OPTIMIZING SILVER THIOSULFATE APPLICATIONS ON CASSAVA TO BLOCK ETHYLENE EFFECTS AND IMPROVE FLOWER RETENTION FOR BREEDING PURPOSES

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ABSTRACT

Cassava is the fourth largest staple crop in the world, and it serves as a main carbon source for human nutrition in Africa, South America, and Southeast Asia. However, its poor flower set and lack of flowering synchrony makes it hard to breed improved genotypes with higher production and superior qualities. Previous findings indicated that applying silver thiosulfate (STS) could improve cassava flower retention and might be useful in cassava breeding. However, at the rates used it might induce severe phytotoxicity on the plants, and there is concern silver may accumulate in the soil. The goal of this research was to find better strategies to apply STS and reduce plant phytotoxicity. Experiments with ethephon showed that ethylene was responsible for cassava flower abortion and senescence. STS inhibited ethylene signaling thus protecting the flowers from abortion and senescence. Compared to wide-scale spraying of STS to mature leaves, localized STS application to the apex was equally effective for flower retention, and it requires less silver. To develop an optimal protocol, tests were conducted to find 1) the best timing for STS applications; 2) the number of applications required for better flowering; 3) the best placement for STS application; and 4) the best concentration for STS application on cassava. Overall, the findings indicate that optimal STS treatment is when it starts at least several days before bud appearance, and with a continued supply

during flowering. STS application to the young leaves in the apex avoids excess application and phytotoxicity. STS dosage requires optimization: it needs to be sufficiently high for substantial flowering benefit, and low enough to avoid phytotoxicity. These findings indicate that STS could serve as a potentially valuable tool for cassava breeders in managing flowering.

BIOGRAPHIC SKETCH

Xian Guan obtained her Bachelor of Science degrees from Beijing Forestry University in Turfgrass Management and from Michigan State University, Department of Crop and Soil Sciences, in 2011. In that same year, she entered University of Massachusetts-Amherst and earned a Master of Science degree in Plant Sciences in 2014. After that, she began her study at Cornell University, and majored in Soil and Crop Sciences. She earned another Master of Science degree in May, 2017. Xian Guan was a member of several scientific societies, including American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, a teaching and research assistant for several courses at both UMass and Cornell, and a member of Department of Crop and Soil Sciences Graduate Student Association at Cornell. In her spare time, Xian Guan loves cooking, traveling and outdoor activities. Upon graduation, she will move to Beijing and start a life there with her husband.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

1.1 Generals about Cassava

Cassava (*Manihot esculenta*), also called yuca, manioc, mandioca and tapioca, originates from Brazil, and was introduced throughout tropical regions of the world by international trade, including into Africa in the 16th century by Portuguese. It has become one of the most important and productive crops in tropical and subtropical regions of the world, especially in Africa (Jones, 1959, Raji et al., 2009). It is also widely spread in South America and Southeast Asia, as well as Indonesia and several tropical islands in Oceania (Burkhill, 1904). Because of the high content of starch in the root, plentiful vitamins in leaves, as well as low input and maintenance requirements for crop production, cassava serves as a major food source for humans and livestock, especially in South America and Africa. According to data reported by the FAO for 2009, about 233 million tons (fresh weight) of cassava are produced each year (http://faostat.fao.org, accessed 1 April, 2017). This represents about 86 million tons of root dry weight, of which about 85% is starch. Its production ranks as the 4th largest staple food crop in the world, after maize (*Zea mays*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*). In Asia, cassava is also used for industrial and energy purposes, such as in the production of ethanol, degradable plastics, and organic acids (Howeler, 2002, FAO, 2009).

Compared to other food and economic crops, cassava is generally more disease and stress

tolerant (Jones, 1959, Cock, 1982), and requires much less input for growth and maintenance. It provides a large share of the food requirements in Africa and South America, and due to its tolerance of high temperatures and drought, it most likely will play a more important role in response to climate changes, and food demands for increasing populations in the future (Jarvis, et al., 2012). Even though cassava is a good crop species due to its productivity and comparably low requirements for mineral nutrient fertilizers, there are still several disadvantages and problems about this crop that need to be addressed. These problems include susceptibility to Cassava Mosaic Disease (CMD) (Bock and Woods, 1983) and Cassava Brown Streak Disease (CBSD) (Winter et al., 2010), pest damage caused by white flies, cassava mealy bug and green mite, as well as the scarcity of micronutrients in starchy roots. According to the data reported by El-Sharkawy in 1993, the potential yield capacity for cassava is 7 times higher than those typical of farmer's fields, as shown in research trials under intense management. Cassava breeders around the world aim to enhance the yield of this crop, make it more disease resistant, and improve the quality and nutrient value of the crop (Ceballos et al., 2010, Sayre et al., 2011). However, breeding of this species for improved characteristics is stagnant and limited (Okechukwu and Dixon, 2008). The slow progress in cassava breeding results from several causes, including biological obstacles (discussed below), management problems, and environmental

constraints (Fregene et al., 2001).

1.2 Breeding Obstacles of Cassava

In general, cassava breeding faces two major problems. The first one is the lengthy breeding cycle. Cassava is primarily vegetatively propagated, with one mature plant producing about five to six stakes that can be used for propagation. The limited number of stakes produced by a single plant slows the process of establishing replicated plots, screening genotypes for desired phenotypes, and identification of superior genotypes in recombinant populations or after genetic modification. Normally, it requires more than five years for a breeding cycle in cassava, and the lengthy breeding cycle thus limits the improvement of favorable characteristics and delays the discoveries of other potential genetic problems (Ceballos et al. 2015; Ceballos et al. 2012; Ceballos et al. 2004; Kawano 2003). Part of this problem lies in the poor flower and seed set in cassava (Ceballos, 2004). Early abortion of cassava flowers occurs frequently, and the seeds produced by a plant are limited. A cassava plant produces both female and male flowers (monecious); female flowers are produced first, at the base of the flower stalks, and male flowers are produced 7-14 days later as the flower stalks continue to develop. One mature plant with high fertility produces seeds at a maximum rate of three

per flower (Perera et al. 2013). In addition, a lack of synchrony in flower timing and poor fertility exist in cassava plants. These flowering problems increase the difficulty in cross-pollination and delay phenotype differentiation. In order to speed up breeding in cassava, flowering and seed set needs to be improved.

Like other Euphorbiaceae plants, cassava flowers are also apetalous (Prenner and Rudall, 2007, Perera et al., 2013). Both female and male flowers consist of a single pistil or stamen surrounded by petal-like bracts or bracteoles. Female flowers are single, and male flowers form an inflorescence that is called cyathia (Prenner and Rudall, 2007). Each cyathia contains several (about 10) male flowers, consisting only of stamens and anthers attached to a disk and surrounded by petal-like bracts such that it resembles a single flower (Perera et al., 2013). In cassava, cyathia align sequentially on branched flower stalks, and about 4 to 5 branches form a raceme (Perera et al., 2013). They are mainly located on the apical part of a flower-stalk branch, where cyathia with female buds have longer pedicels and lie lower than the cyathia with male buds whose pedicels are shorter and are more concentrated on the top part of the flower-stalk branch (Perera et al., 2013). Flower initiation is normally associated with fork-type branching which occurs periodically at the shoot apical meristem and creates a series of sympodia. In general, the flowers that form during the first forking event abort soon after they

develop and the flower stalk produces only a few flower buds. The early abortion of cassava flowers delays possible crossing opportunities, thus it has become a major issue for cassava breeding.

Recent research in Arabidopsis has found that the gene underlying FLOWERING LOCUS T (FT) is the gene that encodes a phloem-mobile protein, which will move from the leaves through phloem to the meristem region where it stimulates flower initiation, and flower formation (Giakountis and Coupland, 2008, Zeevaart, 2008). This finding has been confirmed and extended in numerous flowering plant species including Arabidopsis (An et al., 2004, Abe et al., 2005, Wigge et al., 2005, Corbesier et al., 2007, Mathieu et al., 2007), apple (Kotoda et al., 2010), tomato (Lifschitz et al., 2006, Cao et al., 2016), rice (Tsuji et al., 2011), Populus (Böhlenius et al., 2006, Zhang et al., 2010), Eucalyptus (Klocko et al., 2016) and cassava (Adeyemo, 2016). Recent studies in cassava indicate that plant growth regulator treatments that have favorable effects on flower retention and flower set do not affect the timing of flower initiation (Hyde et al. 2016a), and photoperiod-temperature environments that regulate flower initiation do not affect flower retention/set (Hyde et al. 2016b). This suggests that the flower retention/set is regulated by different factors than those regulating flower initiation.

1.3 Ethylene

Plant hormones are important regulators of many plant growth and development activities. Ethylene is a simple, colorless and odorless gaseous chemical with a formula of C_2H_4. It is recognized as an important plant hormone that participates in many catabolic and metabolic activities that result in fruit ripening and abscission, bud dormancy and germination, floral development and abscission, sex determination, and leaf abscission, chlorosis, and epinasty, pathogen defense and protection, as well as several abiotic stress responses and tolerance (Lipe and Morgan, 1972; Abeles, 1973; Maillette, 1982; Mattoo and Suttle, 1991; Abeles et al., 1992; Kende, 1993; Rademacher, 2015). Because of its characteristics, and the effects it elicits in plants, ethylene is widely used in industries to regulate flower and fruit ripening, and to ensure the timing of plant development during transport to market.

