N) is an essential plant nutrient, and is one of the principal constituents in the formation of many primary and secondary plant metabolites. Although there is an abundance of N in the natural environment, a relatively small amount of plant available N (in the form of nitrate and/or ammonium) is available for plant uptake and utilization. To

remedy this shortcoming, it becomes necessary that agriculturalists apply supplemental N in the form of fertilizers.

Traditionally, N fertility is viewed as one of the most influential cultural practices with regards to leaf quality. Correct levels of applied N are necessary to produce high yielding, quality leaf that possesses smoke characteristics desirable for cigarette production (Flower 1999; Collins and Hawks 2013). Nitrogen must be supplied in adequate amounts to ensure that leaves become physiologically mature, but not in excess quantity which would prevent timely senescence. If leaves are harvested at physiological maturity but N is in excess, they will be difficult to cure, and leaf quality is likely to be diminished. Immature leaves that are nitrogen deficient may appear ripe, but will not obtain maximum yield nor will they contain the appropriate balance of nicotine and reducing sugars (Flower 1999).

Nitrogen fertility has long been recognized as one of the primary influences on nicotine content in flue-cured tobacco. Although most plant essential nutrients (phosphorus, potassium, sulfur, manganese, etc.) have little to no effect on nicotine content, as N rate increases, nicotine content in cured leaf also increases (Woltz and Hutcheson 1953; Collins and Hawks 2013). The direct correlation between N application rate and nicotine concentration is largely due to the role N plays as a primary constituent in the synthesis of nicotine and other alkaloids. While N rate has a direct influence on alkaloid content of tobacco, comparisons of urea-ammonium-nitrate, calcium nitrate, and ammonium nitrate, N source have shown differences with regards to total alkaloid content (Parker 2008; Chaplin and Miner 1980).

2.5 Genetic Control of Nicotine Production

The principal variation in alkaloid type and concentration is due to genetic differences within the *Nicotiana* genus. Alkaloid concentrations in tobacco germplasm reported by Tso (1990) ranged from 0.2 to 7.8%. Maryland and Turkish types of tobacco are generally lower in nicotine content, while flue-cured, burley, Cuban, and Connecticut cigar wrapper types have moderate levels of nicotine, and Pennsylvania and dark fire-cured types are relatively high in nicotine concentration (Tso 1990). Most varieties of commercially produced tobacco (*Nicotiana tabacum*) produce nicotine as their primary alkaloid, although other species such as *Nicotiana glauca* and *Nicotiana sylvestris* produce primarily anabasine and nornicotine, respectively (Weybrew et al. 1953). The overall concentration of alkaloids is also under direct genetic control, as evidenced by *Nicotiana rustica* which produces nicotine as the primary alkaloid, at levels several times more than that of *N. tabacum* (Weybrew et al. 1953)

Genetic regulation of nicotine production is primarily influenced by the *Nic1/Nic2* loci, which are semi-dominant, unlinked loci that behave similarly in regulating alkaloid content (Legg and Collins 1971). Mutants expressing recessive *nic1/nic2* loci have been studied as whole plant and in cell culture extensively. The genes involved with nicotine biosynthesis and transport, such as *ODC, PMT, MPO, QPT, A622, BBL, MATE1/2* and *NUP1,* are classified as *NIC*-controlled genes (Shoji and Hashimoto 2010). *Nic1* and *Nic2* have been found to have varying levels of control over total alkaloid accumulation. *Nic1* has a 2.4 fold greater effect compared to *Nic2*, and their effects are additive (Legg and Collins 1971).

One method to create a broad spectrum of reduced alkaloid content is by genetically manipulating tobacco to down regulate or silence the various enzymes associated with

alkaloid production. Some of the enzymes targeted for nicotine reduction have included *ADC/ODC, PMT, MPO, A622* and *BBL* (Chintapakorn and Hamill 2003; Chintapakorn and Hamill 2007, DeBoer et al. 2011; Shoji and Hashimoto 2008; Kajikawa et al. 2009; Lewis et al. 2015). Transgenic manipulation of *ADC/ODC, PMT, MPO, A622* and *BBL* has produced low nicotine material, however, total alkaloid production was not always as drastically reduced. The reduction in nicotine during suppression of *ADC/ODC, PMT*, and *MPO* was complimented by a large increase in anatabine production in most cases (Dewey and Xie 2013). Increases in anatabine production are likely due to the build-up of nicotinic acid derivatives formed through the pyridine nucleotide cycle (Dewey and Xie 2013). Anatabine requires the joining of two pyridine rings, while nicotine requires the condensation of a pyridine ring and pyrrolidine ring. Enzymes *ADC/ODC, PMT*, and *MPO* are all involved in pyrrolidine ring biosynthesis, which would limit the amount of *N*-methyl-Δ^1-pyrrolinium available for nicotine formation, causing the buildup of anatabine (Dewey and Xie 2013). It is suspected that *A622* is involved late in the nicotine biosynthetic pathway by forming a nicotinic acid derivative common to all tobacco pyridine alkaloids, or in catalyzing the final condensation reaction that couples pyrrolidine and pyridine rings to form nicotine. The *BBL* gene family has been suggested to play a role very late in the nicotine biosynthetic pathway, and potentially the final condensation reaction to form nicotine, anatabine, and anabasine (Lewis et al. 2015). Plants that have been transformed using RNAi constructs to down regulate *BBL* production have been shown to have a 60-83% lower concentration of nicotine in the cured leaf (Lewis et al. 2015).

