GENOME SIZE ESTIMATION AND BREEDING METHODS TO IMPROVE ORNAMENTAL *SALVIA*

by

REBEKAH MAYNARD

(Under the Direction of John Ruter)

ABSTRACT

Several *Salvia* species are desirable for breeding because of their ornamental value and ability to attract pollinators to the landscape. This research was conducted to further describe the diversity in the genus and aid future breeding efforts by determining the genomic content of multiple *Salvia* species through flow cytometric analysis. This research also improved the species *S. coccinea* through intraspecific hybridization. After four generations of breeding, progeny were selected with larger flowers and a more vivid color than the parental selections. Mutation breeding was also used to improve *S. coccinea* by treating seeds with ethyl methanesulfonate (EMS). Improved selections included a variety of chlorophyll mutations and changes to leaf morphology. Finally, this research identified a method to improve *S. uliginosa* through treating vegetative cuttings with gamma radiation. Leaf variation was induced in *S. uliginosa*, and a protocol was established to clonally propagate plant material after treatment with gamma radiation.

INDEX WORDS: *Salvia*, genome size, flow cytometry, propidium iodide, plant breeding, intraspecific hybridization, CIELAB, Nix Color Sensor, plant pigments, heterosis, flower color, mutagens, ethyl methanesulfonate, gamma rays

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CHAPTER 1

INTRODUCTION

Description and Usage of the Genus

Salvia is the largest genus in Lamiaceae, containing over 1000 species (Kew Science, 2019). Over 150 species have ornamental, culinary, or medicinal usage (Drew, 2020). Some species popular for the landscape include *S. nemorosa* 'May Night', which was awarded Perennial Plant of the Year® (Perennial Plant Association, 2021) and All-America Selections winners *S. coccinea* 'Lady in Red' and *S. farinacea* 'Strata' (All-America Selections, 2021). The genus includes two widely used herbs, *S. officinalis* (Cvetkovikj et al., 2015) and *S. rosmarinus* (de Macedo et al., 2020), as well as the seed crop *S. hispanica*, which is rich in omega-3 (Ullah et al., 2016). The perfume industry widely uses *S. sclarea* (Laville et al., 2013), and *S. miltiorrhiza* has long been used as a medicinal plant (Ren et al., 2019).

Salvia are valuable plants for the landscape with a wide variety of flower and leaf colors. The range of flower colors includes the bright-red *S. blepharophylla*, *S. nipponica* with light yellow petals, *S. mexicana* 'Limelight' with a bright green calyx surrounding deep violet flowers, the blue-flowered *S. patens*, lavender *S. canariensis*, bright pink *S. oxyphora*, *S. radula* with pure white petals, and *S. discolor* with nearly black flowers inside a silver calyx. Other selections have been bred with bicolored petals, such as the hybrid *S. greggii* x *microphylla* 'Dyson's Joy' with light pink lower and bright pink upper lobes. *Salvia* are also prized for their leaf colors, such as *S. elegans* 'Golden Delicious' with chartreuse leaves, *S. officinalis* 'Icterina' with variegated green and yellow leaves, or *S. daghestanica* with silver foliage (Whittlessey, 2014).

Salvia make a valuable addition to the landscape with a pleasant fragrance and ability to attract bees, butterflies, and hummingbirds to the garden (Kew Science, 2019; Wester and Claβen-Bockhoff, 2011). Because of the vast diversity in the genus, *Salvia* are well suited to many different growing conditions. Species including *S. cyanescens* and *S. nubicola* are cold tolerant and can survive below -12ºC. Others, such as *S. involucrata* or *S. splendens,* can perform well in high heat and humidity. Species such as *S. dolomitica* and *S. mellifera* are drought-tolerant, and species including *S. discolor* and *S. scabra* perform well in containers. In addition to tolerating a wide range of environments, *Salvia* can also be selected to bloom in different seasons. For example, *S. apiana* and *S. indica* bloom in spring, while *S. broussonetii* and *S. clevelandii* bloom in summer. Others, including *S. blepharophylla* and *S. chiapensis*, bloom in fall, while *S. gersneriiflora* and *S. wagneriana* bloom in winter (Clebsch, 2003).

The genus is found globally and shows phenotypic diversity among geographic regions (Walker et al., 2004). *Salvia* includes both annual and perennial species with herbaceous or shrub-forming habits. The leaves are simple with toothed, lobed, or pinnatifid leaf margins. Inflorescences appear in panicles with 2-lipped corollas. The upper lip is often falcate but sometimes flattened with a broadened lower lip. The calyx is cylindrical to campanulate from two fused lips (Kew Science, 2019). The distinguishing morphological characteristic which separates *Salvia* from other genera of the tribe Mentheae is the presence of two stamens with separated thecae. However, there are differences in the staminal structure within the genus, causing researchers to believe *Salvia* is polyphyletic (Walker et al., 2004).

Based on a phylogenetic study, Salvia was determined to be a descendant from other genera within the Mentheae tribe. The genus was then subdivided into three clades that correlate with their centers of diversity. Clade I mostly comprises plants from Mediterranean regions and southern Africa, Clade II includes plants solely from the Americas, and Clade III contains plants

from eastern Asia (Walker et al., 2004). However, in a broader molecular analysis of the genus, Will and Claβen-Bockhoff (2017) separated *Salvia* into four clades and incorporated species from other related genera.

Pollination Mechanism

The high diversity in phenotypic expression within the genus led researchers to believe that *Salvia* has also undergone adaptive radiation (Claβen-Bockhoff et al., 2004). As a species experiences changes in its environment, the phenotypic expression can be slightly altered without genetic modification. However, when interspecific hybrids are generated, both genetic and epigenetic changes can occur (Radosavljević et al., 2019). Thus, the occurrence of natural hybridization in *Salvia* may have contributed to the high amount of diversity within the genus and genetic erosion among species (Epling, 1947; Radosavljević et al., 2019).

One example of adaptive radiation is the similarity in the reproductive structures of *Salvia* species within a clade but differences among clades. The structural design of the anthers yields insight into the pollination mechanism for pollinator species in a given environment. One anther holds viable pollen under the upper lip of the corolla, and the other, often sterile, blocks access to nectar pooled near the ovaries. As a pollinator reaches towards the base of the corolla, however, the lower theca rotates upwards, providing access to the nectar. Simultaneously, the theca containing viable pollen rotates downwards and deposits pollen on the insect. The accessibility of nectar to only specific pollinators, as well as the location of pollen deposition on the pollinator, may correlate to the success of a certain *Salvia* species in a given environment (Claβen-Bockhoff et al., 2004).

Salvia are classified as melittophilous, ornithophilous, or phychophilous if pollinated by bees, birds, or butterflies. However, some species fall into more than one category, such as *S. eremostachya*, which all groups can pollinate. Pollination syndromes depend on flower length,

flower shape, the position of the lower lip, nectar retention, and thecae extension. These factors determine the accessibility of the pollen and nectar to certain pollinators. For example, any species with long corolla tubes and reduced lower lips are considered inaccessible to pollination by bees. Similarly, flowers with narrow corolla tubes are inaccessible to birds. Flower color also influences pollinator visitation. For example, ornithophilous species often have red, clearly visible flowers, while melittophilous species are often blue or violet (Wester and Claβen-Bockhoff, 2011).

Interspecific Hybridization

Hybridization is generally more successful if the plant species are genetically similar to one another. Crosses made within the primary gene pool are predicted to have greater success than crosses made within the secondary or tertiary gene pools. This relationship is defined by how easily chromosomes are paired during gene transfer (Harlan and de Wet, 1971). Based on this theory, it can be hypothesized that *Salvia* species with the same number of chromosomes will have greater success at generating fertile seed than species with differing numbers of chromosomes. This theory is supported by Epling (1947), who reported homoploid species of section Audiberita hybridizing naturally.

Epling (1938) observed that *Salvia* grown in the same vicinity produce offspring with intermediate morphological characteristics, indicating interspecific hybridization is possible. Hybridization occurs between *S. apiana* and several other California species, including *S. clevelandii*, *S. eremostachya*, *S. leucophylla*, *S. mellifera*, *S. munzii*, *S. pachyphylla*, *S. vaseyi*. Other species within the section Audiberita form interspecific hybrids including *S. eremostachya* x *S. vaseyi* and *S. leucophylla* x *S. clevelandii* (Epling, 1938). Each of these interspecific hybrids are between homoploid species with 15 base chromosomes (2n = 2x = 30) (Epling, 1962).

Hybridization also occurs between the homoploid *S. officinalis* (2n = 2x = 14) and *S. fruticosa* (2n = 2x = 14) (Haque, 1981; Radosavljević et al., 2019; Ranjbar et al., 2015)

However, interspecific hybridization in *Salvia* is not limited by genetic relatedness. Hybridization between *Salvia* with different base chromosomes also occurs. For example, *S. columbariae*, with 13 base chromosomes, hybridizes with and *S. mellifera* (n=15) (Epling, 1962). In a study conducted by Tychonievich and Warner (2011), the phylogenetic relationship of *Salvia* was tested through cross-pollinating nine species with different base chromosome numbers from two separate clades. The researchers proposed that crosses between clades would not produce viable seeds due to the high genetic diversity among regions. However, despite their hypothesis, they were able to generate a successful cross between species from Clade I and Clade II. Additionally, they discovered a greater success in interspecific crosses with differing numbers of chromosomes than interspecific crosses with the same number of chromosomes (Tychonievich and Warner, 2011). Therefore, hybridization can occur in *Salvia* between clades and between species with different base chromosome numbers.

Interspecific hybridization is used to improve the characteristics of commercially available *Salvia*. For example, Purple & Bloom (*S. splendens x guaranitica* PP32,027) has large violet flowers that bloom from spring through fall. It is significantly more compact than similar cultivars, performs well under heat and drought stress, and attracts pollinators to the garden (BallSeed, 2021; Kievit, 2020). Other commercially available interspecific hybrids include Skycraper™ Orange Salvia (*S. buchananii x splendens* PP31,766) with a compact habit and large, uniquely orange flowers (Sapia, 2019; Selecta, 2021) and *Salvia* Arctic Blaze Red® (*Salvia hybrid* PP28,620) with vibrant, red blooms and improved cold hardiness (DarwinPerennials, 2021; Dobres and Janes, 2017).

Genetic Information to Further Breeding Efforts

Despite successful hybridizations between unrelated *Salvia*, genetic information may help predict successful hybridizations between related species (Akbarzadeh et al., 2021). Previously, karyotyping with image analysis was used to determine plant chromosome counts by pairing homologous chromosomes by length (Venora et al., 1991); however, *Salvia* chromosomes are small and difficult to distinguish (Haque, 1981). Due to chromosome size and the number of species present within the genus, only 17% of *Salvia* have recorded chromosome numbers. Of the recorded species, gametophytic chromosome numbers have been reported as x = 6, 7, 8, 9, 10, 11, 13, 15, and 16. Approximately 80% are diploid, 6% are mixoploid, and 14% are polyploid (Ranjbar et al., 2015).

More recently, flow cytometry has been used as a fast and reliable method to assess genetic information, including chromosome number, ploidy, and genome size (Bourge et al., 2018; Hoshino et al., 2019). Flow cytometry assesses genomic content by comparing an unknown sample to a known standard (Hoshino et al., 2019). To analyze plant samples using flow cytometry, nuclear DNA is extracted from the leaf tissue and stained with a fluorochrome. The cells are then suspended in solution and injected thorough sheath fluid into the cytometer. Analysis of single cells is accomplished by passing the suspension through a 50-300 μm orifice by a laser beam. As the cells pass by the light source, the fluorochrome absorbs radiation and re-emits the light at a longer wavelength (Ochatt, 2008). The two samples are then compared based on their relative fluorescent intensities (Coleman et al., 1981).

To compare fluorescent intensities, the sample and the known standard must have similar genome sizes. The standard should be stable, reproducible, and generate distinguishable fluorescent peaks from the sample being analyzed (Coleman et al., 1981). The most accurate way to estimate genome size is simultaneously analyzing the standard and sample, accounting for

extraneous variability from the flow cytometer (Doležel et al., 2007). The estimation of DNA content also depends on the fluorophore used to tag the DNA strand. Fluorophores can be selected to intercalate with the DNA strand or bind preferentially to AT or GC base pairs (Doležel et al., 1992). Fluorophores such as DAPI and mithramycin preferentially bind to AT and GC base pairs, respectively. Preferential binding causes the fluorescent intensity to depend on the percentage of AT and GC base pairs present in the DNA strand. If the AT% and GC% differ between the sample and standard, the analysis will inaccurately estimate genome size (Coleman et al., 1981; Ortega-Ortega et al., 2019).

To determine the degree of error in genome size estimation using base-specific fluorophores, Doležel et al. (1992) compared three types of stains. The intercalating dye, propidium iodide (PI), was compared with 4’ 6-diamidino-2-phenylindole (DAPI), which has AT base pair preference, and mithramycin, which binds preferentially to GC base pairs. Six plant species were analyzed with each stain, and it was found that the calculated nuclear DNA content was significantly different for each stain. Additionally, the researchers found that the genome size estimated by PI within a species was not equal to the sum of AT and GC base pairs estimated by DAPI and mithramycin (Doležel et al., 1992). Therefore, DAPI and mithramycin cannot accurately represent the percentage of AT and GC base pairs present in a DNA strand. Although DAPI is being used to estimate the genome size of various plant species, base-specific fluorophores should not be used as they can yield an inaccurate estimation (Contreras and Shearer, 2018; Ortega-Ortega et al., 2019).

Once the sample and standard have been analyzed with a flow cytometer, the genome size and estimated chromosome number can be determined by comparing the mean fluorescence of the sample to the mean fluorescence of the standard, giving the 2C value (Bourge et al., 2018;

Hoshino et al., 2019). The 2C peak should appear on the far left of the fluorescence histogram; however, if the tissue is haploid, the first peak will represent the 1C value (Doležel et al., 2007).

Multiple peaks appearing on the spectrum can indicate polyploidy (Arumuganathan and Earle, 1991). In a study of mouse liver cells, the fluorescence spectrum indicated polyploidy through the appearance of 2C, 4C, and 8C peaks. The 4C fluorescent peak was approximately twice the absorption of the 2C peak, and the 8C peak was approximately twice the absorption of the 4C peak (Bose et al., 1989). Therefore, fluorescence intensity increases with increasing ploidy for mouse liver cells. Similarly, polyploidy was also observed in *Arabidopsis lyrata* using flow cytometry. Multiple peaks with increasing fluorescent intensity appeared on the histogram. These were found to correspond to the 2C, 4C, and 8C nuclei. In addition to this observation, a triploid peak was identified half-way between the diploid and tetraploid peaks (Dart et al., 2004). These observations indicate that higher ploidy levels occur at greater fluorescent intensities. Additionally, triploid species can be identified on a fluorescence spectrum with respect to both diploid and tetraploid species.

Applications of Mutation Breeding

Due to low success in generating interspecific hybrids of *Salvia*, alternative approaches are needed to select new varieties. One alternative approach is artificially inducing variation through mutation breeding (Tychonievich and Warner, 2011). In mutation breeding, seeds or vegetative tissue can be treated by chemical or physical mutagens to generate desirable characteristics, including improved plant form and alterations to leaf or flower color (Datta and da Silva, 2006). Gaul (1964) describes plant breeding as controlled evolution through gene recombination and selection. Mutation breeding achieves species improvement in a shorter period than cross-pollinations; however, because mutagens induce random alterations to the DNA strand, more breeding material is required to isolate desirable mutants (Gaul, 1964).

Depending on the type of mutation, alterations to the DNA may or may not be immediately visible. Macro-mutations occur in major genes and form visible morphological changes. On the other hand, micro-mutations have a minor effect on gene expression and are only visible in quantitatively inherited traits. Point mutations on a single gene induce heritable mutations. However, most gene mutations are recessive and require further breeding to be phenotypically expressed (Toker, 2007). Furthermore, many visible mutations are lethal or cannot produce viable seeds, which limits breeding with the mutated material (Gaul, 1964).

A common chemical mutagen is ethyl methanesulfonate (EMS). EMS deposits an ethyl group onto electron-rich regions of the DNA strand. These alkylations can cause point mutations and cleavages in the phosphate backbone (Sega, 1984). When the base pair guanine (G) is alkylated, it can no longer pair with cytosine (C) but instead pairs with thymine (T). As the DNA strand is repaired, a point mutation occurs where an A/T base pair is inserted in the place of the G/C pair. The use of EMS results in several mutations along the genome, causing this mutagen to be valuable for developing breeding lines (Kim et al., 2006). Gamma radiation is frequently selected as a physical mutagen for plant breeding. Ionizing radiation forms free radicals, which cause damage to plant cells. The free radicals alter DNA through substitutions, deletions, and chromosomal aberrations (Çelik and Atak, 2017).

Various crops have been improved through mutation breeding, including darkening of fruit color in *Solanum lycopersicum* (Sikder et al., 2013), increased salt tolerance in *Ipomea batatas* (Luan et al., 2007), and higher seed yield in *Trigonella foenum-graecum* (Basu et al., 2007). In ornamentals, plant appearance has been improved with mutation breeding, including induced leaf variegation in *Saintpaulia* (Fang and Traore, 2011), altered flower color in *Delphinium* (Kolar et al., 2015), and increased leaf size in ornamental gingers (Prabhukumar et

al., 2015). Because limited mutation breeding has been done on *Salvia*, this research aims to improve species with EMS and gamma rays.

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CHAPTER 2

GENOME SIZE ESTIMATION IN THE GENUS *SALVIA* (LAMIACEAE)

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Abstract

Salvia is a genetically diverse genus in the Lamiaceae with hundreds of species distributed globally. With base chromosome numbers ranging from 6 to 19 and ploidy levels ranging from diploid to octoploid, the genus has been proposed to be subdivided based on molecular data rather than morphology. However, little information is known about total genomic content across the genus. The DNA content of 141 *Salvia* genotypes were analyzed through flow cytometry to provide additional genetic information on the genus. Samples of *Salvia* were stained with propidium iodide and compared to the internal standards *Pisum sativum* 'Ctirad' and *Solanum lycopersicum* 'Stupické' to generate accurate estimations of genomic content. Genome sizes of the analyzed *Salvia* ranged from 0.63 pg to 6.12 pg. Genomic content did not correlate with chromosome number, ploidy, or clade. The wide distribution of genomic content across the genus further indicates the diversity of *Salvia* and may be useful for future breeding efforts.

Introduction

Salvia is the largest genus in the Lamiaceae, containing 1015 accepted species. It grows natively in Europe, Asia, Africa, Australia, and the Americas (Kew Science, 2019). The genus includes annual and perennial herbs and shrubs which are often highly aromatic and covered in hairs (Kew Science, 2019). Several species, including *S. nemorosa* and *S. miltorrhiza*, have been used for their medicinal properties (Božin et al., 2012; Li et al., 2013), while other species such as *S. hispanica, S. fruticosa,* and *S. officinalis* have been used culinarily (Cvetkovikj et al., 2015; Ullah et al., 2016). Most notably, *Salvia* is used as an ornamental plant to attract pollinators, including bees, butterflies, and birds, to the garden (Wester and Claβen-Bockhoff, 2011).

Great ornamental diversity exists within the genus, making *Salvia* useful for almost any garden. The inflorescences are vibrant and appear in whorls with 2-lipped corollas ranging from

bright red to deep violet, including whites, yellows, and blues. Their visual impact is enhanced by pigmented calyces, formed by two cylindrically fused lips (Kew Science, 2019). The leaves are simple, frequently toothed, and display unique colors, such as *S. argentea* with silver foliage or *S. elegans* 'Golden Delicious' with chartreuse leaves (Whittlesey, 2014). Because of their wide geographic spread, *Salvias* can be selected for a variety of climates. Species such as *S. microphylla* and *S. patens* do well in hot, humid regions, while others like *S. nemorosa* or *S. verticillata* are suited to colder climates. Plants including *S. blepharophylla* and *S. miniata* may be used in shade gardens, and others such as *S. apiana* or *S. officinalis* are useful in areas prone to drought (Clebsch, 2003).

In the last widely accepted classification of *Salvia*, the genus was subdivided into 12 sections based on differences in flower morphology (Bentham and Hooker, 1873; Will and Claβen-Bockhoff, 2017). The distinguishing morphological characteristic that separates *Salvia* from other genera of the tribe Mentheae is the staminal structure. The flowers contain two stamens, with thecae separated by elongated connective tissue. Anterior thecae are often fertile with aborted posterior thecae creating a unique lever mechanism that aids pollination (Walker et al., 2004). The taxonomic classification of the genus as a whole has not been altered since Bentham's arrangement. The staminal structure is highly variable across the genus, leading researchers to believe *Salvia* is polyphyletic. The genus was first confirmed to be polyphyletic using genomic sequencing and phylogenetic analysis. A strong correlation was observed between genetic relatedness and geographic region. Based on this discovery, the genus was proposed to be subdivided into clades (Walker et al., 2004).

Rather than characterizing the genus by morphological characteristics, molecular data provides a way to separate the genus by ancestry. Walker et al. (2007) determined that the lever-like stamens developed multiple times in separate geographic regions and proposed dividing

Salvia into three clades based on their lineage. Clade I and Clade II are monophyletic and include other genera closely related to *Salvia*. The third clade was found to be distinct from the other two clades but was not confirmed to be monophyletic (Walker et al., 2007). In a more recent study, Will and Claβen-Bockhoff (2017) conducted a robust analysis of the genus and identified four lineages. These four clades include other closely related genera and strongly correlate with a geographic region (Will and Claβen-Bockhoff, 2017). Despite various taxonomic revisions, comparatively little research has been done to explore genome evolution.