Ethylene was first realized as a biologically active compound in Neljubow's (1901) etiolated pea seedling growth experiment. It was classified as a plant hormone by subsequent findings that plants produce ethylene endogenously (Crocker, 1932; Gane, 1934). Early studies on ethylene were mainly based on bioassays. Soon, the invention and discovery of gas chromatography made ethylene research simple, fast and easy, and thus there was a rapid advance in understanding how ethylene is synthesized and functions in plant systems (Burg and Burg, 1962；Burg, 1962). In 1966, Lieberman

et al. identified the chemical precursor of ethylene, methionine. Generally, the biosynthesis of ethylene traces the pathway as follows: Firstly, from methionine the intermediate is synthesized: S-adenosylmethionine (AdoMet) by AdoMet synthetase (Adams and Yang, 1977, Yang and Hoffman, 1984, Fluhr et al., 1996). Then, AdoMet is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) via ACC synthase (Yu et al., 1979a. Yang and Hoffman, 1984, Kende, 1989, Fluhr et al., 1996). Finally, ACC is oxidized to ethylene catalyzed by ACC oxidase, which is also known as ethylene forming enzyme (EFE) (John, 1991, Adams and Yang, 1977, Yang and Hoffman, 1984, Fluhr et al., 1996). The ethylene synthetic pathway in many plant species and tissue systems is regulated by feedback loops which in some cases are a negative feedback that keeps the system from becoming excessive, while in other systems it is regulated by a positive feedback loop, which is considered autocatalytic as an initial small increase in ethylene stimulates a much greater subsequent production of ethylene (Serek et al., 2006). In many species, fruit have a climacteric pattern of ripening where, as long as there is ethylene present, no matter endogenously produced or exogenously applied, the ethylene production process is triggered and becomes autocatalytic (Serek et al., 2006). Although the levels of ethylene produced in each species varies greatly in terms of carbon flux, the amount of ethylene produced is small. The responses it induces can be quite substantial and vary greatly

depending on the tissue and developmental stage. For example, depending on other co-occurring factors it helps plants to defend against different biotic and abiotic stresses, such as chilling (McCollum and McDonald, 1991), freezing and hypoxia stress (Lin et al., 2009), wounding (Hyodo and Nishino, 1981), as well as insect (O'Donnell et al., 1996) and pathogen (Achilea et al., 1985) infections. It also promotes maturation, abscission, and senescence (Goldschmit et al., 1993). To regulate ethylene effects on processes that are important in agricultural and post-harvest systems, ethylene-related genes and ethylene antagonists have been investigated for the past several decades, and now they are widely used in industry to ensure the storage and transportation of fruits and flowers.

The production of ethylene is influenced by several external and internal factors (Abeles et al., 1992). Externally, ethylene production is affected by temperature, light, oxygen, carbon dioxide, gravity, mechanical perturbation, as well as diurnal periodicity. Internal differences in genetics, flower sex, developmental stages, organs, feedback regulation systems, and plant hormones balances would also influence the production of ethylene (Abeles et al., 1992).

1.4. Ethylene Response Inhibition

Since the 1930s, physiological responses of ethylene in plants were gradually discovered (Burg, 1962, Burg and Burg, 1962, Crocker, 1932, Crocker, 1948). By knowing its properties and functions on plants, floricultural and agricultural industries became aware of the importance and deleterious effects of ethylene. The inhibition of ethylene production to avoid early abscission and ripening, and to ensure the freshness of fruits, vegetables, and flowers became very important. Over the decades and years, three ways to inhibit ethylene responses in plants were developed: to stop the endogenous ethylene production, to block the ethylene binding receptor, and to inhibit the downstream responses after ethylene binding to the plant receptor (Serek et al., 2006). To inhibit the endogenous production of ethylene, one approach is to find genetic mutations on two steps of the synthetic pathway: ACC synthesis and ACC oxidation, which are catalyzed by ACC synthase and ACC oxidase, respectively. Genetically down regulating the expression of the genes for these two ethylene synthesis enzymes has successfully inhibited the production of ethylene (Hamilton et al., 1990, Oeller et al., 1991). Another way to suppress ethylene synthesis is to apply plant growth regulators (PGR) which inhibit ACC synthase including 1-aminoethoxyvinylglcine (AVG) and aminooxyacetic acid (AOA). In addition, ACC synthesis can also be inhibited by elevated carbon dioxide concentration (Beyer, 1979, Serek et al., 2006). All three methods mentioned above function on ACC synthase, which catalyzes the

synthesis of ACC. By either antisense or suppression of ACC synthase, the ACC synthesis pathway has been suppressed. The inhibition of ethylene production can also be achieved by blocking the ACC oxidation step, which is catalyzed by ACC oxidase. Elevating temperature, or decreasing the oxygen concentration, or applying $CoCl_2$ can successfully deactivate ACC oxidase, thus suppressing the oxidation of ACC, which thereby inhibits the production of ethylene (Serek et al., 2006). Actions can also be taken to reduce the levels of precursors of ethylene: ACC and AdoMet. For example, Klee et al. (1993) found that overexpressing the ACC deaminase enzyme can significantly reduce the amount of ACC, thus limited the production of ethylene. Good et al. (1994) utilized a bacteria gene to encode the AdoMet hydrolase which transferred AdoMet to methylthioadenosine and homoserine, reduced the pool of AdoMet and hence significantly reduced the production of ACC and in turn, ethylene.

Blocking the ethylene binding receptor is another way of blocking ethylene response. Ethylene receptors are dimer proteins composed of histidine kinase encoded by a family of *ETR1*-like genes and embedded in cell membranes (Chang et al., 1993, Hua et al., 1995, Hua et al., 1998, Sakai et al., 1998, Schaller et al., 1995, Bleecker et al., 1998). Generally, receptors encoded by the *ETR1-like* gene family exhibit similar levels of ethylene binding capability (O'Malley et al., 2005). Inactivation or mutations of any member of the gene family could possibly lead to ethylene insensitivity in plants

(Bleecker et al., 1998).

There are two ways to block the ethylene binding receptor (Serek et al., 2006). The first way is to apply chemicals (plant growth regulators). Certain types of chemicals would induce the conformational changes within the plant receptor protein structure therefore inactivates it. For example, in 1967, Burg and Burg first postulated that a metal ion is located in the dimer structure that coordinates and formulates the binding of ethylene, and subsequent work has shown that the metal ion is a copper ion (Thompson et al., 1983). Later on, Beyer (1976) identified that silver ion can block ethylene responses such as abnormal growth (horizontal growth, stem swelling and growth retardation), abscission, and senescence in pea (*Pisum sativum cv. Alaska*), cotton (*Gossypium hirsutum cv.* Stonewille *213*), and hybrid orchid. Beyer (1976b) also discovered that increased concentration of silver nitrate would prolong the protection period, and delay the occurrence of the symptoms listed above. Silver can thus influence ethylene signaling cascade in plants (McDaniel and Binder, 2012). This led to the conclusion that the inhibition is due to the exchange between the silver and the copper ion that lies within the ethylene receptor (Rodrígez et al., 1999). Silver thiosulfate (STS) complex was found to be more easily taken up and transported, giving a greater response than uncomplexed Ag^{+} ion, and it became more commonly used. However, overdose application of silver

may result in damage in plant tissues. Similar responses were also reported in cucumbers and tomatoes (Beyer, 1976b). Since silver is a heavy metal, the use of STS was restricted.

The other way to block ethylene binding receptor is to mutate the receptor protein and disable its ability for binding ethylene; this method involves genetic manipulation using genetic engineering or breeding for the trait. Either way of blocking ethylene receptor can prevent ethylene responses from both endogenously produced and exogenously applied ethylene. Receptor blocking is by far regarded as the most efficient way of inhibiting ethylene responses (Serek and Reid, 1993).

In addition to silver ion, there are several other chemicals that have been found to be able to inhibit ethylene responses at the receptor level, such as 2,5-Norbornadiene, *trans*-cyclooctene, diazocyclopentadiene, and 1-Methylcyclopropene (Abeles et al., 1992, Serek et al., 2006).

1-Methylcyclopropene (1-MCP) is a transparent and odorless gas used for ethylene receptor inhibition. It binds to the ethylene receptors and induces conformational changes that inactivate its ability to induce later ethylene responses. The 1-MCP is a very strong ethylene inhibitor such that a small concentration, for example, 6 nL L^{-1}, is enough to result in ethylene response inhibition (Serek et al., 1994, 1995a,b,c, 2006). Since 1-MCP is colorless and non-toxic, it is the most widely used commercial PGR to ensure the freshness of horticultural products, especially for vegetables, fruits,

and ornamental species. To apply 1-MCP, plants need to be transported to an airtight area with internal air circulation before releasing the treatment to prevent leakage. Since 1-MCP binds to plant receptors irreversibly, in growing tissues repeat applications of 1-MCP is necessary for prolonged protection to block the newly developed ethylene receptors (Serek and Sisler, 2005, Serek et al., 2006).

1.5 Ethylene, STS, and their relation to flowering

Ethylene influences plant growth and development in many ways, depending on the species, tissue, development stage, environmental conditions, and genotype (Abeles et al., 1992, Rademacher, 2015). In 1976, Beyer discovered silver nitrate could be used to delay ethylene symptoms on plants. However, considering its slow mobility within plants and phytotoxicity that may result in potential environmental problems, it did not become a common choice. Instead, silver thiosulfate (STS), with more mobility, becomes more popular in usage, but with restrictions (Rademacher, 2015). Experiments have found that applying STS could release or delay the ethylene induced symptoms on flowers such as abscission and abortion, induce flower opening, and extend vase life (Veen and Van de Geijn, 1978). For example, Cameron and Reid (1981) demonstrated that foliar spray of STS on

Zygocactus could reduce flower abscission, even under the exposure of ethylene. Ichimura and Hiraya (1999) found using STS could induce early flowering, increase floret size, and prolong flower longevity, especially with the addition of sucrose. Davood (2015) found applying STS to *Dianthus caryophyllus* flowers could extend their vase life by inhibiting bacteria growth, and increasing the antioxidant activities. Similar results were also observed in Roses (Reid et al., 1989, Liao et al., 2000), Lilium (Vanmeeteren and Deproft, 1982), and Estoma (Shimizu and Ichimura, 2005).

1.6 Regulation of Ethylene Response in Cassava

Cassava is a staple food crop in the world, mostly consumed in Africa. Improving cassava production quantity and quality could possibly solve hunger problems for millions of people in Africa. However, cassava has very poor flower set. Late flowering and short floral longevity has become a major obstacle for breeding projects. To improve cassava flower set, scientists are trying numerous kinds of methods, including PGR applications. Hyde and Setter, et al. (personal communication) have been conducting experiments with all different PGRs to find those that would work for improving cassava flowering. With all the tests, STS was identified as having promise. Specifically, STS improved the number of flowers/buds produced and their longevity. Their work did not indicate a

significant effect of STS on flower induction or the time until flowering began. It prevented cassava flower/bud abortion from ethephon treatments. It was proposed that STS could be used to improve cassava flower set and benefit breeding. However, there are still questions remaining. For example, when is the best timing to apply STS? To what area on the plant (cassava) should STS be applied? Where is the actual STS functioning place on cassava? To answer these questions, more detailed investigations are needed.

