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CHEMICAL TOPPING BURLEY TOBACCO

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CHEMICAL TOPPING BURLEY TOBACCO

DISSEbATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture, Food and Environment at the University of Kentucky

By
Mitchell Dale Richmond
Lexington, Kentucky

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Dr. Robert C. Pearce, Extension Professor / Tobacco Specialist
Lexington, KY
2018

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ABSTRACT OF DISSERTATION

CHEMICAL TOPPING BURLEY TOBACCO

The act of topping tobacco (Nicotiana tabacum L.) involves the removal of the terminal bud or inflorescence of the tobacco plant. This practice ordinarily is accomplished by manually removing the top of each tobacco plant in an entire field which is labor intensive and costly. Chemical topping utilizes sucker control products to inhibit the terminal bud and axillary bud growth without manually removing the top of the tobacco plant. There were several research objectives in order to determine the utility of a chemical topping system: 1) determine if burley tobacco could be chemically topped with currently registered suckercide products while maintaining control of subsequent sucker growth; 2) compare chemical topping to manual topping for yield and leaf quality; 3) identify burley tobacco varieties that are better suited for chemical topping systems; 4) determine the optimum plant growth stage at which chemical topping treatments should be applied; and 5) identify genes that are differentially expressed following suckercide applications. To pursue our objectives, studies were initiated investigating the optimum timing of application, ideal variety maturity, and efficacy of suckercide applications using combinations of maleic hydrazide (MH), butralin, and fatty alcohols (FA). The terminal bud was not well controlled with FA or butralin alone nor was acceptable sucker control or total yield achieved. Our data suggest that chemically topping burley tobacco with a tank mixture of MH and a local systemic may be a suitable alternative to manual topping, as total yield and leaf quality grade index were not significantly different and total TSNA and MH residues were not significantly higher compared to manual topping. The 10% button and 50% button application timings were best suited for chemical topping practices. Treatments that targeted the 10% bloom stage did not completely halt flower development, but all application timings resulted in excellent sucker control. Medium and late maturity burley varieties were found to be suitable for chemical topping methods; however, timing the suckercide application may be less difficult in later maturing varieties. Chemically topping burley tobacco at 10 to 50% button stages with a tank mixture of MH and a local systemic suckercide was found to be a suitable alternative to manual topping, and would potentially result in labor savings for burley tobacco growers. Expression of genes related to phytohormones, meristem development, cell division, DNA repair and recombination were affected following MH treatment, which likely leads to the inhibition of apical and axillary meristem development.

KEYWORDS: Chemical Topping, Tobacco, Maleic Hydrazide,

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April 10, 2018
CHEMICAL TOPPING BURLEY TOBACCO

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April 10, 2018
This work is dedicated to the memory of my mother and father.

-Mitchell Dale Richmond
April 10, 2018
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Chapter One: Literature Review

Introduction

Kentucky is the leading state for production of type 31 light air-cured burley tobacco (*Nicotiana tabacum* L.), accounting for over 70% of the estimated 150 million pounds produced in the United States (USDA NASS, 2017). The estimated average yield of burley tobacco produced in Kentucky from 2013 to 2017 was 2,180 kg ha\(^{-1}\) (USDA NASS, 2017). Burley tobacco is predominately used as a component in the manufacturing of blended cigarettes (Palmer and Pearce, 1999).

The intensive labor requirement for producing tobacco coupled with fluctuating market prices and increasing costs of inputs leads to uncertainty in profitability. Studies dealing with tobacco production have suggested that it takes 371-494 hours of labor to grow one hectare of burley tobacco, even with increased labor efficiency from changes such as float systems and baling of cured leaves (Snell and Powers, 2013; Duncan and Wilhoit, 2014). Current challenges within the tobacco industry include delivering increasingly regulated and potentially reduced-risk tobacco products to a decreasing number of consumers (Snell, 2017). Maximizing yields and reducing input costs will be vital in maintaining a profitable tobacco operation in the face of a rapidly changing marketplace. Therefore, research to improve the efficiency of production is worth investigating since burley tobacco is still important to Kentucky’s economy.

Removal of the terminal bud or inflorescence of the tobacco plant, commonly known as topping, is usually accomplished by manually removing the top of each tobacco plant in an entire field, which is labor intensive and costly. Removal of the terminal bud or inflorescence prevents reproductive development (i.e. seed head) and results in energy transfer to increased leaf size, weight, nicotine content, and other chemical constituents.
Topping results in a loss of apical dominance and the stimulation of axillary bud growth, known as suckers (Decker and Seltmann, 1971). Controlling sucker growth is positively correlated with yield, where greater sucker control is associated with higher yielding tobacco (Collins and Hawks, 1993).

**Topping and stimulation of axillary bud growth**

Unlike most other row crops, tobacco is valued primarily for the vegetative growth, so the terminal bud or inflorescence is removed from the plant. This operation is known as topping and results in the loss of apical dominance. The plants start to produce axillary buds in the leaf axil region known as suckers (Tso, 1990; Decker and Seltmann, 1971). There are other crops that also benefit from topping. It has been shown in cotton (*Gossypium hirsutum* L.) that a higher number of bolls per plant were retained, and boll growth was increased after plants were topped (Yang et al., 2012). Other experiments have shown an increase in dry weight, plant height, number of branches, and pods and seed yield per plant in okra (*Abelmoschus esculentus*) after topping and spraying gibberellic acid (Marie et al., 2007). It is well documented that topping and control of sucker growth are required to achieve acceptable yields and higher quality tobacco leaf (Douglass et al., 1985; Link et al., 1982; Goins et al., 1993; McKee, 1995; Sheets et al., 1994). In addition to the benefits on yield, quality, and increased alkaloid production, it has been suggested that topping also reduces the potential for wind damage, increases fertilizer use efficiency and drought tolerance, as well as provides a reduction in the population of insects such as aphids and budworms (Bailey et al., 2017; Fisher et al., 1990).
Therefore, topping and control of subsequent sucker growth should be managed in order to avoid detrimental effects on tobacco yield and leaf quality.

In a topping timing study using flue-cured tobacco, it was shown that the highest yields in hand suckered treatments were observed when tobacco was topped in the button or early flower stages, with delays resulting in a yield penalty of around 28 kg ha\(^{-1}\) day\(^{-1}\) (Marshall and Seltmann, 1964). Another study found no significant differences in burley tobacco yield and value when topped at early bloom or mid-bloom stages (Seltmann et al., 1969). However, the number of leaves left on the plant after topping has been shown to be positively related to yield (King, 1986), but value has been shown to have a negative relationship with number of leaves left on the plant (Collins and Hawks, 1993). Topping burley tobacco at ten to twenty-five percent bloom with an optimum leaf number of 22-24 leaves has been shown to provide the best yield, leaf quality, and a better opportunity for an actual tip grade (Bailey et al., 2017).

Topping, which wounds the plant, triggers wound-activated responses in gene expression and metabolism to activate defense mechanisms (León et al. 2001). The phytohormone, jasmonic acid (JA), is well known as a regulator in the wound-signaling pathway (León et al., 2001). Phytohormones, primarily auxin (IAA) and cytokinin (CK), are known to be involved with the initiation of axillary bud growth (Müller and Leyser, 2011). Wang et al. (2018) performed comparative transcriptomic analyses to find differentially expressed genes (DEGs) in untopped and topped tobacco plants. They found that many of the DEGs are involved in starch and sucrose metabolism, glycolysis/glucconeogenesis, pyruvate metabolism, and plant hormone signal transduction, along with other processes. The previously-mentioned processes (starch and sucrose
metabolism) are believed to contribute significantly to the enlargement of axillary bud growth (Wang et al. 2018). Another study found that DEGs in flue-cured tobacco roots after topping are mostly related to secondary metabolism, hormone metabolism, signaling/transcription, stress/defense, protein metabolism and carbon metabolism (Qi et al., 2012).

A number of transcription factors (TFs) belonging to R2R3 MYB (Myeloblastosis), basic helix-loop-helix (bHLH), GRAS (GAI, gibberellic acid insensitive-RGA, repressor of GAI-Scarecrow), NAC (NAM-no apical meristem, ATAF-Arabidopsis transcription activator factor, CUC- cup-shaped cotyledon), homeodomain/leucine zipper (HD/ZIP), and TCP (Teosinte branched1-Cycloidea-Proliferating cell nuclear antigen factor) families have been identified and characterized for their roles in meristem development in Arabidopsis, tomato, pepper, and rice (Wang et al., 2018; Janssen et al., 2014). After investigating the phenotypic and genetic interactions of mutations in the REVOLUTA (REV) gene, it was found that REV is required for lateral meristem and floral meristem initiation and encodes a HD/ZIP TF in Arabidopsis (Otsuga et al., 2001). Schmitz et al. (2001) identified two genes, BLIND and TOROSA belonging to R2R3 MYBs that control lateral meristem (axillary bud) initiation in tomato. Müller et al. (2006) found three R2R3 MYB genes in Arabidopsis, which were homologous to the tomato Blind gene and were designated as REGULATORS OF AXILLARY MERISTEMS (RAX). RAX control axillary bud formation at a very early step of initiation in Arabidopsis. BLIND ortholog was also found to reduce axillary meristem initiation in pepper plants (Jeifetz et al., 2011). The GRAS family TF, lateral suppressor (LAS) has also been shown to play a role in meristem development in
Arabidopsis and tomato (Greb et al. 2003; Schumacher et al. 1999). Double mutant analyses in tomato and Arabidopsis revealed that LAS and MYB TFs control axillary meristem formation through separate pathways (Schmitz et al., 2002; Müller et al., 2006). In tomato, blind mutants did not initiate lateral meristem during shoot and inflorescence development (Schmitz et al. 2002). The lateral suppressor (ls) mutant almost blocked all lateral meristem development during vegetative development (Schumacher et al., 1999); however, during reproductive development, the LS gene is not required for axillary meristem formation (Greb et al., 2003). The TCP family TF, BRANCHED1, is known to be involved in axillary meristem development in plants. Axillary buds in Arabidopsis express only a single BRC1 gene compared to two BRC1-like genes in other Solanaceae species such as tomato (Martin-Trillo et al., 2011). Martin-Trillo et al. (2011) suggested that interplay between these two dimerizing transcription factors might result in a more complex regulation of axillary bud growth patterns in plants like tomato, as two divergent BRC1-like genes are co-expressed. Li et al. (2003) characterized MONOCULM1 (MOC1), which is an important gene for rice tillering and encodes a protein highly homologous to the tomato LAS. MOC1 is a key regulator of tillering and regulates expression of several important genes involved with axillary bud development, namely, OSH1. OSH1 is a rice orthologue of the maize TB1 that is expressed in axillary buds and regulates axillary bud outgrowth (Li et al., 2003).

Each leaf axil of mature tobacco plants can potentially produce three suckers, but it has been noted that only two suckers develop under normal commercial production (Seltmann and Kim, 1964). Increased root growth in response to manual topping and hand suckering has been shown to result in higher potential for the tobacco plant to
absorb water and nutrients as well as an increased ability to synthesize nicotine (Collins and Hawks, 1993). In agreement with the previous statement, Woltz (1955) showed that topping and suckering flue-cured tobacco resulted in better yield and quality and not-topped plants resulted in substantial decreases in nicotine and sugar content. Tso (1990) concluded that topping increased nicotine content and resulted in a net gain in total alkaloid content. It has also been documented that tobacco that is untopped and grown in higher plant populations produce less than 1.5% nicotine (Papenfus, 1987).

Tobacco alkaloids are an important component of leaf quality and provide tobacco consumers a physiological stimulus that makes consumption of tobacco products pleasurable (Bush, 1999). The major carcinogens found in tobacco are tobacco-specific nitrosamines (TSNA), which are formed from tobacco alkaloids and are produced primarily during curing. The amount of specific alkaloid precursor influences the amount of TSNA accumulation and the most prevalent TSNA in burley tobacco is N-nitrosonornicotine (NNN), which is converted from nornicotine (Jack et al, 2017).

**Control of Axillary Bud Growth**

In the broadest sense, three types of chemicals can be used for chemical inhibition of axillary bud growth. These include contact, local systemic, and systemic suckercides (Bailey et al., 2017). Tobacco growers relied on intensive labor to remove suckers by hand prior to chemical sucker control development (Meyer et al., 1987). Therefore, it is no surprise that chemical sucker control methods were readily adopted by tobacco growers.
Contact suckercides are not absorbed, nor translocated by the plant and effective control of suckers requires placement of the chemicals directly on the leaf axil. Local systemic suckercides are absorbed in the leaf axil area to inhibit cell division. Systemic suckercides, unlike contact and local systemic suckercides, do not need to directly contact the suckers as they are absorbed by the leaves and translocated to the leaf axils, where cell division is inhibited. Maleic hydrazide (1,2-dihydro-3,6,-pyridazinedione) is the only true systemic suckercide that is used in tobacco production (Bailey et al. 2017).

Contact suckercides, also known as long chain fatty acids or alcohols (FA), were developed in the early 1960s and destroy differentiating plant cells (Tso 1990; Tso and Chu, 1977). There were many vegetable oils, saturated fatty acids, unsaturated fatty acids and their analogues evaluated for inhibition of axillary bud development in tobacco (Tso 1990). Tso (1964) found that alkyl esters of C$_8$–C$_{12}$ fatty acids were able to inhibit the growth of suckers without detrimentally affecting the leaf when applied after topping tobacco. Within the C$_8$–C$_{12}$ fatty acids, the C$_{10}$ and C$_{11}$ methyl esters were the most effective but the C$_{11}$ compound showed an increased amount of phytotoxicity (Steffens et al., 1967). These products are applied as sprays and as stalk-run down methods to contact and kill immature actively-growing suckers, however, suckers dormant at the time of application are not controlled so multiple applications of suckercides are required (Tso 1990). After an application of an emulsified fatty acid ester onto a plant, the ester is not translocated and is restricted to the general area of application. Therefore, it was suggested that growth of meristematic tissues are inhibited because of selective penetration into rapidly dividing cells (Steffens et al., 1967). Wheeler et al. (1991) studied the mode of action of fatty alcohols and found that the plasma membrane is
broken down after application, followed by dehydration of the cell, which leads to cell death. Cathey et al. (1966) reported that lower alkyl esters of the C_8 – C_{12} fatty acids and the C_8 – C_{10} fatty alcohols in aqueous emulsions were able to selectively kill the terminal meristems without damaging axillary meristems, leaves, or stem tissues in herbaceous, semi-woody, and woody plants. Steffens and Cathey (1969) found that terminal buds or axillary buds of topped tobacco plants were controlled with use of emulsions containing 2:1 ratios of alcohol and surfactant or 3:1 ester to surfactant ratio. Other fatty alcohol chain lengths, namely C_9, C_{10}, and C_{11}, have also been reported to be highly active and selective on inhibiting axillary and terminal bud growth of tobacco when using the proper type and amount of surfactant as these fatty alcohols are nonselective in the absence of surfactants (Steffens et al., 1967). Cathey et al. (1966) observed that the first visible plant response occurs within 15 minutes after application of fatty acid esters or alcohols. Comparable to other agricultural chemicals, there are concerns of fatty alcohol residue levels on the treated tobacco leaves. However, studies have found that residue levels of fatty alcohols were not detected 26 days after treatment (Tso 1990).

Local systemic suckercides need to be applied similarly to contacts so that the solution will contact every leaf axil. Products that are local systemic belong to the dintroanaline family and butralin and flumetralin are the major active ingredients (Bailey et al., 2017). Singh et al. (2015) identified 179 common DEGs between tobacco plants that were topped or topped and treated with local systemic or a contact suckercide. DEGs related to wounding, phytohormone metabolism, and secondary metabolite biosynthesis were upregulated after topping and downregulated after suckercide treatment. This study also found that the application of a local systemic suckercide affected the expression of
auxin and cytokinin signaling pathways, which are likely involved with axillary bud formation (Singh et al. 2015).

Maleic hydrazide (MH) was first synthesized by Curtis and Fosterburg (1895) from maleic anhydride and hydrazine (Hoffman and Parips, 1964; Meyer et al., 1987). However, it was not known that it had an effect on plant growth until the late 1940’s when Schoene and Hoffman reported this effect (Schoene and Hoffman, 1949). Currier and Crafts (1950) suggested that MH could be used as a selective herbicide. In 1951, Peterson investigated the ability to control suckers in tobacco using maleic hydrazide and found that MH provided excellent sucker control with no significant effects on yield, quality, or burning properties of cured leaves (Peterson, 1952).

Maleic hydrazide has provided excellent sucker control and equivalent cured leaf yield as opposed to hand suckering without adversely influencing leaf quality (Chaplin, 1967). Maleic hydrazide has also been shown to reduce total alkaloid levels compared to a hand-suckered control (Cui et al. 1995). Cui et al. (1995) found that alkaloid content was reduced by 9-34%, 4-20%, and 5-29% in the top, middle, and bottom stalk positions after MH treatment, respectively. Treatments applied to burley tobacco that were topped and then treated with MH provided the highest yield and value per acre (Seltmann et al. 1969). Maleic hydrazide was originally applied as a spray over the upper one-third of the plant to cover the upper leaves (Marshall and Seltmann, 1964) and must be absorbed by the leaves to be effective. Absorption was enhanced when MH was applied to rapidly growing plants under conditions of high humidity (Steffens, 1983; Smith et al., 1959). Smith et al. (1959) studied different factors including temperature, light, humidity, plant species, plant turgidity, application rate, and formulation on absorption of MH. All
previously mentioned factors could impact the absorption of MH and efficacy with the exception of light. However, relative humidity had the greatest effect as a three- to five-fold increase in absorption was observed when relative humidity increased from 50% to 100% (Smith et al., 1959). There was only a moderate effect of temperature, possibly due to changes in relative humidity through changes in temperature (Meyer et al., 1987). The rate of MH uptake was decreased as the plant turgidity decreased (Smith et al., 1959), therefore, spraying MH in the morning and evening hours of the day compared to during the afternoon may lead to faster absorption (Smith and Stone, 1957; Meyer et al., 1987). MH was most effective when applied on a crop growing under good moisture conditions, more than likely as a result of less difficult penetration of the leaf cuticle during active plant growth (Collins and Hawks, 1993).