Due to the polyphyletic nature of *Salvia* and vast geographic spread, remarkable variation in chromosome number and ploidy exists across the genus. Ploidy levels range from diploid to octoploid, and chromosome base numbers include x = 6 – 11, 13, 14, 15, 17, and 19 (Delestaing, 1954; Ranjbar et al., 2015). A wide range of chromosome numbers appears to have developed over time through aneuploidy, dysploidy, and polyploidy. The majority of known polyploids come from section *Calosphace,* while the sections *Salvia*, *Drymosphce,* and *Horminum* contain only diploid species. Although polyploidy seems to be the driving factor for speciation in South America, species from China appear to have evolved through differences in karyotype structure. Cytomixis has also been observed in *S. indica* and *S. atropatana*, resulting in gametes with different chromosome numbers (Ranjbar et al., 2015).

Although there is no clear correlation of base chromosome numbers with geographic regions, some chromosome numbers appear more frequently in certain regions. The most commonly occurring base chromosome numbers for species from Europe, Turkey, and the Mediterranean are x = 7, 8, and 11. Species from Iran typically have the base numbers x = 10 and 11, while species from China tend to be x = 8. South and Central American species are usually x = 11, while North American species tend to be x = 15 and 16 (Ranjbar et al., 2015). Despite these trends, not all species within these regions follow the pattern exactly.

Although there are over 1000 species of *Salvia*, there is little reported genetic information making the genus suitable for a genome study (Kew Science, 2019). Analyzing the DNA content across many species may lead to a greater understanding of species development and adaptation. Knowing genome size also provides valuable information for future breeding efforts and can aid the process of genome sequencing (Doležel and Greilhuber, 2010). Genetic information, including nuclear DNA content, can be determined for plant samples through flow cytometry by comparing the fluorescent intensity of a plant sample to the fluorescent intensity of a known standard with a similar genome size (Doležel et al., 2007).

The accuracy of DNA content estimation also depends on the fluorophore used to tag the DNA strand. The fluorophore can either be intercalating or bind preferentially to AT or GC base pairs (Doležel et al., 1992). Studies have shown that fluorophores such as DAPI and mithramycin, which preferentially bind to AT and GC base pairs, respectively, can yield inaccurate estimates of genome size based on differing AT% and GC% between the sample and standard (Coleman et al., 1981; Ortega-Ortega et al., 2019). Although DAPI has been used to estimate the genome size of various plant species, intercalating fluorophores such as propidium iodide (PI) should be preferentially selected to yield more accurate estimates (Contreras and Shearer, 2018; Ortega-Ortega et al., 2019).

As an alternative to counting chromosomes through direct observation, Hoshino et al. (2019) tested the ability of flow cytometry to estimate chromosome numbers for *Lychnis senno* accurately. The chromosome number of the unknown was estimated by comparing the relative fluorescent intensity of the sample to a known internal standard of the same species. These estimated values were confirmed through chromosome counts. Furthermore, a positive correlation was identified when fluorescent intensity was plotted against chromosome number,

suggesting that flow cytometry is a fast and reliable way to estimate taxa with unknown chromosome numbers (Hoshino et al., 2019).

The relationship between ploidy and fluorescent intensity was tested for several *Vaccinium* and *Rubus* species in separate studies. Species with known ploidy were analyzed through flow cytometry with a known standard. The relative fluorescence of each sample was plotted against their known ploidy level resulting in a positive, linear relationship between fluorescent intensity and ploidy. Using this method, researchers were able to estimate ploidy for several unknowns. Furthermore, it was determined that flow cytometry can accurately estimate the ploidy across species (Costich et al., 1993; Meng and Finn, 2002). The aim of the present study was to analyze genome size across clades and test the ability of flow cytometry to estimate chromosome number and ploidy across species of *Salvia.*

Materials and Methods

To enhance the genetic information on the genus *Salvia*, seeds and vegetative cuttings of 141 genotypes, including 92 individual species, were collected and grown for cytometric analysis. These species were obtained from germplasm collections worldwide to represent the wide natural distribution of species in the genus. Figure 2.1 shows the native distribution of species analyzed for this study. The nuclear DNA content was estimated using the CytoFLEX S flow cytometer (Beckman Coulter, Hialeah, Florida) and the CytExpert software. Two internal standards were selected for this study to represent the wide range of genome sizes existing in *Salvia*. These were *Solanum lycopersicum* ‘Stupické’, which has a reported 2C value of 1.96 pg, and *Pisum sativum* ‘Ctirad’ with a 2C value of 9.09 (Doležel and Bartoš, 2005). Propidium iodide was chosen as the fluorochrome to yield an accurate estimation of genome size (Doležel et al., 1992). Sample tissue of *Salvia* and the standards were collected for analysis by selecting young leaves at similar developmental stages.

The Sysmex CyStain PI Absolute P stain kit (Sysmex America, Inc., Lincolnshire, Illinois) was used for this experiment. The staining solution was prepared with 12 μL of PI, 1.0 mL of staining butter, and 6 μL of RNase stock solution per sample. Approximately 0.5 cm^2 leaf samples from *Salvia* and from the standard were placed in a Petri dish with 500 μL of the nuclei extraction buffer. The leaves were then chopped into a fine particle with a clean razor blade and left to incubate for 60 seconds. Next, the solution was passed through a 50 μL CellTrics filter (Sysmex America, Inc., Lincolnshire, Illinois) into a test tube. Then, 1.0 mL of the prepared staining solution was pipetted into a test tube. The sample was then transferred to a refrigerator to incubate in the absence of light for 20 minutes.

After the staining period, 350 μL of the solution was pipetted into a 96-well plate for analysis. Three replicate samples were prepared for each species on separate days to minimize error in sample preparation. The optical filter in the CytoFLEX S flow cytometer was set to select a wavelength range of 488 – 690 nm to include the 535 nm excitation and 617 nm emission maxima for propidium iodide bound to DNA (ThermoFisher Scientific). The suspended cells were drawn into the cytometer at a slow flow rate of 10 μL/s. A minimum of 1,000 events was recorded within the gate of *Salvia* and 1,000 events within the gate of the standard.

The total genomic content (2C) of each species was estimated by comparing the fluorescent peak of *Salvia* to that of the internal standard using Equation 1.

$$\text{2C of sample} = \text{2C value of standard} \times \frac{\text{mean fluorescence value of the sample}}{\text{mean fluorescence value of the standard}}$$

Equation 1: Estimating the genome size of a sample by comparing mean fluorescent peak values to that of a standard (Contreras and Shearer, 2018).

For each species, the 2C value was calculated by averaging three replicate scans. Most of the samples were analyzed with *S. lycopersicum* ‘Stupické’; however, many species had

overlapping fluorescent peaks with tomato, necessitating the use of *P. sativum* ‘Ctirad’. Figure 2.2 includes sample spectra of *Salvia* analyzed with each internal standard. For species with known ploidy, genomic content of the non-replicated genome (1C) was calculated by dividing the experimentally determined 2C value by ploidy (Contreras and Shearer, 2018). DNA content per chromosome was calculated by averaging the 2C value of each species and dividing it by the reported chromosome number (Akbarzadeh et al., 2021). Relationship between 2C, 1C, ploidy, and clade was determined using the linear regression function in RStudio (RStudio, 2018).

Results and Discussion

For each of the analyzed samples, the mean genome size, standard deviation, and internal standard used are listed in Table 2.1. According to the genome size classifications described by Leitch et al. (1998), 45% of the analyzed *Salvia* samples could be classified as having a very small genome and 48% as having a small genome. Genome size does not correlate with the complexity of an organism (Doležel and Bartoš, 2005), but small genomes have been correlated with shorter reproductive cycles and rapid establishment of seedlings. Therefore, the small genomes of *Salvia* may give them a competitive advantage over other plants (Leitch et al., 1998).

Multiple accessions of *S. bucharica*, *S. canariensis*, *S. coccinea*, *S. dorrii*, *S. elegans*, *S. greggii*, *S. guaranitica*, *S. hispanica*, *S. involucrata*, *S. leucantha*, *S. macrophylla*, *S. nemorosa*, *S. pratensis*, *S. prunelloides*, *S. sclarea*, *S. splendens*, and *S. verbenaca* were analyzed for total genetic content. Minimal variation was observed among the accessions within each species. Therefore, these accessions were believed to be genetically representative of their species, which is consistent with Castro et al. (2012) who states that DNA content is constant across a species.

Available hybrids were analyzed by comparing the 2C value of the hybrid to the 2C value of the parental species. The interspecific hybrid *S. mexicana x gesneriiflora*, with an estimated 2C genome size of 1.59 ± 0.002 pg, fell halfway between the parental species *S. mexicana* (2C =

1.21 ± 0.043 pg) and *S. gesneriiflora* (2C =1.96 pg ± 0.055). The same trend was observed for the hybrids *S. fruticosa x officinalis*, *S. x jamensis* 'Elk Cranberry Red', and *S. splendens x guaranitica* 'Purple & Bloom'. Interspecific hybrids having a genome size value between the genome sizes of their parental species has also been observed in other genera such as *Cirsium* (Bureš et al., 2004), *Diphasiastrum* (Hanušová et al., 2014), *Dryopteris* (Ekrt et al., 2010), *Ficaria* (Popelka et al., 2019), and *Sarcococca* (Denaeghel et al., 2017).

Contrary to these examples, however, some of the studied hybrids did not have an intermediary genome size compared to their parents. Rather, *S. x jamensis* 'Red Velvet', a hybrid of *S. microphylla* and *S. greggii*, has a larger genomic content (3.27 ± 0.077 pg) than either parent. *S.* 'Jezebel' is also a hybrid of *S. microphylla* but has an unknown pollen source. Like *S. x jamensis* 'Red Velvet', 'Jezebel' also has a larger genome size (2.48 ± 0.031 pg) than *S. microphylla*. A similar observation was made by Baack et al. (2005) in the study of *Helianthus* hybrids. The interspecific hybrids of *H. annuus* and *H. petiolaris* had a greater total genomic content than either parental species (Baack et al., 2005). Therefore, genetic content is not always reliable evidence of the hybridization of species.

Salvia chromosomes are small and difficult to distinguish resulting in many species lacking chromosome counts (Haque, 1981). Unfortunately, flow cytometry cannot be used to predict chromosome numbers in *Salvia* and can be shown by comparing known chromosome numbers to calculated genome sizes summarized in Table 2.1. For example, *S. macrophylla* 'Purple Leaf' (2n = 2x = 18) had a genome size of 0.93 ± 0.008 pg (Bolkhovskikh et al., 1969). In comparison, *S. hispanica* (2n = 2x = 12) had the same genome size (0.93 ± 0.016 pg) despite having a different base chromosome number (Estilai et al., 1990). Contrary to these findings, flow cytometry has been used to estimate chromosome number within the species *Lychnis senno*

and among species in the genera *Veronica*, *Scrophularia*, and *Verbascum* (Hoshino et al., 2019; Castro et al., 2012).

Inconsistent chromosome reports further complicate chromosome estimation. Different chromosome numbers have been assigned to the same species, as shown in Table 2.1. For example, *S. nemorosa* has been reported to have 12 or 14 base chromosomes, while *S. pratensis* has been observed to have 16, 18, or 20 (Haque, 1981). *Salvia* with the same number of chromosomes also vary in genome size. For example, species with 16 base chromosomes (2n = 2x = 32) range from 0.92 ± 0.003 pg in *S. leucantha* 'Midnight' to 6.07 ± 0.129 pg in *S. carduacea* (Epling, 1962; Haque, 1981). Therefore, flow cytometry is an unreliable method for estimating chromosome counts in *Salvia*. These findings are consistent with the analysis of the genus *Helleborus*. All species in *Helleborus* have the same base chromosome number but range significantly in their genomic content (Zonneveld, 2001). In the case of *Helleborus* and *Salvia*, the inability to predict chromosome numbers based on genome size is caused by each species having a different amount of DNA in their chromosomes.

No relationship was found between the calculated 2C value of *Salvia* and reported ploidy, as shown Figure 2.3 ($R^2 = 0.22$). The lack of relationship may be explained by the variation in chromosome number and size throughout the genus. Therefore, ploidy cannot be estimated among species in *Salvia* based on relative fluorescence. In contrast to these findings, flow cytometry has previously been used to estimate ploidy based on genome size in *Rubus* (Meng and Finn, 2002) and *Vaccinium* (Costich et al., 1993). Although flow cytometry cannot be used to estimate ploidy, it may, however, be a valuable way to confirm induced polyploidy within a species (Bose et al., 1989).

In this study, the genome sizes of *S. farinacea* (2n = 2x = 20) were constant across four varieties (Alberto et al., 2003). The *S. farinacea* Duelberg series were 2.19 ± 0.051 pg and 2.15 ±

0.039 pg for ‘Augusta Duelberg’ and ‘Henry Duelberg’, respectively. In the *S. farinacea* Cathedral® series, genome size was 2.17 ± 0.070 pg and 2.15 ± 0.016 pg for the lavender and purple cultivars, respectively. However, the genome size of *S. farinacea* Unplugged® So Blue™ was roughly double these values at 4.10 ± 0.068 pg. Chromosome counts are needed to confirm polyploidy; however, the doubled genomic content of *S. farinacea* Unplugged® So Blue™ may indicate tetraploidy (2n = 4x = 40) in this cultivar. Higher ploidy levels have been linked to larger genome sizes between cultivars of *Olea europaea* (Besnard et al., 2008). Genetic content is expected to increase proportionally with ploidy and has been observed in many other genera, including *Bougainvillea*, *Narcissus*, and *Tulipa* (Castro et al., 2012; Leitch and Bennett, 2004).

Ploidy has not been reported for *S. discolor*, *S. elegans*, *S. repens*, or *S. sessilifolia*; however, these values can be inferred from their listed chromosome numbers. For example, *S. elegans* and *S. repens* are reported as 2n = 20 (CCDB, 2021; Kumar and Subramaniam, 1987). However, because base chromosome numbers for *Salvia* are only known to be x = 6 – 11, 13, 14, 15, 17, and 19, these species must be diploid with a base chromosome number x = 10 (Ranjbar et al., 2015). By the same logic, *S. discolor*, reported as 2n = 24, must be a tetraploid with base chromosome number x = 6 (Kumar and Subramaniam, 1987). Lastly, *S. sessilifolia*, with 2n = 44, must be tetraploid with 11 chromosomes (CCDB, 2021).

Although *Salvia* was originally subdivided into twelve sections based on Bentham’s arrangement, the most recent treatment of the genus by Will and Claβen-Bockhoff (2017) divides the genus into four clades. The clade assignments are based on phylogenetic data and do not necessarily correspond with older section classifications. Estimated 2C values and calculated 1C values are summarized in Table 2.2 with their section and assigned clades. Most plants obtained from this study were from Clades I and II, a few species were from Clade IV, and none were obtained from Clade III (Will and Claβen-Bockhoff, 2017). Each clade showed a

significant range of genome sizes from a 1C value of 0.20 pg in the tetraploid *S. sessilifolia* to a 1C value of 3.04 pg in the diploid *S. carduacea*. The distribution of 2C and 1C values within each clade is summarized in Table 2.3. There was no relationship between 2C value and clade ($R^2 = 0.01$) or 1C value and clade ($R^2 = 0.01$).

Although genome sizes could not predict chromosome number or ploidy, data collected in this study may benefit future breeding efforts. For example, interspecific hybridization is difficult to achieve in *Salvia*; however, genome size data may be helpful to predict crosses (Tychonievich and Warner, 2011; Akbarzadeh et al., 2021). In a study conducted by Bureš et al. (2004), the cross-compatibility of *Cirsium* was compared to total genomic content. A negative correlation was observed between total genetic content and the ability to hybridize between species. In *Cirsium*, smaller genomes were more likely to form interspecific hybrids (Bureš et al., 2004). Genome size data can also increase understanding of species development and adaptation. For example, larger plant genomes have been related to an increased ability to withstand humid environments and elevated atmospheric CO_2 levels (Jasienski and Bazzaz, 1995; Vesleý et al., 2012). On the other hand, smaller plant genomes are less susceptible to damage by ionizing radiation (Sparrow and Miksche, 1961).

The 141 genotypes of *Salvia* analyzed in this study were intentionally selected from many geographic regions to provide a representative subset of the genus. In addition to having wide geographic distribution, multiple base chromosome numbers, and variation in ploidy across the genus, significant variation in total genomic content was observed across the species screened in this study. This study showed that flow cytometry is not a reliable way to estimate chromosome number or ploidy across species in *Salvia*. Although genome size could not be correlated with Will and Claβen-Bockhoff's (2017) clade assignments, the genetic information

obtained in this study does provide further evidence of species diversity within the genus and may aid in future breeding work.

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Tables and Figures

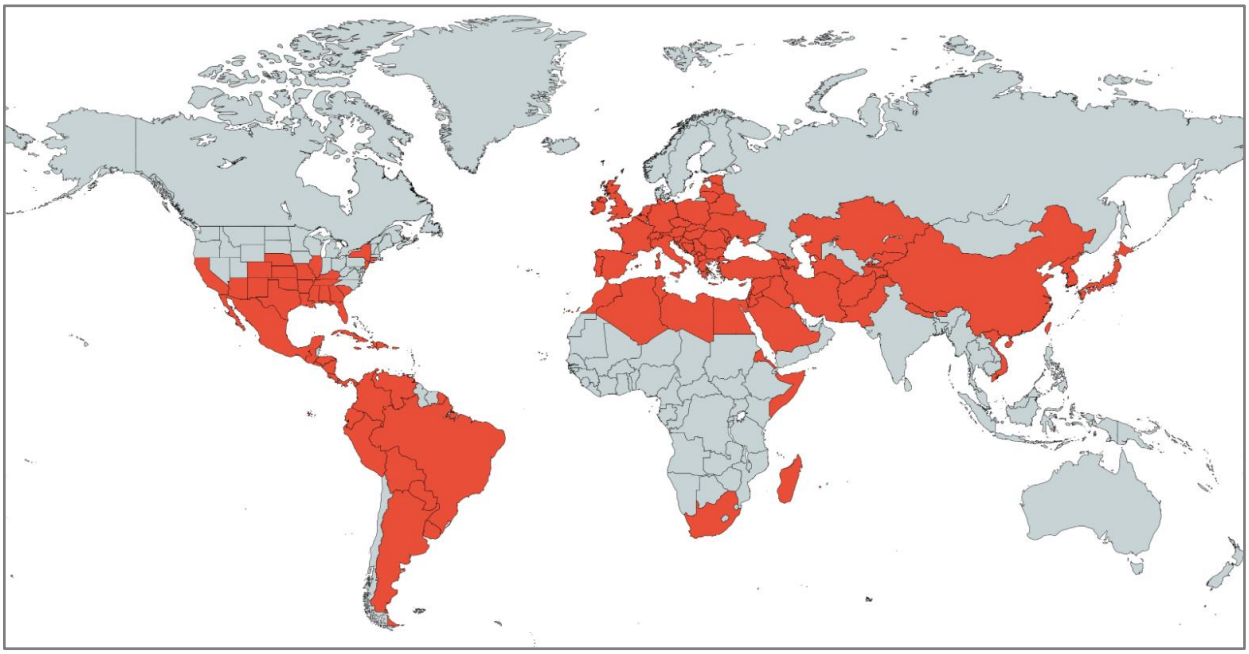

Figure 2.1. Native distribution of *Salvia* species collected for this study.

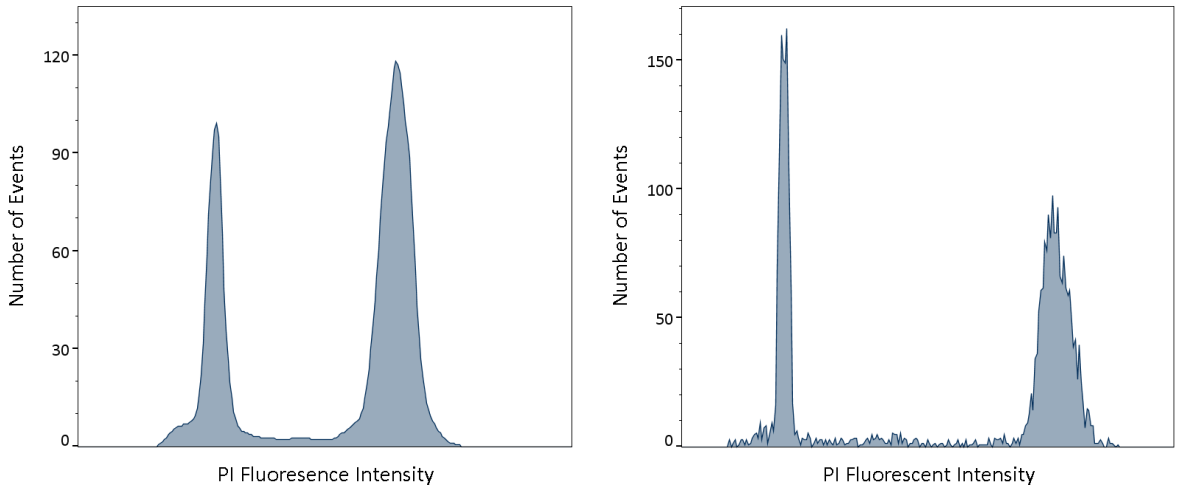

Figure 2.2. (L) Sample spectrum of *S. hispanica* as the left peak analyzed with internal standard *S. lycopersicum* 'Stupické' on the right. (R) Sample spectrum shows *S.* x 'Jezebel' as the left peak with *P. sativum* 'Ctirad' on the right.