Objectives

Building on the findings of Hyde et al. (2016a), the objectives of this thesis research was to determine factors influencing the effectiveness of STS to improve cassava flowering. My focus was on cassava flowering processes after flower induction because previous work in cassava did not indicate that STS induces earlier flowering (Hyde et al., personal communication). This thesis addresses several questions, including: 1. What is the best timing for STS application? 2. What methods of STS application are most effective (foliar spray or swab/wick)? 3. What part of the plant is the best place to apply STS? 4. How many times of application is best for cassava flowering retention? 5. What is the best concentration of STS that we should be using for to improve flowering in cassava? 6. Does STS affect a signaling response that regulates flower retention in cassava that is confirmed as ethylene-induced?

Resolution of the above questions will provide us a better understanding on cassava flowering mechanisms, and a more detailed method to improve cassava flowering.

MATERIALS AND METHODS

Plant Material and Growth Environment

Four cassava genotypes were initially used: TME 204 (204), TMEB 419 (419), NASE 3 (N3; which is essentially the same as TMS 30572), from the biotechnology unit of the National Crop Resources Research Institute (NaCRRI), Namulonge, Uganda, and TMSI980002 (0002) from the cassava germplasm unit of the International Institute for Tropical Agriculture (IITA), in Ibadan, Nigeria. After preliminary tests of these genotypes, further experimentation was with 0002 only, as it flowers earlier and is less susceptible to STS phytotoxicity. The genotypes 204 and 419 are very late flowering and moderately sensitive to STS phytotoxicity; N3 is medium early and is highly susceptible to STS phytotoxity. Cassava were planted from stakes into 11-L pots filled with soil-less growing media containing peat-vermiculite-perlite mix, consisting of 60% peat moss, 20% vermiculite, 15% perlite, 2.2% finely crushed dolomitic limestone, 0.1% wetting agent (AquaGro 2000G, Aquatrols, Paulsboro, NJ), and 2.2% 10-5-10 Jacks Pro Media mix plus III (J.R. Peters, Inc., Allentown, Pennsylvania, USA), by weight (approximately 1:1:1 ratio peat:vermiculite:perlite, by volume). Plants were grown in Guterman Greenhouse, Cornell University, Ithaca, New York. The plants were maintained in greenhouses with natural light supplemented with illumination from either

400 W high pressure sodium lamps or 400 W metal halide lamps, as specified for each experiment below. Lamps were in rows 190cm apart, and lamps were spaced 80cm apart within rows; lamp height above the floor was 220cm. Lamps provided 14-h photoperiod, and thermostats had a set-point of 30°C (day)/25°C (night) , which regulated steam-heating, exhaust fans, and evaporative cooling pads. During the whole time-frame of maintenance and experimental treatments, plants were watered as needed and fertilized weekly.

Plant Growth Regulators and Methods of Preparation

Silver Thiosulfate (STS) Solution

To prepare stock solutions of STS, silver nitrate ($AgNO_3$; Sigma-Aldrich, St Louis, MO) was diluted into distilled water to make 0.1 M silver nitrate, and sodium thiosulfate ($Na_2S_2O_3$)(Sigma-Aldrich, St Louis, MO) was diluted into distilled water to make 0.1 M sodium thiosulfate solution. Then the sodium thiosulfate solution was shaken while slowly adding silver nitrate solution, with a 1:4 molar ratio of Ag:thiosulfate, resulting in an STS stock containing 0.02 M silver thiosulfate. STS treatment solutions (Ag in STS at 0.25, 0.50, 0.75, and 1.0 mM, as needed for each study) were prepared by diluting the 0.02 M STS stock with the appropriate amount of distilled water, and Tween 20 surfactant

was added to create a 0.1 % (w/v) final concentration.

Ethephon solution

Ethephon (2-chloroethylphosphonic acid, Ethephon 2SL, Makhteshim Agan of North America, Inc., Raleigh, NC 27604) solution with 500 ppm (w/v) ethephon was prepared in distilled water.

Methods of Applying PGRs

Overall Foliage Spray Method

PGR solutions (STS or ethephon) were put into a compressed-gas sprayer or a spray bottle (size selection based on the volume of the solution). Plants were grouped into an isolated area to avoid contamination to other plants. PGR solutions were sprayed to all exposed foliage until solutions drenched the leaf surface and began dripping down from the leaves (generally about 100 mL per plant). After spraying, plants were isolated in that area 24 hrs to allow thorough PGR absorption.

Apex Spray Method

This method was designed to target the application to the shoot apex area and its cluster of folded unexpanded leaves surrounding the shoot apical meristem. Aluminum foil ($15\times15cm^2$) was wrapped around the apex system in a upside down cone shape (vortex on the bottom attached to the

stem below the apex). The narrow vortex of the cone was tied to the stem beneath the apex to avoid excess solution drainage onto underlying stem and foliage. PGR solutions were sprayed to the apex system inside of the cone. After spraying, a polyethylene plastic bag was put on the apex system and tied to maintain the humidity inside the bag. The plastic bag was removed after 24hrs.

Cotton Swab Method

The cotton swab method was designed to apply STS or ethephon solution directly at the meristem (apex) or the inflorescence system. Generally, a piece of cotton (5×4cm) was soaked with PGR solution, then wrapped around the target area of application. A plastic bag was then put on the cotton swab and tied at the base to maintain high humidity inside the bag. The plastic bag and cotton swabs were removed after 24hrs.

Flowering Data Recording

Flowering data were recorded weekly or twice per week, as indicated below for each experiment. Counts of floral parts only included non-senesced organs. The date of fork-type branching at the apex was recorded when two or more branches or a cluster of flower buds were first observed. At each date of observation, the following were recorded: 1) number of flower stalks arising off the fork

surface of the stem, 2) length of the longest flower stalk, 3) number of flower buds which exceeded 1 mm in diameter and were not showing signs of senescence, 4) number of open female and male flowers, 5) number of fruits. Duration of floral development for each fork (and associated set of inflorescences) was calculated as the number of days between forking/flower appearance and absence on non-senesced flowers. In some studies (indicated below), the date of inflorescence abortion was scored when ≤ 20% of the maximum flowers remained non-senesced. The inflorescence stalk number was measured by assessing the number of inflorescence stalks in each apex. Among all inflorescence stalks, the inflorescence lengths were measured in cm by a ruler from the bottom of the inflorescence stalk to the bottom of the highest bud located on this stalk. The number of buds was assessed weekly or twice per week (depending on the experiment) by counting the number of non-senesced buds whose diameter was greater than 1 mm in all inflorescence stalks.

Experimental Design and Statistical Analysis

Experiment One: STS sprayed onto mature leaves vs. apices

Plant Material and Growth Environment

TMSI980002 was selected as the cultivar for this experiment because previous work at IITA

indicated it initiates flowering relatively soon after planting, but it usually aborts floral development in the first and second tiers of forking fairly soon after flower appearance. Clones were planted from stakes on Feb. 4th 2015 in 3 gallon pots filled with soilless rooting media. The plants were maintained in greenhouses with natural light supplemented with illumination from 400 W high pressure sodium lamps as described above.

Treatments

A total of three treatments were applied in this experiment, including: 1.Applying STS to old leaves only ($STS_{-Leaves}$). Applications were made with a 1L spray bottle. In this treatment, a plastic bag was used to cover the young apexes to avoid them being sprayed; 2. Applying STS to the apex only (STS_{-Apex}). A piece of aluminum foil (approximately 20 × 20cm), was used to wrap around the apex with the bottom tied up to avoid the liquid flowing to the mature leaves, and spray was made within the apex area. 3. A water control. The STS treatments were applied with STS at 0.25mM every two weeks for 5 times. Preliminary work indicated that a frequency of treatment every two weeks was necessary to avoid severe leaf tissue burning caused by silver ion. More frequent applications would result in more phytotoxicity.

Measurements

Flowering data included the number of buds, number of inflorescence stalks, inflorescence length, number of female and male flowers, number of fruits, and duration of floral development were measured

Experimental Design and Statistical Analyses

This study was conducted as a completely randomized design with four replications. All data were subjected to analysis of variance using the MIXED procedure in the Statistical Analysis System software v. 9.4 (SAS Institute Inc., Cary, NC). Treatment means were separated using Fisher's Protected Least Significant Difference test (p=0.05).

Experiment Two: Ethylene Production

Plant Material and Growth Environment

Four genotypes were used in this experiment: TME 204, NASE 3, TMEB 419, and TMSI980002. Plants were grown in greenhouse conditions as described above with an average of 28°C between 6 am and 6 pm (day) and 21°C between 6 pm and 6 am (night).

Treatments

There were three treatments in this experiment:1). AVG (Aminoethoxyvinylglycine, Retain®, Valent U.S.A. Corporation., Walnut Creek, CA) with 100 mL 250 ppm (w/v) AVG active ingredient. 2). STS (100 mL 0.5 mM STS and 0.1% (v/v) Tween 20 liquid); and 3). Water control. Each treatment was prepared with 0.1% (v/v) Tween 20 liquid). All the treatment solutions were applied by spraying 100 mL of each to the exposed foliage with a compressed-gas sprayer (Solo, 5100 Chestnut Avenue Newport News, VA 23605) with a 0.14 MPa (21 psi) constant flow valve. Applications were sprayed 75 days after planting (DAP), before any plants had forked or flowered. Treatments were reapplied on 89, 103, and 117 DAP.

Ethylene Analysis

Glass test tubes (34 mL) to which a half-piece of filter paper (diameter 70 mm) (Whatman 1001-070) was folded and inserted, and 1 mL of distilled water was added, were sealed with rubber septa (diameter 13.8 mm, Fisher Scientific Co). One or two leaf lobes (depending on the size of the lobe) from a mature leaf were excised with a razor blade, sealed into glass test tubes. Tubes were sealed with rubber septa and incubated at room temperature (about 22°C) in the dark for 12 hours. Then ethylene concentration was measured using gas chromatography. One mL of gas was extracted from

the sealed test tube through rubber septa and injected into a gas chromatograph (Buck Scientific, Model 310, Norwalk CT) fitted with an alumina column with flame ionization detection (FID). The FID signal from the chromatograph was calibrated by injecting 1 mL of standard ethylene gas in air with known ethylene concentration in units of μL/L. After measurement, leaf material was dried at 60°C and weighed. The rate of ethylene production was determined by multiplying the ethylene concentration (μL/L) of the analyzed aliquot by the volume of the test tube (0.033 L), and dividing by the incubation time (12 hrs), and the fresh weight (g) of the plant material.

