## ABSTRACT

TAYLOR, ZACHARY GRAHAM. Effects of the Ethylene Binding Inhibitor 1-methylcyclopropene on Flue-cured Tobacco (*Nicotiana tabacum* L.). (Under the direction of W. D. Smith and L. R. Fisher).

Three experiments were conducted from 2005 to 2008 to determine the effects of 1-methylcyclopropene (1-MCP) on harvest management of flue-cured tobacco. The first experiment evaluated the use of 1-MCP on ripening delay and holding ability in flue-cured tobacco. Treatments consisted of 1-MCP at a rate of 0.026 kg ai $ha^{-1}$ applied at: 14 d prior to normal final harvest, 14 and 7 d prior to normal final harvest, 7 d prior to normal final harvest, and 7 and 1 d prior to normal final harvest. To determine holding ability and the effect of ripening delay, treatments included a normal harvest, and delaying harvest 10 and 20 d after the normal harvest. Holding ability and ripening delay of flue-cured tobacco was not increased by applications of 1-MCP. Value per hectare, grade index, average price, and yield were not affected by applications of 1-MCP, but were reduced when harvest was delayed from the normal.

The second experiment was conducted to determine if applications of 1-MCP could inhibit chemically enhanced senescence from applications of 2-chloroethylphosphonic acid in flue-cured tobacco. Treatments consisted of applications of 1-MCP at 0.026 kg ai $ha^{-1}$ and 0.0129 kg ai $ha^{-1}$ as well as 2-chloroethylphosphonic acid at a rate of 1.68 kg ai $ha^{-1}$. Applications of 1-MCP were applied at: 14 d prior to normal final harvest, 14 and 7 d prior to normal final harvest, 7 d prior to normal final harvest, and 7 and 1 d prior to normal final harvest. Applications of 2-chloroethylphosphonic acid were utilized to induce senescence at

the normal harvest timing for every treatment. Chlorophyll meter value measurements were taken prior to 2-chloroethylphosphonic acid applications (base), then 24, 48, and 72-hours after 2-chloroethylphosphonic acid applications. Neither base nor 24-hour chlorophyll meter values were affected by 1-MCP treatments in any year or location. However, 48 and 72-hour chlorophyll meter values were affected when 1-MCP was applied at 14 d alone and 7 d alone in both 2006 and 2008 at either location. Thus in these years and locations, two of the four 1-MCP treatments were effective at inhibiting chemically enhanced senescence from applications of 2-chloroethylphosphonic acid.

The final experiment was conducted to determine the effective concentrations of ethylene and 1-MCP as well as determined if prior applications of 1-MCP could inhibit chemically induced senescence in flue-cured tobacco. Treatments consisted of applications of 1-MCP at 0.0129 kg ai $ha^{-1}$ applied at: 14 d prior to normal final harvest, 14 and 7 d prior to normal final harvest, 7 d prior to normal final harvest, and 7 and 1 d prior to normal final harvest. Leaves were sampled prior to 1-MCP application and 30-minutes, 1, 2, 4, and 8-hours after 1-MCP applications. All samples were analyzed by gas chromatograph to determine the effective concentrations of both ethylene and 1-MCP. All remaining tobacco in the field was then subjected to an application of 2-chloroethylphosphonic acid at a rate of 1.68 kg ai $ha^{-1}$. Chlorophyll meter value measurements were taken prior to the 2-chloroethylphosphonic acid application (base) then 24, 48, and 72-hours after application. No concentration of 1-MCP was found in this experiment.

Differences in ethylene concentrations suggest that 1-MCP was bound to the receptor site and by 8-hours, 75-97% of the ethylene concentration had evolved from the leaf tissue.

Once 2-chloroethylphosphonic acid had been applied for 48-hours, chlorophyll content in all chemical treatments was significantly reduced from that of the non-treated control. Data suggest that 1-MCP potentially occupied ethylene-binding sites and that new binding sites were generated to accept ethylene in the form of 2-chloroethylphosphonic acid and initiate chemical senescence.

# Effects of the Ethylene Binding Inhibitor, 1-methylcyclopropene, on Flue-cured Tobacco (*Nicotiana tabacum* L.)

by
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Zachary Graham Taylor, son of Jane and Linwood Taylor, was born May 24th 1978, in Franklin Virginia. He was raised in Murfreesboro, NC on a small cotton and peanut farm. After graduating from Ridgecroft School, he attended Chowan College from the fall of 1996 until the fall of 1997. He then transferred to North Carolina State University in the spring of 1998, where he majored in Agronomy. He was awarded a Bachelor of Science in Agronomy in the fall of 2001. Zach completed a Master of Science in Crop Science at North Carolina State University under the direction of Dr. Loren R. Fisher in 2005. Zach then entered a Doctor of Philosophy program in the Crop Science Department at North Carolina State University under the direction of Dr. David Smith and Dr. Loren Fisher in the spring of 2005.

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# LITERATURE REVIEW

Since the elimination of the Federal Tobacco Program and restrictions on production in 2004, flue-cured tobacco (*Nicotiana tabacum* L.) production has increased in North Carolina. In 2007 nearly 171 million kg of tobacco were produced on 67,000 hectares and sold at an average price of $3.36 per kg in North Carolina up from 156 million kg produced on 61,000 hectares in 2004 (NCDA Website). The value of the 2007 North Carolina tobacco crop was approximately 570 million dollars as compared to 2004 when value from tobacco exceeded 637 million dollars (NCDA Website). As a result of the lower price, producers now struggle to decrease production costs and improve yield and quality in order to increase profits to pre Federal Tobacco Program levels (L. R. Fisher, Personal Com.).

Tobacco is a flowering plant but during normal production the inflorescence is removed to stimulate root growth and decrease the rate of decline in net photosynthesis (Papenfus, 1987). As a result growth resources are diverted into leaf rather than seed production thus the size, yield, and quality of cured leaves are increased (Papenfus, 1987). Flue-cured tobacco leaves senesce in sequence from the bottom to the top of the plant with the lowest leaf senescing about 70 days after transplanting (Moseley *et al*., 1963; Weybrew, 1984). Flue-cured tobacco is harvested after the initiation of senescence when leaves are physiologically mature (Walker, 1968). Physiological maturity marks the transition from growth to senescence, identified as the point of maximum dry weight attainment (Weybrew, 1984; Tso, 1990). Leaves become ripe about 12 d after reaching physiological maturity (Weybrew, 1984). Proper ripening of tobacco is determined by rainfall, growth temperatures, variety selection, and decreased nitrogen absorption when the plant is

physiologically mature (Gains, 1959; Fisher *et al*., 2007). Ripeness is a subjective judgment that develops gradually and is difficult to define precisely (Weybrew, 1984). Ripeness describes a level of senescence and because of its widespread use in describing tobacco production and management techniques the term will be used in this manuscript when describing tobacco specifically.

From a quality standpoint harvesting ripe tobacco results in higher grade indices, preference in smoke panel tests, aging, and increased ability to cure, compared to harvesting unripe leaf (Weybrew, 1984; Moseley *et al*., 1963). Tobacco producers use changes in leaf color as a visual key to determine the correct ripeness of tobacco leaves (Moseley *et al*., 1963). Leaves harvested under-ripe are associated with negative smell and taste, dullness, slickness, and sogginess which decreases quality and final receipts (Moseley *et al*., 1963). Leaves harvested over-ripe are often considered unsatisfactory for smoking quality as well (Walker, 1968). Environmental conditions have the greatest influence on ripening rate of tobacco but are difficult to manage (Fisher *et al*., 2007).

Ethylene, the gaseous plant hormone was first discovered in the early 1900's in etiolated pea seedlings (Neljubov, 1901). In 1934, ethylene was identified as a natural product of plant metabolism (Gane, 1934). By 1959 ethylene research was drastically increased with the introduction of the gas chromatograph (Burg and Thimann, 1959). Ethylene in its simplest form is an olefin, an unsaturated open chain hydrocarbon with one or more carbon-carbon double bonds, represented by two carbons and four hydrogens ($C_2H_4$) with one double bond and a molecular weight of 28g (Tiaz and Zeiger, 2006). Ethylene can

be synthesized either photochemically in the atmosphere and upper oceanic levels or enzymatically in plants (Perovic *et al.*, 2001).

Ethylene is a key component of several plant processes including seed germination, seedling development, fruit ripening, senescence, seed and bud dormancy, root hair formation, defense responses, cell expansion, cell differentiation, abscission, flower promotion and production (Buchanan-Wollaston, 1997; Stern, 1994). Ethylene can be produced by most plant tissues and at every stage of plant development (Tso, 1990). Natural ethylene is also stimulated by the environment and other plant hormones (Yang and Hoffman, 1984). Ethylene production in plants is highest in young developing leaves, senescing tissues, and ripening fruits (Yang and Hoffman, 1984). After physiological maturity and the onset of senescence, amino acids are exported from senescing leaves to other plant parts as reserve nutrients ultimately winding up in the developing seeds of the plant (Mattoo and Aharoni, 1988; Buchanan-Wollaston, 1997).

Abiotic and biotic stresses increase production of ethylene (Yang and Hoffman). Examples of these stresses include: drought, flooding, chilling, insect feeding, wounding, salinity, air pollutants, disease, photoperiod, mineral deprivation, and mechanical injury (Rav and Fluhr, 1993; Nooden, 1988; Lieberman, 1979). Ethylene stimulation due to stress results in plant responses of senescence, abscission, wound healing, and disease resistance (Grbic and Bleecker, 1995; Buchanan-Wollaston, 1997; Wasternack *et al*., 2006; van Loon *et al*., 2006). Plants that are subjected to poor growing environments are induced to senesce so that non-essential portions of the plant can abscise to promote the well being of the entire plant as

well as to decrease transpiration and minimize injury to the whole plant (Buchanan-Wollaston, 1997; Mattoo and Aharoni, 1988).

The most profound plant responses caused by ethylene are the senescence of leaves and the ripening of fruit (Mattoo and Aharoni, 1988). Senescence and/or ripening is associated with changes in respiration, membrane permeability, chlorophyll destruction, carbohydrate metabolism, organic acids, proteins, loss of tissue integrity, and flavor development (Sacher, 1973). With the onset of senescence, there is an increase in ethylene biosynthesis (Mattoo and Aharoni, 1988). Leaf senescence is actually regulated by both ethylene and cytokinin where ethylene increases rate and timing factors of senescence instead of acting as a direct component (Buchanan-Wollaston, 1997). Buchanan-Wollaston (1997) suggested that four different pathways regulate senescence, including cytokinin levels, signal perception from developing sinks, ethylene level, and photosynthate and metabolite concentrations. Ethylene's apparent role is to increase the sensitivity of the signaling pathway so that senescence can be initiated by other age-related factors and regulate genetic expression (Buchanan-Wollaston, 1997; Davies and Grierson, 1979). Gene regulation controls the breakdown of the photosynthetic apparatus, degradation of starch and chlorophyll, and the initiation of respiration and subsequent ethylene production, which further promotes the senescence process (McGlasson *et al.*, 1975).

Methionine, an essential amino acid, acts as the precursor to ethylene and is constantly produced by the Yang cycle (Argueso *et al*., 2007; Miyazaki and Yang, 1987). Methionine is first converted to S-Adenosylmethionine (AdoMet) by the enzyme AdoMet synthetase (Adams and Yang, 1979). AdoMet is then converted to 1-aminocyclopropane-1-

carboxylic acid (ACC) by the enzyme ACC synthase (Adams and Yang, 1979). ACC synthase regulates ethylene biosynthesis by forming a rate-limiting phase in the synthesis process (Argueso *et al*., 2007). Activation of ACC synthase is regulated by environmental stress, hormonal signals, and ethylene itself and is encoded by a multigene family, which increases ethylene biosynthesis (Ecker, 1995). In Arabidopsis there are at least eight different ACC synthase genes which are used for different roles in various tissues and cells and are induced by auxin, wounding, and fruit ripening (Liang *et al*., 1995). ACC is then converted to ethylene by the enzyme ACC oxidase (Adams and Yang, 1979; Tso, 1990). ACC oxidase can be rate limiting as well do to a multigene family encoding ACC oxidase where each gene of the family is differentially regulated (Argueso *et al*., 2007; Barry *et al*., 1996).

After the ethylene molecule is produced, it is available for binding to a receptor whereby activating a pathway leading to a cellular response by altering the pattern of gene expression (Sisler, 2006; Ecker, 1995; Evans *et al*., 1982). Nearly 85% of ethylene binding has been associated with the smooth and rough endoplasmic reticulum, while 15% of ethylene binding is associated with cell walls, nuclei, chloroplasts, mitochondria, and amyloplasts (Evans *et al*., 1982). Binding of ethylene to the receptor requires a copper ion for high affinity binding that promotes interaction between the signal and the receptor (Rodriguez *et al*., 1998).

Ethylene is perceived by five ethylene receptors, etr1, etr2, ers1, ers2, and ein4 (Chang *et al*., 1993; Hua *et al*., 1998). The first ethylene receptor discovered, etr1, conveyed ethylene insensitivity and was found on the endoplasmic reticulum (Chang *et al*., 1993).

Research conducted by Bleecker *et al.*, (1988), found that seeds that contained the etr1 mutation had low germination rates. Plants with the etr1 mutation could not be induced to senesce, thus ethylene synthesis as well as wound ethylene could not be induced, compared to wild-type plants (Bleecker *et al*., 1988). Other studies found that etr1 mutants have delayed senescence, a higher expression of photosynthetic genes, and lower induction of senescence-associated genes than did wild-type plants (Grbric and Bleeker, 1995). Senescence-associated genes in wild type plants increased in expression 6 - 7 days before the etr1 mutant plants, however, the level of expression was similar in both plant types (Grbic and Bleeker, 1995). To date, all ethylene receptors have been associated with ethylene binding by dominant missense mutations, which portrays ethylene insensitivity when each receptor is silenced and displays inhibited cell elongation, seed germination, leaf senescence, and feedback suppression of ethylene (Solano and Ecker, 1998; Sakai *et al*., 1998; Hua *et al.*, 1995).

Different receptors regulate different responses to ethylene under different conditions or achieve different sensitivities in different plant tissues (Solano and Ecker, 1998). Evidence supports that the receptors may not possess the same activity, indicating that each receptor is responsible for a different role in the plant (Hall *et al.*, 2000). According to Rodriguez and Bleecker (unpublished data), ethylene can bind to all five receptors with the same high affinity (Wang *et al.*, 2003). When transcript levels were investigated in Arabidopsis, before the application of ethylene, it was found that the receptor etr1 compromised 50%, ers1 37%, while each of the remaining three receptors compromised 6% or less of the total transcript level (Binder *et al*., 2004). After ethylene application there was

no change in transcription level for 30 minutes (Binder *et al*., 2004). Two hours later, etr1 compromised 20%, ers1 54%, ers2 14%, and ein4 and etr2 each compromised 7% of the total transcript level (Binder *et al.*, 2004). Results show the inactivity of the receptors to the initial response to ethylene and diversity in transcription level and receptor activity after ethylene induction (Binder *et al.*, 2004).

The crt1 protein is the initial component that acts downstream of ethylene receptors and promotes the ethylene signaling pathway (Solano and Ecker, 1998). Once ethylene binds to the receptor, ctr1 is inactivated and the signaling pathway is allowed to continue (Solano and Ecker, 1998). When ethylene is not bound to the receptor, the kinase of ctr1 is in an activated state and inactivates downstream targets, namely ethylene responses (Ecker, 1995; Solano and Ecker, 1998). After ctr1 inactivation, a signal is produced and transferred to the nucleus through a series of proteins, ein2, ein5, ein6, and ein7 (Solano and Ecker, 1998). Once the signal enters the nucleus, a family of 'ein' proteins further processes the signal where the expressions of genes that elicit hormonal responses are produced (Solano and Ecker, 1998; Binder *et al*., 2004).