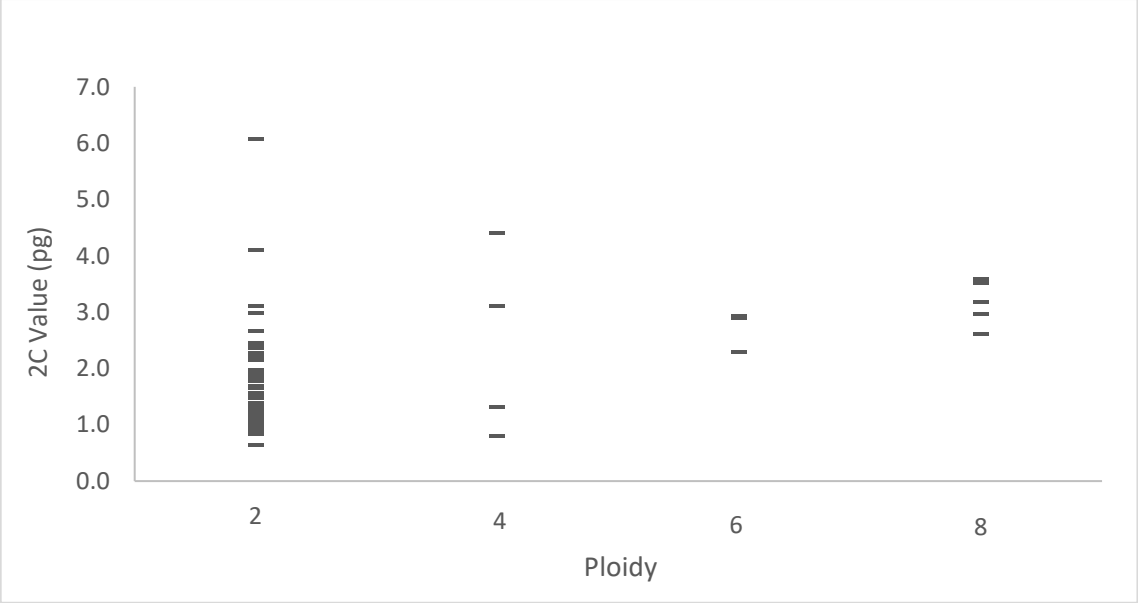

Figure 2.3. The total genomic content of *Salvia* analyzed in this study was plotted against known ploidy to determine if the two values were correlated. No relationship was found between the two parameters (R^2 = 0.22).

Table 2.1. Estimated 2C genome size from the current study and reported ploidy for *Salvia*.

Taxa Family *Lamiaceae* Genus *Salvia*	[a] Estimated 2C genome size (pg)	[b] Internal Standard	Reported Chromosome Number	[c] Chromosome Number Reference	[d] Plant Source
S. aethiopis	2.99 ± 0.011	2	2n = 2x = 22	7	GBG
S. amplexicaulis	1.47 ± 0.013	2	2n = 2x = 20	9	USNA
S. arenaria 'Bau'	2.96 ± 0.036	2			BAA
S. atropatana	2.36 ± 0.032	1	2n = 2x = 22	9	ASC
S. azurea	0.64 ± 0.004	2	2n = 2x = 20	8	GBG
S. azurea var. *grandiflora*	1.77 ± 0.018	2	2n = 2x = 20	8	WRPI
S. barrelieri	3.10 ± 0.042	1	2n = 2x = 38	2	SC
S. biserrata	0.93 ± 0.019	2			BAA
S. blepharophylla	0.89 ± 0.030	2			TG
S. brandegeei 'Pacific Blue'	1.84 ± 0.007	1	2n = 2x = 30	5	DDN
S. broussonetii	1.02 ± 0.020	2			SC
S. buchananii x splendens 'HYBSV18020'	1.38 ± 0.030	2			S
S. bucharica	2.44 ± 0.029	1	2n = 2x = 32	2	GBG
S. bucharica	2.43 ± 0.032	1	2n = 2x = 32	2	JPN
S. bullulata	2.27 ± 0.009	1			BAA
S. cacaliifolia 'Guatemalan Leaf Sage'	1.53 ± 0.004	2			AAP
S. calolophos	1.97 ± 0.055	1			BAA
S. canariensis	1.06 ± 0.027	2	2n = 2x = 22	10	CBG
S. canariensis 'Lancelot'	1.06 ± 0.016	2	2n = 2x = 22	10	PA
S. cardiophylla	1.31 ± 0.005	2	2n = 4x = 44+0-1B	9	BAA
S. carduacea	6.07 ± 0.192	1	2n = 2x = 32	5	WRPI
S. caudata	1.04 ± 0.011	2			BAA
S. chiapensis	1.95 ± 0.030	1			AA

S. coccinea	0.85 ± 0.004	2	2n = 2x = 20, 22	1	TWF
S. coccinea	0.85 ± 0.008	2	2n = 2x = 20, 22	1	FL
S. coccinea 'Yucatan'	0.87 ± 0.007	2	2n = 2x = 20, 22	1	BAA
S. columbariae	1.56 ± 0.009	2	2n = 2x = 26	5	WRPI
S. concolor	0.72 ± 0.007	2			AAP
S. confertiflora	1.70 ± 0.017	1			USNA
S. corrugata	2.33 ± 0.041	1			DDN
S. cuatrecasana	4.31 ± 0.072	2			BAA
S. deserta	1.12 ± 0.006	2	2n = 2x = 14, 16	9	WRPI
S. desoleana	3.11 ± 0.093	2	2n = 4x = 44	9	SC
S. discolor	2.20 ± 0.021	1	2n = 24	8	TG
S. disermas	3.52 ± 0.013	1			BG
S. dolichantha	6.12 ± 0.129	1			BAA
S. dorrii v. carnosa	1.68 ± 0.011	2	2n = 2x = 30	5	ASC
S. dorrii v. dorrii	1.68 ± 0.028	2	2n = 2x = 30	5	ASC
S. dumetorum	1.20 ± 0.017	2	2n = 2x = 14	4	JPN
S. elegans 'Honey Melon'	1.12 ± 0.009	2	2n = 20	3	BAA
S. elegans 'Sonoran Red'	1.11 ± 0.026	2	2n = 20	3	BAA
S. elegans Rockin'® 'Golden Delicious'	1.13 ± 0.014	2	2n = 20	3	PW
S. farinacea 'Augusta Duelberg'	2.19 ± 0.051	1	2n = 2x = 20	1	BAA
S. farinacea 'Henry Duelberg'	2.15 ± 0.039	1	2n = 2x = 20	1	BAA
S. farinacea Cathedral® Lavender	2.17 ± 0.070	1	2n = 2x = 20	1	GFB
S. farinacea Cathedral® Purple	2.15 ± 0.016	1	2n = 2x = 20	1	GFB
S. farinacea Unplugged® So Blue™	4.10 ± 0.068	1			PW
S. fruticosa	1.15 ± 0.013	2	2n = 2x = 14, 16	9	GBG
S. fruticosa x officinalis 'Newe Ya'ar'	1.16 ± 0.005	2			USNA
S. gesneriiflora 'Compact Form'	1.96 ± 0.055	1			BAA
S. greggii 'Cherry Lips'	2.33 ± 0.063	1			RS

S. greggii ‘Stormy Pink’	2.31 ± 0.025	1			BAA
S. guaranitica ‘Argentine Skies’	3.58 ± 0.088	1	2n = 8x = 88	1	TG
S. guaranitica ‘Black and Blue’	3.52 ± 0.060	1	2n = 8x = 88	1	TG
S. guaranitica var. *congestiflora*	3.17 ± 0.014	1	2n = 8x = 88	1	BAA
S. haenkei	4.30 ± 0.040	2			BAA
S. hispanica	0.92 ± 0.004	2	2n = 2x = 12	6	BG
S. hispanica	0.93 ± 0.016	2	2n = 2x = 12	6	GBG
S. indica	1.95 ± 0.040	1	2n = 2x = 22	9	UZC
S. involucrata	1.20 ± 0.003	2	2n = 2x = 22	1	USNA
S. involucrata ‘Bethellii’	1.26 ± 0.012	2	2n = 2x = 22	1	BAA
S. judaica	2.43 ± 0.057	1	2n = 2x = 16, 18	9	GBG
S. lanceolata	1.20 ± 0.009	2			USNA
S. leucantha ‘Danielle’s Dream’	0.95 ± 0.016	2	2n = 2x = 32	7	TG
S. leucantha ‘Midnight’	0.92 ± 0.003	2	2n = 2x = 32	7	USNA
S. leucantha ‘Purple Velvet’	0.97 ± 0.018	2	2n = 2x = 32	7	BAA
S. leucantha ‘White Mischief’	0.93 ± 0.011	2	2n = 2x = 32	7	USNA
S. lyrata ‘Purple Knockout’	2.45 ± 0.025	1	2n = 2x = 36	2	UZC
S. macrophylla	0.94 ± 0.008	2	2n = 2x = 18	2	SC
S. macrophylla ‘Purple Leaf’	0.93 ± 0.008	2	2n = 2x = 18	2	BAA
S. macrophylla ‘Short Form’	0.90 ± 0.032	2	2n = 2x = 18	2	TG
S. madrensis	1.17 ± 0.006	2			TG
S. mexicana ‘Limelight’	1.21 ± 0.043	2	2n = 2x =22	9	USNA
S. mexicana x *gesneriiflora* ‘Raspberry Truffle’	1.59 ± 0.002	2			FBTS
S. microphylla ‘Hot Lips’	2.28 ± 0.068	1	2n = 2x = 22	1	TG
S. miltiorrhiza	1.39 ± 0.004	2	2n = 2x = 16	11	USNA
S. miniata	1.01 ± 0.010	2			TG
S. namaensis	1.17 ± 0.001	2			SC
S. nemorosa ‘New Dimension Blue’	1.15 ± 0.012	2	2n = 2x = 12, 14	7	PA
S. nemorosa ‘Rose Queen’	1.19 ± 0.004	2	2n = 2x = 12, 14	7	GBG

S. nemorosa ‘Salvatore Blue’	1.12 ± 0.014	2	2n = 2x = 12, 14	7	PA
S. nemorosa ‘Salute Blue Improved’	1.11 ± 0.009	2	2n = 2x = 12, 14	7	DO
S. nemorosa subsp. *pseudosylvestris*	1.15 ± 0.009	2	2n = 2x = 12, 14	7	TG
S. nipponica ‘BSWJ5829’	2.42 ± 0.091	1	2n = 2x = 16	9	FBTS
S. nubicola	2.22 ± 0.050	1	2n = 2x = 16	9	JPN
S. nutans	0.99 ± 0.013	2	2n = 2x = 18, 22	9	ULBG
S. officinalis	1.17 ± 0.003	2	2n = 2x = 14	7	GBG
S. orthostachys	1.50 ± 0.016	2			BAA
S. ovalifolia var. *ovalifolia* ‘Blue Form’	2.79 ± 0.036	2			BAA
S. oxyphora	5.57 ± 0.152	1			TG
S. pallida ‘Alba’	2.96 ± 0.053	1	2n = 8x = 88	1	BAA
S. patens ‘Ocean Blue’	1.91 ± 0.041	1	2n = 2x = 18	2	TG
S. pratensis ‘Indigo’	1.12 ± 0.017	2	2n = 2x = 16, 18, 20	7	BAA
S. pratensis subsp. *haematodes*	1.13 ± 0.013	2	2n = 2x = 16, 18, 20	7	SC
S. pratensis subsp. *pratensis*	1.12 ± 0.017	2	2n = 2x = 16, 18, 20	7	JPN
S. prunelloides	2.32 ± 0.008	1			USNA
S. prunelloides	2.35 ± 0.037	1			DDN
S. purpurea ‘Lavender Lace’	0.95 ± 0.017	2			BAA
S. reflexa	0.63 ± 0.009	2	2n = 2x = 20	7	WRPI
S. repens	2.67 ± 0.051	2	2n = 20	8	BAA
S. Rockin’® Blue Suede Shoes™	3.40 ± 0.033	1			PW
S. roscida	1.10 ± 0.010	2			BAA
S. rubescens	1.27 ± 0.010	2			USNA
S. rubescens subsp. *dolichothrix*	1.76 ± 0.027	1			BAA
S. scabiosifolia	1.54 ± 0.003	2	2n = 2x = 14	2	GBG
S. scabra	1.24 ± 0.008	2			BAA
S. schlechteri	1.64 ± 0.015	1			BAA
S. sclarea ‘Kardynal’	1.37 ± 0.010	2	2n = 2x = 22	9	GBG
S. sclarea ‘Vatican White’	1.32 ± 0.005	2	2n = 2x = 22	9	DDN

S. sclarea var. *turkestanica*	1.34 ± 0.021	2	2n = 2x = 22	9	JPN
S. sclarea var. *turkestanica*	1.36 ± 0.008	2	2n = 2x = 22	9	GBG
S. semiatrata	1.09 ± 0.005	2			SC
S. sessilifolia	0.81 ± 0.006	2	2n = 44	3	BAA
S. spathacea	1.65 ± 0.008	2	2n = 2x = 26, 30	2	SC
S. splendens 'Sao Borja'	1.95 ± 0.023	1	2n = 2x = 20	1	BAA
S. splendens Grandstand™ 'Red Lipstick Pink'	1.96 ± 0.058	1	2n = 2x = 20	1	GFB
S. splendens Grandstand™ 'Red'	1.95 ± 0.055	1	2n = 2x = 20	1	GFB
S. splendens Mojave™ Red/White Bicolor	1.98 ± 0.023	1	2n = 2x = 20	1	GFB
S. splendens x guaranitica PP 32027 'Purple & Bloom'	2.55 ± 0.012	1			BFP
S. stachydifolia var. *grandiflora*	2.99 ± 0.026	2	2n = 6x = 66	1	BAA
S. stenophylla	1.59 ± 0.009	2			JPN
S. striata	2.04 ± 0.016	1			BAA
S. subrotunda	1.64 ± 0.019	1			SC
S. texana	5.29 ± 0.058	1			ASC
S. thyrsiflora	0.96 ± 0.012	2			BAA
S. tingitana	2.82 ± 0.026	2			SC
S. trijuga	0.83 ± 0.001	2	2n = 2x = 16	11	BAA
S. tuerckheimii	2.71 ± 0.053	1			BAA
S. uliginosa 'Ballon Azul'	4.40 ± 0.085	1	2n = 4x = 52 + 0-3 B	1	BAA
S. urica	0.90 ± 0.001	2			USNA
S. verbenaca	2.90 ± 0.021	2	2n = 6x = 54	7	ULBG
S. verbenaca	2.91 ± 0.018	2	2n = 6x = 54	7	CBG
S. verbenaca	2.94 ± 0.012	2	2n = 6x = 54	7	UZC
S. verticillata	1.50 ± 0.016	2	2n = 2x = 16	9	JPN
S. viridis	1.06 ± 0.017	2	2n = 2x = 16	9	BG
S. x 'Blue Chiquita'	1.83 ± 0.021	1			BAA

S. x 'Jeans Jewel'	2.61 ± 0.052	1	FBTS
S. x 'Jezebel'	2.48 ± 0.031	1	BAA
S. *x* 'Mulberry Jam'	1.20 ± 0.016	2	FBTS
S. x *jamensis* 'Elk Cranberry Red'	2.23 ± 0.012	1	FBTS
S. x *jamensis* 'Red Velvet'	3.27 ± 0.077	1	USNA

[a] Values are listed as x ± SD, n = 3

[b] 1 = Piscum sativum 'Ctirad'; 2 = Solanum lycopersicum 'Stupické'

[c] 1 = Alberto et al., 2003; 2 = Bolkhovskikh et al., 1969; 3 = CCDB, 2021; 4 = Darlington and Wylie, 1956; 5 = Epling, 1962; 6 = Estilai et al., 1990; 7 = Haque, 1981; 8 = Kumar and Subramaniam, 1987; 9 = Ranjbar et al., 2015; 10 = Suda et al., 2005; 11 = Yang et al., 2009.

[d] AAP = Annie's Annuals & Perennials, Richmond, Clifornia; ASC = ALPLAINS, Kiowa, Colorado; BAA = Salvias.com.ar, Buenos Aires, Argentina; BFP = Ball FloraPlant, Arroyo Grande, California; BG = Botanischer Garten und Rhododendron-Park, Bremen, Germany; CBG = Jardin Botanique de Caen, Caen, France; DDN = Digging Dog Nursery, Albion, California; DO = Dümmen Orange, Columbus, Ohio; FBTS = Flowers by the Sea, Mendocino County, California; FL = Nassau County, Florida; GBG = M.M. Gryshko National Botanical Garden, Kiev, Ukraine; GFB = Green Fuse® Botanicals, Inc., Santa Paula, California; JPN = Jardin des Plantes de Nantes, Nantes, France; PA = Pan American Seed Co, West Chicago, Illinois; PW = Proven Winners, Sycamore, Illinois; RS = Rijnbeek and Sons, Boskoop, Netherlands; S = Selecta, Cham, Switzerland; SC = Seedhunt, Freedom, California; TG = Trial Gardens, Athens, Georgia; TWF = Trade Winds Fruit, Santa Rosa, California; ULBG = University of Lativa Botanical Garden, Riga, Lativa; USNA = United States National Arboretum, Washington, D.C.; UZC = University of Zagreb, Zagreb, Croatia; WRPI = Western Regional Plant Introduction Station, Pullman, Washington.

Table 2.2. Calculated 1C genome size from the current study and assigned clades for *Salvia*.

Taxa	[a] Estimated	Calculated			
Family *Lamiaceae*	2C genome	1C genome			Assigned
Genus *Salvia*	size (pg)	size (pg)	Section	[b] Source	Clade
S. lanceolata	1.20 ± 0.009		Hymenosphace	6	I-A
S. namaensis	1.17 ± 0.001		Heterospace	6	I-A
S. repens	2.67 ± 0.051	1.34	Heterospace	2	I-A
S. scabra	1.24 ± 0.008		Heterospace	2	I-A
S. schlechteri	1.64 ± 0.015		Heterospace	6	I-A
S. sessilifolia	0.81 ± 0.006	0.20	Eusphace	6	I-A
S. stenophylla	1.59 ± 0.009		Heterospace	6	I-A

S. lyrata ‘Purple Knockout’	2.45 ± 0.025	1.23	Heterospace	2	I-B
S. texana	5.29 ± 0.058		Salviastrum	6	I-B
S. aethiopis	2.99 ± 0.011	1.50	Aethiopis	2	I-C
S. amplexicaulis	1.47 ± 0.013	0.74	Plethiosphace	6	I-C
S. atropatana	2.36 ± 0.032	1.18	Aethiopis	6	I-C
S. barrelieri	3.10 ± 0.042	1.55	Plethiosphace	2	I-C
S. broussonetii	1.02 ± 0.020		Aethiopis	2	I-C
S. canariensis	1.06 ± 0.027	0.53	Hymenosphace	2	I-C
S. canariensis ‘Lancelot’	1.06 ± 0.016	0.53	Hymenosphace	2	I-C
S. deserta	1.12 ± 0.006	0.56	Plethiosphace	6	I-C
S. desoleana	3.11 ± 0.093	0.78	Aethiopis	1	I-C
S. disermas	3.52 ± 0.013		Plethiosphace	2	I-C
S. dumetorum	1.20 ± 0.017	0.60	Plethiosphace	2	I-C
S. indica	1.95 ± 0.040	0.98	Aethiopis	2	I-C
S. judaica	2.43 ± 0.057	1.22	Hemisphace	6	I-C
S. nemorosa ‘Salute Blue Improved’	1.11 ± 0.009	0.56	Plethiosphace	2	I-C
S. nemorosa ‘New Dimension Blue’	1.15 ± 0.012	0.58	Plethiosphace	2	I-C
S. nemorosa ‘Rose Queen’	1.19 ± 0.004	0.60	Plethiosphace	2	I-C
S. nemorosa ‘Salvatore Blue’	1.12 ± 0.014	0.56	Plethiosphace	2	I-C
S. nemorosa subsp. *pseudosylvestris*	1.15 ± 0.009	0.58	Plethiosphace	2	I-C
S. nutans	0.99 ± 0.013	0.50	Plethiosphace	2	I-C
S. pratensis ‘Indigo’	1.12 ± 0.017	0.56	Plethiosphace	2	I-C
S. pratensis subsp. *haematodes*	1.13 ± 0.013	0.57	Plethiosphace	2	I-C
S. pratensis subsp. *pratensis*	1.12 ± 0.017	0.56	Plethiosphace	2	I-C
S. sclarea ‘Kardynal’	1.37 ± 0.010	0.69	Aethiopis	2	I-C
S. sclarea var. *turkestanica*	1.34 ± 0.021	0.67	Aethiopis	2	I-C
S. sclarea var. *turkestanica*	1.36 ± 0.008	0.68	Aethiopis	2	I-C
S. sclarea ‘Vatican White’	1.32 ± 0.005	0.66	Aethiopis	2	I-C
S. tingitana	2.82 ± 0.026		Aethiopis	2	I-C