Measurements

Flower data was also measured and recorded in this study by other lab members and will be reported elsewhere; however, in this thesis we only include ethylene production data.

Experimental Design and Statistical Analyses

The study was conducted as a completely randomized design with four replications. All data were subjected to analysis of variance using the MIXED procedure in the Statistical Analysis System software v. 9.4 (SAS Institute Inc., Cary, NC). Treatment means were separated using Fisher's Protected Least Significant Difference test (p=0.05).

Experiment Three: Ethephon Effect

Plant Material and Growth Environment

Plants of cultivar 0002 were grown in two matched growth cabinets (Model 37, Sherer Controlled Environment Chambers, Marshall, Michigan) with 122 cm (wide) × 76 cm (depth) × 116 cm (height) internal dimensions. Chambers were illuminated with sixteen 48-Watt fluorescent lamps (model F48T8 HO, Phillips) and twelve 26-Watt fluorescent lamps (model ELmdT2, minidecorative twister, 4100 K, Phillips) such that the photosynthetically active radiation (400-700 nm) was 300 μmol m^{-2} s^{-1} at the top of the plant canopy; day-length was 12 hours. Temperature was set to 30°C day and 25°C night. Plants were grown in 11-L pots with soilless rooting media and weekly fertilization, as described above. For this experiment it was necessary to have a batch of plants that had formed flower buds and were retaining them in the controls. The plants in these growth chambers were in the second tier and satisfied this criterion.

Treatments

STS solutions were prepared at a concentration of 0.25mM, and the ethephon concentration was 500 ppm (w/v). The treatments were commenced when flower buds were visible, as follows: 1) Plants were sprayed with STS only; 2) Plants were sprayed with STS and followed 2 and 6 days later by

ethephon application; 3) Plants were treated with ethephon only on days 2 and 4, and 4) Control (no STS and no ethephon applied). All the data were recorded the day before and the day after the treatments.

Measurements

The flower senescence status (whether flowers were senesced or not and whether they had abscised) and the number of floral buds were measured one day before the STS treatment, and 4 days after the final ethephon treatment.

Experimental Design and Statistical Analyses

The study was conducted as a 2 × 2 factorial completely randomized design with ten replications. All data were subjected to analysis of variance using the MIXED procedure in the Statistical Analysis System software v. 9.4 (SAS Institute Inc., Cary, NC). Treatment means were separated using Fisher's Protected Least Significant Difference test ($p \leq 0.05$).

Experiment Four: STS Applied with Cotton Swab

Plant Material and Growth Environment

Cultivar 0002 was used 10 days after first tier forking for this experiment.

Treatments

Treatments included: 1) STS at 0.25 mM using cotton swab method; 2) Water control.

Measurements

Flowering data include number of buds, number of inflorescence stalks, inflorescence length, number of female and male buds, number of fruits, duration of floral development were measured.

Experimental Design and Statistical Analyses

The study was conducted as a completely randomized design with four replications. All data were subjected to analysis of variance using the MIXED procedure in the Statistical Analysis System software v. 9.4 (SAS Institute Inc., Cary, NC). Treatment means were separated using Fisher's Protected Least Significant Difference test (p=0.05).

Experiment Five: STS Timing with Cotton Swab

Plant Materials and Growing Conditions

The cultivar 0002 was used for this experiment. Plants were grown to the stage of first tier forking, after which treatments were begun.

Treatments

To understand the best timing of STS application, using the cotton swab method, a total of four treatments were designed, where four weekly applications were given and they differed in the time when STS applications commenced (and concluded): 1). Early (applying STS 24 to 27 d before flowering); 2). Medium Early (applying STS 20 to 23 d before flowering); 3). Late (applying STS 13 to 16 d before flowering; 4). Control (applying water only).

Measurements

Flowering data include number of buds, number of inflorescence stalks, inflorescence length, number of female and male buds, number of fruits, duration of floral development were measured.

Experimental Design and Statistical Analyses

The study was conducted as a completely randomized design with four replications. All data were subjected to analysis of variance using the MIXED procedure in the Statistical Analysis System software v. 9.4 (SAS Institute Inc., Cary, NC). Treatment means were separated using Fisher's Protected Least Significant Difference test (p=0.05).

Experiment Six: STS Multiple Application Test

Plant Materials and Growing Conditions

Cultivar 0002 plants were treated with STS after they had reached the first fork, and flowering data were collected on the second fork.

Treatments

This study was conducted to determine how many STS applications are necessary to extend cassava flower retention time. Therefore, a total of four treatments were given using the cotton swab method, each timed so that the last treatment was applied 2 to 3 weeks before flower appearance : 1). Zero applications (control); 2). One application; 3). Two applications, one week apart; 4). Three weekly applications. All STS solutions were made at 0.25mM concentration. The application was conducted using cotton swab method as described above.

Measurements

Flowering data include number of buds, number of inflorescence stalks, inflorescence length, number of female and male buds, number of fruits, duration of floral development were measured.

Experimental Design and Statistical Analyses

The study was conducted as a completely randomized design with four replications. All data

were subjected to analysis of variance using the MIXED procedure in the Statistical Analysis System software v. 9.4 (SAS Institute Inc., Cary, NC). Treatment means were separated using Fisher's Protected Least Significant Difference test (p=0.05).

Experiment Seven: STS Application Placement Test

Plant Materials and Growing Conditions

Cultivar 0002 plants were treated with STS after they had reached the first fork, and flowering data were collected on the second fork.

Treatments

The aim of this study was to understand whether directly applying STS to the inflorescence would improve STS efficacy as indicated by an increase in flower retention time. Therefore, a total of four treatments were designed where in each case up to six weekly applications were given, depending on the treatment plant received: 1). Applying STS solution to the immature folded leaves and enclosed organs of the shoot apical region until inflorescence stalks grew out and were about 3 cm long such that they could not be wrapped into one cotton piece. Applications were made 4 times, starting 2 to 4 weeks before flowering; 2). Initially applying STS to the apical region, but when

forking occurred, only applying to inflorescences. Applications were made 6 times, starting 3 to 8 weeks before flowering; 3). Applying STS to the apical region only; after the forking event, apply to the newly developed apical regions on each of the new branches. Applications were made 6 times, starting 3 to 4 weeks before flowering; 4). Water Control. All STS applications were made using cotton swab method, and STS concentration was 0.25mM. Application started after the 1st tier forking event occurred, as the 4th node on the second tier branch formed.

Measurements

Flowering data include number of buds, number of inflorescence stalks, inflorescence length, number of female and male buds, number of fruits, duration of floral development were measured.

Experimental Design and Statistical Analyses

The study was conducted as a completely randomized design with four replications. All data were subjected to analysis of variance using the MIXED procedure in the Statistical Analysis System software v. 9.4 (SAS Institute Inc., Cary, NC). Treatment means were separated using Fisher's Protected Least Significant Difference test (p=0.05).

Experiment Eight: STS Concentration Test

Plant Materials and Growing Conditions

Cultivar 0002 plants were treated with STS after they had reached the first fork, and flowering data were collected on the second fork.

Treatments

Three STS treatments using the cotton swab method were used in this experiment: 1) Apply STS at 0.25 mM; 2) Apply STS at 0.5 mM; 3). Water control. We had 4 replicates for each treatment. All treatment were applied using cotton swab method, and plants were treated weekly for a total of 6 weeks.

Measurements

Flowering data include number of buds, number of inflorescence stalks, inflorescence length, number of female and male buds, number of fruits, duration of floral development were measured twice weekly.

Experimental Design and Statistical Analyses

The study was conducted as a completely randomized design with four replications. All data were subjected to analysis of variance using the MIXED procedure in the Statistical Analysis System

software v. 9.4 (SAS Institute Inc., Cary, NC). Treatment means were separated using Fisher's Protected Least Significant Difference test (p=0.05).

RESULTS AND DISCUSSION

Cassava breeding is hampered because many of the most desirable lines for storage root production have sparse flowering and so cannot be used as parents in crosses (Ceballos et al. 2004; Ceballos et al. 2015; Ceballos et al. 2016). Previous studies have shown that cassava flowering is improved by STS when it is applied as a whole plant foliar spray (Hyde et al., 2016a). However, large scale application of silver has potential disadvantages in regulating flowering in cassava breeder's nurseries. Excess use of STS might result in silver residue in soil, leading to environmental contamination. And at high rates, silver causes plant tissue death due to phytotoxicity. Therefore, an improved way of application, in which silver use is more limited would be preferred for future applications, especially in large scale field experiments. Since the target area is the shoot apical region, where flower initiation and development take place, we hypothesized that STS benefit on cassava flowering involves STS disruption of ethylene signaling at the shoot apical meristem. Such application to a small zone at the apex might allow more efficient and effective use of STS as a growth regulator for cassava flowering. My objective was to test this hypothesis, and develop and develop a method for application in breeding programs.

Experiment One. Leaves vs. Apex

Previous studies have shown that when STS is applied to mature leaves silver is transported to sink leaves in the apex (Nichols and Kofranek, 1982, Hyde et al., 2016a, Hyde and Setter, personal communication). According to our hypothesis, foliar-applied silver could be transported to the apical region and affect flower development there. If so, perhaps directly applying STS to the apical region would be equally efficient. Therefore, in this experiment, we tested if directly applying STS to the apex region would serve as an alternative way to improve flower retention on cassava.