Ethylene can inflict either a positive or negative feedback regulation on itself through enzymatic control (Mattoo and Aharoni, 1988). Experiments in mungbean (*Vigna radiate* L.) have shown that exogenous ethylene caused an increase in ACC oxidase transcript levels while decreasing ACC synthase transcript levels, indicating that both enzymes are under positive and negative feedback regulation (Kim *et al.*, 2001). Positive regulations are considered autocatalytic while negative regulation is considered an autoinhibition process (Mattoo and Aharoni, 1988). In fruits, the modulation of ethylene and respiration after

ethylene treatment is termed climacteric and represents the initiation of senescence and/or ripening (Aharoni and Lieberman, 1979). The positive feedback regulation of ethylene is then used to ensure whole fruit ripening as well as treatment with exogenous ethylene induces the fruit to produce more endogenous ethylene (Tiaz and Zeiger, 2006). Fruits that do not respond to additional treatments of ethylene with increases in respiration and ethylene production are considered non-climacteric and ripening cannot be accelerated (Tiaz and Zeiger, 2006).

Concentrations of ethylene in mature tobacco leaves range from 0.1 to 0.2 µl/L (Aharoni *et al*., 1979; Abeles, 1973). In detached tobacco leaves, 0.27 µl/L of ethylene displaced one-half of the labeled ethylene from the binding sites and in tobacco leaves there are $2.1 * 10^4$ binding sites per cell (Sisler, 1979). Internal concentrations of ethylene in tobacco leaves were highest in apical leaves, decreasing during expansion, maturation, and early senescence, but increasing during the later stages of senescence (Aharoni *et al.*, 1979). When tobacco leaf discs senesce there is a rise and decline of ethylene and respiration occurring after the initiation of chlorophyll loss (Aharoni and Lieberman, 1979). In other experiments conducted by Aharoni *et al.*, 1979, there was a decline in ethylene during the first phase of chlorophyll loss. The climacteric modulation of ethylene in the leaf discs does not indicate initiation but instead a later stage of senescence (Aharoni and Lieberman, 1979). When tobacco leaf discs were treated with 10 µl/L of ethylene there was no significant increase in the climacteric-like rise in ethylene production for the first 24 hours (Aharoni and Lieberman, 1979).

Only 0.01 to 0.1µl/L of ethylene is required to start the senescence process in tobacco leaves (Abeles, 1973). Ethylene responses are dependent on the size of leaf, where the amount of binding will increase as leaves increase in size from 11 to 36 cm (Goren and Sisler, 1984; Goren *et al*., 1984). Larger leaves will have more total binding per leaf, but less ethylene binding per gram of fresh weight (Goren and Sisler, 1984). Thus, with increased leaf weight comes additional water and cell wall constituents, opposed to other cellular constituents such as membranes, where ethylene would bind (Goren and Sisler, 1984). However, the amount of ethylene present in the plant system is not as important as the sensitivity of the plant tissue to the ethylene concentrations (Blankenship and Sisler, 1989).

Applications of 2-chloroethylphosphonic acid (ethephon) have been used in commercial tobacco production to facilitate the curing process, increase barn capacity by reducing yellowing time, enhance the color of cured leaves, and give greater flexibility in harvest timing when ripening is delayed (Abeles, 1973; Peedin, 1999). An application of 2-chloroethylphosphonic acid allows growers to harvest slow ripening portions of the crop early and avoid barn shortages if the crop ripens too fast later in the season as well as avoid deleterious weather later in the harvest season (Collins and Hawks, 1993). Discovered in the 1960's, 2-chloroethylphosphonic acid is applied in an aqueous solution where it is degraded to ethylene and absorbed and translocated within the plant (Yang, 1969). By the late 1970's 2-chloroethylphosphonic acid had been identified as an effective ripening agent in tobacco showing activity on mature leaves (Domir and Foy, 1976).

The inability of immature leaves to yellow when subjected to 2-chloroethylphosphonic acid has been attributed to the physiological stage of development

(Domir and Foy, 1976). According to Long *et al.*, (1974) tobacco leaves must be a certain age to be responsive to 2-chloroethylphosphonic acid and is estimated to be between physiological maturity, maximum leaf dry weight, and ripeness. Problems with 2-chloroethylphosphonic acid applications include matching treatment area to curing barn capacity, leaf abscission two to four days after application, and poor conditions between application timing and the leaves readiness to senesce (Walker *et al.*, 1985; Collins and Hawks, 1993). To date the tobacco producer's only chemical application option for ripening management of tobacco leaves is limited to increasing the rate of ripening through 2-chloroethylphosphonic acid applications.

In the late 1980's, 1-methylcyclopropene (1-MCP) was discovered as an ethylene binding inhibitor from research conducted by Blankenship and Sisler (Prange and Delong, 2003). By 1999, 1-MCP was approved by the Environmental Protection Agency for use on ornamental crops (Watkins, 2006). Since 2005, 1-MCP has had a food use registration in the U.S. and in numerous foreign countries (Watkins, 2006). To date, 1-MCP is used extensively in the cut flower and fruit industries to delay senescence and ripening and extend shelf life and quality of plant products (Blankenship and Dole, 2003; Prange and Delong, 2003). Binding of 1-MCP to the ethylene binding receptor is accompanied by an affinity that is ten times greater than that of ethylene (Blankenship and Dole, 2003). Binding of 1-MCP to the ethylene receptor promotes a non-toxic mode of action, low residues, and can inhibit ethylene activity at low concentrations (Watkins, 2006). Binding of 1-MCP to the ethylene receptor allows the ethylene cascade to be inactivated and protects the plant from both

endogenously and exogenously produced ethylene (Serek *et al*., 1994; Serek and Reid, 1993; Serek *et al*., 2006).

In commercialized applications 1-MCP is formulated as a cyclodextrin powder (Blankenship and Dole, 2003). At standard temperature and pressure, 1-MCP is a gas with a molecular weight of 54g and a chemical formula of $C_4H_6$ (Blankenship and Dole, 2003). Upon submersion in water, formulated 1-MCP is completely evolved into a gas within 20-30 minutes (Blankenship and Dole, 2003). However, if the cyclodextrin formulation is not added to water 1-MCP will remain stable for some time (Sisler, 2006; Nanthachai *et al.*, 2007). Absorption through leaf and fruit material is the limiting factor to 1-MCP activity because tissue structure and cuticle resistance limit gas diffusion (Nanthachai *et al*., 2007). Applications of 1-MCP can also be lost when applied to certain produce through either the degradation by enzymatic components or the binding to non-physiological components such as cellulose (Nanthachai *et al.*, 2007). Thus, the effectiveness of 1-MCP to delay ripening and senescence of fruits and vegetables depends upon the concentration applied, method and timing of application, temperature, plant maturity, and commodity (Watkins, 2006; Blankenship and Dole, 2003).

Sisler and Serek, (1997) proposed a model for ethylene agonists and antagonist's interaction with the ethylene receptor. Binding of 1-MCP to the receptor first requires the withdrawal of electrons into the orbital of 1-MCP (Sisler and Serek, 1997). Secondly a ligand on the metal in the receptor, a copper ion for high affinity binding is rearranged in the receptor (Sisler and Serek, 1997; Rodriguez *et al.*, 1999). Thirdly the ligand in the trans position is either substituted or released followed by further rearrangement and then binding

(Sisler and Serek, 1997). The plant will remain insensitive to ethylene for as long as 1-MCP is bound to the receptor (Sisler, 2006). Sisler states that there is not enough information yet available to determine the exact sequence of events of 1-MCP binding (Sisler, 2006).

Most plant material will absorb 1-MCP but the rate of absorption differs, which is based on fresh weight, dry matter, insoluble dry matter, and water weight (Nanthachai *et al.*, 2007). Plants requiring different or higher concentrations of 1-MCP, suggests that new receptors are produced in growing tissue or the receptor has a lower affinity for 1-MCP (Watkins, 2006). Feng *et al*., (2004), postulated that 1-MCP will bind permanently to the ethylene receptors and any recovery of sensitivity to ethylene comes from the appearance of new binding sites. Bananas (sp. *Cavendish*) and tomatoes (*Lycopersicon esculentum*, L. cv Prisca) appear to regenerate ethylene-binding sites after 1-MCP applications (Hoeberichts *et al*., 2002; Jiang *et al*., 1999). Single applications of 1-MCP delay ripening in tomato, but multiple applications spaced apart increase the delay of ripening for longer periods of time (Hoebrichts *et al.*, 2002; Blankenship and Dole, 2003). However, crystallographic evidence supports that ethylene receptors etr1, etr2, and ein4 can dimerize faster than the receptors ers1 and ers2, which leads to the production of active receptors (Muller-Dieckmann *et al*., 1999).

Blankenship and Dole (2003) have found that applications of 1-MCP cause an increase in ethylene production in some species but not others. In penicillin infected grapefruit (*Citrus paradise*), applications of 1-MCP caused an increase in ethylene production (Mullins *et. al*., 2000). These increases in ethylene concentration were linked to the uninhibited expression of the stress associated ACC synthase genes (Mullins *et al*., 2000;

Blankenship and Dole, 2003). Bouquin *et al*., (1997) found that in melon (*Cucumis melo* L.) plants ACC oxidase gene expression was undetectable after 1-MCP application. However, accumulation and expression due to wounding a stress response, was not affected by 1-MCP (Bouquin *et al.*, 1997). This differentiated expression suggests a separate pathway for stress induced ethylene production (Bouquin *et al*., 1997).

Previous research has shown that 1-MCP has activity at reducing respiration with the onset of climacteric, delaying softening, chlorophyll degradation, abscission, and color change in many fruits and vegetables (Blankenship and Dole, 2003; Muller *et al*., 1998). According to Prange and Delong (2003), one side effect witnessed with 1-MCP is the decrease in the production of volatile components which resulted in a loss of flavor and aroma, potentially reducing market value. Applications of 1-MCP to banana (*Musa* sp., AAA group, cv. Zhonggang) caused decreases in fruit firmness, respiration, and ethylene production (Zhang *et al.*, 2006). Further application of exogenous ethylene to banana did not initiate senescence after the previous 1-MCP application (Zhang *et al*., 2006). Watermelons (*C. lanatus* cv.Thunb. Matsum. and Nakai) that received doses of 1-MCP at 0.5 or 1.0 µl/L for 18 hours exhibited a decrease in ethylene mediated quality deterioration (Saftner *et al.*, 2007). When 1-MCP was applied for 3, 6, 12, and 24 h at 0.1 and 1.0 µl/L on zonal geraniums (*Pelargonium x hortorum*, cv. Cotton Candy, Fox, Kim, and Veronica) petal abscission was inhibited even after exogenous ethylene was applied to induce senescence and abscission (Jones *et al*., 2001). Applications of 1-MCP to grapefruit showed an increase in ethylene production, promoted by the loss of negative feedback inhibition without affecting the ripening pattern of fruit (Watkins, 2006; Mullins *et al*., 2000).

Field applications of 1-MCP to citrus trees (*Citrus sinensis* L. Osbeck cv. Valencia and Hamlin) reduced leaf abscission but had little effect on the reduction of fruit detachment in response to 2-chloroethylphosphonic acid (Pozo *et al.*, 2004). Thus the mixture and timing of 1-MCP applications with 2-chloroethylphosphonic acid could control leaf drop allowing for fruit loosening (Pozo *et al.*, 2004). Some cultivars of onion (*Allium cepa* L.) are not responsive to 1-MCP because of a thick outer skin covering that acts as a barrier to water loss (Chope *et al.*, 2007). The onion cultivar SS1 has a thin outer skin and when treated with 1-MCP experienced a decrease in sprout growth when onions were stored at $4^{o}$ and $12^{o}$ C (Chope *et al*., 2007).

While 1-MCP has been evaluated on a wide array of fruits and vegetables, very little research has been completed on non-postharvest processes because of multiple ethylene mediated responses found in plants (Prange and Delong, 2003). Research efforts to date have experimented with 1-MCP applied in a fumigant application method which involves volatilization of 1-MCP upon submersion of cyclodextrin powder in water. In 2005, (MSDS, AFxRD-020 Rohm and Haas) Agro Fresh Inc. released a formulation of 1-MCP designed for field applications.

The 2002 growing season in North Carolina was extremely dry and the 2003 growing season was extremely wet in North Carolina (Fisher *et al.*, 2007). Tobacco grown in 2002 experienced a fast ripening process mainly due to the dry environment and little fertilizer absorption creating a shortage of curing barn space (Fisher *et al.*, 2007). Prolonged drought situations will destroy most plants species but periodic drought with minimal rainfall can speed up senescence and promote an early seed set and a reduced life span (Nooden, 1988;

Buchanan-Wollaston, 1997). The 2004 growing season experienced moderate rainfall accumulation and the ripening process was slower compared to dryer years because of increases in nitrogen uptake and precipitation, extending the vegetative growth phase (Fisher *et al.*, 2007; Tso, 1990). The time required for leaves to properly ripen on the stalk then poses problems in regards to labor shortages, potential hurricanes, and leaf diseases later in the growing season (Moseley *et al.*, 1963).

Effects of 1-MCP on flue-cured tobacco have not been evaluated. If 1-MCP could reduce the ripening rate of tobacco by inhibiting the ethylene signal transduction pathway, reductions in ripening rate would allow for greater harvest flexibility, and better management of curing structures. Applied alone or paired with a 2-chloroethylphosphonic acid system, 1-MCP could help manage weather related issues that affect the overall ripening rate of flue-cured tobacco.

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# The Effects of 1-methylcyclopropene on Ripening Delay and Holding Ability in Flue-cured Tobacco (*Nicotiana tabacum* L.)

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## ABSTRACT

Taylor, Zachary G. The effects of 1-methylcyclopropene on ripening delay and holding ability in flue-cured tobacco (*Nicotiana tabacum* L.) (Under the direction of W. D. Smith and L. R. Fisher).

Ethylene serves as a key component in many plant processes including seed germination, seedling development, fruit ripening, and senescence (Buchanan-Wollaston, 1997). The most profound plant responses that ethylene is responsible for is the senescence of leaves and the ripening of fruit (Mattoo and Aharoni, 1988). The role played by ethylene is to increase the sensitivity of the signaling pathway so that senescence and ripeness can be initiated by other age-related factors (Buchanan-Wollaston, 1997).

In the late 1980's 1-methylcyclopropene (1-MCP) an ethylene binding inhibitor, was developed by Blankenship and Sisler (Prange and Delong, 2003). When 1-MCP binds to the ethylene binding receptor the affinity of 1-MCP for the binding site is ten times greater than that of ethylene for its own receptor (Blankenship and Dole, 2003). Binding of 1-MCP allows the ethylene signal transduction pathway to be inactivated and protects the plant from both endogenously and exogenously produced ethylene (Serek *et al*., 1994; Serek and Reid, 1993; Serek *et al.*, 2006).

Research was conducted in 2005 and 2006 at the Central Crops Research Station (CCRS) near Clayton, NC and at the Border Belt Tobacco Research Station (BBTRS) near Whiteville, NC to evaluate the use of 1-MCP on ripening delay and holding ability in flue-cured tobacco. Treatments consisted of 1-MCP at a rate of 0.026 kg ai $ha^{-1}$ applied prior to when optimal ripeness correlated to normal harvest. Applications of 1-MCP were made 14 d

prior to normal final harvest, 14 and 7 d prior to normal final harvest, 7 d prior to normal final harvest, and 7 and 1 d prior to normal final harvest. To determine holding ability and ripening delay, treatments included a normal harvest, and a 10 and 20 d harvest delay.

Grade index and average price followed the same trend and were significantly reduced in two environments. Value per hectare was reduced as harvest was delayed by 10 or 20 d past normal. In three of the four environments as harvest was delayed yield was significantly reduced. Thus holding ability and ripening delay of flue-cured tobacco was not increased by applications of 1-MCP.

# INTRODUCTION

Flue-cured tobacco (*Nicotiana tabacum* L.) leaves senesce in sequence from the bottom to the top of the plant with the lowest leaf senescing about 70-days after transplanting (Moseley *et al*., 1963; Weybrew, 1984). Flue-cured tobacco is then harvested after the initiation of senescence when leaves are physiologically mature (Walker, 1968). Physiological maturity marks the transition from vegetative growth to senescence, identified as the point of maximum dry weight attainment (Weybrew, 1984; Tso, 1990). Leaves become ripe about 12 d after reaching physiological maturity (Weybrew, 1984). Proper ripening of tobacco is determined by rainfall, growth temperatures, variety selection, and decreased nitrogen absorption when the plant is physiologically mature (Gains, 1959; Fisher *et al*., 2007).