After MH enters the plant, it is readily translocated throughout the plant vasculature, in both phloem and xylem tissues (Hoffman and Parips, 1964; Steffens, 1983; Zukel, 1963). Similar patterns of distribution after translocation were observed when C\textsuperscript{14} MH was applied to the top, middle, or bottom leaves (Smith et al., 1959). Most of the chemical leaving the treated leaves went to actively growing tissues, i.e. apical and axillary bud regions (Smith et al., 1959). Frear and Swanson (1978) also observed a source to sink translocation pattern using foliar absorbed C\textsuperscript{14} MH. When MH was applied to tobacco, it inhibited cell division without affecting cell elongation, thus preventing the growth of newly developing suckers without hindering the growth of more mature leaves (Collins and Hawks, 1993).

MH applications have been shown to be related to an increase in starch accumulation, but these changes with respect to increased photosynthesis or decreased
translocation have been unclear (Bush and Sims, 1974; Seltmann and Nichols, 1984). Callaghan and Norman (1956) concluded that a foliar spray of maleic hydrazide in the cotyledon stage (Swiss chard) or at five to six leaves (tobacco) increased the rate of photosynthesis. However, later studies showed that there was an increase in sucrose and starch accumulation as a result of decreased translocation of assimilate and not due to increased photosynthesis in burley tobacco in response to MH (Crafts-Brandner and Sutton, 1994).

**Chemical Topping**

There are no suckercides that are registered or manufactured specifically for chemical topping of tobacco, but some experiments have evaluated such products for this purpose (Long et al., 1989; Steffens and McKee, 1969; Steffens et al. 1967; Peek, 1995). Chemical topping utilizes sucker control products to inhibit the terminal bud and axillary bud growth without manually removing the top of the tobacco plant. It was found that fatty alcohols (FA) with chain lengths of C₉, C₁₀, and C₁₁ could inhibit the terminal bud if applied before the flowers were open and terminate suckers after the FA contacted leaf axils (Steffens et al., 1967). Another study showed that chemically topped tobacco yielded significantly higher when FA was applied at the button stage compared to manually topped at the full bloom stage, but yield from chemically topped tobacco was not significantly different than manually topped and sprayed with FA at the button stage (Steffens and McKee, 1967). Long et al. (1989) evaluated chemically topping with MH, flumetralin, FA, and tank mixtures and found that suppression of the terminal and axillary buds were successful in all treatments, however, MH alone produced
significantly less yield due to reduced sucker control. Similarly, Peek (1995) found that a tank mixture of MH with flumetralin resulted in the highest total yield and MH alone resulted in the lowest yield of all chemically topped treatments. Other studies have found chemical topping to be successful if applied at earlier button growth stages but the earliest button stages also resulted in the largest yield reduction (Peek, 1995). Peek (1995) found that all chemically topped treatments resulted in reduced yields when compared to a manually topped check, except when a mammoth-type variety was chemically topped at higher leaf numbers.

**Exploring the Mode of Action of MH**

The biochemical processes through which MH affects plant development are still not completely understood even though the mode of action for MH has been studied since 1949 (Bush and Sims, 1974). It has been shown that maleic hydrazide acts as an antimitotic agent in axillary bud tissue (Clapp and Seltmann, 1983). In the early 1970s, there were two different views on how MH worked; those who believed that MH interacted with nucleic acid precursors and thus ultimately with nucleic acid synthesis, and those who did not agree (Coupland and Peel, 1971). Coupland and Peel (1971) showed that for an increase in the concentration of MH, there was a corresponding increase in the inhibition of uracil uptake. Their data supports the hypothesis that MH can inhibit uptake of uracil into cells by a competitive process supporting the claim that MH has a two-fold effect on plant tissues: 1) inhibits uracil uptake into the cell and 2) once inside the cell, MH can become incorporated into RNA. This could be due to the close structural resemblance MH has to uracil (Coupland and Peel, 1971; Cradwick, 1975).
Collins and Hawks (1993) reported that MH is absorbed by the tobacco plant and symplastically translocated to active growing points where the mechanism of action is as a uracil antimetabolite. Weed Science Society of America (WSSA) (2010) also stated that maleic hydrazide may act as a uracil anti-metabolite but the mechanism of action is not well understood. A study conducted by Appleton et al. (1981) showed that MH was incorporated into RNA in yeast cells where it was substituted for cytosine rather than for uracil. However, some evidence indicates that MH inhibits cell division and subsequent sucker growth by inhibiting DNA and RNA synthesis (Nooden, 1969; Nooden, 1972; Zukel, 1963) but does not influence actively growing cells, as they will enlarge and differentiate (Steffens, 1983). Other theories have suggested that MH reacts with sulphhydryl groups (Muir and Hansch, 1953) or a carbonyl reagent (Suzuki, 1966), however Nooden (1973) showed no reactions between MH and sulphhydryl or carbonyl compounds and discounted these theories. To summarize, there is no widely accepted proposed mechanism of action for maleic hydrazide in the literature but it is apparent that cell division is inhibited.

**Fate of Maleic Hydrazide in Plants**

Chemically, maleic hydrazide is a very stable molecule in and on plants as several of the degradation and transfer processes for organic chemicals were not effective on MH (Ponnapalam et al., 1983; Collins and Hawks, 1993; Nooden, 1970). MH was stable under ultraviolet irradiation and decomposed at 260°C (WSSA, 2010), thus field and curing conditions associated with these factors are not likely to influence residual amounts of MH on cured tobacco leaves. In addition to UV and temperature, the vapor
pressure of MH is nearly zero, which leads to insignificant amounts of MH lost to volatilization (Collins and Hawks, 1993). Therefore, there is a higher potential for MH residues to be present in and on the surface of cured tobacco leaf since MH can become fixed and is not believed to be highly metabolized (Collins and Hawks, 1993; WSSA 2010). To address high MH residues, a Maximum Residue Limit (MRL) of 80 µg/g was established in Germany (Weber, 1974; Wittekindt, 1978).

MH as commonly applied is formulated as a potassium salt of MH which possesses a high water solubility. This has a two-fold implications: higher penetration efficiency in the plant (Coresta, 2014), and potential for control of suckers and MH residues to be significantly influenced by rainfall and irrigation (Collins and Hawks, 1993; Seltmann and Sheets, 1987; Fisher et al., 2018).

Seltmann and Sheets (1987) found reduced sucker control with simulated rainfall amounts of 0.2-2.0 cm within 12 hours of MH application. Leaf samples from plots exposed to simulated rainfall 24 hours after application had significantly less MH residue than the control (no simulated rainfall) in one year of their study. Sheets (1978), in Collins and Hawks (1993), observed in flue-cured tobacco that mid-stalk positions of tobacco had decreasing MH residues from harvest through four days after harvest. By day four, after a 2.2-inch rain on day three, there was a 66% reduction in residues from harvest. Dew may also contribute to a reduction in unbound MH on the leaf surfaces as there was a 24% reduction in residues on day three when compared to the initial day of harvest.

After entering the plant, it is believed that MH can exist as unmodified or free MH, become bound with cell wall components such as lignin (Nooden, 1970), or
detoxified through formation of a glycoside (Towers, 1958; Coresta, 2014). The amount of free MH has been shown to decrease after harvest, curing, and storage, which alludes to a gradual conversion to a bound form of MH (Coresta, 2014). Nooden (1970) was able to show that C\textsuperscript{14} MH was bound to the cell wall fragments using an energy requiring process. Towers et al. (1958) concluded that there is formation of glycosides of MH which could serve as a detoxifying mechanism in leaf segments of wheat. In later studies, it was shown that MH can be metabolized with glucose to form two different glucoside conjugates, MH-\(\text{N-}\beta-\text{D-glucoside}\) (Tagawa et al., 1995) or MH-\(\text{O-}\beta-\text{D-glucoside}\) (Frear and Swanson, 1978). Tagawa (1995) showed that ten to thirty percent of the total MH residue found in MH-treated cured tobacco was attributed to MH-\(\text{N-}\beta-\text{D-glucoside}\).

**Conclusions and Dissertation Overview**

The focus of this research involved evaluating the feasibility of eliminating manual topping by utilizing chemical topping to top the plant without sacrificing yield, quality, and other characteristics of tobacco. There were several research objectives in order to determine the utility of a chemical topping system: 1) determine if burley tobacco could be chemically topped with currently registered suckercide products while maintaining control of subsequent sucker growth; 2) compare chemical topping to manual topping for yield and leaf quality; 3) Identify burley tobacco varieties that are better suited for chemical topping systems; 4) determine the optimum plant growth stage at which chemical topping treatments should be applied; and 5) identify genes that are differentially expressed following suckercide applications.
Burley tobacco production is historically and economically important for Kentucky and much research has been dedicated to agronomic practices that promote and maintain good agricultural practices. This review has centered on the act of topping tobacco, formation of axillary buds, controlling axillary buds, chemical topping instead of manual topping, and maleic hydrazide. This review demonstrates that using currently registered suckercides to chemically top burley tobacco has the potential to reduce the cost of labor associated with manual topping. In this dissertation, field experiments were conducted at two locations in Kentucky for three years to evaluate different suckercide products and application rates (Chapter 2) and the optimum application timing and appropriate variety maturity (Chapter 3) for chemical topping of burley tobacco. In Chapter 4, we studied changes in gene expression through use of RNA-sequencing in MH-treated chemically topped burley tobacco and further investigate the mechanisms of how systemic suckercides inhibit apical and axillary shoot formation. A summary of chemical topping findings from this series of field and lab experiments is in Chapter 5.
Chapter Two: The Effect of Suckercide Product and Rate on Chemical Topping of Burley Tobacco

Abstract

The act of topping tobacco (*Nicotiana tabacum* L.) involves the removal of the terminal bud or inflorescence of the tobacco plant. This practice ordinarily is accomplished by manually removing the top of each tobacco plant in an entire field which is labor intensive and costly. The major objectives for this research were to determine which labelled suckercides could effectively chemically top burley tobacco and the effect of suckercide rate on sucker control, yield, leaf quality, MH residues, and leaf chemistry. To pursue our objectives, a study was initiated at Murray, Princeton, and Lexington, KY that investigated the efficacy of suckercide applications using combinations of maleic hydrazide (MH), butralin, and fatty alcohols (FA). The terminal bud was not well controlled with FA or butralin alone nor was adequate sucker control or total yield achieved. A significant reduction in total yield and sucker control was observed when plants were chemically topped with MH alone compared to manually topped or chemically topped with a tank mixture of MH and butralin at Princeton only. At the other locations, all chemically topped plants had similar yield to manually topped plants. Our data suggested that chemical topping of burley tobacco with a tank mixture of MH and a local systemic can be an acceptable alternative to manual topping as total yield and leaf quality grade index were not significantly different at any location. Total tobacco-specific nitrosamine (TSNA) content and MH residues were not significantly higher than manual topping.
**Introduction**

Kentucky is the leading state for production of type 31 light air-cured burley tobacco (*Nicotiana tabacum* L.), accounting for over seventy percent of the estimated 68,000 metric tons produced in the United States (USDA NASS, 2017). The estimated average yield of burley tobacco produced in Kentucky for 2014, 2015, and 2016 was 2,400, 2,000, and 1,950 kg ha\(^{-1}\), respectively (USDA NASS, 2017). Burley tobacco is predominately used as a component in the manufacturing of blended cigarettes (Palmer and Pearce, 1999), along with flue-cured and oriental tobacco.

The intensive labor requirement for producing tobacco coupled with fluctuating market prices and increased costs for labor and other inputs has led to declining profit margins for burley growers. Studies on tobacco production have indicated that it takes 150-200 hours of labor to grow one acre of burley tobacco even with advances that have increased labor efficiency (Snell and Powers, 2013; Duncan and Wilhoit, 2014). Current challenges within the tobacco industry involve delivering increasingly regulated, reduced-risk tobacco products to a decreasing number of consumers (Snell, 2017). Maximizing yields and reducing input costs will be vital in maintaining a profitable tobacco operation in a changing marketplace. Therefore, research on improving the efficiency of production is worth investigating since burley tobacco is significant to Kentucky’s agricultural economy.

One area to focus research effort involves the practice of removing the terminal bud or inflorescence of the tobacco plant. This practice, commonly known as topping, is ordinarily accomplished by manually removing the apical meristem of each tobacco plant in an entire field, which is labor intensive and costly (Swetnam and Walton, 1998).
Removal of the terminal bud or inflorescence prevents reproductive development (i.e. seed head) and results in energy transferred to increased leaf size, weight, nicotine content, and other chemical constituents (Tso, 1990). Subsequently, topping suppresses apical dominance in the plant resulting in axillary bud growth, known as suckers (Decker and Seltmann, 1971). Each leaf axil of a mature tobacco plant can potentially produce three suckers, but it has been noted that only two suckers develop under normal commercial production (Seltmann and Kim, 1964). Effective sucker control and yield are positively correlated (Collins and Hawks, 1993). It is well documented that topping and control of sucker growth is required to achieve acceptable yields and higher quality leaf (Douglass et al., 1985; Link et al., 1982; Goins et al., 1993; McKee, 1995; Sheets et al., 1994). In the broadest sense, there are three types of chemicals that can be used for chemical inhibition of axillary bud growth. These three types are contact (fatty alcohols), local systemic (butralin or flumetralin), and systemic (maleic hydrazide) suckercides (Bailey et al., 2017).

Maleic hydrazide has been shown to result in excellent sucker control and equivalent cured leaf yield, compared to hand suckering, without adversely influencing leaf quality (Chaplin, 1967). Chemically, MH is a very stable molecule in and on plants as several of the degradation and transfer processes for organic chemicals are not effective (Ponnapalam et al., 1983; Collins and Hawks, 1993; Nooden, 1970). MH is stable under ultraviolet irradiation and decomposes at 260°C (WSSA, 2010), thus field and curing conditions associated with these factors are not likely to influence residual amounts of MH on cured tobacco leaves. In addition to UV and temperature, the vapor pressure of MH is nearly zero, which leads to insignificant amounts of MH lost to volatilization (Collins and Hawks, 1993). Therefore, there is a higher potential for MH residues to be present in and
on the surface of cured tobacco leaf since MH can become fixed and is not believed to be highly metabolized (Collins and Hawks, 1993; WSSA 2010). However, MH is formulated as a potassium salt of MH which possesses a high water solubility and has a two-fold implication: higher penetration efficiency in plant (CORESTA, 2014), and control of suckers and MH residues can be significantly influenced by rainfall and irrigation (Collins and Hawks, 1993; Seltmann and Sheets, 1987; Fisher et al., 2018). Nonetheless, higher chemical residues can be explained by the chemical properties of MH molecules and use patterns by tobacco producers (Collins and Hawks, 1993).

Increased root growth in response to manual topping and hand suckering has been shown to increase the potential for the tobacco plant to absorb water and nutrients as well as an increased ability to synthesize nicotine (Collins and Hawks, 1993). Woltz (1955) showed that topping and suckering flue-cured tobacco resulted in better yield and quality and that untopped plants had substantially lower nicotine and sugar content. Tso (1990) concluded that topping increases nicotine content and results in a net gain in total alkaloid content. Cui et al. (1995) found a reduction in total alkaloid levels when MH was applied compared to a hand suckered control. Long et al. (1989) found that chemically topped plants had a reduced percentage of total alkaloids compared to manually topped tobacco plants. It has been shown that applications of MH decreased lamina tobacco-specific nitrosamine (TSNA) content due to altering the precursor-TSNA relationship (Cui et al., 1994). TSNAs are nitrogenous compounds that are formed only from tobacco alkaloids and are detectable in the tobacco leaf and in the particulate phase of tobacco smoke. There are four major TSNAs: N-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosoanatabine (NAT), and N-nitrosoanabasine (NAB)
Suckercides that are currently registered were not intended specifically for chemically topping tobacco, but some experiments have evaluated use of such chemicals for this purpose (Long et al., 1989; Steffens and McKee, 1969; Steffens et al. 1967; Peek, 1995). Fatty alcohols (FA) with chain lengths of C₉, C₁₀, and C₁₁ could inhibit the terminal bud if applied before the flowers were open and terminate suckers after the FA contacted leaf axils (Steffens et al., 1967). Another study showed that chemically topped tobacco yielded significantly higher when FA was applied at the button stage compared to manually topped at the full bloom stage but not significantly different than manually topped and sprayed with FA at the button stage (Steffens and McKee, 1967). Long et al. (1989) evaluated chemical topping with MH, flumetralin, FA, and tank mixtures and found that suppression of the terminal and axillary buds were successful in all treatments, however, MH alone produced significantly less yield due to reduced sucker control. Peek (1995) found that a tank mixture of MH with flumetralin resulted in the highest total yield and MH alone resulted in the lowest yield of all chemically topped treatments. Chemically topping with a tank mixture of MH and flumetralin on photoperiod-sensitive cultivars of flue-cured tobacco resulted no differences in yield compared to manually topped and sprayed (Long et al., 1989). Long et al. (1989) found that split treatments of a half rate of MH or one application of a full rate of MH sprayed without manually topping resulted in reduced yield compared to other treatments due to poor sucker control.