Our findings indicated that inflorescence length was increased significantly ($P \leq 0.05$) with both the $STS_{\text{-Leaves}}$ and $STS_{\text{-Apex}}$ treatments compared to the control (Table 1). For the maximum (peak) number of buds, the $STS_{\text{-Leaves}}$ treatment had five times more ($P \leq 0.05$) flower buds than the control, and the $STS_{\text{-Apex}}$ treatment averaged 34 buds compared to 10 in the control, though this difference was not significantly different ($P \leq 0.05$). The lack of statistical significance in this case might due to the lack of precision of the bud number data as several individual plants were severely burned due to STS phytotoxicity, increasing variability. Both the $STS_{\text{-Leaves}}$ and $STS_{\text{-Apex}}$ treatments significantly ($P \leq 0.05$) increased flower longevity to values of more than four times longer than controls (Table 1, Duration). The $STS_{\text{-Apex}}$ treatment had significantly ($P \leq 0.05$) longer duration than the $STS_{\text{-Leaves}}$ treatment. Hence,

considering all three measures of flower development, the STS_{-Apex} treatment, which involved applying STS to a small zone in the apex, was at least as effective, or more so, than application to the much larger surface area of the fully expanded and mature leaves ($STS_{-Leaves}$). Given that STS_{-Apex} treatment only used about 10% as much silver as was used in the $STS_{-Leaves}$ treatment, the result suggests that STS_{-Apex} might be a better way for field STS applications considering the more efficient usage of STS and its minimal environmental effect (less chemical residue).

Experiment Two. Ethylene Measurement

Previous studies have indicated that silver ion, the active ingredient in STS, blocks ethylene signaling by displacing copper ion from the ethylene receptor and by doing this, preventing ethylene binding by the receptor (Rodrígez et al., 1999). Studies of factors influencing ethylene synthesis have shown that in some plant species and organ/tissues, ethylene is controlled by a positive feedback loop that operates as an accelerator of ethylene synthesis when initially-produced ethylene is detected (Liu et al., 1985, Inaba and Nakamura, 1986, Petruzzeli et al., 2000). This is the regulatory system that operates in climacteric fruits that have a burst of ethylene synthesis when ethylene synthesis is initiated. In other plant species and tissues, ethylene synthesis is controlled by a negative feedback

loop that operates to suppress the rate of ethylene synthesis when ethylene levels are initially elevated (Yang and Hoffman, 1984, Kende, 1993). The latter regulatory system tends toward homeostasis. To determine whether the effects of STS were due to changes in ethylene production, we determined the rate of ethylene synthesis in mature cassava leaves of 4 genotypes that were sprayed with STS. For comparison, we also measured the rate of ethylene production in mature leaves that were sprayed with aminoethoxyvinylglycine (AVG), which is a widely used inhibitor of ethylene synthesis. AVG inhibits the enzyme ACC synthase, which catalyzes the conversion of SAM to ACC, which limits the amount of ACC available to convert to ethylene (Boller et al., 1979, Yu and Yang, 1979, Abeles et al., 1992). This study involved four cassava genotypes to which AVG, STS or control treatments were applied. ANOVA showed there was not a significant ($P \leq 0.05$) treatment X genotype interaction. Therefore, only the main effect of the treatments is shown. As expected, AVG decreased ($P \leq 0.05$) the ethylene synthesis rate by about half (Fig. 1). In contrast, STS increased ($P \leq 0.05$) the rate of ethylene synthesis about 40%. It is known that AVG is an inhibitor of ethylene synthesis by deactivating ACC synthase, an enzyme that is necessary for the production of ethylene precursor, ACC (Boller et al., 1979, Yang and Hoffman,1984). AVG inhibition of ethylene synthesis has been documented in many plant systems, including apple (Silverman et al., 2004, Yuan and Li, 2008), plum (Jobling et al.,

2003), and citrus (Mullins et al., 1999). The stimulation of ethylene synthesis by STS in cassava leaves (Fig. 1) is consistent with ethylene production being controlled by negative feedback inhibition (Sisler et al., 1985, Bleecker et al., 1987, Kende, 1993). It is known that STS prevents downstream ethylene responses by blocking the ethylene receptors on plants. With feedback inhibition, one of the signaling pathways downstream of ethylene signaling, negatively regulates the further production of enzyme activity for ethylene synthesis. This prevents excess ethylene synthesis and modulates it to a more modest rate (Kende, 1993). By blocking the ethylene receptors, STS releases inhibition caused by the downstream ethylene responses, including feedback inhibition of ACC synthesis and/or ACC oxidase. Therefore the production of ethylene was increased. This finding indicates that cassava leaves regulate ethylene synthesis in a way similar to that found in several plant systems, including young pre-climacteric (green) tomato fruits (Nakatsuka et al., 1998, Atta-aly et al., 1987), pre-climacteric banana fruit (Inaba et al., 2007), non-climacteric citrus fruit (Mullins et al., 1999), and non-climacteric strawberry fruit (Atta-aly et al., 2000), where studies indicate there is feedback inhibition of ethylene synthesis. Hence, the favorable effects of STS in Experiment One occurred despite the treatment stimulating ethylene production in leaves. The linkage between ethylene responses and STS will be further examined in Experiment Three.

Experiment Three. Ethephon experiment

Experiment Two indicated that ethylene production increased when STS was applied. As a further test of the hypothesis that the favorable effects of STS on flower production and retention, which were observed in Experiment One, was due to an STS block of ethylene's signaling, we determined the effect of exogenously generated ethylene production, and the effect of STS in this situation. Ethephon is an organic compound that releases ethylene, and it is widely used in horticulture and floriculture to serve as an ethylene generator (Davis et al., 1991). In this experiment, we monitored and recorded bud survival rate under the influence of ethephon and STS applications. First, one group of plants was randomly selected and sprayed with STS, while the other was not. Two days after the STS application, one branch in each plant was randomly selected, sprayed with ethephon and sealed in a plastic bag. Treatments were assigned as described in the Materials and Methods. In this experiment, since our target was the apex, we applied ethephon directly to the apex using a cotton swab to hold the solution in place. As shown in Fig. 2, inflorescence survival rate on plants in the group that was treated with ethephon was substantially decreased ($P \leq 0.05$) compared to controls, indicating that ethephon induced inflorescence abortion. The plants that were only treated with STS had a higher inflorescence survival

rate than the plants which received the control treatment (P≤0.05). For the group that received STS first, followed by ethephon treatment 2 d later, the inflorescence survival rate was substantially higher (P≤0.05) than the plants given ethephon only, and was about the same as the STS-only plants. These data are consistent with the interpretation that STS protected the inflorescences from abortion induced by ethephon-generated ethylene.

The plants in Experiment Three were about three months old and in the 2nd tier flowering stage, which in general produces more flower buds with a higher bud survival rate than the 1st tier flowers (Hyde and Setter, personal communications; Perera et al, 2013). The plants in the control group showed a 70% inflorescence survival rate, which is relatively high compared to controls in other experiments (Table 1). Plants were grown in a growth chamber with cool temperature, high fertility and favorable growing conditions that might have contributed to the high inflorescence survival of controls. There was not a significant (P≤0.05) improvement in flower inflorescence number with the STS-only treatment in this study, probably because the STS-only treatment was applied to some plants on the day of fork and bud cluster appearance and in others just 1 d before forking, which did not give it sufficient time to affect bud formation. However, the STS pre-treatment in advance of ethephon application was able to maintain high survival in the face of ethephon-generated ethylene.

These findings indicate that ethylene results in flower bud abscission and loss of flower longevity, and STS protects flower buds from such effects of ethylene.

Ethephon has been used as an ethylene generator since the 1960s (Davis et al., 1991). Upon hydrolysis under acidic environments it releases ethylene (Abeles et al., 1992). It is widely used to generate ethylene in floriculture, horticulture, pomology, and ornamental crops and is used for many growth regulatory purposes (Abeles et al., 1992). In this experiment, our findings indicate that ethephon-generated ethylene, elicited inflorescence abortion. When ethylene receptors were blocked by pre-treatment with STS, the inflorescences were protected from ethylene-induced abscission and senescence.

Experiment Four. Cotton Swab

Studies have indicated that STS is transported via xylem and phloem vascular systems in plants (Nichols and Kofranek, 1982). Our results from Experiment One showed that spraying STS to the young leaves in the apex area was equally effective as spraying STS to the mature leaves. However, one shortcoming of this method of spraying on the apex is the phytotoxicity it induces to the young leaf and inflorescence tissues, which happened to the plants in Experiment One. It is possible that

phyotoxicity is affected by the liquid residue that remains after spraying and dry-down on this liquid, thereby concentrating STS. In an attempt to limit the concentrating effect of dry-down, and perhaps reduce the phytotoxicity induced by directly spraying to the apex systems, STS was applied using an STS-drenched cotton swab. This method also has the potential advantage that it facilitates movement of the STS solution to regions inside the cluster of folded leaves and to the inner apical tissue via wicking and capillary flow. As is shown in Table 2, using a cotton swab to apply STS increased inflorescence length more than ten times compared to the water-treated controls. It also produced twice as many buds as the controls. The duration over which at least some flowers remained non-senescent was 33.5 d with STS and 23 d in the control, which was not statistically different ($P \leq 0.05$). Based on this study, it appeared that localized STS application to the apex region using a cotton swab improved flower development. Based on visual observation, the cotton swab treatments showed much less burning on the plants compared to the previous experiment where STS was sprayed directly onto leaves. Perhaps STS applied using STS-drenched cotton swab is slowly but continuously transported to the apical meristem, by capillary flow like a wick. Leaves in the apex area would take up adequate STS to provide it to the inflorescence without excess absorption, thus limiting phytotoxicity and burning.

Experiment Five: Timing Study

Previous experience with STS in cassava suggested that if treatments began less than a week before the appearance of forks/flowers, it was ineffective (Hyde et al., 2016a). To conserve STS and avoid phytotoxicity, an appropriate timing of application is desired. Since the actual flowering date is hard to predict, and to ensure on-time application, studies were conducted to test the effect of various timings of STS application, starting several weeks before flower appearance. In this experiment, timing treatments were early, medium, and late. Each treatment had a total of 4 weekly STS applications using the cotton swab method. All treatments were started before the actual flowering date. On average, the early treatment was applied between -25 and -4 d from flowering, the medium treatment was applied between -22 and +1 from flowering, and the late treatment was applied between -15 and +7 d from flowering (Table 3). Compared to the control treatment, both the medium and late treatments increased ($P \leq 0.05$) all measures of flower development: inflorescence length, number of flower buds, duration of non-senescent flowers, the number of open flowers, and the integral of time and flower numbers.

In general, the early treatment was not discernibly different from the control. In this study the duration of floral development was calculated as the period from bud appearance to the time where

<20% of peak number of buds remain on inflorescence stalk. This method of calculating flower longevity (duration) differed from the method used in the previous experiment where it was calculated as the time from flower appearance until the complete absence of non-senescent flowers. The method used in the present experiment is less prone to spurious/anomalous flowers which can add to experimental error.