Harvesting ripe tobacco results in higher grade indices, preference in smoke panel tests, aging, and increased ability to cure, compared to harvesting unripe leaf (Weybrew, 1984; Moseley *et al*., 1963). Tobacco producers use changes in leaf color as a visual key to determine the correct ripeness of tobacco leaves (Moseley *et al*., 1963). Leaves harvested under-ripe are associated with negative smell and taste, dullness, slickness, and sogginess which decreases quality and final receipts (Moseley *et al*., 1963). Leaves harvested over-ripe are often considered unsatisfactory for smoking quality as well (Walker, 1968). Fertilizer application rates and environmental conditions have the greatest influence on the ripening rate of tobacco but only the latter are difficult to manage (Fisher *et al*., 2007).

Ethylene is a gaseous plant hormone that is a component of several plant processes including seed germination, seedling development, fruit ripening, senescence, seed and bud

dormancy, root hair formation, defense responses, cell expansion, cell differentiation, abscission, flower promotion and production (Buchanan-Wollaston, 1997; Stern, 1994). Ethylene can be produced by most plant tissues and at every stage of plant development (Tso, 1990). The most profound plant responses caused by ethylene are the senescence of leaves and the ripening of fruit (Mattoo and Aharoni, 1988). Senescence and/or ripening are associated with changes in respiration, membrane permeability, chlorophyll destruction, carbohydrate metabolism, organic acids, proteins, softening, and flavor development (Sacher, 1973).

Leaf senescence is regulated by both ethylene and cytokinin where ethylene increases rate and timing factors of senescence instead of acting as a direct component (Buchanan-Wollaston, 1997). Buchanan-Wollaston (1997) suggested that four different pathways regulate senescence, including cytokinin levels, signal perception from developing sinks, ethylene level, and photosynthate and metabolite concentrations. Ethylene's role is to increase the sensitivity of the signaling pathway so that senescence can be initiated by other age-related factors and regulate genetic expression (Buchanan-Wollaston, 1997; Davies and Grierson, 1979). Gene regulation then controls the breakdown of the photosynthetic apparatus, degradation of starch and chlorophyll, and the initiation of subsequent respiration and ethylene production, which promotes the senescence process (McGlasson *et al.*, 1975).

In the late 1980's, 1-methylcyclopropene (1-MCP) was discovered as an ethylene binding inhibitor from research conducted by Blankenship and Sisler (Prange and Delong, 2003). Binding of 1-MCP to the ethylene binding receptor is accompanied by an affinity that is ten times greater than that of ethylene (Blankenship and Dole, 2003). Binding of 1-MCP

to the ethylene receptor promotes a non-toxic mode of action with low residues (Watkins, 2006). Binding of 1-MCP to the ethylene receptor allows the ethylene cascade to be inactivated and protects the plant from both endogenously and exogenously produced ethylene (Serek and Reid, 1993; Serek *et al*., 1994; Serek *et al*., 2006).

Absorption through leaf and fruit material is the limiting factor of 1-MCP activity where tissue structure and cuticle resistance limit gas diffusion (Nanthachai *et al.,* 2007). Absorption of 1-MCP is also affected by fresh weight, dry matter, insoluble dry matter, and water content of the target plant (Nanthachai *et al*., 2007). Applications of 1-MCP can be lost when applied to certain produce through either the potential degradation by enzymatic components or the binding to non-physiological components such as cellulose (Nanthachai *et al.*, 2007). Plants that require different or higher concentrations of 1-MCP, suggests that new receptors are produced in growing tissue or the receptor has a lower affinity for 1-MCP (Watkins, 2006). Feng *et al*., (2004), postulated that after applications of 1-MCP any recovery of sensitivity to ethylene comes from the appearance of new binding sites. Crystallographic evidence indicates that ethylene receptors etr1, etr2, and ein4 can dimerize faster than the receptors ers1 and ers2, leading to the production of active receptors (Muller-Dieckmann *et al.*, 1999).

While 1-MCP has been evaluated on a wide array of fruits and vegetables, very little research has been completed on non-postharvest processes because of the multiple ethylene mediated plant responses that are possible (Prange and Delong, 2003). Research efforts to date have experimented with 1-MCP applied in a fumigant application method involving volatilization of 1-MCP upon submergence of cyclodextrin in water. In 2005, Agro Fresh

Inc. released a formulation of 1-MCP (MSDS, AFxRD-020 Rohm and Haas) designed for field applications. Effects of 1-MCP on field grown flue-cured tobacco have never been evaluated.

The objectives of this experiment were to determine if applications of 1-MCP could reduce the ripening rate of tobacco by inhibiting the ethylene signal transduction pathway and increase the holding ability of the plant. Reductions in ripening rate and increases in holding ability would allow for greater harvest flexibility, and better management of curing structures.

## METHODS AND MATERIALS

Research was conducted in 2005 and 2006 at the Central Crops Research Station (CCRS) near Clayton, NC and at the Border Belt Tobacco Research Station (BBTRS) near Whiteville, NC to evaluate the effects of 1-methylcyclopropene (1-MCP) on ripening delay and holding ability of flue-cured tobacco (*Nicotiana tabacum* L.). Soils were a Bibbs sandy loam (Typic Fluvaquents, coarse-loamy, siliceous, acid, thermic) and a Goldsboro fine sandy loam (Aquic Paleudults, fine loamy, siliceous, thermic) at CCRS and BBTRS in both years, respectively. Flue-cured cultivar 'K326' was transplanted on April 20$^{th}$ in 2005 and April 25$^{th}$ in 2006 at CCRS and 'K346' was transplanted on April 21$^{st}$ in 2005 and 'NC 71' was transplanted on April 26$^{th}$ in 2006 at BBTRS. Tobacco was produced using normal production practices for each research station and according to extension recommendations, except for treatments imposed.

Experimental design was a randomized complete block with four replications. Plots were two rows wide, and 13.7 m long. Row spacing was 1.20 m at BBTRS and 1.14 m at CCRS. Applications of 1-MCP were made at 0.026 kg ai ha$^{-1}$ prior to normal final harvest when an average of seven to eight leaves were remaining on the stalk. All treatments were applied with a $CO_2$ powered backpack sprayer with a delivery volume of 467 L ha$^{-1}$ at 138 kPa. Treatments were applied using three nozzles per row, with 26-cm spacing between nozzles. The outside nozzles were a TG[1] 3 and the center nozzle was a TG 5. Applications of 1-MCP were made at: 14 d prior to normal final harvest, 14 and 7 d prior to normal final harvest, 7 d prior to normal final harvest, and 7 and 1 d prior to normal final harvest. Normal

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final harvest date was predicted based on visual estimation of leaf maturity and ripeness. Harvest date treatments after 1-MCP application included, a normal final harvest and a 10 and 20 d delayed harvest.

Cured leaf yield from the final priming, grade index, average price, value $ha^{-1}$, percent total alkaloids, and reducing sugar data were collected from a single treated row allowing for a common border row. Non-treated controls were included for each harvest date to provide a comparison across the various harvest timings. Efficacy of 1-MCP was determined based on yield and quality factor comparisons of each harvest date to its respective non-treated control. The Tobacco Analytical Services Lab at North Carolina State University performed analysis of percent total alkaloids and reducing sugars on a 50-g cured leaf sample, composited from the final priming, for each plot.

All data were subjected to a factorial analysis of variance (ANOVA) (Table 2.2) and treatment means were separated using Fisher's F-protected LSD at $P \leq 0.05$.

# RESULTS

## Grade Index

Grade index was not affected by the environment * treatment * harvest interaction with a P-value of 0.2167 but was affected by the environment * harvest interaction with a P-value of <0.0001 (Table 2.2). When averaged over treatments (Table 2.3), both environments in 2006 had a significant reduction in grade index with delayed harvest. There was no effect on grade index in 2005 (Table 2.3).

## Average Price

The environment * treatment * harvest interaction was not significant at a P-value of 0.1695 while environment * harvest interaction was significant at a P-value of <0.0001 for average price (Table 2.2). When averaged over treatments (Table 2.4), there were no differences in average price when harvest was delayed by 10 or 20 days in both environments in 2005. In 2006 at both environments, delaying harvest caused significant reductions in average price (Table 2.4). Average price followed the same trend as grade index since both factors are based on US government grades.

## Value

The environment * treatment * harvest interaction was significant at a P-value of 0.0385 for value per hectare (Table 2.2). Means are therefore reported by environment with the appropriate main effects reported from the ANOVA of each environment. In all environments (Table 2.5) when harvest was delayed by 20 d there was a significant reduction in value per hectare when compared to the normal harvest. In three of the four environments when harvest was delayed by 10 d a significant reduction in value per hectare was observed

(Table 2.5). In the CCRS 2005 and 2006 environments, delays in harvest from 10 to 20 d produced no differences in value per hectare but each had significantly lower value when compared to value per hectare at normal harvest (Table 2.5). In the BBTRS 2005 environment when harvest was delayed 20 d value per hectare was significantly lower than either the normal or the 10 d harvest delay (Table 2.5).

**Yield**

The environment * treatment * harvest interaction was not significant with a P-value of 0.1714 however, the environment * harvest interactions was significant at a P-value of <0.0001 for yield (Table 2.2). In all environments except CCRS 2006 (Table 2.6), the 20 d delay in harvest reduced yield when compared to the yield from normal harvest. The CCRS 2006 environment shows an actual increase in yield when harvest is delayed from the normal (Table 2.6). Both CCRS 2006 and BBTRS 2005 show no yield reduction when harvest was delayed 10 d (Table 2.6).

**Total Alkaloids**

Total alkaloids were not affected by the environment * treatment * harvest interaction with a P-value of 0.7238 while the environment * harvest interaction did affect total alkaloids with a P-value of 0.0021 (Table 2.2). When averaged over treatments (Table 2.7) no differences were observed in total alkaloids at CCRS in 2006 and BBTRS in 2005. At the CCRS location in 2005, delaying harvest by 20 d increased total alkaloids while at the BBTRS location in 2006, total alkaloids were reduced (Table 2.7).

**Reducing Sugars**

The environment * treatment * harvest interaction was not significant at a P-value of 0.4518, while the environment * harvest interaction was significant at a P-value of <0.0001 for reducing sugars (Table 2.2). When averaged over treatments reducing sugars decreased when harvest was delayed 10 and 20 d past normal at three of the four environments (Table 2.8).

# DISCUSSION

Ripening delay and holding ability of flue-cured tobacco is best determined by factors such as grade index, average price, and total value on a crop-year basis. Grade index is an assigned value from 0 - 100 that corresponds to visually assigned US Government grades and is used to determine quality of cured leaf (Smith *et al*., 2007). Holding ability is best defined as the ability of a variety to hold its ripeness during the harvest period until maximum quality and value can be obtained through appropriate harvest (Smith *et al.*, 2007). Thus varieties that have superior holding ability are those that allow maximum quality and value to be obtained for longer periods of time before declines in quality, value and yield occur (Fisher *et al.*, 2007). Quality, value, and yield data collected determines if treatments imposed increased holding ability or not, by comparing treatments imposed and harvest dates to non-treated controls.

There was no effect of 1-MCP treatment on grade index (Table 2.2). Grade indices were reduced at both the CCRS and BBTRS 2006 environments when harvest was delayed, but no differences were seen in either environment in 2005 (Table 2.3). Differences across environments were likely related to environmental conditions when tobacco reached optimum maturity and ripeness in 2005 compared to 2006. Specifically, conditions were more favorable for holding tobacco in the field in 2005 than in 2006 due to rainfall (Table 2.1).

Average price (Table 2.4) follows the same trend as grade index with decreases in average price with delayed harvest at two of the four environments. Both average price and grade index are based on US government grade therefore similar trends would be expected.

Imposed treatments did not affect value per hectare, but delayed harvest caused differences that were expected (Table 2.5). A general reduction in value per hectare was evident when harvest was delayed by 10 or 20 d, which suggests that when averaging over treatments 1-MCP did not prohibit the ripening process (Table 2.5).

Treatment with 1-MCP had no effect on yield, when harvest was delayed there was a yield reduction at three of the four environments (Table 2.6). At the CCRS 2006 environment, yield increased as harvest was delayed (Table 2.6).

Applications of 1-MCP did not affect total alkaloid levels (Table 2.7). Differences in total alkaloids were seen when harvest was delayed in two of four environments (Table 2.7). At the CCRS 2005 environment total alkaloids increased and at the BBTRS 2006 environment total alkaloids decreased as harvest was delayed (Table 2.7). Differences are likely related to seasonal effects on alkaloid accumulation.

Applications of 1-MCP also did not affect reducing sugars (Table 2.8). There was a significant reduction in reducing sugars at three of the four environments when harvest was delayed by 20 d (Table 2.8). However, at the CCRS 2006 environment when harvest was delayed by 20 d an increase in reducing sugars was observed (Table 2.8).

Under the scope of this experiment, no treatment with 1-MCP delayed ripening or increased holding ability of flue-cured tobacco. Differences in holding ability, based on yield and quality data collected were related to environmental conditions following normal harvest timing. The 1-MCP applied could have volatilized before reaching ethylene binding sites in leaf tissue. Binding sites could have already been occupied with activating ethylene concentrations at the pre-determined application timings. Furthermore, ethylene receptor

sites in attached tobacco leaves may have been generated. Plants that recover ethylene sensitivity appear to reproduce ethylene receptors (Nanthachai *et al.*, 2007; Watkins, 2006; Feng *et al.*, 2004). Crystallographic evidence supports that ethylene receptors can dimerize rapidly leading to new active receptors (Muller-Dieckmann *et al.*, 1999). Generation of new ethylene binding receptors would allow exogenous and endogenous concentrations of ethylene to bind to receptors and initiate the senescence process. The data do not support use of 1-MCP in tobacco as a management tool.

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Table 2.1. Monthly total precipitation from pre-transplant to harvest at Central Crops Research Station (CCRS) and the Border Belt Tobacco Research Station (BBTRS) from 2005 to 2007.

| | CCRS | | | BBTRS | | |
|---|---|---|---|---|---|---|
| Month | 2005 | 2006 | 2007 | 2005 | 2006 | 2007 |
| | .....cm..... | | | | | |
| February | 5.9 | 2.6 | 4.5 | 7.8 | 7.1 | 5.3 |
| March | 12.9 | 2.9 | 8.1 | 6.7 | 2.4 | 3.7 |
| April | 5.0 | 8.9 | 8.4 | 3.6 | 11.4 | 9.1 |
| May | 8.3 | 9.4 | 2.3 | 9.5 | 8.3 | 3.6 |
| June | 7.6 | 1.4 | 6.5 | 11.5 | 22.6 | 8.9 |
| July | 12.6 | 4.7 | 10.4 | 10.0 | 7.5 | 4.7 |
| August | 7.7 | 8.8 | 1.8 | 8.3 | 17.9 | 4.6 |
| September | 3.3 | 8.7 | 8.0 | 3.7 | 8.7 | 2.9 |
| **TOTAL** | **63.3** | **47.4** | **50** | **61.1** | **85.9** | **42.8** |

Table 2.2. Analysis of variance (P-values) for grade index, average price, value per hectare, yield, total alkaloids, and reducing sugars.

| Source | df | Grade index | Average price | Value per hectare | Yield | Total alkaloids | Reducing sugars |
|---|---|---|---|---|---|---|---|
| Environment | 3 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| Rep (Environment) | 12 | 0.1817 | 0.2579 | 0.0039 | <0.0001 | 0.0001 | 0.0142 |
| Treatment | 4 | 0.4829 | 0.2632 | 0.7956 | 0.6131 | 0.1520 | 0.0829 |
| Harvest | 2 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | 0.7493 | <0.0001 |
| Treatment * Harvest | 8 | 0.9757 | 0.9521 | 0.5850 | 0.1194 | 0.1963 | 0.2725 |
| Environment * Treatment | 12 | 0.7747 | 0.5966 | 0.3416 | 0.2582 | 0.4759 | 0.2348 |
| Environment * Harvest | 6 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | 0.0021 | <0.0001 |
| Environment * Treatment * Harvest | 24 | 0.2167 | 0.1695 | 0.0385 | 0.1714 | 0.7238 | 0.4518 |

Table 2.3. Effect of harvest timing on grade index at Central Crops Research Station (CCRS) and Border Belt Tobacco Research Station (BBTRS) in 2005 and 2006.

| | 2005 | | 2006 | |
|---|---|---|---|---|
| Harvest Date | CCRS | BBTRS | CCRS | BBTRS |
| Normal | 72 a | 88 a | 90 a | 70 a |
| 10 day harvest delay | 67 a | 92 a | 64 b | 38 b |
| 20 day harvest delay | 68 a | 91 a | 51 c | 25 c |

[1]Means followed by the same letter within each location and year are not significantly different. Based on US Government grades; 1-100 scale, with 100 being the best.