As the FDA continues to mandate lower levels of nicotine in combustible tobacco products, the need may arise for low alkaloid varieties of tobacco that can provide similar

flavor and leaf characteristics relative to traditional varieties so that favorable smoke characteristics are preserved, while nicotine levels are decreased. Many attempts to alter the alkaloid content of flue cured tobacco varieties have resulted in undesirable leaf quality, yield, and smoke flavor characteristics when compared to traditional commercial varieties. Alteration of plant biosynthetic pathways often leads to buildup of deleterious substrates and byproducts which have a negative impact on overall plant health and performance, as well as cured leaf quality. Plants that have been modified to reduce expression of the BBL gene family show some utility in an alkaloid reduction program due to the fact that the BBL gene family is responsible for one of the final steps in nicotine biosynthesis (Lewis et al. 2015). Since BBL is responsible for one of the final steps in nicotine biosynthesis, it is possible there will be a reduced negative influence on plant physiology and leaf chemistry. The research reported herein was conducted to compare the yield, quality, value, and leaf chemistry of four low alkaloid flue-cured tobacco cultivars to that of two conventional cultivars grown using different nitrogen fertilizer application rates. The nitrogen fertility rates utilized for this research were recommended, and then reduced rates. Reduced rates were evaluated to see if reduced alkaloid lines require less nitrogen to mature and ripen in a timely fashion.

2.6 Materials and Methods

Site and Experimental Design

Research was conducted at the Lower Coastal Plain Research Station (LCPRS) in Kinston, NC, the Upper Coastal Plain Research Station (UCPRS) in Rocky Mount, NC, and the Oxford Tobacco Research Station (OTRS) in Oxford, NC during the 2014 and 2015

growing seasons. With the exception of nitrogen application rate, tobacco cultivars were produced using practices recommended by the North Carolina Cooperative Extension Service (Fisher et al., 2014). Tobacco was produced on a Norfolk sandy loam soil (Fine-loamy, kaolinitic, thermic Typic Kandiudults) in each LCPRS and UCPRS environments and an Appling sandy loam (Fine, mixed, semiactive, thermic Typic Hapludults) in both OTRS environments. Soil pH at all six sites ranged from 5.8-6.2 with soil organic matter being less than one percent within each environment, per recommendations from the North Carolina Cooperative Extension Service (Fisher et al., 2014).

Treatments were replicated four times in each environment and imposed in a split-plot design with a factorial treatment arrangement. The whole plot factor was N application rate and sub-plot factor was cultivar. Individual plots contained two treated rows, each 1.12 m wide by 15.24 m long at the LCPRS and 1.22 m wide by 15.24 m long at the OTRS and UCPRS. Planting density within each environment was 14,820 plants per hectare.

Description of Nitrogen Application Rates and Cultivars

Nitrogen application rates for the experiment were based the recommended rate for each location (78 kg N/ha at UCPRS and OTRS, 84 kg N/ha at LCPRS), -15% the recommended rate (66 kg N/ha at UCPRS and OTRS, 71 kg N/ha at LCPRS), and -30% the recommended rate (55 kg N/ha at UCPRS and OTRS, 59 kg N/ha at LCPRS) (Vann and Smith 2014). Reduced rates of N were evaluated based on the hypothesis that low alkaloid varieties would likely require less nitrogen to mature normally, due to decreased N demand from alkaloid synthesis. Nitrogen was applied at a base rate of 34 kg/ha 10 days after transplanting with the additional N applied at layby (when plant height was approximately 38 cm), in the form of 28% liquid urea ammonium nitrate (UAN). Layby applications of

nitrogen were delivered with a CO_2-pressurized backpack sprayer containing a single TG-3 or TG-5 full cone nozzle or a TX-12 hollow cone nozzle at an operating pressure range of 100-150 kPa, depending upon desired output. Sidedress placement of N was a single band, 10.2 cm away from the row-ridge at a 10.2 cm depth. Post-application cultivation was used to incorporate N into the soil.

Three transgenic low alkaloid cultivars (DH22A DH32, DH16A), one conventional low alkaloid cultivar (LAFC53), and two non-transgenic conventional cultivars (K326 and NC95) were randomized over the three different nitrogen rates, for a total of 18 treatments. Transgenic varieties were altered to reduce expression of the *BBL* gene family, and were selected based on previous field evaluations for low alkaloid production. The transgenic double haploid lines were produced from K326 with RNAi constructs that reduced expression of the *BBL* gene family. K326 was utilized as a check cultivar because it is the parent of the three transgenic varieties. LAFC53 is a low alkaloid variety which was developed through traditional breeding practices. NC95 is a parent line for LAFC53. NC95 and K326 are also check cultivars in the Minimum Standards program, so their yield, quality, and alkaloid characteristics are well recorded.

Data Collection

All plant measurements were averages based upon data collected from 10 random plants within each plot. Leaf length and width were measured from a mid-stalk leaf position after first harvest to ensure maximum expansion. Ten 23.7 mm leaf discs were taken from tip leaves after first harvest, dried to 0% moisture and weighed to estimate relative tip leaf density. Chlorophyll content of leaves was estimated using a Konica Minolta SPAD-502 meter and measurements were taken once on tip leaves to quantify differences in leaf color in

upper stalk positions before second harvest. Both SPAD meter readings and leaf discs were collected approximately three centimeters from the leaf margin and seven centimeters from the leaf tip. Tobacco was hand-harvested four times and cured in a forced-air bulk curing barn. Stalk height was measured and leaf nodes were counted after harvest to estimate total number of leaves per stem. After curing, leaves were weighed and assigned a USDA grade based on leaf maturity and ripeness. Grades were assigned a monetary value that is associated with a grade index value (Bowman et al., 1988). One-hundred gram cured leaf composite samples from each plot were collected and analyzed for reducing sugars and alkaloids.