We also calculated the flower development integral, which is the area under the curve of buds-count with respect to development time (Figure 3). The integral is a putatively more stable measure of flower development because it utilizes all of the week-by-week measurements, rather than only the single point for the maximum number of buds, and individual time-points for the start and end to obtain the duration. As shown in Table 3, flower development integral was 8- to 10-fold greater in the late and medium-late treatments than in the control ($P \leq 0.05$).

In contrast to the medium and late treatments, the early treatment did not significantly ($P \leq 0.05$) increase any of the measures of flower development. The early treatment began between 24 and 27 d before the actual flowering date (average of 25 d before flowering), and ended between 3 and 6 d before flowering (average of 4 d before flowering). It is possible that when STS is applied too early, before the flowering date, it is transported to transpiring leaves that are positioned below the fork and

inflorescences such that STS is used up and there is not sufficient STS remaining to be transported to the inflorescence. In contrast, in the medium and late treatments the last application was after flowering such that the developing flowers could benefit more effectively. This experiment indicates that STS applications that start too early and are not continued through the time of flower appearance are not as effective as treatments that start between 15 and 22 d before flowering and extend to 1 to 7 days after flowering. These results suggest that when using the cotton swab method on the apex region, a continuous supply of STS is needed through the early flower-appearance stage if we are to affect flower development and retain flowers.

Experiment Six: Multiple application test

The evidence in Experiment Five indicated that to be effective the timing of STS application needs to provide STS through the period leading up to, and extending shortly after initial flower appearance. Since the time of flower appearance is highly variable and not known, it is valuable to apply multiple times to ensure a favorable effect of STS on cassava flowering. However, with multiple doses, STS buildup might induce tissue burning, as well as waste and result in loss of silver to the environment. To find an ideal number of applications that would ensure STS's effect on cassava flower retention, an

experiment was designed, with one, two or three weekly applications. The intention was to time these applications so they would begin shortly before flowering. However, given the uncertainty of flowering time, the treatments were timed rather early, well in advance of flowering, ranging from 1) 13 d before (single application), 2) 26 to 19 d before (two applications), and 3) 30 to 16 d before flower appearance (Table 4). The results showed that these early and sparse applications did not have a significant ($P \leq 0.05$) effect on inflorescence length or flower bud production. However, all three STS treatments increased ($P \leq 0.05$) the duration of floral development by about 30%, and increased ($P \leq 0.05$) the integral of flower number x time by about 66%. This finding is illustrated in Figure 4 with a plot of flower bud counts over time. It shows that the STS treatments did not affect the peak number of buds, though it extended the time of bud longevity and the integral of bud counts x time.

As shown in Table 4, in all three treatments of Experiment Six the STS applications were completed well before flower appearance: 13, 19 and 16 days before flowering, in the treatments 1, 2 and 3, respectively. Based on the findings of Experiment Five, we can conclude that the timing of applications did not continue long enough to provide substantial benefit to flower development. In Experiment Five, the early treatment in which the last application was 4 d before flowering did not significantly improve flower development, whereas the medium and late treatments improved all

flower development traits (Table 4).

Experiment Seven. Placement Test

Given the results from Experiment Three and Four, I concluded that directly applying STS to the apex region using cotton swabs is an effective way to improve flower retention in cassava without causing excess phytotoxicity and creating unnecessary loss of silver to the environment. Since our STS target tissue is the inflorescence, we hypothesized that directly applying STS to the target region may be the most efficient mechanism for STS uptake by the target tissue in young inflorescences and flowers. Alternatively, transport of STS from the young leaves in the apex region to the inflorescence is an alternative mechanism. To test these hypotheses, this experiment tested four treatments: I) four weekly applications from 20 d before flower appearance until 4 d after flower appearance, II) the same as in I, (though started at 31 d before flower appearance), and with two additional applications after flowering to the developing floral parts, and III) the same as in I but with two additional applications after flowering and fork-type branching to the young developing leaves in the apex regions of the new developing branches. A water control was also applied. As shown in Table 5, inflorescence length was greater ($P \leq 0.05$) than control for Treatments I and III; Treatment II was not

significantly different from the control. For peak bud counts, all STS treatments had more ($P \leq 0.05$) buds than the control. The duration of floral development and open flowers count data indicated that Treatment III and I were both similarly effective, and had greater ($P \leq 0.05$) flower longevity than controls, while Treatment II was not significantly different from the control. The integral of flower numbers × time was substantially greater in all three STS treatments compared to the control. Treatment III had a larger ($P \leq 0.05$) integral than Treatment II, and Treatment I was intermediate and not significantly ($P \leq 0.05$) different from either Treatment II or III. Overall, the experiment indicated that applications of STS directly onto the floral parts (Treatment II) was not optimal for flower development. It is possible that directly applying STS to the inflorescence might have overwhelmed the floral tissues with STS solution on their exterior, and this might have caused malfunction or burn damage to the young floral parts. It is possible that directly applying STS to the inflorescence did not improve absorption and transport to the interior cells of floral parts that could provide benefit. In contrast, both of the treatments involving applications to the apex regions of the main shoot before floral parts emerged (I and III) were equally promising for retaining cassava flower development. Treatment III, which involved additional applications after flowering to the developing shoot apexes of branches appeared to be the best treatment based on its overall consistency for all measures of

flower development. This suggests that in this treatment, post-flowering STS application to apex regions of branches that develop on forks above the floral node might provide benefit. Given the extended time-frame of inflorescence development (Figure 6), absorption of STS by leaves on these branch shoots and transport from them to the developing inflorescences and flowers via phloem translocation might be an effective way to obtain extended duration of floral development.

Experiment Eight: STS Concentration

This experiment examined the effect of various concentrations of STS, applied using the cotton swab method on the retention of flower development. As shown in Table 6, the 0.5 mM treatment was significantly ($P \leq 0.05$) better than the control for all measures of flower development. The 0.25 mM was less effective than 0.5 mM; relative to the control, it did not significantly affect inflorescence length bud number or integral, but it significantly ($P \leq 0.05$) extended flower longevity (duration) and increased the number of open flowers. By visual observation, it appeared that applying STS at 0.5mM induced more phytotoxicity than 0.25mM STS. To ensure good effect on flower retention, spraying STS at 0.5mM would be recommended; however, care would be needed to avoid phytotoxicity. To find the optimum of STS dosage, investigations involving more STS concentrations will be needed.

GENERAL DISCUSSION

According to previous literature, silver ion is relatively non-translocatable within plants (Korfranek and Paul, 1972; Petterson, 1976). The positively charged silver ions would bind to the anion regions in xylem vessels thereby interfering with its uptake and transport processes (Bell and Biddulph, 1963; Veen and Van de Gejn, 1978). However, the mobility and rate of transport of silver ion is improved by complexing it with thiosulfate (Veen and Van de Geijn, 1978; Veen, 1983). In fact, STS moves much more freely within the xylem compared to silver nitrate (Veen and Van de Geijn, 1978). For the past a few decades, silver thiosulfate has been widely used as a plant growth regulator (PGR) that increases vase life of many plant species (Veen and Van de Geijn, 1978; Nichols and Korfranek, 1982; Cameron and Reid, 1983; Liao et al.,2000; Serek et al., 2006). The finding of STS protection from ethylene effects is consistent with those obtained in other plants systems such as rose (Serek, 1993; Liao et al., 2000), daisy (Serek et al., 1998), Zygocatus (Cameron and Reid, 1981), pea (Ichimura and Hiraya, 1999) and others (Veen, 1983; Serek et al., 1998).

In cassava, our laboratory's previous tests and recent findings also showed that STS could move up towards the meristem and receptacle tissues (Hyde et al. unpublished data, 2017), thus affecting ethylene signaling and flowering retention there. These studies involved tracing the movement of Ag^{+}

with determinations of tissue Ag content using inductively coupled plasma spectroscopy (ICP). In the current research, STS was shown to be effective whether it was applied to mature leaves or just to the young expanding leaves in the apical region (Exp. One). This is consistent with the mechanism of transport from leaves to the apical meristem via the phloem or xylem. However, application of STS to the apex required just one tenth as much STS. Studies in this thesis with the cotton swab approach were intended to apply STS in the location of the apex so that STS could move by capillary flow to the apical meristem.

CONCLUSION

Combining all the experiments above, it can be concluded that direct applications of STS, using the cotton swab method improves inflorescence and flower development so that there is more profuse flowering, and flowering occurs over longer durations such that inflorescences produced more flowers and fully mature flowers are produced before flower senescence. However, given the fact that we only tested these experiments on the genotype 0002, more testing should be done on other cassava genotypes before it is adopted for large scale usage in breeding programs. These studies showed that the timing of applications is critical: treatments should be started 15 and 22 d before flowering and continued to 1 to 7 days after flowering. Treatments started later or earlier than this time range, without continuous supply, might not be helpful for flower retention. These treatments improve the likelihood of successful cassava pollination by increasing the production of buds that do not senesce pre-maturely. Therefore, for large scale cassava breeding, STS could serve as a potentially valuable tool for breeders to use on parent plants before flowering takes place.

Figure 1. Ethylene production rate on cassava mature leaves after being sprayed with AVG (250 ppm), STS (0.5mM), and water control. Samples were incubated for 12 hours at room temperature (20-25˚C) before measuring. Letters represent means separation (P ≤ 0.05).