Table 2.4. Effect of harvest timing on average price at Central Crops Research Station (CCRS) and Border Belt Tobacco Research Station (BBTRS) in 2005 and 2006.

| | 2005 | | 2006 | |
|---|---|---|---|---|
| Harvest Date | CCRS | BBTRS | CCRS | BBTRS |
| | . . . . . $ US / kg. . . . . | | | |
| Normal | 2.55 a | 3.23 a | 3.43 a | 2.18 a |
| 10 day harvest delay | 2.40 a | 3.34 a | 1.65 b | 1.19 b |
| 20 day harvest delay | 2.44 a | 3.26 a | 1.25 c | 0.66 c |

[1]Means followed by the same letter within each location and year are not significantly different.

Table 2.5. Effect of harvest timing on value[1] per hectare at Central Crops Research Station (CCRS) and Border Belt Tobacco Research Station (BBTRS) in 2005 and 2006.

| | 2005 | | 2006 | |
|---|---|---|---|---|
| Harvest date | CCRS | BBTRS | CCRS | BBTRS |
| | . . . . . $ US / ha. . . . . | | | |
| Normal | 3356 a | 6024 a | 5137 a | 2918 a |
| 10 day harvest delay | 2607 b | 5723 a | 2654 b | 1236 b |
| 20 day harvest delay | 22617 b | 2644 b | 2345 b | 292 c |

| Source | df | P-values | df | P-values | df | P-values | df | P-values |
|---|---|---|---|---|---|---|---|---|
| Rep | 3 | 0.0119 | 3 | 0.0190 | 3 | 0.2838 | 3 | 0.3136 |
| Treatment | 4 | 0.1247 | 4 | 0.9936 | 4 | 0.2764 | 4 | 0.2945 |
| Harvest | 2 | 0.0014 | 2 | 0.0001 | 2 | 0.0001 | 2 | 0.0001 |
| Treatment * Harvest | 8 | 0.3988 | 8 | 0.2322 | 8 | 0.1260 | 8 | 0.1966 |

[1]Means followed by the same letter within each location and year are not significantly different.

Table 2.6. Effect of harvest timing on yield[1] at Central Crops Research Station (CCRS) and Border Belt Tobacco Research Station (BBTRS) in 2005 and 2006.

| | 2005 | | 2006 | |
|---|---|---|---|---|
| Harvest date | CCRS | BBTRS | CCRS | BBTRS |
| | . . . . . kg / ha. . . . . | | | |
| Normal | 1305 a | 1858 a | 1493 b | 1363 a |
| 10 day harvest delay | 1091 b | 1710 a | 1623 b | 1068 b |
| 20 day harvest delay | 1063 b | 786 b | 1810 a | 441 c |

[1] Means followed by the same letter within each location and year are not significantly different.

Table 2.7. Effect of harvest timing on total alkaloids[1] at Central Crops Research Station (CCRS) and Border Belt Tobacco Research Station (BBTRS) in 2005 and 2006.

| | 2005 | | 2006 | |
|---|---|---|---|---|
| Harvest date | CCRS | BBTRS | CCRS | BBTRS |
| | . . . . . % . . . . . | | | |
| Normal | 2.86 b | 3.07 a | 2.64 a | 2.98 a |
| 10 day harvest delay | 2.97 ab | 3.16 a | 2.65 a | 2.96 a |
| 20 day harvest delay | 3.22 a | 3.32 a | 2.44 a | 2.59 b |

[1]Means followed by the same letter within each year and location are not significantly different.

Table 2.8. Effect of harvest timing on reducing sugars[1] at Central Crops Research Station (CCRS) and Border Belt Tobacco Research Station (BBTRS) in 2005 and 2006.

| | 2005 | | 2006 | |
|---|---|---|---|---|
| Harvest date | CCRS | BBTRS | CCRS | BBTRS |
| | . . . . . % . . . . . | | | |
| Normal | 15.30 a | 14.5 a | 16.90 ab | 12.78 a |
| 10 day harvest delay | 13.03 b | 13.71 a | 15.86 b | 7.72 b |
| 20 day harvest delay | 11.34 c | 7.34 b | 17.90 a | 5.69 c |

[1]Means followed by the same letter within each location and year are not significantly different.

# The effects of 1-methylcyclopropene (1-MCP) on flue-cured tobacco (*Nicotiana tabacum* L.) with the initiation of chemical senescence from applications of 2-chloroethylphosphonic acid

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## ABSTRACT

Taylor, Zachary G. The effects of 1-methylcyclopropene (1-MCP) on flue-cured tobacco (*Nicotiana tabacum* L.) with the initiation of chemical senescence from applications of 2-chloroethylphosphonic acid (Under the direction of W. D. Smith and L. R. Fisher).

Flue-cured tobacco leaves senesce in sequence from the bottom to the top of the plant with the lowest leaf senescing about 70 d after transplanting (Moseley *et al.*, 1963; Weybrew, 1984). Ethylene the gaseous plant hormone is responsible for the senescence and ripening of tobacco leaves (Mattoo and Aharoni, 1988). In commercial tobacco production, applications of 2-chloroethylphosphonic acid have been used to facilitate the curing process by reducing ripening time, enhancing the color of cured leaves and giving greater flexibility in harvest timing when ripening is delayed (Abeles, 1973; Peedin, 1999). Applied in an aqueous solution, 2-chloroethylphosphonic acid is degraded to ethylene and absorbed and translocated within the plant (Yang, 1969).

In the late 1980's 1-MCP was discovered to be an ethylene binding inhibitor from research conducted by Blankenship and Sisler (Prange and Delong, 2003). Applications of 1-MCP bind to the ethylene binding receptor where the affinity of 1-MCP for the binding site is ten times greater than that of ethylene for its own receptor (Blankenship and Dole, 2003). Binding of 1-MCP allows the ethylene signal transduction pathway to be inactivated and protects the plant from both endogenously and exogenously produced ethylene (Serek *et al*., 1994; Serek and Reid, 1993; Serek *et al.*, 2006).

Research was conducted from 2006 to 2008 at the Central Crops Research Station (CCRS) near Clayton, NC and at the Border Belt Tobacco Research Station (BBTRS) near

Whiteville, NC to determine if applications of 1-MCP could inhibit chemically enhanced senescence from applications of 2-chloroethylphosphonic acid in flue-cured tobacco.

Treatments consisted of applications of 1-MCP at 0.026 kg ai $ha^{-1}$ at both locations in 2006 and at the CCRS location in 2008, and 0.0129 kg ai $ha^{-1}$ at both locations in 2007 as well as applications of 2-chloroethylphosphonic acid at 1.68 kg ai $ha^{-1}$ at both locations and all years. Applications of 1-MCP were applied prior to when optimal ripeness correlated to normal harvest at: 14 d prior to normal final harvest, 14 and 7 d prior to normal final harvest, 7 d prior to normal final harvest, and 7 and 1 d prior to normal final harvest. Applications of 2-chloroethylphosphonic acid were then applied to chemically enhance senescence at the normal harvest timing; 24-hours after the last 1-MCP application had been applied. Chlorophyll meter values were taken prior to (base) and 24, 48, and 72-hours after 2-chloroethylphosphonic acid applications.

Both base and 24-hour spad values were not affected by any chemical treatment regardless of year or environment. Both the 48 and 72-hour chlorophyll meter values had significant reductions in chlorophyll content for all but two chemical treatments when compared to the non-treated check. The 1-MCP treatments of 14 d alone and 7 d alone prevented chemically enhanced senescence from applications of 2-chloroethylphosphonic acid in 2006 and 2008 for both 48 and 72-hour chlorophyll meter values. All treatments containing 1-MCP could not bind to ethylene receptors in such a manner as to consistently inhibit chemically induced senescence from applications of 2-chloroethylphosphonic acid in all years and locations.

# INTRODUCTION

Flue-cured tobacco (*Nicotiana tabacum* L.) leaves senesce in sequence from the bottom to the top of the plant with the lowest leaf senescing about 70 d after transplanting (Moseley *et al*., 1963; Weybrew, 1984). Flue-cured tobacco is then harvested after the initiation of senescence when leaves are physiologically mature (Walker, 1968). Physiological maturity marks the transition from vegetative growth to senescence, identified as the point of maximum dry weight attainment (Weybrew, 1984; Tso, 1990). Leaves become ripe about 12 d after reaching physiological maturity (Weybrew, 1984). Proper ripening of tobacco is determined by rainfall, growing season temperatures, variety selection, applied fertilizer, and decreased nitrogen absorption when the plant is physiologically mature (Gains, 1959; Fisher *et al*., 2007).

Harvesting ripe tobacco results in higher grade indices, greater preference in smoke panel tests, enhanced aging, and increased ability to cure, compared to harvesting unripe leaves (Weybrew, 1984; Moseley *et al*., 1963). Tobacco producers use changes in leaf color as a visual key to determine the correct ripeness of tobacco leaves (Moseley *et al*., 1963). Leaves harvested under-ripe are associated with negative smell and taste, dullness, slickness, and sogginess which decreases quality and value (Moseley *et al*., 1963). Leaves harvested over-ripe are often considered unsatisfactory for smoking quality as well (Walker, 1968). Fertilizer application and environmental conditions have the greatest influence on ripening rate of tobacco but only the latter is difficult to manage (Fisher *et al*., 2007).

Ethylene a gaseous plant hormone is a key component of several plant processes including seed germination, seedling development, fruit ripening, senescence, seed and bud

dormancy, root hair formation, defense responses, cell expansion, cell differentiation, abscission, flower promotion and production (Buchanan-Wollaston, 1997; Stern, 1994). Ethylene can be produced by most plant tissues and at every stage of plant development (Tso, 1990). The most profound plant responses caused by ethylene are the senescence of leaves and the ripening of fruit (Mattoo and Aharoni, 1988). Ethylene acts to increase the sensitivity of the signaling pathway so that senescence can be initiated by other age-related factors and regulate genetic expression (Buchanan-Wollaston, 1997; Davies and Grierson, 1979). Gene regulation then controls the breakdown of photosynthesis, degradation of starch and chlorophyll, and the initiation of subsequent respiration and ethylene production, which promotes the senescence process (McGlasson *et al.*, 1975).

Discovered in the 1960's, 2-chloroethylphosphonic acid is applied in an aqueous solution where it is degraded to ethylene and absorbed and translocated within the plant (Yang, 1969). Applications of 2-chloroethylphosphonic acid (ethephon) have been used in commercial tobacco production to facilitate the curing process, increase barn capacity by reducing yellowing time, enhance the color of cured leaves, and give greater flexibility in harvest timing when ripening is naturally delayed (Abeles, 1973; Peedin, 1999). An application of 2-chloroethylphosphonic acid enhances ripening rate thereby leading to earlier harvest (Collins and Hawks, 1993). This hastening of harvest allows better management of curing space and avoidance of late-season deleterious weather (Collins and Hawks, 1993).

In order for tobacco leaves to be responsive to 2-chloroethylphosphonic acid the leaves must be of a certain age, estimated to be between physiological maturity, maximum leaf dry weight, and ripeness (Long *et al.*, 1974). Use of 2-chloroethylphosphonic acid

requires that the leaves are sufficiently mature to senesce and that there is sufficient curing barn capacity to avoid losses of leaves due to 2-chloroethylphosphoinc acid induced abscission at 2 to 4 d after application (Walker *et al.*, 1985; Collins and Hawks, 1993). To date, the tobacco producer's only chemical application option for ripening management of tobacco leaves is limited to increasing the rate of ripening through 2-chloroethylphosphonic acid applications.

In the late 1980's, 1-methylcyclopropene (1-MCP) was discovered as an ethylene binding inhibitor from research conducted by Blankenship and Sisler (Prange and Delong, 2003). Binding of 1-MCP to the ethylene binding receptor is accompanied by an affinity that is ten times greater than that of ethylene (Blankenship and Dole, 2003). Binding of 1-MCP to the ethylene receptor promotes a non-toxic mode of action, low residues, and can inhibit ethylene activity at low concentrations (Watkins, 2006). Binding of 1-MCP to the ethylene receptor allows the ethylene cascade to be inactivated and protects the plant from both endogenously and exogenously produced ethylene (Serek *et al*., 1994; Serek and Reid, 1993; Serek *et al*., 2006).

Absorption through leaf and fruit material is the limiting factor of 1-MCP activity because tissue structure and cuticle resistance limit gas diffusion (Nanthachai *et al.*, 2007). Absorption of 1-MCP is also affected by fresh weight, dry matter, insoluble dry matter, and water content of the target plant (Nanthachai *et al*., 2007). Applications of 1-MCP can be lost when applied to certain produce through either the degradation by enzymatic components or the binding to non-physiological components such as cellulose (Nanthachai *et al.*, 2007). Some plants that require different or higher concentrations of 1-MCP, suggesting

that new receptors are produced in growing tissue or the receptors have lower affinity for 1-MCP (Watkins, 2006). Feng *et al*., (2004), postulated that 1-MCP will bind permanently to the ethylene receptors and any recovery of sensitivity to ethylene comes from the appearance of new binding sites. Crystallographic evidence supports that ethylene receptors etr1, etr2, and ein4 can dimerize faster than the receptors ers1 and ers2, which leads to the production of new and active receptors (Muller-Dieckmann *et al*., 1999).

While 1-MCP has been evaluated on a wide array of fruits and vegetables, very little research has been completed on non-postharvest processes because of the many ethylene mediated plant responses (Prange and Delong, 2003). Research efforts to date have experimented with 1-MCP applied in a fumigant application method where the cyclodextrin powder formulation is submerged in water and 1-MCP is volatilized. In 2005, Agro Fresh Inc. released a formulation of 1-MCP designed for field applications (MSDS, AFxRD-020 Rohm and Haas). Effects of 1-MCP on field grown flue-cured tobacco have never been evaluated.

The objectives of this experiment were to determine if applications of 1-MCP could inhibit chemically enhanced senescence from applications of 2-chloroethylphosphonic acid. Application of 1-MCP could aid in tobacco harvesting management by increasing harvest flexibility and promoting efficient use of curing facilities. In addition, when applied with 2-chloroethylphosphonic acid, 1-MCP may allow better management of ripening in varied environmental conditions.

## METHODS AND MATERIALS

Research was conducted from 2006 to 2008 at the Central Crops Research Station (CCRS) near Clayton, NC and at the Border Belt Tobacco Research Station (BBTRS) near Whiteville, NC to determine if applications of 1-methylcyclopropene (1-MCP) could inhibit chemically enhanced senescence from applications of 2-chloroethylphosphonic acid[1] in flue-cured tobacco (*Nicotiana tabacum* L.). Soils were a Dothan loamy sand (Plinthic Paleudults fine-loamy, siliceous, thermic) and a Norfolk fine sandy loam (Typic Paleudults fine loamy, siliceous, thermic) at CCRS and BBTRS in all years, respectively. Flue-cured cultivar 'K326'was transplanted on April 25$^{th}$ in 2006 and April 26$^{th}$ in 2007, and NC 71 was transplanted on April 25$^{th}$ in 2008 at CCRS. Variety 'K346' was transplanted on April 26$^{th}$ in 2006 and 'NC 71' was transplanted on April 30$^{th}$ in 2007 at BBTRS. Tobacco was produced using normal production practices for each research station and according to extension recommendations, except for treatments imposed. Rainfall data for each location are shown in (Table 1).