Quantitative determinations of nicotine, nornicotine, anabasine, and anatabine in ground leaf were made via gas chromatographic analyses using an Agilent HP 6890 GC-FID and a 30 m DB-5MS column (J & W 0.53 mm i.d and 1.50 μm film thickness) with a 1 m deactivated guard column in front (Lewis et al. 2015). Percent total alkaloids were reported as the sum of percent nicotine, nornicotine, anabasine, and anatabine. Percent reducing sugars for cured leaf samples was quantified using the method of Davis (1976).

Data Analysis

Analysis of variance was conducted using the PROC MIXED procedure in SAS version 9.4 (SAS Institute, Cary, NC) to test the effects of N application rates and tobacco cultivar on phenotypic characteristics of flue-cured tobacco, leaf yield, quality, value, and chemical constituents (Table 1). Nitrogen application rate and cultivar were considered to be fixed factors, while environment and replication were considered as random factors. Treatment means were reported using least square means. Means were separated using Fisher's Protected LSD at $P \leq 0.05$. Results were pooled across all growing environments

and the main effects of N application rate and cultivar were reported in the absence of a significant interaction between the two factors. Figures were created using SigmaPlot version 12.5 (Systat Software, Inc., San Jose, CA).

2.7 Results and Discussion

The main effect of N application rate was significant for leaf length and reducing sugars. Main effect of variety had a significant effect on all phenotypic measurements as well as reducing sugars, yield, quality, and value. There was a N application rate by variety interaction on all four alkaloids quantified in this research (Table 2.1).

SPAD Measurements

In the field, darkness of leaf color in traditional flue cured tobacco varieties is often associated with higher N rates and consequently higher nicotine content. It has also been observed however that low alkaloid varieties can also exhibit dark green characteristics when compared to check varieties at the same N rate (R. Lewis, personal communication). Low alkaloid varieties LAFC53, DH32, and DH22A had higher SPAD readings than K326 and NC95 (Figure 2.1). DH16A and K326 had similar SPAD meter readings, and NC95 had the lowest SPAD average. Upon visual inspection in the field, NC95 was easy to spot as it always had a much lighter hue of green, so the SPAD measurements tend to reinforce that observation.

Leaf Measurements

Leaf length for low alkaloid varieties ranged between 59.0cm and 60.6cm and tended to be between the longer K326 at 62.4cm, and the shorter NC95 at 57.7cm. Leaves on the three transgenic varieties were significantly narrower than K326, NC95, and LAFC53.

NC95 and LAFC53 had statistically similar leaf widths, and were slightly wider than K326. As illustrated in Figure 2, transgenic varieties shared very similar leaf shapes, and were noticeably narrower than all three non-transgenic varieties. Transgenic Varieties DH16A and DH32 had the highest sectional density at 0.127mg/mm^2, followed by DH22A at 0.122 mg/mm^2. K326 had a slightly lower sectional density at 0.118 mg/mm^2 followed by LAFC53 at 0.106 mg/mm^2 and NC95 with 0.095 mg/mm^2 (Table 2.2).

Node count and stalk height

Leaf number tended to be higher on transgenic varieties ranging from 19.7 to 20.5 nodes per stalk, while leaf number tended to be lower on K326 and NC95 with an average of 19.5 and 18.1 nodes respectively. LAFC53 had the fewest nodes averaging 16.8 nodes per stalk. Transgenic varieties had a stalk height ranging from 95.0cm to 95.3cm, statistically similar to LAFC53 at 95.9cm, but significantly shorter when compared to K326 with an average height of 98.9cm. NC95 had the tallest average stalk height at 105.1cm. By comparing higher average node counts, and shorter overall stalk height of the transgenic varieties, one can begin to see that the internode spacing on the transgenic varieties was slightly less than K326, and much less than NC95. When observed in addition to the other various phenotypic measurements collected over the course of this experiment, one begins to see the outline of a slightly more compact plant, with narrower leaves, a darker hue of green, and a slightly thicker leaf when observing the three transgenic varieties.

Yield, Quality, Value

The main effect of cultivar had the greatest effect on yield, quality, and value. Variety K326 had the highest yield, at 3,164 kg ha^{-1} (Table 2.3). Varieties NC95, LAFC53, DH32, DH22A, and DH16A did not have statistically different yields (Table 3.). Similar to

results from Lewis et al. (2015) varieties K326, NC95, DH32, DH22A, and DH16A had statistically similar quality indices ranging from 82 to 86, with LAFC53 having the statistically lowest quality index of 75. LAFC53 has been shown in the past to exhibit poor quality characteristics. As a function of yield and price associated with a given quality, variety K326 had the highest value per hectare at $12,861. K326 is recognized as a high yielding and high quality flue cured tobacco variety, so it's high performance was not surprising. All three transgenic varieties had statistically similar value per acre and were somewhat intermediate in value per hectare ranging from $9,973 to $10,606, and although the three transgenic varieties were numerically higher than NC-95 which averaged $9,440, there was no statistical difference. LAFC53 had the statistically lowest value at $7,708 per hectare, this was due to the low yield and significantly lower quality associated with this variety.