The primary objective of this research was to determine if burley tobacco could be chemically topped while simultaneously controlling axillary bud growth (suckers) using
currently registered rates of suckercide products without detrimentally impacting yield and leaf quality.

Materials and Methods

Field experiments were conducted in 2016 and 2017 at the Agricultural Experiment Station Spindletop Farm near Lexington, KY and the University of Kentucky Research and Education Center near Princeton, KY. In 2015, this study was conducted at the Spindletop Farm and the West Farm of Murray State University near Murray, KY. Plants of late-maturing burley tobacco (‘KT 210’ or ‘KT 215’ depending on location) were produced in a greenhouse float system according to current University of Kentucky recommendations (Pearce et al., 2017). Tobacco plants were transplanted to the field in late May/early June in all years and locations of these experiments. All field production practices, other than topping, followed University Extension guidelines (Pearce et al., 2017). Prior to harvest, sucker control data and plant measurements were collected from the center two rows of each four row plot.

The experimental design was a randomized complete block with four replications. Suckercides were applied based on product labels with a CO₂-pressurized sprayer calibrated to deliver 468 L ha⁻¹ through an over-the-row three-nozzle row⁻¹ configuration using solid cone spray tips (TG3 - TG5- TG3). Treatments included maleic hydrazide (Royal® MH-30, 0.18 kg L⁻¹, Arysta LifeSciences), butralin (Butralin, 0.36 kg L⁻¹, Arysta LifeSciences), and a fatty alcohol (Off-Shoot-T, 0.31 kg octanol + 0.41 kg decanol + 0.002 kg dodecanol per L, Arysta LifeSciences). All treatments, suckercide application rates, and dates are listed in Table 2.1. There were six chemically topped treatments including applications of MH alone at 2.24 (Full MH) or 1.68 (Reduced MH) kg a.i. ha⁻¹, a tank
mixture of MH and butralin at 2.24 + 0.56 (Full Mix) or 1.68 + 0.56 (Reduced Mix) kg ha\(^{-1}\), respectively. A local systemic (butralin) or contact (FA) alone at 1.12 kg ha\(^{-1}\) or at 10% v/v was also included. There was also a manually topped and not sprayed (Untreated Control or UTC) and a manually topped and sprayed (Grower Standard or G.S.) treatment with the full mixture of MH and butralin. Chemically topped treatments were applied at the pre-bud (10% button) stage and manually topped treatments were imposed at the 10% bloom stage. Button percentage was calculated by dividing the total number of plants in the two center rows of each plot by the number of plants with a visible terminal bud between the apical leaves, or growth stage 51 (Coresta, 2009). Bloom percentage was calculated by dividing the total number of plants in the two center rows of each plot by the number of plants with at least one flower open, or growth stage 60 (Coresta, 2009). All sucker control data were collected within 7 days prior to tobacco harvest and are shown in fresh weight of suckers (grams). All treatment application dates are provided in Table 2.1.

Thirty tobacco plants from the center two rows in each plot were stalk harvested 3 – 4 weeks after manual topping, placed on sticks, and cured in traditional air-curing barns. After curing, tobacco leaves were removed from the stalk, sorted into four stalk positions including flyings (lower stalk), lug (lower mid-stalk), leaf (upper mid-stalk), and tip (upper stalk), and weighed to calculate yield per hectare. MH residue analyses on cured leaf from lower (flyings and lug) and upper (leaf and tip) stalk positions were performed by Global Laboratory Services, Wilson, NC. In 2016 and 2017, a United States Department of Agriculture (USDA) grader evaluated cured leaf to USDA standards for type 31 light air-cured burley tobacco and grades were assigned an index value between 1 and 100 (Bowman et al., 1989). Grade index data are a weighted average of grade across stalk
positions based on the grade received for each stalk position, and the percent contribution of that stalk position to total yield. TSNA samples consisted of 20 leaves, collected from the 4th leaf position from the top of 20 plants in each plot. Samples were then air-dried, ground to 1 mm, and sent to the University of Kentucky Tobacco Analytical Laboratory located at the Kentucky Tobacco Research and Development Center for TSNA analysis following the method described by Morgan et al. 2004. TSNAs are presented as total TSNA in micrograms per gram, which is the sum of all individual TSNAs (NNN, NAT, NAB, NNK). All data were subjected to analysis of variance (ANOVA) with the general linear model procedure (proc GLM), and means were separated using the LS-means multiple comparison procedure at P = 0.10 using SAS 9.4 (SAS Institute Inc., Cary, N.C.).

Results and Discussion

Data for sucker control effectiveness, plant height, tobacco yield, quality grade index, MH residue, and total TSNA are presented by year and location as there were significant environment by treatment interactions.

Sucker Control.

There was a significant treatment effect in each site-year on sucker control. In 2015, there was a significant reduction in sucker control for treatments that did not include MH. Butralin and FA used alone resulted in significantly less sucker control in Murray, and Lexington (Table 2.2). Treatments that included MH (G.S., Full MH, Reduced MH, Full Mix, and Reduced Mix) ranged from 87 to 100% control. Butralin and FA alone treatments were discontinued for 2016 and 2017 as a result of inadequate sucker control and the inability to chemically top the apical meristem observed in 2015.
Sucker growth (grams of fresh weight) ranged from 0 – 60 grams in treated plots in 2016 with the UTC significantly highest at each location. There was a significant reduction in control when MH was used alone as compared to the G.S. and mix treatments Princeton. In Lexington however, only the reduced MH treatment resulted in significantly less sucker control (94%) when compared to all other treated plots. There were no significant differences in sucker control between the G.S. and full or reduced mix treatments at either location in 2016.

The range of sucker control effectiveness in treated plots was 99 to 100% in 2017 at Lexington. Therefore, the addition of butralin in the treatment did not improve the control of axillary bud growth. There was a statistically significant reduction with the reduced MH only treatment at Lexington (5 grams) but this difference is likely not biologically relevant as most MH treated plots controlled all sucker growth. Treatments at Princeton in 2017 followed a similar trend as 2016 with MH alone treatments resulting in reduced sucker control. There was a benefit of using the full rate (119 grams) when compared to the reduced rate (173) of MH, however, only the full and reduced mix treatments provided equivalent sucker control to the G.S. (94 to 100% control).

**Plant Height.**

Investigating the total length of the tobacco plant to be harvested and cured was of interest to determine if there would be limitations with the stalk harvesting and curing as a result of the chemical topping system compared to traditional manual topping. There was a significant treatment effect on plant height in all years and locations (Table 2.3). There was variability in plant height across all environments and treatments ranging from 121 cm to 251 cm. However, all plant heights above 200 cm came from plots with little to no
sucker control in the butralin alone, FA alone, and the UTC treatments in 2015 at each location. Tobacco in chemically topped treatments that included MH was significantly shorter than the G.S. at both locations in 2015, except for the Reduced Mix treatment at Lexington. There was a total range of 12 cm in plant height at Princeton in 2016 across all treatments. Chemically topped treatments that included MH resulted in significantly reduced plant height when compared to the G.S. at Lexington in 2016. The total range in plant height between the G.S. and chemically topped treatments for Princeton in 2017 was 11 cm. However, chemically topped treatments at Lexington resulted in significantly taller tobacco when compared to the G.S. To summarize, differences in plant height between treatments were observed. However, other than treatments without MH, these differences did not result in difficulties in the process of harvesting, handling, and curing.

**Total Yield.**

There was a significant treatment effect on total yield in each year and location combination except in 2016 at Lexington (Table 2.4). Total yield ranged from 1697 to 2252 kg ha\(^{-1}\) at Murray in 2015, with no significant differences in total yield between the G.S. and chemically topped treatments that included MH. Butralin and FA alone were not different from the UTC and resulted in a significant reduction in total yield compared to the G.S. and chemically topped treatments that included MH. These reductions in yield likely resulted from reduced sucker control (Table 2.2). Total yield at Lexington in 2015 ranged from 1817 to 2244 kg ha\(^{-1}\) (Table 2.4). Butralin and FA alone treatments produced significantly lower total yield and MH treated plots grouped with the higher yielding treatments. There were no significant differences between the G.S. and all chemically topped treatments that included MH. The higher yielding treatments also had a
corresponding increase in sucker control effectiveness. The butralin and FA alone treatments were discontinued after the 2015 season as these treatments were not successful in chemically topping the plant, controlling axillary bud growth, and producing yields that were comparable to the G.S.

Total yield ranged from 1884 to 2647 kg ha\(^{-1}\) at Princeton in 2016 (Table 2.4). The G.S., Full Mix, and Reduced Mix treatments resulted in equivalent total yield at Princeton in 2016 and 2017, however, the MH alone (Full MH and Reduced MH) treatments resulted in significantly reduced total yield. The reduction in total yield in the Full MH and Reduced MH treatments at Princeton were accompanied by a significant reduction in sucker control effectiveness (Table 2.2). The addition of butralin (Full Mix and Reduced Mix) provided significantly better sucker control and higher total yield. There were no significant differences in total yield at Lexington in 2016 (P = 0.6447), however, there was a narrow range of 3320 to 3397 kg ha\(^{-1}\) in treated plots. Therefore, chemically topped treatments did not result in a significant decrease in yield as compared to the G.S. This result can be attributed to a high degree of sucker control at Lexington in 2016 (Table 2.2). There were no statistically significant differences between the G.S. and any chemically topped treatment at Lexington in 2017, and sucker control was 99 – 100% across all treated plots.

The Full and Reduced Mix treatments were most consistent and were not significantly different from the G.S. in any year-location combination for total yield and sucker control effectiveness (Tables 2 and 4). Chemical topping with MH alone (Full or Reduced MH) did provide yields that were comparable to the G.S. and tank mix treatments
with butralin in four of the six environments that were tested. Butralin or FA alone was not suitable for chemical topping as yields and sucker control were reduced.

**Quality Grade Index.**

There were no significant effects of chemically topped treatments ($P = 0.3463$) on quality grade index at Murray in 2015 with a range of 38 to 48, as shown in Table 2.5. Federal quality grade index data were not collected at Lexington in 2015. In 2016, there was a significant treatment effect in Lexington ($P = 0.0107$) on quality grade index but this was not observed at Princeton (Table 2.5). There were no significant differences between the G.S. and chemically topped treatments with a range of 70 to 73 with the UTC resulting in significantly higher quality grade index than all other treatments. There were no significant differences at Princeton ($P=0.1884$) or Lexington ($P=0.6712$) in 2017.

**Maleic hydrazide residues.**

MH residue samples for G.S., Full Mix, and Reduced Mix treatments were collected in all years and locations of this experiment. Within all years and locations, MH residues were higher in the upper leaf positions than the lower leaf positions except Lexington, 2015 and Princeton, 2017 (Table 2.6). There was no consistent reduction in MH residues due to the application of a reduced rate of MH, as the Full Mix contained only 25% more product and did not always produce higher MH residues. Generally, precipitation occurring after topping and prior to harvest provided some explanation for differing MH residues in the different environments. The highest rainfall during this period (10.39 cm) resulted in the lowest residues at Princeton in 2016. Higher MH residues were observed in Murray, 2015 and Lexington, 2016 and lower amounts of rainfall after topping and prior to harvest.
At Murray in 2015, the G.S. treatment had numerically higher MH residues (64 ppm) than the Full and Reduced Mix treatments (33 and 59 ppm, respectively), although this was not statistically significant. The Reduced Mix treatment (19 ppm) had significantly less MH residues than the G.S. (49 ppm) with the Full Mix (32 ppm) treatment not significantly different from either at Lexington in 2015 (Table 2.6). In 2016 at Princeton, the G.S. treatment resulted in significantly higher MH residues compared to the Full and Reduced Mix treatments (P = 0.0233). Overall, MH residues at Princeton in 2016 were lower than all other location and year combinations likely due to heavy rainfall (10.39 cm) after topping through harvest. There were no significant differences at Lexington in 2016, however, the G.S. had numerically higher MH residues than chemically topped treatments. Unexpectedly, the Full Mix chemically topped treatment resulted in significantly lower MH residues compared to the Reduced Mix and G.S. at Princeton in 2017. Although not significant, the G.S. had numerically less MH residues compared to chemically topped treatments at Lexington in 2017. This is likely due to a rainfall event that occurred within three to six hours after application. The decision was made not to reapply this treatment as it may have influenced results; however, sucker control and yield were not negatively affected (Tables 2 and 4). Theoretically, chemical topping may result in lower MH residues due to the timing of application as chemical topping applications are typically made about seven days prior to when growers would normally apply MH following manual topping. Assuming both are harvested at the same time, the increased time between application and harvest would allow more time for precipitation and degradation to reduce MH residue levels. There was no clear evidence of a reduction in
MH residues with chemical topping in this study, but MH residues were not higher compared to the current grower standard for sucker control.

**Tobacco-Specific Nitrosamines.**

There were no significant differences in TSNA between manually or chemically topped treatments in 2016 (p=0.8444) or 2017 (p=0.2046) at Princeton as shown in Table 2.7. However, there was a significant treatment effect on total TSNA at Lexington in 2016 (p=0.0019) and 2017 (p=0.0702). The UTC had significantly higher TSNAs than all other treatments in 2016. Chemically topped treatments (Full Mix and Reduced Mix) resulted in significantly lower TSNAs than the UTC and the G.S. There was a topping effect and MH application effect on TSNA. Topping without spraying MH (UTC) resulted in significantly higher total TSNA compared to manually topping and spraying MH (G.S.). A similar significant trend was observed at Lexington in 2017, however, there were no significant differences between the G.S. and UTC. Cui et. al. (1994) suggested that applying MH reduced TSNA in air-cured burley tobacco due to MH altering the precursor-TSNA relationship. Tso (1990) concluded that topping increases nicotine content and results in a net gain in total alkaloid content; therefore, tobacco plants that are not manually topped should be expected to have less alkaloids and therefore less precursor to TSNA formation. This is likely due to a combination of increased root growth leading to an increase in nicotine biosynthesis and upregulated plant defenses due to wound signaling pathways. This may help explain reduced total TSNA in chemically topped treatments (Full Mix and Reduced Mix) at Lexington in 2016, as nicotine content (Table 2.7) was significantly less in chemically topped treatments (p<.0001). Chemically topped treatments resulted in lower nicotine content at Princeton in 2016 and in both years at
Lexington (Table 2.7). Another possible explanation is the timing of MH application between the G.S. and the chemically topped treatments as the chemically topped MH treatments were applied seven days prior to the G.S. (Table 2.1), thus altering the timing of the precursor relationship. Significant reductions in TSNA were only observed in Lexington, however, numerical trends were observed in Princeton.

**Cumulative Distribution Function for Cost Savings**

Chemical topping burley tobacco was found to be a suitable alternative to the traditional manual topping as sucker control, total yield, and leaf quality grade index was not significantly different in all environments tested. A stochastic simulation model was developed to evaluate the potential savings from the use of chemical versus manual topping. The stochastic variables in the model are the number of man-hours required for manual topping (Min=3, Mean=5.5, Max=10), amount of time required to spray (Min=0.4, Mean=0.5, Max=0.6), hourly wage (Min=8, Mean=10, Max 12.5), yield (kg ha\(^{-1}\)) (Min=1905, Mean=2242, Max=3138) and the average price per kilogram (Min=2.71, Mean=3.95, Max=4.41). Minimal research has been conducted on hours to manually top versus spraying a hectare of tobacco and its impact on yield and quality, which impacts price. A GRKS distribution was utilized based on parameters using the preceding minimum, mean, and maximum values in variables. The GRKS\(^1\) distribution is an augmented triangle distribution and is used in situations when minimal information is available (Richardson et al., 2006). The critical difference between manual and chemical topping is labor cost savings potential (Figure 2.1). The foundation for the simulation is

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\(^1\) The Gray, Richardson, Klose, and Schumann (GRKS) distribution is similar to a triangular distribution and was developed to simulate random variables when insufficient historical data is available (Richardson, 2016).
the 2016 burley tobacco budget used to calculate the cost reduction of chemical topping as a function of the reduced labor requirement for topping (Snell et al., 2016). Cost saving (\$ ha\(^{-1}\)) is based on the return over variable cost as opposed to return over total costs. Based on the assumptions of this simulation, an average of $134.45 ha\(^{-1}\) was saved when chemical topping was used if topping required only five man-hours in a manual topping system. The range of cost saving is $28.81 to $288.49 ha\(^{-1}\) based on 500 iterations, with an iteration representing a possible outcome given the assumptions, under the assumptions with the simulation. Another simulation was performed assuming that manual topping required ten man-hours in a manual topping system. An estimated average of $259.23 ha\(^{-1}\) was saved when chemical topping was used to replace the labor associated with topping. The range of cost saving would be $142.21 to $438.11 ha\(^{-1}\) if ten man-hours were required to manually top based off 500 iterations under the assumptions with the simulation.