S. verbenaca	2.90 ± 0.021	0.48	Plethiosphace	2	I-C
S. verbenaca	2.91 ± 0.018	0.49	Plethiosphace	2	I-C
S. verbenaca	2.94 ± 0.012	0.49	Plethiosphace	2	I-C
S. verticillata	1.50 ± 0.016	0.75	Hemisphace	2	I-C
S. viridis	1.06 ± 0.017	0.53	Horminum	2	I-C
S. bucharica	2.44 ± 0.029	1.22	Hymenosphace	6	I-D
S. bucharica	2.43 ± 0.032	1.22	Hymenosphace	6	I-D
S. fruticosa	1.15 ± 0.013	0.58	Eusphace	6	I-D
S. officinalis	1.17 ± 0.003	0.59	Eusphace	2	I-D
S. scabiosifolia	1.54 ± 0.003	0.77	Eusphace	2	I-D
S. arenaria ‘Bau’	2.96 ± 0.036		Calosphace	2	II-A
S. azurea	0.64 ± 0.004	0.32	Calosphace	2	II-A
S. azurea var grandiflora	1.77 ± 0.018	0.89	Calosphace	2	II-A
S. biserrata	0.93 ± 0.019		Calosphace	4	II-A
S. blepharophylla	0.89 ± 0.030		Calosphace	4	II-A
S. bullulata	2.27 ± 0.009		Calosphace	4	II-A
S. cacaliifolia ‘Guatemalan Leaf Sage’	1.53 ± 0.004		Calosphace	4	II-A
S. calolophos	1.97 ± 0.055		Calosphace	4	II-A
S. cardiophylla	1.31 ± 0.005	0.33	Calosphace	4	II-A
S. caudata	1.04 ± 0.011		Calosphace	4	II-A
S. chiapensis	1.95 ± 0.030		Calosphace	4	II-A
S. coccinea	0.85 ± 0.004	0.43	Calosphace	2	II-A
S. coccinea	0.85 ± 0.008	0.43	Calosphace	2	II-A
S. coccinea ‘Yucatan’	0.87 ± 0.007	0.44	Calosphace	2	II-A
S. concolor	0.72 ± 0.007		Calosphace	2	II-A
S. confertiflora	1.70 ± 0.017		Calosphace	2	II-A
S. corrugata	2.33 ± 0.041		Calosphace	2	II-A
S. cuatrecasana	4.31 ± 0.072		Calosphace	4	II-A
S. discolor	2.20 ± 0.021	0.55	Calosphace	4	II-A

S. elegans ‘Honey Melon’	1.12 ± 0.009	0.56	Calosphace	2	II-A
S. elegans Rockin’® ‘Golden Delicious’	1.13 ± 0.014	0.57	Calosphace	2	II-A
S. elegans ‘Sonoran Red’	1.11 ± 0.026	0.56	Calosphace	2	II-A
S. farinacea ‘Augusta Duelberg’	2.19 ± 0.051	1.10	Calosphace	2	II-A
S. farinacea Cathedral® Lavender	2.17 ± 0.070	1.09	Calosphace	2	II-A
S. farinacea Cathedral® Purple	2.15 ± 0.016	1.08	Calosphace	2	II-A
S. farinacea ‘Henry Duelberg’	2.15 ± 0.039	1.08	Calosphace	2	II-A
S. farinacea Unplugged® So Blue™	4.10 ± 0.068	2.05	Calosphace	2	II-A
S. gesneriiflora ‘Compact Form’	1.96 ± 0.055		Calosphace	4	II-A
S. greggii ‘Cherry Lips’	2.33 ± 0.063		Calosphace	4	II-A
S. greggii ‘Stormy Pink’	2.31 ± 0.025		Calosphace	4	II-A
S. guaranitica ‘Argentine Skies’	3.58 ± 0.088	0.45	Calosphace	2	II-A
S. guaranitica ‘Black and Blue’	3.52 ± 0.060	0.44	Calosphace	2	II-A
S. guaranitica var. *congestiflora*	3.17 ± 0.014	0.40	Calosphace	2	II-A
S. haenkei	4.30 ± 0.040		Calosphace	2	II-A
S. hispanica	0.92 ± 0.004	0.46	Calosphace	2	II-A
S. hispanica	0.93 ± 0.016	0.47	Calosphace	2	II-A
S. involucrata	1.20 ± 0.003	0.60	Calosphace	2	II-A
S. involucrata ‘Bethellii’	1.26 ± 0.012	0.63	Calosphace	2	II-A
S. leucantha ‘Danielle’s Dream’	0.95 ± 0.016	0.48	Calosphace	2	II-A
S. leucantha ‘Midnight’	0.92 ± 0.003	0.46	Calosphace	2	II-A
S. leucantha ‘Purple Velvet’	0.97 ± 0.018	0.49	Calosphace	2	II-A
S. leucantha ‘White Mischief’	0.93 ± 0.011	0.47	Calosphace	2	II-A
S. macrophylla	0.94 ± 0.008	0.47	Calosphace	4	II-A
S. macrophylla ‘Purple Leaf’	0.93 ± 0.008	0.47	Calosphace	4	II-A
S. macrophylla ‘Short Form’	0.90 ± 0.032	0.45	Calosphace	4	II-A
S. madrensis	1.17 ± 0.006		Calosphace	4	II-A
S. mexicana ‘Limelight’	1.21 ± 0.043	0.61	Calosphace	2	II-A

S. microphylla ‘Hot Lips’	2.28 ± 0.068	1.14	Calosphace	2	II-A
S. miniata	1.01 ± 0.010		Calosphace	4	II-A
S. orthostachys	1.50 ± 0.016		Calosphace	4	II-A
S. ovalifolia var. *ovalifolia* ‘Blue Form’	2.79 ± 0.036		Calosphace	2	II-A
S. oxyphora	5.57 ± 0.152		Calosphace	4	II-A
S. pallida ‘Alba’	2.96 ± 0.053	0.37	Calosphace	2	II-A
S. patens ‘Ocean Blue’	1.91 ± 0.041	0.96	Calosphace	2	II-A
S. prunelloides	2.32 ± 0.008		Calosphace	2	II-A
S. prunelloides	2.35 ± 0.037		Calosphace	2	II-A
S. purpurea ‘Lavender Lace’	0.95 ± 0.017		Calosphace	2	II-A
S. reflexa	0.63 ± 0.009	0.32	Calosphace	4	II-A
S. roscida	1.10 ± 0.010		Calosphace	4	II-A
S. rubescens	1.27 ± 0.010		Calosphace	2	II-A
S. rubescens subsp. *dolichothrix*	1.76 ± 0.027		Calosphace	2	II-A
S. semiatrata	1.09 ± 0.005		Calosphace	4	II-A
S. splendens Grandstand™ ‘Red’	1.95 ± 0.055	0.98	Calosphace	2	II-A
S. splendens Grandstand™ ‘Red Lipstick Pink’	1.96 ± 0.058	0.98	Calosphace	2	II-A
S. splendens Mojave™ Red/White Bicolor	1.98 ± 0.023	0.99	Calosphace	2	II-A
S. splendens ‘Sao Borja’	1.95 ± 0.023	0.98	Calosphace	2	II-A
S. stachydifolia var. *grandiflora*	2.99 ± 0.026	0.50	Calosphace	4	II-A
S. striata	2.04 ± 0.016		Calosphace	4	II-A
S. subrotunda	1.64 ± 0.019		Calosphace	2	II-A
S. thyrsiflora	0.96 ± 0.012		Calosphace	4	II-A
S. tuerckheimii	2.71 ± 0.053		Calosphace	4	II-A
S. uliginosa ‘Ballon Azul’	4.40 ± 0.085	1.10	Calosphace	2	II-A
S. urica	0.90 ± 0.001		Calosphace	4	II-A
S. brandegeei ‘Pacific Blue’	1.84 ± 0.007	0.92	Audiberita	3	II-C
S. carduacea	6.07 ± 0.192	3.04	Audiberita	3	II-C

S. columbariae	1.56 ± 0.009	0.78	Audiberita	3	II-C
S. dorrii v. carnosa	1.68 ± 0.011	0.84	Audiberita	3	II-C
S. dorrii v. dorrii	1.68 ± 0.028	0.84	Audiberita	3	II-C
S. spathacea	1.65 ± 0.008	0.83	Audiberita	3	II-C
S. dolichantha	6.12 ± 0.129		Eurysphace	5	IV-A
S. nipponica 'BSWJ5829'	2.42 ± 0.091	1.21	Eurysphace	6	IV-A
S. nubicola	2.22 ± 0.050	1.11	Eurysphace	6	IV-A
S. trijuga	0.83 ± 0.001	0.42	Eurysphace	6	IV-A
S. miltiorrhiza	1.39 ± 0.004	0.70	Drymosphace	6	IV-B

[a] Values are listed as x ± SD, n = 3

[c] 1 = Atzei and Picci, 1982; 2 = Bentham, 1832; 3 = Epling, 1962; 4 = Gonzalez-Gallegos, 2020; 5 = Skottsberg, 1934; 6 = Will and Claben-Bockhoff, 2017.

Table 2.3. Summary of genome content in *Salvia* by clade.

Clade	Subclade	2C Range (pg)	2C Mean ± SD (pg)	1C Range (pg)	1C Mean ± SD (pg)	Number Analyzed
Clade I		**0.81 - 5.29**	**1.80 ± 0.93**	**0.20 - 1.55**	**0.75 ± 0.32**	**46**
	I-A	0.81 – 2.67	1.47 ± 0.60	0.20 – 1.34	0.77 ± 0.80	7
	I-B	2.45 – 5.29	3.87 ± 2.10	N/A	1.23	2
	I-C	0.99 – 3.52	1.75 ± 0.83	0.49 – 1.55	0.71 ± 0.29	32
	I-D	1.15 – 2.44	1.75 ± 0.65	0.58 – 1.22	0.88 ± 0.32	5
Clade II		**0.63 - 6.07**	**1.89 ± 1.11**	**0.32 - 3.04**	**0.75 ± 0.48**	**79**
	II-A	0.63 – 5.57	1.84 ± 1.04	0.32 – 2.05	0.68 ± 0.35	73
	II-C	1.56 – 6.07	2.41 ± 1.79	0.78 – 3.04	1.21 ± 0.90	6
Clade IV		**0.83 – 6.12**	**2.60 ± 2.07**	**0.42 - 1.21**	**0.86 ± 0.37**	**5**
	IV-A	0.83 – 6.12	2.90 ± 2.26	0.42 – 1.21	0.91 ± 0.43	4
	IV-B	N/A	1.39	N/A	0.70	1

CHAPTER 3

IMPROVED FLORAL CHARACTERISTIC OF *SALVIA COCCINEA* THROUGH INTRASPECIFIC HYBRIDIZATION

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[1] Maynard, R. and J. Ruter. To be submitted to *HortScience.*

Abstract

Salvia is the largest genus in the family Lamiaceae, with over 1000 species. The species *S. coccinea* used in this study has naturalized in the southeastern United States and is an important plant for pollinators. Because the available varieties have small flowers and a limited range of petal colors, this project aimed to improve phenotypic characteristics of *S. coccinea* for use in the landscape. Two elite accessions were selected for hybridization using the pedigree method. One selection displayed compact habit with bicolored coral and white flowers, while the other was slightly larger with solid red flowers. Selections were made based on improved flower color and larger petal size. The breeding program achieved a 25% increase in petal width and a more vivid petal color for the coral bicolored selections. Additionally, a 60% increase in petal width was achieved for red flowers. These novel selections are attractive plants for the landscape, displaying improved ornamental value and supporting local populations of pollinators.

Introduction

Salvia is the largest genus in Lamiaceae with over 1000 accepted species. With a cosmopolitan native range, *Salvia* species can be found growing in Europe, Asia, Africa, Australia, and the Americas (Kew Science, 2019). Several members of the genus are desirable for their ornamental value and ability to attract pollinators. *Salvia coccinea* is a valuable plant for the landscape with red flowers that support both bees and hummingbirds. Its natural distribution is not well known but likely ranges from Mexico to Central America or Brazil (Wester and Claβen-Bockhoff, 2011). However, it has naturalized in the southeastern United States from South Carolina through Texas (Kew Science, 2019). The species flowers from spring into August and can be used as an annual or herbaceous perennial depending on its geographic location (Clebsch, 2003).

Salvia coccinea is often grown as an annual seed crop in cooler regions and is included in wildflower mixes for the southeastern United States. This species is visited by bees for its valuable nectar reserve but is primarily pollinated by hummingbirds. Nectar guides are usually not present but can exist as small white spots on the corolla with about 3-10 µL of nectar held at the base of the flower (Wester and Claβen-Bockhoff, 2011). The inflorescence appears in panicles with bilabiate corollas and continually blooms throughout the season. Although the flowers are typically red, petal color can vary from coral to white, including bi-colored flowers. The species displays uniform branching with an average height of one meter. The deltoid leaves have crenate margins and increase in size down the stem from 2.5 to 7.0 cm in length (Clebsch, 2003; Starr, 1985).

Salvia coccinea is a diploid species with 11 base chromosomes (2n = 2x = 22) (Alberto et al., 2003). In previous genetic studies, red flowers have been identified as dominant, with pink-colored varieties arising as a recessive trait (Kumar, L.S.S., 1943). Several selections have been made commercially available. The Summer Jewel™ series, developed by Takii & Co., Ltd. (Kyoto, Japan) includes red, pink, white, and lavender flowering cultivars with compact habits. These were winners of All-America Selections marketed as displaying large 1.3 cm blooms (All-America Selections, 2021). Other varieties have been selected for compact growth, such as ‘Brenthurst’ with bi-colored white and coral petals. However, limited breeding work has been done to improve the species further.

Although *S. coccinea* has not been extensively bred, interspecific hybrids have been achieved between other *Salvia* species. In a study conducted by Tychonievich and Warner (2011), nine species of *Salvia* were crossed to determine hybridization ability. Due to autogamy in many *Salvia* species, the seed parent was emasculated before anther dehiscence. Pollination was achieved by touching the pollen parent’s anther to the seed parent’s stigma. All self-

pollinated species successfully developed seeds using this method. However, only a few interspecific crosses resulted in seed set (Tychonievich and Warner, 2011). Therefore, this breeding technique should be a reliable way to generate intraspecific crosses in *S. coccinea*.

Intraspecific hybridization is a breeding tool used to improve the value of many plant species. For example, inbreeding depression was observed in the self-pollinated *Andrographis paniculata*, leading to its classification as an endangered species. However, through intraspecific hybridization, researchers were able to increase the genetic diversity of *A. paniculata* and subsequently support conservation efforts (Valdiani et al., 2012). This breeding technique has also been used to enhanced performance and disease resistance *Poa pratensis* (Pepin and Funk, 1971), increase biomass production in *Chamaecrista fasciculata* (Erickson and Fenster, 2006), and induce variation in flower size in *Plumbago auriculata* (Chen et al., 2021). In this study, interspecific hybridization was used to improve *S. coccinea* in the landscape by selecting unique flower colors from two morphologically distinct accessions.

To describe changes in observable flower color, samples must be compared to a reliable standard (Griesbach and Austin, 2005). One of the most common standards used to describe horticultural specimens is the Royal Horticultural Society Color Chart. In a study conducted by Tucker et al. (1991), several color charts were compared for their use in describing biological samples. The RHS color chart was recommended as the best option based on ease of use (Tucker et al., 1991).

Although RHS values can be used as a standard to report the color of biological samples, variation in hue can be more easily described numerically by using the CIELAB color space (Commission on Illumination, 2004). In this model, L describes the lightness of a color (black to white) on a scale of 0 to 100. The other two parameters, a* and b*, describe mutually exclusive zones of color. For example, channel a gives amounts of red or green where red is a positive

value and green is negative. Similarly, channel b represents yellow or blue, where yellow is positive and blue is negative (Luo, 2015). These three scales of lightness, red to green, and yellow to blue are considered independent of one another and can be used in combination to describe color space (Krauskopf, 1982).

Materials and Methods

To develop an improved hybrid line, an accession of *S. coccinea* with a compact habit and coral bicolored petals was collected from Amelia Island in Nassau County, Florida. Two red accessions were trialed with the coral selection for use in the breeding program. The common species, also known as Scarlet Sage, was obtained from Trade Winds Fruit (Santa Rosa, California). The other, 'Yucatan', was a larger cultivar from Salvias.com.ar (Buenos Aires, Argentina). These three selections were grown from seed in the Trial Gardens at the University of Georgia in Athens, GA (33.944507, -83.375774) to observe overall growth habits in the Summer of 2019. The common Scarlet Sage was selected to hybridize with the coral accession based on its compact, well-branched structure. Vegetative cuttings of both plants were taken to maintain these selections during the winter in a greenhouse at the University of Georgia Horticulture Research Farm (33.886811, -83.419844). The day/night greenhouse conditions were set to 25°C /20 °C and 40% / 30% humidity.

Because red is the dominant flower color, the coral flowered accession was selected as the female parent to easily confirm hybridization in the F_1 population (Kumar, L.S.S., 1943). In January of 2020, controlled crosses were made between the two accessions, using the breeding technique described by Tychonievich and Warner (2011). The female parent was emasculated before anthesis to prevent autogamy. Once the style had fully extended from the female flower, mature anthers were collected from the male parent for pollination. Using forceps, pollen was transferred from the male anther to the stigma of the female parent. Primary pollinators were

considered excluded from plants in the greenhouse, so the inflorescences were not bagged after pollination.

Each flower set a maximum of four seeds. They were harvested when the calyx had dried, and seeds had become visibly brown. All hybrid seeds were individually sown on the surface of PRO-MIX high porosity substrate with biofungicide and mycorrhizae (Premier Tech Horticulture, Quakertown, Pennsylvania) in a 200-cell (22 mL) plug tray. Trays were irrigated from above and kept consistently moist on a 70% shade bench maintained under the same greenhouse conditions mentioned above.

Once seedlings had three sets of true leaves, they were potted up into 280 mL Square Deep Vacuum pots (HC Companies, Twinsburg, Ohio) using the same substrate. They were fertilized with 2.5 g of 8-9-month Osmocote Plus 15-9-12 (15-4.0-10.0 N-P_2O_5-K_2O) (ICL Specialty Fertilizers, Summerville, South Carolina) and moved into full sun. Elite selections in the F_4 generation, were moved into C300 2.8 L pots (Nursery Supplies Inc., Kissimmee, Florida) with substrate consisting of 20% peat moss, 28% 3/8" aged pine bark, 42% 5/8" aged pine bark, and 10% sand (Old Castle, Shady Dale, Georgia) and fertilized with 10.5 g of Osmocote Plus 15-9-12.

The F_1 population was morphologically uniform, so no selections were made from this generation. Instead, each plant was allowed to self-pollinate, and seeds were collected to form the F_2 generation. The pedigree selection method was used in which each plant from the F_2 was kept as a separate breeding line, and selections were made based on phenotypic traits (Love, 1927). Selections were made in the F_2 and subsequent populations based on petal size, petal color, and the number of nodes to the first flower. The pedigree breeding method was continued in the F_3 and F_4 generations to select plants with larger flowers, improved petal color, and compact growth from each breeding line.

One selection criterion, flower color, was used to isolate plants with improved petal pigmentation compared to the parents. Flower color was determined by comparing the upper and lower lobes to the RHS color chart (Royal Horticultural Society, 2001). These RHS values were converted to the CIELAB color space to describe variation in color numerically. The RHS color sample was scanned with the Nix Mini 2 Color Sensor to obtain CIELAB values (Nix Sensor Ltd., Hamilton, Ontario, Canada). The bicolored coral parent had the RHS color 56D (CIELAB: 89, 8, 3) on its upper lobe and a lower lobe color of 43D (CIELAB: 70, 42, 20). The red parent had the same upper and lower lobe color with an RHS value of 44B (CIELAB: 47, 64, 50). Individuals from this breeding program were selected to have improved petal color compared to the parents.

Another selection criterion, petal size, was the measured distance from one edge of the flower's lower lobe to the opposite edge at the widest point. The petal lobe width was 1.3 cm at the widest point for the red parent and 1.6 cm for the bicolored coral parent. Any progeny with equal or lesser petal size than the parents was discarded. Finally, selections were made for a more compact plant by estimating final height based on growth in the 280 mL pots. The number of nodes from the substrate line to the first inflorescence was used to distinguish plants with the same size and color flowers. When a decision was made based on the number of nodes to the first flower, the plant with fewer nodes was selected to remain in the breeding program.

In-ground beds were prepared for trialing the elite selections by forming three rows 30 m in length and 0.9 m in width at the University of Georgia Horticulture Research Farm. The soil type was Cecil association, well-draining soil with a surface layer of sandy clay loam and deep red clay subsoil (Robertson, 1968). Pre-plant fertilizer was applied at a rate of 9 kg N per hectare using 10-4.4-8.3 All Season's Lawn and Garden Fertilizer (Tri County Fertilizer and Specialty Co., Inc., Honea Path, South Carolina). A single line of 16 mm ID and 10 mm wall drip tape

with 30 cm emitter spacing (Rivulis Irrigation Ltd., San Diego, California) was laid on each bed for controlled irrigation. TIF Total Blockade plastic mulch (Berry Global Inc., Evansville, Indiana), 1.5 m width and 1.25 mm thickness, was applied to each row with a Model 2600 Series II raised bed plastic mulch layer (Rian-Flo Irrigation, East Earl, Pennsylvania) to minimize weed pressure.

By June 2021, the elite selections were well established in the 2.8 L pots and were planted in the prepared field rows. Plants were spaced 1.2 m within the row and 0.9 m between rows. The plants were irrigated in the morning for two hours, three times per week. After three months in the field, total height was measured to determine the plant's mature size. Final selections were made based on overall plant form, flower size, and flower color.

The ratio of red to coral flowers in the F_2 was analyzed with a chi-squared test. The mean, standard deviation, and skewness were analyzed for the distribution of lower lobe width and number of nodes to first flower. Finally, relationship between measured parameters was assessed for each of the field-trialed plants using the linear regression function in RStudio. These analyses were completed using the R programming language (RStudio, 2018).