Reducing Sugar Concentration

As suspected, main effect of nitrogen rate had an inverse relationship with reducing sugars, and as N rate went down, reducing sugars increased, similarly observed by Robert Parker, 2008. Variety DH16A had the statistically highest concentration of reducing sugars, with DH22A, DH32 and K326 having statistically similar intermediate concentrations. NC95 and LAFC53 had the lowest reducing sugar concentrations, consistent with varietal descriptions. It is likely that LAFC53 and NC95 had similar reducing sugar concentrations due to the fact that NC95 is a parent line of LAFC53.

Alkaloid Concentration

Nitrogen rate in commercial tobacco production has long been known as one of the most important factors in determining alkaloid content in cured leaf. When averaged over N

rates, nicotine content in DH32, DH16A and DH22A was 75-80% lower than K326, and 80-85% lower than NC95. Alkaloids were the only measurements in which there was an interaction between N application rate and variety. As N rate decreased, nicotine content decreased significantly in both NC95 and K326, which is in line with previous research (Parker 2008, Woltz 1953). In all four low alkaloid varieties decreasing nitrogen rate did not result in significantly lower levels of nicotine in cured leaf although a decreasing trend was noticed. Nornicotine was roughly 1.8x higher in transgenic varieties, compared to NC95 and K326, and 6x higher than LAFC53. Varieties NC95 and DH32 experienced a nitrogen X variety interaction where decreasing nitrogen rate significantly decreased nornicotine content, and although all varieties possessed that trend, no other interactions were significant. Anabasine levels decreased significantly with nitrogen rate in varieties K326, NC95, DH22A, and DH16A, however, N rate had no significant effect on anabasine levels in varieties LAFC53 and DH32. All four low alkaloid varieties had similarly low levels of anatabine regardless of nitrogen rate when compared to K326 and NC95. Anatabine levels in K326 and NC95 decreased with N rate.

2.8 Conclusions

Results from these field trials indicate that suppression of the BBL gene family can provide a 75-80% reduction in alkaloid content across N rates when compared to K326 while leaf quality remained similar. Leaf yield for the three transgenic varieties was numerically higher, and cured leaf quality was significantly higher when compared to LAFC53. The three transgenic varieties provided more desirable yield, and quality characteristics when compared to LAFC53; however, their performance was not equivalent to K326, and only slightly better than NC95. Overall, the transgenic varieties were able to be grown with very

similar agronomic practices, and achieve adequate yields while drastically reducing alkaloid content when compared to K326. Transgenic low alkaloid varieties had shorter internode lengths, shorter overall height, narrower leaf widths, thicker leaf lamina, and a darker green appearance. Lowering N rate had little effect on most parameters in this study with the exception of leaf length, reducing sugars, and alkaloid content. There is no place in the current leaf market for transgenic tobacco, nor is there demand for leaf with such low levels of alkaloids. Through EMS-induced mutagenesis, varieties with the same *BBL* suppression have been developed, but are not considered to be transgenic. It is possible that as regulated levels of nicotine in cigarettes are reduced, these non-transgenic, low alkaloid mutants may hold some promise as a way to maintain smoke flavor, with low levels of addictiveness.

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Table 2.1. Analysis of variance of flue-cured tobacco phenotypic measurements, cured leaf yield, quality, value, and leaf chemistry resulting from cultivar selection and nitrogen application rate[a].

Factor	N app rate x Cultivar	N app rate	Cultivar
SPAD[b]	ns[c]	ns	<.0001
Leaf Length	ns	0.0074	<.0001
Leaf Width	ns	ns	<.0001
Disc Density[b]	ns	ns	<.0001
Leaf Count[b]	ns	ns	<.0001
Stalk Height[b]	ns	ns	<.0001
Yield	ns	ns	0.0065
Quality	ns	ns	<.0001
Value	ns	ns	<.0001
Reducing Sugar Concentration	ns	0.0001	<.0001
Nicotine Concentration	0.0041	<.0001	<.0001
Nornicotine Concentration	0.0036	0.0008	<.0001
Anabasine Concentration	0.0074	<.0001	<.0001
Anatabine Concentration	<.0001	<.0001	<.0001

[a] Data are pooled across six growing environments.
[b] Data are pooled across five growing environments.
[c] ns; not significant at the α=0.05 level.

Table 2.2. Tobacco Plant Characteristics as Influenced by the Main Effects of Nitrogen Application Rate and Cultivar[a]. Data are pooled across six growing environments.

Main Effect	SPAD	Leaf Length	Leaf Width	Density	Leaf Count	Stalk Height
Nitrogen Rate		----------cm----------		Mg/mm^2	Num./plant	cm
Rec	28.05 a	60.7 a	28.3 a	0.115 a	19.1 a	98.1 a
Rec. -15%	26.79 a	59.7 ab	28.1 a	0.115 a	19.2 a	97.8 a
Rec. -30%	26.09 a	58.9 b	27.9 a	0.118 a	19.3 a	96.8 a
Variety						
K326	24.06 d	62.4 a	28.3 b	0.118 c	19.5 b	98.9 b
NC95	20.17 e	57.7 d	31.5 a	0.095 e	18.1 c	105.1 b
LAFC53	28.39 bc	59.9 bc	30.5 a	0.106 d	16.8 d	95.9 bc
DH32	31.52 a	59.0 cd	25.8 c	0.127 a	20.5 a	95.0 c
DH22A	30.76 ab	60.6 b	26.1 c	0.122 b	20.5 a	95.2 c
DH16A	26.96 cd	59.0 cd	26.5 c	0.127 a	19.7 b	95.3 c

[a] Treatment means followed by the same letter within the same column and main effect are not significantly different at α=0.05.