**Conclusion**

Chemical topping of burley tobacco at 10% button stage with a tank mixture of MH and a local systemic suckercide was a suitable alternative to manual topping as sucker control, total yield, and leaf quality grade index were not different in all years and locations of this study. Application of a local systemic or fatty alcohol alone did not inhibit the terminal bud nor control subsequent sucker growth resulting in a reduction in total yield. MH residues for chemically topped tobacco were not consistently higher than residues from manually topped and sprayed tobacco. Total TSNA was not increased due to chemically topped treatments, and at Lexington there was a reduction in total TSNA compared to manually topping. Future work should further investigate these total TSNA reductions that were observed. Chemical topping has the potential to reduce labor input
and production costs without negatively impacting the yield, quality or chemistry of burley tobacco.
Table 2.1. Suckercide application rate and date for manual and chemical topping treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Suckercides</th>
<th>Application Rate</th>
<th>Manually Topped</th>
<th>Treatment Applied</th>
<th>Spindletop</th>
<th>Princeton</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>kg a.i. ha(^{-1})</td>
<td>Yes/No</td>
<td>2015</td>
<td>2016</td>
<td>2017</td>
</tr>
<tr>
<td>UTC(^a)</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
<td>7/27</td>
<td>8/9</td>
<td>7/28</td>
</tr>
<tr>
<td>G.S.</td>
<td>MH + B</td>
<td>2.24 + 0.56</td>
<td>Yes</td>
<td>7/27</td>
<td>8/9</td>
<td>7/28</td>
</tr>
<tr>
<td>Full MH</td>
<td>MH</td>
<td>2.24</td>
<td>No</td>
<td>7/20</td>
<td>8/2</td>
<td>7/20</td>
</tr>
<tr>
<td>Reduced MH</td>
<td>MH</td>
<td>1.68</td>
<td>No</td>
<td>7/20</td>
<td>8/2</td>
<td>7/20</td>
</tr>
<tr>
<td>Full Mix</td>
<td>MH + B</td>
<td>2.24 + 0.56</td>
<td>No</td>
<td>7/20</td>
<td>8/2</td>
<td>7/20</td>
</tr>
<tr>
<td>Reduced Mix</td>
<td>MH + B</td>
<td>1.68 + 0.56</td>
<td>No</td>
<td>7/20</td>
<td>8/2</td>
<td>7/20</td>
</tr>
<tr>
<td>Butralin</td>
<td>B</td>
<td>1.12</td>
<td>No</td>
<td>7/20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FA</td>
<td>C(<em>8)-C(</em>{12})</td>
<td>10% v/v</td>
<td>No</td>
<td>7/20</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) UTC = Untreated control ; G.S. = Grower Standard ; MH = maleic hydrazide; FA = Fatty Alcohol; B = butralin.
* 2015 location was at Murray, KY
Table 2.2. Total weight of suckers per plant prior to harvest for manual and chemical topping treatments\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Treatment\textsuperscript{b}</th>
<th>2015\textsuperscript{c}</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Murray</td>
<td>Lexington</td>
<td>Princeton</td>
</tr>
<tr>
<td>UTC</td>
<td>269 d</td>
<td>454 d</td>
<td>324 c</td>
</tr>
<tr>
<td>G.S.</td>
<td>2 a</td>
<td>2 a</td>
<td>0 a</td>
</tr>
<tr>
<td>Full MH</td>
<td>5 a</td>
<td>30 a</td>
<td>50 b</td>
</tr>
<tr>
<td>Reduced MH</td>
<td>25 ab</td>
<td>43 a</td>
<td>60 b</td>
</tr>
<tr>
<td>Full Mix</td>
<td>0 a</td>
<td>26 a</td>
<td>12 a</td>
</tr>
<tr>
<td>Reduced Mix</td>
<td>1 a</td>
<td>57 a</td>
<td>6 a</td>
</tr>
<tr>
<td>Butralin</td>
<td>96 bc</td>
<td>338 c</td>
<td>-</td>
</tr>
<tr>
<td>FA</td>
<td>121 c</td>
<td>257 b</td>
<td>-</td>
</tr>
</tbody>
</table>

\textit{p-value} \textless .0001 \textless .0001 \textless .0001 \textless .0001 \textless .0001 \textless .0001

\textsuperscript{a} Total weight of suckers per plant calculated from a sample of 10 plants per plot.

\textsuperscript{b} UTC = Untreated control; G.S. = Grower Standard; MH = maleic hydrazide; FA = Fatty Alcohol; B = butralin.

\textsuperscript{c} Means within a column followed by the same letter are not significantly different according to Fisher’s Protected LSD at P = 0.10.
Table 2.3. Plant height following manual topping and chemical topping treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Murray</th>
<th>Lexington</th>
<th>Princeton</th>
<th>Lexington</th>
<th>Princeton</th>
<th>Lexington</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTC</td>
<td>187 cd</td>
<td>209 b</td>
<td>184 c</td>
<td>195 a</td>
<td>196 a</td>
<td>161 a</td>
</tr>
<tr>
<td>G.S.</td>
<td>190 c</td>
<td>169 c</td>
<td>178 d</td>
<td>185 b</td>
<td>142 b</td>
<td>121 c</td>
</tr>
<tr>
<td>Full MH</td>
<td>169 e</td>
<td>151 e</td>
<td>185 bc</td>
<td>168 c</td>
<td>143 b</td>
<td>148 bc</td>
</tr>
<tr>
<td>Reduced MH</td>
<td>179 d</td>
<td>159 d</td>
<td>189 ab</td>
<td>171 c</td>
<td>142 b</td>
<td>149 b</td>
</tr>
<tr>
<td>Full Mix</td>
<td>167 e</td>
<td>161 d</td>
<td>190 a</td>
<td>168 c</td>
<td>132 c</td>
<td>150 b</td>
</tr>
<tr>
<td>Reduced Mix</td>
<td>171 e</td>
<td>168 c</td>
<td>189 abc</td>
<td>166 c</td>
<td>135 c</td>
<td>143 c</td>
</tr>
<tr>
<td>Butralin</td>
<td>242 b</td>
<td>219 a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>251 a</td>
<td>218 a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p-value*  <.0001  <.0001  <.0001  <.0001  <.0001  <.0001

\(^{a}\) UTC = Untreated control; G.S. = Grower Standard; MH = maleic hydrazide; FA = Fatty Alcohol; B = butralin.

\(^{b}\) Means within a column followed by the same letter are not significantly different according to Fisher’s Protected LSD at P = 0.10.
Table 2.4. Effect of manual or chemical topping treatments on burley tobacco yield.

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Murray</th>
<th>Lexington</th>
<th>Princeton</th>
<th>Lexington</th>
<th>Princeton</th>
<th>Lexington</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTC</td>
<td>1720 b</td>
<td>1872 ab</td>
<td>1884 b</td>
<td>3098</td>
<td>2122 b</td>
<td>2005 b</td>
</tr>
<tr>
<td>G.S.</td>
<td>2121 a</td>
<td>2155 ab</td>
<td>2627 a</td>
<td>3371</td>
<td>2725 a</td>
<td>2598 a</td>
</tr>
<tr>
<td>Full MH</td>
<td>2166 a</td>
<td>2229 ab</td>
<td>2074 b</td>
<td>3338</td>
<td>2297 b</td>
<td>2779 a</td>
</tr>
<tr>
<td>Reduced MH</td>
<td>2233 a</td>
<td>2141 ab</td>
<td>2127 b</td>
<td>3397</td>
<td>2112 b</td>
<td>2705 a</td>
</tr>
<tr>
<td>Full Mix</td>
<td>2252 a</td>
<td>2157 ab</td>
<td>2647 a</td>
<td>3356</td>
<td>2690 a</td>
<td>2828 a</td>
</tr>
<tr>
<td>Reduced Mix</td>
<td>2148 a</td>
<td>2244 a</td>
<td>2611 a</td>
<td>3320</td>
<td>2614 a</td>
<td>2576 a</td>
</tr>
<tr>
<td>Butralin</td>
<td>1697 b</td>
<td>1817 b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>1719 b</td>
<td>1817 b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p-value*  
0.0026 0.0371 0.0030 0.6447 <.0001 <.0001

*a UTC = Untreated control ; G.S. = Grower Standard ; MH = maleic hydrazide; FA = Fatty Alcohol; B = butralin.

*b Means within a column followed by the same letter are not significantly different according to Fisher’s Protected LSD at P = 0.10.*
Table 2.5. Effect of manual or chemical topping treatments on quality grade index for type 31 burley tobacco\(^a\).

<table>
<thead>
<tr>
<th>Treatment(^b)</th>
<th>Murray</th>
<th>Lexington</th>
<th>Princeton</th>
<th>2016</th>
<th>Lexington</th>
<th>Princeton</th>
<th>2017</th>
<th>Lexington</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTC</td>
<td>42</td>
<td>-</td>
<td>60</td>
<td>77</td>
<td>a</td>
<td>64</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>G.S.</td>
<td>38</td>
<td>-</td>
<td>61</td>
<td>71</td>
<td>b</td>
<td>57</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Full MH</td>
<td>43</td>
<td>-</td>
<td>61</td>
<td>70</td>
<td>b</td>
<td>67</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Reduced MH</td>
<td>47</td>
<td>-</td>
<td>62</td>
<td>71</td>
<td>b</td>
<td>62</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Full Mix</td>
<td>39</td>
<td>-</td>
<td>61</td>
<td>73</td>
<td>b</td>
<td>63</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Reduced Mix</td>
<td>48</td>
<td>-</td>
<td>62</td>
<td>73</td>
<td>b</td>
<td>68</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Butralin</td>
<td>45</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>41</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

\(p\)-value\(^c\) 0.3463 - 0.1306 0.0107 0.1884 0.6712

\(^a\)Quality grade index is a numerical representation of Federal quality grade index received for tobacco and is a weighted average of grade index for all stalk positions following Bowman et al. 1989.

\(^b\)UTC = Untreated control ; G.S. = Grower Standard ; MH = maleic hydrazide; FA = Fatty Alcohol; B = butralin.

\(^c\)Means within a column followed by the same letter are not significantly different according to Fisher’s Protected LSD at \(P = 0.10\).
Table 2.6. Maleic hydrazide residues as affected by manual or chemical topping, upper and lower stalk positions, and precipitation.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ppm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G.S.</td>
<td>---</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full Mix</td>
<td>33</td>
<td>32 ab</td>
<td>10 b</td>
<td>62</td>
<td>41 a</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced Mix</td>
<td>59</td>
<td>19 b</td>
<td>11 b</td>
<td>51</td>
<td>36 a</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.1886</td>
<td>0.0944</td>
<td>0.0233</td>
<td>0.7038</td>
<td>0.0231</td>
<td>0.1168</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Position</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>78 A</td>
<td>38</td>
<td>13 A</td>
<td>85 A</td>
<td>35</td>
<td>53 A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower</td>
<td>26 B</td>
<td>26</td>
<td>10 B</td>
<td>27 B</td>
<td>24</td>
<td>29 B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.0011</td>
<td>0.1692</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.2279</td>
<td>0.0078</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.92</td>
<td>6.17</td>
<td>10.39</td>
<td>4.47</td>
<td>3.12</td>
<td>7.49</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Means within a column followed by the same uppercase or lowercase letter are not significantly different according to Fisher’s Protected LSD at P = 0.10.

b Full MH and Reduced MH were excluded from residue analysis to make better comparisons to the G.S.

c Total rainfall from topping through harvest.
Table 2.7. Tobacco-specific nitrosamines and nicotine content from differing manual or chemical topping treatments.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg g⁻¹</td>
<td></td>
<td>µg g⁻¹</td>
<td></td>
</tr>
<tr>
<td>UTC</td>
<td>4.72</td>
<td>3.14 a</td>
<td>1.38</td>
<td>1.34 ab</td>
</tr>
<tr>
<td>G.S.</td>
<td>5.04</td>
<td>2.22 b</td>
<td>1.24</td>
<td>1.60 a</td>
</tr>
<tr>
<td>Full Mix</td>
<td>4.23</td>
<td>1.01 c</td>
<td>0.87</td>
<td>0.59 b</td>
</tr>
<tr>
<td>Reduced Mix</td>
<td>4.63</td>
<td>1.15 c</td>
<td>0.85</td>
<td>0.57 b</td>
</tr>
<tr>
<td>p-value</td>
<td>0.8444</td>
<td>0.0019</td>
<td>0.2046</td>
<td>0.0702</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>UTC</td>
<td>3.05 c</td>
<td>6.01 a</td>
<td>4.76</td>
<td>6.03 a</td>
</tr>
<tr>
<td>G.S.</td>
<td>4.65 a</td>
<td>5.34 a</td>
<td>4.18</td>
<td>5.55 a</td>
</tr>
<tr>
<td>Full Mix</td>
<td>4.02 b</td>
<td>2.23 c</td>
<td>4.43</td>
<td>3.58 b</td>
</tr>
<tr>
<td>Reduced Mix</td>
<td>3.56 b</td>
<td>3.00 b</td>
<td>4.41</td>
<td>3.29 b</td>
</tr>
<tr>
<td>p-value</td>
<td>0.0002</td>
<td>&lt;.0001</td>
<td>0.8773</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

a Means within a column and variable followed by the same letter are not significantly different according to Fisher’s Protected LSD at P = 0.10.

b Full MH and Reduced MH were excluded from leaf chemistry analyses to make better comparisons to the G.S.
Figure 2.1. Cumulative Distribution Function for Manual and Chemical Topping Burley Tobacco. The lines represent the cost saved per hectare with the assumption that 5 man-hours (blue) or 10 man-hours (red) was required for manual topping.
Chapter 3: The Effect of Suckercide Application Timing and Variety Maturity on Chemical Topping of Burley Tobacco

Abstract

Experiments were initiated in 2015 to evaluate the efficacy of chemical topping for burley tobacco. The major objectives for this study were to determine the optimum timing of suckercide application and appropriate variety maturity for effective chemical topping. Burley tobacco varieties TN90 (medium maturity), KT210 and KT215 (late maturity) were chemically topped at the 10% button, 50% button, and 10% bloom growth stages. The 10% button and 50% button application timings were best suited for chemical topping practices. Treatments that targeted the 10% bloom stage did not completely halt inflorescence growth, however all application timings resulted in excellent sucker control. Both medium and late-maturing burley varieties proved to be acceptable for chemical topping methods, however, timing the suckercide application may be less difficult with later maturing varieties. Chemically topped treatments generally resulted in shorter, narrower tip leaves than manually topped treatments. There were no significant differences in total yield when comparing tobacco that was manually topped at 10% bloom to chemically topped at 10% button, 50% button, or 10% bloom across all environments in TN90. In four out of six environments, total yield was not significantly different between manual topping and any chemically topped application timing in the late-maturing burley varieties; however, at least one chemically topped application timing was equivalent to manually topped tobacco in all environments.
Introduction

Topping, the removal of the terminal bud or inflorescence of the tobacco (*Nicotiana tabacum* L.) plant, is ordinarily accomplished by manually removing the top of each tobacco plant in an entire field, which is labor intensive and costly. Removal of the terminal bud or inflorescence prevents reproductive development (i.e. seed head) and results in energy transferred to increased leaf size, weight, nicotine, and other chemical constituents (Tso, 1990). Topping eliminates apical dominance in the plant resulting in axillary bud growth, known as suckers (Decker and Seltmann, 1971). It has been shown that controlling sucker growth and yield are positively correlated (Collins and Hawks, 1993).

Topping burley tobacco at ten to twenty-five percent bloom with an optimum leaf number of 22-24 leaves has been shown to provide the best yield, leaf quality, and a better opportunity for a true tip grade (Bailey et al., 2017). Higher yields were observed when flue-cured tobacco was topped in the button or early flower stages in hand-suckered treatments with a yield penalty of around 28 kg\(^{-1}\) day\(^{-1}\) when topping was delayed beyond this point (Marshall and Seltmann, 1964). Other studies have found no significant differences in burley tobacco yield and value when topped at early bloom or mid-bloom stages (Seltmann et al., 1969). However, the number of leaves left on the plant after topping was shown to be positively related to yield (King, 1986), but value has been shown to have a negative relationship with number of leaves left on the plant (Collins and Hawks, 1993). A chemical topping study applying a tank mixture of maleic hydrazide (MH) and flumetralin when the 20\(^{th}\) leaf expanded to 15 cm on photoperiod-sensitive cultivars of flue-cured tobacco found no differences in yield compared to manually topped and sprayed
tobacco (Long et al., 1989). Peek (1995) found that chemical topping at the 25% button stage was the most effective timing of application but resulted in the largest yield reduction. The associated yield reduction was attributed to reduced leaf size, specifically in the upper stalk positions (Peek, 1995). Long et al. (1989) found that chemically topped plants generally resulted in taller plants with shorter, narrower top leaves. The primary objectives of this research was to determine the optimum stage of apical bud growth to target that could chemically top the plant while simultaneously controlling axillary bud growth (suckers) using currently registered suckercide products in medium and late maturing burley tobacco varieties.

**Materials and Methods**

Field experiments were conducted in 2016 and 2017 at the Agricultural Experiment Station Spindletop Farm near Lexington, KY and the University of Kentucky Research and Education Center near Princeton, KY. In 2015, this study was conducted at the Spindletop Farm and the West Farm of Murray State University near Murray, KY. Transplants of burley tobacco varieties ‘KT 210 and KT 215’ (late maturity) and ‘TN90’ (medium maturity) were grown in a greenhouse float system according to current University of Kentucky recommendations (Pearce et al. 2017). Tobacco plants were transplanted to the field in late May/early June in all years and locations of these experiments. All field production practices, other than topping, followed recommendations based on the University Extension guidelines (Pearce et al., 2017).