Experimental design was a randomized complete block with four replications. Plots were two rows wide, and 13.7 m in length. Row spacing was 1.20 m at BBTRS and 1.14 m at CCRS. Treatments consisted of 1-MCP at 0.026 kg ai ha$^{-1}$ at both locations in 2006 and 0.0129 kg ai ha$^{-1}$ at both locations in 2007 with crop oil at 1% v/v (Agri-Dex). Treatments of 1-MCP were also applied at 0.026 kg ai ha$^{-1}$ with crop oil at 1% v/v (Peptoil) only at the CCRS environment in 2008. Applications of 2-chloroethylphosphonic acid at 1.68 kg ai ha$^{-1}$ were made at all locations and years. Treatments of 1-MCP were applied prior to final

[1] Mature XL, Fair Products Inc.

harvest when an average of seven to eight leaves were remaining on the stalk. All treatments were applied with a $CO_2$ powered backpack sprayer with a delivery volume of 467 L $ha^{-1}$ at 138 kPa. Treatments were applied using three nozzles per row, with 26-cm spacing between nozzles. The outside nozzles were a TG[2] 3 and the center nozzle was a TG 5.

Applications of 1-MCP were applied at: 14 d prior to normal final harvest, 14 and 7 d prior to normal final harvest, 7 d prior to normal final harvest, and 7 and 1 d prior to normal final harvest date. Harvest date was predicted based on visual estimation of leaf maturity and ripeness. Applications of 2-chloroethylphosphonic acid were applied to chemically enhance senescence at the normal harvest timing for every 1-MCP treatment, 24-hours after the final 1-MCP application (1-MCP applied at 1d prior to normal final harvest). A non-treated control (no 1-MCP, no 2-chloroethylphosphonic acid) and a 2-chloroethylphosphonic acid only treated control (no 1-MCP but with 2-chloroethylphosphonic acid) was included for comparison.

Leaf chlorophyll content was estimated using a Spad 502 Chlorophyll meter[3]. Chlorophyll meter values were taken prior to (base) and 24, 48, and 72 h after 2-chloroethylphosphonic acid applications. Base chlorophyll meter values were observed at all environments and all years. The 24-hour chlorophyll meter values were observed at all environments and years except at the BBTRS environment in 2006. Both base and 24-hour chlorophyll meter values showed no significant differences in regards to the main effects or interaction (Table 2). The 48-hour chlorophyll meter value data was observed at all

[2] TEEJET
[3] Minolta Camera Co., Ltd. 3-13, 2-Chrome, Azuchi-Macji, Chuo-KU, Osaka 541, Japan.

environments and all years. The 72-hour chlorophyll meter value data was observed at both environments in 2007 and at the CCRS environment in 2008.

The Spad 502 Chlorophyll Meter determines the red region transmittance peak of chlorophyll a and b (660 nm), producing a calculated chlorophyll meter (Spad) value, which corresponds to the amount of chlorophyll in the tested leaf (Anonymous). Higher chlorophyll meter numbers correlate to greener colors while lower chlorophyll meter numbers correspond to more yellow colors.

Data from each year were subjected to a separate factorial analysis of variance (ANOVA) (Table 2) and treatment means from each year were separated using Fisher's F-protected LSD at $P \leq 0.05$.

# RESULTS

## 48-Hour Spad

The environment * treatment interaction was significant at a P-value of 0.0208 for 48-hour chlorophyll meter values in 2006 (Table 3.2), and means are reported for each environment (Table 3.3). In both 2007 and 2008 the 1-MCP treatment main effects were significant with P-values of 0.0089 and 0.0001, respectively (Table 3.2). Chlorophyll meter values were reduced with 2-chloroethylphosphonic acid applications at the CCRS in 2006 and at both locations in 2007 when compared to the non-treated control (Table 3.3). Only one treatment at BBTRS in 2006 and two treatments at CCRS in 2008 inhibited the effects of 2-chloroethylphosphonic acid on chlorophyll meter value (Table 3.3). Both treatments in 2008 were also similar to the non-treated control (Table 3.3). All other chemical treatments in both environments and all years had chlorophyll meter values that were similar to the 2-chloroethylphosphonic acid control and significantly less than the non-treated control (Table 3.3).

## 72-Hour Spad

The environment * treatment interaction was not significant with a P-value of 0.5090 in 2007, while the treatment main effect was significant at 0.0003 in 2007 and 0.0071 in 2008 (Table 3.2). When averaged over both environments in 2007 all chemical treatments were similar and had lower chlorophyll meter values than the non-treated control (Table 3.4). However at the CCRS environment in 2008 when 1-MCP was applied 14 d prior to normal final harvest, chlorophyll meter values were similar to the non-treated control and were significantly greater than the 2-chloroethylphosphonic acid only control (Table 3.4).

## DISCUSSION

Both base and 24-hour chlorophyll meter data showed no differences in chlorophyll meter values regardless of rep(environment), treatment, or the interaction of environment * treatment in all years and environments (Table 3.2).

All but two 1-MCP treatments had significantly lower chlorophyll meter values than the non-treated check 48-hours after 2-chloroethylphosphonic acid application in all three years (Table 3.3). At the BBTRS location in 2006 when 1-MCP was applied at 7 d alone, chlorophyll meter values were significantly higher than the 2-chloroethylphosphonic acid control (Table 3.3). In the CCRS location in 2008 when 1-MCP was applied at 14 d alone and 7 d alone chlorophyll meter values were significantly higher than the 2-chloroethylphosphonic acid control and similar to the non-treated control (Table 3.3). Thus applications of 1-MCP at 14 d alone and 7 d alone inhibited the effects of chemically enhanced senescence from applications of 2-chloroethylphosphonic acid. Because of inconsistencies across all environments, color differences may have been related to environmental factors in the field instead of treatment effects.

When data were averaged over environments in 2007 for 72-hour chlorophyll meter values, all chemical treatments were similar and had lower chlorophyll meter values when compared to the non-treated check (Table 3.4). In 2008, the 14 d 1-MCP treatment was similar to the non-treated control and had higher chlorophyll meter values than the 2-chloroethylphosphonic acid control (Table 3.4). All other chemical treatments in 2008 were similar to the 2-chloroehtylphosphonic acid control and significantly less than the non-treated control (Table 3.4). Applications of 2-chloroethylphosphonic acid resulted in consistent

senescence of flue-cured tobacco in 2007 but not in 2008 for 72-hour chlorophyll meter values (Table 3.4).

Two of the four 1-MCP treatments made prior to 2-chloroethylphosphonic acid applications prevented chemically enhanced senescence from 2-chloroethylphosphonic acid applications. Both treatments were single application treatments, other research supports that multiple applications will increase delays in ripening for longer periods of time (Hoebrichts *et al.*, 2002; Blankenship and Dole, 2003). Based on previous research, multiple application treatments should have been more effective than single application treatments. Differences in effectiveness between 1-MCP treatments could potentially be related to environmental factors in each year and location. However, data support no conclusive reasoning for the difference in effectiveness of 1-MCP treatments.

Potential volatilization of 1-MCP before application, pre-existing ethylene binding in the leaf tissue, and generation of new ethylene binding receptors could explain inactivity of the ineffective 1-MCP treatments. Visual estimation of harvest date could have been estimated prematurely causing significant differences in chlorophyll meter values and explaining higher chlorophyll meter values in 2008 than any other year. Ineffectiveness of 1-MCP treatments in 2007 could have been due to the use of a half rate of 1-MCP compared to the rates used in 2006 and 2008. However, detached tobacco leaves contain $2.1*10^4$ binding sites per cell and 0.27 ppm of ethylene will displace one-half of the labeled ethylene from those binding sites (Sisler, 1979). Applications of 1-MCP made in this experiment were on the magnitude of 27.5 ppm in 2007 and 55 ppm in 2006 and 2008, proving that 1-MCP

concentrations were more than adequate to saturate at least half of the binding sites regardless of rate.

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Table 3.1. Monthly total precipitation from pre-transplant to harvest at Central Crops Research Station (CCRS) and the Border Belt Tobacco Research Station (BBTRS) from 2006 to 2008.

| | CCRS | | | BBTRS | |
|---|---|---|---|---|---|
| Month | 2006 | 2007 | 2008 | 2006 | 2007 |
| | . . . . . .cm. . . . . | | | | |
| February | 2.6 | 4.5 | 4 | 7.1 | 5.3 |
| March | 2.9 | 8.1 | 10 | 2.4 | 3.7 |
| April | 8.9 | 8.4 | 9.8 | 11.4 | 9.1 |
| May | 9.4 | 2.3 | 5.9 | 8.3 | 3.6 |
| June | 1.4 | 6.5 | 6.3 | 22.6 | 8.9 |
| July | 4.7 | 10.4 | 10.6 | 7.5 | 4.7 |
| August | 8.8 | 1.8 | 7.8 | 17.9 | 4.6 |
| September | 8.7 | 8.0 | 13.2 | 8.7 | 2.9 |
| **TOTAL** | **47.4** | **50** | **67.6** | **85.9** | **42.8** |

Table 3.2. Analysis of variance (P-values) for chlorophyll meter value data in 2006, 2007, and 2008.

| 2006 | | | | | | | |
|---|---|---|---|---|---|---|---|
| Source | df | Base spad[1] | df | 24-hour spad[2] | df | 48-hour spad[3] | |
| Environment | 1 | 0.0014 | 0 | -- | 1 | 0.0001 | |
| Rep (Environment) | 6 | 0.9129 | 3 | 0.4231 | 6 | 0.0311 | |
| Treatment | 5 | 0.0715 | 5 | 0.6490 | 5 | 0.0001 | |
| Environment * Treatment | 5 | 0.5469 | 0 | -- | 5 | 0.0208 | |
| **2007** | | | | | | | |
| Source | df | Base spad[1] | df | 24-hour spad[2] | df | 48-hour spad[3] | df | 72-hour spad[4] |
| Environment | 1 | 0.2978 | 1 | 0.0099 | 1 | 0.0075 | 1 | 0.0124 |
| Rep (Environment) | 6 | 0.0191 | 6 | 0.4271 | 6 | 0.0392 | 6 | 0.0415 |
| Treatment | 5 | 0.9765 | 5 | 0.5334 | 5 | 0.0089 | 5 | 0.0003 |
| Environment * Treatment | 5 | 0.5603 | 5 | 0.7133 | 5 | 0.6483 | 5 | 0.5090 |
| **2008** | | | | | | | |
| Source | df | Base spad[1] | df | 24-hour spad[2] | df | 48-hour spad[3] | df | 72-hour spad[4] |
| Rep (Environment) | 3 | 0.5071 | 3 | 0.1483 | 3 | 0.0020 | 3 | 0.1812 |
| Treatment | 5 | 0.1175 | 5 | 0.0697 | 5 | 0.0001 | 5 | 0.0071 |

[1]Base spad measurements obtained before applications of 2-chloroethylphosphonic acid.
[2]24-hour spad measurements obtained 24 hours after 2-chloroethylphosphonic acid application.
[3]48-hour spad measurements obtained 48 hours after 2-chlorethylphosphonic acid application
[4]72-hour spad measurements obtained 72 hours after 2-chloroethylphosphonic acid application.

Table 3.3. Effect of 1-methylcylopropene and 2-chloroethylphosphonic acid applications on 48-hour chlorophyll meter values[1] at Central Crops Research Station (CCRS) and Border Belt Tobacco Research Station (BBTRS) in 2006, 2007, and 2008.

| 1-MCP application timing[4] | 2006 | | 2007[2] | 2008[3] |
|---|---|---|---|---|
| | CCRS | BBTRS | | CCRS |
| | Chlorophyll Meter Value | | | |
| 14 d prior to normal final harvest | 4.30 b | 11.45 bc | 7.74 b | 26.40 ab |
| 14 and 7 d prior to normal final harvest | 9.58 b | 13.19 bc | 8.56 b | 19.50 c |
| 7 d prior to normal final harvest | 5.98 b | 18.35 b | 9.39 b | 25.15 ab |
| 7 and 1 d prior to normal final harvest | 8.98 b | 13.43 bc | 8.29 b | 17.95 c |
| 2-chloroethylphosphonic acid at normal harvest (control) | 5.13 b | 9.80 c | 8.31 b | 16.40 c |
| Non-treated (control) | 21.6 a | 28.23 a | 14.10 a | 28.43 a |

| Source | df | P-values | df | P-values | df | P-values | df | P-values |
|---|---|---|---|---|---|---|---|---|
| Rep | 3 | 0.5276 | 3 | 0.0699 | 6 | 0.0392 | 3 | 0.0020 |
| Treatment | 5 | 0.0007 | 5 | 0.0014 | 5 | <0.0001 | 5 | 0.0001 |

[1]Chlorophyll meter values are calculated, unit-less values that correspond to chlorophyll content in the leaf sample. Means followed by the same letter within each column are not significantly different.

[2]Combined over both environments

[3]CCRS environment only

[4]All 1-MCP treatments were followed by an application of 2-chloroethylphosphonic acid at the normal harvest timing, 24 hours after the final 1-MCP application (1-MCP 1 d prior to normal final harvest)

Table 3.4. Effect of 1-methylcyclopropene and 2-chloroethylphosphonic acid application on chlorophyll meter value[1] 72-hours after the final application of 2-chloroethylphosphonic acid averaged over both locations in 2007 and at the Central Crops Research Station (CCRS) in 2008.

| | 2007[2] | 2008[3] |
|---|---|---|
| 1-MCP application timing[4] | Chlorophyll Meter Value | |
| 14 d prior to normal final harvest | 3.45 b | 21.9 ab |
| 14 and 7 d prior to normal final harvest | 4.99 b | 18.23 bc |
| 7 d prior to normal final harvest | 4.93 b | 20.48 bc |
| 7 and 1 d prior to normal final harvest | 4.53 b | 16.6 bc |
| 2-chloroethylphosphonic acid at normal harvest (control) | 3.80 b | 15.1 c |
| Non-treated (control) | 13.78 a | 27.25 a |

[1]Cholorphyll meter values are calculated, unit-less values that correspond to chlorophyll content in the leaf sample. Means followed by the same letter within each column are not significantly different.
[2]Combined over both environments
[3]CCRS environment only
[4]All 1-MCP treatments were followed by an application of 2-chloroethylphosphonic acid at the normal harvest timing, 24 hours after the final 1-MCP application (1-MCP 1 d prior to normal final harvest)

# The determination of the effective concentrations of ethylene and 1-methylcyclopropene (1-MCP) evolved per gram of fresh weight after timed applications of 1-MCP in flue-cured tobacco (*Nicotiana tabacum* L.)

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## ABSTRACT

Taylor, Zachary G. The determination of the effective concentrations of ethylene and 1-methylcyclopropene (1-MCP) evolved per gram of fresh weight after timed applications of 1-MCP in flue-cured tobacco (*Nicotiana tabacum* L.) (Under the direction of W. D. Smith and L. R. Fisher).

Concentration of ethylene in mature tobacco leaves range between 0.1 and 0.2 µl/L while only 0.01 to 0.1µl/L of ethylene is required to start the senescence process (Aharoni *et al.*, 1979; Abeles, 1973; Mattoo and Aharoni, 1988). In detached tobacco leaves 0.27 µl/L of ethylene displaced one-half of the labeled ethylene from the binding sites and in tobacco leaves there are $2.1*10^4$ binding sites per cell (Goren and Sisler, 1984). However, the amount of ethylene present in the plant system is not as important as the sensitivity of plant tissues to ethylene concentrations (Blankenship and Sisler, 1989).

The ethylene binding inhibitor 1-MCP, binds to the ethylene binding receptor where the affinity of 1-MCP for the binding site is ten times greater than that of ethylene for its own receptor (Blankenship and Dole, 2003). Depending on the time 1-MCP is bound to the receptor, certain plants can remain insensitive to ethylene (Watkins, 2006). Plants that require different or at least higher concentrations of 1-MCP, suggest that new receptors are produced in growing tissue or that the receptor has a lower affinity for 1-MCP (Watkins, 2006).

Research was conducted in 2007 at the Central Crops Research Station (CCRS) near Clayton, NC to determine the effective concentrations of ethylene and 1-MCP evolved per gram of fresh weight after timed applications of 1-MCP in flue-cured tobacco. Treatments

consisted of applications of 1-MCP at 0.0129 kg ai $ha^{-1}$ with a crop oil concentrate at 1% v/v, applied prior to when optimal ripeness correlated to normal harvest. Applications of 1-MCP were made at: 14 d prior to normal final harvest, 14 and 7 d prior to normal final harvest, 7 d prior to normal final harvest, and 7 and 1 d prior to normal final harvest. Leaves were sampled prior to (pre-application) then 30-minutes, and 1, 2, 4, and 8-hours after the final 1-MCP application for each 1-MCP application timing. Headspace gases from all leaf samples were analyzed by gas chromatograph to determine the effective concentrations of both ethylene and 1-MCP.