Table 2.3. Tobacco Yield, Quality, and Value as Influenced by the Main Effect of Nitrogen Application Rate and Cultivar[a]. Data are pooled across six growing environments.

Main Effect	Yield	Quality[b]	Value
Nitrogen Rate	**kg ha^{-1}**		**$ ha^{-1}**
Rec	2,671 a	82 a	10,267 a
Rec. -15%	2,668 a	83 a	10,282 a
Rec. -30%	2,628 a	83 a	10,001 a
Variety			
K326	3,164 a	86 a	12,861 a
NC95	2,493 b	82 a	9,440 b
LAFC53	2,336 b	75 b	7,708 c
DH32	2,704 b	84 a	10,606 b
DH22A	2,598 b	85 a	10,517 b
DH16A	2,637 b	83 a	9,973 b

[a] Treatment means followed by the same letter within the same column and main effect are not significantly different at α=0.05.

[b] Quality is assigned on a scale of 1-100, with 100 having the highest quality

Table 2.4. Tobacco Reducing Sugar Content as Influenced by the Main Effect of Nitrogen Application Rate and Cultivar[a]. Data are pooled across six growing environments.

Main Effect	Reducing Sugars
Nitrogen Rate	**%**
Rec	16.89 c
Rec. -15 lbs/a	18.00 b
Rec. -30 lbs/a	19.09 a
Variety	
K326	18.93 b
NC95	16.60 c
LAFC53	15.27 c
DH32	18.29 b
DH22A	18.67 b
DH16A	20.50 a

[a] Treatment means followed by the same letter within the same column and main effect are not significantly different at α=0.05.

Table 2.5. Tobacco Chemistry as Influenced by the Interaction of Nitrogen Application Rate and Cultivar[a]. Data are pooled across six growing environments.

Treatment		Nicotine	Nornicotine	Anabasine	Anatabine
Cultivar	N app. rate	----------	mg/g	----------	
K326	**Rec. N**	25.93 c	0.6438 d	0.1459 c	1.118 d
K326	**-15 %**	25.54 c	0.6050 d	0.1417 c	1.084 d
K326	**-30 %**	23.63 d	0.5524 d	0.1284 d	0.982 e
NC95	**Rec. N**	32.85 a	0.7545 c	0.2131 a	2.064 a
NC95	**-15 %**	32.65 a	0.6179 d	0.2150 a	1.997 b
NC95	**-30 %**	30.18 b	0.6001 d	0.1974 b	1.799 c
LAFC53	**Rec. N**	3.90 ef	0.1574 e	0.0221 g	0.117 f
LAFC53	**-15 %**	3.84 ef	0.1588 e	0.0220 g	0.102 f
LAFC53	**-30 %**	3.23 f	0.1695 e	0.0222 g	0.102 f
DH32	**Rec. N**	5.60 ef	1.0982 a	0.0928 e	0.052 f
DH32	**-15 %**	5.26 ef	1.0577 ab	0.0885 ef	0.040 f
DH32	**-30 %**	4.91 ef	0.9910 b	0.0849 ef	0.033 f
DH22A	**Rec. N**	5.90 ef	1.0899 ab	0.0920 e	0.049 f
DH22A	**-15 %**	5.80 ef	0.9020 b	0.0819 f	0.070 f
DH22A	**-30 %**	5.15 ef	0.9198 b	0.0796 f	0.041 f
DH16A	**Rec. N**	6.44 e	1.0549 ab	0.0914 e	0.064 f
DH16A	**-15 %**	6.20 ef	0.9864 b	0.0862 ef	0.068 f
DH16A	**-30%**	5.78 ef	0.9043 b	0.0804 f	0.057 f

[a] Treatment means followed by the same letter within the same column and main effect are not significantly different at α=0.05.

Table 2.6. Rainfall for each growing environment compared to monthly average

Month	LCPRS[a]			UCPRS[b]			OTRS[c]		
	cm								
	2014	2015	Average[d]	2014	2015	Average[d]	2014	2015	Average[d]
April	11.9	10.5	8.1	14.7	13.3	8.1	16.1	15.1	7.8
May	8.3	14.6	9.8	6.7	6.0	9.7	20.2	3.4	10.6
June	29.4	13.9	11.4	13.1	8.4	10.0	13.5	19.7	8.5
July	28.7	16.4	13.4	16.9	5.4	12.4	15.0	23.9	11.4
August	15.2	9.8	13.6	23.5	8.2	11.2	14.4	6.6	11.5
September	16.6	9.0	14.3	11.8	16.7	13.5	18.0	17.3	10.7
October	3.8	14.8	8.4	6.6	13.7	7.7	11.3	10.4	9.4
Total	113.9	89.0	79.0	93.3	71.7	72.6	108.5	96.4	81