The experimental design was a randomized complete block with treatments replicated four times. Suckercides were applied with a CO$_2$-pressurized sprayer calibrated to 468 L ha$^{-1}$ with a directed three-nozzle row$^{-1}$ configuration (TG3-TG5-TG3). Maleic
hydrazide (Royal MH-30®, 0.18 kg a.i. L⁻¹, Arysta LifeScience) tank mixed with butralin (Butralin®, 0.36 kg a.i. L⁻¹, Arysta LifeScience) was used as the suckercide application. Chemical topping treatments were applied at either the 10% button, 50% button, or 10% bloom stages. There was also a manually topped and not sprayed (Untreated Control or UTC) and a manually topped and sprayed (Grower Standard G.S.) imposed at the 10% bloom stage (Figure 3.1). Button percentage was calculated by dividing the total number of plants in the two center rows of each plot by the number of plants with a visible terminal bud between the apical leaves, or growth stage 51 (Coresta Guide #7, 2009). Bloom percentage was with the total number of plants in the two center rows of each plot with at least one flower open, or growth stage 60 (Coresta Guide #7, 2009). All sucker control data were collected within 7 days before tobacco harvest and are shown in percent control of fresh weight of suckers compared to fresh weight of sucker in the manually topped untreated control that did not receive suckercide treatment.

Thirty tobacco plants from the center two rows in each plot were stalk harvested three-four weeks after manual topping, placed on sticks, and cured in traditional air-curing barns. Prior to harvest, sucker control data and plant height measurements were collected from the center two rows of each four-row plot. After curing, tobacco leaves were removed from the stalk, sorted into 4 stalk positions including flyings (lower stalk), lug (lower mid-stalk), leaf (upper mid-stalk), and tip (upper stalk), and weighed to calculate yield per hectare. A sample of 25 leaves from the tip grade of each plot was measured to determine leaf length and leaf width. A United States Department of Agriculture (USDA) grader evaluated cured leaf to USDA standards for type 31 light air-cured burley tobacco and grades were assigned an index value between 1 and 100. Grade index data are a weighted
average of grade across stalk positions based on the grade received for each stalk position, and the percent contribution of that stalk position to total yield (Bowman et al., 1989). All data were subjected to analysis of variance (ANOVA) with the general linear model procedure (proc GLM), and means were separated using the least-square means multiple comparison procedure at $P = 0.10$ using SAS 9.4 (SAS Institute Inc., Cary, N.C.).

**Results and Discussion**

Data for sucker control effectiveness, plant height, tip leaf stalk position length, tobacco yield, and quality grade index are presented by year, location, and variety maturity as there were significant environment by treatment interactions.

**Sucker Control.**

There was a significant application timing effect on percent sucker control in each environment as shown in Table 3.2. Overall, sucker control ranged from 89 to 100% control in treated plots across all environments. In 2015 at Murray, there was a significant reduction in sucker control when suckercides were applied at the 10% bloom stage in the late maturity group compared to all other timings, however, this difference was only one percent. There were no significant differences between application timings in the medium maturity TN 90 at Murray. In 2015 at Lexington, there was a significant reduction in sucker control when applications were made at the 10% bloom stage in TN 90 but this difference was only five percent in comparison with the G.S. There was a significant three percent reduction in sucker control in the 10% button timing as compared to the G.S. in the late maturing KT 210.

The range of sucker control effectiveness across application timings in treated plots for medium and late maturing varieties was 91 to 100% and 89 to 100%, respectively.
2016 at Princeton. There were no significant differences in the medium-maturing TN 90 between the G.S. and any application timing at Lexington in 2016 as excellent sucker control was observed in all treated plots. There was a significant four percent reduction in sucker control when late-maturing varieties were chemically topped at the 10% bloom application timing. In 2017, there was a significant two percent reduction in sucker control in the 50% button application timing when compared to the G.S. at Princeton in the medium-maturing TN 90. There were no significant differences between the G.S. and any chemical topping application timing in the late-maturing varieties. In 2017 at Lexington, the 10% and 50% button chemical topping application timings resulted in significantly higher sucker control than the G.S. and the 10% bloom stage in the medium maturing TN 90. The 50% button application timing resulted in a significant nine percent reduction in sucker control compared to all other treated plots.

In summary, excellent sucker control was achieved in all chemical topping application timings. Peek (1995) observed reduced sucker control when suckercides were applied at later maturity stages. Chemical topping at 10% bloom resulted in around 10% flower spikes present at harvest as the blooms were not manually removed. Therefore, we concluded that the 10% or 50% button application timings were better suited for chemical topping of burley tobacco.

**Plant Height.**

Investigating the height of the tobacco plants to be harvested and cured was of interest to determine if there would be harvest difficulties encountered when using chemical topping compared to traditional manual topping. Plant height was measured while plants were still in the field and was determined by measuring from the ground to
the uppermost plant part. There was a significant timing effect on plant height in all years and locations (Figure 3.3). There was variability in plant height across all environments, maturity, and application timings ranging from 123 to 231 cm. The UTC had significantly higher plant height compared to all other application timings for the medium maturity TN 90, which was due to no sucker control applied after topping. Within the medium maturity TN 90, the 10% bloom application timing resulted in significantly lower plant height than the UTC but significantly higher plant height compared to the G.S. and 10% or 50% button timings within each environment. There was a 1 to 13 cm difference in plant heights across all environments when comparing the chemical topping application timings at 10% button and 50% button to the G.S. within the medium maturity TN 90. Therefore, 10% and 50% button application timings appeared to be more suitable target timings for chemical topping when comparing plant height for the medium maturity variety used in these experiments.

Unlike in the medium maturity variety, the UTC did not always result in significantly higher plant height in all years and locations for the late maturing variety. Either the UTC or 10% bloom application timing had significantly higher plant height compared to all other application timings and the G.S within each environment (Figure 3.3) for the late maturing varieties used in these experiments. In three of the six environments within the late-maturing varieties (Murray, 2015; Lexington, 2016; and Princeton, 2017), there were no significant differences between 10% button application timing and the G.S. The 10% button application timing resulted in significantly lower plant height at Lexington, 2015 and Princeton, 2016 but significantly higher plant height in Lexington, 2017 compared to the G.S. Within the late-maturing varieties, the 50% button application timing resulted in significantly higher plant height compared to the G.S. and 10% button
in all environments with the exception of 2016 at Lexington where there were no significant differences between either timing. There were no undesirable plant heights that caused problems in harvesting or curing except no sucker control within the UTC and the existence of blooms within the 10% bloom application timing. The UTC was trimmed immediately prior to harvest to meet the size requirements of the curing facility; however, the plots were not suckered. Therefore, we concluded that the 10% or 50% button application timings should be targeted.

**Leaf Dimensions.**

Leaf dimension data were collected from a 25-leaf sample of cured-leaf from the tip stalk position. There was a significant application timing effect on leaf length in each environment except Princeton in 2017 as shown in Figure 3.4. The range of tip leaf length for medium maturity was 32 – 54 cm across all environments and treatments. The G.S. resulted in significantly longer tip leaves than any chemically topped application timing within the medium maturity variety in four of five environments where tip leaf length was measured. In the late-maturing varieties, either the UTC or the G.S. resulted in significantly longer tip leaves when comparing all treatments within each environment. Chemical topping in the late maturity variety resulted in significantly shorter tip leaf length at Lexington in all years of this study. However, only the 10% button application timing resulted in significantly shorter tip leaves at Princeton in 2016 with the 50% button and 10% bloom timings not significantly different than the G.S. Significant differences in 2017 at Princeton were likely not biologically relevant as the total range in tip leaf length was only two cm when comparing all treatments excluding the UTC. The total difference
between the 10% button, 50% button, and 10% bloom application timings within each environment and maturity ranged from one to three cm.

There was a significant application timing effect on tip leaf width within each environment (Figure 3.5). The range of tip leaf width for medium maturity was 13 – 24 cm across all environments and treatments. Within the medium maturity TN 90, the G.S. had significantly wider tip leaves compared to all chemically topped application timings except 10% bloom at Lexington in 2016. The range in tip leaf width for the late-maturing varieties was 15 – 28 cm across all environments and treatments. Within the late-maturing varieties, the G.S. had significantly wider leaves than all chemically topped application timings with the exception of 50% button at Princeton in 2016. The 10% button application timing was grouped with the significantly narrowest leaf in all environments for each maturity, except for late maturing varieties at Lexington in 2016.

Generally, chemically topped plants resulted in shorter, narrower leaves in the tip stalk position compared to treatments that were manually topped, which is comparable to other previous results (Long et al. 1989; Peek, 1995). It would be expected that tip leaf length in chemically topped burley tobacco would be equal to or less than manually topped. Thus, the marketable cured tip leaf stalk position would be expected to have a higher likelihood to meet the leaf length requirement for tip grade in chemically topped burley tobacco.

**Total Yield.**

There was a significant application timing effect on total yield in each year and location combination except in the late maturing KT 215 at Murray in 2015 (Figure 3.6). As expected, the UTC resulted in the lowest total yield within each environment, maturity,
and application timing as there was no sucker control applied to these plots. There were no significant differences between the G.S. and chemically topped application timings in the medium maturity TN 90 at either location in any year. Within chemically topped treatments, the 10% bloom application timing resulted in significantly higher total yield compared to chemically topped at 10% button timing at Murray in 2015 (p=0.0040). Chemically topped at the 50% button application timing resulted in significantly higher total yield compared to the 10% bloom timing at Lexington in 2015 within the medium maturity (p=0.0026). Each location in 2016 and 2017 for the medium maturity TN 90 followed the same trend with the G.S. not significantly different than any chemically topped application timing, which is similar to sucker control effectiveness data.

Within the late maturing varieties, there were no significant differences between the G.S., 10% button, 50% button, and 10% bloom at Lexington in 2015 and 2017 or Princeton in 2016. The G.S. resulted in significantly higher total yield than the 50% button and 10% bloom application timings at Lexington in 2016 but was not different than the 10% button (p=0.0206). The 10% button application timing at Princeton in 2017 had significantly lower total yield compared to the G.S. and 10% bloom application timing (p=0.0059). Sucker control effectiveness data does not exclusively explain differences in total yield for the late maturing varieties, as sucker control across all treated plots ranged from 89 – 100%, especially considering the excellent sucker control with all treatments at Princeton in 2017 (Figure 3.2). It should be noted that later maturing/flowering varieties might be better suited for adopting chemical topping methods, as the transition between reproductive growth stages is slower than in earlier maturing varieties. To summarize, there were no significant differences in total yield when comparing the G.S. to tobacco that
was chemically topped at 10% button, 50% button, and 10% bloom across all environments in the medium maturity TN 90. With the exception of two environments, total yield was not significantly different between the G.S. and any chemically topped application timing in the later maturing varieties; however, at least one chemical topping timing was equivalent to the G.S. in all environments.

**Quality Grade Index.**

There was no significant effect of treatment across all environments and maturities on quality grade index (Figure 3.7). Quality grade index data were not collected at Lexington in 2015. There was a difference of 11 grade index points between all treatments within the medium maturity TN 90 at Murray; however, there was only two grade index points difference between the G.S. and all chemically topped application timings for quality grade index. Within TN 90, there was a difference of 3 and 13 grade index points across all treatments in 2016 and 3 and 9 grade index points in 2017 at Princeton and Lexington, respectively. There was a difference of 12 grade index points across all treatments within the late maturing KT 215 at Murray. Within the late maturing varieties, there was a difference of 5 and 6 grade index points across all treatments in 2016 and 2 and 10 grade index points in 2017 at Princeton and Lexington, respectively. Therefore, our data suggested that no application timing detrimentally influences quality grade index as there were no significant differences across manually or chemically topped treatments.

**Conclusion**

Chemical topping burley tobacco at 10% button (pre-bud) to 50% button (early-bud) is ideal as application of suckercides at 10% bloom did not completely halt the development of reproductive growth. Most chemically topped application timings
included in these experiments provided similar sucker control, total yield, and leaf quality compared to manually topping. Chemically topped treatments also appeared to have shorter tip leaves which may contribute to an increased amount of marketable tip grades compared to manually topping. Although there were no outstanding differences in yield and quality between the medium and late maturing varieties used in this experiment, later maturing varieties tended to yield higher and may be better suited for chemical topping due to less rapid change from vegetative to reproductive growth, which would result in a wider window for making chemical topping applications at the most appropriate timings.
Table 3.1. Suckercide application date for manual topping and chemical topping application timings.

<table>
<thead>
<tr>
<th>Maturity</th>
<th>Treatment</th>
<th>Timing</th>
<th>Manually Topped</th>
<th>Treatment Applied</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yes/No</td>
<td>Spindletop</td>
</tr>
<tr>
<td>Medium</td>
<td>UTC\textsuperscript{a}</td>
<td>10% Bloom</td>
<td>Yes</td>
<td>7/24</td>
</tr>
<tr>
<td></td>
<td>G.S.</td>
<td>10% Bloom</td>
<td>Yes</td>
<td>7/24</td>
</tr>
<tr>
<td>Medium</td>
<td>10% Button</td>
<td>10% Button</td>
<td>No</td>
<td>7/20</td>
</tr>
<tr>
<td>Medium</td>
<td>50% Button</td>
<td>50% Button</td>
<td>No</td>
<td>7/20</td>
</tr>
<tr>
<td>Medium</td>
<td>10% Bloom</td>
<td>10% Bloom</td>
<td>No</td>
<td>7/20</td>
</tr>
<tr>
<td>Medium</td>
<td>UTC</td>
<td>10% Bloom</td>
<td>Yes</td>
<td>7/27</td>
</tr>
<tr>
<td>Medium</td>
<td>G.S.</td>
<td>10% Bloom</td>
<td>Yes</td>
<td>7/27</td>
</tr>
<tr>
<td>Late</td>
<td>10% Button</td>
<td>10% Button</td>
<td>No</td>
<td>7/20</td>
</tr>
<tr>
<td>Late</td>
<td>50% Button</td>
<td>50% Button</td>
<td>No</td>
<td>7/24</td>
</tr>
<tr>
<td>Late</td>
<td>10% Bloom</td>
<td>10% Bloom</td>
<td>No</td>
<td>7/27</td>
</tr>
</tbody>
</table>

\textsuperscript{a}UTC = Untreated control; G.S. = Grower Standard

\textsuperscript{b}2015 location was at Murray, KY.
Table 3.2. Sucker control effectiveness as percent of the control for manual and chemical topping application timings for medium and late maturing varieties.

<table>
<thead>
<tr>
<th>Maturity</th>
<th>Timing</th>
<th>2015&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Murray</td>
<td>Lexington</td>
<td>Princeton</td>
</tr>
<tr>
<td>Medium</td>
<td>UTC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 B</td>
<td>0 C</td>
<td>0 B</td>
</tr>
<tr>
<td></td>
<td>G.S.</td>
<td>100 A</td>
<td>100 A</td>
<td>97 A</td>
</tr>
<tr>
<td></td>
<td>10% Button</td>
<td>100 A</td>
<td>97 AB</td>
<td>100 A</td>
</tr>
<tr>
<td></td>
<td>50% Button</td>
<td>100 A</td>
<td>97 AB</td>
<td>91 A</td>
</tr>
<tr>
<td></td>
<td>10% Bloom</td>
<td>99 A</td>
<td>95 B</td>
<td>96 A</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;p-value&lt;/i&gt;</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Late</td>
<td>UTC</td>
<td>0 c</td>
<td>0 c</td>
<td>0 b</td>
</tr>
<tr>
<td></td>
<td>G.S.</td>
<td>100 a</td>
<td>100 a</td>
<td>97 a</td>
</tr>
<tr>
<td></td>
<td>10% Button</td>
<td>100 a</td>
<td>97 b</td>
<td>100 a</td>
</tr>
<tr>
<td></td>
<td>50% Button</td>
<td>100 a</td>
<td>100 a</td>
<td>89 a</td>
</tr>
<tr>
<td></td>
<td>10% Bloom</td>
<td>99 b</td>
<td>100 a</td>
<td>94 a</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;p-value&lt;/i&gt;</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

<sup>a</sup>Means within a column followed by the same uppercase or lowercase letter are not significantly different according to Fisher’s Protected LSD at P = 0.10.

<sup>b</sup>UTC = Untreated control; G.S. = Grower Standard.
Table 3.3. Plant height following manual topping and chemical topping application timings for medium and late maturing varieties.

<table>
<thead>
<tr>
<th>Maturity</th>
<th>Timing</th>
<th>2015&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Murray</td>
<td>Lexington</td>
<td>Princeton</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cm</td>
<td>cm</td>
<td>cm</td>
</tr>
<tr>
<td>Medium</td>
<td>UTC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>231 A</td>
<td>199 A</td>
<td>216 A</td>
</tr>
<tr>
<td></td>
<td>G.S.</td>
<td>150 D</td>
<td>160 C</td>
<td>166 C</td>
</tr>
<tr>
<td></td>
<td>10% Button</td>
<td>137 E</td>
<td>164 C</td>
<td>153 D</td>
</tr>
<tr>
<td></td>
<td>50% Button</td>
<td>162 C</td>
<td>164 C</td>
<td>166 C</td>
</tr>
<tr>
<td></td>
<td>10% Bloom</td>
<td>185 B</td>
<td>176 B</td>
<td>188 B</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Late</td>
<td>UTC</td>
<td>198 c</td>
<td>191 a</td>
<td>177 c</td>
</tr>
<tr>
<td></td>
<td>G.S.</td>
<td>179 d</td>
<td>161 c</td>
<td>187 b</td>
</tr>
<tr>
<td></td>
<td>10% Button</td>
<td>178 d</td>
<td>144 d</td>
<td>167 d</td>
</tr>
<tr>
<td></td>
<td>50% Button</td>
<td>207 b</td>
<td>176 b</td>
<td>201 a</td>
</tr>
<tr>
<td></td>
<td>10% Bloom</td>
<td>220 a</td>
<td>180 b</td>
<td>206 a</td>
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<td>p-value</td>
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<td>&lt;.0001</td>
<td>&lt;.0001</td>
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</tbody>
</table>

<sup>a</sup>Means within a column followed by the same uppercase or lowercase letter are not significantly different according to Fisher’s Protected LSD at P = 0.10.