Results

Uniform flower color was observed in the F_1 population, with all plants having red petals. Because the female parent had coral-colored flowers, the occurrence of all red progeny confirmed a successful cross. In the subsequent generation, the observance of 517 red-flowered plants and 155 coral-flowered plants (Figure 3.1) indicated a strong 3:1 ratio of red to coral flowers. This ratio was confirmed with a chi-squared test indicating that flower color is inherited in a Mendelian fashion, with red expressing complete dominance X^2 (1, N = 672) = 1.34, P > .05. These findings are consistent with observations made by Kumar (1942) in intraspecific hybrids of *S. coccinea*.

Although the F1 population was morphologically uniform, one plant from this generation did not set seed in the greenhouse. This selection did not appear to produce any pollen and was unable to be bred through later generations. However, this plant was maintained in the breeding program to determine fertility in the field trial. Despite all other *S. coccinea* selections setting seed in the field, this selection did not set seed through pollinator visitation. Additionally, it could not be induced to set seed by hand-crossing. Therefore, this selection is believed to be both male and female sterile, which has not previously been reported in *S. coccinea*.

No variation in petal color was observed in the red-petaled progeny; however, in the F_2 and subsequent generations, wide variation was observed in the bicolored progeny from pale pink to deep coral. Therefore, progeny with a deeper orange color than the parent were considered elite selections to be maintained in the breeding program. The measured RHS color values and associated CIELAB values for the elite coral-colored progeny are summarized in Table 3.1. All a* and b* are positive, indicating that with the CIELAB system, flower color is represented by a combination of red and yellow alone.

For the upper lobe of the bicolored parent, the CIELAB values were 89, 8, 3. The first parameter, L* = 89 indicates the upper lobe of the parent had a significant amount of white in the pigment. Comparatively, the upper lobe of the red parent had CIELAB values of 47, 64, 50. All selected progeny listed in Table 3.1 have L* values between the two parents, indicating their pigmentation was darker than the bicolored parent, but lighter than the red parent. With a* and b* values of 8 and 3, the bicolored parent was found to have minimal red or yellow coloration. However, the red parent had comparatively high values with a* = 64 and b* = 50. All selected coral progeny had high a* values indicating a significant increase in red compared to the bicolored parent, with variations in yellow indicated by the b* value.

For the lower lobe of the bicolored parent, the CIELAB values were 70, 42, 20. Comparatively, these values were 47, 64, 50 for the red parent. Thus, when looking at the progeny, it was evident that they had lightness values between the two parents. Additionally, the elevation of both a* and b* values compared to the bicolored parent resulted in a more orange color in the progeny. Overall, the lower lobe of selected progeny was coral in color with intermediate darkness between the two parents.

The second measured trait, petal width, was also uniform in the F_1, with the entire population having a lower lobe width of 1.6 cm. In the F_2, however, petal width varied, as shown in Figure 3.2. The median lower lobe width was 1.60 with a mean value of 1.58 cm (± 0.12 cm) and slightly negative skewness (-0.24). The red and coral parents had a lower lobe width of 1.4 cm and 1.6 cm, respectively. Therefore, a mean value of 1.58 cm and slight left skew in the F_2 indicates most of the population fell below the lobe width of the female parent. However, the lobe width of some progeny exceeded the width of either parent. The red-flowered progeny ranged in size from 1.2 – 2.0 cm, and selections were made to maintain flowers 1.6 cm or larger. The coral-flowered progeny had lower lobe widths of 1.2 – 1.8 cm in size. Because the coral-colored progeny also showed variance in petal color, selections were made for improved pigmentation and size.

By the F_4 generation, size improvements had been made in both the coral and red flowers. A side-by-side comparison of the original bicolored parent and an elite coral-colored selection from the F_4 is shown in Figure 3.3. With a 2.0 cm bottom lobe width, elite coral selections had up to a 25% increase in size compared to the 1.6 cm parent. Because selections were not limited by flower color, an even more significant improvement in lobe width was achieved for the red flowers. Pictured on the left in Figure 3.4 is the original red parent with a

bottom lobe width of 1.3 cm. A 60% increase in petal size was achieved through the breeding program by selecting red flowers up to 2.1 cm in size.

Selections were made primarily on petal size and color; however, the number of nodes to the first flower was used as the final selection criterion. This measurement was used to project the mature height of a plant and make selections for compactness. In the F_2 population, the number of nodes to the first flower varied widely, as shown in Figure 3.5. The average number of nodes to first flower was 9 nodes in the F_2 with a standard deviation of 1 node. The distribution was almost perfectly symmetrical with a slight right skew (0.04). When a decision had to be made between plants with indistinguishable flowers, plants with eight or fewer nodes were selected to remain in the breeding program. However, final height measurements taken after the three-month growth in the field indicated no relationship between height and number of nodes to the first flower ($R^2 = 0.09$), as shown in Figure 3.6. Additionally, no relationship was identified between final height and lower petal lobe width ($R^2 = 0.04$), shown in Figure 3.7. Final selections were made for plants with large flowers, improved petal color, and compact habit in the field and are summarized in Table 3.2.

Discussion

Variation in flower color of the *S. coccinea* hybrids can be explained by the presence of anthocyanins. These pigments impact colors ranging from orange to blue in plant tissue. The shift from orange to blue depends mainly on the number of hydroxyl groups attached to the anthocyanin B-ring (Tanaka et al., 2008). Two anthocyanins identified in *S. coccinea* flowers are pelargonidin 3-caffeoylglucoside-5-dimalonylglucoside and pelargonidin 3-*p*-coumaroylglucoside-5-dimalonylglucoside (Tomás-Barberán et al., 1987). Only one hydroxyl group is present on the B-ring in these pelargonidin anthocyanins, resulting in a red appearance. In a study conducted by Xue et al. (2016), white and pink-petaled strawberries were hybridized

to observe color inheritance. Five categories of petal colors were observed with the emergence of a deeper red color. Pigment analysis revealed that anthocyanins were responsible for the red coloration, with a positive correlation between a* CIELAB values and anthocyanin concentration (Xue et al., 2016).

The number of nodes to the first flower did not correlate with plant height in *S. coccinea*; however, this is not the case for other species. In a study conducted by Torres and Lopez (2011), the plant architecture of *Tecoma stans* was observed in response to changes in the daily light integral. Total plant height and number of nodes were found to increase together with an increase in light (Torres and Lopez, 2011). Plant height was also positively correlated with the number of nodes in *Ocimum basilicum* in different growing temperatures (Walters and Currey, 2019). In these studies, the number of nodes was a positive predictor of plant height, despite changes in environmental factors. However, the number of nodes to the first flower did not correlate with plant height in *S. coccinea*, and therefore, it is not a reliable way to select for compact growth.

Future research may improve the selections in at least two ways. The first objective would be to develop a selection with reduced fertility to inhibit self-seeding in the landscape. For example, dense populations of *S. coccinea* have spread from gardens to uncultivated areas of South Africa, leading researchers to believe it has become invasive (Moshobane et al., 2020). Intraspecific hybridization is believed to contribute to the invasive spread of species, including *Phalaris arundinacea* (Lavergne and Molofsky, 2007), *Pyrus calleryana* (Culley and Hardiman, 2009), and *Schinus terebinthifolius* (Williams et al., 2005). For example, *Pyrus calleryana* is self-incompatible but readily sets seeds and forms thickets when crossed with cultivars of the same species (Culley and Hardiman, 2009). Although *S. coccinea* is beneficial to pollinators and is not currently on a list of invasive species for the United States, its ability to re-seed may be undesirable.

A second objective for future research would be to create resistance to the southern pink moth, *Pyrausta inornatalis* (Crambidae). The larvae of the southern pink moth bore into and feed on unopened flowers exclusively in the genus *Salvia* (Oregon Department of Agriculture, 2021). Moths of the family Crambidae have successfully been repelled through treatment with the essential oil of *Salvia officinalis* (Göttig et al., 2017). Essential oil content is low in *S. coccinea*; however, increasing oil production may have environmental benefits and reduce maintenance costs of *S. coccinea* by deterring *Pyrausta inornatalis*.

Essential oil production is related to the ploidy level. For example, chromosomes in *Salvia leriifolia* were induced to double from n = 11 to n = 22 using colchicine. As a result, while the diploid species contained 14 distinct essential oils, the tetraploid developed with eight additional compounds (Estaji et al., 2017). An increase in aromatic compounds was also reported in *S. muticaulis* (Tavan et al., 2021) and other polyploid-induced plants (Dhawan and Lavania, 1996). Therefore, the quantity and diversity of essential oils may be increased by inducing polyploidy in *S. coccinea*.

By crossing two accessions of *Salvia coccinea,* improved selections were isolated with characteristics superior to either parent. A 25% increase in petal size was achieved for coral-petaled flowers, and a 60% increase was achieved for red-petaled flowers. Additionally, deeper orange, bicolored petals were isolated from this breeding program. From the field trial, plants were found to have a uniform shape and compact habit. Overall, the selections from this breeding program bring improved growth habit, larger flowers, and a more vivid color pallet to the available *S. coccinea.* These selections can be used as attractive flowering plants in the landscape to support local pollinator populations and are available for commercial release by vegetative propagation.

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Tables and Figures

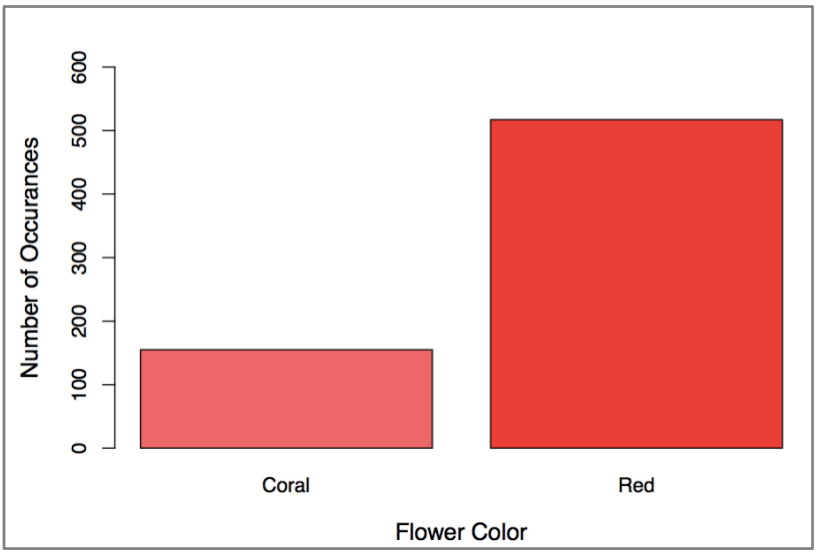

Figure 3.1. A 3:1 ratio of red to coral flowers observed in the F_2 population of intraspecific hybrid *Salvia coccinea* X^2 (1, N = 672) = 1.34, P > .05.

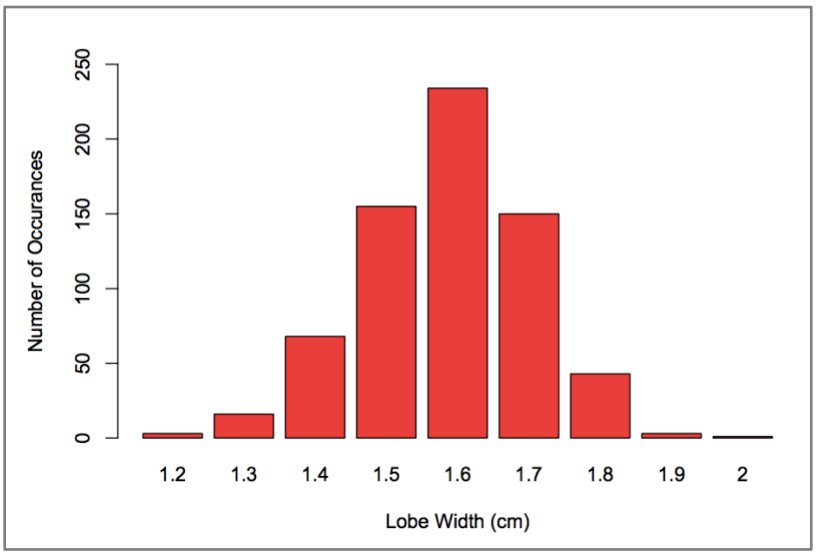

Figure 3.2. Distribution of petal lobe width in the entire F_2 population of *S. coccinea*. The average lobe width was 1.58 cm with a standard deviation of 0.12 cm and skewness of -0.21.

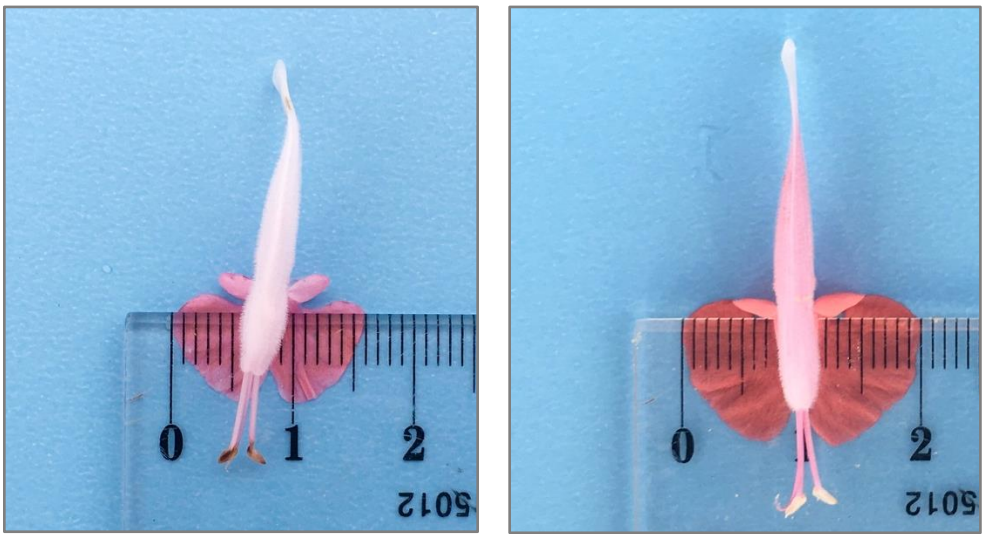

Figure 3.3. Selection improvements in coral-petaled *Salvia coccinea*. (Left) original bicolored parent with a bottom lobe width of 1.6 cm. (Right) Improved selection from the F_4 population with a 2.0 cm bottom lobe width and more vivid coral pigmentation.

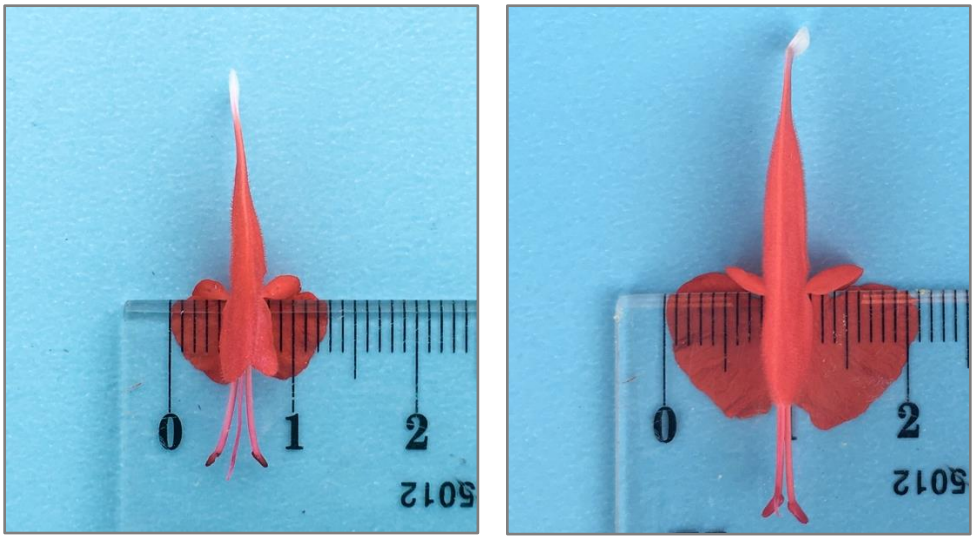

Figure 3.4. Selection improvements in red-petaled *Salvia coccinea*. (Left) original red parent with a bottom lobe width of 1.3 cm. (Right) Improved selection from the F_4 population with a 2.1 cm bottom lobe width.

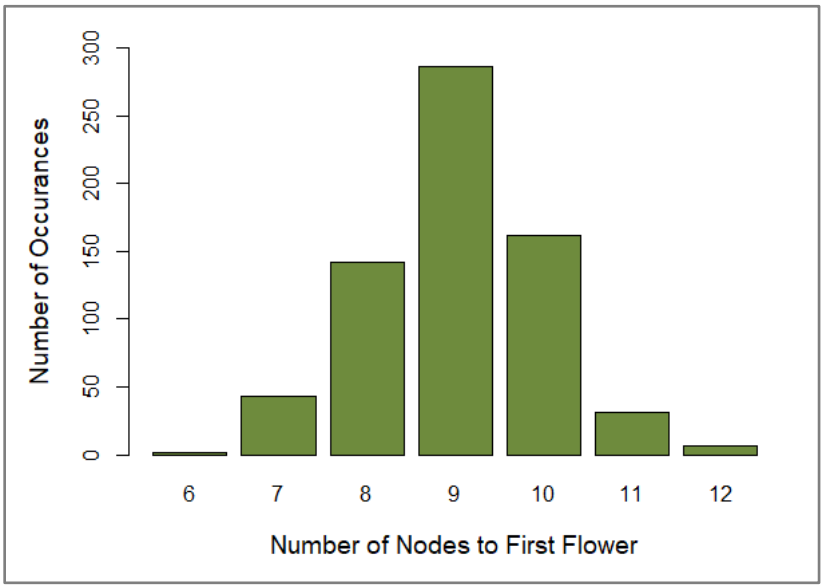

Figure 3.5. Distribution of the number of nodes to the first flower in the entire F_2 population of *S. coccinea*. The average number of nodes to first flower was 9 nodes with a standard deviation of 1 node. The data is fairly symmetrical with a skewness of 0.04.

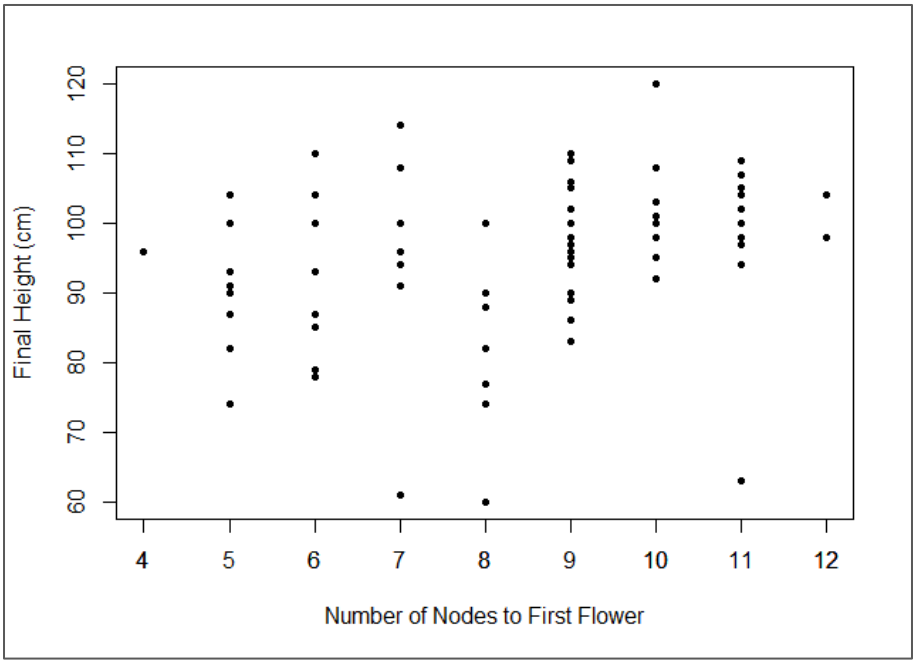

Figure 3.6. Comparison of the number of nodes to the first flower with overall height of *Salvia coccinea* hybrids. No relationship was identified ($R^2 = 0.09$).

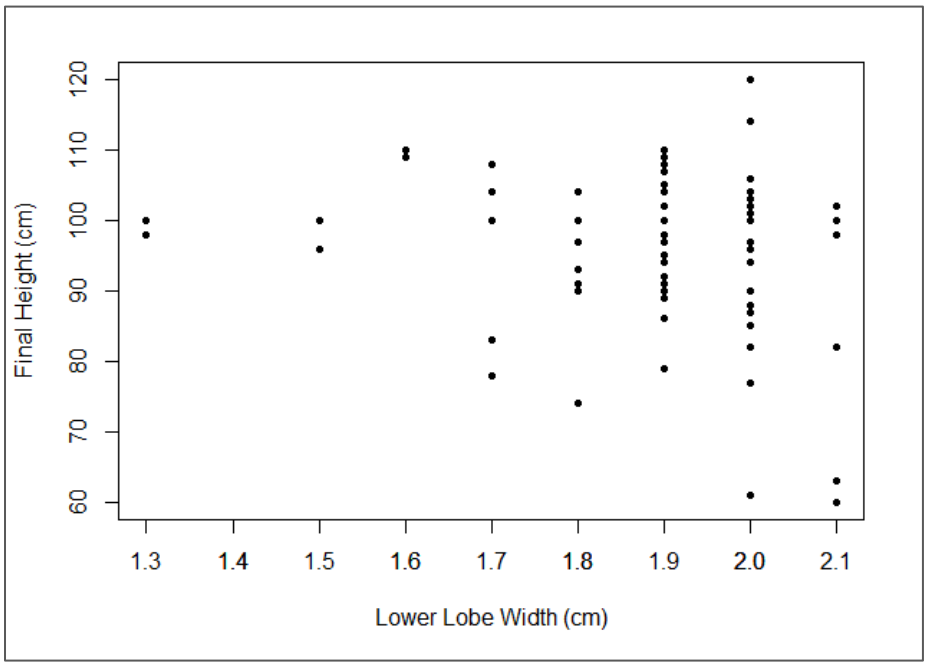

Figure 3.7. Comparison of the lower petal lobe width with overall height of *Salvia coccinea* hybrids. No relationship was identified ($R^2 = 0.04$).