[a] Lower Coastal Plain Research Station

[b] Upper Coastal Plain Research Station

[c] Oxford Tobacco Research Station

[d] Averages based on 30 year, 1971-2000 data

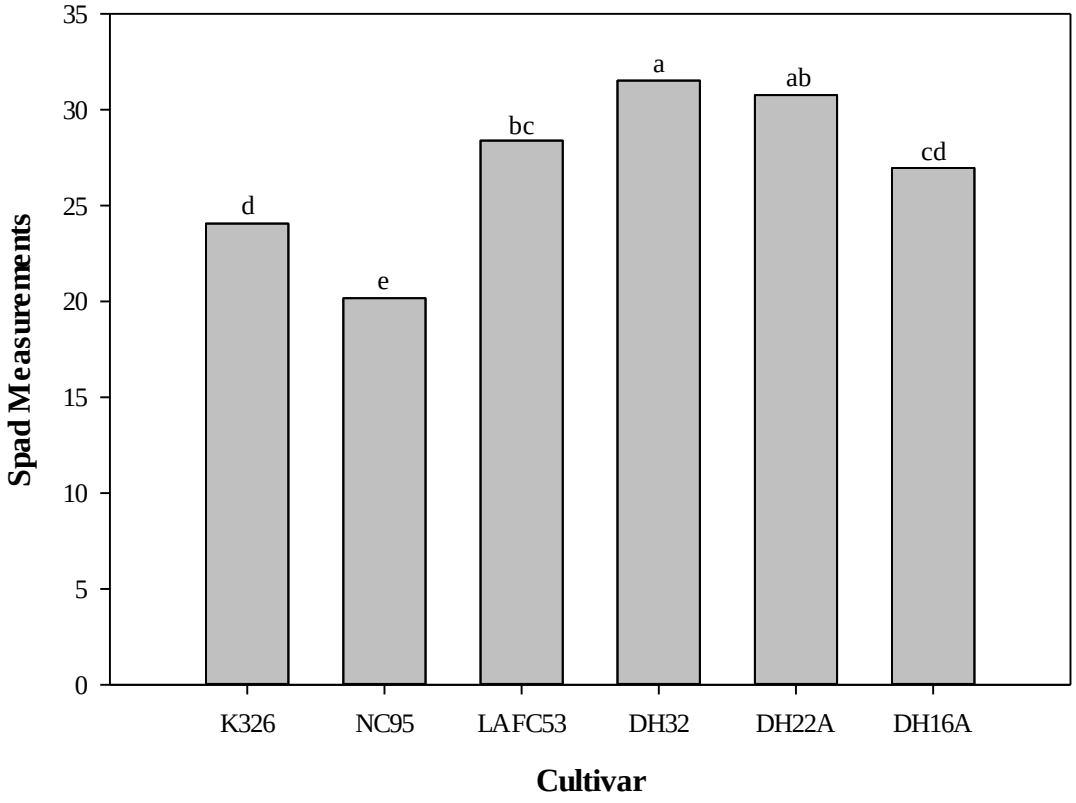

Figure 2.1**.** SPAD measurements as influenced by the main effect of flue-cured tobacco cultivar. Data are pooled across five growing locations and the main effect of nitrogen application rate. Means followed by the same letter are not significantly different at $P \leq 0.05$ based on Fisher's Protected LSD.

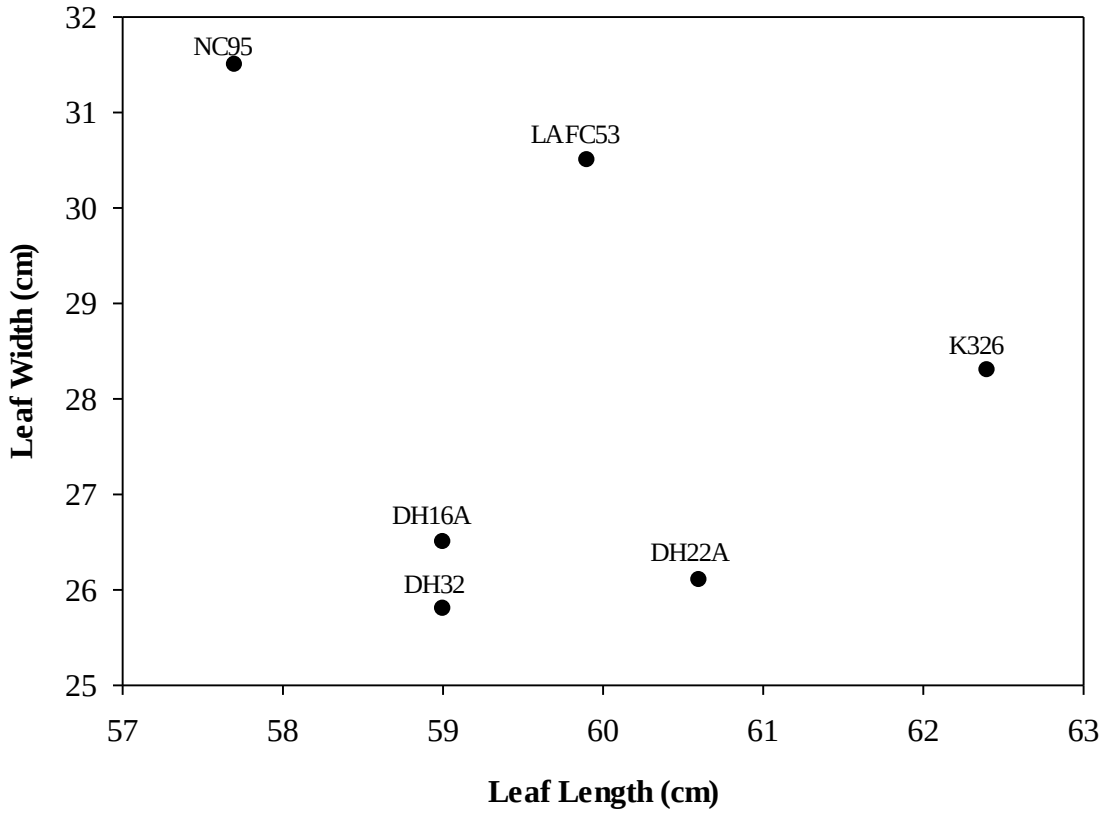

Figure 2.2. Mid-stalk leaf length and width as influenced by the main effect of flue-cured tobacco cultivar. Data were recorded following first harvest and are pooled across six growing environments and the main effect of nitrogen application rate.