<sup>b</sup>UTC = Untreated control; G.S. = Grower Standard.
Table 3.4. Leaf length for tip stalk position following manual topping and chemical topping application timings for medium and late maturing varieties.

<table>
<thead>
<tr>
<th>Maturity</th>
<th>Timing</th>
<th>2015&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Murray&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Lexington</td>
<td>Princeton</td>
<td>Lexington</td>
</tr>
<tr>
<td>Medium</td>
<td>UTC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>52 A</td>
<td>42 A</td>
</tr>
<tr>
<td></td>
<td>G.S.</td>
<td>-</td>
<td>54 A</td>
<td>41 A</td>
</tr>
<tr>
<td></td>
<td>10% Button</td>
<td>-</td>
<td>44 C</td>
<td>39 B</td>
</tr>
<tr>
<td></td>
<td>50% Button</td>
<td>-</td>
<td>47 B</td>
<td>40 B</td>
</tr>
<tr>
<td></td>
<td>10% Bloom</td>
<td>-</td>
<td>45 BC</td>
<td>39 B</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>-</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

| Late     | UTC      | -     | 56 a | 51 a | 48 b | 40 a | 40 b |
|          | G.S.     | -     | 51 b | 48 b | 50 a | 37 b | 45 a |
|          | 10% Button | -     | 45 c | 44 c | 44 c | 36 bc| 36 c |
|          | 50% Button | -     | 44 c | 47 b | 43 d | 35 c | 36 cd|
|          | 10% Bloom | -     | 43 c | 47 b | 41 d | 35 c | 35 d |
|          | p-value   | -     | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |

<sup>a</sup>Means within a column followed by the same uppercase or lowercase letter are not significantly different according to Fisher’s Protected LSD at P = 0.10.

<sup>b</sup>UTC = Untreated control; G.S. = Grower Standard.

<sup>c</sup>Leaf length data not collected at Murray in 2015.
Table 3.5. Leaf width for tip stalk position following manual topping and chemical topping application timings for medium and late maturing varieties.

<table>
<thead>
<tr>
<th>Maturity</th>
<th>Timing</th>
<th>2015&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Murray</td>
<td>Lexington</td>
<td>Princeton</td>
</tr>
<tr>
<td>Medium</td>
<td>UTC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>22.7 A</td>
<td>22.4 A</td>
</tr>
<tr>
<td></td>
<td>G.S.</td>
<td>-</td>
<td>23.5 A</td>
<td>21.3 B</td>
</tr>
<tr>
<td></td>
<td>10% Button</td>
<td>-</td>
<td>17.4 C</td>
<td>18.2 D</td>
</tr>
<tr>
<td></td>
<td>50% Button</td>
<td>-</td>
<td>19.1 B</td>
<td>19.4 C</td>
</tr>
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<td></td>
<td>10% Bloom</td>
<td>-</td>
<td>20.0 B</td>
<td>19.8 C</td>
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<td>p-value</td>
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<td>&lt;.0001</td>
</tr>
<tr>
<td>Late</td>
<td>UTC</td>
<td>-</td>
<td>24.2 a</td>
<td>28.2 a</td>
</tr>
<tr>
<td></td>
<td>G.S.</td>
<td>-</td>
<td>22.9 a</td>
<td>26.7 b</td>
</tr>
<tr>
<td></td>
<td>10% Button</td>
<td>-</td>
<td>18.1 b</td>
<td>22.8 d</td>
</tr>
<tr>
<td></td>
<td>50% Button</td>
<td>-</td>
<td>18.9 b</td>
<td>26.2 b</td>
</tr>
<tr>
<td></td>
<td>10% Bloom</td>
<td>-</td>
<td>18.7 b</td>
<td>25.1 c</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>-</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

<sup>a</sup>Means within a column followed by the same uppercase or lowercase letter are not significantly different according to Fisher’s Protected LSD at P = 0.10.

<sup>b</sup>UTC = Untreated control; G.S. = Grower Standard.

<sup>c</sup>Leaf width data not collected at Murray in 2015.
Table 3.6. Total yield following manual topping and chemical topping application timings for medium and late maturing varieties.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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<sup>a</sup>Means within a column followed by the same uppercase or lowercase letter are not significantly different according to Fisher’s Protected LSD at P = 0.10.

<sup>b</sup>UTC = Untreated control; G.S. = Grower Standard.
Table 3.7. Quality grade index following manual topping and chemical topping application timings for medium and late maturing varieties.

<table>
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<th>2017</th>
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<td>65</td>
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<td>-</td>
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<td>-</td>
<td>0.1124</td>
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\(^a\)Means within a column followed by the same uppercase or lowercase letter are not significantly different according to Fisher’s Protected LSD at \(P = 0.10\).

\(^b\)UTC = Untreated control; G.S. = Grower Standard.
Chapter 4: Global Transcriptomic Changes in Chemically Topped Burley Tobacco

Abstract

Information on the influence of suckercides on tobacco gene expression and their molecular mechanism of action is limited. Therefore, the primary objectives of this experiment were to study global changes in gene expression in apical (ApB) and axillary buds (AxB) of “chemically topped” (untopped plants treated with MH) burley tobacco using RNA-sequencing (RNA-seq) and to propose a possible molecular mechanism of action of MH on sucker control. Sequencing of RNA libraries from ApB and AxB of control and MH-treated tobacco generated a total of 450 million (M) clean reads and more than 75% of the total reads were mapped to reference tobacco genome. Analysis of the RNA-seq libraries revealed that compared with the control, chemical topping (CT) significantly altered gene expression in ApB and AxB; 573 (132 upregulated, 441 downregulated) and 2,632 (2,174 upregulated, 458 down-regulated) genes were found to be differentially expressed in chemically topped ApB and AxB, respectively. Gene ontology (GO) enrichment analysis was performed on differentially expressed genes (DEGs) both in ApB and AxB. In MH-treated ApB, upregulated genes were enriched for phosphorelay signal transduction, leaf proximal/distal pattern formation and regulation of timing of transition from vegetative to reproductive phase whereas GO terms related to meristem maintenance, cytokinin metabolism, cell wall synthesis, photosynthesis and DNA metabolism were enriched in downregulated genes. In MH-treated AxB, GO terms related to defense response and oxylipin metabolism were enriched in upregulated genes whereas GO terms related to cell cycle and DNA metabolism, cytokinin metabolism were enriched in downregulated genes. Genes encoding proteins
essential for cell division control and DNA replication were downregulated. Expression of a number of transcription factor genes known to play crucial roles in apical and axillary meristem development were downregulated in ApB and AxB after MH treatment. MH negatively affects the expression of a number of MADS-box family TFs in ApB known to determine floral organ identity in plants. In addition, MH-treatment induces defense and secondary metabolism genes in axillary buds. TFs belonging WRKY, AP2/ERF and NAC families were mostly affected in MH-treated AxB. Furthermore, genes related to biosynthesis and signaling of a number of phytohormones including CK, JA, ethylene (ET), abscisic acid (ABA), and gibberellic acid (GA), were affected by MH-treatment in ApB and AxB of tobacco. In summary, MH profoundly influenced gene expression in ApB and AxB of tobacco. The number of DEGs were higher in AxB compared to ApB. In both ApB and AxB the expression of genes related to phytohormones, meristem development, cell division, DNA repair and recombination were affected following MH treatment, which likely leads to the inhibition of apical and axillary shoot growth. Collectively, RNA-seq analysis provides insights into the possible molecular mechanism of action of MH on apical and axillary buds of tobacco.

**Introduction**

Removal of the terminal bud or inflorescence of the tobacco (*Nicotiana tabacum* L.) plants, commonly known as topping, is usually accomplished by manually removing the top of each tobacco plant in an entire field, which is labor intensive and costly. There are other crops that also benefit from topping. It has been shown in cotton (*Gossypium spp.*) that a higher number of plants retain cotton bolls and show increased boll growth after topping (Yang et al., 2012). Topping of okra (*Abelmoschus esculentus*) has been
shown to result in an increase in seed yield per plant (Marie et al., 2007). Removal of the terminal bud or inflorescence in tobacco prevents reproductive development (i.e. seed head) and results in energy transferred to increased leaf size, weight, nicotine, and other chemical constituents (Tso, 1990). Topping also eliminates apical dominance in the plant resulting in axillary bud growth, known as suckers (Decker and Seltmann, 1971). Topping, which wounds the plant, triggers wound-responsive gene expression and metabolism to activate defense mechanisms (León et al. 2001). The phytohormone, jasmonic acid (JA) and its methyl esters, methyl jasmonate (MeJA), are well known elicitors of the wound-signaling pathway in plants (León et al., 2001). Wang et al. (2018) performed comparative transcriptomic analyses to find differentially expressed genes (DEGs) in untopped and topped tobacco plants. They found that many of the DEGs are involved in starch and sucrose metabolism, glycolysis/ gluconeogenesis, pyruvate metabolism, and plant hormone signal transduction, along with other processes. The starch and sucrose metabolism processes are believed to contribute significantly to the enlargement of axillary bud growth (Wang et al. 2018). Another study found that DEGs in flue-cured tobacco roots after topping are mostly related to secondary metabolism, hormone metabolism, signaling/transcription, stress/defense, protein metabolism and carbon metabolism (Qi et al., 2012).

Phytohormones, primarily auxin (IAA) and cytokinin (CK), are known to be involved with the initiation of axillary bud growth in plants (Müller and Leyser, 2011). A number of transcription factors (TFs) belonging to R2R3 MYB, basic helix-loop-helix (bHLH), GRAS (GAI, gibberellic acid insensitive-RGA, repressor of GAI-Scarecrow), NAC (NAM, no apical meristem-ATAF, Arabidopsis transcription activator factor-CUC,
cup-shaped cotyledon), homeodomain/leucine zipper (HD/ZIP), and TCP (Teosinte branched1-Cycloidea-Proliferating cell nuclear antigen factor) families have been identified and characterized for their roles in meristem development in Arabidopsis, tomato, pepper, and rice. After investigating the phenotypic and genetic interactions of mutations in the REVOLUTA (REV) gene, it was found that REV is required for lateral meristem and floral meristem initiation and encodes a HD/ZIP TF in Arabidopsis (Otsuga et al., 2001). Schmitz et al. (2001) identified two genes, BLIND and TOROSA belonging to R2R3 MYBs that control lateral meristem (axillary bud) initiation in tomato. Müller et al. (2006) found three R2R3 MYB genes in Arabidopsis, which were homologous to the tomato Blind gene and were designated as REGULATORS OF AXILLARY MERISTEMS (RAX). RAX control axillary bud formation at a very early step of initiation in Arabidopsis. A BLIND ortholog was also found to reduce axillary meristem initiation in pepper plants (Jeifetz et al., 2011). The GRAS family TF, lateral suppressor (LAS) has also been shown to play role meristem development in Arabidopsis and tomato (Greb et al. 2003; Schumacher et al. 1999). Double mutant analyses in tomato and Arabidopsis revealed that LAS and MYB TFs control axillary meristem formation through separate pathways (Schmitz et al., 2002; Müller et al., 2006). In tomato, blind mutants did not initiate lateral meristems during shoot and inflorescence development (Schmitz et al. 2002). The lateral suppressor (ls) mutant blocked almost all lateral meristem development during vegetative development (Schumacher et al. 1999); however, during reproductive development the LS gene is not required for axillary meristem formation (Greb et al., 2003). The TCP family TF, BRANCHED1, is known to be involved in axillary meristem development in plants. Axillary buds in Arabidopsis express only a single BRC1 gene compared to two BRC1-like
genes in other Solanaceae species such as tomato (Martin-Trillo et al., 2011). Martin-Trillo et al. (2011) suggested that interplay between these two dimerizing transcription factors might result in a more complex regulation of axillary bud growth patterns in plants like tomato, as two divergent BRC1-like genes are co-expressed. Li et al. (2003) characterized MONOCULM 1 (MOC1), which is an important gene for rice tillering and encodes a protein highly homologous to the tomato LAS. MOC1 is a key regulator of tillering and regulates expression of several important genes involved with axillary bud development, namely, OSH1. OSH1 is a rice orthologue of the maize TB1 that is expressed in axillary buds and regulates axillary bud outgrowth (Li et al., 2003).

Sucker growth control and yield are positively correlated (Collins and Hawks, 1993). Studies dealing with tobacco production have indicated that it takes 150-200 hours of labor to grow one acre of burley tobacco even with advances that have come with increased labor efficiency (Snell and Powers, 2013; Duncan and Wilhoit, 2014). Therefore, non-traditional methods of topping are of interest to eliminate the need for manual topping to reduce the labor requirement. There are three major types of chemicals (suckercides) that are typically used for chemical inhibition of axillary bud growth. These three types consist of contact, local systemic, and systemic suckercides (Bailey et al., 2017). Contact suckercides are not absorbed, nor translocated by the plant and effective control of suckers requires placement of the chemicals directly on the leaf axil (Bailey et al., 2017). Local systemic suckercides are absorbed in the leaf axil area to inhibit cell division (Bailey et al., 2017). Singh et al. (2015) identified 179 common DEGs between tobacco plants that were topped, and treated after topping with a local systemic or contact suckercide. DEGs related to wounding, phytohormone metabolism, and secondary
metabolite biosynthesis were upregulated after topping and downregulated after suckercide treatment. This study also found that the application of a local systemic suckercide affected the expression of auxin and cytokinin signaling pathways, which are likely involved with axillary bud formation.

Systemic suckercides, unlike contact and local systemic suckercides, do not need to be in direct contact with the suckers as they are absorbed by the leaves and translocated to the leaf axils, where they inhibit cell division (Bailey et al., 2017). Maleic hydrazide (MH, 1,2-dihydro – 3,6-pyridazinedione) is the only true systemic suckercide that is used in tobacco production (Bailey et al. 2017). MH is readily translocated throughout the plant vasculature, in both phloem and xylem tissues (Hoffman and Parips, 1964; Steffens, 1983; Zukel, 1963) and inhibits cell division without affecting cell elongation, thus preventing the growth of newly developing suckers without hindering the growth of more mature leaves (Collins and Hawks, 1993). The molecular mechanism through which MH affects bud growth is still not completely understood even though the mode of action for MH has been studied since 1949 (Bush and Sims, 1974). It has been shown that MH acts as an antimitotic agent in axillary bud tissue (Clapp and Seltmann, 1983). In the early 1970’s, there were two different views on how MH works; those who believed that MH interacts with nucleic acid precursors and thus ultimately with nucleic acid synthesis, and those who did not agree. Coupland and Peel (1971) showed that for an increase in the concentration of MH, there is a corresponding increase in the inhibition of uracil uptake. Their data supports the hypothesis that MH can inhibit uptake of uracil into cells by a competitive process eluding to the claim that MH has a two-fold effect on plant tissues: 1) inhibits uracil uptake into the cell and 2) once inside the cell, MH can become incorporated into
RNA. This could be due to the close structural resemblance of MH with uracil (Coupland and Peel, 1971; Cradwick, 1975). Collins and Hawks (1993) reported that MH is absorbed by the tobacco plant and symplastically translocated to active growing points where the mechanism of action is a uracil antimetabolite. A study conducted by Appleton et al. (1981) showed that MH was incorporated into RNA in yeast cells where it was substituted for cytosine rather than for uracil. However, some evidence indicates that MH inhibits cell division and subsequent sucker growth by inhibiting DNA and RNA synthesis (Nooden, 1969; Nooden, 1972; Zukel, 1963) but does not affect actively growing cells, as they enlarge and differentiate (Steffens, 1983). Other theories have suggested that MH reacts with sulfhydryl groups (Muir and Hansch, 1953) or a carbonyl reagent (Suzuki, 1966); however, Nooden (1973) showed no reactions between MH and sulfhydryl or carbonyl compounds and discounted these theories. To summarize, there is no widely accepted mechanism of action for MH in the literature since the mid-20th century but it is apparent that cell division is inhibited after MH application. Technology, such as RNA-sequencing, can be used to address this knowledge gap.

There are no suckercides that are registered or intended specifically for chemical topping of tobacco but some experiments have evaluated products for this purpose (Long et al., 1989; Steffens and McKee, 1969; Steffens et al. 1967; Peek, 1995). Field experiments conducted at the University of Kentucky found that chemically topping burley tobacco can be achieved with use of MH. Manually topping followed by an application of suckercides are common agronomic practices in the production of burley tobacco. A few studies have investigated DEGs prior to and after topping, as well as after suckercide treatment in tobacco (Wang et al., 2018, Singh et al. 2015). However, the impact of
chemical topping on gene expression has not been investigated. The primary objective of this experiment was to study global changes in gene expression through use of RNA-sequencing in MH-treated chemically topped burley tobacco and to expand the knowledge on how systemic suckercides can inhibit apical and axillary shoot formation.