All remaining tobacco in the field was then subjected to an application of 2-chloroethylphosphonic acid at a rate of 1.68 kg ai $ha^{-1}$ to determine if prior applications of 1-MCP could prohibit chemically enhanced senescence. After application of 2-chloroethylphosphonic acid, chlorophyll measurements in the form of chlorophyll meter values were taken randomly from the treated row prior to (base) then 24, 48, and 72-hours after 2-chloroethylphosphonic acid applications.

Data suggest that three out of four 1-MCP treatments did reduce ethylene concentrations 8-hours after 1-MCP application. However, applying 1-MCP before the onset of senescence had no long lasting effect regardless of 1-MCP application timing. Applications of 1-MCP could not bind to ethylene receptors in such a manner as to prohibit chemically enhanced senescence 48 to 72-hours after the application of 2-chloroethylphosphonic acid.

## INTRODUCTION

Ethylene, the gaseous plant hormone is a key component of several plant processes including seed germination, seedling development, fruit ripening, senescence, seed and bud dormancy, root hair formation, defense responses, cell expansion, cell differentiation, abscission, flower promotion and production (Buchanan-Wollaston, 1997; Stern, 1994). Ethylene can be produced by most plant tissues and at every stage of plant development (Tso, 1990). Ethylene production in plants is highest in young developing leaves, senescing tissues, and ripening fruits (Yang and Hoffman, 1984).

The most profound plant responses caused by ethylene are the senescence of leaves and the ripening of fruit (Mattoo and Aharoni, 1988). Leaf senescence is actually regulated by both ethylene and cytokinin where ethylene increases rate and timing factors of senescence instead of acting as a direct component (Buchanan-Wollaston, 1997). Ethylene's role then is to increase the sensitivity of the signaling pathway so that senescence can be initiated by other age-related factors and regulate genetic expression (Buchanan-Wollaston, 1997; Davies and Grierson, 1979). Gene regulation then controls the breakdown of photosynthesis, degradation of starch and chlorophyll, and the initiation of subsequent respiration and ethylene production, which further promotes the senescence process (McGlasson *et al.,* 1975).

Nearly 85% of ethylene binding has been associated with the smooth and rough endoplasmic reticulum, while 15% of ethylene binding is associated with cell walls, nuclei, chloroplasts, mitochondria, and amyloplasts (Evans *et al.*, 1982). Ethylene is perceived by five ethylene receptors, etr1, etr2, ers1, ers2, and ein4 (Chang *et. al*., 1993; Hua *et. al*., 1998).

Different receptors regulate different responses to ethylene under different conditions or achieve different sensitivities in different plant tissues (Solano and Ecker, 1998). The ctr1 protein is the initial component that acts downstream of ethylene receptors and promotes the ethylene signaling pathway (Solano and Ecker, 1998). Once ethylene binds to the receptor, ctr1 is inactivated and the signaling pathway is allowed to continue where the expression of genes that elicit ethylene responses are produced (Solano and Ecker, 1998). When ethylene is not bound to the receptor the kinase of ctr1 is in an activated state and inactivates downstream targets, namely ethylene responses (Ecker, 1995; Solano and Ecker, 1998).

Concentration of ethylene in mature tobacco (*Nicotiana tabacum* L.) leaves range from 0.1 to 0.2 µl/L (Aharoni *et al*., 1979; Abeles, 1973). Internal concentrations of ethylene in tobacco leaves were highest in apical leaves, decreasing during expansion, maturation, and early senescence, but increasing again during the later stages of senescence (Aharoni *et al.*, 1979). Only 0.01 to 0.1 µl/L of ethylene is required to start the senescence process in tobacco leaves (Abeles, 1973). In detached tobacco leaves, 0.27 µl/L of ethylene displaced one-half of the labeled ethylene from the binding sites and in tobacco leaves there are 2.1 * $10^4$ binding sites per cell (Sisler, 1979).

Ethylene responses are dependent on the size of leaf, where the amount of binding will increase as leaves increase in size from 11 to 36 cm (Goren and Sisler, 1984; Goren *et al*., 1984). Larger leaves will have more total binding per leaf, but less ethylene binding per gram of fresh weight (Goren and Sisler, 1984). Increased leaf weight is due to additional water and cell wall constituents as opposed to other cellular constituents such as membranes, where ethylene would bind (Goren and Sisler, 1984). However, the amount of ethylene

present in the plant system is not as important as the sensitivity of the plant tissue to ethylene concentrations (Blankenship and Sisler, 1989).

In the late 1980's, 1-methylcyclopropene (1-MCP) was discovered as an ethylene binding inhibitor from research conducted by Blankenship and Sisler (Prange and Delong, 2003). By 1999, 1-MCP was approved by the Environmental Protection Agency for use on ornamental crops (Watkins, 2006). Since 2005, 1-MCP has had a food use registration in the U.S. and in numerous foreign countries (Watkins, 2006). To date, 1-MCP has been used extensively in the cut flower and fruit industries to delay senescence and ripening, extend shelf life, and improve the quality of plant products (Blankenship and Dole, 2003; Prange and Delong, 2003). Binding of 1-MCP to the ethylene binding receptor is accompanied by an affinity that is ten times greater than that of ethylene (Blankenship and Dole, 2003). Binding of 1-MCP to the ethylene receptor allows the ethylene cascade to be inactivated and protects the plant from both endogenously and exogenously produced ethylene (Serek *et al*., 1994; Serek and Reid, 1993; Serek *et al*., 2006).

Most plant material will absorb 1-MCP but the rate of absorption differs, which is based on fresh weight, dry matter, insoluble dry matter, and water content (Nanthachai *et al.*, 2007). Plants that require different or higher concentrations of 1-MCP, suggests that new receptors are produced in growing tissue or the receptor has a lower affinity for 1-MCP (Watkins, 2006). Feng *et al*., (2004), postulated that 1-MCP will bind permanently to the ethylene receptors and any recovery of sensitivity to ethylene comes from the appearance of new binding sites. Crystallographic evidence supports that ethylene receptors etr1, etr2, and

ein4 can dimerize faster than the receptors ers1 and ers2, which leads to the production of new and active receptors (Muller-Dieckmann *et al*., 1999).

Blankenship and Dole (2003) have found that applications of 1-MCP cause an increase in ethylene production in some species of plants but not others. In penicillin infected grapefruit (*Citrus paradise*), applications of 1-MCP caused an increase in ethylene production (Mullins *et. al*., 2000). These increases in ethylene concentration were linked to the uninhibited expression of the stress associated ACC synthase genes (Mullins *et al*., 2000; Blankenship and Dole, 2003). Bouquin *et al*., (1997) found that melon (*Cucumis melo* L.) plants showed that ACC oxidase gene expression was undetectable after 1-MCP application. However, accumulation and expression of ACC oxidase in response to wounding, a stress response, was not affected by 1-MCP (Bouquin *et al.*, 1997). This differentiated expression suggests a separate pathway for stress induced ethylene production (Bouquin *et al*., 1997).

Previous research has shown that 1-MCP has activity at reducing respiration with the onset of climacteric, delaying softening, chlorophyll degradation, abscission, and color change in many fruits and vegetables (Blankenship and Dole, 2003; Muller *et al*., 1998). However, while 1-MCP has been evaluated on a wide array of fruits and vegetables, very little research has been completed on non-postharvest processes because of the many ethylene mediated plant responses (Prange and Delong, 2003). Research efforts to date have experimented with 1-MCP applied in a fumigant application method with volatilization of 1-MCP upon submergence of cyclodextrin in water. In 2005, Agro Fresh Inc. released a formulation of 1-MCP designed for field applications (MSDS, AFxRD-020 Rohm and Haas).

The objective of this experiment was to determine the effective concentrations of ethylene and 1-MCP evolved from mature tobacco leaves following timed applications of 1-MCP. A secondary objective included applying 2-chloroethylphosphonic acid to remaining tobacco to determine if prior applications of 1-MCP could inhibit chemically enhanced senescence.

## METHODS AND MATERIALS

Research was conducted in 2007 at the Central Crops Research Station (CCRS) near Clayton, NC to determine the effective concentrations of ethylene and 1-methylcyclopropene (1-MCP) evolved per gram of fresh weight after applications of 1-MCP to flue-cured tobacco (*Nicotiana tabacum* L.). Soil type was a Dothan loamy sand (Plinthic Paleudults fine-loamy, siliceous, thermic). Flue-cured cultivar 'K326' was transplanted on April 26$^{th}$. Tobacco was produced using normal production practices and according to extension recommendations, except for treatments imposed. Rainfall data for the CCRS environment is shown in (Table 1).

Experimental design was a randomized complete block with four replications. Plots were two rows wide, 13.7 m in length and 1.14 m between rows. Treatments consisted of applications of 1-MCP at 0.0129 kg ai ha$^{-1}$ with a crop oil concentrate at 1% v/v (Agri-Dex). Chemical treatments with 1-MCP were applied prior to final harvest when an average of seven to eight leaves remained on the stalk. All treatments were applied with a $CO_2$ powered backpack sprayer with a delivery volume of 467 L ha$^{-1}$ at 138 kPA. Treatments were applied using three nozzles per row, with 26-cm spacing between nozzles. The outside nozzles were a TG[1] 3 and the center nozzle was a TG 5. Applications of 1-MCP were made at: 14 d prior to normal final harvest, 14 and 7 d prior to normal final harvest, 7 d prior to normal final harvest, and 7 and 1 d prior to normal final harvest. Application timing was considered a point in time reference for the level of senescence of the tobacco prior to normal final harvest. Harvest date was predicted based on visual estimation of leaf maturity and ripeness.

[1] TEEJET

Leaves were then sampled prior to (pre-application) and 30 min, 1, 2, 4, and 8 h after the final 1-MCP application at each individual 1-MCP application timing. All applications of 1-MCP were initiated between 6-8 a.m., samples were collected throughout the day with the final 8-hour samples being collected between 2 and 4 in the afternoon. Pre-application samples serve as a baseline comparison for all other samples collected and is not to be considered a non-treated control. Approximately one half of a leaf randomly selected from the treated row was sampled for each leaf sampling time, and placed into a 950 ml glass jar with an installed neoprene septum in the lid to facilitate headspace sampling. Sealed samples were then placed into a cooler with a core temperature of approximately 15$^{o}$C, in order to slow metabolism of the leaf sample. Samples were weighed subsequent to chromatograph analysis.

A gas chromatograph (GC, Varian 3400; Varian Inc, Walnut Creek, CA) fitted with a glass column (Porapak Q, 80-100 mesh, 183cm * 2mm) running at 120$^{o}$C injector, 120$^{o}$C column, and 130$^{o}$C detector (flame ionization) was used to measure ethylene concentrations. The same gas chromatograph and setup was used at 100$^{o}$C injector, 130$^{o}$C column, and 200$^{o}$C detector to measure concentrations of 1-MCP. Flow rates for $He^{+}$, $H_2$, and $O_2$ were 30, 16, and 90 ml min$^{-1}$. Ethylene quantification was based on a response factor generated using a 1 µl/L ethylene standard. Similarly, a 1 µl/L butylene standard was used for 1-MCP quantification. Injection volume into the gas chromatograph was 1 ml of headspace gas drawn via a neoprene septum from the sample jar lid

Twenty-four hours after the final 1-MCP application (1-MCP applied at 1 d prior to normal final harvest), 2-chloroethylphosphonic acid[2] was applied at a rate of 1.68 kg ai $ha^{-1}$ to determine if prior applications of 1-MCP could prohibit chemically enhanced senescence. A non-treated control (no 1-MCP, no 2-chloroethylphosphonic acid) and a 2-chloroethylphosphonic acid only treated control (no 1-MCP but with 2-chloroethylphosphonic acid) was included for comparison. Twenty-four hours after application of 2-chloroethylphosphonic acid, chlorophyll meter values were collected to evaluate changes in leaf color. Chlorophyll meter values were taken prior to (base) then 24, 48, and 72 h after 2-chloroethylphosphonic acid applications.

The Spad 502 (Minolta) Chlorophyll Meter[3] determines the red region transmittance peak of chlorophyll a and b (660 nm), producing a calculated chlorophyll meter value, which corresponds to the amount of chlorophyll in the tested leaf (Anonymous). Higher chlorophyll meter values correlate to greener colors while lower chlorophyll meter values correlate to more yellow colors. Non-treated plots allowed for a direct comparison in regards to normally senesced tobacco.

[2] Mature XL, Fair Products Inc.
[3] Minolta Camera Co., Ltd. 3-13, 2-Chrome, Azuchi-Machi, Chuo-KU, Osaka 541, Japan.

# RESULTS

Data are reported for the appropriate main effects and interactions based on the ANOVA (Table 4.2). Only the sample nested in application and the application main effect were significant for ethylene concentration at P-values of 0.0001 and 0.0014 respectively (Table 4.2). No concentration of 1-MCP was found at any 1-MCP application timing or at any leaf sampling time. All means were separated using Fisher's F-protected LSD at $P \leq 0.05$.

Concentrations of ethylene evolved per gram of fresh weight generally decreased or remained the same as leaf sampling time increased after 1-MCP application (Table 4.3). At each 1-MCP application timing there was a reduced ethylene concentration level when comparing 30-minute sample timing to the 8-hour sample timing (Table 4.3). There was no difference in concentration of ethylene 30-minutes after 1-MCP application regardless of application timing when compared to the pre-application concentration (Table 4.3). At the 2-hour leaf sampling time, all ethylene concentrations were similar to the pre-application concentration (Table 4.3).

At the 4-hour leaf sampling time all ethylene concentration were similar to the pre-application concentration (Table 4.3). At the 8-hour leaf sampling time, three of four 1-MCP treatments had ethylene concentrations significantly lower than the pre-application concentration (Table 4.3). When 1-MCP was applied at 7 and 1 d prior to normal final harvest, ethylene concentrations were significantly higher for the 1 and 2-hour leaf sampling times when compared to any other 1-MCP application timing at those same leaf sampling times (Table 4.3).

When ethylene concentrations were averaged over leaf sampling times only the 14 and 7 d and 7 d alone 1-MCP application timings were significantly lower in ethylene concentrations than all other application timings (Table 4.4). The pre-application concentration was similar to both 1-MCP applied at 14 d and 1-MCP applied at 7 and 1 d prior to normal final harvest (Table 4.4). However, it must be noted that the pre-application sample was not assessed at every leaf sampling time, thus comparison to chemical treatments with leaf sampling times can be misleading and averaging over these data causes responses that are transient and removes treatment effects. What can be surmised when data is averaged over leaf sampling times is that the 14 d and 7 and 1 d applications of 1-MCP had more of an effect on evolved ethylene on average than did the other 1-MCP application timings (Table 4.4).

The ANOVA of the linear relationships between leaf sampling time and concentration of ethylene evolved per gram of fresh weight, for each 1-MCP application timing shows that only the 14 d application timing had a non-significant slope main effect with a P-value of 0.1986 (Table 4.5, Fig. 1). All means were separated using Fisher's F-protected LSD at $P \leq 0.05$. A non-significant slope main effect suggests that the rate of ethylene evolution was no different regardless of when leaf samples were taken thus 1-MCP caused no difference in ethylene evolution rate at this application timing (Table 4.5). All other 1-MCP application timings had significant slope main effects (Table 4.5, Fig 2, 3, 4). Based on these linear relationships, applications of 1-MCP effected ethylene evolution over an 8-hour time period in three of the four treatments (Table 4.5).

Slopes of the remaining 1-MCP treatments with significant slope main effects were then analyzed and reported based on the ANOVA, with means separated by Fisher's F-protected LSD at $P \leq 0.05$ (Table 4.6). The application main effect was significant with a P-value of 0.0019 (Table 4.6).

When averaged over the slopes of the linear relationships all 1-MCP application timings had similar slopes except when 1-MCP was applied at 7 and 1 d prior to normal final harvest (Table 4.7; Fig. 2, 3, 4). Thus two of three 1-MCP treatments caused a similar rate of decline in ethylene evolution (Table 4.7; Fig. 2, 3). Only the 7 and 1 d application of 1-MCP had a greater rate of decline in ethylene evolution over an 8-hour time period than any other 1-MCP treatment (Table 4.7, Fig. 4). Differences in the rate of decline in ethylene evolution between 1-MCP treatments suggest that applications of 1-MCP reduced ethylene evolution over an 8-hour time period.