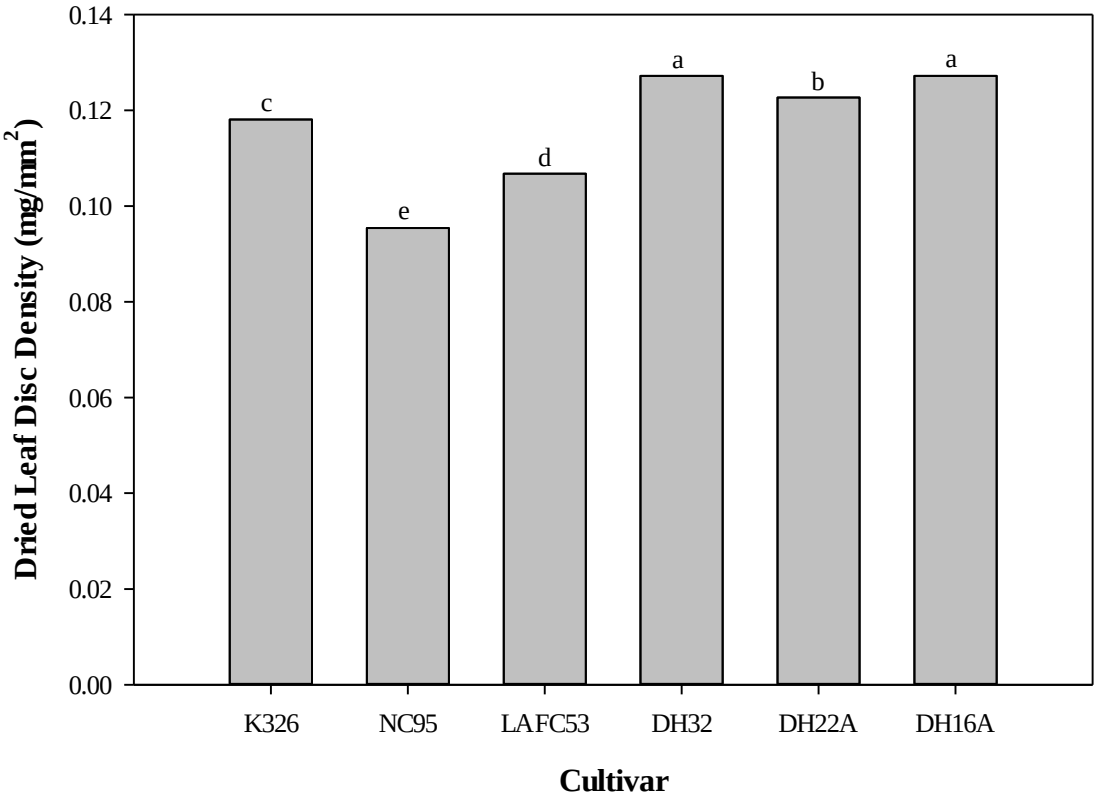

Figure 2.3. Leaf disc density after topping as influenced by the main effect of flue-cured tobacco cultivar. Data are pooled across five growing locations and the main effect of nitrogen application rate. Means followed by the same letter are not significantly different at $P \leq 0.05$ based on Fisher's Protected LSD.