Table 3.1. Summary of RHS and CIELAB colors of each elite coral selection from the F_2, F_3, and F_4 populations.

Lower Lobe Width (cm)	RHS Color Upper Lobe	CIELAB Upper Lobe	RHS Color Lower Lobe	CIELAB Lower Lobe	Selection Number
1.6	55B	69, 40, 2	40A	54, 65, 51	Z13-1-1
1.7	52D	73, 38, 13	41B	57, 56, 37	L1-2
1.7	55B	69, 40, 2	41B	57, 56, 37	AD9-2
1.7	52D	73, 38, 13	41C	66, 50, 32	S12-5
1.7	52C	64, 50, 15	40B	56, 62, 51	AD9-5-5
1.7	55B	69, 40, 2	40B	56, 62, 51	K15-3-5
1.7	55B	69, 40, 2	41B	57, 56, 37	L1-2-4
1.7	55C	77, 28, 2	41B	57, 56, 37	Z9-4-2
1.7	55C	77, 28, 2	43C	58, 56, 31	Z12-4-1
1.8	52C	64, 50, 15	41B	57, 56, 37	F18-1
1.8	52D	73, 38, 13	41B	57, 56, 37	AF1-1
1.8	52D	73, 38, 13	41B	57, 56, 37	Z13-1
1.8	55C	77, 28, 2	41C	66, 50, 32	Z9-4
1.8	55C	77, 28, 2	43C	58, 56, 31	Z12-4
1.8	55B	69, 40, 2	41A	54, 62, 44	Y14-2-3
1.8	55C	77, 28, 2	41B	57, 56, 37	AF1-4-3
1.8	55B	69, 40, 2	43B	57, 56, 37	AF1-1-1

1.8	55B	69, 40, 2	43C	58, 56, 31	J18-4-2
1.9	55B	69, 40, 2	41A	54, 62, 44	J18-4-4
1.9	55C	77, 28, 2	41A	54, 62, 44	AD9-1-1
1.9	55B	69, 40, 2	41B	57, 56, 37	AD9-5-1
1.9	55B	69, 40, 2	41B	57, 56, 37	J18-1-3
1.9	55C	77, 28, 2	43C	58, 56, 31	E11-5-2
1.9	55C	77, 28, 2	43C	58, 56, 31	Z12-4-5
2.0	55B	69, 40, 2	43B	57, 56, 37	AD9-2-5

Table 3.2. Description of final selections.

Selection	RHS Upper Lobe Color	RHS Lower Lobe Color	Lower Lobe Width (cm)	Final Plant Height (cm)	Final Plant Width (cm)
AD9-2	55B	41B	1.7	104	168
Z13-1	52D	41B	1.8	74	100
Z9-4	55C	41C	1.8	100	169
AD9-2-5	55B	43B	2.0	85	138
AK10-4-3	44B	44B	2.1	60	54
AK10-2-4	44B	44B	2.1	82	117

CHAPTER 4

MUTATION BREEDING OF *SALVIA COCCINEA* WITH ETHYL METHANESULFONATE (EMS)

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[1] Maynard, R. and J. Ruter. To be submitted to *HortScience.*

Abstract

Salvia coccinea is a valuable flowering annual that attracts hummingbirds and bees to the garden. Unfortunately, few varieties are commercially available. There is a limited range of petal colors and no leaf variegation. This research aimed to improve the ornamental value of *S. coccinea* by inducing mutations with ethyl methanesulfonate (EMS). The standard, red-flowered species was selected for treatment by exposing seeds to 0, 0.4, 0.8, or 1.2% EMS for 8, 12, or 24 hours. The optimal treatment rate was determined to be 1.2% EMS for 8 hours, which generated desirable mutations near the LD_{50}. The M_1 population had a 53% germination rate and was completely morphologically uniform. By the M_2, mutations included differences in leaf shape and flower size in addition to *albina*, *chlorina*, *virescens*, and chimeral chlorophyll changes. A 1% mutation rate was achieved in this breeding program with seven unstable mutations and six stable mutations. The normalized difference vegetation index (NDVI) values were measured to determine differences in chlorophyll content between lethal *albina* mutations, chartreuse *chlorina* and *virescens* mutations, and typical leaf color. Future work will investigate the stability and heritability of chlorophyll variegation by hybridizing these selections with coral-flowered accessions of *S. coccinea*.

Introduction

Salvia coccinea is an attractive flowering plant used as a self-seeding annual or herbaceous perennial in the landscape. On average, it grows to one meter in height with uniform branching, pubescent green leaves, and red to white flowers. (Clebsch, 2003). *S. coccinea* is a vital pollinator plant for hummingbirds and has naturalized in the southeastern United States. Its native range is uncertain but is believed to extend from Mexico to Central America or Brazil (Wester and Claβen-Bockhoff, 2011). Compact varieties have been selected, such as ‘Lady in

Red' with crimson flowers and 'Summer Jewel Pink' with bicolored white and pink petals. However, limited breeding work has been done to improve the species further.

There is a continual market demand for ornamental plants with new characteristics. If limited natural variation exists, mutation breeding can improve a crop by artificially inducing genetic variation. This breeding technique has been shown to cause phenotypic variations in color, flower shape, plant height, and leaf chimeras (Datta and da Silva, 2006). Chlorophyll mutations are the most common and reliable way to determine the efficacy of treatments (Patial et al., 2017). However, most induced mutations are recessive and cannot be segregated until the M_2 generation (Toker et al., 2007).

Although unique traits can be isolated using mutation breeding, the occurrences are random, and induction treatments can reduce germination and subsequent growth of seedlings. Therefore, large populations must be treated to increase the chances of achieving a plant with desirable characteristics (Toker et al., 2007). Nine categories and sub-categories of chlorophyll mutations were described by Gustafsson (1940). These categories can be used to distinguish a mutated population-based on leaf coloration and pattern. Unfortunately, because of a disruption in chlorophyll production, many mutants are lethal. However, observing chlorophyll mutations helps determine an appropriate mutagen concentration to maximize induced genetic variation (Singh et al., 2019).

The color differences caused by chlorophyll mutations can be described numerically using spectrophotometer readings. Chlorophyll absorbs solar radiation and fluoresces in the red (685 – 690 nm) and far-red (730-740 nm) regions of the electromagnetic spectrum. Therefore, chlorophyll content, and subsequently leaf greenness, can be determined by comparing the ratio of red and far-red reflectance (Buschmann, 2007). The normalized difference vegetation index (NDVI) describes relative amounts of red and far-red light reflected from leaves. Leaves with

low chlorophyll content reflect more red light and lower the NDVI value (Glen and Tabb, 2019). Therefore, NDVI readings can be used to assess leaf greenness and photochemical activity.

Mutations can be induced through radiation or exposure to a chemical mutagen (Datta and Teixeira da Silva, 2006). In a mutation study conducted on *Arabidopsis thaliana*, eleven different physical and chemical mutagens were tested to determine which had the most significant effect. The chemical mutagen ethyl methanesulfonate (EMS) was shown to have the highest mutation and survival rate compared to the other treatments (McKelvie, 1963). In similar studies, EMS was found to generate the most significant mutations in *Abelmoschus esculentus* (Gupta et al., 2017), *Delphinium malabaricum* (Kolar et al., 2015), and *Vigna umbellata* (Patial et al., 2017) compared to other chemical and physical mutagens.

The LD_{50} is commonly used to generate many mutated plants without significantly reducing the population from the toxic effects of the mutagen (Yadav et al., 2016). However, prolonged exposure to EMS may have deleterious effects on phenotypic characteristics without reducing seed germination. For example, in research conducted by Jiang and Wilde (2014), an increase in EMS concentration and exposure time was determined to reduce flower production in *Petunia* without reducing seed germination (Jiang and Wilde, 2014). Furthermore, Khalatkar (1976) studied the effect of EMS uptake in dry *Hordeum vulgare* seeds compared to seeds pre-soaked in water. It was found that dry seeds did not develop with chlorophyll chimeras, but pre-soaking resulted in a 9% chlorophyll mutation rate without a significant decrease in germination. Therefore, it was recommended to pre-soak seeds prior to treatment with EMS to increase mutation frequency (Khalatkar, 1976).

EMS mutagenesis has been used to improve the appearance of several ornamental plants. These improvements included the introduction of golden and white foliage in *Delphinium* (Kolar et al., 2015), novel and stable petal colors in *Dendranthema* (Latado et al., 2004), increased

flowering ability in *Gladiolus* (Bahajantri and Patil, 2013), and unique petal fringe in *Saintpaulia* (Fang and Traore, 2011). Although this breeding technique is widely used, it has not been well documented for *Salvia*. This study aimed to induce genetic variation in *S. coccinea* by exposure to EMS and isolate plants with improved phenotypic characteristics for use in the landscape.

Materials and Methods

Experiment 1

An experiment was designed to determine the optimum treatment parameters to induce mutations in *S. coccinea.* Twelve different treatment groups were tested by varying exposure time and concentration of the chemical mutagen EMS to form a two-way factorial. The first factor, with three levels, tested the mutagenic impact of exposure time by using an 8, 12, or 24-hour treatment period. The second factor, with four levels, tested the impact of mutagen concentration by using 0, 0.4, 0.8, or 1.2% EMS. Finally, the experiment had three replicates of 18 seeds in each treatment group.

Mature seeds were harvested from a stock plant maintained under greenhouse conditions in January 2020. Seeds were pre-soaked in deionized water for 12 hours to increase their ability to imbibe EMS solution. For the control group, a beaker was prepared with only DI water. EMS was diluted with DI water to form the appropriate solution concentrations for treatment groups. Once the solutions were created, seeds were randomly assigned into their treatment groups. The beakers were covered with Parafilm and continuously agitated on a mechanical shaker for 8, 12, or 24 hours at 200 RPM (New Brunswick Scientific, Edison, New Jersey). After the treatment period, the beakers were removed from the shaker, and their solution was decanted off. Seeds were rinsed for five seconds with DI water and decanted three times to remove residual EMS.

The treated seeds were individually sown on PRO-MIX HP substrate with biofungicide and mycorrhizae (Premier Tech Horticulture, Quakertown, Pennsylvania) in a 128-cell (45 mL)

plug flat. Each flat contained four treatment groups. The flats were arranged in a randomized complete block design across three benches in a greenhouse at the University of Georgia Horticulture Research Farm in Athens, GA (33.8870, -83.4201). The flats were left under 70% shade with no supplemental lighting and irrigated from below. Daytime temperatures were set to 24°C, and nighttime temperatures were set to 19°C.

Cotyledon emergence was recorded through daily observation. Once the seedlings had two sets of true leaves, they were stepped up into 1.05 L square pots with PRO-MIX HP substrate, fertilized with 3.5 g of 8-9-month Osmocote Plus 15-9-12 (15-4.0-10.0 N-P_2O_5-K_2O) (ICL Specialty Fertilizers, Summerville, South Carolina), and moved into full sun. The significance of EMS concentration and exposure time effects on seed germination was tested with a two-way ANOVA using the programming language R (RStudio, 2018). The M_1 population was self-pollinated, and 60 seeds were collected from each treatment group to form the M_2 generation. Mutations were described by their leaf color, leaf shape, and floral structures.

Experiment 2

Based on the LD_{50} of the M_1 population and the observed mutations in the M_2 population from the first experiment, 1.2% EMS for 8 hours was selected as the optimum treatment for inducing mutations in *S. coccinea*. Therefore, this treatment concentration and exposure time were used to treat a large population of seeds. In January 2021, seeds were harvested from a *S. coccinea* greenhouse stock plant and randomly assigned into treatment groups. The treated population consisted of six replicates of 100 seeds, while the control had six replicates of 20 seeds. All seeds were pre-soaked and treated with EMS as previously described. After the treatment period, seeds were rinsed three times with DI water and sown individually in 200-cell (22 mL) plug flats on PRO-MIX HP substrate. Each flat had one treatment group and one control

and was left under a shaded bench with the same greenhouse conditions as the first part of the experiment.

A 53% germination rate was observed in the M_1 population. Once the surviving seedlings had two sets of true leaves, they were stepped up into 1.05 L square pots, fertilized with 3.5 g of 8-9-month Osmocote Plus 15-9-12, and moved into full sun. The plants were self-pollinated, and four seeds were collected from each plant to form the M_2 population. These seeds were sown individually in 200-cell (22 mL) plug flats on PRO-MIX HP substrate. They were left to germinate on a shaded bench and treated with the same greenhouse conditions from the first experiment. Mutations in the M_2 population were described using the same parameters as the first experiment. The PolyPen RP 410 UVIS (Photon Systems Instruments, Drásov, Czech Republic) was used to measure the spectral reflectance of leaves with uniform chlorophyll mutations. Five representative leaves of each mutation were averaged to form mean and standard deviation values of NDVI.

Results and Discussion

Experiment 1

In the M_1 population, a statistically significant difference was found in germination for both EMS concentration ($f(3)=141.59$, $p<0.001$) and exposure time ($f(2)=101.50$, $p<0.001$). In addition, a significant interaction appeared between the two factors at higher concentrations of EMS ($f(6)=37.87$, $p<0.001$). Figure 4.1 shows a significant decline in germination with increasing EMS concentration and exposure time. A decline in seedling germination with increasing EMS concentration has been well documented with many other species, including *Abelmoschus esculentus* (Baghery et al., 2016), *Oryza sativa* (Talebi et al., 2012), and *Sarcococca confusa* (Hoskins and Contreras, 2020).

In this study, all plants in the M_1 were morphologically uniform. This result was expected because most mutations are recessive (Toker et al., 2007). The mutation effect of EMS on *S. coccinea* was observed in the M_2 generation through chlorophyll mutations, leaf deformation, and changes in floral structures. The leaf chlorophyll mutations were described using classification by Gustafsson (1940). These mutations included *albina, chlorina*, *virescens,* and other non-uniform chlorophyll mutations not described by Gustafsson. The *albina* mutation is characterized by a lack of chlorophyll and carotenoids resulting in completely white leaves. In *chlorina* mutants, leaves develop with a uniform, stable chartreuse color. *Virescens* mutants are similar to *chlorina* mutants at the early developmental stages; however, leaf color returns to normal in the mature plant (Gustafsson, 1940).

Two stable mutated plants were observed in the M_2 population from separate treatment groups. These mutations are shown in Figures 4.2B and 4.2C. The first mutated plant was observed in the group treated with 1.2% EMS for 8 hours. This plant had a visible *chlorina* mutation, the overall growth habit was significantly more compact, and both the leaves and flowers were smaller than the species. The leaf shape became more ovate than the parent but maintained serrated leaf margins and typical reproductive structures. Reduction in growth has also been observed in a yellow-green leaf mutant of *Betula*. This reduced growth was believed to be the result of lower chlorophyll production (ShuoQi, 2018).

The second mutated plant was found in the M_2 population from seeds exposed to 0.8% EMS for 12 hours. No chlorophyll mutations were observed in this plant; however, the leaves were densely covered with fine hairs, giving a hazy blue-gray appearance. This mutation displayed significantly deformed leaves with revolute leaf margins. The flowers lacked anthers and had reduced floral lobes. Changes in leaf shape and male sterility were also observed in EMS mutants of *Arabidopsis* (McKelvie, 1963). No chlorophyll mutations occurred below 0.8%

EMS in this experiment. Although mutations were observed at 1.2% EMS for 8 hours and 0.8% EMS for 12 hours, no mutations were observed at longer exposure times for either of these concentrations.

Experiment 2

In the second study, mutations began to appear at the seedling stage of the M_2. The greatest number of chlorophyll mutations in this population were unstable *virescens* mutations shown in Figures 4.3B, 4.3C, and 4.3D. The leaves initially were chartreuse but later matured to the typical leaf color associated with *S. coccinea*. *Virescens* mutations were also the most commonly occurring chlorophyll mutation in *Vigna umbellata* (Gustafsson, 1940; Patial et al., 2017). Two lethal *albina* mutants, shown in Figures 4.3E and 4.3F, were observed with no production of chlorophyll or carotenoids. These did not survive beyond two sets of true leaves. A*lbina* mutants have been described as entirely lethal in other species, including *Vigna umbellata* (Patial et al., 2017), *Cajanus cajan* (Etther et al., 2019), and *Capsicum annum* (Kumar et al., 2000).

The lethal nature of *albina* mutants can be explained by their lack of chlorophyll. Photochemical reflectance measurements are commonly used to determine variations in vegetation and subsequent photosynthetic activity (Chen et al., 2014; Chu et al., 2019; Gamon et al., 2015). This study compared the chartreuse leaves observed in the *viridis* and *virescens* mutants with white leaves of the *albina* mutant and normal *S. coccinea* leaves by measuring their NDVI. The difference in leaf color is shown in Figure 4.4. The NDVI values of the green, chartreuse, and white leaves were 0.722 ± 0.009, 0.442 ± 0.003, and 0.016 ± 0.002, respectively. Lower NDVI values correspond with lower chlorophyll content, as observed in the less pigmented leaves (Glen and Tabb, 2019). These NDVI readings explain the difference in

pigmentation among the mutations and the inability of the *albina* mutant to survive past the seedling stage with reduced photosynthetic activity.

Three other non-uniform but stable chlorophyll mutations were isolated from this population, which Gustafsson's (1940) classifications cannot describe. As shown in Figure 4.2D, one plant appeared to be a sectorial chimera with half of an entire leaf lacking chlorophyll. Sectorial chimeras form when a mutation in the chloroplast DNA creates blocks of distinct tissue in all three apical layers (Marcotrigiano, 1997). The other two stable, non-uniform mutations, shown in Figures 4.2E and 4.2F, had splotches of light green and white tissue dispersed across the leaf. Because these mutations did not show a pattern relating to the apical cell layers, they were likely caused by a mutation in the chloroplast. Cells with both mutated and normal chloroplasts are known as heteroplastic cells. As the cell divides, the chloroplasts are sorted into cells containing either mutated or normal DNA, resulting in a mosaic appearance (Marcotrigiano, 1997).

In addition to chlorophyll mutations, three plants with leaf deformations were observed in the M_2. One leaf mutation was stable and resulted in larger, more cordate leaves compared to the untreated species, as shown in Figure 4.2G. The other two mutations were unstable leaf deformations. The altered leaves were lanceolate with entire leaf margins and are shown in Figures 4.3G and 4.3H. Eventually, the mature plants reverted to typically shaped leaves. Changes in leaf shape from EMS exposure have been described in *Abelmoschus esculentus* (Gupta and Sood, 2019), *Chrysanthemum morifolium* (Nasri et al., 2021), and *Cucumis sativus* (Chen et al., 2018).

Six stable mutants were isolated from the M_2 populations of both experiments and are summarized in Table 4.1. This research may be continued by self-pollinating and seed propagating plants from the M_2 to look for mutations in later generations. In research conducted

by Qiaojuan et al. (2015), variations in flower color were only found after reaching the M_4 of EMS mutated *Salvia splendens*. In *Chrysanthemum indicum*, mutations from EMS exposure were not isolated until the M_3 (Purente et al., 2020). Future work could look at mutation frequency through the M_4 to check for mutations in the later generations.

These mutant populations may be further improved by testing the heritability and stability of selected mutants. Early studies in maize indicated that chlorophyll is inherited maternally (Anderson, 1923). In a more recent study, however, maternal inheritance was not detected in mutant *Arachis hypogea.* Chlorophyll mutants reciprocally crossed with a normal green-leaved accession produced mutants that were recessive but heritable. In the F_2 population, progeny segregated in 3:1 ratio of normal to *albino-virescent* leaves (Branch and Brown, 2019). *S. coccinea* mutants from this study may be further improved through reciprocal crosses to accessions with different flower colors. Mutants were autogamous, indicating they were male and female fertile. If chlorophyll mutations are heritable, improved lines may be selected for leaf variegation in multiple flower colors.

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Tables and Figures

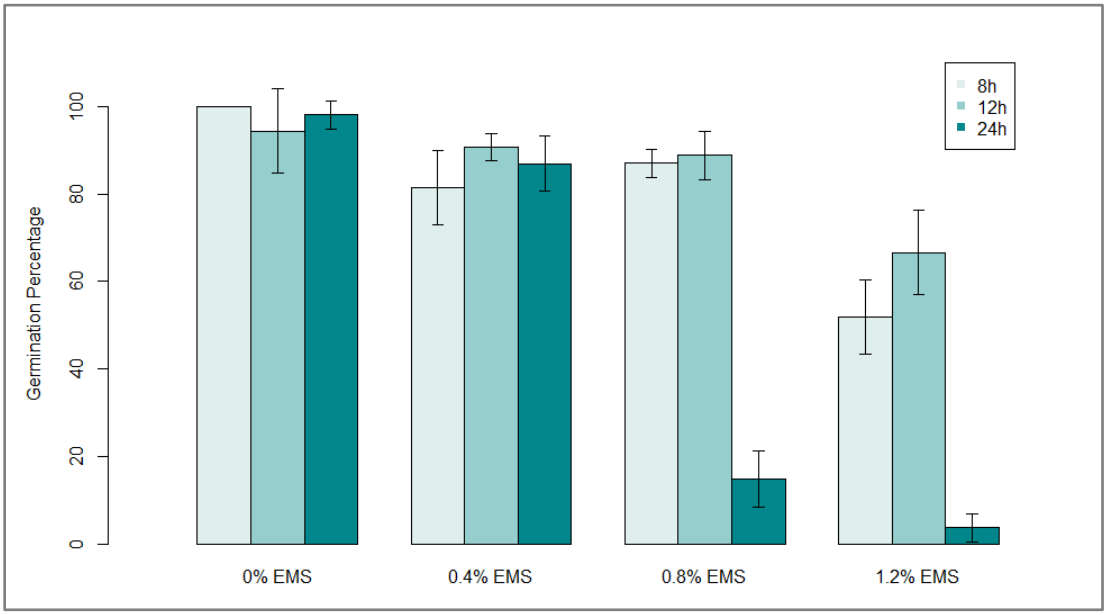

Figure 4.1: Germination percentage was measured after treating a population of *S. coccinea* seeds with 0, 0.4, 0.8, or 1.2% EMS for 8, 12, or 24 hours. Cotyledon emergence was found to decline significantly with higher levels of EMS and longer exposure times.