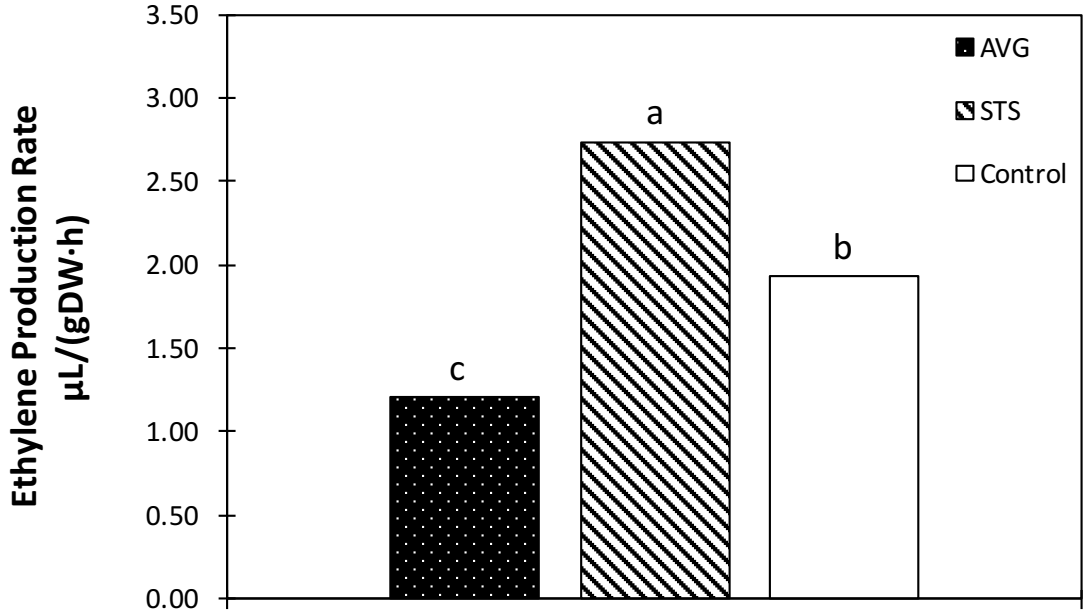

Figure 2.The survival rate of cassava buds at the 2nd tier fork after different STS treatments : 1) Control (no STS and no ethephon applied); 2) Applied ethephon only; 3) Sprayed with STS only; 4). Sprayed with STS and followed 2 days later by ethephon application.

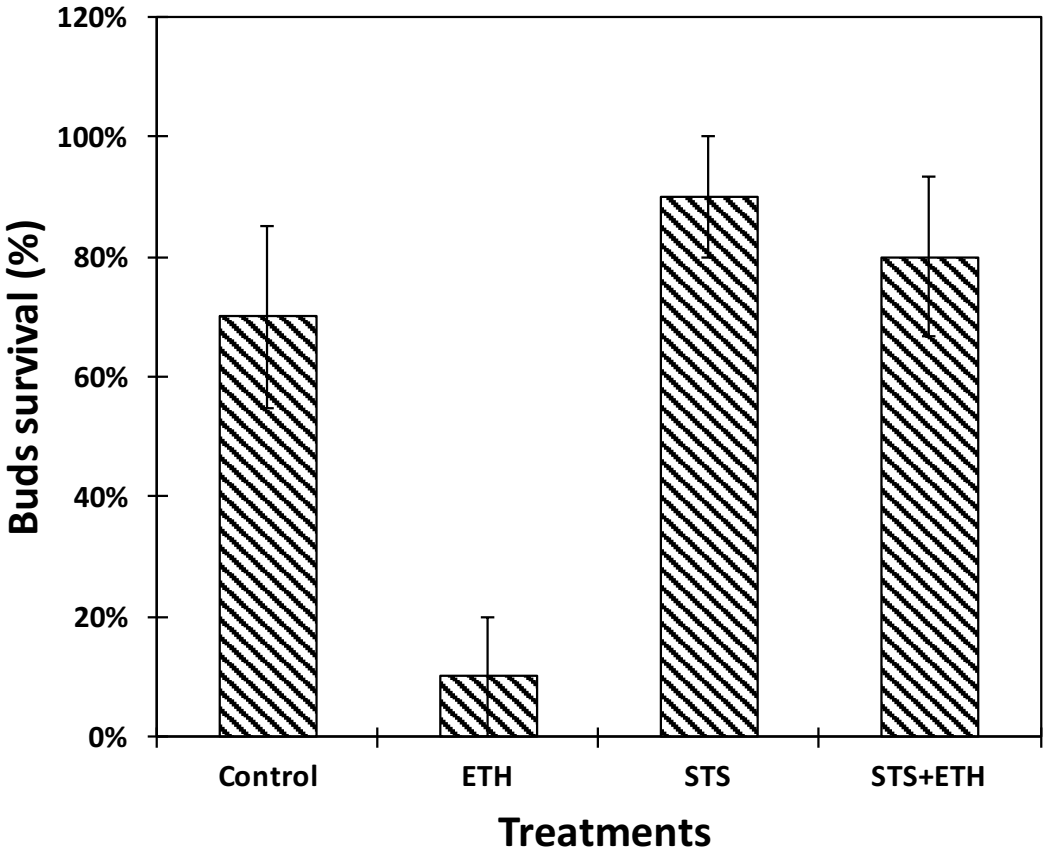

Figure 3. Flower-buds count with respect to development time after the 2nd tier forking event of cassava plants. STS was applied with different timing of STS treatments including: 1) Early; 2) Medium; 3) Late; and 4) Control.

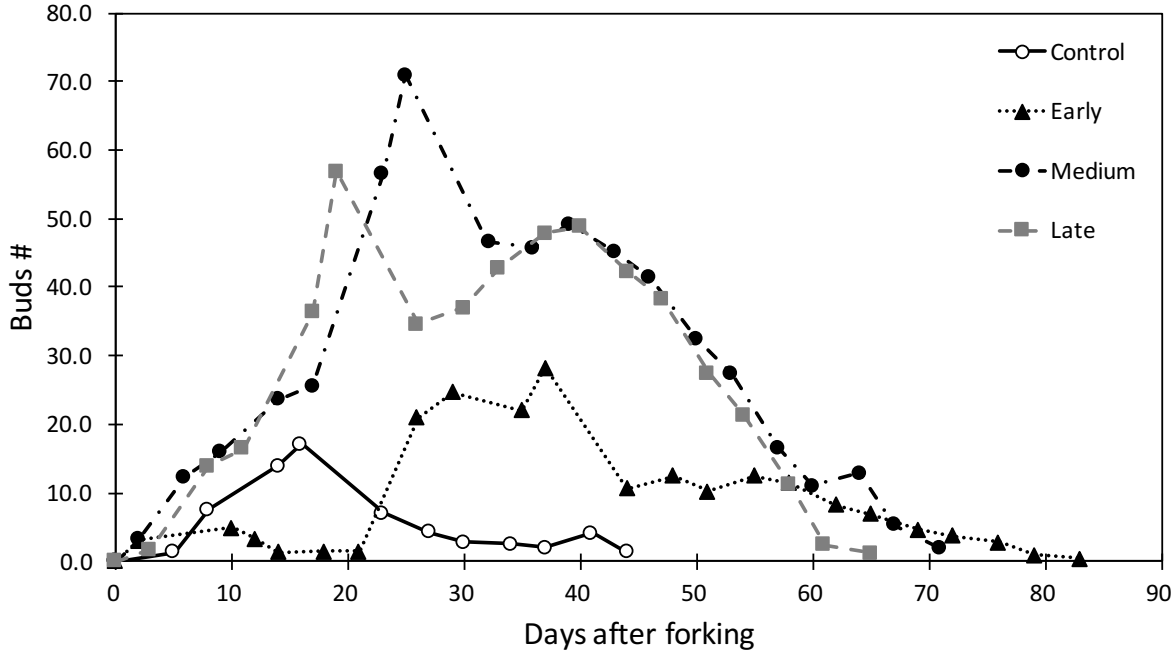

Figure 4. Flower-buds count with respect to development time after the 2nd tier forking event. Plants were treated with STS (0.25mM) for different times including: 1) Once (One); 2) Twice(Two); 3) Three times (Three) and 4) Control.

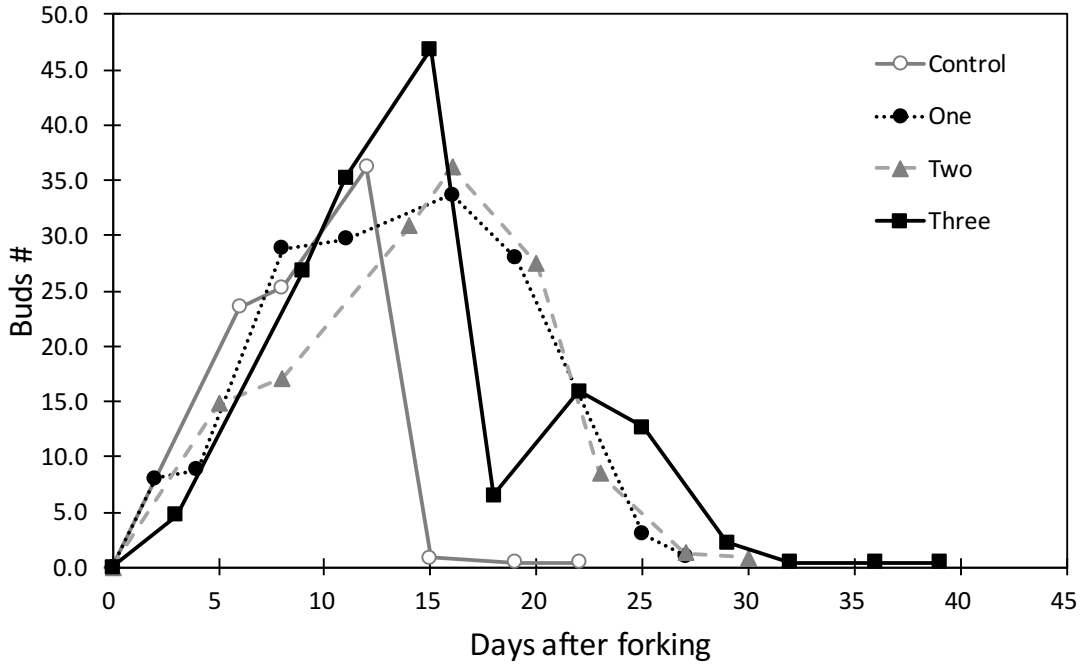

Figure 5. Flower-buds count with respect to development time after the 2nd tier forking event. Buds were counted on plants that were treated with STS at different locations, including: 1) Treatment I; 2) Treatment II; 3) Treatment III and 4). Control. (see text for details)