**Materials and Methods**

**Tissues and RNA Sequencing**

Plants of a late-maturing burley tobacco variety were produced in 2015 at the Agricultural Experiment Station Spindletop Farm near Lexington, KY. Tobacco plants were transplanted to the field in late May/early June and all field production practices, other than topping, were standard based on the University Extension guidelines (Pearce et al., 2017). The experimental design was a randomized complete block with treatments replicated four times. Maleic hydrazide (Royal MH-30, 180 g liter\(^{-1}\), Arysta LifeSciences), a systemic suckercide, was applied with a CO\(_2\)-pressurized sprayer calibrated to 468 L ha\(^{-1}\) with a directed three-nozzle row\(^{-1}\) configuration (TG3 - TG5 - TG3). Chemically topped and manually topped treatments were applied at the pre-bud (10% button) stage. Button percentage was calculated by dividing the total number of plants in the two center rows of each plot by the number of plants with a visible terminal bud between the apical leaves, or growth stage 51 (Coresta Guide #7, 2009). The axillary (AxB) and apical (ApB) meristems were collected 24 h after treatment from the control (not-topped, not-sprayed), chemically-topped (not-topped, sprayed with MH), topped without sprayed, and the grower standard (topped and sprayed with MH) and were frozen immediately in liquid nitrogen and stored at −80 °C until RNA extraction.
Total RNA was isolated from 100 mg of AxB and ApB tissues using the RNeasy Plant Mini Kit (Qiagen, Chatsworth, USA) following manufacturer’s instructions. A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to determine RNA quantity. RNA quality was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and only samples with RNA integrity number (RIN) above eight were used for library preparation. Two micrograms from each RNA sample was sent to the Sequencing and Genomic Technologies Shared Resource facility at Duke University for RNA-Seq library preparation and sequencing.

**Data processing and gene expression quantification**

Raw Illumina sequence reads were processed as described previously (Singh et al., 2015). In summary, raw Illumina sequence reads were filtered for low-quality reads using the prinseq-lite-0.20.426 (Schmieder and Edwards, 2011). The preprocessed reads were assessed for quality control with systemPipeR (Backman and Girke, 2016). Read mapping was performed by Bowtie2 (Langmead and Salzberg, 2012) using the reference sequence downloaded from Solgenomics Network database (Bombarely et al., 2011; Sierro et al., 2014). Finally, differential gene expression analysis was carried out using the DESeq2 Bioconductor package in R (Love et al, 2014). Differentially expressed genes (DEGs) were identified using the following two criteria: (i) log2 fold-change $\geq 1$ and (ii) false discovery rate (FDR) p-value correction of $\leq 0.05$. The heatmap was constructed using the ComplexHeatmap (Gu et al, 2013) function in R through the Bioconductor package (Team, 2013).
Functional annotation and gene ontology (GO) analysis

Functional annotation of DEGs was performed with eggNOG 4.5 (Huerta-Cepas et al., 2016) and InterPro (Mitchell et al., 2015) databases. GO analysis of enriched functional categories was performed using BiNGO (version 2.44) (Maere et al., 2005) and visualized in Cytoscape (Shannon et al., 2003). The hypergeometric test with Benjamini & Hochberg’s false discovery rate (FDR) correction was used to calculate overrepresented GO categories among differentially expressed genes, using a P-value<0.05. Results from the gene list analyzed using BiNGO were summarized with REViGO by removing redundant GO terms (Supek et al., 2011). For pathway analysis, a MapMan mapping file was specifically generated for the tobacco genes by the Mercator tool, which bins all genes according to hierarchical ontologies after searching a variety of databases and, finally, MapMan v.3.5.1 was used to visualize DEGs on different pathways (Thimm et al., 2004).

Quantitative RT-PCR

Gene specific primers for the six candidate genes were designed using Primer3 software3 (Untergasser et al., 2012). RNA isolated from control, topped, MH-treated samples were reverse-transcribed using the Superscript III Reverse Transcriptase (Invitrogen, USA), following the manufacturer’s instructions. Quantitative PCR was performed as described by (Pattanaik et al., 2010). All PCR reactions were performed in triplicate and repeated two times. The comparative cycle threshold (Ct) method (bulletin no. 2; Applied Biosystems, http://www.appliedbiosystems.com) was used to measure transcript levels. In addition to tobacco α-tubulin (GenBank accession number AJ421411), tobacco elongation factor-1α (GenBank accession number D63396) was also used as a reference gene.
Results and Discussion

RNA-seq analysis of MH-treated apical and axillary buds

RNA-seq analysis was performed to study the influence of MH on gene expression in apical (ApB) and axillary buds (AxB) of chemically topped tobacco plants and to propose a molecular mechanism of action of MH on sucker control. A total of 12 samples comprised of two different tissues (ApB and AxB) and two different treatments (control and MH) were used for library preparation and sequencing using the Illumina HiSeq2500 system (Table 4.1). Sequencing of RNA libraries from ApB and AxB of tobacco generated a total of 450 million (M) clean reads (Table 4.1). Each biological sample (control and MH-treated) was represented by an average of more than 100 M reads (Table 4.1). On average, more than 75% of the total reads from control and MH-treated libraries were successfully mapped to the reference tobacco genome sequence (Table 4.1).

For our analysis, we considered a transcript as ‘detected’ if the Fragments Per Kilobase of gene per Million reads mapped (FPKM) value was ≥1. Total number of transcripts varied from approximately 44,000 to 47,000 in all analyzed samples which were further divided into three categories, low (1-5 FPKM), moderate (5-20 FPKM), or high (>20 FPKM), based on transcript abundance. Both control and MH-treated samples had similar distribution of low, moderate, and highly expressed mRNAs (Figure 4.2A). We selected 10,000 mRNAs which are most abundant and show distinct accumulation pattern between the two tissue and treatments for further analysis (Figure 4.2B).
MH-treatment significantly alters gene expression both in apical and axillary buds

Compared with the control, chemical topping (CT; untopped plants treated with MH) significantly altered gene expression in ApB and AxB; 573 (132 upregulated, 441 downregulated) and 2,632 (2,174 upregulated, 458 down-regulated) genes were found to be differentially expressed in chemically topped ApB and AxB, respectively (Figure 4.3A). A total of 87 genes were commonly affected by MH-treatment in both ApB and AxB (Figure 4.3B). Among the 87 common genes, 8 were upregulated whereas 18 were downregulated. The other 61 genes showed contra-regulation (opposite regulation) in ApB and AxB. These commonly affected genes were enriched for meristem development and cytokinin (CK) metabolism. The member of KNOX family and BTB-POZ domain transcription factors were downregulated in both ApB and AxB by MH-treatment. Among the contra-regulated genes, genes related to secondary metabolism and defense, such as terpene synthase, pathogenesis-related (PR) genes, chitinase and hormone biosynthesis (jasmonate and ethylene biosynthetic genes), were upregulated in MH treated AxB but downregulated in ApB. Genes related to translation (such as member of ribosomal protein L22p/L17e family) were upregulated in MH treated ApB only.

Gene ontology (GO) enrichment analysis highlights influence of MH on different developmental and metabolic pathways in ApB and AxB

To gain further insight into the implications of MH-treatment, we performed GO enrichment analysis on genes that were upregulated and downregulated both in ApB and AxB. In MH-treated ApB, upregulated genes were enriched for phosphorelay signal transduction system (GO:0000160), leaf proximal/distal pattern formation (GO:0010589), regulation of timing of transition from vegetative to reproductive phase
(GO:0048510) and vernalization (GO:0010048) while GO terms related to meristem maintenance, cytokinin metabolism, cell wall synthesis, photosynthesis and DNA metabolism were enriched in downregulated genes (Figure 4.4A). In MH-treated AxB, GO terms related to defense response and oxylipin metabolism were enriched in upregulated genes whereas GO terms related to cell cycle and DNA metabolism, cytokinin metabolism were enriched in downregulated genes (Figure 4.4B). Genes from several protein families such as mini-chromosome maintenance (MCM2/3/5) family protein, origin recognition complex (ORC) proteins family and cell division control protein which are essential for initiation of DNA replication were downregulated (Shultz et al., 2007). Genes with known function in DNA repair and recombination such as Replication Protein A 1B, RAD21.2, ARABIDOPSIS HOMOLOG OF YEAST CDT1 A, BREAST CANCER ASSOCIATED RING 1 (BARD1) and RECQ helicase 11 were also suppressed by MH treatment (Singh et al., 2010). The cytokinin metabolism is affected in both ApB and AxB. Collectively, these findings suggest that MH suppresses the DNA repair and recombination machinery which, leads to inaccurate DNA replication and cell cycle arrest. Moreover, MH inhibits apical and axillary shoot development possibly by affecting cytokinin metabolic processes in tobacco.

MH perturbs expression of transcription factor genes involved in meristem maintenance and development

Transcription factors (TFs) belonging to the R2R3MYBs, bHLH, GRAS, HD/ZIP, KNOX and BTB/POZ families are known to play crucial role in meristem maintenance and development in plants. The R2R3 MYB, BLIND/RAX, bHLH TF ROX, GRAS family TF LAS and HD/ZIP TF REV are positive regulators of axillary meristem development in
Arabidopsis, tomato and pepper. Our transcriptome analysis revealed that expression of these TFs were downregulated in ApB and AxB after MH treatment (**Figure 4.5**). The KNOX genes comprise a small family of TALE homeobox TFs that are found in all plant species and can be divided into two major subclasses (Gao et al., 2015). Class I KNOX genes are most similar to maize knotted1 (kn1) gene and are predominantly expressed in the shoot apical meristem (SAM) (Hake et al., 2004; Gao et al., 2015), whereas Class II KNOX genes show diverse expression patterns (Gao et al., 2015). KNOX ARABIDOPSIS THALIANA MEINOX (KNATM) genes are relatively new members of the KNOX family that encodes a MEINOX domain but not a homeodomain. In Arabidopsis, KNATM is expressed in proximal-lateral domains of organ primordia and at the boundary of mature organs and is involved in leaf proximal-distal patterning (Magnani and Hake, 2008). We identified 19 members of the KNOX family in tobacco and phylogenetic analysis revealed three major clades, the KNOX I, KNOX II and KNATM, as described previously (Gao et al., 2015) (**Figure 4.6A**). MH-treatment repressed the expression of most of the members of class I KNOX genes in ApB and AxB (**Figure 4.6B**), whereas members of KNOX II subfamily were not significantly affected. In Arabidopsis, the Class I KNOX gene SHOOT MERISTEMLESS (STM) has been shown to play key role in shoot and floral meristem maintenance (Endrizzi et al., 1996). In addition, STM is shown to activate CK biosynthesis genes and, consequently, CK accumulation (Yanai et al., 2005). Therefore, it is reasonable to hypothesize that MH affects CK accumulation in ApB and AxB by repressing the class I KNOX genes. Unlike typical KNOX family members, KNATM encodes a MEINOX domain without homeodomain and interacts with TALE-class homeodomain proteins to modulate their activities (Magnani and Hake, 2008). We identified two KNATM family
members in tobacco and both copies of KNATM were upregulated in ApB but not in AxB (Figure 4.6B).

In MH-treated ApB, expression of a number of MADS-box family TFs were downregulated (Figure 4.7A). The members of the MADS-box family are well known for their function in flower and fruit development (Ng and Yanofsky, 2001; Theißen et al., 2016). MADS-box family genes regulate both flowering time and vegetative to reproductive phase transition (Borner et al., 2000; Putterill et al., 2004). The vegetative to reproductive phase transition in plants is accurately controlled by environmental conditions and endogenous developmental cues. In Arabidopsis, flowering has been proposed to be regulated by four genetic pathways, photoperiod, autonomous, vernalization, and gibberellin induced pathways (Boss et al., 2004; Bäurle and Dean, 2006). The current ABCDE model for flower development proposes that floral organ identity is specified by five classes of homeotic genes, A (APETALA1, AP1), B (PISTILATA, PI), C (AGAMOUS, AG), D (SEEDSTICK/AGAMOUS-LIKE11,STK/AGL11) and E (SEPALLATA1s, SEPs) (Rijpkema et al., 2010). Different combinations of these homeotic genes determine the identities of the floral organs: sepals (A + E), petals (A + B + E), stamens (B + C + E), carpels (C + E), and ovules (D + E). In Arabidopsis, most of the members of class A, B, C, D, E belong to the MADS-box TF family. Since a large number of MADS-box genes were differentially expressed in MH-treated ApB, we looked into the expression of the homologs of well characterized MADS-box family members in our transcriptome. SEPALLATA1 (SEP1), SEP2 and SEP3, and SEP4 are required to specify petals, stamens, and carpels (Ditta et al., 2004). We found that expression of several homologs of SEPs were repressed by MH application. In addition, expression of genes
required for floral organ identity such as *PISTILLATA*, *AGAMOUS*, *API*, and *AP3* were also repressed by MH treatment. In *Arabidopsis*, AGAMOUS-like 22 (AGL22) regulates flowering time by negatively regulating the expression of the floral integrator, *FT*, via direct binding to the CArG motifs in the FT promoter region (Lee et al., 2007). AGL22 was induced in ApB by MH-treatment (Hartmann et al., 2000). A MADS-box TF, AGL6, which is reported to be a positive regulator of axillary meristem formation and flowering is also repressed by application of MH (Koo et al., 2010; Huang et al., 2012). In *Arabidopsis*, the BTB/POZ domain TFs BLADE ON PETIOLE 1 (BOP1) and BOP2, act redundantly to control leaf and floral patterning by modulating the meristematic activity. BOP2 is highly expressed in young floral meristem (Xu et al., 2010). Expression of BOP2 was repressed by MH-treatment. Barley homolog (*Cul4*) of *Arabidopsis* BOP2 has also been shown to express in axil and leaf boundary regions to positively control axillary bud (Tavakol et al., 2015). Downregulation of multiple MADS-box and BTB/POZ family genes after MH application was consistent with previous results that MH treatment delays or inhibits the flower initiation in several plants including tobacco (Naylor, 1950; Klein and Leopold, 1953).

**MH-treatment induces defense and secondary metabolism genes in axillary buds**

We found that TFs belonging to WRKY, AP2/ERF and NAC families were mostly affected in MH-treated AxB (*Figure 4.7B*). WRKYs are well studied plant-specific TFs which are involved in diverse biotic and abiotic stress responses as well as in developmental/physiological processes (Phukan et al., 2016). Expression of several WRKYs TF genes including *WRKY2*, *WRKY6*, *WRKY7*, *WRKY11*, *WRKY23*, *WRKY28*, *WRKY33*, *WRKY38*, *WRKY40*, *WRKY41*, *WRKY45*, *WRKY50*, *WRKY51*, *WRKY53*,
WRKY70 were found to be upregulated in AxB in response to MH treatment. Previous studies suggest that in Arabidopsis, WRKY50 and WRKY51 act as positive regulators of SA-mediated signaling and negative regulators of JA signaling (Gao et al., 2011) whereas WRKY28 and WRKY70 are involved in both SA and JA-signaling pathways in plants (Li et al., 2004; Chen et al., 2013). WRKY33 is a key regulator of camalexine biosynthesis and is required for resistance to necrotrophic fungal pathogens in Arabidopsis (Zheng et al., 2006; Liu et al., 2016). Notably, WRKY13, which is known to activate lignin biosynthesis-related genes (Li et al., 2015) and repress flowering (Li et al., 2016) in Arabidopsis, was upregulated by MH treatment in AxB.

**MH-treatment affects expression of phytohormone biosynthesis and signaling genes in apical and axillary buds**

Phytohormones play a crucial role in ApB and AxB development (Yang and Jiao, 2016). We identified phytohormone metabolism and signaling related genes in tobacco as described previously (Prasad et al., 2016). Genes related to biosynthesis and signaling of a number of phytohormones including CK, SA, JA, ethylene (ET), abscisic acid (ABA), and gibberellic acid (GA), were affected by MH-treatment in ApB and AxB of tobacco (Tables 4.2-4.3). RNA-seq analysis revealed that compared with the control, 20 and 57 genes related to phytohormone metabolism and signaling were significantly differentially expressed in CT-ApB and CT-AxB, respectively. In addition, seven differentially expressed genes were common to CT-ApB and CT-AxB. These seven genes belong to ET, JA and CK metabolism, which are downregulated in AxB but upregulated in ApB. Among seven common genes, 4 are homologs of Arabidopsis ACO4, key gene in ET biosynthetic pathway. Two (i.e. AOS) are related to JA biosynthesis. One gene belongs to UDP-
glycosyltransferase superfamily and was also common between MH-treated ApB and AxB. UDP-glycosyltransferase superfamily mediate the transfer of glycosyl residues from activated nucleotide sugars to acceptor molecules (such as hormone) and thus regulate the homeostasis (Ross et al., 2001). CT-AxB specific genes are related to SA, JA, GA and ET metabolism (Table 4.3). Interestingly, genes related to ET biosynthesis including 1-amino-cyclopropane-1-carboxylate (ACC) synthase and ACC oxidase were upregulated by MH-treatment in AxB. ET induces the expression of ET signaling pathway genes such as members of ET insensitive (EIN) family. In CT-AxB dataset, EIN homologs of Arabidopsis, EIN3, was upregulated. ET is known to inhibit cell division, DNA synthesis, and growth of AxB.