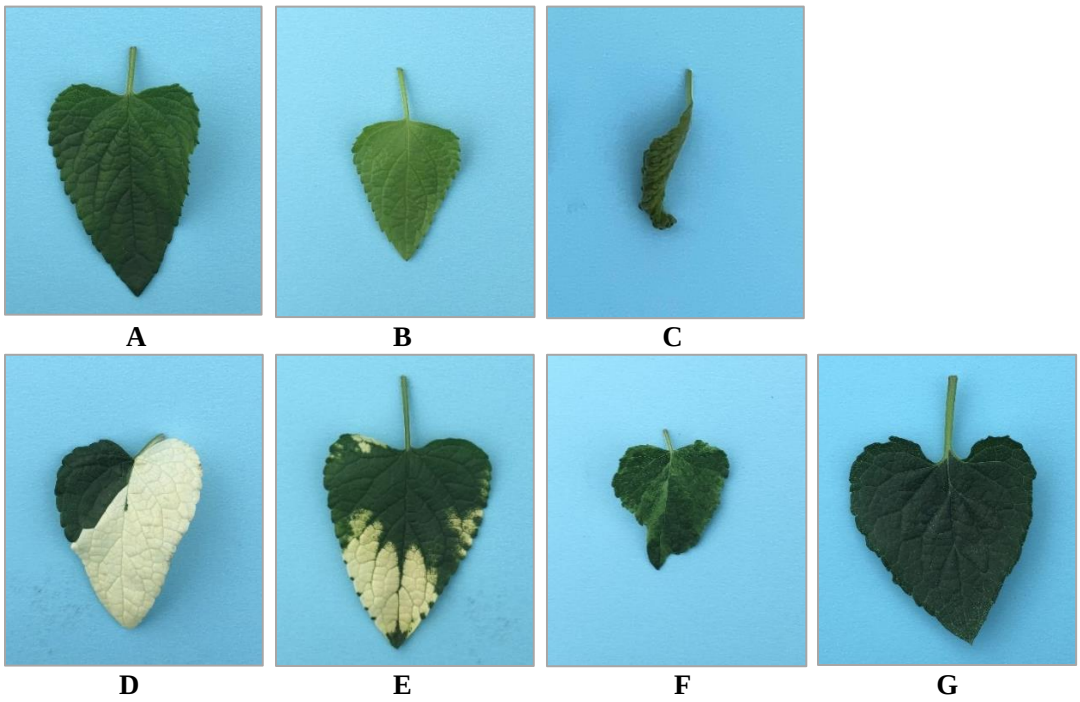

Figure 4.2: Stable mutations observed in the M_2 population from treatment with EMS. (**A**) *Salvia coccinea* untreated control, (**B**) leaf deformation from the first study with exposure to 0.8% EMS for 12 hours, and (**C**) *chlorina* mutation from the first study with exposure to 1.2% EMS for 8 hours. (**D**-**G**) mutations from the second study with exposure to 1.2% EMS for 8 hours. (**D**) sectorial chimera, (**E** & **F**) differential gene expression, and (**G**) leaf deformation.

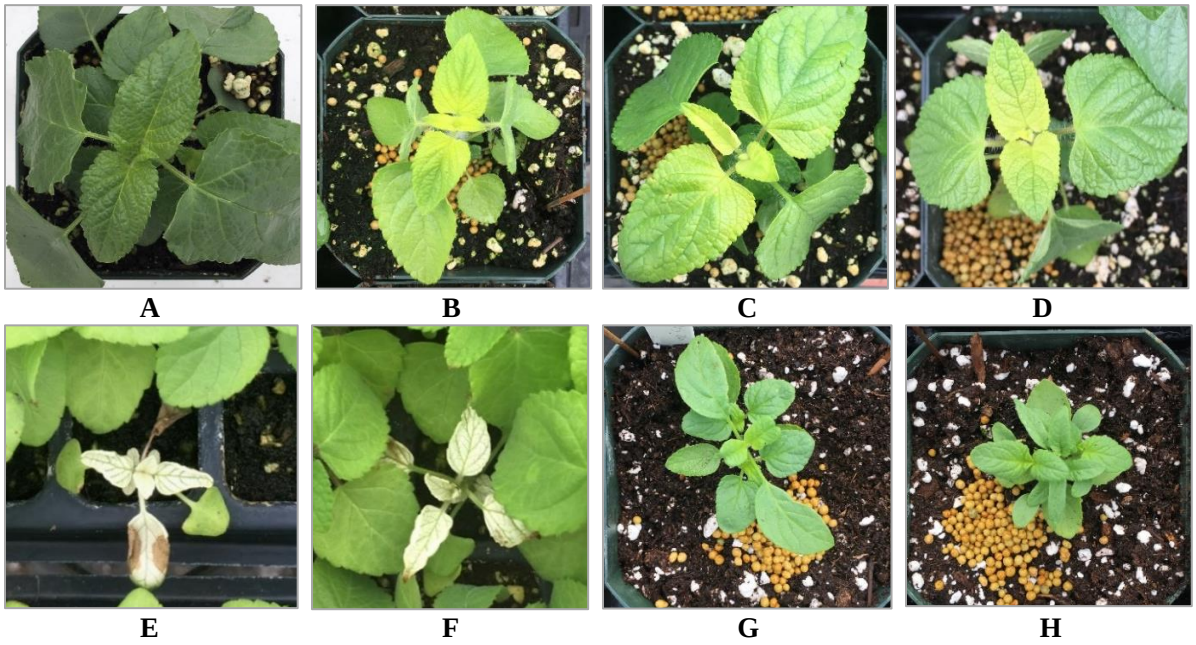

Figure 4.3: Unstable mutations observed in second study from exposure to 1.2% EMS for 8 hours. (**A**) *Salvia coccinea* untreated control, (**B** – **D**) *virescens* chlorophyll mutations, (**E** – **F**) lethal a*lbina* chlorophyll mutations, and (**G** – **H**) leaf deformations.

Figure 4.4. Mutants from the M_2 population of *S. coccinea* treated with 1.2% EMS for 8 hours. (L) *Albina* chlorophyll mutation, (C) *chlorina* leaf mutation, and (R) untreated control.

Table 4.1: Summary of stable mutations from Figure 4.2 isolated in the M_2 population from treatment with EMS. (**A**) *Salvia coccinea* untreated control, (**B**) leaf deformation from the first study with exposure to 0.8% EMS for 12 hours, and (**C**) *chlorina* mutation from the first study with exposure to 1.2% EMS for 8 hours. (**D-G**) mutations from the second study with exposure to 1.2% EMS for 8 hours. (**D**) sectorial chimera, (**E** & **F**) differential gene expression, and (**G**) leaf deformation.

Plant	Leaf Length and Width (cm)	Leaf Shape	Chlorophyll Mutation	Flower Lobe Width (cm)
A	4.6, 3.1	Deltate	N/A	1.4
B	3.6, 2.3	Ovate	*chlorina*	1.0
C	4.5, 0.8	Deltate	none	0.8
D	3.0, 3.9	Deltate	chimera	1.4
E	3.6, 4.6	Deltate	variegated	1.4
F	2.6, 3.0	Deltate	variegated	1.2
G	4.5, 4.5	Cordate	none	1.9

CHAPTER 5

DETERMINATION OF AN OPTIMAL GAMMA IRRADIATION TREATMENT RATE FOR MUTATING *SALVIA ULIGINOSA*

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[1] Maynard, R. and J. Ruter. To be submitted to *HortScience.*

Abstract

Salvia uliginosa is desirable for the landscape with blue flowers and the ability to attract pollinators. However, limited variation is commercially available in this species. Mutation breeding is a valuable tool to induce variation in ornamental species. However, many deleterious effects are associated with mutation breeding, including reduced rooting ability of vegetative cuttings. Cuttings of *S. uliginosa* were exposed to 0, 10, 20, 30, 40, or 50 Gy of gamma rays from a cobalt-60 source to determine an appropriate treatment rate. Root quality, survival, and plant height were reduced at higher levels of gamma radiation in the M_1V_1. However, rooting ability was not impacted in M_1V_2 selections. A follow-up experiment in which *S. uliginosa* cuttings were treated at 35 Gy resulted in the isolation of one mutant with variegated leaves. This study determined the optimal treatment rate for inducing mutations in *S. uliginosa* and minimizing the deleterious effect of gamma radiation.

Introduction

Salvia uliginosa is an attractive flowering plant for the landscape with distinctly blue flowers. It reaches 1-2 m in height and is used as an herbaceous perennial with cold hardiness to -9°C. It grows natively in southern Brazil, Uruguay, and Argentina and thrives in various environmental conditions, including wet soils (Clebsch, 2003; Kew Science, 2019). The flowers have a short corolla tube and blue petals marked with a white nectar guide (Clebsch, 2003; Wester and Claβen-Bockhoff, 2011). Based on its floral characteristics, *S. uliginosa* is classified as melittophilous, indicating the flowers are morphologically designed to be pollinated by bees. However, pollinators are often inhibited by sterile thecae that block access to nectar reserves, limiting natural seed set (Wester and Claβen-Bockhoff, 2011). The cultivar ‘Ballon Azul’ has been selected for its compact habit; however, limited breeding work has been done to improve the species further.

Breeding programs are continually seeking new plant characteristics to meet the market demand. Mutation breeding is a common technique to induce variation in plants through chemical or physical mutagens. Chemical mutagens may have poor uptake into plant tissue and cause DNA point mutations. Physical mutagens, however, induce more extensive DNA changes. Physical mutagens include X-rays, gamma rays, and ion beams; however, among the physical mutagens, gamma rays cause more mutations and are less damaging to plant tissue. Changes in phenotypic characteristics are easily detectible, making mutation breeding an easy and effective way to select new cultivars (Ibrahim et al., 2018).

Mutation breeding is used to rapidly improve the value of commercial and ornamental crops. In a study conducted by Jamboonsri et al. (2012), gamma irradiation was used to induce early flowering in *Salvia hispanica*. Mutants had an extended production period, resulting in an increased yield of omega-rich chia seeds. Gamma irradiation also increased fruit production in *Hibiscus sabdariffa* (Sherif et al., 2011) and essential oil production in *Rosa hybrida* (Ryu et al., 2020). In ornamental species, mutated selections include changes in flower shape, flower color, and leaf chlorophyll variegation (Datta and da Silva, 2006). New flower colors have been isolated from gamma-irradiated *Catharanthus roseus* (Kannabiran et al., 2017), *Chrysanthemum* (Setia et al., 2020), and *Delphinium malabaricum* (Kolar et al., 2015).

Despite these advantages, mutation breeding can have deleterious effects. Numerous studies have shown that gamma irradiation negatively affects plant growth and development including reduced seed germination, survival, rooting, and plant height (Abdullah et al., 2009; Jan et al., 2012; Ramesh et al., 2013). *Salvia uliginosa* ‘Ballon Azul’ was selected for treatment with gamma rays to determine the optimum exposure for inducing mutations and minimizing adverse effects. With a future aim of using gamma irradiation to select for desirable mutations, this study determined the impact of gamma irradiation on rooting ability in *S. uliginosa* cuttings.

Materials and Methods

Experiment 1

Due to the minimal natural seed set in *S. uliginosa*, vegetative cuttings were selected for mutation breeding. Stem cuttings were collected from a healthy stock plant of *Salvia uliginosa* 'Ballon Azul' for treatment with a ^{60}Co source. The cuttings were trimmed to two-nodal segments, and leaves were stripped from the lower node of each segment. Cuttings were randomly assigned into groups of 10 for treatment with 0, 10, 20, 30, 40, or 50 Gy at an exposure rate of 2.58 Gy/min. Each group of cuttings was wrapped in a moistened paper towel to prevent desiccation during treatment. The control group was subjected to the same conditions as the other cuttings without exposure to the radioactive material.

After irradiation, the cuttings were treated with a 5-second quick dip in a 1,000 ppm KIBA solution and left to air-dry for 10 minutes. They were then stuck in 280 mL Square Deep Vacuum pots (HC Companies, Twinsburg, Ohio) with a 1:1 mixture of pearlite and PRO-MIX high porosity substrate with biofungicide and mycorrhizae (Premier Tech Horticulture, Quakertown, Pennsylvania). The cuttings were left to root on a 70% shade bench with 21ºC bottom heat and mist every five minutes for five seconds. After four weeks, the cuttings were removed from their pots and measured for root length and overall root quality. Root quality was measured on a scale of zero to five, with zero having no roots and 5 having a strong root system.

After each rooted cutting was measured, they were potted up into C300 2.8 L pots (Nursery Supplies Inc., Kissimmee, Florida) with substrate consisting of 20% peat moss, 28% 3/8" aged pine bark, 42% 5/8" aged pine bark, and 10% sand (Old Castle, Shady Dale, Georgia). The pots were placed in full sun and fertilized with 10.5 g of Osmocote Plus 15-4.0-10.0 (ICL Specialty Fertilizers, Summerville, South Carolina). After six weeks when the plants were well

established in the 2.8 L pots, the height of the mature plants was measured from the substrate line to the top of the vegetative growth.

Root length, root quality, the height of the mature plant, and survival were assessed with ANOVA. Treatment groups were compared using a Tukey HSD test to determine which exposures were significantly different from the control. The linear regression function in RStudio was used to determine the relationship between measured parameters and radiation exposure. Each of these analyses were carried out using the R programming language (RStudio 2018).

Experiment 2

To measure the re-rooting ability of plants after irradiation, 10 sub-terminal cuttings were collected from each plant in the control, 10, 20, and 30 Gy treatment groups. Due to reduced growth and survival, only four plants from the 40 Gy and three plants from the 50 Gy were available as sources for cuttings. Cuttings were rooted in the same manner described in Experiment 1. To conserve greenhouse space, cuttings were rooted in 73-cell (126 mL) plug flats and arranged in a randomized complete block design. After four weeks, each cutting was removed from its cell and measured for root length and root quality as previously described.

Root length and root quality were assessed with ANOVA. Treatment groups were compared using a Tukey HSD test to determine which exposures were significantly different from the control. The linear regression function in RStudio was used to determine the relationship between measured parameters and radiation exposure. Each of these analyses were carried out using the R programming language (RStudio 2018).

Experiment 3

Based on the rooting ability, survival, and final height of cuttings irradiated in Experiment 1, a third experiment was designed to measure the performance of cuttings treated with 35 Gy. An additional 25 cuttings of *S. uliginosa* ‘Ballon Azul’ from the original stock plant

were treated with 35 Gy from the ^{60}Co source as described in Experiment 1. The aim of this experiment was to induce desirable morphological changes without significantly impacting rooting ability or survival. After irradiation, cuttings were rooted as described in Experiment 1. After four weeks, cuttings were observed for morphological changes.

Results and Discussion

Experiment 1

An ANOVA indicated treatment had a significant impact on overall root quality ($f(5)=71.92$, $p<0.001$) using the rooting scale in Figure 5.1. A post hoc Tukey test indicated only the 30, 40, and 50 Gy treatment groups were significantly different from the control. Figure 5.2 illustrates that root quality decreased with increasing gamma irradiation, while no decrease in rooting quality occurred in the 10 or 20 Gy treatment groups compared to the control. Although root quality decreased linearly with increased exposure ($R^2 = 0.87$), a Tukey test indicated only the 40 Gy treatment group had a significant decrease in overall root length ($p<0.01$). However, as shown in Figure 5.3, there was significant variation in root length in both the 40 and 50 Gy treatment groups. Because mutations are random, not all treated cuttings were expected to have a uniform response to irradiation (Toker et al., 2007). Due to prolonged exposure with gamma radiation, 30% of cuttings treated at 40 and 50 Gy never developed roots.

Although root length did not decrease uniformly with increasing exposure in *S. uliginosa*, root quality decreased with increasing exposure and may be a better indicator of overall rooting ability. Root number and root length decreased with higher doses of gamma radiation in *Coffee arabica* (Dada et al., 2018) and *Musa* (Abdulhafiz et al., 2018). Contrary to observations made on *S. uliginosa*, the number of roots increased in *Chrysanthemum morifolium* (Sadhukhan et al., 2015) and *Fragaria* (Gupta et al., 2018) treated with 10 Gy compared to the control. However, at

higher rates of gamma exposure, the number of roots decreased with increased exposure to gamma rays (Gupta et al., 2018; Sadhukhan et al., 2015).

Height of mature *S. uliginosa* also significantly decreased with increased exposure to ^{60}Co ($f(5)=17.48$, $p<0.001$). A post hoc Tukey test indicated that only the 40 and 50 Gy treatment groups had a significant reduction in height compared to the control. Figure 5.4 shows that overall plant height decreased with increasing exposure to irradiation. Height reduction was observed in *Triticum aestivum* (Singh and Datta, 2010) and *Vigna radiata* (Rukesh et al., 2017) with exposure to gamma radiation. In *Abelmoschus esculentus,* although plant height decreased with increasing levels of gamma radiation, at 500 Gy, overall height was significantly greater than the control (Amir et al., 2018).

Exposure to higher levels of ^{60}Co resulted in undesirable traits in *S. uliginosa*. First, treatment level significantly impacted survival of the rooted cuttings ($f(5)=5.48$, $p<0.001$). A post hoc Tukey test indicated the group treated with 50 Gy had a significantly lower survival rate compared to the control. Survival was not impacted for the control, 10, 20, or 30 Gy treatment groups; however, only 70% of cuttings treated with 40 Gy and 50% of cuttings treated with 50 Gy survived. Increased doses of gamma radiation also decreased survival of *Chrysanthemum morifolium* and *Musa* (Abdulhafiz et al., 2018; Sadhukhan et al., 2015).

A second undesirable trait was the generation of brittle stems. At 40 and 50 Gy treatment rates, the stems of irradiated *S. uliginosa* were easily broken, which is unsuitable for introduction to the landscape. Exposure to gamma radiation also generated brittle stems in *Glycine max* (Killion and Constantin, 1974). Future research could investigate calcium uptake as a potential contributing factor to brittle stem development at higher treatement rates.

Experiment 2

In contrast to observations made on the M_1V_1, the re-rooting experiment showed no treatment effect on overall root quality of M_1V_2 cuttings ($f(5)=1.06$, $p>0.1$). As shown in Figure 5.5, root quality was not treatment-induced; however, root quality of all groups, including the M_1V_2 control, was reduced compared to the M_1V_1 control. This is likely caused by a reduction in pot volume used to root the M_1V_2. Because the re-rooting experiment analyzed approximately 80 cuttings per treatment group, 126 mL pots were used to conserve greenhouse space instead of the 360 mL pots used to root the M_1V_1. Therefore, M_1V_2 cuttings had significantly less room to form a strong root system. Nevertheless, there was no relationship between treatment exposure and root quality ($R^2 = 0.01$). There was also no treatment effect on overall root length in the M_1V_2 ($f(5)=1.56$, $p>0.1$). Figure 5.6 shows root length varied across all treatment groups. However, a post hoc Tukey test indicated root length was not significantly different in any treatment group compared to the control. Altered root development in M_1V_2 and normal root development in all M_1V_2 selections indicates that radiation affected the existing tissue but not the development of new cells.

Experiment 3

Cuttings of *S. uliginosa* should be treated with less than 40 Gy to ensure the development of a strong root system, increase the chance of survival, and prevent the formation of delicate stems. However, compact growth is a desirable trait and was only observed above 30 Gy of irradiation. Therefore, 35 Gy of irradiation may be the optimal treatment for *S. uligionsa*. In the second study, 25 cuttings of *S. uligionsa* ‘Ballon Azul’ were treated with 35 Gy to select for desirable mutations. Out of the 25 treated cuttings, one cutting developed a leaf chlorophyll variegation. This variegation appeared as large patches of light green tissue with small, dark-green spots of unaltered chlorophyll as shown in Figure 5.7. This mutation is likely caused by alteration to the chloroplast DNA. Mutated cells in the chloroplast divide and eventually sort into

sections of normal and altered chlorophyll content, generating a mosaic appearance (Marcotrigiano, 1997). Chlorophyll mutations have also been observed in *Bougainvillea* 'Arijuna', *Lantana depressa*, and *Polianthus tuberosa* by exposure to gamma radiation (Datta and da Silva, 2006).

Although chlorophyll variegation was isolated from the M_1 in *S. uliginosa*, most mutations are recessive (Toker et al., 2007). Therefore, future work with *S. uliginosa* could allow a mutated population to self-pollinate, followed by selection for improved morphological characteristics in the M_2 and later generations. Future work with the species could also breeding with the variegated selection to determine the stability and heritability of the variegation. Although this study did not continue breeding into later generations, it determined that rooting ability was not impacted for M_1V_2 selections and that 35 Gy was the appropriate gamma rate for inducing mutations in *S. uliginosa* 'Ballon Azul'.