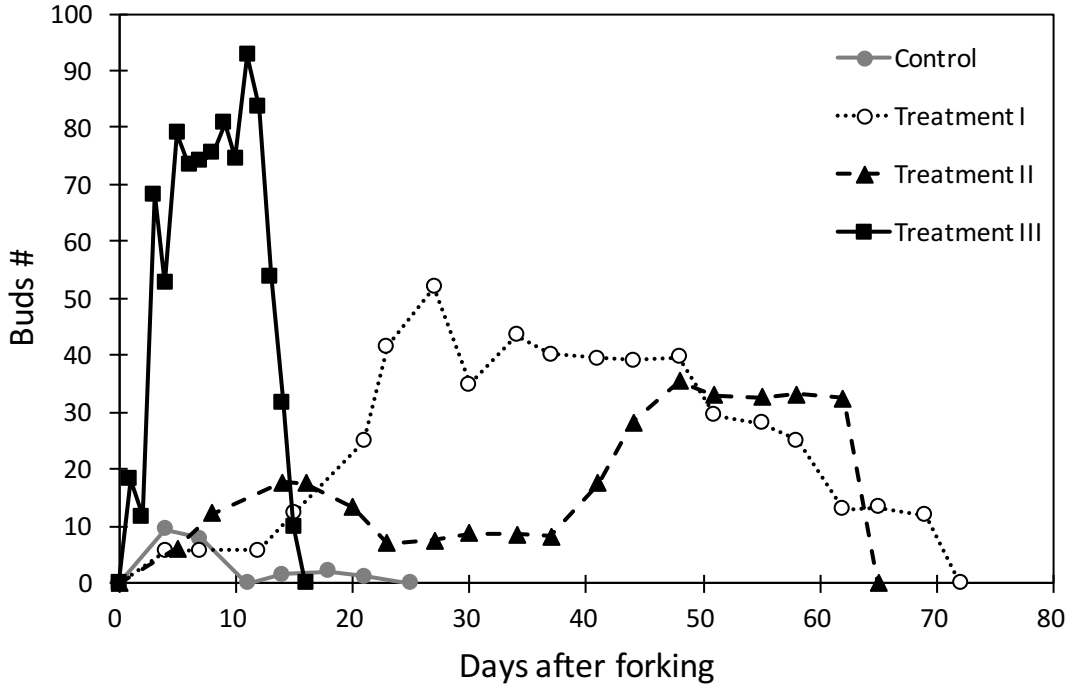

Figure 6. Flower-buds count with respect to development time after the 2nd tier forking event. Treatments: 1) Applying STS at 0.25mM; 2) Applying STS at 0.5mM, and 3) Water Control.

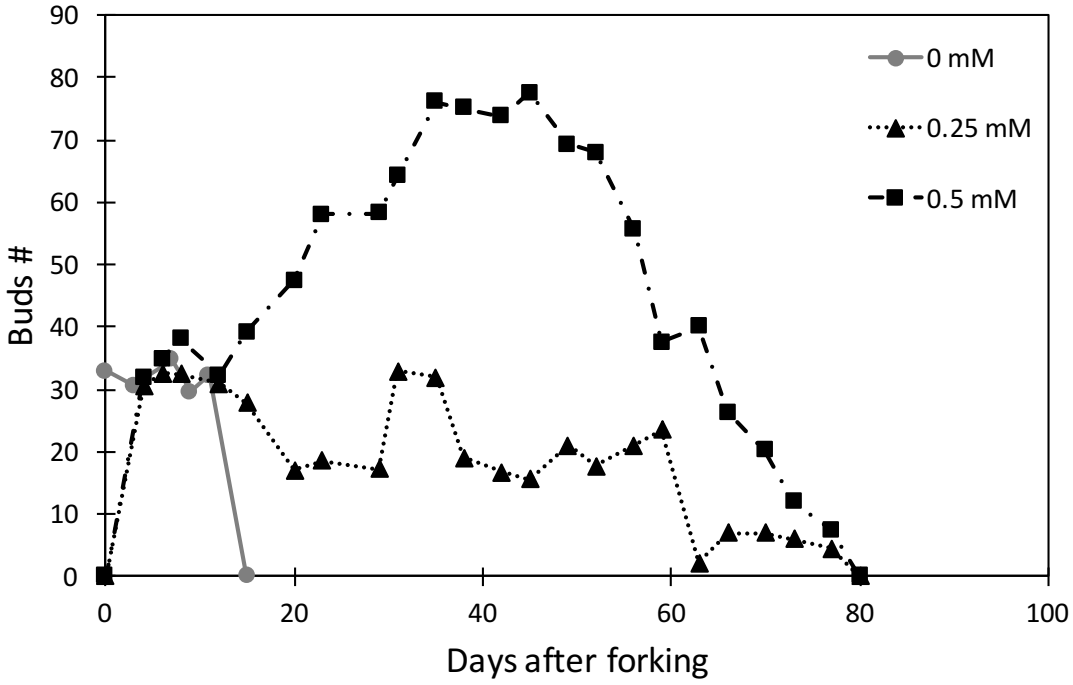

Table 1. Effect of STS treatments (Trt) applied to the young apex (STS_{-Apex}) vs. mature leaves ($STS_{-Leaves}$) on the length of flower stalks, maximum number of flower buds, and duration of flower development. Treatments began 1 to 2 weeks before flower appearance and were applied bi-weekly until 6 to 7 weeks after flower appearance.

Trt	Length	Buds	Duration
	— cm —	— # —	— day —
H_2O Control	1.4 b	10.0 b	15 c
STS_{-Apex}	9.5 a	34.8 b	72 a
$STS_{-Leaves}$	8.9 a	51.8 a	67 b
LSD‡	4.5	32	4
SE	1.4	7.7	1.2
	ANOVA		
	**	*	***

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability level, respectively. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to the multiple range test.

Table 2. Effect of STS treatment applied using the cotton swab method on the length of flower stalks, maximum number of flower buds, and duration of flower development. Treatments began 3 to 5 weeks before flower appearance (WBF) and were applied weekly until 2 to 3 weeks after flower appearance.

Treatment	Length	Buds	Duration
	—— cm ——	—— # ——	—— day ——
H_2O Control	0.7 b	29.7 b	23.0
STS	8.2 a	66.3 a	33.5
LSD‡	1.7	23.9	
SE	1.0	6.5	3.4
	———— ANOVA ————		
	**	*	NS

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability level, respectively. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to the multiple range test.

Table 3. Effect of STS treatments started either early, medium or late relative to flower appearance on the length of flower stalks, maximum number of flower buds, duration of flower development, peak number of open flowers, flower development integral, and timing of the first and last of weekly treatment applications (app) to the apical region expressed in days before flowering.

Trt	First app to flower	Last app to flower	Length	Buds	Duration	Open Flowers	Integral†
	– day –	– day –	— cm —	— # —	– day –	— # —	#-d
H_2O							
Control	NA	NA	2.0 b	27.8 c	21.0 c	0.0 b	213.9 b
Early	-25	-4	6.0 ab	47.8 bc	20.3 bc	7.0 ab	733.2 b
Medium	-22	+1	12.8 a	86.0 ab	43.3 ab	19.3 a	2053.7 a
Late	-15	+7	11.5 a	95.0 a	49.0 a	18.3 a	1783.2 a
LSD‡			7.7	41.0	13.6	14.9	888.2
SE			2.5	13.3	4.4	4.8	312.6
	ANOVA						
Timing			*	**	**	*	**

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability level, respectively. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to the multiple range test.

† Flower development integral is the area under the curve of buds-count with respect to development time.

Table 4. Effect of STS treatments involving one, two or three weekly applications (app) started at the indicated number of days before flower appearance on the length of flower stalks, maximum number of flower buds, duration of flower development, and flower development integral.

Trt: no. of STS applications	First app to flower	Last app to flower	Length	Buds	Duration	Integral[†]
	— day —	— day —	— cm —	— # —	— day —	—#⋆d—
0 (H_2O Control)	NA	NA	12	41	19 b	270 b
1	-13	-13	2	42	25 a	447 a
2	-26	-19	3	47	25 a	494 a
3	-30	-16	13	48	25 a	439 a
LSD[‡]					3	127
SE			3	4	1	
	ANOVA					
Number of applications			NS	NS	***	**

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability level, respectively. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to the multiple range test.

† Flower development integral is the area under the curve of buds-count with respect to development time.

Table 5. Effect of STS treatments on the length of flower stalks, maximum number of flower buds, duration of flower development, number of open flowers, and flower development integral. Treatment I: STS applied to the shoot apex beginning 2 to 3 weeks before flower appearance and continuing for a total of four weekly applications; Treatment II: STS applied as I with two additional weekly applications on the flower parts only after flower appearance; Treatment III: STS applied to the shoot apex as in I, with two additional weekly applications to the newly-formed vegetative shoot apices (not including flower parts).

Trt	First app to flower	Last app to flower	Length	Buds	duration	Open Flower	Integral†
	– day –	– day –	— cm —	— # —	– day –	— # —	—#⋆d—
H_2O							
Control	NA	NA	1.4 c	0.0 b	20.8 b	0.5 b	86 c
I	-19	2	11.5 ab	93.8 a	48.3 a	17.8 a	2591 ab
II	-23	15	7.6 bc	64.8 a	29.8 b	10.0 b	1348 b
III	-28	11	16.6 a	112.3 a	53.3 a	39.5 a	3185 a
LSD‡			7.9	51.9	10.5	21.2	1223
SE			2.6	16.9	3.4	6.9	
				ANOVA			
Placement			*	**	***	**	***

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability level, respectively. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to the multiple range test.

† Flower development integral is the area under the curve of buds-count with respect to development time.

Table 6. Effect of STS treatments at several concentrations on the length of flower stalks, maximum number of flower buds, duration of flower development, number of open flowers, and flower development integral.

Trt	First app to flower	Last app to flower	Length	Buds	duration	Open Flower	Integral†
	– day –	– day –	— cm —	— # —	– day –	— # —	—#⋆d—
H_2O							
Control	NA	NA	1.4 c	0.0 b	20.8 b	0.5 b	86 c
I	-19	2	11.5 ab	93.8 a	48.3 a	17.8 a	2591 ab
II	-23	15	7.6 bc	64.8 a	29.8 b	10.0 b	1348 b
III	-28	11	16.6 a	112.3 a	53.3 a	39.5 a	3185 a
LSD‡			7.9	51.9	10.5	21.2	1223
SE			2.6	16.9	3.4	6.9	
	ANOVA						
Placement			*	**	***	**	***

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability level, respectively. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to the multiple range test.

† Flower development integral is the area under the curve of buds-count with respect to development time.

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