Only the 48 and 72-hour chlorophyll meter values had significant treatment main effects while base and 24-hour values showed no difference in regards to chlorophyll meter value (Table 4.8). All chemical treatments had significantly lower chlorophyll meter values than the non-treated check for both 48 and 72-hour sample times (Table 4.9). All 1-MCP treatments had statistically similar chlorophyll meter values when compared to one another and the 2-chloroethylphosphonic acid control for both the 48 and 72-hour sample times (Table 4.9).

# DISCUSSION

Internal concentrations of ethylene in mature tobacco leaves range between 0.1 and 0.2 µl/L while only 0.01 to 0.1µl/L of ethylene is required to start the senescence process (Aharoni *et al.*, 1979b; Abeles, 1973; Mattoo and Aharoni, 1988). In detached tobacco leaves, 0.27 µl/L of ethylene displaced one-half of the labeled ethylene from the binding sites and in tobacco leaves there are $2.1*10^4$ binding sites per cell (Goren and Sisler, 1984). Applications of 1-MCP made in this experiment were applied at a concentration of 27.5 ppm making the application of 1-MCP nearly 100 times more concentrated than what was required to displace half of the ethylene binding sites in flue-cured tobacco (*Nicotiana tabacum* L.) leaves.

Concentrations of ethylene that evolved per gram of fresh weight were reduced when leaf samples were analyzed at the 8-hour sampling time compared to the 30-minute sampling time (Table 4.3). Reductions in ethylene concentrations can suggest that 1-MCP was bound to the receptor site and by 8-hours 75-97% of the original concentration of ethylene had evolved from the leaf tissue. Three of four 1-MCP treatments had lower ethylene concentrations than the pre-application concentration when leaves were sampled at the 8-hour sampling time (Table 4.3). At the 30-minute, 2-hour, and 4-hour leaf sampling times all ethylene concentrations were similar to the pre-application concentration regardless of 1-MCP application timing (Table 3).

Previous research supports that ethylene concentrations increase during later stages of senescence (Aharoni *et al.*, 1979; Aharoni and Lieberman, 1979). Ethylene evolution is also affected by circadian rhythms but has yet to be determined in tobacco. In both cotton

(*Gossypium hirsutum*) and *Arabidopsis*, ethylene evolution follows a circadian rhythm were ethylene evolution peaks at mid-day then decreases until the initiation of the next light cycle (Jasoni *et al.*, 2000; Thain *et al*., 2004). Assumptions are made that tobacco follows the same trends where at mid-day ethylene evolution is higher than in pre-dawn hours. All 8-hour leaf samples were taken between 2 and 4 in the afternoon at each 1-MCP application timing. Research supports that ethylene concentrations should not be reduced to the extent witnessed in this experiment during that time in the afternoon under natural conditions. Decreased ethylene concentrations over 8-hours in this experiment, suggest that 1-MCP was bound to ethylene receptors.

When ethylene concentrations were averaged over leaf sampling times the pre-application concentration was similar to 1-MCP applied at 14 d and at 7 and 1 d prior to normal final harvest having higher concentrations than all other treatments (Table 4.4). What can be surmised is that a difference between treatments exists and the rate of decline in ethylene evolution averaged over time can be related to applications of 1-MCP (Table 4.4).

The ANOVA for the linear relationship between leaf sampling times and evolved ethylene concentrations further determines the effect of 1-MCP treatments on ethylene emission. Only the 14 d prior to normal final harvest 1-MCP application had a non-significant slope main effect which supports, that regardless of leaf sampling time ethylene evolution was the same (Table 4.5). However, all other 1-MCP application timings had significant slope main effects which suggest that 1-MCP had an effect on ethylene evolution (Table 4.5). Increases in the rate of decline in ethylene evolution and total ethylene evolution

suggest that 1-MCP was bound to ethylene receptors and ethylene was being evolved off of the leaf sample over time.

Analysis of the resulting significant slopes determined that the 7 and 1 d prior to normal final harvest timing had a greater decline in ethylene evolution than any other 1-MCP treatment based on the differences in slope (Table 4.7). Similar results were seen when the 7 and 1 d 1-MCP application timing was compared to the other application timings at the 1 and 2-hour leaf sampling times (Table 4.3). These significant differences in slope suggest that greater rates of ethylene were evolved at specific leaf sampling times and that 1-MCP was bound to receptor sites within 8-hours of application (Fig. 2, 3, 4).

Differences in the slopes of 1-MCP treatments may be due to environmental conditions at the time of application, or the ability of individual plants to generate new key ethylene receptor sites. Research supports that 1-MCP will permanently bind to the ethylene receptors and any recovery of sensitivity to ethylene comes from the appearance of new binding sites (Feng *et al*., 2004; Tassoni *et al*., 2006). Crystallographic evidence supports that the ethylene receptors etr1, etr2, and ein4 can dimerize faster than the receptors ers1 and ers2, which leads to the production of new and active ethylene receptors (Muller-Dieckmann *et al*., 1999).

The increased rate of decline in ethylene evolution at the 7 and 1 d application timing could have coincided with the increase of ethylene production during later stages of senescence. Final applications of significant 1-MCP treatments were separated by 7 d, of which changes in natural senescence were 7 d closer to complete senescence at 1 d prior to harvest than at 7 d prior. Ethylene concentrations increase during later stages of senescence

(Aharoni *et al.*, 1979; Aharoni and Lieberman, 1979). On the other hand, final applications of 1-MCP could have stressed tobacco more when applied at 1 d prior to normal final harvest allowing for an increase in ethylene emission when 1-MCP was applied at the 7 and 1 d timing. Applications of 1-MCP can cause an increase in ethylene production in some species of plants which is linked to the uninhibited expression of the stress associated ACC synthase genes (Mullins *et al*., 2000; Blankenship and Dole, 2003).

After gas chromatograph analysis, all remaining tobacco in the field was then subjected to applications of 2-chloroethylphosphonic acid. When chlorophyll meter values were measured before 2-chloroethylphosphonic acid application (base) and 24-hours after, no differences in chlorophyll content was evident between all treatments (Table 4.8). However, when chlorophyll meter values were taken 48 and 72-hours after 2-chloroethylphosphonic acid application all chemical treatments had significantly less chlorophyll content than the non-treated control (Table 4.9).

Data suggest that three out of four 1-MCP treatments did reduce ethylene concentrations 8-hours after 1-MCP application. Applications of 1-MCP at 7 and 1 d prior to normal final harvest experienced a greater decline in evolved ethylene over sampling times than any other 1-MCP treatment. However, applying 1-MCP before the onset of senescence had no long lasting effect regardless of the timing of 1-MCP application. Applications of 1-MCP could not bind to ethylene receptors in such a manner as to prohibit chemically induced senescence 48 to 72-hours after the application of 2-chloroethylphosphonic acid.

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Table 4.1. Monthly total precipitation from pre-transplant to harvest at Central Crops Research Station (CCRS) from 2005 to 2007.

| Month | CCRS 2005 | 2006 | 2007 |
|---|---|---|---|
| | | .....cm..... | |
| February | 5.9 | 2.6 | 4.5 |
| March | 12.9 | 2.9 | 8.1 |
| April | 5.0 | 8.9 | 8.4 |
| May | 8.3 | 9.4 | 2.3 |
| June | 7.6 | 1.4 | 6.5 |
| July | 12.6 | 4.7 | 10.4 |
| August | 7.7 | 8.8 | 1.8 |
| September | 3.3 | 8.7 | 8.0 |
| **TOTAL** | **63.3** | **47.4** | **50** |

Table. 4.2. Analysis of variance (P-values) for ethylene concentrations per gram of green leaf tissue.

| Source | df | (P-values) |
|---|---|---|
| Rep | 3 | 0.1493 |
| Application | 3 | 0.0014 |
| Rep * Application | 9 | 0.2923 |
| Sample (Application) | 5 | 0.0001 |

Table 4.3. Effect of ethylene concentration per gram of green leaf tissue following 1-methylcyclopropene application at the Central Crops Research Station (CCRS) in 2007.

| Time after 1-MCP application | Timing of 1-MCP application | | | | |
|---|---|---|---|---|---|
| | 14 d prior to normal final harvest | 14 and 7 d prior to normal final harvest | 7 d prior to normal final harvest | 7 and 1 d prior to normal final harvest | |
| | . . . . . ppm[1] . . . . . | | | | |
| Pre application | | | | | 0.018 bcd |
| 30 minutes | 0.027 ab | 0.017 b-e | 0.018 bcd | 0.029 ab | |
| 1 hour | 0.016 b-f | 0.017 b-e | 0.013 c-g | 0.034 a | |
| 2 hour | 0.01 c-g | 0.009 d-g | 0.012 c-g | 0.027 ab | |
| 4 hour | 0.023 abc | 0.006 d-g | 0.006 d-g | 0.009 d-g | |
| 8 hour | 0.007 d-g | 0.0004 g | 0.0007 g | 0.0007 g | |

[1]Means followed by the same letter are not significantly different.

Table 4.4. Effect of ethylene concentration per gram of green leaf tissue following 1-methylcyclopropene application at the Central Crops Research Station (CCRS) in 2007 averaged over leaf sampling times.

| Timing of 1-MCP application | CCRS 2007 |
|---|---|
| | . . . . . ppm[1] . . . . . |
| 14 d prior to normal final harvest | 0.0165 a |
| 14 and 7 d prior to normal final harvest | 0.0103 b |
| 7 d prior to normal final harvest | 0.0099 b |
| 7 and 1 d prior to normal final harvest | 0.0198 a |
| Pre-application | 0.0176 a |

[1]Means followed by the same letter are not significantly different.

Table. 4.5. Analysis of variance (P-values) for the regression of leaf sampling time and ethylene concentration per gram of fresh weight.

| Treatment | Source | df | (P-values) |
|---|---|---|---|
| 14 d prior to normal final harvest | Intercept | 1 | 0.0004 |
| | Slope | 1 | 0.1986 |
| 14 and 7 d prior to normal final harvest | Intercept | 1 | <0.0001 |
| | Slope | 1 | 0.0002 |
| 7 d prior to normal final harvest | Intercept | 1 | <0.0001 |
| | Slope | 1 | <0.0001 |
| 7 and 1 d prior to normal final harvest | Intercept | 1 | <0.0001 |
| | Slope | 1 | <0.0001 |

Table. 4.6. Analysis of variance (P-values) for the slopes of the regression interaction between leaf sampling timing and concentration of ethylene evolved per gram of fresh weight for three 1-methylcyclopropene application timings.

| Source | df | (P-values) |
|---|---|---|
| Rep | 3 | 0.1946 |
| Application | 2 | 0.0019 |

Table 4.7. Effect of 1-methylcyclopropene application timing on slope of the regression line of leaf sample timing and concentration of ethylene evolved per gram of fresh weight at the Central Crops Research Station (CCRS) in 2007.

| Timing of 1-MCP application | CCRS 2007 |
|---|---|
| | . . . . . slope[1] . . . . . |
| 14 and 7 d prior to normal final harvest | -0.0022 a |
| 7 d prior to normal final harvest | -0.0021 a |
| 7 and 1 d prior to normal final harvest | -0.0044 b |

[1]Means followed by the same letter are not significantly different.

Table. 4.8. Analysis of variance (P-values) for chlorophyll meter value data at the Central Crops Research Station (CCRS) location in 2007.

| Source | df | Base chlorophyll meter value[1] | df | 24-hour chlorophyll meter value[2] | df | 48-hour chlorophyll meter value[3] | df | 72-hour chlorophyll meter value[4] |
|---|---|---|---|---|---|---|---|---|
| Rep | 3 | 0.0242 | 3 | 0.6264 | 3 | 0.0944 | 6 | 0.0253 |
| Treatment | 5 | 0.8933 | 5 | 0.4984 | 5 | 0.0020 | 5 | 0.0001 |

[1]Base chlorophyll meter measurements obtained before applications of 2-Chloroethylphosphonic acid
[2]24-hour chlorophyll meter measurements obtained 24 hours after 2-Chloroethylphosphonic acid application.
[3]48-hour chlorophyll meter measurements obtained 48 hours after 2-Chloroethylphosphonic acid application
[4]72-hour chlorophyll measurements obtained 72 hours after 2-Chloroethylphosphonic acid application.

Table 4.9. Effect of 1-methylcyclopropene on leaf chlorophyll meter readings 48 and 72-hours after application of 2-chloroethylphosphonic acid at Central Crops Research Station (CCRS) in 2007.

| Timing of 1-MCP application | Chlorophyll Meter Value[1] | |
|---|---|---|
| | 48 hour | 72 hour |
| 14 d prior to normal final harvest | 6.2 b | 2.6 b |
| 14 and 7 d prior to normal final harvest | 6.6 b | 3.4 b |
| 7 d prior to normal final harvest | 8.4 b | 4.9 b |
| 7 and 1 d prior to normal final harvest | 7.6 b | 4.3 b |
| 2-chloroethylphosphonic acid | 7.4 b | 3.2 b |
| Non-treated | 14.2 a | 13.1 a |

[1]Chlorophyll meter values are calculated, unit-less values that correspond to relative chlorophyll content in the leaf sample. Means followed by the same letter within each column are not significantly different.

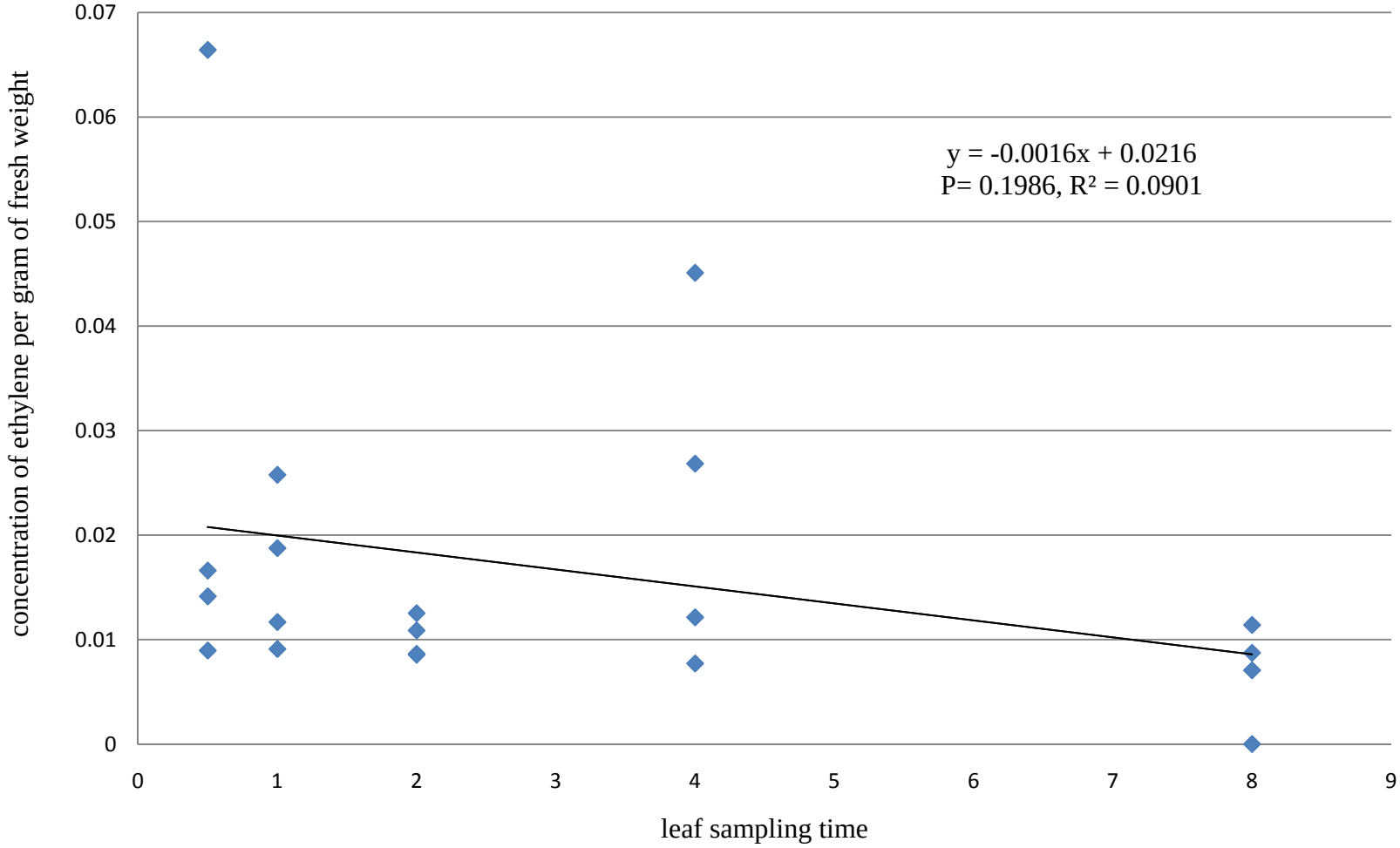


Figure 4.1. Rate of ethylene evolution when 1-methylcyclopropene is applied at 14 d prior to normal final harvest.