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Tables and Figures

Figure 5.1: Scale of rooting quality in *Salvia uliginosa* 'Ballon Azul' with zero having no roots and five having a strong root system with long roots and significant branching.

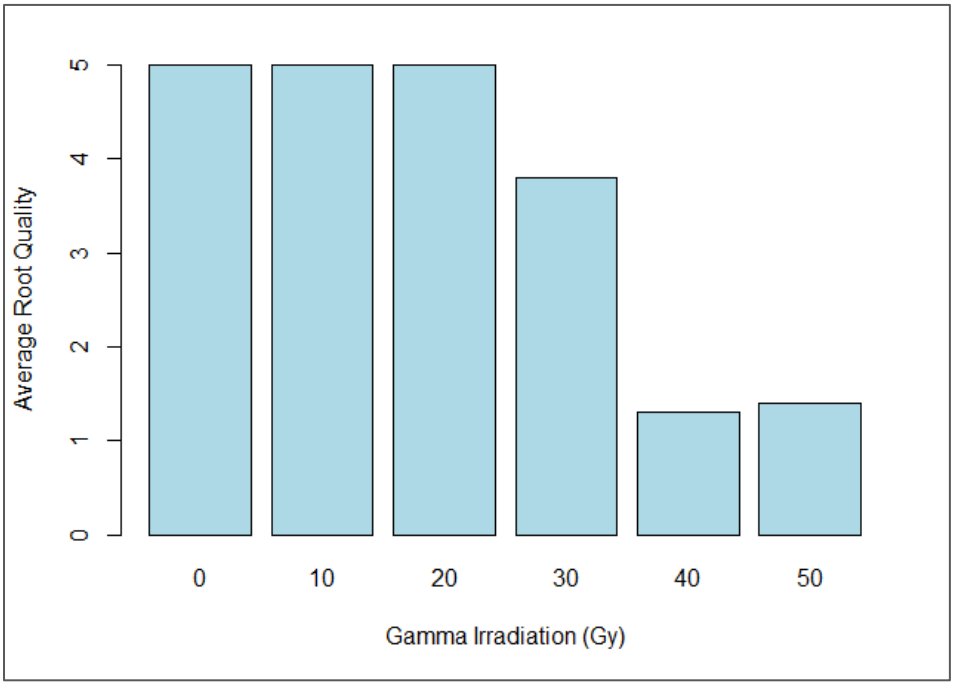

Figure 5.2: Average rooting quality of M_1V_1 cuttings from *Salvia uliginosa* 'Ballon Azul' treated with 10, 20, 30, 40 or 50 Gy of irradiation.

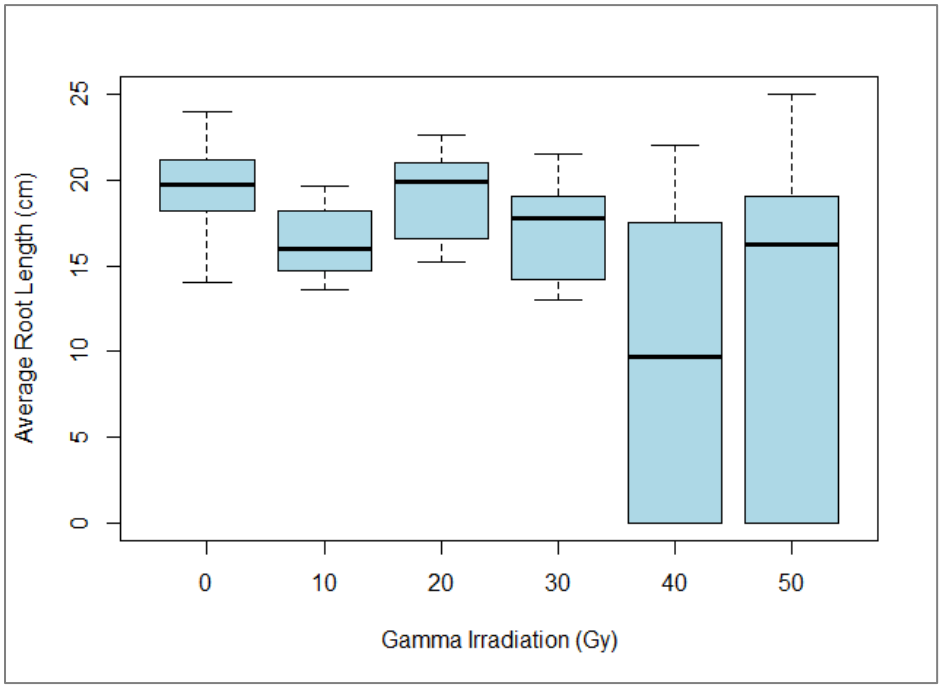

Figure 5.3: Average rooting length of M_1V_1 cuttings from *Salvia uliginosa* 'Ballon Azul' treated with 10, 20, 30, 40 or 50 Gy of irradiation.

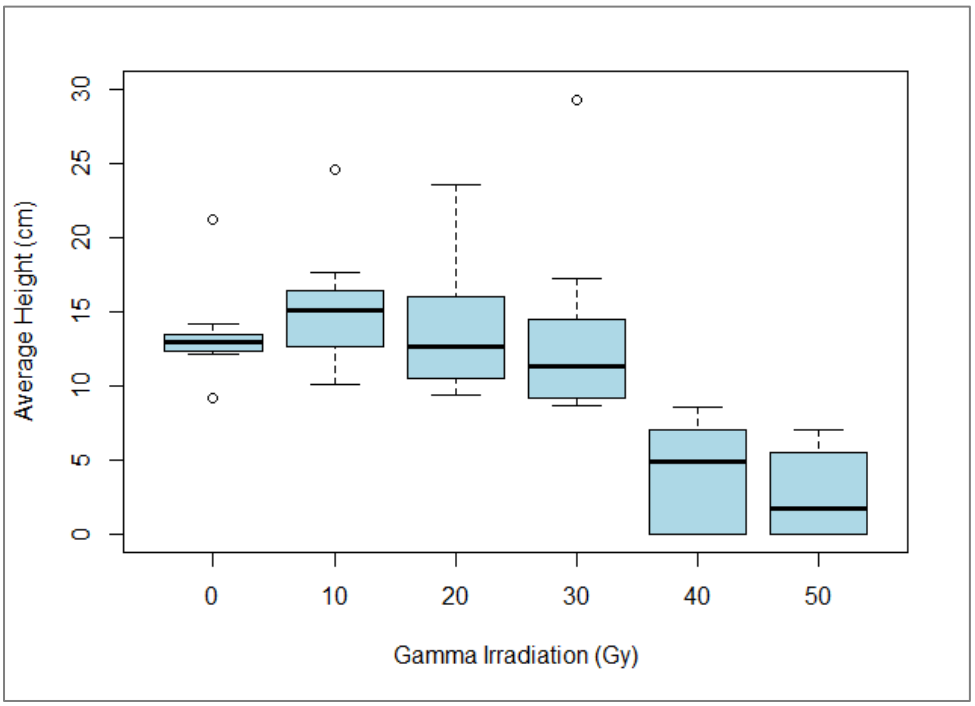

Figure 5.4: Average height of M_1V_1 cuttings from *Salvia uliginosa* 'Ballon Azul' treated with 10, 20, 30, 40 or 50 Gy of irradiation.

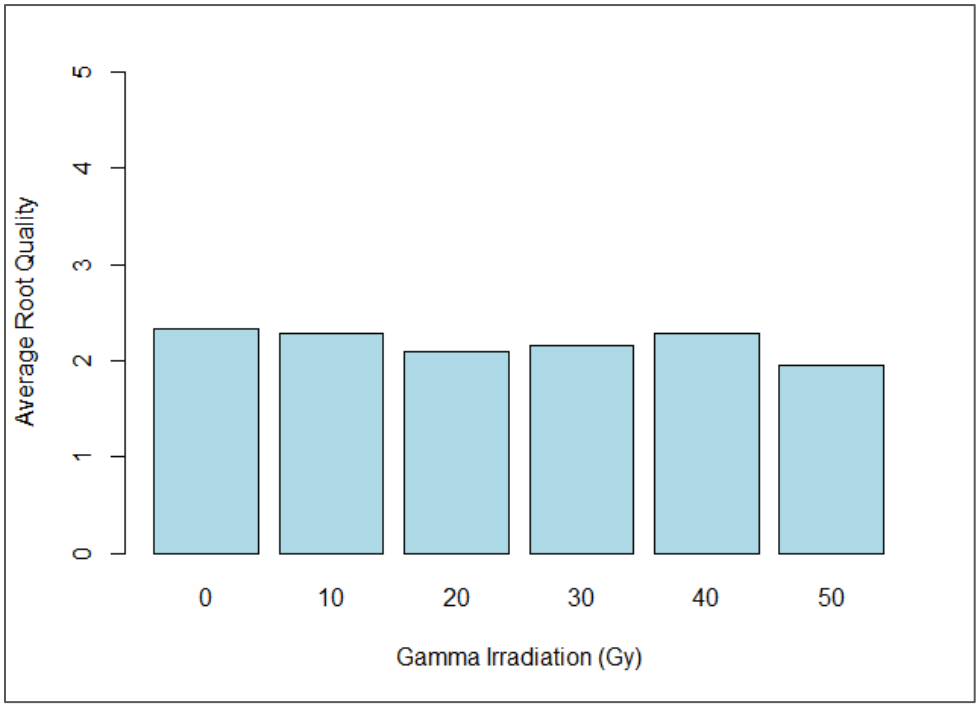

Figure 5.5: Average rooting quality of M_1V_2 cuttings from *Salvia uliginosa* 'Ballon Azul' treated with 10, 20, 30, 40 or 50 Gy of irradiation.

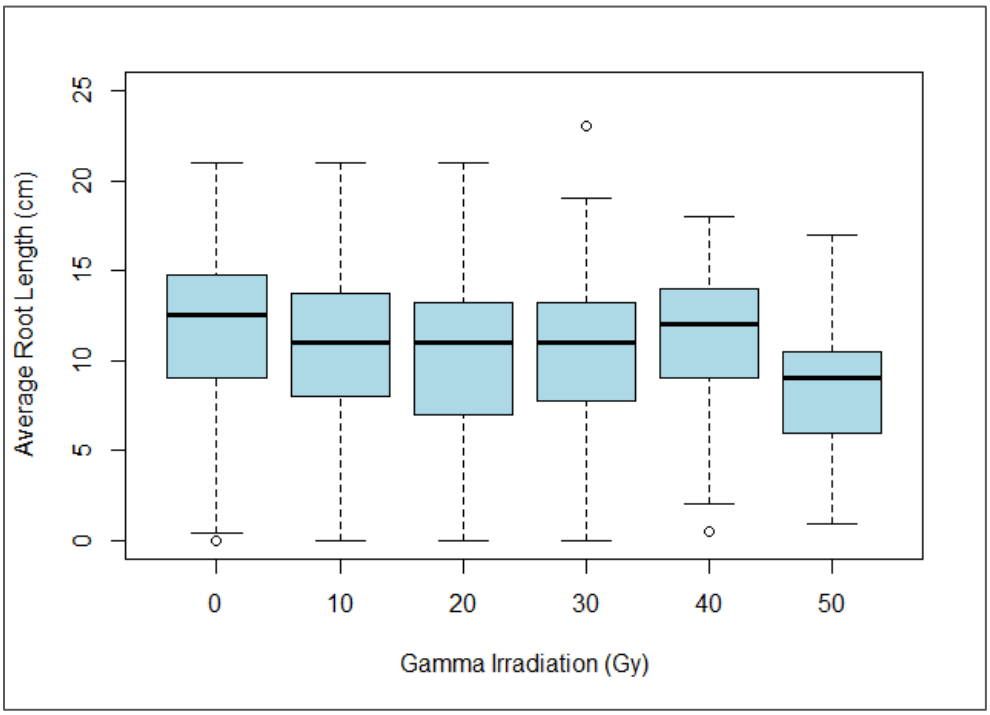

Figure 5.6: Average rooting length of M_1V_2 cuttings from *Salvia uliginosa* 'Ballon Azul' treated with 10, 20, 30, 40 or 50 Gy of irradiation.

Figure 5.7: Chlorophyll variegation in *Salvia uliginosa* 'Ballon Azul' treated with 35 Gy from a cobalt-60 source.

APPENDICES

APPENDIX A

GENERATING ROOTED CUTTINGS OF *SALVIA ULIGINOSA* IN VITRO

Introduction

Generating viable tissue culture propagules using vegetative growth in vitro requires the establishment of a successful surface disinfestation protocol. Various species of *Salvia* have been surface-sterilized for tissue culture using either mercuric chloride or sodium hypochlorite as the disinfestation agent (Jan et al., 2015; Cuena and Amo-Marco 1999; Hosoki and Tahara, 1993). However, mercuric chloride is acutely toxic with mutagenic properties. Therefore, sodium hypochlorite was selected as the disinfectant for this experiment. Tween 20 can also be used to increase the disinfestation agent's surface contact, thereby increasing the efficacy in eliminating microorganisms (Oyebanji et al., 2009). In addition to tissue surface-sterilization, all surfaces should be wiped down with 70% EtOH prior to use to prevent sample contamination (McGarrity and Coriell, 1971).

Due to a limited amount of natural seed production in *Salvia uliginosa*, mutation experiments with this species required the use of vegetative tissue. *S. leucantha* was regenerated in vitro by Hosoki and Tahara (1993). The tissue surface sterilization and culture media for shoot regeneration of S. *uliginosa* for this experiment was based on the protocol described by Hosoki and Tahara (1993). This experiment was designed to determine the appropriate sterilization technique and culture media required to generate rooted cuttings of *S. uliginosa* in vitro.

Materials and Methods

Experiment 1

A laminar flow hood (NuAire, Plymouth, Minnesota) was used to carry out tissue sterilization and culture initiation. Internal surfaces of the hood were wiped down with 70% EtOH before use. All glassware was covered with aluminum foil and heated in an Amsco Lab 250 laboratory steam sterilizer (STERIS, Mentor, Ohio) for 20 minutes at 121°C. Forceps, scissors, and a scoopula were sterilized at 250°C for thirty seconds in a Steri 350 dry bead sterilizer (Inotech Bioscience, LLC, Rockville, Maryland). Young shoot tips of *S. uliginosa* were collected from mature stock plants and kept in a cooler until ready to use. Cuttings were stripped of leaves, placed in a mesh bag, and rinsed continuously under tap water for 10 minutes to remove any surface particles. The cuttings were then transferred to the laminar flow hood, trimmed into 1-nodal segments, and placed in a beaker with sterile DI water to prevent the cuttings from drying out.

Fifteen segments were randomly selected from the beaker for each treatment group. Selected cuttings were agitated for 3 minutes in a 30 mL solution of 0.6% sodium hypochlorite with three drops of Tween 20. Segments were then transferred to a 50 mL beaker with 30 mL sterilized water and agitated for 30 seconds to remove residual sodium hypochlorite. The rinsing process was repeated twice more in separate beakers of sterile water, with the control group agitated in sterile DI water for the same duration as the treated groups.

Sterile growing media was prepared by forming a solution of 4.4 g/L Murashige and Skoog (MS) basal salts, 20.0 g/L sucrose, and 8.0 g/L agar (Murashige and Skoog, 1962). The pH was adjusted to 5.6, adding NaOH or HCl to raise or lower the pH, respectively. Containers were capped and autoclaved for 20 minutes at 121°C. Next, 25 mL of the partially cooled sterile media was dispensed into 9 cm Petri dishes. The Petri dishes were sealed with Parafilm and left to solidify. Five segments from each treatment group were plated on the sterile solid media with

three replicates in each group. Plates were then sealed with Parafilm and maintained at 24°C with a 12-hour photoperiod.

Due to contamination using the 0.6% sodium hypochlorite, the experiment was repeated with higher bleach concentrations. Cuttings were treated with a 1.5% sodium hypochlorite solution with 3 drops of Tween 20 for 1 or 3 minutes. A control group was treated with sterile DI water alone. Three replicates of five cuttings were plated for each treatment group. Plates were maintained at 24°C with a 12-hour photoperiod.

Experiment 2

Full-strength MS, half-strength MS, and woody plant media (WPM) were prepared following the protocol described above to test the success of shoot regeneration on different media types (McCown and Lloyd, 1981; Murashige and Skoog, 1962). Full-strength MS media was formed with 4.4g/L MS Basal salts, 20.0 g/L sucrose, and 8.0 g/L agar. Half-strength MS media was formed with 2.2g/L MS Basal salts, 20.0 g/L sucrose, and 8.0 g/L agar. Finally, the WPM was prepared with a WPM stock solution, 20.0 g/L sucrose, and 8.0 g/L agar. After the media had been sterilized, 0.1mg/L benzyladenine was pipetted into each solution to prevent thermal degradation. Then, 75 mL of the partially cooled media was dispensed into Magenta GA-7 culture vessels.

Shoot tips of *S. uliginosa* were collected from healthy stock plants and kept in a cooler until ready to use. Leaves were stripped off the lower portion of the cuttings leaving only one set of leaves at the top. Cuttings were trimmed into 3-nodal segments, placed in a mesh bag, and rinsed under tap water for 10 minutes. They were then submerged in a beaker of sterile DI water until ready to use to prevent desiccation. All glassware and instruments were sterilized before use, following the protocol mentioned above.

Four cuttings were randomly assigned to each culture vessel and two culture vessels were prepared for each media type. Plant samples were surface sterilized in 50 mL of a 1.5% sodium hypochlorite solution with three drops of Tween 20. Cuttings were agitated for three minutes in the sodium hypochlorite solution, then rinsed in three separate beakers with sterile DI water. Cuttings were stuck vertically in the culture vessels with the bottom node pressed into the solid media. Four cuttings were stuck per culture vessel, and two culture vessels were prepared for each media type. This process was repeated to generate a total of 16 cuttings per media type. The culture vessels were arranged in a randomized complete block design on separate shelves of a clean room. Cuttings were maintained at 24°C with a 12-hour photoperiod from a LED bulb. After 30 days, the treated cuttings were destructively harvested and measured for their fresh weight.

Results

Experiment 1

In the first tissue sterilization experiment, cuttings were treated with a 0.6% sodium hypochlorite solution for 1, 5, or 10 minutes. All samples became contaminated after 20 days, necessitating the use of a more concentrated bleach solution. The second sterilization experiment treated cuttings with 1.5% sodium hypochlorite for 1 or 3 minutes. After 20 days, two plates in the 1-minute treatment group became contaminated. However, no contamination was observed in the groups treated with 1.5% sodium hypochlorite for 3 minutes. Therefore, this concentration and exposure time was selected for the second phase of the experiment.

Experiment 2

The cuttings stuck in full-strength MS medium had the most significant amount of axillary shoot multiplication with little browning on the leaves. All cuttings formed callus, but only rooting only occurred on some cuttings. The half-strength MS media had less axillary shoot

multiplication than the full-strength; however, more leaf browning occurred. Callus formed on all the cuttings, and significant root growth occurred on this medium. Finally, the segments cultured on WPM had little, if any, axillary shoot multiplication with significant leaf browning occurring on most cuttings. All cuttings formed callus, and some root production was observed. The amount of vegetative growth was confirmed through fresh weight measurements, which was greatest on full-strength MS medium and least on WPM. The findings are summarized in Table 6.1 and pictured in Figure 6.1.

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Tables and Figures

Table 6.1: Summary of tissue formation in *Salvia uliginosa* cuttings grown in vitro.

Media	Vegetative Growth	Callous Formation	Root Formation	Fresh Weight (g)
MS	Yes	Yes	Minimal	9.64
½ MS	Yes	Yes	Yes	8.61
WPM	Minimal	Yes	Minimal	3.74

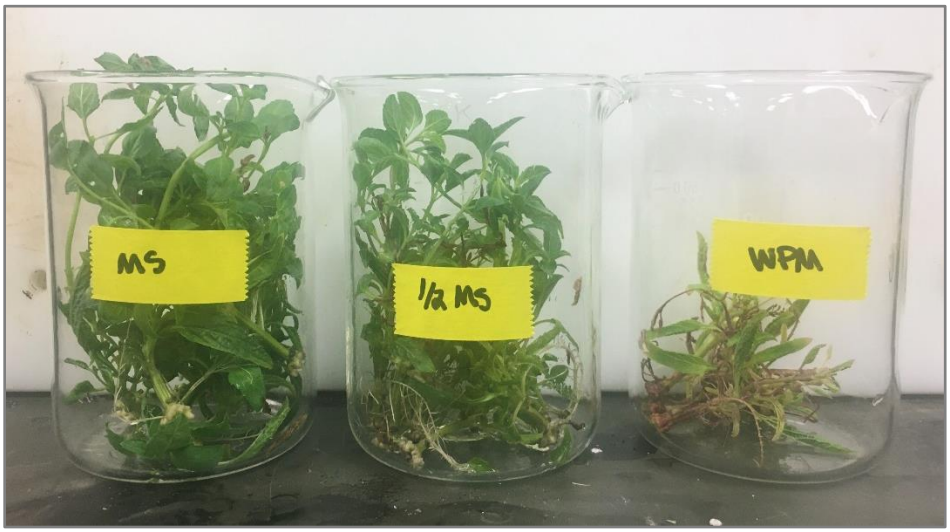

Figure 6.1: Vegetative production in *Salvia uliginosa* grown in vitro. (L) Murashige and Skoog media; (C) half-strength MS media; (R) woody plant media (McCown and Lloyd, 1981; Murashige and Skoog, 1962).