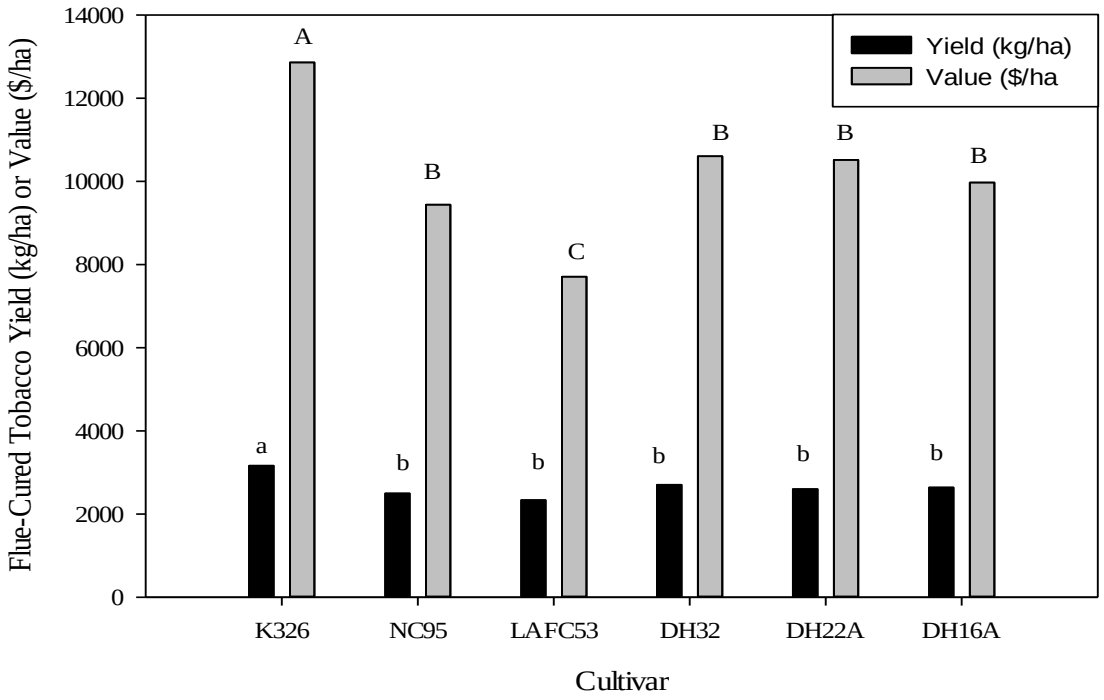

Figure 2.4. The yield and value of flue-cured tobacco as influenced by the main effect of cultivar. Data are pooled across six environments and the main effect of nitrogen application rate. Means followed by the same lowercase or uppercase letter within the same parameter are not significantly different at $P \leq 0.05$ based on Fisher's Protected LSD.

APPENDIX

Appendix A

Entomology Section

Dr. Hannah Burrack and Aurora Toennisson collected insect counts from the Kinston and Rocky Mount locations in 2014. Ratings were taken for TSWV, hornworms, budworms, aphids, flea beetle holes, and flea beetles on the highest and lowest N rates only.

Extra plots of each variety were produced at the Kinston location in 2015 for use in a bioassay. The extra plots received the full, recommended rate of nitrogen (84kg N/Ha), and they were not sprayed with any insecticides for the entirety of the season. Leaf samples were taken from each variety at layby and again after 2nd harvest. Extra care was taken during the second round of sampling so that the upper most leaf with the least insect feeding was utilized. Leaf stems were placed in water for 30 minutes to rehydrate, after which, 15cm leaf discs were excised that contained no pre-existing insect feeding. Leaf discs were placed in 15cm petri dishes containing a 1% agar solution to keep the leaf viable for feeding. Budworms in their third instar were placed on the leaf discs and the edges of the petri dishes were sealed with parafilm to prevent budworm escape. Frass counts were taken at 24, 72, and 168 hours to estimate leaf consumption. The remaining leaf after 15 cm discs were removed was dried and analyzed for alkaloids and reducing sugars using gas chromatography.

Results:

As expected, frass production was much higher for the layby sampling than for the late season sampling, most likely due to a tougher leaf, with generally higher concentrations of alkaloids for all varieties. No significant difference was observed after 24 hours for the layby sampling interval, however there were significant differences at 72 and 168 hours. Low alkaloid variety DH32 had the numerically highest rate of feeding at both 72 and 168 hours, and was statistically higher than DH22a and DH16a. Curiously, low alkaloid varieties DH22a and DH16a showed statistically lower rates of feeding compared to traditional varieties K326, NC95 and low alkaloid varieties DH32 and LAFC53.

Table A.1. Frass production and alkaloid content; Layby

Variety	24 hrs	72 hrs	168 hrs	RS	Nicotine	Nornic.	Anabas.	Anata.
	--------num. frass/disc--------			----------%----------				
K326	14 a	142 ab	219 ab	2.85 bc	0.5835 a	0.0110 c	0.0021 bc	0.0150 b
NC95	13 a	134 ab	243 ab	3.66 a	0.5931 a	0.0110 c	0.0028 ab	0.0208 a
LAFC53	17 a	161 ab	238 ab	2.92 bc	0.0675 e	0.0048 c	0.0000 d	0.0025 c
DH22A	17 a	75 bc	166 b	2.21 d	0.2381 c	0.0398 a	0.0028 ab	0.0028 c
DH32	22 a	169 a	334 a	3.28 ab	0.1471 d	0.0205 b	0.0016 c	0.0018 c
DH16A	7 a	23 c	35 c	2.51 cd	0.3051 b	0.0350 a	0.0030 a	0.0041 c

[a] Treatment means followed by the same letter within the same column and main effect are not significantly different at α=0.05.

Table A.2. Frass production and alkaloid content; After 2nd harvest

Variety	24 hrs	72 hrs	168 hrs	RS	Nicotine	Nornic.	Anabas.	Anata.
	-----num. of frass per disc-----			----------%----------				
K326	1 a	13 bc	18 c	2.13 b	2.3385 a	0.0356 c	0.0126 b	0.0463 b
NC95	0 a	6 c	10 c	2.21 b	2.3580 a	0.0343 c	0.0156 a	0.0750 a
LAFC53	6 a	36 ab	84 ab	3.02 a	0.2348 c	0.0080 d	0.0014 e	0.0050 c
DH22A	6 a	25 abc	40 bc	1.89 b	0.3840 bc	0.0628 b	0.0058 d	0.0028 c
DH32	5 a	49 a	96 a	1.96 b	0.4770 b	0.0921 a	0.0088 c	0.0028 c
DH16A	5 a	8 c	10 c	2.08 b	0.3395 bc	0.0451 c	0.0060 d	0.0023 c

[a] Treatment means followed by the same letter within the same column and main effect are not significantly different at α=0.05.