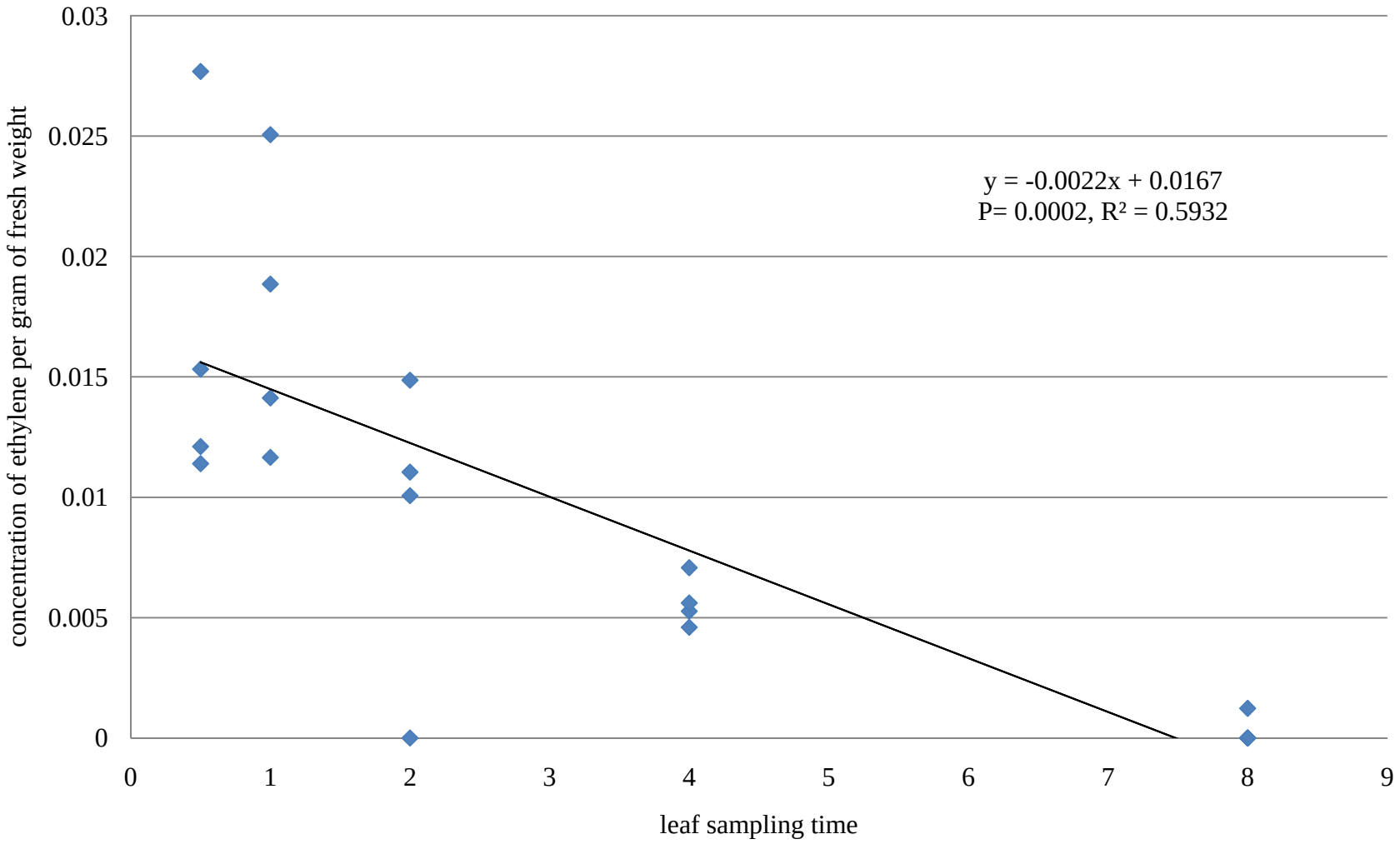


Figure 4.2. Rate of ethylene evolution when 1-methylcyclopropene is applied at 14 and 7 d prior to normal final harvest.

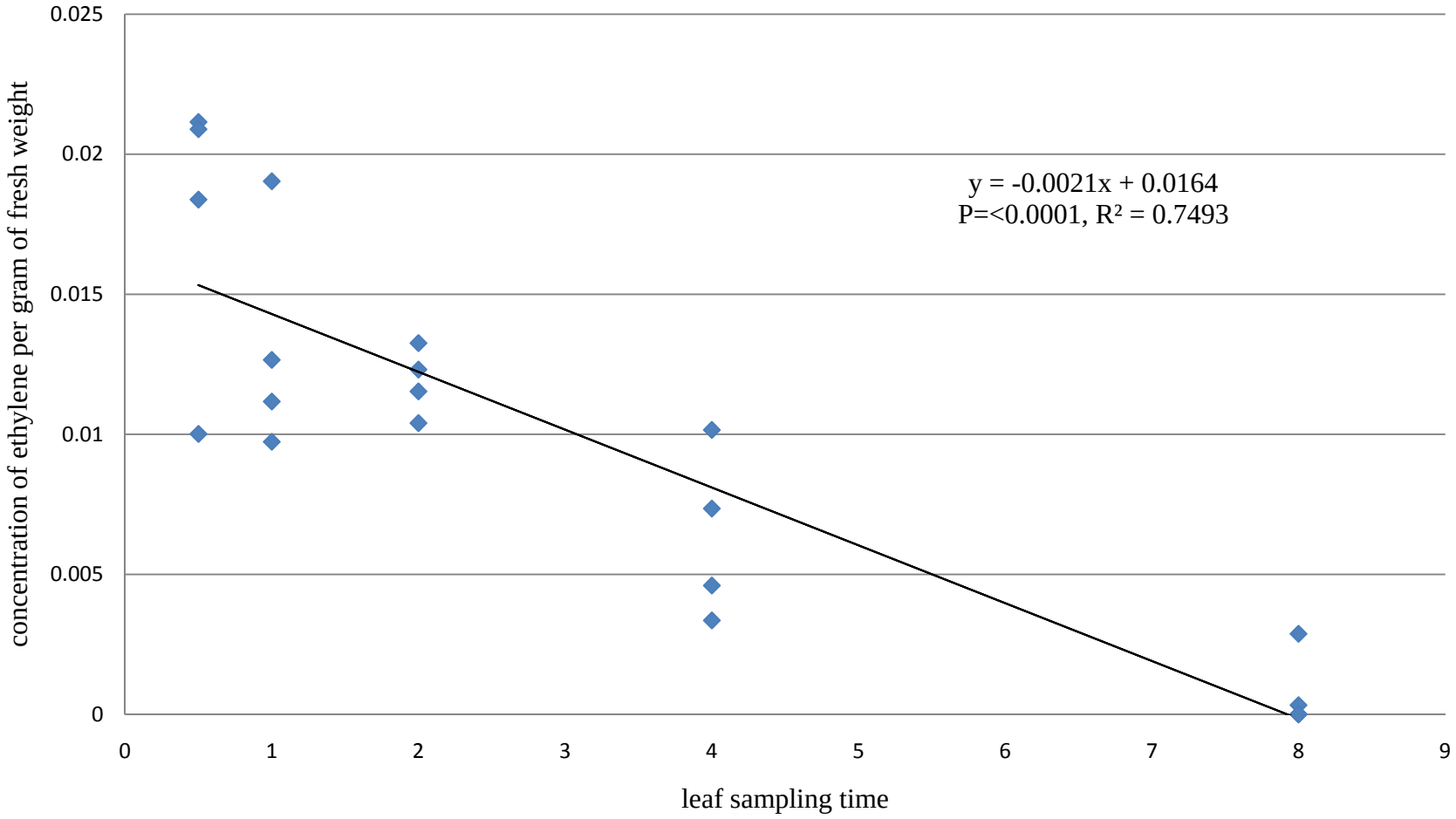


Figure 4.3. Rate of ethylene evolution when 1-methycyclopropene is applied at 7 d prior to normal final harvest.

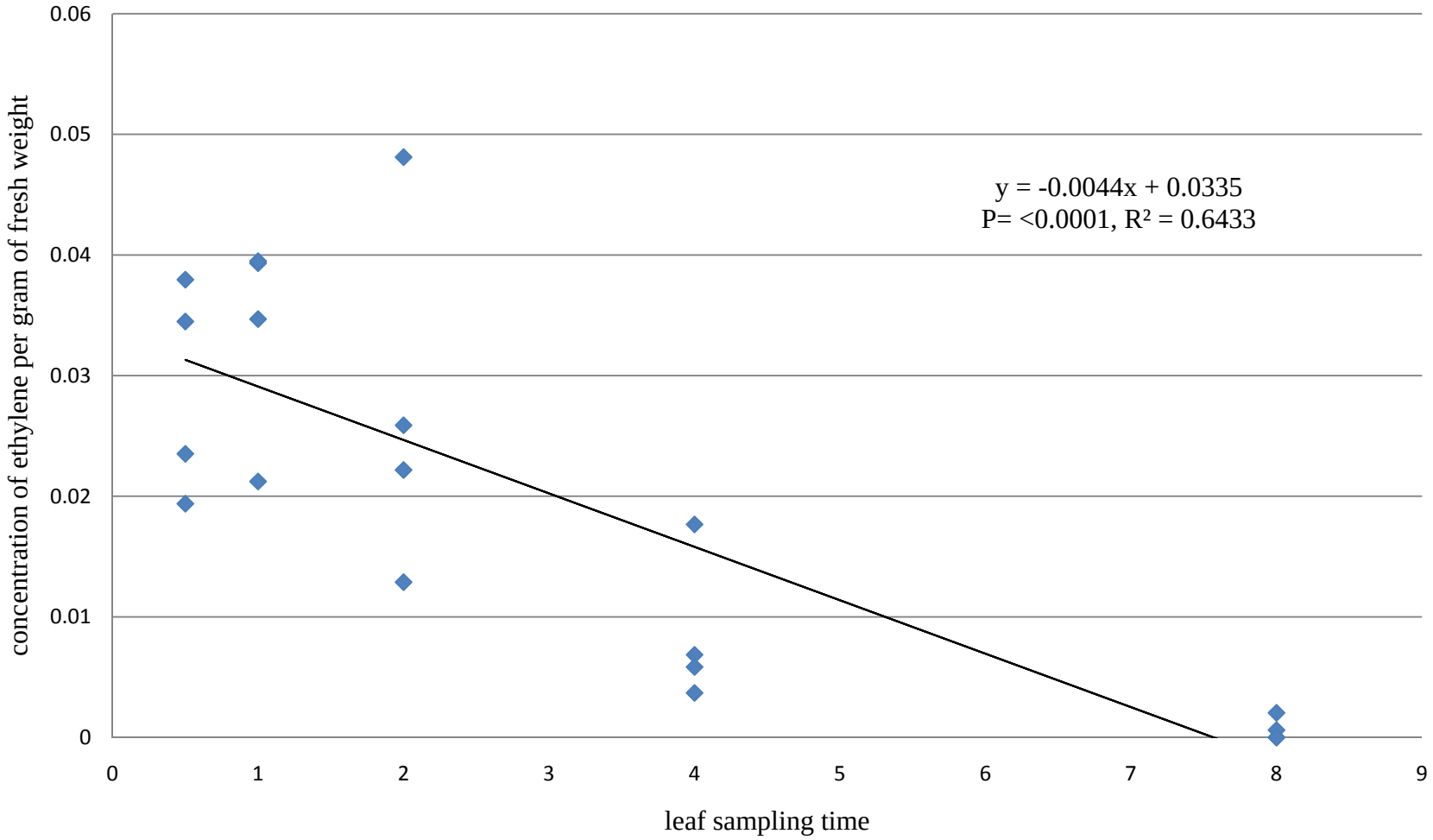


Figure 4.4. Rate of ethylene evolution when 1-methylcyclopropene is applied at 7 and 1 d prior to normal final harvest

# FINAL CONCLUSIONS

Applications of 1-MCP are extremely effective on ripening delay of cut flowers and fruit (Blankenship and Dole, 2003; Prange and Delong, 2003). However, applications of 1-MCP on attached leaves of flue-cured tobacco (*Nicotiana tabacum* L.) were not consistently effective in delaying natural senescence or inhibiting chemically enhanced senescence.

No treatment with 1-MCP delayed ripening or increased holding ability of flue-cured tobacco. Differences in holding ability, based on yield and quality data collected were related to environmental conditions following normal harvest timing. Applications of 1-MCP may have volatilized before reaching ethylene binding sites in leaf tissue. Binding sites may have already been occupied with activating ethylene concentrations prior to application of 1-MCP. Furthermore ethylene receptor sites in attached tobacco leaves may have been regenerated allowing ethylene to bind and initiate the senescence process.

In the second experiment, applications of 1-MCP at 14 d alone and 7 d alone made prior to 2-chloroethylphosphonic acid applications prevented chemical senescence enhanced by 2-chloroethylphosphonic acid application. However, applications of 1-MCP at 14 and 7 d and 7 and 1 d could not inhibit chemically enhanced senescence. Ineffectiveness of non-significant 1-MCP treatments could be related to potential volatilization of 1-MCP before application, pre-existing ethylene binding in the leaf tissue, and regeneration of ethylene binding receptors, as in the first experiment could explain inactivity of 1-MCP. Detached tobacco leaves contain $2.1*10^4$ binding sites per cell and 0.27 ppm of ethylene will displace one-half of the labeled ethylene from those binding sites (Sisler, 1979). Applications of 1-MCP made in this experiment were on the magnitude of 27.5 to 55 ppm proving that 1-MCP

concentrations were more than adequate to saturate binding sites. Data support no conclusive reasoning for the differences in effectiveness of 1-MCP treatments.

In the third experiment concentrations of 1-MCP were never found at any level of application or leaf sampling time. Data suggest that ethylene concentrations were reduced when leaf samples were analyzed 8-hours after 1-MCP application. Reductions in concentrations of ethylene evolved from sampled leaves suggest that 1-MCP was bound to ethylene receptors. Tobacco that remained in the field was then subjected to applications of 2-chloroethylphosphonic acid to enhance chemical senescence. Forty-eight hours after the final 1-MCP application chlorophyll content in all chemical treatments was significantly reduced when compared to the non-treated control. Chemically induced senescence was initiated 48 to 72-hours after the final 1-MCP application which suggests that either new receptors where produced or that 1-MCP had disassociated from the receptor after 8-hours.

Under the scope of these experiments, 1-MCP did not delay ripening or increase holding ability of flue-cured tobacco in our first experiment. Applications of two 1-MCP treatments did prohibit the induction of chemically induced senescence by 2-chloroethylphosphonic acid in our second experiment. However, remaining 1-MCP treatments could not inhibit chemically enhanced senescence. Reduced ethylene concentrations in our third experiment suggest that 1-MCP did indeed bind to ethylene receptors 8-hours after application. However, 48 hours after 2-chloroethylphosphonic acid application and the final 1-MCP application, ethylene was bound to receptors and chemical senescence was induced. At the current rate of 8-hours to reduce ethylene stores and remain bound to receptors, avoidance of ethylene sensitivity in flue-cured tobacco would require

multiple applications of 1-MCP. Applications of this magnitude would be an ineffective option for producers in regards to ripening management of flue-cured tobacco.

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