GAs play fundamental roles in plant growth and development. Three classes of enzyme, i.e. terpene synthases (TPSs), CYP450s and GA oxidases (GAoxs), are required for the biosynthesis of bioactive GAs from geranylgeranyl diphosphate (GGDP), and the pathway can be divided into two main steps. The early steps are catalyzed by a series of genes encoding enzymes such as ENT-COPALYL DIPHOSPHATE SYNTHASE (CPS), ENT-KAURENE SYNTHASE (KS), ENT-KAURENE OXIDASE (KO), and ENTKAURENOIC (KAO). The enzymes catalyzing later steps, such as GA2 oxidase (GA2ox), GA20 oxidase (GA20ox), and GA3 oxidase (GA3ox), belong to the 2OG-Fe (II) oxygenase superfamily and are encoded by different gene families (Hedden and Phillips, 2000). The genes involved in the later steps of GA biosynthesis are differentially regulated by developmental and environmental cues and play crucial but antagonistic roles in the accumulation of bioactive GA levels. For instance, upregulation of GA20ox and GA3ox increase the GA level whereas higher expression of GA2ox decreases the GA level.
GA is involved in AxB development in different plants including tomato, rice and aspen (Lo et al., 2008; Martínez-Bello et al., 2015; Rinne et al., 2016). In rice, GA negatively regulates expression of two TFs, homeobox 1 and TEOSINTE BRANCHED1 (TB1), which control meristem initiation and AxB outgrowth, respectively, and inhibits tillering (Lo et al., 2008). Two homologs of Arabidopsis GA2ox were found to be upregulated in response to MH-treatment in AxB in our dataset (Table 4.3), which likely lowered the concentration of GA and inhibited AxB development.

MapMan visualization highlights the influence of MH-treatment on different plant metabolic pathways

Pathway-based analysis was performed to associate biological functions with the genes differentially expressed in response to MH treatment. We used a comprehensive tool, the MapMan, to visualize the pathways affected by MH-treatment in ApB and AxB tissues in tobacco. We overlaid the log2 fold change of DEGs to identify and visualize affected pathways. The number of genes in AxB affected by MH treatment were significantly higher compared to ApB, indicating a broader impact of MH on AxB. Genes related to defense such as secondary metabolites, proteolysis, pathogenesis related genes, and heat shock protein were downregulated in MH-treated ApB (Figure 4.8). However, unlike ApB, genes related to defense pathway and hormone biosynthesis were upregulated in AxB by MH treatment (Figure 4.9). In AxB, several genes in the JA biosynthesis pathway such as lipoxygenase and allene oxidase were upregulated in response to MH treatment. Genes related to auxin homeostasis (IAA-amino acid hydrolase and GH3 family), ethylene biosynthesis and signaling (ethylene responsive factor1 (ERF1), ERF2, ERF5, ERF4, 1-aminocyclopropane-1-carboxylate oxidases and
1-aminocyclopropane-1-carboxylate synthase) were also induced by MH treatment in AxB. Plant glutathione S-transferases (GSTs) comprise a large, complex gene family in plants. For instance, there are 25 GST genes in Glycine max, 42 in Zea mays, and 47 in Arabidopsis thaliana. Plant GST gene family are divided by sequence similarity into three categories (I, II, and III) or, alternatively, into six classes (tau, phi, zeta, theta, lambda, and DHAR), with the tau and phi classes being prevalent. GSTs catalyze the conjugation of toxic xenobiotics and oxidatively produced compounds to reduced glutathione, which further facilitates their metabolism, sequestration, or removal (Dalton et al., 2009). Expression of GSTs are also known to be induced by auxin and ethylene in plants including tobacco (Van der Zaal et al., 1991; Itzhaki and Woodson, 1993; Droog et al., 1995; van der Kop et al., 1996). Unlike ApB, several homologs of auxin-responsive GSTs were found to be induced by MH treatment in AxB (Figure 4.9).

Pathogenesis-related (PR) proteins play numerous roles in plant development and defense. The PR proteins are highly conserved proteins and have been classified into 17 classes based on their amino acid sequence, serological relationship, and biological activities (Van Loon and Van Strien, 1999). PR proteins are involved in plant immune responses (Stintzi et al., 1993) and enhance plants tolerance to both biotic and abiotic stresses (Wu et al., 2016). For instance, overexpression of PR proteins, such as PR-1, PR-5, or PR-10, in plants enhances tolerance to a number of pathogens such as Rhizoctonia solani, Phytophthora nicotianae, Ralstonia solanacearum, and Pseudomonas syringae (Datta et al., 1999; Sarowar et al., 2005). Those PR proteins have also been reported to have multiple roles in adaption to abiotic stresses such as salt and heavy metal tolerance (Sarowar et al., 2005; de las Mercedes Dana et al., 2006). MH treatment was found to activate the expression of
several PR genes and secondary metabolite related genes in AxB (Figure 4.9). Taken together, these results indicate that ApB and AxB respond differently to MH treatment (Figures 4.8-4.9).

**Quantitative RT-PCR analysis of selected DEGs validates the RNA-seq data**

To validate the RNA-seq results, expression of six differentially expressed genes were analyzed by qRT-PCR (Figure 4.10). A list of primers used to conduct RT-qPCR analysis is shown in Table 4.4. These genes encode the KNOX (KNOX1 and KNOX12) genes, AGL (AGL6 and AGL20) genes, 1-aminocyclopropane-1-carboxylate oxidase 4 (ACO4) and SALICYCLIC ACID CARBOXYL METHYLTRANSFERASE (SAMT). The qRT-PCR results complemented the RNA-seq data, confirming the reliability and accuracy of our RNA-seq in this study.

In summary, MH has profound influence on gene expression in ApB and AxB of tobacco. The number of differentially expressed genes were higher in AxB compared to ApB. In both ApB and AxB, the expression of genes related to a number of phytohormones, meristem development, cell division, DNA repair and recombination were affected following MH treatment, which likely leads to the inhibition of apical and axillary shoot growth. In addition, MH elicits defense responses in plants by inducing the expression genes involved in oxylipin biosynthesis, secondary metabolism and defense-related genes. In addition, MH-treatment induces the expression of a number of GSTs, which are possibly involved in detoxification processes. Collectively, our RNA-seq analysis reveals a possible molecular mechanism of action of MH on apical and axillary buds of tobacco (Figure 4.11).
Table 4.1. Summary of sequencing and read mapping in different RNA-seq libraries.

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<th>Treatment</th>
<th>Symbol</th>
<th>Experiment</th>
<th>Total raw reads</th>
<th>Total clean reads</th>
<th>Total raw reads per biological sample</th>
<th>Total clean reads mapped to reference transcriptome</th>
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<td>109</td>
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Table 4.2. Expression of different phytohormone biosynthesis and signaling genes in MH-treated apical buds of tobacco.

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<th>Function</th>
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<th>Gene symbol</th>
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Table 4.3. Expression of different phytohormone biosynthesis and signaling genes in MH-treated axillary buds of tobacco.

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Figure 4.1. Schematic diagram of the experimental design for chemical topping and gene expression analysis. C-ApB, control apical bud; CT-ApB, chemically topped apical bud; C-AxB, control axillary bud; CT-AxB, chemically topped axillary bud.
Figure 4.2. Overview of RNA sequencing analysis (A) Distribution of FPKM normalized transcripts across the four treatments. (B) Clustered heat-map of top 10,000 highly abundant mRNAs. C-ApB, control apical bud; CT-ApB, chemically topped-apical bud; C-AxB, control axillary bud; CT-AxB, chemically topped-axillary bud.
Figure 4.3. Differentially expressed genes in maleic hydrazide (MH)-treated apical and axillary buds. (A) Number of upregulated (Red) and downregulated (Blue) genes. (B) Venn diagram depicting the overlap of differentially expressed genes (DEGs) between MH-treated apical and axillary bud. CT-ApB, chemically topped-apical bud; CT-AxB, chemically topped-axillary bud.
Figure 4.4. Gene Ontology (GO) analyses of DEGs in maleic hydrazide (MH)-treated apical and axillary buds. GO analysis of DEGs in apical (A) and axillary bud (B). Upregulated terms are colored in ‘red’ while downregulated terms are in ‘blue’. Each circle represents one GO term. Circle size represents the number of genes in each GO category while color represents the significance level.
Figure 4.5. Effect of maleic hydrazide on expression of key transcription factors involved in apical and axillary bud development. LAS, Lateral suppressor; RAX, Regulator of axillary meristem; ROX, Regulator of axillary meristem formation; REV, Revoluta; CT-ApB, chemically topped-apical bud; CT-AxB, chemically topped-axillary bud.
Figure 4.6. Phylogenetic and gene expression analysis of KNOX gene family from tobacco. (A) A neighbor-joining phylogenetic tree of members of KNOX gene family from Arabidopsis thaliana and Nicotiana tabacum (tobacco) was constructed using ClustalX and MEGA7.0 software with 1000 bootstraps. Nodes belong to A. thaliana are represented by ‘blue’ circles while ‘red’ circles represent the genes from tobacco. (B) Heat map showing the FPKM values KNOX genes obtained by RNA-seq analysis. Rows are probes and columns are samples. The differential expression of each class of KNOX genes is annotated in the right bar. NSC, not significantly changed.
Figure 4.7. Differentially expressed transcription factor (TF) genes in chemically topped axillary and apical buds. The X-axis represents the names of differentially expressed TF families and Y-axis indicates the number of transcription factors. (A) apical bud (B) axillary bud.
Figure 4.8: MapMan visualization of differential gene expression in chemically topped apical bud compared with control. Each dot denotes a gene. ‘Blue’ color indicates downregulation while ‘red’ upregulation. The log2 fold changes of significantly differentially expressed genes were imported and visualized in MapMan for the chemically topped apical bud sample with regard to pathogen/pest attack.
Figure 4.9: MapMan visualization of differential gene expression in chemically topped axillary bud compared with control. Each dot denotes a gene. ‘Blue’ color indicates downregulation while ‘red’ upregulation. The log2 fold changes of significantly differentially expressed genes were imported and visualized in MapMan for the chemically topped axillary bud sample with regard to pathogen/pest attack.
Figure 4.10. Validation of RNA-seq results using quantitative real-time PCR (qRT-PCR). Six differentially expressed genes were selected for qRT-PCR. Tobacco tubulin was used an internal control for normalization. Data represents mean±SD of three biological replicates.
Figure 4.11. A model depicting the effects of maleic hydrazide on different developmental and metabolic processes in apical (ApB) and axillary (AxB) buds of tobacco. Solid arrows represent positive regulation; solid T-bars represent negative regulation. Dashed arrow or T-bars represent possible regulation through combined effects of up- or down-regulated genes.
Conclusion

This research has shown that chemical topping burley tobacco at 10% button stage with a tank mixture of MH and a local systemic suckercide was a suitable alternative to manual topping as sucker control, total yield, and leaf quality grade index were not different between manually topped and chemically topped tobacco (Chapter 2 and 3). However, applications of a local systemic or fatty alcohol alone did not inhibit the terminal bud or control sucker growth, resulting in reduced yield. Chemical topping with MH alone did not provide adequate sucker control and equivalent yields when compared to manual topping in all years and locations of these studies. MH residues for chemically topped tobacco were not consistently different from residues from manually topped and sprayed tobacco, and often were observed to be lower within an environment. Total TSNA was not increased due to chemically topped treatments, and at Lexington there was a significant reduction in total TSNA compared to manually topping, a similar result was also shown in nicotine content. Future work should further investigate these total TSNA and nicotine content reductions that were observed. Chemical topping has the potential to reduce labor input and production costs without negatively impacting the yield, quality or chemistry of burley tobacco (Chapter 2).

Applications of MH plus Butralin at 10% button (pre-bud) to 50% button (early-bud) was found to be an ideal application timing for applying suckercides to chemically top burley tobacco as applications at 10% bloom did not completely halt the development of reproductive growth (Chapter 3). Most chemically topped application timings included in these experiments provided similar sucker control, total yield, and leaf quality compared to manually topping. Chemically topped treatments also appeared to have shorter tip leaves which may contribute to an increased amount of marketable tip grades compared to
manually topping. Later maturing varieties may be better suited for chemical topping due to less rapid change from vegetative to reproductive growth, which would result in a wider window for making chemical topping applications at the most appropriate timings.

MH has a profound influence on gene expression in apical and axillary buds of tobacco (Chapter 4). The number of differentially expressed genes were higher in axillary buds compared to apical buds. Expression of genes related to a number of phytohormones, meristem development, cell division, DNA repair and recombination were affected following MH treatment in both apical and axillary buds, which likely leads to the inhibition of apical and axillary shoot growth. In addition, MH elicits defense responses in plants by inducing the expression genes involved in oxylipin biosynthesis, secondary metabolism and defense-related genes. Collectively, RNA-sequencing analysis may have revealed a possible molecular mechanism of action of MH on apical and axillary buds of tobacco.

Chemical topping is a viable labor saving alternative to manual topping without negatively affecting the yield, quality, or chemistry of burley tobacco.
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EDUCATION

University of Kentucky 2014-2018
PhD Integrated Plant and Soil Science emphasis in Crop Science

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Work Study, Derrickson Agricultural Complex, Morehead, Kentucky 2009-2012

Work Study, IKON Document Services, Morehead, Kentucky 2008-2009

Assistant Farm Manager/Labor, Paradise Valley Farms, Grayson, Kentucky 2005–2012

TEACHING, ADVISING, and MENTORSHIP

Guest lecturer, Senior Seminar, Morehead State University 2017

Guest lecturer, Integrated Weed Management, University of Kentucky 2017

Guest lecturer, Senior Seminar, Morehead State University 2016

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Guest lecturer, Senior Seminar, Morehead State University 2015

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Agriculture Education Student Teaching Practicum, West Carter High School 2012
SERVICE AND RECOGNITION

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Graduate Senator-at-Large, Student Government, University of Kentucky 2017
President, UK IPSS GSA 2017
Summer Senate, Student Government Association, University of Kentucky 2017
Executive Board, Graduate Senator-at-Large, Graduate Student Congress 2017
Plant and Soil Science Department Head Search Committee, University of Kentucky 2017
Certified Non-Commercial Pesticide Applicator, Category 10 R&D 2017
Department of Plant and Soil Science, Seminar Committee 2016
Plant and Soil Science Graduate Student Spotlight 2016
National FFA Convention Judge, Agriscience Fair-Plant Systems 2015
Gamma Sigma Delta, the Honor Society of Agriculture. 2013-2015
Carter County Fair Board 2005-2018
Science Fair Judge for Fayette County Schools 2014
UK Graduate Student Weed Science Team 2013
Morehead State University Outstanding Agricultural Sciences Student 2012
Agriculture Ambassadors, Senior Coordinator 2009-2012
Morehead State University FFA Collegiate Chapter, President 2008-2012
Morehead State University Deans List 2008-2012
Delta Tau Alpha honorary fraternity, Morehead State University 2010-2012
West Carter FFA Alumni, Treasurer 2009-2011
American FFA Degree 2011
Appleseed Scholarship 2009-2011
Farm Credit Services Scholarship 2010
Center for Regional Engagement, Gateway Homeless Shelter Grant, $1,500 2010
Kentucky State FFA Officer Candidate 2008
West Carter FFA, President 2004-2008

GRANTS/FELLOWSHIPS

Altria Client Services Graduate Fellowship Recipient. 18,000/year, tuition, and health insurance 2017-2018

Altria Client Services Graduate Fellowship Recipient. 18,000/year, tuition, and health insurance 2016-2017

Jeffrey’s Fellowship Recipient. 15,000/year, tuition, and health insurance. 2015-2016

CORESTA Study Grant Recipient. 15,000/year. Internationally competitive. 2012-2014

PUBLICATIONS
Refereed Journal Articles


Thesis


Abstracts Published in Proceedings


Richmond, M.D., W.A. Bailey, and R.C. Pearce. 2014. Evaluation of Correlation between Within-Barn Curing Environment and TSNA Accumulation in Dark Air-Cured Tobacco.


**Popular Press**


Interviewed by Tobacco Farm Quarterly magazine on September 7, 2012 describing importance of tobacco research, current research project, and future tobacco research.

**Reviewer Service**

Assisted in review of articles for *Tobacco Science* journal.

Assisted in review of one article for *American Society of Agricultural and Biological Engineers* journal.

**MEETINGS AND PRESENTATIONS**

Moderator, First Annual IPSS 3-Minute-Thesis 2018
Moderator and presenter, IPSS Graduate Student Symposium 2017
University of Kentucky Tobacco Field Day, Presenter 2017
Twilight Tour Field Day, Murray State University, Presenter 2017
Tobacco, Beef, and More Field Day, Presenter, University of Tennessee 2017
Twilight Tour Field Day, Murray State University, Presenter 2016
University of Kentucky Tobacco Field Day, Presenter 2016
University of Kentucky Research and Education Center Field Day, Presenter 2016
Woodford County Field Day, Presenter 2016
Moderator and presenter, IPSS Graduate Student Symposium 2016
Twilight Tour Field Day, Murray State University, Presenter 2015
University of Kentucky Research and Education Center Field Day, Tour Guide 2015
Evaluation of Curing Environment and TSNA Accumulation Within Barns in Dark Air-cured Tobacco, Graduate Student Day at the Capitol. Frankfort, Kentucky 2015
TSNA Subgroup meeting, CORESTA 2014
Residue Field Trial Task Force meeting, CORESTA 2014
Moderator, Integrated Plant and Soil Science Graduate Student Symposium 2014
Evaluation of Curing Environment and TSNA Accumulation Within Barns in Dark Air-cured Tobacco. IPSS Mini-Symposium. 2014
Tobacco Specific Nitrosamine (TSNA) Content in Dark Air-Cured Tobacco can be Correlated to Variability in Curing Conditions. IPSS Mini-symposium. 2013
University of Kentucky Research and Education Center Field Day, Tour Guide 2013